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MOLECULAR CLONING AND EXPRESSION PROFILE OF β-KETOACYL-ACP SYNTHASE GENE FROM TUNG TREE (*Vernicia fordii* Hemsl.)

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Tung tree (*Vernicia fordii*) is an important woody oil tree. Tung tree seeds contain 50-60% oil with approximately 80 mole α-eleostearic acid (9 cis, 11 trans, 13 trans octadecatrienoic acid). Fatty acid synthesis is catalyzed by the concerted action of acetyl-CoA carboxylase and fatty acid synthase, a multienzyme complex including β-ketoacyl-acyl-carrier-protein synthase (KAS). Little is known about KAS in tung tree. The objective of this study was to clone KAS genes and analyze their expression profiles in tung tree. A full-length cDNA encoding KAS III and a partial cDNA encoding KAS II were isolated from tung tree by PCR cloning using degenerate primers and rapid amplification of cDNA ends system. The full-length cDNA of *VfKAS III* was 1881 bp in length with an open reading frame of 1212 bp. *VfKAS III* genomic DNA was also isolated and sequenced, which contained 8 exons in 5403 bp length. The deduced VfKAS III protein shared approximately 80% identity with homologous KAS IIIs from other plants. Quantitative PCR analysis revealed that KAS II and KAS III were expressed in all of the tissues and organs tested but exhibited different expression patterns in tung tree. The expression levels of KAS II in young tissues were much lower than those in mature tissues, whereas the highest expression levels of KAS III were observed in young stem and young leaf. These results should facilitate further studies on the regulation of tung oil biosynthesis by KAS in tung tree.

Key words: β-ketoacyl-ACP synthase, expression profile, molecular cloning, *Vernicia fordii*

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INTRODUCTION

Tung tree (*Vernicia fordii*) is an economically important non-edible woody oil tree originated in China. It is mainly distributed in subtropical areas of China, Argentina, Paraguay, Africa and southeastern region of the United States (POTTER, 1986; TAN *et al.*, 2011; CAO *et al.*, 2014). It has been grown in China for the production of tung oil for centuries (ZHANG *et al.*, 2014). Tung tree seeds contain 50-60% oil with about 80 mole α-eleostearic acid (9cis, 11trans, 13trans octadecatrienoic acid) (SHOCKEY *et al.*, 2006; CAO *et al.*, 2012b). Because of the three special conjugated double bonds in eleostearic acid, tung oil is easily oxidized and forms a unique polymer after being exposed to air, to form a tough, glossy, waterproof, insulative and anticorrosion surface on the object it is coating (BROWN *et al.*, 2005). Tung oil is widely used in paints, varnishes, coatings and finishes due to these excellent properties (BROWN and KEELER, 2005). Tung oil is also the high-quality raw material for producing biodiesel (PARK *et al.*, 2008; CHEN *et al.*, 2010; MANH *et al.*, 2012). It has been explored as polyurethane and wood flour composites, thermosetting polymer, cleaning and polishing compounds, waterproofing for paperand ceramic products, repairing agent for self-healing epoxy coatings (SAMADZADEH *et al.*, 2011; ARANGUREN *et al.*, 2012; LIU *et al.*, 2013). Tung tree is also an important ornamental plant (TIMOTHY *et al.*, 2013).

Researchers have focused on understanding the genetic control of tung oil biosynthesis. Many tung oil biosynthetic genes have been identified, including those coding for diacylglycerol acyltransferases (DGAT) (SHOCKEY *et al.*, 2006; CAO *et al.*, 2013b), delta-12 oleic acid desaturase (FAD2) and delta-12 fatty acid conjugase (FADX) (DYER *et al.*, 2002), cytochrome b5 and cytochrome b5 reductase (HWANG, *et al.*, 2004; SHOCKEY *et al.*, 2005), acyl-CoA binding proteins (PASTOR *et al.*, 2013), oleosins (LONG *et al.*, 2010; CAO *et al.*, 2014), glycerol-3-phosphate acyltransferase (GPAT) and plastid-type omega-3 fatty acid desaturase (TnDES2) (SHEPHERD *et al.*, 2000; GIDDA *et al.*, 2009). Quantitative PCR, northern blot and western blot have been used to study the expression of these tung genes (DYER *et al.*, 2002; SHOCKEY *et al.*, 2006; LONG *et al.*, 2010; CAO *et al.*, 2012a; CAO *et al.*, 2013b; PASTOR *et al.*, 2013; CAO *et al.*, 2013a; CAO *et al.*, 2014). However, it is difficult to select target genes for genetic engineering of plant oil because of the complexity of oil biosynthetic pathway in which at least 10 enzymatic steps and each step is catalyzed by multiple isozymes (DYER *et al.*, 2008; CAO, 2011a). Moreover, study on tung oil biosynthesis at the protein level is difficult because these enzymes are mostly hydrophobic and membrane localized proteins (CAO *et al.*, 2011b; CAO *et al.*, 2012b).

Fatty acid synthesis is catalyzed by the concerted action of acetyl-CoA carboxylase and fatty acid synthase (FAS) (JONES *et al.*, 2000). In plants, FAS is located in the plastids where it exists as a type II multienzyme complex (HARWOOD, 1996). Several protein components in FAS exist as isoforms, including the condensing enzyme β-ketoacyl-[acyl-carrier-protein (ACP)] synthase (KAS). Four forms of KAS designated KAS I, KAS II, KAS III and KAS IV are found in plants. KAS I and KAS II were first purified from spinach (WU *et al.*, 2009; WU and XUE, 2010), barley and parsley (JONES *et al.*, 2000). KAS I and KAS II are condensing enzymes for the elongation of the carbon chain from C4 to C18. KAS I is capable of condensing malonyl-ACP with acyl-ACP primers of between 2 and 14 carbons (RUTGER and JOHN, 2000). It yields palmitate as its longest chain product. Some studies have indicated that KAS I is related to the regulation of chloroplast division and embryo development (WU and XUE, 2010). KAS II mainly uses palmitoyl-ACP as the substrate to produce stearoyl-ACP (WU *et al.*, 2009; WU and XUE, 2010). KAS III is responsible for the condensation reaction of malonyl-ACP and acetyl-ACP (WU and XUE, 2010),

controlling the speed of the initial reactions of FAS (DEHESH *et al.*, 1998; STOLL *et al.*, 2006; CHI *et al.*, 2010). As the initiator of elongation, KAS III determines the number of fatty acids that will be produced by the pathway, and the substrate specificity of KAS III is a major determining factor in membrane fatty acid composition (WHITE *et al.*, 2005). To date, KAS III cDNA from *Pisum sativum*, *Jatropha curcas* and *Helianthus annuus* have been cloned (JONES *et al.*, 2003; LI *et al.*, 2008; GONZÁLEZ-MELLADO *et al.*, 2010; GU *et al.*, 2012). The substrate specificity of KAS III is an important determinant of branched-chain fatty acid production (CHOI *et al.*, 2000). Overexpression of KAS III in tobacco, *Arabidopsis* and rapeseed increases the amounts of C16:0 fatty acids (DEHESH *et al.*, 2001). A decreased amount of C18:1 fatty acids and increased amounts of C18:2 and C18:3 fatty acids are observed in the *B. napus* overexpressing *KAS III*. *KAS III* plays a vital role in fatty acid biosynthesis because the concentrations of C8-C14 medium-chain fatty acids are increased in the *KAS III* regulation knockout *B. napus* strain (STOLL *et al.*, 2006). The *KAS IV* cDNA was isolated from the seed of *Cuphea* (DEHESH *et al.*, 1998). The protein sequence of this enzyme is different from the previously characterized KAS I and KAS III, but with 85% identity to KAS II, and implicated in synthesis of intermediate chain lengths from C8 to C14 (SLABAUGH *et al.*, 1998).

Here we report the cloning and expression of two cDNAs isolated from *V. fordii*, designated *VfKAS II* (partial sequence) and *VfKAS III* (full-length sequence), which encodes a putative β-ketoacyl ACP synthases likely involved in carbon-chain elongation. Sequence analysis showed that the full-length *VfKAS III* shared close identity with *KAS III* of other plants. QPCR analysis indicated that *VfKAS III* was expressed in all tissues examined, which was consistent with *JcKAS III* (LI *et al.*, 2008). Molecular analysis of *VfKAS III* provided insights into the regulation of fatty acid biosynthesis and would help to improve oil content of *V. fordii* through the development of transgenic plants.

MATERIALS AND METHODS

Plant Materials

The seeds of 4-year old tung tree (*Vernicia fordii*) cultivar 'Dui nian tong' were collected from Central South University of Forestry and Technology (CSFU) campus, Changsha, Hunan Province, China. Total RNA was isolated from the seeds and used for gene cloning subsequently. For real-time fluorescence quantitative PCR (qPCR), various tissues (young leaf, mature leaf, young stem, mature stem, young petiole, maturepetiole, carpopodium, root, sepal, ovary, stigma, petals, and stamens) were collected on May 15, 2013. Tung tree seeds at nine developmental stages (50 DAF-days after flowering, 65 DAF, 80 DAF, 95 DAF, 110 DAF, 125 DAF, 140 DAF, 155 DAF, 170 DAF) were also collected for qPCR analyses. All plant samples were immediately frozen in liquid nitrogen and stored at -80°C.

RNA Isolation and cDNA Cloning

Total RNA was extracted from different tung tree tissues using PureLink^{TM} RNA Mini Kit (Life Technologies, USA). The RNase-free DNaseI (Promega, USA) was used to remove potential contaminating DNA from the total RNA. Complementary DNA (cDNA) was synthesized using RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA) according to the manufacturer's instructions. Degenerate primers (KII-F, KII-R) were used to synthesize *KAS II* fragment under the following conditions: 94 ℃ for 5 min, 35 cycles of 94 ℃ for 40 s, 55 ℃ for 30 s, 72 ℃ for 1 min, and 72 ℃ for 7 min. The *KAS III* (KIII-F, KIII-R) fragment were amplified

under the condition: 94 ℃ for 5 min, 35 cycles of 94 ℃ for 40 s, 60 ℃ for 30 s, 72 ℃ for 1 min, and 72 ℃ for 7 min. The degenerate primers were designed based on the conserved *KAS II* sequences of *Jatropha curcas* (DQ987700.2), *Ricinus communis* (XM_002516182.1), *Populus trichocarpa* (XM_006381514.1), *Glycine max* (XM_006593247.1), and the conserved *KAS III* sequences of *Jatropha curcas* (DQ987701.1), *Ricinus communis* (XM_002529743.1), *Populus trichocarpa* (XM_006368118.1), *Vitis vinifera* (XM_003631390.1). The PCR-amplified products were ligated into the pMD18-T cloning vector (TaKaRa, Japan). After the fragment were confirmed to be part of *KAS II* and *KAS III* genes by DNA sequencing and database search with NCBI blast, the 5′ and 3′ ends of the full-length cDNAs were further amplified according to the instruction of Rapid Amplification of cDNA Ends kit (Invitrogen, USA). Gene specific primers and nested primers were designed according to the core cDNA sequences. They are as follows: KⅡ-3GSPN, KⅢ-3GSPN, KⅢ-5GSPN, as shown in Table 1. The PCR products were separated by 1.2% agarose gel electrophoresis, purified by Gel Purification Kit (TIANGEN, China), and ligated into the pMD18-T vector (TaKaRa, Japan) for sequencing. Finally, the above comfirmed sequences were spliced and assembled into the full-length cDNA, which was designated as *VfKAS II* (partial sequence) and *VfKAS III*. Finally, the primers KIII-G-F, KIII-G-R were designed according to the assembled full length cDNA of *VfKAS III* to verify the CDS under the condition: 94 ℃ for 5 min, 35 cycles of 94 ℃ for 40 s, 63 ℃ for 30 s, 72 ℃ for 2 min, and 72 ℃ for 7 min. The amplified products were ligated into the pMD18-T cloning vector for sequencing (TaKaRa, Japan).

Table 1. The Primers used in this research

Primer	Sequence (5'-3')
KII-F	GTGTCTT(A/C)CTTGTGTGA(A/T)TCGAGCT
KII-R	ATCCC(G/T)ATTCATATCCCAAGGTCGT
KIII-F	AGCTCTT(C/G)A(A/G)ATGGCAGAGGTTGA
KIII-R	AAGCGGAARAC(C/T)TC(C/T)TT(A/G/T)CC(A/G)TT
KII-3GSP1	AGCAAACCACATCATTAGAGGCGAAGC
KII-3GSP2	GCCGCAATTATACCTATTGGCTTGGGA
KIII-3GSP1	TGTTGACTGGACGGATAGAGGAAGTTGTA
KIII-3GSP2	AGTTTTAAGTTTCCCTCCCAAGCGCTCT
KIII-5GSP1	CTTGGGAGGGAAACTT
KIII-5GSP 2	TGAACGAGCACAGCACCTGCAGCAT
KIII-5GSP 3	CCACTACATGCAGCTGTAATATCATAAG
KIII-5GSP 4	CATCAAGACTAGGTCCACATCATCAGGT
KIII-G-F	TACACAGATAGGGAGAGCATTGGAGTTT
KIII-G-R	CAACATCTCAGAATATCTGGTAGAAAGG
UBQ-F, UBQ-R	CCGTGGTGGCTGTTAAGTTT, AAGGCCATTTCAACATCCTG
KII-Q-F, KII-Q-R	CAAAGAAGAAACCCACTATGA, CTCTATCTGACTTATGCCACT
KIII-Q-F, KIII-Q-R	GCATTGATTGGTTACTGCT, GCCTTCACCTTCCCACTTC

Genomic DNA cloning

Genomic DNA was isolated from leaves of tung tree cultivar 'Dui nian tong' by plant genomic DNA quick Plant System kit (TIANGEN, China). The primers KIII-G-F, KIII-G-R (Table 1) were used to amplify the *KAS III* gene from genomic DNA template. The long PCR amplification (BARNES, 1994) conditions were as follows: 94 °C for 2 min, 20 cycles of 92 °C for 30 s, 64 °C for 30 s, 68 °C for 12 min, another 20 cycles of 92 °C for 30 s, 64 °C for 30 s, 68 °C for 12 min 20 s (each cycle increase 20 s), and 68 °C for 10 min. The PCR products were analyzed through 1.0% agarose gel electrophoresis, purified by gel Purification Kit (TIANGEN, China) and sequenced directly.

Real-time fluorescence quantitative PCR (qPCR)

The gene specific primers KII-Q-F, KII-Q-R, KIII-Q-F, KIII-Q-R, designed by Primer Primer 5, were used to amplify 136 base pair (bp), 180bp cDNA fragment for the qPCR of *KAS II* and *KAS III*, respectively. *Ubiquitin* (*UBQ,* JQ680041) gene was used as an internal reference gene (HAN *et al.*, 2012), and the primers UBQ-F, UBQ-R were designed for the *UBQ* cDNA. QPCR was performed using the CFX96™ Real-time PCR System (BIO-RAD, USA) with SYBR Premix Ex Taq™ (TaKaRa, Japan). The amplification fragments were extracted and sequenced before qPCR.

The total volumes of the qPCR reaction were 25 µl, and consisted of 12.5 µl of 2×SYBR Premix ExTaq ™ (Tli RNaseH Plus, TaKaRa, Japan), 9.5 µl RNase free water, 0.5 µl of each primer (10 µM), and 2 µl of 10-fold cDNA dilutions as PCR templates. The cyclings consisted of 95℃ for 30 s, 39 cycles of 95℃for 5 s, 60℃ for 30 s, and 72 ℃ for 30 s. Each reaction was repeated three times. Two replicate reactions of non- template negative controls were included in each run to ensure that reagents were free of contaminants. The $2^{-\Delta\Delta CT}$ method was used to analyze the quantitative PCR data (LIVAK *et al.*, 2001).

Data Analyses

DNA and protein sequences were analyzed using NCBI server (http://www.ncbi.nlm.nih.gov), GENEDOC and Vector NTI 10.3.0. The analyses of protein properties were performed by ProtParam (http://web.expasy.org/protparam/), Signal P4.1sever (http://www.cbs.dtu.dk/services/SignalP/), and TMpred (http://www.ch.embnet.org/software/TMPRED_form.html). The secondary structure was predicted by SOPMA (http://npsapbil.ibcp.fr/cgibin/npsa_automat.pl?page=/NPSA/npsa_sopma.html) and PredictProtein (https://www.predictprotein.org/). Subcellular location was predicted using the WoLF PSORT Protein Subcellular Localization Prediction (http://wolfpsort.seq.cbrc.jp) (PAUL *et al.,* 2007). The phylogenetic tree was constructed from the amino acid sequences by the neighbor joining method using Mega4.1. The qPCR data were analyzed by Bio-Rad CFX Manager software.

RESULTS

Cloning and identifying of KAS II and KAS III cDNA

We cloned a full length of *KAS III* gene (GenBank ID: KP025795) and partial sequence of *KAS II* gene from *Venicia fordii.* We used degenerate primers and rapid amplification of cDNA ends system (RACE). Two fragments were amplified by degenerate PCR, 895 bp of *VfKAS II* gene and 526 bp of *VfKAS III* gene (Figure 1a). The 3′ RACE and 5′ RACE fragments of *VfKAS III* were 630 bp and 870 bp, respectively, but we only obtained 1156 bp 3′ RACE fragment of *VfKAS*

II (Figure 1b and 1c). The full-length cDNA of *VfKAS III* was 1881 bp (Figure 1d), consisting of 5′-untranslated region (5′UTR) of 444 bp, an open reading frame (ORF) of 1212 bp, and 3′ untranslated region (3′UTR) of 225 bp (Figure 2). It also contained the polyadenylation signal (AATAA), and poly (A) tail in the 3′UTR (Figure 2). The predicted ORF of *VfKAS III*encodes a protein of 403 amino acids. In addition, the partial cDNA sequence of *VfKAS II* is 1458 bp with 5′ end lacked of 261 bp (Figure 2). The genomic DNA sequence of *VfKAS III* was amplified by KIII-G-F/KIII-G-R primers (Figure 1e). The result showed that the *VfKAS III* genomic DNA sequence (GenBank ID: KP025796) was 5403 bp containing 8 exons and 7 introns with different sizes. The numbers of exons and introns are consistent with *Ricinus communis* and *Glycine max KAS III* genomic DNA sequence. The length of each exon of the 3 plants is the same except the first exon, but the length and position of each intron is different.

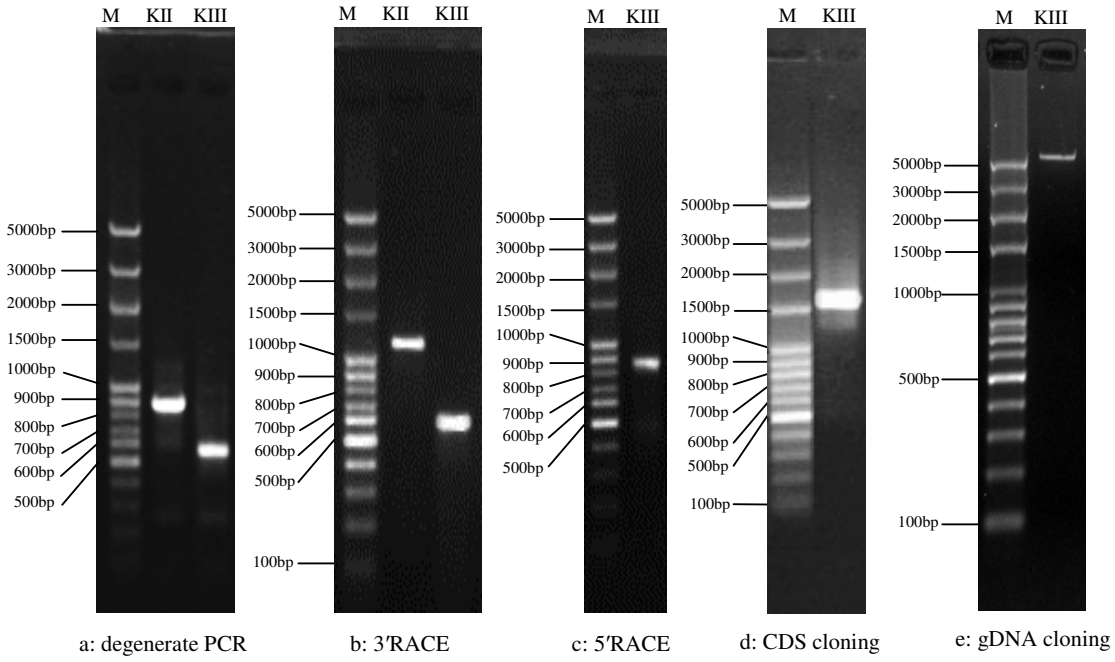


Figure 1. PCR products of *KAS II* and *KAS III* cDNA from *V. Fordii.* (a) Degenerate PCR result of *KAS II* (895 bp) and *KAS III* (526 bp). (b) 3′RACE PCR result of *KAS II* (1156 bp) and *KAS III* (630 bp). (c) 5′RACE PCR result of *KAS III* (870 bp). (d) CDS cloning of *KAS III* (1881 bp). (e) Genomic DNA amplification of *KAS III* (5403 bp) . M: 100bp plus DNA ladder, KII and KIII are the abbreviations for *KAS II* and *KAS III*, respectively.

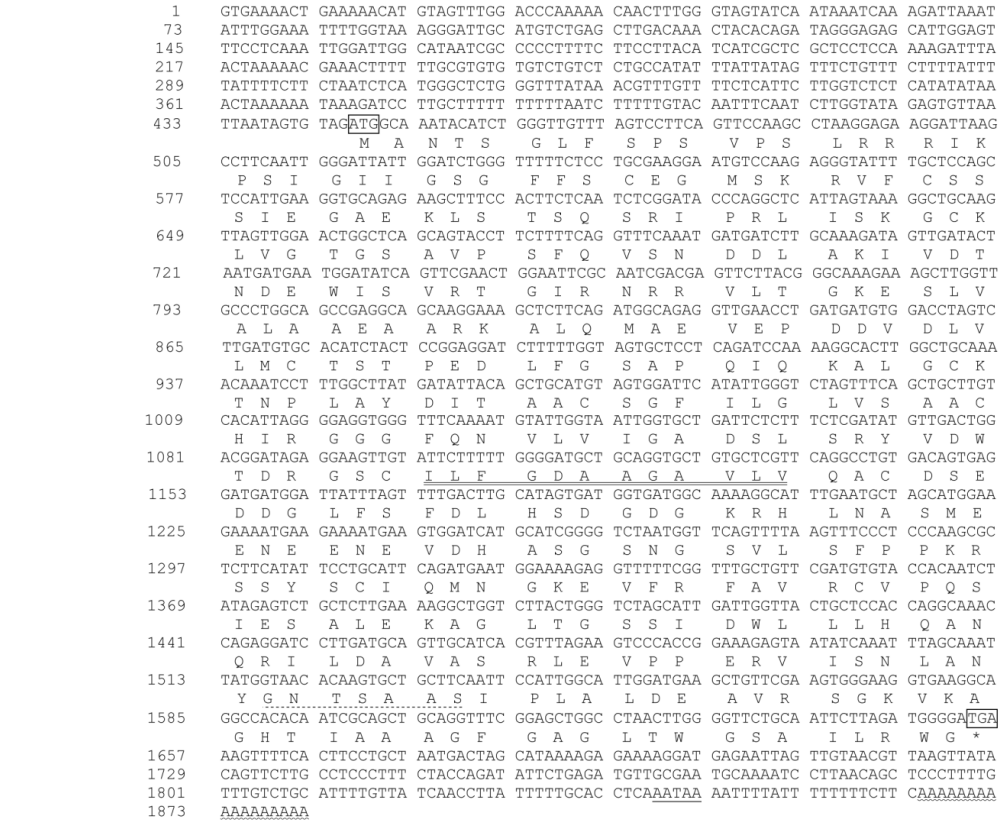


Figure 2. The nucleotide and deduced amino acid sequences of full-length *VfKAS III* cDNAs of *V. fordii.* The translational initiation codon (ATG) and termination codon (TGA) are boxed. The polyadenylation signal AATAA is underlined. The polyadenylation tail is underscored with a wavy line. The stop codon is indicated by an asterisk (*). The putative acetyl-CoA binding site (ILFGDAAGAVLV) is shown by a double underline (PAUL *et al.*, 2007). The putative acyl-ACP binding site (GNTSAAS) is shown by a dotted line (ABBADI *et al.*, 2000).

Bioinformatics Analysis of VfKAS III Properties

Conserved domain analysis using CDD of NCBI revealed that the putative VfKAS III protein belongs to the cond_enzymes superfamily (Figure 3). This family of enzymes catalyzes a (decarboxylating or non-decarboxylating) Claisen-like condensation reaction. The chemical properties of VfKAS III protein were analyzed by bioinformatics software. These analyses showed that the predicted protein had a chemical formula of $C_{1858}H_{2979}N_{531}O_{585}S_{17}$ with a calculated molecular weight of 42.7 kDa and the theoretical isoelectric point (pI) of 5.78. This protein had a predicted instability index of 46.12 and aliphatic index of 89.60. The grand average of hydropathicity of VfKAS III protein was -0.013, indicating that the KAS III is a hydrophilic protein. No signal peptide was found in the putative polypeptide, which suggests that it probably

codes a non-secretary protein. It contained four putative transmembrane domains with one of the strongest transmembrane domains at its C-terminal region. The analysis further showed that the predicted polypeptide possessed alpha helix of 31.0%, beta sheet of 18.1%, and beta turn and coil of 50.9%. It had 12 alpha helices and 15 beta sheet (Figure 4). Subcellular location prediction showed that the KAS III protein is probably located in chloroplast.

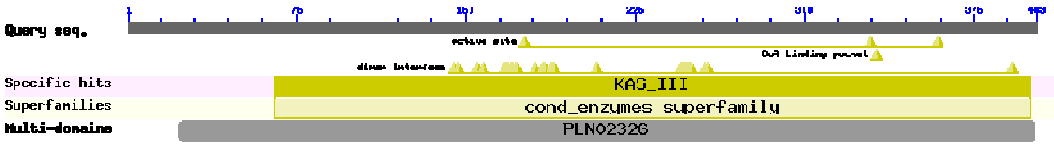


Figure 3. Analysis of conserved domains in VfKAS III

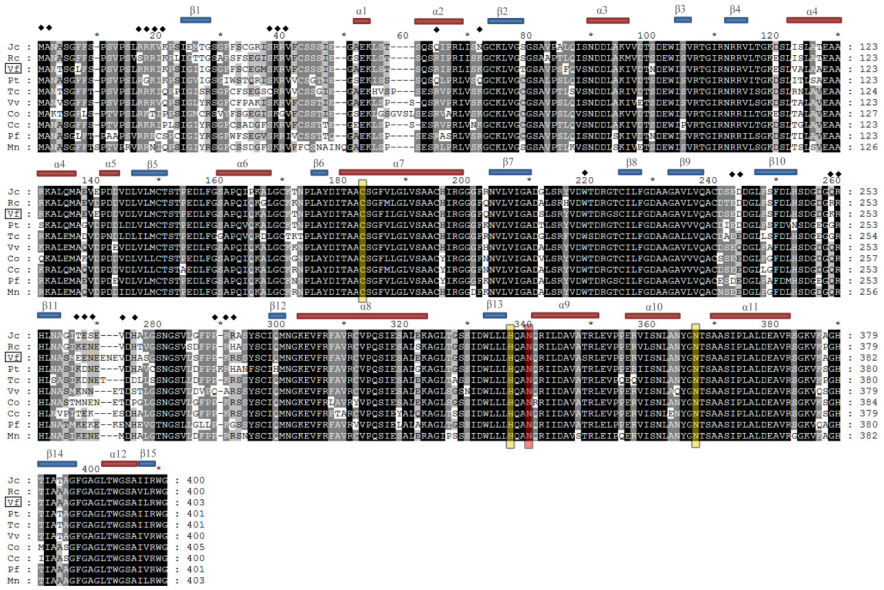


Figure 4. Multiple sequence alignment of VfKAS III and other KAS III homologs derived from *Jatropha curcas* (Jc, ABJ90470.1), *Ricinus communis* (Rc, XP_002529789.1), *Populus trichocarpa* (Pt, XP_006368180.1), *Theobroma cacao* (Tc, XP_007039910.1), *Vitis vinifera* (Vv,XP_003631438.1), *Camellia oleifera* (Co, AFK31316.1), *Camellia chekiangoleosa* (Cc, AGH32918.1), *Perilla frutescens* (Pf, AAC04693.1) and *Morus notabilis* (Mn, EXB54472.1). Multiple sequence alignment was carried out by Vector NTI 10.0 and displayed by GeneDoc. The β-strands (drawn as blue rectangles) and α-helices (drawn as brown rectangles) are shown with assigned labels above the sequences. The symbol '◆' represents protein binding regions. The conserved cysteine (C), histidine (H) and asparagine (N) active site residues are highlighted in yellow and the conserved asparagine (N) CoA binding site residues is in red. Identical peptides highlighted in black.

Multiple sequence alignments demonstrated that the deduced amino acid sequence of VfKAS III was 88%, 85%, 84%, 84%, 83%, 83%, 82% and 80% identical to those homologues from *Ricinus communis*, *Vitis vinifera*, *Populus trichocarpa*, *Camellia oleifera*, *Camellia chekiangoleosa*, *Morus notabilis*, *Perilla frutescens* and *Theobroma cacao*, respectively (Figure 4). The highest degree of sequence similarity reached almost 90% between VfKAS III and *Jatropha curcas* KAS III. The deduced VfKAS III protein contained 24 protein binding regions (Figure 4) with the amino acid residues $G^{355}NTSAAS^{361}$ that are responsible for the binding of regulatory acyl-ACPs (ABBADI *et al.*, 2000). The result indicated that the KAS III protein contained a more conserved C-terminal regions than the N-terminal regions.

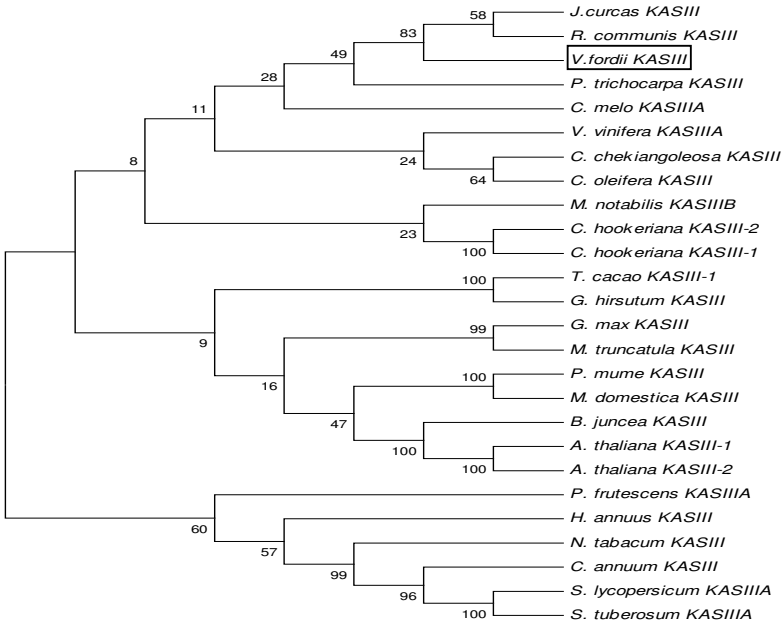
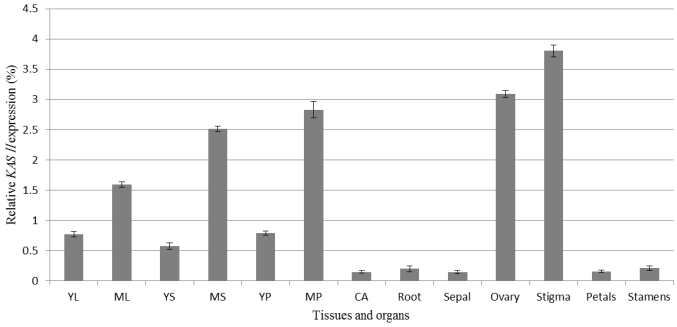


Figure 5. Phylogenetic analysis of the deduced amino acid sequences of *V. fordii* KAS III ortholog and other plants. The tree was constructed by the neighbor-joining method of ClustalX 2.0 and Mega 4.1. The numbers at the nodes indicate boostrap values from 1000 replications. The following KAS III sequences were used: *Jatropha curcas* (ABJ90470.1), *Ricinus communis* (XP_002529789.1), *Vitis vinifera* (XP_003631438.1), *Populus trichocarpa* (XP_006368180.1), *Camellia chekiangoleosa* (AGH32918.1), *Morus notabilis* (EXB54472.1), *Camellia oleifera* (AFK31316.1), *Perilla frutescens* (AAC04693.1), *Theobroma cacao* (XP_007039910.1), *Prunus mume* (XP_008238595.1), *Glycine max* (NP_001237735.1), *Gossypium hirsutum* (ADK23941.1), *Cuphea hookeriana* (AAF61399.1), *Capsicum annuum* (ACF17661.1), *Solanum lycopersicum* (XP_004235286.1), *Solanum tuberosum* (XP_006347624.1), *Malus domestica* (XP_008363277.1), *Cucumis melo* (XP_008437123.1), *Arabidopsis thaliana* (NP_176452.1), *Cuphea hookeriana* (AAF61398.1), *Arabidopsis thaliana* (AAA61348.1), *Helianthus annuus* (ABP93352.1), *Nicotiana tabacum* (AFE88231.1), *Medicago truncatula* (KEH39644.1), *Brassica juncea* (ABP38071.1).

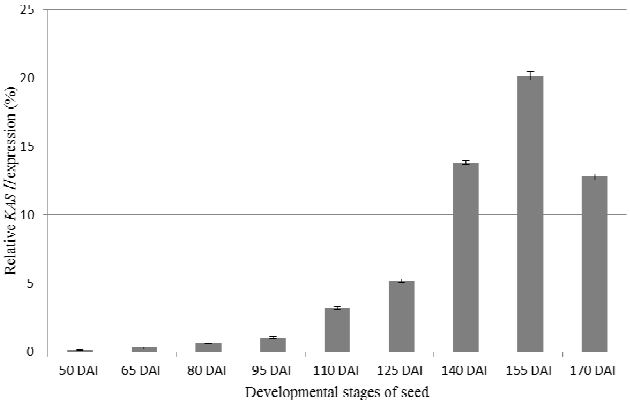
Phylogenetic analysis of some β-ketoacyl-ACP synthases from various plants showed that VfKAS III formed a cluster with other plant KAS Ⅲ homologs and was most closely related to those of the *Jatropha curcas* (ABJ90470.1) and *Ricinus communis* (XP_002529789.1), which belongs to the euphorbiaceae (Figure 5). These results indicate that we successfully cloned a *KAS III* ortholog from *V. fordii*.

KAS II Expression in Different Tissues and Seed Developmental Stages

Quantitative PCR analysis revealed that *VfKAS II* was expressed in all tissues tested (Figure 6a), but there were obvious differences in the expression levels in various tissues.



(a)



(b)

Figure 6. Expression patterns of *KAS II* in various tissues and seed developmental stages. Relative transcript levels were measured using qPCR with *ubiquitin* transcript as an internal control. (a) *KAS II* transcript levels in various tissues and organs (YL: young leaf; ML: mature leaf; YS: young stem; MS: mature stem; YP: young petiole; MP: mature petiole; CA: carpopodium). (b) *KAS II* transcript levels during seed development (DAF: days after flowering). The vertical bars indicate means±standard error of three biological replications.

The highest expression of *VfKAS II* was detected in stigma, followed by ovary. The lowest expression of *VfKAS II* was observed in carpopodium, sepal and petals. The overall expression levels in young tissues were much lower than those observed in mature tissues, such as the expression levels in young leaf were lower than mature leaf, in young stem was lower than mature stem and in young petiole was lower than mature petiole. There was a strongest expression level of *VfKAS II* in155 DAF (days after flowering) during developmental stages of seeds (Figure 6b). From 80 DAF to 155 DAF, the expression level of *VfKAS II* increased gradually, and the expression peaked at 155 DAF, then decreased at 170 DAF. From 110 DAF, expression level of *VfKAS II* increased obviously, the expression levels at 110 DAF was 3.19 times of 95 DAF, the expression levels at 155 DAF was 20.15 times of 95 DAF.

KAS III Expression in Different Tissues and Seed Developmental Stages.

We investigated the expression patterns of *KAS III* in different tissues (Figure 7a). *KAS III* transcripts were identified in all tissues and organs, like *KAS II*, significant difference expression level existed among these tissues. The highest expression level was observed in young stem and young leaf, followed by stigma. In contrast to *KAS II*, *KAS III* showed much higher level of expression in young tissues, compared to mature tissues. During seed development, *KAS III* express level increased rapidly at 110 DAF, highly expressed at 125 DAF and sharply down regulated at 140 DAF (Figure 7b). The lowest expression was detected at 50 DAF and 170 DAF.

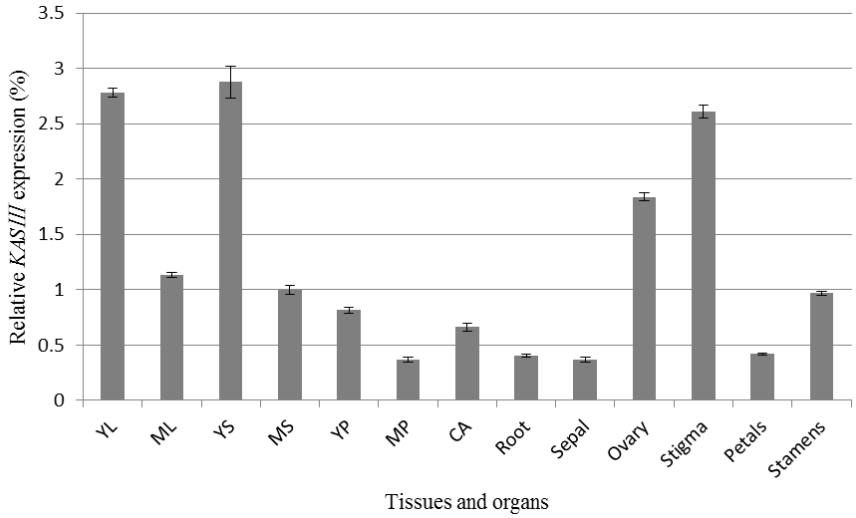


Figure 7a. Expression patterns of *KAS III* in various tissues and seed developmental stages. Relative transcript levels were measured using qPCR using *ubiquitin* transcript as an internal control. *KAS III* transcript levels in various tissues and organs (YL: young leaf; ML: mature leaf; YS: young stem; MS: mature stem; YP: young petiole; MP: mature petiole; CA: carpopodium).

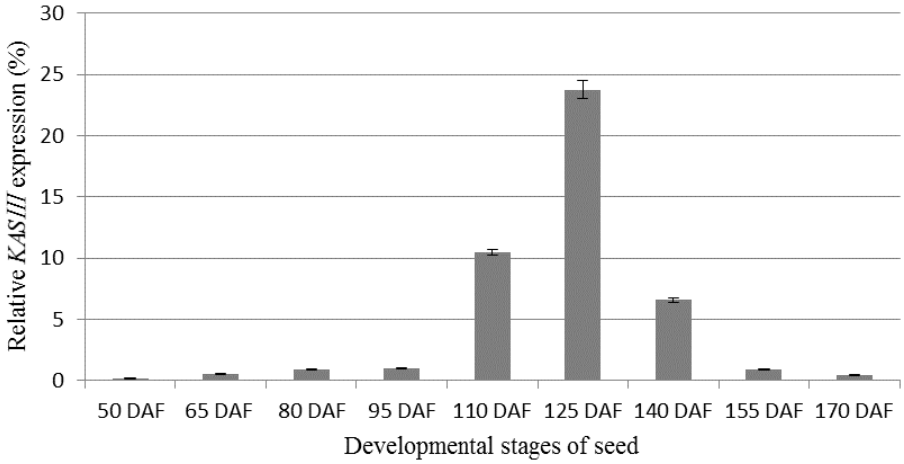


Figure 7b. Expression patterns of *KAS III* in various tissues and seed developmental stages. Relative transcript levels were measured using qPCR using *ubiquitin* transcript as an internal control. *KAS III* transcript levels during seed development. The vertical bars indicate means±standard error of three biological replications.

DISCUSSION

In higher plants, the fatty acid biosynthesis pathway is catalyzed by a set of nuclear-encoded enzymes in plastid. C16 to C18 fatty acids are synthesized by the consecutive addition of C2 units. Each cycle of C2 addition is initiated by a reaction catalyzed by a β-ketoacyl-ACP synthase (KAS) and involves in the condensation of a malonyl-ACP with an acyl acceptor. KAS III isoform catalyzes the first condensation step in the pathway using acetyl-CoA as the primer and malonyl-ACP as the acceptor (WHITE *et al.*, 2005). KAS I extends C4 to C16 in six rounds of elongation, and KAS II catalyzes the elongation of 16:0-ACP to 18:0-ACP (OLSEN *et al.*, 2001).

In this study, we cloned a full-length cDNA from *V. fordii* that encoded KAS III. Sequence analysis of VfKAS III revealed that the predicted protein contained the motif ILFGDAAGAVLV for acetyl-CoA binding and the motif G^{355}NTSAAS^{361} for regulatory acyl-ACPs binding (ABBADI *et al.*, 2000). VfKAS Ⅲ shared about 80% identity with KAS IIIs from other plants. These active site and conserved domains indicate that VfKAS III probably perform similar biochemical functions with other known plant KAS IIIs. VfKAS III was predicted to be localized in the chloroplasts, similar to KAS protein from peanut (LI *et al.,* 2009). Previous research has shown that plant mitochondrial fatty acid synthesis is also catalysed by the type II FAS system, and that plant type II FAS in mitochondria is more closely related to *E. coli* type II FAS than plastidic KASs (LI *et al.,* 2009; YASUNO *et al.*, 2004].

Quantitative real-time PCR result showed that *VfKAS III* and *VfKAS II* gene were expressed in all tung tree tissues and organs tested but both exhibited different expression patterns in various tissues. *KAS II* expression levels in young tissues were much lower than those in mature tissues. During seed development, the expression level of *VfKAS II* increased from 50 DAF to 155 DAF. However, *VfKAS III* showed much higher level of expression in young tissues than mature

tissues, and its expression levels increased from 50 DAF to 125 DAF and decreased sharply in more mature tung tree seeds. KAS III is the initiator of elongation in type II fatty acid synthesis and mainly functions in the first condensation reaction of the fatty acid synthesis. Fatty acid synthesis in tung tree seeds starts from 65 DAF approximately (CAO *et al.*, 2013b; ZHANG *et al.*, 2014). *VfKAS III gene* expression level was increased at the early stages of seed development. Less KAS III is needed to accomplish the first step condensation reaction with fatty acids accumulated, and *KAS III* expression level was decreased and more *KAS II* is needed in C16 to C18 condensation reaction, so *KAS II* expression increased gradually. When the fatty acid content of tung tree seed reached the maximum at 155 DAF, the *KAS II* expression level reached the peak and then decreased at 170 DAF. We speculate that fatty acid synthesis mainly at the first step of condensation reaction in young tissues, and fatty acid synthesis mainly at the later stage of condensation in mature tissues, so *KAS II* expression levels in young tissues were lower than those observed in mature tissues, and *VfKAS III* showed higher expression in young tissues than mature tissues.

Previous research has shown that KAS I affects multiple developmental processes in *Arabidopsis.* A deficiency in *KAS I* significantly suppresses chloroplast division in rosette leaves and arrests embryo development (WU and XUE, 2010). A decrease in the proportion of the 16-carbon fatty acids in lipids was observed at low positive temperatures, possibly due to the regulation of *Betula pendula KAS II* expression (MARTZ *et al.*, 2006). Some experiments have demonstrated that modulation of KAS II levels in *Arabidopsis* is sufficient to convert its temperate oilseed composition to that of the palm-like tropical oil (PIDKOWICH *et al.*, 2007). In the *KAS III* transgenic tobacco leaves and *Arabidopsis* seeds, the relative levels of oil content and lipid synthesis rate are reduced simultaneously with the increases of C16:0, C18:2 and C18:3 fatty acids and decreases of C18:1 fatty acids. It is indicated that overexpressing the *KAS III* resulted in changes in the activity of a series of enzymes in the FAS multienzyme complex. Higher concentrations of middle-chain fatty acids are obtained following knockout of *KAS III* gene in rape seeds. Overexpressing or restraining *KAS III* expression can alter the cocentrations of fatty acids, irrespective of the plant species from which it is derived or the tissue in which it is expressed (LI *et al.*, 2008). Therefore, overexpression or knock out of the *VfKAS III* gene could be exploited to increase C16:0 fatty acids, the middle-chain fatty acids and/or the unsaturated fatty acids in *V. fordii* seeds. However, de novo fatty acid synthesis is a complicated process involving multiple enzymatic steps. Further research is needed to comprehend the regulation machenism of fatty acid biosynthesis in *V. fordii* and to provide foundation of improving oil content via biotechnology in tung tree.

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MOLEKULARNO KLONIRANJE I EKSPRESIJA PROFILA GENA KOJI KONTROLIŠE β-KETOACIL-ACP SYNTAZE KOD TUNG DRVETA (*Vernicia fordii* Hemsl.)

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Izvod

Tung drvo(*Vernicia fordii*) je značajno drvenasto uljano stablo, čije seme sadrži 50 – 60 % ulja - sa oko 80 *mola* is an important woody oil tree. Tung tree seeds contain 50-60% oil with approximately 80 mole α-elostearinske kiseine (9 cis, 11 trans, 13 trans octadecatrienolna kiselina). Multienzimatski sisstem za sintezu masnih kiselina je katalizovan usklađenom *acetyl-CoA carboxylase* i sintaze masnih kiselina, uključujući β-ketoacil –acil - nosač proteina sintaze (KAS). Cilj ovih istraživanja je bio kloniraanje KAS gena i analiza ekspresije njihovih profila u tung stablu. Izolovana je cDNK kompletne dužine, koja kodira KAS III i parcijalna cDNK, koja kodira KAS IIA full-length cDNA encoding KAS III and a partial cDNA encoding KAS II iz tung stabla PCR kloniranjen, uz korišćenje degenerativnih prajmera i RAPID amplifikacije krajeva cDNK sistema. Dužina celog lanca cDNK *VfKAS III* je 1881 bp (baznih parova) sa jednim okvirom otvorenog dešifrovanja (*open reading frame)* dužine 1212 bp. Izolovan je i sekvencioniran *VfKAS III* genomske DNK, koji sadrži 8 egzona dužine 5403 bazna para (bp) Dedukcijonom analizom je utvrđeno da protein VfKAS III ima oko 80% identičnosti sa homologom KAS IIIs drugih biljaka. Kvantativnom PCR analizom je utvrđeno da KAS II i KAS III imaji ekspresiju u svim ispitivanim tkivima i organima ali imaju različitu ekspresiju kod tung stabla. Nivo ekspresije KAS II je bio mnogo niži u mladim tkivima u poređenju sa zrelim tkivom , dok je najviši nivo ekspresije KAS III utvrđen u mladim listovima i mladim stabljikama.

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