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ORIGINAL ARTICLE

Multigene engineering of medium-chain fatty acid biosynthesis in transgenic *Arabidopsis thaliana* by a *Cre/LoxP* multigene expression system

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Abstract
Medium-chain fatty acids (MCFAs) are an ideal feedstock for biodiesel and a range of oleochemical products. In this study, different combinations of *CnFATB3*, *CnLPAAT-B* and *CnKASI* from coconut (*Cocos nucifera* L.) were coexpressed in transgenic *Arabidopsis thaliana by* a Cre/LoxP multigene expression system. Transgenic lines expressing different combinations of these genes were designated FL (*FatB3* + *LPAAT-B*), FK (*FatB3* + *KASI*) and FLK (*FatB3* + *LPAAT-B* + *KASI*). The homozygous seeds of transgenic *Arabidopsis thaliana* expressing high levels of these genes were screened, and their fatty acid composition and lipid contents were determined. Compared with its content in wild-type *A. thaliana*, the lauric acid (C12:0) content was significantly increased by at least 395%, 134% and 124% in FLK, FL and FK seeds, respectively. Meanwhile, the myristic acid (C14:0) content was significantly increased by at least 383%, 106% and 102% in FL, FLK and FK seeds, respectively, compared to its level in wild-type seeds. Therefore, the FLK plants exhibited the best effects to increase the level of C12:0, and FL expressed the optimal combination of genes to increase the level of 14:0 MCFA.

Keywords Medium-chain fatty acids (MCFAs) · *CnKASI* · *CnFatB3* · *CnLPAAT-B* · Combinatorial expression effect · *Cre/lox* combination system

Introduction

Medium-chain fatty acids (MCFAs), defined as fatty acids with fewer than 18 carbons, most of which are 12, 14, and 16 carbons in length, are natural compounds present in plant tissues (Schönfeld and Wojtczak 2016). In industries, MCFAs are an ideal source for biodiesel and a wide range of oleochemical feedstocks, including pharmaceuticals, personal care products, lubricants and detergents (Martínez-Vallespín et al. 2016; Jovancevic et al. 2017). The metabolic engineering of high biomass crops for increased lipid content and desired fatty acid compositions has been proposed as a novel platform to produce low-cost, energy-dense lipids such as triacylglycerol (TAG) (Chapman et al. 2013; Troncoso-Ponce et al. 2013). However, the impact of increasing MCFA levels by genetic transformation is theoretically limited.

Fatty acid (FA) and TAG synthesis in plants is complex and regulated by different factors in different cellular compartments, and each intermediate biochemical reaction is catalyzed by specialized enzymes (Durrett et al. 2008). The first committed step of de novo FA biosynthesis in plastids is catalyzed by acetyl-coenzyme A (CoA) carboxylase (ACCase), which converts acetyl-CoA to malonyl-CoA, which is then condensed by a set of beta-ketoacyl-acyl carrier protein synthases (KASs) that are involved in FA chain elongation from C4 to C18 (Gornicki and Haselkorn 1993). Among the several types of *KASs*, *CnKASI*, which was cloned from the coconut endosperm, is the most essential

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and catalyzes the production of C16:0-ACP from C4:0-ACP in six iterative cycles of C2 condensation (Yuan et al. 2014; Yang et al. 2016).

After de novo FA synthesis occurs in the plastid, the transfer of nascent FA to the endoplasmic reticulum (ER) for TAG assembly requires the hydrolysis of acyl-ACP to release free FA. This reaction is catalyzed by acyl-ACP thioesterases. Plant acyl-ACP thioesterases are separated into two classes, termed FatA and FatB3. Generally, FatA enzymes preferentially hydrolyze 18:1-ACP; whereas, saturated acyl-ACP is the preferential substrate of FatB3 thioesterases (Dussert et al. 2013a). Many reports revealed high *FatB3* transcription levels in lipid-rich tissues containing high saturated FA levels, which implied a connection between *FatB3* function and saturated FA biosynthesis (Joët et al. 2009). In fact, *CnFatB1* and *CnFatB2*, which are two paralogs of *FatB3* from the coconut endosperm, showed specificity toward 14:0 and 16:1 MCFAs; whereas, CnFatB3 exhibited a substrate preference for C12:0 and C14:0 (Jing et al. 2011). Most importantly, in plants that store MCFAs, *CnFatB3* is the only paralog that encodes an MCFA-specific thioesterase, which is able to prematurely truncate the elongating acyl-ACP within the plastid and allow its export into the cytoplasm (Toni and Anthony 2001).

One of the two main routes of TAG assembly in the ER leads to the accumulation of TAG acylated with MCFAs. Through this assembly pathway, lysophosphatidic acid acyltransferase (LPAAT), which is separated into two classes of genes termed *LPAAT-A* and *LPAAT-B*, is involved in the transfer of acyl-CoA to the second position of glycerol. LPAAT-Bs display a preference for distinct, unusual saturated or unsaturated acyl groups that are mostly expressed in storage organs and have been reported in several different plants, including meadowfoams (*Limnanthes alba and Limnanthes douglasii*) (Brough et al. 1997) and garden nasturtium (*Tropaeolum majus*) (Taylor et al. 2010). Consistent with this, *CnLPAAT-B* from the coconut endosperm also efficiently transfers medium-chain fatty acyl-CoA substrates to the sn-2 position during the biosynthesis of TAG (Dussert et al. 2013b; Yuan et al. 2015a).

Regarding MCFA engineering, previous studies have made some progress on the modification of seed oils to increase MCFA content and predominantly focused on the engineering of lauric acid (C12:0) (Reynolds et al. 2017). In the MCFA biosynthesis process, specialized thioesterase (FatB3) is an essential enzyme that prematurely truncates the standard fatty acid elongation cycle within the plastid and allows the export of MCFAs into the cytoplasm. In the cytoplasm, MCFAs are incorporated into TAG by three key acyltransferases: glycerol-3-phosphate acyltransferase (GPAT), lysophosphatidic acid acyltransferase (LPAAT) and diacylglycerol acyltransferase (DGAT), which avoid the incorporation of lipid head groups into the cell membrane. Even so, the synergistic combination of multiple genes is needed to increase the incorporation of MCFAs into TAG.

Cre/LoxP multigene expression system is a site-specific DNA excision system, which originated from *Escherichia coli* phage P1 and mediated by the *Cre* recombinase, recognizes and interacts with the two 34-bp lox sites to direct the excision, integrate or inverse the intervening DNA sequence depending on the orientation of lox sites towards each other (Hoess and Abremski 1985). Proper functioning of *Cre*-mediated recombination is independent of host cofactor or accessory proteins. Additionally, *Cre*-recombinase specifically recognize the *loxP* sequence of 34 bp in length and excises a DNA segment flanked by two direct repeats of loxP, leaving a single copy of *loxP* (Kasai and Harayama 2016). This system has been proven to be a powerful marker rescue tool, and overcomes the limitations of sexual crossing in eukaryotic cells (Dohlemann et al. 2016).The function of the *Cre/LoxP* system was recently well documented and considered as a more efficient multigene recombination system (Mizutani et al. 2012).

The coconut (*Cocos nucifera* L.) endosperm accumulates a high saturated oil content (approximately 91.4%), and more than 60% of its total fatty acid composition is MCFAs; the coconut endosperm is especially rich in lauric acid (C12:0; 47.8%) and myristic acid (C14:0; 19.4%) (Patil and Benjakul 2018). In this study, *CnKASI*, *CnFATB3* and *CnLPAAT-B* from coconut palm were introduced into a *Cre/LoxP* multigene expression system and transformed into *Arabidopsis*. The lauric acid (C12:0) content increased in all FL, FK and FLK transgenic *Arabidopsis* seeds when compared with that in wild-type seeds. The myristic acid (C14:0) content also increased. These results suggested that *CnKASI*, *CnFatB3* and *CnLPAAT-B* synergistically improve the MCFA yield. Additionally, the lipid content was increased in all FL, FK and FLK transgenic *Arabidopsis* seeds compared with that in the wild-type seeds. The combination of *CnFatB3* and *CnKASI* had the optimum combinatorial expression effect on lipid yield. As far as we know, this is the first report to reveal synergy among MCFA-associated genes from coconut palm. This study will provide a potential approach to achieve high MCFA content by metabolic engineering in plants.

Materials and methods

Plant materials

Coconuts (*Cocos nucifera* L.) were obtained from the Coconut Research Institute, Chinese Agricultural Academy of Tropical Crops, Hainan, PR China (Yuan et al. 2015b). The samples were randomly harvested, immediately frozen in liquid nitrogen, and stored at − 70 ℃ for RNA extractions. All chemicals, endonucleases and other required enzymes

were obtained from Sigma (St. Louis, MO, USA) or TaKaRa (Dalian, China) unless otherwise stated. *Arabidopsis thaliana* ecotype Columbia was used in this study. *Arabidopsis* plants were cultivated in a growth chamber at 23 ℃ with a 16-h photoperiod (16 h of 150 μE m^2 sec^1 light and 8 h of darkness).

Construction of target genes in donor vectors

The acyl-ACP thioesterases *FatB3* (AEM72521.1), *LPAAT-B* (Q42670.1) and *KASI* (JX275887) from coconut, named *CnFatB3*, *CnLPAAT-B* and *CnKASI*, respectively, were cloned as in our previous studies (Yuan et al. 2017, 2015a, b). The three genes were then subcloned into the pYL322-d1 and pYL322-d2 vectors. The seed-specific promoter *napin, Pro-at5g5400* and *Pro-at5g1646* (Jeong et al. 2014) were employed to drive the expression of *CnFatB3*, *CnLPAAT-B* and *CnKASI*, respectively. The PCR primers used to amplify these genes and promoters are shown in Supplementary Table 1.

Construction of multigene expression vectors

To generate the p*FatB3–KASI–380DTH*, p*FATB3–LPAAT-B–80DTH* and p*FatB3–KASI–LPAAT-B–380DTH* vectors to produce transgenic plants, the *CnFatB3*, *CnLPAAT-B* and *CnKASI* gene expression cassettes were introduced into the multiple gene expression vector pYLTAC380DTH by the homologous recombination method (Lin et al. 2003). In brief, a mixture of the donor plasmid, pYL322-d1/NAPIN-FATB3, and the receptor plasmid, pYLTAC380DTH, was introduced into *E. coli* strain NS3529, which expresses *Cre* recombinase. The obtained recombinant plasmids were digested using the restriction enzyme *NotI* (Thermo Fisher, USA) to verify target gene assembly into the receptor plasmids. The receptor plasmid *FatB3–380DTH* and the donor plasmid pYL322-d2/*16460-KASI* were mixed and transformed into competent NS3529 cells on the second round. Colonies were screened, and recombination plasmids were extracted for digestion with *PI-Sce*I. The digested plasmids were transformed into competent TransT1 cells. Recombinant colonies were selected for PCR determination. The donor plasmid PYL322-d1/*5400-LPAAT-B* and the second recombination plasmid *FatB3–KASI–380DTH* were assembled by the same method on the third round.

Arabidopsis transformation and selection

The recombined vectors were transported into *Agrobacterium* strain GV3101 cells by electroporation. The transformants were verified by PCR and used for plant transformation. *Arabidopsis* was transformed by the floral dip method (Bent and Clough 1998). The transformants were first selected by growth with hygromycin (30 mg/L) (Zhang et al. 2006) and then confirmed by PCR amplification of the genomic DNA using the following primers: *Hpt-F*: TGT CCTGCGGGTAAATAGC and *Hpt-R*: ATGTTGGCGACC TCGTATT. The conditions for PCR amplification were 98 ℃ for 30 s, 32 cycles of 98 ℃ for 10 s, 69 ℃ for 30 s, 72 ℃ for 40 s and a final extension step of 72 ℃ for 7 min. Homozygous lines in the T_3 generation were identified by segregation analyses.

Quantitative real-time PCR (qRT-PCR) analysis

The seeds of wild-type and transgenic Arabidopsis were collected after 40 flowering when the seeds were fully matured. Total RNA was extracted from seeds using the instructions with the RNA extraction kit (Takara, Dalian, China). The transcript levels of the three genes in *Arabidopsis* seeds were determined using qRT-PCR. qRT-PCR amplification was performed by the use of a SYBR® PremixEx Taq™II Kit according to the manufacturer's instructions (TaKaRa, Dalian, China) with *β-actin* as an internal control. The reactions were performed in triplicate, including a template-free reaction and a reverse transcriptase-free reaction. The qRT-PCR primers used to amplify *CnFatB3*, *CnKASI* and *CnLPAAT-B* are shown in Supplementary Table 2.

Fatty acid composition detection

Total lipids were extracted in triplicate from the mature seeds of single transgenic *Arabidopsis* and wild-type *Arabidopsis* plantlets using dichloromethane:methanol (2:1). Fatty acid methyl esters were recovered using N-hexane. Analysis of the fatty acid methyl esters was performed using gas chromatography (GC), and methyl heptadecanoate (C17:0) obtained from Sigma (St. Louis, MO, USA) was used as an internal standard. FAMEs were analyzed using an Agilent Technologies GC 19091 N-1331 column with HP-INNO-Wax (30.0 m × 0.25 mm × 0.25 um) and a flame ionization detector. FAMEs in the mixture were identified by comparing their retention times with those of 15 FAME standards (Nu-Check Prep, USA).

Statistical analysis

Three biological triplicates were used for every sample to ensure reproducibility, and the data provided as mean ± standard deviation. Student's *t* test was used to analyze significant differences between the transgenic and nontransgenic (with empty vector) groups. Differences with $p < 0.05$ and $p \leq 0.01$ were regarded as statistically significant and extremely significant, respectively.

Results

Construction of a Cre/LoxP recombination system carrying *CnFatB3*, *CnLPAAT-B* and *CnKASI* multiple genes inserts

The *CnFatB3*, *CnLPAAT-B* and *CnKASI* genes were amplified from the previously described plasmids (Yuan et al. 2017, 2015a, b). The seed-specific promoters *napin*, *At5400* (defined as Pro-at5g5400) and *At16460* (defined as *Pro-at5g16460*) were cloned from *Arabidopsis thaliana* genomic DNA. In our study, *napin* was used to promote *CnFatB3* expression, and *Pro-at5g5400* and Pro-at5g1646 were used to promote *CnLPAAT-B* and *CnKASI* expression, respectively (Fig. 1a–c). The *NOS* terminators were derived from the plant expression vector pCAMBIA1300S. The *CnFatB3* and *CnLPAAT-B* expression modules were recombined to form a combinatory dual-gene coexpression system (*CnFatB3–LPAAT-B*, designated FL). The *CnFatB3* and *CnKASI* expression modules were constructed as a dual-gene coexpression system (*CnFatB3–KASI*, FK) as well. Similarly, the *CnFatB3–LPAAT-B* expression system and the *CnKASI* expression module were used to produce a triple gene coexpression system (*CnFatB3–LPAAT-B–KASI*, FLK) (Fig. 1 d–f).

Heterologous expression of multigene combinations in *A. thaliana*

Three multigene combinations, FL, FK and FLK, were transformed into *Arabidopsis thaliana* using the floral dip method. T1 generation transgenic plants were screened based on hygromycin resistance. PCR was employed to identify the genes in T2 generation transgenic *Arabidopsis*. As shown in Fig. 2a–c, the three multigene combinations were successfully integrated into the *Arabidopsis* genome. Hygromycin screening was then applied to further confirm homozygous T2 generation transgenic plants.

To reveal the expression levels of three genes in different transgenic plants, qRT-PCR was employed to detect the expression *of CnFatB3*, *CnKASI* and *CnLPAAT-B* in the three transgenic lines. The results indicated that *CnFatB3*, *CnKASI* and *CnLPAAT-B,* were significantly overexpressed in the three transgenic lines compared with their expression in wild-type *Arabidopsis* (Fig. 2d–f). Interestingly, *CnLPAAT-B* showed the highest expression level in the three transgenic lines. Finally, the three lines with the highest expression levels of each combination of genes were chosen for fatty acid profile analysis. These were lines 3, 5 and 10 from the FL plants; lines 9, 12 and 75 from the FK plants; and lines 3, 17, and 2 from the FLK plants.

Multigene combinations improve total fatty acids content

The percentage of the total fatty acid content in 50-mg seeds was calculated and regarded as the content relative to that of C17:0, which was used as an internal standard. As expected, the relative total fatty acid content was significantly increased when seeds from transgenic *Arabidopsis* plants were compared to those from wild-type, and the total fatty acid content was increased by at least 47%, 70% and 56% in the FL, FK and FLK seeds, respectively, compared to that in the wild-type seeds. Moreover, several lines exhibited an extremely significant increase in total fatty acid content. Line 12 from the FK plants had the highest fatty acids content among all of the measured lines and contained 9.687 ± 1.102 μg total fatty acid/50 mg seeds (while, wild-type content is 4.821 ± 0.241) (Fig. 3).

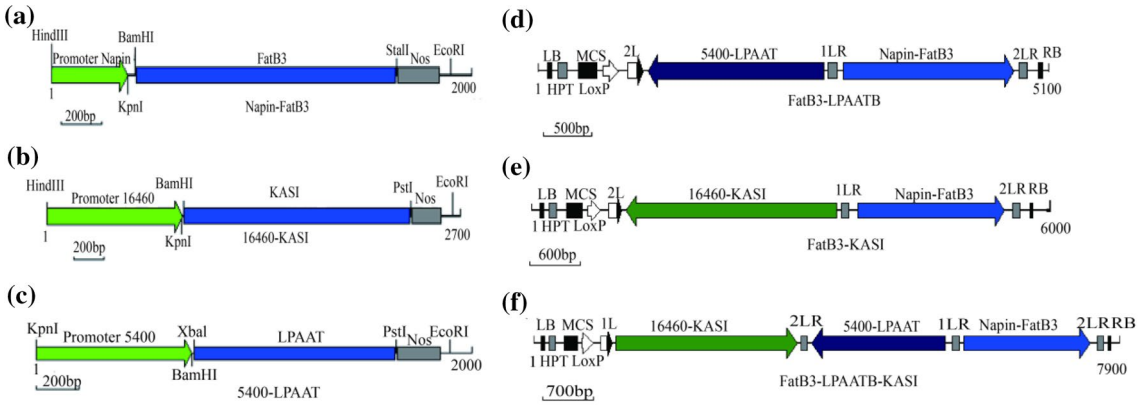

Fig. 1 Construction map of CnFatB3, CnLPAAT-B and CnKASI by *Cre/LoxP* multigene expression system. **a–c** The CnFatB3, CnLPAAT-B and CnKASI expression cassettes. **d–f** Multiple-gene vector map of CnFatB3–LPAAT-B, CnFatB3–KASI and CnFatB3–LPAAT-B–KASI

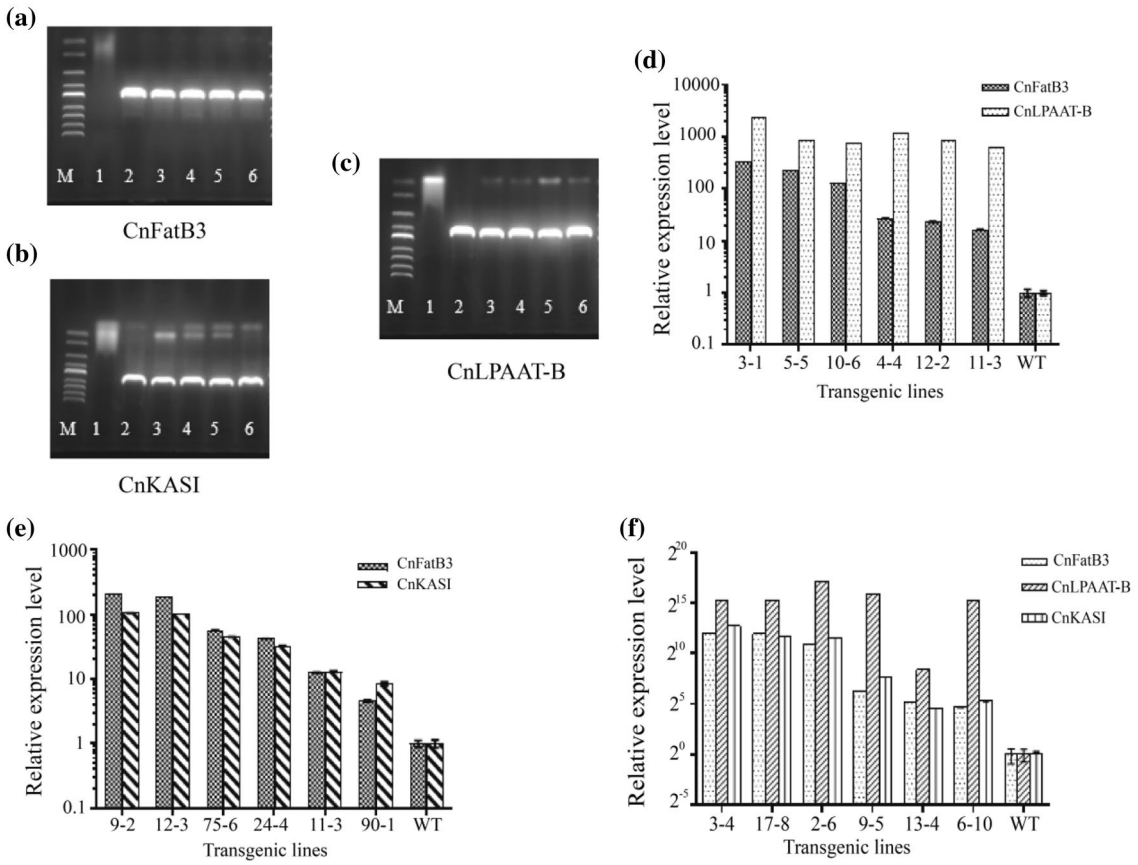

Fig. 2 Identification of transgenic plants by PCR and qRT-PCR. **a–c** Agarose gel electrophoresis showing the PCR identification of T1 generation transgenic *Arabidopsis*. M: DNA marker. Lane 1: Wild type. Lane 2: FKL. Lanes 3 and 4: FKL. **a** Lane 5: FK. Lane 6: FL. **b** Lanes 5 and 6: FK. **c** Lanes 5 and 6: FL. **d–f** Relative expression levels of *CnFatB3, CnLPAAT-B* and *CnKASI* mRNA in the transgenic FL, FK and FLK plants, respectively

Multigene combinations alter fatty acid composition

The total fatty acid composition of mature seeds from the T3 homozygous transgenic lines expressing three combinations of genes and wild-type *Arabidopsis* was analyzed by gas chromatography (GC). The results (Fig. 3) showed that the levels of lauric acid (C12:0) and myristic acid (C14:0) were significantly increased in seeds from all three transgenic lines compared to those in seeds from wild-type plants. The relative amount of lauric acid (C12:0) increased by at least 395%, 134%, and 124% in seeds from the FLK, FL and FK transgenic *Arabidopsis* lines, respectively, compared with that in seeds from the wild type. Similarly, the myristic acid (C14:0) levels increased by at least 383%, 106% and 102%, respectively, in the FL, FLK and FK transgenic lines (Fig. 4a–c). Notably, there was also a significant difference in the medium-chain fatty acid content between different transgenic lines expressing the same combination of genes. Different sites of integration during genetic transformation and the unequal expression levels of each gene could be the main causes for this phenomenon (Table 1).

In addition, the levels of other fatty acids in the transgenic lines expressing three gene combinations were also determined. Unlike the consistent increase in C12:0 and C14:0 across the transgenic lines, the content of the other fatty acids was different in the three transgenic lines. For example, in the FL lines, the very-long-chain fatty acid (VLFA) content, including C22:1, C24:0 and C24:1, was significantly increased compared to that in the wild type. In the FK transgenic line, the C18:1 and C18:2 fatty acid content was significantly increased in two transgenic lines compared to that in the third transgenic line and the wild type (Fig. 4a–c). However, in the FLK lines, the change of VLFA and UFA was not significant.

Combinatorial expression effect of *CnFatB3, CnLPAAT-B* and *CnKASI*

To further unravel the optimal combinatorial expression effect among *CnFatB3, CnLPAAT-B* and *CnKASI*, the

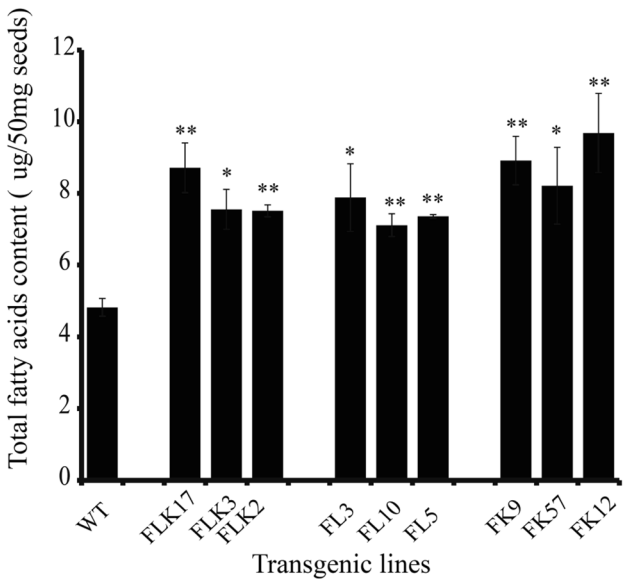

Fig. 3 Analysis of the differences in total fatty acids content in different transgenic plants compared with that in wild-type. FKL: *FatB3–KASI–LPAAT-B*, FL: *FatB3–LPAAT-B*, FK: *FatB3–KASI*. All experiments were performed in triplicate. *Significant difference according to Student's *t* test at $p < 0.05$. **Extremely significantly different, $p < 0.01$

transgenic lines expressing each combination of genes with the most pronounced effect were selected for further comparison. These were lines 17, 8, and 3; lines 3, 1, and 2 and lines 9, 2, and 3 from the FLK, FL and FK transgenic lines, respectively. Interestingly, the highest lauric acid (C12:0) content was detected in the FLK transgenic lines. The lauric acid content was 19% and 116% higher than that in the FL and FK transgenic lines, respectively. In contrast, the C14:0 content was highest in the FL transgenic line and at least 58% and 92% higher than that in the FLK and FK transgenic lines, respectively (Fig. 5). Above all, with regard to the biosynthesis of C12:0, the FLK transgenic line expressed the optimal combination of the genes, and to increase the C14:0 content, the combination of genes in the FL lines should be used. Additionally, the FLK lines showed increased C18:1 fatty acid levels, and the FL lines exhibited C16:0, C16:1, C24:0 and C24:1 accumulation.

Discussion

The endosperm of coconut (*Cocos nucifera* L.), an important oil-yielding tropical crop, is rich in MCFAs. This peculiar property makes the coconut endosperm an attractive model to unveil MCFA biosynthesis mechanisms. *CnFatB3, CnLPAAT-B* and *CnKASI* play vital roles in the metabolism of MCFAs (Yuan et al. 2015a, b, 2017); however, increasing the expression of a single gene in a complex metabolic pathway such as fatty acid synthesis is relatively ineffective at changing the final product unless concomitant changes are made in other enzymes (Dehesh et al. 2001). In this study, three genes, *CnFatB3*, *CnLPAAT-B* and *CnKASI*, were introduced to construct different Cre/LoxP multigene combination transgenic lines, designated *CnFatB3–LPAAT–KASI* (FLK), *CnFatB3–LPAAT* (FL), and *CnFatB3–KASI* (FK), to determine their combinatorial expression effects on MCFA and lipid biosynthesis. To our knowledge, this is the first time the harmony between MCFA-associated enzymes in coconut has been clarified.

The expression levels of *CnFatB3, CnLPAAT-B* and/or *CnKASI* were examined in FLK, FL and FK transgenic *Arabidopsis*. The gene expression levels were extensively fluctuant in the seeds of plants expressing different combinations of the genes. Different lines of expressing same combination of genes also showed different expression patterns. These results suggested that combinatorial vectors may be inserted into disparate genome sites, resulting in diverse gene expression profiles. The expression of *CnLPAAT-B* was the highest in all FLK and FL lines and the expression of *CnFatB3* was similar in all FK lines. Moreover, the transcription levels of any particular gene were different with different combinations of genes as well. For instance, the *CnFatB3* mRNA level was highest in FK seeds and, in contrast, relatively low in FL seeds. Differences in the transcription of a particular gene might be due to the interaction of multiple coexpressed genes in the same vector.

In addition, the correlation between gene expression level and the effects on fatty acid and lipid biosynthesis has been unraveled. In general, high expression levels were accompanied by a remarkable increase in MCFA yield. For example, line #17-8-3; line# 3-1-2; and line #9-2-3, which had the highest gene transcription levels in the FLK, FL and FK transgenic plants, respectively, expressed high levels of 12:0 and 14:0 fatty acids. Coincidently, the other two lines (line #3-4-5, line #10-6-4) with decreased mRNA levels exhibited relatively low 12:0 and 14:0 fatty acid content. Further analysis revealed that the transcription level and lipid content were also positively correlated. To some extent, when the transcription levels of a particular gene were positively correlated with the corresponding enzyme level, this influenced catalysis.

The use of a seed-specific promoter with high efficiency is a valid way to improve crop seed production. In this study, napin, *Pro-at16460* and *Pro-at5400* were employed to promote *CnFatB3*, *CnKASI* and *CnLPAAT-B* gene expression, respectively. High expression levels of *CnFatB3*, *CnKASI* and *CnLPAAT-B* suggested that these promoters were highly efficient and seed specific, which is concordant with the previous reports of expression in embryos and endosperms (Jeong et al. 2014). Specifically, the expression of *CnLPAAT-B* was still relatively high in FLK and FL, two

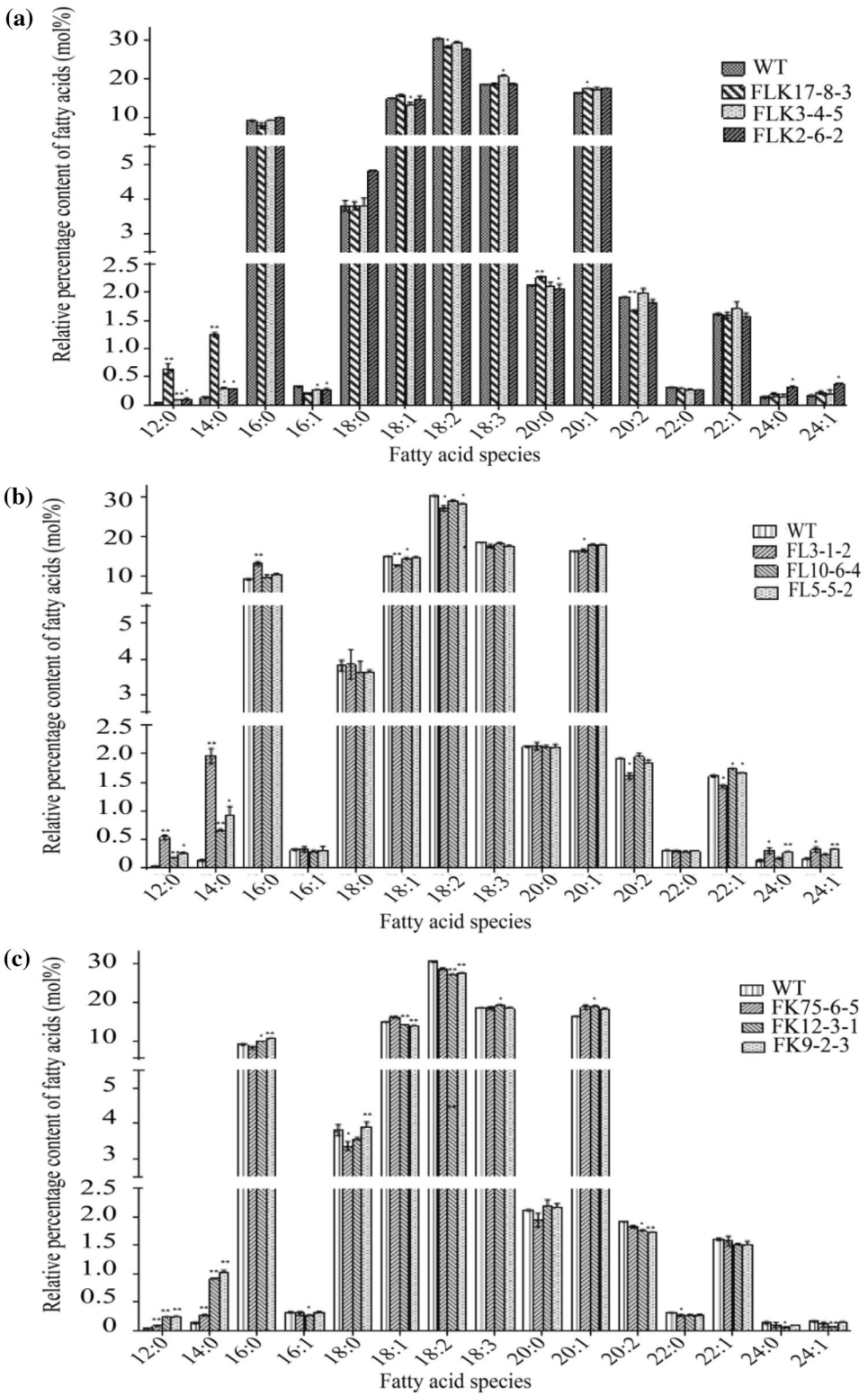

Fig. 4 Compositions analysis of individual fatty acid content in transgenic plants. FLK (**a**), FL (**b**) and FK (**c**) compared with that of wild-type plants. FKL: *FatB3–KASI–LPAAT-B*, FL: *FatB3–LPAAT-B*, FK: *FatB3–KASI*. All experiments were performed in triplicate. *Significant difference according to Student's *t* test at $p < 0.05$. **Extremely significantly different, $p < 0.01$

different transgenic *Arabidopsis* plants expressing different combinations of genes. This implied that Pro-at5400 was a more powerful promoter than the *napin* and *Pro-at16460* promoters.

To verify the combinatorial expression effects of *CnFatB3, CnLPAAT-B* and *CnKASI* on fatty acid biosynthesis, 15 kinds of fatty acids species were examined in all FLK, FL and FK transgenic plants. Instructively, the

Table 1 Content of lauric acid and myristic acid in transgenic *Arabidopsis* and wild type

Gene type	Line no	Content of lauric acid (12:0)	Content of myristic acid (14:0)
Wild type		0.035% ± 0.005	0.133% ± 0.014
FLK combination type	Line 17-8-1	0.639% ± 0.089**	0.247% ± 0.037**
	Line3-4-5	0.083% ± 0.004**	0.276% ± 0.033*
	Line2-6-2	0.099% ± 0.022*	0.282% ± 0.001
FL combination type	Line3-1-2	0.538% ± 0.038**	1.961% ± 0.130**
	Line 10-6-4	0.176% ± 0.013**	0.644% ± 0.036**
	Line 5-5-2	0.244% ± 0.037*	0.915% ± 0.163*
FK combination type	Line 75-6-5	0.080% ± 0.003**	0.270% ± 0.012**
	Line 12-3-1	0.244% ± 0.001**	0.913% ± 0.001**
	Line 9-2-3	0.248% ± 0.006**	1.017% ± 0.043**

Values are expressed as the mean ± s.d. (standard deviation)

* and ** denote a significant difference ($p < 0.05$) and a highly significant difference ($p < 0.01$), respectively

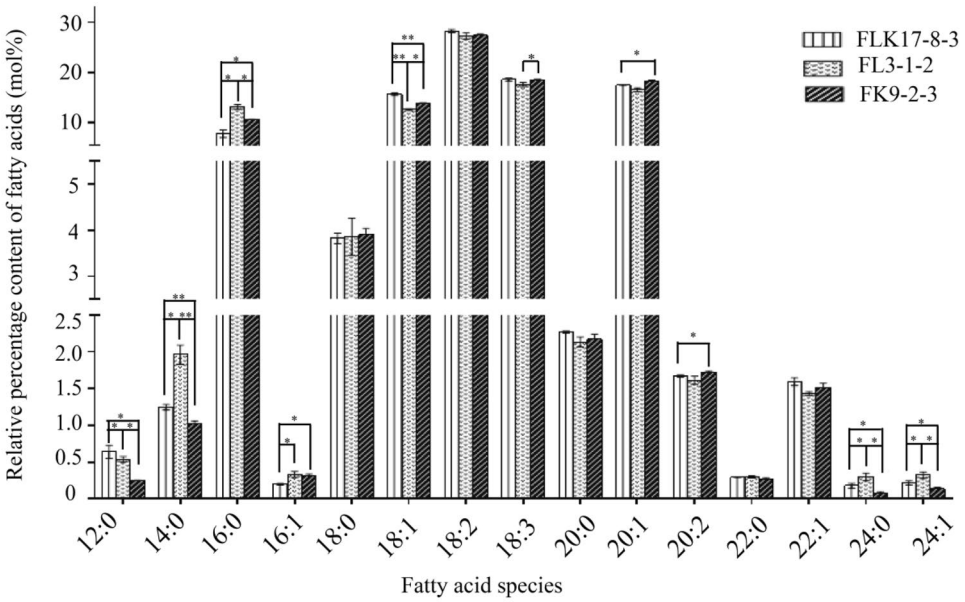

Fig. 5 Comparison of individual fatty acid among different transgenic combination. FKL: *FatB3–KASI–LPAAT-B*, FL: *FatB3–LPAAT-B*, FK: *FatB3–KASI.* All experiments were performed in triplicate. *Significant difference according to Student's *t* test at $p < 0.05$. **Extremely significantly different, $p < 0.01$

levels of 12:0 and 14:0 fatty acids, two important MCFAs, were significantly increased in transgenic lines expressing all three combinations of genes compared to their levels in wild-type plants. Although the overexpression of the single *CnFatB3, CnLPAAT-B* and *CnKAS*I genes improves the 12:0 and 14:0 fatty acid content, e.g., the amount of 12:0 fatty acid increased by at least 104.1%, 133% and 30% and that of 14:0 fatty acid increased by 29.6%, 13% and 80% in the *CnLPAAT-B, CnKASI* and *CnFatB3* transgenic plants, respectively, when compared to their expression in wild-type plants, their increased levels in plants overexpressing single genes were lower than those in the FLK, FL and FK combination plants (Yuan et al. 2015a, b, 2017). These results suggest the combinatorial expression effect of the *CnLPAAT-B, CnKASI* and *CnFatB3* enzymes on MCFA biosynthesis. Among the FLK, FL and FK transgenic plants, the FLK plants, which synchronously expressed the *CnFatB3, CnLPAAT-B* and *CnKASI* genes, exhibited the most effective increase in 12:0 fatty acid levels. In addition, the FL plants, which expressed the *CnFatB3* and *CnLPAAT-B* genes, exhibited the most vigorous enhancement of the 14:0 fatty acid yields. These results suggest that the *CnFatB3, CnLPAAT-B* and *CnKASI* enzymes have a strong combinatorial expression effect on 12:0 fatty acid biosynthesis and that the functions of *CnFatB3* and *CnLPAAT-B* are concordant with the increase in the 14:0 fatty acid content. Based on previous reports, the accumulation of C12:0 and C14:0 in FKL of this study should be due to the combination of these three determinants, which play crucial roles on MCFAs biosynthesis.

Except for the C12:0 and C14:0 fatty acids, the levels of other fatty acids, such as C20:2, C22:1, C24:0 and 24:1 fatty acids, were significantly different in the FLK, FL and FK plants compared with the wild-type plants. These findings are consistent with previous reports. For instance, CnFatB3 exhibited a preference for C16:0 and C18:0 fatty acids (Yuan et al. 2017). The expression of transgenic *CnKASI* and *CnLPAAT-B* increased the levels of C6:0, C8:0 and C16:0 fatty acids (Yuan et al. 2015a, b). Unexpectedly, there is a decrease in C18:2 with an increase in C18:3 appearing in several transgenic lines, since unsaturated fatty acids are more concentrated on sn-2 position in typical vegetable oil, the exact reason need to be clarified further. Moreover, total fatty acid content in the FLK, FL and FK plants were significantly increased compared with those in the wild-type plants. When analyzing the harmony of *CnFatB3, CnLPAAT-B* and *CnKASI* in lipid assembly, the lipid contents of the FLK, FL and FK plants were compared, and the highest lipid content was found in the FL transgenic plants. Therefore, *CnFatB3* and *CnKASI* are indispensable genes that drive lipid assembly. Hence, *CnFatB3, CnLPAAT-B* and *CnKASI* are crucial enzymes involved for not only MCFA biosynthesis but also lipid assembly.

In conclusion, in this work, the combinatorial expression effects of gene expression in plants stably transformed with *CnFatB3, CnLPAAT-B* and *CnKASI* in a constitutive expression system carrying tissue-specific and inducible promoters were determined. A significant combinatorial expression effect on MCFA levels, especially 12:0 and 14:0 fatty acid biosynthesis, and TAG accumulation were shown when *CnFatB3, CnLPAAT-B* and/or *CnKASI* were coexpressed in *Arabidopsis*. This work revealed that the incorporated *CnFatB3, CnLPAAT-B* and *CnKASI* genes worked in harmony in the 12:0 fatty acid biosynthesis pathways; while, the combination of *CnFatB3* and *CnLPAAT-B* expression was optimal for 14:0 fatty acid biosynthesis. Thus, *CnFatB3, CnLPAAT-B* and *CnKASI* could be underlying powerful candidate genes used in the MCFA production industry. When multiple genes were stably coexpressed, the accumulated MCFA levels significantly exceed their currently reported levels, and these results could provide new insight into genes with distinct roles in fatty acid and TAG metabolism and assist in technology to increase in the levels of particular fatty acids by the addition or knockdown of other genes.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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