12-12-2008

A high-oleic-acid and low-palmitic-acid soybean: agronomic performance and evaluation as a feedstock for biodiesel

George L. Graef

Bradley J. LaVallee

Patrick Tenopir

Mustafa Tat

Bruce Schweiger

See next page for additional authors

Authors

George L. Graef, Bradley J. LaVallee, Patrick Tenopir, Mustafa Tat, Bruce Schweiger, Anthony J. Kinney, Jon H. Van Gerpen, and Thomas E. Clemente

Plant Biotechnology Journal

Plant Biotechnology Journal (2009) **7**, pp. 411–421 doi: 10.1111/j.1467-7652.2009.00408.x

A high-oleic-acid and low-palmitic-acid soybean: agronomic performance and evaluation as a feedstock for biodiesel

George Graef[1], Bradley J. LaVallee[2], Patrick Tenopir[2], Mustafa Tat[3], Bruce Schweiger[4], Anthony J. Kinney[4], Jon H. Van Gerpen[5] and Tom E. Clemente[1,2,6,*]

[1]*Department of Agronomy & Horticulture, University of Nebraska-Lincoln, Lincoln, NE 68583, USA*
[2]*Center for Biotechnology, University of Nebraska-Lincoln, Lincoln, NE 68588, USA*
[3]*Current address: Department of Mechanical Engineering, Eskisehir Osmangazi University, Meselik, 26480 Eskisehir, Turkiye*
[4]*Crop Genetics Research, DuPont Experimental Station, Wilmington, DE 19880, USA*
[5]*Department of Biological and Agricultural Engineering, University of Idaho, Moscow, ID 83844, USA*
[6]*Center for Plant Science Innovation, University of Nebraska-Lincoln, Lincoln, NE 68588, USA*

Received 25 October 2008;
revised 2 December 2008;
accepted 12 December 2008.
*Correspondence (fax 402-472-3139;
e-mail tclemente1@unl.edu)

Keywords: cold flow, *Glycine max*, oxidative stability, vegetable oil, yield.

Summary

Phenotypic characterization of soybean event 335-13, which possesses oil with an increased oleic acid content (> 85%) and reduced palmitic acid content (< 5%), was conducted across multiple environments during 2004 and 2005. Under these conditions, the stability of the novel fatty acid profile of the oil was not influenced by environment. Importantly, the novel soybean event 335-13 was not compromised in yield in both irrigated and non-irrigated production schemes. Moreover, seed characteristics, including total oil and protein, as well as amino acid profile, were not altered as a result of the large shift in the fatty acid profile. The novel oil trait was inherited in a simple Mendelian fashion. The event 335-13 was also evaluated as a feedstock for biodiesel. Extruded oil from event 335-13 produced a biodiesel with improved cold flow and enhanced oxidative stability, two critical fuel parameters that can limit the utility of this renewable transportation fuel.

Introduction

Acreages planted to soybean in the USA over the last 8 years averaged more than 70 million, which translates into an annual supply of roughly 8.6 million metric tonnes of vegetable oil for use in food or industrial applications (http://www.nass.usda.gov). This volume accounts for over 50% of the world's supply of vegetable oil. Less than 4% of this renewable oil supply is used for industrial applications, with the major industrial product from soybean oil being biodiesel. Biodiesel is commonly generated by transesterification of the oil using ethanol or methanol in the presence of a catalyst. Biodiesel can be used in neat form (100%) or blended with petroleum diesel, and can be employed in diesel engines without modification to the engine design.

Renewable sources of transportation fuels have attracted much attention over the last few years and, although other feedstocks are available for biodiesel, expanding the use of a commodity crop must be evaluated in terms of the environmental impacts of the cropping system. For example, the increase in production of palm oil has come at the expense of precious forest land and potential extinction of species (Schmidt, 2007). However, soybean as a feedstock for biodiesel possesses one significant attribute: as it is a legume, it does not require nitrogen fertilizer. This, in turn, drastically improves its net energy balance, with an estimated 93% more energy in the derived biodiesel than required for its production (Hill *et al.*, 2006).

Soybean seed is approximately 18% total oil, on a 13% moisture basis, with a fatty acid profile primarily composed of 13% palmitic acid (16:0), 4% stearic acid (18:0), 18% oleic acid (18:1), 55% linoleic acid (18:2) and 10% linolenic acid (18:3). Because of this high proportion of polyunsaturated fatty acids, soybean oil is oxidatively unstable and an oxidized

biofuel can compromise engine performance (Canakci *et al*., 1999). Although enhancing the oxidative stability of oils for food applications has been addressed historically through partial hydrogenation, this approach to the enhancement of oxidative stability will have a negative impact on the cold flow properties of biodiesel. To maximize the fuel characteristics of a biodiesel, Duffield *et al*. (1998) suggested the development of an oil high in oleic acid and low in saturated fatty acids, thereby simultaneously improving oxidative stability whilst augmenting cold flow.

In the developing soybean, oleic acid is converted to linoleic acid in a single desaturation step. This reaction is catalysed by a Δ12 desaturase enzyme encoded by the *FAD2* gene (Heppard *et al*., 1996). There are two *FAD2* gene families in soybean, designated as *FAD2-1* and *FAD2-2*; the former is primarily expressed during embryogenesis, and the latter is constitutive. In addition, at least two different *FAD2-1* genes are present in the soybean genome, designated as *FAD2-1a* and *FAD2-1b* (Tang *et al*., 2005). Significant efforts have been made to introgress an elevated oleic acid phenotype into elite soybean varieties through conventional breeding by exploiting variation in oleic acid levels in soybean germplasm (Takagi and Rahman, 1996; Rahman *et al*., 2001; Alt *et al*., 2005a). Although this approach has led to some success in developing mid-oleic-acid genotypes (approximately 30%–70% oleic acid), it has certain significant drawbacks. First, the novel trait is influenced by the environment, requiring growth in warmer climates for stability of the mid-oleic-acid phenotype. This is mainly caused by the temperature effect on Δ12 desaturase activity (Heppard *et al*., 1996; Tang *et al*., 2005). Second, the novel fatty acid profile in the mid-oleic-acid germplasm tends to be linked to a reduced yield (Primomo *et al*., 2002a). In addition, multiple genetic loci are associated with the mid-oleic-acid phenotype in soybean (Alt *et al*., 2005a,b; Oliva *et al*., 2006), which can complicate breeding.

In soybean, seed palmitic acid content is influenced by the expression of the *FatB* gene (Kinney, 1997), a palmitoyl thioesterase. Low-palmitic-acid soybean genotypes have been reported previously (Bubeck *et al*., 1989; Primomo *et al*., 2002b), and the novel oil phenotype has recently been shown to be associated with mutations in alleles of *FatB* (Cardinal *et al*., 2007). Although variation in palmitic acid content in the low-palmitic-acid soybean lines is influenced by temperature, the environmental impact is less severe than that observed for oleic acid content in soybean (Rebetzke *et al*., 2001). However, similar to the mid-oleic-acid soybean genotypes, reduced palmitic acid lines of soybean have been reported to suffer a decrease in yield (Rebetzke *et al*., 1998).

The implementation of the tools of biotechnology to directly target perturbation of *FAD2-1* expression in soybean has been shown to produce a high-oleic-acid phenotype that is stable across environments (Kinney and Knowlton, 1997; Mazur *et al*., 1999). Buhr *et al*. (2002) described the development of transgenic soybean events in which the expression of *FAD2-1* and *FatB* was simultaneously down-regulated in a seed-specific fashion, thereby generating soybean oil with a reduced level of palmitic acid content (< 5%) and significantly increased oleic acid content (> 85%). This phenotype matches that suggested by Duffield *et al*. (1998) as ideal for a biodiesel source.

In this article, we report yield and agronomic performance data collected from field trials conducted with soybeans containing this unique trait, and the utility of the new oil as a renewable source for biodiesel.

Results

Selection of event 335-13 for characterization

Four transgenic events, designated 294-5, 325-61, 333-7 and 335-13, that harbour genetic elements designed to either down-regulate *FAD2-1* alone (event 294-5) or simultaneously down-regulate *FAD2-1* and *FatB* (events 325-61, 333-7 and 335-13) have been described previously (Buhr *et al*., 2002). Monitoring of the fatty acid profile over generations was initially conducted under glasshouse conditions to select homozygous lines from each event prior to field trials. Each event displayed a high-oleic-acid phenotype under glasshouse conditions, and subsequent field trials were initiated in 2002 in Nebraska at two locations. Fatty acid profiles were ascertained from a minimum of 10 random samples per event from the field harvest (Table 1). Event 335-13 displayed the least variation in both oleic acid and palmitic acid under field conditions, and was selected for further characterization.

A Southern blot analysis on event 335-13 (Figure 1) was conducted to determine the complexity of the transgenic locus. Total genomic DNA was restriction digested with either *Hin*d*III* or *Sst*I. The former enzyme is expected to release ~2.5-kb and 1.7-kb hybridizing fragments when probed with the *FAD2-1* and *bar* open reading frames (ORFs), respectively. There is a single *Sst*I site located in the centre of the T-DNA element of the binary vector pPTN303, which is harboured in event 335-13 (Buhr *et al*., 2002). Hence, probing with *FAD2-1* and *bar* ORFs will hybridize to junction fragments about the right and left border elements, respectively. Hybridizing signals with the *Sst*I digestion are expected to be

Table 1 Fatty acid profiles of events 294-5, 325-61, 333-7 and 335-13

Event	Location	Palmitic acid (%)	Stearic acid (%)	Oleic acid (%)	Linoleic acid (%)	Linolenic acid (%)
294-5	GH	7.5 ± 0.4	3.5 ± 0.2	83.8 ± 1.1	1.3 ± 0.5	3.0 ± 0.4
294-5	NE	10.3 ± 1.7	3.1 ± 0.3	51.8 ± 18.9	24.2 ± 16.0	10.6 ± 1.9
325-61	GH	2.4 ± 0.2	2.6 ± 0.4	90.7 ± 0.8	0.9 ± 0.1	2.5 ± 0.3
325-61	NE	5.4 ± 1.1	3.7 ± 0.4	72.4 ± 11.2	11.1 ± 8.8	7.3 ± 1.4
333-7	GH	4.9 ± 1.1	3.0 ± 0.2	78.5 ± 7.l	7.4 ± 5.2	5.9 ± 1.2
333-7	NE	4.7 ± 1.5	2.6 ± 0.2	82.2 ± 7.2	4.2 ± 4.2	6.3 ± 1.7
335-13	GH	2.6 ± 0.2	3.1 ± 0.2	88.0 ± 1.4	1.8 ± 05	4.4 ± 0.8
335-13	NE	3.8 ± 0.3	2.7 ± 0.2	87.1 ± 1.4	2.3 ± 0.5	41 ± 0.6

Location column shows seed collected from either glasshouse (GH) or field environments (NE). Numbers within the respective fatty acid columns indicate the mean percentage of the corresponding fatty acid ± standard deviation.

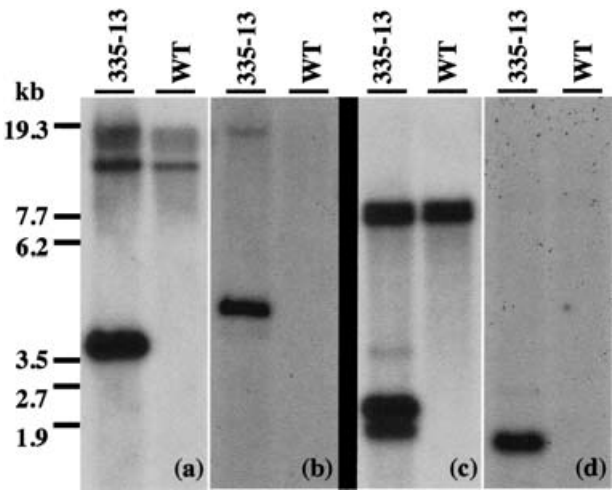

Figure 1 Southern blot analysis on event 335-13. WT, wild-type. (a) Total genomic DNA digested with *Sst*I, and probed with *Fad2-1* open reading frame (ORF). (b) Membrane from (a) stripped and reprobed with *bar* ORF. (c) Total genomic DNA digested with *Hin*dIII and probed with *Fad2-1* ORF. (d) Membrane from (c) stripped and reprobed with *bar* ORF.

greater than 3.4 kb and 2.2 kb, with the *FAD2-1* and *bar* ORF probes, respectively (Figure 1).

Inheritance of the foreign allele in event 335-13 was monitored over generations to identify homozygous lines. The segregation pattern observed in the derived siblings was that of a single dominant functional locus displaying a 3 : 1 goodness of fit (data not shown), which is consistent with the integration pattern observed in the Southern blot analysis (Figure 1).

Agronomic performance of event 335-13

During 2004 and 2005, event 335-13 was grown in one location in Juana Diaz, Puerto Rico and at two locations in Nebraska. The Puerto Rico and Nebraska sites allowed for monitoring of the effect of environment on the novel fatty acid profile, whereas the two Nebraska sites were used to study the agronomic performance of the event.

Table 2 Fatty acid profile observed from event 335-13 across two environments

Location	16:0	18:0	18:1	18:2	18:3
PR2004	4.1 ± 0.5	2.3 ± 0.2	86.4 ± 2.5	2.7 ± 1.7	3.5 ± 0.4
NE2004	3.9 ± 0.7	2.4 ± 0.2	85.5 ± 2.7	2.9 ± 1.5	4.2 ± 0.9
PR2005	3.8 ± 0.3	1.7 ± 0.1	87.9 ± 1.5	2.6 ± 1.3	2.8 ± 0.2
NE2005*	4.3	2.9	85.2	3.2	3.1

Location column shows site from which seeds were harvested. PR2004 and PR2005 denote Puerto Rico harvests, whereas NE2004 and NE2005 denote Nebraska harvests.

*Nebraska 2005 analysis (NE2005) data reflect fatty acid analysis conducted on approximately 12 bushels of extruded oil from harvested plots.

Soybean plantings were harvested in March 2004 and April 2005 from the Puerto Rico sites and during the second week of October for the Nebraska plantings in 2004 and 2005. The dataset reveals that the stability of the novel fatty acid profile is not influenced by harvest location. The percentage of oleic acid averaged between 85.2% and 85.5% in the Puerto Rico environments, and 86.4% and 87.9% in the Nebraska environments (Table 2). The percentage of palmitic acid in commodity soybean oil is approximately 13%, and down-regulation of FAD2-1 alone will result in a slight decrease in saturates, primarily palmitic acid (Buhr *et al*., 2002). What differentiates events down-regulated in FAD2-1 alone from events simultaneously down-regulated in both FAD2-1 and FatB, at the phenotypic level, is the greater decrease in palmitic acid, resulting in a greater increase in the percentage of oleic acid (Table 1).

Table 3 Means are over eight environments, four replications per environment. Means for fatty acid composition are from two replications per environment over five environments. The least significant difference (LSD) is for comparison among 9 means at $\alpha = 0.05$

Line	Yield (kg/ha)	Maturity (days)	Lodging score	Plant height (cm)	Seed weight (g/100)	Protein (% DM)	Oil (% DM)	Palmitic (% of oil)	Stearic (% of oil)	Oleic (% of oil)	Linolenic (% of oil)	Linolenic (% of oil)
335-13-51-6	3554	35	1.4	87	15.2	43.0	19.1	3.4	2.5	86.1	1.6	3.6
335-13-51-9	3531	35	1 5	88	15.4	42.7	19.2	3.1	2.5	87.0	1.2	3.2
335-13 63 13	3505	34	1.5	88	15.5	43.1	19.1	3.1	2.4	87.0	1.3	3.2
335-13-63-8	3508	35	1.3	88	15.5	43.0	19.1	3.2	*2.5*	86.3	1.3	3.3
A3237	3521	35	1.3	91	15.3	41.4	19.0	12.0	3.4	20.8	52.0	9.5
NE2801	3842	29	1.3	76	16.7	40.6	18.9	12.7	3.5	21.2	49.2	11.2
NE3001	4120	29	1.3	72	18.5	40.0	19.2	12.1	3.4	21.7	49.3	10.3
RMLPC1-311-128	3481	31	1.8	85	15.8	38.4	20.6	11.8	3.3	19.8	52.8	9.5
U98-307917	4156	32	1.5	89	16.5	40.7	19.1	13.0	3.7	21.5	49.8	9.9
LSD ($\alpha = 0.05$)	299	3	0.4	6	0.9	0.6	0.3	0.3	0.3	2.3	2.4	0.6

Attempts to use existing genetic variation and induced mutations in soybean to increase oleic acid levels in seed oil have proved to be difficult. In addition, the mid-oleic-acid phenotype is not stable over environments as a result of genetic effects and effects of temperature during seed development (Alt *et al*., 2005a; Oliva *et al*., 2006; Byfield and Upchurch, 2007). Even in the mid-oleic-acid parental line M23, which has a deletion in the Fad2-1 gene, oleic acid levels in seeds produced in Puerto Rico environments were significantly higher than those in seeds of the same line produced in Iowa (Alt *et al*., 2005a). In contrast, the oleic acid level in the seed oil from event 335-13 remained between 85% and 88% in the two Puerto Rico environments and two Nebraska environments (Table 2). The oil from event 335-13 also contains less than 7% saturated fatty acids (16:0 + 18:0), which would provide health benefits, given that the Food and Drug Administration defines a low-saturated-fat food as one that contains less than 1 g of saturated fat per serving (http://www.fda.gov) and the typical serving size for soybean oil is 14 g (http://www.thumboilseed.com/soy-oil.htm). Therefore, the maximum percentage saturates allowed in refined soybean oil to qualify as a low saturate is 7% (equivalent to 0.98 g per serving). Under these guidelines, soybean oil derived from event 335-13 would be classified as a low-saturate oil.

The fatty acid profile was also evaluated for all entries from five Nebraska yield test environments (Table 3). The combined analysis of variance indicated significant differences among the environments for palmitic, linoleic and linolenic acids for the wild-type (WT) lines, and for palmitic, stearic and oleic acids for the homozygous lines derived from event 335-13 (data not shown). The WT lines differed for palmitic, linoleic and linolenic acid content, whereas no differences in fatty acid composition were detected among the four homozygous lines from event 335-13. No significant line × environment interactions were observed for the WT lines or for the four homozygous 335-13 event-derived lines (data not shown). The mean oleic acid composition of the four high-oleic-acid lines derived from event 335-13 averaged from 84.6% in the LD04 environment to 88.4% in the MI05 environment (Figure 2). The high-oleic-acid phenotype was stable across the Nebraska environments, and averaged more than four times the oleic acid content of 20.8% observed for the A3237 WT parental line (Figure 2).

Another consideration in the development of transgenic crops is that their performance should be equal to that of non-transformed material, except for the desired modified trait(s). Over eight Nebraska environments, the four T_1-derived lines from event 335-13 did not differ significantly from parental line A3237 in terms of yield (Figure 3). The high-oleic-acid lines and A3237 also did not differ in maturity, lodging, plant height or seed weight averaged over the eight environments (Table 3). The data indicate that the novel fatty acid profile in event 335-13 is stable across environments, and that the agronomic performance of lines with a high oleic acid content is not adversely affected.

Total oil and protein were determined by near-infrared (NIR) analysis of seed samples taken from each plot in the eight Nebraska environments. Total protein levels, based on NIR analysis, were significantly enhanced in the high-oleic-acid lines, without a concomitant decrease in total oil content (Table 3). To further investigate this unexpected result, seed samples from a random subset of progeny derived from a cross between event 335-13 and RMLPC1-311-128 (designated as population UX1625) were taken for analysis. The plots that were sampled were from replicated yield tests

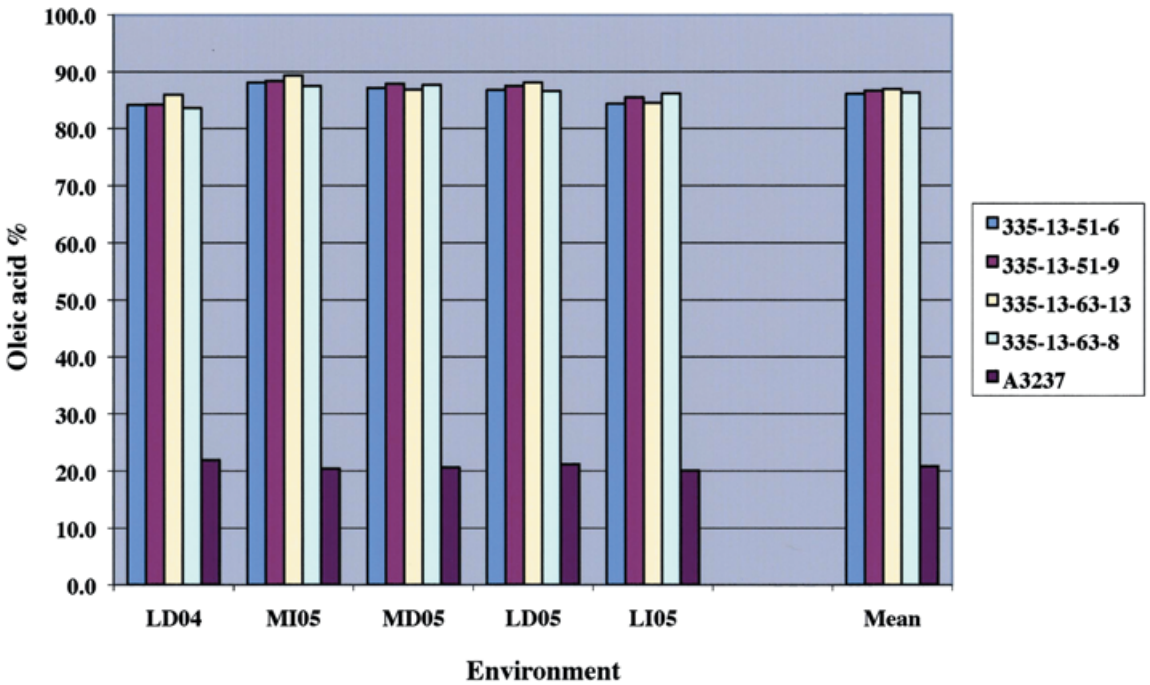

Figure 2 Oleic acid percentage of four T_1-derived soybean lines from event 335-13 and the wild-type parental line A3237. Environmental designations: D, non-irrigated; I, irrigated; L, Lincoln, NE; M, Mead, NE; 04, 2004; 05, 2005.

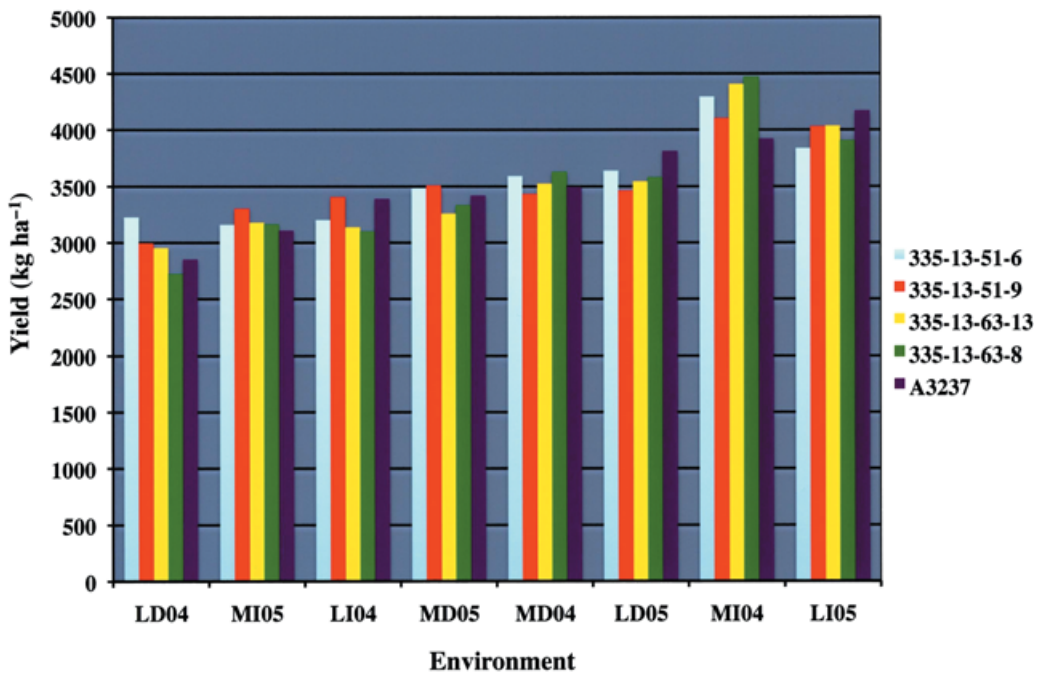

Figure 3 Yield of four homozygous T_1-derived lines from event 335-13 and the wild-type parental line A3237. Environments are ordered from lowest to highest average yield for A3237. Environmental designations: D, non-irrigated; I, irrigated; L, Lincoln, NE; M, Mead, NE; 04, 2004; 05, 2005.

conducted during 2006. A comparison of the results for seed protein and oil composition from proximate analysis and indirect measurement using NIR was conducted using the same seed sample for each analysis. The data collected from this study comparing total oil/protein as determined by wet-bench proximate analysis vs. the rapid NIR readings indicated that the NIR estimate of total oil, in general, mirrored that observed for the wet-bench approach. However, NIR tended to artificially elevate total protein levels of the high-oleic-acid seed samples (data not shown). Hence,

Table 4 Properties of the respective test fuels

Property	Soybean methyl esters	HO soybean methyl esters	Canola methyl esters	HO soybean isopropyl esters	No. 2 diesel
Lower heating value (Btu/lb)	16 100	16 208	16 188	18 267	18 518
Carbon (%)	77.2	77.0	77.3	79.6	86.8
Hydrogen (%)	11.9	12.2	12.0	13.1	12.8
Oxygen (%)	11.3	11.1	11.1	7.3	N/A
Cetane number	47.2	51.5	49.4	57.1	44.0
Specific gravity	0.883	0.881	0.879	0.870	0.846
Cloud point (°C)	–1	–5	–2	–10	–6
Pour point (°C)	0	–9	–9	–18	–9
Total glycerol (% wt.)	0.097	0.068	0.004	0.01	N/A
Kinematic viscosity (cSt at 40 °C)	4.012	4.780	4.783	5.907	2.686

Property column indicates the physical parameter measured. Test fuels include conventional soybean biodiesel ('Soybean methyl esters' column), high-oleic-acid biodiesel ('HO soybean methyl esters' column), canola biodiesel ('Canola methyl esters' column), high-oleic-acid isopropyl esters ('HO soybean isopropyl esters' column) and No. 2 diesel. N/A, not applicable.

although the systematic scan with NIR to monitor total oil/protein from the yield plots planted in 2004 and 2005 indicated a significant increase in total protein in seed, with a drastic shift in the fatty acid profile towards oleic acid (Table 3), this was probably not caused by the perturbation of seed metabolism, but rather a misread from the NIR machine calibrated to meet the specifications for standard commodity soybean.

Total amino acid profiles were determined from random samples taken from the 2004 and 2005 harvests of the parental genotype A3237, plantings of event 335-13 from 2004, 2005 and field trials conducted in 2006, together with genotype NE3001 and F_4-derived lines from the UX1625 cross planted in 2006 under field conditions in Nebraska. The mean percentage, based on a 100-g sample, of 18 amino acids determined from the respective soybean seed samples was ascertained. The data revealed no significant variation in the levels of amino acids across all samples assayed (data not shown), demonstrating that the modulation of the fatty acid oil profile, triggered by the down-regulation of the fatty acid biosynthetic genes *Fatb* and *FAD2-1*, did not influence the amino acid composition of the soybean meal.

Biodiesel derived from oil high in oleic acid and low in palmitic acid

In 2004, a bulk planting of event 335-13 was conducted to secure sufficient material to allow for testing of a biodiesel derived from the novel oil. The oil was extruded from two separate batches totalling over 350 bushels. Both methyl and isopropyl esters were prepared from the high-oleic-acid soybean event 335-13 and compared with standard soybean- and canola-derived biodiesels, together with a commercial grade No. 2 petroleum-based diesel. Studies were conducted with a John Deere 4045T engine coupled to a General Electric DC dynamometer (TLC 2524) (Tat *et al.*, 2007b), a 1994 Dodge Ram 2500 with a 5.9-L Cummins diesel engine (B series) tested on an SF 602 Superflow Chassis Dyno or a John Deere 3150 tractor attached to a PTO dynamometer.

A comparison of the fatty acid profiles of the respective feedstocks is shown in Figure 4a, and the corresponding fuel properties are listed in Table 4. Methyl esters derived from the high-oleic-acid soybean displayed improved cold flow properties, with both cloud and pour points drastically reduced when compared with the corresponding methyl esters derived from commodity soybean (Table 4). The cold flow parameters of methyl esters derived from canola oil were more closely aligned with those of the high-oleic-acid soybean oil biodiesel than those of the commodity soybean oil biodiesel. These data reflect the impact of the fatty acid profile on these two parameters, namely both the high-oleic-acid, low-palmitic-acid oil produced from soybean event 335-13 and the elevated oleic acid canola oil showed a reduced saturated fatty acid content when compared with commodity soybean oil (Figure 4a).

Brake-specific NO_x ($BSNO_x$) emissions for the methyl esters derived from canola, soybean event 335-13 and commodity soybean oil were monitored in a test with the Cummins 5.9-L engine over various load conditions. A reduction in $BSNO_x$ was observed at low load across the biodiesel fuels tested, when compared with petroleum diesel, whereas, at full load, the two conventional biodiesel fuels, soybean and canola, exhibited a higher $BSNO_x$ than that emitted from the

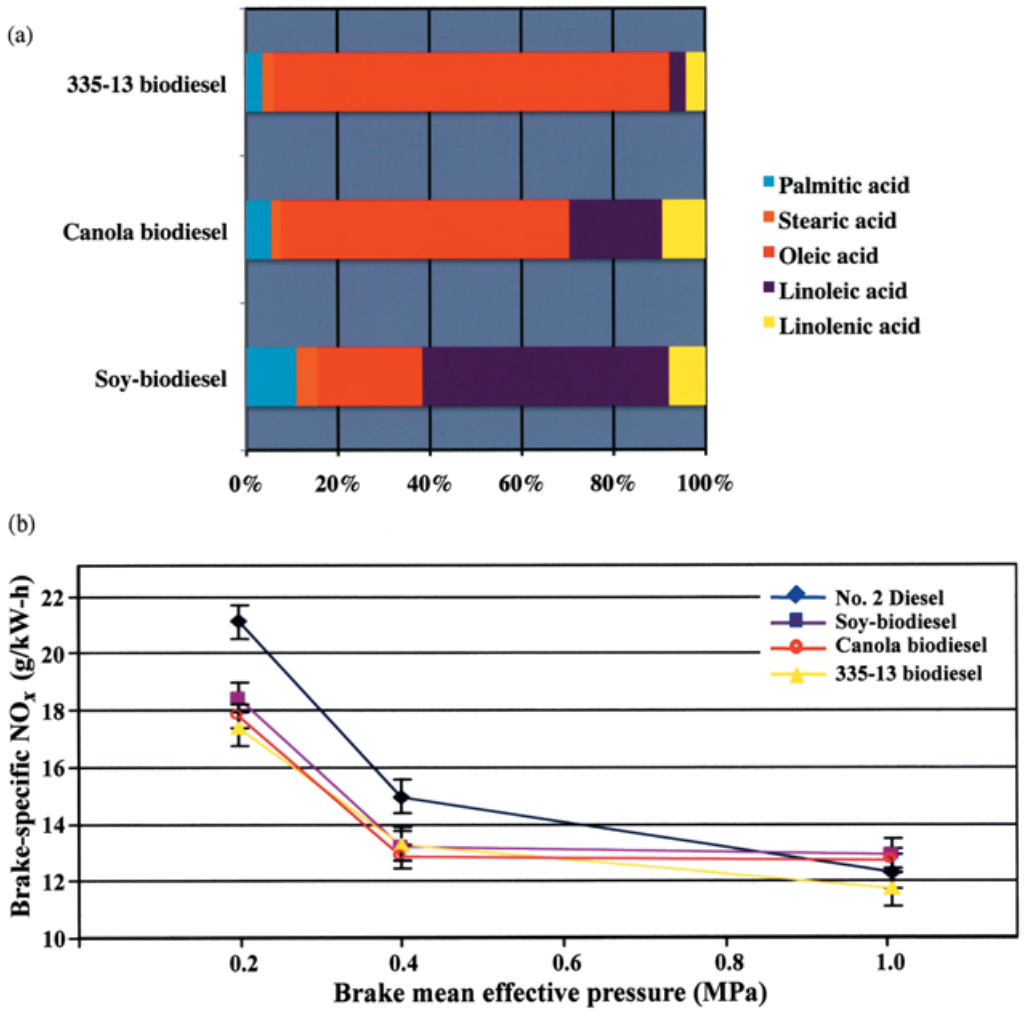

Figure 4 Fatty acid profiles of the respective test biofuels and brake-specific NO_x emissions across different engine loads. (a) Percentage of the respective fatty acids in the test biofuels. (b) Brake-specific NO_x emissions across different engine loads. Brake-specific NO_x emissions (g/kW-h) measured across three engine loads in the Cummins engine set-up fuelled with the various biodiesels and No. 2 diesel.

petroleum-based diesel, and the 335-13-derived fuel displayed the lowest $BSNO_x$ (Figure 4b).

Monitoring of $BSNO_x$ emission using the John Deere 3150 set-up was conducted with fuels derived from soybean event 335-13 and canola oils, together with No. 2 diesel. With this engine set-up at full load, the increases in $BSNO_x$ over No. 2 diesel were 15.2% and 21.1% for the high-oleic-acid soybean- and canola-derived biodiesel fuels, respectively.

Isopropyl esters prepared from oil derived from event 335-13 resulted in a biodiesel with improved cold flow properties, enhanced lower heating value and increased cetane number (Table 4). However, the increase in cetane number did not translate to a significant decrease in $BSNO_x$ when compared with the high-oleate-derived methyl ester biodiesel on engine runs with the John Deere 3150 set-up, with values of 248.7 and 253.0 g/kW-h with the engine fuelled with isopropyl and methyl esters derived from high-oleate biodiesel, respectively.

Discussion

Improving the shelf-life of soybean oil for food and feed applications historically has been addressed via partial hydrogenation. As a result of the production in this process of *trans*-fatty acids, which have been linked to cardiovascular disease, a genetic approach has been used to develop soybean germplasm low in linolenic acid as a means to augment oxidative stability and thereby circumvent the need for partial hydrogenation. Although these strategies have been valuable for addressing some of the oxidative stability issues faced by oil processors, they do not meet the requirements necessary for optimal biodiesel performance. Moreover, low-linolenic-acid

soybean oil possesses comparable levels of saturated fatty acids, palmitate and stearate, and hence would be expected to have similar cold flow issues to biodiesel derived from standard commodity soybean oil. Employing a partial hydrogenation approach to improve the oxidative stability of biodiesel would lead to reduced cold flow properties, together with poorer viscosity and lubricity (Moser *et al*., 2007). Moreover, high-oleic-acid oils, such as that described here, are far superior in terms of oxidative stability to low-linolenic-acid soybean oil (Erhan *et al*., 2006).

As mentioned above, improvement of the oil characteristics of soybean for food, feed or industrial applications, through either conventional or biotechnological approaches, must take into consideration the potential impact of the novel trait on the agronomics of soybean. The primary value of soybean lies in protein and oil, and these parameters are usually inversely related in soybean, with the former typically increasing by 2% for every 1% decrease in the latter. Any trait that has an impact on the total production of either protein or oil must be of sufficient value to negate the potential decrease in profitability associated with the overall loss in either of these seed components. A high-oleic-acid/low-saturated/low-polyunsaturated fatty acid soybean oil is clearly a valuable trait. However, this value would be significantly decreased if the protein or oil yield per hectare were significantly reduced. We have shown that a soybean event in which fatty acid biosynthetic genes *FatB* and *FAD2-1* are simultaneously down-regulated, resulting in the accumulation of oleic acid and a concomitant decrease in polyunsaturated and saturated fatty acids, is not compromised in agronomic performance, including the overall yield, total protein/oil and amino acid profile. No significant variation was observed between event 335-13 and its parental genotype A3237 for a number of other parameters, including days to maturity, height and lodging (Table 3).

The stability of the fatty acid profile and the agronomic performance of the high-oleic-acid event 335-13 are in stark contrast with the reports by Bachlava *et al*. (2008) and Scherder and Fehr (2008), with both indicating significantly lower yields for soybean lines with increased oleate content. Scherder and Fehr (2008) evaluated 27 mid-oleate (MO) lines, with a mean oleate content of more than 50% of the oil, and 27 conventional oleate (CO) lines, with an average oleate content of less than 28% of the oil, from three soybean populations. The lines were homozygous for the *fan1* (A5), *fan2* and *fan3* alleles, resulting in a linolenic acid content of 1%, and the *ol* allele from M21 for increased oleic acid content. In all three populations studied, the mean yield of the MO lines was significantly lower than the average yield of the CO lines (Scherder and Fehr, 2008). They also observed significant differences between CO and MO groups in all three populations for maturity date and lodging, with the MO lines being earlier in maturity and showing poorer lodging than the CO lines (Scherder and Fehr, 2008). Likewise, Bachlava *et al*. (2008) reported significant negative genotypic and phenotypic correlations between yield and oleate content. In addition, they found significant positive genotypic and phenotypic correlations in two populations between maturity and oleate content, and significant negative correlations between oleate content and oil content in two populations. Significant genotype × environment interactions were also reported for oleic acid content (Bachlava *et al*., 2008). Oliva *et al*. (2006) showed that mid-oleic-acid genotypes were less stable for oleic acid content across environments than genotypes with normal oleic acid content. The data for the high-oleic-acid lines derived from event 335-13 and reported here indicate stable expression of 86%–87% oleic acid content in the oil, with no negative effects on yield, maturity, plant height, lodging, seed weight or oil content (Table 3).

Transgenic event 335-13, a high-oleic-acid, low-saturate soybean oil, not only possesses properties important for food and feed applications, but also meets the criteria highlighted by Duffield *et al*. (1998) for improving the properties of biodiesel, namely improved oxidative stability and cold flow. In addition, biodiesel derived from oil high in oleic acid and low in saturated fatty acids has been shown previously to mitigate NO_x emissions when tested using a John Deere 4045T engine (Tat *et al*., 2007b). The emission tests conducted with the Cummins engine on the three test biodiesel fuels prepared from standard soybean oil, canola oil and event 335-13-derived oil included monitoring of $BSNO_x$ over various engine loads (Figure 4b). At the full load scenario, standard soybean- and canola-derived biodiesel displayed increases in $BSNO_x$ of approximately 5.5% and 3.5%, respectively, relative to No. 2 diesel, whereas 335-13-derived biodiesel emitted 4.7% less $BSNO_x$.

The cetane number is a parameter determining the ignition quality of diesel fuel; moreover, the cetane number and NO_x emissions tend to show a negative correlation (Tat *et al*., 2007a). Knothe *et al*. (2003) reported that isopropyl oleate has a cetane number of 86.6; hence, we explored the impact of isopropyl esters derived from 335-13 oil on the cetane number and NO_x emissions relative to the corresponding methyl esters derived from 335-13 soybean oil. As shown in Table 4, transesterification with isopropanol of the 335-13 soybean oil enhanced the cold flow properties, cloud and pour points, together with an increase in the energy content of the fuel, relative to the methyl esters derived from 335-13

oil. However, although the cetane number of isopropyl esters was determined to be 57.1, compared with a value of 51.5 for methyl esters, no significant variation in NO_x emissions from the John Deere 3150 engine set-up was observed.

The increase in NO_x emissions is often exaggerated when engines are fuelled with 100% biodiesel; however, the NO_x effect is not as pronounced when blends of biodiesel are used (McCormick *et al*., 2006). Monitoring of NO_x emissions from the John Deere 3150 tractor engine fuelled with neat forms of canola- and event 335-13-derived biodiesel at full load revealed 21.1% and 15.2% increases, respectively, in $BSNO_x$ relative to that of No. 2 diesel. The increases in NO_x emissions, relative to No. 2 diesel, observed from the John Deere 3150 engine were higher than those monitored from either the Cummins B5.9 or John Deere 4045T engines fuelled with the various biodiesel-derived feedstocks, suggesting that engine design *per se* has a significant impact on the level of NO_x emissions. Indeed, Sharp *et al*. (2000) reported that the Cummins B5.9 engine produced minimal NO_x variation when fuelled with biodiesel when compared with No. 2 diesel. Hence, although a major attribute of biodiesel, either neat or as a blend, is the observed decrease in emissions, namely hydrocarbons, carbon monoxide and particulate matter, with respect to NO_x there is an observed increase. Although the NO_x effect in engines fuelled with biodiesel is not fully understood, insights into the origin of NO_x release were recently discussed in a communication by Ban-Weiss *et al*. (2007). Nonetheless, the engine tests described herein, and previously reported by Tat *et al*. (2007b), reflect a general mitigation of the NO_x effect of a biodiesel derived from the transesterfication of oil high in oleic acid and low in poly-unsaturated and saturated fatty acids. Therefore, through a combination of engine design, blending and fatty acid modification, the NO_x factor can be effectively negated in engines fuelled with biodiesel.

Clearly, the current soybean oil supply will not meet the market demand for total displacement of diesel fuel. For example, in 2007, the estimated on-highway diesel fuel consumption for the state of Nebraska alone was 420.5 million gallons (http://www.neo.ne.gov/statshtml/37b.html). The 2007 soybean harvest in Nebraska was approximately 190 million bushels (http://www.nass.usda.gov). Given that one bushel of soybean roughly translates into 1.5 gallons of oil, the Nebraska soybean harvest would only be able to displace about 68% of the diesel fuel consumed in the state. For this reason, a stronger argument can be made for the targeted usage of biodiesel blends, not with the intent for immediate displacement of petroleum, but rather to capture the environmental and health attributes associated with the use of biodiesel. For instance, the decrease in gaseous emissions is a strong justification for use in school buses and in the mining industry, where engine power may be required in confined areas. Moreover, the enhanced biodegradation of biodiesel (Zhang *et al*., 1998) should provide encouragement to use blended fuels in the marine industry.

From the perspective of sustainability, it would be unwise to rely on a single feedstock source to meet diesel fuel demand, even if supply were sufficient to meet demand. The uncertainties surrounding the production of an oilseed crop, as a result of environmental and biotic imposed stresses, could have a significant impact on yield, and thus supply, in a very unpredictable manner. Hence, a more strategic approach would be to create multiple oilseed crops in which oil metabolism has been manipulated for uniform fatty acid profile, thereby creating a supply scenario in which a single biofuel production facility could source plant oil derived from multiple crops. This would help to ensure a plentiful, safe and sustainable oil supply to meet the demands for food, feed and fuel.

Experimental procedures

Plant materials

The development of the transgenic soybean events 294-5, 325-61, 333-7 and 335-13 has been reported previously (Buhr *et al*., 2002); the parental genotype of these transgenic events is either A3237 (Asgrow® Seed Company, Dekalb, IL, USA) or Thorne (McBlain *et al*., 1993). Soybean genotypes NE2801, NE3001, RMPLC1-311-128 and U98-307917 were developed at the University of Nebraska's soybean breeding programme.

Field trials with transgenic soybeans

Field testing of transgenic soybean events was carried out in accordance with the United States Department of Agriculture/Animal and Plant Health Inspection Service (USDA/APHIS) guidelines governing the movement and release of regulated soybeans. Field trials in Puerto Rico were conducted in Juana Diaz at the Illinois Crop Improvement Association, and the Nebraska field plantings were conducted at nurseries located on the Lincoln campus of the University of Nebraska and the University's Agricultural Research and Development Center, near Mead. The yield trials carried out from the Nebraska plantings were conducted under both irrigated and non-irrigated environments. Plots consisted of four rows, 3.6 m long, with 0.7-m spacing between rows. The experimental design was a randomized complete block with four replications in each environment. For the high-oleic-acid event 335-13, four homozygous T_2-derived lines, tracing to two different T_1 individuals, were included as entries in the test, together with the A3237 untransformed parent, and yield and seed composition checks NE2801, NE3001, RMLPC1-311-128 and U98-307917. Data were collected from the middle two

rows of each plot for yield, date to maturity, height and lodging, as well as post-harvest seed composition data.

Fatty acid analysis

Fatty acid profiles were ascertained from the initial field study conducted in 2002 in Nebraska, monitoring seed chips derived from events 294-5, 325-61, 333-7 and 335-13, following the procedure described by Butte (1983) (Table 1). To monitor the effect on growth in Puerto Rico vs. Nebraska on the oleic acid content (Table 2), fatty acid analysis was conducted on 219 randomly selected 335-13 seeds from the Puerto Rico harvest in 2004; the samplings from the Nebraska site in 2004 and the Puerto Rico harvest in 2005 were conducted on 11 and 18 random bulk-extracted 335-13 samples, respectively. From the harvest in Nebraska in 2005, fatty acid analysis was conducted on extruded oil derived from approximately 325 kg of seed from event 335-13 (Table 2). To monitor the impact on the fatty acid profile across the Nebraska environments during 2004 and 2005, data were gathered on seed chip samples taken from two of the four replications for all the genotypes in the test plots. The fatty acid methyl esters were monitored by gas chromatography.

Total oil and protein determination

Total oil and protein determination was carried out by NIR spectroscopy using an Infratec (FOSS North America, Inc, Eden Prairie, MN 55344) 1255 whole-grain analyser on soybean plantings in Nebraska from seed samples taken from each plot. To compare the results obtained by wet-bench proximate analysis with NIR determination for total protein and oil, random seed samples taken from field plots planted in 2006 were analysed via NIR and subsequently sent for proximate analysis at the University of Missouri-Columbia's Agricultural Experiment Station Chemical Laboratories. Control, standard fatty acid profile samples were assayed from three U98-307917 plots, three RMPLC1-311-128 plots and 14 NE3001 plots, whereas high-oleic-acid samples were analysed from plots planted with lineages of the RMPLC1-311-128 × 335-13 cross (Ux1625), designated Ux1625-183 (16 samples) and Ux1625-12 (10 samples), together with three samples from the 335-13 parental event.

Amino acid profile determination

Total amino acids were determined from random samples from the 2005 planting in Nebraska. Data were collected from nine plots of Ux1625 cross populations, nine plots of NE3001, three plots of A3237 and six plots of 335-13. Total amino acid profiles were determined at the University of Missouri-Columbia's Agricultural Experiment Station Chemical Laboratories.

Southern blot analysis

Total genomic DNA was isolated from glasshouse-grown material representing the homozygous lineage of 335-13-63-8, together with control soybean DNA, following a modification of the protocol described by Dellaporta *et al*. (1983). Genomic DNA was digested with restriction enzymes *Sst*I and *Hin*dIII and separated on 0.8% agarose gels. The gels were processed and transferred to a nylon membrane (Zeta probe GT; Bio-Rad, Hercules, CA, USA) following standard procedures (Sambrook *et al*., 1989). Membranes were hybridized with *bar* and *FAD2-1* ORF probes. The probes were labelled with [α-^{32}P]-deoxycytidine triphosphate ([α-^{32}P]-dCTP) by random prime synthesis using Stratagene's (Strategene, La Jolla, CA, USA) Prime It II Kit (Cat # 300385), following the manufacturer's protocol. Membranes were hybridized in 0.5 M Na_2HPO_4 (pH 7.2), 7% sodium dodecylsulphate (SDS), 1% bovine serum albumin (BSA) solution at 65 °C overnight. Hybridized membranes were washed twice for 30 min in 5% SDS, 40 mM Na_2HPO_4, followed by a single wash for 30 min in 1% SDS, 40 mM Na_2HPO_4. Washing steps were conducted at 65 °C.

Biodiesel evaluation

Soybean oil extruded from approximately 350 bushels of event 335-13 was transesterified to produce methyl and isopropyl ester biodiesel fuels. Standard commodity soybean oil-derived biodiesel was purchased from Air Energy (Creston, WA, USA), whereas canola oil was purchased at Seattle Biodiesel (Seattle, WA, USA) and methyl esters were prepared at the University of Idaho. The No. 2 diesel was purchased commercially.

Emissions testing was conducted on a 1994 Dodge ram 2500 with a 5.9-L Cummins diesel engine (B series) tested on an SF 602 Superflow Chassis Dyno (Colorado Springs, CO, USA) or a John Deere (John Deere Moline, IL, USA) 3150 tractor attached to a PTO dynamometer. Both engines are inline six cylinder. The brake mean effective pressure (BMEP) of the Cummins engine set-up is 2, 4 and 10 bar at 1800 r.p.m., whereas BMEP of the John Deere 3150 set-up is 0.26 MPa at 1400 r.p.m. and 0.84 MPa at 2200 r.p.m.

Engine emissions are reported on a brake-specific basis (g/kW-h). The measured emissions were carbon monoxide (CO), carbon dioxide (CO_2), unburned hydrocarbons (HC) and nitric oxides (NO_x). All steady state conditions were replicated three times. Datasets were analysed using analysis of variance, with Tukey's means separation ($P = 0.05$).

Acknowledgements

Funding for this project was provided through the Department of Energy award number DEFG36-04GO14245, United Soybean Board, Nebraska Soybean Board and the Nebraska Research Initiative.

References

Alt, J.L., Fehr, W.R., Welke, G.A. and Shannon, J.G. (2005a) Transgressive segregation for oleate content in three soybean populations. *Crop Sci*. **45**, 2005–2007.

Alt, J.L., Fehr, W.R., Welke, G.A. and Sandu, D. (2005b) Phenotypic and molecular analysis of oleate content in the mutant soybean line M23. *Crop Sci*. **45**, 1997–2000.

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.

Ban-Weiss, G.A., Chen, Y.Y., Buchholz, B.A. and Dibble, R.W. (2007) A numerical investigation into the anomalous slight NOx increase when burning biodiesel; a new (old) theory. *Fuel Process. Technol*. **88**, 659–667.

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-regulates seed fatty acid genes in transgenic soybean. *Plant J*. **30**, 155–163.

Butte, W. (1983) Rapid method for the determination of fatty acid profiles from fats and oils using trimethylsulphonium hydroxide for transesterification. *J. Chromatogr*. **261**, 142–145.

Byfield, G.E. and Upchurch, R.G. (2007) Effect of temperature on microsomal omega-3 linoleate desaturase gene expression and linolenic acid content in developing soybean seeds. *Crop Sci*. **47**, 2445–2452.

Canakci, M., Monyem, A. and Van Gerpen, J. (1999) Accelerated oxidation processes in biodiesel. *Trans. Am. Soc. Agric. Eng*. **42**, 1565–1572.

Cardinal, A.J., Burton, J.W., Camacho-Roger, A.M., Yang, J.H., Wilson, R.F. and Dewey, R.E. (2007) Molecular analysis of soybean lines with low palmitic acid content in the seed oil. *Crop Sci*. **47**, 304–310.

Dellaporta, S.L., Wood, J. and Hicks, J.B. (1983) A plant DNA mini-preparation: Version II. *Plant Mol Biol. Rep*. **1**, 19–21.

Duffield, J., Shapouri, H., Graboski, M., McCormick, R. and Wilson, R. (1998) *US Biodiesel Development: New Markets for Conventional and Genetically Modified Agricultural Products*. Washington, DC, USA: Economic Research Service/United States Department of Agriculture (USDA).

Erhan, S.V., Sharma, B.K. and Perez, J.M. (2006) Oxidation and low temperature stability of vegetable oil-based lubricants. *Ind. Crops Prod*. **24**, 292–299.

Heppard, E.P., Kinney, A.J., Stecca, K.L. and Miao, G.H. (1996) Developmental and growth temperature regulation of two different microsomal ω-6 desaturase genes in soybeans. *Plant Physiol*. **110**, 311–319.

Hill, J., Nelson, E., Tilman, D., Polasky, S. and Tiffany, D. (2006) Environmental, economic, and energetic costs and benefits of biodiesel and ethanol biofuels. *Proc. Natl. Acad. Sci. USA*, **103**, 11 206–11 210.

Kinney, A.J. (1997) Genetic engineering of oilseeds for desired traits. In: *Genetic Engineering* (Setlow, J.K., ed.), pp. 149–166. New York, NY: Plenum Press.

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., Matheaus, A.C. and Ryan, T.W.I. (2003) Cetane numbers of branched and straight-chain fatty esters determined in an ignition quality tester. *Fuel*, **82**, 971–975.

Mazur, B., Krebbers, E. and Tingey, S. (1999) Gene discovery and product development for grain quality traits. *Science*, **285**, 372–375.

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

McCormick, R.L., Williams, A., Ireland, J., Brimhall, M. and Hayes, R.R. (2006) Effects of biodiesel blends on vehicle emissions. *Milestone Report NREL/MP-540-40554*. National Renewable Energy Laboratory, Golden, CO, USA.

Moser, B.R., Haas, M.J., Jackson, M.A., Erhan, S.V. and List, G.R. (2007) Evaluation of partially hydrogenated methyl esters of soybean oil as biodiesel. *Eur. J. Lipid Sci. Technol*. **109**, 17–24.

Oliva, M.L., Shannon, J.G., Sleper, D.A., Ellersieck, M.R., Cardinal, A.J., Paris, R.L. and Lee, J.D. (2006) Stability of fatty acid profile in soybean genotypes with modified oil composition. *Crop Sci*. **46**, 2069–2075.

Primomo, V.S., Falk, D.E., Ablett, G.R., Tanner, J.W. and Rajcan, I. (2002a) Genotype × environment interactions, stability, and agronomic performance of soybean with altered fatty acid profiles. *Crop Sci*. **42**, 37–44.

Primomo, V.S., Falk, D.E., Ablett, G.R., Tanner, J.W. and Rajcan, I. (2002b) Inheritance and interaction of low palmitic and low linolenic soybean. *Crop Sci*. **42**, 31–36.

Rahman, S.M., Kinoshita, T., Anai, T. and Takagi, Y. (2001) Combining ability in loci for high oleic and low linolenic acids in soybean. *Crop Sci*. **41**, 26–29.

Rebetzke, G.J., Burton, J.W., Carter, T.E. Jr and Wilson, R.F. (1998) Changes in agronomic and seed characteristics with selection for reduced palmitic acid content in soybean. *Crop Sci*. **38**, 297–302.

Rebetzke, G.J., Pantalone, V.R., Burton, J.W., Carter, T.E. Jr and Wilson, R.F. (2001) Genetic background and environment influence palmitic content of soybean seed oil. *Crop Sci*. **41**, 1731–1736.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor, New York, NY: Cold Spring Harbor Laboratory Press.

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

Schmidt, C.W. (2007) Biodiesel: cultivating alternative fuels. *Environ. Health Perspect*. **115**, A87–A91.

Sharp, C.A., Howell, S.A. and Jobe, J. (2000) The effect of biodiesel fuels on transient emissions from modern diesel engines, Part I. Regulated emissions and performance. In: *SAE Technical Paper No. 2000-01-1967*. Society of Automative Engineers, Warrendale, PA, USA.

Takagi, Y. and Rahman, S.M. (1996) Inheritance of high oleic acid content in the seed oil of soybean mutant M23. *Theor. Appl. Genet*. **92**, 179–182.

Tang, G.-Q., Novitzky, W.P., Griffin, H.C., Huber, S.C. and Dewey, R.E. (2005) Oleate desaturase enzymes of soybean: evidence of regulation through differential stability and phosphorylation. *Plant J*. **44**, 433–446.

Tat, M.E., Van Gerpen, J. and Wang, P.S. (2007a) Fuel property effects on injection timing, ignition timing, and oxides of nitrogen emissions from biodiesel-fueled engines. *Trans. Am. Soc. Agric. Eng*. **50**, 1123–1128.

Tat, M.E., Wang, P., Van Gerpen, J. and Clemente, T. (2007b) Exhaust emissions from an engine fueled with biodiesel from high-oleic soybeans. *J. Am. Oil Chem. Soc*. **84**, 865–869.

Zhang, X., Peterson, C., Reece, D., Haws, D. and Möller, G. (1998) Biodegradation of biodiesel in the aquatic environment. *Trans. Am. Soc. Agric. Eng*. **41**, 1423–1430.

