Cloning and functional characterization of an acyl-acyl carrier protein thioesterase (JcFATB1) from *Jatropha curcas*

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Summary A full-length cDNA of an acyl-acyl carrier protein (ACP) thioesterase (TE) (EC 3.1.2.14), named JcFATB1, was isolated from the woody oil plant *Jatropha curcas* L. The deduced amino acid sequence of the cDNA shares about 78% identity with FATB TEs, but only about 33% identity with FATA TEs from other plants. The deduced sequence also contains two essential residues (H317 and C352) for TE catalytic activity and a putative chloroplast transit peptide at the N-terminal. Southern blot analysis revealed that a single copy of JcFATB1 is present in the *J. curcas* genome, and semi-quantitative PCR analysis showed that JcFATB1 was expressed in all tissues that were examined, most strongly in seeds, in which its expression peaked in late developmental stages. Seed-specific overexpression of the JcFATB1 cDNA in *Arabidopsis* resulted in increased levels of saturated fatty acids, especially palmitate, and in reduced levels of unsaturated fatty acids. The findings suggest that JcFATB1 from this woody oil plant can function as a saturated acyl-ACP TE and could potentially modify the seed oil of *J. curcas* to increase its levels of palmitate.

Keywords: fatty acid, palmitate.

Introduction In higher plants, fatty acid (FA) biosynthesis is catalyzed by a small family of β-ketoacyl-acyl carrier protein (ACP) synthases in the plastids, which are referred to as the KAS family (Shimakata and Stumpf 1982). More specifically, acyl chains that are esterified to an ACP are successively elongated, in two-carbon increments from malonyl-ACP, by the action of three discrete, monofunctional enzymes: KASIII condenses acetyl-Coenzyme A (CoA) with malonyl-ACP, forming 4:0-ACP; KASI catalyzes the elongation of 4:0- to 16:0-ACP and KASII catalyzes the elongation of 16:0- to 18:0-ACP. Acyl chain elongation can be terminated by either of the two mechanisms. In the prokaryotic pathway, the acyl group is transferred to glycerol 3-phosphate by acyltransferase and retained in plastids, whereas in the eukaryotic pathway, acyl-ACP thioesterase (TE) hydrolyzes the thioester bond of the acyl-ACP, releasing free FA and ACP. Then, the free FAs are exported from the plastid to the cytosol and re-esterified to CoA, and the acyl-CoAs are transferred into the endoplasmic reticulum (ER), thereby forming the acyl-CoA pool that is required for glycerolipid synthesis (Lohden and Frentzen 1988, Browse and Somerville 1991). Thus, acyl-ACP TEs play an essential role in determining the carbon chain length of storage lipids.

Plant acyl-ACP TEs are nuclear-encoded proteins that are plastid-targeted by a transit peptide at the N-terminus (Voelker et al. 1992) and function as dimers (McKeon and Stumpf 1982, Hellyer et al. 1992). According to their amino acid sequences, there are two isoforms of acyl-ACP TEs: FATA and FATB (Jones et al. 1995, Mekhedov et al. 2000). The FATA class has high specificity for 18:1-ACP and a lower activity toward 18:0- and 16:0-ACP (Knutzon et al. 1992, Hawkins and Kridl 1998, Serrano-Vega et al. 2005), whereas the FATB class more efficiently catalyzes the reaction with ACPs carrying saturated fatty acyl chains with 8–18 carbons (Voelker et al. 1997, Salas and Ohlrogge 2002). The Arabidopsis genome encodes two FATA and a single FATB (Beisson et al. 2003). Several FATB cDNAs have been cloned, and their substrate specificities have been characterized in vitro following recombinant expression in *Escherichia coli*. In addition, the potential scope for modifying FA metabolism by expressing these FATBs in vivo (and thus exploiting their differences in chain length specificities) in several species has been demonstrated. Notably, the expression of *UcFATB1*, from (12:0)-producing oilseeds of California bay [*Umbellularia californica* (Hook. & Arn.)] in oilseeds of *Arabidopsis*...
**Materials and methods**

**Plant materials**

Mature seeds of *J. curcas* were collected from plants growing naturally in Qianxi (106.04° E and 27.03° N), Guizhou Province, China, then these were planted and grown on a farmland in Guangzhou, Guangdong Province, China. Two years later, in autumn, various tissues were obtained for the described analyses.

**Cloning and isolation of *JcFATB1* gene**

Total RNA was extracted using Trizol (Invitrogen, Carlsbad, CA) following the manufacturer’s instructions, and then first-strand cDNA was synthesized from 3 μg of the RNA using Superscript II (Invitrogen). To isolate part of the *JcFATB1* gene, two degenerate PCR primers, designated FATB-1 and FATB-2 (Table 1), were designed corresponding to the amino acid sequences that were widely conserved in plant FATB proteins according to the alignment of AtFATB1 (Doermann et al. 1995), GhFATB1 (Jones et al. 1995), GhFATB2 (Pirtle et al. 1999) and UcFATB1 sequences using ClustalW (see Figure 1A, degenerate positions arrowed). The PCR was performed as follows: 94 °C for 3 min; 34 cycles of 94 °C for 30 s, 52 °C for 30 s and 72 °C for 1 min; and a final extension step of 72 °C for 7 min. The PCR product was purified using an Agarose Gel DNA Purification Kit (Biotek, Beijing, China), ligated into the pMD 18-T vector (TaKaRa, Otsu, Japan) and sequenced by Invitrogen (Shanghai). The 5’- and 3’-ends of *JcFATB1* were obtained using a SMART+ RACE cDNA Amplification Kit (Clontech, Palo Alto, CA) according to the manufacturer’s instructions, with FATB-3/FATB-4 and FATB-5/FATB-6 primers for 5’-RACE and 3’-RACE, respectively (Table 1). The PCR fragments obtained were cloned into the pMD 18-T vector and sequenced by Invitrogen (Shanghai).

**Southern hybridization**

Genomic DNA was extracted from *J. curcas* leaves using the CTAB method. Briefly, 10 μg of total DNA was

**Table 1. Primer sequences used in the experiments.**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5‘–3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>FATB-1</td>
<td>MNTAYCCNACNTGNGGNA</td>
</tr>
<tr>
<td>FATB-2</td>
<td>RTTRRNCRTTYGRTT</td>
</tr>
<tr>
<td>FATB-3</td>
<td>GCATTTTTCTGCACCATCTCATCAC</td>
</tr>
<tr>
<td>FATB-4</td>
<td>CACTGGAGGCCTCTGTTAGGTTTCACC</td>
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<tr>
<td>FATB-5</td>
<td>CCCTCCGTTGTTGGATATGGT</td>
</tr>
<tr>
<td>FATB-6</td>
<td>ACACCTGGCATATCTGGTC</td>
</tr>
<tr>
<td>FATB-7</td>
<td>ATGTTGCTAGCTGCTGCTAC</td>
</tr>
<tr>
<td>FATB-8</td>
<td>TTGATGCAACACTGGGATAT</td>
</tr>
<tr>
<td>FATB-9</td>
<td>CGACAAAGAAGAGGGGTGA</td>
</tr>
<tr>
<td>FATB-10</td>
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</tr>
<tr>
<td>FATB-11</td>
<td>TCGAGCTTCTAGGACCCTTTCACT</td>
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<td>CAATTTCACCAGCATCCTCATC</td>
</tr>
<tr>
<td>FATB-16</td>
<td>GCTCTAGAATGGTGCTACTGCTG</td>
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<tr>
<td>FATB-17</td>
<td>CCTGAACCTCTCTAGGAAAT</td>
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<td>Actin-F</td>
<td>AGTACGTCTGAGTTGCA</td>
</tr>
<tr>
<td>Actin-R</td>
<td>ATGGCTTACATCTGAGTCTGCT</td>
</tr>
<tr>
<td>At2s3-F</td>
<td>CCAAGCTTCCAGAAGTGGGAT</td>
</tr>
<tr>
<td>At2s3-R</td>
<td>CCTCTAGGTTTGTCAATGTTGATAGT</td>
</tr>
<tr>
<td>β-tubulin-F</td>
<td>GACGCTTACACCTGCTGCTG</td>
</tr>
<tr>
<td>β-tubulin-R</td>
<td>ACACAGCATACTAGCAGAAATCAG</td>
</tr>
</tbody>
</table>
Figure 1. Sequence analysis of \( JcFATB1 \) and related proteins. (A) Amino acid alignment of \( JcFATB1 \) homologs by the ClustalW program. AtFATB1 and ChFATB1 have TE specificity toward 16:0-ACP, while ChFATB2, UcFATB1 and CcFATB1 are specific for 8:0/10:0-, 12:0- and 14:0 ACP, respectively. Conserved amino acids are indicated by shaded squares, dashes indicate gaps and horizontal arrows indicate the domains used for designing the degenerate primers FATB-1 and FATB-2. The putative N-terminus of the mature \( JcFATB1 \) TE, as proposed for ChFATB1 (Doermann et al. 1995) and AtFATB1 (Jones et al. 1995), is indicated by the vertical arrowhead. Two essential residues (H 317 and C 352) for TE catalytic activity are marked with asterisks under the sequences.

(B) Phylogenetic tree showing relationships among \( JcFATB1 \) and other acyl-ACP TEs, according to ClustalX-derived protein alignments, generated by MEGA 4.1. The proteins AtFATB1 and AtFATA1 come from \( A. \) thaliana, ChFATB1 and ChFATB2 from \( C. \) hookeriana, GarmFATB1 and GarmFATA1 from \( G. \) mangostana \( L. \), UcFATB1 from \( U. \) californica, GhFATB1 from \( G. \) hirsutum \( L. \) and CcFATB1 from \( C. \) camphora \( P. \) resl.
digested with *Hind*III, *Xho*1 and *EcoRV*, separated on a 0.8% agarose gel and transferred to a Hybond N+ membrane (Amersham Biosciences, Buckinghamshire, UK). A digoxigenin (DIG-11-dUTP)-labeled probe of a 748-bp

brane (Amersham Biosciences, Buckinghamshire, UK). A digoxigenin (DIG-11-dUTP)-labeled probe of a 748-bp 5’ portion of the Jc*FATB1* gene was prepared using the

primers FATB-7 and FATB-4 (Table 1), and used in Southern blotting with a PCR DIG Probe Synthesis Kit and a DIG Luminescent Detection Kit (Roche Diagnostics, Mannheim, Germany) according to the manufacturer’s instructions.

**Gene expression analysis by semi-quantitative reverse-transcriptase mediated (RT)-PCR**

For tissue-specific expression analysis, total RNA was isolated from roots, stems, leaves, flowers and seeds of 29 days after flowering (DAF). In addition, for temporal-expression studies, total RNA was extracted from seeds 23, 26, 29 and 32-DAF (Li et al. 2008). The isolated RNA was subsequently treated with RNase-free DNase I (TaKaRa, Otsu, Japan), and first-strand cDNA was synthesized from 3 μg of total RNA using Superscript II (Invitrogen, Carlsbad, CA). The reverse-transcribed cDNA samples were then used for semi-quantitative RT-PCR, by amplifying a Jc*FATB1* cDNA fragment (408 bp) using the gene-specific primers FATB-8 and FATB-9 (Table 1), and a *J. curcas* *Actin* gene, amplified with the primers Actin-F and Actin-R (Table 1), yielding a 180-bp product, as a reference for normalizing the Jc*FATB1* cDNA levels.

**Plant transformation and gene expression in transgenic plants**

For seed-specific overexpression in plants, the seed-specific promoter of the At2s3 gene (Guerche et al. 1990) from Arabidopsis, amplified using the At2s3-F and At2s3-R primers shown in Table 1, was engineered to replace the CaMV 35S promoter in the binary plant transformation vector pBI121, using *Hind*III and *Xho*I, resulting in 25:GUS. The coding region (including the chloroplas targetting peptide) of Jc*FATB1* cDNA was amplified with the primers FATB-10 and FATB-11 (Table 1), then ligated into the 2s:GUS plasmid vector, using *Xba*I/SceI to place the *gusA* gene behind the seed-specific promoter. The 25:Jc*FATB1* construct was then transferred into *Agrobacterium tumefaciens* GV3101, and Arabidopsis plants (Col-0 ecotype) were transformed by the floral dip method (Clough and Bent 1998). Expression levels of the endogenous gene At*FATB1* and transgene Jc*FATB1* were examined by RT-PCR, using the gene-specific primer pairs FATB-12/FATB-13 and FATB-14/FATB-15, respectively (Table 1). In addition, two highly conserved sequences between Jc*FATB1* and At*FATB1* were used as primers, designated FATB-16 and FATB-17 (Table 1), to detect the whole FATB transcripts. β-tubulin-F and β-tubulin-R (Table 1), amplifying a 165-bp cDNA fragment or a 668-bp genomic fragment, respectively, were used as a control.

**Lipid analysis**

To analyze the lipid composition of seeds from the transgenic plants, the FA content of T4 seeds from homozygous and non-segregating transgenic plants was measured, according to the method described by Li et al. (2006) with little changes. Briefly, about 10 mg of seeds was weighed in a glass tube (1.5 x 10 cm) that had been thoroughly pre-rinsed with chloroform and dried to remove any contaminating lipid residues. To this tube, 1 ml of 5% (v/v) sulfuric acid in MeOH (freshly prepared for each analysis) was added, and 300 μl of toluene were then added, supplemented with 170 μl as an internal standard. The mixture was vortex-mixed for 30 s, and then heated at 90 °C for 1.5 h. After cooling to room temperature, 1.5 ml of 0.9% NaCl (w/v) was added and FA methyl esters (FAMEs) were extracted with 2 ml of hexane three times. Pooled extracts were evaporated under nitrogen and then dissolved in 400 μl of hexane.

The FAME extracts were analyzed by gas chromatography (GC) using an Agilent 7890A GC system equipped with a flame ionization detector (FID) and an HP-88 column (30 m x 0.25 mm I.D., 0.20 μm film thickness). The GC conditions were carrier gas (hydrogen) flow rate 1 ml min⁻¹; split injection (1:10); injector and FID temperature 250 and 300 °C; oven temperature program 150 °C for 3 min, then increasing by 10 °C min⁻¹ to 200 °C, then (after holding 200 °C for 5 min) 5 °C min⁻¹ to 230 °C, held for 3 min. The peaks were identified by mass spectrometry. Statistical analysis of the data was performed using Student’s *t* test.

**Results**

**Identification of a gene encoding an acyl-ACP TE from J. curcas**

Based on the alignment of sequences of proteins encoded by *AtFATB1* (Doermann et al. 1995), *ChFATB1* (Jones et al. 1995), *GhFATB1* (Pirtle et al. 1999) and *UcFATB1* (Voeler et al. 1992) (Figure 1A), two degenerate primers, FATB-1 and FATB-2, were designed. Using these primers, a 340-bp fragment was amplified from cDNA generated from the extracts of developing *J. curcas* seeds. Then, the 5’ and 3’ parts of the cDNA were obtained by RACE-PCR. The gene was named Jc*FATB1* and its sequence was deposited in GenBank under Accession No. EU106891. The full-length Jc*FATB1* cDNA has an ORF encoding a deduced 418-amino acid pre-protein, which has a calculated molecular mass of 48.08 kDa, a predicted pI of 6.57 and a putative chloroplast transit peptide at the N-terminal (identified by ChloroP neural network analysis). Alignment of Jc*FATB1* with several other FATB TE whose substrate specificities had been experimentally demonstrated showed that they shared a conserved C-terminal, but had variable N-terminals (Figure 1A). According to the
information obtained from ChFATB1 (Jones et al. 1995) and AtFATB1 (Doermann et al. 1995), the cleavage site of the transit peptide is likely to be at amino acid 89 just before the LPDW sequence of JcFATB1.

Proteins with similar amino acid sequences to JcFATB1 were sought by BLASTp searches of entries in the NCBI total databank. The JcFATB1 protein was found to have 78% identity to GhFATB1, but only 50% identity to UcFATB1. In addition, phylogenetic analysis indicated that JcFATB1 has the highest similarity to TEs whose preferred substrate is 16:0-ACP (Figure 1B), such as GarmFATB1, GhFATB1 and AtFATB1. It showed a lower similarity to ChFATB2, UcFATB1 and CcFATB1, which have preferences for 10:0-, 12:0- and 14:0-ACP, respectively, and still lower identity (ca. 33%) to FATA TEs with high specificity for 18:1-ACP, like AtFATA1 and GarmFATA1 (data not shown). Thus, these findings indicate that JcFATB1 was a palmitoyl-ACP TE.

Copy number of JcFATB1 in J. curcas

The copy number of the JcFATB1 gene in the J. curcas genome was assessed by Southern blotting, using a 748-bp probe comprising the 5' variable portion of JcFATB1 (to ensure accuracy of the assessment) amplified from the J. curcas genome, following digestion with HindIII, XbaI and EcoRV (for which no restriction sites were present in the probe sequence). As shown in Figure 2, only one band was discovered after the hybridization, which strongly suggested that only one copy of JcFATB1 gene is present in the genome.

Expression patterns of the JcFATB1 gene

The expression levels of the JcFATB1 gene in a range of tissues were analyzed by semi-quantitative RT-PCR using gene-specific primers. Transcripts were detected in every tissue examined, but most strongly in seeds and most weakly in roots (Figure 3A). The expression of the JcFATB1 gene in seeds at several developmental stages (23-, 26-, 29- and 32-DAF) was also examined by RT-PCR (Figure 3B), and the results showed that JcFATB1 transcript levels increased during their later developmental stages.

Expression of JcFATB1 in transgenic Arabidopsis seed leads to the accumulation of palmitate

To elucidate the substrate specificity of JcFATB1 in planta, the JcFATB1 cDNA was expressed in Arabidopsis under the control of a seed-specific promoter. The growth, development and 1000-seed weight of the transgenic Arabidopsis lines were not significantly different from those of wild-type plants (data not shown). Expression levels of the endogenous gene AtFATB1, transgene JcFATB1 and whole FATBs in three individual transgenic lines and wild-type plants were determined by RT-PCR. As shown in Figure 4A, all plants that were examined showed a moderate expression of AtFATB1, while JcFATB1 transcripts were only detected in the transgenic lines (most strongly in line 1, then successively less strongly in lines 2 and 3). In addition, higher levels of whole FATB transcripts were detected in all three transgenic lines than in wild-type controls, most abundantly in line 1.

Fatty acid content in the seeds of the transgenic and wild-type plants was determined, and compared, using GC
In all three transgenic lines, the levels of the saturated FAs, C16:0, C18:0, and C20:0, were increased. More specifically, the C16:0 content was increased several fold, from about 9.0 mol% in wild-type plants to ca. 40.9, 34.2, and 30.4 mol% in transgenic lines 1, 2, and 3, respectively, while their proportions of unsaturated FAs (such as C18:1, C18:2, C18:3, C20:1, C20:2, and C22:1) decreased.

**Discussion**

Since acyl-ACP TEs terminate FA synthesis and allow the export of FAs from plastids, these enzymes may be important determinants of FA composition in plants; the chain length of FAs is determined by both FATA and FATB depending on their specificity and activity level. Several FATBs have been characterized, including the representatives not only from model plants such as Arabidopsis (Doermann et al. 1995), but also from some medium-chain FA-rich plants, such as U. californica (Voelker et al. 1992) and 8:0-rich C. hookeriana (Dehesh et al. 1996). In addition, the structures of a few FATBs and the amino acids that affect their substrate specificity have been explored (Mayer and Shanklin 2005, 2007). However, more FATBs need to be characterized to exploit their potential uses, notably to adjust the fatty composition of oil from various plants.

In this study, a full-length TE cDNA, designated JcFATB1, was isolated from J. curcas. Sequence alignment showed that the protein it encodes, JcFATB1, has a high identity with other characterized FATBs, up to 78% with GhFATB1 (Figure 1). An apparent chloroplast transit peptide was detected at the N-terminal with a cleavage site at amino acid 89, before the marker sequence LPDW, and two essential residues (H317 and C352) for TE catalytic activity appear in the mature protein (Doermann et al. 1995, Jones et al. 1995). These sequence characteristics suggest that JcFATB1 may function as a chloroplast-localized stearate-ACP TE, with high specificity for 16:0-ACP TE.

In C. hookeriana, which accumulates caprylate (8:0) and caprate (10:0) up to 75% in its seed oil, there are two FATBs, named ChFATB1 (C16:0-specific) and ChFATB2 (C8:0- and C10:0-prefering). ChFATB1 transcripts have been detected in various tissues (Jones et al. 1995), while ChFATB2 expression appears to be seed specific (Dehesh et al. 1996). However, in Arabidopsis, Brassica and cotton, FATB always appears to be encoded by a single gene, i.e., expressed in various tissues. In this work, the JcFATB1 gene was shown to be present in a single copy according to DNA gel hybridization (Figure 2), and as in common plants such as Arabidopsis, JcFATB1 transcripts were detected in various tissues, most abundantly in seeds, especially in late developmental stages.

The seed-specific expression of JcFATB1 in Arabidopsis led to a 3–4-fold increase in the proportion of accumulated palmitate (up to 40.9 mol%) and moderate increases in the proportions of other saturated FAs, with associated reductions in the proportions of unsaturated FAs. The FA composition turned out in the transgenic Arabidopsis indicates that JcFATB1 showed to be a saturated-ACP specific TE, especially to the palmitate-ACP. These findings are very similar to the reported results of overexpressing FATBs from C. hookeriana and Arabidopsis in transgenic plants (Jones et al. 1995, Doermann et al. 2000). Our results suggest that JcFATB1 is a key determinant of the saturation and chain length of FAs synthesized in J. curcas. Further, JcFATB1 could be used to modify the FA composition of J. curcas seed oil. Methods for the Agrobacterium-mediated transformation of J. curcas have been established (Li et al. 2008b), which could be used in such efforts, but additional work will be needed to obtain genetically engineered J. curcas.

**Acknowledgments**

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