

Research Article

Cloning of Acyl-ACP Thioesterase FatA from *Arachis hypogaea* L. and Its Expression in *Escherichia coli*

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In this study, a full-length cDNA of the acyl-ACP thioesterase, *AhFatA*, was cloned from developing seeds of *Arachis hypogaea* L. by 3'-RACE. Sequence analysis showed that the open reading frame encodes a peptide of 372 amino acids and has 50–70% identity with FatA from other plants. Real-time quantitative PCR analysis revealed that *AhFatA* was expressed in all tissues of *A. hypogaea* L., but most strongly in the immature seeds harvested at 60 days after pegging. Heterologous expression of *AhFatA* in *Escherichia coli* affected bacterial growth and changed the fatty acid profiles of the membrane lipid, resulting in directed accumulation towards palmitoleic acid and oleic acid. These results indicate that *AhFatA* is at least partially responsible for determining the high palmitoleic acid and oleic acid composition of *E. coli*.

1. Introduction

In higher plants, fatty acid biosynthesis is catalyzed by the action of a type II fatty acid synthase, located in plastids [1–4]. The reaction includes the condensation of malonyl-ACP (acyl carrier protein) with acyl-ACP derivatives resulting in the acyl-ACP chain successively elongated with two carbon units [5, 6]. The final acyl chain elongation product is terminated by acyl-ACP thioesterases (Fats) that hydrolyze the thioester bond of the acyl-ACP and release free fatty acids, which are quickly exported to the cytosol via acyl-CoA synthetase [3, 7, 8].

Plant acyl-ACP thioesterases are plastid-targeted and nuclear-encoded proteins. Based on their sequence identity and substrate specificity, there are two gene families: FatA and FatB [9–11]. The FatA gene is one of the key genes involved in the plastidial fatty acid biosynthesis pathway and encodes thioesterase, with a higher specificity for 18:1-ACP and a lower activity for 18:0-ACP and 16:0-ACP [5, 12–15]. The FatA thioesterase determines which fatty acids are available for the biosynthesis of membrane lipids and allows

the transport of fatty acids out of the plastids to incorporate into glycerolipids. On the other hand, the FatB gene encodes thioesterases with a preference for saturated fatty acids with 8–18 carbons [4, 5, 7, 16].

Recently, several FatA and FatB cDNAs have been cloned and characterized following recombinant expression in *E. coli* and in plants [4, 5, 13, 17–20]. However, there has not yet been a similar report regarding peanut Fat genes. In the present study, we report the isolation of the *AhFatA* gene and the characterization of the mechanisms and expression levels of FatA in *A. hypogaea* L. We believe this is the first such work reported for *AhFatA* and that it will provide information for the genetic manipulation of *A. hypogaea* L. fatty acid. We also demonstrate significant changes in fatty acid profiles as a result of heterologous expression in *E. coli*.

2. Materials and Methods

2.1. Plant Materials. Peanut cultivar “Luhua 14” was used in this study. Roots, stems, leaves, flowers, and seeds of

TABLE 1: Primer sequences used in this study.

Primer name	Sequence (5'-3')
3' RACE primer	GCTTGGAGTGATGTAGTTGAGAT
3' RACE outer primer	TACCGTCGTTCCACTAGTGATTT
3' RACE inner primer	CGCGGATCCTCCACTAGTGATTTCACTATAGG
AhFatA-outer-F	GCTTGGAGTGATGTAGTTGAGAT
AhFatA-inner-F	ATGGTGTCAAGGGGAAGGGAGGGT
AhFatA-F	CTACCATGGAATGTTGAAGGTTTCAT
AhFatA-R	CATCACGTGGATCATAATCTTGAAGCT
Actin-F	GTCCATCAGGCAACTCGTAGC
Actin-R	GCCCTCGACTATGAGCAAGAG
AhFatA-F	AGGCCTCATATGGGTCCTG
AhFatA-R	TGACTTGATCGGTCGCATAG

“Luhua 14” were harvested at various developmental stages of the immature seeds at 10, 20, 30, 40, 50, 60, and 70 days after pegging (DAP). All tissues and seeds were immediately frozen in liquid nitrogen and then stored at -80°C until further use.

2.2. RNA Isolation and cDNA Synthesis. Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, CA) following the manufacturer’s instructions. First strand cDNA was synthesized using M-MLV reverse transcriptase and modified oligo (dT) following the manufacturer’s instructions (TaKaRa, Dalian, China).

2.3. Analysis of the FatA Sequence in *A. hypogaea* L. On the basis of expression sequence tags (ESTs) of peanut (G0260003, G0340956, and G0268105) from Genbank, sequence assembly and alignment showed that these three ESTs that contained the start codon were AhFATA fragments. To amplify 3'-end cDNA, a 3' RACE primer (Table 1) was designed based on the ESTs, and 3' RACE PCR was performed using a 3'-Full RACE Core Set Ver.2.0 Kit (TaKaRa) according to the manufacturer’s instructions. PCR primers were then designed to obtain full length AhFATA, AhFATA-F, and AhFATA-R (Table 1). The PCR program consisted of an initial denaturation cycle at 94°C for 3 min followed by 35 cycles of 94°C for 40 s, 60°C for 35 s, 72°C for 90 s, and then final extension at 72°C for 7 min. The amplified products were separated on a 1.0% agarose gel and visualized after ethidium bromide staining. The PCR products were then cloned into a pGEM-T Easy Cloning Vector (Promega, Madison, WI) and sequenced by the High-Tech Research Center, Shandong Academy of Agricultural Science (Jinan, China).

2.4. Sequence Analysis of AhFatA. The AhFatA coding sequence and putative amino acid sequence were analyzed by DNAMAN software (Lynnon Biosoft, Vaudreuil, Quebec, Canada). Multisequence alignment was carried out by

CLUSTALX. The phylogenetic tree for *A. hypogaea* L. and other plant FatAs was constructed by DNAMAN 6.0.

2.5. Expression Analysis of AhFatA in Different Tissues and Seed Developmental Stages by Quantitative Real-Time PCR. Quantitative real-time PCR (qRT-PCR) examination of AhFatA expression was carried out with a Bio-Rad iQ5. Peanut β -actin (primers of Actin-F and Actin-R, Table 1) was used as an internal control for normalization of the cDNA. We also designed the AhFatA qRT-PCR primers, AhFatA-F and AhFatA-R (Table 1). Reactions were prepared following the manufacturer’s instructions, and qRT-PCR was performed using the Bio-Rad iQ5. Each PCR was repeated four times in a total volume of $20\ \mu\text{L}$ containing $2\times$ SYBR Green I PCR Master Mix (TaKaRa), 100 nM of each primer, and $1\ \mu\text{L}$ diluted (1:20) template cDNA. Reactions were carried out in 96-well optical-grade PCR plates and the matched optical-grade membrane (TaKaRa). The amplification program was as follows: an initial denaturation step consisting of 1 min at 95°C , followed by 42 cycles of 10 s at 95°C , 30 s at 60°C and 30 s at 72°C , and an additional cycle of 10 s at 95°C , 30 s at 58°C and 5 min at 72°C , and 10 s at 95°C for melting curve analysis. The data obtained were analyzed with Bio-Rad iQ5 software. The relative expression of AhFatA in different tissues and at various developmental stages of the immature seeds was calculated using the relative $2^{-\Delta\Delta\text{Ct}}$ method [21]; the error bars indicate SD ($n = 4$).

2.6. Recombinant AhFatA Expression in *E. coli*. The 1119-bp EcoRI-SalI DNA fragment encoding AhFatA from pGEM-T Easy/AhFatA was ligated at the same sites into pGEX-4T-1 (Biovector Science Lab, Beijing, China), generating pGEX-4T-1-FATA. The control plasmid and pGEX-4T-1-FATA were transformed into *E. coli* BL21 (DE3) (TransGen, Beijing, China). *E. coli* BL21 (DE3) cells harboring the control pGEX-4T-1 and recombinant pGEX-4T-1-FATA plasmids were grown at 37°C in LB liquid medium containing 100 mg/mL ampicillin. The cultures were induced 1.5 hours after inoculation with 1 mM IPTG and were under constant shaking for defined periods of time. Induction experiments were also performed with the same set of cultures at 25°C and 1 mM IPTG.

2.7. Lipid Extraction and Fatty Acid Methyl Ester (FAME) Analysis of *E. coli* Samples. Bacterial membrane lipid extraction was carried out as described by Bligh and Dyer [22], with modifications. Wet cell samples were heated at 40°C to obtain 300 mg dry cell paste. The dry cell paste was diluted with 4 mL chloroform/methanol (1:10, v/v), and a suspension of 1 mL hexane containing C19:0 internal standard (1 mg/mL) was added. The mixture was heated at 80°C for 2 hours in a water bath, and then after cooling, 5 mL of 7% potash was added and mixed. After 10 min, the mixture was centrifuged at $10,000\times g$ for 10 min. The supernatants (bacterial sample FAME eluate) were subjected to gas chromatography (GC) using the Elite-wax column in a Perkin-Elmer instrument (ASXL). The flame-ionization detection (FID) temperature was 250°C , and the operating temperature was maintained

at 220°C. The data presented in this paper are the average of three experiments for each sample.

3. Results

3.1. Isolation and Sequence Analysis of a Cloned Thioesterase Gene from *A. hypogaea* L. Based on sequence assembly (G0260003, G0340956, and G0268105) and alignment (Supplementary Figure 1 of the Supplementary Material available online at doi:10.1155/2012/652579), four nested primers were designed: 3' RACE outer primer, 3' RACE inner primer, *AhFATA*-outer-F, and *AhFATA*-inner-F (Table 1). Using these primers, a 750-bp DNA fragment was obtained from the 3'-RACE from the extracts of developing *A. hypogaea* L. seeds. The full-length primers, *AhFATA*-F and *AhFATA*-R (Table 1), were used to obtain a full-length cDNA of 1650 bp that was named *AhFatA* (deposited to GenBank GU324446). Sequence analysis showed that the open reading frame (ORF) encodes 372 amino acids, with a calculated molecular mass of 40 kDa and a pI of 6.73. CLUSTALX was used to align the amino acid sequence of *AhFatA* with that from other plant species. This alignment (Supplementary Figure 1A) showed that the amino acid sequence has the highest identity to *VvFatA* (*Vitis vinifera*, 225470104c) (71%) and 58% identity to *TaFatA* (*Triticum aestivum*, 21262149). Phylogenetic analysis (Supplementary Figure 1B) indicated that *AhFatA* has a higher similarity to *FatAs*, such as *GarmFatA* (*Garcinia mangostana*, 1930076) and *RcFatA* (*Ricinus communis*, 152206073), which have preference for 18:1-ACP and 16:1-ACP [4, 13]. *AhFatA* showed a lower similarity to *AtFatA* (*Arabidopsis thaliana*, 186510396), *CsFatA* (*Coriandrum sativum*, 457209), and *CtFatA1* (*Carthamus tinctorius*, 404027), which have high thioesterase activity towards 18:1-ACP and lower activity towards 16:0-ACP and 18:0-ACP [5, 12].

3.2. Expression Patterns of the *AhFatA* Gene. To investigate the expression patterns of the *AhFatA* gene in a range of organs and at different growth stages of the peanut, the relevant samples were analyzed by qRT-PCR. Results showed that in wild-type *A. hypogaea* L., transcripts were detected in every tissue, but most strongly in seeds and most weakly in roots (Figure 1). The expression of the *AhFatA* gene in seeds at different developmental stages (10, 20, 30, 40, 50, 60, and 70 DAP) was also examined by qRT-PCR, and results showed that the *AhFatA* transcript levels were higher at 60 DAP than at other stages (Figure 2). These results showed that *AhFatA* allows fatty acid accumulation in the seeds of *A. hypogaea* L., indicating that the seeds may have thioesterase activity that is different from that of plants with a shortage of fatty acids.

3.3. Overexpression of Recombinant *AhFatA* in *E. coli* Leads to the Accumulation of Palmitoleic Acid and Oleic Acid. The *E. coli* cells with pGEX-4T-1 and pGEX-4T-1-*AhFatA* plasmids were induced by 1 mM IPTG. A GST protein with a molecular mass of 26 kDa and a fusion protein with a molecular mass of 72 kDa were expressed in *E. coli* strains as determined by SDS-PAGE (Figure 3). Our results indicated

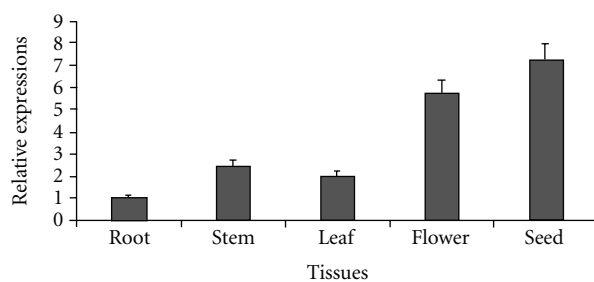


FIGURE 1: Real-time quantitative PCR analysis expression patterns of *AhFatA* in different tissues of *A. hypogaea* L. *Actin* from *A. hypogaea* L. was used as an internal control. Error bars SD. The transcript level of root was used as the calibrator whose *AhFatA* mRNA level was given as 1.

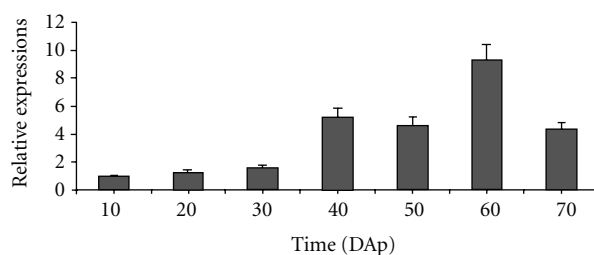


FIGURE 2: Real-time quantitative PCR analysis expression patterns of *AhFatA* at different periods of seeds development in *A. hypogaea* L. The cDNA were isolated from developing seeds at 10, 20, 30, 40, 50, 60, 70 DAP. *Actin* from *A. hypogaea* L. was used as an internal control. Error bars SD. The transcript level of 10 DAP was used as the calibrator whose *AhFatA* mRNA level was given as 1.

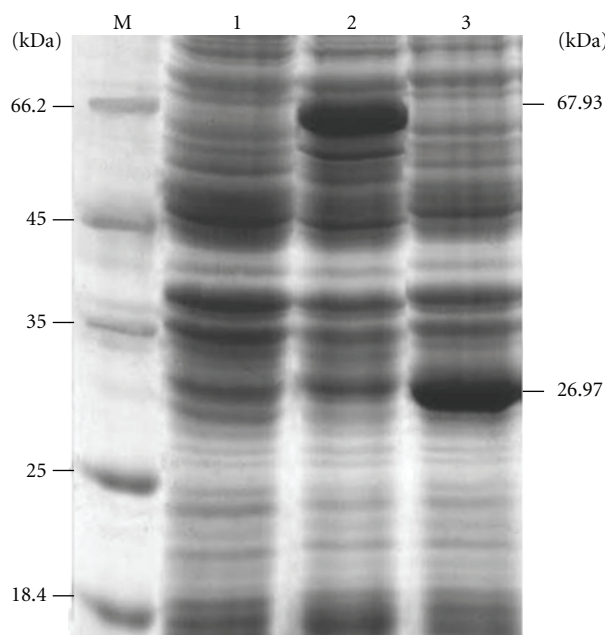


FIGURE 3: SDS-PAGE analysis of the fusion protein produced in the recombinant *E. coli* BL 21 (DE3). M: Protein Marker; (1) *E. coli* strain; (2) *E. coli* strain harboring pGEX-4T-1-*AhFatA*; (3) *E. coli* strain harboring pGEX-4T-1.

that *AhFatA* had the highest expression levels, and the fusion protein GST-*AhFatA* was soluble after being induced by IPTG for 4 h at 37°C. The *E. coli* cells with the pGEX-4T-1 plasmid also had the same expression levels of *AhFatA* (Figure 3).

It has been reported that thioesterases of type A can improve the palmitoleic acid and oleic acid composition of plants such as *Garcinia mangostana* [13], *Arabidopsis thaliana*, *Coriandrum sativum* [5], *Brassica campestris* [23], and *Ricinus communis* L. [4]. To study the changes in fatty acid composition caused by recombinant *AhFatA* activity in vivo, we performed GC analysis of the FAMES prepared from *E. coli* expressing heterologous *AhFatA*. At 37°C we observed approximate increases by 149%, 20%, and 157% in C16:1, C18:0, and C18:1 fatty acid content, respectively, in bacteria, and 18%, 28%, 0.6%, and 31.4% decreases in C12:0, C14:0, C16:0, and others (such as C14:1, C17:0, C17:1, C18:2, C18:3, C20:2), respectively, compared to vector controls (Supplementary Figure 2A). When the bacteria were grown at 25°C, C12:0, C14:0, C16:0, C18:0, and others were reduced by 33%, 27%, 7%, 23%, and 13.8%, respectively, along with a 26% increase in C16:1 and a 146% increase in C18:1 content compared to vector controls (Supplementary Figure 2B). These results demonstrate that the fatty acid composition of recombinant bacteria changed greatly towards C18:1 when the *AhFatA* gene was expressed in *E. coli* BL21 (DE3).

3.4. Overexpression of *AhFatA* in *E. coli* Affects Bacterial Growth. The *AhFatA* gene was expressed from the lac promoter in *E. coli* BL21 (DE3) and induced by IPTG. Our results showed no significant change in bacterial growth rate of either control cells or transformants before IPTG induction in 37°C. In contrast, after IPTG induction, the growth rate of *E. coli* BL21 (DE3) with pGEX-4T-1-*AhFatA* decreased over time compared to the cells with pGEX-4T-1, and cells with pGEX-4T-1 plasmid maintained the higher growth rate at 37°C (Supplementary Figure 3A). Approximately 5 hours after-induction, growth of the *E. coli* cells harboring pGEX-4T-1-*AhFatA*, or pGEX-4T-1 slowed and eventually stopped. The growth rate of the *E. coli* cells (control and transformants) showed the same trends at 25°C (Supplementary Figure 3B) as at 37°C, although growth was slower at 25°C.

4. Discussion

In this study, we cloned a 1119-bp gene from *A. hypogaea* L. and showed that it had high similarity with the *FatA* genes from other plants. The gene, called *AhFatA*, represents the first *FatA* from *A. hypogaea* L. to be studied. Real-time quantitative PCR analysis of the *AhFatA* expression pattern revealed that *AhFatA* was expressed in all tissues and was quite similar to *AtFatA* from *A. thaliana* and many other plants [1, 24]. Of all plant thioesterases, *FatA* is essential for plant viability and plays an important role in transferring acyl chains to the extraplastidial glycerolipid and determining the metabolic flux into triacylglycerols. *AhFatA* transcript levels were higher in seeds and higher at 60 DAP

than at other stages, showing that this gene functions largely in fatty acid accumulation, which is in accordance with the oil accumulation in seeds.

It has been reported that thioesterase types A have a higher specificity for 18:1-ACP. We expressed *AhFatA* in *E. coli* BL21 (DE3) using the pGEX-4T-1 vector with the lac promoter and demonstrated high levels of expression of *AhFatA*. Our results showed that the fatty acid composition of the recombinants changed greatly towards C18:1 and C16:1. There is also a relative increase in the accumulation of C18:2 in direct of the proportion of 18:1. Therefore, it could be hypothesized that the bacteria compensate for the available fatty acids by increasing the saturated fatty acids. The results are similar to those in other plants such as *Garcinia mangostana* [13], *Arabidopsis thaliana*, *Coriandrum sativum* [2], *Brassica campestris* [23], and *Ricinus communis* L. [4]. Therefore, *AhFatA* may function mainly to provide palmitoleic acid and oleic acid.

The effect of *AhFatA* gene overexpression on cell growth at different temperature was examined. The specific growth rates of *E. coli* BL21 (DE3) harboring pGEX-4T-1-*AhFatA* during the exponential phase were much lower than the host with pGEX-4T-1 plasmid. This phenomenon may due to the following reasons. The accumulation of *AhFatA* protein affected the lipid metabolism in the *E. coli* BL21 (DE3) harboring pGEX-4T-1-*AhFatA*, and then the release of free fatty acids limited the cells growth. The presented results demonstrate that the transformed cells may use the plasmid-encoded *FatA* gene to produce fatty acids.

5. Conclusions

In conclusion, we cloned the *AhFatA* gene of *A. hypogaea* L., a member of the acyl-ACP thioesterases and described the functional characterization of *AhFatA* in *E. coli* BL21 (DE3). Our results showed that the expression of *AhFatA*, which was higher in seeds than other tissues, has a high specificity for 18:1-ACP and 16:1-ACP. Overexpression of *AhFatA* in *E. coli* BL21 (DE3) leads to the accumulation of palmitoleic acid and oleic acid. This research provides the basis not only for the cloning and expression of the *AhFatA* gene, but also for modifying fatty acid composition through genetic engineering of the acyl-ACP thioesterases in plants and microorganisms. We are currently developing transgenic *A. hypogaea* L. and cyanobacteria to enhance downstream fatty acid production by termination of fatty acyl chain-elongation with *AhFatA*. We believe that the *AhFatA* gene will be helpful in transgenic lines and that it will be a suitable tool for genetic modification of oil crops to generate improved crops in the future.

Abbreviations

IPTG: Isopropyl- β -D-thiogalactoside
 ORF: Open reading frame
 PCR: Polymerase chain reaction
 RACE: Rapid amplification of cDNA ends.

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