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Functional heterologous expression of a lysophosphatidic acid acyltransferase from coconut (*Cocos nucifera* L.) endosperm in *Saccharomyces cerevisiae* and *Nicotiana tabacum*

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ABSTRACT

The unique properties of oils from the coconut (*Cocos nucifera* L.) endosperm, which contains a remarkable amount of saturated medium-chain fatty acids (MCFAs), has garnered significant attention because of the increasing daily demand for these fatty acids. In this paper, lyso-phosphatidic acid acyltransferase (LPAAT), which can esterify acyl-CoA into the *sn*-2 position of the glycerol backbone during the triacylglycerol (TAG) biosynthesis, has been isolated from coconut endosperm, designated CnLPAAT. Quantitative real-time PCR showed that the *CnLPAAT* transcript was present in both leaves and endosperms, with the higher level in mature leaves, and the transcript level was constant during coconut endosperm development. Heterologous expression in yeast was used to confirm the *in vivo* function of *CnLPAAT*. The fatty acid analysis showed that the levels of C12:0 and C14:0 in a *CnLPAAT*-pYES2 transformant increased significantly compared with a pYES2 transformant. Additionally, to detect the effect of *CnLPAAT* expression in plants, it was heterologously overexpressed in transgenic tobacco (*Nicotiana tabacum* L.) under the control of the seed-specific napin promoter. The transcript level of *CnLPAAT* varied 28-fold among different transgenic lines, with 11 lines displaying detectable levels of *CnLPAAT* expression. However, an analysis of the fatty acid composition of transgenic tobacco seeds showed that the levels of C12:0 and C14:0 decreased diversely in comparison with untransformed tobacco plants. These findings provide new insights into the activities of *CnLPAAT* and assist in genetically applying this functional mechanism in other plants to accumulate high levels of MCFAs.

1. Introduction

In higher plants, triacylglycerol (TAG) is the major storage lipid that accumulates in seeds or fruits, and other tissues, such as senescing leaves, flower petals and pollen grains (Zhang et al., 2009; Li-Beisson et al., 2013). TAG from plants is an important renewable source of reduced carbon used as food, industrial feedstocks and biofuels (Durrett et al., 2008; Dyer et al., 2008; Bates and Browse, 2012). *De novo* fatty acid (FA) synthesis occurs exclusively in plastids and produces various free FAs, which are exported as acyl-CoA esters to the endoplasmic reticulum as substrates for glycerolipid synthesis (Ohlrogge and Browse, 1995). An increasing number of studies have revealed that TAG synthesis can be more complex than the previously recognized and that different plants use the various alternative pathways or combinations of pathways from TAG assembly (Chapman and Ohlrogge, 2011; Bates and Browse, 2012). The simplest pathway, and also first well-known one, is referred to as the Kennedy pathway, which is the constitutive, sequential esterification of the *sn*-1 and *sn*-2 positions of glycerol-3-phosphate producing lyso-phosphatidic acid, and subsequently phosphatic acid, by acyl-CoA:glycerol-3-phosphate acyltransferase (GPAT) and acyl-CoA:lyso-phosphatidic acid acyltransferase (LPAAT), respectively. Then, the removal of the phosphatidate group from the *sn*-3 position of the glycerol backbone catalyzed by phosphatidic acid phosphatase yields diacylglycerol (DAG), which can be used for the final acylation of TAG synthesis by diacylglycerol acyltransferase (DGAT) (Bates and Browse, 2012; Bates et al., 2009; Zhang et al., 2009). In addition, this pathway overlaps with the synthesis of

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membrane phospholipids because PA and DAG are also precursors for the major membrane lipids in all cells (Chapman and Ohlrogge, 2011).

The acyltransferases involved in the synthesis of TAG have been studied extensively to decipher their corresponding contributions to lipid synthesis. For LPAAT, five genes encoding LPAATs have been identified from the *Arabidopsis* genome database, and plastid LPAAT is essential for embryo development (Kim and Huang, 2004; Yu et al., 2004). A knockout mutant of the plastid LPAAT displayed embryo lethality, which could be repaired by the heterozygous expression of *LPAAT* (Kim and Huang, 2004). The other two LPAAT activities have been confirmed. LPAAT2 is necessary for female gametophyte development, and LPAAT3 is essential for the survival of the male gametophyte (Kim et al., 2005). In addition, the *in vitro* activities of the plastid LPAATs from *Brassica napus* and *Arabidopsis* both showed a preference for 16:0- over 18:1-CoA, which is a characteristic of lipids synthesized by the prokaryotic pathway (Bourgis et al., 1999; Kim and Huang, 2004; Yu et al., 2004). In contrast, the cytoplasmic LPAAT in microsomal fractions prefers 18:1-CoA and discriminates strongly against saturated 16:0- and 18:0-CoA, a characteristic of eukaryotic enzymes involved in lipid synthesis, which are designated as class A and can be ubiquitously expressed in the plant (Ohlrogge and Browse, 1995; Bourgis et al., 1999; Kim et al., 2005; Maisonneuve et al., 2010). The overexpression of *B. napus* microsomal LPAAT isozymes enhanced the lipid content and TAG accumulation in *Arabidopsis* seeds (Maisonneuve et al., 2010).

Species that can accumulate unusual acyl moieties at the *sn*-2 position of TAG in seeds, possess additional seed-specific LPAATs that are reactive toward the uncommon acyl-CoAs (Cao et al., 1990; Laurant and Huang, 1992; Brown et al., 1995; Frentzen, 1998; Kim et al., 2005). These members of the class B LPAATs display preferences for distinct, unusual saturated or unsaturated acyl groups, which are dominantly expressed in storage organs, have been reported in several different plants previously, including coconut (*Cocos nucifera* L.) (Davies et al., 1995; Knutzon et al., 1995), meadowfoams (*Limnanthes alba* and *Limnanthes douglasii*) (Lassner et al., 1995; Brown et al., 1995; Brough et al., 1996), and garden nasturtium (*Tropaeolum majus*) (Taylor et al., 2010). LPAATs from Meadowfoams, which accumulate rich fatty acids longer than 18 carbons (VLCFAs), can incorporate erucic acid into the *sn*-2 position of TAG in transgenic rape seeds to generating trierucin (Lassner et al., 1995; Brough et al., 1996). Similarly, the LPAAT from the coconut endosperm shows a preference for medium-chain-length fatty acyl-coenzyme A, substrates at *sn*-2 during the biosynthesis of TAGs, which have *in vivo* activities and substrate specificities that were confirmed in both *Escherichia coli* and plants (Davies et al., 1995; Knutzon et al., 1995, 1999). These LPAATs are of interest for genetically engineering the content of uncommon fatty acids found in various oil crop plant species (Bourgis et al., 1999).

Unlike the major vegetable oil crops (soybean, palm, canola and sunflower), which have FA compositions limited to primarily five FAs, saturated 16:0 and 18:0, monounsaturated 18:1, and polyunsaturated FA (PUFA) 18:2 and 18:3, the coconut endosperm accumulates abundant levels of saturated medium-chain fatty acids (MCFA), especially lauric acid (about 50%) (Ceniza et al., 1991; Bhatnagar et al., 2009). To understand the accumulation of rich MCFAs that occurs in the coconut endosperm, researchers have studied its fatty acid mechanisms. Several pivotal proteins involved in fatty acids synthesis have been indicated to be necessary for the rich MCFA accumulation during the development of coconut endosperm. Three acyl–acyl carrier protein (ACP) thioesterases, which terminate acyl chain elongation by hydrolyzing acyl-ACP into free fatty acids in fatty acid biosynthesis, have been isolated from the coconut endosperm and their *in vivo* functions have been tested in *E. coli* (Jing et al., 2011). CnFatB1 and CnFatB2 showed specificity toward 14:0 and 16:1, whereas CnFatB3 exhibited a substrate preference for 12:0 and 14:1 (Jing et al., 2011). Over expressing CnFatB1 in transgenic tobacco increased the level of 14:0, 16:0 and 18:0 in seeds (Yuan et al., 2014). Recently, β-ketoacyl-(acyl-carrier protein) synthase I (KASI), which is responsible for elongating 4:0-ACP to 16:0-ACP in six iterative cycles of C2 condensation during de novo fatty acid synthesis, was cloned from the coconut endosperm, and its *in vivo* role was verified by heterogenous over expression in transgenic tobacco seeds (Yuan et al., 2015). LPAAT from the coconut endosperm was cloned years ago and is well-known because of its unusual substrate specificity. However, previous studies merely showed its *in vivo* activities and substrate specificities in *E.coli*, or when co-expressed with California bay laurel (*Umbellularia californica*) 12:0-ACP thioesterase (BTE) in a transgenic oilseed rape (*B. napus*) (Davies et al., 1995; Knutzon et al., 1995, 1999). To date, however its function in a plant with a small, or even negligible, amount of 12:0-ACP has not yet be unknown.

In this research, we show the expression pattern of *CnLPAAT* during coconut ripening and confirm its function and substrate specificity in *Saccharomyces cerevisiae*. Additionally, the *CnLPAAT* gene was heterologously expressed in transgenic tobacco (*Nicotiana tabacum* L.) under the control of the seed-specific napin promoter (Kridl et al., 1991). These results will provide new insights into the activities of *CnLPAAT* and aid in genetically applying this functional mechanism in other plants to produce high levels of MCFAs.

2. Materials and methods

2.1. Plant materials

Coconut endosperms (8 and 15 months old) and mature leaves were obtained from the Coconut Research Institute, Chinese Agricultural Academy of Tropical Crops, Hainan, PR China (Yuan et al., 2014). These samples were harvested randomly, frozen in liquid nitrogen immediately, and stored at −70 °C for RNA extractions. The pCAMBIA1300S vector was donated by Yongjun Lin (Professor of Huazhong Agricultural University). All chemicals, endonucleases and other required enzymes were obtained from Sigma (St. Louis, MO, USA) or TaKaRa (Dalian, China), unless otherwise stated.

2.2. RNA extraction and CnLPAAT isolation

For the gene expression analysis and cDNA cloning, total RNAs were isolated from coconut pulps and leaves using cetyltrimethylammonium bromide (CTAB)-based methods described by Li and Fan (2007). Then, total RNA (2 μg) of each sample was used to synthesize the first strand cDNA using TIANScript OneStep RT-PCR Kits (Tiangen, Beijing, China). The resulting cDNA was used as a template to amplify the coding sequence of the coconut endosperm LPAAT gene with primers LPAAT-F and LPAAT-R (Table 1), which were designed based on the full-length *CnLPAAT* cDNA (GenBank accession no. U29657.1) reported by Knutzon et al. (1995). *Bam*HI and *Pst*I were added at the 5′ and 3′ ends, respectively. PCR amplification was performed for 30 cycles of 94 °C for 45 s, 48 °C for 45 s and 72 °C for 1 min. PCR products were analyzed by 1.2% (w/v) agarose gel electrophoresis. Then, the PCR fragment of the correct size was cloned into the pMD18-T vector (TaKaRa, Dalian, China) and sequenced. The sequencing result was analyzed using the BLAST program of the NCBI website http://www.ncbi.nlm.gov A phylogenetic tree of LPAAT proteins from different plants, including *C. nucifera* (Q42670.1), *Populus trichocarpa* (XP_002317596.1), *Ricinus communis* (XP_002522947.1), *Vitis vinifera* (CBI26630.3), *Oryza sativa Indica Group* (CAH66825.1)