Molecular cloning and expression analysis of a gene encoding a putative β-ketoacyl-acyl carrier protein (ACP) synthase III (KAS III) from Jatropha curcas

JUN LI,1 MEI-RU LI,1 PING-ZHI WU,1 CHANG-EN TIAN,2 HUA-WU JIANG1 and GUO-JIANG WU1,3

1 South China Botanical Garden, Chinese Academy of Sciences, Guangzhou 510650, P.R. China
2 Research Center for Functional Genomics and Microarray, Guangzhou University, Guangzhou 510405, P.R. China
3 Corresponding author (wugj@scbg.ac.cn)

Received January 1, 2008; accepted February 18, 2008; published online April 1, 2008

Summary A cDNA clone encoding a putative β-ketoacyl-acyl carrier protein (ACP) synthase III (KAS III) was isolated from Jatropha curcas L., a woody oil plant. The cDNA clone (named JcKAS III) contained a 1203-bp open reading frame coding for 400 amino acids with a predicted molecular mass of about 42 kDa. The deduced amino acid sequence of the cDNA clone shares about 80% identity to KAS III from other plants, and contains a conserved Cys176 in the active site and the amino acid motif G355NTSAAS361 which is responsible for binding acyl-ACP thioesterase or desaturated by stearoyl-ACP desaturase. The activity of KAS III was first detected in bacteria (Jaworski et al. 1989, Walsh et al. 1990, Clough et al. 1992, Tai et al. 1993) and Jaworski et al. 1995, Chen and Post-Beittenmiller 1996) and in higher plant species (Jaworski et al. 1989, Walsh et al. 1990, Clough et al. 1992, Tai et al. 1993, Schuch et al. 1994, Tai et al. 1994, Slabaugh et al. 1995, Chen and Post-Beittenmiller 1996) and red algae (Reith 1993).

Previous studies indicate that KAS III activity matches the rate of fatty acid synthesis in spinach homogenates, suggesting that KAS III may have a rate-limiting role in fatty acid synthesis (Jaworski et al. 1989, Dehesh et al. 2001). Overexpression of KAS III in tobacco, Arabidopsis and rapeseed increased the amounts of C16:0 fatty acids (Dehesh 2001). Similar studies were carried out in Escherichia coli and Brassica napus L. (Verwoert 1995). A higher yield of C14:0 fatty acids and a lower yield of C18:1 fatty acids were detected in E. coli overexpressing KAS III. Similarly, a decreased amount of C18:1 fatty acids and increased amounts of C18:2 and C18:3 fatty acids were observed in the B. napus strain (Stoll et al. 2006), demonstrating that KAS III plays an essential role in fatty acid biosynthesis. These results indicate that KAS III has a universal role in fatty acid biosynthesis.

Introduction

β-Ketoacyl-acyl carrier protein (ACP) synthase III (KAS III) is thought to catalyze the first elongation reaction of type II fatty acid synthesis in bacteria and plant plastids. The enzyme catalyzes the condensation of acetyl-CoA with malonyl-ACP to produce 3-ketobutyryl-ACP, which is reduced by a consecutive set of enzymes to butyryl-ACP. The product of the first chain-extension cycle is the substrate for condensation with acetyl-CoA, and His258 not only contributes to malonyl-ACP decarboxylation in addition to binding regulatory acyl-ACPs. Southern blotting analysis indicated that JcKAS III is a single copy gene in the J. curcas genome. Quantitative real-time PCR analysis showed that JcKAS III was expressed in all tissues examined with highest expression in roots, and that expression of JcKAS III increased as seeds developed.

Keywords: carbon chain elongation, fatty acid synthesis, seed oil.
ids, of which oleic acid and linoleic acid represent about 40 and 30%, respectively, the C16 fatty acids concentration is about 15%, whereas middle-chain fatty acid concentrations are low. Most research on J. curcas has focused on techniques for extracting oil from seed (Wilhelm et al. 2000, Shweta et al. 2004). Recently, however, some genes encoding fatty acid synthesis enzymes in J. curcas have been cloned (Luo et al. 2006).

Here we report the cloning and characterization of a cDNA from J. curcas, designated as JcKAS III, which codes for a putative β-ketoacyl ACP synthase likely involved in carbon-chain elongation. Sequence analysis showed that JcKAS III shares a close identity with KAS III from other plants. Quantitative real-time PCR analysis revealed that JcKAS III was expressed in all tissues examined, with highest expression in roots, and the expression of JcKAS III increased during seed development. Molecular analysis of JcKAS III provides insight into the regulation of fatty acid biosynthesis and carbon-chain elongation, and ultimately may help to improve oil production of J. curcas through the development of transgenic plants.

Materials and methods

Plant materials

In autumn, matured seeds of J. curcas were collected from 2-year-old trees growing in Guizhou province, P.R. China. The seeds were planted in farmland in Guangzhou, P.R. China.

Cloning and sequencing of JcKAS III gene

Total RNA was extracted with Trizol (Invitrogen, Carlsbad, CA) following the manufacturer’s instructions. First-strand cDNA was synthesized from 3 µg of total RNA with SuperScript II (Invitrogen). A specific fragment of JcKAS III was amplified with the primer pair KAS31 and KAS32 (Table 1), designed based on the conserved regions of the corresponding genes from other higher plants. The PCR was performed as follows: 94 °C for 3 min; 30 cycles of 94 °C for 30 s, 50 °C for 1 min and 72 °C for 2 min; and a final extension step of 72 °C for 5 min. The PCR fragment was purified with an Agarose Gel DNA Purification Kit, and ligated into the pMD 18-T Vector (TaKaRa, Otsu, Japan). Sequence analysis was performed by Invitrogen in Shanghai. The 5′- and 3′-ends of JcKAS III were obtained with a BD SMART RACE cDNA Amplification Kit (Clontech, Palo Alto, CA) according to the manufacturer’s instructions and the specific primers KAS33, KAS34, KAS35 and KAS36 (Table 1).

Protein sequence comparisons and phylogenetic analysis

The nucleotide sequence, deduced amino acid sequence and ORF (open reading frame) encoded by JcKAS III were analyzed and a sequence comparison was conducted with BLAST (http://www.ncbi.nlm.nih.gov). From among the best BLAST hits, genes for which there were published reports were selected for comparison. The sequences homologous to JcKAS III were Perilla frutescens (L.) Britton, AAC04694; Cuphea wrightii A. gray, AAAT97533; Glycine max (L.) Merr., AAF70509; Cuphea hookeriana Walp., AAF61399; Pisum sativum L., CAC08184; Arabidopsis thaliana (L.) Heynh., NP_176452; Spinacia oleracea L., CAA80452; Elaeis oleifera (Kunth) Cortés, ABE73470; Elaeis guineensis Jacq., ABE73469; Allium porrum L., AAB61310; and Jatropha curcas, DQ987701. The phylogenetic analysis of KAS III from other species was aligned with Clustal W (http://align.genome.jp/) program. The neighbor-joining method (Saitou and Nei 1987) was used to construct the tree.

Real-time PCR

Total RNA was extracted from different tissues and seeds of J. curcas at different developmental stages and treated with RNase-Free DNase I (TaKaRa). First-strand cDNA was synthesized from 3 µg of total RNA using SuperScript II (Invitrogen). The reverse transcribed cDNA samples were used for real-time-PCR, which was performed on an ABI PRISM 7000 sequence detection system (Applied Biosystems). A JcKAS III cDNA fragment (136 bp) was amplified with gene-specific primers KAS39 and KAS310 (Table 1). A J. curcas actin gene, amplified with the primers Actin-F and Actin-R (Table 1), giving a product of 180 bp, was used as a reference for normalizing the JcKAS III cDNA amounts. Each PCR was performed in a 25-µl reaction mix containing 1 µl of template cDNA or the standard, 1× SYBR Premix Ex Taq (TaKaRa) and 0.3 µM of each primer. Thermal cycling conditions were: 95 °C for 10 s; 40 cycles of 95 °C for 5 s, 60 °C for 30 s; then 95 °C for 15 s, 60 °C for 20 s and 95 °C for 15 s for the dissociation stage. After the real-time PCR, the absence of unwanted by-products was confirmed by automated melting curve analysis and agarose gel electrophoresis of the PCR product. The relative expression ratio of JcKAS III was calcu-
lateral based on real-time PCR efficiencies and the crossing point differences of each sample versus a control sample. Crossing point is defined as the cycle number at which the fluorescence rises above the background fluorescence and the efficiency of the reaction reveals the increase in amplification at each cycle. \( JcKAS \) cDNA levels were normalized with those of \( \alpha \) actin in the same samples qualified in the same manner, and the final relative cDNA amounts of \( JcKAS \) were the means of three replicates. Statistical differences in expression between the mean values of control and experimental samples were analyzed by one-way analysis of variance (ANOVA) and the LSD test.

**Southern hybridization**

Genomic DNA was extracted from \( J. \) *curcas* leaves by the CTAB method. Briefly, 10 \( \mu \)g of total DNA was digested with \( EcoRI \) and \( HindIII \), separated in a 0.8% agarose gel and transferred to a Hybond N+ membrane (Amersham Biosciences, Buckinghamshire, U.K.). Digoxygenin (DIG-11-dUTP)-labeled 633 bp \( JcKAS \) probe was prepared using the primers KAS37 and KAS38. Southern blotting was performed using the PCR DIG Probe Synthesis Kit and DIG Luminescent Detection Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer’s instructions.

**Results**

**Cloning and characterization of gene \( JcKAS \)**

A number of \( KAS \) genes have been cloned from many plants and sequence analysis revealed many conserved regions (Jaworski et al. 1989, Walsh et al. 1990, Clough et al. 1992, Tai and Jaworski 1993, Schuch et al. 1994, Tai et al. 1994, Slabaugh et al. 1995, Chen and Post-Beittenmiller 1996). These conserved regions were used to design degenerate primers to clone the \( KAS \) gene from \( J. \) *curcas* (Figure 1). A 726-bp fragment was amplified from the cDNA of \( J. \) *curcas* embryos. The full-length cDNA, named \( JcKAS \), was obtained by RACE-PCR and consists of 1451 bp nucleotides including 18-bp 5′-untranslated and 230-bp 3′-untranslated regions (Figure 2). The cloned \( JcKAS \) has an ORF encoding a polypeptide of 400 amino acids with a calculated molecular mass of 42.18 kDa and an isoelectric point of 6.50 (Figure 2). The protein encoded by \( JcKAS \) III contains an active site Cys 176 (Tai et al. 1994) and the amino acid residues, G355NTSAAS361, responsible for the binding of regulatory acyl-ACPs (Abbadi et al. 2000) (Figure 2). Sequence alignment of the deduced amino acids (Figure 1) showed that the \( JcKAS \) encoded protein shares about 80% identity with \( KAS \) III from \( Perilla frutescens \), \( Cuphea wrightii \), \( Glycine max \), \( Cuphea hookeriana \), \( Pisum sativum \) and \( Arabidopsis thaliana \). The N-terminal regions of \( KAS \) III from \( J. \) *curcas* and other species have low sequence identity (Figure 1). Phylogenetic analysis indicated that \( JcKAS \) is closely related to \( KAS \) III from \( Perilla frutescens \) (Figure 3). The full-length cDNA sequence of \( JcKAS \) has been deposited in the NCBI GenBank under Accession number DQ987701.

**Comparison of expression levels of gene \( JcKAS \)**

Quantitative real-time PCR analysis indicated that the \( JcKAS \) gene was expressed in all \( J. \) *curcas* tissues examined (root, stem, cotyledon, leaf, flower and seed at 29 days after flowering (DAF)). The expression of \( JcKAS \) was significantly higher in roots than in other tissues (Figure 4A). Real-time PCR analysis of the expression of \( JcKAS \) during seed development (23-, 26-, 29- and 32-DAF) showed that the expression of \( JcKAS \) increased as the seed developed (Figure 4B).

**Southern blotting analysis of gene \( JcKAS \)**

We performed a Southern blot analysis to identify the genomic organization of the \( JcKAS \) gene. \( Jatropha curcas \) genomic DNA was digested with restriction enzymes \( EcoRI \) and \( HindIII \) — neither enzyme has a cutting position inside the \( JcKAS \) gene. The results showed that when the genomic DNA was digested with \( EcoRI \) and \( HindIII \), only one hybridization band was detected (Figure 5), indicating that \( JcKAS \) is a single copy gene in the \( J. \) *curcas* genome.

**Discussion**

Fatty acid biosynthesis in higher plants is a plastid-localized pathway catalyzed by a set of nuclear-encoded enzymes. Fatty acids are synthesized by the sequential addition of \( C_2 \) units, resulting in \( C_{16} \) to \( C_{18} \) fatty acids. Each cycle of \( C_2 \) addition is initiated by a reaction catalyzed by a KAS and involves the condensation of a malonyl-ACP with an acyl acceptor. \( KAS \) III initiates fatty acid synthesis using acetyl-CoA and malonyl-ACP as substrates (Jaworski et al. 1989, Clough et al. 1992, Schuch et al. 1994).

We isolated a full-length \( JcKAS \) cDNA from \( J. \) *curcas*. Sequence alignment of \( JcKAS \) III with \( KAS \) III from other plants revealed that \( JcKAS \) contains the active site \( Cys^{176} \) (Tai et al. 1994) and the amino acid residues, G355NTSAAS361, responsible for the binding of regulatory acyl-ACPs (Abbadi et al. 2000; Figure 2). \( JcKAS \) shares about 80% identity with other reported \( KAS \) III proteins. These conserved regions and the active site suggest that \( JcKAS \) III likely has the same function as other reported \( KAS \) III proteins. Quantitative real-time PCR analysis showed that the \( JcKAS \) gene is expressed in all \( J. \) *curcas* tissues examined, and that expression of \( JcKAS \) increased during seed development.

Analysis of the in vivo levels of acyl-ACPs in plants and bacteria (Jackowski and Rock 1987, Post-Beittenmiller et al. 1991, Clough et al. 1992) suggests that \( KAS \) III plays a regulatory role in fatty acid biosynthesis. Altering the levels of expression of this enzyme has helped to define more clearly the influence of \( KAS \) III on regulation of fatty acid biosynthesis in
Figure 1. Sequence alignment of JcKAS III from *Jatropha curcas* with KAS III from other organisms. The amino acids shaded in black indicate identity. Degenerate primers of KAS31 and KAS32 were designed according to conserved regions denoted by arrows.
plants. For example, in tobacco leaves and *Arabidopsis* and rapeseed seeds, overexpression of *KAS III* results in increased concentrations of C16:0 fatty acids. Also, decreased amounts of C18:1 fatty acids and increased amounts of C18:2 and C18:3 fatty acids were observed when expression of *KAS III* was upregulated. In *Brassica napus*, higher concentrations of middle-chain fatty acids were obtained following knockout of *KAS III* regulation. These results indicate that *KAS III* has a universal role in fatty acid biosynthesis; overexpressing or restraining its expression can alter the concentrations of fatty acids, irrespective of the plant species from which it is derived or the tissue in which it is expressed. Thus, overexpression or knockout of the *JcKASIII* gene could potentially be exploited to increase the amount of middle-chain fatty acids and C16:0 fatty acids or the extent of unsaturation of fatty acids in *J. curcas*. Further characterization of the *JcKAS III* gene is needed to understand the regulation of fatty acid biosynthesis in *J. curcas* and provide insights on how to improve oil production using transgenic *J. curcas* plants.

**Acknowledgments**

The authors thank Dr. H.G. Ding, Department of Biological Sciences, Louisiana State University for critical reading of the manuscript. This research was supported by the NSFC-Guangdong Joint Fund of Natural Sciences (U0733005), the Key Innovation Programs of the Chinese Academy of Sciences (KSCX2-YW-G-027), the Natural Science Foundation of Guangdong Province (07118251) and The CAS “100 Talents” program.

**Figure 2.** Nucleotide and deduced amino acid sequences of *KAS III* cDNA from *Jatropha curcas*. The start and stop codons are marked by underlines. The active site Cys176 is denoted by an asterisk (Tai et al. 1994). The putative acetyl-CoA binding site between residues 219 and 230 is shown by a double underline (Choi et al. 2000) and the putative acyl-ACP binding site GNTSAAS by a dotted line (Abbadi et al. 2000).

**Figure 3.** Phylogenetic analysis of *JcKAS III* proteins. The phylogenetic tree was constructed with Clustal W based on neighbor-joining method.

**Tree Physiology Online**

References


