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MODIFYING THE FATTY ACID PROFILE OF SOYBEAN OIL FOR NUTRITIONAL AND INDUSTRIAL APPLICATIONS

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MODIFYING THE FATTY ACID PROFILE OF SOYBEAN OIL FOR
NUTRITIONAL AND INDUSTRIAL APPLICATIONS

By

Hyunwoo Park

A DISSERTATION

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MODIFYING THE FATTY ACID PROFILE OF SOYBEAN OIL FOR NUTRITIONAL AND INDUSTRIAL APPLICATIONS

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University of Nebraska, 2012

Advisor: Tom Clemente

The objectives of this study are: 1. To develop of soybean oil low in palmitic acid and elevated in stearic and oleic acids for margarine source. 2. To produce the high omega-3 fatty acid and astaxanthin in soybean seed as a feedstock for aquaculture. Over expression of mangosteen stearoyl-ACP thioesterase gene increased stearic acid content up to 10% to 15%. The identified elevated stearic acid events were crossed with our high oleic acid/low palmitic acid event. Average stearic and oleic acid content ranged 14 to 19% and 68 to 73% respectively. Stearic acid content of elevated stearic acid soybean event and cross line was stable over generation. However, in generation six and seven under field conditions the oleate levels decreased to 30-40%.

Decline of marine fish stocks due to over-fishing and the use of wild fish for diets of farmed fish pushed for development of sustainable land based alternative diets for farming fish such as soybean with respect to levels of very long chain omega-3 fatty acids, EPA and DHA. To generate EPA and the high value carotenoid, astaxanthin in soybean, we first created a two gene stack in soybean combining Δ6 and Δ15 desaturase genes, respectively. The resultant event designated 535-9 accumulates stearidonic acid in seed oil up to 35%. We have subsequently generated transgenic events that carry Δ6
elongase and Δ5 desaturase genes. Selected events derived from the latter have been crossed with event 535-9. EPA level in seed oil was up to 4%. In regards to astaxanthin production, we generated transgenic event carrying phytoene synthase, crtZ and crtW genes. Transgenic soybean produced up to 25µg/g astaxanthin. We crossed the four-gene EPA stack with the three gene astaxanthin stack to create a prototype soybean-based feedstock for aquaculture. We identified that the longer chain omega-3 fatty acids ETA and EPA, combined ranged from approximately 3-5% of the oil, along with astaxanthin and β-carotene accumulation from 23 to 44.8 ug/gr and 527 to 1139 ug/gr seed, respectively.
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I would like to acknowledge the contribution of my mother, sister and my wife, Yeonjung. Finally, I dedicate this thesis to the memory of my father and my beatiful daughter, celestyna.
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Soybean – Introduction

Soybean [Glycine max (L.) Merr.] is one of the major crops in the world, providing protein and oil. Soybean belongs to regume family. Regume plant fixes atmospheric nitrogen via symbiosis with nitrogen fixing rhizobia bacteria. Chromosome number of soybean is 2n=40. Recently Soybean (Glycine max var. Williams 82) genome was sequenced by whole-genome shotgun approach. The number of predicted protein coding genes is 46,430. This is 70% more than that of Arabidopsis (Schmutz et al, 2012). U.S. production of soybean in 2011 was 3,329 million bushels, which accounts for over 30% of the total world soybean production (NASS, 2012). Soybean seed contains about 20% oil and 40% protein. Soybean is a good source of protein, oil and tocopherol. Soybeans have been used for human diet, source of margarine and shortening, livestock feed and biodiesel feed stock. Soybeans are about 90 percent of United States total oilseed production and soybean oil is one of the world’s most widely used edible oils. Soybean oil occupies 65% of US edible vegetable oil market. Canola, palm and corn oil occupies 12, 8 and 7% respectively in 2010 (USDA, ERS, 2010). Most of shortening and margarine produced in the united state containing soybean oil. Soybean oils are primarily composed of 13% palmitic acid, 4% stearic acid, 18% oleic acid, 55% linoleic acid, and 10% linolenic acid. Soybean oil is one of the non-fish sources of omega 3 fatty acid.
Oxidation stability of soybean oil and hydrogenation

Oil crop store the lipids as energy source in form of TAGs. TAGs are synthesized by an extension of the membrane-lipid synthesis pathway. Storage TAGs are existed as small organelles called oil bodies. The fatty acid composition in TAGs is one of the important factors determining the quality of oils. Commercial soybean oil contains 55% linoleic acid (18:2) and 13% linolenic acid (18:3). Relatively high content of polyunsaturated fatty acids (PUFAs) results in low oxidative stability. The olefinic bonds of PUFAs are labile to oxygen. The spontaneous oxidation rate of 18:3 is twice the rate for 18:2. Oxidation rates become greater under high temperature frying condition (Wilson, 2004). To enhance the oxidative stability and shelf life, soybean oil is often partially hydrogenated. Although hydrogenation increases the oxidative stability, during hydrogenation, trans fatty acids are produced. It has been known that trans fatty acid associated with an elevated risk of coronary heart disease (Mensink et al., 1994). Therefore, to enhance the oxidative stability of soybean oil without hydrogenation, polyunsaturated fatty acid content should be reduced or saturated fatty acids content should be increased (Clemente and Cahoon, 2009).

Soybean oil is composed of 10% palmitic acid, 4% stearic acid. It has been reported that dietary saturated fatty acid effect on plasma lipoprotein. Most of study showed that stearic acid (18:0) has a neutral effect on LDL (low-density lipoprotein)-cholesterol level on plasma whereas lauric acid (12:0), myristic acid (14:0) and palmitic acid (16:0) strongly increases LDL-cholesterol (Liu et al., 2002). The LDL-cholesterol level of plasma is one of the major risk factors for coronary heart disease. Therefore, increasing the stearic acid content rather than palmitic acid content not only will increase
the oxidative stability by reducing the need of hydrogenation but also reduce the formation of trans fatty acid. So, ideal type of fatty acid profile of soybean oil for margarine or shortening is trans fatty acid free oils low in palmitic acids with high in stearic acid and oleic acid.

**Alteration of fatty acid profile**

Beside chemical modification of oil by hydrogenation or interesterification, many researchers tried to alter the fatty acid composition by breeding, mutation or transgenic approach because oil crop cannot produce abundant amount of stearic acid and oleic acid. Among all enzymes involved in FA synthesis, 3-keto-acyl-ACP II (KAS-II), steryl-ACP-thioesterase or Δ9-steroyl-ACP-desaturase has been known to be able to change the stearic acid content. KAS-II catalyzes the elongation of 16:0-ACP to 18:0-ACP, steryl-ACP-thioesterase convert 18:0-ACP to 18:0 and ACP and Δ9-steroyl-ACP-desaturase desaturates 18:0-ACP to 18:1-ACP (Pantalone et al., 2002). Steroyl-ACP-thioesterase (Gram FatA1) has been cloned from mangosteen (*Garcinia mangostana*) and their expression in *Bassica* seeds results in the accumulation of stearate up to 22% in seed oil (Hawkins and kridl, 1998). Expression of this gene in soybean seed showed the similar level of accumulation in seed oil (Kridl J, 2009). The site directed mutagenesis of Gram FatA1 increase the enzyme activity 13 fold in vitro and their expression in *Bassica* seeds led to 55-68% more accumulation of stearate than plants expressing wild type enzyme (Facciotti et al., 1999). Recently three soybean Δ9-steroyl-ACP-desaturase genes, designated SACPD-A, SACPD-B and SACPD-C have been reported (Byfield et al., 2006; Byfield and Upchurch, 2007; Zhang et al., 2008). Unlike the other two genes, high level of SACPD-C gene expression was only detected in developing seed. This result indicates
that SACPD-C is controlled by seed specific promoter and this gene might involve in elevated 18:0 phenotype in seed. It was revealed that elevated stearic acid soybean mutant A6 (30% 18:0) and FAM94-41(9% 18:0) lines were due to deletion of SACPD-C gene and amino acid substitution in SACPD-C gene respectively (Zhang et al., 2008).

Oil high in 18:1 and low in polyunsaturated fatty acids has oxidative stability as well as nutritional value. The Δ12 fatty acid desaturase (FAD2) convert oleic acid to linoleic acid which is the first step for polyunsaturation. FAD2 is located in the endoplasmic reticulum(ER) membrane. More than 90% of polyunsaturated fatty acid in nonphotosynthetic tissue are synthesized through ER located FAD2 (Miquel MF and Browse JA 1994). It was reported that FAD2 gene family of soybean is consisted of at least five members in four regions of the genome (Schlueter et al., 2007). FAD2-1A and FAD2-1B genes are primarily expressed in developing seeds, whereas FAD2-2A and FAD2-2B genes are expressed in developing seeds and vegetative tissues. Recently new FAD-2 gene, designated FAD2-2C, was found. Semiquantitative RT-PCR result showed that only FAD2-2C transcript level was increased in developing seed grown in cooler conditions compare to those grown in warm conditions (Schlueter et al., 2007).

In plant cell, lower temperature lead to increase in the production of PUFAs such as linoleic and linolenic acids, and a decrease in saturated and monounsaturated fatty acid, such as oleic acid. The increase of both PUFAs at lower temperature thought to maintain the membrane fluidity, because their melting temperature is low (Schlueter et al., 2007; O’Quin et al., 2010).

Mid oleic soybean having 40 to 70% oleic acid or high oleic soybean having up to 80% oleic acid have been developed through conventional breeding or transgenic...
approaches. Rahman et al., (1994) developed mid oleic soybean with 46% oleic acid, designated M23, through x-ray irradiation. Recently study reveals that mid oleic phenotype was due to deletion of FAD2-1A locus (Sandhu et al., 2007). Through conventional breeding, N98-4445A soybean line with 50% oleic acid has been developed (Burton et al., 2006). The yield of both M23 and N98-4445A lines were significantly lower than that of wild type. Their oleic acid content was largely affected by temperature change (Scherder and Fehr, 2008; Bachlava et al., 2008). Soybean with 80% oleic acid has been developed through down regulation of FAD2-1A and 1B gene expression by posttranscriptional gene-silencing (PTGS). In case of this event, Oleic acid content of in seed oil and yield were not influenced by environment (Buhr et al., 2002; Graef et al., 2009). Pham et al., (2010) developed soybean with 80% oleic acid by crossing of soybean lines having missense mutation in the FAD2-1B allele with FAD2-1A gene deletion line, M23, or 17D line having missense mutation in FAD2-1A allele. High oleic acid trait of this line was stable across three different environments of Costa Rica, Portageville, MO and Columbia, MO.

Hoffer et al., (2011) reported that down regulation of FAD2-1 gene with FAD2-1A intron or 3’UTR region does not affect transcription rate of target genes but strongly reduced the transcript level in nucleus. This result showed that nuclear precursor mRNA can be a target for PTGS and RNA degradation can be occurred in the nucleus. Wagner et al., (2011) also generated soybean with up to 80% oleic acid by down regulation of FAD2-1 gene with 420bp RNAi trigger of FAD2-1A intron. They observed a gradual depression of FAD2-1 transcripts level by increasing the trigger fragment size.
In this study, we generated the elevated stearic acid soybeans by over expression of mangosteen stearoyl-ACP thioesterase gene under the control of the seed specific promoter element, β-conglycinin. The identified elevated stearic acid soybean events were crossed with our high oleic acid/low palmitic acid soybean event. Stearic acid content of elevated stearic acid soybean event and cross line was stable over generation. But oleic acid content of cross line was reduced to 34 to 39% over generation in field condition. Finally, we developed a soybean with 15-16% stearic acid and 34-39% oleic acid.

**Astaxanthin and coloration for farming fish**

Astaxanthin (3,3’-dihydroxy-β,β’-carotene-4,4’-dione) is an orange color carotenoid pigment. It is widely distributed in nature and is found in certain aquatic animal, plant, algae and bird. All photosynthetic organism, some bacteria and fungi synthesized carotenoids. Molecular structure of astaxanthin is closely related to β-Carotene, lutein, zeaxanthin and canthaxanthin and they shares with physiological functions (Hussein et al., 2006). β-carotene is one of the most widely known carotenoids which is a major substrate for astaxanthin synthesis in plants. Astaxanthin is synthesized from β-Carotene by 3-hydroxylation and 4-ketolation at both ionone groups. One of major biological function of astaxanthin is a light harvesting role in the antenna complexes of the chloroplast. Another function is the protecting role from the harmful photooxidative effect from light (Wataru M, 1991). It have been reported that antioxidant activity of astaxanthin is 10 times stronger than those of any known carotenoids and 100 times greater than those of α-tocopherol (Wataru M, 1991). Also it have been reported that astaxanthin has anti-inflammatory properties and anti cancer properties. It also effect
on immune system and diabetes (Hussein et al., 2006). As animal cannot synthesize carotenoid de novo, they should obtain from diet. It have been reported that mammals cannot convert dietary astaxanthin into Vitamin A. Thus, unlike β-Carotene, astaxanthin dosen’t has pro-vitaminA activity (Goswami et al., 2010; Jyonouchi et al., 1995).

Carotenoid pigments contribute to yellow, orange and red coloration of many types of seafood (Shahidi et al., 1998). Because of consumers have preferences for red colored salmonid fishes. Color of flesh is the most important factor to determine the acceptance and price of salmonid fishes by consumer (Bjerkeng B, 2000). Color of flesh is determined by carotenoid type. Astaxanthin and canthaxanthin are the most commonly used carotenoids for pigmentation of salmonoids fishes. Canthaxanthin pigmented salmon has a more yellowish color than astaxanthin pigmented salmon (Shahidi et al., 1998). Since astaxanthin is stronger red and pink coloring source than canthaxanthin, it has been used for pigmentation in aquaculture, specifically salmon, trout, and red sea beam (Guerin M et al., 2003). Synthetic astaxanthin have been mainly used in fish farming. Natural sources of pigment such as shrimp residues, krill, green algae, red yeast, red pepper and paprika also has been used.

Production of astaxanthin through metabolic engineering

The estimated market value of commercially used carotenoids was nearly $1.2 billion in 2010. The market value of beta–carotene, lutein and astaxanthin were $261 million, $233 million and $225 million in 2010 (The global market for carotenoid, BBC research, 2011). Market price of astaxanthin is above $2000/kg. Most of the astaxanthin used in fish farming is synthetic astaxanthin. One of the problems of synthetic
astaxanthin is the contamination with reaction intermediates or by-products. During chemically synthesis of astaxanthin, stereoisomer by-products (3S.3’R) and (3R.3’R) are produced, in addition to the naturally occurring (3S.3’S). By-products may have an inhibitory effect on the bioactivity of natural astaxanthin (Hasunuma et al., 2008). The high production cost of synthetic astaxanthin and consumer’s demand for natural astaxanthin made researcher try to produce the astaxanthin from natural sources (Li et al., 2011). Astaxanthin can be produced algae *Haematococcus pluvialis* and red yeast *xanthophyllomyces dendrorhous*. Because yeast has 0.4% astaxanthin by dry weight, algae is more widely used for production of astaxanthin, which contain 3% astaxanthin by dry weight (Milledge, 2011). Although algae can produce more astaxanthin, algae needs high light intensities, special growing condition and grows slowly (Yuan and Chen, 2000). Also, researchers tried to produce the ketocarotenoids in plant. Ketocarotenoid is a group of oxygenated carotenoids. Astaxanthin is synthesized by introduction of keto and hydroxyl group at the 4, 4’ and 3, 3’ position of β-ionone rings of β-carotene. These reactions are achieved by β-carotene ketorase (CrtW, BKT or CrtO) and β-carotene hydroxylase (CrtZ or BHY). It is proposed that ketolating of Zeaxanthin is major limitation of astaxanthin production in transgenic plant. Over expression of algal β-carotene oxygenase under seed storage protein promoter napA in arabidopsis seed result in accumulation of free and esterified forms of ketocarotenoids. Crossing of this plant with another transgenic plant over expressing phytoene synthase increased total pigment up to 4.6 fold and increased the three major ketocarotenoids, 4-keto-lutein, adonirubin and canthaxanthin up to 13 fold compared to transgenic plants expressing β-carotene oxygenase (Stålberg et al., 2003). Over expression of bacterial phytoene synthase under
seed specific promoter napin in Brassica accumulated the 50 fold increased carotenoids (Shewmaker et al., 1999). Seven key genes \{\(\beta\)-carotene ketorase (crtW), Isopentenyl pyrophosphate isomerase (idi), Geranylgeranyl pyrophosphate synthase (crtE), \(\beta\)-carotene hydroxylase (crtZ), Phytoene synthase (crtB), Phytoene desaturase (crtI) and Lycopene \(\beta\)-cyclase (crtY)\} involved in ketocarotenoid synthesis were over expressed in brassica napus. This transgenic plant accumulated 19- to 30- fold increased carotenoids and total amount of carotenoids in transgenic seed was 412-657ug/g of fresh weight (Fujisawa et al., 2009). Gerjets et al., (2006) showed possibility of production of astaxanathin in potato tubers by introducing cyanobacterial ketorase into transgenic line inactivated zeaxanthin epoxidase. Carrot root is important source of dietary \(\alpha\)-carotene, \(\beta\)-carotene and lutein. Jayaraj et al., (2008) overexpressed \(\beta\)-carotene ketorase of \textit{Haematococcus pluvialis} and \(\beta\)-carotene hydroxylase of Arabidopsis in carrot. Up to 70% of total carotenoids were converted to novel ketocarotenoids with accumulation of up to 2,400ug/g root dry weight. Astaxanthin, adonirubin and canthaxanthin were accounted for 57% of the total ketocarotenoid in transgenic roots. Astaxanthin content of root and leaf was 91.6ug/g and 34.7ug/g dry weight respectively (Jayaraj et al., 2008). Expression of CrtW and CrtZ genes form a marine bacterium \textit{Brevundimonas sp} in the tobacco chloroplast by plastid transformation lead to accumulate the astaxanthin up to 5.4mg/g dry weight. It is correspond to 74% of the total carotenoids (Hasunuma et al., 2008). Schmidt et al., (2020) expressed soybean codon optimized bacterial phytoene syntase under seed specific promoter. They observed that 870ug/g of \(\beta\)-carotene was accumulated in seed. Recently several algal \(\beta\)-carotene ketolase (BKTs) were functionally characterized by using \textit{Escherichia coli} system to verify ketolation of zeaxanthin is major limiting step for
production of astaxanthin in transgenic plant. Three identified BKT genes, *Chlamydomonas reinhardtii* (CrBKT), *Chlorella zofingiensis* (CzBKT), and *Haematococcus pluvialis* (HpBKT3), with high, moderate and low conversion rate were transformed to *Arabidopsis thaliana* respectively. Transgenic Arabidopsis expressing CrBKT and CzBKT accumulated astaxanthin up to 2mg/g and 0.24mg/g dry weight respectively. Transgenic Arabidopsis expressing HpBKT3 was unable to synthesis astaxanthin (Zhong et al., 2011)

In this study, we generated soybeans producing astaxanthin up to 25ug/g dry weight by co-expressing maize phytoene synthase and *Brevundimonas sp* CrtW and CrtZ genes under seed specific promotor. These transgenic soybeans have crossed with another transgenic soybean producing very long chain omega-3 fatty acids, eicosapentaenoic acid (EPA) to produce astaxanthin and EPA for alternative land-based fish feed stock for aquaculture.

**Plant membrane lipids and storage lipids**

The control of membrane lipid composition is essential for normal plant growth and development. The cell membrane is mainly composed of lipids and proteins and lipid molecules constitute about 50% of the mass of most cell membranes. The three major classes of membrane lipids are phospholipids, glycolipids, and cholesterol. The major structural lipids in eukaryotic membranes are the phospholipids: phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), phosphatidylinositol (PI), and phosphatidic acid (PA). PC accounts for >50% of the phospholipids in most eukaryotic membranes (Meer GV et al., 2008). Interestingly, desatulation, hydroxylation,
epoxyation, acetylation and conjugation of fatty acid occurred on PC in endoplasmic reticulum (ER).

16-carbon and 18-carbon fatty acids having up to three double bonds are the main membrane lipids of plant. Most seeds contain the same acyl groups that are found in membrane lipids but the lipid composition in seed oils of some plant species is significantly different (Millar AA et al., 2000). Sometimes unusual fatty acid (ricinoleic acid, calendic acid, α-elestearic acid and vernolic acid) accumulate up to 80% of the total fatty acid content in the seed triacylglycerol(TAG), but these unusual fatty acids are generally excluded from the membrane lipids of the plant (Cahoon EB et al., 2006). For example, the leaves of castor bean contain no detectable ricinoleic acid (James AT et al., 1965).

There are two distinct pathways for the synthesis of membrane lipids in higher plants. One is prokaryotic pathway which occurs in the chloroplast inner envelope and the other is eukaryotic pathway which occurs in the ER. In both pathways, membrane lipids are synthesized by the Kennedy pathway. In seeds, diacylglycerol is also further acylated to produce triacylglycerol. Therefore, the synthesis of membrane and storage lipids shares the Kennedy pathway (Ohlrogge JB and Browse J 1995).

In seeds, lipids accumulate as TAGs which are formed by an extension of the membrane-lipid biosynthetic pathway common to all plant tissues. Storage TAG in seeds presents in small discrete intracellular organelles called oil bodies. An oil body has a matrix of TAG, which is surrounded by phospholipids (PL) and oleosins (Lu C et al., 2009). Because unusual fatty acids are stored in oil bodies, plants can accumulate high levels of unusual fatty acids in storage lipids without deleterious effects on membrane.
Discrimination in plant membrane lipids

Sometimes heterologous expression of genes responsible for unusual fatty acid (very long chain fatty acid, ricinoleic acid and α-eleostearic acid etc.) in transgenic plants has resulted in low accumulation of the desired fatty acid product in addition to changes in plant morphology. Transgenic seed display wrinkled morphology and chloroplast from transgenic plant have altered thylakoid membrane shape. Such alterations of morphology must be solved for the commercial production of unusual fatty aids in genetically engineered oil seeds (Millar AA., 1998; Lu C et al., 2006; Cahoon EB et al., 2006). For example, the over expression of Arabidopsis Fatty acid elongase1 under 35S promoter in Arabidopsis produced only up to 30% VLC-PUFC in leaf oils. The High proportion of VLC-PUFC was accumulated in PC and PE. But there was a correlation between the levels of VLC-PUFC and stunt type phenotype and short shoot. It seems that host plant doesn’t efficiently screen out the membrane lipids.

The physical and chemical properties of different lipid acyl chains can remodel the membrane structure. Many unusual fatty acids have special physical and chemical properties. Insertion of an unusual fatty acid into the membrane bilayer disrupts the structural integrity and will induce deleterious effects on the cell. For example, the polar oxygenated functional group of ricinoleic acid would be incompatible with the hydrophobic environment of the membrane. It should be screened out from membrane. This screening process will result in different fatty acid compositions between storage and membrane lipids (Millar AA et al., 2000).
Momordica charantia, and Vernicia fordii accumulate conjugated fatty acids α-eleostearic and vernolic acid to 80% of the total fatty acids in seed (Liu et al., 1997; Liu et al., 1998). Ricinus communis accumulate ricinoleic acid up to approximately 90% of the total fatty acid in seed. But conjugated fatty acids and ricinoleic acid content in PC is lower than 5% of total fatty acid (Erp HV et al., 2011). In seeds, storage lipids (TAGs) are formed by an extension of the membrane-lipid biosynthetic pathway which is common to all plant tissues. DAG used for TAG synthesis is mostly derived from PC, whereas de novo synthesized DAG is mostly used for PC synthesis. Almost 60% of the newly synthesized fatty acid first enters glycerolipids through PC acyl editing largely at the sn-2 position (Bates PD et al., 2009). PC is also a membrane lipid but storage lipids are mostly derived from PC. Therefore, screening and moving mechanisms of unusual fatty acids from PC to storage lipids must be extremely selective and efficient. But how plants prevent the accumulation of unusual fatty acids in membrane lipids is not completely understood (Millar AA et al., 2000). Understanding of membrane lipids discrimination mechanism will give a chance to increase more VLC-PUFC content in plant.

**Omega-3 fatty acids and alternative diets for farming fish**

One of the major goals of plant biotechnology is the production of important oils in the oil seed crop. For several years, scientists have been trying to produce high homogeneity of novel fatty acids in oil seeds through genetic engineering (Dyer and Mullen, 2008).
Marine sources of long chain omega-3 fatty acids are EPA and DHA. Plant sources of omega 3 fatty acids are ALA. The long chain omega-3 fatty acids such as EPA and DHA play important role in brain function and normal growth and development. Also, research suggested that EPA and DHA prevent cardiovascular disease such as heart attacks and strokes. Human body does not synthesize EPA and DHA from ALA, so we must obtain them from diet (Damude HG and Kinney AJ, 2008)

Demand of fishmeal and fish oil has continuously increased over past decade. However marine fish stocks have been severely declining due to pollution of the marine ecosystem and over fishing. Aquaculture has been considered as alternative method to fit the demand. But use of wild fish for diets of farmed fish resulted in reduction of wild fish population which is low down on the food chain. Consequently, aquaculture didn’t fulfill well the demand of fish. This result pushes the developing of sustainable land based alternative diets for farming fish such as soybean with respect to levels of very long chain omega-3 fatty acids, eicosapentaenoic acid (EPA), and docosahexaenoic acid (DHA)(Damude and Kinney, 2008; Petrie et al., 2010; Venegas-Calerón et al., 2010; Ruiz-Lopez., et al 2012)

Production of VLC-PUFA through metabolic engineering

Δ12 and Δ15 desaturases play important role in the synthesis of membrane lipid to support the photosynthesis and these enzymes exist in all higher plant. Therefore, higher plants are rich in LA and ALA that serve as metabolic precursors for VLC-PUFA synthesis. But VLC-PUFA are almost completely absent in higher plants because of lack of enzyme to synthesize VLC-PUFA (Venegas-Calerón et al., 2010). Three enzymatic
steps of Δ6 desaturation, Δ6 elongation and Δ5 desaturation are required to convert poly unsaturated C18 fatty acid into arachidonic acid (ARA) or EPA. During the last decade, all primary genes involved in VLC-PUFA synthesis have been isolated from various organisms and transgenic plants have been generated to synthesize the VLC-PUFA in seed oil.

Expression of Δ6 and Δ5 desaturase from *Phaeodactylum tricornutum* and Δ6 elongase from *Physcomitrella patens* in linseed under UPS promoter accumulated up to 1.5% ARA and 1% EPA. Also, they found that high proportion of Δ6 desaturated C18-fatty acid didn’t efficiently elongate to C20-PUFA. This result maybe due to slow exchange of Δ6 desaturated acyl groups between PC and the acyl CoA pool (Abbadi et al 2004).

Δ6 desaturase, Δ6 elongase and Δ5 desaturase from *Mortierella alpine* along with an *Arabidopsis* Δ15 desaturase and *Saprolegnia diclina* Δ17 desaturase were expressed under seed-specific promoters in soybean. EPA content in seed oil was reached to 20% with significantly no level of intermediate fatty acid, ALA. This is due to Δ17 desaturase highly converted ALA into EPA (kinney et al., 2004).

Alternative Δ9 elongation/Δ8 desaturation pathway was applied to produce the VLC-PUFA. *Isochrysis galbana* Δ9 elgongase, protest *Euglena gracilis* Δ8 desaturase and *M. alpina* Δ5 desaturase were expressed in Arabidopsis under 35S promoter. This try resulted in accumulation of 6.6% ARA and 3.0% EPA in total lipids of leaf tissue. This result showed omega3 and omega6 Δ8 desaturation for the VLC-PUFA production is working in higher plant as well as possibility of bypassing rate-limiting steps associated
with poor elongation rates in conventional Δ6 desaturase/ elongase pathway (Qi et al., 2004).

Series of *Brassica juncea* transformations with increasing numbers of genes involved in VLC-PUFA synthesis resulted in the accumulation of ARA up to 25% and EPA up to 15% of total seed fatty acids respectively. Compare to transgenic flax and tobacco (Abbadi et al., 2004) it seems like that fatty acid was more efficiently exchanged between the acyl-PC and acyl-CoA pools. They found no germination problem and over 90% of total ARA and total EPA was unexpectedly accumulated in TAG (Wu et al, 2005).

Previous study to produce the VLC-PUFA by introducing conventional Δ6 desaturation or alternative Δ8 desaturation pathway showed low level of production of ARA and EPA. This results attributed to inefficient transfer of fatty acid substrates between the the acyl-PC, where desaturation occurs and acyl-CoA pools, where elongation occurs. To avoid this bottle neck, acyl-CoA dependent dual-purpose Δ5/Δ6 desaturase from zebrafish was introduced along with nematode Δ6 elongase into Arabidopsis. Expression of these genes under seed specific napin prompter produce 4 fold higher level of EPA in seed than that of in transgenic linseed (Abbadi et al 2004). Precusor for EPA, ALA, level was less than a third of that present in transgenic linseed. Through this attempt, they showed acyl-CoA dependent desaturase is more efficiently operating than acyl-PC dependent desaturase (Robert et al 2005).

Another acyl-CoA dependent Δ6 and Δ5 desaturase were isolated from the microalga *Mantoniella squamata*. These two genes were coexpressed with Δ6 elongase of the *Physcomitrella paten* in Arabidopsis. It was referred as triple-MS plant. To directly compare the efficiency of EPA production by different desaturase, they generated two
more transgenic plants expressing acyl-CoA dependent Δ6 and Δ5 desaturase of the
*Ostreococcus tauri* with same elongase which is referred as triple-Ot plant and expressing
lipid-CoA dependent Δ6 and Δ5 desaturase of the *Phaeodactylum tricornutum* with same
elongase which is referred as triple-Pt plant. The most abundant new fatty acid in triple-
MS and Ot seed was ω3-20:4 Δ8,11,14,17. In case of triple-Pt seed, ω6-18:3 Δ6,9,12, ω3-
20:3 Δ11,14,17 and first desturation product of the ω3-pathway, 18:4 Δ6,9,12,15 were
most abundant. The accumulation of both Δ6 desaturased fatty acids indicates that
presence of bottle neck in triple-Pt seed expressing lipid-CoA dependent desaturase. They
observed no or small amount of accumulation of Δ6 desaturation product 18:4 Δ6,9,12,15
in triple-MS and Ot plants and nearly all 18:4 Δ6,9,12,15 was elongated to ω3-20:4
Δ8,11,14,17 with high efficiency (97% conversion) by Δ6 elongase in triple-MS plant.
These results showed that acyl exchange bottle neck between the acyl-PC and acyl-CoA
pool was avoided by the use of acyl-CoA dependent desaturase. Nevertheless, EPA
content was still low. This was due to inefficient Δ5 desaturation. The distribution
analysis of ALA and EPA in various lipid classes showed the higher accumulation of
VLC-PUFA in TAG produced through acyl-CoA dependent pathway than those produced
through acyl-PC dependent pathway. Higher incorporation of VLC-PUFA produced
through acyl-CoA pathway into TAG rather than into phospholipid would be explained
by preference of the acyl-CoA: diacylglycerol acyltransferase and phospholipid:
diacylglycerol acyltransferase for conversion of ω3-substrates. (Hoffmann et al 2008).

Recently, two novel genes, an 18-carbon ω3 desaturase (CpDesX) and 20-carbon
ω3 desaturase (Pir-omega3) were identified from *Claviceps purpurea* and *Pythium
irregulare*. The expression of these genes with *Pythium irregular* Δ6 desaturase,
*Thraustochytrium sp* Δ5 desaturase from *Thalassiosira pseudonana* Δ6 elongase and *Calendula officinalis* Δ12 desaturase in zero or high-erucic acid *Brassica carinata* accumulated 9 and 20% EPA in total lipid of seed. This result showed that new noble genes are very effective in increasing EPA level and zero-erucic acid *Brassica carinata* is a good host plant for EPA production. This study showed that importance of gene as well as host plant to produce the VLC-PUFA (Cheng et al 2010).

Acyl-CoA dependent Δ6-desaturase (Micpu-d6D) was identified from the marine microalga *Micromonas pusilla*. They identified that this gene has a high ω3 substrate preference by yeast feeding experiment. They proved that this gene is acyl-CoA dependent desaturase through distribution analysis of STA in various lipids. Transient expression of *Micromonas pusilla* Δ6-desaturase with *Pyramimonas cordata* Δ6-elongase and *Pavlova salina* Δ5-desaturase under 35 promoters in *Nicotiana benthamiana* leaf resulted in accumulation of 26% EPA in leaf oil. Also very no level of intermediate fatty acid was found in leaf oil. This result indicates that the strong preference of ω3 substrate would simplify the metabolic pathway to produce the VLC-PUFA by reducing the additional Δ17-desaturation step (Petrie et al 2010).

In this study, to produce the EPA in soybean seed, we first created a two gene stack in soybean combining a Δ6 and Δ15 desaturases, respectively. The resultant event designated 535-9 accumulates stearidonic acid in seed oil up to 35% (Eckert et al., 2006). We have subsequently generated transgenic events that carry a Δ6 elongase and Δ5 desaturase genes. Selected events derived from the latter have been crossed with event 535-9 to produce EPA. We identified that F1 seed accumulates EPA in seed oil up to 4%. We crossed the four-gene EPA stack with the three gene astaxanthin stack to create a
prototype soybean-based feedstock for aquaculture feeds. We identified that F2 seed accumulates EPA and astaxanthin in seed oil up to 2% and 34ug/g respectively. Soybean seed producing astaxanthin and EPA can be used as a new alternative fish feed stock to displace fish oil based feed with land-based feed in a cost-effective environmentally friendly manner.
References


Pham AT, Lee JD, Shannon JG and Bilyeu KD (2010) Mutant alleles of FAD2-1A and FAD2-1B combine to produce soybeans with the high oleic acid seed oil trait. BMC Plant Biology.10:1-13


The global market for carotenoid, BBC research (2011) report code: FOD025D
The plant journal. 20: 401-412


CHAPTER TWO

Stacking of stearoyl thioesterase with a dual silenced palmitoyl-ACP thioesterase and Δ12 fatty acid desaturase in transgenic soybean

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Abstract

Soybean (*Glycine max* (L.) Merr) is valued for both its protein and oil components, which the seed is composed 40% and 20% of each storage component respectively. Given its high percentage of polyunsaturated fatty acids, linoleic acid and linolenic acid, soybean oil oxidative stability is relatively poor. Historically food processors have employed a partial hydrogenation process as a means to improve both the oxidative stability of soybean oil and functionality in solid oil end use applications. However the hydrogenation process leads to the formation of trans-fats, which are associated with negative cardiovascular health. As a means to circumvent the need for hydrogenation genetic engineering approaches are actively being pursued to improve oil quality in oilseeds. In this regard we report here on the introduction of the mangosteen (*Garcinia mangostana*) stearoyl-ACP thioesterase into soybean and its stacking with an event that is dual silenced palmitoyl-ACP thioesterase and Δ12 fatty acid desaturase expression in a seed specific fashion. Phenotypic analysis on transgenic soybean expressing the mangosteen stearoyl-ACP thioesterase revealed increases in seed stearic acid levels up to 17%. This transgene was subsequently stacked with a soybean event silenced in both palmitoyl-ACP thioesterase and Δ12 fatty acid desaturase activity, which resulted in a seed lipid phenotype of approximately 11-19% stearate and approximately 70% oleate. This seed oil profile created by the stack was maintained for four generations under greenhouse conditions and a fifth generation under a field environment. However, in generation six and seven under field conditions the oleate levels decreased to 30-40%, while the stearic level remained elevated.
Introduction

Soybean (*Glycine max* (L.) Merr.) is a major feedstock for protein and oil in the world. In 2011 an estimated 76.3 million acres of soybean were harvested in the US resulting in a production of approximately 3.1 billion bushels with a market value of $35.8 billion (nass.usda.gov). Soybean primary storage components protein and oil accumulate at 40% and 20%, respectively. The protein component of the seed, referred to as the meal, is utilized extensively in poultry and swine feeds, while soybean oil occupies approximately 65% of the US edible vegetable oil market (USDA economic research service, 2010). Implementing genetic approaches to improve functional and nutritional qualities of oils has attracted significant attention over the past decade. The fatty acid profile of an oil has a significant influence on functionality, and nutritional aspects in food and feed applications.

Commodity soybean oils is composed of five major fatty acids at percentages of approximately, 10% palmitic acid (16:0), 4% stearic acid (18:0), 20% oleic acid (18:1), 55% linoleic acid (18:2) and 10% linolenic acid (18:3). Due to the relatively high percentage of polyunsaturated fatty acids, linoleic and linolenic, soybean oil possesses poor oxidative stability which is an important parameter associated with shelf-life of food and feed products and engine performance when bio-based oil is used as a liquid transportation fuel (Clemente and Cahoon, 2009; Kinney and Clemente, 2011). Food processors have relied upon a hydrogenation process as a means to chemically improve the oxidative stability of soybean oil. This chemical approach will reduce the percentage
of polyunsaturated fatty acids in the oil with a concomitant increase in monounsaturated and saturated fatty acids. However, this chemical approach leads to the formation of so-called trans-fatty acids, which have been linked to negative cardiovascular health (Minihane and Harland, 2007), and can also compromise engine performance if the hydrogenated oil is subsequently converted to a biodiesel (Graef et al., 2009; Kinney and Clemente, 2011).

In baking applications oils are preferred that possess reduced polyunsaturated fatty acids, combined with elevated saturated fatty acids, so to address both oxidative stability and high melting temperatures. The two most prevalent saturated fatty acids in plant-based commodity oils are palmitic and stearic. Most dietary recommendations suggest limiting the amount of saturated fat intake due to the relationship to cardiovascular health. However, stearic acid has been shown to more cardiovascular neutral in comparison to palmitic acid in many studies (Bonanome and Grundy, 1988; Schwab et al., 1996; Kris-Etherton et al., 2005; Stanley, 2009). Hence, genetic approaches to simultaneously reduce polyunsaturated fatty acids and enhance steric acid levels in soybean oil would be expected to have value in food application as a margarine replacement, and for use as a biodiesel in warmer climates (Duffield et al., 1998).

A number of genetic approaches have been pursued as a means to elevate stearic acid levels in oil seeds, including soybean (Clemente and Cahoon, 2009). As a means to simultaneously elevate stearic acid and reduce polyunsaturated fatty acids in soybean we pursued a strategy similar to the biotechnology design implemented by Liu et al (Liu et
al., 2002) to raise stearic acid and oleic acid levels in cotton seed oil. To this end, we first introduced into soybean the mangosteen (*Garcinia mangostana*) stearoyl-ACP thioesterase (Hawkins and Kridl, 1998), and subsequently stacked this transgene with a soybean event designated 335-13 that is down-regulated in both palmitoyl-ACP thioesterase activity and Δ12 fatty acid desaturase activity in a seed specific fashion (Buhr et al., 2002). We report herein on the molecular and phenotypic characterizations of soybean carrying the mangosteen steryl-ACP thioesterase alone and stacked with the dual silenced event 335-13.

**Results**

*Transgenic soybean events carrying the mangosteen stearoyl-ACP thioesterase*

A two T-DNA binary vector was assembled that carries a codon-optimized version of the stearoyl-ACP thioesterase and the vector is designated pPTN811 (Fig 1). Simultaneous delivery of two T-DNAs from a single binary plasmid is a means to derived transgenic plants free of the marker gene, including soybean (Xing et al., 2000). We generated a total of 20 transgenic soybean events from transformations conducted with *A. tumefaciens* transconjugants carrying pPTN811. Segregation analysis on T₁ progeny derived from these transgenic events is shown in Table 1. The T₁ individuals were categorized into four phenotypic classes, herbicide tolerant (HT) with a elevated stearic acid phenotype (HT/18:0), herbicide sensitive (HS) with the 18:0 phenotype (HS/18:0), HT with normal 18:0 percentage designated as wild type (WT) given phenotypic category of HT/WT and the double null category of HS/WT.
As can be seen in Table 1 from three events we identified marker-free individuals, phenotype as HS/18:0, these events are designated as 683-2, 687-2 and 688-5. Fatty acid profiles of all the derived events are listed in Table 2. The data shows that grown under greenhouse conditions mean stearic acid levels were increased higher than the control seed grown at the same time, except in two of the events 679-11 and 679-13. In the remaining events mean stearic acid levels ranged from 8.4% up to just over 17% (Table 2) in T1 seed, wherein the nulls were not used to calculate the mean.

Three events were selected to propagate further, these are 680-2, 688-5 and 683-2. Fatty acid profiles on either T2 or T3 generations from these events are shown in Table 3. As can be seen from the data stearic acid levels, under a greenhouse environment, from these events range from approximately 12% up to just over 20%, hence, revealing the phenotype is stable over generations, with stearate levels capping at just over 20%.

**Stacking of the elevated stearate phenotype with high oleic acid and low palmitic acid**

The selected events were subsequently crossed with event 335-13, a soybean event high in oleic acid, and low in palmitic acid (Table 3) (Graef et al., 2009). Fatty acid profiles of derived F1 seed from the crosses are listed in Table 3. The creation of this gene stack manifested a fatty acid profile in the seed with oleate percentages in the mid 60s to upper 70s, combined with stearate percentages from approximately 7% to just over 11% (Table
3). Molecular analysis of the stack, and corresponding parents, is shown in Fig 2, confirming the presence of both transgenic alleles in the stack.

These F₁ seeds were followed to the F₂ population (Table 4), and fatty acid levels monitored, in which mean percentages were calculated on seed carry both transgenic alleles determined by PCR using primer sets that specifically amplify the targeted transgenes in the stack. In the F₂ population the gene stack led to oleic acid percentages in the 70s and stearic acid percentages just over 5% to over 13% (Table 4). Based on these data populations derived from the cross of HO (335-13) X 680-2 were carries on to homozygosity under greenhouse conditions.

Homozygous lineages at the F₄ generation were identified from the stack created by crossing the high oleic acid (HO) event with the elevated stearic acid event 680-2. Fatty acid profiles of five homozygous lineages are shown in Table 5, wherein the oleic acid and stearic acid percentages of the oil ranged from 67.7% to 70.5% and 16.5% to 19.7%, respectively.

**Field evaluations of transgenic event 680-2 and stack**

Field releases of the populations derived from 680-2, 335-13 and stack thereof, was carried out initially in the 2009 growing season to gain insight on the stability of the phenotype under field conditions and to secure sufficient seed to carry out small scale agronomic testing in subsequent years.
Fatty acid profiles of seed samples taken following the 2009 harvest are shown in Table 6, which tended to mirror that observed under greenhouse conditions of the parents (Table 3), but slightly lower stearate levels in the stack under field conditions (Table 6) as compared to the populations grown under greenhouse environment (Table 5).

Isolated oil from the 2009 harvest was also analyzed for oxidative stability, cloud and melting points. The data from these analyses are shown in Table 7. The oxidative onset temperature (OOT) was higher in both HO event 335-13 and stack (Table 7) as compared to the elevated stearate parent 680-2 and control oil, which undoubtedly reflects the reduction in polyunsaturated fatty acids in the former two. Moreover, the cloud point, temperature at which crystals initiate, is increased in the oils with the higher level of stearate, where cloud point is approximately 0°C, while cloud point of standard soybean oil is about -12°C (Table 7). Change in melting temperatures appears to be influenced by both stearate levels and reduction in polyunsaturated fatty acids in the oil, where in the soybean oil tested two endothermic peaks were observed in the heating curve (Table 7).

The change in melting temperature across the oils is visually represented in Fig 3, where the respective oil samples were placed at -20°C for 1 hour and removed, where in the degree of melting visualized more closely parallels melting point 1 data of the oil.

A second field trial was conducted in 2010, with sufficient plot replications to monitor both fatty acid profiles and insight on agronomics. However, due to hail event which
severely damaged the leaves approximately 30 days prior to harvest, agronomic parameters were not measured, only fatty acid profiles from the across the plots. In 2011, plots were replicated under both irrigated and non-irrigated conditions.

Fatty acid profiles from the harvest are shown in Table 8. Cropping under simulated dry land conditions or irrigation did not influence oil composition across the genotypes. However, in the gene stack, the percentage of oleate fell below 50%, with a relatively high standard deviation about the means. Northern blot analysis on immature embryos sampled from the respective plots approximately 15-20 days post flowering revealed the presence of FAD2-1 transcript in the stack (Fig 4), which was not observed in the HO event 335-13, where the high oleate phenotype was maintained. Moreover, in the 680-2 plots and stack plots the transcript level of steroyl-ACP thioesterase was comparable, with similar levels of stearate produced at harvest (Table 8).

A third field trial was carried out in 2011, in this trial only the stack, control plots and HO event were planted. Replicated plots were planted under both irrigated and non-irrigated environments. Data was ascertained on various yield parameters including yield, days to maturity, plant height, 100 seed weight, and total protein and oil, in addition to fatty acid profiles. The 2011 growing season was a relatively wet year, hence, simulated dry land conditions under non-irrigated conditions was not met. Nonetheless, within each environment no significant difference was observed with exception of days to maturity (Table 9).
However, in a similar fashion to what was observed in 2010, oleate levels in the gene stack were significantly lower (Table 10), with FAD2-1 transcript accumulation observed in the gene stack from sampled immature embryos (Fig 5). While in the HO event, 335-13 parent, remained silenced in FAD2-1, and the steroyl-ACP thioesterase transcript accumulating in the stack (Fig 5), which these results were reflected in the elevated stearate levels in the latter, and high oleic acid in the former (Table 10).

**Discussion**

The influence of fatty acid profile on end use nutritional and functionality attributes of oils for food, feed and industrial applications is well documented (Kinney and Knowlton, 1997; Kinney, 2003; Cahoon et al., 2007; Damude and Kinney, 2008; Lu et al., 2010). When considering applications for oils in deep frying, margarine type uses or as a liquid transportation fuel, i.e. biodiesel, two critical parameters are oxidative stability, and functionality. Oxidative stability impacts shelf-life of a food/feed product, and can negatively effect engine performance if the stability of the biodiesel is low (Knothe, 2005). In regards to functionality, fatty acid composition of oils will need to shift towards more saturated fatty acids to create a more solids for such end uses in baking or confectionary type applications.

The majority of commodity plant oils the saturated fatty acids present are palmitic acid, found as the predominant saturated fatty acid in palm oil (*Elaeis* sp.), and stearic acid, present in high percentages in cocoa butter (*Theobroma cacao* L.), along with palmitic
acid. Given that high palmitic acid consumption and chemical approaches such as hydrogenation as a means to improve oxidative stability while increasing solid content of oils both tend to be linked with cardiovascular health problems researchers have been actively pursuing genetic approaches to simultaneous raise stearate levels, with a concomitant reduction in polyunsaturated fatty acid, as a means to produce a low cost high volume vegetable oil that addresses the functionality requirements for margarine type applications.

As a means to raise stearate levels in seed oil researchers have implemented two approaches, a biotechnology avenue (Hawkins and Kridl, 1998; Merlo et al., 1998) and a mutational breeding strategy (Bubeck et al., 1989; Pérez-Vich et al., 2006; Salas et al., 2008; Zhang et al., 2008). In soybean high stearate mutants have been identified (Rahman et al., 1995; Spencer et al., 2003), the elevated stearate mutants of soybean, as with those for high oleate (Bachlava et al., 2008; Scherder and Fehr, 2008; Pham et al., 2010), tend to be recessive alleles, which can complicate breeding of the phenotype into elite germplasm, especially when stacks of recessive alleles need to be assembled to create the desired trait. Moreover, the recessive alleles governing high oleic acid levels in soybean tend to be linked with a yield drag (Scherder and Fehr, 2008). For this reason, we pursued the stacking of two dominant transgenic alleles, combining expression of the stearoyl-ACP thioesterase, with a dual silencing element to create soybean oil with elevated stearate and high oleate. A similar approach was used to create the same phenotype in cotton oil with the exception dual silencing of the cotton FAD2-1 was stacked with a silenced ∆9-steroyl-ACP desaturase (Liu et al., 2002).
Our approach took a two-step process whereby we first introduced the mangosteen stearoyl-ACP thioesterase gene into soybean. We produced a total of 20 transgenic events, in which 3 events produced marker-free progeny (Table 1), which is a higher percentage than previously observed in soybean (Xing et al., 2000; Sato et al., 2004). While we monitored for the presence of the bar gene cassette in the marker-free individuals, we did not conduct a deeper molecular analysis to determine what elements beyond the second, gene of interest T-DNA, integrated within the soybean genome.

Monitoring of the fatty acid profile of the transgenic events the maximum percentage of stearate observed was just over 20% under greenhouse conditions, and 10% to 13% under field conditions (Table 3 & 6). The stearate level under greenhouse conditions is comparable to what was observed when this approach was used to raise stearic acid levels in canola (Hawkins and Kridl, 1998). We assume the environmental impact, most likely temperature (Fernández-Moya et al., 2002), led to differences observed under field conditions.

To create the target phenotype, a reduction in polyunsaturated fatty acids, combined with high stearic acid, we subsequently crossed the stearoyl-ACP thioesterase transgenic plant with a event 335-13. This resulted in a soybean oil with oleic acid levels at approximately 70% and stearic acid levels at approximately 17%, with total saturates of 20%. Such an oil composition displayed changes in oxidative stability, cloud point and melting point (Table 7). The change in physical properties observed is not sufficient to
meet the parameters necessary for end use in baking or margarine type applications. However, such an oil may serve as a feedstock in a combined blending with other higher saturated lipids or be suitable for interesterification of TAG, if the stearate levels can be obtained and maintained in the mid 20%, (Neff and List, 1999).

The stacked lineage developed in this study did not maintain the high oleic acid phenotype after five generations (Table 8), yet the elevated stearic acid phenotype was still observed. Northern blot analysis on the reduced oleic acid observed in the stack line revealed accumulation of the FAD2-1 transcript (Fig 4 & 5). This apparent loss in silencing was not observed in parental 335-13 grown under the same environments. These results were not expected, for we initially assumed it would be difficult to maintain stearic acid levels when stacked with the silencing element in 335-13. Nonetheless, our data does demonstrate that a novel soybean oil, elevated in stearic and high in oleic acid can be achieved through biotechnology. However a more strategic design of the transgenic elements may need to be assembled to allow for the phenotype to be stable over generations. For example, one potential design may include hair-pin element targeting FAD2-1, embedded in a intron, placed just upstream of the strearoyl-ACP thioesterase, under control of a heterologous seed specific promoter. Thereby, creating a single cassette that can simultaneously down regulate FAD2-1 with enhanced thioesterase activity. This in turn will produce an oil with the oleate and stearate levels, with a slight increase in palmitate thereby producing the required levels of saturates, with oxidative stability to meet the requirements for margarine type applications.
Materials and Methods

Construction of two T-DNA binary vector

The mangosteen stearoyl-ACP thioesterase (Genbank accession AAB51523.1) was codon optimized (GenScript, Piscataway, NJ) for soybean. The open reading frame (ORF) was fused to the tobacco etch virus translational enhancer element (Carrington and Freed, 1990) and subsequently assembled into a expression cassette under control of the soybean seed-specific β-conglycinin promoter (Allen et al., 1989) and terminated by the cauliflower mosaic virus 35S transcription terminator. The resultant cassette was subcloned into the binary vector pPZP101 (Hajdukiewicz et al., 1994) and the plasmid referred to as pPTN802. The T-DNA element of pPTN802 was excised as a Sca I fragment and cloned into the binary vector pPTN200, which carries a single Sca I site outside of a T-DNA element harboring a bar gene (Thompson et al., 1987) cassette under control of the Pnos promoter from A. tumefaciens. The resultant two T-DNA binary vector is designated pPTN811 (Fig 1). The two T-DNA vector pPTN811 was mobilized into A. tumefaciens strain EHA101 (Hood et al., 1986) via tri-parental mating. The integrity of pPTN811 was confirmed by plasmid rescue and the resultant transconjugant used to transform soybean.

Soybean transformation
Soybean transformations were conducted as previously described (Zhang et al., 1999; Xing et al., 2000) using the genotype Thorne (McBlain et al., 1993). The derived transgenic events were established and grown to maturity in the greenhouse for subsequent molecular and phenotypic characterizations. The targeted gene stack phenotype of combining elevated stearic acid and high oleic acid was carried out via sexual crossing of selected pPTN811 derived transgenic events with an event designated 335-13 which carries a dual silencing element designed to simultaneously down-regulate the soybean *fatB* gene, a palmitoyl-ACP thioesterase and *FAD2-1* a Δ12 fatty acid desaturase gene (Buhr et al., 2002; Graef et al., 2009).

**Molecular characterizations of transgenic events**

Southern and northern blot analyses on the selected event and stack was carried out as previously described (Buhr et al., 2002). Briefly, for DNA hybridizations total genomic DNA was isolated from leaves (10 µg) and restriction digested with *Sst* I and separated on 0.8% agarose gel. RNA was isolated from immature embryos using TRIzol reagent following the manufacturer’s protocol. A total of 15 µg of RNA was separated on a 1% formaldehyde agarose gel. The separated DNA and RNA were subsequently transferred to a nylon membrane (Zeta Probe GT, Bio-Rad, Hercules, CA) and fixed by UV crosslinking. Probes, approximately 50 ng, were labeled with dCT$^{32}$P by random prime synthesis (Stratagene Prim- It II ). Membranes were hybridized in a 1mM EDTA, 0.5 M Na$_2$HPO$_4$ (pH 7.2), 7% SDS, and 1% BSA at 65°C over night. Following the
hybridization step membranes were washed twice with 5% SDS, 40 mM \( \text{Na}_2\text{HPO}_4 \) solution for 30 min at 65°C, with a subsequent third wash with 1% SDS, 40 mM \( \text{Na}_2\text{HPO}_4 \) solution for 30 min at 65°C. Membranes were exposed on x-ray film for 1-3 days at -80°C.

**Phenotypic analyses of transgenic events and stack**

Segregation analysis on \( T_1 \) individuals derived soybean transformations with the two T-DNA binary vector pPTN811 was conducted using dual scoring. Fatty acid profile of individual seeds was determined from cotyledon chips through gas chromatography as previously described (Buhr et al., 2002). Derived methyl esters were analyzed on a 6890N gas chromatograph (Agilent Technologies, Santa Clara, CA) fitted with a 30 M X 250 µM HP-INNOWAX column (Agilent Technologies Cat# 19091N-133). A leaf painting technique (Zhang et al., 1999) was subsequently used to score for presence of the *bar* gene. Leaf paining was carried on V2 stage plants grown from the corresponding seed. \( T_1 \) individuals were subsequently placed into one of four phenotypic categories, herbicide tolerant (HT) / elevated stearic acid (18:0); HT/ wild type stearate level < 4% (WT); herbicide sensitive (HS)/ 18:0; or double the double null HS/WT. The phenotypic classifications were re-confirmed via PCR using the primer set Stear5: 5’-CCATGGCACCTAAAACCTCCTCATCC-3’ and Stear3: 5’-TCTAGATCATCTTGTTGGTTTCTTCCCTCC-3’ to amplify the stearoyl-ACP thioesterase transgene and primer set Pnos: 5’-AGTTGACCGTGCTGTCTCGATGT-3’
and bar3: 5’-CGTTTGGAACTCACAGAACCGCAA-3’ to amplify an element with the bar gene cassette.

**Monitoring of physical properties of derived oils**

Soybean oil samples with elevated stearic acid, high oleic/low palmitic and stack phenotype of elevated stearic high oleic were monitored for oxidative stability and cold properties, cloud and melting points. Oxidative onset temperatures (OOT) of the tested oils were determined by differential scanning calorimetry (DSC, Mettler Columbus, OH) using the ASTM E 2009-02 method (2009-02, 2002). Approximately 3 mg of test oil sample was placed in an aluminum pan. The pan was heated at a rate of 10°C per min in an aerobic environment. Heat flow was monitored as a function of temperature until oxidative reaction was triggered by heat evolution on the thermal curve.

Cloud and melting points of the test oils were determined with a DSC. Oils samples, 35-40 mg were cooled from 10°C to -40°C at a cooling rate of 1°C per min and stayed at -40°C for 2 min, followed by heat rate of 1°C per min to 10°C. Cloud point reflects the onset of temperature of the initial small exothermic peak on the cooling curve, while melting point refers to the onset temperature at which the initial small endothermic peak on the heating curve is observed. Samples were run in duplicate.

**Field trials with selected transgenic soybean event and stack**
Filed tests on selected homozygous lineages derived from transgenic event (680-2) and stack were conducted in 2009, 2010 and 2011. Plots consisted of four 10 feet rows, with data collected from the inner two rows. Limited seed in 2009 and storm damage in 2010 limited the value of agronomic measurements in those years, hence, only fatty acid phenotypic analysis was tabulated. In 2011 in addition to fatty acid analysis of the harvest data was ascertained on estimated yields, 100 seed weight, days to maturity, plant height, along with total protein and oil determinations by using NIR spectroscopy, Infratec, (FOSS North America, Inc, Eden Prairie, MN 55344).
References


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


Scherder CW, Fehr WR (2008) Agronomic and seed characteristics of soybean lines with increased oleate content. Crop Sci. 48: 1755-1758


The abbreviations for promoter, gene and terminator are as follows: β-con, β-conglycinin promoter; FatB, palmitoyl-thioesterase; FAD2-1, Δ12 desaturase; RZ, ribozyme; 35S, 35S promoter; TEV, tobacco etch translational enhancer; bar, bar gene; T35S, 35S terminator; RB and LB refer to the right border and left border elements (Buhr et al., 2002). Ster, Stearoyl-ACP thioesterase; Pnos, nopaline synthase promoter; Tnos, nopaline synthase terminator.
**Fig 2-2.** Southern blot analysis on F\textsubscript{1} generation of soybean.

10\mu g of genomic DNA was digested with \textit{Sst}1 and hybridized with Steroyl-ACP thioesterase (left panel) and FAD2-1 (right panel). Lane 1, HO (335-13). Lane 2, 680-2. Lane 3, HO X 680-2. Lane 4, HO X 680-2. Lane 5, 6 and 7 refer to wild type control Thorne DNA, 100pg of Pptn811 digested with \textit{Sst}1 and 100pg of Pptn303 digested with \textit{Sst}1 respectively.
Fig 2-3. Phenotype of oil after freezing at -20 °C and then thawed

Photo was taken 5 minutes after thawing. Oil was extracted from control and transgenic seeds from 2009 field trial. A, thorne B, 335-13 C, 680-2 D, HO X 680-2.
**Fig 2-4.** Northern blot analysis on immature embryo derived from 2010 field trial.

RNA was extracted from immature embryo and 15ug of RNA was used. Top panel, northern blot. Lower panel, ribosomal RNA. Lane 1 and 2, wild type soybean immature Embryo. Lane 3 thru 6, immature embryos derived from 680-2, 335-13 and 680-2 x 335-13 lines. FAD2-1 and steroyl-ACP thioesterase(TE) genes were used as a probe.
**Fig 2-5.** Northern blot analysis on immature embryo derived from 2011 field trial.

RNA was extracted from immature embryo and 15ug of RNA was used. Top and middle panel, northern blot. Lower panel, ribosomal RNA. Lane 1and 2, wild type soybean immature embryo. Lane 3 thru 6, immature embryos derived from 335-13 and 680-2 x 335-13 lines. FAD2-1 and steroyl-ACP thioesterase(TE) genes were used as a probe.
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### Table 2-2: Fatty acid profile on T₁ seed derived from pPTN811

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Table 2-4: Fatty acid profile in F$_2$ seed of gene stacks

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Table 2-5: Fatty acid profile of homozygous lineages derived from HO X 680-2 stack

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### Table 2-6: Fatty acid profile of soybean events under field conditions in 2009

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<td>11.3±0.5</td>
<td>3.0±1.9</td>
<td>15.4±1.7</td>
<td>51.0±1.7</td>
<td>14.3±1.2</td>
</tr>
<tr>
<td>680-2</td>
<td>8.4±0.6</td>
<td>10.2±1.4</td>
<td>15.1±1.8</td>
<td>48.0±1.5</td>
<td>15.2±1.6</td>
</tr>
<tr>
<td>HO × 680-2</td>
<td>4.1±0.9</td>
<td>12.7±2.2</td>
<td>67.3±5.4</td>
<td>4.1±2.6</td>
<td>8.0±1.9</td>
</tr>
</tbody>
</table>
Table 2-7: Oxidative stability and cold properties of derived oils

<table>
<thead>
<tr>
<th>Event/Stack</th>
<th>Oxidative (°C)</th>
<th>Cloud Point (°C)</th>
<th>Melting Point1 (°C)</th>
<th>Melting Point2 (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT (Thorne)</td>
<td>173.7</td>
<td>-12.3</td>
<td>-34.7</td>
<td>-15.7</td>
</tr>
<tr>
<td>HO (335-13)</td>
<td>194.2</td>
<td>-15.6</td>
<td>-13.4</td>
<td>-0.9</td>
</tr>
<tr>
<td>680-2</td>
<td>174.5</td>
<td>-0.3</td>
<td>-19.1</td>
<td>-1.8</td>
</tr>
<tr>
<td>HO X 680-2</td>
<td>207.6</td>
<td>0.4</td>
<td>-15.6</td>
<td>-6.7</td>
</tr>
</tbody>
</table>
**Table 2-8**: Fatty acid profile of soybean events under field conditions in 2010

<table>
<thead>
<tr>
<th>Event/Stack</th>
<th>Palmitic%</th>
<th>Stearic%</th>
<th>Oleic%</th>
<th>Linoleic%</th>
<th>Linolenic%</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT (Thorne)</td>
<td>10.6±0.3</td>
<td>3.6±0.1</td>
<td>21.6±3.0</td>
<td>53.9±2.5</td>
<td>8.6±0.4</td>
</tr>
<tr>
<td>680-2</td>
<td>8.1±0.2</td>
<td>13.1±0.6</td>
<td>15.4±0.7</td>
<td>51.6±0.8</td>
<td>9.1±0.3</td>
</tr>
<tr>
<td>HO</td>
<td>4.0±0.4</td>
<td>2.3±0.1</td>
<td>82.8±2.9</td>
<td>3.6±2.2</td>
<td>2.0±0.3</td>
</tr>
<tr>
<td>HO X 680-2</td>
<td>5.7±0.5</td>
<td>13.6±1.0</td>
<td>46.9±6.8</td>
<td>22.8±5.6</td>
<td>8.1±0.6</td>
</tr>
<tr>
<td>WT (Thorne)</td>
<td>11.6±0.2</td>
<td>3.3±0.1</td>
<td>19.0±0.7</td>
<td>55.2±0.7</td>
<td>8.9±0.3</td>
</tr>
<tr>
<td>680-2</td>
<td>8.2±0.2</td>
<td>12.8±0.4</td>
<td>16.8±0.5</td>
<td>50.2±0.5</td>
<td>8.8±0.3</td>
</tr>
<tr>
<td>HO</td>
<td>3.7±0.1</td>
<td>2.2±0.2</td>
<td>84.0±1.1</td>
<td>2.8±0.8</td>
<td>4.0±0.2</td>
</tr>
<tr>
<td>HO X 680-2</td>
<td>5.7±0.6</td>
<td>14.6±0.9</td>
<td>44.0±8.4</td>
<td>24.0±6.9</td>
<td>8.0±0.9</td>
</tr>
<tr>
<td>Event/Stack</td>
<td>Yield (kg/ha)</td>
<td>Maturity (Days)</td>
<td>Plant Height (cm)</td>
<td>100 Seed Wt (g)</td>
<td>Protein %</td>
</tr>
<tr>
<td>-------------</td>
<td>--------------</td>
<td>----------------</td>
<td>-------------------</td>
<td>-----------------</td>
<td>-----------</td>
</tr>
<tr>
<td>HO</td>
<td>4071.3±290</td>
<td>31.2±1.6</td>
<td>108.3±5.8</td>
<td>14.9±0.2</td>
<td>34.5±0.3</td>
</tr>
<tr>
<td>HO X 680-2</td>
<td>4272.2±326</td>
<td>31±1.4</td>
<td>108.8±5.3</td>
<td>14.1±0.2</td>
<td>34.7±0.2</td>
</tr>
<tr>
<td>WT(Thorne)</td>
<td>4131.7±328</td>
<td>27.2±0.5</td>
<td>108.8±8.2</td>
<td>15.4±0.6</td>
<td>35.5±0.7</td>
</tr>
<tr>
<td>HO</td>
<td>5119±326</td>
<td>31.4±1.8</td>
<td>114.8±14.1</td>
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<td>35.0±0.2</td>
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<tr>
<td>HO X 680-2</td>
<td>4801±386</td>
<td>32.0±1.2</td>
<td>108.2±2.3</td>
<td>15.2±0.6</td>
<td>35.1±0.3</td>
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<tr>
<td>WT(Thorne)</td>
<td>4744±330</td>
<td>27.2±0.5</td>
<td>107.2±8.3</td>
<td>16.5±0.4</td>
<td>35.8±0.3</td>
</tr>
<tr>
<td>LSD(α=0.05)</td>
<td>287.0</td>
<td>1.0</td>
<td>6.2</td>
<td>0.4</td>
<td>0.3</td>
</tr>
</tbody>
</table>
**Table 2-10**: Fatty acid profile of soybean events under field conditions in 2011

<table>
<thead>
<tr>
<th>Event/Stack</th>
<th>Palmitic%</th>
<th>Stearic%</th>
<th>Oleic%</th>
<th>Linoleic%</th>
<th>Linolenic%</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT (Thorne)</td>
<td>10.9±0.3</td>
<td>3.6±0.2</td>
<td>19.7±0.7</td>
<td>55.1±0.9</td>
<td>8.3±0.4</td>
</tr>
<tr>
<td>HO</td>
<td>3.4±0.2</td>
<td>2.4±0.2</td>
<td>84.6±1.2</td>
<td>2.2±0.3</td>
<td>3.2±0.3</td>
</tr>
<tr>
<td>HO X 680-2</td>
<td>5.6±0.6</td>
<td>15.2±1.1</td>
<td>38.5±9.5</td>
<td>29.8±9.0</td>
<td>8.3±1.1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Event/Stack</th>
<th>Palmitic%</th>
<th>Stearic%</th>
<th>Oleic%</th>
<th>Linoleic%</th>
<th>Linolenic%</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT (Thorne)</td>
<td>11.1±0.8</td>
<td>3.4±0.2</td>
<td>19.4±1.9</td>
<td>54.5±2.0</td>
<td>8.1±0.6</td>
</tr>
<tr>
<td>HO</td>
<td>3.7±0.4</td>
<td>2.3±0.1</td>
<td>82.5±3.7</td>
<td>3.6±2.6</td>
<td>3.5±0.4</td>
</tr>
<tr>
<td>HO X 680-2</td>
<td>5.8±0.7</td>
<td>16.4±1.3</td>
<td>33.9±7.8</td>
<td>33.5±7.6</td>
<td>8.0±0.8</td>
</tr>
</tbody>
</table>
CHAPTER THREE

Assembly of a seven-gene stack in soybean for the simultaneous production of eicosapentaenoic acid and the high value carotenoid astaxanthin in seed oil

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Abstract

Soybean (*Glycine max* (L.) Merr.) is sought after for both its oil and protein components. Genetic approaches to add value to either component are ongoing efforts in soybean breeding and molecular biology programs. The former is the primary vegetable oil consumed in the world. Hence, its primary usage is in direct human consumption. As a means to increase its utility in feed applications, thereby expand the market of soybean oil, we pursued a multi-gene stack strategy for the targeted synthesis of two value-added traits, eicosapentaenoic acid (EPA) and the carotenoid, astaxanthin. With the long-term goal of designing an optimal identity preserved soybean-based feedstock for aquaculture. To this end we describe herein the systematic introduction of seven transgenic cassettes into soybean, and the molecular and phenotypic evaluation of the derived novel lines.
Introduction

Consumption of fish products has rapidly increased over the past 20 years, and remains a major source of protein in the human diet throughout the world. It has been estimated that in the near future over 50% of the fish harvest will be derived from aquaculture (Tidwell and Allan, 2001). The underlying concern is the feedstocks presently supplying the protein and oil to the aquaculture industry rely upon wild caught fisheries. While significant efforts are being put forth to properly manage the world’s fisheries, the ever increasing demand placed upon them to provide protein and oil to the aquaculture industry is not sustainable in the long-run (Naylor et al., 2000; Naylor et al., 2008). Hence, alternative, renewable feedstocks for the aquaculture industry need to be developed. Clearly, land-based commodities are viable candidates to meet the ever increasing demand for protein and oil in the aquaculture industry. However, when considering the attributes a potential terrestrial feedstock should possess to meet the requirements for the aquaculture industry, soybean should be one taken under consideration. The rationale for soybean as a terrestrial feedstock for aquaculture is the following; it is one of the very few commodities that partitions a significant amount of its storage reserves as protein and oil, with approximately 40% and 20% accumulating, respectively, in the mature seed. Secondly, soybean is the most widely available feedstock for vegetable oil and quality protein. Third, being a legume its nitrogen footprint is a positive. Lastly, the wealth of enabling technologies available to the crop, including a complete draft genome sequence (Schmutz et al., 2010), genetic and physical maps (Shoemaker et al., 2008) along with a reliable transformation system (Parrott and
Clemente, 2004) makes it an ideal system for targeted genetic improvements in end use traits.

Major steps towards the goal of designing land-based commodities for use in aquaculture feeds were the elucidation of the metabolic pathways governing the synthesis of very long chain polyunsaturated fatty acid along with the high value carotenoids and the translation of these findings to demonstrations that metabolic engineering of the respective steps could be carried out in higher plants (Abbadi et al., 2004; Gerjets and Sandmann, 2006; Graham et al., 2007; Hasunuma et al., 2008; Jayaraj et al., 2008; Lu et al., 2010).

We report here on the development of a prototype soybean-based feedstock for aquaculture in which the creation of a systematic seven-gene stack led to the simultaneous accumulation of the EPA and astaxanthin in the seed of transgenic soybean.

**Results**

*Genetic constructs and characterization of transgenic soybean events*

The T-DNA elements of the two binary vectors pPTN809D5 and pASTA are shown in Fig 1. We screened progeny derived from 15 events and 21 events from pPTN809D5, and pASTA, respectively. In regards to the former two independent transgenic events where carried on for further characterizations these are designated 824-1, 824-8 (clone)
and 826-4, while 21 events were originally phenotype for carotenoid accumulation, wherein these were selected based on yellowish colored seed phenotype (Fig 2).

Molecular analysis of the pPTN809D5 events is shown in Fig 3. Event 826-4 carries three loci, while the event 824-1 harbors two transgenic alleles. However, the fatty acid profile changes triggered by the dual expression of the introduced fatty acid elongase and Δ5 desaturase included accumulation of only the elongation products of oleic acid, linoleic acid, and linolenic acid, eicosenoic acid, eicosadienoic acid, and eicosatrienoic acid, respectively. Moreover, the phenotype of the apparent clones slightly differed from each other (Table 1 & 2). We elected to move forward with the 824 event due to presence of fewer transgenic loci in the genome (Fig 3). To confirm expression of both the elongase and Δ5 desaturase a northern blot analysis was conducted on immature seeds (Fig 4), wherein an abundance of the corresponding transcripts were observed in developing seed.

*Stacking of fatty acid elongase and Δ5 desaturase with Δ6 and Δ15 fatty acid desaturase transgenic cassettes.*

The 824 clones (824-1/824-8) were subsequently crossed with a soybean event designated 535-9 (Eckert et al., 2006) that accumulates stearidonic acid (STA) due to the dual expression of the borage Δ6 fatty acid desaturase (Sayanova et al., 1997) along with the Arabidopsis Δ15 fatty acid desaturase. Fatty acid profiles of F₁ seeds derived from this four-gene stack are shown in Table 2, where the accumulation of EPA ranged from
approximately 1% up to just over 4% in the seed, and its immediate precursor, eicosatetraenoic acid (ETA) was present in levels up to just over 11% (Table 2). The fatty acid profiles of F₃ populations derived from the F₁ seeds revealed that the relative level of EPA in the seed was maintained over two generations, however, the major 20 carbon omega-3 fatty acid present in the oil was ETA (Table 3). Moreover, fidelity of the expression of all four transgenes was confirmed in F₂ immature seed (Fig 5).

**Phenotyping of carotenoid accumulation in transgenic soybean events carrying pASTA T-DNA**

A subset of the derived transgenic events produced from transformations conducted with the binary vector pASTA (Fig 1) were selected based on yellowish phenotype coloration of the mature seed (Fig 2). These events were subsequently characterized at the molecular level (Fig 6 & 1S). As can be seen in the Southern blot analysis in some cases not all the transgenes were integrated within the genome. For example, event 806-14 only carries the phytoene synthase, 806-12 carries the phytoene synthase and crtZ, while event 807-5 harbors phytoene synthase and crtW (Fig 6). Based on northern analysis on selected transgenic events derived from pASTA, events were identified that expressed only phytoene synthase (806-14, Fig 7A), phytoene synthase and CrtZ (806-12, Fig 7B), phytoene synthase and CrtW (807-5, Fig 7B). However, the majority of these events screened accumulated transcripts corresponding to the three transgenes, and these results reflect the corresponding the Southern data (Fig 6 & 1S).
Quantification of two carotenoids, astaxanthin and β-carotene, were ascertained across the set of 21 selected events (Fig 8). For example, soybean event 806-14, which only carries phytoene synthase cassette (Fig 6 & 7A), accumulated only β-carotene at just over 800 ug/gr seed (Fig 8). While a the soybean event 807-2 which harbors all three transgenic cassettes (Fig 1S), with faithful co-expression of the respective transgenes (Fig 7C), accumulated astaxanthin at app. 25 ug/gr seed, with a concomitant production of β-carotene at 500 ug/gr seed (Fig 8). Phenotyping of events that differentially expressed CrtZ or CrtW in conjunction with phytoene synthase revealed some level of accumulation of other carotenoids down-stream of β-carotene. However, in the absence of both of these additional transgene products only the production of β-carotene was observed. Importantly, co-expression of both CrtW and CrtZ maximized astaxanthin synthesis. Additional carotenoids detected in soybean events in which minimally phytoene synthase, CrtW and/or CrtZ were being expressed include echinenone, canthaxanthin, phenicoxanthin and β-carotene (Table 4). While analysis of non-transgenic soybean seed reveals the presence of only a single carotenoid, lutein (Table 4).

Small plots were planted in Nebraska during the 2011 growing season to monitor accumulation of carotenoids in the seed under field conditions. While these plots were too small to ascertain agronomic parameters, no obvious impact on germination, lodging, and seed numbers per plant were observed (Data not shown). Levels of β-carotene and astaxanthin determined are shown in Fig 9, which tended to mirror that observed from the previous greenhouse grown harvest.
Given expression of phytoene synthase is expected to pull carbon down-stream towards carotenoids and therefore, competing away the substrate geranylgeranyl diphosphate (GGPP) for tocopherols production, we hypothesized that shifting carbon flux down to carotenoids would reduce tocopherol accumulation in the oil. However monitoring of tocopherol levels in the transgenic seed did not reveal a trend correlating tocopherol levels with carotenoid accumulation (Fig 10A). Interestingly, accumulation of the three forms of tocotrienol was observed in the transgenic events, which in soybean this form of vitamin E is not present (Fig 10B)

Combining of the four gene EPA stack with the three gene carotenoid stack

To create a soybean that simultaneously accumulates high value carotenoids along with enhanced omega-3 fatty acid profiles in the lipids, we crossed selected soybean events carrying pPTN809D5 combined with borage Δ6- and Arabidopsis Δ15- fatty acid desaturase genes with the pASTA T-DNA element. Thereby assembling a seven gene-of-interests transgene stack. Progeny (F₁) derived from the cross were initially selected based on yellowish color, suggestive of presence of pASTA T-DNA in the genome, and fatty acid profile of chip from the F₁ seed was subsequently ascertained. Fatty acid profiles of the 13 confirmed crosses are listed in Table 5, with subsequent F₂ generation dataset shown in Table 6. As can be seen in the analysis, the longer chain omega-3 fatty acids ETA and EPA, combined ranged from approximately 3-5% of the oil, along with astaxanthin and β-carotene accumulation from 23 to 44.8 ug/gr and 527 to 1139 ug/gr seed, respectively.
Discussion

A number of novel output traits have been under development for many agricultural plants. Such traits targeted for end-use applications for food, feed or industrial products require some level of identity preservation in order to capture value. For instance, soybean producing stearidonic acid (STA) will be launched under the trade name Soymega™ as an omega-3 fatty acid enriched oil for targeted food and feed applications. Albeit there are exceptions, for example in the case of high oleic acid soybean developed through down-regulation of a Δ12 fatty acid desaturase alone or in combination with a silenced palmitoyl thioesterase, stacked with mutant alleles of FAD3, being marketed under trade names Plenish™ and Vistive Gold™, respectively, should command a sufficient market demand to ultimately become the commodity soybean.

Given the significant costs associated with identity preservation, coupled with the enormous expense of regulatory hurdles of transgenic derived traits, production of multiple output traits through genetic stacking of transgenic alleles is an avenue that can enhance the economic attractiveness of an agriculture biotechnology product. To this end, we have designed and assembled a prototype soybean-based feedstock for aquaculture with two output traits, a high omega-3 fatty acid oil, coupled with production valuable carotenoids, which reflect two critical components of fish feed. In regards to the latter astaxanthin has annual sales upward of $200 million (Li et al., 2011), commanding a greater than $1,000 per kg market value. This ingredient is incorporated in many aquaculture feeds for example, salmon, trout and shrimp, for coloration of the harvested product (Higuera-Ciapara et al., 2006; Hussein et al., 2006).
The three-gene stack introduced into soybean herein, was sufficient to accumulate from approximately 25 to 38 µg/gr seed astaxanthin in one of the selected events 807-2 (Table 6, Fig 8). We assume the variation in astaxanthin level observed is likely due to environment, with a later greenhouse propagation leading to an increased level of the carotenoid (Table 6). Considering the lower range observed, 25 µg/gr seed accumulation, would require approximately 4-6 million acres of such soybean to be identity preserved in order to meet the estimated annual consumption of 100,000kg of astaxanthin used in feed and nutraceutical applications. Hence, further optimizations of genetic designs for astaxanthin accumulation in soybean are required, so to reduce the required acreage below a threshold for economic viability.

The four-gene stack assembled in soybean for the targeted synthesis of EPA led to accumulation of this high value long-chain polyunsaturated omega-3 fatty acid of levels up to 5%, with total omega-3 fatty acid levels exceeding 60% (Table 3). While this change in fatty acid profile is impressive, to maximize value as an aquaculture feedstock EPA levels will need to be improved, to better mirror that present in fish oil, which tend to range from 9% to 15%.

Nonetheless the study communicated herein, demonstrates that indeed a metabolic engineering strategy can be implemented that will lead to the production of two critical aquaculture feed ingredients in a plant genotype, thereby creating a framework for a sustainable terrestrial feedstock for the aquaculture industry. Soybean as such a platform
for such a metabolic engineering approach is highly desirable due to its attributes as a legume, along with its well-established cropping system and utility in feed applications.

Clearly, some biological hurdles remain. However, by exploiting the tools of synthetic biology (Jenkins et al., 2011; Collins, 2012; Facchini et al., 2012), will permit a facile strategy for the assembly of an array genetic cassettes linked on a single T-DNA or an DNA element suitable for direct DNA delivery, incorporating a diverse set of promoter and terminator elements for optimal coordination of gene expression. The stacks created here were assembled by crossing, resulting in duplication of many genetic elements, along with multiple copies of the selectable marker cassette. While northern blot analysis on the three and four gene stacks did not suggest silencing of the stacked transgenic cassettes (Figs 5 & 7), duplication of genetic elements, and lack of attention to cassette orientation is not ideal for stability of the phenotype over generations (Bhullar et al., 2003; Yang et al., 2005; Gudynaite-Savitch et al., 2009; Singer et al., 2011; Singer et al., 2012). Moreover, investigations into metabolic networks leading to the synthesis of these two targets, EPA and carotenoids have led to the identification alternative routes, than the ones pursued herein, that have potential to enhance the accumulation of the desired products (Petrie et al., 2010; Venegas-Calerón et al., 2010; Cunningham Jr. and Gantt, 2011; Ruiz-López et al., 2012). Hence, through combination of optimal genetic designs, exploitation of synthetic biology and novel metabolic engineering strategies a terrestrial feedstock for aquaculture is technically feasible. Questions on economic viability of such a feedstock will ultimately dictate if such a product enters the marketplace.
Materials and methods

Construction of binary vectors

A bifunctional fatty acid elongase cDNA (gift from A.J. Teale) (Agaba et al., 2004), and a cDNA of a ∆5 fatty acid desaturase gene (Pereira et al., 2004) were assembled into seed specific plant expression cassettes under control of the soybean β-conglycinin promoter. The respective elements were subsequently subcloned into the binary vector pPTN200 which carries a bar gene cassette (Thompson et al., 1987) under control of the Pnos promoter from A. tumefaciens. The resultant binary vector is designated pPTN809D5 (Fig 1A).

A three-gene stack was designed in a separate binary vector referred to as pASTA. Here, the β-carotene hydroxylase (CrtZ), β-carotene ketolase (CrtW) genes from Brevundimonas sp., along with the maize phyteone synthase gene placed under control of seed specific promoters, and terminated by either the soybean glycinin terminator or common bean transcriptional terminator (Fig 1B).

Soybean transformation

The respective binary vectors, pPTN809D5 and pASTA were mobilized into A. tumefaciens strain EHA101 (Hood et al., 1986) and NTL4/pKPSF2 (Luo et al., 2001), respectively, via tri-parental mating. The derived transconjugants were used for soybean
transformations as previously described (Zhang et al., 1999; Xing et al., 2000) using the soybean genotype Thorne (McBlain et al., 1993). Primary transgenic events were established and grown to maturity under greenhouse conditions.

Molecular characterization of transgenic soybean events

Southern blot analysis on transgenic events was carried out as previously described (Buhr et al., 2002). Total genomic DNA was isolated from young fully expanded leaves following a modification of the protocol outlined by Dellaporta et al (Dellaporta et al., 1983). Ten µg of genomic DNA was restriction digested with either EcoR I, pASTA events or Pst I, pPTN809D5 events, and subsequently separated on 0.8% agarose gel. The separated DNAs were transferred to a nylon membrane (Zeta Probe GT, Bio-Rad, Hercules, CA) by capillary transfer and fixed via UV crosslinking. Probes were prepared by random prime synthesis incorporating dCT32P using Stratagene’s Prime-It II kit following the manufacturer’s protocol (Cat #300385). Membranes were hybridized in 1 mM EDTA, 0.5 M Na₂HPO₄ (pH 7.2), 7% SDS, 1% BSA at 65°C overnight. Membranes were subsequently washed twice in 5% SDS, 40 mM Na₂HPO₄ solution for 30 min each, followed by a 30 min wash in 1% SDS, 40 mM Na₂HPO₄ solution at 65°C. For re-probing membranes, were stripped in 0.1N NaOH, 0.2% SDS solution for 20 min at room temperature and washed twice in 0.2 M Tris (pH 7.5), 0.1X SSC, 0.2% SDS solution for 20 min at 95°C.
Northern blot analysis was carried out on immature embryos as described by Buhr et al (Buhr et al., 2002). RNA was extracted using TRIzol reagent (Cat #10296) following manufacturer’s instructions. Fifteen micrograms of RNA was separated on a 1% formaldehyde agarose gel, and transferred to a nylon membrane. Hybridization and washing conditions were performed as described above. Membranes were exposed to X-ray film at -80°C to visualize signals.

**Fatty acid analysis**

Fatty acid analysis was conducted on cotyledon chips following the procedure of Butte et al. (Butte, 1983). Fatty acid profiles on the derived methyl-esters were monitored on a 6890N gas chromatography flame ionization detector (Agilent Technologies, Santa Clara, CA) fitted with a 30m X 250µm HP-INOWAX column (Agilent Technologies Cat # 19091N-133). Fatty acids are reported as percentages of total fatty acids.

**Carotenoid analysis**

Total lipids were extracted from mature seed by the Bligh and Dyer method (Bligh and Dyer, 1959). Extractions were conducted on 5 colored seeds per plant, with 5 µg of trans-beta-apo-8’-carotenal added to 50 mg ground sample as an internal standard for quantification. Three milliliters of methanol/chloroform (2:1) was added and incubated at room temperature for 30 min. Followed by the addition of 1 ml chloroform and 1.8 ml water and mixed. The mixture was subsequently centrifuged for 2 min at 3800 rpm, and
the lower organic phase was transferred to a new tube. The previous steps were repeated twice, and the organic layer dried under N2 and dried sample resuspended in 3 ml acetone. The suspension was briefly centrifuged and 1 ml of the top layer used for HPLC analysis. HPLC analysis was carried out on an Agilent 12000 series system, fitted with a protonsil 200-5-C30 column. The elution was carried out with methanol:t-butyl methyl ether (80:20, v/v) solvent system at room temperature, with a flow rate of 1.4 ml/min.

_Tocopherol and tocotrienol analysis_

Powder derived from soybean samples was spiked with the internal standard 5,7-dimethyl tocopherol, in a 5µg per 50 mg powder ratio. The powdered mixture was then combined with 600 µl methanol/dichloromethane (9:1) gently mixed and incubated at room temperature for 30 min. The mixture was subsequently centrifuged for 5 min at 2500 rpm, and 35 µl of the upper layer subjected to HPLC analysis. HPLC analysis was conducted on an Agilent technologies 1200 series system, fitted with a Eclipse XDB-C18 column, eluted with a two solvent system, methanol/water (19:1) at a flow rate of 1.5 ml/min.

_Creation of gene stacks_

For the targeted production of EPA, a cross was made using two pPTN809D5 events designated 824-1 and 824-8, with a transgenic soybean event that produces high levels of stearidonic acid (STA) referred to as 535-9 (Eckert et al., 2006) which carries the borage $\Delta_6$ fatty acid desaturase gene (Sayanova et al., 1997), and the Arabidopsis $\Delta_{15}$ fatty acid desaturase gene. Thereby creating a four-gene stack in soybean.
Lineages of the derived four-gene stack producing EPA were subsequently crossed with pASTA events previously determined to producing asatxanthin, 818-3, 807-2, and 806-14. This in turn manifested a seven-gene stack for the simultaneous production of EPA and astaxanthin.
Acknowledgements

This research was supported by United Soybean Board and Nebraska Soybean Board. I would like to say thank you to Dr Alan J. Teale for giving me a fatty acid elongase gene.
References


Fig 3-1. pPTN 809D5(A) and pASTA(B) Binary vector map.

The abbreviations for promoter, gene and terminator are as follows: BC-P, β-conglycinin promoter; TEV, tobacco etch translational enhancer; D6 Elo, Δ6 elongase; T35S, 35s terminator; Gly-p, Glycinin promoter; D5 des, Δ5 desaturase; Gly-T, Glycinin terminator; Pnos, nopaline synthase promoter; Bar, bar gene; Tnos, nopaline synthase terminator; CrtW, β-carotene ketolase; Phy, phytoene synthase; CrtZ, β-carotene hydroxylase; P3U, Phaseolin 3’UTR. RB and LB refer to the right border and left border elements.
**Fig 3-2.** Comparision of carotenoid and seed color of wild type and transgenic soybean seeds having various combinations of Phytoene synthase, CrtZ and CrtW genes.

806-12 habors Phytoene synthase and CrtZ. 807-5 habors Phytoene synthase and CrtW. 806-14 habors only Phytoene synthase. 807-2 and 818-3 habor all three genes. Top panel, extracted carotenoid from wild type and transgenic T2 seeds. Lower panel, color of wild type and transgenic T2 seeds after peeling off the seed coat. Total carotenoids were extracted from ground soybean powder of 5 orange color mature seeds by the method of Bligh and Dyer(1959).
Fig 3-3. Southern blot analysis on T1 generation of soybean having Δ6 desaturase and Δ5 desaturase.

10ug of genomic DNA was digested with PstI and hybridized with Δ6 desaturase (left panel) and Δ5 desaturase (right panel). Lane 4 and 5 refer to wild type control Thorne DNA and 100pg of pPTN 809D5 digested with PstI respectively.
Fig 3-4. Northern blot analysis on immature embryo derived from 824-1 T1-45 and 824-8 T1-37 events.

RNA was extracted from immature embryo and 15ug of RNA was used. A and B panels, Northern blot. C panel, ribosomal RNA. Lane 1 and 2, wild type control Thorne immature Embryo. Lane 3 thru 6, immature embryos derived from 824-1 T1-45. Lane 7 thru 10, immature embryos derived from 824-8 T1-37. Δ6 elongase(A) and Δ5 desaturase(B) genes were used as a probe.
**Fig 3-5.** Northern blot analysis on immature embryo derived from STD x 824-1-T1-45 F₁ #1 cross line.

RNA was extracted from immature embryo and 15μg of RNA was used. A, B, C and D panels, Northern blot. E panel, ribosomal RNA. Lane 1and 2, wild type soybean immature Embryo. Lane 3 thru 9, immature embryos derived from STD x 824-1-T1-45 F₁ #1 cross line. Δ6 desaturase(A), Δ15 desaturase(B), Δ6 elongase(C)and Δ5 desaturase(D) genes were used as a probe.
**Fig 3-6.** Southern blot analysis on T1 generation of soybean plant producing astaxanthin.

10ug of genomic DNA was digested with *EcoRI* and hybridized with Phytoene synthase(A), CrtZ (B) and CrtW(C) respectively. Lane 1 thru 9, T1 progeny transformants. Lane 10 and 11 refer to wild type control Thorne DNA and 100pg of pASTA vector digested with *EcoRI* respectively.
Fig 3-7. Northern blot analysis on immature embryos derived from T1 soybean plant producing astaxanthin.

1, 2 and 3 panel, Northern blot hybridized with Phytoene synthase(A), CrtZ(B) and CrtW(Z) respectively. 4 panel, 25s ribosomal RNA. Lane 1, 2, 11 and 12, wild type soybean immature embryo. Lane 3 thru10 and 13 thru 20, immature embryos derived from T1 generation of each transgenic line.
**Fig 3-8.** astaxanthin(A) and β-carotene content of transgenic mature soybean T2 seeds expressing Phytoene synthase, CrtZ and CrtW genes.

5 orange colored mature seeds were collected from each event and ground with liquid nitrogen. Total carotenoids were extracted from 50 mg of grinded soybean powder by the method of Bligh and Dyer (1959). Astaxanthin and β-carotene content were measured by HPLC with c30 column.
Fig 3-9. Astaxanthin(A) and β-carotene(B) content of transgenic mature soybean T3 or T4 seeds from field in 2011.

5 orange colored mature seeds were collected and ground together and then the powder was used for measuring the astaxanthin and β-carotene content.
Fig 3-10. tocopherol(A) and tocotrienol(B) content of transgenic mature soybean T2 seeds expressing Phytoene synthase, CrtZ and CrtW genes.

5 orange colored mature seeds were collected from each event and ground with liquid nitrogen. Tocopherol and tocotrienol were extracted from 50mg of the soybean powder. Ethanol-dichloromethane (9:1 v/v) was used to extract the tocopherol and tocotrienol. Tocopherol and tocotrienol content were measured by HPLC with c18 column.
Fig 3-S1. Southern blot analysis on T1 generation of soybean producing astaxanthin.

10μg of genomic DNA was digested with *EcoRI* and hybridized with Phytoene synthase(A), CrtZ(B) and CrtW(C) respectively. Lane 1 thru 10, T1 progeny transformants. Lane 11 and 12 refer to wild type control Thorne DNA and 100pg of pASTA vector digested with *EcoRI* respectively.
Fig 3-S2. Southern blot analysis on F₁ generation of soybean producing EPA and astaxanthin.

10ug of genomic DNA was digested with EcoRI and hybridized with Δ6 desaturase(A), Δ15 desaturase(B), Δ6 elongase(C), Δ5 desaturase(D), Phytoene synthase(E), CrtZ(F) and CrtW(G) respectively. ETP refers to EPA producing event 824-1 X 535-9. Lane1, ETP F₁ #1 F₂ #2. Lane2, 807-2 T1-2. Lane3, 818-3 T1-2. Lane4, (ETP F₁ #1 X 807-2 T1-2) F₁ #9. Lane5, (ETP F₁ #1 X 818-3 T1-2) F₁ #2. Lane 5, 6, 7, 8 and 9 refer to wild type control Thorne DNA, 100pg of pPTN373, 100pg of pPTN382, 100pg of pPTN 809D5 and 100pg of pASTA digested with EcoRI respectively.
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<th>18:1</th>
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<td>2.55 ± 0.05</td>
<td>14.54 ± 2.19</td>
<td>51.73 ± 1.11</td>
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<td>51.97 ± 4.27</td>
<td>9.32 ± 2.01</td>
<td>0.84 ± 0.51</td>
<td>1.76 ± 1.64</td>
<td>0.48 ± 0.44</td>
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<tr>
<td>824-8</td>
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<td>5.32 ± 0.50</td>
<td>12.13 ± 1.90</td>
<td>43.16 ± 2.88</td>
<td>10.77 ± 1.01</td>
<td>2.47 ± 0.69</td>
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Table 3-1. Fatty acid profiles on T₁ seed derived from pPTN809D5
**Table 3-2.** Fatty acid profiles on F1 populations

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<th>STA</th>
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**Table 3-4.** Carotenoids content(%) of selected events having all three genes or lacking either crtZ or crtZ and crtW genes

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<td>0</td>
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<td>1.9</td>
<td>1.6</td>
<td>1.2</td>
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<tr>
<td>Canthaxanthin</td>
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<tr>
<td>Phenicoxanthin</td>
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<td>0</td>
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<td>2</td>
<td>1.4</td>
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</tr>
<tr>
<td>Astaxanthin</td>
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<td>0</td>
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<td>2</td>
<td>2</td>
<td>3.3</td>
</tr>
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<table>
<thead>
<tr>
<th></th>
<th>phy</th>
<th>crtZ</th>
<th>crtW</th>
</tr>
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<tr>
<td><strong>phy</strong></td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>crtz</strong></td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><strong>crtw</strong></td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
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</table>
Table 3.5. Fatty acid profiles on selected orange color F1 seeds producing ALA, STA or EPA

<table>
<thead>
<tr>
<th>Cross line</th>
<th>16:0</th>
<th>18:0</th>
<th>18:1</th>
<th>18:2</th>
<th>GLA</th>
<th>ALA</th>
<th>STA</th>
<th>20:1</th>
<th>20:2</th>
<th>UN</th>
<th>ARA</th>
<th>20:3</th>
<th>ETA</th>
<th>EPA</th>
<th>Phenotype</th>
</tr>
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<tbody>
<tr>
<td>(ETP(F1;1) X 818-3) (F1,1)</td>
<td>10.51</td>
<td>2.69</td>
<td>9.98</td>
<td>8.95</td>
<td>0.00</td>
<td>51.85</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>ALA/Carotenoids</td>
</tr>
<tr>
<td>(ETP(F1;1) X 818-3) (F1,2)</td>
<td>11.21</td>
<td>3.91</td>
<td>11.23</td>
<td>9.91</td>
<td>4.88</td>
<td>28.15</td>
<td>11.22</td>
<td>1.28</td>
<td>0.89</td>
<td>2.97</td>
<td>0.85</td>
<td>2.04</td>
<td>4.62</td>
<td>0.42</td>
<td>EPA/Carotenoids</td>
</tr>
<tr>
<td>(ETP(F1;1) X 818-3) (F1,3)</td>
<td>10.85</td>
<td>3.02</td>
<td>8.40</td>
<td>5.76</td>
<td>5.87</td>
<td>27.57</td>
<td>28.52</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>STA/Carotenoids</td>
</tr>
<tr>
<td>(ETP(F1;1) X 807-2) (F1,2)</td>
<td>9.79</td>
<td>3.30</td>
<td>16.73</td>
<td>13.92</td>
<td>4.94</td>
<td>27.84</td>
<td>7.94</td>
<td>1.64</td>
<td>1.11</td>
<td>2.63</td>
<td>0.58</td>
<td>2.14</td>
<td>3.20</td>
<td>0.84</td>
<td>EPA/Carotenoids</td>
</tr>
<tr>
<td>(ETP(F1;1) X 807-2) (F1,5)</td>
<td>9.82</td>
<td>2.73</td>
<td>12.88</td>
<td>9.92</td>
<td>8.24</td>
<td>32.08</td>
<td>21.78</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>STA/Carotenoids</td>
</tr>
<tr>
<td>(ETP(F1;1) X 807-2) (F1,2)</td>
<td>10.27</td>
<td>2.84</td>
<td>13.51</td>
<td>13.60</td>
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<td>55.81</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
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<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>ALA/Carotenoids</td>
</tr>
<tr>
<td>(ETP(F1;1) X 807-2) (F1,3)</td>
<td>10.88</td>
<td>3.43</td>
<td>10.58</td>
<td>8.02</td>
<td>4.48</td>
<td>32.92</td>
<td>13.31</td>
<td>0.92</td>
<td>0.44</td>
<td>2.10</td>
<td>0.63</td>
<td>2.00</td>
<td>5.27</td>
<td>1.51</td>
<td>EPA/Carotenoids</td>
</tr>
<tr>
<td>(ETP(F1;1) X 807-2) (F1,9)</td>
<td>10.21</td>
<td>3.14</td>
<td>12.58</td>
<td>10.23</td>
<td>3.77</td>
<td>34.03</td>
<td>11.10</td>
<td>0.71</td>
<td>0.38</td>
<td>1.76</td>
<td>0.65</td>
<td>1.82</td>
<td>4.73</td>
<td>1.71</td>
<td>EPA/Carotenoids</td>
</tr>
<tr>
<td>(807-2 X ETP(F1;1)) (F1,1)</td>
<td>9.86</td>
<td>2.76</td>
<td>11.51</td>
<td>14.27</td>
<td>0.00</td>
<td>58.79</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>ALA/Carotenoids</td>
</tr>
<tr>
<td>(806-14 X ETP,F1;1) (F1,1)</td>
<td>9.70</td>
<td>2.55</td>
<td>19.24</td>
<td>11.53</td>
<td>5.50</td>
<td>33.93</td>
<td>14.78</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>STA/Carotenoids</td>
</tr>
<tr>
<td>(806-14 X ETP,F1;1) (F1,3)</td>
<td>9.95</td>
<td>2.51</td>
<td>17.57</td>
<td>14.93</td>
<td>0.00</td>
<td>51.41</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>ALA/Carotenoids</td>
</tr>
<tr>
<td>(806-14 X ETP,F1;1) (F1,4)</td>
<td>9.45</td>
<td>3.30</td>
<td>16.29</td>
<td>10.99</td>
<td>4.01</td>
<td>29.77</td>
<td>10.34</td>
<td>1.35</td>
<td>0.65</td>
<td>1.95</td>
<td>0.44</td>
<td>2.64</td>
<td>4.33</td>
<td>1.09</td>
<td>EPA/Carotenoids</td>
</tr>
<tr>
<td>(806-14 X ETP,F1;1) (F1,6)</td>
<td>9.76</td>
<td>2.46</td>
<td>14.47</td>
<td>11.25</td>
<td>6.95</td>
<td>32.39</td>
<td>19.02</td>
<td>0.00</td>
<td>0.00</td>
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<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>0.00</td>
<td>STA/Carotenoids</td>
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</table>
Table 3-6. Fatty acid profiles, total oil and carotenoid content on selected orange color F2 seeds producing EPA

<table>
<thead>
<tr>
<th>crossline</th>
<th>16:0</th>
<th>18:0</th>
<th>18:1</th>
<th>18:2</th>
<th>GLA</th>
<th>ALA</th>
<th>STA</th>
<th>ETA</th>
<th>EPA</th>
<th>total oil</th>
<th>astaxanthin</th>
<th>β-carotene</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT(Thorne)</td>
<td>11.8 ± 0.8</td>
<td>3.6 ± 0.2</td>
<td>15.8 ± 2.2</td>
<td>53.9 ± 1.6</td>
<td>0.0 ± 0.0</td>
<td>12.9 ± 0.9</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>22.2 ± 1.1</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>806-14</td>
<td>10.9 ± 0.2</td>
<td>3.2 ± 0.2</td>
<td>18.6 ± 3.5</td>
<td>52.6 ± 1.8</td>
<td>0.0 ± 0.0</td>
<td>12.8 ± 2.1</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>20.3 ± 2.1</td>
<td>0.0 ± 0.0</td>
<td>1834.8 ± 264.6</td>
</tr>
<tr>
<td>818-3</td>
<td>11.8 ± 0.6</td>
<td>3.4 ± 0.1</td>
<td>16.9 ± 1.7</td>
<td>51.8 ± 0.9</td>
<td>0.0 ± 0.0</td>
<td>14.2 ± 1.7</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>20.3 ± 2.1</td>
<td>19.0 ± 3.2</td>
<td>931.2 ± 5.8</td>
</tr>
<tr>
<td>807-2</td>
<td>11.1 ± 0.4</td>
<td>3.3 ± 0.1</td>
<td>18.0 ± 1.8</td>
<td>51.6 ± 1.0</td>
<td>0.0 ± 0.0</td>
<td>14.0 ± 1.1</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>18.2 ± 0.8</td>
<td>38.7 ± 1.5</td>
<td>1751.6 ± 98.4</td>
</tr>
<tr>
<td>ETP(F1)</td>
<td>12.2 ± 0.6</td>
<td>3.4 ± 0.3</td>
<td>10.2 ± 1.3</td>
<td>11.9 ± 7.4</td>
<td>7.3 ± 5.8</td>
<td>29.1 ± 13.2</td>
<td>11.5 ± 7.1</td>
<td>4.7 ± 3.1</td>
<td>1.6 ± 0.7</td>
<td>21.8 ± 0.3</td>
<td>N/D</td>
<td>N/D</td>
</tr>
<tr>
<td>ETP(F1)</td>
<td>11.8 ± 0.6</td>
<td>3.6 ± 0.2</td>
<td>16.6 ± 4.9</td>
<td>5.3 ± 1.6</td>
<td>3.8 ± 1.0</td>
<td>27.8 ± 7.7</td>
<td>17.2 ± 4.4</td>
<td>6.6 ± 1.7</td>
<td>1.5 ± 0.2</td>
<td>22.7 ± 0.9</td>
<td>N/D</td>
<td>N/D</td>
</tr>
<tr>
<td>(806-14 X ETP(F1,1)) (F1,1) (F2)</td>
<td>10.1 ± 0.5</td>
<td>3.9 ± 0.1</td>
<td>13.7 ± 3.3</td>
<td>12.2 ± 4.2</td>
<td>5.2 ± 6.6</td>
<td>35.8 ± 16.7</td>
<td>8.1 ± 3.3</td>
<td>3.3 ± 1.5</td>
<td>1.0 ± 0.2</td>
<td>18.7 ± 0.0</td>
<td>0.0 ± 0.0</td>
<td>1440.7 ± 182.3</td>
</tr>
<tr>
<td>(ETP(F1,1) X 818-3) (F1,2) (F2)</td>
<td>12.0 ± 0.7</td>
<td>3.9 ± 0.4</td>
<td>12.6 ± 1.9</td>
<td>11.7 ± 5.7</td>
<td>5.4 ± 5.3</td>
<td>31.7 ± 11.0</td>
<td>10.3 ± 5.3</td>
<td>4.2 ± 2.2</td>
<td>1.3 ± 0.6</td>
<td>21.4 ± 1.2</td>
<td>25.8 ± 4.1</td>
<td>396.9 ± 8.8</td>
</tr>
<tr>
<td>(ETP(F1,1) X 807-2) (F1,3) (F2)</td>
<td>10.2 ± 0.7</td>
<td>2.9 ± 0.5</td>
<td>22.4 ± 3.7</td>
<td>18.9 ± 11.2</td>
<td>3.6 ± 3.4</td>
<td>29.1 ± 14.4</td>
<td>5.0 ± 3.6</td>
<td>2.0 ± 1.5</td>
<td>0.7 ± 0.4</td>
<td>17.7 ± 0.7</td>
<td>26.8 ± 2.7</td>
<td>833.0 ± 23.8</td>
</tr>
<tr>
<td>(ETP(F1,1) X 807-2) (F1,9) (F2)</td>
<td>10.4 ± 0.4</td>
<td>3.2 ± 0.6</td>
<td>23.2 ± 7.8</td>
<td>18.0 ± 11.9</td>
<td>2.3 ± 1.6</td>
<td>28.4 ± 6.5</td>
<td>5.6 ± 3.4</td>
<td>2.5 ± 1.6</td>
<td>1.0 ± 0.5</td>
<td>17.3 ± 0.5</td>
<td>44 ± 6.9</td>
<td>1139.4 ± 181.5</td>
</tr>
<tr>
<td>(ETP(F1,1) X 807-2) (F1,9) (F2)</td>
<td>9.6 ± 0.8</td>
<td>3.5 ± 0.5</td>
<td>15.7 ± 6.9</td>
<td>23.0 ± 12.5</td>
<td>4.2 ± 3.1</td>
<td>28.4 ± 12.3</td>
<td>5.5 ± 5.5</td>
<td>2.4 ± 2.5</td>
<td>0.9 ± 0.5</td>
<td>14.4 ± 0.3</td>
<td>23 ± 1.0</td>
<td>527 ± 46.7</td>
</tr>
</tbody>
</table>
Appendix 1. biosynthetic pathway of very long chain poly unsaturated fatty acid. The solid and dashed arrows indicate the reaction catalyzed by the elongase and desaturase.
Appendix 2. Astaxanthin biosynthetic pathway.

The abbreviations for intermediates and genes are as follows: GA3P, glyceraldehyde-3-phosphate; IPP, isopentenyl pyrophosphate; GGPS, Geranylgeranyl pyrophosphate synthetase; GGPP, geranylgeranyl diphosphate; PSY(crtB), phytoene synthase; PDS(crtI), phytoene desaturase; LCYb(crtY), lycopene β-cyclase; BHY(crtZ), β-carotene hydroxylase; BKT(crtW), β-carotene ketolase. The blue and red arrows indicate the reactions catalyzed by the enzymes encoded by *crtZ* and *crtW* respectively. Figure is modified from Giovanni *et al.* (2008)