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Isolation and characterization of the Δ^{12}-fatty acid desaturase in peanut (*Arachis hypogaea* L.) and search for polymorphisms for the high oleate trait in Spanish market-type lines

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Abstract An understanding of the molecular mechanisms that are responsible for increased oleic acid accumulation would open avenues to alter peanut fatty acid composition and allow detection of polymorphic regions which can be used for marker assisted selection (MAS). Δ^{12}-Fatty acid desaturase (FAD) was isolated and characterized from genotypes having a low or high oleic to linoleic acid O/L ratio – genotypes, Tamspan 90 (T-90) and F435–2-2 (F435), respectively. Southern blots showed three to four copies per haploid genome, and no major differences in organization between the two parental lines. Approximately 3525 bp was isolated from both genotypes, including a genomic walk toward the promoter region. The Δ^{12}-Fad contains a putative intron, the coding region at the 3′ end, and an open reading frame (ORF) of 1140 bp encoding 379 amino acids. Comparisons of the coding sequences from the high and low oleic acid genotypes revealed several single nucleotide polymorphisms (SNPs). Two polymorphisms appear to be associated with the high O/L trait. The first is an "A" insertion 442 bp after the start codon. The "A" insertion shifts the amino acid reading frame, probably resulting in a truncated, inactive protein and the loss of one of three histidine boxes believed to be involved in metal ion complexation required for the reduction of oxygen. Another polymorphism at 448 bp from the start codon results in an amino acid change. The region containing the polymorphisms was amplified from leaf tissue of several independently derived backcross lines (IDBLs). Most high O/L lines had either the "A" insertion or the amino acid substitution.

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Introduction

Peanut (*Arachis hypogaea*) is a global crop, providing a high-protein, high-energy food source. The peanut seed is comprised of 45–51% oil, of which approximately 80% consists of oleic and linoleic acids. Whether the peanut is used as a source of oil or nuts, the quality of the product depends on the properties of the oil. Oxidized lipids have a significant impact on storage stability and development of off-flavors, making fatty acid composition an important component of oil quality. Oils with higher ratios of oleic to linoleic acids are less prone to oxidation and the development of off-flavors. The ratio of oleic to linoleic acid (O/L) is a measure of oil stability (Holley and Hammons 1968; Worthington and Hammons 1977). In most commercial peanuts, the O/L ratio varies from 1:1 to 2.5:1, with Spanish types typically at the low end of the scale. The development of varieties with higher O/L ratios will help maintain whole seed and oil quality of peanut.

Increased knowledge of lipid metabolism in other crops has led to an understanding of cellular development and function and, ultimately, to the modification of oil composition. Our understanding of the mechanisms and regulation of chloroplast and endoplasmic reticulum desaturases is primarily based on the characterization of *Arabidopsis* mutants for fatty acid desaturation and acylation (Browse and Somerville 1991; Ohlrogge 1994). This information has led to the genetic modification of oil composition for several oil seed crops and other organisms. These include: rapeseed (Weier et al. 1998), *Brassicaceae* (Zou et al. 1997), *Petunia hybrida* (Choudhary et al. 1994), *Synechococcus* (Reddy et al. 1993), and *Synechocystis* PCC6803 (Wada et al. 1992). Several genes involved in the fatty acids pathway have also been cloned and characterized, including fatty acid

desaturase 2 (fad2) from *Arabidopsis* (Okuley et al. 1994), biotin carboxylase and carboxyl transferase with two subunits from *B. napus* (Elborough et al. 1995); FAD2-1 and FAD-2, which encode microsomal soybean ω–6 desaturases (Heppard et al. 1996), three genes coding for different multi-functional acetyl-CoA carboxylase enzymes from *Brassica napus* (Wolfgang et al. 1997), Δ^9 fatty acid desaturase from *Cryptoccus curvatus* (Meesters et al. 1997), and Δ^6 fatty acid desaturase from borage (Sayanova et al. 1997).

Membrane and reserve lipids of plants contain fatty acids with varying degrees of unsaturation which are controlled by the action of different desaturases (Schmidt et al. 1994). An important class of enzymes involved in the synthesis of polyunsaturated fatty acids are the fatty acids desaturases (Fads) that catalyze the introduction of double bonds into the hydrocarbon chain. In plant membrane lipids, the major locations for double bonds are at the Δ^9, $-^{12}$, and $-^{15}$ (ω-3) positions of 18 carbon acyl chains (Browse and Somerville 1991). Δ^{12}-Oleate Fad desaturates oleoyl phosphatidychloline (PC) to 18:2-PC, thereby converting oleic acid to linoleic acid (Harwood and Page 1994). One highly conserved feature of all membrane-bound desaturases is the presence of three histidine boxes, with the general sequence HXXXH. These boxes may be involved in metal ion complexation required for oxygen reduction (Schmidt et al. 1994).

Δ^{12}-Fads have been targeted as logical candidates controlling the high oleate trait. In sunflower, genes encoding Δ^{12}-oleate Fad were used as candidates for the dominant mutation (*Ol*) producing high and low oleic germplasm lines (Hongtrakul et al. 1998). They isolated a full-length cDNA clone (OLD-7) from a developing seed cDNA library of sunflower. Restriction fragment analysis showed that OLD-7 is duplicated, rearranged in mutant lines and linked to the *Ol* locus. Thus, increased oleic acid content seems to be caused by DNA changes affecting OLD-7 *cis* regulatory sequences. In peanut, Δ^{12}-Fad activity was tenfold lower in a high oleic acid line than in its normal oleic acid isoline (Ray et al. 1993). They also demonstrated that the Δ^{12}-Fad activity was localized in the microsomes. No changes in other enzyme activities associated with Δ^{12}-Fad were detected.

In an evolutionary nascent species like cultivated peanut, it is likely that simple nucleotide substitutions, rather than gross differences, account for variation among genotypes (Guohao and Prakash 1997). Sequence information for the genes involved in the fatty acid pathway might lead to the development of molecular markers useful for accelerating selection of high O/L plants. In the study described here, sequences responsible for C18 fatty acid desaturation (Δ^{12}) were retrieved from the GenBank and used as probes to amplify the Δ^{12}-Fad gene from the low and high O/L Spanish peanut lines, T-90 and F435, respectively. The genomic sequence of the Δ^{12}-Fad gene was determined for both parental lines. The search for molecular polymorphisms and a genome walk toward the promoter region are discussed.

Materials and methods

Plant material

Two parental lines, T-90 (Smith et al. 1991) and F435 (Norden et al. 1987), were grown in the greenhouse (24°–30°C average temperature and sandy loam soil). T-90, the low O/L (approx. 1.3:1.0) parent, is a Spanish variety widely grown in Texas and parts of Oklahoma. It is resistant to sclerotinia blight (*Sclerotinia minor*) and pod rot (a complex caused mainly by *Pythium myriotylum* Drechs. and *Rhyzoctonia solani* Kuhn). Line F435, the high O/L (approx. 34.0:1.0) parent, is a Spanish-type breeding line from Florida. All crosses were made under greenhouse conditions at College Station, Texas, following standard artificial hybridization procedures for peanut (Knauft et al. 1987). T-90 was crossed to F435 in 1990. A backcross toward T-90 was then made in 1991. Plant-row populations from the BC_1 were planted in College Station, Texas. Samples consisting of ten seeds were analyzed for the O/L ratio from each plot. In the F_4 generation, individual plants from BC_1 plant-rows having an intermediate O/L ratio (2.7–9.0) were selected for low and high O/L ratio, selfed, and advanced to the F_9 generation to form independently derived backcross lines (IDBLs). Off-season production in Puerto Rico was included to hasten the formation of the IDBLs. Unopened leaves were collected from T-90, F435, eight high O/L IDBLs and eight low O/L IDBLs at 20–40 days after planting. The leaves were immediately frozen in liquid N_2 and stored at –80°C for further analysis.

Oil analysis

For each sample, a piece of cotyledon from the non-embryo end of the seed was detached, peeled from the testa, and stored in a 0.5 ml micro-centrifuge tube (VWR, Scientific Products, West Chester, Pa.) at –20°C. The embryo portion was saved for planting. Samples were extracted by adding 2 ml hexane with 0.01% butylated hydroxytoluene (BHT) and vortexed. A boiling chip was added, along with 2 ml H_2SO_4:methanol (1:99), and vortexed. Samples were placed in a heat block at 90°C until 0.5 ml of solution was left in the tube and then allowed to cool to room temperature. Two milliliters of hexane with 0.01% BHT was added, and the samples were again vortexed and held at room temperature for 30 min. The organic phase was transferred to a pre-weighed, capped vial. The solvent was evaporated under a fume hood with a stream of nitrogen gas. The tubes with the extracted oil were weighed, and the exact weight of the oil sample was calculated. Each sample was dissolved in ethyl acetate to a concentration of 1 ug/ml in a 2-ml borosilicate glass vial.

Chromatography profiles

A 540 Tremetric GC (Tremetric™, Austin, Tex.), with an automatic sample injector was used for analysis of the methyl ester fatty acids. Two-microliter aliquots were injected for each analysis. Injector and detector temperatures were set at 250°C. The oven was programmed for an initial temperature setting of 95°C for 2 min, then increased at a rate of 1.8°C per minute until a temperature of 130°C was obtained, which was held for 3 min. The temperature was then increased at a rate of 10°C per minute to a maximum of 150°C. The column was an Rtx 2330 (30 m×0.53 mm, and 0.2-µm film thickness; Restek, Belefonte, Pa.). Standard methylester fatty acid mixtures (AOCS-2, Sigma, St. Louis, Mo.) were used to identify the fatty acids by comparison of retention times. The fatty acids are reported as the proportion of oleic to linoleic.

DNA isolation

Total DNA was isolated and purified from the frozen tissue following the method described by Chen and Dellaporta (1994).

Table 1 Oligonucleotide primer names (Oligo), sequences, and function in the amplification of Δ^{12}-fatty acid desaturase in peanut and genome walk toward the 5′ end

Oligo	Nucleotide sequence (5′–3′)	Function
AH12F	GCGAGATTCATCATAGGAGAAGGACTC	Δ^{12}-Fad
AH12R	TCAGAACTTGTTCTTCTACCAATAAACACC	Δ^{12}-Fad
P12I	GAATTATATAGAAGAATACAATGAGCAAAGCAGAATGC	Genomic walk 5′ end
P12II	GCTGAACAAAGAAATAATGAAAGTTTATAATAAAAGG	Genomic walk 5′ end
SF2	CATTAACTCCTTCTTGGGTG	Sequencing forward
SF3	CAGCAAGTACCAACTTCTTGA	Sequencing forward
SR2	CTTGGTATACCATGATACCTT	Sequencing forward
SR3	AGATATGTTACAGCAAAGAC	Sequencing reverse
SR4	CTACAAAGCTAATGGTTCCTG	Sequencing reverse
SR5	CACATGACACATGCACAG	Sequencing reverse
M13F	TGTAAAACGACGGCCAGT	Sequencing
M13R	AGCGGATAACAATTTGACACAGG	Sequencing
AP1	GGGATCCTAATACGACTCACTATAGGGC	Adaptor
AP2	AATACGACTCACTATAGGGCTCGAGCGGC	Adaptor

Candidate gene approach: amplification and cloning Δ^{12}-Fad from genomic DNA

Primers were designed based on published Δ^{12}-Fad sequences retrieved from GenBank for *Arachis hypogaea* L. Genomic DNA from T-90 and F435 (50 ng/μl) was used as a template for the amplification of Δ^{12}-Fad using the primers AH12F and AH12R (Table 1). [Reaction mixture $MgCl_2$ and 1 U elongase enzyme, (GIBCO/BRL Life Technologies, Baltimore, Md.)]. Polymerase chain reaction (PCR) conditions were: 1 min at 94°C followed by 35 cycles of 30 s at 94°C, 3 min at 68°C, 7 min at 72°C, an a final 7-min cycle at 72°C. The original PCR products and PCR products digested with *Sau*3A and *Hae*III were run on a 1% agarose gel containing 1 mg/ml of ethidium bromide for 20 h at 0.5 v/cm.

The amplified PCR product (2.8 kb, Fig. 1) was eluted from the gel using the Gel Extraction Kit (Qiagen, Valencia, Calif.) and cloned using the Topo Zero Blunt Cloning Kit (Invitrogen, Carlsbad, Calif.). Several positive clones containing the 2.8-kb fragment were obtained from each genotype. Clone no. 12 for T-90 and Clone no. 14 for F435 were selected and used for initial sequencing and genomic walk experiments. The 2.8-kb insert was sequenced using primers M13F and M13R (Table 1). In order to obtain the sequence of the full-length clones, we designed additional forward and reverse sequence primers (sequentially designed as SF2, SF3, SR2, SR4, and SR5; Table 1). PCR conditions were: 1 min at 96°C followed by 45 cycles of 10 s at 96°C, 10 s at 50°C, 4 min 60°C. Nucleotide sequences were determined by dideoxy automated sequencing using the ABI 373 sequencer (Applied Biosystems, Foster City, Calif.). Sequences were compiled and analyzed using the Sequencher version 3.0 software package from the Gene Codes (Madison, Wis.). Amino acid alignment was conducted using the software package ClustalW 1.7 (Baylor College of Medicine, Human Genome Sequencing Center, Houston, Tex.). Sequence comparisons were done using the basic local alignment search tool (BLAST, Altschul et al. 1990).

Genome walking to 5′ end

The technique of Siebert et al. (1995) was used to characterize 5′ flanking sequences. *Dra*I and *Hind*III digests were chosen to initiate the genome walk. Two primers (P12I and P12II, Table 1) were designed and used to amplify 5′ flanking sequences. Cloning and sequencing were performed as previously described.

Amplification of region of clustered SNPs for the parents and the IDBLs

To confirm the polymorphisms found between the two parental lines, a region of clustered SNPs was amplified from 14 clones of each of the two parental lines and 1 of each of the 16 IDBLs using *Taq* enzyme (2.5 U) and primers SF3 and AhR (Table 1). PCR conditions were: 30 s at 94°C followed by 35 cycles of 15 s at 94°C, 30 s at 58°C, 1 min at 68°C and a final 5-min cycle at 68°C. Previously described cloning and sequencing procedures were followed.

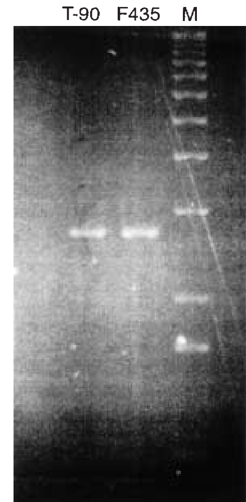

Fig. 1 A 2.8-kb-amplified PCR product from T-90 (left) and F435 (right). M Marker

Southern Blotting

Genomic DNA of T-90 and F435 was completely digested with *Eco*RI and *Dra*I. The fragments were extracted with phenol and run on a 1% agarose gel for 16 h at 26 V with standards. Southern blotting was performed following standard methods (Sambrook et al. 1989).

Results and discussion

Candidate gene approach

The initial objective of the candidate gene approach was to check for polymorphisms in size or restriction sites between the low (T-90) and high (F435) O/L ratio genotypes. Differences found between the two parental genotypes would have been useful for the development of a technique to distinguish low versus high O/L ratio genotypes. The Δ^{12}-Fad-specific primers amplified a 2.8-kb fragment from each genotype (Fig. 1). Digestion with

Fig. 2 Genomic sequence of Δ^{12}-fatty acid desaturase from Tamspan 90. Potential TATA box is in *bold*, intron is in *lower-case* print; stop and start codons are *underlined*

1	TGTGATGGATATCTGCAGAATTCGCCCTTAATACGACTCA
41	CTATAGGGCTCGAGCGGCCGCCCGGGCAGGTAATTCCTTG
81	AATCATCCTTAATAGTTTATTTCTCATATATATTTTTTAG
121	TCATTTCTCACACACACATATATAGAGAAACCCAAAACTC
161	CTAATCTTTATTTGCATAACCCTTCTTCAATTCAGAGATC
201	ACTAAAGTTGTCCATACTCTATCCAACCTCTCTTGCAGCC
241	ACCCCCTCGTCACCCTTCTCACCGGCATCATCACACCTCA
281	CCGTGCCATCACCCTTACTGCGTCGTAATTTCTATTTGTG
321	GCGTTCCTCTTCGTAACTTTGTCGCGTGTCCATTCTCATT
361	CTCCTCTCTGTTGCCGCGTCTCTCACCTCGTCCATTGTTT
401	TATCCTTTGGCTCGTCGTTGCGTCCCCCCATTGCGTTATT
441	TCGAAAGAAGTCACCATCGTATCATTTAGACTTTTTGTAT
481	AAAATCTTGCAGTTTTTGTATAAGATAGATACTTGCAGCT
521	CTATTCATATGAAAATTAATAATTAGTTAGAACTATTATG
561	TAGATGGGGAATGGGGTCCCCGCGGGGAATGGGGCTCCGT
601	GGGGAATGAGGATGGGGACCAATATTCCCCCACGGCGGGG
641	AATGGGGGCGGGGATGGGGAGCAAATCTGAGGGCGGGAAT
681	GGCAGGGAGGCATCCCCCGCCCCTGCCCTGCCCCGTTGAC
721	ATCCCTAGCCGCGGGTTCGGATAG**TATTATATA**AAAACCC
761	AATGTGAGTGAGACAACAACTTAACACATTACATAAAACC
801	TTAAACGTGGCTGCGAGATTCATCATAGGAGAAGCACTCA
841	TTTCTCTTCTCTCTGTTGGAATTGCTTTCACGgtttgcac
881	tatgtcccttttattataaaactttcattatttctttgtt
921	ctgcacttctgcttctgctcattgtattcttctatataat
961	tcatgcaaatcgctctaaaaaattgaactcgtgttgctgg
1001	tttcttcttgtgtccatttctataacatcaacatgcatgc
1041	ttggataacttcttattttgatcttttataataaccttgg
1081	attttctgaattctgaagtaagggttggtgaagtttcttt
1121	ccatgttatgtctactggatttcagattctgcattaaaca
1161	atatcaatgagaatgctgacaaatttcttttatcctcaga
1201	cacacagttttgttatctcagctggtttcatagccgccgt
1241	ttttcatttttctttttatttatttatttatttcttttggaatc
1281	ctttggaatctctgaagattttgaactccttcttggaaat
1321	gacacctatcatttatatctttacatatatgacatgaaaa
1361	ttgtcatatagaaataggaatatctcttaaaccataagat
1401	tatttctgttgcaaaatgttttcttctccccatcactttttt
1441	cttttttaatattttttattttctggctccaagtccaagcaa
1481	taattaatgggaccctttttactctttcatggatagtaaa
1521	tatataaataaggaatgtaatgtaatacaaaaactccaca
1561	acttctcttttggagtatagttgcaatttttttatcca
1601	taatcttttccttgaggttaatattcttttctaatttgcc
1641	ttgtaaatgactgaattatatttgatttttatttttttctgt
1681	tagtatgaattactaaaccagaaatccattgccatttcct
1721	ttagtagtataattcattctggttgaagagaaaaattaga
1761	agaaaatatttgcatacattaaggcattagtaattgcttc
1801	tgggccctcactgatatatgattttgtcattgtgtatttg
1841	tgtttggtttatgatagtttttgcatgtcaacctcacacg
1881	tatactaatacaagtgaacctcagaatcatgccccttaca
1921	actatatatatagtgtgaaattaatgtttctgtgcatgtg
1961	tcatgtgagtctgtgacactgctaccaccatccaaaatcc
2001	aaacagttgtgtgtgttgtagtggttaacccctggcacac
2041	tcttcaagctcttttgctctgtccctcattatagaagatg
2081	catgctttcttgtcaaatgaagggtgcaaaacatcatcac
2121	tcatactagtcacttggcatgataagctaaattgtgatat
2161	ttgaatgccacatgtgtttggaaattggtgtctattgtgt
2201	taggcattttcacatcctttcttttcctttctctattttt
2241	ataggattcccaaggcataatagaacttcaaataactggt
2281	tgtaatatcagaaatcctttctttgctgtttttctggtca

*Sau*3A and *Hae*III also revealed no differences between the parents. Since no differences in size or restriction pattern were found, we searched for differences at the nucleotide level by determining the genomic sequences of both parents. We also initiated a genome walk toward the 5' end of the gene.

Genomic walking to the 5′ end

Analysis of the genomic sequence of T-90 revealed one intron of 1564 bp, with the entire coding region at the 3′ end. This is consistent with the observations of Hongtrakul et al. (1998) in sunflower.

Using the technique of Siebert et al. (1995), we were able to isolate and sequence an upstream region of approximately 2229 bp from each parental genotype. The total length of the genomic sequence for T-90 (Genbank accession no. AF248739) was 3522 bp while the F435 (Genbank accession no. AF248740) sequence spanned 3529 bp. The sequenced fragment contains the putative promoter, intron, and coding regions, including a potential TATA box at 119 bp upstream from the intron (Fig. 2).

The nucleotide sequence of Δ^{12}-Fad from T-90 revealed an open reading frame (ORF) of 1140 bp encoding a peptide of 379 amino acids. The peptide has a predicted molecular weight of 41000 and contains the three conserved histidine boxes. Five amino acid differences between the published Δ^{12}-Fad (g2613050) sequence and the T-90 Δ^{12}-Fad were detected and are shown in Fig. 3.

When the BLAST search was used, the accession with the highest level of homology to the T-90 sequence (98%) is an *A. hypogaea* omega-6 desaturase (gi 2613051). Additionally, high levels of homology were also found with the Δ^{12}-Fad *of Gossipium hirsutum*

Fig. 2 Continued

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2321 t g g t t t a a g t c a c t c t c a t c t g c a a t g a c t a t c a t t c a t t
2361 c a t t t t c t t a g a t a t c a g a a c c a t t a g c t t t g t a g t a g t g
2401 c a a a g t g c t a a c t c t t t c t t t c t a c a t t g g t a a c a g G A G C
2441 T T T A A C A A C A C A A C A A T G G G A G C T G G A G G G C G T G T C A C T A
                                   M     G     A     G     G     R     V     T     K
2481 A G A T T G A A G C T C A A A A G A A G C C T C T T T C A A G G G T T C C A C A
         I     E     A     Q     K     K     P     L     S     R     V     P     H
2521 T T C A A A C C C T C C A T T C A G T G T T G G C C A A C T C A A G A A A G C A
       S     N     P     P     F     S     V     G     Q     L     K     K     A
2561 A T T C C A C C A C A T T G C T T T G A A C G T T C T C T T T T C A T A T C A T
     I     P     P     H     C     F     E     R     S     L     F     I     S     F
2601 T C T C A T A T G T T G T C T A T G A T C T C T T A A T G G C C T A C T T A C T
         S     Y     V     V     Y     D     L     L     M     A     Y     L     L
2641 C T T C T A C A T T G C C A C C A C T T A T T T C C A C A A G C T T C C A T A C
       F     Y     I     A     T     T     Y     F     H     K     L     P     Y
2681 C C A T T T T C C T T C C T T G C T T G G C C A A T C T A T T G G G C C A T C C
     P     F     S     F     L     A     W     P     I     Y     W     A     I     Q
2721 A A G G C T G C A T T C T C A C C G G T G T T T G G G T G A T T G C T C A T G A
         G     C     I     L     T     G     V     W     V     I     A     H     E
2761 G T G T G G C C A C C A T G C C T T C A G C A A G T A C C A A C T T G T T G A T
       C     G     H     H     A     F     S     K     Y     Q     L     V     D
2801 G A C A T G G T T G G T T T G A C C C T T C A C T C T T G T C T A T T A G T T C
     D     M     V     G     L     T     L     H     S     C     L     L     V     P
2841 C T T A T T T C T C A T G G A A A A T C A G C C A C C G C C G C C A C C A C T C
         Y     F     S     W     K     I     S     H     R     R     H     H     S
2881 C A A C A C A G G T T C C C T C G A C C G C G A C G A A G T G T T T G T C C C G
       N     T     G     S     L     D     R     D     E     V     F     V     P
2921 A A A C C A A A A T C A A A G G T A T C A T G G T A T A A C A A G T A C A T G A
     K     P     K     S     K     V     S     W     Y     N     K     Y     M     N
2961 A C A A T C C A C C A G G G A G G G C T A T T T C C C T T T T C A T C A C A C T
         N     P     P     G     R     A     I     S     L     F     I     T     L
3001 C A C A C T A G G A T G G C C C T T G T A C T T G G C C T T C A A T G T T T C T
       T     L     G     W     P     L     Y     L     A     F     N     V     S
3041 G G C A G A C C C T A T G A T A G A T T T G C A A G C C A C T A T G A C C C T T
     G     R     P     Y     D     R     F     A     S     H     Y     D     P     Y
3081 A T G C T C C C A T A T A C T C T A A C A G G G A A A G G C T T C T A A T T T A
         A     P     I     Y     S     N     R     E     R     L     L     I     Y
3121 T G T C T C A G A T T C A T C T G T C T T T G C T G T A A C A T A T C T G C T A
       V     S     D     S     S     V     F     A     V     T     Y     L     L
3161 T A T C A C A T A G C A A C T T T G A A A G G T T T G G G T T G G G T G G T A T
     Y     H     I     A     T     L     K     G     L     G     W     V     V     C
3201 G T G T T T A T G G G G T G C C A T T G C T C A T T G T G A A T G G G T T T C T
         V     Y     G     V     P     L     L     I     V     N     G     F     L
3241 A G T T A C C A T A A C C T A T T T G C A G C A C A C A C A T G C A T C A T T G
       V     T     I     T     Y     L     Q     H     T     H     A     S     L
3281 C C T C A C T A T G A T T C A T C C G A A T G G G A C T G G T T A A G A G G A G
     P     H     Y     D     S     S     E     W     D     W     L     R     G     A
3321 C A T T G G C A A C A G T G G A C A G A G A T T A T G G G A T A C T G A A T A A
         L     A     T     V     D     R     D     Y     G     I     L     N     K
3361 G G C A T T T C A T C A T A T A A C T G A T A C G C A T G T G G C T C A T C A T
       A     F     H     H     I     T     D     T     H     V     A     H     H
3401 T T G T T C T C A A C A A T G C C T C A T T A C C G T G C A A T G G A A G C A A
     L     F     S     T     M     P     H     Y     R     A     M     E     A     T
3441 C C A A T G C A A T A A A G C C A A T A T T G G G T G A T T A C T A C C A A T T
         N     A     I     K     P     I     L     G     D     Y     Y     Q     F
3481 T G A T G G C A C C C C A G T T T A C A A A G C A T T G T G G A G A G A A G C C
       D     G     T     P     V     Y     K     A     L     W     R     E     A
3521 A A A G A G T G C C T C T A T G T G G A G C C A G A T G A T G G A G C T T C T C
     K     E     C     L     Y     V     E     P     D     D     G     A     S     Q
3561 A G A A G G G T G T T T A T T G G T A C A A G A A C A A G T T C T G A
         K     G     V     Y     W     Y     K     N     K     F     .
```

(X97016), *Glycine max* (gi/904154), *Gossipium hirsutum* (Y10112), and *Crepis palaestina* (CAA76157). The deduced amino acid sequence of the peanut Δ^{12}-Fad is eight amino acids shorter than the soybean omega-6 Fad (gi/904152) and four amino acids shorter than D12 oleate desaturase in *Solanum commersonii* (X92487).

Comparison of genomic sequences of Δ^{12}-Fad of T-90 versus F435

A comparison of the two genomic sequences revealed several SNPs. One SNP was detected in the flanking region at –229 bp upstream of start codon. A cluster of four SNPs were detected in the coding region. The first (412 bp) results in a change from T (T-90) to C (F435) but does not change the amino acid sequence. The third SNP, a shift from G (T-90) to A (F435), at 972 bp is also silent. The second SNP, an "A" insertion in F435 relative to T-90, is found 442 bp from the start codon. This insertion shifts the amino acid reading frame and is found approximately 15 bp after the second histidine box. The shift in the amino acid reading frame results in the creation of several stop codons and likely produces a truncated, inactive protein (Fig. 3). The frame shift also causes the elimination of the third histidine box. A fourth single nucleotide change was found at position 448 in clones from high O/L genotypes that lacked the "A" insertion. This polymorphism results in an amino acid change 7 bp after the "A" insertion. In low O/L lines a guanine is found in position 448, but this is changed to an adenosine in high O/L genotypes. This results in the replacement of aspartic acid with asparagine. Figure 4 summarizes these two SNPs. These results are in agreement with the findings of Ray et al. (1993), who reported that Δ^{12}-Fad activity of peanut was tenfold lower in a high oleic acid line than in its normal oleic acid isoline.

Fig. 3 Amino acid sequence alignment of the published Δ^{12}-fatty acid desaturse (-FadS, accession g26130150), the peanut Δ^{12}-fatty acid desaturase from T-90 (low O/L), and Δ^{12}-fatty acid desaturase from F435 (high O/L). The three histidine boxes are *underlined*. Amino acid differences between T-90 and FadS are indicated by *dark* circles. An *A* insertion shifts the ORF for F435 after amino acid no. 148 resulting in the loss of the third histidine box. Also note that there are several stop codons (*letter Z*) in the altered F435 sequence

```
        1                                          44
T-90    MGAGGRVTKIEAQKKPLSRVPHSNPPFSVGQLKKAIPPHCFERS
F435    MGAGGRVTKIEAQKKPLSRVPHSNPPFSVGQLKKAIPPHCFERS
AhFads  MGAGGRVTKIEAQKKPLSRVPHSNPPFSVGQLKKAIPPHCFERS

        45                                         88
T-90    LFISFSYVVYDLLMAYLLFYIATTYFHKLPYPFSFLAWPIYWAI
F435    LFISFSYVVYDLLMAYLLFYIATTYFHKLPYPFSFLAWPIYWAI
AhFads  LFISFSYVVYDLLVAYLLFYIATTYFHKLPYPFSFLAWPIYWAI
                     •
        89                                        132
T-90    QGCILTGVWVIAHECGHHAFSKYQLVDDMVGLTLHSCLLVPYFS
F435    QGCILTGVWVIAHECGHHAFSKYQLVDDMVGLTLHSCLLVPYFS
AhFads  QGCILTGVWVIAHECGHHAFSKYQLVDDMVGLTLHSCLLVPYFS

        133            148                        176
T-90    WKISHRRHHSNTGSLDRDEVFVPKPKSK------VSWYNKYMNN
F435    WKISHRRHHSNTGSLRPRRSVCPETKIKGIMVZQVHEQSTREGY
AhFads  WKISHRRHHSNTGSLDRNEVFVPKPKSK------VSWYNKYMNN
                         •
        177                                       220
T-90    PPGRAISLFITLTLGWPLYLAFN-VSGRPYDRFASHYDPYAPIY
F435    FPFHHTHTRMALVLGLQCFWQTLZZICKPLZPLCSHILZQGKAS
AhFads  PPGRAISLFITLTLGWPLYLAFN-VSGRPYDRFASHYDPYAPIY

        221                                       264
T-90    SNRERLLIYVSDSSVFAVTYLLYHIATLKGLGWVVCVYGVPLLI
F435    NLCLRFICLCCN--ISAISHSNFERFGLGGM----CLWGAIAHC
AhFads  SNRERLLIYVSDSSVFAVTYLLYHIATLKGLGWVVCVYGVPLLI

        265                                       308
T-90    VNGFLVTITYLQHTHASLPHYDSSEWDWLRGALATVDRDYGILN
F435    EWVSSYHNLFAAHTCIIASLZ-FIRMGLVKRSIGNSGQRLWDTE
AhFads  VNGFLVTITYLQHTHASLPHYDSSEWDWLRGALATVDRDYGILN

        309                                       352
T-90    KAFHHITDTHVAHHLFSTMPHYRAMEATNAIKPILGDYYQFDGT
F435    ZGISSYNZYACGSSFVLNNASLPCNGSNQCNKANIGZLLPIZWH
AhFads  KAFHHITDTHVAHHLFSTMPHYHAMEATNAIKPILGDYYQFDGT
                              •
        353                          386
T-90    PVYKALWREAKECLYVEPDDGASQKGVYWYKNKF. 379
F435    PSLQSIVERSQRVPLCGARZWSFSEGCLLLQEQGVY?
AhFads  PFYKALWREAKECLYVEPDDGASKKGVYWYKNKF. 379
         •                     •
```

Genotype	Start codon		"A" insertion	Amino acid change
T-90:	ATG…CCCTC	:	GACCGC	GAC
F435:	ATG…CCCTC	A	GACCGC	GAC
F435:	ATG…CCCTC	:	GACCGC	AAC
F435:	ATG…CCCTC	:	GACCGC	GAC
BP no.	123	442		448

Fig. 4 Two single nucleotide polymorphisms were very consistent between the low and high O/L ratio peanut genotypes. One is the "A" insertion at position 442 after the start codon. The other is an amino acid change, G to A, changing aspartic acid in the low-O/L ratio lines to asparagine in the high O/L ratio lines

Table 2 Oleic to linoleic (O/L) ratio values for the IDBLs and parents included in this study

ID IDBL no.	O/L	ID IDBL no.	O/L
26	1.3	25	35.5
129	1.3	30	20.0
117	1.2	115	24.0
39	1.3	101	24.0
59	1.3	23	22.0
31	1.3	27	22.0
108	1.3	29	30.0
638	1.5	642	18.0
24	1.4	644	19.0
T-90	1.3	F435	34.0

Amplification of the polymorphic region from the parental lines and the IDBLs

The region containing the cluster of SNPs was sequenced from 14 clones of each parental line and 1 clone from each of the 16 IDBLs (eight low O/L ratio and eight high O/L ratio, Table 2). The amplified fragment was approximately 800 bp. The "A" insertion was found in 7 of the 14 clones of F435 and seven of the eight high O/L ratio IDBLs. The amino acid change was detected in six of the F435 clones and in one of the eight high O/L IDBLs. One clone of F435, and one clone of a high O/L IDBL were similar to T-90, the low O/L genotype (Fig. 4). This observation is likely due to the presence of

Table 3 Genomic sequences found for the Δ^{12}-Fad from genomic DNA of Tamspan 90, F435, eight low O/L IDBLs, and eight high O/L IDBLs

Genotype	O/L	Wild Type	"A" insertion	Amino acid change
T-90	1.2	14	0	0
F435	34.0	1	7	6
8 IDBLs	1.2–1.5	8	0	0
8 IDBLs	18–35.0	0	7	1

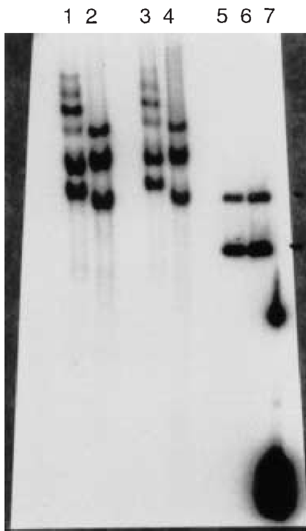

Fig. 5 Southern blot analysis for Tamspan 90 and F435 revealed three to four copies per haploid genome. From *left to right*: line *1* T-90 digested with *Eco*RI, line *2* T-90 digested with *Dra*I, line *3* F435 digested with *Eco*RI, line *4* F435 digested with *Dra*I, lines *5–7* standards

multiple copies of the gene. None of the 14 clones sequenced for T-90 or the eight clones of the low O/L IDBLs had the "A" insertion or G to A change at 448 bp. In summary, data on miniprep sets of gene variants found for T-90, F435, and the IDBLs amplified showed a mixed population (Table 3). The different clones fell into several groups, which have different forms of the Δ^{12}-Fad. The association of these molecular polymorphisms with the low and high oleate trait in peanut should allow us to move toward the development of a molecular assay for the high O/L trait. Such polymorphisms also suggested the possibility that multiple copies of the gene are present. Southern blotting revealed three to four copies per genome (Fig. 5), and no major differences in organization between the low and high O/L genotypes studied.

In addition to marker-assisted selection (MAS) applications, the outcomes of this study help to better understand the tetraploid condition and furnish knowledge about the molecular biology of cultivated peanut. Constructs to be used in an antisense technique were assembled based on this results, and transformation experiments are in progress.

While the high O/L trait is desirable from a quality perspective, we must also consider other factors in the development of high O/L ratio lines. The acclimation of higher plants to high or low temperatures is often accompanied by changes in their fatty acid composition. Some examples of this are the following (1) Thermotolerance of photosynthesis increases relative to that of the wild type due to the lack of trienoic fatty acid (in the fad3fad7–2fad8 *Arabidopsis* mutant) (Routaboul et al. 1996). (2) The highly frost resistant Bolivian potato has higher levels of polyunsaturated fatty acids in both high and low growth temperature environments, suggesting its frost-resistant phenotype may correlate with the degree of unsaturation (Guerra et al. 1996). Linoleic acid has also been reported to play an important role in key fitness factors such as resistance to cold temperatures for germination, and pest and disease resistance for different crops (Kirsch et al. 1997; Reinold and Hahlbrock 1996; Hamada et al. 1996; Yamamoto et al. 1992). While high oleic acid content is important for a better shelf-life of peanut, maintaining a certain level of linoleic acid could be of agronomic importance. The results reported here may be used further to develop techniques and define the relationship between fatty acid composition and fitness factors in peanut. Ultimately, this may aid in the development of a peanut that maintains a balance between food quality and agronomic fitness.

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