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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).

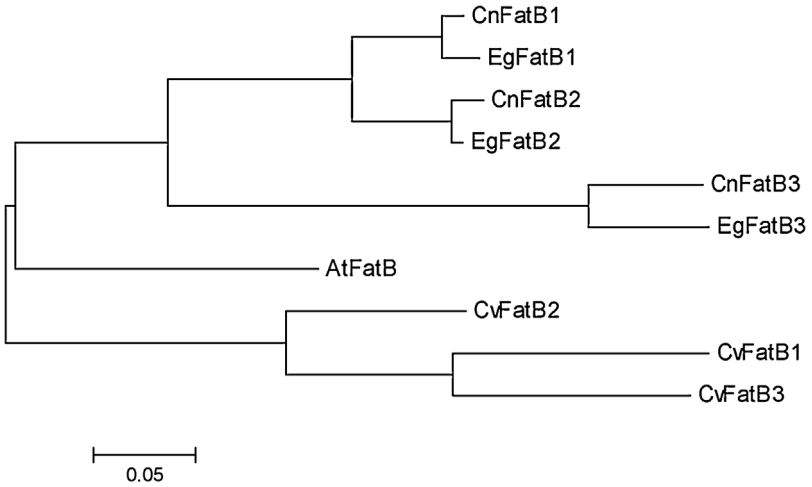

Fig. 1. Phylogenetic analysis of CnFatB3 and thioesterases from other plant species. A neighbor-joining tree was generated by MEGA5. GenBank accession numbers of sequences used are as follows: *Cocos nucifera* CnFatB1, 2, 3 (AEM72519, AEM72520, AEM72521); *Elaeis guineensis* EgFatB1, 2, 3 (XP_010925300.1, XP_010915014.1, XP_010925110); *Arabidopsis thaliana* AtFatB (NP_172327.1); *Cuphea viscosissima* CvFatB1, 2, 3 (AEM72522.1, AEM72523.1, AEM72524.1).

3.2. Heterologous expression of CnFatB3 in Arabidopsis

In order to verify the *in vivo* activity of CnFatB3 in plant, *CnFatB3* was cloned into pCAMBIA1300s-napin vector, which is driven by seed-specific promoter napin (Kridl et al., 1991). The recombinant plasmid was introduced into *Agrobacterium tumefaciens* and used to transform wild-type Arabidopsis. The transgenic lines were first selected by hygromycin in 1/2 MS medium, then transplanted to soil to maturity. Then, nine positive transgenic plants were obtained and confirmed by PCR using genome DNA from T1/T2 transgenic Arabidopsis seedling as template and *CnFatB3* gene specific primers. *CnFatB3* transcript expression in seed varied up to 4000-fold between different T3 homozygous transgenic plants, with three of them (3, 4 and 5) exhibiting over 500-fold of *CnFatB3* expression (Fig. 2). Three transformant lines (4, 5 and 9) with different levels of *CnFatB3* transcript were selected for further analysis.

3.3. Over-expression of CnFatB3 in Arabidopsis leads to MCFAs accumulation in seeds

Total fatty acid composition of mature seeds from T3 homozygous transgenic lines and wild-type Arabidopsis was analyzed by gas chromatography (GC). The results (Fig. 3) showed that the levels of 12:0 and 14:0 in CnFatB3 over-expression plant seeds, were increased by 30 and 80 folds up to 1.6% and 8.6%, respectively, which were barely detectable in wild-type Arabidopsis seeds. Moreover, the contents of 16:0 and 18:0 in transgenic seeds were separately elevated by 4 and 2 folds, compared to untransformed seeds (Fig. 3). Contrarily, the levels of 18:1, 18:2, 18:3, 20:1 and 20:2 in transgenic seeds were decreased by 7%, 12%, 8%, 5%, 6% and 0.8%, respectively, compared with that from wild-type seeds (Fig. 3). In addition, there was no obvious difference of seed oil content between transformed and untransformed Arabidopsis (data not shown).

4. Discussion

In the present study, we characterized the *in vivo* function of CnFatB3 from coconut endosperm by heterologous expression in transgenic Arabidopsis driven by a seed-specific promoter napin. Coconut is famous for the unique capability of accumulating rich MCFAs in endosperm, especially 12:0 (50%). CnFatB3 is the only thioesterase that the transcripts during pulp development were posi-

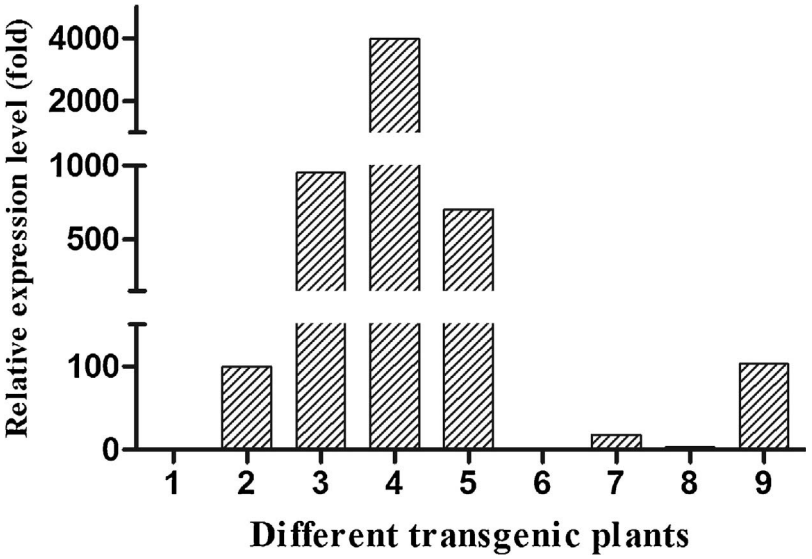

Fig. 2. Expression levels of *CnFatB3* in the seeds of nine individual transgenic lines.

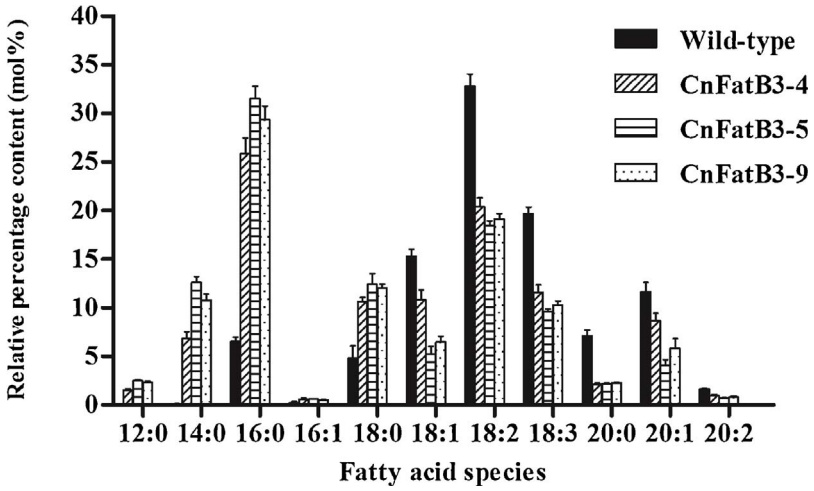

Fig. 3. Total fatty acid composition of mature seeds from three overexpressing CnFatB3 transgenic plants and wild-type Arabidopsis.

tively correlated with the content of 12:0 and MCFAs in coconut endosperm (Liang et al., 2014). So it could be a potential MCFA-producing enzyme when expressed in other temperate plants. As we expected, CnFatB3 exhibited the strong substrate activities toward 12:0 and 14:0, and also moderate preferences for 16:0 and 18:0 in Arabidopsis.

The activities of these three CnFatBs from coconut endosperm have been separately demonstrated by expression in *E.coli* K27 (Jing et al., 2011) and transient expression in tobacco (*N. benthamiana*) leaves (Reynolds et al., 2015). Although the results from these two experiments were not exactly the same, the substrate specificities of CnFatB1 and CnFatB2 were always consistent preferring 14:0 and 16:0, while only CnFatB3 had one more substrate preference towards 12:0 (Jing et al., 2011; Reynolds et al., 2015). Therefore, our result from CnFatB3 expression in Arabidopsis is consistent with results from previous expression studies in *E.coli* or tobacco.

From phylogenetic analysis of FatBs from several species, we can see three FatBs from coconut and oil palm were very identical. Similarly, among these three EgFatBs, EgFatB3 was predominantly expressed in oil palm endosperm, which is the most proportionally correlated to MCFA accumulation (Dussert et al., 2013). The results from transient expression of EgFatBs in tobacco leaves indicated that the activities of EgFatB1 and EgFatB2 were very similar with preference towards 16:0 and little 14:0, whereas EgFatB3 exhibited substrate specificities for 12:0, 14:0 and 16:0 (Dussert et al., 2013). So FatBs from coconut and oil palm (originated from the same genus family) are very similar in genetic evolution, expression pattern and substrate preference.

As for the substrate specificities of CnFatB3, it exhibited preference to 12:0, 14:0 and 16:0 −ACPs with the strongest activity towards 14:0-ACP (80-fold increase) in transgenic Arabidopsis, although expected to be 12:0-ACP-specific. The possible reason might be the amount of CnFatB3 preference-substrates MCFA-ACPs is very limited in wild-type Arabidopsis. Previous studies about CwFatB from *Cuphea wrightii* A. Gray that accumulates 30% 10:0 and 54% 12:0 in seed oil also verified this hypothesis: expression of CwFatB in Arabidopsis resulted in the increase of 12:0, 14:0, and 16:0 in seeds, with the greatest accumulation at 14:0 instead of 12:0 (Leonard et al., 1997). Even heterologous expression of the same MCFA-producing thioesterase in different plant species might lead to diverse effects. For example, expression of a 12:0-ACP thioesterase from *Umbellularia californica* in oilseed rape (*Brassica napus*) led to the accumulation of rich laurate (up to 50%), whereas its transient expression in tobacco leaves only increased the level of 12:0 from 0.2 to 1.6%, 14:0 from 0.1% to 1.1% (Eccleston et al., 1996; Reynolds et al., 2015; Voelker et al., 1996). Specific thioesterase may be a necessary prerequisite but not sufficient for those unusual plant accumulating high level of unusual fatty acids in storage oil (Leonard et al., 1997).

To enhance the expression system for producing MCFAs, coexpression MCFA-preferring FatBs with other genes encoding enzymes involved in oil biosynthesis could further increase the level of MCFAs, such as β-ketoacyl-(acyl-carrier protein) synthase I (KASI), lyso-phosphatidic acid acyltransferase (LPAAT). Coexpression of a coconut 12:0-CoA-preferring CnLPAAT with a California bay laurel 12:0-ACP thioesterase led to the accumulation of trilaurin and also further increased the total laurate levels (Knutzon et al., 1999). Coexpressing CwKASI from *Cuphea wrightii* with medium chain-specific thioerstrases CwFatB1 or CwFatB2 also led to the increase of MCFAs when compared to FatB parent (Leonard et al., 1998). Recently, coexpression combinations including 12:0/14:0/16:0-ACP thioesterase, CnLPAAT, and other key enzymes were carried out in tobacco leaves to produce rich MCFA-containing oil in non-seed tissue for plant metabolic engineering (Reynolds et al., 2015). Thus, CnFatB3 could be an underlying and powerful candidate gene used in MCFAs-producing engineering industry.

In summary, we demonstrated the *in vivo* function of CnFatB3 by heterologous expression in transgenic Arabidopsis, which showed a strong preference for 12:0, 14:0, 16:0 and 18:0-ACPs. The expression of CnFatB3 in Arabidopsis could increase the amounts of MCFAs, 16:0 and 18:0 of seed oil, without changing the oil production of seeds comparing with that of wild-type Arabidopsis. This research will be helpful for deciphering the mechanism of accumulating MCFAs in coconut endosperm and other medium-chain phenotype species. Moreover, it provides us another choice for engineering other temperate oil crops to produce rich MCFAs in seed or non-seed organs.

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