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Update on Oilseed Biotechnology

Design of New Plant Products: Engineering of Fatty Acid Metabolism

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PLANTS AS CHEMICAL FACTORIES

Manipulation of biosynthetic pathways in transgenic plants offers a number of exciting opportunities for plant biochemists and molecular biologists to redesign plant metabolism toward production of specific higher-value products. Here I review several recent examples that have demonstrated that plant fatty acid metabolism can be manipulated in useful ways to produce new and potentially more valuable vegetable oils.

A number of factors have converged to make metabolic engineering of fatty acid metabolism particularly attractive. Although for centuries plant breeders have manipulated plant metabolism, the primary goal has been to produce better yields of crops for human and animal consumption, and in the past 100 years, efforts to increase yields of the basic food crops have had dramatic success. Through a number of technological advances, yields per hectare have risen many-fold, resulting in substantial surplus capacity worldwide. These surpluses are a reflection of the high yields that can be obtained when optimum agronomic practices are applied to highly developed crop varieties. As a result, the United States spends $20 to $30 billion annually on a variety of programs designed to deal with farm overproduction capacity.

One strategy to exploit this excess capacity is to design new products that can provide expanded or new markets for excess agricultural output. For example, two recent successes have been the use of a major portion of the U.S. maize crop for production of high-fructose corn sweeteners and for fermentation to ethanol. Further progress is likely to be most effective if plant metabolism in the highly productive elite crop varieties can be redirected from production of food to synthesis of new industrial feedstocks or other chemicals. Fortunately, the combined germplasm within the plant kingdom represents a vast reservoir of genes coding for enzyme catalysts that can produce many valuable chemicals.

Plant metabolism has evolved the ability to produce an incredibly diverse range of structures, including more than 20,000 different terpenoids, flavonoids, alkaloids, and fatty acids. Of these, the fatty acids have already been extensively exploited for industrial uses in products such as lubricants, plasticizers, and surfactants. In fact, approximately one-third of vegetable oils produced in the world are already used for nonfood purposes; some of these are summarized in Table I. Furthermore, there is a rich heritage of knowledge in the chemical industry of the properties and chemical potential of fatty acids and their derivatives. Thus, in addition to providing food, oilseed crops can be seen as efficient, low-polluting chemical factories that are able to harness energy from sunlight and transform it into a variety of valuable chemical structures with a multitude of nonfood uses.

Success in the design of new plant oils is expected to have multiple benefits, including reduction of government price supports and subsidies. In addition, some new plant products may provide renewable chemical feedstocks that could replace nonrenewable and imported petroleum-derived products. Thus, benefits can accrue both in local agricultural economies and more broadly by generating a more favorable overall balance of trade. Furthermore, the two-thirds of vegetable oils that are used for food can now be improved in their nutritional qualities with resulting long-term health benefits. Although engineered vegetable oils are not yet on the market, several have had extensive field trials. A summary of recently reported successes in modifying plant lipid metabolism is provided in Table II.

THE PATHWAY FOR FATTY ACID SYNTHESIS

The predominant plant fatty acids found in nature consist of just six or seven structures that have chain lengths of 16 or 18 carbons and one to three double bonds. (Fatty acid structures are designated by a shorthand notation [for example, 18:2], where the number before the colon indicates the chain length and the number after the colon indicates the number of double bonds.) These fatty acids are synthesized from acetyl-CoA by a series of reactions that are localized in the plastids (Fig. 1). The assembly of fatty acids and the introduction of the first double bond occurs while these structures are attached to ACP. After their release from ACP by the action of a specific thioesterase, fatty acids can cross the plastid envelope membrane, after which they are reesterified to CoA. Subsequent metabolism and modification of the fatty acids is believed to occur primarily by membrane-bound enzymes in the ER. These reactions include desaturation to introduce additional double bonds and assembly, via acyltransferase reactions, of three fatty acyl chains onto a glycerol backbone to yield triacylglycerol, the storage form of fatty acids in seeds.

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Abbreviation: ACP, acyl carrier protein.

Table I. *Some nonfood uses of plant fatty acids and oils*

Lipid Type	Example	Major Sources	Major Uses	Approximate U.S. Market Size (10^6 $)
Medium chain	Lauric acid (12:0)	Coconut, palm, kernal	Soaps, detergents, surfactants	350
Long chain	Erucic acid (22:1)	Rapeseed	Lubricants, anti-slip agents	100
Epoxy	Vernolic acid	Epoxidized soybean oil, *Vernonia*	Plasticizers	70
Hydroxy	Ricinoleic acid	Castor bean	Coatings, lubricants	50
Trienoic	Linolenic acid (18:3)	Flax	Paints, varnishes, coatings	45
Wax esters	Jojoba oil	Jojoba	Lubricants, cosmetics	10

ENGINEERING OF FATTY ACIDS FOR INDUSTRIAL USE

In addition to the common fatty acids mentioned above, some plants produce storage oils, which contain high levels of fatty acids of "unusual" structure. In fact, several hundred different fatty acid structures have been found to occur in seed oils. These structures include variations in chain length (both shorter and longer) and the addition of hydroxy, epoxy, acetylenic, cyclopropane, and other functional groups. In many cases these unusual fatty acids have valuable chemical or physical properties that give them potential as industrial commodities. For example, erucic acid (22:1) is a major constituent of *Brassica napus* seeds that, when converted to erucamide, is used extensively as a slip agent in plastic film manufacture. Erucic acid might also have a larger potential as a precursor to nylon 13,13, a high-temperature thermoplastic. However, the current costs of purifying this fatty acid from *B. napus* oil makes this source less economic than alternative routes from petroleum. If the level of erucic acid in seeds was increased from its current level of about 50 to >90%, costs of erucic acid could fall substantially and thereby supplant the petrochemical alternatives. This example serves to illustrate that products from plants frequently compete economically with petrochemicals for many nonfood markets.

Currently, the major nonfood market for vegetable oils is

Table II. *Modifications of lipid metabolism in transgenic plants*

Modification Achieved	Enzyme Engineered	Source of Gene	Reference
Lauric acid production	Acyl-ACP thioesterase	California bay	Voelker et al., 1992
Increased stearic acid	Antisense of stearoyl-ACP desaturase	*Brassica napus*	Knutzon et al., 1992
	Stearyl-CoA desaturase	Rat	Grayburn et al., 1992
Reduced saturated fatty acids	Stearyl-CoA desaturase	Yeast	Polashock et al., 1992
	3-Ketoacyl-ACP synthase II	Castor	Bleibaum et al., 1993
	Acyl-ACP thioesterase	Soybean	Yadav et al., 1993
Reduced saturated fatty acids in phosphatidylglycerol	Acyl-ACP:glycerol-3-phosphate acyltransferase	Squash, *Arabidopsis*	Murata et al., 1992
Increased saturated fatty acids in phosphatidylglycerol	Acyl-ACP:glycerol-3-phosphate acyltransferase	*Escherichia coli*	Wolter et al., 1992
Petroselinic acid production	Acyl-ACP desaturase	Coriander	Cahoon et al., 1992
Increased α-linolenic acid	ω3 desaturase	*Arabidopsis*	Arondel et al., 1992; Yadav et al., 1993
Cyclopropane fatty acid production	Cyclopropane synthase	*Escherichia coli*	Schmid, 1993
γ-Linolenic acid production	Linolenic Δ^6 desaturase	*Synechocystis*	Reddy et al., 1993
Increased oleic acid	ACP:protein A fusion	Spinach	Lee et al., 1993

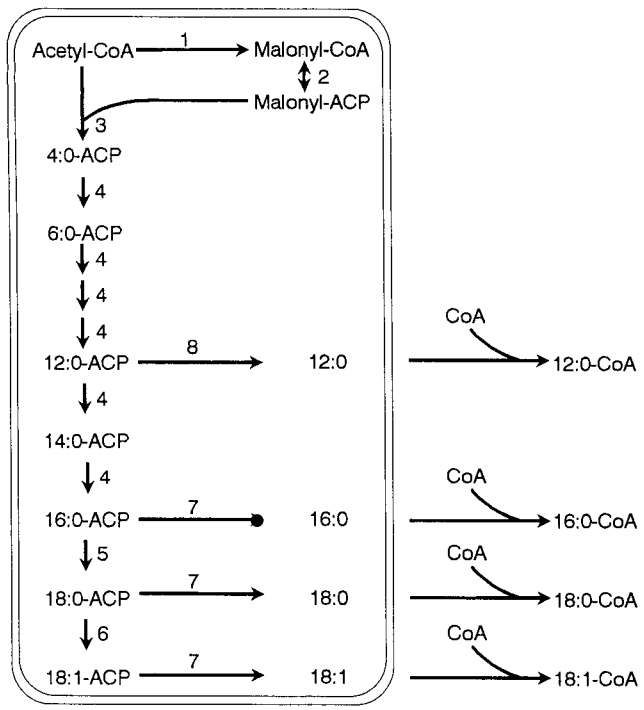


Figure 1. Simplified scheme of plastid fatty acid metabolism. Malonyl-ACP is also the co-substrate and two-carbon donor for each condensing enzyme reaction (3–5). The enzymes represented by numbers are: 1, acetyl-CoA carboxylase; 2, malonyl-CoA:ACP transacylase; 3, 3-ketoacyl-ACP synthase III; 4, 3-ketoacyl-ACP synthase I; 5, 3-ketoacyl-ACP synthase II; 6, stearoyl-ACP desaturase; 7, oleoyl-ACP thioesterase; 8, medium-chain acyl-ACP thioesterase. In addition to the 3-ketoacyl-ACP synthase, each two-carbon addition also requires the action of 3-ketoacyl-ACP reductase, 3-hydroxyacyl-ACP dehydrase, and enoyl-ACP reductase.

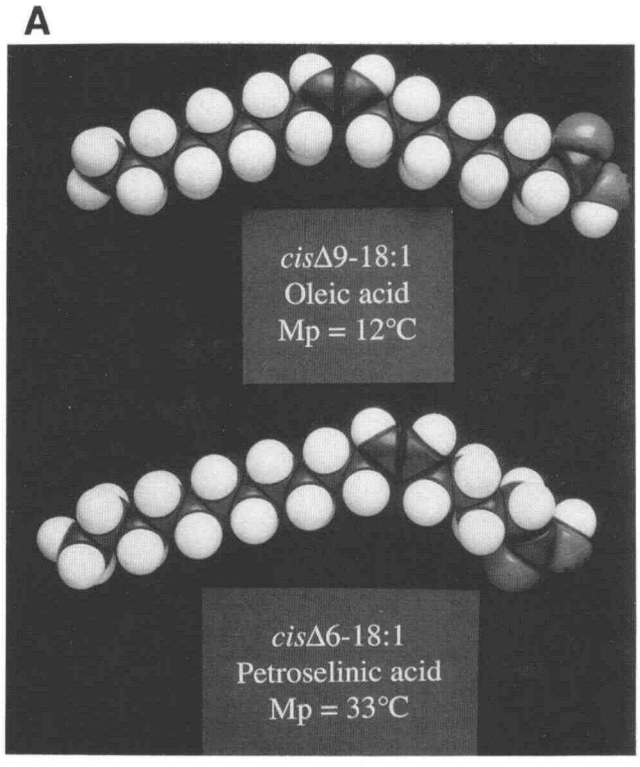
the production of soaps, detergents, and other surfactants. It is in this market where a newly designed plant oil is about to emerge. Saturated fatty acids with a 12-carbon chain length (lauric acid) have ideal properties as surfactants because this chain length provides a balance of solubility in both aqueous and nonaqueous environments. Although medium-chain length fatty acids are produced in a number of plant species, the major commercial sources are the coconut and palm trees of the tropics. Currently, the United States imports approximately $400 million per year of high-lauric tropical oils, which provide the predominant source of lauric acid for the surfactant industry.

To provide a domestic source of lauric acid with more stable prices, several industry and academic laboratories have attempted to engineer the production of lauric acid into domestic crops. This goal has recently been achieved. The first step was to determine the mechanism by which some plants are able to produce high levels of medium-chain length fatty acids. A key observation was that seeds of the California bay tree (*Umbellularia californica*), which produces 70% medium-chain fatty acid in its seed oils, contain a special acyl-ACP thioesterase that is specific for lauroyl-ACP (Pollard et al., 1991). Thus, this enzyme leads to termination of fatty acid synthesis after the acyl chain on ACP has reached 12 carbons rather than after 16 or 18 carbons, as in most plant species. Purification and amino acid sequencing of this thioesterase allowed a cDNA clone to be isolated from seeds of the California bay tree. Confirmation of the pivotal role played by this enzyme in controlling lauric acid production was obtained when transgenic *Arabidopsis* plants expressing this thioesterase were shown to produce up to 25% lauric acid in their seed oils (Voelker et al., 1992). More recently, rapeseed plants expressing the same thioesterase have produced over 40% lauric acid, and field trials of these engineered oilseeds are currently in progress. It now seems assured that these or similar oilseeds will provide an economic alternative to imported coconut and palm kernel oils for the soap and detergent industry.

INCREASING SATURATED FATTY ACIDS

In addition to industrial applications, there is also substantial interest and progress in altering the composition of oils used for human consumption. Vegetable oils have gradually replaced animal fats as the major source of lipids in human diets and they now constitute 15 to 20% of total caloric intake by industrialized nations. About half of human consumption of vegetable oils is in the form of margarines and shortenings. Because most vegetable oils are liquid at room temperature, the production of margarines and shortenings from such oils requires alteration of their physical properties. This is most frequently achieved by catalytic hydrogenation of the oil, a process that reduces the double bonds and thereby increases the melting point of the oil. Thus, hydrogenation substantially increases the saturated fat content of the oil. An additional side reaction that occurs during hydrogenation is the conversion of much of the naturally occurring *cis* double bonds to the *trans* configuration. Almost all unsaturated fatty acids found in nature have double bonds that are in the *cis* rather than the *trans* configuration. As indicated by the structures shown in Figure 2A, the *cis* double bond introduces a bend in the fatty acid that prevents close packing of the acyl chains and thereby reduces the melting point of the fatty acid. Although no convincing evidence for a deleterious effect of *trans* isomers in the diet has been shown, some nutritionists consider the reduction of *trans* double bonds in the diet to be advantageous. Another disadvantage to hydrogenation is the additional 2 to 3 cents per pound cost to the price of the oil. Thus, for several reasons, the creation of an alternative to vegetable oil hydrogenation is desirable in the manufacture of margarines and shortenings.

Soybean and other mutant crop varieties have been available for some time with high stearic acid (18:0) content (and high melting point), but these varieties do not yet have desirable agronomic properties. Recently, progress has been made toward reducing the need for hydrogenation by using a molecular genetic approach. The introduction of the first double bond in plant fatty acids occurs by the action of the enzyme stearoyl-ACP desaturase (Fig. 1). An obvious route to alter the activity of this enzyme in oilseeds is to use antisense RNA. This objective was recently achieved in *B. napus* and *Brassica rapa*, where the antisense expression of stearoyl-ACP desaturase mRNA reduced the enzyme activity



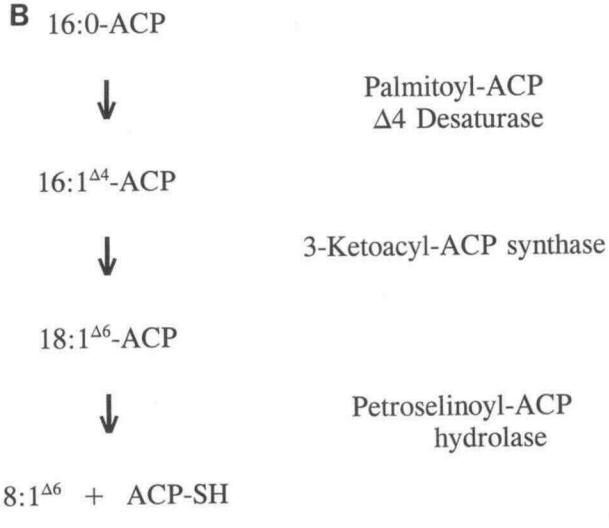


Figure 2. A, Structure of oleic acid (*cis*Δ^9-18:1) and petroselinic acid (*cis*Δ^6-18:1). The positioning of the double bond three carbons nearer to the carboxyl terminus of the acyl chain results in a markedly higher melting point. B, Pathway for the petroselinic acid biosynthesis in coriander. The biosynthesis of this unusual fatty acid may require the action of at least three specialized enzymes. The initial step is the introduction of a *cis* double bond in the Δ^4 position of palmitoyl-ACP by an acyl-ACP desaturase. Next, the 16-carbon *cis*Δ^4-16:1 is elongated to produce petroselinoyl-ACP. Finally, a specialized acyl-ACP thioesterase, which is highly selective for petroselinoyl-ACP, releases petroselinic acid from ACP so that it can be exported from the plastid for further incorporation into storage triacylglycerols.

and desaturase protein to barely detectable levels. As a result, the content of stearic acid in the seed oil was increased up to 20-fold (from 2–40%). Although seed germination and oil content were reduced in the *B. rapa* plants with the highest levels of stearic acid, seeds of *B. napus* with stearic acid levels of 39% appeared to have normal oil and viability.

These two examples provide convincing evidence that plant oil composition can be substantially altered by either inserting or deleting the expression of a single enzyme activity. Prior to these results, several questions about the feasibility of such modifications had been raised. First, it was considered possible that the introduction of a new fatty acid structure such as lauric acid to an oilseed might result in problems with its metabolism by any of the several enzymes (such as acyltransferases) needed for fatty acid incorporation into triacylglycerols. Second, oilseeds that produce unusual fatty acids almost always "sequester" these structures in the storage triacylglycerols and exclude them from the membrane glycerolipids. It was not known if *B. napus*, for example, would possess mechanisms that would "target" an engineered unusual fatty acid, such as lauric acid, toward the storage pathway and away from the membranes. Such a mechanism might be important to prevent the unusual fatty acids from disrupting membrane structure. Finally, it was unclear if oil modification might lead to reductions in oil content or crop yield. The preliminary answer to each of the above questions is that the engineered plants appear to have accepted the altered fatty acid composition without major negative consequences.

REDUCING SATURATED FATTY ACIDS IN DIETARY OILS

A second example of the modification of plant oils for food concerns the dietary goal of reducing saturated fatty acid intake. Because vegetable oils generally contain far less saturated fatty acids than the 40 to 50% found in animal fats, the replacement of animal fats by vegetable oil is considered to be beneficial in reducing cholesterol levels. However, most vegetable oils still contain 10 to 20% saturated fatty acids. Furthermore, the hydrogenation of liquid vegetable oils to produce margarines and shortenings substantially increases this level of saturation. Breeding programs have successfully reduced saturated fatty acid content in some oilseeds, but in most cases high-yielding varieties are not yet available.

An additional approach to reducing saturated fat content is by molecular genetic manipulation of the enzymes of fatty acid biosynthesis. The major saturated fatty acid in most plant oils is palmitic acid (16:0). As shown in Figure 1, palmitic acid (attached to ACP) is at a branch point in fatty acid metabolism. The palmitoyl-ACP can either be further elongated (by 3-ketoacyl-ACP synthase II) or, alternatively, it can be released from ACP by an acyl-ACP thioesterase. Release from ACP allows the palmitic acid to enter into storage oils without further modification. Thus, the flux of saturated fatty acids into most plant oils is controlled in large part by the relative activities of the elongation and thioesterase reactions. Recognition that this branch point may control saturated fatty acid content of vegetable oils has allowed the design of molecular genetic strategies to improve the nutri-

tional value of plant oils. In one case, the strategy chosen was to increase the level of the enzyme that catalyzes the elongation of palmitoyl-ACP to stearoyl-ACP. Very recent results have suggested that overexpression of the 3-ketoacyl-ACP synthase II in transgenic *B. napus* seeds can result in reduced levels of palmitic acid (Bleibaum et al., 1993).

A second approach has been to reduce the activity of the acyl-ACP thioesterase, and this has been achieved in soybeans by the phenomenon of co-suppression. Transformation of soybean with an additional acyl-ACP thioesterase gene led to reduction of the thioesterase activity and an approximately 2-fold reduction in saturated fatty acid levels in somatic embryos (Yadav et al., 1993). A third approach to improving the unsaturation level is to transform plants with additional membrane-bound desaturases that can convert saturated fatty acids to unsaturated. This route also appears to have succeeded. Both a rat and a yeast gene for the enzyme stearoyl-CoA desaturase have been introduced into tobacco. In both cases, the level of saturated fatty acids was slightly reduced and the levels of palmitoleic acid (16:1) were increased severalfold (Grayburn et al., 1992; Polashock et al., 1992).

ENGINEERING OF PETROSELINIC ACID PRODUCTION

Although it is clear from the examples of high lauric acid and high stearate acid production in *Brassica* that addition or modification of a single enzyme can result in major fatty acid compositional changes, it is likely that some other desired modifications will require more extensive metabolic engineering. For example, a fatty acid modification that might simultaneously increase unsaturation in diets and reduce the need for hydrogenation is the production of petroselinic acid-rich vegetable oils. Petroselinic acid is an isomer of oleic acid that has a *cis* double bond at the sixth carbon from the carboxyl end of the molecule rather than at the ninth carbon. As shown in Figure 2A, this minor modification of the structure alters the melting point of the fatty acid such that petroselinic acid melts at 33°C, whereas oleic acid melts at 12°C. This property means that petroselinic acid might provide the means to produce an unsaturated vegetable oil that is also a solid at room temperatures and is therefore ideal for the manufacture of margarine and shortening. Petroselinic acid is a major fatty acid of seed oils of some species of the Umbelliferae, Araliaceae, and Garryaceae families, where it can account for as much as 85% of the total fatty acids. Although species such as coriander and carrot produce a high percentage of petroselinic acid in their seed oils, these crops have a low yield of oil per hectare. Until recently, the biosynthetic pathway for petroselinic acid was unknown. However, a series of biochemical experiments indicated that the synthesis of petroselinic acid involves introduction of the double bond while the acyl chain is still esterified to ACP (Cahoon and Ohlrogge, 1994). Based on this information, a cDNA has been isolated from coriander that codes for an acyl-ACP desaturase involved in petroselinic acid biosynthesis. When this cDNA was used to transform tobacco, the transgenic plants produced petroselinic acid, but only at a level of about 5% of total fatty acids synthesized (Cahoon et al., 1992). Although a number of factors may contribute to the low yield of petroselinic acid in the transgenic plants, recent results suggest that the pathway for petroselinic acid involves at least three enzymes. The first is the acyl-ACP desaturase, which is now known to act on palmitoyl-ACP and to introduce a double bond at the Δ^4 position in this substrate (Cahoon and Ohlrogge, 1994). The second enzyme is postulated to be a modified condensing enzyme (3-ketoacyl-ACP synthase), which is specific for the elongation of *cis*Δ^4–16:1-ACP to *cis*Δ^6–18:1-ACP. The existence of this enzyme was inferred from the observation that the transgenic tobacco plants produce as much or more *cis*Δ^4–16:1 in their lipids as *cis*Δ^6–18:1. Furthermore, analysis of the acyl-ACP pools of the transgenic tobacco indicated that the major acyl group found on ACP was *cis*Δ^4–16:1. The accumulation of this fatty acid in the acyl-ACP pool suggested that elongation of *cis*Δ^4–16:1 was a slow step in acyl-ACP metabolism in the transgenic plants and therefore may be limiting to petroselinic acid production. A third enzyme that may be essential for high petroselinic acid yield is the acyl-ACP thioesterase. This enzyme is required to release fatty acids from ACP so that they can leave the plastid and be incorporated into storage triacylglycerols. Coriander and other Umbelliferae species that produce high levels of petroselinic acid have recently been found to have a novel acyl-ACP thioesterase, which is specific for petroselinoyl-ACP (Dörmann et al., 1994). Considered together, the data described above have led us to propose the pathway shown in Figure 2B for petroselinic acid biosynthesis. Although the original assumption was that petroselinic acid production could be engineered in transgenic plants by the expression of a single acyl-ACP desaturase, it now appears that at least three genes might be needed to obtain the high levels required for economic production of this fatty acid in transgenic oilseeds.

CONCLUSIONS AND FUTURE PROSPECTS

The examples above provide an impressive demonstration of how far our understanding of how to engineer plant oil composition has evolved. Although fatty acid biosynthesis is a complex pathway, almost all of the steps are known and clones have been obtained for most of the enzymes that produce the common fatty acids. It is now possible to begin with a biochemical examination of the likely enzymes involved in controlling fatty acid composition and to intervene in that process by either adding new enzymes, overexpressing existing enzymes, or using antisense RNA to reduce expression of endogenous enzymes. The success already achieved with lauric acid suggests that there are no fundamental barriers toward making substantial modifications in plant oil biosynthesis. In many cases, modification of a single enzyme will be sufficient. In others, as exemplified by petroselinic acid, transfer of more than one gene may be essential for efficient production of the desired fatty acid. The major limitation to increasing the number of engineered oils is that for many desirable fatty acid modifications that lead to economically valuable "unusual" fatty acid structures, we have not yet isolated the genes responsible for production of these fatty acids. However, the examples cited in Table II indicate that efforts in this area need not be limited by the availability

of genes from the plant kingdom. Bacterial, animal, and yeast genes for membrane-bound fatty acid-modifying enzymes have all been shown to function in transgenic plants. Two other future developments are likely to lead to expanded opportunities in the use of plants as chemical factories. First, it should be possible to produce seeds that direct essentially all of their incoming photosynthate toward a single high-value storage component such as oil rather than also producing storage proteins and carbohydrates. Such seeds might not be viable and therefore genes controlling this trait would only be induced by chemical treatments or by crossing two strains. Second, as our understanding of enzyme structure expands, it is likely that new enzymes can be designed by site-directed mutagenesis that will allow synthesis of structures tailor-made for specific chemical applications. Such enzyme redesign has already been successful in several microbial model systems. Based on the inherent efficiency of using sunlight and photosynthesis to produce renewable chemicals and on our increasing ability to specifically engineer plant metabolism, continued expansion in the use of transgenic plants as alternative chemical factories can be expected.

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