5-1-2014

Stacking of a stearoyl-ACP thioesterase with a dual-silenced palmitoyl-ACP thioesterase and ▲12 fatty acid desaturase in transgenic soybean

Hyunwoo Park
University of Nebraska-Lincoln, dnahyun@yahoo.com

George L. Graef
University of Nebraska - Lincoln, ggraef1@unl.edu

Yixiang Xu
University of Nebraska-Lincoln, yixu@vsu.edu

Patrick Tenopir
University of Nebraska-Lincoln, ptenopir2@unl.edu

Thomas E. Clemente
University of Nebraska - Lincoln, tclemente1@unl.edu

Plant Biotechnology Journal

Plant Biotechnology Journal (2014) **12**, pp. 1035–1043 doi: 10.1111/pbi.12209

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

Hyunwoo Park[1,2], George Graef[1], Yixiang Xu[3,†], Patrick Tenopir[1] and Tom E. Clemente[1,2,*]

[1]*Department of Agronomy and Horticulture, University of Nebraska-Lincoln, Lincoln, NE, USA*
[2]*Center for Plant Science Innovation, University of Nebraska-Lincoln, Lincoln, NE, USA*
[3]*Department of Biological Systems Engineering, University of Nebraska-Lincoln, Lincoln, NE, USA*

Received 22 January 2014;
revised 9 April 2014;
accepted 1 May 2014.
**Correspondence* (Tel 402 472 1428;
email tclemente1@unl.edu)
[†]Present address: Agricultural Research Station, Virginia State University, VA, USA.

Keywords: stearic acid, soybean oil, *Agrobacterium*, *Glycine max*.

Summary

Soybean (*Glycine max* (L.) Merr) is valued for both its protein and oil, whose seed is composed of 40% and 20% of each 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 to soybean oil as a means to improve both the oxidative stability and functionality in 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 the hydrogenation process, genetic approaches are 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 the subsequent stacking with an event that is dual-silenced in palmitoyl-ACP thioesterase and Δ12 fatty acid desaturase expression in a seed-specific fashion. Phenotypic analyses on transgenic soybean expressing the mangosteen stearoyl-ACP thioesterase revealed increases in seed stearic acid levels up to 17%. The subsequent stacked with a soybean event silenced in both palmitoyl-ACP thioesterase and Δ12 fatty acid desaturase activity, resulted in a seed lipid phenotype of approximately 11%–19% stearate and approximately 70% oleate. The 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 United States 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 in seeds to 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, www.ers.usda.gov). Implementing genetic approaches to improve functional and nutritional qualities of oils has attracted significant attention over the past decade. To this end, the fatty acid profile of oil has a significant influence on functionality and nutritional aspects in food and feed applications.

Commodity soybean oil 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 acid and stearic acid. 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 be more cardiovascular neutral in comparison with palmitic acid in many studies (Bonanome and Grundy, 1988; Kris-Etherton *et al.*, 2005; Schwab *et al.*, 1996; Stanley, 2009). Hence, genetic approaches to simultaneously reduce polyunsaturated fatty acids and enhance stearic acid levels in soybean oil would be expected to

have value in food application as a margarine replacement, as well 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.* (2002) to raise stearic acid and oleic acid levels in cotton seed oil. Here, 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 steroyl-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 (Figure 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 *Agrobacterium tumefaciens* transconjugants carrying pPTN811. Segregation analysis on T_1 progeny derived from these transgenic events is shown in Table S1. The T_1 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 S1 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 1. The data show 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% to 17.3% (Table 1) in T_1 seed. The nulls were not used in the calculation the respective means.

Three of the pPTN811 soybean events were selected to propagate further. These are 680-2, 688-5 and 683-2. Fatty acid profiles on either T_2 or T_3 generations from these events are shown in Table 2. As can be seen from the data mean stearic acid levels, under a greenhouse environment, from these events range

Table 1 Fatty acid profile on T_1 seed derived from pPTN811

Event	Palmitic%	Stearic%	Oleic%	Linoleic%	Linolenic%
679-2	8.1 ± 3.2	9.4 ± 1.7	8.0 ± 1.0	48.5 ± 2.1	10.4 ± 1.5
679-5	12.0 ± 0.4	12.0 ± 1.7	14.2 ± 1.2	54.3 ± 1.3	14.6 ± 1.3
679-11	11.3 ± 0.6	5.4 ± 1.7	11.8 ± 0.6	57.0 ± 1.3	10.4 ± 0.6
679-13	11.8 ± 1.0	4.4 ± 1.2	12.3 ± 1.3	56.6 ± 0.6	10.9 ± 1.2
680-2	9.3 ± 4.7	9.2 ± 3.5	10.1 ± 1.0	56.0 ± 0.6	10.9 ± 1.2
680-3	10.0 ± 0.5	9.0 ± 2.3	11.0 ± 1.5	54.4 ± 2.0	9.6 ± 1.3
680-9	10.3 ± 0.5	8.4 ± 1.3	13.2 ± 1.7	54.9 ± 3.4	10.1 ± 1.1
681-9	8.7 ± 0.5	11.1 ± 1.9	12.9 ± 0.9	53.7 ± 1.0	9.3 ± 0.8
681-10	9.6 ± 0.6	8.8 ± 2.4	14.3 ± 3.0	54.0 ± 1.1	8.9 ± 1.2
681-14	9.2 ± 0.6	9.6 ± 1.0	13.1 ± 0.6	54.4 ± 1.1	9.6 ± 0.9
683-2	8.7 ± 0.8	17.3 ± 4.4	8.2 ± 0.3	47.7 ± 2.7	12.1 ± 0.6
686-5	10.8 ± 1.3	8.6 ± 2.7	13.7 ± 2.0	51.3 ± 1.7	10.8 ± 1.4
686-6	9.4 ± 0.3	10.6 ± 1.6	15.2 ± 1.6	50.8 ± 1.2	9.2 ± 0.7
686-8	9.4 ± 0.3	10.4 ± 1.9	13.3 ± 2.1	51.3 ± 1.5	10.8 ± 1.4
687-1	10.1 ± 1.1	9.6 ± 3.2	11.9 ± 2.5	52.7 ± 2.0	9.8 ± 1.2
687-2	9.9 ± 1.0	10.2 ± 3.2	10.1 ± 1.3	53.6 ± 1.7	11.2 ± 1.2
687-3	10.0 ± 0.6	10.7 ± 2.7	10.1 ± 1.2	53.2 ± 1.1	10.8 ± 0.7
687-8	9.0 ± 0.8	11.4 ± 2.9	13.8 ± 1.3	52.1 ± 1.3	9.2 ± 2.9
688-4	9.1 ± 0.7	10.8 ± 3.1	13.9 ± 3.6	51.8 ± 1.7	10.4 ± 1.4
688-5	9.2 ± 0.8	10.8 ± 2.8	9.4 ± 1.1	52.0 ± 1.9	13.7 ± 1.2
WT	10.9 ± 0.7	3.3 ± 0.2	16.0 ± 1.5	54.8 ± 0.6	10.7 ± 0.7

Numbers within the respective fatty acid columns reflect mean percentage of the corresponding fatty acid ± standard deviation. Means were tabulated on seed chips from 2 to 11 seeds per event confirmed to be carrying the stearoyl-ACP thioesterase transgene.

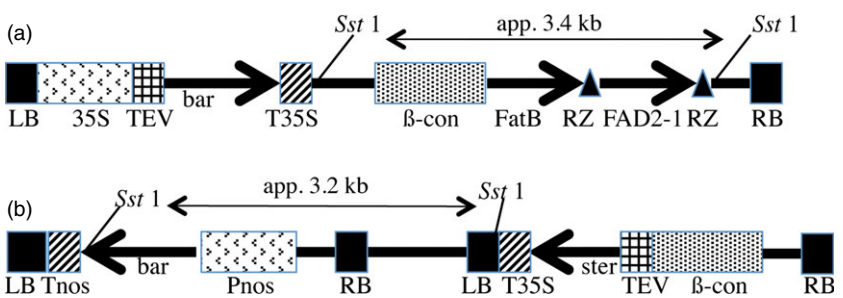

Figure 1 Diagrammatic representation of T-DNA elements in binary plasmids pPTN303 and pPTN811. (a) T-DNA element within pPTN303 and (b) two T-DNA elements in pPTN811. The location of the *Sst* 1 restriction sites used in Southern analysis (Figure S1) is shown. LB and RB refer to left and right border, respectively. Elements 35S and T35S indicate 35S CaMV promoter and terminator regions, respectively. Pnos and Tnos designate nopaline synthase promoter and terminator elements, respectively. TEV refers to tobacco etch translational enhancer element. β-con designates the seed-specific beta-conglycinin promoter, while *bar*, *FAD*2-1, *fat*B and *ster* refer to the *bar* resistance gene Δ12 fatty acid desaturase, palmitoyl-ACP thioesterase and stearoyl-ACP thioesterase, respectively. RZ designates the self-cleaving ribozyme (Buhr *et al.*, 2002).

from 13.4% to 17.4%, hence, revealing the phenotype is stable over generations.

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

The selected events were subsequently crossed with event 335-13, a soybean event high in oleic acid and low in palmitic acid (Graef *et al.*, 2009). The silencing allele in 335-13 resided initially in an Asgrow® (Asgrow® Seed Company, Dekalb, IL) genotype, A3237 (Buhr *et al.*, 2002), this event was developed at the University of Nebraska in January 2000. The A3237 event designated 335-13 was backcrossed into high oil/yielding germplasm developed at the University of Nebraska's soybean breeding programme, with the genotype designated Ux1625-83-165. The soybean genotype Ux1625-83-165 is referred to by the transgenic event 335-13. The 335-13 event used for stacking with the stearoyl-ACP thioesterase was a lineage derived from the backcrossing. Fatty acid profiles of derived F_1 seed from the crosses are listed in Table 2. 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 2). Molecular analysis of the stack, and corresponding parents from one of these crosses, is shown in Figure S1, confirming the presence of both transgenic alleles in the stack.

These F_1 seeds were followed to the F_2 population (Table S2), 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 F_2 population, the gene stack led to oleic acid percentages in the 70s and stearic acid percentages of 5.2%–13.6% (Table S2). Based on these data (stearate and oleate percentages), populations derived from the cross of 335-13 × 680-2 were carried on to homozygosity under greenhouse conditions.

Homozygous lineages at the F_4 generation were identified from the stack created by crossing event 335-13 with the elevated stearic acid event 680-2. Fatty acid profiles of five homozygous lineages are shown in Table 3, wherein the oleic acid and stearic acid percentages of the oil ranged from 67.7% to 70.5% and 16.7% to 19.7%, respectively.

Field evaluations of transgenic event 680-2 and stack

Field releases of the populations derived from 680-2, and 335-13 × 680-2 stack, 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. Field trials were conducted at the University of Nebraska's Plant Biotechnology Field Facility located outside Mead, Nebraska.

Fatty acid profiles of seed samples taken following the 2009 harvest are shown in Table 4, which tended to mirror that observed under greenhouse conditions of the parents (Table 2), but slightly lower stearate levels in the stack under field

Table 2 Fatty acid profiles of parental and F_1 seed stacks

Event/Stack	Palmitic%	Stearic%	Oleic%	Linoleic%	Linolenic%
680-2 (T_2)	8.9 ± 0.5	13.6 ± 1.7	10.9 ± 1.2	43.9 ± 2.0	12.3 ± 1.4
688-5 (T_3)	8.0 ± 0.4	14.7 ± 0.6	11.7 ± 0.9	47.7 ± 1.4	10.9 ± 1.4
683-2 (T_3)	8.7 ± 0.7	17.8 ± 1.9	10.5 ± 0.8	45.4 ± 1.9	13.0 ± 2.3
683-2 (T_{3a})	9.5 ± 0.5	13.4 ± 1.8	9.0 ± 0.8	49.0 ± 1.2	14.4 ± 1.0
335-13	3.3 ± 0.4	2.8 ± 0.5	82.1 ± 2.2	1.6 ± 0.3	3.8 ± 0.9
335-13 × 680-2 (F_{1-4})	3.9	11.5	70.9	2.8	4.5
335-13 × 680-2 (F_{1-5})	5.3	10.5	65.8	4.9	6.7
335-13 × 688-5 (F_{1-1})	3.6	9.1	76.0	1.7	5.3
335-13 × 688-5 (F_{1-2})	3.4	8.5	75.0	4.1	4.4
335-13 × 688-5 (F_{1-3})	2.8	7.3	79.9	1.2	3.9
683-2 × 335-13 (F_{1-1})	4.0	9.4	74.0	1.6	6.3
683-2 × 335-13 (F_{1-2})	4.4	10.2	73.3	1.9	6.6
683-2 × 335-13 (F_{1-4})	4.0	9.8	73.3	2.0	6.8
335-13 × 683-2 (F_{1-1})	4.0	10.2	74.3	1.2	5.4
335-13 × 683-2 (F_{1-4})	3.5	10.6	75.8	1.2	4.7

Event/stack column indicates the parental events used for crossing. 335-13 refers to high oleic acid event 335-13. Event listed first in stacks indicates parent used as male. Numbers within each column refer to the percentage of corresponding fatty acid as determined by GC analysis.

Table 3 Fatty acid profile of homozygous lineages derived from 335-13 × 680-2 stack

Stack	Palmitic%	Stearic%	Oleic%	Linoleic%	Linolenic%
335-13 × 680-2	3.7 ± 0.3	16.7 ± 1.4	70.5 ± 2.4	1.7 ± 0.6	3.7 ± 0.5
335-13 × 680-2	3.7 ± 0.4	18.0 ± 2.2	68.4 ± 3.8	1.9 ± 1.2	4.0 ± 0.9
335-13 × 680-2	3.8 ± 0.5	18.2 ± 1.7	67.9 ± 3.5	2.4 ± 1.4	4.1 ± 0.7
335-13 × 680-2	3.6 ± 0.3	16.5 ± 0.9	69.7 ± 1.6	2.2 ± 0.8	4.9 ± 0.6
335-13 × 680-2	3.7 ± 0.3	19.7 ± 0.9	67.7 ± 1.9	1.5 ± 0.3	3.8 ± 0.2

Stack column is five F_4-derived populations from two F_1 seed of a cross between the high oleic acid event 335-13 and elevated stearic acid event 680-2.

Table 4 Fatty acid profile of soybean events under field conditions in 2009

Event/Stack	Palmitic%	Stearic%	Oleic%	Linoleic%	Linolenic%
WT (Thorne)	11.3 ± 0.5	3.0 ± 1.9	15.4 ± 1.7	51.0 ± 1.7	14.3 ± 1.2
680-2	8.4 ± 0.6	10.2 ± 1.4	15.1 ± 1.8	48.0 ± 1.5	15.2 ± 1.6
335-13 × 680-2	4.1 ± 0.9	12.7 ± 2.2	67.3 ± 5.4	4.1 ± 2.6	8.0 ± 1.9

Event/Stack column refers to control seed (genotype Thorne), event 680-2 and stack line with high oleic acid event 335-13. Numbers within the columns are mean percentages of corresponding fatty acid ± standard deviations based on 50–90 samples.

conditions (Table 4) as compared to the populations grown under greenhouse environment (Table 3).

Isolated oil from the 2009 harvest, along with oil extracted from 335-13, was analysed for oxidative stability, cloud and melting points. The data from these analyses are shown in Table 5. The oxidative onset temperature (OOT) was higher in both event 335-13 and stack (Table 5) 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 5). 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 5).

The change in melting temperature across the oils is visually represented in Figure S2, where the respective oil samples were placed at −20 °C for 1 h and removed, the degree of melting visualized closely parallels melting point 1 data of the oil (Table 5).

A second field trial was conducted in 2010 at the University of Nebraska's Plant Biotechnology Field Facility, with sufficient plot replications to monitor both fatty acid profiles and gain 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 2010, plots were replicated under both irrigated and nonirrigated conditions.

Fatty acid profiles from the 2010 harvest are shown in Table S3. 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 postflowering revealed the presence of *FAD*2-1 transcript in the stack (Figure 2), which was not observed in the parental 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 S3).

Table 5 Oxidative stability and cold properties of derived oils

Event/Stack	Oxidative (°C)	Cloud Point (°C)	Melting Point 1 (°C)	Melting Point 2 (°C)
WT (Thorne)	173.7	−12.3	−34.7	−15.7
335-13	194.2	−15.6	−13.4	−0.9
680-2	174.5	−0.3	−19.1	−1.8
335-13 × 680-2	207.6	0.4	−15.6	−6.7

All values are means of derived from duplicate tests. Oxidative column refers to the oxidative onset temperature (OOT). Cloud point indicates temperature at which a cloud is formed in the oil reflecting first stage of crystallization. Melting point 1 and 2 columns are the onset of endothermic peaks in the heating curve, in which two such peaks were observed in the analysis.

A third field trial was carried out in 2011 at the University's Plant Biotechnology Field Facility, and in this trial, the stack, control plots and 335-13 event were planted. Replicated plots were planted under both irrigated and nonirrigated environments. Data were 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 nonirrigated conditions were not met. Nonetheless, within each environment, no significant difference was observed with exception of days to maturity (Table S4).

However, in a similar fashion to what was observed in 2010, oleate levels in the gene stack were significantly lower (Table 6), with *FAD*2-1 transcript accumulation observed in the gene stack from sampled immature embryos (Figure 3), while in the parent event, 335-13, remained silenced in *FAD*2-1, and the steroyl-ACP thioesterase transcript accumulating in the stack (Figure 3). These results were reflected in the elevated stearate levels in the latter and high oleic acid in the former (Table 6).

To confirm the identity preservation of the stacked plots, we randomly selected 50 seed from 2011 harvest, genotyped each for the presence of the silencing allele and subsequently monitored fatty acid profile on each seed. The results showed all 50 seed had elevated stearic acid levels in the oil with means ranging from 14.7% to 16.9%, reflecting the steroyl-ACP thioesterase activity during seed development. In regard to oleate percentages, 15 of the seed were high (>60%), 26 seed were categorized as mid-oleic (35%–50%), and nine contained wild-type levels of oleic acid. The subsequent genotyping of the seed confirmed the presence of the silencing allele. Hence, these data combined with those shown in Figure 3 reflect that in this stack, we are observing silencing of the silencing allele.

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 (Cahoon *et al.*, 2007; Damude and Kinney, 2008; Kinney, 2003; Kinney and Knowlton, 1997; Lu *et al.*, 2010). When considering applications for oils in deep-frying,

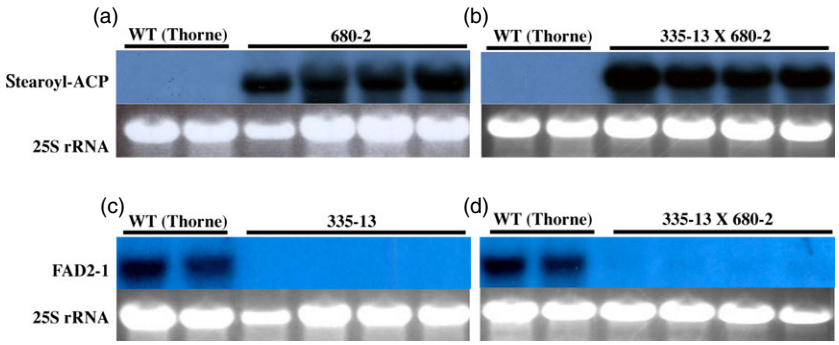

Figure 2 Northern blot analyses on immature embryos from 2010 field trial. Total RNA was isolated from immature embryos (15 μg). (a) first two lanes, control Thorne immature embryos, and last four lanes, immature embryos from 680-2 plots. Membrane hybridized with stearoyl-ACP thioesterase probe. Bottom panel 25S rRNA image. (b) first two lanes, control Thorne immature embryos, and last four lanes, immature embryos from 335-13 × 680-2 stack. Membrane hybridized with stearoyl-ACP thioesterase probe. Bottom panel 25S rRNA image. (c) first two lanes, control Thorne immature embryos, and last four lanes, immature embryos from 335-13 plots. Membrane hybridized with the *FAD2*-1 probe. Bottom panel 25s rRNA image. (d) first two lanes, control Thorne immature embryos, and last four lanes, immature embryos from 335-13 × 680-2 stack plots. Membrane hybridized with *FAD2*-1 probe. Bottom panel 25s rRNA image.

Table 6 Fatty acid profile of soybean events under field conditions in 2011

Event/Stack	Palmitic%	Stearic%	Oleic%	Linoleic%	Linolenic%
WT (Thorne)	10.9 ± 0.3	3.6 ± 0.2	19.7 ± 0.7	55.1 ± 0.9	8.3 ± 0.4
335-13	3.4 ± 0.2	2.4 ± 0.2	84.6 ± 1.2	2.2 ± 0.3	3.2 ± 0.3
335-13 × 680-2	5.6 ± 0.6	15.2 ± 1.1	38.5 ± 9.5	29.8 ± 9.0	8.3 ± 1.1
WT (Thorne)	11.1 ± 0.8	3.4 ± 0.2	19.4 ± 1.9	54.5 ± 2.0	8.1 ± 0.6
335-13	3.7 ± 0.4	2.3 ± 0.1	82.5 ± 3.7	3.6 ± 2.6	3.5 ± 0.4
335-13 × 680-2	5.8 ± 0.7	16.4 ± 1.3	33.9 ± 7.8	33.5 ± 7.6	8.0 ± 0.8

Event/stack column refers to control seed (genotype Thorne), 335-13 refers to high oleic acid event 335-13 and stack line 680-2 with high oleic acid event 335-13. Top three rows plots were under irrigated conditions, and bottom three rows plots were under nonirrigated conditions. Numbers within the columns are mean percentages of corresponding fatty acid ± standard deviations based on 10 ground bulk seed samples from plots.

margarine type uses or as a liquid transportation fuel, that is, biodiesel, two critical parameters are oxidative stability and functionality. Oxidative stability impacts shelf life of a food/feed product and can negatively affect engine performance if the oxidative stability of the biodiesel is low (Knothe, 2005). In regard to functionality, fatty acid composition of oils will need to shift towards more saturated fatty acids to create a solid/semi-solid state at room temperature for such end uses in baking- or confectionary-type applications.

Among 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 (Stanley, 2009), researchers have been actively pursuing genetic approaches to simultaneously 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; Pham *et al.*, 2010; Scherder and Fehr, 2008), tend to be recessive alleles, which may require combination of recessive alleles to create the desired trait into elite germplasm. Moreover, the recessive alleles governing high oleic acid levels in soybean tend to be linked with a yield drag (Scherder and Fehr, 2008). However, more recently, new mutant *FAD2* alleles have been identified that have been shown to mitigate the negative agronomics seen in earlier characterized *FAD2* mutant soybean lines (Pham *et al.*, 2012). These new mutant *FAD2* alleles, along with novel sources of *FAD2* mutations that can be obtained through genome editing tools (Gaj *et al.*, 2013; Voytas, 2013), offer promising approaches to broaden the soybean germplasm with an elevated oleate trait for soybean breeders to draw upon. The study communicated herein, however, 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

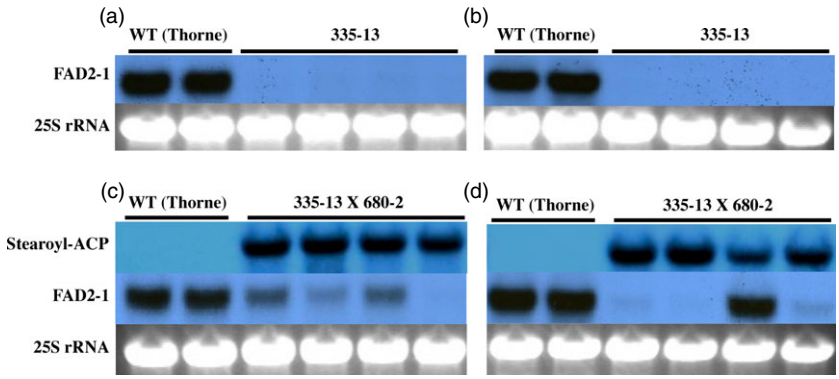

Figure 3 Northern blot analyses on immature embryos from 2011 field trial. Total RNA was isolated from immature embryos (15 μg). (a) first two lanes, control Thorne immature embryos, and last four lanes, immature embryos from 335-13 nonirrigated plots. Membrane hybridized with *FAD*2-1 probe. Bottom panel 25S rRNA image. (b) first two lanes, control Thorne immature embryos, and last four lanes, immature embryos from 335-13 irrigated plots. Membrane hybridized with *FAD*2-1 probe. Bottom panel 25S rRNA image. (c) first two lanes, control Thorne immature embryos, and last four lanes, immature embryos from 335-13 × 680-2 stack nonirrigated plots. Membrane hybridized with stearoyl-ACP thioesterase probe (top) and *FAD*2-1 probe (middle). Bottom panel 25s rRNA image. (d) first two lanes, control Thorne immature embryos, and last four lanes, immature embryos from 335-13 × 680-2 stack irrigated plots. Membrane hybridized with stearoyl-ACP thioesterase probe (top) and *FAD*2-1 probe (middle). Bottom panel 25s rRNA image.

silencing of the cotton *FAD* was stacked with a silenced Δ9-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 three events produced marker-free progeny (Table S1). This is a higher frequency of unlinked integration events than previously observed in soybean (Sato *et al.*, 2004; Xing *et al.*, 2000). 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 under 20% under greenhouse conditions and 10% to approximately 13% under field conditions (Tables 3 and 4). 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, reduction in polyunsaturated fatty acids, combined with high stearic acid, we subsequently crossed the stearoyl-ACP thioesterase transgene with event 335-13 (Buhr *et al.*, 2002; Graef *et al.*, 2009). This resulted in a soybean oil with oleic acid levels at approximately 70% and stearic acid levels greater than 17%, with total saturates over 20% under greenhouse conditions (Table 3), with slightly reduced saturates under a field environment (Table 4). The fatty acid profile of the oil extracted from the stack (Table 4) displayed changes in oxidative stability, cloud point and melting point (Table 5). The change in physical properties observed (Table 5) is likely not sufficient to meet the parameters necessary for end use in baking- or margarine-type applications (Cahoon *et al.*, 2009). A critical parameter that was not met with the specific gene stack described here is the oil did not remain in a semi-solid state at room temperature, which likely would require total saturated fatty acid composition >25%, coupled with reduced polyunsaturated fatty acids. However, an oil with the physical characteristics manifested by the gene stack created herein 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% or greater, (Neff and List, 1999) or in a fractionation process similar to is utilized for palm oil (Kellens *et al.*, 2007).

The stacked lineage developed in this study did not maintain the high oleic acid phenotype after five generations (Tables S3 and 6), 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 *FAD*2-1 transcript (Figures 2 and 3), and subsequent random genotyping and phenotyping of the 2011 field harvest confirmed identity preservation of the stack lineages. 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. The stacking potential of the soybean event 335-13 may be compromised due to the insertion complexity of the transgenic allele. Molecular data conducted on this event suggest two linked inserts are present in the genome of this event (Buhr *et al.*, 2002); however, the elucidation of the structure of this transgenic locus will require sequence analysis about the insertion site. Nonetheless, our data do demonstrate that a novel soybean oil elevated in stearic and high in oleic acid can be achieved through biotechnology. Perhaps a path forward towards the development of stable high stearate combined with elevated oleate soybean oil is a more strategic design of the transgenic elements. For example, one potential design may include hairpin element targeting *FAD*2-1, embedded in a intron, placed just upstream of the stearoyl-ACP thioesterase, under control of a heterologous seed-specific promoter, thereby creating a single cassette that can simultaneously down-regulate *FAD*2-1, with enhanced stearoyl-ACP thioesterase activity, while reducing the number of gene cassettes and thus duplication of genetic elements in the transgenic alleles. This in turn will theoretically produce an oil with the oleate and stearate levels, with a slight increase in palmitate leading to the synthesis of the required levels of saturates, with oxidative

stability to meet the requirements for margarine-type applications.

Experimental procedures

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 (not shown). 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 harbouring 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 (Figure 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 (Xing *et al.*, 2000; Zhang *et al.*, 1999) 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 *fat*B gene, a palmitoyl-ACP thioesterase and *FAD*2-1 a Δ12 fatty acid desaturase gene (Buhr *et al.*, 2002). The silencing allele in 335-13 was originally characterized in soybean genotype A3237 (Asgrow Seed Company, Dekalb, IL), which was subsequently backcrossed into high yielding/oil genotypes at the University of Nebraska's soybean breeding programme (Graef *et al.*, 2009). A genotype from this backcrossing effort designated Ux1625-83-165 was utilized in this study, referred to as merely 335-13 herein.

Molecular characterizations of transgenic events

Southern and Northern blot analyses on the selected event and stack were 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 (Ambion; Life Technologies, Carlsbad, CA). 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 cross-linking. Probes, approximately 50 ng, were labelled with $dCT^{32}P$ by random prime synthesis (Prime-It II Random Synthesis Kit; Agilent Technologies, La Jolla, CA, Cat # 300385). Membranes were hybridized in a 1 mM EDTA, 0.5 M Na_2HPO_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 Na_2HPO_4 solution for 30 min at 65 °C, with a subsequent third wash with 1% SDS, 40 mM Na_2HPO_4 solution for 30 min at 65 °C. Membranes were exposed on X-ray film for 1–3 days at −80 °C.

Confirmation screening for seed identity preservation of the 2011 field plots planted with the gene stack lineages was carried out by genotyping seed for the presence of the silencing allele in the stack via PCR. Primers used in the PCR were designated FatBFad2-F and FatBFad2-R, which specifically hybridized to *fatB* and *fad2-1* gene regions within the silencing element carried in soybean event 335-13 (Buhr *et al.*, 2002). Primer sequences are; FatB-Fad2-F: 5′-GCCACGGCGACCTGACATGC and FatB-Fad2-R: 5′AGTGATGGCGGCGATGGCTT-3.

Phenotypic analyses of transgenic events and stack

Segregation analysis on T_1 individuals derived from transgenic soybean events carrying the two-T-DNA binary vector pPTN811 was conducted using a dual-scoring approach. 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 analysed on a 6890N gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) fitted with a 30 M × 250 μM HP-INNOWAX column (Agilent Technologies Cat# 19091N-133). A leaf painting technique (Zhang *et al.*, 1999) was subsequently used to score for the 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, HT/elevated stearic acid (18 : 0); HT/wild-type stearate level <4% (WT); HS/18 : 0; or double the double null HS/WT. The phenotypic classifications were re-confirmed via PCR using the primer set Stear5: 5′-CCATGGCACTTAAACTCTCCTCATCC-3′ and Stear3: 5′-TCTAGATCATCTTGTTGGTTTCTTCCTCC -3′ to amplify the stearoyl-ACP thioesterase transgene and primer set Pnos: 5′-AGTTGACCGTGCTTGTCTCGATGT-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 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 aluminium 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

Field tests on selected homozygous lineages derived from transgenic events and stacks described 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 were ascertained on estimated yields, 100 seed weight, days to maturity, plant height, along with total protein and oil determinations via proximate analysis (Eurofins, Des Moines IA, USA).

Acknowledgements

Funding support for this project was provided by the United Soybean Board, Nebraska Soybean Board and the University of Nebraska's Center for Biotechnology. Gratitude is extended to Amy Hilske for greenhouse care of plants.

References

2009-02, A.S.E. (2002) *Standard Test Method for Oxidation Onset Temperature of Hydrocarbons by Differential Scanning Calorimetry*. pp. 734–738. West Conshohocken, PA: American Society for Testing Materials.

Allen, R.D., Bernier, F., Lessard, P.A. and Beachy, R.N. (1989) Nuclear factors interact with a soybean β-conglycinin enhancer. *Plant Cell*, **1**, 623–631.

Bachlava, E., Burton, J.W., Brownie, C., Wang, S., Auclair, J. and Cardinal, A.J. (2008) Heritability of oleic acid content in soybean seed oil and its genetic correlation with fatty acid and agronomics traits. *Crop Sci.* **48**, 1764–1772.

Bonanome, A. and Grundy, S.M. (1988) Effect on dietary stearic acid on plasma cholesterol and lipoprotein levels. *N. Engl. J. Med.* **318**, 1244–1248.

Bubeck, D.M., Fehr, W.R. and Hammond, E.G. (1989) Inheritance of palmitic and stearic acid mutants of soybean. *Crop Sci.* **29**, 652–656.

Buhr, T., Sato, S., Ebrahim, F., Xing, A., Zhou, Y., Mathiesen, M., Schweiger, B., Kinney, A.J., Staswick, P. and Clemente, T. (2002) Ribozyme termination of RNA transcripts down-regulate seed fatty acid genes in transgenic soybean. *Plant J.* **30**, 155–163.

Cahoon, E.B., Shockley, J.M., Dietrich, C.R., Gidda, S.K., Mullen, R.T. and Dyer, J.M. (2007) Engineering oilseeds for sustainable production of industrial and nutritional feedstocks: solving bottlenecks in fatty acid flux. *Curr. Opin. Biotechnol.* **10**, 236–244.

Cahoon, E.B., Clemente, T.E., Damude, H.G. and Kinney, A.J. (2009) Modifying vegetable oils for food and non-food purposes. In *Oil Crops, Handbook of Plant Breeding 4* (Vollmann, J. and Rajcan, I., eds), pp. 31–56. New York: Springer Science + Business Media, LLC.

Carrington, J.C. and Freed, D.D. (1990) Cap-independent enhancement of translation by a plant potyvirus 5′ nontranslated region. *J. Virol.* **64**, 1590–1597.

Clemente, T.E. and Cahoon, E.B. (2009) Soybean oil: genetic approaches for modification of functionality and total oil content. *Plant Physiol.* **151**, 1030–1040.

Damude, H.G. and Kinney, A.J. (2008) Engineering oilseeds to produce nutritional fatty acids. *Physiol. Plant.* **132**, 1–10.

Duffield, J., Shapouri, H., Graboski, M., McCormick, R. and Wilson, R. (1998) *U.S. Biodiesel Development: New Markets for Conventional and Genetically Modified Agricultural Products*. Agricultural Economic Report No. 770. Economic Research Service/USDA, Washington DC.

Fernández-Moya, V., Martínez-Force, E. and Garcés, R. (2002) Temperature effect on a high stearic acid sunflower mutant. *Phytochemistry*, **59**, 33–37.

Gaj, T., Gersbach, C.A. and Barbas, C.F. III. (2013) ZFN, TALEN, and CRISPR/CAS-based methods for genome engineering. *Trends Biotechnol.* **31**, 397–405.

Graef, G., LaVallee, B.J., Tenopir, P., Tat, M.E., Schweiger, B.J., Kinney, A.J., Van Gerpen, J. and Clemente, T.E. (2009) A high oleic acid and low palmitic acid soybean: agronomic performance and evaluation as a feedstock for biodiesel. *Plant Biotechnol. J.* **7**, 411–421.

Hajdukiewicz, P., Svab, Z. and Maliga, P. (1994) The small, versatile *pPZP* family of *Agrobacterium* binary vectors for plant transformation. *Plant Mol. Biol.* **25**, 989–994.

Hawkins, D.J. and Kridl, J.C. (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.

Hood, E.E., Helmer, G.L., Fraley, R.T. and Chilton, M.-D. (1986) The hypervirulence of *Agrobacterium tumefaciens* A281 is encoded in a region of pTiBo542 outside of T-DNA. *J. Bacteriol.* **168**, 1291–1301.

Kellens, M., Gibon, V., Hendrix, M. and De Greyt, W. (2007) Palm oil fractionation. *Eur. J. Lipid Sci. Technol.* **109**, 336–349.

Kinney, A.J. (2003) Engineering soybeans for food and health. *AgBioForum*, **6**, 1–5.

Kinney, A.J. and Clemente, T.E. (2011) Soybean. In *Energy Crops* (Halford, N.G. and Karp, A., eds), pp. 148–164. Cambridge, UK: Royal Society of Chemistry.

Kinney, A.J. and Knowlton, S. (1997) Designer oils: the high oleic soybean. In *Genetic Engineering for Food Industry: A Strategy for Food Quality Improvement* (Harander, S. and Roller, S., eds), pp. 193–213. London: Blackie Academic.

Knothe, G. (2005) Dependence of biodiesel fuel properties on the structure of fatty acid alkyl esters. *Fuel Process. Technol.* **86**, 1059–1070.

Kris-Etherton, P.M., Griel, A.E., Psota, T.L., Gebauer, S.K., Zhang, J. and Etherton, T.D. (2005) Dietary stearic acid and risk of cardiovascular disease: intake, sources, digestion, and adsorption. *Lipids*, **40**, 1193–2001.

Liu, Q., Singh, S. and Green, A. (2002) High-oleic and high-stearic cottonseed oils: nutritionally improved cooking oils developed using gene silencing. *J. Am. Coll. Nutr.* **21**, 205S–211S.

Lu, C., Napier, J.A., Clemente, T.E. and Cahoon, E.B. (2010) New frontiers in oilseed biotechnology: meeting the global demand for vegetable oils for food, feed, biofuel, and industrial applications. *Curr. Opin. Biotechnol.* **22**, 1–8.

McBlain, B.A., Fioritto, R.J., St. Martin, S.K., Calip-Dubois, A.J., Schmitthenner, A.F., Cooper, R.L., Martin, R.J. (1993) Registration of 'Thorne' soybean. *Crop Sci.* **33**, 1406.

Merlo, A.O., Cowen, N., Delate, T., Edington, B., Folkerts, O., Hopkins, N., Lemeiux, C., Skokut, T., Smith, K., Woosley, A., Yang, Y., Young, S. and Zwick, M. (1998) Ribozymes targeted to stearoyl-ACP delta 9 desaturase mRNA produce heritable increases of stearic acid in transgenic maize leaves. *Plant Cell*, **10**, 1603–1621.

Minihane, A.M. and Harland, J.I. (2007) Impact of oil used by the frying industry on population fat intake. *Crit. Rev. Food Sci. Nutr.* **47**, 287–297.

Neff, W.E. and List, G.R. (1999) Oxidative stability of natural and randomized high-palmitic-and high-stearic-acid oils from genetically modified soybean varieties. *J. Am. Oil Chem. Soc.* **76**, 825–831.

Pérez-Vich, B., Leon, A.J., Grondona, M., Velasco, L. and Fernández-Martinez, J.M. (2006) Molecular analysis of the high stearic acid content in sunflower mutant CAS-14. *Theor. Appl. Genet.* **112**, 867–875.

Pham, A.-T., Lee, J.-D., Shannon, J.G. and Bilyeu, K.D. (2010) Mutant alleles of FAD2-1A and FAD2-1B combine to produce soybeans with high oleic acid seed oil trait. *BMC Plant Biol.* **10**, 195–208.

Pham, A.-T., Shannon, J.D. and Bilyeu, K.D. (2012) Combinations of mutant FAD2 and FAD3 genes to produce high oleic acid and low linolenic acid soybean oil. *Theor. Appl. Genet.* **125**, 503–515.

Rahman, S.M., Takagi, Y., Miyamoto, K. and Kawakita, T. (1995) High stearic acid soybean mutant induced by X-ray irradiation. *Biosci. Biotech. Biochem.* **59**, 922–923.

Salas, J.J., Youssar, L., Martinez-Force, E. and Garcés, R. (2008) The biochemical characterization of a high-stearic acid sunflower mutant reveals the coordinated regulation of stearoyl-acyl carrier protein desaturases. *Plant Physiol. Biochem.* **46**, 109–116.

Sato, S., Xing, A., Ye, X., Schweiger, B., Kinney, A., Graef, G. and Clemente, T. (2004) Production of γ-linolenic acid and stearidonic acid in seeds of marker-free transgenic soybean. *Crop Sci.* **44**, 646–652.

Scherder, C.W. and Fehr, W.R. (2008) Agronomic and seed characteristics of soybean lines with increased oleate content. *Crop Sci.* **48**, 1755–1758.

Schwab, U.S., Maliranta, H.M., Sarkkinen, E.S., Savolainen, M.J., Kesäniemi, Y.A. and Uusitupa, M.I.J. (1996) Different effects of palmitic and stearic acid-enriched diets on serum lipids and lipoproteins and plasma cholesteryl ester transfer protein activity in healthy young women. *Metabolism*, **45**, 143–149.

Spencer, M.M., Pantalone, V.R., Meyer, E.J., Landau-Ellis, D. and Hyten, D.L. Jr. (2003) Mapping the fas locus controlling stearic acid content in soybean. *Theor. Appl. Genet.* **106**, 615–619.

Stanley, J.C. (2009) Stearic acid or palmitic acid as a substitute for *trans* fatty acids? *Lipid Technol.* **21**, 195–198.

Thompson, C.J., Movva, N.R., Tizard, R., Crameri, R., Davies, J.E., Lauwereys, M. and Botterman, J. (1987) Characterization of the herbicide-resistance gene *bar* from *Streptomyces hygroscopicus*. *EMBO*, **6**, 2519–2523.

Voytas, D.F. (2013) Plant genome engineering with sequence-specific nucleases. *Annu. Rev. Plant Biol.* **64**, 327–350.

Xing, A., Zhang, Z., Sato, S., Staswick, P. and Clemente, T. (2000) The use of the two T-DNA binary system to derive marker-free transgenic soybeans. *In Vitro Cell Dev. Biol.* **36**, 456–463.

Zhang, Z., Xing, A., Staswick, P.E. and Clemente, T.E. (1999) The use of glufosinate as a selective agent in *Agrobacterium*-mediated transformation of soybean. *Plant Cell, Tissue Organ Cult.* **56**, 37–46.

Zhang, P., Burton, J.W., Upchurch, R.G., Whittle, E., Shanklin, J. and Dewey, R.E. (2008) Mutations in a Δ9-stearoyl-ACP-desaturase gene are associated with enhanced stearic acid levels in soybean seeds. *Crop Sci.* **48**, 2305–2313.

Supporting information

Additional Supporting information may be found in the online version of this article:

Figure S1 Southern blot analysis on parental and F_1 gene-stack soybean events.

Figure S2 Melting variation across the various oils with modified fatty acid profiles.

Table S1 Segregation analysis on soybean events derived from pPTN811.

Table S2 Fatty acid profile in F_2 seed of gene stacks.

Table S3 Fatty acid profile of soybean events under field conditions 2010.

Table S4 Agronomic parameters measured in 2011 field trial.

