

Modification of Plant Lipid Synthesis

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Genetic engineering of new storage oils and fats has produced oil crop plants with fatty acid compositions unattainable by plant breeding alone. The combination of classical breeding methods with molecular techniques provides new ways for designing oils for food and nonfood uses. Alterations in the position and number of double bonds, variation in fatty acid chain length, and the introduction of desired functional groups have already been achieved in model systems. Short-term prospects include crops such as rapeseed or soybean engineered to have greater than 70 to 80 percent medium-chain fatty acids by content, greater than 90 percent oleic acid, and high erucic acid content, and engineered to form ricinoleic acid in seed storage tissues.

Oils and fats, which chemically are glycerol esters of fatty acids [triacylglycerols (TAGs)], play a major role in human nutrition because of their high energy content. Ninety percent of the vegetable oil produced is used for human consumption, predominantly in margarines, shortenings, salad oils, and frying oils (1). Breeding efforts have traditionally focused on improving oils for these uses. Selected plants had considerably altered seed oil compositions, which indicated that plants could tolerate a wide variation in fatty acid composition of storage lipids (2, 3). Examples of progress through breeding efforts include the development of rapeseed with a low erucic acid content (canola), which has established rapeseed oil as a prime edible oil (4, 5), and the development of sunflower varieties with high oleic acid content whose seed oils have better frying properties (6). However, by classical breeding methods it was impossible to increase the oleic acid content of rapeseed above 80% without obtaining undesired agronomic properties such as reduced cold tolerance (7, 8).

Currently, only 10% of the vegetable oils produced are used in nonfood applications such as lubricants, hydraulic oil, biofuel, or oleochemicals for coatings, plasticizer, soaps, and detergents (Table 1) (2). The economic requirements for industrial raw materials are quite different from those for nutritional oils. The ideal oil for an oleochemical application would consist of a particular type of fatty acid that could be supplied constantly at a competitively low price as compared with raw materials based

on mineral oil products. Furthermore, such a fatty acid should have a reactive group in addition to the carboxyl function to provide an additional target for chemical modifications. It is expected that such fatty acids can be produced in plants (9). This challenge can best be met by using genetic engineering techniques.

The oils of the six major oil crops (soybean, oil palm, rapeseed, sunflower, cotton seed, and groundnut), which account for 84% of worldwide vegetative oil production (2), consist mainly of palmitic, stearic, oleic, linoleic, and linolenic acids (Fig. 1). The majority of the 210 known types of fatty acids synthesized by plants (9), however, are not available for economic uses because they occur in noncrop plants. But genetic engineering provides the means to tap this vast capacity for the production of specific oils in agriculturally and economically attractive crop plants. For both food and nonfood uses, transgenic and traditional breeding approaches have been used [for reviews see (3, 10–12)] to change the degree of desaturation and to reduce or increase the chain length of fatty acids (Fig. 1). Tissue-specific promoter elements from genes encoding seed storage proteins can be used to direct gene expression to the desired storage tissue, thus avoiding possible deleterious effects that could result from expression of the transgene throughout the plant. Moreover, progress has been such that field trials can be under way within 2 years after a gene has been isolated, thus substantially accelerating the breeding process with the insertion of transgenes into elite breeding lines.

Biosynthesis of Storage Lipids

In plants *de novo* fatty acid biosynthesis occurs exclusively in the stroma of plastids, whereas, with the exception of plastidial desaturation, modification of fatty acyl residues and TAG assembly are localized in

the cytoplasm (Fig. 2) [reviewed in (13–16)]. With a few exceptions such as palm kernel, coconut, or several *Cuphea* species which form fatty acids of C_8 to C_{14} carbon chain length, C_{16} and C_{18} acyl chains are the primary products of *de novo* fatty acid biosynthesis. The intermediate molecules $C_{16:0}$ -ACP, $C_{18:0}$ -ACP, and $\Delta 9C_{18:1}$ -ACP (where the number after the colon refers to the number of double bonds and ACP is acyl-carrier protein) are used in the plastidic pathway for membrane lipid synthesis or diverted to the cytoplasmic pathway after conversion to coenzyme A (CoA)-esters for the synthesis of membrane lipids or storage TAGs.

The first committed step in fatty acid biosynthesis in the plastid is the formation of malonyl-CoA from acetyl-CoA and HCO_3^- in an adenosine triphosphate (ATP)-dependent reaction catalyzed by acetyl-CoA carboxylase (ACCase, E.C. 6.4.1.2, Fig. 2) (13). ACCase is considered to be a rate-limiting step in fatty acid biosynthesis (17, 18). Complete coding sequences have been cloned from various plant species and may help provide more insight into the regulatory role of this enzyme (19–22).

Individual enzymes constituting the type II fatty acid synthase (FAS) consecutively add two carbon units derived from malonyl-CoA to a growing acyl chain that is bound to the small cofactor-like ACP. The first condensation of acetyl-CoA and malonyl-ACP is catalyzed by β -ketoacyl-ACP synthase III (KASIII) (23). In a series of seven condensation cycles a C_{16} acyl thioester, palmitoyl-ACP, is formed by the action of a second condensing enzyme (KASI) (24). In most lipid-accumulating plant tissues palmitoyl-ACP is elongated to $C_{18:0}$ stearyl-ACP by a specific KASII (25). The subsequent desaturation is catalyzed by soluble $\Delta 9$ -stearyl-ACP desaturase ($\Delta 9$ DES, E.C. 1.14.99.6) which is localized in the stroma of plastids and converts most of the stearyl-ACP to oleoyl-ACP (26). The primary fatty acids formed in the plastid (palmitic, stearic, and oleic acid) are released from the ACP by the action of thioesterases (TEs) such as oleoyl-ACP hydrolase (E.C. 3.1.2.14) (27). In certain species such as elm, coconut, and camphor, these enzymes play an important role in controlling the chain length of fatty acids smaller than 18 carbon atoms (28). The acyl residues are subsequently exported to the cytosol by an unknown mechanism and converted to acyl-CoA esters by an acyl-CoA synthetase located in the outer envelope of the plastids (29).

In the endoplasmic reticulum, TAGs are formed by the stepwise acylation of glycerol-3-phosphate according to the Kennedy pathway (Fig. 2, gray circles) (30). The glycerol-3-phosphate backbone, which is

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sequentially acylated, is probably provided by a soluble glycerol-3-phosphate dehydrogenase (GPDH, E.C. 1.1.1.8) (31, 32). The first acyltransferase involved in this pathway is glycerol-3-phosphate acyltransferase (G3PAT, E.C. 2.3.1.15) which generates lysophosphatidic acid that can then be converted into diacylglycerol (DAG)-phosphate by lysophosphatidic acid acyltransferase (LPAAT, E.C. 2.3.1.51). LPAAT is substrate-specific (33–35). In rapeseed, for example, it is most efficient with C₁₈ unsaturated fatty acids but does not incorporate erucic acid or fatty acids of medium chain length into the sn2 position during TAG synthesis (36, 37). DAG-phosphate is converted to DAG by phosphatidic acid phosphatase (PAP, E.C. 3.1.3.4). DAG has two fates. It can be used to form TAG or it can be used in a freely reversible phosphati-

dylcholine (PC)-DAG interconversion to enter membrane lipid synthesis. It is an open question as to whether this reaction might control TAG synthesis (38).

Finally, DAG acyltransferase (DAGAT, E.C. 2.3.1.20), in the only enzymatic step unique to TAG synthesis, adds the third acyl chain. The TAG thus formed is deposited in oil bodies. DAGAT generally has a broad specificity for fatty acids (39) and may control the flux over the pathway (40, 41).

The addition of functional groups to fatty acids takes place primarily after they are esterified to various glycerophosphatides. Fatty acids with double bonds in addition to the $\Delta 9$ double bond (polyunsaturated) are produced by membrane-bound desaturases that use as substrates acyl residues esterified to phospholipids. One of the most efficient substrates for desaturation is PC (42, 43).

PC with desaturated fatty acid residues, most often at positions $\Delta 9$, $\Delta 12$, and $\Delta 15$, or $\Delta 6$, may be converted to a variety of DAGs by acyl exchange or PC-DAG interconversion (44). In castor seeds, the pathway for the introduction of a functional hydroxy group at the $\Delta 12$ position of oleic acid proceeds similarly to that of fatty acid polyunsaturation (45). In rapeseed (*Brassica napus*) and other *Brassica* species, which are rich in erucic acid, another pathway leads to erucyl-CoA ($\Delta 13C_{22:1}$) by means of a two-step elongation of oleoyl-CoA ($\Delta 9C_{18:1}$) (10). A variety of plant genes coding for enzymes involved in fatty acid biosynthesis and TAG assembly, such as desaturases, thioesterases, and others, have been cloned [for review see (46, 47)]. Some of the targets used for modification of storage lipid biosynthesis are indicated in Fig. 2 by colored circles.

Desaturation of Fatty Acids

A major challenge in modifying the composition of plant storage oil was to change the degree of fatty acid desaturation. The first success was an increase in the stearic acid (C_{18:0}) content of canola at the expense of the oleic acid ($\Delta 9C_{18:1}$) content. This was achieved by Knutson and coworkers (48) who down-regulated the $\Delta 9DES$ activity by antisense repression (Fig. 3). A complementary DNA (cDNA) encoding $\Delta 9DES$ from *Brassica rapa* was expressed in the opposite orientation (antisense) under the control of a napin gene promoter in seeds of rapeseed. The amount of desaturase protein present in these transgenic seeds was drastically reduced, resulting in a decreased formation of C_{18:1}. As a consequence, up to 40% of the fatty acid content was in the form of stearate, making the oil potentially useful as a cocoa butter substitute (49).

In another study, an increase in stearic acid content to 11% was achieved by over-expression of a long chain-specific thioesterase (TE, see Fig. 2) gene from soybean. Crossing this line with a transgenic line producing 13% stearate by down-regulation of $\Delta 9DES$ resulted in cultivars with about 45% stearate (50). This result indicates that thioesterase and $\Delta 9DES$ compete for the stearyl-ACP substrate and that crossing the two different transgenic lines produces combined effects that achieve the breeding goal.

For oleochemical applications, an increase of oleic acid (Fig. 1) content to over 90% would be of considerable value because processing costs for homogenous or near-homogenous starting materials would be reduced. Also, for food purposes, oxidation stability could be improved by reducing the content of polyunsaturated fatty acids, thus avoiding partial hydrogenation of the oil

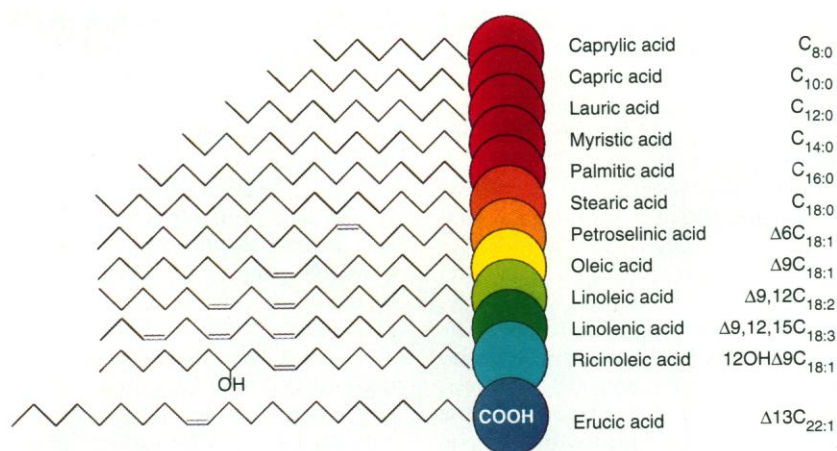


Fig. 1. Chemical formula, trivial names, and abbreviations of important fatty acids for food and nonfood applications (see Table 1). (The color code applies also to Figs. 2 and 3.)

Table 1. Examples of present and potential uses for fatty acids and their derivatives (2, 6). Dash indicates not applicable.

Chain length	Use	
	Food	Nonfood
C ₈ to C ₁₀ C ₁₂ to C ₁₄	Dietary margarine Confectionary, synthetic creams	Soaps, detergents, cosmetics Detergents, laundries, cosmetics
C ₁₆ C _{18:0} $\Delta 6C_{18:1}$	Shortenings Confectionary —	Soaps, candles, lubricant grease Cosmetics, pharmaceuticals, candles Detergents, polymers, cosmetics, pharmaceuticals
$\Delta 9C_{18:1}$	Margarine, frying oil, salad oil	Soaps, detergents, coatings, plasticizer, cosmetics, pharmaceuticals, polymers
$\Delta 9,12C_{18:2}$ * $\Delta 9,12,15C_{18:3}$ * $12OH-\Delta 9C_{18:1}$	Salad oil, margarine Salad oil, margarine —	Coatings, drying oils Varnishes, coatings, linoleum, drying oils Lubricants, plasticizer, coatings, pharmaceuticals, cosmetics, printing inks, textile dyes, leather manufacture, medicinal applications
$\Delta 13C_{22:1}$	—	Polymers, cosmetics, lubricants, plasticizer, surfactants, detergents, pharmaceuticals

*Essential fatty acids in human diets.

and the concomitant formation of *trans*-fatty acids, which are considered to be unhealthy (51). Similarly, the reduction of saturated fatty acids with cholesterologenic properties would be beneficial (51, 52). Oils with a very high oleic acid content can also be processed after harvest for nonfood applications. Ozone oxidizes oleic acid to produce azelaic acid, which is a C_9 straight-chain, saturated, dibasic acid that can be used as a plasticizer and in the manufacture of polymers, fibers, films, and adhesives (53).

Classical breeding techniques produced mutant rapeseed lines with close to 80% oleic acid. Analysis of such mutant lines and experiments with *Arabidopsis* as a model indicated that further increase in oleate content was accompanied by an undesired reduction of cold tolerance during seed germination, possibly due to the lack of unsaturated fatty acids in the membranes of these plants (3, 54). An alternative approach would be to achieve very low amounts of unsaturated fatty acids only in rapeseed embryos, thus producing lines with a very high content of oleic acid in the seeds but with normal amounts of unsaturated fatty acids in the rest of the plant (3). Antisense repression of the $\Delta 12$ -oleate desaturase ($\Delta 12$ DES, Fig. 2) in transgenic rapeseed resulted in an increase in oleic acid of up to 83%. Crossing this line with the mutant IMC129, which accumulates 78% oleic acid, produced a rapeseed line with 88% oleic acid in the TAG fraction (55). A similar result was obtained by cosuppression of $\Delta 12$ DES, which produced lines with 87% oleic acid content (Fig. 3) (50). This is a substantial increase of oleic acid in rapeseed.

Similar results (79% $C_{18:1}$ as compared with 22% in controls) have been obtained with antisense expression of a $\Delta 12$ DES in soybean (55). Also in soybean, linoleic acid content has been increased 10% by antisense expression of linolate desaturase ($\Delta 15$ DES see Fig. 2). All of these results indicate that it might be possible to increase oleic acid content to 90% by seed-specific antisense inhibition of the respective desaturase.

As already mentioned, there is an excess of palmitic, stearic, oleic, linoleic, and linolenic acid in vegetable oils derived from conventional oil crops. Certain species of the *Umbelliferae* such as coriander contain petroselinic acid ($\Delta 6C_{18:1}$, Fig. 1) which is an isomer of oleic acid that has a double bond at the $\Delta 6$ position. Petroselinic acid could contribute to nonfood uses (Table 1) if it were available in a productive oil crop. Oxidation of petroselinic acid by ozone forms lauric acid, which is used in detergents, and adipic acid, a $C_{6:0}$ dicarboxylic acid which is used as a monomer for nylon 6,6 formation. In addition, petroselinic acid may have useful properties for cosmetic and

pharmaceutical applications (49) because fats consisting of tri-petroselinate melt at a higher temperature (33°C) than fats containing the more common isomer oleic acid (12°C).

An alternative to growing coriander for petroselinic acid would be the production of rapeseed engineered for a high content of petroselinic acid in the seed oil. That this is possible is indicated by the transformation of tobacco with a cDNA from coriander that expresses an acyl-ACP desaturase involved in petroselinic acid formation. The fatty acid content of the transgenic tobacco

calli was 5% petroselinic acid (56). As small amounts of $\Delta 4C_{16:1}$ were also observed in these calli, it was reasoned that the coriander desaturase might act on palmitic acid ($C_{16:0}$) rather than stearic acid ($C_{18:0}$), and that petroselinic acid might be produced by elongation of $\Delta 4C_{16:1}$. In the biosynthetic pathway of petroselinic acid in coriander, elucidated by Ohlogge and co-workers (11, 57), a specific desaturase ($\Delta 4$ -palmitoyl-ACP desaturase) introduces a *cis* double bond in the $\Delta 4$ position of palmitoyl-ACP. The 16-carbon *cis* $\Delta 4C_{16:1}$ is thought to be elongated by a specific condensing enzyme

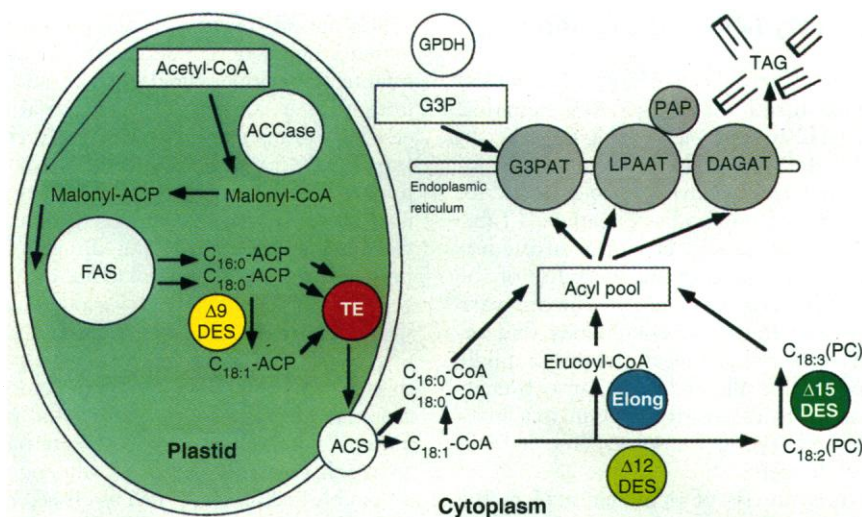


Fig. 2. Schematic outline of the biosynthesis of storage lipids. De novo fatty acid biosynthesis from acetyl-CoA occurs exclusively in the plastid. Products of de novo fatty acid biosynthesis are exported into the cytoplasm where they can be further modified. Triacylglycerol (TAG) assembly is catalyzed by the enzymes of the Kennedy pathway (gray circles). Colored circles indicate key steps in the synthesis of distinct fatty acids and targets for modifying the pathway. ACCase, acetyl-CoA carboxylase; ACP, acyl-carrier protein; ACS, acyl CoA synthetase; CoA, coenzyme A; DAGAT, diacylglycerol acyltransferase; $\Delta 9$ DES, $\Delta 9$ -stearoyl-ACP desaturase; $\Delta 12$ DES, $\Delta 12$ -oleate desaturase; $\Delta 15$ DES, $\Delta 15$ -linolate desaturase; Elong, elongase; FAS, fatty acid synthase; G3P, glycerol-3-phosphate; G3PAT, G3P acyltransferase; GPDH, G3P dehydrogenase; LPAAT, lysophosphatidic acid acyltransferase; PAP, phosphatidic acid phosphatase; PC, phosphatidylcholine; and TE, acyl-ACP-thioesterase. Color code as in Fig. 1.

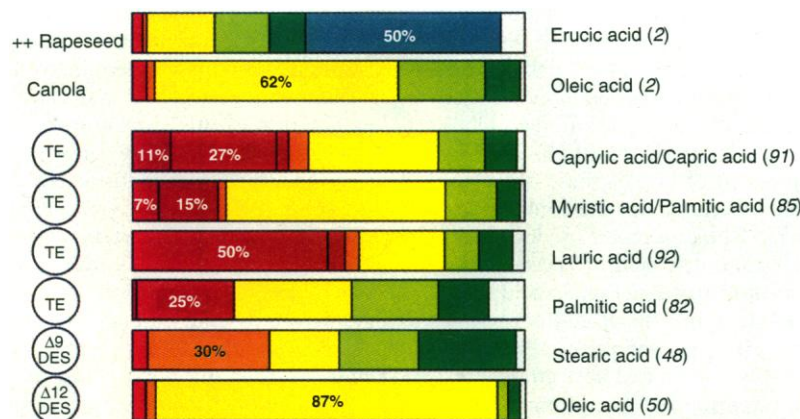


Fig. 3. Fatty acid composition in rapeseed cultivars (++ rapeseed, canola) and transgenically engineered canola lines. The enzymatic target for engineering is indicated in circles (TE, thioesterase; $\Delta 9$ DES, $\Delta 9$ -stearoyl-ACP desaturase; $\Delta 12$ DES, $\Delta 12$ -oleate desaturase). Total fatty acid composition of each seed type is normalized to 100 mole percent. The fatty acid targeted by bioengineering and its subsequent contribution to the total is indicated. The reference for the experiment is given in parentheses. Colors indicate the type of fatty acid as in Fig. 1; white indicates "other."

to form petroselinoyl-ACP. A specialized acyl-ACP thioesterase with high selectivity for petroselinoyl-ACP is responsible for the release of petroselinic acid from its carrier (13). Therefore, to genetically engineer crop plants for the production of petroselinic acid, one may have to introduce at least three different genes from coriander that code for (i) a $\Delta 4$ -palmitoyl-ACP desaturase, (ii) a specific condensing enzyme, and (iii) a specific thioesterase. This strategy does not take into consideration possible necessary alterations within the Kennedy pathway needed to obtain tripetroselinate TAGs preferentially.

Fatty Acid Hydroxylation

Seeds of castor bean (*Ricinus communis*) produce an oil with up to 90% ricinoleic acid ($\Delta 12$ -hydroxyoleic acid, Fig. 1). In 1992 ~460,000 metric tons of castor oil were produced worldwide (58) and used for a number of nonfood applications (Table 1). Major drawbacks of castor oil production include the high susceptibility of the castor bean crop to divergent climatic variations and the toxic compounds that remain in the residual meal: ricinine, a mildly toxic pyridine alkaloid; ricin, an extremely poisonous heat-sensitive protein that inactivates eucaryotic ribosomes; and a heat-stable allergen.

The synthesis of ricinoleic acid in the developing castor endosperm occurs by means of direct hydroxylation of an oleic acid moiety (59) bound to phosphatidylcholine at the endoplasmic reticulum (45, 60, 61). To isolate cDNAs that code for this hydroxylase, van de Loo and co-workers partially sequenced 468 clones obtained from a castor bean cDNA library enriched by differentially screening for cDNAs expressed in endosperm but not in leaves (62). The sequence of the hydroxylase was expected to contain the protein consensus motifs His-X-X-His-His or His-X-X-X-His-His found in functionally related membrane-bound desaturases and mono-oxygenases, respectively (63, 64). Three cDNAs were found to carry the motif His-X-X-His-His. Expression of one of these cDNAs in transgenic tobacco under the control of the CAMV 35S RNA promoter produced seeds with 0.1% ricinoleic acid. It is conceivable that in tobacco tissue ricinoleic acid is rapidly catabolized and therefore does not accumulate. High phospholipase A activity that removes oxygenated acyl groups (65), or acyltransferases that discriminate against ricinoleoyl groups (45), may restrict accumulation of ricinoleic acid in storage lipids (62). Nevertheless, the low level of expression in tobacco indicates that the pathway of ricinoleic acid synthesis probably requires additional enzymes for its stable formation

in high abundance. The cDNA for hydroxylase is now available and may allow for the modification of crops that normally produce high amounts of oleic acid to produce ricinoleic acid in TAGs.

Termination of Fatty Acid Chain Length

A second challenge in modifying the fatty acid composition of oil crop plants is to understand the mechanism responsible for chain length termination during the synthesis of medium-chain fatty acids. Current sources of medium-chain fatty acids are coconut and palm kernel oils. Dietary fats, which are an important energy source for patients deficient in fat resorption or for infants, are manufactured from C_8 and C_{10} fatty acids in an expensive industrial process that forms medium-chain triglycerides (66). These fatty acids are also desirable for nonfood applications because they are soluble in both aqueous and nonaqueous environments, thus providing an excellent raw material for the manufacture of detergents (Table 1). Seeds of various *Cuphea* species contain up to 93% of specific C_8 to C_{14} medium-chain fatty acids (67). Although domestication of these plants as crops might provide a new source for medium-chain fatty acids (68), an alternative approach would be to genetically engineer an established oil crop, such as oilseed rape, to produce them.

The elucidation of the mechanism by which some plants are able to produce seeds with high percentages of medium-chain fatty acids was therefore of interest. Seeds of the California bay tree (*Umbellularia californica*) contain up to 70% lauric acid ($C_{12:0}$) in TAGs. Pollard and co-workers (69) provided biochemical evidence indicating that a specific acyl-ACP thioesterase could be responsible for laurate production. A cDNA encoding this enzyme was isolated and expressed under the control of an early seed-specific napin promoter-terminator cassette in transgenic *Arabidopsis* and canola. The key function of this enzyme in the premature termination of the growing ACP-bound acyl chain was confirmed by showing that seeds of transgenic *Arabidopsis* plants expressing the bay thioesterase had an altered fatty acid profile with up to 25% lauric acid (70). Field trials with similarly modified transgenic canola plants, having a fatty acid content in the seed oil of 45% laurate, showed that these transgenic plants had a normal appearance, produced normal yields of seed oil, and had no apparent change in other agronomic traits (71). This genetically engineered high-laurate canola line was recently given nonregulated status in the United States, and market-oriented cultivation on a limited acreage was started

in southern Georgia (72). Because laurate is poorly incorporated into the sn2 position of TAG in transgenic high-laurate rapeseed (73), expression of a gene from coconut encoding a specific LPAAT in these high-laurate rapeseed lines (74, 75) may increase the content of laurate in TAG (76).

Several other acyl-ACP thioesterases have been isolated from various plants, and some were introduced and expressed in rapeseed (Fig. 3) (70, 77–82). On the basis of significant differences in their deduced primary amino acid sequences, such as characteristic amino acid deletions in the vicinity of the active cysteine site, these thioesterases can be classified into two types (82, 83). Jones and co-workers have termed $C_{18:1}$ -specific thioesterases FatA and thioesterases preferring saturated acyl-ACPs FatB (the laurate-specific bay thioesterase is designated UCFatB1). In *Cuphea lanceolata*, which accumulates up to 83% capric acid (C_{10}) in its seed storage oil, a gene family with at least four members was found to encode FatB thioesterases (83). When two of these genes responsible for embryo-specific expression in *C. lanceolata* (84) were transferred and expressed in transgenic rapeseed lines, distinct altered fatty acid profiles were observed in mature T2 seeds. Transgenic plants with CITEg100 (product of gene ClFatB3) produced seeds containing 1 and 3% caprylic (C_8) and capric acid (C_{10}), respectively, whereas expression of CITEg200 (product of gene ClFatB4) resulted in seeds with 7% myristic acid (C_{14}) and 15% palmitic acid in their oil (Fig. 3). These two genes, although derived from a species that accumulates predominantly capric acid, apparently encode two distinct traits, catalysis of the synthesis of either C_8 and C_{10} fatty acids or C_{14} and C_{16} fatty acids in transgenic rapeseed, respectively (85). As summarized in Fig. 3, various thioesterase genes were introduced into rapeseed to produce desired medium-chain fatty acids (see examples in Fig. 3).

Elongation of Fatty Acids

Erucic acid ($\Delta 13C_{22:1}$), a monoene fatty acid, is found naturally in high erucic acid rapeseed (HEAR) (about 50% $C_{22:1}$, Fig. 3) varieties and other *Cruciferae*. It is used as an additive to lubricants and solvents, a softener for textiles, and an amide derivative in polymer synthesis. Cleavage of erucic acid at the double bond in position 13 by ozone oxidation produces pelargonic acid (with nine carbon atoms) and brassylic acid (a dicarboxylic acid with 13 carbon atoms) which is used in the perfume industry (49). Brassylic acid could also be used as the starting material for the production of high-temperature thermoplastics, but the limited supply of erucic acid prevents such a

development. If the content of erucic acid in rapeseed oil could be increased from its current level of about 50% to >90%, HEAR oil could become an economically more attractive source for erucic acid and a desirable alternative to petrochemicals (12).

In erucic acid-rich rapeseed, the sn2 position of the glycerol backbone in TAG is rarely occupied by erucic acid, whereas seeds of *Limnanthes* species accumulate trierucin (TAGs with erucic acid in all three positions of the glycerol molecule), probably as a result of the activity of a specific microsomal acyl-CoA-sn1-acylglycerol-3-phosphate acyltransferase or lysophosphatidic acid acyltransferase (LPAAT) (87). It would therefore be desirable to transfer this trait from *Limnanthes* to existing HEAR varieties. Hanke and co-workers have isolated cDNAs from *Limnanthes douglasii* encoding an erucoyl-CoA-specific LPAAT (87). They plan to overexpress this cDNA from *Limnanthes* in rapeseed and to inhibit expression of the rapeseed-specific LPAAT gene by an antisense RNA approach.

In addition to exchanging LPAAT enzyme specificities, it may be necessary to increase the amount of erucoyl-CoA produced to obtain rapeseed oils with an erucic acid content of up to 90%. This might be achieved by the introduction and expression in rapeseed of fatty acid elongation functions such as the $C_{18:1}$ -CoA or $C_{20:1}$ -CoA synthase or synthases. The elongase is likely to consist of four enzymatic functions similar to those of the fatty acid synthase (88). The *fae1* gene from *Arabidopsis*, which is presumed to encode a condensing enzyme of the elongase, has been cloned recently by transposon tagging (89), making the gene available for heterologous expression in transgenic oilseeds. That this approach is feasible was demonstrated by the introduction of a jojoba elongase gene, which also encodes the condensing enzyme function, into a canola-quality rapeseed, resulting in a 20% erucic acid content (90). However, the limits of trierucin biosynthesis need to be determined. Important genes such as LPAAT and the condensing enzyme of the elongase are available, which might help in answering this question.

Conclusion and Prospects

Molecular genetics has provided scientists and plant breeders with powerful tools, not only for the study of complex metabolic pathways, but also for the production of novel crop varieties with economically valuable food and nonfood uses. The progress made thus far should further encourage the development of crops containing new oils with medium-chain triglycerides and high oleate or high erucate content. The elucidation of pathways for new

types of fatty acids such as epoxy, acetylenic, and branched chain or cyclic fatty acids could lead to the production of new oils of economic value, particularly in the nonfood sector as oleochemical raw material. However, further progress depends on the elucidation of the regulatory mechanisms and identification of the key steps limiting TAG synthesis as well as on understanding the mechanisms that partition photosynthate into proteins, carbohydrates, or lipids in different agronomically relevant plant organs. When these questions are answered, it may become possible to control photosynthate flux and thus increase yield by redirecting carbon flux into oil. The knowledge gained by studying these questions will substantially contribute to our understanding of the synthesis of distinct fatty acids and of the regulation of the storage lipid synthesis pathway and allow us to move from empirical technology to predictable oil design.

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Cosuppression, Flower Color Patterns, and Metastable Gene Expression States

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In plants, transgenes often induce rapid turnover of homologous endogenous transcripts. This "cosuppression" of homologous genes is an extremely nonlinear response to small increases in gene expression or dosage, inversely amplifying them into dramatic phenotypic alterations. Pigment transgenes elicit metastable cosuppression patterns organized by flower morphology. Pattern organization and metastability reflect regulatory states (probably transgene transcription states) that respond to morphological features and are labile to physiology and development. Shifts between regulatory states can be highly ordered; for example, a shift may be imposed on a population of cells defining a meristem, which then stably maintains and transmits the new state throughout growth.

When plant biologists began to investigate the control of gene expression and to over-express genes and their products by introducing transgenes into the plant genome, they encountered a great deal of variability in transgene expression that was at first attributed to inferred "position effects." However, it was soon learned that at least two kinds of trans effects contribute to this variability: one that appears to cause transcriptional silencing and another that acts posttranscriptionally. These effects are collectively termed "homology-dependent, or repeat-induced, gene silencing." Transcriptional silencing is usually a directional outcome whereby one gene silences another, as in the phenomenon of paramutation, known also as epigenetic conversion (1). Posttranscriptional silencing is usually reciprocal, resulting in the "cosuppression" of ex-

pression of homologous genes (2). Insights into the mechanisms behind cosuppression and epigenetic conversion have come from analyses of many plant genes, and this body of work has been extensively reviewed (3). Cosuppression is common in plants but has yet to be reported in fungi or animals. Possible mechanistic relations between epigenetic conversion and various transcriptional silencing phenomena in fungi and animals have been widely discussed (3–5).

This paper discusses cosuppression mainly in the context of physiological and developmental controls that can influence the cosuppression outcome. The purpose is to show how a hierarchy of mechanisms can explain the organization, diversity, and plasticity of cosuppression-based phenotypes and how this hierarchy can behave as a dynamic regulatory system, capable of orderly shifts in regulatory states, as in normal physiology and development. The emphasis is on a petunia flower color gene, chalcone

synthase (CHS), because its visible phenotype allows us to monitor gene expression on a cell-by-cell basis, and loss of flower pigments has no pleiotropic effect on petal development. An important feature of petunia is its herbaceous, freely branching growth habit, which allows us to monitor the influence of physiology on gene expression and so to explore not only individual mechanisms but also the ensemble of mechanisms that together create complex ordered patterns of control.

CHS is necessary for the biosynthesis of anthocyanin pigments that are responsible for the blue, purple, and red coloration of many flowers. Introduction of a chimeric CHS transgene (in which a sense-oriented CHS coding sequence is driven by a heterologous promoter) results in white flowers that exhibit cosuppression of endogenous and introduced CHS transcripts (6, 7). The cosuppression state is defined by contrast with the "coexpression" of these transcripts in purple "revertant" flowers retaining the transgene, which shows that endogenous gene suppression is not caused simply by a high steady-state concentration of transgene transcript. Cosuppression is often referred to as "sense suppression" to avoid confusion with unrelated methods exhibiting similar outcomes (8). The mechanism of sense suppression is still unknown. The possibility of feedback regulation by the overexpressed enzyme or its biochemical product (7) has been ruled out in several systems, including CHS, by demonstration of the fact that truncated nonfunctional genes are sufficient to induce cosuppression (9–11). Organisms control transcript concentration by modulating transcript production and degradation. Through analysis of transcripts in isolated nuclei, sense suppression was found to occur posttranscriptionally (12, 13). Also, transcript turnover depends on transcription of the endogenous gene (9) and on transgene dosage (12, 14). In eukaryotes, transcription is controlled not only by the action of trans-acting factors on promoter elements but also by local effects of DNA sequence organization patterns and chromatin states, as in position-effect variegation (PEV) (15). Plant transgenes frequently exhibit variable expression patterns reminiscent of PEV that could account for the metastability of some cosuppression phenotypes (16).

Sense suppression of CHS in petunia produces, besides white flowers, an array of flower color patterns (6, 17, 18). However, unlike PEV, these patterns are organized according to three primary features of corolla morphology: veins, junctions between petals, and corolla edges (Fig. 1). Boundaries between white and purple sectors are many cells wide and exhibit steep gradients of pigment intensity from zero to wild-type

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