J Am Oil Chem Soc (2010) 87:857–864
DOI 10.1007/s11746-010-1576-z

ORIGINAL PAPER

Gene Expression Profiles of Soybeans with Mid-Oleic Acid Seed Phenotype

Robert G. Upchurch · Martha E. Ramirez

Received: 13 August 2009 / Revised: 23 December 2009 / Accepted: 14 March 2010 / Published online: 1 April 2010

Abstract Seeds of the mid-oleic acid soybean mutant M23 accumulate higher levels of oleic acid (50–60% oleate) by virtue of a deletion of *GmFAD2-1A*, an allele of the microsomal omega-6 oleate desaturase gene. In other less well characterized soybean varieties that are phenotypically mid-oleic, little is known about the expression levels of omega-6 desaturase *GmFAD2* genes and other candidate genes that determine seed oleic acid content. We compared the steady-state transcript abundance during seed development of the oleate-ACP thioesterase (*GmFATB1a*), delta-9 stearoyl acyl carrier protein desaturase (*GmSACPD*) and the omega-6 fatty acid desaturase (*GmFAD2*) genes in five natural mid-oleic varieties and mutant M23 to gene expression data for the conventional non mid-oleic cultivar Dare. We found that, relative to Dare, there were instances where lower expression of *GmFATB1a*, *GmFAD2-1A*, *GmFAD2-1B*, *GmFAD2-2*, and *GmFAD2-3* and higher expression of the *GmSACPD-C* might be associated with the mid-oleic seed phenotype. This finding suggests that of the several soybean genomic loci known or suspected to be involved in oleic acid phenotype, some are likely to encode genes involved in regulation of transcription of the oleate biosynthetic genes.

Keywords *GmFATB1a* · *GmSACPD-A*, *-B*, *-C* · *GmFAD2-1A*, *-1B* · *GmFAD2-2* · *GmFAD2-3* · Allele · Oleic acid · qRT-PCR

R. G. Upchurch (✉) · M. E. Ramirez
Soybean and Nitrogen Fixation Unit, Plant Pathology Department, USDA-ARS, North Carolina State University, Raleigh, NC 27695, USA
e-mail: greg.upchurch@ars.usda.gov

Introduction

Soybean [*Glycine max* (L.) Merr] is the largest oilseed crop produced and consumed worldwide, accounting for 56% of the world oilseed production [1], yet soybean oil is lacking in several quality characteristics. The average fatty acid composition of soybean oil is 110 mg g^{-1} palmitic acid (16:0), 40 mg g^{-1} stearic acid (18:0), 230 mg g^{-1} oleic acid (18:1), 530 mg g^{-1} linoleic acid (18:2), and 100 mg g^{-1} linolenic acid (18:3) [2]. These five fatty acids are the precursors for the synthesis of triacylglycerols which constitute the storage lipid reserves of developing oilseeds and their relative contents largely determine oil quality. Both the nutritional value and oxidative stability of soybean oil would be improved if the content of the monounsaturate, oleic acid, were increased. Oil higher in oleic acid possesses increased oxidative stability which negates the need for hydrogenation and eliminates the production of *trans*-fats, a byproduct of hydrogenation and a significant concern for cardiovascular health. In the past decade, concerted efforts have been made to incorporate stable expression of the high-oleic-acid trait into soybean germplasm using both plant breeding [3] and molecular genetic [4] approaches.

The discovery and characterization of soybean omega-6 oleate desaturase genes has considerably enhanced understanding and efforts to identify and produce higher oleic acid soybean germplasm. Omega-6 oleate desaturases are microsomal enzymes in the primary route of polyunsaturated lipid production in plants that catalyze the first extraplastidial desaturation that converts oleic acid esterified to phosphatidylcholine to α-linoleic acid. Three different soybean omega-6 desaturase genes, *GmFAD2-1*, *GmFAD2-2* [5] and *GmFAD2-3* [6] have been reported. Gm*FAD2-1* is specifically expressed during lipid synthesis in developing seeds and not in vegetative tissues while *GmFAD2-2* is

constitutively expressed in both vegetative tissue and developing seeds. Although *FAD2-2* contributes to the production of linoleic acid in all tissues, transcript expression analysis suggests that *GmFAD2-1* plays the major role in the conversion of oleic acid to linoleic acid in developing seed tissues. Two seed-specific alleles, A and B, of *GmFAD2-1* encoding omega-6 enzymes that differ in stability at high temperatures have been described [7]. Recent soybean genomic analyses have shown that the *GmFAD2-2* gene exists as three alleles, *GmFAD2-2A*, *GmFAD2-2B*, and *GmFAD2-2C* [8]. Transcript abundance of *GmFAD2-2*C was observed to increase approximately eightfold in pods grown in cool conditions relative to those in warmer conditions. The third gene, *GmFAD2-3*, is also constitutively expressed in both vegetative and developing seed tissues, but shows no significant changes in transcript levels in cold-stressed leaves. Several years ago, a soybean transgenic approach involving self-cleaving ribozyme transcript termination was successfully employed to downregulate *GmFAD2-1* and *GmFATB* (16:0 acyl-thioesterase) expression in seeds. Transgenic plants exhibiting dual downregulation were recovered that exhibited oleic acid levels greater that 85% and saturated fatty acid (palmitate plus stearate) levels less that 6% (4). The mid-oleic (50–60% 18:1) mutant soybean line M23, developed by X-ray irradiation of dry seeds of the normal oleate cultivar Bay [9] has recently been molecularly characterized. PCR and DNA dot blot analyses have shown that the enhanced seed oleate content in this mutant is associated with deletion of the entire *GmFAD2-1A* allele [10, 11].

Manipulation of other fatty acid biosynthetic genes potentially has use for achieving higher oleic acid content in soybean seeds. Soluble delta-9 stearoyl-ACP desaturases (SACPDs), found in all plant cells, introduce the first double bond into stearoyl-ACP (18:0-ACP) to produce oleoyl ($18:1^{\Delta 9}$)-ACP [12]. Thus, delta 9-stearoyl-ACP-desaturase occupies a key position in C_{18} fatty acid biosynthesis since perturbation of SACPD gene expression and/or enzyme activity may modulate the relative levels of both stearic and oleic acid in oilseeds. Three alleles of *SACPD* have been identified and characterized from soybean. Transcripts of the *GmSACPD-A* and *-B* were detected most abundantly in developing seeds, however, differences in transcript abundance between A and B were not dramatic [13]. Recently, the characterization of a unique third allele, *GmSACPD-C*, was reported [14]. High levels of *SACPD-C* transcripts are expressed only in developing seeds and mutations in this gene from two soybean germplasm sources, mutants A6 (30% stearate) and FAM94-41 (9% stearate) resulted in elevated seed stearic acid. These results suggest that germplasm with high SACPD-C gene expression and/or enzyme activity may contribute to, conversely, elevated seed oleic acid levels.

Plastid localized acyl-acyl carrier protein (ACP) thioesterase enzymes terminate chain elongation by hydrolyzing newly formed acyl-ACP into free fatty acids and ACP [12]. Subsequently, the free fatty acids may exit the plastid to take part in further lipid biosynthesis. Currently there are thought to be four *FATB* alleles of the soybean 16:0-ACP thioesterase and cDNAs of three of the four have been isolated. One of these, *GmFATB1a*, was found to be deleted in low palmitic acid lines possessing the fap_{nc} locus. Allele specific PCR primers for *FATB1a* were effective in accounting for 62–70% of the genotypic variation in palmitate content in two soybean lines examined [15]. *GmFATB* hydrolyzes both palmitoyl-ACP and stearoyl-ACP to, respectively, palmitic and stearic acid. Therefore, *FATB1a* expression and or enzyme activity may modulate oleate as well as palmitate and stearate content since the enzyme competes with SACPD for the 18:0-ACP substrate. Germplasm with low *GmFATB1a* expression and/or enzyme activity might be expected to have in addition to lower seed palmitic acid content, potentially higher oleic acid content.

Many of the higher oleic acid soybean lines currently under development are progeny of crosses with the M23 mutant. Recent field trials have uncovered environmental instability in the expression of this trait in the M23-derived lines [16], as well as reductions in seed yield, protein, and oil [17]. To ensure that stable, mid-oleic germplasm without yield penalty is forthcoming, even as the issues with current mid-oleic lines are addressed, additional efforts to identify and characterize new sources of the mid-oleate trait seem justified. To further this aim, we selected five natural varieties, reported as phenotypically mid-oleic, for study in tandem with the conventional cultivar Dare and mutant M23. We previously determined that each of these soybean varieties, with the exception of *GmFAD2-1A* deleted in M23, produced transcripts for each of the eight genes under investigation in this study (data not shown). Our goal was to determine whether expression levels of any of the genes could be associated with the accumulation of oleic acid during seed development. We employed real time, quantitative reverse transcription PCR (qRT-PCR) to measure and compare normalized gene expression in seeds of the conventional cultivar Dare to expression in six mid-oleic varieties. Seed fatty acid composition was analyzed at 22, 28, 35, and 55 DAF so that we could compare and contrast fatty acid accumulation profiles of the mid-oleic varieties with that of the Dare cultivar.

Experimental Procedures

Plant Materials and Growth Conditions

Seeds of six soybean varieties with elevated seed oleic acid content ranging from 33 to 50% or greater and Dare

[18], a cultivar with ca. 20% seed oleic acid were supplied by J. W. Burton, USDA-ARS, Raleigh, NC. The elevated oleic acid varieties, also called mid-oleics, were the X-ray-induced mutant M23 [9], lines N0304-303-3 [19], N01-3544 [3], PI592941, PI417360, and PI506582 [20]. Plants were grown in a semicontrolled chamber at the Southeastern Plant Environment Laboratory (Phytotron) at North Carolina State University, Raleigh, NC. Pregerminated seeds were planted in 25-cm pots containing a potting mix of 1/3 peat-lite and 2/3 gravel. One week after planting, the plants were thinned so that the four pots for each soybean variety contained one robust plant. Each day the pots were watered in the morning with deionized water and in the afternoon with a nutrient solution. Chamber temperature was maintained at D/N = 26/22 °C throughout plant growth. For the initial 14 days after planting, the D/N cycle was 14 h of incandescent light and 10 h of darkness with a 1-h incandescent light interruption of the dark to aid in synchronizing flowering. On day 15, the light interruption was discontinued and chambers were reset to D/N = 9/15 h to induce flowering. Four plants of each soybean variety were grown. All four plants of each variety were sampled at four developmental stages between R5 (beginning of seed set) and R6 (mature bean), corresponding to 22, 28, 35, and 55 days after flowering (DAF). Five seeds from each of the plants of a particular soybean variety were harvested at each of the four seed developmental stages, pooled, and quickly frozen in liquid nitrogen and stored at −80 °C until further analyses.

Analysis of Seed Fatty Acid Content

Seeds were ground in a small mortar and pestle under liquid nitrogen. Fatty acid methyl esters (FAMEs) of a sub sample of each seed pool were prepared by acid methanolysis. Frozen, ground seed tissue was heated to and held at 85 °C for 90 min in a 5% HCl–95% methanol solution. FAMEs were partitioned two times into hexane and transferred to 2-ml vials for analysis. The FAMES were separated by gas chromatography using an HP 6890 GC (Agilent Technologies, Inc., Wilmington, DE) equipped with a DB-23 30 × 0.53 mm column (Agilent Technologies, Inc.). Operating conditions were 1-µl injection volume, a 20:1 split ratio, and He carrier gas flow of 6 ml min^{-1}. Temperatures were 250 °C, 200 °C, and 275 °C for the injector, oven, and flame ionization detector, respectively. Peak areas of the chromatograms were analyzed using HP ChemStation software. Fatty acid contents, as percentages, were calculated as mg fatty acid g^{-1} oil.

Analysis of Gene Expression

PCR primers (Table 1) were designed to amplify transcripts of eight soybean fatty acid biosynthesis candidate genes potentially involved in oleic acid biosynthesis as well as four housekeeping genes for normalization of transcript abundance using gene sequences from the National Center for Biotechnology Information (NCBI) GenBank and Clone manager software (Sci-Ed Software, Cary, NC). Each primer pair, with the exception of the primers for *GmFAD2-2*, was designed to amplify a single amplicon from soybean cDNA. Primers for *GmFAD2-2* were designed to a region identical in each of the three alleles of this gene (*GmFAD2-2A*, *-2B* and *-2C*) so that the amplified product represents the combined transcript abundance of these three constitutively expressed alleles. Amplicon fidelity and uniqueness were verified by electrophoretic, melt curve and sequence analyses (data not shown). Seed samples were ground under liquid nitrogen and total RNA was extracted using the RNeasy Plant mini kit and protocol from Qiagen (Valencia, CA). Traces of genomic DNA were eliminated by treatment of the RNA with the on-column DNase digestion system from Qiagen. RNA yield and quality were estimated by measuring absorbance at 260 and 280 nm with a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE). First strand cDNA synthesis was carried out using the High Capacity cDNA Reverse Transcription kit with MultiScribe reverse transcriptase (RT) and random primers (Applied Biosystems, Foster City, CA). RNA (2.5 µg) in 12.5 µl nuclease free water were mixed with the same volume of 2× RT master mix. The reaction conditions were: 10 min at 25 °C, then 120 min at 37 °C, followed by 5 s at 85 °C. qRT-PCR assays with SYBR green were performed in triplicate with an i-Cycler Thermocycler (BioRad, Hercules, CA). The PCR volume was 25 µl and contained 200–300 nM of each forward and reverse primer, 1× SYBR green PCR master mix (Applied Biosystems, Foster City, CA), and template cDNA equivalent to 40 ng of total RNA. As a control, amplifications were also conducted using water instead of the nucleic acid templates. In addition, PCR with RNA templates (RT minus control) were performed to ensure that there was no DNA contamination and no amplification of non-specific products. The amplification conditions were 1 cycle at 95 °C for 15 min, followed by 35 cycles of 94 °C for 15 s, the annealing temperature (T_a in Table 1) for 30 s and 72 °C for 30 s. Finally, melt curve analysis was conducted. Absolute quantification of transcript abundance was calculated using standard curves obtained for each gene [21]. For this purpose, a tenfold dilution series of a total of five dilutions was prepared using a cloned

Table 1 Real time PCR primers employed for this work

Gene	Forward/reverse primers	Target[a] sequence	T_a[b] (°C)	Amplicon (bp)	Description	References
FATB1a	5′ACAGCCAATCTTGGAGAGTC 5′GATACAGCAGTCAGGGAATC	DQ861997	57	99	Fatty acid thioesterase B1a	[15]
SACPD-A	5′GAAGGAGCATGGTGACATAA 5′CATCAGGATCAACCTCAAAC	AY885234	57	119	Stearoyl-acyl carrier protein desaturase-A	[13]
SACPD B	5′CCTGTTTGATAGCTACTCTTCG 5′GTTAGCTGCTCCACCTTCC	AY885233	60	111	Stearoyl-acyl carrier protein desaturase-B	[13]
SACPD-C	5′CATCTTACGCGCTCGCTCAT 5′CGTTGAACGGTGGAGATTGG	EF113911	60	132	Stearoyl-acyl carrier protein desaturase-C	[14]
FAD2-1A	5′CCAATGGGTTGATGATGTTG 5′GTTGTTTAAGTACTTGGAAA	L43920	52	178	Microsomal ω-6 fatty acid desaturase 2-1A	[7]
FAD2-1B	5′TTGACCGTTCACTCAGCAC 5′GGTTGTTCAGGTACTTGGTGT	AB188251	59	154	Microsomal ω-6 fatty acid desaturase 2-1B	[7]
FAD2-2	5′GCAATGGAGGCTACAAAGG 5′CCTTTGCTCTCGGTACTTTG	L43921	56	140	Microsomal ω-6 fatty acid desaturase 2-2	[5]
FAD2-3	5′AGGCGACAAAGGCAATAAAG 5′CCTTTGCTCTGAGTACTTTG	DQ532371	55	133	Microsomal ω-6 fatty acid desaturase 2-3	[6]
Actin	5′GAGCTATGAATTGCCTGATGG 5′CGTTTCATGAATTCCAGTAGC	GMU60500	58	118	Soy 57 actin	[22]
GM16 F	5′TTCCTTTATCAGAAATAACGAGTTTAGCT 5′ TGTCTCATTTTGCGGCAGC	D10717	57	116	Phosphoenolpyruvate carboxylase	[25]
EF1-F	5′CTGTAACAAGATGGATGCCACTAC 5′CAGTCAAGGTTRGTGGACCT	X56856	61	176	Elongation factor 1a	[23]
UBi3-F1	5′GTGTAATGTTGGATGTGTTCCC 5′ACACAATTGAGTTCAACACAAACCG	D28123	59	108	SUBI-3 ubiquitin	[24]

[a] NCBI accession number

[b] Primer annealing temperature

DNA fragment of each gene. C_T values and i-Cycler software were used to plot a standard curve that allowed quantification of the target gene in each sample. A normalization factor was derived from expression values obtained for *Soy 57 actin* [22], elongation factor *EF1-F* [23], ubiquitin *UBi3-F1* [24] and *Gm16F* [25] using geNorm software [26]. Normalized expression of each fatty acid biosynthesis gene was then calculated as a ratio of the expression level of the respective gene in the conventional Dare cultivar.

Statistical Analysis

Fatty acid and normalized gene expression data were subjected to analysis of variance using the general linear model procedure (PROC GLM) of SAS, version 9.1, SAS Institute, Cary, NC, USA. Significant differences between conventional cultivar Dare and mid-oleic varieties were determined using Dunnett's *t* test ($P = 0.05$).

Results and Discussion

Changes in Fatty Acid Content During Seed Development

The seed palmitic, stearic, oleic, linoleic and linolenic acid contents of conventional cultivar Dare, mid-oleic mutant M23 and the five natural mid-oleic acid varieties under investigation are shown for each of four sampling dates in Fig. 1. Significant differences ($P = 0.05$) were detected in the accumulation patterns of the five fatty acids in the six mid-oleic varieties compared to Dare as would be expected and also more subtle differences in the accumulation patterns of oleate and linoleate among the mid-oleic varieties themselves. Palmitate (Panel A) decreased in seeds of all varieties during the 22–55 DAF interval with Dare highest (mean 117 mg g^{-1}) and N0304-303-3 lowest (mean 40 mg g^{-1}) and the others between 88 and 104 mg g^{-1} palmitate at 55 DAF. Stearate (Panel B) also decreased in

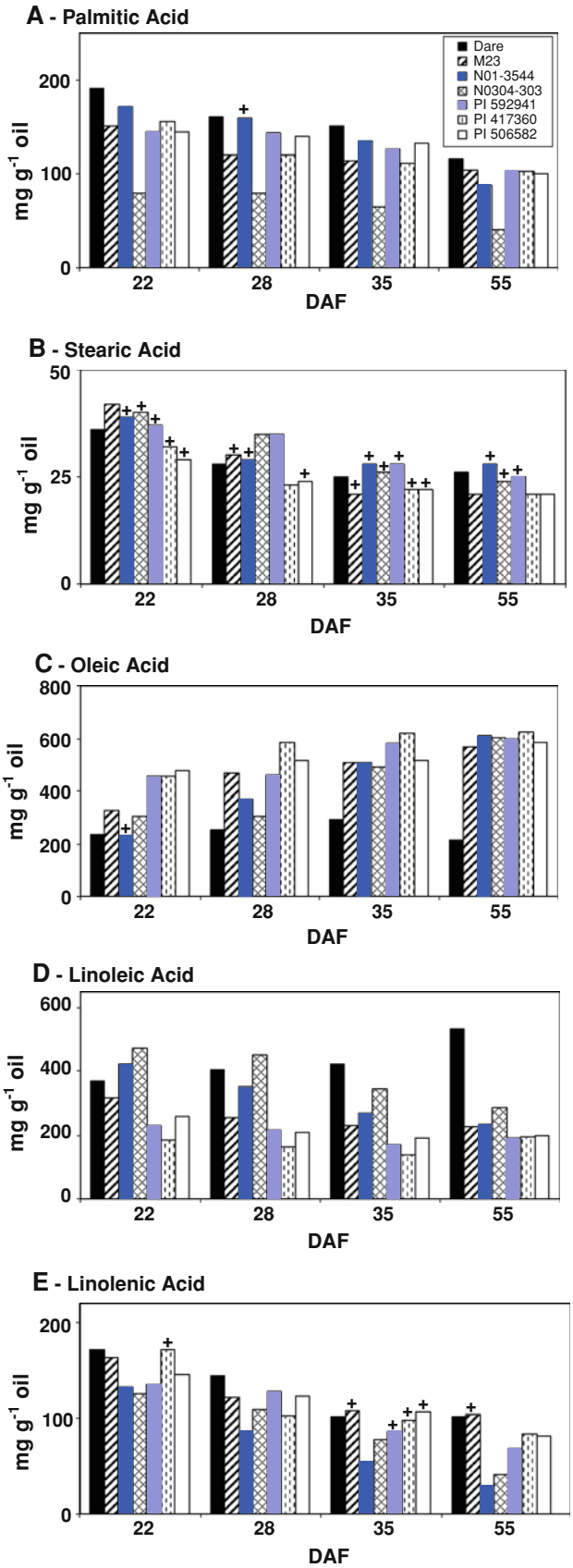

Fig. 1 Accumulation of palmitate **a**, stearate **b**, oleate **c**, linoleate **d** and linolenate **e** in seeds of cultivar Dare and six mid-oleic soybean varieties during seed development at 22, 28, 35, and 55 days after flowering (DAF). Fatty acid values not significantly different from Dare by Dunnett's *t* test ($P = 0.05$) at the same sampling date are indicated by a cross (+)

seeds of all varieties during the 22–55 DAF interval, though the stearate levels of several varieties were not significantly different from Dare. At 55 DAF, N01-3544 stearate was highest (mean 28 mg g^{-1}) and M23, PI417360 and PI506582 lowest (means = 21 mg g^{-1}) and the others between 24 and 26 mg g^{-1}. Oleate (Panel C) increased in seeds of the mid-oleic mutant M23 and the other five mid-oleic varieties during the interval with PI417360 highest (mean 624 mg g^{-1}) and M23 lowest (mean 567 mg g^{-1}) at 55 DAF. Oleate content in Dare seeds initially increased from 235 at 22 DAF to 291 mg g^{-1} at 35 DAF and then decreased to 217 mg g^{-1} by 55 DAF. Differences in oleate accumulation patterns were evident among the six mid-oleic varieties. Mean oleate levels in N01-3544, N0304-303-3 and M23, increased more steeply over the interval to their levels at 55 DAF. Seed oleate in PI592941, PI417360 and PI506582 was relatively high at 22 DAF and increased less steeply over the interval to their levels at 55 DAF. Linoleate (panel D) decreased reciprocally with oleate increase in seeds of the mid-oleic mutant M23 and the other five mid-oleic varieties during the interval, with PI592941 lowest (mean 191 mg g^{-1}) and N0304-303-3 highest (mean 289 mg g^{-1}) at 55 DAF. Linoleate content in Dare seeds increased from a mean of 371 at 22 DAF to a mean of 533 mg g^{-1} at 55 DAF. Differences in linoleate accumulation patterns also were evident among the six mid-oleic varieties. Mean linoleate levels in M23, N01-3544, N0304-303-3 at 22 DAF decreased more steeply, while seed linoleate in PI592941, PI417360 and PI506582, lower at 22 DAF (185–260 mg g^{-1}), decreased less steeply over the interval. Linolenate (Panel E) decreased in seeds of all varieties during the 22–55 DAF interval with Dare and M23 highest (mean 102 and 104 mg g^{-1}, respectively) and N01-3544 and N0304-303-3 lowest (means 30 and 41 mg g^{-1}, respectively) and the others between 69 and 84 mg g^{-1} linolenate at 55 DAF. In summary, data form Fig. 1 shows that by 55 DAF, seed oleate content in the six mid-oleic acid varieties had increased to levels nearly threefold higher than the oleate content measured in Dare seeds. By 55 DAF, seed linoleate content in the six mid-oleic acid varieties had decreased to levels nearly half or lower than the linoleate content measured in Dare seeds. Seed palmitate content decreased in Dare and the six mid-oleic soybean varieties during the 22–55 DAF interval,

with N0304-303-3 lowest, with less than half the seed palmitate of the next lowest variety. Seed stearate decreased in all seven varieties during the interval but the stearate content of some mid-oleic varieties was not always significantly different ($P = 0.05$) from stearate content measured in Dare seeds. Seed linolenate content also decreased during the interval in all seven varieties (although not always significantly different from Dare), however; seeds of mid-oleic varieties N01-3544 and N0304-303-3 contained significantly very low levels of this fatty acid by 55 DAF.

Gene Expression during Seed Development

Table 2 shows gene expression ratios (relative to Dare) for the six mid-oleic varieties that differed significantly ($P = 0.05$), either higher or lower, from Dare at 22, 28, and 35 DAF for *GmFATB1a*, the three alleles of *GmSACPD*, the two alleles of *GmFAD2-1*, and genes *GmFAD2-2* and *GmFAD2-3*. As expected, no *GmFAD2-1A* transcript was detected in the mid-oleic deletion mutant, M23 at any sampling date. For *GmFATB1a*, at 22 and 28 DAF, one mid-oleic variety (N0304-303-3) had a transcript ratio significantly <1.0, and by 35 DAF, it and two other mid-oleic varieties, M23 and N01-3544 had ratios <1.0. For *GmSACPD-A*, two mid-oleic varieties at 22 DAF, four at 28 DAF, but none at 35 DAF had transcript ratios >1.0. For *GmSACPD-B*, two at 22 DAF, four at 28 DAF, but again none at 35 DAF had transcript ratios >1.0. For *GmSACPD-C*, two mid-oleic varieties at 22 DAF, three at 28 DAF, and five at 35 DAF had transcript ratios >1.0, with the highest ratio (6.35) for PI592941 at 35 DAF. For *GmFAD2-1A*, with the exception of the deletion mutant M23, none of the other mid-oleic varieties had a transcript ratio <1.0 at 22 and 28 DAF, but by 35 DAF, two other varieties (N0304-303-3 and PI 506582) had ratios <1.0. For *GmFAD2-1B*, two mid-oleic varieties (M23 and N01-3544) at 22 DAF, none at 28 DAF, but five including M23 at 35 DAF had ratios <1.0. For *GmFAD2-2*, one mid-oleic variety at 22 DAF (PI417360), none at 28 DAF and none at 35 DAF had ratios <1.0. For *GmFAD2-3*, four mid-oleic varieties at 22 DAF, none at 28 DAF, and two at 35 DAF had ratios <1.0.

Seed fatty acid composition is probably under the control of multiple mechanisms, both genetic and biochemical that ultimately determine the amount of enzyme

Table 2 Expression ratios relative to cultivar Dare of genes governing palmitate, stearate, oleate and linoleate in seeds of six mid-oleic soybean varieties at 22, 28, and 35 days after flowering (DAF)

DAF	Variety	Mean gene expression ratio relative to Dare							
		FATB1a	*SACPD-A*	*SACPD-B*	*SACPD-C*	*FAD2-1A*	*FAD2-1B*	*FAD2-2*	*FAD2-3*
22	Dare	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	M23	NS	NS	0.61	NS	0.00	0.70	NS	0.58
	N01-3544	1.35	1.63	0.62	NS	NS	0.76	NS	0.63
	N0304-303-3	0.30	NS	0.70	NS	NS	NS	1.54	NS
	PI592941	1.61	1.56	NS	2.00	1.99	1.64	1.64	NS
	PI417360	2.24	NS	1.67	2.41	2.18	NS	0.56	0.54
	PI506582	1.51	NS	2.37	NS	1.66	NS	NS	0.61
28	Dare	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	M23	1.28	1.91	1.55	NS	0.00	1.40	NS	1.45
	N01-3544	NS	NS	NS	NS	NS	1.51	1.64	NS
	N0304-303-3	0.30	NS	NS	NS	NS	1.41	NS	NS
	PI592941	1.76	2.74	1.55	3.91	2.89	2.23	3.11	2.50
	PI417360	2.09	1.70	3.20	2.44	2.80	1.98	NS	NS
	PI506582	1.54	2.03	2.93	2.44	2.55	1.63	NS	NS
35	Dare	1.00	1.00	1.00	1.00	1.00	1.00	1.00	1.00
	M23	0.71	NS	0.60	2.37	0.00	0.53	NS	NS
	N01-3544	0.47	0.59	0.48	2.15	NS	0.44	NS	0.71
	N0304-303-3	0.10	0.31	0.25	NS	0.46	0.27	NS	0.76
	PI592941	NS	NS	NS	6.35	1.88	NS	1.88	2.08
	PI417360	NS	NS	NS	2.72	NS	0.52	NS	1.21
	PI506582	NS	NS	0.23	2.63	0.14	0.19	4.71	NS

Ratios <1.0 for lower, and ratios >1.0 for higher expression than in the conventional cultivar Dare. All values given are significantly different from Dare at the same sampling date (Dunnett's *t* test, $P = 0.05$). Values not significantly different from Dare are denoted by NS

protein and enzyme activity produced as well as the fatty acid fluxes through the desaturase pathway. Thus, the elevated accumulation of seed oleate in mid-oleic soybeans could be the result of several phenomena including mutations or deletions in the promoters or coding sequences of these genes that alter or abolish enzyme activity or the production of enzyme protein (as in mutant M23), mutations in the post-translational machinery that alter enzyme activities, or mutations in the components of transcription complexes that control the rates of gene transcription, transcript stability, and transcript accumulation. In this study we measured the steady-state transcript accumulation patterns of eight fatty acid biosynthetic genes in five natural mid-oleic soybean lines and mid-oleic mutant M23 and compared their gene expression pattern with the expression pattern of the non mid-oleic cultivar Dare to determine possible relationships between the levels of gene expression and the mid-oleic phenotype. Using Dare for base line gene expression levels, we hypothesized that if gene expression levels control the accumulation of oleic acid in the seeds of mid-oleic varieties, then we might expect to detect a lower expression (ratio <1.0) of *GmFATB1a* and/or lower expression of one or more of the *GmFAD2s* and/or higher expression (ratio >1.0) of one or more of the *GmSACPDs* since these kinds of gene expression patterns, if synonymous with enzyme content and activity, could result in the accumulation of higher levels of oleate in developing seed. Interpretation of our data presents some difficulty since we do not know what threshold is required for a change in transcript level to be biologically relevant, nor do we know that transcript levels measured always reflected enzyme content and activity. None the less, we did find that the expression ratios of one, two, or three of the *GmFAD2s* were significantly lower (0.0–0.76) compared to Dare by 35 DAF in the five mid-oleic varieties M23, N01-3544, N0304-303-3, PI417360, and PI506582. For *GmFATB1a*, by 35 DAF, three of the six mid-oleic varieties, M23, N01-3544, and N0304-303-3 had expression ratios significantly lower (0.1–0.71) than Dare. If lower *GmFATB1a and GmFAD2* transcript levels are reflected in enzyme activities, then these mid-oleic varieties may accumulate higher levels of seed oleate because of reduced rates of saturate and linoleate synthesis. For *GmSACPD-C*, gene expression was significantly higher (2.15–6.35) than Dare in five of the six mid-oleic varieties by 35 DAF. If higher Gm*SACPD-C* transcript levels reflect enzyme activity, then these five mid-oleic varieties may accumulate higher levels of seed oleate because of higher rates of oleate synthesis. For *SACPD-A* and *-B*, significantly higher expression ratios for two and three of the mid-oleic varieties were detected at 22 and 28 DAF, respectively, but by 35 DAF expression levels of these genes was not significantly different or significantly lower compared to Dare.

Changes in unsaturated fatty acid content in seeds have been observed in low palmitic acid lines including the phenotype of increased oleic acid. For example, progenies inheriting the major fap_{nc} allele from the cross of line N87-2122-4 to two high yielding normal palmitic acid lines had a 4–10% increase in oleic acid content [15]. N0304-303-3, a mid-oleic variety included in our study, accumulated the lowest levels (40 mg g^{-1}) of seed palmitate and consistently had the lowest (0.10–0.30) *GmFATB1a* transcript ratio at the three sampling dates of all the mid-oleic varieties examined, however; by 35 DAF, three other mid-oleic varieties had *GmFATB1a* ratios significantly <1.0 but with normal palmitate accumulation. This suggests that low *GmFATB1a* expression, if synonymous with decreased thioesterase activity, may contribute to the mid-oleic phenotype by a redirection of stearate into oleate in the low palmitate variety N0304-303-3 as well as in the other two normal palmitate, mid-oleic varieties M23 and N01-3544.

As shown previously (Table 2), we found that the mid-oleic varieties N01-3544 and N0304-303-3 had the lowest linolenate seed content of all the varieties at 35 and 55 DAF. We did not measure the transcript levels of the three seed expressed ω-3 *GmFAD3* genes encoding the enzymes for desaturation of linoleic to linolenic acid, but previous research has shown a strong positive association between decreased *GmFAD3* transcript abundance and substantial reductions in linolenate content in soybean seeds [27].

In summary, we did uncover instances where lower expression levels of *GmFATB1a*, *GmFAD2-1A*, *GmFAD2-2*, *GmFAD2-2*, and *GmFAD2-3*, and higher expression levels of *GmSACPD-C* appear to be associated with the mid-oleic phenotype. These findings suggests that, of the many soybean genomic loci either confirmed or suspected of involvement in the mid-oleic fatty acid phenotype, one or more likely contain genes that direct and regulate the expression pattern of the oleate biosynthetic genes during seed development. Six Quantitative Trait Loci (QTLs) for oleic acid have been mapped and confirmed in areas of the soybean genome associated with oleic acid content in the mid-oleic soybean line N00-3350 [28]. Two QTLs were mapped to LG-G, two to LG-L, and one each to LG-A1 and LG-D2 that contribute between 4% (Satt 211) to 25% (Satt 561) to oleic content. Mapping has also recently shown that *GmFAD2* alleles cosegregate with QTLs affecting oleate content [29]. *GmFAD2-1A* mapped to LG-O, *GmFAD2-1B* to LG-I and *GmFAD2-2A* and *GmFAD2-2B* each to LG-L. In addition, oleate QTLs with minor effects were detected in the proximity of *GmFAD2-1B* and possibly *GmFAD2-2B* on LG-I and LG-L, respectively [29]. Continued efforts to map, characterize, and compare these and other QTLs for seed oleate content in segregating soybean populations

[30] may result in the identification of transcription promoting and regulating genes that control oleic acid content.

Acknowledgments We thank William P. Novitzky for the fatty acid analyses and the staff of the Southeastern Plant Environmental Laboratory at North Carolina State University for growth chamber space. Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture.

References

1. American Soybean Association (2009) SoyStats 2009. Available at http://www.soystats.com American Soybean Association, St. Louis
2. Wilson RF (2004) Seed composition. In: Boerma HR, Specht JE (eds) Soybeans: improvement, production, and uses. ASA, Madison, pp 621–677
3. Burton JW, Wilson RF, Novitzky W, Rebetzke GJ, Pantalone VR (2005) Registration of N98-4445A, a mid-oleic soybean germplasm line. Crop Sci 46:1010–1011
4. Buhr T, Sato S, Ebrahim F, Xing A, Zhou Y, Mathiesen M, Schweiger B, Kinney A, Staswick P, Clemente T (2002) Ribozyme termination of RNA transcripts down-regulate seed fatty acid genes in transgenic soybean. Plant J 30:155–163
5. Heppard EP, Kinney AJ, Stecca KL, Miao GH (1996) Developmental and growth temperature regulation of two different microsomal omega-6 desaturase genes in soybeans. Plant Physiol 110:311–319
6. Li L, Wang X, Gai J, Yu D (2007) Molecular cloning and characterization of a novel microsomal oleate desaturase gene from soybean. J Plant Physiol 164:1516–1526
7. Tang G-Q, Novitzky WP, Griffin HC, Huber SC, Dewey RE (2005) Oleate desaturase enzymes of soybean: evidence of regulation through differential stability and phosphorylation. Plant J 44:433–446
8. Schlueter JA, Vasylenko-Sanders IF, Deshpande S, Yi J, Siegfried M, Roe BA, Schlueter SD, Scheffler BE, Shoemaker RC (2007) The FAD2 gene family of soybean: insights into the structural and functional divergence of a paleopolyploid genome. Crop Sci 47(S1):S14–S26
9. Takagi Y, Rahman SM (1996) Inheritance of high oleic acid content in the seed oil of soybean mutant M23. Theor Appl Genet 92:179–182
10. Alt JL, Fehr WR, Welke GA, Sandhu D (2005) Phenotypic and molecular analysis of oleate content in the mutant soybean line M23. Crop Sci 45:1997–2000
11. Sandhu D, Alt JL, Scherder CW, Fehr WR, Bhattacharyya MK (2007) Enhanced oleic acid content in soybean mutant M23 is associated with the deletion in the Fad2-1a gene encoding a fatty acid desaturase. J Amer Oil Chem Soc 84:229–235
12. Ohlrogge J, Browse (1995) Lipid biosynthesis. Plant Cell 7:957–970
13. Byfield GE, Xue H, Upchurch RG (2006) Two genes from soybean encoding soluble Δ9stearoyl-ACP desaturases. Crop Sci 46:840–846
14. Zhang P, Burton JW, Upchurch RG, Whittle E, Shanklin J, Dewey RE (2008) Mutations in a Δ^9-stearoyl-ACP-desaturase gene are associated with enhanced stearic acid levels in soybean seeds. Crop Sci 48:2305–2313
15. Cardinal AJ, Burton JW, Camacho-Roger AM, Yang JH, Wilson RF, Dewey RE (2007) Molecular analysis of soybean lines with low palmitic acid content in the seed oil. Crop Sci 47:304–310
16. Scherder CW, Fehr WR, Shannon JG (2008) Stability of oleate content in soybean lines derived from M23. Crop Sci 48:1749–1754
17. Scherder CW, Fehr WR (2008) Agronomic and seed characteristics of soybean lines with increased oleate content. Crop Sci 48:1755–1758
18. Brim CA (1966) Registration of Dare soybeans. Crop Sci 6:95
19. Xue HQ, Upchurch RG, Kwanyuen P (2008) Relationships between oleic and linoleic acid and seed colonization by *Cercospora kikuchii* and *Diaporthe phaseolorum*. Plant Dis 92:1038–1042
20. USDA, ARS, National Genetic Resources Program. Germplasm Resources Information Network-(GRIN). National Germplasm Resources Laboratory, Beltsville, MD. Available: http//www.ars-grin.gov/cgi-bin/npgs/acc/abs
21. Pfaffl MW (2004) Quantification strategies in real-time PCR, pp 90–120. In: Bustin SA (ed) A–Z of quantitative PCR. International Univ. Line, La Jolla
22. Moniz de Sa M, Drouin G (1996) Phylogeny and substitution rates of angiosperm actin genes. Mol Biol Evol 13:1198–1212
23. Aguilar F, Montandon PE, Stutz E (1991) Two genes encoding the soybean translation elongation factor eEF-1 alpha are transcribed in seedling leaves. Plant Mol Biol 17:351–360
24. Trevaskis B, Wandrey M, Colebatch G, Udvardi MK (2002) The soybean *GmN6L* gene encodes a late nodulin expressed in the infected zone of nitrogen-fixing nodules. Mol Plant Microbe Interact 15:630–636
25. Sugimoto T, Kawasaki T, Kato T, Whittier RF, Shibata D, Kawamura Y (1992) cDNA sequence and expression of a phosphoenolpyruvate carboxylase gene from soybean. Plant Mol Biol 20:743–747
26. Vandesompele J, De Preter K, Pattyn F, Poppe B, Van Roy N, De Paepe A, Speleman P (2002) Accurate normalization of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. Genome Biol 3:1–12
27. Byfield GE, Upchurch RG (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
28. Monteros MJ, Burton JW, Boerma HR (2008) Molecular mapping and confirmation of QTLs associated with oleic acid content in N00-3350 soybean. Crop Sci 48:2223–2234
29. Bachlava E, Dewey RE, Auclair J, Wang S, Burton JW, Cardinal AJ (2008) Mapping genes encoding microsomal ω-6 desaturase enzymes and their cosegregation with QTL affecting oleate content in soybean. Crop Sci 48:640–650
30. Bachlava E, Dewey RE, Burton JW, Cardinal AJ (2009) Mapping and comparison of quantitative trait loci for oleic acid seed content in two segregating soybean populations. Crop Sci 49:433–442