Chapter 1

Genetic Modification of Seed Oils for Industrial Applications

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Introduction

While most vegetable oils are produced for food and feed uses, up to 15% of soy (as well as other food oils) and up to 100% of certain commodity oils are used for industrial purposes. Most food oils, such as soybean or canola, are composed primarily of five fatty acids (FA): palmitic, stearic, oleic, linoleic, and linolenic; these oils are used to produce surfactants, lubricants, inks, coatings, and polymers. Commodity oils containing uncommon FA, such as castor (90% 12-hydroxyoleate) and tung (up to 80% conjugated FA), have no nutritive value, but due to the unusual properties of the FA, they prove very useful for industrial applications. It is the chemical functionality of a vegetable oil that can make it useful to industry; chemical functionality can alter physical properties or allow chemical precursors or useful derivatives to be made. For example, ricinoleate, the FA from castor oil, has a mid-chain hydroxyl group that enhances its viscous properties for use as grease and also enables production of an extensive range of chemical derivatives (1). Coconut oil contains laurate (12:0) which has excellent foaming properties and is used to make anionic surfactants. Hydroformylation of petroleum provides an equivalent surfactant (2). The possibility of replacing such petroleum products with plant-derived FA is a major goal of seed oil utilization research.

There are hundreds of FA with unusual functionalities, at least some of which would have immediate application if readily available from a suitable crop. To the extent that uncommon FA are produced in a given plant, these are a result of evolution, perhaps providing selective advantage as a result of toxic or other protective effects of the FA on pathogens. Though it operates on a long time scale, evolution has provided an unusual array of genetic material for production of useful FA. However, many of these FA are produced in plants that are unsuitable as crops.

Traditional breeding techniques can alter levels of FA present in the oil and, with suitable germ plasm, can reduce or eliminate one or more of the FA normally present, as was the case in the development of canola (low-erucic acid rapeseed) (3,4). Breeding has been used to develop plant selections with a high proportion of a single component, e.g., such as high oleic safflower. High enrichment of a single component such as oleate represents another industrially useful feature, as it

reduces the expense of purifying the desired component. But breeding cannot be used to introduce a FA not already present in one of the crossed plants. Random mutagenesis using chemical or radiation agents to alter the genome followed by screening and breeding has also produced varieties with altered FA composition in oil (5). Genetic identification and chemical characterization of FA biosynthetic mutants in mutated *Arabidopsis thaliana* has provided an extensive genetic map of FA and lipid biosynthetic steps during plant growth and development (6), in many cases providing null mutants lacking a specific enzymatic activity. Since the mutagenic approach is geared toward eliminating genes, this approach has been used as part of breeding programs to reduce levels of undesirable FA components such as high polyunsaturates from linseed oil (7) or to increase levels of a desired FA, e.g., oleate in sunflower by eliminating the enzyme that normally converts it to linoleate (8). A recent innovation in this approach is TILLING (Targeting Induced Local Lesions IN Genomes), which uses a mutagenic approach, but introduces high-throughput screening of the M_2 generation (the second generation of self-pollinated, mutated lines) in order to identify specific genes that have been altered or inactivated by mutagenic events (9). Plant selections carrying these mutated genes can then be screened directly for desired characteristics. The TILLING process thus moves most of the screening effort into the laboratory, considerably reducing the population that would otherwise have to be grown in the field for phenotypic screening.

With the advent of genetic engineering, the technology needed to introduce novel traits became available to breeders. A driving force behind development of genetically engineered oils is the perennial surplus of oils produced. The unused inventory of soybean oil may reach nearly two billion pounds in any year. Crops with altered oil composition hold the promise of reducing or preventing annual inventory carryover, thus stabilizing or improving farm income. This chapter will explain the biochemistry underlying the alteration of FA composition, briefly describe some oils that have been developed through genetic engineering and mention some of the "target" FA of interest for production in transgenic oilseed crops.

FA Biosynthesis

FA biosynthesis in plants proceeds from acetylCoA, which initiates a set of condensation reactions with malonyl-ACP through six or seven additional condensations with malonyl-ACP. This yields the saturated FA palmitate or stearate, respectively, as depicted in Figure 1.1, which depicts the pathway of FA biosynthesis to linoleic acid, with the reactions leading to palmitate, stearate, and oleate occurring in the plastid, separate from reactions leading to oil biosynthesis. Given the dependence of FA production on malonyl-CoA production (to provide malonyl-ACP), the acetyl-CoA carboxylase (ACCase) is generally thought to play a regulatory role in FA production and oil biosynthesis (10). This hypothesis is supported by research in which ACCase from *Arabidopsis* was overexpressed in potato, leading to an increase in FA production and a fivefold increase in triacylglycerol levels in the tuber (11).

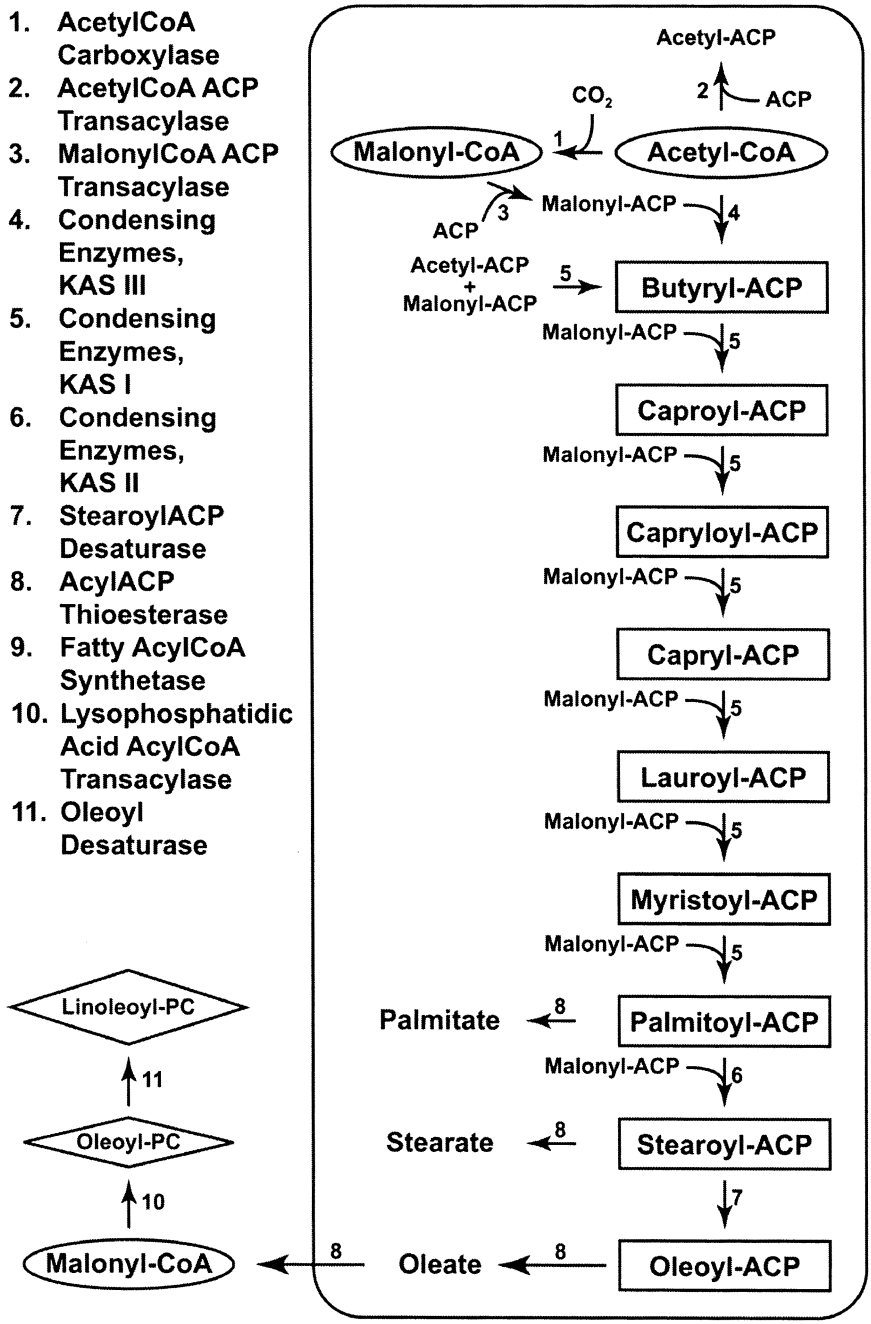

Fig. 1.1. The pathway of fatty acid biosynthesis to linoleic acid.

Medium Chain-Length FA Biosynthesis

In seeds of certain plants such as coconut, palm kernel, bay laurel, and cuphea, the flow of carbon to the long-chain saturated FA is disrupted, and this occurs as the result of an acyl-ACP thioesterase (product of the FAT B gene), which removes the ACP from the elongating FA chain prior to achieving full length. This produces a medium chain-length FA which is transported from the plastid and enters the oil biosynthetic pathway. This approach copied from nature led to the development of the first transgenic oilseed modified to produce an industrial oil product, namely Laurate Canola (12). By inserting into Canola the cDNA for a medium-chain specific acyl-ACP thioesterase (13) from California bay laurel, a plant which produces seeds containing >60% laurate(dodecanoate) in its oil, plastidial FA synthesis was diverted to the production of laurate, which was incorporated into the seed oil (14). Although this achievement was a key early success in the contribution of genetic engineering to agriculture, the underlying science also pointed to a number of technical problems that have since been widely recognized. The production of a FA not normally produced by the seed may trigger a "counter-reaction." In the case of laurate, considerable amounts of the laurate were β-oxidized, since the cytoplasmic lauroyl-CoA used to acylate glycerolipid is also an intermediate in β-oxidation (15,16). While increased carbon flux through the FA biosynthetic pathway enhanced laurate production, the overall outcome was a canola cultivar with reduced oil yield, since some of the carbon incorporated into laurate production was oxidized through the futile cycle.

The laurate canola oil produced also lacked laurate in the *sn*-2 position of the triacylglycerol (TAG) (17). The canola seed lacked a lyso-phosphatidic acid acyltransferase (LPAAT) that could use lauroyl-CoA as an acyl donor for the *sn*-2 position of glycerolipid. Researchers at Calgene solved this problem by crossing a canola plant containing an LPAAT gene from coconut (17), with a laurate canola plant (18). The resulting plant produced an oilseed in which laurate is distributed among all three positions of the TG. The resulting "High-Laurate Canola" had a laurate content of up to 70%. The successful design of a novel, temperate-climate industrial crop provided a great impetus to follow this approach for other industrially useful products, especially oils. It also provided a foreshadowing of the difficulties to be encountered in engineering production of uncommon FA in oilseeds.

Monounsaturated FA Biosynthesis

In general, once saturated FA are released from acyl-ACP, they are incorporated into oil without any apparent modification except, to a minor extent, elongation. In the plastid, though, the saturated fatty acyl-ACP can be desaturated by the Δ9-desaturase, a class of soluble enzymes (as opposed to membrane-bound) formerly identified as the stearoyl-ACP desaturase, which is the type present in most oilseeds. These enzymes share a considerable degree of amino acid sequence homology and the same type of active site in which the desaturation is carried out.

In most plants, the Δ9-desaturase produces oleate which, for purposes of oil biosynthesis, is transported from the plastid to the endoplasmic reticulum and incorporated into CoA, phospholipid, and acylipid. Oils high in oleic acid content have been considered desirable both for food and nonfood uses. A high-oleate soybean oil containing greater than 80% oleic acid was developed by suppressing expression of the desaturase enzyme that converts oleate to linoleate in soybean. This oilseed has been commercialized and for industrial purposes find applications as a stable, biodegradeable hydraulic oil and is likely useful for developing other bio-based lubricant applications (19).

Some plants produce monounsaturated FA of differing chain-length or with the double-bond in a different position on the carbon chain, or both. Many such desaturases have been cloned, the crystal structure of the soluble desaturase from castor (*Ricinus communis*) has been determined, and considerable insight on factors involved in chain-length and positional specificity of the desaturase reaction have been revealed (20,21). The ability to engineer this type of enzyme to introduce a *cis*-double bond at a specific position on a selected chain-length represents a bench chemist's dream for saturated hydrocarbon chemistry. However, despite the apparent similarity of some products to oleate, e.g., 18:1 Δ6 (petroselenate), their production can differ from that of oleate, resulting in limited amounts of the product when introduced into a transgenic plant (10). It has been shown that, in some cases, co-factors such as ferredoxin and ACP isoforms that interact specifically with the enzyme are required. Moreover, the FA may also require altered lipid metabolism to be suitably incorporated into TAG (10). Thus, further understanding of lipid biochemistry leading to TAG production will underly successful attempts to engineer oil composition.

Modification of Oleate

In most temperate climate oilseeds, the oleate may be further desaturated to linoleate and α-linolenate. In rapeseed, crambe and na-sturtium, the oleate may be elongated to erucic acid by the action of an acylCoA based elongation reaction, mediated in part and possibly regulated by expression of a keto-acyl synthase (KAS) specific to elongation of long-chain FA. The products of elongation, usually 20:1 Δ11 and 22:1 Δ13 are incorporated into the TAG fraction (oil). In some plants, the oleate is oxidized to uncommon FA. For example, in *Vernonia*, 18:1 Δ9, 12-13 epoxy (vernolate) is formed and then incorporated into TAG (21,22). The possibilities resulting from oleate production provided the basis for the original concept of oleate as the central substrate in plant FA biosynthesis (23). The set of modification reactions that can alter oleate is unusual, in that it comprises a family of homologous enzymes that have evolved from the FAD2 genes, which encode the oleoyl desaturase in oilseeds. Enzymes that have evolved from the FAD2 have been found to carry out an unusual array of conversions, using an oleoyl-phosphocholine (oleoylPC)-based substrate. These reactions include hydroxylation, epoxi-

dation, desaturation-conjugation and desaturation to a triple bond (22,24,25). In the case of hydroxylation and desaturation, changes in as few as 4–6 amino acid residues result in an interchange of the two types of activity (24,26). In fact, the oleate 12-hydroxylase from *Lesquerella* has mixed functionality, and can introduce a hydroxyl group or double bond (27). Interestingly, as with some other uncommon FA described in previous sections, introduction of genes with suitable production of these uncommon FA in the oil of a transgenic plant has also proven difficult. The next section will elaborate on this theme by describing the biochemistry of castor oil, an important commodity oil with numerous applications (1).

Castor Oil Biosynthesis

Castor oil is a product of great interest to plant lipid scientists. It is an established commercial product with a significant market and a cost of 45–50 cents per pound versus soybean oil at 15–25 cents, yet is entirely imported by most industrialized nations. Because castor seed contains noxious proteins, it is problematic as a crop. Therefore, producing castor oil transgenically represents an enticing target and a long-term challenge. Understanding the basis for the regulation of seed oil yield is also a major research goal and castor, at 60% oil, has served as a benchmark for high oil content.

Interest in castor oil biochemistry precedes the genetic engineering revolution. In the 1960s, both the Stumpf research group at University of California, Davis, and the Morris group at Unilever Research in Great Britain, carried out basic research investigating the hydroxylation reaction that converts oleate to ricinoleate (28,29). These early biochemical developments were followed by the research groups of Stymne at Uppsala and Somerville and colleagues from MSU. These groups contributed greatly to current understanding of ricinoleate production, and the latter two groups elucidated the genetic basis for castor oil production by identifying and cloning two of the key genes (30,31)

The oleoyl-12-hydroxylase enzyme proved challenging to purify (32–36). Although the enzyme has not been purified to date, the cDNA for its gene was cloned by a genomics approach (30). Based on the hypothesis that the hydroxylation reaction is analogous to, or the first step in, the desaturation reaction, this research group proposed that the hydroxylase would share sequence elements in common with FA desaturases. Using this approach, hundreds of cDNAs from developing castor seed were sequenced, prospective hydroxylase cDNAs expressed in tobacco seed, and the seed oil assayed for hydroxy FA. Although ricinoleate production was low, 0.1%, it was sufficient to show that the hydroxylase had been cloned and successfully expressed in a transgenic plant. However, to date, oilseeds transformed to express the gene for oleoyl-12-hydroxylase produce much less than the 90% present in castor oil, with most transgenes producing less than 20% hydroxy FA content in oil (37). It has been hypothesized that the ricinoleate incorporated in lipid inhibits membrane function in most plants, so it may

be eliminated from the membrane by endogenous phospholipases (38) and beta-oxidized (39) by analogy to laurate. On the other hand, castor has evolved biochemically to produce and incorporate ricinoleate into oil. This led to the approach of identifying additional enzyme components in castor that enable it to produce an oil with 90% ricinoleate. Based on a considerable body of research (31,33,38,40–43), a number of enzymes have been identified that appear to be involved in high ricinoleate production, ricinoleate incorporation into oil, or maximizing oleate conversion to ricinoleate (44). The latter role is clearly fundamental, since the final content of oleate in castor oil is less than 4%, and the castor oil biosynthetic pathway is 96% efficient in converting oleate.

This research has been aided by development of methods for "metabolic profiling" castor oil biosynthesis. In an effort to develop an alternative oilseed that could produce castor oil, a microsomal system that carries out the biosynthesis of castor oil in microsomes prepared from immature castor seed endosperm and embryo has been developed (36,42). The microsomal system is effective in synthesizing the TAG produced by the intact seed and provides a realistic model system for investigating castor oil biosynthesis. Using this system and analysis of lipid metabolites by high-performance liquid chromatography with selected columns and solvent conditions, intermediates that accumulate during castor oil biosynthesis can be separated and identified (44). This approach has enabled the identification of additional enzymes that provide the unique basis for biosynthesis of castor oil, since the gene for FA hydroxylation by itself is not sufficient to produce high levels of ricinoleate in other oilseeds (37). Based on these research results and other published research, the pathway in Figure 1.2 has been proposed.

The following narrative of the pathway summarizes these findings, with key reactions and their role described briefly:

(i) The lyso-phosphatidylcholine acyltransferase (LPCAT) transfers the oleoyl-moiety from oleoyl CoA into the *sn*-2 position of PC for hydroxylation.

(ii) The oleoyl-12-hydroxylase hydroxylates the *sn*-2 oleate to form *sn*-2 ricinoleoyl-PC.

(iii) The phospholipase A_2 preferentially removes ricinoleate from the *sn*-2 position of PC and releases lyso-PC for reincorporation of oleate by LPCAT.

(iv) The free ricinoleate is preferentially incorporated into ricinoleoyl-containing diacylglycerols by the diacylglycerol acyltransferase (DGAT) to form diricinoleins and triricinolein, which make up castor oil.

(v) The phospholipid-diacylglycerol acyltransferase (PDAT) incorporates the *sn*-2 ricinoleate directly from the ricinoleoyl-PC product of the hydroxylase reaction into the TAG end product.

The final step in oil biosynthesis (Fig. 1.2) shows a high degree of selectivity for incorporating ricinoleate preferentially. Based on *in vitro* results, both the DGAT and PDAT (45) appear to be active in carrying out the incorporation of ricinoleate

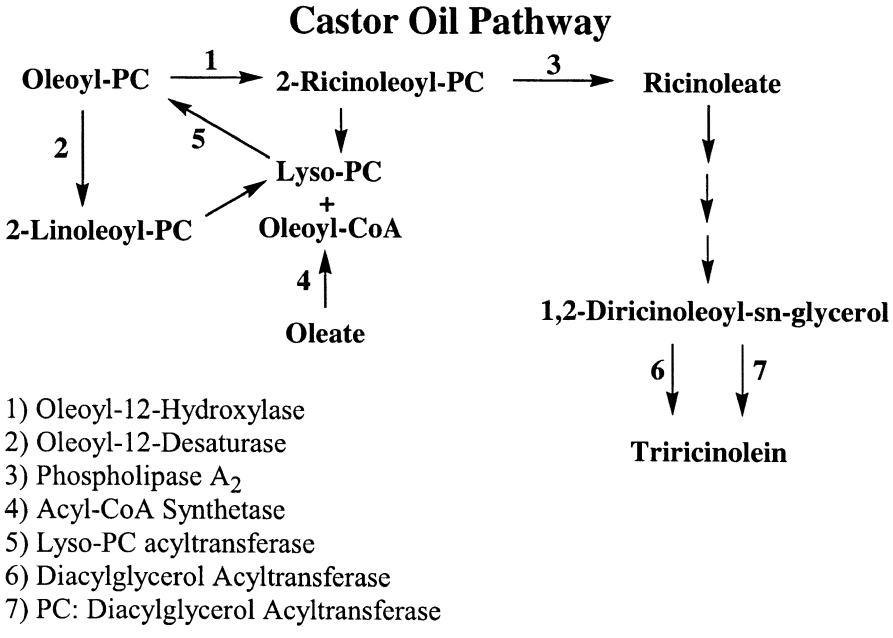

Fig. 1.2. Castor oil pathway.

into castor oil. The DGAT cloned from castor shows a preference for using diricinolein as a substrate in comparison to the DGAT from Arabidopsis, a plant that does not produce hydroxy FA in its seed oil (46).

New and Improved Crops

The production of industrially useful FA in transgenic crops is complicated by the need for greater understanding of how such FA are efficiently made in the plants that make them, and how their incorporation into oil is directed. Table 1.1 lists a number of FA and related products that are of interest to researchers seeking to expand the role of seed oils in the "hydrocarbon economy." The plants developed would be renewable resources, enhance opportunities for rural development, and contribute to the improvement of the environment. Current research efforts are on the appropriate control of gene expression, elucidating the synthesis of the FA, and controlling its "destiny"—assuring its incorporation in oil and preventing it from being further metabolized.

Another application of transgenic technology is the development of oilseeds with improved agronomic characteristics. In fact, this has been the primary goal of agricultural chemical producers that have initiated programs to produce GM crops. Currently, the four genetically engineered crops that have been adopted are all oilseed crops: soy, corn, cotton and canola. They account for 99% of transgenic crops planted

TABLE 1.1
Industrially Useful Fatty Acids for Transgenic Plant Production

Fatty acid	Functionality	Source	Use
Eleostearic Octadeca-9*c*,11*t*,13*t*-trienoic	Conjugated double bonds	Tung, bitter melon	Drying oil
Erucic Docosa-13*c*-enoic	Very long-chain (VLC)	Rapeseed, crambe	Lubricants, anti-slip agent
γ-Linolenic Octadeca-6*c*,9*c*,12*c*-trienoic	Polyunsaturate	Borage, blackberry	Nutraceutical
Caproic to Myristate 6 to 14 carbons	Medium chain-length	Cuphea, coconut, bay laurel	Detergents
Oleic Octadeca-9*c*-enoic	Monounsaturate	Many	Hydraulic oil, oleochemicals
Petroselenic Octadeca-6*c*-enoic	Monounsaturate isomer	Coriander	Nylon 6,6
Ricinoleic Octadeca-9*c*,12-OH-enoic	Hydroxylated	Castor	Lubricants, polymers
Vernolic Octadeca-9*c*, 12,13-O-enoic	Epoxy	*Vernonia, Euphorbia lagascae*	Coatings, plasticizer
Docosahexaenoic	VLC polyunsaturated	Algae	Nutraceutical
Nervonyl Erucate	VLC wax ester	Jojoba	High-temperature lubricant

worldwide. Over 70% of the soy grown in the U.S, 50% of the corn and 70% of the cotton are genetically engineered. Most of the canola grown in Canada, a leading producer, is transgenic. An increasing number of countries have adopted the technology. The U.S., Argentina, Canada, Brazil, China, and South Africa account for 99% of the transgenic crops produced, with an additional 12 countries adopting the technology (47). The growth in planting of transgenic crops is remarkable in that it has all occurred in the last eight years, from the time the first transgenic crops were introduced in 1996. At this time, each of these crops has been modified for "input" traits, reducing or eliminating the need for chemical applications by the introduction of genes encoding herbicide tolerance (soy, canola), insect resistance (corn, cotton), or both (cotton). As plant genomics and proteomics programs identify other agronomically useful genes, other transgenic traits will also be incorporated. These can range from elimination of noxious components (48) to introduction of dwarfing genes for greater plant efficiency. Small volume crops, such as papaya and squash, have already been genetically modified for viral resistance. Crop genetic engineering holds great promise as a means for developing oilseed crops with unique characteristics that add both commercial and nutritive value, increase utilization, and benefit the environment.

Summary

Oilseeds are an important source of chemicals for industry. Most temperate climate oilseeds produce oils containing the same five FA (palmitate, stearate, oleate, linoleate, and α-linolenate) in different proportions. In addition to nutritive uses, these FA are used to produce soaps and detergents, coatings, lubricants, cosmetics, plastics, plasticizers, and numerous chemical derivatives. For specific uses, certain FA are more desirable. For example, the conjugated double bond system present in FA of tung oil gives it excellent properties as a drying oil. Lauric acid from coconut provides a chemical feedstock for producing detergents. Laurate canola was the first commercial crop that was genetically designed to produce an industrial FA. The ability to manipulate FA composition in oilseeds resulted from a combination of three approaches. First, biochemical characterisation has identified most of the steps in FA biosynthesis. Secondly, genetic identification and chemical characterization of *Arabidopsis thaliana* mutants has provided an extensive genetic map of FA and lipid biosynthetic steps during plant growth and development. Finally, the additional information needed to broaden the spectrum of FA available from oilseeds has been provided by the identification, characterization, and cloning of unusual enzyme activities from plants that produce uncommon, industrially useful FA.

Hundreds of uncommon FA, with unusual chemical functionalities, are produced by one or more oilseed plants. A considerable amount of research has gone into elucidating the biosynthetic process by which such FA are made; much of the enzymology underlying the introduction of unsaturation, conjugated unsaturation, and hydroxyl, acetylenic, and epoxy functionality is now understood. As knowledge of the mechanistic and structural knowledge of these enzymes expands, there is potential for engineering production of FA that are not yet known. The specificity of the chemistry carried out on what is essentially a straight hydrocarbon chain is unprecedented for the bench chemist, and presents the possibility of "green" chemistry carried out in green plants to produce a wide array of chemicals designed for industrial applications.

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