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Expression of the heterologous *Dunaliella tertiolecta* fatty acyl-ACP thioesterase leads to increased lipid production in *Chlamydomonas reinhardtii*

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ABSTRACT

Biofuel production from genetically-engineered microalgae is currently among the most widely studied strategies in generating renewable energy. However, microalgae currently suffer from low oil yields which limit the commercial feasibility of industrial-scale production. A major bottleneck in cost-efficient biofuel production from microalgae is the dilemma between biomass productivity and lipid accumulation. When grown under stressful culture conditions such as nitrogen depletion, microalgae accumulate large amounts of neutral lipids, but it comes at the expense of growth which negatively impacts overall lipid productivity. Overexpression of acyl-ACP thioesterases (TE) had been successful in increasing the production of fatty acids (FA) in prokaryotes such as *E. coli* and cyanobacteria, but has not been effectively tested in microalgae. In this study, we introduced a TE from *D. tertiolecta* (DtTE) into *C. reinhardtii* to investigate its effects on FA production without compromising growth. The results indicate that *C. reinhardtii* transformants were able to produce 63 and 94% more neutral lipids than the wild-type, which translates to an approximately 56% improvement in total lipids, without compromising growth. These findings demonstrate the cross-species functionality of TE, and provide a platform for further studies into using TE as a strategy to increase biofuel production from microalgae.

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1. INTRODUCTION

Diminishing fossil fuel reserves, rising oil prices, and concerns over climate change due to increasing atmospheric CO_2 levels have renewed support for alternative and renewable energy sources (Stephens et al., 2010). In particular, biofuels derived from photosynthetic microorganisms such as microalgae have received considerable interest because compared to plants, they require smaller land area that does not need to be arable and can be cultivated in saltwater systems which will not directly compete with resources necessary for agricultural food production (Wigmosta et al., 2011). A major bottleneck in cost-efficient biofuel production from microalgae is the dilemma between biomass productivity and lipid accumulation. Microalgae can accumulate large amounts of energy-rich lipids, predominantly triacylglycerols (TAGs), under stressful culture conditions such as nitrogen depletion (Tan and Lee, 2016). However, the accumulation of storage lipids under such circumstances comes at the expense of cellular growth and biomass productivity. For biotechnological applications where large-scale production of lipids is desired, utilizing an approach involving nitrogen depletion is unfavorable as the culture will suffer from low biomass production which negatively impacts overall lipid productivity. Therefore, it is essential to obtain an ideal strain which possess high lipid production with no compromise to growth (Tan and Lee, 2016). A strategy to improve the productivity of lipid synthesis in microalgae is through modification of their fatty acid (FA) synthesis pathways. Rate-limiting enzymes of FA synthesis were potential targets for gene manipulation with the aim of improving overall lipid synthesis (Radakovits et al., 2010). Overexpression of the enzyme acetyl-CoA carboxylase (ACCase), believed to catalyze the important rate-limiting step in FA synthesis, has been performed in diatoms (Dunahay et al., 1995) and plants (Kindle, 1990; Roesler et al., 1997) but no increase in lipid content were observed. Increasing the expression of another enzyme in FA synthesis, 3-ketoacyl-acyl carrier protein synthase (KAS) III, was also not successful in improving lipid content in three species of plants (Dehesh et al., 2001). *De novo* FA synthesis and elongation occurs within an algal plastid by the action of ACCase and KAS, and ends with the production of 16–18 C fatty acyl groups esterified to acyl carrier protein (ACP), a central protein that carries the growing FA

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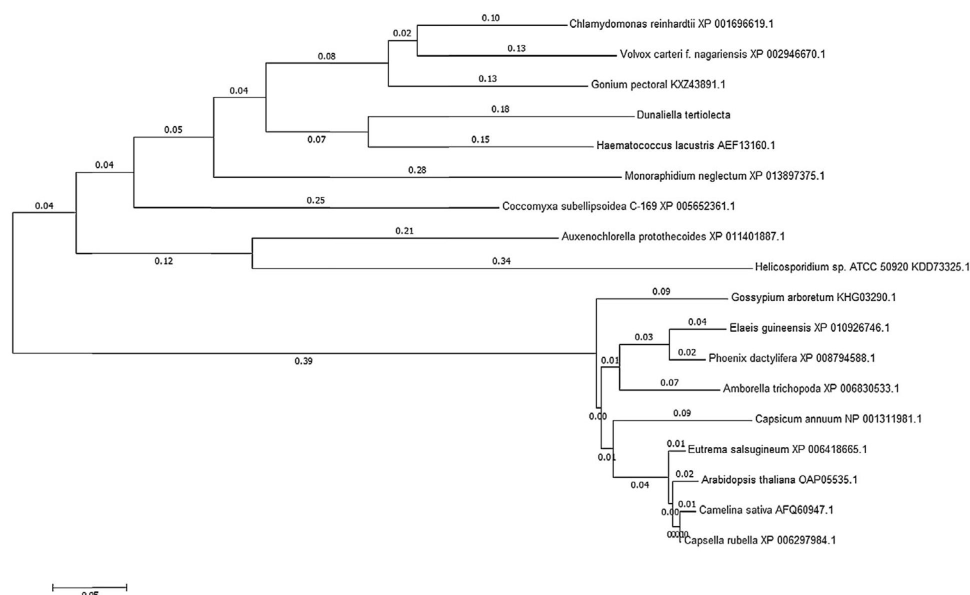

Fig. 1. Phylogenetic relationships of DtTE with related species based on BLAST. Evolutionary analyses were inferred with the neighbor-Joining method on the MEGA7 software using 18 amino acid sequences. Branch lengths are in units of the number of amino acid substitutions per site.

chain for elongation (Chen and Smith, 2012). To finally off-load the cargo from the ACP, the cells utilize an acyl-ACP thioesterase (TE) to hydrolyze the mature FA chain from the ACP (Beld et al., 2014).

The buildup of fatty acyl-ACPs can regulate the rate of FA synthesis by feedback inhibition of ACCase (Davis and Cronan, 2001), KAS, and enoyl-ACP reductase activities (Heath and Rock, 1996a, 1996b). TEs can relieve this inhibition by hydrolyzing the acyl-ACP into free FAs (FFAs), which are subsequently converted into acyl-CoA and released from the chloroplast to be incorporated into TAGs (Chen and Smith, 2012). Recently, the expression of endogenous TEs such as oleoyl-ACP hydrolase and acyl-ACP thioesterase A were found to be up-regulated in microalgae subjected to nitrogen limiting conditions (Miller et al., 2010; Rismani-Yazdi et al., 2012). In addition, expression of TE showed a linear relationship with FA synthesis in *Haematococcus pluvialis*, indicating that TE could be involved in a key rate-limiting step for FA synthesis (Lei et al., 2012). Based on this principle, increasing the activity of TEs appears to be a good strategy to promote the continuous production of FAs and channeling them to storage lipids rather than membrane lipids. Indeed, the overexpression of TEs has been shown to increase total FAs in *E. coli* (Lu et al., 2008), cyanobacteria (*Synechocystis* sp. PCC6803) (Liu et al., 2011), and diatoms (*Phaeodactylum tricornutum*) by up to 72% (Gong et al., 2011).

While plants develop specific TEs for FAs of different chain lengths and saturation, FatA and FatB (Salas and Ohlrogge, 2002), microalgae use one broad-specificity TE, termed FAT1 (Blatti et al., 2012), which could allow for cross-species expression of microalgal TEs to influence FA production and profile. While plant TEs have been heterologously engineered into a variety of species to effectively alter their oil content (Liu et al., 2011; Zhang et al., 2011), introducing plant TEs into *C. reinhardtii* did not alter the FA profile, suggesting that plant TEs may not functionally interact with CrACP to affect FA synthesis. The aim of this study is to genetically engineer a heterologous TE into *Chlamydomonas reinhardtii* to increase FA production. We chose to investigate *C. reinhardtii* as it is one of the microalgal species which can be easily transformed with current transformation techniques (Berthold et al., 2002), and heterologous promoters have been developed for robust expression of transgenes in the nuclear and plastid genomes (Díaz-Santos et al., 2013; Specht et al., 2010). In this study, we sequenced a TE gene from *Dunaliella tertiolecta*, which encodes a rate-limiting enzyme in FA synthesis, leading to enhanced levels of total lipids in *C. reinhardtii* without compromising its growth. The results suggest that genetic modification could substantially improve FA productivity in microalgae.

2. MATERIALS AND METHODS

2.1. Microalgae strain and culture conditions

The *Chlamydomonas reinhardtii* strain used was CC-424 (cw15, arg2, sr-u-2-60, mt^-) obtained from the Chalmydomonas Resource Center. The cells were grown in 50 mL batch cultures with Tris-acetate-phosphate (TAP) medium on a rotary shaker at 25 °C under continuous light (approximately 30 μmol photons m^2/s). Cell densities were determined using an automated cell counter (TC20™ automated cell counter, Biorad Laboratories). Transformants were maintained in TAP medium containing 10 μg/mL hygromycin B.

2.2. Cloning full length cDNA of TE from D. tertiolecta using RACE

The 1176 bp full length coding region of DtTE was amplified from total RNA by Rapid Amplification of cDNA ends (RACE) using the SMARTer™ RACE cDNA amplification kit (Clontech). Gene specific RACE primers were designed by looking for conserved nucleotide regions between the gene sequences of *C. reinhardtii* and *D. tertiolecta*. For example, primers used for RACE of DtTE were designed by comparing with the acyl carrier protein thioesterase from *C. reinhardtii* (NCBI RefSeq: XP_001696619.1). *C. reinhardtii* mRNA sequences were obtained from NCBI and subjected to a BLAST on the Sequence Read Archive (SRA) database (http://www.ncbi.nlm.nih.gov/sra) of *D. tertiolecta* under the accession number SRX029446 (Rismani-Yazdi et al., 2011). Overlapping sequences conserved between *C. reinhardtii* and *D. tertiolecta* were then identified and used to design the RACE primer. When unspecific bands were observed from the initial PCR, nested PCR was done using internal primers to achieve higher target specificity. Open reading frames (ORFs) were identified using ORF Finder (http://www.ncbi.nlm.nih.gov/gorf/) and the full length coding sequences were confirmed using a high fidelity Taq polymerase (platinum Taq DNA poly-

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