

## Enhancing Plant Seed Oils for Human Nutrition

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### SEED OILS AND THE WESTERN DIET

The age of true designer plant oils has arrived. Using the tools of biotechnology, it is now possible to modify the fatty acid content of oilseed plants to change the relative abundance of individual fatty acids in seed oil for health purposes or to produce nutritional fatty acids not normally found in crop plants.

Fats and oils are an essential part of the human diet, and as a species we consume a lot of them, on average 25 kg per person per year, mostly (80%) from plant sources (Subar et al., 1998; USDA, 2008). Since at least the 1930s, it has been realized that not all fats are created equal in terms of human nutrition. Studies in rats determined that certain polyunsaturated fatty acids (PUFAs) derived from plants, namely, linoleic and linolenic acids, were necessary to sustain life in animals (Burr et al., 1932). Other unsaturated fatty acids, such as oleic and eleostearic acids, could not substitute for these omega-6 and omega-3 PUFAs. It is now known that while animals, including humans, can make monounsaturated fatty acids from sugar, they do not have the necessary desaturases to convert oleic into linoleic and linolenic acids and must derive them from their diet (Wallis et al., 2002). These PUFAs, it turns out, are further converted in humans to arachidonic acid (ARA) and eicosapentaenoic acid (EPA), which are in turn precursors to important molecules that regulate the immune response and to docosahexaenoic acid (DHA), an important component of membranes, especially in the brain (Tocher et al., 1998).

ARA (an omega-6 fatty acid) and EPA (an omega-3 fatty acid) play a yin-yang type of role in human metabolism; ARA is a precursor molecule for the proinflammatory molecules necessary for an immune response and EPA provides balance by being the precursor for antiinflammatory molecules. The human body is not very efficient at synthesizing very-long-chain PUFAs and it has been postulated that this is because humans have evolved with diets abundant in fish and game meats, which are quite rich in these types of fats (Cordain et al., 2000, 2005). Modern grain-fed meat and grain-rich diets are particularly abundant in omega-6 fatty acids, and it is thought that a

deficiency of omega-3 fatty acids, especially the EPA and DHA found in fish oils, can be linked to many of the inflammatory diseases of the western diet, such as cardiovascular disease and arthritis (Hibbeln et al., 2006). DHA has been recognized as being vitally important for brain function, and a deficiency of this fatty acid has been linked to depression, cognitive disorders, and mental illness (Conklin et al., 2007).

Over the past 50 years, various other lipid components of the foods we eat, such as cholesterol as well as saturated and trans fatty acids, have been correlated with an increased risk of cardiovascular disease (Van Horn et al., 2008). These correlations are usually confounded by other lifestyle aspects, such as smoking, and by genetic differences among individuals. Nevertheless, there is a general consensus that the healthiest oils are those rich in monounsaturated fatty acids, such as olive oil, and those rich in long-chain omega-3 fatty acids, such as fish oils (Hardin-Fanning, 2008). Olive oil, although stable, is too expensive for many food applications, and the need for heat-stable oils has been addressed in the past through the use of chemical hydrogenation of common vegetable oils such as soy (*Glycine max*) and canola (*Brassica napus*). The desire to replace hydrogenated oils in these food applications, and thus eliminate trans fatty acids from the diet, has led to the development of new vegetable oils that are inexpensive, stable during cooking, and rich in monounsaturated fatty acids.

The increasing popularity of fish oils has placed a huge demand on natural ecosystems, and the current demand cannot be met in a sustainable way. The nutritional needs of humans for long-chain omega-3 fatty acids can also be addressed in a safe and sustainable manner by the genetic modification of common oilseed crops, such as soybean.

### Healthy Seed Oils for the World's Food Supply

The most abundant plant oils are palm (*Elaeis guineensis*), soy, and rapeseed (*Brassica napus*); together, these account for 75% of the world vegetable oil production. These oils are used in the preparation of many kinds of foods, both for retail sales and in the restaurant industry. Palm oil has the advantage of being very resistant to oxidation, and thus resistant to off-flavor production, during the cooking process. Unfortunately, palm oil is very rich in saturated fatty acids, the consumption of which has been linked to cardiovascular disease in humans (Vega-Lopez et al., 2006). Regular soy and rapeseed oils are usually hydrogenated for high-

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temperature cooking, and this results not only in an increased stability but also the formation of trans fatty acids, which have also been linked to cardiovascular disease (Mozaffarian and Willett, 2007). Both saturated and trans fatty acid also provide solid-fat functionality to cooking oils, which is very important for baking. For this reason, baked goods are second only to animal fats as a source of saturated fats and are a major source of trans fats in the human diet (Dupont et al., 1991; Subar et al., 1998).

For a number of years, food scientists have been looking for healthy replacements for palm and hydrogenated oils that are similarly resistant to oxidation and also provide the desired solid fat functionality. Oils rich in oleic acid meet some of these needs, and high oleic mutants, with an oleic content ranging from 60% to 90%, have been developed in corn (*Zea mays*), peanut (*Arachis hypogaea*), canola, and sunflower (*Helianthus annuus*). These mutants all have defective *FAD2* genes (Pérez-Vich et al., 2002; Patel et al., 2004; Hu et al., 2006; Beló et al., 2008). The *FAD2* gene encodes an omega-6 desaturase that converts oleic acid to linoleic acid. The problem with mutants is that the fatty acid content of nonseed organs is also changed, often resulting in agronomic issues (Miquel and Browse, 1994). In addition, many plants have a number of *FAD2* genes, all of which contribute to seed linoleic content (Tang et al., 2005). This means that development of mutant lines with useful oleic acid contents often requires combining several mutant loci and provides a breeding challenge (Mikkilineni and Rocheford, 2003).

The *FAD2* gene was cloned from many crop plants more than 10 years ago and the efficacy of seed-specific *FAD2* gene silencing to increase seed oleic acid content was demonstrated (Kinney and Knowlton, 1998). After many years of development and safety testing, one of the first genetically modified crops to directly benefit the consumer, a high oleic soybean line, will be commercialized in 2009 (Reuters, 2008).

High oleic soybean oil also has the added health benefit of having a saturated fatty acid content that is reduced by 20%. For some uses, such as salad oils, it is desirable to reduce saturated fatty acid as much as possible. It has been shown that the FATB class of acyl:ACP thioesterases control the release of saturated fatty acids into the cytoplasm, making them available for oil biosynthesis (Dörmann et al., 2000). Seed-specific silencing of *FATB* genes in soybean, for example, leads to a major reduction in total saturated fatty acids, from about 15% to <6% (Kinney, 1996). When this gene is combined with a silenced *FAD2*, oleic acid contents of >90% have been observed (Buhr et al., 2002). It is conceivable that soybean lines with very low saturated fatty acids and very high oleic acid contents could become the next generation of commodity oil.

For some food applications, especially baking, a solid fat is needed to provide a healthy replacement for animal fats, palm oil, and hydrogenated vegetable oils. Plant oils rich in stearic and oleic acids could provide this important function without compromis-

ing human health (Dirienzo et al., 2008). The combination of stearic and oleic acids confers a solid fat functionality, and stearic acid, unlike other saturated fatty acids, is not thought to play a significant role in cardiovascular disease (Mensink, 2005).

There are a number of potential molecular strategies for increasing the stearic acid content of plant oils. Soybeans seeds have several delta-9 desaturase (*SAD*) genes expressed in their seeds, and silencing one of these genes (*SAD3*) in a seed-specific manner results in soy oils containing 20% to 30% stearic acid when compared with just a few percent in commodity soy (Booth et al., 2006). Another approach is to express a thioesterase with a high specificity for stearic acid. One such thioesterase, normally involved in the production of stearate in the seeds of mangosteen (*Garcinia mangostana*), in which stearate is the predominant fatty acid (45%–50% of total fatty acids), was expressed in transgenic canola seeds with reported stearate contents of 20% to 30% (Hawkins and Kridl, 1998). Similar increases in stearate have been observed when this gene was expressed in soybean seeds (Kridl, 2002).

To replace the solid-fat functionality of many hydrogenated oils, the stearate needs to be a major component of the seed oil, approximately 30% of the total fatty acids (Lumor et al., 2007). As one might imagine, living oilseeds do not take too kindly to being stuffed full of margarine, and germination problems are often seen when the stearic acid content of seeds is elevated to these levels (Roberts et al., 2006). Most seeds, however, are tolerant of high oleic acid contents, and by combining the silencing of *SAD3* with silencing of *FAD2* in soybean, it has been possible to make viable seeds that have an oleic content in the 50% to 60% range with stearic content approximately 15% to 20% (Booth et al., 2002). Improved functionality can also be obtained by combining stearic oils with low linolenic acid oils (Dirienzo et al., 2008). Healthy oils from these types of seeds have the potential to replace solid fats in a wide range of baking and heavy-duty frying applications and are currently in the research pipelines of several major companies (Booth et al., 2002; Dirienzo et al., 2008).

The introduction of modified seeds oils such as high oleic acid soybean oil and high oleic/high stearic oils will go a long way to removing the undesirable saturated fatty acids and trans fatty acids from the human diet and help promote cardiovascular health (Lichtenstein et al., 2006).

#### Renewable Sources for Some Important Fatty Acids

In addition to containing trans fatty acids and excessive amounts of certain saturated fats, the western diet is often also deficient in omega-3 (n-3) LCPUFAs (for long-chain PUFAs), the kind of fatty acids normally found in fish oils and some organ meats (Hibbeln et al., 2006). These are made in the body from linolenic acid (18:3 n-3) in the diet, but most vegetable oils are rich in the omega-6 PUFA linoleic acid (18:2 n-6) and are a poor

source of 18:3 n-3. The widespread use of plant seed oils and grain-fed meat during this century has increased the n-6 intake of humans at the expense of n-3 (Cordain et al., 2005). This reduction in n-3 intake by humans has been cited as one of the possible deleterious effects of the modern western diet on health (Hibbeln et al., 2006). Not surprisingly then, consumption of n-3 LCPUFAs can reduce the risk of cardiovascular disease. Indeed, clinical intervention studies with fish oil-derived n-3 LCPUFAs have been shown to dramatically decrease the risk of mortality in patients with cardiovascular disease (Kris-Etherton et al., 2002). These fatty acids have also been used as nonsteroidal antiinflammatory supplements and as therapeutic agents for autoimmune diseases (Simopoulos, 2002).

The benefits of long-chain omega-3 fatty acids have not gone unnoticed by health-conscious consumers, resulting in an increased demand for fish and fish oils, as well as LCPUFA-containing foods. Because landings of wild-caught fish have been at a plateau for the past 10 years, the rising demand for fish is being met by an increased supply of farmed fish (Pauly et al., 2005). Most fish, however, obtain and concentrate n-3 LCPUFAs from the microorganisms in their diet. For farmed fish to contain an abundance of n-3 LCPUFAs, they are fed n-3 LCPUFAs, usually in the form of fish oil. Currently, most of the wild-caught fish landings in the world are used to provide food to farmed fish, hardly a sensible or sustainable situation (Naylor et al., 2000; Dalton, 2004).

There is strong need then for a land-based, renewable, and sustainable source of n-3 LCPUFAs for the human diet.

#### Transgenic Oilseed Plants as Sources of n-3 Fatty Acids

Because ALA from plant sources is converted by the body to EPA, consuming a high proportion of n-3 oils, such as flax oil, may be a useful dietary strategy. The first step in the human EPA pathway, the conversion of ALA to stearidonic acid (SDA; a reaction catalyzed by a delta-6 desaturase), is rate-limiting in humans (Burdge et al., 2002; James et al., 2003). It has been shown that ALA in the diet is converted to EPA, with only a fraction of the efficiency of SDA in healthy subjects (James et al., 2003; Miles et al., 2004). Further, delta-6 activity has been shown to decline with age (Bourre et al., 1990) and with a significant number of clinical conditions, such as cancer, making utilization of dietary ALA even less efficient (Lane et al., 2003).

Some plants, such as *Echium* (from the borage family), hemp (*Cannabis* sp.), and black currant (*Ribes nigrum*), produce SDA in their seed oils. These oils are currently marketed and sold for their proposed health benefits (Barre, 2001), but in addition to the problem that they also contain a relatively high abundance of n-6 fatty acids, their cultivation is carried out on a scale that is relatively small with poor yields, making the oils produced very expensive. However, it is possible to take the delta-6 desaturase gene from one of these

species and express it in plant that does not normally produce GLA or SDA.

An early example of this kind was the constitutive expression of a *Synechocystis* delta-6 desaturase in tobacco (*Nicotiana tabacum*; Reddy and Thomas, 1996). These investigators were able to record 1% GLA and 3% SDA in the leaves but no detectable amounts in the seeds of these transgenic plants. Higher concentrations in leaves (13% GLA, 10% SDA) were observed when a borage delta-6 desaturase gene was constitutively expressed in tobacco (Sayanova et al., 1997), but only a trace (2% GLA) of new fatty acid was seen in the seed.

The use of seed-specific promoters resulted in more meaningful amounts of modified fatty acids in the seed oil. Delta-6 desaturase genes from borage, *Pythium irregulare*, and *Saprolegnia diclina* have been seed specifically expressed in soybean, *Brassica juncea*, and safflower (*Carthamus tinctorius*), with resulting GLA contents in the seed oil as high as 70% of total fatty acids (Hong et al., 2002; Qiu et al., 2002; Sato et al., 2004; Knauf et al., 2006). Because most oilseed crops are predominantly omega-6 fatty acid-containing plants, it is necessary to express an omega-3 desaturase gene along with the delta-6 gene to produce SDA. Expression in soybean seeds of a delta-6 desaturase gene from *Mortierella alpina* along with an omega-3 desaturase resulted in an SDA content of >20%, and when a borage delta-6 desaturase gene was expressed with an *Arabidopsis* (*Arabidopsis thaliana*) omega-3 desaturase gene in soybean seed, SDA contents of as high as 30% were observed (Eckert et al., 2006).

Soybean oil containing SDA is currently in commercial development and is likely to be the first transgenic food oil containing n-3 PUFAs available to consumers (Monsanto, 2008).

#### Dietary EPA and DHA: Cutting Out the Middle Fish

SDA is an important stepping stone on the path to EPA in humans, although it needs to be consumed in larger amounts to have the same efficacy as EPA (Miles et al., 2004). In addition, SDA does not appear to be converted by the body to DHA, an n-3 LCPUFA that is very important for cognitive function (James et al., 2003). Even with a potential source of SDA in the diet, we will still need a source of DHA. Algal oils rich in DHA can be produced through a fermentation process, but this is a very expensive process, and the use of DHA from these sources is currently limited to infant formula and medical foods (Boswell et al., 1996). Modified plant oils are an attractive alternative. Consumption of fish oils provides both EPA and DHA, and thus the ideal transgenic plant oil would contain both these fatty acids in a ratio similar to that found in fish.

Most of the wild-caught fish we eat obtain their n-3 LCPUFAs from eating smaller fish, which in turn obtain their n-3 LCPUFAs from the eukaryotic marine microbes in their diet. These microbes, which include algae, fungi, and protists, synthesize their own EPA,

DHA, or often both. Three distinct pathways for n-3 LCPUFA synthesis have evolved in these organisms (Wallis et al., 2002). Some marine fungi, such as those from the genus *Saprolegnia*, make n-3 LCPUFAs by a pathway analogous to that found in humans (Pereira et al., 2004a). The pathway begins with a delta-6 desaturation of ALA, followed by an elongation reaction and a delta-5 desaturation to EPA. Certain protists, such as some *Euglena* species, begin EPA synthesis by elongating ALA with a delta-9 elongase reaction, followed by a delta-8 desaturation and then a delta-5 desaturation common to both pathways (Wallis and Browse, 1999). In both pathways, EPA is converted to DHA by a delta-5 elongation followed by a delta-4 desaturation (Pereira et al., 2004b). This is in contrast to humans, in which the pathway for DHA synthesis from EPA is a complex, four-step, multi-organelle affair (Wallis et al., 2002).

A third route to n-3 LCPUFAs has been discovered in some marine algae, such as *Schizochytrium*. In these organisms, DHA is synthesized by a polyketide synthase (PKS) complex. This type of pathway is also found in some prokaryotic marine microbes, such as *Shewanella*, which also synthesize EPA or DHA (Metz et al., 2001).

These marine organisms provide a useful source of genes for recreating these pathways in land plants and thus for providing a dietary source of EPA and DHA that completely by-passes fish consumption.

### Engineering New Fatty Acid Biosynthetic Pathways into Crop Plants

An early report of recreating the microbial synthesis of n-3 LCPUFAs in a plant described assembly in nonseed tissue of a delta-9 elongase pathway to generate EPA. The genes for this pathway were obtained from a grab-bag of marine organisms, including the microalga *Isochrysis galbana* (delta-9 elongase), the protist *Euglena gracilis* (delta-8 desaturase), and the microbial fungus *M. alpina* (delta-5 desaturase). The pathway was expressed in nonseed tissue of the model plant *Arabidopsis* (Qi et al., 2004), and an EPA content in leaves of about 3% was observed. The leaves also contained a similar amount of the n-6 LCPUFA ARA, because the enzymes expressed by these genes are effective on both n-3 and n-6 fatty acid substrates. As in the SDA work described above, expressing an additional n-3 desaturase might help move things away from n-6 fatty acids to n-3 fatty acids.

In addition to EPA in these transgenic *Arabidopsis* leaves, certain pathway intermediates and fatty acid by-products were also observed. These by-products included sciadonic acid and juniperonic acid resulting from competition between the delta-8 and delta-5 desaturases for elongated LA or ALA. Interestingly, the various microbes that make LCPUFAs by this pathway do not produce significant levels of sciadonic acid and juniperonic acid, probably because there is a more efficient flux through the endogenous pathway.

This study was a groundbreaking first step toward producing EPA in plants, but production of 3% in *Arabidopsis* leaves is hardly a commercially viable alternative to fish oil. In addition, the ARA and by-product content would also need to be reduced for commercial food oil.

The first demonstration of heterologous expression of an EPA pathway in a plant seed is described in a published patent application (Kinney et al., 2004). In this study, a delta-6 fatty acid pathway from *Mortierella* was expressed in soybean embryos; in addition, n-3 desaturases from *Arabidopsis* (Yadav et al., 1993) and from the freshwater mold *S. diclina* (Pereira et al., 2004b) were included in the gene set. EPA contents in seeds as high as 10% were observed, and this could be increased to 20% by replacing the delta-6 desaturase gene from *Mortierella* with one from *Saprolegnia*. To date, this is the highest abundance of EPA achieved in any plant tissue. These transgenic soybean seeds contained only trace amounts of ARA due to the presence of the highly efficient *Saprolegnia* n-3 desaturase that has a substrate preference for ARA, converting it to EPA (Pereira et al., 2004b).

Quite unpredictably, the soybean seeds also contained the DHA precursor n-3 docosapentaenoic acid (DPA). Because no additional delta-5 elongase had been added to the mix (and endogenous elongation factors were not responsible), the DPA must have been formed from the activity of the *Mortierella* delta-6 elongase on EPA. This was quite unexpected, because this elongase had no delta-5 EPA-elongating activity when previously expressed in yeast (Parker-Barnes et al., 2000). When a delta-4 desaturase gene from *Schizochytrium aggregatum* (Mukerji et al., 2002) was added to this pathway in soybean embryos, over 3% DHA was observed in the embryo oil. This is the highest DHA content seen in any plant oil to date.

In a later study, the *Arabidopsis* n-3 desaturase was replaced with an n-3 desaturase from *Fusarium moniliforme* (Damude et al., 2006). This desaturase has been shown to convert oleic acid to LA and LA to ALA and is very active when expressed in soybean seeds (Damude et al., 2006). Seeds expressing this desaturase have been shown to have a total n-3 fatty acid content of over 70%. When combined with the *Saprolegnia* desaturase, the total n-6 content of EPA soybean seeds was negligible.

A more recent attempt (Chen et al., 2006) at expressing the *Mortierella* delta-6 desaturase pathway in soybean seed to produce ARA (the endogenous n-3 desaturase was downregulated in these experiments) resulted in only trace amounts ARA (0.5%–0.8% of seed oil fatty acids). The authors suggested that their use of the same promoter multiple times caused poor expression of the transgenes, although they show no data to support this hypothesis. However, in the soy EPA project described above (Kinney et al., 2004), in events where the added n-3 desaturases were not functional, ARA contents of up to 20% were observed in soy seeds using the same *Mortierella* delta-6 pathway.

Trace amounts of EPA (<2%) were also demonstrated in transgenic flax seeds, a relative abundance much less than that observed in soy (Abadi et al., 2004). The delta-6 pathway genes, from the diatom *Phaeodactylum tricorutum* and the fungus *Phycomitrella patens*, were expressed under the control of seed-specific promoters, and the poor end-product accumulation was attributed to problems with pathway flux rather than poor gene expression. Because the transgenic flax seeds accumulated large amounts of GLA and SDA but not the elongated products of these fatty acids, the authors suggested that elongation GLA and SDA was limiting EPA accumulation.

While GLA and SDA are formed from LA and ALA when they are part of a phospholipid, they are elongated as acyl-CoAs. In the transgenic flax seeds, it appeared that the GLA and SDA were stuck in the membranes and were not being exchanged into the acyl-CoA pool where they could be elongated (Abadi et al., 2004). These fatty acids were being incorporated into the seed triacylglycerol by direct conversion of the phosphatidylcholine containing the GLA/SDA into diacylglycerol, which in turn was converted to triacylglycerol.

This observation led the authors to search for an acyltransferase that might facilitate the exchange of LCPUFA from phospholipids into the acyl-CoA pool, resulting in the identification of a lysophosphatidyl acyltransferase (LPAT) from an uncharacterized species of the marine fungus *Thraustochytrium* (Wu et al., 2005). This new gene was included in a more recent attempt to produce EPA in *Brassica juncea* seeds (Wu et al., 2005) by using a delta-6 pathway and the methods of pathway assembly previously shown to be effective in soy (Kinney et al., 2004). As in the soybean study, addition of an n-3 desaturase with a substrate preference for ARA was shown to be important for optimizing EPA content of the seed oil (up to 15% of total fatty acids). Curiously, the addition of the *Thraustochytrium* LPAT did not significantly boost flux through the pathway into EPA, suggesting the pathway assembly techniques described in the soy study were more important for achieving target amounts of n-3 LCPUFAs.

In the same *Brassica* study, the authors were able to produce small amounts of DHA (1.5% of total fatty acids) by the addition of an EPA elongase from a fish *Oncorhynchus mykiss* and a delta-4 desaturase from a *Thraustochytrium*, the successful use of a fish gene demonstrating that the source of transgenes for plant production of n-3 LCPUFAs need not be restricted to marine microbes. Indeed, some investigators have suggested that using acyl-CoA desaturases, such as those found in fish LCPUFA pathways, may help improve EPA synthesis in transgenic plants by eliminating the need for acyl chains to move between phospholipid and acyl-CoA pools for various desaturation and elongation steps and thus improve flux into EPA (Domergue et al., 2005; Robert et al., 2005). This hypothesis was tested by assembling a delta-6 desaturase pathway in *Arabidopsis* seeds using a bifunctional delta-6/delta-5 desaturase from a species of zebra fish

(*Danio rerio*), which utilizes acyl-CoA substrates, along with an acyl-CoA elongase from the nematode *Caenorhabditis elegans* (Robert et al., 2005). The highest EPA content observed in the seed oil was 2.5% of total seed fatty acids. When these EPA plants were retransformed with delta-5 elongase and a delta-4 desaturase genes from *Pavlova salina*, trace DHA amounts of approximately 0.5% were observed in the seed oil.

Just as in the *Brassica* LPAT study (Wu et al., 2005), these results support the conclusion that exchange between phospholipid and acyl-CoA pools may not be the limiting factor for obtaining optimal flux into EPA in a transgenic LCPUFA pathway. Indeed, the authors conclude that the pathway assembly methods described in the soy study (Kinney et al., 2004) should be used to obtain optimal coexpression of all the pathway genes in an LCPUFA pathway (Robert et al., 2005).

### Engineering n-3 LCPUFA Polyketide Pathways into Crop Plants

An attractive alternative to assembling LCPUFA pathways with multiple enzymes, all of which must work in concert to produce EPA and/or DHA, is the concept of expressing PKS complexes in plant seeds. Most of the activities necessary for EPA or DHA synthesis by a PKS mechanism reside on a single polypeptide, theoretically making concerns and issues with coexpressing multiple new pathway genes in a seed redundant. In addition, n-3 LCPUFA oil produced from a PKS complex should be completely free from pathway intermediates and thus less of a regulatory concern for food use.

Successful expression of PKS genes in yeast and plants has been now been reported (Metz et al., 2006). This undertaking involved expressing in *Arabidopsis* three genes from a *Schizochytrium* encoding subunits of a PKS enzyme that catalyzes the synthesis of DHA from malonyl-CoA. It was also necessary to coexpress a phosphopantetheinyl transferase from the fresh water cyanobacteria *Nostoc*, which it turns out is essential for activating the ACP domains of the DHA-synthase PKS. The genes were targeted to the plastid by fusing them with a *Brassica* acyl-ACP thioesterase plastid-targeting signal.

The resulting *Arabidopsis* seeds had a DHA content of a bit less than 1% and also contained approximately 2% of a n-6 isomer of DPA. These same investigators (Metz et al., 2007) found that the DHA content of the seeds could be boosted, up to approximately 2.5% of seed fatty acids, by coexpressing the PKS/phosphopantetheinyl transferase with a *Schizochytrium* acyl-CoA synthetase (ACSI or ACSII) along with an RNAi construct to silence one or more of the acyl-ACP condensing enzyme activities of the seed (which presumably compete with the PKS for malonyl-CoA).

At this point, the transgene count is up there with the fatty acid (desaturase/elongase) LCPUFA pathways, losing some of the suggested advantages of the PKS route. Nevertheless, the successful expression of a

microbial PKS complex of any kind in a transgenic plant is a significant technical achievement in itself; persuading such a PKS to make significant amounts of DHA in a plant seed is quite a tour-de-force of bioengineering.

### The New Oilseed Plants: Sowing Seeds of Happiness

In the course of these adventures in metabolic pathway engineering, a lot has been learned about moving chains of biosynthetic activities from various marine organisms into plants and about the basic biochemistry of the enzymes themselves. It seems remarkable that the fatty acid composition of a plant seed, such as soybean, could be radically shifted so that one of its minor fatty acids, oleic acid, becomes the predominant species (greater than 80%) without affecting the viability or oil content of the seed. And yet such seed is now being grown on thousands of acres, and its oil will soon find its way into some of the foods we consume.

It is perhaps even more remarkable that the fatty acid composition of oilseeds can be radically reconfigured by advanced pathway engineering to produce vital fatty acids previously only obtainable from non-sustainable sources. The bottom line from the studies with fatty acids and polyketide biosynthetic pathways is that it is now possible to assemble complete n-3 LCPUFA pathways in transgenic oilseed plants to produce EPA and DHA for human food uses. Consumption of EPA and DHA has been found in clinical studies not only to improve cardiovascular health, but also to reduce depression, aggression, anger, and improve mental well-being. In a fascinating and entertaining survey, Reis and Hibbeln (2006) have shown that fish have been potent symbols of emotional well-being in many different cultures across thousands of years. They suggest there is a link between the positive psychotropic properties of EPA and DHA consumption and the cultural perception of fish as an emotionally healing food. We are now well on the road to new plant sources of these important, nutritional fatty acids and perhaps to a healthier, maybe even happier, human population.

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### LITERATURE CITED

- Abbadì A, Domergue F, Bauer J, Napier JA, Welti R, Zähringer U, Cirpus P, Heinz E (2004) Biosynthesis of very-long-chain polyunsaturated fatty acids in transgenic oilseeds: constraints on their accumulation. *Plant Cell* **16**: 2734–2748
- Barre DE (2001) Potential of evening primrose, borage, black currant, and fungal oils in human health. *Ann Nutr Metab* **45**: 47–57
- Beló A, Zheng P, Luck S, Shen B, Meyer DJ, Li B, Tingey S, Rafalski A (2008) Whole genome scan detects an allelic variant of *fad2* associated with increased oleic acid levels in maize. *Mol Genet Genomics* **279**: 1–10
- Booth JR, Broglie RM, Hitz WD, Kinney AJ, Knowlton S, Sebastian SA, inventors. July 30, 2002. Gene combinations that alter the quality and functionality of soybean oil. U.S. Patent US6426448
- Booth JR, Cahoon RE, Hitz WD, Kinney AJ, Yadav NS, inventors. March 8, 2006. Nucleotide sequences of a new class of diverged delta-9 stearoyl-ACP desaturase genes. European Patent Publication EP1311659
- Boswell K, Koskelo EK, Carl L, Glaza S, Hensen DJ, Williams KD, Kyle DJ (1996) Preclinical evaluation of single-cell oils that are highly enriched with arachidonic acid and docosahexaenoic acid. *Food Chem Toxicol* **34**: 585–593
- Bourre JM, Piciotti M, Dumont O (1990) Delta 6 desaturase in brain and liver during development and aging. *Lipids* **25**: 354–356
- Buhr T, Sato S, Ebrahim F, Xing A, Zhou Y, Mathiesen M, Schweiger B, Kinney AJ, Staswick P, Clemente T (2002) Ribozyme termination of RNA transcripts down-regulate seed fatty acid genes in transgenic soybean. *Plant J* **30**: 155–163
- Burdge GC, Jones AE, Wootton SA (2002) Eicosapentaenoic and docosapentaenoic acids are the principal products of alpha-linolenic acid metabolism in young men. *Br J Nutr* **88**: 355–363
- Burr GO, Burr MM, Miller ES (1932) On the fatty acids essential in nutrition. *J Biol Chem* **97**: 1–9
- Chen R, Matsui K, Ogaw M, Oe M, Ochiai M, Kawashima H, Sakuradani E, Shimizu S, Ishimoto M, Hayashi M, et al (2006) Expression of  $\Delta 6$ ,  $\Delta 5$  desaturase and GLELO elongase genes from *Mortierella alpina* for production of arachidonic acid in soybean [*Glycine max* (L.) Merrill] seeds. *Plant Sci* **170**: 399–406
- Conklin SM, Harris JI, Manuck SB, Yao JK, Hibbeln JR, Muldoon MF (2007) Serum omega-3 fatty acids are associated with variation in mood, personality and behavior in hypercholesterolemic community volunteers. *Psychiatry Res* **152**: 1–10
- Cordain L, Brand-Miller J, Eaton SB, Mann N, Holt SHA, Speth JD (2000) Plant-animal subsistence ratios and macronutrient energy estimations in worldwide hunter-gatherer diets. *Am J Clin Nutr* **71**: 682–692
- Cordain L, Eaton SB, Sebastian A, Mann N, Lindeberg S, Watkins A, O'Keefe JH, Brand-Miller J (2005) Origins and evolution of the Western diet: health implications for the 21st century. *Am J Clin Nutr* **81**: 341–354
- Dalton R (2004) Aquaculture: fishing for trouble. *Nature* **431**: 502–504
- Damude HG, Zhang H, Farrall L, Ripp KG, Tomb JF, Hollerbach D, Yadav NS (2006) Identification of bifunctional  $\Delta 12/\omega 3$  fatty acid desaturases for improving the ratio of  $\omega 3$  to  $\omega 6$  fatty acids in microbes and plants. *Proc Natl Acad Sci USA* **103**: 9446–9451
- Dirienzo MA, Lemke SL, Petersen BJ, Smith KM (2008) Effect of substitution of high stearic low linolenic acid soybean oil for hydrogenated soybean oil on fatty acid intake. *Lipids* **43**: 451–456
- Domergue F, Abbadì A, Zähringer U, Moreau H, Heinz E (2005) In vivo characterization of the first acyl-CoA Delta6-desaturase from a member of the plant kingdom, the microalga *Ostreococcus tauri*. *Biochem J* **389**: 483–490
- Dörmann P, Voelker TA, Ohlrogge JB (2000) Accumulation of palmitate in *Arabidopsis* mediated by the acyl-acyl carrier protein thioesterase FATB1. *Plant Physiol* **123**: 637–644
- Dupont J, White PJ, Feldman EB (1991) Saturated and hydrogenated fats in food in relation to health. *J Am Coll Nutr* **10**: 577–592
- Eckert H, LaVallee B, Schweiger BJ, Kinney AJ, Cahoon EB, Clemente T (2006) Co-expression of the borage D6 desaturase and the *Arabidopsis* D15 desaturase results in high accumulation of stearidonic acid in the seeds of transgenic soybean. *Planta* **224**: 1050–1057
- Hardin-Fanning F (2008) The effects of a Mediterranean-style dietary pattern on cardiovascular disease risk. *Nurs Clin North Am* **43**: 105–115
- Hawkins DJ, Kridl JC (1998) Characterization of acyl-ACP thioesterases of mangosteen (*Garcinia mangostana*) seed and high levels of stearate production in transgenic canola. *Plant J* **13**: 743–752
- Hibbeln JR, Nieminen LRG, Blasbalg TL, Riggs JA, Lands WEM (2006) Healthy intakes of n-3 and n-6 fatty acids: estimations considering worldwide diversity. *Am J Clin Nutr* (Suppl) **83**: 1483s–1493s
- Hong H, Datla N, Reed DW, Covello PS, MacKenzie SL, Qiu X (2002) High-level production of  $\gamma$ -linolenic acid in *Brassica juncea* using a  $\Delta 6$  desaturase from *Pythium irregulare*. *Plant Physiol* **129**: 354–362
- Hu X, Sullivan-Gilbert M, Gupta M, Thompson SA (2006) Mapping of the loci controlling oleic and linolenic acid contents and development of *fad2* and *fad3* allele-specific markers in canola (*Brassica napus* L.). *Theor Appl Genet* **113**: 497–507
- James MJ, Ursin VM, Cleland LG (2003) Metabolism of stearidonic acid in human subjects: comparison with the metabolism of other n-3 fatty acids. *Am J Clin Nutr* **77**: 1140–1145
- Kinney AJ (1996) Development of genetically engineered soybean oils for food applications. *J Food Lipids* **3**: 273–292
- Kinney AJ, Cahoon EB, Damude HG, Hitz WD, Kolar CW, Liu ZB, inventors.

- August 10, 2004. Production of very long chain polyunsaturated fatty acids in oilseed plants. International Patent Publication WO2004071467
- Kinney AJ, Knowlton S** (1998) Designer oils: the high oleic soybean. In S Roller, S Harlander, eds, Genetic Modification in the Food Industry. Blackie, London
- Knauf VC, Shewmaker C, Flider FJ, Emlay D, Ray E, inventors.** November 30, 2006. Safflower with elevated gamma-linolenic acid. International Patent Publication WO2006127789
- Kridl JC, inventor.** April 2, 2002. Methods for increasing stearate content in soybean oil. U.S. Patent US6365802
- Kris-Etherton PM, Harris WS, Appel LJ** (2002) Fish consumption, fish oil, omega-3 fatty acids, and cardiovascular disease. *Circulation* **106**: 2747–2757
- Lane J, Mansel RE, Jiang WG** (2003) Expression of human delta-6-desaturase is associated with aggressiveness of human breast cancer. *Int J Mol Med* **12**: 253–257
- Lichtenstein AH, Matthan NR, Jalbert SM, Resteghini NA, Schaefer EJ, Ausman LM** (2006) Novel soybean oils with different fatty acid profiles alter cardiovascular disease risk factors in moderately hyperlipidemic subjects. *Am J Clin Nutr* **84**: 497–504
- Lumor SE, Jones KC, Ashby R, Strahan GD, Kim BH, Lee GC, Shaw JF, Kays SE, Chang SW, Foglia TA, et al** (2007) Synthesis and characterization of canola oil-stearic acid-based trans-free structured lipids for possible margarine application. *J Agric Food Chem* **55**: 10692–10702
- Mensink RP** (2005) Effects of stearic acid on plasma lipid and lipoproteins in humans. *Lipids* **40**: 1201–1205
- Metz JG, Flatt JH, Kuner JM, inventors.** December 21, 2006. The genes for the enzymes of the polyunsaturated fatty acid polyketide synthase of *Schizochytrium* and their use in the manufacture of polyunsaturated fatty acids. International Patent Publication WO 2006135866
- Metz JG, Kuner JM, Lippmeier JC, inventors.** September 20, 2007. Polyunsaturated fatty acid production in genetically modified organisms using PUFA polyketide synthase systems. International Patent Publication WO 2007106903
- Metz JG, Roessler P, Facciotti D, Levering C, Dittrich F, Lassner M, Valentine R, Lardizabal K, Domergue F, Yamada A, et al** (2001) Production of polyunsaturated fatty acids by polyketide synthases in both prokaryotes and eukaryotes. *Science* **293**: 290–293
- Mikkilineni V, Rocheford TR** (2003) Sequence variation and genomic organization of fatty acid desaturase-2 (*fad2*) and fatty acid desaturase-6 (*fad6*) cDNAs in maize. *Theor Appl Genet* **106**: 1326–1332
- Miles EA, Banerjee T, Maaik MB, Dooper MM, M'Rabet L, Graus YMF, Calder PC** (2004) The influence of different combinations of gamma-linolenic acid, stearidonic acid and EPA on immune function in healthy young male subjects. *Br J Nutr* **91**: 893–903
- Miquel ME, Browse JA** (1994) High-oleate oilseeds fail to develop at low temperature. *Plant Physiol* **106**: 421–427
- Monsanto** (2008) Monsanto and the Solae Company to collaborate on soy-based omega-3 portfolio. Monsanto Company. [www.monsanto.com/features/omega.asp](http://www.monsanto.com/features/omega.asp) (April 16, 2008)
- Mozaffarian D, Willett WC** (2007) *Trans* fatty acids and cardiovascular risk: a unique cardiometabolic imprint? *Curr Atheroscler Rep* **9**: 486–493
- Mukerji P, Huang YS, Das T, Thurmond JM, Leonard AE, Pereira SL, inventors.** November 14, 2002. Protein and cDNA sequences of delta-4 desaturases isolated from fungi and therapeutical uses thereof. International Patent Publication WO 2002090493
- Naylor RL, Goldburg RJ, Primavera JH, Kautsky N, Beveridge MC, Clay J, Folke C, Lubchenco J, Mooney H, Troell M** (2000) Effect of aquaculture on world fish supplies. *Nature* **405**: 1017–1024
- Parker-Barnes JM, Das T, Bobik E, Leonard AE, Thurmond JM, Chaung LT, Huang YS, Mukerji P** (2000) Identification and characterization of an enzyme involved in the elongation of n-6 and n-3 polyunsaturated fatty acids. *Proc Natl Acad Sci USA* **97**: 8284–8289
- Patel M, Jung S, Moore K, Powell G, Ainsworth C, Abbott A** (2004) High-oleate peanut mutants result from a MITE insertion into the *FAD2* gene. *Theor Appl Genet* **108**: 1492–1502
- Pauly D, Watson R, Alder J** (2005) Global trends in world fisheries: impacts on marine ecosystems and food security. *Philos Trans R Soc Lond B Biol Sci* **360**: 5–12
- Pereira SL, Huang YS, Bobik EG, Kinney AJ, Stecca KL, Packer JC, Mukerji P** (2004a) A novel omega3-fatty acid desaturase involved in the biosynthesis of eicosapentaenoic acid. *Biochem J* **378**: 665–671
- Pereira SL, Leonard AE, Huang YS, Chuang LT, Mukerji P** (2004b) Identification of two novel microalgal enzymes involved in the conversion of the omega3-fatty acid, eicosapentaenoic acid, into docosahexaenoic acid. *Biochem J* **384**: 357–366
- Pérez-Vich B, Fernández-Martínez JM, Grondona M, Knapp SJ, Berry ST** (2002) Tearoyl-ACP and oleoyl-PC desaturase genes cosegregate with quantitative trait loci underlying high stearic and high oleic acid mutant phenotypes in sunflower. *Theor Appl Genet* **104**: 338–349
- Qi B, Fraser T, Mugford S, Dobson G, Sayanova O, Butler J, Napier JA, Stobart AK, Lazarus CM** (2004) Production of very long chain polyunsaturated omega-3 and omega-6 fatty acids in plants. *Nat Biotechnol* **22**: 739–745
- Qiu X, Hong H, Datla N, MacKenzie SL, Taylor DC, Thomas TL** (2002) Expression of borage  $\Delta 6$  desaturase in *Saccharomyces cerevisiae* and oilseed crops. *Can J Bot* **80**: 42–49
- Reddy AS, Thomas TL** (1996) Expression of a cyanobacterial delta 6-desaturase gene results in gamma-linolenic acid production in transgenic plants. *Nat Biotechnol* **14**: 639–642
- Reis LC, Hibbeln JR** (2006) Cultural symbolism of fish and the psychotropic properties of omega-3 fatty acids. *Prostaglandins Leukot Essent Fatty Acids* **75**: 227–236
- Reuters** (2008) Research confirms better oil from new DuPont high oleic soybean trait. Reuters. <http://www.reuters.com/article/pressRelease/idUS177160+18-Mar-2008+PRN20080318> (April 16, 2008)
- Robert S, Singh SP, Zhou XR, Petrie JR, Blackburn SI, Mansour PM, Nichols PD, Liu Q, Green A** (2005) Metabolic engineering of *Arabidopsis* to produce nutritionally important DHA in seed oil. *Funct Plant Biol* **32**: 473–479
- Roberts CA, Ren C, Beuselinck PR, Benedict HR, Bilyeu K** (2006) Fatty acid profiling of soybean cotyledons by near-infrared spectroscopy. *Appl Spectrosc* **60**: 1328–1333
- Sato S, Xing A, Ye X, Schweiger B, Kinney AJ, Graef G, Clemente TE** (2004) Production of  $\gamma$ -linolenic acid and stearidonic acid in seeds of marker-free transgenic soybean. *Crop Sci* **44**: 646–652
- Sayanova O, Smith MA, Lapinskas P, Stobart AK, Dobson G, Christie WW, Shewry PR, Napier JA** (1997) Expression of a borage desaturase cDNA containing an N-terminal cytochrome b5 domain results in the accumulation of high levels of D6-desaturated fatty acids in transgenic tobacco. *Proc Natl Acad Sci USA* **94**: 4211–4216
- Simopoulos AP** (2002) Omega-3 fatty acids in inflammation and autoimmune diseases. *J Am Coll Nutr* **21**: 495–505
- Subar AF, Krebs-Smith SM, Cook A, Kahle LL** (1998) Dietary sources of nutrients among US adults, 1989 to 1991. *J Am Diet Assoc* **98**: 537–547
- Tang GQ, Novitzky WP, Carol Griffin H, Huber SC, Dewey RE** (2005) Oleate desaturase enzymes of soybean: evidence of regulation through differential stability and phosphorylation. *Plant J* **44**: 433–446
- Tocher DR, Leaver MJ, Hodgson PA** (1998) Recent advances in the biochemistry and molecular biology of fatty acyl desaturases. *Prog Lipid Res* **37**: 73–117
- USDA** (2008) What we eat in America: source of data on food and nutrient intakes of Americans. U.S. Department of Agriculture. <http://www.ars.usda.gov/Services/> (April 16, 2008)
- Van Horn L, McCain M, Kris-Etherton PM, Burke F, Carson JA, Champagne CM, Karmally W, Sikand G** (2008) The evidence for dietary prevention and treatment of cardiovascular disease. *J Am Diet Assoc* **108**: 287–331
- Vega-Lopez S, Ausman LM, Jalbert SM, Erkkila AT, Lichtenstein AH** (2006) Palm and partially hydrogenated soybean oils adversely alter lipoprotein profiles compared with soybean and canola oils in moderately hyperlipidemic subjects. *Am J Clin Nutr* **84**: 54–62
- Wallis JG, Browse J** (1999) The Delta8-desaturase of *Euglena gracilis*: an alternate pathway for synthesis of 20-carbon polyunsaturated fatty acids. *Arch Biochem Biophys* **365**: 307–316
- Wallis JG, Watts JL, Browse J** (2002) Polyunsaturated fatty acid synthesis: what will they think of next? *Trends Biochem Sci* **27**: 467–473
- Wu G, Truksa M, Datla N, Vrinten P, Bauer J, Zank T, Cirpus P, Heinz E, Qiu X** (2005) Stepwise engineering to produce high yields of very long-chain polyunsaturated fatty acids in plants. *Nat Biotechnol* **23**: 1013–1017
- Yadav NS, Wierzbicki A, Aegerter M, Caster CS, Perez-Grau L, Kinney AJ, Hitz WD, Booth JR, Schweiger BJ, Stecca KL, et al** (1993) Cloning of higher plant w-3 fatty acid desaturases. *Plant Physiol* **103**: 467–476