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Functional Expression of the *Arachis hypogaea* L. Acyl-ACP Thioesterases *AhFatA* and *AhFatB* Enhances Fatty Acid Production in *Synechocystis* sp. PCC6803

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Abstract: Palmitoleic acid (C16:1) and stearic acid (C18:0) are precursors of polyunsaturated fatty acids, which are the focus of intensive global research due to their nutritional value, medicinal applications, and potential use as biofuel. Acyl-acyl carrier protein (ACP) thioesterases are intraplastidial enzymes that determine the types and amounts of fatty acids produced in plants and release fatty acids into the cytosol to be incorporated into glycerolipids. Based on amino acid sequence identity and substrate specificity, these enzymes are classified into two families, *FatA* and *FatB*. In this study, we cloned *FatA* and *FatB* thioesterases from *Arachis hypogaea* L. seeds and functionally expressed these genes, both individually and in tandem, in a blue-green alga *Synechocystis* sp. PCC6803. The heterologous expression of these genes in *Synechocystis* altered the fatty acid composition of lipids, resulting in a 29.5–31.6% increase in palmitoleic acid production and a 22.5–35.5% increase in stearic acid production. Moreover, the transgenic *Synechocystis* cells also showed significant increases in levels of oleic acid (C18:1, OA), linoleic acid (C18:2, LA), and α-linolenic acid (C18:3n3, ALA). These results suggest that the fatty acid profile of algae can be significantly improved by the heterologous expression of exogenous genes. This study not only provides insight into fatty acid biosynthesis, but also lays the foundation for manipulating the fatty acid content of cyanobacteria.

Keywords: Fatty acids; gene expression; acyl-acyl carrier protein thioesterases; *Synechocystis* sp. PCC6803

1. Introduction

In higher plants, de novo fatty acid (FA) biosynthesis mainly takes place in plastids. In this pathway, malonylacyl carrier protein (ACP) is condensed with acyl-ACP derivatives to form two-carbon units, which are successively added to the acyl-ACP chain [1–3]. Acyl chain elongation

is terminated by an acyl-ACP thioesterase (FAT), which hydrolyzes the thioester bond of acyl-ACP and releases free fatty acids, which are quickly transported to the cytosol via acyl-CoA synthetase in prokaryotic and eukaryotic cells [4–6]. Therefore, acyl-ACP FAT proteins are important regulators of lipid storage. Based on their amino acid sequence identity and substrate specificity, acyl-ACP FAT proteins are classified into two families: *FatA* and *FatB* [2,7]. Whereas *FatA* has a higher specificity for 18:1-ACP substrates and a lower specificity for 18:0-ACP and 16:0-ACP [1,8], *FatB* has a preference for saturated FAs containing 8–18 carbons [1,7,9].

FAs are carboxylic acids with hydrocarbon chains of 4 to 36 carbon atoms. Unsaturated fatty acids (UFAs) are hydrocarbon chains that have at least one double bond in their backbone structure, and are 18 to 22 carbons in length. Polyunsaturated fatty acids (PUFAs) are UFAs that possess two or more double bonds. Based on the position of the first double bond from the methyl end of the molecule, PUFAs are classified as omega-3 (ω-3) or omega-6 (ω-6) FAs. ω-3 PUFAs are essential dietary molecules with potential medicinal and nutritional applications, and have recently become the focus of intensive research due to their perceived benefits to human health [10]. Furthermore, PUFAs were found to have potential applications in biofuel production, and may thus represent an alternative energy source to meet the world's increasing energy demands.

Genetic engineering provides an effective approach for enhancing the production of FAs, and the FA biosynthetic pathways in higher plants and microbes have been well studied. Much research has focused on developing metabolic engineering methods to increase the FA content in oilseed crops [11,12] and microalgae [10,13–15]. Cyanobacteria are photosynthetic prokaryotes that produce valuable metabolites using solar energy and carbon. These microorganisms grow readily in a broad range of environments, exhibit rapid growth rates, and produce high yields of metabolites. The unicellular cyanobacterium *Synechocystis* sp. PCC6803 is a facultative phototroph that is considered a model organism for studying FA biosynthesis [10,16].

Recently, several Fat genes have been cloned and characterized [1,3,8,9]. It has been reported that overproduction of thioesterase stimulates fatty acid synthesis by decreasing feedback inhibition and reducing the cellular acyl-ACP concentrations [17,18]. In cyanobacteria, acyl-CoA synthetases are essential for the incorporation of exogenously supplied free fatty acids into cellular lipid metabolism [19]. However, the study of peanut Fat genes in microalgae has not previously been reported. The primary objective of this work was to create a strain of *Synechocystis* sp. PCC6803 that effectively produces useful FAs. Here, we cloned the genes encoding *FatA* and *FatB* thioesterases (*AhFatA*and *AhFatB*) from *Arachis hypogaea*L. seeds and functionally expressed these genes in the blue-green alga, *Synechocystis* sp. PCC6803. By heterologously expressing exogenous thioesterases in *Synechocystis* sp. PCC6803, the accumulation of the PUFAs palmitoleic acid and stearic acid in this cyanobacterium were markedly increased.

2. Results

2.1. Integration of AhFatA and AhFatB into Synechocystis 6803 and Their Expression Analysis

We constructed three homologous recombination plasmids, harboring *AhFatA* (SD*FatA*), *AhFatB* (SD*FatB*), and both *AhFatA* and *AhFatB* (SD*FatAB*; Figure 1). We selected *psbA2* as the integration site, because this gene belongs to the *psbA* multi-gene family, and inactivation of psbA2 has no effect on *Syenchocystis* [20,21]. Therefore, to construct the homologous recombination vectors, we used the 500-bp fragment upstream of the *psbA2* ORF and the *psbA2* ORF as the upstream and downstream arms, respectively, and cloned *AhFatA*, *AhFatB*, or both *AhFatA* and *AhFatB* between these arms. The fragment upstream of the *psbA2* ORF was used as the promoter to drive the expression of *AhFatA*, *AhFatB*, or both *AhFatA* and *AhFatB* in *Synechocystis* sp. PCC6803. *psbA2* was replaced with the target gene(s) (i.e., *AhFatA*, *AhFatB*, or both *AhFatA* and *AhFatB*) and an antibiotics selection gene by double homologous recombination. To isolate transgenic *Synechocystis* cells, the transformants were subcultured in BG-11 solid medium in the presence of kanamycin (50 µg/mL). To verify that *AhFatA*, *AhFatB*, or both *AhFatA*

and *AhFatB* were stably integrated into the genome, the complete segregation of the transformants was confirmed by PCR. Genomic DNA from the wild-type strain was used as a control. In the transgenic *Synechocystis* cells, the DNA fragment containing *AhFatA*, *AhFatB*, or *AhFatAB* with the *psbA2*promoter were amplified using the promoter-*Sal*I-F and *psbA2*-*Sac*I-R primers (Figure 2A). The PCR products were recycled and sequenced. Sequence analysis showed the amplified fragments were the same as the recombinant fragments. No DNA fragment corresponding to the wild-type *psbA2* gene and its promoter (1.5 kb) was detected, confirming that we had indeed isolated *Synechocystis* transformants.

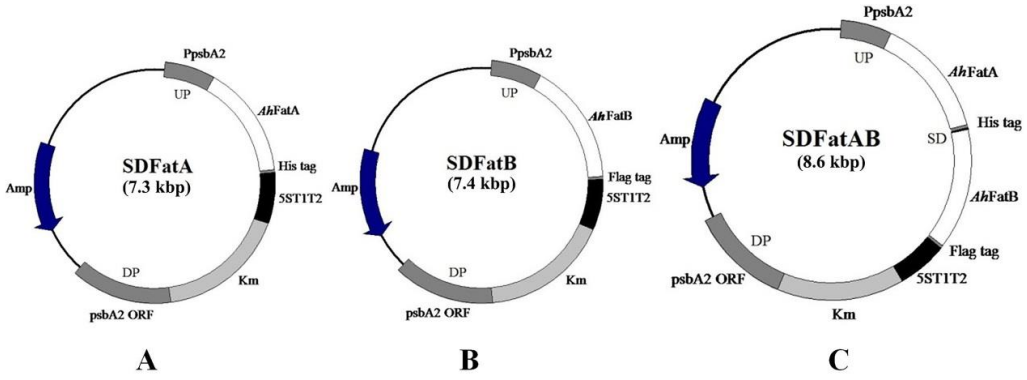

Figure 1. Structure of homologous recombination vectors harboring thioesterase genes from *Arachis hypogaea* L. Fat genes (*AhFatA*, *AhFatB*, or both *AhFatA* and *AhFatB*) were cloned into the pBluescript II SK (+) plasmid. The *psbA2* promoter (P*psbA2*), consisting of the 0.5-kb fragment upstream (UP) of psbA2, the kanamycin resistance cassette (Km), the 1.0-kb fragment downstream of *psbA2* (*psbA2* ORF, DP), and the *E. coli* 5ST1T2 terminator were also cloned into the plasmids. Amp: ampicillin resistance cassette. (**A**) SD*FatA*, for the heterologous expression of *AhFatA* tailed with a His tag; (**B**) SD*FatB*, for the heterologous expression of *AhFatB* tailed with a Flag-tag; (**C**) SD*FatAB*, tandem expression of *AhFatA* tailed with a His tag and *AhFatB* tailed with a Flag-tag.

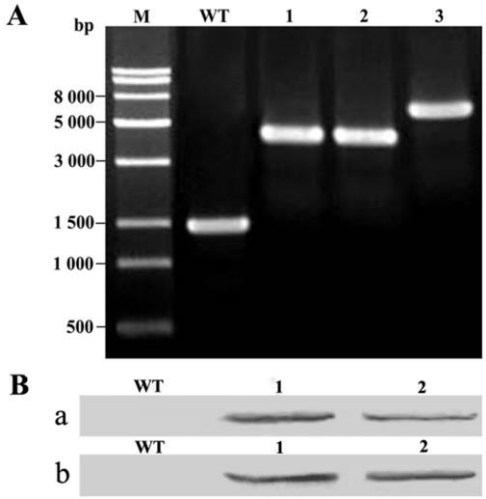

Figure 2. PCR and immunoblot analysis of wild-type *Synechocystis* and thioesterase transformants. (**A**): PCR analysis of transgenic *Synechocystis* in which a fragment of *psbA2* was deleted and replaced with various exogenous genes. M: Trans15k DNA Marker; WT: wild type *Syenchocystis* sp. PCC6803; Lane 1: pSD*FatA*; Lane 2: pSD*FatB*; Lane 3: pSD*FatAB*; (**B**): Immunoblot analysis of *Synechocystis* wild type and thioesterase transformants grown at 30 °C under mixotrophic conditions. a: Immunoblot analysis using His-tag antibodies. WT: wild type *Synechocystis* sp. PCC6803; Lane 1: pSD*FatA Synechocystis* transformant; Lane2: pSD*FatAB Synechocystis* transformant; b: Immunoblot analysis using Flag-tag antibodies. WT: wild type *Synechocystis* sp. PCC6803; Lane 1: pSD*FatB Synechocystis* transformant; Lane 2: pSD*FatAB Synechocystis* transformant.

To detect the expression of *AhFatA* or *AhFatB* in the transformants, we incubated the wild-type and SD*FatA*/SD*FatB*/SD*FatAB* transformant cells at 30 °C. Immunoblot analysis confirmed the expression of *AhFatA* and *AhFatB*, cloned in-frame with the *psbA2* promoter and the T1T2 terminator, in the *Synechocystis* transformants. Furthermore, immunoblot analysis using antibodies to the His tag and Flag-tag confirmed that *AhFatA* and *AhFatB*, respectively, were localized to the cell extracts of transgenic pSD*FatA*, pSD*FatB*, and pSD*FatAB Synechocystis* cell extracts (Figure 2B). Reverse transcription PCR (RT-PCR) analysis showed that both *AhFatA* and *AhFatB* were indeed expressed at 20 °C and 30 °C in these transformants (Figure 3). Furthermore, we found that *AhFatA* expression was greater at 20 °C than at 30 °C in transgenic *Synechocystis* cells expressing *AhFatA*. However, there was no significant difference in *AhFatB* expression when cells were cultured under 20 °C or 30 °C.

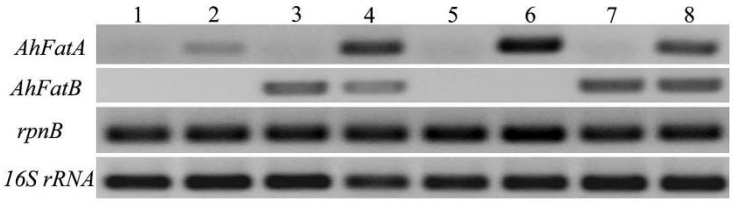

Figure 3. Semi-quantitative RT-PCR analysis of Acyl-ACP thioesterases from *Arachis hypogaea* L. in *Synechocystis* transformants. Lane 1: wild type *Syenchocystis* sp. PCC6803; Lane 2: pSD*FatA Synechocystis* transformant; Lane 3: pSD*FatB Synechocystis* transformant; Lane 4: pSD*FatAB Synechocystis* transformant; Lane 5: WT *Syenchocystis* sp. PCC6803; Lane 6: pSD*FatA Synechocystis* transformant; Lane 7: pSD*FatB Synechocystis* transformant; Lane 8: pSD*FatAB Synechocystis* transformant. All samples were cultured under mixotrophic culture conditions. Those in Lanes 1 to 4 were cultured at 20 °C, whereas those in Lanes 5 to 8 were cultured at 30 °C.

Growth rates of the *AhFatA*/*AhFatB* transformants and wild-type strains were measured in BG-11 medium at 20 °C and 30 °C. The growth rates of the transformant strains were similar to those of wild-type cells (Figure 4). These results indicate that the exogenous expression of *AhFatA* and/or *AhFatB* and the interruption of the neutral site (*psbA2* promoter and *psbA2* gene) have no negative effect on the cell physiology and growth of *Synechocystis*.

2.2. GC Analysis of Fatty Acids in the Synechocystis 6803 Strain Expressing AhFatA and AhFatB

Next, we cultivated wild-type and transgenic *Synechocystis* sp. PCC6803 cells under mixotrophic conditions at 20 °C and 30 °C and then analyzed the FA content in membranes by GC (Figure 5, see Supplementary Materials, Table S1). The FA profiles of the *Synechocystis* 6803 strains expressing *AhFatA*, *AhFatB*, or *AhFatAB* exhibited striking changes.

In wild-type cells grown under mixotrophic conditions at 20 °C, the total FA content was about 60.66 mg/g, but the heterologous expression of *AhFatA* or *AhFatB* increased the total FA content to 74.79 mg/g and 72.73 mg/g, respectively (i.e., resulted in increases of 23.3% and 19.9%). The FA content of cells heterologously expressing both *AhFatA* and *AhFatB* was 81.94 mg/g, which was 35.1% more than that of the wild type. These results suggest that the heterologous expression of *AhFatA* has a greater effect than that of *AhFatB* or both *AhFatA* and *AhFatB* on the FA content under mixotrophic conditions at 20 °C.

Gas chromatography (GC) analysis revealed that the cultivated temperature affected the FA content. Temperature has a large effect on the composition of FAs produced by microalgae which, inturn, affects the physiology of the organism. Several studies indicate that the exponential growth rates of many microalgal species increase in response to elevated growth temperatures, up to an optimal temperature. In response to cold stress, several microalgal species enhance the biosynthesis and accumulation of total FAs [10,22,23]. The higher temperature (30 °C) enhanced the expression of *AhFatA*, and increased the FA content of transgenic *Synechocystis* expressing *AhFatA* or *AhFatAB*,

which is in accordance with the results of reverse transcription PCR (Figure. 3). The FA content of transgenic *Synechocystis* expressing *AhFatA* was 3.1% greater under mixotrophic conditions at 30 °C than at 20 °C, whereas the FA content of transgenic *Synechocystis* expressing *AhFatB* and *AhFatAB* were decreased 10.3% and 4.7% under mixotrophic conditions at 30 °C than at 20 °C.These results suggest that transgenic *Synechocystis* cells expressing *AhFatA* should be cultured under mixotrophic conditions at 30 °C to increase the yield of FAs. While for transgenic *Synechocystis* cells expressing *AhFatB* and *AhFatAB* should be cultured under mixotrophic conditions at 20 °C to increase the yield of FAs.

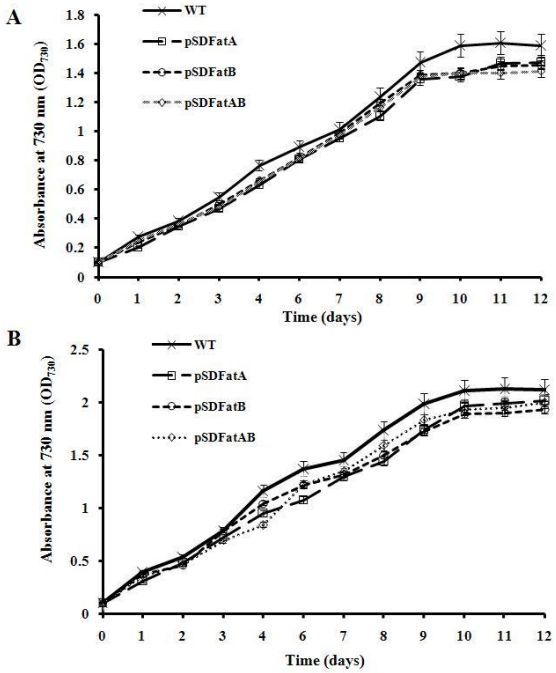

Figure 4. Growth curves of wild-type and transgenic *Synechocystis*. Cells were grown under mixotrophic conditions at (**A**) 20 °C or (**B**) 30 °C. Cultures were grown in BG-11 medium and bubbled with air under an illumination of 40 µmol photon m^{-2} s^{-1}. The optical density of cells at 730 nm was measured at the indicated time points. Values are means ± SD (bars) of three independent experiments conducted on different days. Absence of a bar indicates that the SD falls within the symbol.

In wild-type cells grown under mixotrophic conditions at 30 °C, the most abundant FAs are C14:1 (0.8%), C16:0 (51.5%), C16:1 (3.4%), C18:0 (0.8%), and C18:1 (7.1%). By contrast, at 20 °C, C14:1 (0.8%), C16:0 (47.1%), C16:1 (5.0%), C18:0 (1.4), and C18:1 (3.9) were most abundant.

The heterologous expression of *AhFatA* in *Synechocystis* 6803 increased the content of palmitoleic acid (C16:1) and stearic acid (C18:0) significantly. Under mixotrophic cultivation, the concentration of C16:1 and C18:0 in cells heterologously expressing *AhFatA* was about 3.36 mg/g and 0.76 mg/g at 30 °C, respectively, which was 31.2% and 22.5% greater than the corresponding concentrations in wild-type cells, and 3.39 mg/g and 1.08 mg/g at 20 °C, which was 12.7% and 22.7% greater than the corresponding concentrations in wild-type cells. The content of C14:0 and C18:1 in cells heterologously expressing *AhFatA* was about 0.14mg/g and 3.05 mg/g at 20 °C under mixotrophic conditions, which was 27.2% and 27.6% greater than those of wild-type cells grown under the same conditions.

The heterologous expression of *AhFatB* in *Synechocystis* 6803 markedly increased the content of C16:1, C18:0, and C18:3n3 at 30 °C under mixotrophic condition, resulting in concentrations of about 3.13 mg/g, 0.84 mg/g, and 1.51 mg/g, respectively, which were 29.5%, 35.5%, and 38.5% greater than those of the wild type. Similarly, the heterologous expression of *AhFatB* in *Synechocystis* 6803 increased the content of C14:0, C16:1, and C18:3n6, at 20 °C under mixotrophic cultivation, by 31.4, 24.0, and 23.6%, respectively.

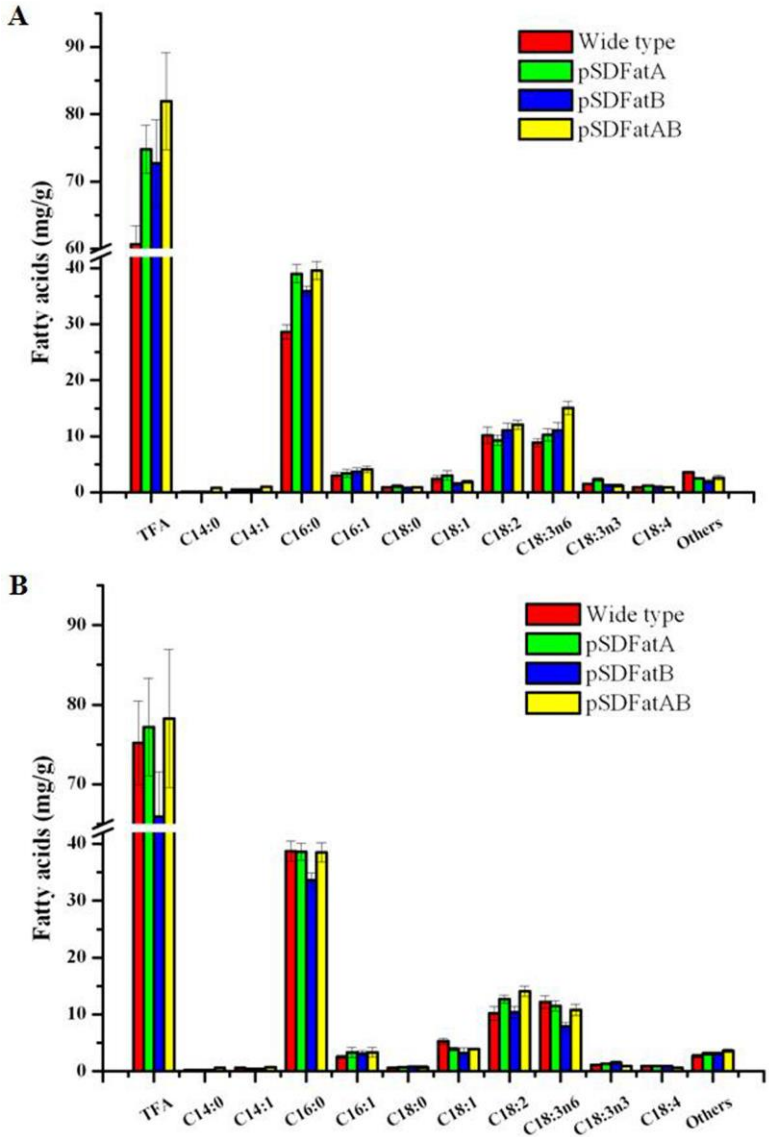

Figure 5. Fatty acid content of wild-type and transgenic *Synechocystis* sp. PCC6803 [a] cultured under mixotrophic growth conditions at (**A**) 20 °C and (**B**) 30 °C. TFA: Total fatty acids; FA: fatty acids. [a] Values are means and S.D. of triplicate experiments. Cells were grown under a light intensity of 40 μmol photon m^{-2} s^{-1} for 10 d in BG-11 medium. The membrane lipids were extracted from the wild-type and genetically engineered *Synechocystis* sp. PCC6803. The enzymes overexpressed are indicated in parentheses (*FatA*: Acyl-ACP Thioesterase A from 'Luhua 14' cultivar of *Arachis hypogaea* L.; *FatB*: Acyl-ACP Thioesterase B from 'Luhua 14' cultivar of *Arachis hypogaea* L.).

Tandemly expressed *AhFatA* and *AhFatB* increased the content of C16:1, C18:0, and C18:2, to about 3.37 mg/g, 0.78 mg/g, and 13.12 mg/g, respectively, at 30 °C under mixotrophic conditions, which was 31.6%, 25.8%, and 28.4% greater than the content in wild-type cells, while the content of C16:1, C18:0, and C18:2 was about 4.07 mg/g, 0.92 mg/g, and 12.08 mg/g, at 20 °C under mixotrophic cultivation, which was 35.7, 4.5, and 18.9% greater than levels in wild-type cells grown under the same conditions, respectively.

The heterologous expression of *AhFatA* in *Synechocystis* 6803 decreased the content of C14:1 and C18:1. At 30 °C under mixotrophic cultivation, the concentration of C14:1 and C18:1 in cells heterologously expressing *AhFatA* was about 0.44 mg/g and 3.94 mg/g, respectively, which was 27.9% and 25.8% lower than that in the wild type. Furthermore, the content of C14:1 and C18:2 was

about 0.48 mg/g and 9.24 mg/g, respectively, at 20 °C under mixotrophic conditions, which was 4.3% and 9.1% lower than that of wild-type cells grown under the same conditions. While heterologously expressing of *AhFatB* in *Synechocystis* 6803 decreased the content of C14:1, C18:1, and C18:3n6, by 29.5%, 38.6%, and 35.5% at 30 °C under mixotrophic cultivation, and decreased the content of C18:0, C18:1, and C18:3n3, by 1.1%, 37.7%, and 12.4% at 20 °C under mixotrophic cultivation. Tandem expression of *AhFatA* and *AhFatB* decreased the content of C18:1 and C18:3n6 to 3.96 mg/g and 10.82 mg/g, which was 25.4% and 11.5% lower than the corresponding levels in wild-type cells grown at 30 °C under mixotrophic cultivation, while the content of C18:1 and C18:3n3 decreased by 20.9% and 19.0%, respectively, at 20 °C under mixotrophic cultivation, respectively.

Therefore, *Synechocystis* lines heterologously expressing *AhFatA* and *AhFatB* exhibit similar changes in FA profiles. For example, they both had increased levels of C14:0, C16:1, C18:0, and C18:2, and decreased levels of C14:1, C16:0, C18:1, and C18:3n6 at 30 °C under mixotrophic cultivation, and increased levels of C14:0, C16:0, C16:1, and C18:3n6 at 20 °C under mixotrophic cultivation. However, there were also some differences in FA content between cells expressing *AhFatA* and those expressing *AhFatB*. For example, *AhFatA* expression increased the content of C18:3n3 and C18:4, whereas *AhFatB* expression increased the content of C18:3n3, but tandem expression of *AhFatA* and *AhFatB* decreased the content of C18:3n3 and C18:4 at 30 °C under mixotrophic cultivation. While the expression of *AhFatA* increased the content of C18:3n3 and C18:4, that of *AhFatB* decreased the content of C18:4 at 20 °C under mixotrophic conditions.

3. Discussion

Thioesterase play an important role intransferring acyl chains to the extraplastidial glycerolipid and determine the metabolic flux into triacylglycerols [3]. Over the past few decades, several thioesterases have been cloned and functionally characterized, including representatives from *Jatropha curcas*, *Arachis hypogaea* L., *Synechocystis*, *Populus tomentosa*, *Ricinus communis* L., and so on [2,3,5,21]. However, more thioesterases need to be characterized to exploit their potential uses, especially to adjust the fatty acid composition from microorganisms and various plants.

In cyanobacteria, fatty acids are synthesized by the type II fatty acid synthase complex, which interacts with acyl carrier protein [24]. The products of fatty acid synthase are released as acyl-ACPs and incorporated into membrane lipids [25]. Acyl-ACP thioesterase hydrolyzes the thioester bond of acyl-ACP, releaseing free fatty acids, which are quickly transported into the cytosol via acyl-CoA synthetase in prokaryotic and eukaryotic cells [4–6]. However, such a mechanism would be detrimental to cyanobacteria, causing a loss of fatty acids; as expected, no homologs of acyl-ACP thioesterases have been identified in cyanobacteria [19].

In this study, we sought to develop a method to increase the FA content in *Synechocystis* sp. PCC6803. We cloned the genes encoding *FatA* and *FatB* thioesterases from *Arachis hypogaea* L. seeds and then functionally expressed these genes in a blue-green alga *Synechocystis* sp. PCC6803, both individually and in tandem. We then analyzed the expression of exogenous genes (*AhFatA* and *AhFatB* thioesterases) in transgenic *Syenchocystis* under mixotrophic conditions at 30 °C and 20 °C Immunoblot analysis confirmed the presence of *AhFatA* and *AhFatB* proteins in the soluble fraction of *Synechocystis* transformant cell extracts. We also used RT-PCR analysis to detect the expression of *AhFatA* and *AhFatB*. Both *AhFatA* and *AhFatB* were indeed expressed at 20 °C and 30 °C, demonstrating that the presence of *AhFatA* and *AhFatB* mRNA in the transformants.

The heterologous expression of these genes altered the FA profiles of the transgenic *Synechcystis* cells, increasing the content of palmitoleic acid (C16:1, PA) and stearic acid (C18:0, SA). Moreover, the altered FA profiles in transgenic *Syenchocystis* also showed striking increases in the levels of oleic acid (C18:1, OA) and linoleic acid (C18:2, LA), along with increases in the content of α-linolenic acid (C18:3n3, ALA). These results suggest that the heterologous expression of *Fat* genes can alter FA production. Our finding that transgenic *Syenchocystis* cells heterologously expressing *AhFatA* had significantly higher levels of C16:0, C18:0, and C18:1 at 20 °C under mixotrophic cultivation conditions

is in accordance with the finding that *FatA* encodes a thioesterase with a higher specificity for 18:1-ACP substrates and lower specificities for 18:0-ACP and 16:0-ACP [1,8]. Furthermore, under mixotrophic cultivation, transgenic *Syenchocystis* cells heterologously expressing *AhFatB* had significantly higher levels of C16:0 at 20 °C and of C18:0 at 30 °C, which is in accordance with the conclusion that *FatB* encodes a thioesterase with a preference for saturated FAs containing 8-18 carbons [1,7,9]. It is interesting that the tandem expression of *AhFatA* and *AhFatB* in *Syenchocystis* decreased the content of C18:1 and increased the content of C18:2 at 30 °C and increased the content of C18:2 and C18:3n6 at 20 °C under mixotrophic cultivation. It is possible that the tandem expression of *AhFatA* and *AhFatB* increased the activity of delta-9 and delta-6 fatty acid desaturases during FA biosynthesis.

The increase in the world's population coupled with dwindling supplies of fossil fuel and environmental degradation have prompted intensive research aimed at developing renewable and potentially carbon neutral liquid, solid, and gaseous biofuels as alternative energy resources [26]. Among many potential sources of biodiesel fuel, the FA methyl esters derived from triglycerides have emerged as a promising alternative to diesel fuels. Although current biodiesels, generally produced by transesterification of vegetable oils and animal fats, are considered to be a renewable transportation fuel, they have the disadvantage of having low volatilities, high viscosities, and poor cold flow properties [27]. Microalgae, which have simple growth requirements and grow rapidly, are photosynthetic microorganisms that are able to produce lipids, proteins, and carbohydrates in large amounts. These products can be processed into both biofuels and other valuable co-products [27–29]. As the third generation of biofuel feedstocks, microalgae are considered to be a technically viable alternative energy resource.

4. Materials and Methods

The cyanobacterium *Synechocystis* sp. PCC6803 was cultivated in BG-11 medium (5 mM glucose) at 30 °C [30]. The culture was bubbled with air under a light intensity of 40 μmol photon m^{-2} s^{-1}. For growth on solid medium, cells were cultured on plates containing 1.5% (w/v) DifcoBacto Agar (Becton Dickinson, Sparks, MD, USA), 0.3% (w/v) sodium thiosulfate, and 10 mM TES (pH8.2). For resistance selection of the transformed strains, 50μg/mL kanamycin (Dingguo Company, Beijing, China) was added to the liquid and solid BG-11 medium. Cell density was determined by measuring the optical density (OD) of the suspension at 730 nm (OD_{730}) with a spectrophotometer (DU-70, Beckman Coulter, Brea, CA, USA).

For heterologous expression of *AhFatA* and *AhFatB* (individually or in tandem) in *Synechocystis* sp. PCC6803, the 1.1-kb *AhFatA* gene (GenBank: GU324446) and 1.2-kb *AhFatB* gene (GenBank: GU324447) were amplified from *Arachis hypogaea* L. cultivar 'Luhua 14' using primers *Ah*FatA-F and*Ah*FatA-*Sal*I-His-R and *AhFatB*-F and *AhFatB*-*Sal*I-Flag-R primers, respectively (Table 1). For fusion PCR of *AhFatA* and *AhFatB*, the *Ah*FatA-F and *Ah*FatA-R′ promoters and *AhFatB*-F and *AhFatB*-*Sal*I-Flag-R primers were used, and then the *AhFatA* and *AhFatB* fragments (*AhFatAB*) were combined by fusion PCR. The termination codon of *AhFatA* was deleted and a 17-bp Shine-Dalgarno (SD) sequence, 5′-TTGGTTATAATTCCTTA-3′, was added to the 3′ end of *AhFatA*. For heterologous expression of *AhFatA* and *AhFatB* (individually or in tandem) in *Synechocystis* sp. PCC6803, plasmid constructs were generated in which a His-tag was added to the 3′ end of *AhFatA* and a Flag-tag to the 3′ end of *AhFatB*. The resulting plasmid constructs (i.e., SD*FatA*, SD*FatB*, and SD*FatAB*) were used to replace the *psbA2* gene of *Synechocystis* with *AhFatA*, *AhFatB*, or both of these genes via double homologous recombination. The *psbA2* promoter (0.5-kb fragment) in *Synechocystis* genomic DNA upstream of the *psbA2*, ORF was amplified by PCR as the upstream region and the promoter. To fuse the *psbA2* promoter to *AhFatA* (*AhFatB*/*AhFatAB*), the *psbA2* promoter was amplified using promoter-*Sal*I-F and promoter-R, and the 1.0-kb fragment of *Synechocystis* genomic DNA that encodes the *psbA2* ORF was amplified by PCR as the downstream region of the homologous recombination vector, using the primers *psbA2*-*Sac*II-F and *psbA2*-*Sac*I-R. The downstream fragment was cloned into the *Sac*II and *Sac*I sites of pBluescript SK+T1T2, to form plasmid p5ST1T2*psbA2*. Then the kanamycin resistance cassette

carrying *npt* was cloned into the single *Bam*HI site of p5ST1T2*psbA2*, to form p5ST1T2*psbA2*npt. Then the fragments of *AhFatA*, *AhFatB*, and *AhFatAB* that fused with promoter were cloned into the *Sal*I site of p5ST1T2*psbA2*npt, to form the pSD*FatA*, pSD*FatB*, and pSD*FatAB* plasmids.

Synechocystis sp. PCC 6803 was grown in liquid BG-11 medium at 30 °C under a light intensity of 40 μmol of photon m^{-2} s^{-1} until the OD_{730} reached 0.6, and the cells were harvested by centrifugation and resuspended in fresh BG-11 medium to an OD_{730} value of 4.8. Then, 2 mg plasmid DNA was added to 500 μL of cell suspension and gently mixed, and the mixture was incubated at 30 °C under low light for 6 h and then spread on BG-11 agar plates containing kanamycin (20μg/mL). Transformants were isolated after about 10 days of incubation, and subcultured on BG-11 agar plates containing 50μg/mL kanamycin. The transformants were then grown in liquid culture for analysis.

Wild-type and transformant cell lines were cultured and harvested in the exponential growth phase, and total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA, USA), following the manufacturer's instructions. First strand cDNA was synthesized using M-MLV reverse transcriptase and modified oligo (dT) following the manufacturer's instructions (TaKaRa, Dalian, China). The resulting cDNA molecules were amplified by PCR using the following gene-specific primers: rnpB-F and rnpB-R for amplification of the rnpB gene and 16S rRNA-F and 16S rRNA-R for amplification of the 16S rRNA gene, which were used as loading controls; *AhFatA*-RT-F and *AhFatA*-RT-R for amplification of *AhFatA*; *AhFatB*-RT-F and *AhFatB*-RT-R for amplification of *AhFatB*. The PCR products were analyzed by 1.0% agarose gel electrophoresis. The PCR products were then cloned in to pGEX-T Easy Cloning Vector (Promega, Madison, WI, USA) and sequenced by the Biotechnology Research Center, Shandong Academy of Agricultural Science (Ji'nan, China).

The suspension cultures grown at 30 °C were diluted to an OD_{730} nm of 0.1, and were further incubated at 20 °C and 30 °C. The growth of wild-type and transformant were measured by monitoring absorbance of the culture medium at 730 nm after a regular interval of 1 day using SP-723 UV–VIS Spectrophotometer (Shanghai Spectrum Instruments Co., Ltd., Shanghai, China).

Table 1. Primer sequences used in this study [a].

Primer	Sequence (5′–3′)
promoter-*Sal*I-F	GAT*GTCGAC*GCTTTAGCGTTCCAGTG
promoter-R	CATTTGGTTATAATTCCTTATGTAT
AhFatA-F	GGAATTATAACCAAATGTTGAAGGTTTCATGCAACG
*AhFatA-Sal*I-His-R	CCG*GTCGAC*TCAATGATGATGATGATGATGTAATCTTGAAGCTTTCTTTC
AhFatB-F	GGAATTATAACCAAATGGCAACTGCTGCTACTGCT
*AhFatB-Sal*I-Flag-R	TTC*GTCGAC*TTACTTATCGTCGTCATCCTTGTAATCGATGCTTTCGGCTGGAACC
AhFatA-R′	TTGGTTATAATTCCTTATCAATGATGATGATGATGAT
AhFatB-F′	TAAGGAATTATAACCAAATGGCAACTGCTGCTACTGCT
*psbA2-Sac*II-F	CTT*CCGCGG*ATGACAACGACTCTCCAAC
*psbA2-Sac*I-R	AGT*GAGCTC*TTAACCGTTGACAGCAGG
rnpB-F	GTTAGGGAGGGAGTTGCGG
rnpB-R	AAGAGAGTTAGTCGTAAGCCG
16S rRNA-F	AGCGTCCGTAGGTGGTTATG
16S rRNA-R	CTACGCATTTCACCGCTACA
AhFatA-RT-F	CAATAAGACTGCCACCGT
AhFatA-RT-R	TCAAGAACCCAACCAAT
AhFatB-RT-F	TTCTTGGTGATGGCTTTG
AhFatB-RT-R	GACCCGTGCGAATGTAAT

[a] Italicized, underlined text indicates restriction enzyme sites.

Crude extracts of wild-type and transformant cells were collected and dissolved in lysis buffer (1 mL of 40 mM Tris–HCl pH 8.0). After sonication, incubation, and centrifugation, the insoluble material was removed and the supernatants were subjected to immunoblot analysis. The soluble proteins were separated on 12% SDS-PAGE gels and then blotted onto 0.45 μm PVDF membranes (Beijing CoWin Biotech Co., Ltd., Beijing, China), stained with antibody to His tag or FLAG tag

(1: 5000, Beijing CoWin Biotech Co., Ltd., Beijing, China) for 1 h, and then incubated with goat anti-rabbit IgG HRP at 1: 5000 for 1 h. The cross-reactions between protein bands and antibodies were detected using an HRP-DAB Color Development Kit (Tiangen Biotech, Beijing, China), according to the manufacturer's instructions.

Membrane lipid extractions of wild-type cyanobacteria *Synechocystis* sp. PCC6803 and genetically engineered cyanobacteria *Synechocystis* sp. PCC6803 were carried out as described by Bligh and Dyer [31]. The colonies were collected and transferred to flasks containing 400 mL sterile BG-11 medium, respectively, and grown for 10 days at a light intensity of 40 μmol photons m^{-2} s^{-1} and a constant temperature of 30 °C. Cultures were harvested when OD_{730} reached 3.0, and were then washed with distilled water by centrifugation (6000× *g* for 10 min at room temperature). The wet cell pellets were heated at 40 °C to obtain 600 mg dry cell paste. The dry cell paste was diluted with 4 mL chloroform/methanol (1:10, v/v), and a suspension of 1 mL hexane containing C19:0 internal standard (1 mg/mL) was added. The mixture was heated at 80 °C for 2 h in a water bath, and then after cooling, 5 mL of 7% potash was added and mixed. After 10 min, the mixture was centrifuged at 10,000 × *g* for 10 min. The supernatants (bacterial sample FAME eluate) were subjected to gas chromatography (GC) using the Elite-WAX column in an ASXL instrument (Perkin-Elmer, Waltham, MA, USA). The flame-ionization detection (FID) temperature was 250 °C, and the operating temperature was maintained at 220 °C. The data presented in this paper are the average of three experiments for each sample.

5. Conclusions

In this study, we cloned two thioesterase genes from *Arachis hypogaea* L. seeds and then functionally expressed these genes in a blue-green alga *Synechocystis* sp. PCC6803 individually and in tandem. The heterologous expression of *AhFatA* and *AhFatB* altered the FA profiles of the transgenic cells. This study lays the foundation for increasing the content of desirable FAs.

Supplementary Materials: The following are available online at www.mdpi.com/1996-1073/10/12/2093/s1, Table S1: Fatty acid content of wild-type and transgenic *Synechocystis* sp. PCC6803 [a] cultured under mixotrophic growth conditions at 30 °C and 20 °C.

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Conflicts of Interest: The authors declare no conflict of interest.

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