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Strategies for Modifying Fatty Acid Composition in Transgenic Plants

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Introduction

One of the major goals of agricultural biotechnology is to increase the value of traditional crops by the addition of novel and desirable traits. An area in which significant progress has been made toward this goal is the modification of seed oils. Vegetable oils are important agricultural commodities; worldwide, they contribute significantly to human caloric intake and their composition can have a major effect on cardiovascular health. In addition, a large proportion (approximately one-third) of vegetable oil production is directed toward non-food uses (Ohlrogge, 1994). For example, plant oil-derived fatty acids are used industrially in surfactants, lubricants, coatings, pharmaceuticals, cosmetics, and plasticizers (Ohlrogge, 1994; Töpfer *et al.*, 1995; Mackenzie, 1995). However, the limited variability of the fatty acid composition of seed oils obtained from current oilseed crops restricts the number of applications that are economically feasible. Thus, significant effort has recently focused on the modification of seed oils (Ohlrogge, 1994; Töpfer *et al.*, 1995; Mackenzie, 1995; Murphy, 1996; Budziszewski *et al.*, 1996; Metz and Lassner, 1996).

Vegetable oils are composed chiefly of triacylglycerols (TAGs), three fatty acids esterified to the three hydroxyls of glycerol. The value of an oil and its uses are largely determined by the fatty acid composition of the component TAGs. The fatty acid composition that is desirable for use in diesel fuel is significantly different from that of an ideal cooking oil. Yet another composition is required for use as a feedstock for polymer synthesis. The major oilseed crops, historically, were selected for food use and contain chiefly five fatty acids (oleic, linoleic, α-linolenic, stearic, and palmitic) in a limited range of concentrations (Budziszewski *et al.*, 1996). On the other hand, the plant kingdom produces an array of fatty acids in widely varying amounts and proportions. Over 200 different plant-synthesized fatty acids have been identified (van de Loo *et al.*, 1993). Structural variations among fatty acids occur in chain length, the number and positions of double bonds, and the presence and position of modifications such as hydroxylations or epoxidations. While most of the plants that produce these fatty acids do not possess good agronomic traits, they represent an extensive genetic

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resource that can be tapped to engineer desired oil compositions into existing oilseed crops.

Conventional plant breeding has improved the fatty acid profile of oil to address some of the consumer demands or market requirements for altered fatty acid content. However, breeding is limited by the natural variability that can be derived from existing germplasm. In contrast, the biotechnological approach to plant improvement via the construction of transgenic plants has the ability to alter or introduce specific functions within a defined genetic background and has the capability to introduce genetic traits from other species, even from non-plants, that could not be introduced by traditional breeding methods.

In addition to addressing consumer demands, the ability to impart new enzymatic functions to transgenic oilseed crops affords the opportunity to produce inexpensively valuable fatty acids for industrial applications. For example, high lauric acid rapeseed oil, produced by transgenic rapeseed, is targeted for the manufacture of detergents, soaps, and related products (Metz and Lassner, 1996; Voelker *et al.*, 1996). Although lauric acid currently is obtained from plant oils, i.e. coconut and palm kernel oils, the ability to produce it in an oilseed crop will broaden the agricultural base for lauric acid production. Should climatic conditions affect production of an oil crop in one region, the ability to produce the oil in multiple crops would enable its continued production, thus serving to stabilize world supplies of important oil commodities.

Biosynthesis

The intermediary metabolism of plant fatty acid biosynthesis has been, and continues to be, extensively investigated. The general pathways are known and specific details continue to be filled in (Harwood, 1996; Browse and Somerville, 1991; Ohlrogge *et al.*, 1991). This knowledge provides extensive insight into appropriate areas for genetic manipulation of fatty acid content.

In plants, the synthesis of fatty acids up to 18 carbons long occurs in plastids; in seeds, synthesis is in the undifferentiated plastid. The synthesis is catalysed by a group of enzymatic functions collectively referred to as fatty acid synthase, during which the growing carbon chain is attached via a thioester linkage to acyl carrier protein (ACP).

Initially, malonyl-ACP is formed in a short series of reactions by carboxylation of acetyl-CoA. Subsequently, a cycle of reactions results in the sequential addition of two-carbon units to the growing acyl chain. These include a condensation reaction in which the carbon–carbon bond is formed yielding a β-ketoacyl-ACP. This is followed, in order, by a reduction of the carbonyl, a dehydration, and a second reduction, thereby converting the β-ketoacyl-ACP to an acyl-ACP that is two carbons longer than before the cycle began. Three different β-ketoacyl-ACP synthases (KAS) catalyse the condensation reactions. The first, KASIII, employs acetyl-CoA and malonyl-ACP as substrates. Substrates for the second enzyme, KASI, are acyl-ACPs (acyl chains of 4 to 14 carbons) and malonyl-ACP. The third enzyme, KASII, catalyses the final condensation reaction between palmitoyl-ACP (C16-ACP) and malonyl-ACP producing stearoyl-ACP (C18-ACP).

Additional plastidial reactions frequently include desaturation (between carbons 9 and 10 from the carboxyl end) of stearoyl-ACP forming the monounsaturated oleoyl-ACP (C18:1Δ^9-ACP). This double bond characteristically is in the *cis* configuration.

Terminating fatty acid synthesis in the plastid, chain length-specific thioesterases release free fatty acids from the ACP. The specificities of the thioesterases for different acyl-ACPs influence the ratio of fatty acids produced; in seed oil crops, most of the fatty acids are either C16 or C18. The free fatty acids then pass out of the plastid and, in the process, are esterified to coenzyme A (Budziszewski *et al.*, 1996; Harwood, 1996; Töpfer *et al.*, 1995).

The remainder of fatty acid biosynthesis, fatty acid modification, and storage oil TAG synthesis occurs outside the plastid. Incorporation of fatty acids into other lipids, such as membrane phospholipids, also occurs primarily outside of the plastid. In some plant species, one possible modification is elongation, which, when it occurs, is apparently catalysed by a membrane bound microsomal multienzyme complex in a reaction very similar to the two-carbon additions that occur in the plastids (Bessoule *et al.*, 1989).

Additional desaturation of monounsaturated oleic acid is a common modification and is catalyzed by membrane bound desaturases thought to be localized in the endoplasmic reticulum. Desaturation occurs following incorporation of oleic acid at the *sn*-2 position of phosphatidyl choline (PC) (Budziszewski *et al.*, 1996) and most commonly occurs between fatty acid carbons 12 and 13 and between carbons 15 and 16, forming double bonds referred to as Δ^{12} and Δ^{15}, respectively. Oleoyl desaturase converts the PC *sn*-2 oleoyl group to linoleic acid (C18:2 $\Delta^{9,12}$), which can then be further desaturated by linoleoyl desaturase to α-linolenic acid (C18:3 $\Delta^{9,12,15}$). The polyunsaturated fatty acids can be incorporated into TAGs following release from PC via the free fatty acyl-CoA pool or by enzymatic conversion of PC to a diacylglycerol and then to a TAG.

Less common fatty acid modifications, but of definite commercial interest, are those such as hydroxylation and epoxidation. In many cases, these modifications appear to be catalyzed by single enzymes. These complex biosynthetic pathways provide numerous points where biotechnological methods may be employed to modify the final outcome of seed oil TAG synthesis.

Core technologies

In the last 30 years increasing emphasis has been placed on modifying the seed oil composition of major crops. Initial efforts employed classic plant breeding techniques (mutagenesis, screening, crosses between compatible germ lines) with impressive results. Notable were the breeding of low erucic acid rapeseed, high oleic acid sunflower, high stearic acid soybean, and low linolenic acid flax varieties (Loof and Appleqvist, 1972; Stefansson *et al.*, 1961; Urie, 1985; Graef *et al.*, 1985; Tonnet and Green, 1987). The advent of plant genetic engineering technology has greatly increased both the rate at which modifications can be made and the range of modifications that are possible. The genetic engineering of fatty acid composition involves three interdependent technologies. The cloning of genes encoding proteins involved in fatty acid biosynthesis, the transgenic expression of these genes, and recently, the modification of cloned genes in order to engineer the expressed protein.

Numerous plant genes encoding proteins and enzymes involved in fatty acid synthesis have been cloned (Töpfer and Martini, 1994; Budziszewski *et al.*, 1996). A variety of methods have been employed from whole gene synthesis (Beremand *et al.*,

1987) to T-DNA tagging (Yadav *et al.*, 1993) to expressed sequence tag (EST) analysis (Nunberg *et al.*, van de Loo *et al.*, 1995). Confirmation that the correct gene has been cloned usually involves expressing the cloned gene transgenically and demonstrating the proper acquired activity. This often involves extraction and gas chromatographic analysis of the fatty acids produced by the transgenic plant.

Transgenic expression is critical for more than just confirming cloned gene identity. Appropriately expressing the cloned gene in transgenic plants is essential for genetically engineering the desired trait. In plants, fatty acids serve several roles in addition to their use as energy reserves in seed oils. These include a structural role in all cellular membranes and in protective surface layers such as waxes (Harwood, 1996). In order to avoid adversely affecting these other functions, it is desirable to express the cloned fatty acid synthesis or modification gene in such a way that only the fatty acid profile of the seed lipids is altered. Furthermore, high level production of the desired fatty acid usually requires expression at the appropriate developmental stage. Thus, the choice of an appropriate promoter is important. Complicating this choice is the observation that some promoters are more effective in some host plants than in others. In practice, optimized expression of genes influencing seed oil composition is greatly facilitated by the availability of an array of seed-specific promoters. Numerous other factors such as the presence or absence of untranslated regions of the gene may also be important.

The progress seen in cloning and expressing plant fatty acid synthesis genes has been paralleled by progress in understanding the structure and function of the proteins involved. In some cases these proteins have been purified and directly analysed (Lindqvist *et al.*, 1996). Often, however, the membrane associated nature of the proteins has thwarted efforts at purification. Nevertheless, the cloning of the genes encoding many of these proteins has allowed their amino acid sequence to be deduced. For example, several different fatty acid desaturase genes, from a variety of plants and cyanobacteria, have been cloned and sequenced (Wada *et al.*, 1990; Arondel *et al.*, 1992; Iba *et al.*, 1993). Analysis of the derived amino acid sequences has revealed several conserved histidine rich motifs critical for catalysis (Sakamoto *et al.*, 1994; Shanklin *et al.*, 1994).

Recently, this growing knowledge of plant fatty acid synthesis enzyme structure has been applied to engineering substrate specificity in recombinant enzymes (Yuan *et al.*, 1995; Cahoon *et al.*, 1997). In one striking example, Cahoon *et al.* replaced various portions of a Δ^6-palmitoyl-ACP desaturase with the corresponding portions of Δ^9-stearoyl-ACP desaturase. The hybrid enzymes created were shown to have altered specificities for chain length, altered positions for double bond formation, or both. These data were correlated with X-ray crystallographic data on the Δ^9-desaturase. Then, the active site model of the Δ^9-desaturase based on X-ray crystallography was used to predict changes that would convert a Δ^9-stearoyl-ACP desaturase to a Δ^6-palmitoyl-ACP desaturase. Changing two specific residues resulted in the anticipated change (Cahoon *et al.*, 1997). These results suggest the exciting possibility that not only can crop plants be engineered to express fatty acid synthesis genes encoded by other organisms, but that one also may construct 'designer' genes specifically modified to produce a desired product.

Genetically engineered modifications in seed oil quality

One aspect of oil quality that has been targeted for modification is fatty acid chain length. While the majority of fatty acids from oilseed crops are either C16 or C18, numerous applications exist for fatty acids of chain lengths ranging from C6 to C24. To date, one of the most successful examples of modifying plant oil composition has been the production of high laurate canola oil. Lauric acid (C12:0) is used extensively in the manufacture of surfactants such as soaps and detergents (Ohlrogge, 1994). Traditional commercial sources of oil rich in lauric acid have been tropical oils such as coconut and palm kernel oil. The hundreds of millions of dollars that have been spent annually importing these oils has provided major economic incentive to develop domestic crops enriched in lauric acid.

As mentioned above, the thioesterase enzymes in plastids release fatty acids from acyl-ACP molecules during fatty acid synthesis and, by their specificities, regulate the proportions of fatty acid that are present in the seed oil. A thioesterase was discovered during investigations of medium chain length fatty acid biosynthesis in *Ubellularia californica* (California bay tree) seeds that has a strong preference for lauroyl-ACP (Pollard *et al.*, 1991; Davies *et al.*, 1991). This thioesterase was purified and partial amino acid sequences were obtained (Davies *et al.*, 1991). This information was used to design polymerase chain reaction (PCR) primers to clone the bay thioesterase gene. Expression of this gene in *Arabidopsis* under the control of the seed-specific napin promoter resulted in the production of seed oils containing 25% lauric acid (Voelker *et al.*, 1992). This gene was next expressed in canola. Using tandem gene constructs and creating plants with multiple copies of the lauroyl-ACP thioesterase gene allowed the accumulation of lauric acid to 60% of fatty acids derived from seed TAGs (Voelker *et al.*, 1996).

Further increases in lauric acid content have been hampered because canola does not normally put lauric acid onto the middle carbon of TAGs (Frentzen, 1993). Coconut oil does contain lauric acid in the middle position, due to the presence of a lysophosphatidic acid acyltransferase (the enzyme responsible for acyl transfer to the middle position of TAGs) with a specificity for lauric acid. This enzyme has recently been purified and a cDNA clone encoding the enzyme has been isolated (Knutzon *et al.*, 1995). Thus, it may soon be possible to increase the content of lauric acid even further in appropriately constructed canola lines.

Similar approaches are being used to engineer other medium chain fatty acids into the oil of seed crops. A cDNA generated from *Cuphea hookeriana* seed mRNA, encoding an acyl-ACP thioesterase with a specificity for C8:0 and C10:0, was identified and introduced into *Brassica napus* resulting in significant accumulation of these fatty acids in the seed oil of the recipient plants (Dehesh *et al.*, 1996). Work has also begun on engineering the substrate specificity of thioesterase enzymes. For example, site directed mutagenesis of the lauroyl-ACP thioesterase gene from bay seeds resulted in the creation of a gene that encodes a thioesterase specific for myristate (C14:0) (Yuan *et al.*, 1995).

The engineering of oils containing fatty acids longer than C18 (very long chain fatty acids or VLCFA) is also being pursued. Erucic acid (C22:1 Δ^{13}) is used extensively in industry. The primary use is as a 'slip agent' that allows the free flow of polyethylene (Walker, 1995). Other uses that have been described include the manufacture of

paints, corrosion inhibitors, cosmetics, and lubricants (Johnson and Fritz, 1989). High erucic oils have also been used as diesel fuels (Johnson and Fritz, 1989). The chief commercial plant source for erucic acid is the high erucic acid rapeseed (HEAR) line of *Brassica napus* (Metz and Lassner, 1996). In this line the seed oil contains 50–60% erucic acid. Increasing the erucic acid content to 90% would improve the economics of the current applications and make additional applications feasible (Mackenzie, 1995). Two reactions that could limit the overall levels of erucic acid in TAGs have been identified (Metz and Lassner, 1996). The first is the rate-limiting step in the microsomal cycle of extension of fatty acids over C18, catalysed by β-ketoacyl-CoA synthase (Lassner *et al.*, 1996). The second is the reaction noted above catalysed by LPAAT an enzyme that esterifies fatty acids to the middle position of glycerol. In rapeseed this enzyme has a strong preference for C18 substrates and a very low affinity for VLCFAs, thus limiting erucic acid content in TAGs of rape seed oil to a maximum of about 67% of total fatty acid (Metz and Lassner, 1996).

Genes have recently been cloned from other plant sources to be used in attempts to enhance these activities in favor of increased erucic acid content in rape seed oil. A ß-ketoacyl-CoA synthase was cloned from jojoba (*Simmondsia chinensis*) a plant that produces high levels of VLCFAs as components of waxes in its seed oil (Lassner *et al.*, 1996). Genes encoding LPAAT were cloned from meadowfoam (*Limnanthes alba alba*) a species in which approximately 90% of the seed oil fatty acids are VLCFAs and from the related species *Limnanthes douglassie* (Lassner *et al.*, 1995; Brown *et al.*, 1995). PCR of cDNA was used to generate a probe to identify the respective full length cDNA for the synthase gene and the *L. alba alba* LPAAT gene. For the synthase gene primers were designed from a partially sequenced purified protein (Lassner *et al.*, 1996). A degenerate primer was designed for the *L. alba alba* LPAAT gene based on a region conserved in other known LPAATs (Lassner *et al.*, 1995). The LPAAT from *L. Douglassie* was isolated by complementing a mutant strain of the bacterium *Escherichia coli*. (Brown *et al.*, 1995). The genes encoding these enzymes were then expressed in *B. napus* under the control of the napin promoter.

The jojoba β-ketoacyl-CoA synthase was introduced into low erucic acid rape, a line that has a significantly reduced ability to carry out the microsomal fatty acid elongation process (Lassner *et al.*, 1996; Stumpf and Pollard, 1983). Seeds of some of the resulting transformants had erucic acid levels as high as 40% of seed derived fatty acids (Lassner *et al.*, 1996). The same construct was expressed in a HEAR strain of *Brassica napus* and resulted in little change in erucic acid levels relative to the non-transformed HEAR strain (Lassner *et al.*, 1996). This suggests that increased levels of β-ketoacyl-CoA synthase are not sufficient for increased erucic acid levels in the HEAR strain.

HEAR strains expressing either of the *Limnanthes* LPAAT genes resulted in significant production of TAGs with erucic acid esterified to the middle carbon. In both cases some trierucic acid was produced. In neither case, however, did expression result in the production of higher total levels of erucic acid in the HEAR seed oils (Lassner *et al.*, 1995; Brough *et al.*, 1996). This indicates that adding LPAAT activity specific for erucic acid is also not sufficient to increase the levels of erucic acid in these strains. Strains are now being created in which the jojoba ß-ketoacyl-CoA synthase and the *L. alba alba* LPAAT are both expressed simultaneously in the HEAR background. These strains will be tested to see if the combination of these traits can

increase overall erucic acid content of TAG (Metz and Lassner, 1996). Recently, a VLCFA-specific LPAAT encoding gene isolated from *Saccharomyces cerevesiae* was introduced into a line of *Brassica* resulting in a significant increase in erucic acid content from 45% to 56% (Zou *et al.*, 1997). Introduction of this yeast gene also had an interesting effect on the overall seed oil quantity, as discussed below.

The presence or absence of double bonds in a fatty acid greatly influences the potential uses and value of that fatty acid. The presence, number, position, and conformation of double bonds influence physical properties (such as melting temperature), chemical properties, and nutritional value of fatty acids. As a result, modification of the degree of fatty acid desaturation in TAGs is another active area for genetic engineering (Töpfer *et al.*, 1995; Ohlrogge, 1994; Budziszewski *et al.*, 1996).

Stearic acid, C18:0, is present in most vegetable oils at low levels relative to the unsaturated fatty acids, C18:1, C18:2, and C18:3, derived from it. A high stearic acid-containing oil has potential use as a substitute for cocoa butter and could substitute for partially hydrogenated plant oils in margarine production (Ohlrogge, 1994; Töpfer *et al.*, 1995). Typically, most margarines are created by partially hydrogenating polyunsaturated vegetable oil in order to raise the melting point and enable the margarine to solidify (Ohlrogge, 1994). This process has the unfortunate side effect of converting many of the double bonds from the *cis* configuration to the *trans* configuration. Consumption of *trans* unsaturated fatty acids has been associated with increased risk of cardiovascular disease (Willett *et al.*, 1993; Stender *et al.*, 1995; Booyens and van der Merwe, 1992). Oils with increased relative stearic acid content would have an increased melting temperature providing a desirable alternative to the process. Other reported potential industrial uses for stearic acid include production of cosmetics, pharmaceuticals, and candles (Töpfer *et al.*, 1995).

Increasing the stearic acid content of seed oil was one of the first applications of genetic engineering made in the area of plant lipid metabolism (Knutzon *et al.*, 1992). In this case, a cDNA encoding stearoyl-ACP desaturase (Δ^9-desaturase) was isolated from a cDNA library prepared from *Brassica rapa* embryo mRNA. This cDNA was expressed in *B. rapa* and *B. napus* in the antisense orientation under the control of seed-specific promoters. Increased levels of stearic acid and decreased levels of oleic acid (C18:1Δ^9) were found in the transgenic seeds. This correlated with decreased levels of Δ^9-desaturase measured both by enzymatic activity and Western blot. In some cases the total amount of seed oil was reduced and germination was impaired. However, some *B. napus* lines that produced seed TAGs with up to 40% stearic acid germinated normally and contained normal quantities of seed oil (Knutzon *et al.*, 1992).

One of the most common uses for vegetable oils is for cooking. However, the high level of polyunsaturation in most vegetable oils used for this purpose leads to decreased shelf-life and instability during cooking. Thus these oils are also partially hydrogenated to reduce the polyunsaturation and minimize these difficulties (Kinney, 1996). Oils with increased levels of oleic acid and decreased levels of polyunsaturated fatty acids would have the desired properties of a cooking oil without the added expense and potential health risks of hydrogenation (Kinney, 1996). Oils with a high oleic acid content would also be valuable to the chemical industry because oleic acid can be readily converted to azelaic acid, a compound used in the manufacture of a number of products (Töpfer *et al.*, 1995). Expression of an antisense oleate desaturase

(Δ^{12}-desaturase) gene in rapeseed resulted in a transgenic plant that produced TAGs with 83% oleic acid (compared to approximately 13% in standard rapeseed) presumably by blocking the conversion of oleic acid to linoleic acid (Harwood and Russell, 1984; Hitz *et al.*, 1995). Combining the benefits of classical breeding and genetic engineering this line was crossed with a mutant line of rapeseed that accumulates 78% oleic acid in TAGs. The newly produced line produced TAGs with 88% oleic acid (Hitz *et al.*, 1995). Antisense repression of Δ^{12}-desaturase has also resulted in the production of soybean oil with a 79% oleic acid content (Hitz *et al.*, 1995). In addition, Kinney (1997) reports soybean oil with up to 88% oleic acid content obtained from plants in which an introduced sense copy of the seed specific Δ^{12}-desaturase gene co-suppressed Δ^{12}-desaturase activity.

The above examples demonstrate the ability of genetic engineering to modify the profile of saturated and unsaturated fatty acids that normally are produced by an oilseed crop. Recently, genetic engineering techniques have also been applied to introduce new unsaturated fatty acids to the TAGs of plant seed oils (Reddy and Thomas, 1996; Beremand *et al.*, 1997).

The fatty acids γ-linolenic acid (GLA) (C18:3 $\Delta^{6,9,12}$) and octadecatetraenoic acid (OTA) (C18:4 $\Delta^{6,9,12,15}$) are two valuable polyunsaturated fatty acids that are important in animal and human nutrition. These fatty acids serve as precursors to several prostaglandins and thromboxanes (Lands, 1979; Lands, 1986; Weber *et al.*, 1986; Phillips and Huang, 1996). GLA production is catalyzed by Δ^6-desaturase acting on linoleic acid (LA) (C18:2 $\Delta^{9,12}$) while OTA is formed through the action of the same enzyme on α-linolenic acid (ALA) (C18:3 $\Delta^{6,9,15}$). A growing body of clinical evidence suggests that consumption of GLA has a positive effect on many conditions that correlate with decreased Δ^6-desaturase activity or decreased formation of n-6 desaturated fatty acids. These conditions include rheumatoid arthritis, high blood pressure, hyperlipidemia, and diabetic neuropathy (Phillips and Huang, 1996). GLA has also been shown to be hypocholesterolemic in both animals and humans (Takayasu and Yoshikawa, 1971; Horrobin and Manku, 1983; Sugano and Ikeda, 1996). Reported potential medical uses for OTA include treatment of cardiovascular and thromboembolic diseases associated with platelet aggregation, inhibition of leukotriene biosynthesis, and prevention or treatment of nephrocalcinosis (Lagarde *et al.*, 1992; Guichardant and Rigaud, 1992; Horrobin and Reynolds, 1996). GLA is also used in the production of cosmetics while OTA can be used in fast drying films, special waxes, and plastics (Gross and Dorrell, 1976; Craig and Bhatty, 1964).

GLA and OTA synthesis is fairly common in lower plants such as moss, algae, and fungi (Hansen and Rossi, 1990; Gellerman *et al.*, 1972; Ratledge, 1987). It is also produced by some cyanobacteria (Murata and Nishida, 1987). Neither of these fatty acids is found in the seed oils of major crop plants due to the absence of Δ^6-desaturase activity. In higher plants this activity has chiefly been observed in some members of the Boraginaceae, Onagraceae, and Saxifragaceae families (Phillips and Huang, 1996). Many crop plants do produce abundant amounts of LA and ALA, however. If Δ^6–desaturase activity could be introduced into these crops, they should be able to make GLA and/or OTA. Efforts to engineer GLA production have focused on cloning and expressing genes for Δ^6-desaturase in plants.

A gene encoding Δ^6-desaturase was first isolated from the cyanobacterium

Synechocystis. Cosmid clones carrying this gene were isolated from a *Synechocystis* genomic library. They were identified by their ability to confer GLA and OTA production on another cyanobacterium, *Anabaena* (Reddy *et al.*, 1993). Sequence analysis of the gene showed homology to other known fatty acid desaturase genes (Reddy *et al.*, 1993). This gene was expressed in tobacco under the control of the cauliflower mosaic virus 35S (CaMV 35S) promoter. GLA and OTA were identified in extracts from the leaves but not the seeds of the transgenic tobacco (Reddy and Thomas, 1996).

The low level of expression of the cyanobacterial Δ^6-desaturase gene in transgenic plants turned interest towards isolating a Δ^6-desaturase gene from a higher plant source. Borage (*Borago officinalis*) produces seed oil with a high GLA content and was chosen as a likely source for a Δ^6-desaturase gene. Expressed sequence tag (EST) analysis was carried out on a borage embryo cDNA library. Common non-desaturase sequences were identified and eliminated from the pool of clones being analysed. A database of 500 ESTs was established. One of these was a truncated clone that showed sequence homology to the *Synechocystis* Δ^6-desaturase gene (Nunberg *et al.*, unpublished data ; Beremand *et al.*, 1997). This clone was used to identify a full-length gene that when expressed in tobacco and *Arabidopsis* resulted in GLA and OTA production in seeds and leaves (Nunberg *et al.*, unpublished data; Beremand *et al.*, 1997). Various promoters were used in these studies. The highest levels of seed expression were obtained with the sunflower 2S albumin promoter in transgenic *Arabidopsis*. GLA levels approached 10% of the seed derived C18 pool (Nunberg *et al.*, unpublished data), similar to that of evening primrose, a current commercial source for GLA. Additional promoters and host plants are currently being tested to optimize GLA and OTA production (Thomas *et al.*, unpublished observation).

Another desaturated fatty acid with potential value as a biotechnology product is petroselinic acid (C18:1Δ^6). This fatty acid is similar to oleic acid except for the placement of the double bond. This results in a higher melting temperature, 33° for petroselinic acid vs. 12° for oleic acid (Ohlrogge, 1994). Thus, oil rich in petroselinic could also be used in the production of margarines without the need for hydrogenation. In addition, petroselinic acid can be chemically processed to lauric acid and adipic acid. Adipic acid is used in nylon manufacture (Töpfer *et al.*, 1995). Oil seed crops do not synthesize petroselinic acid although it is abundant in the seeds of carrot and coriander. Synthesis of petroselinic acid starts with the desaturation of palmitic acid (C16:0) by Δ^4–palmitoyl-ACP desaturase. The hexadecaenoic acid (C16:1Δ^4) is then elongated to petroselinic acid by a two-carbon addition. A coriander embryo cDNA expression library was screened with antibody prepared against stearoyl-ACP desaturase (Δ^9-desaturase) from avocado (Shanklin and Somerville, 1991; Cahoon *et al.*, 1992). The sequence of one of the positive clones identified by this method was similar to, yet distinct from, that of Δ^9-desaturase. A full-length cDNA selected from the library with this clone was expressed in tobacco callus leading to the production of Δ^4-hexadecaenoic acid and petroselinic acid. Petroselinic acid was present at about 4% of total fatty acids (Cahoon *et al.*, 1992). Thus, the transgenic plant cells were capable of elongating hexadecaenoic acid, once formed, to petroselinic acid. However, studies in coriander indicate that the formation of high levels of petroselinic acid in seed TAGs involves the action of a specific ketoacyl-ACP synthase and a specific petroselinoyl-ACP thioesterase (Dormann *et al.*, 1994; Cahoon and Ohlrogge, 1994). Thus,

engineering high levels of petroselinic acid in the seed oil of crops like rapeseed will probably require the isolation of these genes and their coordinated expression with the Δ^4-palmitoyl-ACP desaturase.

Another factor that can influence the value of an oil is the presence of hydroxylated fatty acids. Oils with a high content of hydroxylated fatty acids have uses as plasticizers and wetting agents (Mackenzie, 1995). Such oils are also useful because of their high viscosity (Mackenzie, 1995). The fatty acid content of castor oil is between 85 and 90% ricinoleic acid (12 hydroxy C18:1 Δ^9) (Töpfer *et al.*, 1995). This oil is produced from the seed of *Ricinus communis* (castor bean) and is the only commercial source of hydroxylated fatty acid. However, castor bean is a less than ideal crop with limited climatic adaptation. It also produces a number of very toxic compounds (Töpfer *et al.*, 1995). Members of the genus *Lesquerella* also produce hydroxylated fatty acids. They may serve as precursors for nylon production including lesqueroleic acid (14 hydroxy C20:1 Δ^{11}), auricoleic acid (14 hydroxy C20:2 $\Delta^{11,17}$), and densipoleic acid (12 hydroxy C18:2 $\Delta^{9,15}$) (Hayes *et al.*, 1995). No member of this genus has been adapted for agriculture. Thus modifying oil seed crops to produce hydroxylated fatty acids is another goal of agricultural biotechnology.

Ricinoleic acid is synthesized by hydroxylation of oleic acid. Biochemical and physiological studies suggested that the fatty acid hydroxylase involved in catalysing the reaction is very similar to fatty acid desaturase enzymes (Harwood, 1996; van de Loo *et al.*, 1995). It was therefore predicted that the hydroxylase protein would possess certain amino acid sequence motifs (van de Loo *et al.*, 1995). An EST database was derived from an *R. communis* embryo cDNA library and screened for the presence of these motifs. Clones identified by this method were expressed in tobacco plants under the control of the CaMV 35S promoter. In some cases this led to the production of low levels of ricinoleic acid in transgenic tobacco seeds (0.1% of seed fatty acids) (van de Loo *et al.*, 1995). The identified hydroxylase gene was subsequently introduced into *Arabidopsis* either under the control of the CaMV 35S promoter or the seed specific napin promoter. In both cases the transgenic *Arabidopsis* seed oil contained significantly higher levels of ricinoleic acid than were seen in tobacco. In addition, these oils also contained significant levels of lesqueroleic acid and densipoleic acid as well as a trace of auricolic acid. Constructs using the napin promoter gave the highest levels with ricinoleic acid accounting for up to 8% of seed fatty acids. Hydroxylated fatty acids taken together accounted for up to 17% of seed fatty acids (Broun and Somerville, 1997). The exact route of synthesis for the additional hydroxylated fatty acids is not yet clear.

There are many more novel fatty acids that would be desirable to introduce into oilseed crops. Epoxy fatty acids are particularly good candidates. They are used in epoxy coatings and plasticizers and also have potential for use in paints, lubricants adhesives and insecticides (Budziszewski *et al.*, 1996). *Vernonia galamensis* and *Euphorbia lagascae* produce seed oil that contains high levels of vernolic acid (12,13 epoxy C18:1 Δ^9) (Pascual and Correal, 1992; Thompson *et al.*, 1994). In *Euphorbia*, synthesis of this epoxy fatty acid is catalyzed by an epoxygenase that uses linoleic acid as a substrate (Bafor *et al.*, 1993).This would seem to be an ideal gene to target for expression in oilseed crops.

The above examples illustrate a number of points with respect to modifying the

levels of fatty acids in seed oils. First of all, while one might be concerned about possible deleterious effects from changes in fatty acid metabolism, the results to date indicate that oilseed plants in general tolerate a wide range of fatty acid profiles. It seems likely that, in most cases, it will be possible to achieve the desired oil composition. For many applications this will mean producing oils with the highest possible content of a given fatty acid. Theoretically, as long as an altered fatty acid composition has no adverse biological consequences, one might expect to attain relative levels of engineered fatty acids comparable to or approaching the highest, naturally occurring relative levels of seed storage fatty acids. For example, ricinoleic acid (12-hydroxyoleic acid) represents 90% of total fatty acid in the castor bean (Töpfer *et al.*, 1995), and petroselinic acid (C18:1 Δ^6) accumulates up to 85% of total fatty acids in seeds of various members of the *Umbelliferae*, Apiaceae, *Araliaceae*, and *Garryaceae* families (Ohlrogge, 1994). The highest reported single fatty acid level in a genetically engineered oilseed crop is 88% oleic acid in a soybean line in which the seed specific Δ^{12}-desaturase gene is transgenically co-suppressed (Kinney, 1997). However, it is clear that in many cases attaining the highest relative level of that novel fatty acid may require the manipulation of multiple genes, as illustrated by the efforts noted above to produce high lauric, high erucic, and high petroselinic acid producing crops.

Genetically engineered changes in seed oil quantity

The commercial production of novel fatty acids in plants will be feasible only if they can be produced in sufficient quantities to make their harvesting, pressing, processing, and purification economically competitive with current sources. Although there are many variables in this regard – from segregating and storing different, novel oil-containing seeds that are outwardly indistinguishable, to finding markets for by-products – it will be critical to genetically engineer the plants for maximum production, in absolute as well as relative terms, of the desired fatty acid.

Regarding the absolute yield that oilseed crops may be engineered to produce, the observation of seed oils comprising 60% to 75% of the dry weight of some seeds, e.g. candlenut (57%–69%), oiticica (62%), tea seed oil (*Thea sasanqua* 56%–70%), and ucuhaba (65%–76%) (Padley *et al.*, 1994), indicates that there is no biological barrier to attaining such levels in transgenic crops. Achieving this may require derepressing or up-regulating the rate limiting step(s) of fatty acid and TAG synthesis. The first committed step in fatty acid biosynthesis, i.e. the carboxylation of acetyl-CoA by acetyl coenzyme A carboxylase (ACCase), has traditionally been identified as a rate limiting step in fatty acid biosynthesis. This reaction occurs in the plastid catalysed by a heteromeric, 'prokaryotic', form of the enzyme composed of four protein subunits. In tobacco suspension cells, Shintani and Ohlrogge (1995) have demonstrated that feed back inhibition is involved in the regulation of plastid ACCase. A second, homomeric form of the enzyme, HO-ACCase, is composed of a dimerized >220 kD polypeptide and is localized to the cytoplasm. The malonyl-CoA it produces is used for fatty acid chain elongation, forming the VLCFA.

To increase native fatty acid yields in *B. napus* seed oil, Roeseler *et al.* (1997) overexpressed *Arabidopsis ACC1*-encoded HO-ACCase fused to a chloroplast transit peptide from RUBISCO small subunit. This allowed them to engineer a single gene

rather than the four genes encoding the heteromeric plastid ACCase. When the transit peptide-HO-ACCase fusion was expressed in rapeseed under the control of the seed-specific napin promoter, Roesler *et al.* (1997) found that the HO-ACCase was transported into the chloroplast in an enzymatically active form. Based on *in vitro* enzyme assays of isolated plastids the authors estimated that embryos expressing the transit peptide-HO-ACCase fusion possessed one- to two-fold higher ACCase activity in their plastids. These transgenic plants also exhibited increased oleic acid content in the seed TAGs and ~5% greater seed oil amounts on a dry weight basis than control plants. The authors suggested that the plastidial localization of the cytosolic HO-ACCase may have protected it from normal cytosolic protein turnover and the HO-ACCase may be unaffected by the regulatory mechanism controlling the activity of the plastidial ACCase. If so, both of these features would benefit this approach to boosting seed oil production.

One other recently published study reported increases in the relative and absolute yields of fatty acids in *Arabidopsis* and *Brassica*. Zou *et al.* (1997) tested the effects of expression of a yeast LPAAT on the accumulation of erucic acid (C22:1) in *Arabidopsis* and *B. napus* cv Hero. Zou *et al.* (1997) confirmed that the *Saccharomyces cerevisiae SLC1* gene encodes a protein with LPAAT activity and demonstrated that the variant *SLC1-1* gene product had a greater preference for very long chain (VLCFA) fatty acids than the *SLC1* gene product. In both plant species tested, constitutive expression of the yeast *SLC1-1* gene increased the proportion of VLCFA fatty acids at the *sn*-2 position of TAGs, increased the relative proportion of VLCFA fatty acids among total TAG, and, surprisingly, increased the total amount of TAG per seed. In *B. napus*, the LPAAT activity was elevated from two-fold to five-fold (measured for 18:1-CoA). Erucic acid (C22:1) increased from an average of 45% of total fatty acid in controls to as high as 56% of total fatty acid, while at the *sn*-2 position in particular, erucic acid content increased slightly, from <1% in control plants to 4.1% and 3.5% in the two transgenic rapeseed lines reported for this trait. With one exception, the oil content of several transgenic *B. napus* lines increased by 8% to 22%, or from an average of ~34% oil by dry weight in controls up to 41% oil by dry weight in the transgenics.

The increased oil content resulting from the expression of the yeast *SLC1-1* gene contrasts with the results of Lassner *et al.* (1995) and Brough *et al.* (1996) in which *B. napus* expressing a meadowfoam LPAAT did not exhibit increased total oil amounts, although *sn*-2 erucic acid content of the oils increased. Zou *et al.* (1997) proposed that the *sn*-2 acylation to form diacylglycerophosphate may represent a regulated step in plant TAG synthesis, and that the yeast LPAAT is not subject to that regulation. This is similar to the proposed mechanism to explain the elevated ACCase activity when the HO-ACCase is targeted to the plastid and may prove to be a useful general strategy to increase the activity at regulated steps in fatty acid and TAG synthesis.

It is clear that there are multiple strategies to increase seed oil content, both in relative terms and absolute terms. Although some of these strategies will yield only incremental increases, such increases are sometimes sufficient to improve the economics involved in production. Ultimately, the combination of various strategies should yield transgenic oilseed crops that approach or supersede the examples of natural plant oils exhibiting high relative or absolute amounts of specific fatty acids.

Expectations and limitations

Significant advances have been made in understanding the complex pathways involved in plant fatty acid and TAG synthesis and modification, in obtaining clones of the genes involved, and in refining the molecular genetic tools with which to genetically manipulate plants. Efforts to produce a seed oil with a modified fatty acid composition were realized with the first commercial crop of high lauric acid rapeseed in 1995 (Murphy, 1996). During the next ten years, the rate of new field trials of transgenic plants will accelerate and several more commercial crops with altered seed oil compositions should become available.

The economic feasibility of replacing an oil or hydrocarbon with a bioengineered fatty acid will depend on the present source and the application. Many of the engineered fatty acids currently in development would replace plant-produced fatty acids, e.g. substituting high-lauric rapeseed oil for coconut and palm kernel oil. In such a case, the processing requirements may be similar. The ability to produce these oils in a temperate climate would have to be balanced with the cost of importing tropical oils having a higher lauric acid content. In the case of GLA production, engineered rapeseed oil with a GLA content of just 10% to 15% is likely to be favored over oil from evening primrose, with a 7% to 14% GLA content (Hudson, 1984), due to the agronomic advantages of *Brassica* over *Oenothera*.

In contrast, substituting a plant-produced, bioengineered fatty acid for a petroleum-derived hydrocarbon will have different economic criteria. Use as a chemical feedstock, will likely require hydrolysis of the TAG and purification of the desired fatty acid. The associated cost may be minimized, though not eliminated, if relative yields of the desired fatty acid can be boosted to 85% to 90%. As petroleum supplies diminish, the economics of production of bioengineered, substitute fatty acids will become more favourable. In the long term, certainly, oils and fatty acids obtained from renewable resources will be favored.

Manipulations of fatty acid metabolism have the potential of affecting not only the fatty acid composition of the seed storage oils, but other fatty acid-containing lipids as well. As a general rule, the fatty acid composition of oils reflects that of the membrane lipids. However, there exist many diverse genera and species of plants that synthesize unusual fatty acids for exclusive deposition in the seed storage TAGs (van de Loo *et al.*, 1993). Examples include erucic acid (C22:1)-rich rapeseed oil and the high ricinoleic (C18-OH) acid content of castor bean oil. How these plants regulate the relative fatty acid compositions of their various lipids is not well understood. Therefore, the effects which alterations in the composition of the fatty acyl-CoA pool may have on plant viability or vigor are difficult to predict. For example, high-oleate sunflower obtained by mutagenesis and breeding were suitable for commercial growing (Urie, 1985), but high oleate *fad2*-mutant *Arabidopsis thaliana* were found to be cold sensitive and showed poor germination at low temperature (6°C) (Miquel and Browse, 1994), an undesirable trait in oilseed crops.

Additional practical and biological considerations will affect the economics and ultimate success of producing novel fatty acids in oilseed crops. Practical considerations include government regulation, consumer acceptance of genetically engineered products, and finding markets and applications for the by-products of oilseed production, such as seed meal.

Also, the infrastructure for handling novel seed oil crops will have to respond to multiple small harvests, which will require capital expenditures. Novel seed crops may need to be cultivated under contract so that the various novel oils produced in indistinguishable plants can be managed and segregated. Seed pressing facilities will need to modify their equipment and procedures for processing distinct novel oil-producing harvests. And, just as climate and soil condition can affect the seed oil composition of non-genetically engineered crops, so it will likely affect the oil composition of engineered crops. This may require assay of every harvest prior to payment. A potential difficulty that has been noted with classically bred lines of rapeseed is that the growth of volunteers can reduce the yield (Walker, 1995). Growers will need to take extra precautions to minimize this problem.

Although strategies such as using seed-specific promoters are intended to avoid detrimental effects to the growth of the plants, extreme levels of some novel fatty acids may produce nonviable seeds. Such a finding may necessitate the use of hybrid seed, where each parent provides an engineered trait which, in combination in the hybrid, produces seed oil possessing the desired fatty acid composition. Also, if the crop for a particular fatty acid is based on one or only a few transgenic lines, the lack of genetic diversity may make the crop susceptible to disease and pests. Protection from this could be provided by extending the production base to multiple plant species, such as soybean, sunflower, peanut, and cottonseed. Furthermore, by having this important technology in several cultivars suited for different climates, novel fatty acid production in oilseed crops would be on a firm foundation.

Novel fatty acid production in plants holds the promise of generating fatty acids and oils for improved human nutrition, chemical feedstocks, consumer products, pharmaceuticals, and for specialized industrial applications. A great benefit of this is that plants represent a renewable resource. With continuing advances in specialized fatty acid engineering and the eventual depletion of fossil hydrocarbons, plant-produced oils will be positioned to supplement many petroleum-derived products.

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