

Identification and Molecular Characterization of Homeologous $\Delta 9$ -Stearoyl Acyl Carrier Protein Desaturase 3 Genes from the Allotetraploid Peanut (*Arachis hypogaea*)

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Abstract The stearoyl-acyl carrier protein (ACP) desaturase (SAD) is a nuclear-encoded, plastid-localized soluble desaturase that catalyzes the conversion of stearoyl-ACP to oleoyl-ACP and plays a key role in the determination of the properties of the majority of cellular glycerolipids. *Sad* genes from a variety of plant species have been cloned and characterized. However, in peanut (*Arachis hypogaea*), an important edible and oilseed crop, these genes have not yet been characterized. By searching peanut expressed sequence tag (EST) and parallel sequencing (454) libraries, we have identified three members of the *ahSad* gene family. Among them, only one gene, *ahSad3*, was exclusively expressed during seed development and in a manner fully corresponding to oil accumulation. Both *ahSad3* homeologous genes (*ahSad3A* and *ahSad3B*) were recovered from the allotetraploid peanut, and their mRNA expression levels were characterized. The open reading frames for *ahSad3A* and *ahSad3B* are 98% identical and consist of 1,158 bp, encoding a 386-full-amino-acid protein, with one intron in the coding sequence. Comparisons of the sequences of these two homeologous genes revealed seven single-nucleotide polymorphisms and one triplet insertion in the coding region. Southern blot analysis indicated that there are only two copies of the *ahSad3* gene in the peanut genome. Homeolog-specific gene expression analysis showed that both *ahSad3* homeologs are expressed in developing seeds, but gene expression is significantly biased toward the B genome. Our results point to *ahSad3*

as a possible target gene for manipulation of fatty acid saturation in *A. hypogaea*.

Keywords *Arachis hypogaea* · Real-time PCR · Southern blot · Homeologous genes · Stearoyl-acyl carrier protein desaturase

Abbreviations

high O/L	high oleic/linoleic
ACP	acyl carrier protein
SAD	stearoyl-ACP desaturase
SNP	single-nucleotide polymorphism
FA	fatty acid

Introduction

Peanut (*Arachis hypogaea*) is a major source of protein and edible oil and is ranked as the second most important cultivated grain legume and the fourth largest edible oilseed crop in the world. It is grown in at least 100 countries, of which India (13%), China (9%), Nigeria (5%), and the United States (2.3%) are the largest producers. This crop covers 23 million hectares, which produce about 50 million metric tons, with an average yield 1.6 tons/ha (FAOSTAT 2010). The peanut is grown primarily for human consumption. There are several uses for its whole seeds, and it is also processed to make peanut butter, oil, and other products. Peanut seed contains 26% protein, 49% oil, 16% carbohydrate, and 2.3% ash (USDA 2010). There are four basic types of peanuts: Runner, Virginia, Spanish, and Valencia. Each of these peanuts has a distinctive size and flavor.

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The fatty acid content of conventional peanut seed mainly comprised oleic acid (45%) and linoleic acid (36%) (C18:1 and C18:2, respectively; Liu et al. 2009). In recent years, there has been an effort to increase the size of the oleic acid fraction in commercial peanuts because this fatty acid is more saturated and, therefore, more resistant to oxidation both at ambient storage temperatures and at the high temperatures, which prevail during roasting, cooking, and frying. The first high oleic/linoleic (high O/L) peanut germplasm (F435), which contained up to 80% oleate, was described by Norden et al. (1987). Since then, numerous high O/L lines and cultivars have been developed (summarized by Chu et al. 2009). Today, commercial high O/L varieties comprise the majority of peanuts grown in west Texas, and the expectation is that high O/L peanut varieties will become the standard in the United States in the near future. Some high O/L peanut lines have been found to be more resistant to oxidation (Zainuddina et al. 2004) and discoloration (Uematsu et al. 2002) than the seeds of commonly grown peanut cultivars, with no adverse flavor effects (Pattee et al. 2002).

In addition to oleic acid (C18:1), stearic acid (C18:0) is also regarded as a desirable dietary component of seed oil. Stearic acid is a saturated fatty acid with a higher melting point than oleic acid (68°C vs. 16°C, respectively). It is solid at ambient temperatures, so it can impart the solidity and plasticity required for the manufacture of spreads and shortening. Stearic acid behaves differently from its shorter-chain saturated fatty acid counterparts, such as palmitic acid and myristic acid, in that it does not raise low-density lipoprotein cholesterol or total cholesterol (Liu et al. 2002). It is, therefore, regarded as a nutritionally nonharmful saturate. The seeds of both standard and high O/L peanut genetic lines contain only small fractions of stearic acid (~3%–7%; Liu et al. 2009). Also, in contrast to the situation for other oilseed crops such as sunflower (*Helianthus annuus*; Hamdan et al. 2009), soybean (*Glycine max*; Bubeck et al. 1989), and rapeseed (*Brassica napus* L.; Knutzon et al. 1992), there have not been any reports of high stearic acid peanut germplasm. Novel peanut germplasm with oil rich in stearic acid could be used as the hard stock for the manufacture of margarines, resulting in a more stable oil with a concomitant reduction in the need for artificial hydrogenation, a process that often produces *trans*-fatty acid isomers that have been associated with a number of detrimental effects on human health (Katan 1998). In addition, peanut seeds with high levels of stearic acid should be even more resistant to oxidation than high O/L seeds, at both ambient and elevated temperatures.

In plants, the conversion of stearic acid into the monounsaturated oleic acid is catalyzed by the Δ^9 -stearoyl-acyl carrier protein (ACP) desaturase (SAD). This nuclear-encoded, plastid-localized soluble enzyme uses

stearoyl-ACP as a substrate and, for activation, needs nicotinamide adenine dinucleotide phosphate oxidase and ferredoxin, which act as a reducing agent and cofactor, respectively (Thompson et al. 1991). The product of the reaction, oleoyl-ACP, can serve as a precursor for the synthesis of polyunsaturated fatty acids (Shanklin and Cahoon 1998). Therefore, SAD plays a key role in the determination of the properties of the majority of cellular glycerolipids. Genes encoding SAD enzymes have been cloned and characterized from a variety of plant species (Byfield et al. 2006; Cahoon et al. 1998; Kachroo et al. 2007; Lou et al. 2009; Shanklin and Somerville 1991; Thompson et al. 1991; Tong et al. 2006; Whittle et al. 2005). Genetic correlations have been observed between mutations in *Sad* genes and elevated stearic acid levels in soybean seeds (Zhang et al. 2008). Also, antisense expression studies in *Brassica* (Knutzon et al. 1992) and cotton (Liu et al. 2002) have shown the SAD enzyme to be pivotal in determining stearic acid levels in oilseeds. On the other hand, overexpressing the full-length *Sad1* gene from *Lupinus luteus* in tobacco dramatically increased the level of oleic acid, as compared with the wild type (Zaborowska et al. 2002). As a result, down-regulation or inactivation of *Sad* genes has been suggested as a preferred strategy for the manipulation of oilseed germplasm with elevated levels of saturated fatty acids (e.g., Liu et al. 2009).

While peanut is a major oil source, genetic research regarding fatty acid biosynthesis and oil metabolism in this crop is relatively undeveloped. This also includes the SAD gene family that has yet been characterized. In this study, we identified three members of the *Sad* gene family from peanut cDNA libraries based on their expected homology with previously characterized *Sad* genes from soybean. One of the genes, *ahSad3*, was further analyzed based on its mRNA expression levels and its association with oil accumulation in peanut seeds. Both *ahSad3* homologous genes (*AhSad3A* and *AhSad3B*) were cloned from the allotetraploid peanut and characterized at the genomic and mRNA levels. Our results indicate that *AhSad3* is a possible target gene for manipulation of fatty acid saturation in *A. hypogaea*.

Material and Methods

Plant Material and Growing Conditions

Samples of seeds at different developmental stages [20, 30, 40, 50, 60, and 70 days post anthesis (DPA)] as well as five different tissue types (leaf, peg, root, stem, and flower) were obtained from Virginia-type peanut plants (*Arachis hypogaea* cv. Chanoch) grown under stable greenhouse conditions (25–30°C, 30% RH; 14-h photoperiod with

fluorescent light of intensity $35 \mu\text{mol m}^{-2} \text{s}^{-1}$) in Bet-Dagan, Israel. Three replications of three plants each were sampled and bulked, and the fresh tissue samples were frozen immediately in liquid nitrogen until RNA extraction.

RNA Isolation and cDNA Synthesis

Total RNA was extracted using the hot borate (sodium borate decahydrate) method, as previously described (Brand and Hovav 2010). In brief, tissue samples were ground in liquid nitrogen and were combined with 8 mL of borate buffer [0.2 M sodium borate decahydrate; 30 mM ethylene glycol tetraacetic acid; 1% (wt/vol) sodium dodecyl sulfate; 1% sodium deoxycholic acid; 10 mM dithiothreitol; 1% IGEPAL CA-630 (Nonidet P-40, NP-40); 2% (wt/vol) polyvinylpyrrolidone (PVP)-40] at 65°C. The homogenate was then incubated for 1.5 h in a 42°C incubator/shaker, and 1 mL of 2 M potassium chloride was added to each sample. Subsequent to centrifugation, the supernatant was transferred to a 50-mL tube containing 8 M lithium chloride and incubated on ice overnight. Following a second centrifugation, the supernatant was discarded and the pellet was washed a few times with 2 M lithium chloride. The pellet was then suspended in 250 μL $1\times$ Tris-EDTA and warmed to room temperature. Each sample was centrifuged again, and the supernatant was transferred to a 1.5-mL tube containing 2 M potassium acetate. After an additional centrifugation, the pellet was discarded and the supernatant was transferred to a tube containing 3 M sodium acetate and $2.5\times$ cold 100% ethanol. Following centrifugation, the supernatant was discarded, the pellet was washed with 1 mL of 70% ethanol, and the RNA was resuspended in 100 μL of diethylpyrocarbonate-treated water and stored at -80°C . Only RNA samples that had a 260:280 ratio between 1.9 and 2.1 and a 260:230 ratio greater than 2.0 were used for subsequent analyses. The integrity of the RNA samples was assessed using 1.0% agarose/formaldehyde gel electrophoresis.

For reverse transcription, 2 μg of total RNA were treated with RNase-free DNase (Promega, Madison, WI). Treated RNA was reverse-transcribed using the AccuPower RT PreMix kit for reverse transcriptase polymerase chain reaction (PCR; Bioneer, Alameda, CA) with oligo-(dT)-15 primer and hexameric primer (both from Promega). The concentration of cDNA for each sample was determined using the Nano-Drop ND-1000 spectrophotometer (Nano-Drop Technologies, Wilmington, DE).

Quantitative PCR

Primers for quantitative real-time PCR were designed based on EST sequences for *AhSad* genes. Primers were [5'-GAAGTTGGCACAAATATGTGG-3'/5'-GGCATAGCTATTTTCTTCCTC-3'], [5'-CTCCGAGGAGAATAGGC-

3'/5'-TTGTTTTTCAGTTTTTCACATCCAC-3'] and [5'-GCATGATTAACAACCTAGATGG-3'/5'-CCATCTAGGTTGTTAATCATGC-3'] for *ahSad1/2*, *ahSad3*, and *ahSad4*, respectively. The *alcohol dehydrogenase class III* gene (*adh3*) that was previously found to be the preferred control gene for quantitative PCR studies in developing peanut seeds (Brand and Hovav 2010) was used as the reference gene (primers: [5'-GACGCTTGGC GAGATCAACA-3'/5'-AACCGGACAACCACCACATG-3']). For the genome-specific quantitative PCR of the *AhSad3* gene, two genome-specific pairs of primers were designed: AhSAD3A [5'-TATGGCCGCCACATCATGC-3'/5'-TCCAGTGACTTGAAAATCTCGAT-3'] and AhSAD3B [5'-TATGGCCGCCACATCATGG-3'/5'-TCCAGTGACTTGAAAATCTCGAT-3']. Each of the primer pairs produced a single product and amplified the target transcript with efficiency values ranging between 0.93 and 1.02 over a 1,000-fold range of input material.

The quantitative PCR mixture contained 3 μL of a 1:100 dilution of the synthesized cDNA (equivalent to 20 ng input RNA), primers to a final concentration of 0.4 μM each, 5 μL of the SYBR Green PCR Master Mix (Takara, Saint-Germain-en-Laye, France), and PCR-grade water to a total volume of 10 μL . Each reaction was performed in triplicate. The quantitative PCRs were performed in the Rotor Gene 6000 Real-Time PCR cycler (Qiagen, Valencia, CA). All PCR reactions were performed in a 72-well rotor (Qiagen) under the following conditions: 10 min at 95°C, 40 cycles of 5 s at 95°C, 15 s at 55°C, 10 s at 60°C, and 20 s at 72°C. Confirmation of amplicon specificity was based on the dissociation curve produced at the end of each run and visualization of the electrophoresis products on an 8% polyacrylamide gel.

DNA Analyses

Genomic DNA of the tetraploid line and the diploid progenitors (*A. duranensis* and *A. ipaensis*) was prepared from young leaves using the standard cetyl trimethylammonium (CTAB) method. These DNAs were used as the template for the PCR reaction with the primer pair [5'-ATGCAAATGCAAATAAGTTCCTTG-3']/[5'-TGAGTTGAGAGTTTAGACATGA-3'], in order to isolate the entire genomic portion of the *AhSad3* genes.

For Southern blot analysis, a 20- μg portion of DNA was completely digested by *Xba*I, *Cla*I, *Dra*I, *Ssp*I, *Eco*RI, and *Hind*III, separately. The digested products were loaded onto a 0.7% agarose gel and transferred to a nylon membrane, as described by Sambrook et al. (1989). The probe for the Southern blot was a 400-bp fragment from the beginning of the *AhSad3* gene. It was recovered from PCR products and **P32**-labeled using the **NEBlot kit** (Ipswich, MA), according to the manufacturer's directions. The hybridization to the membrane was conducted over night (O.N.) at 65°C, and the

hybridized membrane was washed with saline sodium citrate (SSC), as described by Sambrook et al. (1989).

Determination of Oil Content

The oil content of developing peanut seeds was determined as follows. From each of the developmental stages, one to ten seeds (depending on the size of the seeds) were weighed and kept overnight in a 60°C oven. Samples were then reweighed and ground with a mortar and pestle. Samples were placed in preweighed 2-mL tubes and 1.5 mL of hexane were added to each tube. Samples were mixed vigorously with a vortex for 20 s and incubated with shaking (Intellii-Mixer RM-2, ELMI Ltd., Moscow, Russia) for 1 h. Following incubation, the samples were centrifuged for 10 min at 13,000 rpm, and the upper suspensions were transferred to new preweighed tubes. This process was repeated for the peanut residue, followed by O.N. evaporation of the hexane and a final weighing of the tubes.

Results and Discussion

Characterization of the *ahSad* Gene Family in Peanut

In order to identify the number of independent *ahSad* genes that are actively expressed in *Arachis*, homologs from *Arabidopsis*, *Glycine*, and *Medicago*, sequences from GenBank were BLASTed against a peanut cDNA dataset (http://www.peanut.uga.edu/staging/blast_index.html). This analysis identified nine different contigs whose sizes varied between 226 and 1852 bp (Table 1). Predicted amino-acid sequence homology showed that these contigs can be clearly divided

Table 1 BLAST results of *ahSad* genes from *G. max*, *M. truncatula*, and *A. thaliana* against *A. hypogaea* cDNA sequences

Unigene name	Length (bp)	Gene name (with regard to soybean)
CL1Contig1443	711	SAD3
CL1Contig6893	1,339	SAD3
nmva.EVYLRH003FX3CH	204	SAD3
nc12c.EVJWSIG04H8BXU	226	SAD3
CL12963Contig1	253	SAD4? (new?)
CL1Contig4167	1,648	SAD1/2
CL1Contig14813	1,852	SAD1/2
DurSNP_c5858	545	SAD1/2
CL1Contig2691	389	SAD1/2

Only contigs with E-value for sequences level of similarity $\leq 1e-10$ are presented. The table shows the names of the *A. hypogaea* unigenes that were found, the length of unigene in base pairs, and the name of corresponding *Sad* genes from *G. max* (as resulted from the analysis presented in Fig. 1)

into two groups (Fig. 1). One group shares ~92% homology with both the SAD1 and SAD2 proteins from soybean (GenBank entries AY885234 and AY885233, respectively), while the second clusters best with soybean SAD3 (GenBank no. EF113911), with 80% homology. The two *Arachis* groups were only 65% homologous, demonstrating that these groups are more similar to the corresponding soybean proteins than to each other. Within each group, predicted protein sequences were over 98% homologous, indicating subgenome (homeolog-specific) differences or sequencing errors. One excluded contig was CL12963Contig1, which was only 90% homologous with the SAD3 group (Fig. 1, in black) and was, therefore, considered as a third group. Based on these results, we identified three *Sad* homologous genes in our *A. hypogaea* system. These genes are referred to as *AhSad1/2*, *AhSad3*, and *AhSad4*.

Quantitative reverse transcriptase PCR analysis was conducted to determine the relative abundance of *AhSad1/2*, *AhSad3*, and *AhSad4* at different seed developmental stages (20, 30, 40, 50, 60, and 70 DPA), as well as in five different tissues (leaf, peg, root, stem, and flower). As shown in Fig. 2, *AhSad3* is mostly expressed in developing peanut seeds and its expression corresponds fully to oil accumulation. On the other hand, *AhSad1/2* is expressed in developing seeds as well as all of the other examined tissues. Its level of expression in the examined flowers was even higher than that observed in the seeds. In all of the examined tissues, only trace levels of *AhSad4* mRNA were detected.

Our mRNA expression results are in full correspondence with the situation in soybean, a closely related and very important legume oilseed crop. In contrast to the situation in peanut, the high stearic trait in soybean is well established (summarized by Zhang et al. 2008). As presented in Fig. 1, three *Sad* genes (*SACPD-A*, *SACPD-B*, and *SACPD-C*) have been reported in soybean. While both *SACPD-A* and *SACPD-B* were found in all soybean lines surveyed, including high stearic acid types, no association was reported between these genes and the elevated stearic acid trait (Byfield and Upchurch 2007; Byfield et al. 2006). On the other hand, *SACPD-C* was found to be expressed only during seed development and at no other time, and changes in the *SACPD-C* gene sequences were found to correlate with elevated 18:0 levels in two high stearic soybean lines (A6 and FAM94-41) (Zhang et al. 2008). Our similar expression results also identify *ahSAD3* as a key target gene in developing peanut seeds. Therefore, we investigated *AhSad3* gene/s more closely, at the genomic and mRNA levels.

Genomic and Subgenomic Characterization of *AhSad3*

To recover full-length cDNA clones of the *AhSad3* genes in allotetraploid peanut, PCR amplifications were conducted

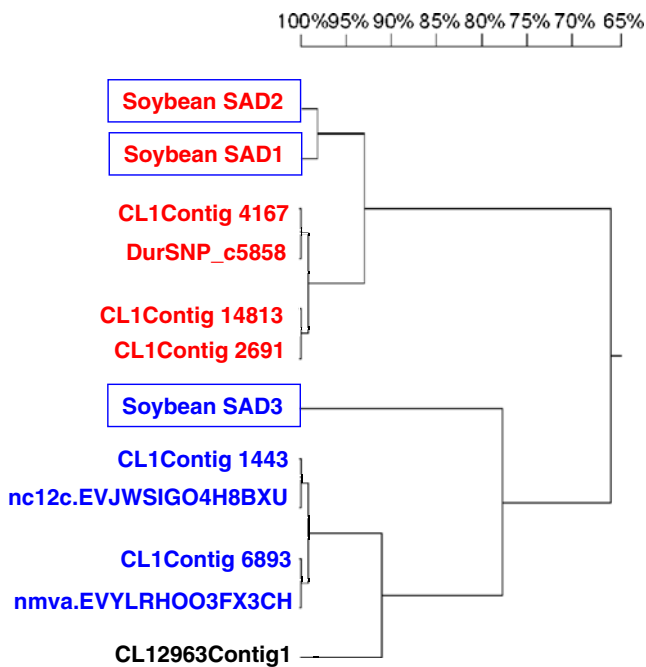


Fig. 1 Analysis of the homology of the deduced amino acid sequences of nine *Arachis* cDNA contigs and three known soybean SAD proteins. The homology is based on a sequence alignment analysis performed using the DNAMAN software (Lynnon; Pointe-Claire, Quebec, Canada). Homology rates are presented at the top of the figure. Colors indicate different SAD groups

on cDNA derived from 60-DPA developing seeds. Primers were designed based on the putative 5'- and 3'-untranslated regions of the CL1Contig6893 unigene. The ~1,160-bp-long product was cloned into plasmid vectors, and a set of clones (~20) was sequenced to evaluate its amplicon pool.

Two types of sequences corresponding to CL1Contig1443 and CL1Contig6893 unigenes were identified (Table 1). These genes were found to be homeologous, as evidenced by the results of the sequencing of the full-length *AhSad3* genomic region from the normal tetraploid peanuts and the A and B subgenomic diploid progenitors (*A. duranensis* and *A. ipaensis*, respectively; data not shown). A comparison of these two genes is shown in Fig. 3a. The open reading frames (ORFs) for both are 98% identical with seven single-nucleotide polymorphisms (SNPs) and one triplet insertion/deletion in the coding region. Both genes encode hypothetically active enzymes with no shifts in reading frame or truncated product. Structurally, both the A genome (*AhSad3A*) and B genome (*AhSad3B*) homeologs are similar to the soybean *Sad3* gene, with a sole intron located in the middle of the gene (Fig. 3b). In order to check for the presence of more copies of *AhSad3* in the peanut genome, a Southern blot analysis was conducted using five different restriction enzymes. High-stringency hybridization revealed one or two intense hybridizing bands (Fig. 4), indicating that there are no more than two copies of the *AhSad3* gene in the peanut tetraploid genome. We suggest that these two copies represent the A and B genomes. Some less intense hybridizing bands appeared on the blot as well, indicating some other closely related genes encoding SAD family proteins, perhaps *AhSad4*, which showed ~90% homology with *AhSad3* (Fig. 1).

Homeolog-Specific Gene Expression Analysis of *AhSad3*

Homeolog-specific gene expression analysis was performed to reveal the expression ratios between *AhSad3A* and

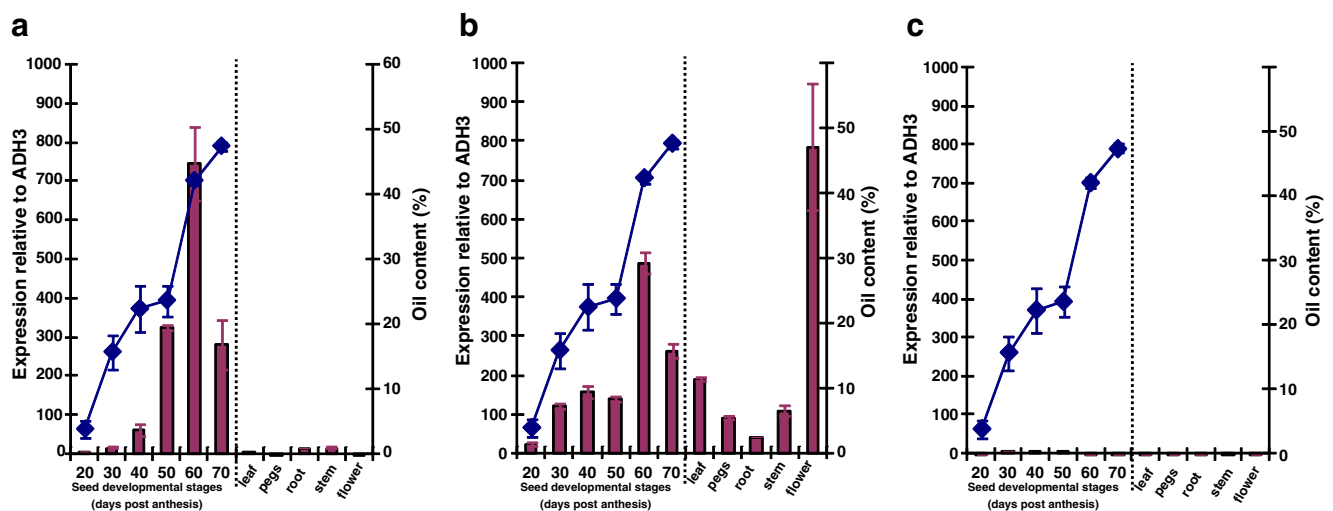


Fig. 2 Quantitative real-time PCR analyses of three *ahSad* genes using *adh3* as the reference gene. Numbers on the x-axis indicate the seed developmental stage (DPA indicates days post anthesis). Three

biological replications were sampled from each examined tissue. The blue line represents the percentage oil content. **a** *ahSad3*. **b** *ahSad1/2*. **c** *ahSad4*

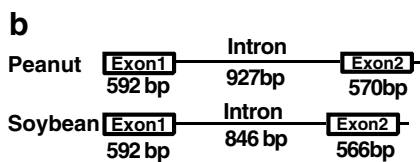
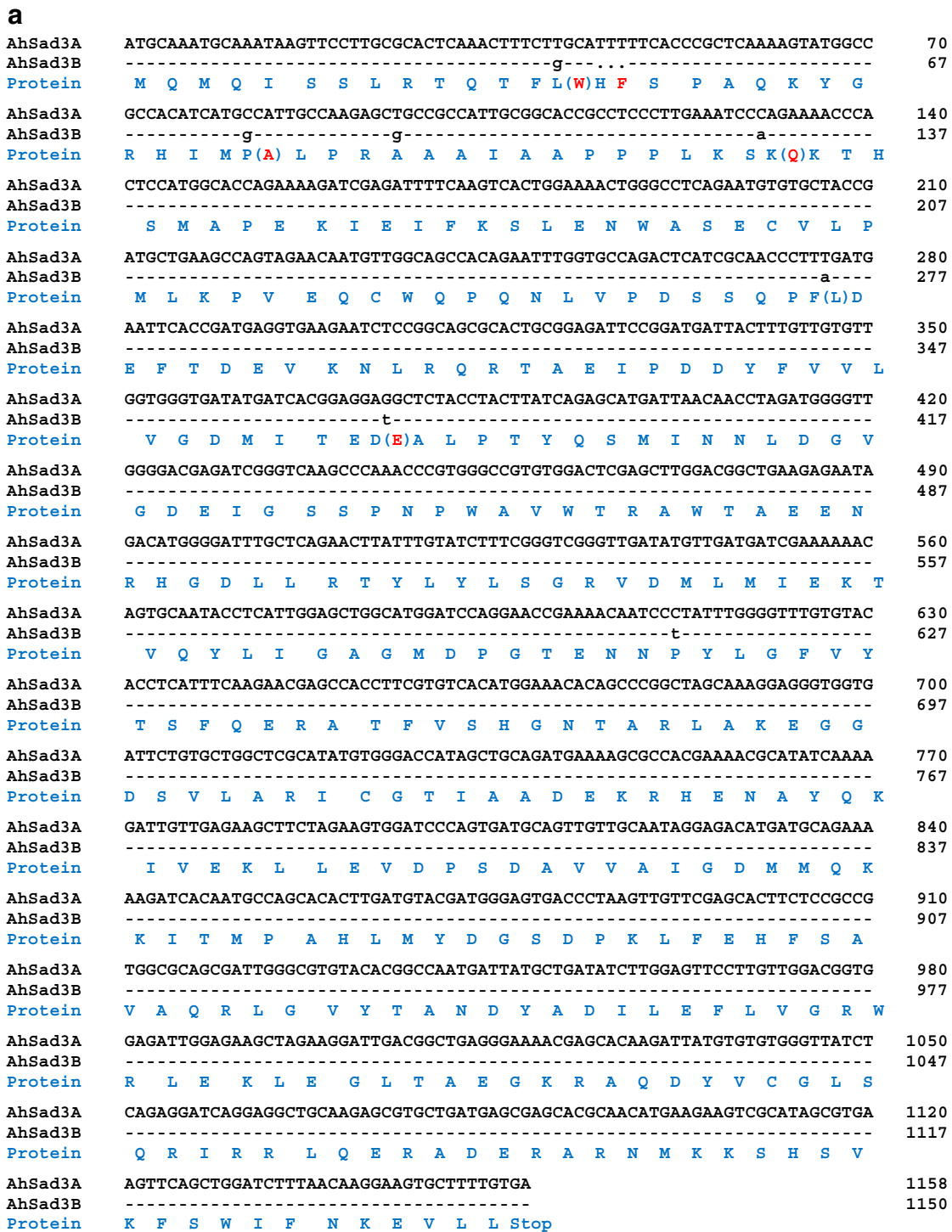


Fig. 3 Gene structure and predicted protein sequence of the *AhSad3* genes of peanuts. **a** Alignment of the two homologous peanut *AhSad3* genes. SNPs and an insertion are presented. The predicted protein is

presented as well (differences between the homologous proteins are indicated with red letters). **b** Comparison of the structure of the *AhSad3* gene with that of the soybean *Sad3* (*SACPD-C*) (Zhang et al. 2008)

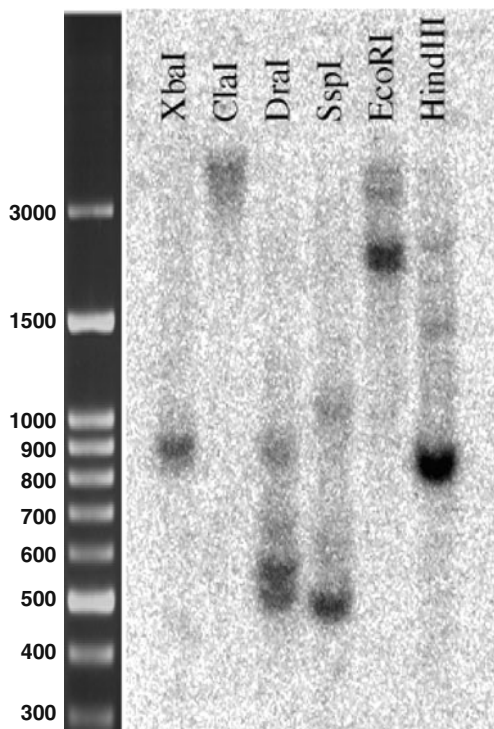


Fig. 4 Very stringent Southern blot analysis to determine the number of *AhSad3* copies in the peanut genome

AhSad3B in developing peanut seeds and other developing tissues. For this purpose, a quantitative real-time PCR procedure was developed. Based on the SNP information for *AhSad3A* and *AhSad3B*, several SNP-specific primers were designed. Initially, each set of primers was checked against the A- and B-diploid genomic DNAs (derived from *A. duranensis* and *A. ipaensis*, respectively). This was done to make sure that *AhSad3A*-specific primers would amplify only A-genome products and that *AhSad3B*-specific primers would amplify only B-genome DNA. Two pairs of primers met this condition (Fig. 5a) and, therefore, were used in the expression analysis. The genome-specific quantitative real-time PCR analysis showed that both *AhSad3* homeologs are expressed in developing seeds, but gene expression is significantly biased toward the B genome (Fig. 5b). Also, gene expression changes during seed development, while at 30, 40, and 70 DPA; the bias toward the B genome was significantly higher than that observed 20, 50, and 60 DPA. Note that at 50 and 60 DPA, when global *AhSad3* expression was highest (Fig. 2), the bias toward the B genome was smallest. This may indicate both genes' recruitment for stearyl-ACP desaturation in these oil filling-related developmental stages. The bias toward the B genome was also observed in other examined tissues. However, in the stem, expression was significantly biased toward the A genome. The results were validated by directly cloning and testing PCR products from cDNA

extractions derived from two extremely biased tissues. Twenty-three *AhSad3* clones were derived from 40 DPA seeds and from stems, representing B-biased and A-biased tissues, respectively. As shown in Fig. 5c, in developing seeds, 16 of the 23 clones were defined as *AhSad3B*; whereas in stems, only 8 of the 23 clones were defined as B genome, validating the real-time PCR results.

The results of alternations in expression ratios between *AhSad3A* and *AhSad3B* genes during seed development and among different tissue types are not surprising. In allopolyploid plants, it has been widely shown that patterns of gene expression are massively altered by both genome merger and subsequent genome doubling. This has been demonstrated in many angiosperm groups, including *Arabidopsis* (Ni et al. 2009), *Senecio* (Hegarty et al. 2009), *Triticum* (Pumphrey et al. 2009), *Tragopogon* (Buggs et al. 2009), maize (*Zea mays*; Riddle et al. 2009), *Brassica* (Gaeta et al. 2009), and *Gossypium* (Hovav et al. 2008a). In the latter (*Gossypium*), studies encompassing temporal scales ranging from immediate to longer evolutionary periods, and developmentally within the life of individual plants, have demonstrated expression alteration among different conditions, organs and tissues (Adams et al. 2003; Chaudhary et al. 2009; Liu and Adams 2007), and even over the course of the development of single cells (Hovav et al. 2008b).

In peanuts, differences in expression among homeologous genes have yet to be characterized. Some homeolog-specific changes in gene expression have been demonstrated in the *haFAD2* gene that encodes for delta-12-desaturase (oleoyl-PC desaturase), which catalyzes the addition of the second double bond onto oleic acid to produce linoleic acid. The absence of the activity of this enzyme is primarily responsible for the high O/L trait in peanut (Ray et al. 1993). As in *AhSAD3*, two homeologous genes, *ahFAD2A* and *ahFAD2B*, which share 99% sequence homology, encode for the ahFAD2 enzyme (Jung et al. 2000). In an attempt to clone the mutant allele of *ahFAD2B* in a high O/L peanut line (8–2,122) using mRNA extracted from stage 2 immature peanut seeds, Jung et al. (2000) found a very strong bias toward the A-genome homeolog. Of 13 clones detected, none belonged to the *ahFAD2B* allele. In another study (Chu et al. 2009), 23 clones from stage 2 seeds from a similar high O/L line were screened, and only 1 clone was identified as the *ahFAD2B* allele. This strong bias in expression toward the *ahFAD2A* genome was explained by the presence of nonsense-mediated mRNA decay, a universal cellular system that prevents truncated mRNA from producing detrimental proteins (Muhlemann et al. 2008). Because the *ahFAD2B* alleles of the high O/L lines studied are mutated at the 441_442insA position, which produces a premature stop codon for translation, transcripts from the mutant allele would be subject to nonsense-mediated mRNA decay, and their steady-state expression

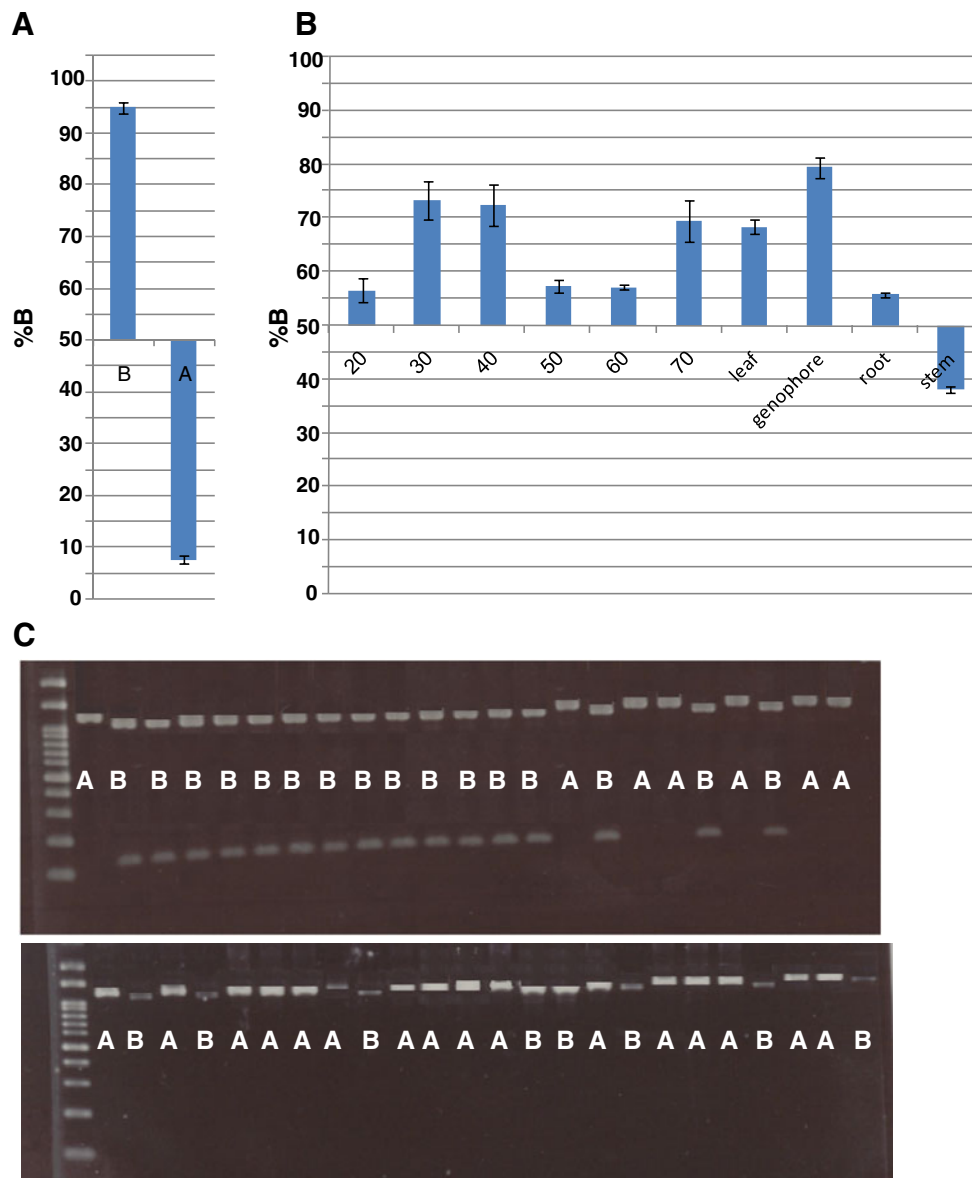


Fig. 5 Analysis of homolog-specific mRNA expression of *AhSad3* genes. **a** Calibration of real-time PCR primers on genomic DNA. A set of A genome and B genome-specific primers was designed for the *AhSad3* gene (*AhSad3A* and *AhSad3B*, respectively). Afterward, real-time PCR analyses were performed using each specific primer set, in which the templates were the genomic DNA of the diploid peanuts' progenitors (A—*A. duranensis*; B—*A. ipaensis*). The expression of *AhSad3B* relative to *AhSad3A* was calculated. For convenience, the graph shows the relative expression of the B genome in percentages. **b** Analysis of homolog-specific mRNA expression of *AhSad3* genes in developing seeds and in four other tissues (same procedure as in

section **a**). **c** Validation of the homolog-specific real-time PCR analysis in two samples (40 DPA developing seeds and stem). PCR reactions were conducted with primers for the entire *AhSad3* gene. The PCR was halted at different cycles, and the first cycle to yield visible bands was selected. From this cycle, a PCR band was excised from the gel, cloned into a pGEMT vector, and transformed into *Escherichia coli*. From each sample, 23 random colonies were picked, and another PCR reaction was performed. The PCR products were cut with the *NotI* restriction enzyme, which cut only the *AhSad3B* sequence. *Upper photograph*—40 DPA developing seeds; *lower photograph*—stems

levels would be subsequently reduced. In our study, however, both *ahSad3* homeologs are predicted to encode for fully functional enzymes, and this can explain the smaller bias toward the B genome.

Interestingly, *AhSAD3* and *ahFAD2* are conjugated in the same fatty acid metabolic pathway and are both located

within chloroplasts. In an initial study, we also noticed that both *ahSad3A* and *ahSad3B* are expressed in 50 DPA developing seeds of a high O/L peanut line (GRIN PI599592), with some bias toward the B genome (data not shown). The differences in homeolog-specific expression patterns of these two genes in developing seeds may

indicate the existence of independent evolutionary systems that shaped the subgenomic expression patterns of fatty acid metabolism-related genes during polyploidy formation and the evolution of *A. hypogaea*. This evolutionary question, however, should be further examined in experiments containing additional genes in the duplicated fatty acid metabolic pathway and including more tissue types/developmental stages and more diploid and tetraploid *Arachis* lines.

In summary, we have identified three members of the *ahSad* gene family. Only one of these genes, *ahSad3*, was mainly expressed during the development of seeds and with full correspondence to oil accumulation. Both *ahSad3* homeologous genes (*ahSad3A* and *ahSad3B*) were characterized at the genomic and mRNA expression levels. Homeolog-specific gene expression analysis showed that both *ahSad3* homeologs are expressed in developing seeds and that gene expression is significantly biased toward the B genome. Collectively, these results support our hypothesis that silencing or inactivating both homeologous copies of the *ahSad3* gene should increase the 18:0 levels in peanut seeds.

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References

- Adams KL, Cronn R, Percifield R, Wendel JF (2003) Genes duplicated by polyploidy show unequal contributions to the transcriptome and organ-specific reciprocal silencing. *Proc Natl Acad Sci USA* 100:4649–4654
- Brand Y, Hovav R (2010) Identification of suitable internal control genes for quantitative real-time PCR expression analyses in peanut (*Arachis hypogaea*). *Peanut Sci* (in press)
- Bubeck D, Fehr W, Hammond E (1989) Inheritance of palmitic and stearic-acid mutants of soybean. *Crop Sci* 29:652–656
- Buggs RJ, Doust AN, Tate JA, Koh J, Soltis K, Feltus FA, Paterson AH, Soltis PS, Soltis DE (2009) Gene loss and silencing in *Tragopogon miscellus* (*Asteraceae*): comparison of natural and synthetic allotetraploids. *Heredity* 103:73–81
- Byfield G, Upchurch R (2007) Effect of temperature on delta-9 stearoyl-ACP and microsomal omega-6 desaturase gene expression and fatty acid content in developing soybean seeds. *Crop Sci* 47:1698–1704
- Byfield G, Xue H, Upchurch R (2006) Two genes from soybean encoding soluble delta 9 stearoyl-ACP desaturases. *Crop Sci* 46:840–846
- Cahoon EB, Shah S, Shanklin J, Browse J (1998) A determinant of substrate specificity predicted from the acyl-acyl carrier protein desaturase of developing cat's claw seed. *Plant Physiol* 117:593–598
- Chaudhary B, Flagel L, Stupar RM, Udall JA, Verma N, Springer NM, Wendel JF (2009) Reciprocal silencing, transcriptional bias and functional divergence of homeologs in polyploid cotton (*Gossypium*). *Genetics* 182:503–517
- Chu Y, Holbrook CC, Ozias-Akins P (2009) Two alleles of *ahFAD2B* control the high oleic acid trait in cultivated peanut. *Crop Sci* 49:2029–2036
- FAOSTAT (2010) <http://faostat.fao.org/default.aspx>
- Gaeta RT, Yoo SY, Pires JC, Doerge RW, Chen ZJ, Osborn TC (2009) Analysis of gene expression in resynthesized *Brassica napus* allopolyploids using arabidopsis 70mer oligo microarrays. *PLoS ONE* 4:e4760
- Hamdan Y, Perez-Vich B, Fernandez-Martinez J, Velasco L (2009) Novel safflower germplasm with increased saturated fatty acid content. *Crop Sci* 49:127–132
- Hegarty MJ, Barker GL, Brennan AC, Edwards KJ, Abbott RJ, Hiscock SJ (2009) Extreme changes to gene expression associated with homoploid hybrid speciation. *Mol Ecol* 18:877–889
- Hovav R, Chaudhary B, Udall JA, Flagel L, Wendel JF (2008a) Parallel domestication, convergent evolution and duplicated gene recruitment in allopolyploid cotton. *Genetics* 179:1725–1733
- Hovav R, Udall JA, Chaudhary B, Rapp R, Flagel L, Wendel JF (2008b) Partitioned expression of duplicated genes during development and evolution of a single cell in a polyploid plant. *Proc Natl Acad Sci USA* 105:6191–6195
- Jung S, Powell G, Moore K, Abbott A (2000) The high oleate trait in the cultivated peanut [*Arachis hypogaea* L.]: I. Isolation and characterization of two genes encoding microsomal oleoyl-PC desaturases. *Mol Gen Genet* 263:796–805
- Kachroo A, Shanklin J, Whittle E, Lapchuk L, Hildebrand D, Kachroo P (2007) The Arabidopsis stearoyl-acyl carrier protein-desaturase family and the contribution of leaf isoforms to oleic acid synthesis. *Plant Mol Biol* 63:257–271
- Katan MB (1998) Health effects of *trans* fatty acids. *Eur J Clin Invest* 28:257–258
- Knutzon DS, Thompson GA, Radke SE, Johnson WB, Knauf VC, Kridl JC (1992) Modification of *Brassica* seed oil by antisense expression of a stearoyl-acyl carrier protein desaturase gene. *Proc Natl Acad Sci USA* 89:2624–2628
- Liu Q, Singh S, Chapman K, Green A (2009) Bridging traditional and molecular genetics in modifying cottonseed oil. In: Paterson AH (ed) *Genetics and genomics of cotton*. Plant genetics and genomics: crops and models 3. Springer Science Business Media, New York, pp 1–30
- Liu Q, Singh S, Green A (2002) High-oleic and high-stearic cottonseed oils: nutritionally improved cooking oils developed using gene silencing. *J Am Coll Nutr* 21:205–211
- Liu Z, Adams K (2007) Expression partitioning between genes duplicated by polyploidy under abiotic stress and during organ development. *Curr Biol* 17:1669–1674
- Lou T, Deng WY, Zeng J, Zhang FL (2009) Cloning and characterization of a stearoyl-acyl carrier protein desaturase gene from *Cinnamomum longepaniculatum*. *Plant Mol Biol Rep* 27:13–19
- Muhlemann O, Eberle AB, Stalder L, Zamudio Orozco R (2008) Recognition and elimination of nonsense mRNA. *Biochim Biophys Acta* 1779:538–549
- Ni Z, Kim ED, Ha M, Lackey E, Liu J, Zhang Y, Sun Q, Chen ZJ (2009) Altered circadian rhythms regulate growth vigour in hybrids and allopolyploids. *Nature* 457:327–331
- Norden AJ, Gorbet DW, Knauff DA, Young CT (1987) Variability in oil quality among peanut genotypes in the Florida breeding program. *Peanut Sci* 14:7–11
- Pattee HE, Isleib TG, Gorbet DW, Moore KM, Lopez Y, Baring MR, Simpson CE (2002) Effect of the high-oleic trait on roasted peanut flavor in backcross-derived breeding lines. *J Agric Food Chem* 50:7362–7365
- Pumphrey M, Bai J, Laudencia-Chingcuanco D, Anderson O, Gill BS (2009) Nonadditive expression of homologous genes is established upon polyploidization in hexaploid wheat. *Genetics* 181:1147–1157

- Ray TK, Holly SP, Knauff DA, Abbott AG, Powell GL (1993) The primary defect in developing seed from the high oleate variety of peanut (*Arachis hypogaea* L.) is the absence of Δ 12-desaturase activity. *Plant Sci* 91:15–21
- Riddle NC, Jiang H, An L, Doerge RW, Birchler JA (2009) Gene expression analysis at the intersection of ploidy and hybridity in maize. *Theor Appl Genet* 120:341–353
- Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular cloning: a laboratory manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor
- Shanklin J, Cahoon EB (1998) Desaturation and related modifications of fatty acids. *Annu Rev Plant Physiol Plant Mol Biol* 49:611–641
- Shanklin J, Somerville C (1991) Stearoyl-acyl-carrier-protein desaturase from higher plants is structurally unrelated to the animal and fungal homologs. *Proc Natl Acad Sci USA* 88:2510–2514
- Thompson GA, Scherer DE, Foxall-Van Aken S, Kenny JW, Young HL, Shintani DK, Kridl JC, Knauf VC (1991) Primary structures of the precursor and mature forms of stearyl-acyl carrier protein desaturase from safflower embryos and requirement of ferredoxin for enzyme activity. *Proc Natl Acad Sci USA* 88:2578–2582
- Tong L, Shu-Ming P, Wu-Yuan D, Dan-Wei M, Ying X, Meng X, Fang C (2006) Characterization of a new stearyl-acyl carrier protein desaturase gene from *Jatropha curcas*. *Biotechnol Lett* 28:657–662
- Uematsu T, Parkányiová L, Endo T, Matsuyama C, Yano T, Miyahara M, Sakurai H, Pokorný J (2002) Effect of the unsaturation degree on browning reactions of peanut oil and other edible oils with proteins under storage and frying conditions. *Int Congr Ser* 1245:445–446
- USDA (2010) <http://www.nal.usda.gov/fnic/foodcomp/search>
- Whittle E, Cahoon EB, Subrahmanyam S, Shanklin J (2005) A multifunctional acyl-acyl carrier protein desaturase from *Hedera helix* L. (English ivy) can synthesize 16- and 18-carbon monoene and diene products. *J Biol Chem* 280:28169–28176
- Zaborowska Z, Starzycki M, Femiak I, Swiderski M, Legocki AB (2002) Yellow lupine gene encoding stearyl-ACP desaturase—organization, expression and potential application. *Acta Biochim Pol* 49:29–42
- Zainuddina A, Parkanyiova J, Parkanyiova L, Pokorhy J, Sakurai H (2004) Comparison of oxidative resistance of traditional and high-oleic peanut oils in emulsions. *Czech J Food Sci* 22:136–139
- Zhang P, Burton JW, Upchurch RG, Whittle E, Shanklin J, Dewey RE (2008) Mutations in a Δ 9-stearyl-ACP-desaturase gene are associated with enhanced stearic acid levels in soybean seeds. *Crop Sci* 48:2305–2313