

Full Length Research Paper

# Molecular cloning and characterization of an acyl-ACP thioesterase gene (*AhFatB1*) from allotetraploid peanut (*Arachis hypogaea* L.)

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Accepted 18 May, 2012

Acyl-acyl carrier protein (ACP) thioesterase is a nuclear encoded plastid localized enzyme which plays an essential role in chain termination during *de novo* fatty acid synthesis in plant. *FatB* genes coding for this enzyme from a variety of plant species have been isolated and characterized. However, there are few reports on such genes in peanut (*Arachis hypogaea*), an important edible and oilseed crop. In this study, full-length cDNA of an acyl-acyl carrier protein thioesterase (EC 3.1.2.14), designated as *AhFatB1*, was isolated from peanut cDNA libraries. The putative open reading frames consist of 1239 bp with five introns spliced from the corresponding genomic sequence, encoding a 413 amino acid protein, two homologous genes, *AhFatB1A* and *AhFatB1B*, with sequence difference at the 5' non-coding regions were characterized at the nucleotide level from different cultivated peanut genotypes, and the two genes have their origin in different diploid progenitor which was evidenced by the characterization of *AhFatB1* genes from *Arachis duranensis* and *Arachis ipaensis*, the putative A-genome donor and B-genome donor respectively. *AhFatB1* genes are constitutively expressed in peanut tissues and the total *FatB1* transcript accumulations are temporally regulated during peanut seed development.

**Key words:** Peanut thioesterase, palmitic acid oilseed.

## INTRODUCTION

Higher plants synthesize fatty acid *de novo* in the stroma compartment of plastids, chloroplasts and to a lesser extent, in mitochondria (Rawsthorne, 2002). Hydrolysis of the acyl-ACP thioester bond by an acyl-ACP thioesterase enzyme terminates acyl chain elongation during fatty acid biosynthesis. Acyl-ACP thioesterases are the key enzymes at determining which fatty acids are available for export to extraplastidic compartments and thus,

incorporated into glycerolipids synthesized in the endoplasmic reticulum (ER), which include the triacylglycerols (TAG) present in oil seeds (Voelker, 1996). The fatty acid desaturases in the ER, modifies the number of double bonds in the fatty acyl chains. Thus, the acyl-ACP thioesterases and fatty acid desaturase together determines the types of fatty acids (fatty acyl chain length and degree of unsaturation, respectively), which are incorporated into membrane or oil glycerolipids.

The *FatB* thioesterases show a vast array of specificities and are further divided into two subgroups depending on their preference for saturated substrates of various chain lengths.

*FatB1* thioesterases, which is present in all plant tissues and shows preference towards long-chain acyl-ACPs, such as the thioesterase isolated from *Arabidopsis* (Dörmann et al., 1995) *Madhuca butyracea* (Jha et al., 2006), *Garcinia mangostana* (Hawkins and Kridl, 1998),

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**Abbreviations:** ACP, Acyl-acyl carrier protein; CTAB, cetyl trimethylammonium bromide; ER, endoplasmic reticulum; FAT, fatty acyl-acyl carrier protein thioesterase; NCBI, National Center for Biotechnology Information; ORF, open reading frame; PCR, polymerase chain reaction; SNP, single nucleotide polymorphism; TAG, triacylglycerol.

*Cuphea hookeriana* (Jones et al., 1995), *Brassica juncea* (Jha et al., 2010), *Jatropha curcas* (Srikanta et al., 2011; Wu et al., 2009), cotton (Pirtle et al., 1999), castor oil seed (Sánchez-García et al., 2010) and macadamia (Moreno-Pérez et al., 2011) and FatB2 thioesterases, which shows preference towards short/medium chain acyl-ACPs in the C8 to C14 range are mainly found in medium chain fatty acids accumulating species of plant, such as the *Cuphea* species (Dehesh et al., 1996; Leonard et al., 1998; Filichkin et al., 2006; Leonard et al., 1997), *Ulmus americana* and *Myristica fragrans* (Voelker et al., 1997), California bay tree (Voelker et al., 1992), *Sorghum bicolor* (L.) and *Iris germanica* (Jing et al., 2011). The disruption of the *FatB* gene in *Arabidopsis* led to abnormal fatty acid composition of various tissues with an obvious reduction of palmitic acid (Bonaventure et al., 2003). On the contrary, over expression of the AtFATB1 thioesterase resulted in the accumulation of palmitate. These results show that the AtFATB1 thioesterase contributes to 16:0 productions in *Arabidopsis* (Dörmann et al., 2000). The QTL-pal9 (Yang et al., 2010), which accounts for 42% of the phenotypic variation of palmitic acid content in maize grain has been mapped to a candidate gene *Zea mays FatB* (*ZmFatB*) using linkage and association analysis. In addition, an 11 bp insertion in the last exon of *ZmFatB* has been reported to be an attribute to the palmitic acid content and concentration between different *Z. mays* population and lines (Li et al., 2011). The fact that FatB thioesterases determine the fatty acids exported from plastids makes them an important target for the manipulation of fatty acid composition of the seed oil. Ectopic expression of FatB1 from *Umbellularia californica* in rapeseed resulted in oil enriched in lauric acid (Voelker et al., 1992; Eccleston et al., 1996; Eccleston and Ohlogge, 1998). When a medium-chain thioesterase from *C. hookeriana* was introduced into rapeseed, enhancement of caprylate (8:0) accumulation was observed (Dehesh et al., 1996). Moreover, expression of elm or nutmeg FatB thioesterase in rapeseed could redirect the fatty acid biosynthesis to short chains (Voelker et al., 1997), but the content of short-chain fatty acids were significantly lower in transgenic hosts than donor species. However, the transgenic lines carrying both a 3-ketoacyl-ACP synthase and a thioesterase from *Cuphea* had higher levels of short-chain fatty acids than the single transgenic lines (Leonard et al., 1998). Consequently, the modification of thioesterase enzymes expressed in developing oilseeds determines to a great extent the available pool of fatty acids that can be incorporated into TAGs. The interest of acyl-ACP thioesterases in lipid biotechnology has gained more and more attention from a variety of sources, especially, in oil seeds (Mandal et al., 2000; Othman et al., 2000; Ghosh et al., 2007; Sánchez-García et al., 2010; Camacho-Roger et al., 2007; Martínez-Force et al., 1999; Josefa et al., 2003).

Peanuts (*A. hypogaea* L.), which originated in South

America (Simpson et al., 2001), are the fourth largest edible oilseed crop and are grown worldwide in many sub-tropical and tropical regions. Peanut oil consists mainly of palmitic, oleic and linoleic acids which are present at levels of 10%, 36 to 67% and 15 to 43%, respectively (Norden et al., 1987). Palmitic acid (C16:0) is regarded as an important dietary component of seed oil, but dietary palmitic acid increase the risk of developing coronary heart disease (Astrup et al., 2011) and cardiovascular disease (Lefevre et al., 2004). Therefore, implementation of the tools of biotechnology to directly reduce the relative amounts of palmitic acid in oil crops seed or to create and release novel cultivars with low-palmitic-acid phenotype has gained significant attention. There have not been any reports of peanut mutant with altered level of palmitic acid, and this situation limits the genetic analysis of the inheritance of palmitic acid content in the seed oil of peanut. Therefore, characterization of genes and enzymes which are responsible for palmitic acid accumulation in peanut seed, including the FatB thioesterases is necessary for the study of the fatty acid biosynthesis and oil metabolism in peanut.

In this study, we report the isolation and the characterization of two FatB thioesterases genes from the cultivated tetraploid peanut; each having its origin in different diploid progenitor species. This is evidenced by the characterization of the FatB thioesterases genes at the DNA level from the two putative diploid progenitor species *A. duranensis* and *A. ipaensis* (Foncéca et al., 2009).

To understand the nature of the FatB thioesterases in peanut, comparison and alignment of the deduced amino acid sequence with the homology proteins from the other plant species were conducted. We also examined the expression pattern of its genes in different peanut tissues and at different development stages of peanut seed. Sequence variations were obtained in peanut genotypes with normal palmitate phenotype and with high palmitate phenotype.

## MATERIALS AND METHODS

### Plant materials

Four cultivated peanut genotypes '06-4104', '10-2310', 'zhonghua No.12', and 'Kainong No. 58' with different fatty acid content (Table 1) in the seeds were used as the plant material in this study. Samples of seeds from '06-4104' at six different developmental stages as well as four different tissue types (leaf, gynophore, root, and flower) were obtained from field grown plants with nature condition. Seed development at stage 1 represents pod formation begin phase, stage 2 to 4 represent immature pod at the R5 (beginning seed), R6 (full seed) and R7 (beginning maturity) stages respectively (Boote, 1982).

Seeds at stage 5 gained fully-colored seed coat and textured pod shells, and in stage 6, seeds filled the space in pods that have attained their final size (Munger et al., 1998). Three replications of three plants each were sampled, and the fresh tissue samples were

**Table 1.** Fatty acids composition (mg g<sup>-1</sup>) in the seed of the peanut genotypes.

Genotype	16: 0	18: 0	18: 1	18: 2	20: 0	20: 1	22: 0	24: 0
06-4104	12.15	2.74	45.87	32.59	1.42	1.00	2.60	1.62
10-2310	6.35	2.35	81.47	2.60	1.28	1.84	2.58	1.53
Zhonghua No. 12	10.47	3.49	50.90	27.15	1.71	1.20	3.21	1.87
Kaihua No. 58	7.38	2.86	79.09	2.89	1.45	1.87	2.74	1.72

**Table 2.** Primer sequences used for *AhFatB1* cloning and expression analysis.

Primer name	Sequence(5'-3')
FatB-F1	GGCTTCGAAATGACGGAATAGC
FatB-F2	CTCGTGTGTCTCTCTGACGGCTA
FatB-F3	ATTCCGCTCCAATTCTGGAAGAGG
FatB-F4	GATTGGCTTCTGCGTGAAGTCAA
FatB-R1	CCGTGCGAATGTAATCAGCGGTA
FatB-R2	ACAGCCTAGCAGTAGCCCAACCA
FatB-R3	GATGCTTTCGGCTGGAACCTG
Ubiquitin-F	AAGCCAAGAAGATCAAGCACA
Ubiquitin-R	GGTTAGCCATGAAGGTTCCAG

frozen immediately in liquid nitrogen until RNA extraction. Seeds used for germination were surface-sterilized for 5 min in 0.2% (w/v) mercuric chloride HgCl<sub>2</sub> and then rinsed three times in sterile deionized water.

#### Germination and growth condition

Surface-sterilized peanut seeds were planted on the basal medium in the dark for three to five days or when the seedling showed 3 to 4 cm of radicle.

The basal medium consisted of Murashige and Skoog salts (Murashige and Skoog, 1962) supplemental with B5 vitamins, 3% sucrose and 0.4% agarose; culture media were adjusted to pH 5.8 and autoclaved at 121°C for 20 min. After germination, the seedlings were transferred from 50 to 100 mL of Hoagland solution (Jones, 1982), and cultured in a light incubator chamber with 16 h light/8 h dark condition at 28°C.

#### Fatty acid composition analysis in peanut seed

Three samples each containing ten seeds from each cultivar and different development stage of seeds from '06-4104' were examined for fatty acid composition. Fatty acid methyl esters were prepared by transmethylation with a 3% solution of potassium hydroxide in methanol. The fatty acid methyl esters of total lipids were analyzed on an Agilent Technologies 7890A gas chromatograph equipped with a flame ionization detector. Column temperature was programmed from 210 to 230°C (10°C min<sup>-1</sup>). Injector temperature was 260°C.

The carrier (nitrogen) had a flow rate of 25 ml min<sup>-1</sup>. A standard fatty acid methyl ester mixture (Sigma Chemical Co, St Louis, MO, USA) was run in order to use retention times in identifying sample peaks. Fatty acid levels were estimated on the basis of peak areas of known concentrations of the standards.

#### Nucleic acid extraction and cDNA synthesis

Genomic DNA of the tetraploid line and the diploid progenitors (*A. duranensis* and *A. ipaensis*) were prepared from young leaves using the modified Cetyl trimethylammonium (CTAB) method (Porebski et al., 1997). These DNAs were used as the template for the PCR reaction with the primer pairs FatB-F1, FatB-R1 and FatB-F3, FatB-R2, in order to isolate the entire genomic portion of the *AhFatB1* genes. Total RNA was isolated from peanut leaf, flower, stem and fruit using the RNA extraction kit (RNeasy Plant Mini Kit, Qiagen and Stanford, CA) according to the manufacturer's instructions. First strand cDNA was synthesized according to the manual enclosed in the cDNA synthesis kit (Life Technologies superscript cDNA synthesis system). The concentration of DNA and total RNA for each sample was quantified with Biotek Epoch Gen5 Take3 Module (Biotek, Winooski, VT).

#### Isolation of FatB thioesterase gene from peanut

In order to identify genes encoding of FatB thioesterase that are actively expressed in peanut, homologue genes with high identity to putative *FatB* cDNA sequences from *Arachis* (Genbank ID: EF117305-EF117309) were screened against full length cDNA libraries from different peanut line '06-4104' tissues. Eight corresponding clones were identified and clustered into two contigs with the same predicted amino acid sequence. Primer pairs (Table 2) were designed for full length cDNA and genomic DNA sequence amplification on the basis of these two contigs. The full length of cDNA was cloned through amplification with the primer pair FatB-F2 and FatB-R3. Amplification was performed in a total reaction volume of 20 µL with 10 pmol each of forward and reverse primer, 2 µL of the single-stranded cDNA, and 10 µL of 2×PCR Master Mix (MBI Fermentas, USA, catalog: K0171); PCR conditions was initial incubation at 94°C for 5 min, 32 cycles of 95°C for 45 s, 55°C for 1 min, 72°C for 2 min and a final incubation at 72°C for 10 min. PCR products were purified with EZNA™ gel extraction kit (Omega) and cloned into the pMD19-T vector (Takara, Japan) and sequenced. DNA sequencing and Oligonucleotides primers synthesis were undertaken by a commercial facility (BGI, China).

#### Sequence analysis

The BLAST analysis confirmed the sequence identity to FatB cDNAs. The N-terminal sequence of 120 amino acids was screened for the presence of a transit peptide using TargetP1.1 server. This was followed by predictions using ChloroP1.1, which is a neural network-based method for predicting chloroplast transit peptides and their cleavage sites. The theoretical molecular weight and isoelectric point of deduced polypeptide were predicted using the ProtParam program (<http://www.expasy.ch/tools/protparam.html>), and Exon-Intron analysis was made with the FGENESH program (<http://linux1.softberry.com/>). Sequence comparisons were conducted using the ClustalX 1.83 program. Amino acid multiple alignments were made with the ClustalW program



AhFatB1 and other FatB thioesterases. Additionally, a conserved acyl-thio N super family and two Helix/4-Stranded sheet (4HBT) domains containing residues that affect specificity in the N-terminal and catalytic residues in the C-terminal (Figure 2A), both of which proved to be typical domains for FatB thioesterases were observed in the deduce amino acid sequence obtained. Moreover, a host of confirmed FatB thioesterases sequences from various plant species displaying high sequence similarity with AhFatB1 were downloaded from NCBI, and a phylogenetic tree was generated based on these sequences according to ClustalW-derived protein alignments (Figure 2B). The putative AhFatB1 was found to have the highest identity with the FatB thioesterase from *Glycine max*, which also belongs to leguminosae. In addition, phylogenetic analysis indicate that AhFatB1 has the highest similarity to thioesterases whose preferred substrate is 16: 0-ACP (Figure 2B), such as GmaFatB, AtFatB1 and GhFatB. It showed a lower similarity to EgFatB, UcFatB and CcFatB, which have preferences for 10: 0-, 12: 0- and 14: 0-ACP, respectively. Thus, these findings indicate that the *AhFatB1* gene cloned was probably coding for a palmitoyl-ACP thioesterase.

### Genomic structure and organization of *AhFatB1* genes

Two pairs of primer were designed to amplify the full length genomic sequence from different peanut genotypes. A 3383-bp region containing the 5'-UTR and 5' reading frame region was amplified from *A. hypogaea* genomic DNA using FatB-F1 and FatB-R1 as primers; the 3' region amplification was conducted with the primer pair FatB-F4 and FatB-R2 (Table 2 and Figure 3). Both *AhFatB1A* and *AhFatB1B* genes could be amplified in each allotetraploid peanut line, which was evidenced by the results of direct sequencing of the PCR amplification products (data not shown). Based on these results, the PCR products amplified from the 5' region and 3' region were sub-cloned, and the sub-clones that correspond to *AhFatB1A* and *AhFatB1B* were identified by sequencing the reading frame of the inserts. The region of overlap between the cDNA sequence and the genomic sequence of *AhFatB1A* was 100% identical, confirming that the genomic sequences obtained by PCR were derived from the *AhFatB1A* gene. In this study, an intron in the 5'-UTR, a 9-bp insertion in *AhFatB1A*, or a 9-bp deletion in *AhFatB1B* were identified and characterized (Figure 4). Since the cultivated peanut is an allotetraploid (AABB genome;  $2n = 4x = 40$ ), it was examined whether *AhFatB1A* and *AhFatB1B* were putative alleles of the same locus, represented duplicated genes, or were possible homologues derived from the original diploid progenitor species. The 3383-bp region of the gene sequences covering the 5'UTR were amplified from genomic DNA of the two putative diploid progenitor species; *A. duranensis* (AA genome;  $2n = 2x = 20$ ) and *A.*

*ipaensis* (BB genome;  $2n = 2x = 20$ ). *AhFatB1B* appears to originate from the ancestral B genome, since both *AhFatB1B* and the *A. ipaensis* sequences lack the 9-bp insertion in the 5'UTR. In contrast, the 9-bp insertion in the 5'UTR shown in *AhFatB1A* is conserved in *A. duranensis*. Results from direct sequencing of the amplification product from the region containing the polymorphism sites also indicate the presence of two homologous *AhFatB1* genes in genomes of cultivated peanut, but a single variant in the diploid progenitors (data not shown).

### Examination of the transcript level of peanut FatB thioesterase

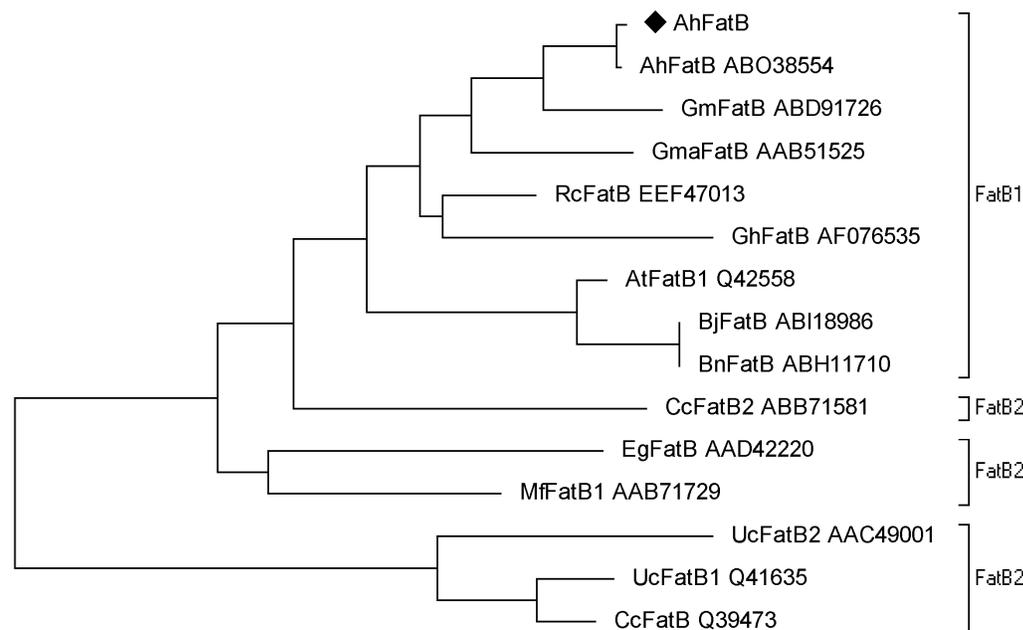
The expression levels of *AhFatB1* gene were studied by real time RT-PCR in developing seeds and vegetative tissues of peanut. Results in Figure 5 show that *AhFatB1* gene is constitutively expressed in peanut and the transcript level was high in flowers and matured seeds, lowest in root and considerably lower in the gynophore and leaf. The expression of *AhFatB1* gene in seeds at several developmental stages was also determined; the transcript accumulation profile was temporally regulated during seed development. The steady-state transcript level was highest in developing seeds at stage 6 which corresponds to the highest level of 16: 0 content, lower in stages 3 and 4, and considerably lower in stages 1 and 2 (Figure 5).

### DISCUSSION

The selection of FatB thioesterases by plant breeders was worked out for many years; the first *FatB* gene 'TE 3-2' in *Arabidopsis* was cloned and characterized to encode for type 1 FatB enzyme, which showed thioesterase activity with a broad substrate specificity toward 14: 0 to 18: 0-ACP accepting 16: 0-ACP as the preferred substrate (Dörmann et al., 1995). A type 2 FatB enzyme was purified from seeds of undomesticated California bay which accumulate laurate (12: 0), and expression of complementary DNA in the seeds of *Arabidopsis thaliana* resulted in the accumulation of medium chain fatty acids at the expense of long chain fatty acid (greater than or equal to 16: 0) normally accumulated in *Arabidopsis* seeds (Voelker et al., 1992). In addition, by comparison of deduced amino acid sequences of FatB from different plant species, regions of possible functional importance on determining substrate specificity were revealed (Salas and Ohlrogge, 2002; Mayer and Shanklin, 2005, 2007). The existence of multiple FatB thioesterase encoding genes in the peanut genome was documented, but their contribution to fatty acid metabolism in peanut seed is yet to be determined. In this study, we isolated the full length *FatB* clone from *A. hypogaea* coding for 413 amino acids with high sequence similarity to putative *FatB* genes

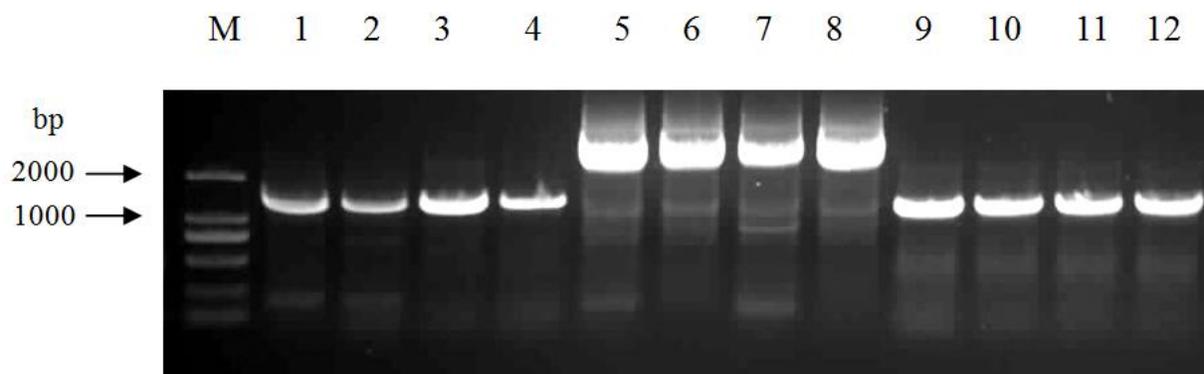


A

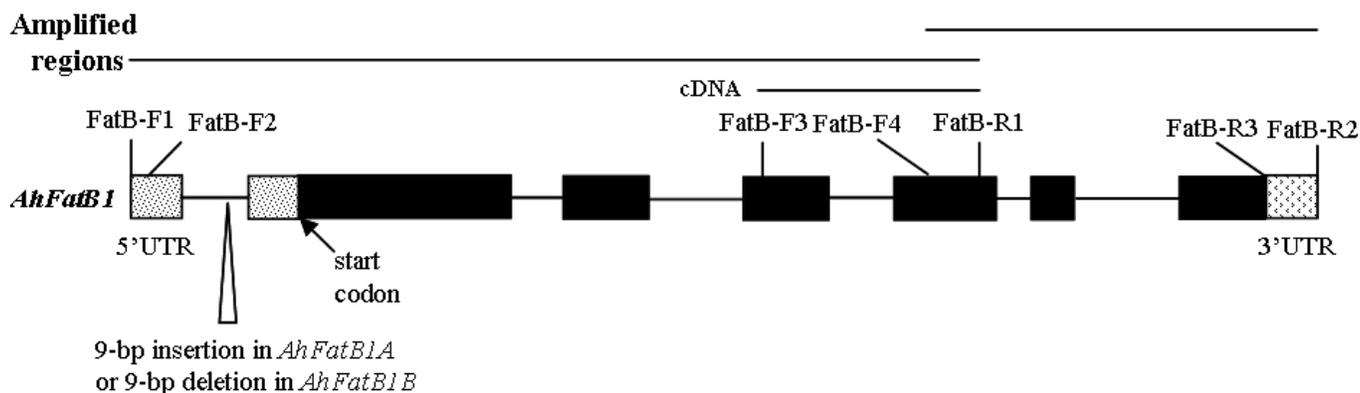


B

**Figure 2.** Sequence analysis of AhFatB1 and its homology proteins. (A) Alignment of FatB thioesterases from the model plant *Arabidopsis* and oil crops. The conserved domains (acyl-thio N super family domain and 4HBT domain respectively) regions for thioesterase are underlined. Two essential catalytic residues (H317 and C352) and the putative chloroplast transit cleavage site are indicated by the vertical arrowhead. (B) Phylogenetic tree showing relationships among AhFatB1 and other plant acyl-ACP thioesterases, according to ClustalW-derived protein alignments, generated by MEGA5. Plant species included in the figure are: At, *Arabidopsis thaliana*; Bj, *Brassica juncea*; Bn, *Brassica napus*; Cc, *Cinnamomum camphora*; Eg, *Elaeis guineensis*; Gh, *Gossypium hirsutum*; Gm, *Glycine max*; Gma, *Garcinia mangostana*; Mf, *Myristica fragans*; Rc, *Ricinus communis*; Uc, *Umbellularia californica*.



**Figure 3.** Amplification of *AhFatB1* at the mRNA and genomic DNA level. Lane M, a 1 kb DNA ladder; lane 1-4, full length cDNA amplification with primer pair FatB-F2 and FatB-R3; lane 5-8, partial DNA sequence amplification with primer pair FatB-F1 and FatB-R1; lane 9-12, partial DNA sequence amplification with primer pair FatB-F4 and FatB-R2. Four different peanut genotypes mentioned in Table 1 were used as templates for PCR amplification.

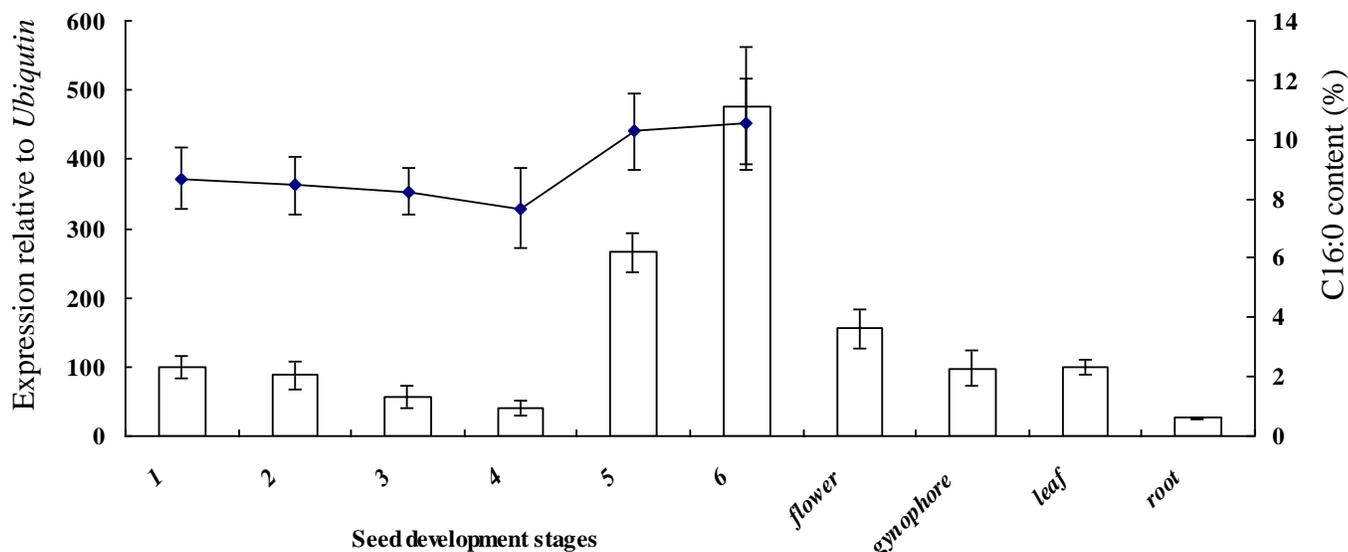


**Figure 4.** Structure of the FatB thioesterase genes of *A. hypogaea*. Two homologous genes (*AhFatB1A* and *AhFatB1B*) were analyzed by sequencing both cDNA and genomic clones. The primers used in this study are shown. The PCR amplified regions designed for genomic sequence assembly and gene expression analysis (at the cDNA level) mentioned in the text are also indicated with straight lines. Black box mark the exons, and introns are indicated by the black lines.

deposited in NCBI. Alignment of the *AhFatB1* with other FatB thioesterases isolated from different taxa revealed that *AhFatB1* could be grouped into type 1 FatB thioesterases which showed substrate specificity toward long chain fatty acid ACPs.

Some sequence heterogeneity was observed at the nucleotide level when compared to the *AhFatB1* gene sequences obtained from peanut genotypes with different fatty acid composition. Seven SNPs in the coding region and the corresponding genomic loci of *AhFatB1* gene were discovered, and the *AhFatB1* gene cloned was classified into two homologous genes, *AhFatB1A* and *AhFatB1B*, each having its origin in different diploid progenitor species. This is evidenced by the characterization of the *AhFatB1* gene from the two putative diploid progenitor species *A. duranensis* and *A. ipaensis*. The major difference between the two

homologous genes was in the 5' non-coding regions where *AhFatB1A* has an additional 9-bp insertion. It is not known if the size differences arise from differential processing of the 5' of pre-mRNAs or other factors. The existence of *AhFatB1A* and *AhFatB1B* in the peanut genome is not surprising, as has previously been reported for some other fatty acid metabolism genes in peanut (Shilman et al., 2011; Jung et al., 2000) but the different functions of *AhFatB1A* and *AhFatB1B* in the peanut fatty acid metabolism are yet to be characterized. The *AhFatB1* cloned was constitutively expressed in all the tissues examined with a relative higher expression level in flower and seed than other tissues. The total *FatB1* gene transcript is temporally regulated at different seed developmental stages, this might explain the difference between young seeds with relative low palmitic acid accumulation and matured peanut seeds with higher



**Figure 5.** Quantitative real-time PCR analyses of *AhFatB1* gene in various peanut tissues and seed development stage using *Ubiquitin* as the reference gene. Numbers on the x-axis indicate the seed developmental stages (as described in material and method).

palmitate accumulated. In this study, the total *FatB1* gene including *AhFatB1A* and *AhFatB1B* expression was measured, though differences in homolog-specific expression patterns of these two genes are yet to be determined.

In summary, one of the acyl-acyl carrier protein thioesterase coding gene *AhFatB1* from allotetraploid and diploid progenitors peanut was identified. Both *AhFatB1* genes (*AhFatB1A* and *AhFatB1B*) were characterized at the nucleotide and amino acid level, and sequence difference was observed between the A-genome origin diploid progenitor *A. duranensis* and the B-genome origin progenitor *A. ipaensis*. Both *AhFatB1A* and *AhFatB1B* was expressed in peanut leaf and seed while the total *AhFatB1* transcript mainly accumulated during the development of seeds and with full correspondence to fatty acid accumulation.

## ACKNOWLEDGMENT

This work was supported by a grant from National Natural Science Foundation of China (No. 31071456).

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