ABSTRACT

Acyl-CoA:diacylglycerol acyltransferase (DGAT) catalyzes the final step in acyl-CoA-dependent synthesis of triacylglycerols (TAGs), a major fatty acid reserve needed for membrane construction and energy metabolism in eukaryotes. Out of two distinct types of DGAT enzymes (type 1 and type 2) ubiquitously present in eukaryotes, the structure-function relationship of DGAT2 remains poorly understood due to the lack of experimentally determined structures. The present study investigates the structure, catalytic mechanism, and substrate specificity of CtDGAT2, a type 2 DGAT from oleaginous yeast *Candida tropicalis*, through *in silico* prediction and *in vivo* validation in *Saccharomyces cerevisiae*, followed by its biotechnological application in the microalga *Chlorella vulgaris* for enhanced lipogenesis. Molecular docking of the AlphaFold-predicted 3D structure of CtDGAT2 with palmitoyl-CoA, a random acyl-donor substrate, suggested His^{237} and Thr^{289} are the key active site residues involved in acyl-CoA binding and/or acyltransferase activity. Phylogenetic analysis of 42 eukaryotic DGAT2s revealed two distinct clades based upon the presence of the conserved motif 'H**PH**G' in animals, fungi, and yeasts, including *C. tropicalis*, and the homologous motif 'E**PH**S' in plants; whereas algal DGAT2 orthologues are distributed in both the clades. Notably, the dipeptide $^{289}TL^{290}$ (of the CtDGAT2 active site) conserved in the 'H**PH**G' clade is replaced by homologous 'AS' in the 'E**PH**S' clade, highlighting an evolutionary divergence. The wild-type CtDGAT2 enzyme and its three mutant versions were functionally characterized by heterologous expression in the *S. cerevisiae* TAG-deficient mutant (H1246). Expression of the codon-optimized *CtDGAT2* gene in H1246 cells restored TAG synthesis, lipid droplet accumulation, while also increasing total lipid and fatty acid methyl ester (FAME) yields. The CtDGAT2 enzyme displayed a broad range of substrate specificity towards saturated fatty acids of varying chain lengths (C12:0, C16:0, C18:0, C20:0, and C24:0). Site-directed mutagenesis validated the critical role of the conserved His^{237} (within the ^{235}H**PH**G^{238} motif located on the putative second transmembrane segment) and Thr^{289} (of the dipeptide $^{289}TL^{290}$ situated on the hydrophilic segment following the putative second transmembrane domain) in the catalytic activity of DGAT2. In order to harness the biotechnological potential, the codon-optimized *CtDGAT2* was constitutively expressed in *C. vulgaris*. The transgenic algal lines significantly boosted lipogenesis under various nutritional conditions, with increased lipid and FAME yields and an abundant accumulation of lipid droplets. Notably, the highest lipid and FAME yields were observed under mixotrophic conditions, reaching up to 352.1±23.5 µg/mg dry cell weight (DCW) and 163.83±11.7 µg/mg DCW, respectively, surpassing 238.5±21.2 µg/mg DCW and 107.94±7.5 µg/mg DCW, respectively, in the untransformed control algal strain. Our findings not only underscore new insights into the molecular aspect of CtDGAT2 acyltransferase activity but also demonstrate that CtDGAT2 is a potent biocatalyst for the development of sustainable biofuel feedstock.

Keywords: Acyltransferase activity, *Candida tropicalis*, catalytic residues, *Chlorella vulgaris*, CtDGAT2, genetic engineering, lipogenesis, site-directed mutagenesis