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Seed-specific expression of an acyl-acyl carrier protein thioesterase CnFatB3 from coconut (*Cocos nucifera* L.) increases the accumulation of medium-chain fatty acids in transgenic Arabidopsis seeds

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ABSTRACT

Coconut (*Cocos nucifers* L.) can accumulate up to nearly 80% of medium-chain fatty acids (MCFAs) in its endosperm. A previous study about suppression subtractive hybridization (SSH) libraries of coconut endosperm indicated that only one acyl-acyl carrier protein (ACP) thioesterase *CnFatB3* was identified and significantly up-regulated during fruit development. Overexpression of *CnFatB3* cDNA in Arabidopsis under control of the seed-specific *napin* promoter increased the amounts of 12:0 (lauric acid), 14:0 (myristic acid), 16:0 (palmitic acid) and 18:0 (stearic acid) by 30, 80, 4, and 2-fold, respectively, although *CnFatB3* transcript levels varied 4000-fold in Arabidopsis seeds from different transgenic lines. These data suggested that CnFatB3 had a preference for 12:0, 14:0, 16:0 and 18:0-ACPs in plant. The results provide new insights into the accumulation of MCFAs in higher plants, and will contribute to the metabolic engineering of MCFAs-producing to meet energy demands.

1. Introduction

Plant oils are an important renewable resource from nature for various daily essential applications, such as food nutrition, chemical industrial feedstocks, and renewable energy biofuels (Dyer et al., 2008). The properties and applications of oils largely depend on their fatty acid composition, while most plant oils contain just five common fatty acids, including: palmitic (16:0), stearic (18:0), linoleic (18:2) and linolenic (18:3) acids. However, some plant species can accumulate oil with high amounts of medium-chain fatty acids (MCFAs), namely fatty acids in the range of 8–14 carbons in length, which are important for industrial production of detergents, soaps, cosmetics, surfactants, flavoring and lubricants, and potential biofuels as well (Dyer et al., 2008; Kim et al., 2015a). For example, the most commercially important plant-derived MCFAs are from tropical crops oil palm (*Elaeis guineensis* Jacq.) kernel and coconut (*Cocos nucifers* L.) endosperm, which produce predominantly lauric acid (12:0; 46% – 52%) and myristic acid (14:0; 16%–19%) (Dyer et al., 2008; Kim et al., 2015b). Likewise, California bay (*Umbellularia californica*) produces a lauric-acid-rich (70%) oil in seeds (Davies et al., 1991; Pollard et al., 1991). Besides, many species of the genus *Cuphea* also can store rich MCFAs in their seeds, such as *Cuphea viscosissima* (64% 10:0 and 25% 8:0), *Cuphea pulcherrima* (95% 8:0), *Cuphea hookeriana* (75% 8:0), *Cuphea lanceolate* (83% 10:0), *Cuphea wrightii* (54% 12:0 and 29% 10:0), and so on (Dehesh et al., 1996a; Dörmann et al., 1993; Kim et al., 2015b; Leonard et al., 1997).

Acyl-acyl carrier protein (acyl-ACP) thioesterases (Fats), which terminate the elongation of acyl chains in *de novo* fatty acid biosynthesis, are therefore considered to be major determinants of carbon chain lengths of fatty acids (Li-Beisson et al., 2013). Thioesterases have been typically classified into two general families, termed FatA and FatB, based on amino acid sequence alignments and substrate specificities (Jones et al., 1995). FatAs show substrate preference towards oleoyl-ACP (C18:1$^{\Delta 9}$-ACP), while FatBs primarily hydrolyze saturated acyl-ACPs with 8–18 carbons (Jones et al., 1995; Sánchez-García et al., 2010; Voelker and Davies, 1994). Numerous FatBs have been identified and characterized from plants, especially from those species producing unusual fatty acids, such as MCFAs. The first FatB was identified from developing seed tissues of California bay (*Umbellularia Californica*), and demonstrated to be a medium (12:0-ACP) chain specific thioesterase (Davies et al., 1991; Pollard et al., 1991). Similarly, species from the genus *Cuphea* (*C. lanceolate*, *C. wrightii*, *C. hookeriana*, and *C. palusris*) all possess at least one FatB showing substrate specificity towards MCFA-ACPs (Dehesh et al., 1996a,b; Dörmann et al., 1993; Leonard et al., 1997). Three FatBs also have been separately identified from oil palm and coconut, and at least one of them (FatB3) from both species was shown to be preference to MCFAs (Dussert et al., 2013; Jing et al., 2011;

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Yuan et al., 2014). When expression in *Escherichia coli* K27, *CnFatB1* and *CnFatB2* enzymes appeared to be predominantly specific for C14:0 and C16:1, while *CnFatB3* enzyme was mainly specific for C12:0 and C14:1 (Jing et al., 2011). Moreover, heterogeneous expression of *CnFatB1* in transgenic tobacco seeds showed specific active mainly for C18:0-ACP and C16:0-ACP (Yuan et al., 2014).

From our previous research, *CnFatB3* was the only ACP thioesterase identified from suppression subtractive hybridization (SSH) libraries of coconut endosperm, and exhibited obvious up-regulation pattern during pulp development, which might be associated with the accumulation of 12:0 (about 50%) in pulp (Liang et al., 2014). Lately, activities of three CnFatBs were also detected by transient expression in tobacco leaves (Reynolds et al., 2015). Three of them all showed very similar activity with preference towards 14:0 and 16:0, whereas CnFatB3 also showed preference for 12:0 but less activity towards 16:0 comparing to the others (Reynolds et al., 2015). In this paper, we reported the function analysis of CnFatB3 by heterologous expression in Arabidopsis driven by a seed-specific promoter napin to explore the potential applications in metabolic engineering of producing MCFAs in temperate plants.

2. Materials and methods

2.1. Plant materials and growth conditions

Coconut (*Cocos nucifera* L.) pulps were obtained from Coconut Research Institute, Chinese Agricultural Academy of Tropical Crops, Wenchang, Hainan Province, China. Pulp about seven months after pollination was collected and quickly frozen in liquid nitrogen, and then stored at −70 °C. Wild-type *Arabidopsis thaliana* ecotype Columbia was used in this study. Arabidopsis plants were grown in a growth chamber at 23 °C with a 16-h photoperiod (16 h of 150 μE m^{-2} sec^{-1} light and 8 h of darkness).

2.2. Bioinformatic analysis and gene cloning

The translated protein sequence from *CnFatB3* was analyzed by protein BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). A phylogenetic tree of CnFatB3 with FatBs from other species was constructed by the neighbor-joining method using MEGA 5.0 software (Tamura et al., 2011).

Total RNA from coconut endosperm was extracted using cetyltrimethylammonium bromide (CTAB)-based method described by Li and Fan (2007). First-strain cDNA was synthesized from 1 μg of total RNA using FastQuant RT Kit (Tiangen, Beijing, China) according to manufacturer's instructions. The coding sequence of CnFatB3 was amplified using Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific, USA) and gene specific primers with specific restriction sites (indicated by underlines and brackets). The sequences of primers were as follows: *CnFatB3*-F: 5′- TAGGTACCATGGTCGCCTCCGTTGCTGCCTCA −3′ (*Kpn*I) and *CnFatB3*-R 5′- CTGGATCCTCATTTACTCTCAGTTGGGTGCA −3′ (*BamH*I). The conditions for PCR amplification were 98 °C for 30 s, 32 cycles of 98 °C for 10 s, 69 °C for 30s, 72 °C for 40 s and a final extension step of 72 °C for 7 min. The PCR product was cloned into the pEASY-Blunt vector (Transgen biotech, Beijing, China) and sequenced.

2.3. Plant transformation and selection for homozygous transgenic lines

To generate a binary plant overexpression construct, *CnFatB3* fragments were released from pEASY vector by a *Kpn*I − *BamH*I double digestion and subcloned into pCAMBIA1300 s vector under the control of a seed-specific napin promoter (Yuan et al., 2014; Kridl et al., 1991). pCAMBIA1300s-napin-*CnFatB3* expression vector was transformed into *Agrobacterium* strain GV3101 (Koncz and Schell, 1986) by electroporation and introduced into *Arabidopsis thaliana* (ecotype Columbia) wild-type by floral dip method (Clough and Bent, 1998). Transformants were first selected by hygromycin (30 mg/L; Zhang et al., 2006), and then confirmed by PCR of genomic DNA using primers as follows: *CnFatB3*-F: 5′-ATGGTCGCCTCCGTTGCTGCCT-3′; *CnFatB3*-R: 5′-TCATTTACTCTC-AGTTGGGTGCAGA-3′. PCR was carried out using Thermo Taq DNA polymerase (Thermo Fisher Scientific, USA) under the following thermal program: an initial denaturation at 94 °C for 5 min followed by 30 cycles of 94 °C for 30 s, 57 °C for 30 s, and 72 °C for 1 min 20 s, with a final extension at 72 °C for 7 min. PCR products were checked by electrophoresis on a 1% agarose gel. Homozygous lines were identified in T3 generation by segregation analyses.

2.4. Real-time quantitative PCR (RT-qPCR) analysis

Total RNA from Arabidopsis seeds was extracted using RNAprep pure plant kit (Tiangen, Beijing, China). First-strand cDNA was synthesized from 1 μg of total RNA as mentioned before. The housekeeper gene ubiquitin-conjugating enzyme was used as an internal control for expression analysis (UBQ; Czechowski et al., 2005). Two pairs of primers used in RT-qPCR are as follows: UBQ-F/R: 5′-GTTGATTTTTGCTGGGAAGC-3′/5′-GATCTTGGCC-TTCACGTTGT-3′ and q*CnFatB3*-F/R: 5′-GAGGATGCCGCTTCAACG-3′/5′-A-AATTCTGCCTGTAAACAAGTCC-3′. RT-qPCR was carried out using a CFX Connect™ Real-Time PCR Detection System (Bio-Rad Laboratories) and SYBR® premix Ex Taq™ II (Tli RNaseH Plus) Kit (Takara, Tokyo, Japan) according to the manufacturer's instructions. Expression was quantified as comparative threshold cycle (Ct) using $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001). Reactions were in triplicate including template-free and no-reverse-transcriptase negative controls.

2.5. Lipid extraction and gas chromatography analysis

Total lipids from Arabidopsis seeds including wild-type and homozygous transgenic lines were extracted by chloroform/methanol (2:1; v/v) as described previously, and transmethylated with KOH-methanol (2%; m/v) at 80 °C for 2 h. The fatty acid methyl esters (FAMEs) were recovered using *n*-hexane, and analyzed by gas chromatography as described previously (Yuan et al., 2015).

3. Results

3.1. Bioinformatics analysis and cloning of CnFatB3 gene

From our previous study, only one acyl-ACP thioesterase (CnFatB3, GenBank accession: JF338905) was identified from coconut suppression subtractive hybridization (SSH) libraries (Liang et al., 2014), though three CnFatBs have been identified from coconut and their *in vivo* activities have been verified in *E.coli* K27 (Jing et al., 2011). The full-length cDNA (1245 bp) of *CnFatB3* was isolated from total RNA of coconut endosperm using gene specific primers. BLAST analysis indicated that CnFatB3 (GenBank accession: AEM72521) has 66% and 63% amino acid identity with the other two acyl-ACP thioesterases from coconut: CnFatB1 (GenBank accession: AEM72519) and CnFatB2 (GenBank accession: AEM72520), respectively. Moreover, CnFatB3 also has 87% identity with a predicted palmitoyl-ACP thioesterase, thereafter referred to as EgFatB3 (GenBank accession: XP_010925110) from oil palm (*Elaeis guineensis* Jacq.) (Dussert et al., 2013).

Phylogenetic analysis of CnFatB3 with several FatBs from other species revealed that CnFatB3 clusters EgFatB3 from oil palm, which showed preference towards MCFAs in tobacco leaves (Dussert et al., 2013). CnFatB3 is also close to CnFatB1/2 and EgFatB1/2 (GenBank accessions: XP_010925300.1 and XP_010915014.1) (Fig. 1). In addition, CnFatB3 is more related to 16:0/18:0-ACP preference AtFatB from Arabidopsis (GenBank accession: NP_172327.1) than to CvFatBs (GenBank accessions: AEM72522.1, AEM72523.1, and AEM72524.1) from *Cuphea viscosissima*, even both CvFatBs and CnFatB3 share substrate specificity towards MCFA-ACPs (Jing et al., 2011).

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