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ORIGINAL PAPER

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High-oleate peanut mutants result from a MITE insertion into the *FAD2* gene

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Abstract A high-oleate trait in the cultivated peanut (*Arachis hypogaea* L.) was reported to rely on the allelic composition of the two homeologous, microsomal oleoyl-PC desaturase genes (*ahFAD2A* or *ahFAD2B*). The enzyme activity of either *ahFAD2A* or *ahFAD2B* is sufficient for a normal oleate phenotype, and a significant reduction in the levels of *ahFAD2B* and a mutation in *ahFAD2A* were reported to be responsible for the high-oleate phenotype in one chemically induced mutant (M2-225) and one derived from a naturally occurring (8-2122) mutant. Here, we report an insertion of the same miniature inverted-repeat transposable element (MITE) in the *ahFAD2B* gene in another chemically induced mutant (Mycogen-Flavo) and the previously characterized M2-225 mutant. In both cases, this MITE insertion in *ahFAD2B* causes a frameshift, resulting in a putatively truncated protein sequence in both mutants. The insertion of this MITE in *ahFAD2B*, in addition to the point mutation in *ahFAD2A*, appears to be the cause of the high-oleate phenotype in Mycogen-Flavo and M2-225 mutants. Utilizing sequences of the MITE, we developed a DNA marker strategy to differentiate the two insertion-containing mutants from the normal oleate peanut variety (AT-108) and the naturally occurring, high-oleate mutant 8-2122. Reverse transcript-PCR/differential digestion results reveal the expression of both *ahFAD2A* and *ahFAD2B* genes in Mycogen-Flavo mutant. This result is in contrast to the observation that *ahFAD2B* transcripts are greatly reduced in the M2-225 mutant having the MITE insertion further 3′ in *ahFAD2B* gene.

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Introduction

Cultivated peanut (*Arachis hypogea* L.) is a globally important oilseed crop. Eighty percent of the total fatty acid of peanut oil consists of varying amounts of oleic acid (36–67%) and linoleic acid (15–43%), depending on the variety (Moore and Knauft 1989). Like other crop plants (soybean, Martin and Rinne 1986; sunflower, Garcés and Mancha 1991; and rapeseed, Lee and Guerra 1994), a naturally occurring high-oleate mutant of peanut (variety F435) was identified in germplasm material (Norden et al. 1987). This high-oleate mutant exhibits seed oil comprised of 80% oleate and as little as 2% linoleate (Norden et al. 1987).

The final quality of the peanut oil depends on its chemical properties. One of the major factors influencing peanut oil quality is the polyunsaturated fatty acid composition. Polyunsaturated fatty acyl residues are more susceptible to oxidation, which adversely impacts the oil stability and increases the development of off-flavors commonly associated with rancidity in stored oil (St. Angelo and Ory 1973). Oxidized products have also been reported to have antherogenic effects (Brown et al. 1999). In contrast, oils rich in monounsaturated fatty acids are more suitable and beneficial for improved oil stability, flavor, and nutrition. Unlike saturated fatty acids, which tend to raise blood cholesterol levels, monounsaturates (e.g., C18:1) have cholesterol-lowering properties (Grundy 1986). Even though linoleic acid (C18:2) is an essential fatty acid and has low-density-lipoprotein-lowering properties, its oxidative instability shortens the oil's shelf life. Several studies have indicated that linoleic acid can selectively stimulate development of a pro-inflammatory environment and marked injury to vascular endothelial cells. However, oleic acid helps to decrease

this inflammation to endothelial cells (Toberek et al. 2002). Dietary intake of oleic acid can also protect endothelial cells against hydrogen peroxide oxidative stress and thus reduce susceptibility of low-density lipoproteins to oxidative modifications (Toberek et al. 2002). Therefore, one of the major goals for oilseed research is to improve the nutritive value of oilseeds by increasing the ratio of oleic acid to linoleic acid. Depending on the oilseed crop, improvements have been achieved by conventional breeding techniques utilizing naturally occurring fatty acid-desaturation mutants or through the use of transgenic technologies (Norden et al. 1987; Kinney 1998a; Brown et al. 1999; Napier et al. 1999; Stoutjesdijk et al. 2000).

Microsomal oleoyl-PC (phosphatidyl choline) desaturase is responsible for the conversion of oleate to linoleate in non-photosynthetic tissues and developing seeds. Activity of microsomal oleoyl-PC desaturase is significantly lower in some high-oleate peanut seeds (Ray et al. 1993). Studies of high-oleate mutants from sunflower, soybean, and rapeseed have also shown the activity of the oleoyl-PC desaturase to be lost in developing seeds (Martin and Rinne, 1986; Garcés and Mancha 1989; Lee and Guerra 1994). The steady-state level of oleoyl-PC desaturase gene transcripts is significantly reduced in high-oleate sunflower seeds (Kabbaje et al. 1996; Hongtrakul et al. 1998). The high-oleate trait in sunflower is due to a dominant mutation (Martinez-Rivas et al. 1998), whereas for peanut, it has been suggested to be recessive (Moore and Knauft 1989). Inactivation of all δ^{12}-desaturases in developing seeds without effecting gene expression in other tissues can be achieved by the implementation of transgenic technologies. Oleic acid levels were increased to 85% in *B. napus* using antisense gene constructs (Töpher et al. 1995), 85% in soybean (Kinney 1998b), and up to 89% in *B. napus* using co-suppression constructs (Stoutjesdijk et al. 2000).

In the previous work from Jung et al. (2000b) two homeologous oleoyl-PC desaturase genes (*ahFAD2A* and *ahFAD2B*), each having its origin in different diploid progenitor species, were isolated and characterized from the cultivated peanut. The expression patterns of both genes were examined in peanut varieties with normal oleate and high-oleate phenotypes. Both homeologous genes are expressed in normal oleate peanuts, whereas the expression of one gene (*ahFAD2B*) is severely reduced or absent in high-oleate mutants (8-2122 and M2-225). Also, there is a mutation in the *ahFAD2A* gene in normal oleate and high-oleate lines that encodes a protein of reduced function (Jung 2000a). Therefore, a significant reduction in the transcript levels of *ahFAD2B* and a mutation in *ahFAD2A* are responsible for the high-oleate phenotype in these peanut varieties, and the expression of one gene encoding a functional enzyme appears to be sufficient for the normal oleate/linoleate phenotype. In this work, however, the nature of the mutation responsible for the reduction in expression of the *ahFAD2B* was not determined.

To further our understanding of the high-oleate phenotype of peanut, it was important to examine in detail both the genes and their regulatory elements in the high-oleate mutants and the normal varieties. In this report, we present a detailed study of the gene structure and putative promoter regions for three high-oleate peanut mutants: one (8-2122) derived from a naturally occurring mutant, two chemically induced (Mycogen-Flavo, M2-225), and a normal line (AT-108). Our results demonstrate that in the two chemically induced, high-oleate mutants of peanut, an insertion of the same miniature inverted repeat transposable elements (MITE) has occurred in the *ahFAD2B* gene at base position 656 in the Mycogen-Flavo (MF), and at 998 in M2-225 mutants. This MITE imposes premature stops in both mutants. In all mutant lines, the *ahFAD2A* gene contains the point mutation previously described in Jung et al. 2000a. Studies of the putative promoter region of the *ahFAD2A* and *ahFAD2B* genes, in both the normal and mutant lines, reveal no obvious significant changes.

Materials and methods

Plant material

Four peanut (*Arachis hypogaea* L.) varieties were used in this project: one normal oleate phenotype, AT-108 (52/28,%oleic/linoleic), and three independently generated oleate mutant phenotypes, 8-2122 81/4, M2-225 80/5, and MF 80/5. 8-2122 is a hybrid issued from a cross between the normal oleate line GK7 and the high-oleate line F435 [spontaneous mutant of F78–1339 (Norden et al. 1987)]. M2-225 and MF runner were generated by a diethyl sulfate mutagenesis (Ashri 1988).

Seeds were soaked in water for 1–2 nights and planted for germination or directly germinated at 28°C in wet paper towels for 3–4 days. Young leaves from the seedlings (10–15 days) or young shoots from the germinated seeds were used for DNA extractions. All the plants were maintained in the greenhouse.

RNA isolation

Total RNA of MF runner was isolated from stage-2 seeds (20–40 days after flowering) by a phenol/SDS method as described by Ausubel et al. (1994). The samples were treated with DNase (Promega) at 37°C for 30 min. The RNA concentration was estimated by spectrophotometry.

DNA isolation

Genomic DNA was extracted from young leaves using the sweet potato protocol (Jarret and Austin 1994). High-molecular weight DNA was also extracted from young shoots according to the method of ten Lohuis (1993). The DNA samples were separated by electrophoresis on a 0.8% agarose gel in 1× TAE buffer, stained in 0.5 μg/ml ethidium bromide, visualized on a UV transilluminator, and their concentrations were estimated by comparison with DNA molecular markers (pGEM or λ*Hin*dIII).

RFLP analysis

Approximately, 8–9 μg of genomic DNA was digested with 32–40 U of the endonucleases *Hin*dIII and *Ssp*eI for 3 h at 37°C. The resulting restriction fragments were separated on a 0.8% agarose

gel in 1× TAE buffer for 12–16 h, stained in an ethidium bromide solution and visualized on a UV transilluminator. The digested DNA was transferred to a nylon membrane (Amersham) by Southern blotting (Southern 1975). Prehybridization was performed for 2 h in 6× SSPE (1.08 M NaCl, 0.06 M NaH_2PO_4, and 6 mM EDTA pH 7.7), 5× Denhardt's solution, 1% SDS, and 20 μg/ml boiled, sonicated Salmon sperm DNA at 65°C. (Sambrook et al. 1989) A PCR-amplified product covering the coding region of *FAD2* gene from a λFix II genomic clone (Jung et al. 2000b) was used as a probe. Thirty to fifty nanograms of the probe was labeled with 15μci [α- ^{32}P] dCTP by random primer extension (Sambrook et al. 1989). Hybridization was performed at 65°C for 24 h. Filters were rinsed 2 times in 2× SSC (0.3 M NaCl, 0.03 M sodium citrate), 0.1% SDS at 65°C for 20 min, and one rinse with 0.1% SSC, and 0.1% SDS at 65°C for 20 min. Filters were autoradiographed at −80° C for 3–4 days exposure using X-Omat AR X-ray film (Kodak).

PCR

PCR amplifications were performed on genomic DNA, cDNA, and bacterial lysates. The amplifications conditions were as follow: 94°C for 4 min; 35 cycles consisted of 94°C for 30 s, 53–60°C for 1 min, 72°C for 2 min; and a final extension of 74°C for 4 min. The annealing temperature was adjusted depending on the primer combinations. The reactions were performed in a 50-μl volume containing 100–300 ng of DNA, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 20 pmol of each primer, 2.5 U of *Taq* polymerase (Fisher Scientific), and 1× assay buffer provided with the enzyme. $MgCl_2$ concentration was varied according to the primer sets and DNA templates. The primer combinations used for the amplifications are presented in Table 1. All PCR products were checked on 2% agarose gels.

Reverse-transcript PCR

The first strand cDNA was synthesized from MF runner total RNA using Super Script Preamplification System (Life Technologies) following the manufacturer's instructions. Two negative controls, one without RNA and one without the reverse transcriptase, were included in the experiment. Primers and restriction enzyme digestions of the reverse transcript (RT)-PCR products were done according to Jung et al. 2000b. Products were displayed on 2% agarose TAE gels.

Table 1 Primer used in amplification and sequencing of δ^{12}-desaturase gene (oleoyl-PC-desaturase) genes, *ahFAD2A* and *ahFAD2B*, in three high-oleate mutant peanut varieties

Primer name	5′ position	Sequence (5′ to 3′)
GF1	−576	CCTCAGAATCATGCCCC
aF19[a]	−88 (*ahFAD2A*)	GATTACTGATTATTGACTT
bF19[b]	−80 (*ahFAD2B*)	CAGAACCATTAGCTTTG
F0.7	21	CACTAAGATTGAAGCTC
F2.2	559	CCCTTGTACTTGGCCTTCAA
F2.4	631	CCCATATACTCTAACAGGG
F3.5	965	CCATGCAATGGAAGCAACC
MITE-1[c]		GGATGATGGATTGTATGG
R1	1,136	CTCTGACTATGCATCAG
aR19[a]	−69 (*ahFAD2A*)	AAGTCAATAATCAGTAATC
bR19[b]	−63 (*ahFAD2B*)	CAAAGCTAATGGTTCTG
R1.6	1,085	GTAATCACCCAATATTGGC
R2.5	668	GCAAAGACAGATGAATCTGAG
R3	503	CCCTGGTGGATTGTTCA

[a] Specific for the *ahFAD2A* gene, containing the 19-bp insertion region
[b] Specific for the *ahFAD2B* gene, spanning the 19-bp deletion region
[c] Primer within the 205-bp transposon insert sequence in Mycogen-Flavo and M2-225 mutants

Cloning of PCR products

The PCR products from the genomic DNA amplifications were ligated into the pGEM-T easy vector (Promega), and then introduced into the *Escherichia coli* strain JM109 (Promega) following the manufacturer's instructions. Plasmid DNA was isolated using the alkaline lysis method followed by a PEG precipitation (Sambrook 1989).

DNA sequencing and analysis

Sequencing was performed using either dye primer Thermo Sequenase fluorescent-labeled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Life Sciences) and sequences resolved on a LiCor model 4200 sequencer (LiCor), or using ABI PRISM Dye terminator cycle sequencing ready reaction kit (Perkin Elmer) and an ABI 373 stretch sequencer (Applied Biosystem). GeneWorks (nucleic acid and protein sequence analysis software), version 2.0 (IntelliGenetics) was used to analyze the DNA sequences.

Results

RFLP analysis of normal oleate and high-oleate peanut varieties

Two mutant varieties, MF and M2-225, were isolated after diethyl sulfate (DES) mutagenesis and one variety, 8-2122, is a naturally occurring mutant. To determine if there were DNA polymorphisms among these three high-oleate mutants and the normal oleate peanut variety (AT-108) in the region carrying the peanut oleoyl-PC desaturase orthologous genes, we performed an RFLP analysis choosing enzymes (*Hin*dIII and *Ssp*I) that had a unique restriction site in the coding region. Genomic DNA from all three mutants and the normal variety were digested with *Hin*dIII or *Ssp*I. The restriction endonuclease-digested fragments were subjected to Southern-blot hybridization analysis using a PCR product (1,133 bp) amplified with the primer set F0.7/R1 (Table 1) covering the coding region from a peanut oleoyl-PC desaturase *ahFAD2A* genomic clone (designated *ahFAD2ag*, Jung, unpublished results) as a probe. The results with both restriction enzymes showed that the banding pattern of 8-2122 was similar to that of the normal AT-108, whereas the patterns of MF and M2-225 were similar to each other, but contrasted to that of AT-108 and 8-2122 (Fig. 1). *Hin*dIII showed the presence of an extra band, 3.0 kb in the MF and M2-225 mutants, which was not present in AT-108 and in 8-2122. In the case of *Ssp*I, MF and M2-225 patterns had a 1.9-kb fragment, and AT-108 and 8-2122 had a 1.7-kb fragment. From these results, the polymorphisms observed suggested that there were differences in the organization of the DNA sequences in these orthologous gene regions among these peanut lines. More specifically, the two DES mutants displayed a

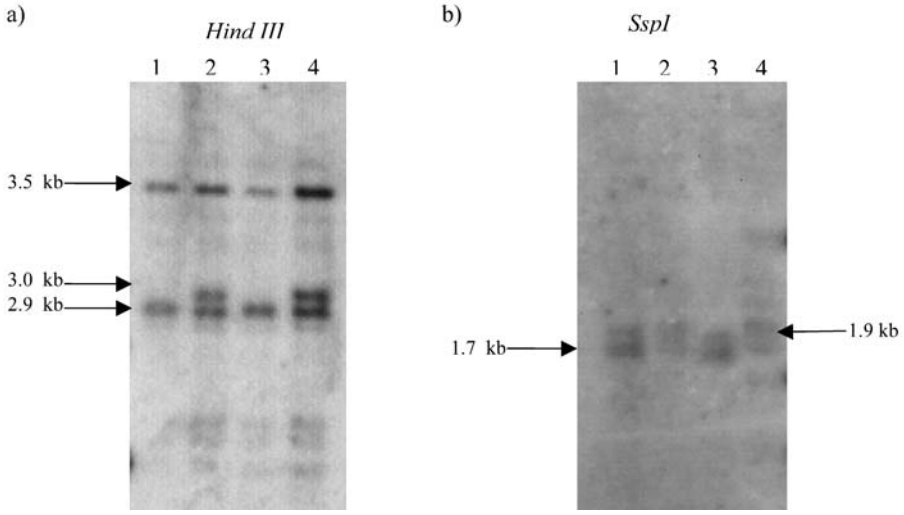

Fig. 1a, b RFLP analysis of oleoyl-PC desaturase in normal (AT-108) and three high-oleate peanut lines [Mycogen-Flavo (*MF*), 8-2122 and M2-225]. Genomic DNA digested with **a** *Hind*III: *lane 1* AT-108, *lane 2* MF, *lane 3* 8-2122, and *lane 4* M2-225; and **b** *Ssp*I: *lane 1* AT-108, *lane 2* MF, *lane 3* 8-2122, and *lane 4* M2-225

Table 2 Primer sets used to amplify PCR products

Primer set	Annealing temperature	Product size (bp)	Peanut varieties from which amplified Products were obtained	Location
F0.7/R3	55.0°C	499	MF[a]	Coding region
GF1 /aR19	58.5°C	577	AT-108, all mutants	Promoter, 5′ UTR
GF1 / bR19	58.1°C	494	AT-108, all mutants	Promoter, 5′ UTR
aF19 /R1	58.0°C	1228	AT-108, all mutants	Coding region
bF19 / R1	58.0°C	1220	AT-108 and 8-2122	Coding region
		1434	MF and M2-225	
GF1 / R3	60.0°C	1095	AT-108, all mutants	Promoter, 5′UTR, half of the coding region
F2.2 / R1	58.5°C	595	AT-108 and 8-2122	Coding region
		809	MF and M2–225	
MITE-1/ R1	58.0°C	639	MF	Coding region
		307	M2-225	
F2.4/R2.5[b]	57.0°C	373	MF	Coding region

[a] Mycogen-Flavo
[b] Primer set amplifying the MITE insertion in *ahFAD2B* gene of MF mutant, used as a probe in Southern blot to show the repetitive sequence

similar genomic polymorphism, distinguishing them from the normal and spontaneous mutant lines.

Sequence comparison of the promoter and coding regions between three high-oleate peanut varieties and a normal oleate peanut variety

To further examine the nature of the genomic changes resulting in RFLP differences among the mutant and normal lines, the coding region and the promoter region of both homeologous oleoyl-PC desaturase genes (*ahFAD2A* and *ahFAD2B*) were amplified from all three mutants (MF, M2-225, and 8-2122) and sequenced (Tables 1, 2; Fig. 2).

The primers were generated from the sequences of *ahFAD2A* and *ahFAD2B* genes of the normal peanut line previously obtained by Jung et al. (2000b). Some primers were specific to each gene, and others amplified products from both *ahFAD2A* and *ahFAD2B*. All the amplified products were sub-cloned and sequenced. Comparing these insert sequences to those of the normal *ahFAD2A* and *ahFAD2B* gene sequences (Jung et al. 2000a) identified sub-clones corresponding to *ahFAD2A* and *ahFAD2B* in the mutants.

The sequences of *ahFAD2A* from the three mutant lines were 100% identical to the normal oleate *ahFAD2A* sequence that was shown by Jung et al. (2000b) to contain a point mutation in a region important to enzymatic activity. In comparison to the *ahFAD2A* gene, the *ahFAD2B* gene sequence of MF, M2-225, 8-2122, and normal AT108 lines shows a 19-bp deletion in the 5′UTR region and 11-bp differences in the coding region as previously noted by Jung et al. (2000b) (Fig. 2). All three mutants show a 1-bp change (G to C) in the promoter region of *ahFAD2B*. However, in addition to these differences, the sequences for *ahFAD2B* gene in MF and M2-225 mutants also showed an additional major difference in the coding region. The same insertion of 205 bp is present in both MF and M2-225, but at a

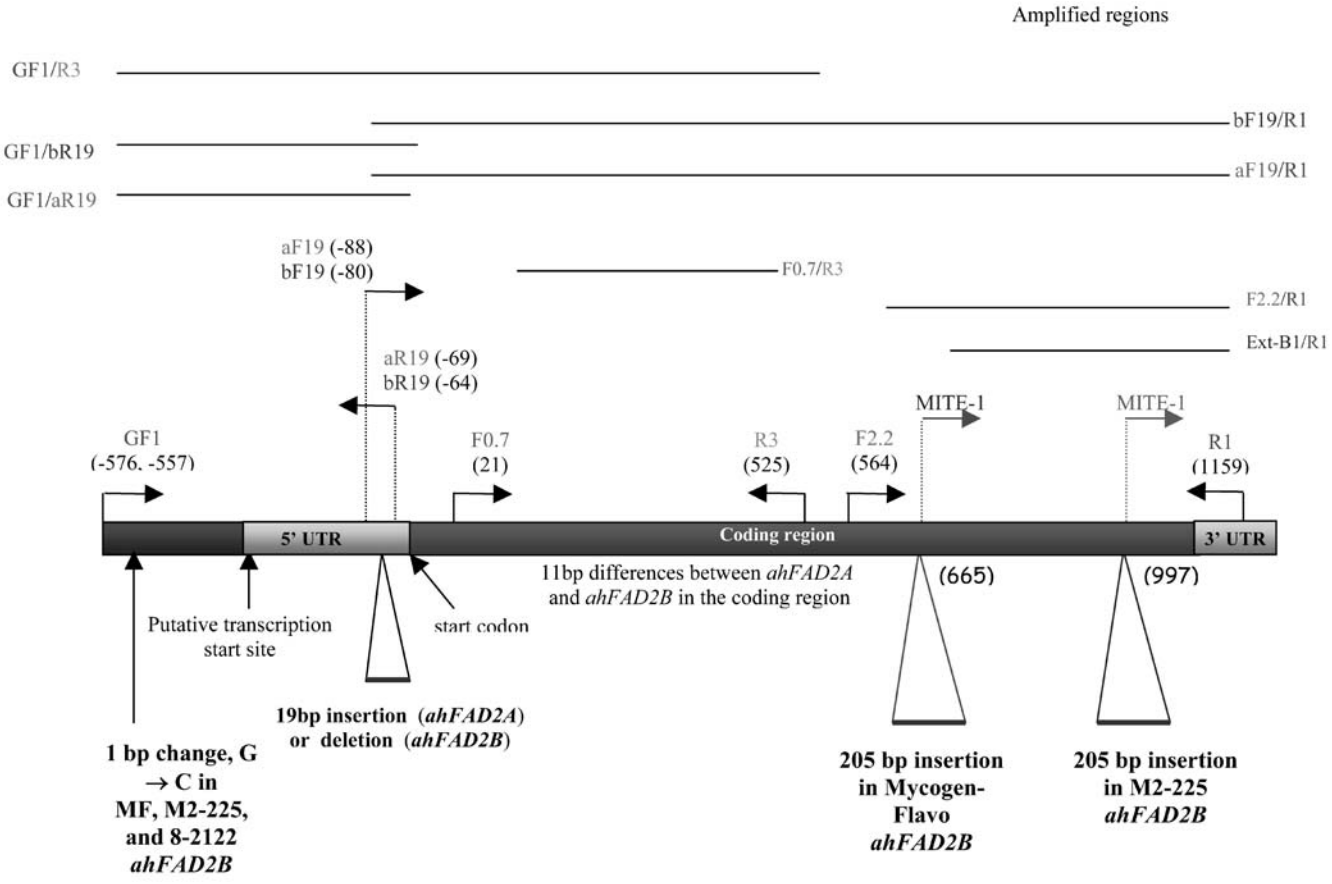

Fig. 2 The structure of two homeologous oleoyl-PC desaturase genes (*ahFAD2A* and *ahFAD2B*). These intronless genes were characterized by sequencing both cDNA and genomic clones, Jung et al. (2000a). The primers for PCR amplifications used in this study are shown, and the numbers in *parentheses* indicate the positions of the primers relative to the transcription start site (0). The PCR amplified regions mentioned in the text are shown

different location: at position 665 near the center of the coding region in the case of MF, and at position 997 in the case of M2-225 (Fig. 2). This 205-bp insertion is a 68.7% AT-rich sequence with no significant matches in the sequence databases. In both cases, the insertion of these sequences has created a target site duplication of AT-rich 9 bases (8/9 AT in MF, and 7/9 AT in M2-225) unique in each case. These target site duplications suggested the footprint of a transposable element insertion, and sequence analysis of the inserted sequence revealed the presence of a 25-bp, perfect inverted repeats on the ends (Fig. 3). Thus, this inserted sequence has characteristics of a miniature inverted-repeat transposable element (MITE) (Wessler et al. 1995).

In both the MF and M2-225 mutants, the 205-bp MITE insertion causes a frameshift, resulting in a putatively truncated protein sequence (Fig. 4). This result suggests that the point mutation in *ahFAD2A* and MITE insertion in *ahFAD2B* are responsible for the high-oleate phenotype in MF and M2-225.

As MITEs described in other systems are interspersed throughout the genome, we were interested to know if this peanut MITE sequence was similarly present in other parts of the peanut genome. Therefore, we performed a genomic Southern-hybridization analysis of peanut utilizing the MITE sequences as a probe. For this purpose, the MITE was amplified from a clone of the MF *ahFAD2B* gene using the primer sets F2.4/R2.5 (Table 2). When the amplified product was used as a probe in a Southern blot of restriction enzyme digested peanut genomic DNA, multiple fragments of varying size hybridized, suggesting that this sequence is a repetitive element in the peanut genome (Fig. 5).

Examination of steady state levels of microsomal oleoyl-PC genes (*ahFAD2A* and *ahFAD2B*) in the MF mutant

From Jung et al. (2000b), it was evident that both homeologous genes, *ahFAD2A* and *ahFAD2B*, were expressed in the normal oleate peanut varieties examined; however, in the high-oleate mutants 8-2122 and M2-225, no transcripts for the *ahFAD2B* were detected in developing seeds. To examine the expression of these homeologous genes in the MF high-oleate mutant, we utilized the

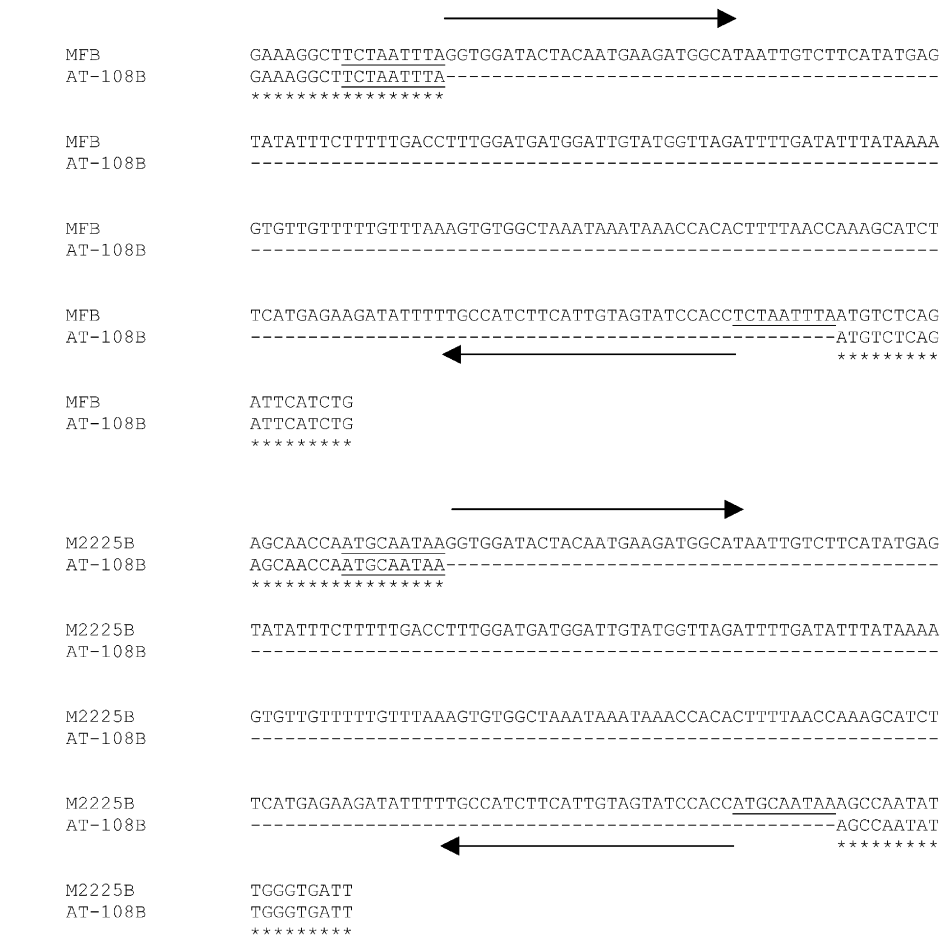

Fig. 3 Miniature inverted-repeat transposable element (MITE) sequences (205 bp) located in the *ahFAD2B* gene of MF and M2-225 mutant lines in comparison with the normal peanut line. Both mutant lines have a unique AT-rich, 9-bp duplication, shown in *box* (8/9 AT) for MF and (7/9 AT) for M2-225. The *underline* in both mutants show the 25-bp perfect inverted repeats, and the *arrows* show the orientation of the repeats

Fig. 4 Comparison of the predicted amino acid sequence for the *ahFAD2B* gene in the two chemically induced mutants lines (MF and M2-225 and the normal peanut line (AT-108). Identical amino acid residues are indicated by an *asterisk* (*)

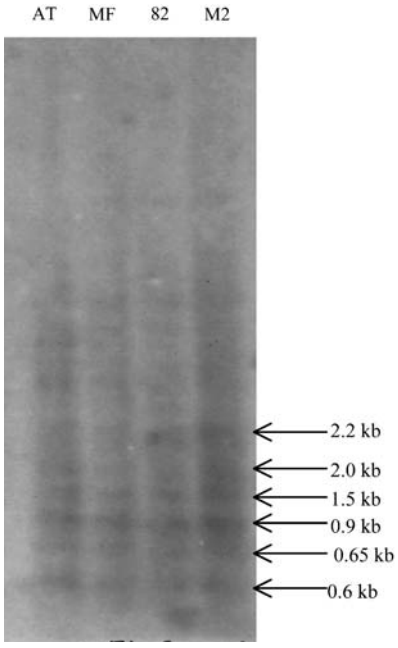

Fig. 5 Southern blot showing repetitive sequence of the 205-bp MITE. Genomic DNA digested with *Ssp*I: *lane 1* Normal (AT-108), *lane 2* MF, *lane 3* 8-2122, *lane 4* M2-225. The probe was a PCR product derived from genomic DNA of the MF mutant using the primer set F2.4/R2.5 (Tables 1, 2), which spans the MITE sequences in this mutant

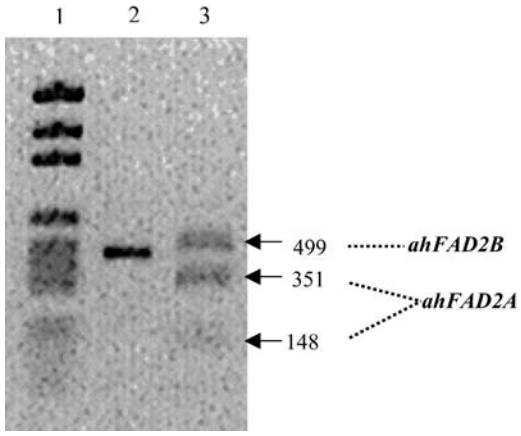

Fig. 6 Reverse transcript (RT)-PCR/*Dde*I digestion analysis of oleoyl-PC desaturase transcripts in the MF mutant. A 2% agarose gel of the RT-PCR/*Dde*I digestion products amplified using the primer set F.07/R3 (Tables 1, 2; Fig. 2). *Lane 1* pGEM molecular weight marker, *lane 2* undigested RT-PCR product, *lane 3* aliquot of the same RT-PCR product in *lane 2* digested with *Dde*I

RT-PCR/*Dde*I digestion strategy as described in Jung et al. (2000b). This strategy takes advantage of a restriction-site polymorphism between the *ahFAD2A* and *ahFAD2B* genes. The targeted transcripts were amplified from RNA of developing seeds using the F0.7 and R3 primers (Tables 1, 2; Fig. 2). With this primer set, a 499-bp region was amplified that potentially contains a single *Dde*I restriction-site polymorphism (the *Dde*I site is present in *ahFAD2A,* but absent from *ahFAD2B*). The initial RT-PCR products of both *ahFAD2A* and *ahFAD2B* were indistinguishable. Digestion of the amplified product with *Dde*I produced three different sized products: two fragments of sizes 148 bp and 351 bp, the expected sizes corresponding to the *ahFAD2A* transcript, and a third uncut fragment of size 499 bp corresponding to the *ahFAD2B* transcript (Fig. 6). This is in contrast to the other DES-generated, high-oleate mutant, M2-225, where the RT-PCR products from *ahFAD2B* transcripts appeared to be severely reduced or absent (Jung et al. 2000b).

Differentiation of mutants by PCR

As the high-oleate character in peanut relates to the allelic composition of the two homeologous *ahFAD2* loci, having markers for each locus/allele would greatly streamline the breeding process. For this reason, sequence differences between the normal and high-oleate mutant alleles are useful for the development of molecular markers to readily genotype the progeny of breeding crosses for the high-oleate character.

In order to distinguish the MF and M2-225 mutants from the normal, we utilized a PCR-amplification strategy. Since the 205-bp MITE is present in the *ahFAD2B* gene in MF and M2-225 mutants, we utilized the primer set F2.2/R1 (Fig. 2), which amplifies the insertion region in both mutants and yields a product distinguishable from the normal and the 8-2122 mutants. Amplifications with this primer set yield a single 595-bp fragment that is composed of identically sized products from the *ahFAD2A* and *ahFAD2B* genes in the normal AT-108 and 8-2122 mutants. However, for MF and M2-225, two fragments, one 809 bp corresponding to the *ahFAD2B* gene containing the insertion of 205 bp (Fig. 7a) and one of 595 bp corresponding to the *ahFAD2A* gene product, were amplified. Another primer set, MITE-B1/R1, was used to distinguish the MF and M2-225 mutant alleles from each other. MITE-B1 is within the 205-bp insertion in the MF and M2-225 mutants (Tables 1, 2; Fig. 2). Primer R1 is common to all the mutant and normal alleles. Since the normal AT-108 and 8-2122 mutants do not contain the insertion in the *ahFAD2B* gene, there was no amplification product; in contrast, amplification products with unique sizes were generated from MF and M2-225 due to the position of the insertion in the *ahFAD2B* in each mutant. As the insertion in M2-225 (at 997 bp) is more towards the 3′UTR compared to that in MF mutant (at 665 bp), a smaller product of 307 bp was amplified from the M2-225 mutant and a larger product of 640 bp from the MF mutant (Fig. 7b). Thus, this primer set can distinguish these mutants. Therefore, by using a two-PCR-reaction strategy, where the first amplification demonstrates that the PCR reactions worked and distinguishes the MITE-induced mutants from the normal and 8-2122 mutant, and the second amplification separates the two MITE-induced mutants, we are able to unequivocally

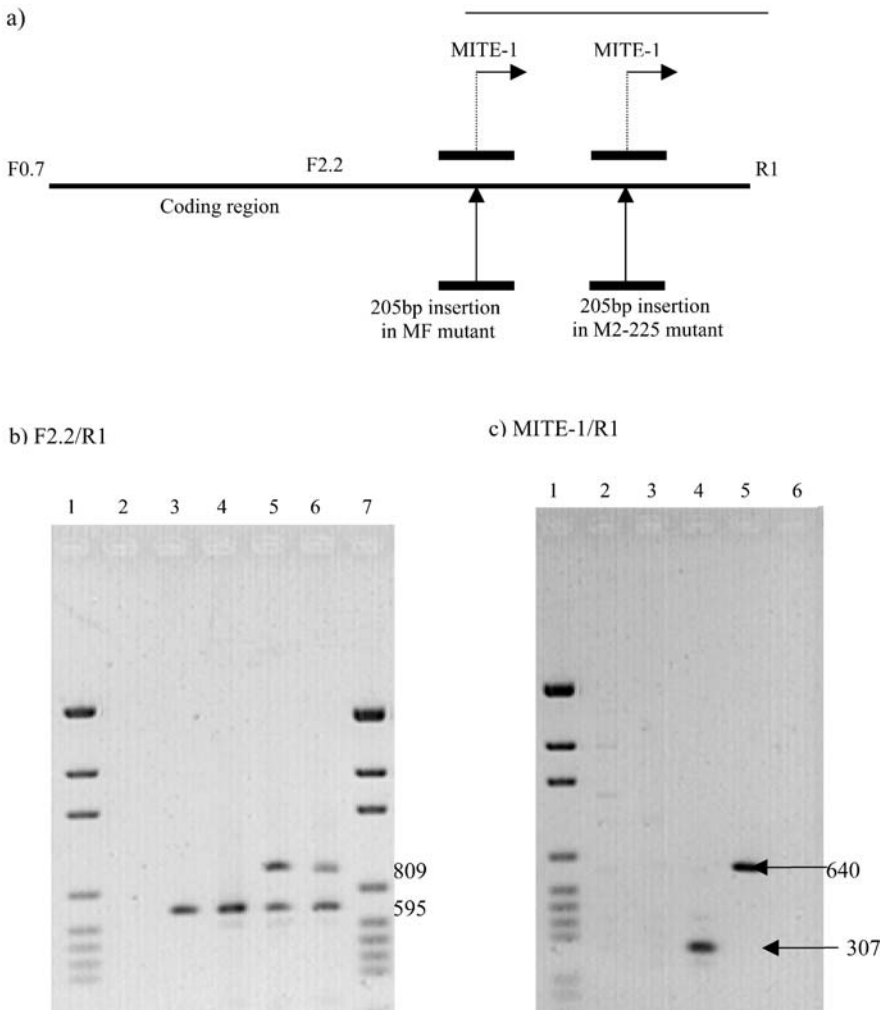

Fig. 7 **a** Figure showing the MITE-1 primer location in the *ahFAD2B* gene-coding region of MF and M2-225 mutants. **b** PCR amplification from genomic DNA with the primer set F2.2/R1. Two percent agarose gel: *lane 1* pGEM marker, *lane 2* control (negative, without any DNA), *lane 3* AT-108, *lane 4* 8-2122, *lane 5* M2-225, *lane 6* MF. **c** PCR amplification from genomic DNA with the primer set MITE-1/R1. Two percent agarose gel: *lane 1* pGEM marker, *lane 2* AT-108, *lane 3* 8-2122, *lane 4* M2-225, *lane 5* MF, *lane 6* control (negative, without any DNA)

separate the high-oleate mutants. However, since the naturally occurring high-oleate mutant (8-2122) does not contain a MITE in the *ahFAD2B* gene using MITE PCR, we cannot distinguish it from the normal oleate line.

Discussion

In this communication, we present evidence that two DES-generated, high-oleate mutants of peanut have resulted from the insertion of the same miniature inverted repeat-transposable element in different parts of the *ahFAD2B* gene. To our knowledge, these results are the first description of this type of transposable element in peanut. Our evidence further suggests the element was activated by DES treatment. It was previously reported in barley that DES mutagenesis yields a significantly higher frequency of mutations than other alkylating agents tested (Heiner et al. 1960). However, the nature of the mutations was not addressed. Our results suggest that stress caused by chemical mutagenesis may have promoted mobilization of these elements in peanut.

Recent work from genomic sequencing efforts have led to the discovery of MITEs in a number of systems; however, whether these are capable of transposition and, if so, how, is still not well understood. The structure and sequence of the group of elements that are classified as MITEs are distinct from other transposable elements (such as LINEs or SINEs), but have some properties of class II elements. MITEs are families of generally short (80–500 bp) interspersed elements with terminal repeats (17–25 bp), generally no coding potential, and AT-richness. Most of the MITEs studies show that they have target-site preference for TAA, or TA, probably resulting from target-site duplication of 2–3 bp upon insertion of the element (Kidwell and Lisch 1997; Tu 2001). In our case, the peanut MITE shows a 9-bp target-site duplication as previously reported for the *Bigfoot* family of MITEs in *Medicago* (Charrier et al. 1999). The peanut MITE does not display any significant similarities when compared to other MITE sequences, except that the insertion site duplication is AT rich (7–8 bp out of 9 bp for MF and M2-225, respectively, are A or T). Thus, the duplicated target site in MITEs of peanut and *Medicago*

indicates that the 9-bp duplication may be a characteristic of the Leguminosae family.

In plants, MITEs are present in high copy number (3,000–10,000) per genome. They were discovered first in the genome of grasses (Bureau and Wessler 1992, 1994). Transposable elements with similar properties were noted in a wide range of organisms including plants: maize, sorghum (Wessler et al. 1995) and *Arabidopsis thaliana* (Feschotte and Mouchès 2000); insects: mosquitoes (Tu 1997 2001), beetles (Braquart et al. 1999); and some vertebrates: human (Morgan 1995; Smith and Riggs 1996), *Xenopus* (Unsal and Morgan 1995), and fish (Izsvàk et al. 1999).

In plants and mosquitoes, MITEs are frequently near genes, indicating a potential role for these elements in gene regulation, defining chromatin domains, and genome organization (Wessler et al. 1995; Tu 1997). The *Tourist* element in maize and the *Stowaway* element in sorghum (Wessler et al. 1995) are found frequently in the 5′ and 3′ non-coding regions of genes and are frequently associated with regulatory regions of diverse flowering plants. Since MITEs have terminal repeats and generally generate a short duplication upon insertion, it has been suggested that MITEs could be non-autonomous elements mobilized by transposase activity encoded by class II elements (Bureau and Wessler 1994; Unsal and Morgan 1995; Smith and Riggs 1996). Recent studies from Feschotte and Mouchès (2000) propose that one of the MITE families (*Emigrant*) from *A. thaliana* is derived from a larger element named *Lemi* I (class II transposon,) encoding a putative transposase. Also, *Lemi* I potentially encodes a product related to the *pogo* family (class II transposon from *Drosophila melanogaster*) transposase (Tudor et al. 1992). For these reasons, it was important to ascertain if this peanut MITE was present at other locations in the peanut genome. The results of genomic, Southern-hybridization studies reported here suggest that sequences complementary to this peanut MITE are dispersed in the peanut genome.

Our results further suggest that the position of the MITE in *ahFAD2B* may be related to the steady-state transcript level of the gene. In the M2-225 peanut mutant where the MITE insertion (at 988 position) in the *ahFAD2B* gene is towards the 3′ end of the coding region, the gene transcript was significantly reduced/absent (Jung et al. 2000b). In contrast, MITE insertion in the *ahFAD2B* gene of the MF mutant is in the center of the coding region, and no significant reduction in transcript was noted. In both cases, the insertion creates a premature nonsense codon.

Reports suggest that transcript levels are additionally regulated at post-transcriptional events such as splicing, mRNA degradation, or translation. It appears that translation and mRNA degradation can be tightly coupled (van Hoof and Green 1996). Several studies in plants suggest a link between translation and mRNA stability. Premature nonsense codons caused by insertion mutations resulted in decreased transcript accumulation (Voelker et al. 1986; Jofuku et al. 1989). Several studies with plant genes such as phytohemagglutinin (*PHA*) in common bean (*Phaseolus vulgaris*) (Voelker et al. 1990; van Hoof and Green 1996), Kunitz trypsin inhibitor (*Kti 3*) in soybean (Jokufu et al. 1989), and ferrodoxin (*FED1*) in pea (Dickey et al. 1994; Petracek et al. 2000), and studies with other genes from organisms such as yeast (Jacobson and Peltz 1996) and *Caenorhabditis elegans* (Cali and Anderson 1998; Arnoff et al. 2001) have demonstrated significantly reduced mRNA accumulation associated with the presence of a premature nonsense codon. Our studies suggest that in the M2-225 mutant, NMD may play a role in reduction of the *ahFAD2B* transcript in the seed.

Since up to now, we have not found any differences in the *ahFAD2B* gene of the 8-2122 mutant and that of the normal, we are still unable to explain the high-oleate phenotype of this mutant. From Jung et al. (2000b), we know that the transcripts of *ahFAD2B* are severely reduced or absent from seed RNA preparations of this naturally occurring mutant. Lopéz et al. (2000, 2002) have published mutation analysis of the original high-oleate parental line, F435, from which the 8-2122 mutant was derived. Their results in most cases concur with those presented in Jung et al. (2000a, 2000b); however, in their study, they report a point mutation causing a frameshift and a premature stop codon in what we believe from their report is the *ahFAD2B* gene. Studies of the expression of this gene in the F435 peanut demonstrate that the RNA from single seeds of high-oleate progeny derived from crosses with this mutant display transcripts containing this point mutation (Lopéz et al. 2002). At the moment, we are unable to explain this discrepancy in the data on the natural mutation; however, as pointed out in Lopéz et al. (2002), the F435 plants appear to display heterogeneity in reference to this mutation. As the 8-2122 mutant is a derivative of the original mutant, it may not display the mutation. Alternatively, Lopéz et al. (2002) suggest that since our RT-PCR-identification strategy-amplified transcript portions downstream of the point mutation, that we may have missed the presence of the transcript; however, if this is the case, then the point mutation would seem to cause a truncation/or biased degradation of the transcript. Clearly, further study of *ahFAD2B* gene region is warranted to uncover a unifying explanation for the high-oleate character in F435 peanut.

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