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Complex lipid biosynthesis and its manipulation in plants

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1. Introduction

In all living organisms lipids play several roles and, according to their structures, can be divided into two main groups: the non-polar lipids (acylglycerols, sterols, free fatty acids, wax and steryl esters) and polar lipids (phosphoglycerides, glycosylglycerides, and sphingolipids). Triacylglycerols act as compact, easily metabolised and non-hydrated energy stores. They are important storage products especially in plants producing oilseeds and in oily fruits such as avocado, olive and oil palm. Waxes are commonly extracellular components such as surface coverings, which function both to reduce water loss and to protect plants from noxious environmental conditions. They also act as an energy store in jojoba.

Polar lipids and sterols are important structural components of cell membranes with many diverse functions. The membrane lipids act as permeability barriers for cells and organelles (Gurr et al. 2002). They provide the matrix for assembly and function of a wide variety of catalytic processes as well as directly participating in metabolism and in a multitude of membrane fusion events. Moreover, the membrane lipids actively influence the functional properties of membrane-associated processes (Gurr et al. 2002). In additional to a structural role, lipids act as key intermediates

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in cell signalling pathways (e.g. inositol lipids, sphingolipids, oxidative products) and play a role in sensing changes in the environment.

Plant fats and oils are utilised for many food and industrial applications. They include edible oils, processed ingredients for the food industry and feedstocks for chemical processes such as formulation of paints, inks, resins, varnishes, plasticizers and biodiesel production (Kridl 1998).

Over the last decades, research into plant lipid metabolic pathways has expanded considerably and it has been influenced by an increasing impact arising from molecular genetic approaches. Many genes encoding lipid-related enzymes have been isolated/cloned and this has allowed the manipulation of plant lipid metabolism for commercial purposes (Slabas and Sanda 1998; Murphy 2005). In theory, this genetic engineering approach required cloning of the genes controlling certain steps of biosynthesis, regulating the genes for proper expression in the seeds of plants and a transformation and regeneration system for the oil-seed of choice (Kridl 1998). In this chapter we will summarize how this approach has been used for oil improvements and modulations of lipid biosynthesis.

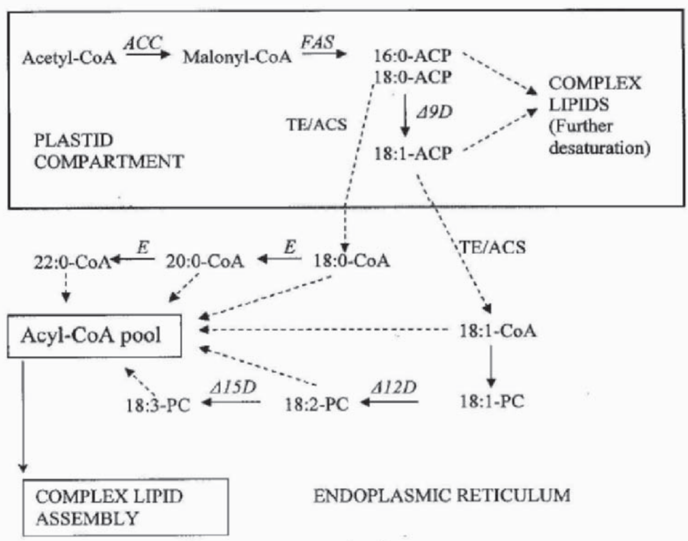

Fig. 9.1. Simplified depiction of fatty acid biosynthesis in plants. Fatty acids are abbreviated with the number before the colon indicating the number of carbon atoms and the number afterwards showing the number of double bonds. Thus 16:0 = palmitic acid, 18:1 = octadecenoic acid (oleic acid in this case) etc. Abbreviations: *ACC*, acetyl-CoA carboxylase; *FAS*, fatty acid synthase; *D*, desaturase (e.g. *Δ9D*, Δ9-desaturase); *E*, fatty acid elongase; TE, acyl-ACP thioesterase; ACS, acyl-CoA synthase.

2. Summary of lipid metabolism

Before summarising molecular biological attempts to manipulate lipid metabolism in plants we must write a few words about the pathways concerned. A detailed discussion is not appropriate here and the reader is referred to Gurr et al. (2002) for a simple description and Murphy (2005) for more details. Appropriate references will be found in these books.

Basically, de novo synthesis uses acetyl-CoA carboxylase and fatty acid synthase to produce palmitic and stearic acids. The latter can be further modified by desaturation and elongation reactions and these processes use enzymes in different compartments of plant cells (Fig. 9.1).

Complex lipids, usually based on a glycerol backbone, are made by the basic Kennedy pathway, together with additional reactions (Fig. 9.2).

3. Fatty acid manipulation

Fatty acid biosynthesis is one of the primary pathways of lipid metabolism and an exclusive source of the acyl chains of complex lipids (Harwood 1996; 2005). The major fatty acids from the world oil supply (mainly from soybean, palm and canola) are palmitic, linoleic and oleic acids (Hildebrand et al. 2005). Many unusual fatty acids have been identified in seed oils as major components. In some cases, these unusual acids comprise more than 90% of the seed oil (Hildebrand et al. 2005). Fatty acids may differ in terms of their chain length, degree of saturation, configuration of double bonds, positional isomers, conjugation of double bonds or additional chemical groups such as hydroxy, allenic, epoxy, acetylenic, cyclo, fluoro and keto. The discovery of many biological activities (as well as industrial applications) of some unusual fatty acids has led to an increased utilization of these molecules and to an attempt to domesticate several unusual fatty acid-producing oilseeds. Since domestication of most of these unusual oilseeds faces a number of diverse problems (e.g. low yield, climatic requirement), genetic engineering approaches often seem to be a more promising route for the development of such resources (Hildebrand et al. 2005).

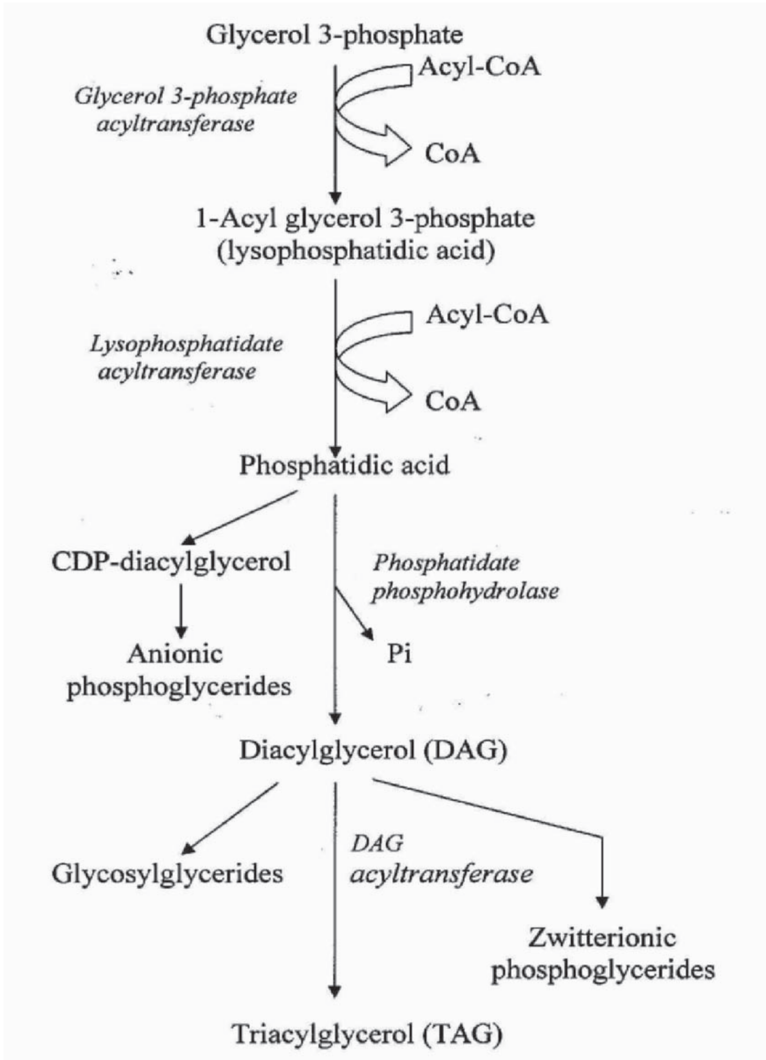

Fig. 9.2. The basic Kennedy pathway for glycerolipid synthesis in plants. Important anionic phosphoglycerides in plants are phosphatidylglycerol, phosphatidylinositol and diphosphatidylglycerol (cardiolipin). Zwitterionic phosphoglycerides include phosphatidylethanolamine and phosphatidylcholine. For additional reactions that can be involved in phosphoglyceride and triacylglycerol formation, refer to Dörmann (2005) and Weselake (2005).

Plants synthesize 18-carbon fatty acids through a pathway located in the plastid that begins with acetyl-CoA and then uses malonyl-acyl carrier protein (ACP) as the two-carbon donor (Fig. 9.1). The acyl-CoA needed for this synthesis comes ultimately from photosynthesis. The actual process of de novo synthesis to produce long-chain fatty acids involves the participation of two enzymes, acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) which are usually multi-protein complexes containing a number of enzymes. ACC is a soluble Class 1 biotin-containing enzyme that catalyses the ATP-dependent formation of malonyl-CoA from bicarbonate and acetyl-CoA. This malonyl-CoA is used for de novo synthesis of fatty acids inside plastids. In addition, malonyl-CoA is needed for elongation of fatty acids on the endoplasmic reticulum as well as for synthesis of various secondary metabolites in the cytosol. As expected from such requirements, two isoforms of ACC are found in plants, the second of which is extra-chloroplastic (presumed to be cytosolic) and is a multifunctional protein. These isoforms have distinct properties which give rise to their different susceptibility to herbicides (Harwood 2005).

FAS is the second major enzyme complex involved in de novo fatty acid formation. The plant FAS is a type II dissociable multiprotein complex, like the *E. coli* system but unlike that of animals, i.e. the individual proteins that make up FAS can be isolated and their function demonstrated separately. The first condensation reaction in fatty acid synthesis is catalysed by β-ketoacyl-ACP synthase III (KAS III) that uses acetyl-CoA and malonyl-ACP substrates to give a 4C-keto-intermediate. Successive reduction, dehydration, and a second reduction then produce a 4C fatty acid, butyrate, which is attached to ACP. The next six condensations are catalysed by KAS I to produce 6-16C fatty acids. The final reaction between palmitoyl-ACP and malonyl-ACP uses KAS II and results in synthesis of stearate. The remaining enzymes of FAS are β-ketoacyl-ACP reductase, β-hydroxylacyl-ACP dehydrase and enoyl-ACP reductase (Fig. 9.3).

Completion of de novo fatty acid synthesis is made in one of three ways. Either the product of acyl-ACP is hydrolysed by a thioesterase, the palmitate or stearate products are desaturated or the acyl-ACPs are used directly for complex lipid formation using plastid acyltransferases. Through the action of thioesterases, non-esterified fatty acids are made available for conversion to acyl-CoAs which provide the acyl chains for assembly into complex lipids in the extra-plastid compartment (Fig. 9.1).

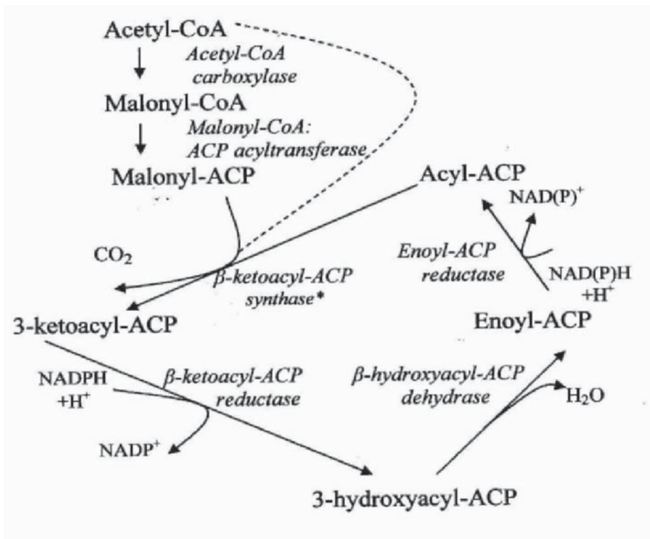

Fig. 9.3. Reactions of fatty acid synthase. *The first condensation reaction is catalysed by β-ketoacyl-ACP synthase III (KAS III) that uses acetyl-CoA and malonyl-ACP substrates. The next six condensations are calalysed by KAS I and the final reaction between palmitoyl-ACP and malonyl-ACP uses KAS II.

3.1 β-ketoacyl-ACP reductase modification

This enzyme has been isolated and purified from several plant tissues and the monomer from *Brassica napus* has a molecular mass of 28 kDa (Slabas et al. 1992). cDNAs have been isolated from a number of plants and show 55-81% identity for their derived sequences (Harwood 1996). The *Arabidopsis* library shows only one gene for β-ketoacyl-ACP reductase so far identified (Mekhedov et al. 2000). The *B. napus* β-ketoacyl-ACP reductase was down-regulated using an antisense approach (O'Hara et al. 2000). Only during the rapid phase of leaf expansion (days 4-7 after emergence) was it thought that β-ketoacyl-ACP reductase could contribute to any significant effects on total fatty acid synthesis rates. At other times, the major control of flux appeared to reside elsewhere in the pathway. Nevertheless, seeds gathered from antisensed *Brassica* lines showed some distinct morphologies and a reduced lipid content (O'Hara et al. 2000).

3.2 β-ketoacyl-ACP synthase modification

Expression of cDNA encoding KAS III showed that this enzyme has a universal role in fatty acid biosynthesis, irrespective of the plant species from which it is derived or the tissue in which it is expressed (Dehesh et al. 2001). An increase in the levels of C16:0 was observed in tobacco (*Nicotiana tabacum,* WT-SR) leaves overexpressing KAS III from spinach (*Spinacia oleracea*) when under the control of the cauliflower mosaic virus-35S promoter and in arabidopsis and rapeseed (*Brassica napus*) seeds overexpressing either of the *Cuphea hookeriana* KAS IIIs when expression was driven by napin. The transgenic seeds contained lower levels of oil as compared with the wild-type levels. In addition, the rate of lipid synthesis in transgenic rapeseed seeds was notably slower than that of the wild-type (Dehesh et al. 2001). The levels of the acyl-ACP intermediates as well as any changes in levels of other fatty acid synthase enzymes have been measured and it was suggested that malonyl-ACP, the carbon donor utilised by all the 3-ketoacyl-ACP synthases, was limiting in the transgenic plants. Malonyl-CoA has been further suggested to be a potential limiting factor affecting the final oil content and C16:0 extension (Dehesh et al. 2001).

In order to understand the contribution to chain length regulation that might be made by β-ketoacyl-ACP synthase, Cw KAS A1, derived from *Cuphea wrightii* (a species that accumulates 30% C10:0 and 54% C12:0 in its seed oil) was investigated by Leonard et al. (1998). Expression of this gene in *Arabidopsis* seeds, especially when combined with *C. wrightii* thioesterase (which has good activity with medium chain fatty acids), allowed a doubling of the production of C10:0 (Leonard et al. 1998).

Decreased amounts of C18:1 and increased amounts of C18:2 and C18:3 acids were observed as compared to control plants when *E. coli fabH* gene, which encodes a KAS III-equivalent enzyme, was overexpressed in *B. napus* (Verwoert et al. 1995). Thus, expression of KAS genes cannot always be predicted to give a simple change in fatty acid composition.

3.3 Acyl-ACP thioesterase modification

Since medium-chain fatty acids are valuable renewable resources, attempts have been made by researchers to produce high-yielding annual crops using genetic transformation. Expression of FatB1 cDNA encoding 12:0-acyl-ACP thioesterase (BTE), isolated from the seeds of undomesticated California bay, in the seeds of *Arabidopsis thaliana* and *Brassica napus* resulted in BTE activity, and in the accumulation of medium chain fatty

acids at the expense of long-chain FAs (Voelker et al. 1992; Yuan et al. 1995). Laurate became the most abundant FA and was deposited in the storage lipids (up to 60% of the triacylglycerol acyl groups) indicating that the "foreign" fatty acid was exported from the plastids and utilised by the enzymes of the Kennedy pathway for the assembly of triacylglycerides (TAGs) (Voelker et al. 1992). Non-destructive analysis of the oil composition of single seeds enabled high-lauric lines to be selected from the best events and, through subsequent breeding and performance trials, a cultivar has been developed. This cultivar is known as "Laurate Canola" (Laurical™) and a very acceptable yield of oil was obtained from seeds grown in Michigan in the summer of 1995 and a crop in North Dakota in November 1996. The oil is being used for the manufacture of soaps and detergents (Kridl 1998; Davies 1996). The possibility of Laurical™ use as a food ingredient is also under investigation.

Production of high levels of caprylate (C8:0) and caprate (C10:0) has also been achieved in transgenic canola, which normally does not accumulate any short chain FAs, by overexpression of Ch FatB2, a thioesterase cDNA isolated from the Mexican shrub *Cuphea hookeriana* (Dehesh et al. 1996). A dramatic increase in the levels of these two fatty acids was accompanied by a preferential decrease in the levels of linoleate and linolenate (Dehesh et al. 1996).

Eccleston and co-workers transformed *B. napus* using a medium-chain acyl-ACP thioesterase isolated from *Umbellularia californica* (California bay). Although laurate accumulated in seeds, none was detectable in leaves despite very high levels of the medium-chain thioesterase. If this thioesterase was expressed in seeds using a napin promoter, then up to 60% of the total seed fatty acids were laurate. At the same time β-oxidation was increased and this was believed to be responsible for the limited accumulation of seed laurate when the constitutive CaMV 35S promoter was used. In support of this hypothesis, isocitrate lyase activity was found to be significantly increased in plants transformed with FatB1. In the high-laurate seeds, the levels of acyl carrier protein and several enzymes of fatty acid synthesis were increased, perhaps to compensate for the lauric acid lost through β-oxidation (Eccleston et al. 1996; Eccleston and Ohlrogge 1998).

The tropical tree species mangosteen (*Garcinia mangostana*) has been found to store stearate (C18:0) in its seed oil in amounts of up to 56% by weight (Hawkins and Kridl 1998). Expression of mangosteen thioesterase (Garm FatA1) in *Brassica* seeds led to the accumulation of stearate up to 22% in seed oil suggesting that Garm FatA1 is, at least, partially responsible for determining the high stearate composition of mangosteen seed oil (Hawkins and Kridl 1998).

3.4 β-ketoacyl-CoA synthase modification

This enzyme catalyzes the condensation of malonyl-CoA with long-chain acyl-CoA and this reaction is the initial step of the microsomal fatty acyl-CoA elongation pathway responsible for formation of very long chain fatty acids (VLCFAs, fatty acids with chain length > 18 carbons) (Fig. 9.1). Manipulation of this pathway is significant for agriculture, because this was the basis for the conversion of high erucic acid (C22:1) rapeseed (HEAR) into canola cultivars. Although canola varieties are used for food purposes, there is an interest in HEAR cultivars to provide lubricant oils. TAGs from HEAR varieties of rapeseed lack erucoyl residues in the sn-2 position, and there has been considerable interest in raising the erucate levels further by overcoming this compositional limitation (Davies 1996). This objective has been approached in the following way by Lassner et al. (1996). First, they cloned a gene involved in an elongation reaction, β-ketoacyl-CoA synthase (KCS), from the jojoba plant, *Simmondsia chinensis.* In its native species this enzyme is part of the "elongase" system that produces the C20, C22 and C24 acyl groups that predominate in the stored wax esters. The introduction of this gene into canola resulted in the production of TAGs containing up to 58% of VLCFAs. The KCS gene will now be used to isolate the homologous gene from HEAR, in order to overexpress it and, thus, obtain an oil containing higher than the typical HEAR value of 40-50% erucate (Davies 1996).

From *Limnanthes douglasii*, cDNAs that encoded a homolog of KCS involved in production of VLCFA synthesis in this species have been isolated (Cahoon et al. 2000). Expression of FAE1 homolog in somatic soybean embryos showed the accumulation of C20 and C22 fatty acids, principally as eicosanoic acid, up to levels of 18% (w/w) of the total fatty acids of single embryos.

An 8-fold increase in erucic acid proportion in *Arabidopsis* seed oil has also been seen as a result of seed-specific expression of nasturtium (*Tropaeolum majus*) KCS (or elongase) gene (Mietkiewska et al. 2004).

3.5 β-ketoacyl-CoA reductase modification

Metz and co-workers purified an alcohol-forming fatty acyl-CoA reductase (FAR) from developing embryos of the jojoba, *S. chinensis*, plants (Metz et al. 2000). Jojoba is known to produce another kind of reserve lipid in seeds, namely esters of long-chain alcohols and fatty acids (waxes), which have an important use in cosmetics. When the jojoba FAR cDNA was expressed in embryos of *B. napus*, long-chain alcohols could be detected in

transmethylated seed oils showing that approximately 4% of the acyl groups were reduced to alcohol groups. It is interesting that, in addition to free alcohols, novel wax esters were identified in the transgenic seed oil (Metz et al. 2000). An endogenous fatty acyl-CoA: fatty alcohol acyl-transferase activity, that could account for this wax synthesis, has been identified using *B. napus*. Thus, introduction of a single cDNA into *B. napus* resulted in a redirection of a portion of seed oil synthesis from TAGs to waxes (Metz et al. 2000). Lardizabal et al. (2000) combined these two cDNAs with a KCS elongase cDNA from *Lunaria annua* (*Brassicaceae*) for coexpression in *A. thaliana* under control of a napin promoter. In some seeds, as much as 70% of the oil was accounted for by wax and the proportion of VLCFAs and alcohols in some of these seeds was increased to 42% as compared to 28% in the wild type (Lardizabal et al. 2000).

3.6 Desaturase manipulation

18:1-Δ12-desaturase and related enzymes

The greatest functional diversity within a family of fatty-acid-modifying enzymes has been observed within the Δ12-oleic acid desaturase or Fatty Acid Desaturase 2 (FAD2) family (Jaworski and Cahoon 2002). This enzyme, which is typically found in nearly all higher plants, catalyzes the insertion of a *cis* double bond between the Δ12 and Δ13 carbon atoms of oleic acid (C18:1-Δ9) to form linoleic acid (C18:2-Δ9,12). By downregulation of FAD2 (which blocked the flux from oleic acid into polyunsaturated fatty acids) in soybean, lines with 85% of oleic acid in seed oil have been produced (Kinney 1998a). Cotton transgenic lines transformed with the microsomal ω-6 (Δ12-) desaturase ghFAD2-1 inverted-repeat construct have been shown to exhibit the increased level of oleic acid from about 15% in untransformed cotton plants up to a range of 26-77% in the mature seeds of primary transgenic plants (Liu et al. 2000). Chapman and co-workers also reported the development of transgenic cotton plants with higher seed oleic acid contents (Chapman et al. 2001). A binary vector was designed to suppress expression of the endogenous cottonseed Δ12 desaturase (FAD2) by subcloning a mutant allele of a rapeseed FAD2 gene downstream from a heterologous, seed-specific promoter (phaseolin). Increased seed oleic acid content ranged from 21 to 30% (by weight) of total fatty acids and was at the expense of linoleic acid, consistent with reduced activity of cottonseed FAD2. The integration of the canola transgene into the cotton genome has been confirmed by molecular analysis of nuclear DNA from transgenics (Chapman et al. 2001).

Co-suppression plasmids carrying oleate desaturase genes from each species have been constructed and transferred into Australian elite breeding lines of *B. napus* and *B. juncea* using *Agrobacterium tumifaciens* plant-transformation techniques (Stoutjesdijk et al. 2000). Silencing of the endogenous oleate desaturase genes resulted in substantial increases in oleic acid levels, of up to 89% in *B. napus* and 73% in *B. juncea* (Stoutjesdijk et al. 2000).

Some plants have evolved new functions for their FAD2 and many variant forms of this enzyme have been identified, e.g. hydroxylases, epoxygenases, acetylenases and conjugases (Fig. 9.4).

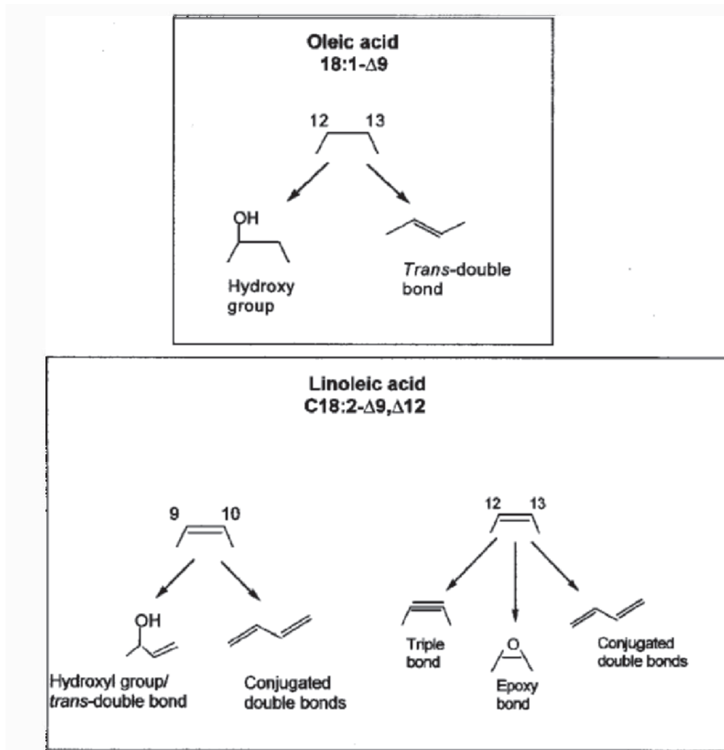

Fig. 9.4. Summary of reactions catalyzed by FAD2-related enzymes (see text for further details).

Analysis of the phylogenetic relationships of acetylenase, conjugase, epoxygenase, hydroxylase and desaturase amino acid sequences indicated that acetylenases, conjugases, epoxygenases and hydroxylases group semi-randomly among Δ12 desaturase sequences suggesting that these enzymes

arose independently many times from pre-existing desaturases during plant evolution (Hildebrand et al. 2005).

The Δ12-oleic acid hydroxylase from the seeds of castor (*Ricinus communis*) was described as the first divergent form of FAD2 (Van de Loo et al. 1995). This enzyme introduces a hydroxyl group at the Δ12 position of oleic acid to produce the industrially valuable ricinoleic acid (12-OH-18:1-Δ9). Hydroxy fatty acids from castor seeds can be used in a wide range of commercial products including plastics, foams, surfactants, cosmetics and lubricants (Jaworski and Cahoon 2003; Kinney and Clemente 2005). Oleate 12-hydroxylase genes have been cloned from developing endosperm of the castor-oil plant and *Lesquerella fendleri* (Brassicaceae) (Van de Loo et al. 1995; Broun and Somerville, 1997; Broun et al. 1998). A cDNA encoding the oleate 12-hydroxylase from castor bean has been shown to have approximately 67% sequence homology to microsomal oleate desaturase from *Arabidopsis* and to direct the synthesis of small amounts of ricinoleic acid in seeds of transgenic tobacco plants (Van de Loo et al. 1995). Expression of the cDNA under control of a napin promoter in transgenic *Arabidopsis thaliana* plants resulted in the accumulation of up to 17% of seed fatty acids as ricinoleate and two novel fatty acids, lesquerolic (14-OH-20:1-Δ11) and densipolic (12-OH-18:2-Δ9,Δ15) acids (Broun and Somerville 1997). These results suggested that, either the castor hydroxylase can utilize oleic acid and eicosenoic acid for substrates for ricinoleic and lesquerolic acid biosynthesis, respectively, or that *Arabidopsis* contains an elongase that accepts ricinoleic acid as a substrate. The discovery of a condensing enzyme from the seeds of *L. fendleri* that specifically elongated hydroxyl fatty acids supported the latter suggestion (Moon et al. 2001). It is also interesting, that expression of the castor bean oleate 12-hydroxylase in *A. thaliana* led to a concomitant increase in oleic acid in the seed oil, from 14.7% to 24.1% (Broun and Somerville 1997). Expression of the *L. fendleri* oleate 12-hydroxylase in transgenic plants of a FAD2 mutant of *Arabidopsis,* which was deficient in cytoplasmic oleate Δ12 desaturase activity, resulted in partial suppression of the mutant phenotype in roots (Broun et al. 1998a). Thus, both hydroxylase and desaturase activities have been suggested for the *L. fendleri* enzyme (Broun et al. 1998a). Moreover, expression in yeast indicated that the castor hydroxylase itself has a low level of desaturase activity (Smith et al. 2000).

Partitioning between desaturation and hydroxylation activities has been achieved by exchanging the identity of amino acids at four key locations within the *A. thaliana* FAD2 and the *L. fendleri* hydroxylase/desaturase (Broun et al. 1998b). Broadwater and co-workers reported that four analogous substitutions in the FAD2 sequence by their equivalents from the castor oleate hydroxylase resulted in hydroxy fatty acid accumulation in *A.*

thaliana to the same levels as for the wild-type castor hydroxylase (Broadwater et al. 2002). Interestingly, control experiments showed that the wild-type *A. thaliana* FAD2 desaturase has inherent, low level, hydroxylation activity. Moreover, fatty acid desaturases from different kingdoms and with different regiospecificities exhibited similar intrinsic hydroxylase activity, underscoring the fundamental mechanistic similarities between desaturation and hydroxylation (Broadwater et al. 2002).

A cDNA (Cpal1) encoding a Δ12-epoxygenase that can catalyse the synthesis of 12,13-epoxy-18:1-Δ9 (C18:1E, vernolic acid) acid from linoleic acid has been isolated from *Crepis palaestina* (Asteraceae) (Lee et al. 1998; Singh et al. 2001). When the Cpal1 gene was expressed under the control of a napin promoter in *A. thaliana*, the seed lipids accumulated only low levels (6.2% of the total fatty acids) of C18:1E and also 12,13-epoxy-18:2-Δ9,Δ15 (C18:2E) acid. The level of oleic acid was increased significantly in these plants, whereas the levels of linoleic and linolenic acids were decreased indicating that endogenous Δ12-desaturation was greatly reduced. Coexpression of a Δ12-desaturase from *C. palaestina* in Cpal2 transgenic *Arabidopsis* returned the relative proportion of C18 seed fatty acids to normal levels and resulted in an almost twofold increase in total epoxy fatty acids (Singh et al. 2001). Seed oil of *Stokesia laevis* has been shown to contain 60-70% vernolic acid and an epoxygenase gene has been cloned from *S. laevis* and expressed in *Arabidopsis* (Hatanaka et al. 2004). The average content of vernolic acid in seeds of these transgenic *Arabidopsis* plants was 5.8 μg/mg dry weight (2.4% of total fatty acids on average) (Hatanaka et al. 2004).

In contrast to *C. palaestina*, results from metabolic experiments suggested the involvement of a cytochrome P450 enzyme in vernolic acid synthesis in seeds of the Euphorbiaceae species *Euphorbia lagascae* (Bafor et al. 1993). An expressed sequence tag strategy has been successfully used by Cahoon and co-workers to identify a cytochrome P450 cDNA (designated *CYP726A1*) that corresponded to a gene that was highly expressed in *E. lagascae* seeds (Cahoon et al. 2002). Expression of this cDNA in tobacco callus and somatic soybean embryos resulted in the production of Δ12-epoxy fatty acids (C18:1E and C18:2E) at up to 15% and 8% (w/w) of the total fatty acids of these transgenic tissues, respectively (Cahoon et al. 2002).

The results described above illustrate opportunities for the production of industrially-valuable epoxy fatty acids in transgenic oilseeds. Furthermore, applications of acids such as vernolic acid as plasticizers of polyvinyl chloride, adhesives, coating materials and a precursor of monomeric components of nylon-11 and nylon-12 have already been demonstrated (Cahoon et al. 2002).

Vegetable oils that contain fatty acids with conjugated double bonds, such as tung oil, are valuable drying agents in paints, varnishes and inks. Cahoon and co-workers first demonstrated the ability of transgenic plants (e.g. somatic soybean embryos) to produce fatty acid components of these oils when they expressed conjugase cDNAs isolated from developing seeds of *Momordica charantia* and *Impatiens balsamina*. These species accumulate large amounts (as much as 17% w/w of the total fatty acids) of α-eleostearic (C18:3-Δ9*cis*, Δ11*trans*, Δ13*trans*) and α-parinaric acids (C18:4-Δ9*cis*,Δ11*trans*,Δ13*trans*, Δ15*cis*), respectively (Cahoon et al. 1999). A class of FAD-related enzymes that modified Δ9-double bonds to produce the conjugated *trans*, *trans*-Δ10-double bonds found in calendic acid (C18:3-Δ8*trans*, Δ10*trans,* Δ12*cis*) from the seed oil of *Calendula officinalis* have been studied by Cahoon et al. (2001). In somatic soybean embryos expressing these genes, calendic acid accumulated at up to 22% (w/w) of the total fatty acids.

A gene encoding a bifunctional fatty acid Δ12 conjugase/desaturase has been cloned from the tung (*Aleurites fordii*) tree (Dyer et al. 2002). The cDNAs that encoded a class of conjugases, associated with the formation of *trans*-Δ11, *cis*-Δ13 double bonds, have also been isolated from *Trichosanthes kirilowii* and *Punica granatum* (Hornung et al. 2002; Iwabuchi et al. 2003). Expression of these genes in *Arabidopsis* seeds under transcriptional control of a napin promoter showed the accumulation of punicic acid (C18:3-Δ9*cis*, Δ11*trans*, Δ13*cis*) at up to 10% (w/w) of the total seed oil (Iwabuchi et al. 2003). It is interesting, that the conjugase was also found to be bifunctional and exhibited Δ12-oleate desaturase activity (Iwabuchi et al. 2003).

Cahoon and Kinney used an expressed sequence tag (EST) analysis of developing *Dimorphotheca sinuate* seeds to provide direct evidence for the biosynthetic origin of dimorphecolic acid (9-OH-C18:2-Δ10*trans*, Δ12*trans*) which is unusual in containing a C-9 hydroxyl group, Δ10,Δ12-conjugated double bond and *trans*-Δ12 unsaturation (Cahoon and Kinney 2004). EST analysis revealed the occurrence of two structurally divergent forms of FAD2 in *D. sinuate* seeds that were designated DsFAD2-1 and DsFAD2-2. Expression of DsFAD2-1 in soybean somatic embryos resulted in the accumulation of the *trans*-Δ12 isomer of linoleic acid (C18:2-Δ9*cis*,Δ12*trans*) rather than the more typical *cis*-Δ12 isomer. When coexpressed with DsFAD2-1 in soybean embryos, DsFAD2-2 converted C18:2-Δ9*cis*,Δ12*trans* into dimorphecolic acid. When DsFAD2-2 was expressed alone in soybean embryos or together with a typical *cis*-Δ12-oleic acid desaturase in yeast, trace amounts of the *cis*-Δ12-isomer of dimorphecolic acid were formed from *cis*-Δ12-linoleic acid (Cahoon and Kinney 2004).

Recently, production of *trans*-10, *cis*-12 conjugated linoleic acid has been reported in tobacco seeds and rice after transformation with the linoleate isomerase gene from *Propionibacterium acnes* (Hornung et al. 2005; Kohno-Murase et al. 2006). Although the amount of this conjugated acid was relatively low (e.g. up to 1.3% w/w of the total fatty acids in the seeds of transgenic rice), these results demonstrated the potential ability of this simple genetic transformation for the production of such a conjugated fatty acid which has been shown to have a number of biological effects (e.g. anti-carcinogenic and anti-atherosclerosis) (Kohno-Murase et al. 2006).

In *Crepis rubra*, oleate is a substrate in the synthesis of the acetylenic acid, 9-octadecen-12-ynoic acid (crepenynic acid). Lee et al. (1998) characterized an enzyme involved in the synthesis of this acid in *C. alpina* (Lee et al. 1998). When the acetylenase gene was expressed in *Arabidopsis*, total fatty acids from seeds of individual T_0 transgenic plants contained up to 25% (w/w) crepenynic acid in contrast to control plants where it was not found.

18:0-Δ9-ACP-desaturase

This enzyme catalyzes the first desaturation step in plant lipid and seed oil biosynthesis, converting stearoyl-ACP to oleoyl-ACP (Fig. 9.1). Seed-specific antisense gene constructs of *B. rapa* stearoyl-ACP desaturase were used to reduce the protein concentration and enzyme activity of steroyl-ACP desaturase in developing rapeseed embryos during storage lipid biosynthesis (Knutzon et al. 1992). The resulting transgenic plants showed dramatically increased stearate levels (up to 45%) in the seeds of transgenic *B. napus* plants (Knutzon et al. 1992). The similar technique of stearoyl-ACP downregulation has been applied to soybean to increase its stearic acid content (Kinney 1998a,b). It is interesting that, when soybean germplasm with increased stearic acid content was sexually crossed to the FAD2-1 downregulated high oleic acid germplasm, the resultant progeny produced an oil with stearic acid and oleic acid at 30% and 60%, respectively (Kinney 1996).

16:0-Δ4-ACP-desaturase

Cahoon and co-workers expressed a cDNA encoding a putative acyl-ACP desaturase from coriander into tobacco by *Agrobacterium tumifaciens*-mediated transformation (Cahoon et al. 1992). Accumulation of petroselinic acid (18:1-Δ6*cis*) and Δ4-hexadecenoic acid, both of which were absent from control callus, was observed. Later, these workers provided metabolic evidence for the involvement of this 16:0-Δ4-ACP-desaturase in

normal petroselinic acid synthesis by coriander endosperm and also by transgenic tobacco cells (Cahoon and Ohlrogge 1994).

A desaturase with 83% sequence identity to coriander 16:0-Δ4-ACP-desaturase was isolated from developing seeds of *Hedera helix* (English ivy) (Whittle et al. 2005). Expression of the ivy desaturase in *Arabidopsis* resulted in the accumulation of C16:1-Δ4 and its expected elongation product C18:1-Δ6 (petroselinic acid). In vitro desaturation reactions also revealed that 16:1-Δ9-ACP and C18:1-Δ9-ACP can be further metabolised to 16:2-Δ4,Δ9 and C18:2-Δ4,Δ9, respectively, by the ivy desaturase showing a capacity of this enzyme to perform two desaturations on the saturated and monounsaturated substrates. This is a new finding for the soluble class of desaturases (Whittle et al. 2005).

20:0-Δ5-desaturase

The cDNAs for enzymes involved in the biosynthesis of C20:1-Δ5 have been identified from developing *Limnanthes douglassii* seeds (Cahoon et al. 2000). Expression of a cDNA for the *L. douglassii* acyl-CoA desaturase homolog in somatic soybean embryos, behind a strong seed-specific promoter, resulted in the accumulation of Δ5-hexadecenoic acid in amounts of 2% to 3% (w/w) of the total fatty acids of single embryos. It is interesting to note that the authors also coexpressed cDNAs for *L. douglassii* acyl-CoA desaturase and FAE1 in order to partially reconstitute the biosynthetic pathway of C20:1-Δ5 in transgenic plant tissue. These transformations resulted in production of C20:1-Δ5 and Δ5-docosenoic acid which comprised up to 12% of the total fatty acids in transgenic embryos (Cahoon et al. 2000).

In the further study, a *L. douglassii* seed-specific cDNA (Lim Des5) encoding a homolog of acyl-CoA desaturase found in animals, fungi and cyanobacteria was expressed in *B. carinata*, which resulted in the accumulation of C22:2-Δ5,Δ13 acid (up to 10%) in the seed oil (Jadhav et al. 2005). In conclusion, these results demonstrated the potential utility of soybean and *B. carinata* for the production of vegetable oils containing novel C20 and C22 fatty acids, and confirmed the preferred substrates of the Lim Des5 as C20:0 and C22:1-Δ13 (Jadhav et al. 2005).

Expression of a Δ5-desaturase cDNA from a filamentous fungus *Mortierella alpina* in transgenic canola seeds resulted in the production of taxoleic acid (C18:2-Δ5,Δ9) and pinolenic acid (C18:3-Δ5,Δ9,Δ12) in amounts ranging from 0.9 to 6.2% of the total fatty acids in the seeds (Knutzon et al. 1998).

Δ6-desaturase

To enable the production of γ-linolenic acid (C18:3-Δ6,Δ9,Δ12; GLA) in conventional oilseeds, Sayanova and co-workers isolated a cDNA encoding the Δ6-fatty acid desaturase from developing seeds of borage (*Borago officinalis*) (Sayanova et al. 1997). Functional expression of this cDNA in transgenic tobacco plants showed the accumulation of GLA and octadecatetraenoic acid (C18:4-Δ6,Δ9,Δ12,Δ15) at levels of 13.2% and 9.6% of the total fatty acids, respectively. The borage Δ6-fatty acid desaturase differs from other desaturase enzymes by the presence of an N-terminal domain related to cytochrome b_5 (Sayanova et al. 1997). It is interesting that Δ6-unsaturated fatty acids were found in both plastidic and microsomal lipids and positional analysis revealed that these fatty acids accumulated predominantly at the *sn*-2 position of the glycerolipids unlike other unusual plant fatty acids (Sayanova et al. 1999). As the borage Δ6-desaturase is most probably located in the endoplasmic reticulum (ER), a possible import of Δ6-unsaturated fatty acids into plastids after desaturation in the ER was suggested (Sayanova et al. 1999).

Expression of a Δ6-desaturase from the oleaginous fungus, *Pythium irregulare*, in *Brassica juncea* (under the control of a napin promoter) resulted in production of three Δ6 unsaturated fatty acids (C18:2-Δ6,Δ9; C18:3-Δ6,Δ9,Δ12; and C18:4-Δ6,Δ9,Δ12,Δ15) in seeds (Hong et al. 2002). Among them, GLA was the most abundant and accounted for up to 40% of the total seed fatty acids. It has also been shown that GLA was incorporated into the TAG fraction (98.5%) with only trace amounts found in the other lipids (Hong et al. 2002). In another study, introduction of borage Δ6-desaturase into flax (*Linum usitatissimum*), under the control of a constitutive (35S) promoter, showed the accumulation of two Δ6-unsaturated fatty acids, C18:3-Δ6,Δ9,Δ12; and C18:4-Δ6,Δ9,Δ12,Δ15 (Qiu et al. 2002). The level of these fatty acids was up to 22% of the total fatty acids in the stem, 19% in the root and 11% in the leaf. Introduction of this desaturase in *B. juncea* under the control of a napin promoter resulted in synthesis of Δ6-fatty acids at levels of up to 13% of the total fatty acids in mature seeds (Qiu et al. 2002).

An increase in GLA and octadecatetraenoic acid in leaf tissues of a commercial variety of evening primrose (*Oenothera* sp.) has been achieved through a robust *Agrobacterium*-mediated transformation procedure to deliver a cDNA encoding a Δ6-desaturase from borage (De Gyves et al. 2004).

As an alternative to fish oil, oilseed plants have been also considered as a potential source of very-long chain polyunsaturated fatty acids (VLCPUFAs) if they are gene engineered (Alonso and Maroto 2002; Drexler et al. 2003; Abbadi et al. 2004). The accumulation of substantial amounts of

arachidonic and eicosapentaenoic acids has been achieved in *Arabidopsis thaliana* (Qi et al. 2004). This involved the use of genes encoding enzymes of the ω3/6-Δ8-desaturation biosynthetic pathways for the formation of C20 PUFAs. *A. thaliana* was transformed sequentially with genes encoding a Δ9-specific elongation activity from *Isochrysis galbana*, a Δ8-desaturase from *Euglena gracilis* and a Δ5-desaturase from *Mortierella alpina* (Qi et al. 2004). It was suggested that *I. galbana* C18-Δ9-elongation activity was important in the successful reconstitution of this pathway since it may bypass rate-limiting steps present in the conventional Δ6-desaturase/elongase pathway (Qi et al. 2004).

In transgenic tobacco and linseed, high accumulation of Δ6-desaturated C18 fatty acids and up to 5% of C20 PUFAs, including arachidonic and eicosapentaenoic acids, has been reported as a result of heterologous expression of three genes encoding a Δ6-desaturase, a Δ6-elongase and Δ5-desaturase (Abbadi et al. 2004).

An interesting aspect of the modulation of fatty acid desaturation has been suggested by Zhang and co-workers in causing an increased tolerance to various abiotic stresses in transgenic tobacco cells and plants (Zhang et al. 2005). They presented evidence that overexpression of either FAD3 or FAD8 led to increased tolerance to drought in tobacco plants and to osmotic stress in cultured cells. In both cultured cells and whole plants, much greater heat sensitivity was noted when the tissues were overexpressed with FAD8 rather than with FAD3 (Zhang et al. 2005).

4. Complex lipid manipulation

In spite of significant progress in genetic engineering of fatty acid synthesis in oilseeds to meet the increasing demand of industry, the high levels of these fatty acids which are necessary for industrial applications have not been achieved very often. The highest content of a potentially useful industrial oil (with the exception of lauric acid) accumulated in the seed oil of transgenic plants is 25% (Jaworski and Cahoon 2003). Taking into account a need for purification of the oils which involves some additional expense, levels of unusual fatty acids at up to 90-95% of total seed fatty acids have been suggested as desirable.

Analysis of the fractional distribution of unusual fatty acids in seed oils showed that they are located almost exclusively in TAGs. The main pathway for the synthesis of TAGs is the Kennedy pathway (Fig. 9.2) where the first two reactions are the formation of phosphatidic acid by stepwise acylation of glycerol 3-phosphate. It is known now that these reactions are

catalysed by two distinct enzymes specific for positions 1 and 2. Membrane-bound glycerol 3-phosphate acyltransferase (GPAT) initiates the process by transferring the acyl chain from CoA to the sn-1 position of glycerol-3-phosphate with the formation of lysophosphatidic acid (Voelker and Kinney 2001). No gene has been identified in plants for the membrane-bound form of GPAT, which is believed to have a low selectivity for different acyl chains. (The soluble chloroplast form of GPAT has, however, been well studied (Frentzen and Wolter 1998). The transfer of acyl chains from acyl-CoAs to the sn-2 position to form phosphatidic acid, is catalyzed by lysophosphatidic acid acyltransferase (LPAAT) which, in plants, prefers unsaturated acyl chains (Voelker and Kinney 2001). The phosphatidic acid is then dephosphorylated to produce diacylglycerol (DAG). The final step in the pathway is the addition of a final fatty-acyl group to the sn-3 position of DAG to produce TAG (Fig. 9.2). It is catalyzed by diacylglycerol acyltransferase (DGAT), an enzyme unique to TAG biosynthesis. In plants, two unrelated genes have been shown to potentially encode DGAT enzymes. One form is related to acyl-CoA:cholesterol acyltransferase, whereas a second form does not resemble any other known genes (see Voelker and Kinney 2001).

Recent studies provide evidence for alternative reactions for TAG synthesis in plants. In one of these reactions, a fatty acid residue is directly transferred from the sn-2 position of phosphatidylcholine (PC) to diacylglycerol forming lyso-PC and TAG (Stobart et al. 1997). There is also a reaction involving acyl transfer between two molecules of DAG (see Weselake 2005). Each of above-mentioned reactions in the TAG biosynthetic pathway may be a limiting step for the incorporation of fatty acids into TAG. Thus, in addition to the enzymes necessary for the synthesis of an unusual fatty acid, it may be necessary to introduce multiple genes to properly channel the fatty acids into TAG (Jaworski and Cahoon 2003).

In order to increase the erucic acid content in rapeseed oil, the cDNA encoding LPAAT from developing seeds of meadowfoam (*Limnanthes alba alba*) was expressed in developing seeds of transgenic high-erucic-acid rapeseed (Lassner et al. 1995). Although the total erucic acid content did not change, it was present at the sn-2 position of TAGs in transgenic plants in contrast to control plants, and trierucin was accumulated in the seed oil from modified plants (Lassner et al. 1995). Similar results were obtained when a cDNA encoding a 1-acyl-sn-3-phosphate acyltransferase (LPAAT) from *Limnanthes douglasii* was introduced into oilseed rape (Brough et al. 1996). Moreover, when a coconut (*Cocos nucifera*) LPAAT (preferring 12:0-CoA) was coexpressed with a 12:0-ACP thioesterase from California bay in developing seeds of oilseed rape, efficient laurate deposition

at the sn-2 position of TAGs and trilaurin accumulation were found (Knutzon et al. 1999).

Zou et al. (1997) transformed the model oilseed arabidopsis and a high-erucic acid cultivar of *Brassica napus* with the yeast sn-2 acyltransferase gene. They reported substantial increases in seed oil content in transgenic plants as well as increases in both the overall proportions and the amounts of VLCFAs in seed TAGs. The proportion of these acids at the sn-2 position of TAGs was also increased in transgenic plants (Zou et al. 1997). Enhancement of seed oil content has been achieved in *A. thaliana* when transformed with a plastidial safflower glycerol 3-phosphate acyltransferase (GPAT) and an *Escherichia coli* GPAT (Jain et al. 2000).

The potential application of acyl-CoA:diacylglycerol acyltransferase-transformed plants has been demonstrated when transformation of tobacco with the DGAT gene isolated from *A. thaliana* was performed (Bouvier-Nave et al. 2000). In several primary transformants, a marked increase of TAG content, which correlated with the DGAT mRNA expression, has been observed (Bouvier-Nave et al. 2000). Moreover, it was shown that seed-specific overexpression of the DGAT cDNA in wild-type arabidopsis enhanced oil deposition and average seed weight which were correlated with DGAT transcript levels (Jako et al. 2001). This study confirmed the important role of DGAT in regulating the quantity of seed TAGs (Jako et al. 2001).

5. Summary remarks

While there is clearly enormous potential for the production of important foodstuffs and renewable chemical sources by use of genetic manipulation of plants, there has been little realisation of this potential so far. A notable exception is laurate canola. Two major problems have been encountered. First, achievement of high levels of the desired fatty acids in TAG has often been limited by the substrate selectivity of endogenous enzymes. This means that, frequently, it appears necessary to introduce novel acyltransferases in order to reduce constraints encountered in the Kennedy pathway. Second, when trying to produce products such as VLCPUFAs, there is an inherent problem in that the desaturation and elongation reactions use different types of substrates and it is constantly necessary to swap the acyl groups between these during synthesis. Such additional reactions severely limit the efficiency of the syntheses achieved so far.

There are two further problems. One of these is our lack of information about control mechanisms for lipid synthesis in different plants (Ohlrogge

and Jaworski 1997). In the first attempts to address this deficiency, flux control analysis is being used to yield quantitative information about the regulation of lipid accumulation (e.g. Ramli et al. 2002; 2005). Such experiments are very necessary in order to identify which enzymes may be worth manipulating in order to achieve increased oil yields.

A second problem is that of consumer resistance to the use of genetically-modified crops. At present such problems are more serious in Europe then many other parts of the World. Nevertheless, they have had a major effect on the development of new crops. However, for the production of new crops for industrial use there is somewhat less public disquiet and it is probable that advances in the production of renewable resources for chemicals are likely to be very significant in the near future.

Abbreviations used

ACC, acetyl-CoA carboxylase
ACP, acyl carrier protein
BTE, fatty acyl thioesterase type B (FatB c.f. FatA)
DAG, diacylglycerol
DGAT, diacylglycerol acyltransferase
E, fatty acid elongase
FAD, fatty acid desaturase
FAE, fatty acid elongase
FAR, fatty acyl-CoA reductase
FAS, fatty acid synthase
GLA, gamma-linolenic acid
GPAT, glycerol 3-phosphate acyltransferase
HEAR, high erucic acid rape
KAS, β-ketoacyl-ACP synthase
KCS, β-ketoacyl-CoA synthase
LPAAT, lysophosphatidic acid acyltransferase
PC, phosphatidylcholine
PUFA, polyunsaturated fatty acid
TAG, triacylglycerol
VLCFA, very long chain (>18C) fatty acid

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