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Cloning and Expression Analysis of β-ketoacyl-ACP Reductase, β-hydroxyacyl-ACP Dehydrase and Enoyl-ACP Reductase from *Arachis hypogaea* L.

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Abstract Fatty acid biosynthesis is catalysed in most bacteria and plants by a group of highly conserved proteins known as the Type II fatty acid synthase (FAS) system. In this study, genes for β−ketoacyl-ACP reductase (KR), β−hydroxyacyl-ACP dehydrase (HD), and enoyl-ACP reductase (ENR) of Type II FAS have been cloned from peanut (*Arachis hypogaea* L.). The results showed that the ORF of the three genes were 972 bp, 651 bp and 1 170 bp in length, encoding 323, 216 and 389 amino acids, respectively. The predicted amino acid sequences of *AhKR*, *AhHD*, *AhENR* shared high sequence identity of 86.3%, 81.2% and 87.2% to the corresponding ones in *Glycine max*, respectively. Further investigation of quantitative real-time RT-PCR analysis suggested that *AhKR*, *AhHD* and *AhENR* were expressed with higher levels in leaf and seed than those in root and stem tissues. Moreover, *AhKR* and *AhENR* genes reached a maximum expression level at 25 DAP (days after pegging) and showed a downward trend thereafter. In contrast, *AhHD* gene expressed in an irregular course during seed development. Overall, the information generated by this study will facilitate the manipulation of the quality of oils produced in seeds of oil crops.

Keywords Peanut (*Arachis hypogaea* L.); Fatty acid biosynthesis; β−ketoacyl-ACP reductase (KR); β−hydroxyacyl-ACP dehydrase (HD); Enoyl-ACP reductase (ENR); Expression analysis

Background

Fatty acid synthesis is essential for the formation of membranes and hence for the viability of all organisms (Massengo-Tiassé and Cronan, 2007). A complex enzyme system, fatty acid synthetase (FAS), is used throughout nature for the *de novo* biosynthesis of fatty acids from acetyl-CoA and malonyl-CoA (Fisher et al., 2000). In general, FAS is organized into two different systems (FAS I and FAS II) based on the architecture of the enzymes involved. In FAS I system, the involved synthases found in fungi and mammals are large multifunctional enzymes with multiple domains that catalyze various reactions of FAS (Smith et al., 2003; Schweizer and Hofmann, 2004). In contrast, the FAS of bacteria, chloroplasts, apicoplasts and mitochondria belongs to the FAS II system, where the acyl chain covalently attached to the acyl carrier protein (ACP) is elongated with four enzymes catalyzing consecutively (Campbell and Cronan, 2001; Olsen et al., 2004).

In bacteria and plants, the enzymes which catalyze the four successive steps of the pathway are as follows: β−ketoacyl-acyl carrier protein (ACP) synthetases I, II and III; β−ketoacyl-acyl carrier protein (ACP) reductase; β−hydroxyacyl-acyl carrier protein (ACP) dehydratase; and enoyl-acyl carrier protein (ACP) reductase (Rafferty et al., 1995). Chain termination is mediated by the hydrolysis of acyl-ACP, catalyzed by an acyl-ACP thioesterase. The fatty acyl chain lengthens by two carbons each time it cycles through the enzymatic reactions (Tai et al., 2007). Most fatty acids reach a chain length of 16 or 18 carbons and then are targeted for glycerolipid synthesis in the plastid or in the endoplasmic reticulum (Browse and Somerville, 1991).

β−ketoacyl-ACP reductase (KR) catalyzes the pyridine-nucleotide-dependent reduction of a β−oxoacyl form of acyl carrier protein (ACP), the second step in *de novo* fatty acid biosynthesis and a reaction often

performed in polyketide biosynthesis (Fisher et al., 2000). Two isoforms of the enzyme, one NADPH-linked and the other NADH-linked, have been reported (Caughey and Kekwick, 1982). The function of the NADH-linked enzyme is unknown. It is the NADPH-linked β−ketoacyl-ACP reductase that functions in fatty acid biosynthesis (Slabas et al., 1992). KR is a member of the Short-chain Dehydrogenase/Reductase (SDR) superfamily (Price et al., 2001), and thereby displays the amino acid signature of this family, Ser-X_{12}-Tyr-X_3-Lys (Persson et al., 2003).

The third step in the elongation cycle is catalyzed by β−hydroxyacyl-ACP dehydratase (HD). There are two isoforms. FabZ, which catalyzes the dehydration of (3R)-hydroxyacyl-ACP to trans−2−acyl-ACP, is a universally expressed component of the type II system. FabA, the second isoform, as has more limited distribution in nature and, in addition to dehydration, also carries out the isomerization of trans−2− to cis−3−decenoyl-ACP as an essential step in unsaturated fatty acid biosynthesis (Heath and Rock, 1996; Kimber et al., 2004). The catalytic site is hydrophobic except for a histidine and a glutamine (aspartic acid), which together are proposed to catalyze the reactions (Leesong et al., 1996).

The enoyl-ACP reductase (ENR) catalyses the last step in the fatty acid elongation cycle (Marrakchi et al., 2003). Four types of ENR, namely, FabI, fabK, FabL and FabV, have been reported (Bergler et al., 1994; Heath et al., 2000; Marrakchi et al., 2003; Massengo-Tiassé and Cronan, 2007). Most ENRs (FabI, FabL and FabV) are distant members of the SDR superfamily. Other classes of ENRs are the TIM barrel flavin containing enzyme, FabK, the ENRs found in mitochondria and the ENR domains of the mammalian and fungal megasynthases. Both NADH and NADPH are used as the hydride source in the reactions of SDR-type ENRs (Massengo-Tiassé and Cronan, 2007). FabI and FabL are atypical in that the key residues are a diad consisting of a Tyr-X_6-Lys motif in contrast with the Tyr-X_3-Lys in prototypical SDRs (Baker, 1995; Parikh et al., 1999). FabV is considerably larger than either FabI or FabL, and contains a Tyr-X_8-Lys active site motif (Massengo-Tiassé and Cronan, 2007).

Cultivated peanuts (*Arachis hypogaea* L.) are important oilseed crops worldwide, because these allotetraploids (2n=4x=40) typically contain 50% oil in the seed. The flavor and quality of either the seed or the oil is immensely dependent on the fatty acid composition of the extracted oil (Andersen and Gorbet, 2002; Jung et al., 2000b; Lopez et al., 2000; Yu et al., 2008). It would be of great importance to study the fatty acid biosynthesis pathway for improving oil quality and increasing oil content of peanut. In this study, we isolated and characterized cDNAs containing the complete coding region of *AhKR*, *AhHD* and *AhENR* genes, and analyzed their expression in different organs and at different development stages of seeds. The identification of these novel genes in this study will be helpful for the reconstruction of the pathways involved in fatty acid biosynthesis and the metabolic engineering of fatty acid synthesis in peanut.

1 Results

1.1 Isolation and analysis of *AhKR*, *AhHD* and *AhENR* genes

Three full-length genes namely β−ketoacyl-ACP reductase (KR), β−hydroxyacyl-ACP dehydrase (HD), and enoyl-ACP reductase (ENR) were isolated from a peanut seedling full-length cDNA library (unpublished data). The ORF of the three genes were 972 bp, 651 bp and 1 170 bp in length, encoding 323, 216 and 389 amino acids, respectively (Table 1). Prediction of subcellular location by two programs, TargetP Server and Predotar, suggested that these three proteins probably located in chloroplast. The first 73, 43 or 70 amino acids at the N-terminal end of the deduced protein for *AhKR*, *AhHD* or *AhENR* had a high proportion of hydroxylated and small, hydrophobic amino acids, which was typical of chloroplast transit peptide. A Blast search revealed that the primary structure of *AhKR*, *AhHD*, *AhENR* shared high sequence identity of 86.3%, 81.2% and 87.2% to the corresponding ones in Glycine max, respectively. The deduced amino acid sequences of *AhKR*, *AhHD*, *AhENR* showed 37.3%, 30.2%, and 20.9% identity with *EcFabG*, *EcFabZ*, and *EcFabI*, respectively.

Table 1 *AhKR*, *AhHD* and *AhENR* genes in peanut

Protein	GenBank accession No.	The length of aa	5' upstream region (bp)	3' downstream region (bp)	Molecular mass (kD)	pI	Localization	The length of CTP
KR	FJ768728	323	197	399	33.855	9.22	Chloroplast	73
HD	FJ768727	216	39	134	23.422 2	8.91	Chloroplast	43
ENR	FJ768731	389	200	330	41.505 4	8.64	Chloroplast	70

Note: CTP represents chloroplast transit peptide

1.1.1 Cloning and phylogenetic analysis of *AhKR* gene

In Figure 1, the peanut β-ketoacyl-ACP reductase protein sequence was compared for similarity with those of plants and bacteria using ClustalW. A triad of Ser217, Tyr230 and Lys234 residues involved in catalysis and substrate binding was revealed by sequence alignments, characteristic of the SDR family. The tyrosine and lysine residues are involved in actual catalysis, whereas serine participates in substrate binding and alignment (Price et al., 2001; Li et al., 2009). To examine the relationships among different sources of *KR* genes, the neighbour-joining method was used to construct the phylogenetic tree (Figure 2). As shown in the phylogenetic tree, all of the *KR* genes fell into two subfamilies: the bacteria subfamily and the cyanobacteria/green algae/mosses/higher plants subfamily. The *AhKR* gene from peanut clustered with those from higher plants, and the genes from cyanobacteria may be the origin of genes from higher plants, mosses and eukaryotic algae.

1.1.2 Cloning and phylogenetic analysis of *AhHD* gene

As shown in Figure 3A, multiple sequence alignment indicated that AhHD protein contained a histidine at amino acid 116 and a glutamine at amino acid 130, which were in positions that were conserved in the FabZ of *Escherichia coli* (Cronan et al., 1988). The location of these residues corresponded to the catalytically active site of the enzymes in *Plasmodium faliciparum* and *Escherichia coli*. (Cronan et al., 1988; Heath and Rock, 1996; Sharma et al., 2003). Specifically, there were distinct differences in the active site residues, an Asp in FabA and a Glu in FabZ (Figure 3B). In addition, similar to PaFabZ (Kimber et al., 2004), five key motifs, LPHRFPFLLVD, GHFP, PGVL, EAMAQ, and AGD, were also conserved in *AhHD*. Phylogenetic analysis suggested that *fabZ* and *fabA* genes fell into two separate subfamilies (Figure 4). The *AhHD* gene from peanut clustered together with genes from higher plants, and were separated from those of green algae. The *FabZ* genes from cyanobacteria may be the origin of genes from higher plants and eukaryotic algae.

1.1.3 Cloning and phylogenetic analysis of *AhENR* gene

FabV, FabI and FabL were distant members of the SDR superfamily, although FabV aligned only weakly with FabI or FabL even when many gaps were allowed (Figure 5A). FabI and FabL contained a Tyr-X_6-Lys active site motif. In FabV other variation was seen, a Tyr-X_8-Lys motif (Figure 5A). The

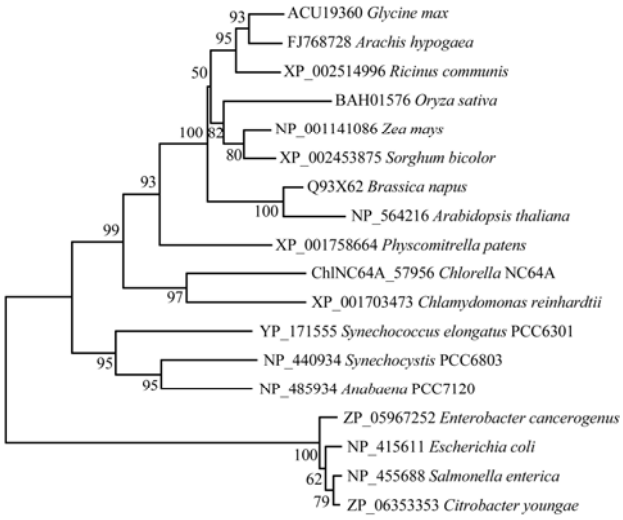

Figure 2 Neighbor-joining tree based on the deduced amino acid sequences of *KR* homologs

Note: Sequences were shown by their accession numbers (locus tags) and strain names; Bootstrap values from neighbor-joining analyses were listed to the left of each node, with values more than 50 were shown

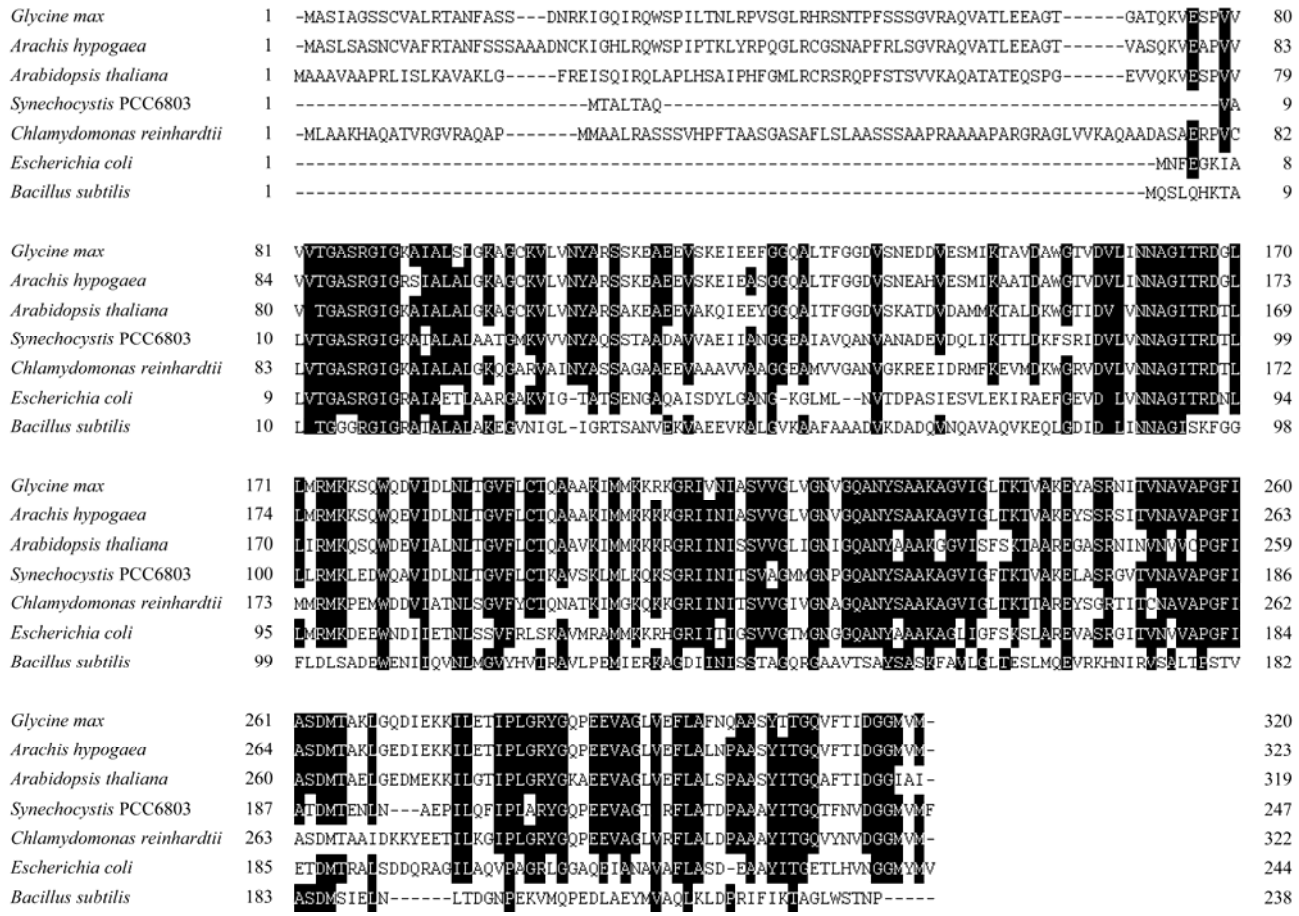

Figure 1 Multiple sequence alignments of *KR* homologs

Note: The three conserved residues of *A. hypogaea* KR corresponding to *E. coli* FabG Ser138, Tyr151 and Lys155 were highlighted in asterisks; GenBank accession numbers were respectively as follows: *Arachis hypogaea* (FJ768728), *Glycine max* (ACU19360), *Arabidopsis thaliana* (NP_564216), *Ricinus communis* (AAA33873), *Synechocystis* PCC6803 (NP_440934), *Chlamydomonas reinhardtii* (XP_001703473), *Escherichia coli* (AAC67304), and *Bacillus subtilis* (NP_389732)

spacing between the putative FabV active site tyrosine and lysine residues was eight residues, two more than FabI and FabL and one more than the maximum reported for other SDR proteins. FabK, first discovered in Streptococcus pneumoniae, was refractory to triclosan and was a flavoprotein unrelated to the SDR isozymes (Marrakchi et al., 2003). The alignment of the predicted FabK-like proteins from this subset of organisms demonstrated that they possessed a conserved nucleotide-binding domain in the N-terminus and a conserved flavin-binding domain at the centre of the protein (Figure 5B).

AhENR and *AhKR* were members of the SDR superfamily, and *AhENR* showed 16.1% sequence identity with *AhKR*, compared with 25% in *Brassica napus* (Fisher et al., 2000). However, *KR* and *ENR* catalyzed distinctly different chemical reactions, namely a carbon–oxygen double-bond reduction and a carbon–carbon double-bond reduction, respectively. Fisher et al. (2000) reported that striking similarities existed in fold, mechanism and substrate binding of *BnKR* and *BnENR*. Thus, these two enzymes may have diverged from a common ancestor during the evolution of the biosynthetic pathway. Phylogenetic analysis suggested that the *AhENR* gene from peanut formed a group with the genes from higher plants and green algae, and set apart from the groups of cyanobacteria and bacteria (Figure 6).

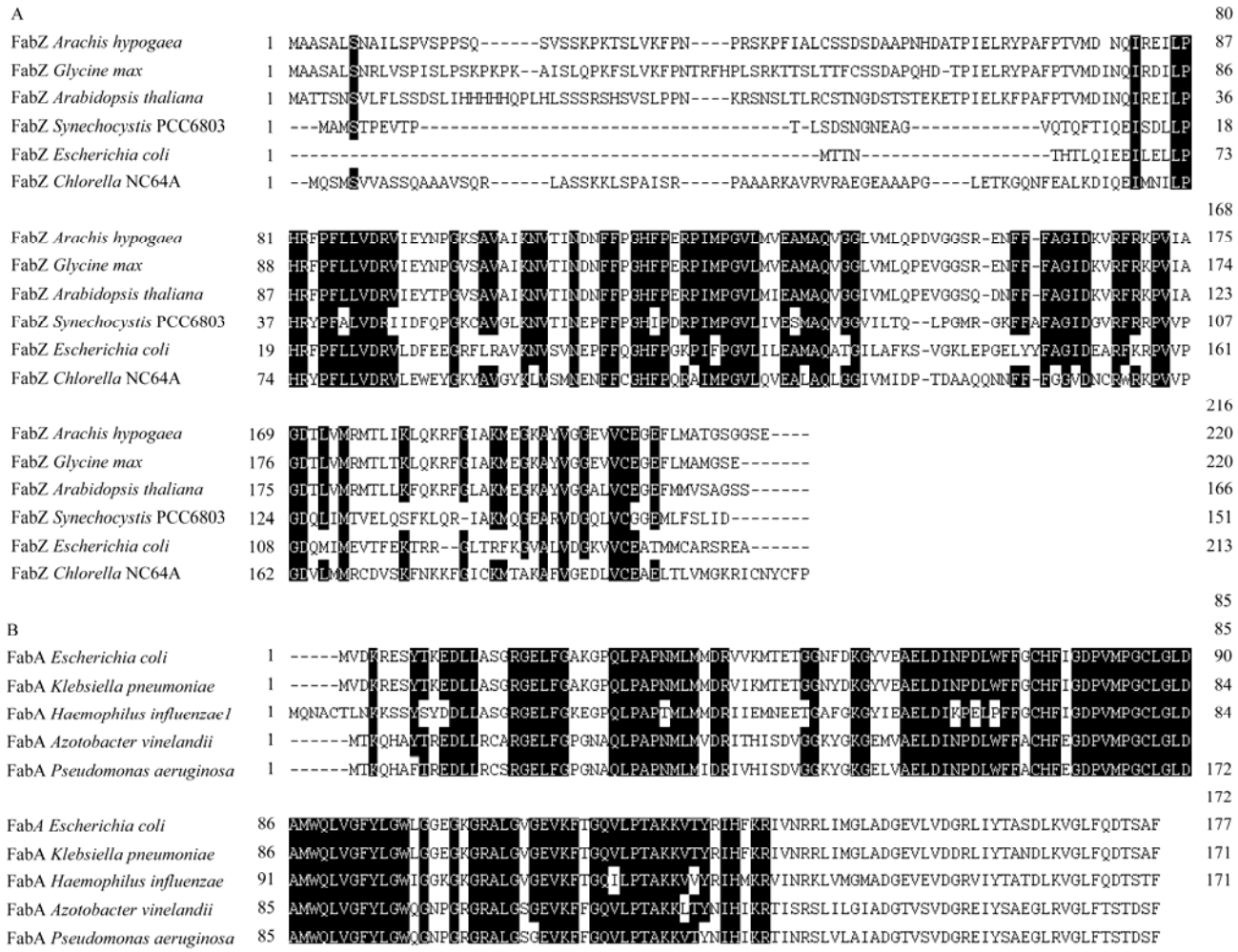

Figure 3 Multiple sequence alignments of *HD* homologs

Note: The two conserved residues of *A. hypogaea* HD corresponding to *E. coli* FabZ His54, Glu68 and *E. coli* FabA His70, Asp84 were highlighted in asterisks; GenBank accession numbers (locus tags) for FabZ were respectively as follows: *Arachis hypogaea* (FJ768727), *Glycine max* (ACU23629), *Arabidopsis thaliana* (NP_565528), *Synechocystis* PCC6803 (NP_441227), *Chlorella NC64A* (ChlNC64A_50849), *Escherichia coli* (AAC36917); GenBank accession numbers (locus tags) for FabA were respectively as follows: *Escherichia coli* (NP_415474), *Klebsiella pneumoniae* (YP_001334649), *Haemophilus influenzae* (NP_439476), *Azotobacter vinelandii* (YP_002800049), and *Pseudomonas aeruginosa* (AAG04999)

1.2 Quantitative real-time RT-PCR analysis

The quantitative real-time RT-PCR (qRT-PCR) was employed to confirm the expression patterns of *AhKR*, *AhHD*, and *AhENR* genes in four peanut tissues and at different developmental stages of seeds. β−actin was used as an internal reference control for total RNA input. β−actin PCR product was not detected when reverse transcriptase was omitted, indicating that the RNA template was free of genomic DNA. The results revealed that the three genes were expressed dominantly in leaf among four tissues tested, and expressed at the lowest level in stem (Figure 7). In addition, the expression level of these genes in seed was also relatively high. The expression patterns of the three genes across six developmental stages of seed were illustrated in Figure 8. *AhKR* and *AhENR* genes reached a maximum expression level at 25 DAP and showed a downward trend thereafter. In contrast, *AhHD* gene expressed in an irregular course during seed development and had high expression at 25 and 60 DAPs. These results indicated that these genes may have different biochemical functions during vegetative growth and seed development.

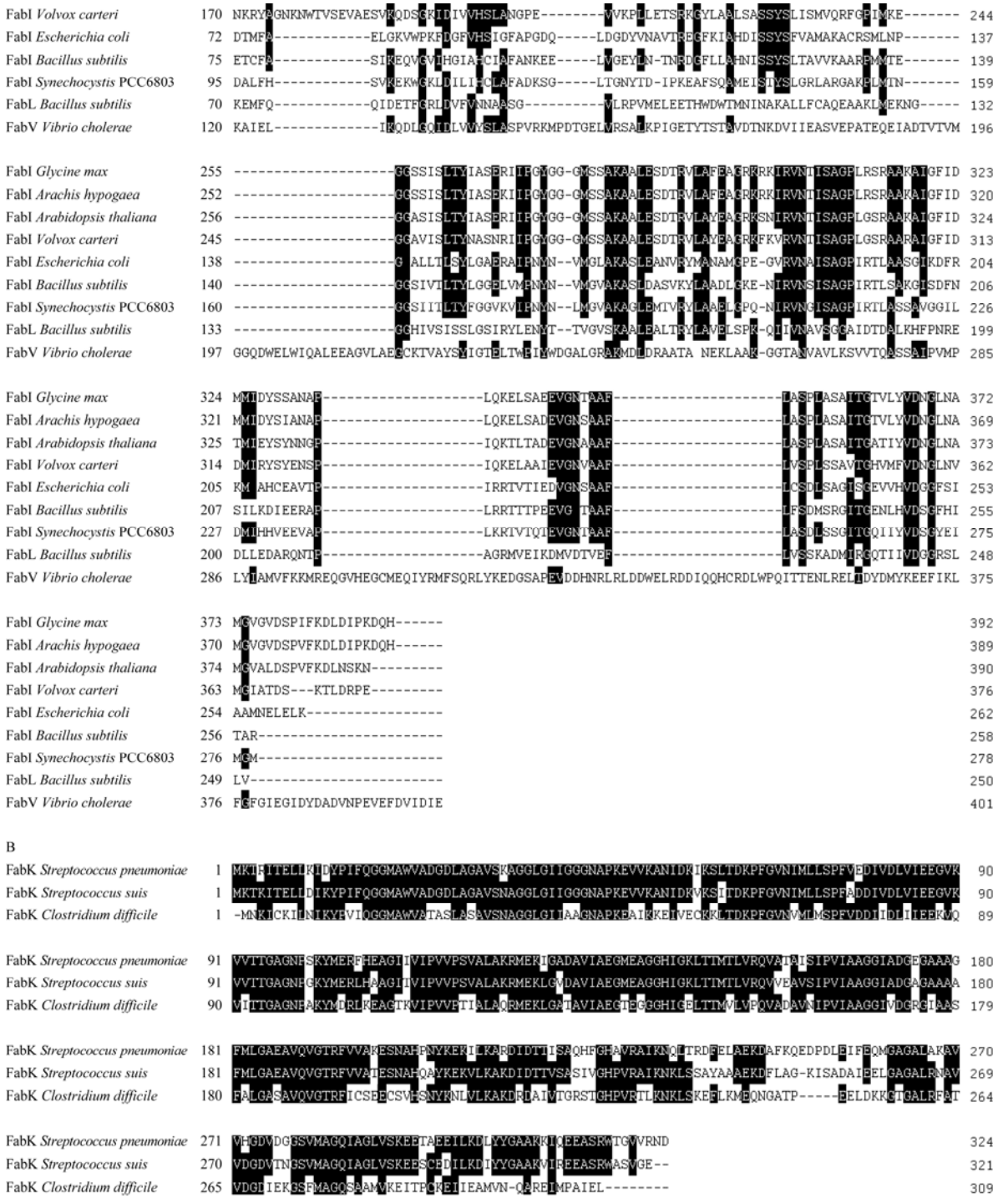

Figure 5 Multiple sequence alignments of ENR homologs

Note: The sequences are denoted by their strain names; The two conserved residues of *A. hypogaea* ENR corresponding to *E. coli* FabI Tyr156 and Lys163 were highlighted in asterisks; The central region of the proteins containing a consensus flavin-binding site was underlined; GenBank accession numbers (locus tags) for FabI, FabL and FabV were respectively as follows: *Arachis hypogaea* (FJ768731), *Glycine max* (ACU23403), *Arabidopsis thaliana* (NP_565331), *Synechocystis* PCC6803 (NP_440356), *Volvox carteri* (Volca1_83516), *Escherichia coli* (AP_001914), *Bacillus subtilis* (NP_389054), *Bacillus subtilis* (NP_388745), and *Vibrio cholerae* (ABX38717); GenBank accession numbers for FabK were respectively as follows: *Streptococcus pneumoniae* (AAF98273), *Clostridium difficile* (YP_003217517), and *Streptococcus suis* (YP_003027441)

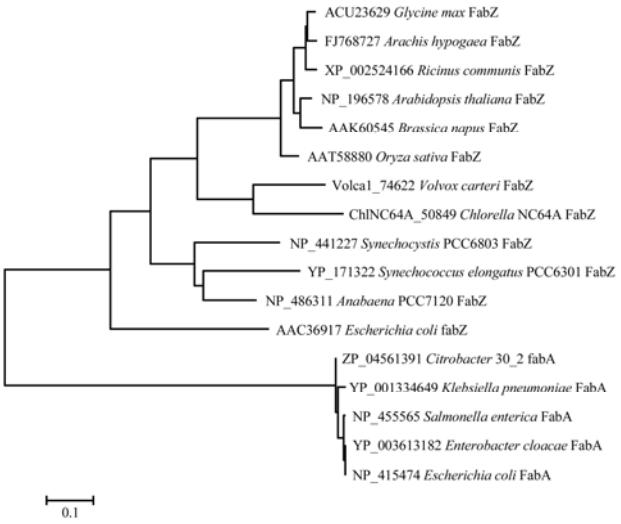

Figure 4 Neighbor-joining tree based on the deduced amino acid sequences of *HD* homologs

Note: Sequences were shown by their accession numbers (locus tags) and strain names; Bootstrap values from neighbor-joining analyses were listed to the left of each node, with values more than 50 were shown

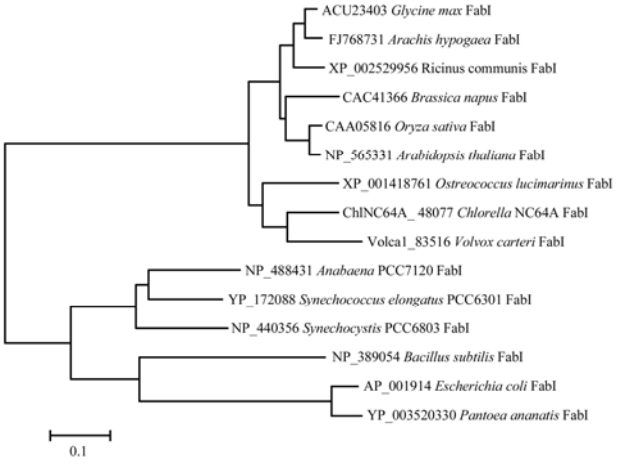

Figure 6 Neighbor-joining tree based on the deduced amino acid sequences of *ENR* homologs

Note: Sequences were shown by their accession numbers (locus tags) and strain names; Bootstrap values from neighbor-joining analyses were listed to the left of each node, with values more than 50 were shown

2 Discussion

Peanut is an important crop internationally for both food and oil production (Luo et al., 2005). One of the major factors influencing peanut oil quality is the

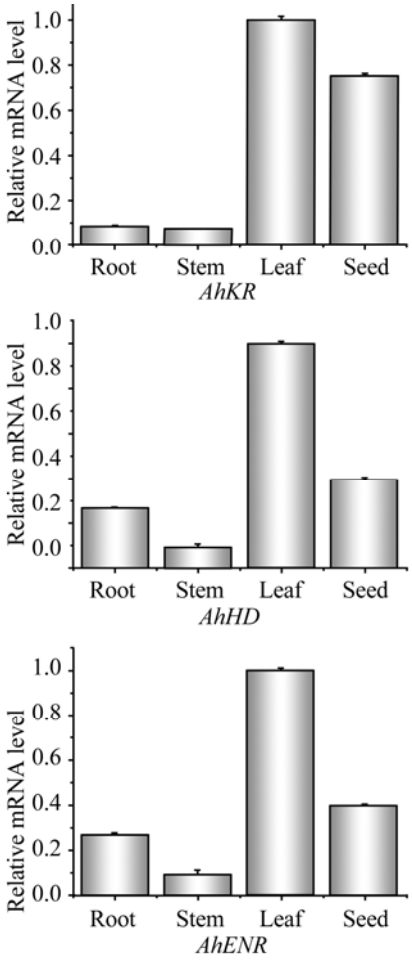

Figure 7 Expression analysis of *AhKR*, *AhHD* and *AhENR* genes in four different tissues

composition of fatty acid. So validating the mechanism of the fatty acid synthesis and metabolism is the central goal to increase peanut quality. Fatty acid biosynthesis in higher plants is carried out by type II FAS. In the present study, we isolated *AhKR*, *AhHD* and *AhENR* orthologues in peanut seedling. Sequence comparisons revealed that the primary structure of plant plastid type II FAS enzymes (KR, HD, ENR) was strictly conserved, especially the catalytic residues, which suggested that these enzymes may have similar functions in higher plants as those in *E. coli*. Real-time RT-PCR analysis revealed that the three genes were expressed dominantly in leaf among four tissues tested, and expressed at the lowest level in stem. *AhKR* and *AhENR* genes shared similar expression behaviors over the developmental stages compared to that of *AhHD* gene. These results indicated

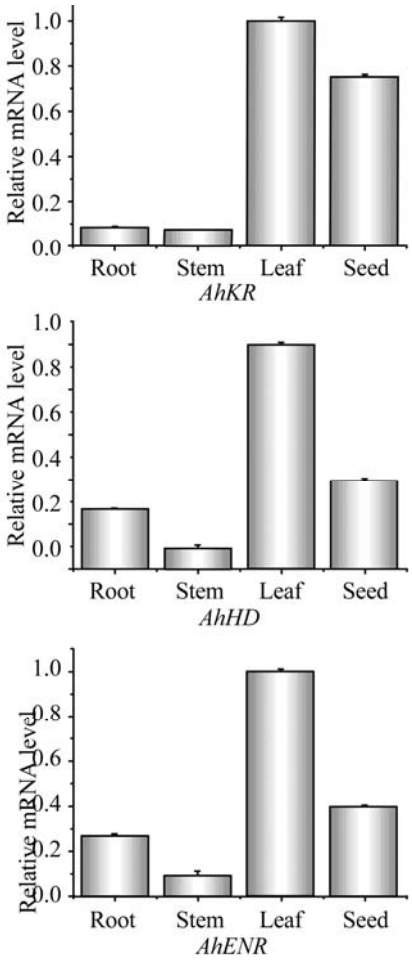

Figure 8 Expression analysis of *AhKR*, *AhHD* and *AhENR* genes in seed at different developmental stages

that the three genes may have different biochemical functions during vegetative growth and seed development. To improve peanut oil quality should coordinate the expression of the three enzymes involved in the fatty acid synthesis pathway. This work may provide a basis for elucidating the molecular mechanism of fatty acid synthesis and provide candidate genes for modifying oil quality via transgenic plants.

3 Materials and methods

3.1 Plant materials

Peanut seeds (*Arachis hypogaea* L. cultivar Huayu19) were sown in sand and soil mixture (1:1), grown in a growth chamber under a 16~8 h light-dark cycle at 26°C and 22°C, respectively. Three kinds of 12−day-old tissues including root, stem and leaf were collected as experimental materials for quantitative real-time RT-PCR analysis. In addition, the immature peanut seeds from 25 to 60 days after pegging (DAP) were also collected for expression analysis.

3.2 Nucleic acid manipulation

Total RNA was extracted from samples using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. The RNA samples were used for real-time RT-PCR after RQ1 RNase-free DNaseI (Promega, Wisconsin, USA) treatment to remove genomic DNA. The first-strand cDNA was synthesized with RT-PCR kit (Promega, Wisconsin, USA) using 500 ng of total RNA according to the manufacturer's instructions. Controls received water instead of reverse transcriptase to assess any contamination from genomic DNA as described by Zhou et al. (2007).

3.3 Full-length cDNA sequence isolation

PCR was performed with the LA PCR system (Takara) using 2.5 μL of 10×PCR buffer with $MgCl_2$, 1 μL of 10 μM each primer, 4.0 μL of 10 mM dNTPs, 1 μL cDNA samples and 0.5 μL LA *Taq*™ DNA polymerase, and 15 μL double distilled water. The PCR products were run on 1% agarose gel and purified with Gel Extraction Kit (Takara) according to the manufacturer's protocol. The purified products were then cloned into the pMD18−T Easy vector (Takara) and sequenced (Shangon, Shanghai) (Table 2).

3.4 Multiple sequence alignment and phylogenetic analysis

Physicochemical properties of the deduced protein were predicted by Protparam (http://www.expasy.ch/tools/protparam.html). The putative subcellular localizations of the candidate proteins were estimated by TargetP (http://www. cbs.dtu.dk/services/TargetP/) and Predotar (http://urgi.versailles.inra.fr/ predotar/predotar.html). The potential N-terminal presequence cleavage site was predicted by ChloroP (http://www.cbs.dtu.dk/services/ChloroP/). Amino acid sequences were aligned using ClustalX program with the implanted BioEdit (Thompson et al., 1994). The neighbor-joining (NJ) method in MEGA4 (Tamura et al., 2007) was used to construct the phylogenetic tree. Bootstrap with 1000 replicates was used to establish the confidence limit of the tree branches. Default program parameters were used.

Table 2 Primers used in experiment

Usage	Primer name	Oligonucleotide sequence (5'–3')
Full-length cDNA sequence cloning	KR-F	ATGGCTTCTCTCTCCGCTTC
	KR-R	TTACATCACCATACCTCCATCAATG
	HD-F	ATGGCAGCCTCTGCTCTCTC
	HD-R	CTATTCACTCCCTCCCGAGC
	ENR-F	ATGGCAACAACACCGTTTTCT
	ENR-R	CTAATGCTGGTCCTTGGGAATG
Real-time RT-PCR	qActin-F	TTGGAATGGGTCAGAAGGATGC
	qActin-R	AGTGGTGCCTCAGTAAGAAGC
	qKR-F	GAACTGCCAACTTCTCCTCCTC
	qKR-R	CTGCCTCCTCAAGCGTAGC
	qHD-F	CCTCTGATTCCGATGCTGCTC
	qHD-R	TGCGGGATACCTTAGTTCAATGG
	qENR-F	AGACTTCGGCACCATAGACATC
	qENR-R	GCGGATAAAGCAGCAAGATACC

3.5 Quantitative real-time RT-PCR

The real-time RT-PCR analysis was performed by using a LightCycler 2.0 instrument system (Roche, Germany). β−actin gene was taken as reference gene. Three pairs of gene-specific primers (Table 1) were designed according to the AhKR, AhHD and AhENR gene sequences. The real-time RT-PCR reactions were performed by using the SYBR Premix Ex *Taq* polymerase (TaKaRa, Japan) according to the manufacturer's instructions. The expression of the gene was calculated relative to the calibration sample and the β−actin to normalize the sample input amount. All the experiments were performed in triplicate to ensure the data accuracy.

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