

Chapter 2

Modifying Vegetable Oils for Food and Non-food Purposes

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2.1 Introduction

Oils and fats are an important source of energy for the human diet and also contribute significantly to the sensory characteristics of food. Many oils are also used for non-food applications, although industrial use currently accounts for only a small proportion of the world vegetable oil production, less than 5% of total production, mostly for biodiesel. About 80% of edible oils are derived from plant sources and temperate annual oil seeds (soy, rapeseed, sunflower and peanut) account for about 60% of this total. Soybean oil is by far the dominant oil in this category, accounting for over half of the world vegetable oil production.

Improving the functional and nutritional qualities of vegetable oils has garnered much attention over the last 15 years or so. This chapter will describe some of the attempts to genetically improve plant seed oils, with special emphasis on soybean oil, for food and non-food uses.

2.2 Modulating the Fatty Acid Content of Plant Oils for Food Uses

2.2.1 Fatty Acid Profile and Oil Functionality

The fatty acid profile plays a significant role in both the nutritional properties and end-use functionality of edible plant oils. With its high percentage of polyunsaturated fatty acids (>65%), the major food oils derived from seeds are relatively oxidatively unstable, which limits their utility in food applications. Historically, oil processors have used partial hydrogenation as a means to improve upon the oxidative stability of plant oils. This process chemically shifts the fatty acid profile towards increased saturated and monounsaturated, and

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concomitantly reduces the polyunsaturated fatty acids. Partial hydrogenation also significantly improves solid fat functionality, which is especially useful for baking and similar applications. This improved functionality comes at a cost to nutritional quality due to the presence of high concentrations of *trans* fatty acids found in partially hydrogenated oils. *Trans* fatty acids have been correlated with cardiovascular disease (Mensink and Katan 1990; Korver and Katan 2006). Intake of other dietary fatty acids can have a strong influence on overall health. For example, saturated fatty acids such as palmitic acid, tend to raise LDL-cholesterol whereas stearic acid has neutral effect (Minihane and Harland 2007). Hence, the development of alternative vegetable oil feed stocks that can simultaneously improve functionality while maintaining nutritional quality holds great market potential to both the frying and baking industries.

Commodity soybean oil presently is composed of 14% saturated fatty acids, 18% monounsaturated fatty acid, and 65% polyunsaturated fatty acids. Through targeted genetic alterations, designer soybean oils are being developed to address both oxidative stability and nutritional quality. These include oils high in oleic acid and low in saturated and polyunsaturated fatty acids, oils elevated in stearic acid, and combinations of reduced polyunsaturate content with elevated stearic acid.

2.2.2 Conventional Approaches to Fatty Acid Modification

Oleic acid is metabolized to linoleic acid by a single desaturation step carried out by a $\Delta 12$ -desaturase encoded by the *FAD2* gene (Heppard et al. 1996). In soybean there are at least six *FAD2* genes that fall into two classes, *FAD2-1* and *FAD2-2*. The *FAD2-1* class is primarily embryo-specific, while the *FAD2-2* class is generally constitutive, expressed during both vegetative and seed developmental stages (Tang et al. 2005). Soybean breeders have made great strides to move an elevated oleic acid phenotype into elite genotypes by exploiting natural variation in oleic acid levels among various sources of soybean germplasm (Takagi and Rahman 1996; Rahman et al. 2001; Alt et al. 2005a,b). Conventional approaches to raise the oleic acid content in soybean oil has led to the development of “mid-oleic” phenotype, in which seed storage lipids range in oleic acid from 30 to 70%. The conventional approach to develop the mid-oleic phenotype has some drawbacks. First, the genetics of the phenotypes require the stacking of multiple loci (Alt et al. 2005a, b), which may complicate the breeding process. Secondly, the “mid-oleic” phenotype is affected by environment, typically requiring growth in warmer climates for stability of the elevated oleic acid trait to be maintained. This is due to the temperature effect on the desaturase activity and expression (Heppard et al. 1996; Tang et al. 2005). The third drawback associated with the conventional breeding approach for a “mid-oleic” soybean is the germplasm that expresses this phenotype is associated with yield drag (Primomo et al. 2002).

In soybean seed the content of palmitate is regulated by a palmitoyl thioesterase encoded by *FatB* genes (Kinney 1997). Low palmitic acid soybean genotypes have been reported (Bubeck et al. 1989; Primomo et al. 2002). The low palmitic acid phenotype in soybean has recently been associated with allelic variation in *FatB* (Cardinal et al. 2007). Importantly, as the conventionally bred “mid-oleic” soybean, the low palmitic acid genotypes seem to suffer from yield penalty (Rebetzke et al. 1998; Cardinal and Burton 2007).

The concentration of the cardiovascular “neutral” saturated fatty acid, stearate, in soybean is controlled by both desaturase (Cheesbrough 1990) and thioesterase activities (Pantalone et al. 2002). The genetic locus controlling elevated stearic acid levels in soybean, is designated *Fas*, and allelic variation at this locus manifests stearic acid levels ranging from wild type concentrations of 3%, up to high stearate germplasm with 35% (Bubeck et al. 1989; Rahman et al. 1995; Pantalone et al. 2002).

Significant amount of progress has been made in developing novel soybean germplasm, with altered fatty acid profiles, using conventional breeding strategies. However, as mentioned above there tends to be a yield drag associated with these improved oil traits. This may be due to the allelic variants selected for altered fatty acid profile during both vegetative and embryogenesis development, thereby increasing the probability of a negative agronomic effect associated with the mutant allele governing the novel oil phenotype. Moreover, while soybean output traits, such as “mid-oleic”, low palmitic acid, and elevated stearate have value, there are applications in which stacking of these traits would offer expanded functionality. Given the genetic complexity regulating the “mid-oleic” trait in soybean, combining this phenotype with low palmitic and/or elevated stearic acid traits, into elite soybean genotypes, would be challenging. High oleic acid mutants, with an oleic content ranging from 60 to 90%, have also been developed in corn, peanut, canola and sunflower. These mutants all have defective *FAD2* genes (Perez-Vich et al. 2002; Patel et al. 2004; Hu et al. 2006; Belo et al. 2008). As in soybean, many of these plants have a number of *FAD2* genes all which contribute to seed linoleic acid content (Tang et al. 2005), which means that the development of mutant lines with useful oleic acid contents often requires combining several mutant loci (Mikkilineni and Rocheford 2003).

2.2.3 Novel Fatty Acid Profiles in Soybean Derived from the Tools of Biotechnology

Implementing the tools of biotechnology novel oil traits can be achieved in a seed-specific fashion, with a single dominant allele. This approach simplifies breeding and reduces the probability of agronomic performance being compromised.

Targeted perturbation of *FAD2* alleles in a seed-specific fashion in soybean has been shown to produce a high oleic acid (75–85%) phenotype in the seed oil (Kinney and Knowlton 1997; Mazur et al. 1999). Modulation of *FAD2* expression using this transgenic approach was carried out by introducing transgenic elements designed to induce post-transcriptional gene silencing (Cerutti 2003).

High oleic acid soybean derived from down-regulating *FAD2*, concomitantly reduces polyunsaturated fatty acids to below 6% (Kinney and Knowlton 1997), in addition palmitic acid was reduced to approximately 7–8% (a 20% reduction in palmitate).

For some uses, such as salad oils, it is desirable to reduce saturated fatty acids, especially palmitic acid as much as possible. It has been shown that the *FATB* class of acyl:ACP thioesterases control the release of some saturated fatty acids, including palmitic, into the cytoplasm, making them available for oil biosynthesis (Dörmann et al. 2000). Seed-specific silencing of *FATB* genes in soybean leads to a major reduction in total saturated fatty acids, from about 15% to less than 6% (Kinney 1996). When this gene was combined with a silenced *FAD2*, oleic acid contents of over 90% were observed (Buhr et al. 2002). Importantly, field trials with soybean having this novel oil phenotype, conducted across multiple environments, revealed that the fatty acid content was not affected by temperature and the agronomic performance was not compromised.

For certain food applications, such as baking, solid fat functionality is needed. This functionality is currently provided by animal fats, palm oil and hydrogenated vegetable oils, and biotechnology has the potential to provide a healthy alternative to these fats. It is thought that oils rich in stearic acid could provide this important function without compromising human health because, unlike other saturated fatty acids, stearic is not considered to play a significant role in cardiovascular disease (Mensink 2005; DiRienzo et al. 2008).

High stearic acid vegetable oil has been developed by targeting either down-regulation of desaturase activity (Liu et al. 2002) or heterologous expression of a stearoyl-ACP thioesterase (Hawkins and Kridl 2002). 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 resulted in soybean oils containing 20–30% stearic acid when compared with just a few percent in commodity soybean (Booth et al. 2006). The seeds of mangosteen (*Garcinia mangostana*) contain stearate as the predominant fatty acid (45–50% of total fatty acids), a result of a stearate-specific thioesterase. When the mangosteen gene was expressed in transgenic canola seeds, stearate contents of 20–30% were reported (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, around 30% of the total fatty acids (Lumor et al. 2007) and germination problems are often seen when the stearic acid content of seeds is elevated to these levels (Roberts et al. 2006; Clemente

unpublished data). However, 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 50–60% range with stearic around 15–20% (Booth et al. 2002). This combination of stearic and oleic confers the desired solid fat functionality without compromising the seed. Improved functionality has also been obtained by combining increased stearic acid 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.

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 (Korver and Katan 2006; Lichtenstein et al. 2006; Mozaffarian and Willett 2007).

2.3 Next Generation Edible Oils: Producing Long Chain ω -3 Fatty Acids in Seed Oils

2.3.1 Engineering Complex Pathways into Plant Seeds

The efforts toward genetic engineering plants to alter seed oil compositions described above focused on modifying existing biosynthetic pathways at a single enzyme step. With advancements in plant transformation and genomic technologies, expression of significantly more complex gene combinations, including whole metabolic pathways from heterologous sources, are now being explored and thus, the flexibility to produce highly functionalized, higher-value metabolic end-products is also becoming possible. A good example of valuable but metabolically complex end products that are of current significant interest are the long-chain polyunsaturated fatty acids (LCPUFA) including the ω -3 LCPUFA eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). The importance of these fatty acids in human health and nutrition and current efforts to produce them in oil seed crops will be discussed.

EPA is a 20 carbon fatty acid having 5 double bonds while DHA is a 22 carbon fatty acid having 6 double bonds. Both are considered ω -3 fatty acids in that they have a double bond occurring between the third and fourth carbon from the methyl end. Arachidonic acid (ARA) is similar to EPA in that it is a 20 carbon fatty acid but lacks the ω -3 double bond and the double bond closest to the methyl end of the fatty acid occurs between the sixth and seventh carbon, thus making ARA an ω -6 fatty acid. Most naturally occurring fatty acids, and all that are relevant to this discussion, have double bonds having a *cis* configuration. The chemical structure of the fatty acid (i.e. chain length; number, position and stereochemistry of double bonds) is directly related to its effect on human physiology including potential health properties and a list of the fatty acids relevant to this discussion along with their chemical structures is shown in Table 2.1

Table 2.1 Nomenclature and chemical structures of fatty acids

Saturation	Common name	Abbreviation	Carboxyl nomenclature	ω nomenclature
Saturated	myristic	MA	14:0	14:0
	palmitic	PA	16:0	16:0
	stearic	SA	18:0	18:0
Monounsaturated	oleic	OA	18:1(Δ 9)	18:1 ω -9
	eicosanoic	EA	20:1(Δ 11)	20:1 ω -9
Polyunsaturated	taxoleic	TXA	18:2(Δ 5,9)	18:2 ω -9
	linoleic	LA	18:2(Δ 9,12)	18:2 ω -6
	γ -linolenic	GLA	18:3(Δ 6,9,12)	18:3 ω -6
	pinolenic	PNA	18:3(Δ 5,9,12)	18:3b ω -6
	α -linolenic	ALA	18:3(Δ 9,12,15)	18:3 ω -3
	stearidonic	STA	18:4(Δ 6,9,12,15)	18:4 ω -3
	eicosadienoic	EDA	20:2(Δ 11,14)	20:2 ω -6
	dihomo- γ -linolenic	DGLA	20:3(Δ 8,11,14)	20:3 ω -6
	sciadonic	SCI	20:3(Δ 5,11,14)	20:3b ω -6
	eicosatrienoic	ERA	20:3(Δ 11,14,17)	20:3 ω -3
	arachidonic	ARA	20:4(Δ 5,8,11,14)	20:4 ω -6
	eicosa-tetraenoic	ETA	20:4(Δ 8,11,14,17)	20:4 ω -3
	juniperonic	JUP	20:4(Δ 5,11,14,17)	20:4b ω -3
	eicosa-pentaenoic	EPA	20:5(Δ 5,8,11,14,17)	20:5 ω -3
	docosa-tetraenoic	DTA	22:4(Δ 7,10,13,16)	22:4 ω -6
	docosa-pentaenoic	DPAn-6	22:5(Δ 4,7,10,13,16)	22:5 ω -6
	docosa-pentaenoic	DPA	22:5(Δ 7,10,13,16,19)	22:5 ω -3
	docosa-hexaenoic	DHA	22:6(Δ 4,7,10,13,16,19)	22:6 ω -3
	tetracosapentaenoic	TPA	24:5(Δ 9,12,15,18,21)	24:5 ω -3
	tetracosahexaenoic	THA	24:6(Δ 6,9,12,15,18,21)	24:6 ω -3

Although the effects of fatty acids on human health may be variable in different human populations depending on age, sex, race and genetic background, studies have shown that consumption of fats high in short-chain, saturated fatty acids leads to increases in low-density lipoprotein (LDL) and may lead to increased risk of cardiovascular disease (Schaefer 1997). Stearic acid (SA), an 18 carbon saturated fatty acid, is neutral in its effect on blood lipids (Kris-Etherton et al. 2005; Davis and Kris-Etherton 2003; Schaefer 1997) and monounsaturated and polyunsaturated fatty acids are inversely correlated with coronary heart disease (Binkoski et al. 2005; Davis and Kris-Etherton 2003; Kris-Etherton 1999). Given these trends, reduced consumption

of short-chain, saturated fat and increased consumption of foods rich in mono- and polyunsaturated fats and oils should lead to an overall healthier state.

Vegetable oils such as soybean and canola oils are rich sources of polyunsaturated fatty acids (ω -3 and ω -6). The polyunsaturated fatty acids linoleic acid (LA) and α -linolenic acid (ALA) commonly found in many oilseeds are not only considered healthy but are, in fact, essential fatty acids since humans lack the enzymes necessary to produce them. Humans are biosynthetically capable of producing oleic acid (OA) *de novo* from glucose or other basic carbon sources but are incapable of further desaturating OA to LA, and LA to ALA as they are missing the Δ 12 and Δ 15 desaturase (ω -3 desaturase) enzymes, respectively. Oilseed plants have Δ 12 and Δ 15 desaturases which act on phospholipid-bound OA. In the body, LA and ALA are further converted to ARA and EPA, respectively and EPA is a precursor to DHA (Sprecher 2000).

The eicosanoid family of metabolites which include prostaglandins, leukotrienes and thromboxanes (Funk 2001; Smith 2005) are formed directly from ARA and EPA. These molecules are key control points for metabolic processes such as inflammatory responses, blood clot induction and regulation of blood pressure (Yaqoob 2003). Inflammatory response is regulated by the balance of these types of eicosanoids with ARA- and EPA-derived eicosanoids producing a pro- and anti-inflammatory response, respectively (Calder 2003; Simopoulos 2006; Smith 2005). When EPA is not consumed directly in the diet, the ratio of LA to ALA consumed has a direct affect on the concentration of ARA and EPA because humans lack an ω -3 desaturase that would convert LA to ALA or ARA to EPA. Therefore, the essential need for LA and ALA in the diet is in part due to their importance as intermediates of LCPUFAs and eicosanoid biosynthesis, which are crucial for normal human physiology.

Although inflammatory responses are critical in many human defense and healing mechanisms, prolonged induction of an inflammatory state could lead to health problems. In Western society, consumption of foods rich in LA, such as vegetable oils or grain-fed meat and poultry, has increased substantially over the past 60 years (Hibbeln et al. 2004). This increase combined with increased consumption of hydrogenated fats where ω -3 fatty acids have been selectively removed, has resulted in a substantial shift in the balance of ARA and EPA in the blood stream with numerous potential negative consequences (Hibbeln et al. 2004; Simopoulos 1999; Wada et al. 2007) including those disease states related to excessive inflammation or auto-immune conditions. For example, inflammatory bowel disease has been linked to increased concentrations of ARA (Ramakers et al. 2007).

Consumption of EPA and/or DHA has also been shown to be beneficial in other areas of human health such as cardiovascular disease and mental health (Ruxton et al. 2007). Benefits of EPA/DHA in decreasing death rates after a

myocardial infarction have been described while consumption of ALA is not as effective (Breslow 2006; von Schacky and Harris 2007). EPA and DHA are protective at doses <1 g/d due to suppression of fatal arrhythmias and at doses >3 g/d, EPA plus DHA can improve cardiovascular disease risk factors, including decreasing plasma triacylglycerides, blood pressure, platelet aggregation, and inflammation, while improving vascular reactivity (Breslow 2006; von Schacky and Harris 2007). Low levels of EPA and/or DHA have been linked to higher instances of depression and increased consumption of EPA and/or DHA can attenuate symptoms of depression and bipolar disorder (Sontrop and Campbell 2006). DHA is an important component of cell membrane phospholipids (Horrocks and Farooqui 2004). Mammalian retinal and brain membranes are enriched in DHA and it is important for the cognitive development of infants (Fleith and Clandinin 2005; Iribarren et al. 2004; Stoll et al. 2001; Willatts and Forsyth 2000). The use of DHA in treating brain-related diseases such as Alzheimer's disease have also been suggested (Oksman et al. 2006).

The best direct source of EPA and DHA in our diet comes from the consumption of marine fish or their oils. In fact, the American Heart Association has recommended at least two weekly servings of fish as part of a heart-healthy diet. Many consumers are also complementing diet with fish oil supplements. But, while increasing consumer awareness towards the health benefits of fish oil, ω -3 fatty acids, EPA and DHA is generally good from a health perspective, the increasing demand for fish and fish oil products is placing further stress on limiting fish populations, many of which are already in jeopardy (Pauly et al. 2002). Additionally, many fish species and resulting fish oil derived from them are tainted with contaminants and some consumption restriction recommendations have been made (Mozaffarian and Rimm 2006). In fact, the main source of LCPUFA in fish originates in marine microorganisms such as diatoms, golden-brown algae, green algae, blue-green algae, microbial fungi and dinoflagellates, all of which are rich in LCPUFA (Radwan 1991; Shaw 1966). These organisms are ingested by small fish which in turn are eaten by larger fish and other animals. Sustainable alternatives to fish oils where LCPUFAs are produced through fermentation of microalgae are free from the contaminants found in fish oils but their widespread use is limited to high value applications such as infant formula and medical foods (Boswell et al. 1996) due to their high cost of manufacture.

2.3.2 LCPUFA Production in Plants

In nature, there exist two main biochemical pathways to ARA, EPA and DHA. These are the aerobic desaturase/elongase-type pathway and the anaerobic polyketide synthase (PKS) pathway (Sperling et al. 2003; Bentley and Bennett 1999). The aerobic desaturase/elongase-type pathway can further be divided into two classes based on whether the first step of the pathway is elongation or desaturation.

2.3.3 EPA Production in Plants via the $\Delta 6$ Desaturase Pathway

In animals, including fish and humans, and the majority of marine micro-organisms studied thus far, EPA is generated by a $\Delta 6$ desaturase pathway (Sayanova and Napier 2004) where a double bond is first added to ALA by a $\Delta 6$ desaturase to form stearidonic acid (STA), followed by elongation of STA to eicosatetraenoic acid (ETA) catalyzed by a $\Delta 6$ fatty acid elongase and lastly the formation of another double bond in ETA by a $\Delta 5$ desaturase to form EPA. Some micro-organisms that produce ARA do so by an analogous pathway as for EPA but the desaturases and elongases utilize the ω -6 forms of these substrates when they are present. Therefore, LA can be converted to γ -linolenic acid (GLA) by the $\Delta 6$ desaturases, GLA can be converted to dihomo- γ -linolenic acid (DGLA) by the elongases and DGLA can be converted to ARA by the $\Delta 5$ desaturases. The substrate preference for either ω -6 or ω -3 substrates varies depending on the species from which the enzymes are derived and some species produce both ω -6 and ω -3 fatty acids (Sayanova et al. 2006; Sayanova and Napier 2004). In addition, some organisms can convert ω -6 fatty acids (C18 and/or C20) to ω -3 fatty acids through the action of an ω -3 fatty acid desaturase (Damude et al. 2006; Oura and Kajiwarra 2004; Pereira et al. 2004b; Sakuradani et al. 2005; Spychalla et al. 1997). The ratio of products produced in any given pathway will, therefore, be a function of both the substrate specificity of the enzymes used and the concentrations of either ω -3 or ω -6 substrates available in the host.

Most oilseed plants that produce polyunsaturated fatty acids also produce a very high concentration of LA, which results in a ratio of ARA to EPA that is too high when converted in the body. One exception to this is linseed oil, which has ALA concentrations of approximately 50–60% of the total fatty acids. A transgenic approach to make higher levels of ALA in soybean by expressing a bifunctional $\Delta 12/\Delta 15$ desaturase from *Fusarium moniliforme* resulted in ALA concentrations as high as 72% in seed (Damude et al. 2006). In a similar attempt to make pork healthier, transgenic pigs were produced, which expressed a “humanized” *Caenorhabditis elegans* ω -3 desaturase (Lai et al. 2006) and this resulted in pork fat with higher concentrations of ALA.

The first step of the $\Delta 6$ desaturase pathway is the conversion of ALA to stearidonic acid (STA). In humans, the $\Delta 6$ desaturase is rate-limiting (Burdge et al. 2002; James et al. 2003). Thus, even when ALA concentrations in the diet are high, ALA is poorly converted to EPA 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).

By-passing the ineffective $\Delta 6$ desaturase by consuming oils rich in STA directly has been proposed as an indirect way of more effectively obtaining needed levels of EPA and DHA. Some plants such as hemp, borage, black

currant and evening primrose express a $\Delta 6$ desaturase and produce GLA and STA in their seed oils naturally, and these oils are currently marketed and sold for their proposed health benefits (Barre 2001). But, their cultivation is carried out on a scale that is relatively small and yields are poor making the oils produced expensive.

Producing STA in commercial oilseed crops has also been proposed and requires the minimal expression of a single $\Delta 6$ desaturase. The synthesis of GLA and STA in a plant (tobacco) was first demonstrated using the $\Delta 6$ desaturase from *Synechocystis* expressed constitutively under control of the 35S promoter (Reddy and Thomas 1996). Subsequent expression of other $\Delta 6$ desaturases in tobacco, *Brassica juncea* and soybean further improved yields of both GLA and STA (Sayanova et al. 1997; Hong et al. 2002; Qiu et al. 2002; Sato et al. 2004). STA production was further optimized in soybean using seed-specific expression of the borage $\Delta 6$ desaturase and the *Arabidopsis* $\Delta 15$ desaturase giving STA contents of as high as 30% (Eckert et al. 2006). Both GLA and STA production in oilseeds are under active commercial development with reports of GLA synthesis in safflower yielding up to 73% GLA (Knauf et al. 2006) and STA synthesis in soybean giving greater than 20% STA with about 5–6% GLA (Wilkes 2007).

Producing GLA and STA in the seeds of commercial oilseed plants are significant achievements in their own right, but STA still needs to be consumed in larger amounts to have the same efficacy as EPA (Miles et al. 2004). In addition STA 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). Thus, direct consumption of EPA and DHA in the diet remains preferred and requires the expression of the remaining pathway genes in plants.

In the $\Delta 6$ pathway to EPA, STA is elongated by two carbons to ETA via a microsomal fatty acid elongation complex (Metz et al. 2001), which is similar to that in the plastid but which uses acyl-CoA substrates instead of acyl-ACP substrates. With the exception of a recently characterized elongase from the marine parasitic protozoan *Perkinus marinus*, all of the elongases that are involved in LCPUFA biosynthetic pathways are of the ELO/SUR4 gene family (Venegas-Caleron et al. 2007). Of the remaining proteins involved in elongation, putative beta-ketoacyl-CoA reductases (Beaudoin et al. 2002; Han et al. 2002) and enoyl-CoA reductases (Gable et al. 2004; Paul et al. 2007) have been suggested for yeast and plants. Recently, the PHS1 gene has been suggested to be responsible for the dehydration reaction in yeast (Denic and Weissman 2007). Further desaturation of ETA by a $\Delta 5$ desaturase generates the final EPA product.

Production of EPA in a plant using the $\Delta 6$ desaturase pathway was first demonstrated in soybean (Kinney et al. 2004) and is, to date, the highest abundance of EPA achieved in any plant tissue. In the study, a $\Delta 6$ desaturase, elongase and $\Delta 5$ desaturase gene from *Mortierella alpina* were used along with an *Arabidopsis* fad3 gene (Yadav et al. 1993) and a

S. diclina $\Delta 17$ desaturase (Pereira et al. 2004b) and each were expressed under the control of different, strong, seed-specific promoters. EPA contents in embryos as high as 13% in embryos and 20% in seed were achieved with little to no ARA produced due mainly to the presence of the highly efficient $\Delta 17$ desaturase used. The ω -3 to ω -6 fatty acid ratio increased from 0.2:1 (the normal soybean ratio) to 1.5:1 (a ratio close to that of many fish oils) and overall elongation was 65% suggesting a highly efficient transfer to CoA pools for subsequent elongation. Unexpectedly, docoasapentaenoic acid (DPA), DHA precursor, was also found in the high EPA lines as abundant as 4%. DPA resulted from the additional activity of the *M. alpina* $\Delta 6$ elongase towards the $\Delta 5$ fatty acid EPA. This same elongase had almost no $\Delta 5$ EPA-elongating activity when expressed in yeast (Parker-Barnes et al. 2000). In the same study, some events where the *S. diclina* $\Delta 17$ desaturase was not functioning contained ARA concentrations as high as 26% in seed were obtained (Damude unpublished data).

ω -3 to ω -6 ratios were further improved when the *Arabidopsis* ω -3 desaturase was replaced with a novel, bifunctional $\Delta 12/\Delta 15$ desaturase from *Fusarium moniliforme* (Damude and Yadav 2005; Damude et al. 2006). The *Fusarium* $\Delta 15$ desaturase had broad substrate specificity for numerous ω -6 fatty acids including LA>GLA>DGLA>ARA (Damude et al. 2006), and when co-expressed with the LCPUA pathway, led to an overall ω -3 content as high as 57% in soybean embryos with up to 16% EPA.

Interestingly, a recent similar attempt at producing ARA using the $\Delta 6$ desaturase pathway in soybean (Chen et al. 2006) resulted in low concentrations of ARA (2.1% of total lipids) in embryos and even lower concentrations (0.5–0.8%) in seed. In this approach, a *FAD3* down-regulation cassette was combined with the *Mortierella alpina* $\Delta 6$ desaturase, elongase and $\Delta 5$ desaturase genes, under control of the strong seed-specific β -conglycinin promoter. Use of the same promoter multiple times was suggested to cause poor expression of the genes and led to low ARA concentrations.

The initial demonstration of EPA in soybeans using a $\Delta 6$ desaturase pathway (Kinney et al. 2004) was followed closely by another report (Abbadi et al. 2004) where ARA and EPA were produced (less than 2% total) in tobacco and flax seeds using genes from the diatom *Phaeodactylum tricornutum* and the moss *Physcomitrella patens* (Abbadi et al. 2004). In this study, the EPA pathway intermediates GLA and STA predominated at approximately 30% with only low concentrations of elongated fatty acids such as DGLA, ETA, ARA and EPA. Results indicated the elongation step was severely limited (10% total elongation) in both tobacco and flax. The elongation bottleneck was shown to be the result of low incorporation of the substrates for elongation, GLA and STA, into the acyl-CoA pools from the phospholipid pools from where they were produced. This poor exchange resulted in the incorporation of GLA directly into triglyceride mostly through the direct conversion of phosphatidylcholine (PC) containing

GLA to DAG followed by acylation to TAG. A similar direct incorporation of phospholipid backbones containing the unusual fatty acid eleostearic acid directly into TAG was seen when a *Momordica charantia* fatty acid conjugase was expressed in *Arabidopsis* (Cahoon et al. 2006).

In another study, a full complement of $\Delta 6$ desaturase pathway genes has been expressed in *Brassica juncea* (Wu et al. 2005) and inclusion of a $\Delta 17$ desaturase significantly increased EPA production (up to 15%) with a substantial decrease in ARA. In all of these studies described above, the $\Delta 6$ and $\Delta 5$ desaturases included utilized acyl-phospholipid substrates and in some cases transfer of the acyl groups to acyl-CoA pools was shown to be limiting. One other $\Delta 6$ desaturase pathway study in *Arabidopsis* seed used a $\Delta 6/\Delta 5$ desaturase from a species of zebrafish (*Danio rerio*) which utilizes acyl-CoA substrates along with a *Caenorhabditis elegans* elongase (Robert et al. 2005). It was thought that a complete pathway with preference for acyl-CoA substrates would improve flux since reduced exchange between acyl-CoA and phospholipid pools would be required. Using this approach, an EPA content of only 2.5% was achieved.

2.3.4 EPA Production in Plants via the $\Delta 9$ Elongase Pathway

Species from the *Prymnesiophyceae* (e.g. *Pavlova*, *Isochrysis*) *Acanthamoebidae* (e.g. *Acanthamoeba*) and *Euglenophyceae* (e.g. *Euglena*) families of LCPUFA-producing microorganisms, have been shown to use a slightly different aerobic pathway to produce EPA (Sayanova and Napier 2004). In this alternate $\Delta 9$ elongase pathway, ALA is first elongated by a $\Delta 9$ -specific elongase to eicosatrienoic acid (ERA), followed by $\Delta 8$ desaturation to ETA. As in the more common $\Delta 6$ pathway, these fatty acids are then desaturated to EPA respectively by a $\Delta 5$ desaturase.

The earliest published report of synthesis of EPA in plants described expression of this alternate $\Delta 9$ elongase pathway in *Arabidopsis* leaf tissue. A $\Delta 9$ elongase from the microalgae *Isochrysis galbana*, a $\Delta 8$ desaturase from the *Euglenophyceae*, *Euglena gracilis*, and a $\Delta 5$ desaturase from the microbial fungus *Mortierella alpina* were expressed constitutively in the model plant *Arabidopsis* (Qi et al. 2004). EPA contents as high as 3.0% with ARA contents up to 6.6% were achieved in *Arabidopsis* leaves with ratios of ω -3 to ω -6 ratio fatty acids (2.2:1) similar to that commonly found in fish oils (Sargent 1997). Total elongation of fatty acids was good at 36% demonstrating a relatively high availability of LA and ALA in the acyl-CoA pool of *Arabidopsis* leaves. It was suggested that the bottleneck of acyl-transfer between acyl-CoA and phospholipid pools was relieved by placing the acyl-CoA-requiring elongation step first in the pathway followed by two acyl-phospholipid-requiring desaturation steps.

In a case of premature desaturation, the fatty acid by-products sciadonic acid (SCI) and juniperonic acid (JUP) were also formed in this $\Delta 9$ elongase pathway. This resulted from the direct action of the $\Delta 5$ desaturase on EDA and ERA before the $\Delta 8$ desaturation step. Interestingly, the species from which the $\Delta 9$ elongase and $\Delta 8$ desaturase were taken do not produce significant levels of these fatty acid by-products suggesting different specificities of the host $\Delta 5$ desaturase or more efficient flux through the pathway.

Some plant gymnosperm species (e.g. *Pinaceae* family) naturally produce seeds with twenty carbon fatty acids having $\Delta 5$ double bonds such as sciadonic acid (SCI) and juniperonic acid (JUP), suggesting the presence of C20 elongases and $\Delta 5$ desaturases (Wolff et al. 2000).

2.3.5 DHA Production in Plants via the Aerobic Elongation/Desaturation Pathways

In animals, generation of DHA from EPA occurs by the Sprecher pathway whereby EPA is elongated twice by the fatty elongation complex (Sprecher et al. 1999) and the first enzyme of elongation is specific for the co-acylated form of EPA. The 24 carbon fatty acid tetracosapentaenoic acid (TPA) generated by elongation is then desaturated by the rate-limiting $\Delta 6$ desaturase to produce tetracosahexaenoic acid (THA). Chain reduction by 2 carbons via beta-oxidation forms DHA (Wallis et al. 2002).

In microorganisms, DHA is synthesized by a much simpler pathway from EPA where initial elongation to docosapentaenoic acid (DPA) by the fatty acid elongation complex is followed by a $\Delta 4$ desaturase to directly produce DHA without the need for further elongation, $\Delta 6$ desaturation or beta-oxidation (Sayanova and Napier 2004).

The first successful production of DHA in plants was carried out in soybean (Kinney et al. 2004). The $\Delta 5$ elongase from *Pavlova* sp. (Pereira et al. 2004a) and the $\Delta 4$ desaturase from *Schizochytrium aggregatum* (Mukerji et al. 2002) was added alongside the $\Delta 6$ desaturase EPA biosynthetic pathway, and soybean somatic embryo oil with up to 3.3% DHA was achieved. To this date this continues to be the highest concentration of DHA made in any plant. It was difficult to separate whether the additional *Pavlova* elongase or the dual specificity of the *M. alpina* $\Delta 6$ elongase for $\Delta 5$ substrates contributed to the elongation of EPA but given the high levels of DPA achieved with only the *M. alpina* elongase, it is most likely the latter. The *S. aggregatum* $\Delta 4$ desaturase used was highly active in plants with, in some cases, close to 100% conversion of DPA to DHA.

Subsequently, DHA has been produced in other plants including up to 1.5% in *Brassica* seed by combining EPA biosynthetic genes ($\Delta 6$ desaturase pathway) with the $\Delta 4$ desaturase from *Thraustochytrium* sp., and an elongase from the fish *Oncorhynchus mykiss* (Wu et al. 2005) and up to 0.5% in

Arabidopsis seeds by re-transforming an *Arabidopsis* plant making EPA with a $\Delta 5$ elongase and a $\Delta 4$ desaturase from *Pavlova salina* (Robert et al. 2005).

2.3.6 DHA Production in Plants via the Anaerobic Polyketide Synthase Pathway

Many marine microbes synthesize EPA or DHA using the anaerobic polyketide synthase pathway (Metz et al. 2001), which are similar to the fatty acid synthase complex in plants. These enzymes can be formed from multiple large proteins which contain many multi-domained subunits and where each domain carries out a different chemistry. Double bonds are produced in the growing fatty acid chain by a dehydrase-isomerase mechanism similar to *FabA* in *E. coli* and do not require oxygen as do fatty acid desaturases (Metz et al. 2001). Interestingly, in some organisms both a PKS and a fatty acid synthase pathway for EPA or DHA synthesis may be present. For example, a complete PKS-type DHA-synthase has been cloned and characterized from a number of *Thraustochytrid* species, as have various fatty acid desaturases and elongases (Metz et al. 2004; Qiu et al. 2001).

Successful expression of PKS genes in yeast and plants has been reported (Metz et al. 2006, 2007). Genes encoding the three subunits (ORFA, B, C) of a *Schizochytrium* PKS were co-expressed with a phosphopantetheinyl transferase (PPT) from *Nostoc*, essential for activating the ACP domains of the DHA-synthase PKS individually under control of the linin seed-specific promoter from flax. Expression in the plastid was achieved by fusion with a *Brassica* acyl-ACP thioesterase plastid targeting signal. *Arabidopsis* seeds were obtained having up to 0.8% DHA with an additional 1.7% DPAn-6, which is also observed in *Schizochytrium*. Further increases in DHA content in *Arabidopsis* seed were made by co-expressing a PKS with either an acyl-CoA synthetase (ACSI or ACSII) from *Schizochytrium*, or RNAi constructs for down-regulation of the *Arabidopsis* KASII or KASIII genes. Analysis of the fatty acid profiles of single seed expressing the PKS, PPT and the KASIII RNAi construct showed levels of DHA and DPAn-6 as high as 2.4 and 1.8%, respectively.

2.4 Modifying Vegetable Oils for Non-food Purposes

2.4.1 Non-food Uses of Plant Oils

Consumption of soybean oil for industrial uses has undergone a dramatic increase between 2003 and 2006. During this period, the use of soybean oil for industrial applications increased from 552 million pounds to 2379 million pounds in the United States (SoyStatsTM 2007). Currently, 12% of U.S.

soybean oil consumption is directed to industrial uses. The large majority of this increase has resulted from demand for soybean oil for biodiesel production. Given the rising petroleum prices, it is likely that this demand will increase into the foreseeable future, not only for biodiesel but for the production of industrial materials such as lubricants, paints, and plastics that have historically been derived from crude oil.

2.4.2 High Oleic Acid Soybean Oil

Nearly all of the soybean oil that is now used for industrial applications is conventional soybean oil that lacks any genetic modification of its fatty acid composition. However, through the use of breeding and biotechnology, it is possible to generate fatty acid profiles that improve the functionality of soybean oil for industrial uses, including biodiesel. Soybeans with increased oleic acid content have received the most attention for use in a variety of industrial applications (Kinney and Clemente 2005). Enhancement of oleic acid content can be achieved by breeding different mutant alleles for $\Delta 12$ oleic acid desaturase (*FAD2*) genes (Burton et al. 2004). Typical *FAD2* enzymes convert oleic acid to linoleic acid (Okuley et al. 1994). This enzyme uses an oleic acid molecule principally to phosphatidylcholine (PC) as its substrate. By mutating or suppressing the expression of *FAD2* genes, soybean seeds accumulate oleic acid, rather than linoleic acid, as the major component of the seed oil. The largest increases in oleic acid have been obtained through a biotechnological approach by suppression of the *FAD2-1* gene in combination with down regulation of palmitoyl-acyl carrier protein thioesterase (*FatB*) genes (Buhr et al. 2002). Through the use of this approach, high oleic (HO) acid oils containing as much as 90% oleic acid have been achieved. The suppression of *FatB* expression also reduced the palmitic acid content of these oils to <4% of the total fatty acids (Buhr et al. 2002), resulting in oils with less saturated fatty acids.

HO soybean oils are not only enriched in oleic acid but also have reduced amounts of the polyunsaturated fatty acids linoleic and linolenic acids. For example, HO oils with as much as 90% oleic acid have <4% each of polyunsaturated fatty acids and palmitic acid (Buhr et al. 2002). It is likely that most of the remaining polyunsaturated fatty acids can be removed by breeding into 1% linolenic acid soybean mutant lines (Ross et al. 2000). The combination of high oleic acid and low polyunsaturated fatty acid content results in a liquid oil with greatly improved oxidative stability. HO soybean oil with 85% oleic acid, for example, has an oxidative stability index value that is nearly 12-fold higher than that of conventional soybean oil (Knowlton 1999). This property is critical for the use of vegetable oils in lubricants, including motor and hydraulic oils (Erhan et al. 2006; Sharma et al. 2005). HO soybean oil also displays superior

properties in biodiesel formulations compared to conventional soybean oil (Kinney and Clemente 2005; Tat et al. 2007). The reduced content of polyunsaturated fatty acids in HO soybean oil results not only in enhanced oxidative stability but also less emission of nitrogen oxides (NO_x) (Tat et al. 2007). In addition, the cold point properties are also improved in HO soybean oil due primarily to its lower content of palmitic acid (~4% in HO soybean oil versus ~12% in conventional soybean oil) (Tat et al. 2007). For example, methyl esters generated from HO soybean oil have cloud and pour points of -5 and -9°C, respectively. By comparison, the cloud and pour points of methyl esters produced from conventional soybean oil are 1 and 0°C, respectively (Tat et al. 2007). Undoubtedly, soybean oils with additional novel industrial functionalities can be generated by crossing of HO soybeans derived from biotechnology with soybeans with altered contents of other fatty acids (e.g. high stearic acid) derived from breeding of mutant alleles.

2.4.3 Metabolic Engineering of Soybean for the Production of Oils with High-Value Industrial Fatty Acids

The improved properties of HO soybean oils for industrial applications demonstrate the utility of altering the relative proportions of the five fatty acids typically found in soybean seeds. Another approach for generating soybean oil with improved industrial functionality is to introduce genes that produce fatty acids with novel structures. Such genes have typically been isolated from non-agronomic species that accumulate unusual types of fatty acids in their seed oils (Voelker and Kinney 2001). The enzymes encoded by such genes have been useful for producing fatty acids with carbon chain modifications such as hydroxy or epoxy groups or for producing fatty acids with novel double bond positions and configurations or for even producing fatty acids with chain lengths greater than 18 carbon atoms. Many of these genes encode enzymes that are related to FAD2 but have evolved new enzymatic properties (Cahoon and Kinney 2005). One example is the oleic acid hydroxylase from castor (*Ricinus communis*) (van de Loo et al. 1995). This enzyme uses the same oleoyl-phosphatidylcholine substrate as the typical FAD2 but introduces a hydroxyl group rather than a *cis* double bond at the Δ12 position. The hydroxylated fatty acid product ricinoleic acid (OH-18:1Δ9) has a wide-range of useful industrial properties. These include uses in lubricants, hydraulic fluids, surfactants, cosmetics, and nylon production. The hydroxyl group of ricinoleic acid may also increase the lubricity of fatty acid methyl esters in biodiesel applications (Kinney and Clemente 2005). In an attempt to capture these desirable industrial properties in soybean, research has been conducted to introduce the castor hydroxylase gene under control of a seed-specific promoter to soybean (Kinney and Clemente 2005). These studies have resulted in the generation of soybeans that accumulate ricinoleic acid to approximately 15% of

the total fatty acids of the seed oil. Oils extracted from these engineered seeds are currently being evaluated to determine the value of the increased lubricity associated with fatty acid hydroxylation and the improved oxidative stability associated with the increased oleic acid content (Clemente unpublished results).

Fatty acid epoxidation is another modification that has received interest for the improvement of the industrial value of soybean oil. Chemical epoxidation of soybean oil is currently used to generate plasticizers and precursors such as polyols for the production of coatings, adhesives, and biopolymers (Liu et al. 2006). Epoxidation involves the reaction of the double bonds of the fatty acids of soybean oil with hydrogen peroxide under acidic conditions (Vlcek and Petrovic 2006). This reaction is non-specific for conversion of the $\Delta 9$, 12, and 15 double bonds that can be found in the fatty acids of soybean oil. A number of non-agronomic plant species have evolved enzymes that allow for the specific conversion of the $\Delta 12$ double bond of linoleic acid to form the epoxy fatty acid vernolic acid (Voelker and Kinney 2001). Vegetable oils enriched in vernolic acid have received interest not only for existing applications for of epoxidized soybean oil but also for paint solvents with low content of volatile organic compounds (VOCs) solvent for paint (Bhardwaj et al. 2007). Novel chemistries for conversion of vernolic acid to industrially useful materials have also been explored (Ayorinde et al. 1997). The $\Delta 12$ epoxy group of vernolic acid can be produced from linoleoyl-PC by the activity of a divergent FAD2 epoxxygenase (Lee et al. 1998) or from a structurally unrelated cytochrome P450 epoxxygenase (Cahoon et al. 2002). A gene from *Vernonia galamensis* for a FAD2 epoxxygenase has been introduced into soybean to produce oils containing approximately 7% vernolic acid (Hitz 1998). Similar levels of vernolic acid have been produced in soybean somatic embryos by expression of a cytochrome P450 epoxxygenase from *Euphorbia lagascae* (Cahoon et al. 2002).

A major industrial use of soybean oil is as a component of soy ink (Erhan and Bagby 1991). Soy ink is widely used for the color print of newspapers. A limitation of soybean oil for newspaper print ink is its relatively slow drying rate. To improve its drying properties, soybean oil can be supplemented with tung oil, which contains high levels of fatty acids with conjugated double bonds (or “conjugated fatty acids”). The double bonds of conjugated fatty acids are positioned at adjacent carbon atoms whereas the double bonds of linoleic and linolenic acids, the major polyunsaturated unsaturated fatty acids of soybean oil, are separated by methylene groups. To date, soybeans have been engineered to produce two isomers of conjugated fatty acids eleostearic acid and calendic acid (Cahoon et al. 1999, 2006). Both fatty acids are produced by the activity of FAD2-related enzymes termed “fatty acid conjugases” (Cahoon et al. 1999, 2001; Qiu et al. 2001). These enzymes convert an existing *cis*-double bond of linoleic acid bound to PC into two conjugated *trans*-double bonds to generate a conjugated trienoic fatty acid (Cahoon and Kinney 2005). The fatty acid conjugase that produces calendic acid converts the $\Delta 9$ double bond of linoleic acid to $\Delta 8$ -*trans* and $\Delta 10$ -*trans* double bonds; whereas, the fatty acid conjugase that generates eleostearic acid converts the $\Delta 12$ double bond of

linoleic acid into $\Delta 11$ -*trans* and $\Delta 13$ -*trans* double bonds (Cahoon and Kinney 1995). A $\Delta 9$ -modifying conjugase cDNA from *Calendula officinalis* has been engineered in soybean to produce calendic acid at levels of 10–25% of the fatty acids of the seed oil (Cahoon et al. 2006). Similarly, $\Delta 12$ -modifying conjugase cDNAs from *Momordica charantia*, *Impatiens balsamina*, and *Chrysobalanus icaco* have been introduced into soybean somatic embryos to generate oils with up to 20% eleostearic acid (Cahoon et al. 1999, 2006, 2007a).

From a technical standpoint, the metabolic engineering of genes from other species has successfully resulted in the production of soybean oil with novel fatty acid compositions. However, in most all cases to date, the amounts of these fatty acids obtained in soybean oil have been considerably lower than those in oils from seeds that naturally accumulate unusual fatty acids. For example, castor bean accumulates ricinoleic acid to levels of approximately 90% of its seed oil. However, soybeans engineered to express the castor bean hydroxylase accumulate ricinoleic acid to amounts of about 15% of the seed oil (Kinney and Clemente 2005). The inability to achieve high levels of unusual fatty acid accumulation in engineered soybean seeds has been the major technical hurdle that has limited the adoption of this technology for producing new types of industrial oils in soybean. In the case of fatty acids derived from divergent FAD2s, bottlenecks associated with unusual fatty acid accumulation in engineered oilseeds appears to result from defects in the efficient flux of unusual fatty acids from PC following their synthesis on this lipid (Cahoon et al. 2006, 2007b). This is exemplified by phenotypes observed in seeds engineered to express fatty acid conjugases. In *Arabidopsis* and soybean seeds that express $\Delta 9$ - and $\Delta 12$ -type conjugases, conjugated fatty acids not only accumulate in TAG but are also present in PC at the same or higher relative amounts (Cahoon et al. 2006). For example, soybean seeds that express the *C. officinalis* conjugase accumulate calendic acid to amounts of about 20% of total fatty acids in triacylglycerols (Cahoon et al. 2006). Calendic acid also aberrantly accumulates to levels of about 25% of the fatty acids in PC in these seeds. By contrast, calendic acid is found in *C. officinalis* seeds at levels of approximately 55% of TAG fatty acids, but <1% of the fatty acids of PC. As such, it appears that *C. officinalis* has evolved a mechanism for limiting calendic acid aberrantly accumulation in PC that is absent in soybean seeds. Seeds of other species that naturally produce conjugated fatty acids have also apparently evolved the metabolic capacity to efficiently remove these fatty acids from PC, as conjugated fatty acids are rarely found at levels of >2% in PC, even in species that accumulate conjugated fatty acids to >80% of the fatty acids of their seed oil (Cahoon et al. 2006). Current research is focusing on the identification of divergent types of phospholipolytic enzymes from plants that naturally accumulate unusual fatty acids from the activity of FAD2-related enzymes. It is presumed that such enzymes have evolved for the efficient metabolism of unusual fatty acids, and that co-expression of these metabolic enzymes

along with FAD2 biosynthetic enzymes will enable the production of economically-relevant levels of industrial fatty acids in soybean seeds.

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