



Accumulation of medium chain fatty acids in *Nannochloropsis oceanica* by heterologous expression of *Cuphea palustris* thioesterase FatB1

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ABSTRACT

Medium chain fatty acids (MCFAs) are compounds of considerable commercial interest that have applications in food, feed, and the production of industrially relevant chemicals. Due to their antipathogenic and health-promoting effects, they are further discussed as potential therapeutic agents for disease treatment and infection prevention and control. Domesticated agricultural crops that can grow in temperate climates lack MCFAs, and increased cultivation of tropical MCFA-rich species such as oil palm is associated with deforestation and a decrease in biodiversity. Alternative sources for more sustainably produced MCFAs are non-domesticated plants such as *Cuphea* spp., as well as oil crops and oleaginous microalgae that can be genetically engineered to accumulate MCFAs by expression of acyl-acyl carrier protein thioesterases (TEs) from MCFA-producing plants. Here, we report the heterologous expression of a TE from *Cuphea palustris* in the industrially relevant microalga *Nannochloropsis oceanica*. Using a recently developed gene expression system, we engineered transformant strains that accumulated up to 2.84 and 4.15% of C8:0 and C10:0 in storage lipids, respectively, and we observed no effect on growth. We further show that MCFA accumulation was negatively correlated with total neutral lipid content, suggesting the presence of regulatory mechanisms that limit MCFA accumulation in *Nannochloropsis*.

1. Introduction

The medium chain fatty acids (MCFAs) C8:0, C10:0 and C12:0 are versatile chemicals with potential application in food [1–3], feed [4], and the production of various compounds such as detergents [5], surfactants [6], antibiotics [7] and insect pheromones [8]. As part of the human diet, MCFAs are preferentially used for beta-oxidation in the liver as a source for quick energy, whereas long chain fatty acids (LCFAs) more readily trigger lipogenesis, making MCFAs a potential agent for treatment of obesity [1,9,10]. MCFAs further have potential for prevention of neurological disorders, such as Alzheimer's, due to their neuroprotective and cognition-enhancing properties, which may be linked to their hepatic metabolism and the production of ketone bodies, or to the stimulation of antioxidant activity [11–13]. MCFAs, moreover, have antibacterial, anticoccidial and antiviral properties, making them potential therapeutic agents for treatment and prevention of infections [4,14–16]. Due to their immune-health-promoting and antipathogenic effects, MCFAs and their monoglyceride derivatives have

huge potential as feed additives to combat feedborne pathogens and to replace in-feed antibiotics in animal industries [4].

Currently, commercial sources of MCFAs are the milk of some animals, including cows, sheep and goats [17–19], and oils from tropical plant sources such as coconut and palm kernel oil, which predominantly contain C12:0 and C14:0 [20,21]. Due to the increasing interest in C8:0 and C10:0 over the past decades, and due to negative environmental impacts of oil palm expansion [22–24], considerable efforts have been undertaken to develop new agricultural sources for these MCFAs. Notably, the temperate genus *Cuphea* accumulates high amounts of C8:0 and C10:0, exceeding 90% of total fatty acids (TFAs) in seed oils [25]. However, *Cuphea* spp. are currently almost irrelevant as an agricultural crop because of low harvest yields and the need for specialized cultivation equipment [26]. Efforts to improve crop performance of *Cuphea* by breeding [27] have met with limited success, and the genus remains a specialty crop that is currently studied mainly for use in crop rotation to improve the performance of corn or soybean [28,29]. An alternative approach to agricultural production of C8:0 and C10:0 is to confer the

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ability to produce these fatty acids (FAs) upon agronomically more relevant plants by genetic engineering. Notably, genetic engineering has allowed the generation of MCFA-accumulating varieties of domesticated plants, including *Arabidopsis thaliana* [30], *Brassica napus* [31,32], *Nicotiana benthamiana* [33], and *Camelina sativa* [34], with promising results. All of these studies were based on heterologous expression of acyl-acyl carrier protein (ACP) thioesterase (TE) genes from MCFA-producing plants such as *Cuphea*, as this type of enzyme is essential for chain termination during de novo fatty acid synthesis, and therefore determines FA chain length [35].

A relatively novel and particularly promising oil crop is oleaginous microalgae. These plant-like aquatic organisms can achieve areal biomass and lipid productivities that significantly exceed those of current oil crops [36], and they can be cultivated independent of freshwater supply and on non-arable land. Therefore, microalgae have attracted considerable attention as a source for sustainably produced oils with application in food and feed, and moreover for the production of oleochemicals and biofuels [37]. Similar to most higher plants, most microalgae do not produce relevant levels of MCFAs, because they usually contain a general-purpose substrate-promiscuous TE that drives production of predominantly LCFAs and very long chain fatty acids [38]. Generation of strains that are able to accumulate MCFAs would substantially increase the commercial potential of microalgae and widen the range of applications for microalgal oils. Several studies have been devoted to this goal, and heterologous expression of MCFA-biased TEs from higher plants in different microalgae has produced mixed results. Whereas expression of TEs from *Umbellularia californica* and *Cuphea hookeriana* in the model microalga *Chlamydomonas reinhardtii* did not affect FA composition [38], expression of *U. californica* and *Cinnamomum camphora* TEs in the diatom *Phaeodactylum tricornutum* [39] and in the chlorophyte *Dunaliella tertiolecta* [40] resulted in increased contents of C12:0 and C14:0 among other MCFAs. C12:0 contents of transgenic strains were less than 0.4% of TFAs in *D. tertiolecta* and between ~1 to 6.2% in *P. tricornutum*, which is substantially lower compared with 45% found in coconut and palm kernel oil [21].

The marine microalga *Nannochloropsis oceanica* is an emerging model organism for research on the sustainable production of high-value lipids and bulk commodities due to its comparably high growth rates and exceptional lipid contents, which can exceed 60% per dry cell weight (DCW) [41–44]. Widespread research has generated a versatile toolbox for genetic engineering of *Nannochloropsis* [45], including high quality genomic and transcriptomic datasets. Despite this, the economic potential of this genus has yet to be exploited. To this end, transforming *N. oceanica* into a platform for MCFA production may improve its current application in aquaculture hatcheries [46], and moreover help to unlock its potential as a fishmeal substitute [47,48] and as feed for industrial livestock production in general, as well as for the production of nutraceuticals, therapeutic agents and biodiesel feedstocks [49,50]. Recently, expression of a *Cuphea palustris* TE was reported for *N. oceanica* [51], which has delivered the proof of concept for MCFA production in this species. However, MCFA contents of transgenic strains were low compared to those obtained for transgenic plants overexpressing MCFA-biased TEs, suggesting room for improvement. Radakovits and co-workers have shown that MCFA contents of transgenic *P. tricornutum* strains were tightly linked to expression levels of the heterologous TE [39], which highlights the importance of gene expression levels for improving MCFA contents in microalgae.

We have recently reported the development of a novel system for gene overexpression in *N. oceanica* [52], which facilitates expression levels that are several-fold higher compared to conventional expression systems. Here, we compare the effects of heterologously expressing *C. palustris* TE in *N. oceanica* using either the novel system or a strong benchmark promoter. We show that the production of heterologous protein and MCFA contents are significantly enhanced when using the novel expression system, and we show that transformant strains are not impaired in growth. We further report a negative correlation between

high levels of MCFA accumulation and neutral lipid contents in *N. oceanica*, and we suggest potential strategies to remedy this phenotype in future genetic engineering studies.

2. Results

2.1. Generation of *N. oceanica* mutants expressing a *Cuphea palustris* TE gene

The thioesterase (TE) FatB1 from *C. palustris* (CpTE) contains two domains, an N-terminal Acyl-thio_N domain (pfam12590) and a C-terminal Acyl-ACP-TE domain (pfam01643) (Fig. 1a). The C-terminal domain is highly conserved among different classes of TEs, and it is responsible for substrate specificity of the enzyme [53]. The N-terminal domain is less conserved and only found in association with pfam01643. The C-terminal Acyl-ACP-TE domain contains a region of high similarity to a conserved domain (cd00586) of 4-hydroxybenzoyl-CoA thioesterase (4HBT) proteins, including 7/7 conserved residues of the active site. The N-terminal domain contains a disordered region, comprising a target peptide that directs localization of CpTE to the chloroplast in *C. palustris*. A previous study revealed that this domain is not essential for enzyme activity and may interfere with heterologous gene expression [54]. The study of Lozada and colleagues [54] has shown that an N-terminal 94 amino acid truncation improved enzyme performance upon heterologous expression in *Escherichia coli*, suggesting interference of the disordered domain or the chloroplast target peptide with protein solubility or activity. Therefore, we codon-harmonized the truncated CpTE variant (CpTE_{No}), and added a myc tag to allow protein detection (Fig. 1a), as well as an endogenous chloroplast target peptide that was previously shown to direct heterologous proteins to the chloroplast stroma of *N. oceanica* [55]. The modified CpTE_{No} was inserted into two different expression constructs (ECs, Fig. 1b). The first construct, EC-CpTE-Pvcp, comprised the strong endogenous promoter of the *VCP1* gene and the transcriptional terminator of the α -tubulin gene, driving expression of CpTE_{No}, together with an antibiotic resistance cassette. The second construct, EC-CpTE-PpolI was designed based on a recently developed expression system [52] and comprised an RNA polymerase I (Pol I) promoter, artificial IRES and the α -tubulin terminator to drive CpTE_{No} expression, together with an antibiotic resistance cassette and homology arms. The homology arms were complementary to the sequence surrounding the rDNA cistron on chromosome 3 of *N. oceanica* because this locus was previously identified as a genomic safe harbor for gene expression using Pol I. EC-CpTE-Pvcp was used to transform wild type *N. oceanica*, whereas EC-CpTE-PpolI was used to transform a previously constructed landing pad strain [52]. The landing pad strain contains and expresses the fluorescent reporter gene *tdTomato* at the safe harbor locus on chromosome 3, which causes cells to emit high levels of yellow fluorescence upon excitation with green light. Using this strain as a parental strain for transformation with EC-CpTE-PpolI, allowed simple selection of transformants with EC integration at the safe harbor locus, by screening transformant colonies for loss of *tdTomato* fluorescence, which indicated replacement of the *tdTomato* gene by the new construct.

After transformation and antibiotic selection, we selected 23 colonies per construct, either randomly (CpTE-Pvcp), or by screening for low *tdTomato* fluorescence emission on agar plates (CpTE-PpolI). Presence of the ECs was confirmed by genotyping PCR (Fig. 1c, ctrl rxn). The CpTE_{No} gene was present in 22/23 CpTE-Pvcp transformants (hereafter referred to as mutants) and in 23/23 CpTE-PpolI mutants. Integration of CpTE-PpolI at the target site was confirmed using two primer pairs specific for the safe harbor locus (Fig. 1c, 5' rxn and 3' rxn). CpTE-PpolI was likely integrated at the safe harbor locus in 23/23 mutants, indicated by a successful 3' reaction for 23/23 mutants, and a successful 5' reaction in 20/23 mutants.

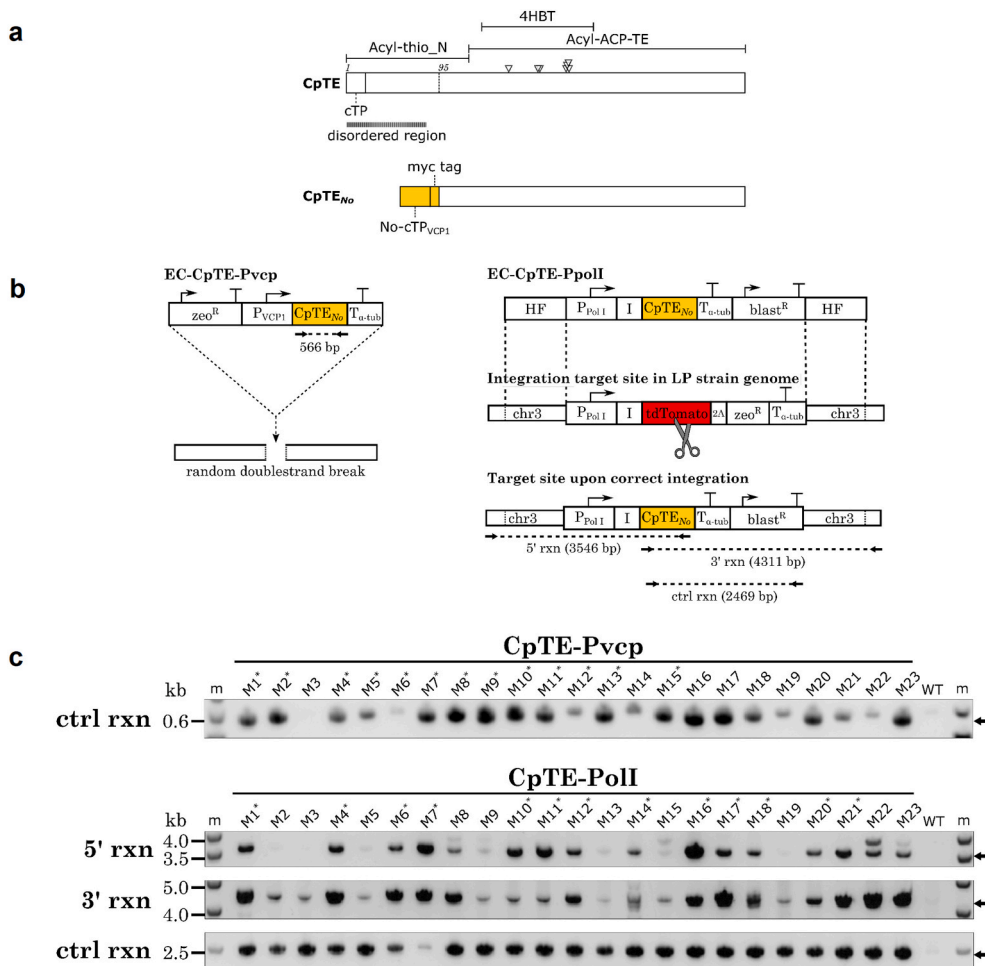


Fig. 1. Design and generation of *N. oceanica* strains expressing a TE gene from *C. palustris*. **(a)** Schematic of the *C. palustris* FatB1 TE (CpTE). The C-terminal Acyl-ACP-TE domain (pfam01643) is highly conserved among TEs and responsible for substrate specificity. This domain in CpTE has similarity with 4-hydroxybenzoyl-CoA thioesterase proteins, and shows 7/7 conserved residues (inverted triangles) of the active site (cd00586). The N-terminal domain (pfam12590) is less conserved, but sometimes found in association with pfam01643. It contains a disordered domain and the chloroplast target peptide responsible for subcellular localization of CpTE in *C. palustris*. CpTE was codon-harmonized for *N. oceanica*, the coding sequence for the N-terminal part was removed, and replaced with a sequence encoding the chloroplast target peptide of an endogenous *N. oceanica* gene (No-cTP_{VCP1}), and a myc tag coding sequence (highlighted yellow), forming CpTE_{No}. **(b)** Production of transformant strains using two different CpTE_{No} expression constructs. In EC-CpTE-Pvcp, CpTE_{No} (yellow) was inserted between the VCP1 promoter (P_{VCP1}) and α -tubulin terminator (T _{α -tub}), and fused to an antibiotic resistance cassette (zeo^R). The construct contained no homology arms and therefore inserted into the genome of *N. oceanica* by non-homologous end joining. In EC-CpTE-PpolII, CpTE_{No} was inserted into a recently reported expression system [52], utilizing an RNA polymerase I promoter (P_{PolII}) and artificial IRES (I), together with T _{α -tub} as an expression enhancer. EC-CpTE-PpolII contained homology arms, directing the construct to the rDNA cistron on chromosome 3. This construct was used to transform a landing pad (LP) strain of *N. oceanica*, which contains a *tdTomato* gene (red) at the safe harbor locus. Primer pairs that were

used for genotyping PCR are displayed as solid horizontal arrows. **(c)** Genotyping PCR for 23 mutants (M1–23) per construct, using the primer pairs depicted in (b). Mutants that were selected for MCFA screening are marked with an asterisk. Arrows on the right side of the gel images denote the expected size, based on comparison with the marker (m) bands. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

2.2. Screening of mutants for medium chain fatty acid production

Based on genotyping PCR results, 13 (CpTE-PpolII) and 14 (CpTE-Pvcp) mutants were chosen for MCFA content screening. C8:0 and C10:0 FAs were quantified per total fatty acids (TFA) using gas chromatography (Fig. 2a–b). Mutants of both constructs contained significantly higher fractions of C8:0 and C10:0 per TFA compared with the wild type and the landing pad strain (i.e. the parental strain of CpTE-PpolII mutants), confirming successful expression and activity of CpTE_{No}. On average, CpTE-PpolII mutants showed higher average MCFA contents compared to CpTE-Pvcp mutants. These differences are likely due to stronger CpTE_{No} gene expression in CpTE-PpolII mutants, which was suggested by Western blot analysis, conducted for the six best MCFA producers per construct (Fig. 2b). CpTE_{No} protein levels were highly similar between different CpTE-PpolII strains, while all CpTE-Pvcp mutants, except for CpTE-Pvcp-M11, produced only faint signals. We confirmed these results by an additional Western blot analysis, using the two best MCFA producers per construct in triplicates (Fig. 2c). These observations are in agreement with our previous study [52], which has shown that gene expression using Pol I at the genomic safe harbor locus is highly consistent among different transformants, and stronger

compared to expression using Pol II.

2.3. Physiologic characterization of selected mutants

Subsequently, we characterized the growth and photosynthetic performance of selected mutants. We chose the two best performers, per construct, based on the MCFA screening results and Western blot analysis (Fig. 2), namely CpTE-Pvcp-M6 and 11, and CpTE-PpolII-M7 and 20. We found no significant difference in growth performance during N-replete and N-depleted cultivation. All strains displayed comparable and exponential growth over a 4-day N-replete cultivation (Fig. 3a), with similar maximum specific growth rates (Fig. 3c), and similar maximum efficiency of photosystem II photochemistry (F_v/F_m , Fig. 3d). To test growth performance under stress conditions, cultures were harvested after 4 days of N-replete cultivation, washed and resuspended in N-depleted media (N-, Fig. 3b). After 2 days of cultivation under N-depleted conditions, F_v/F_m values were comparable between mutant strains and the wild type (Fig. 3e).

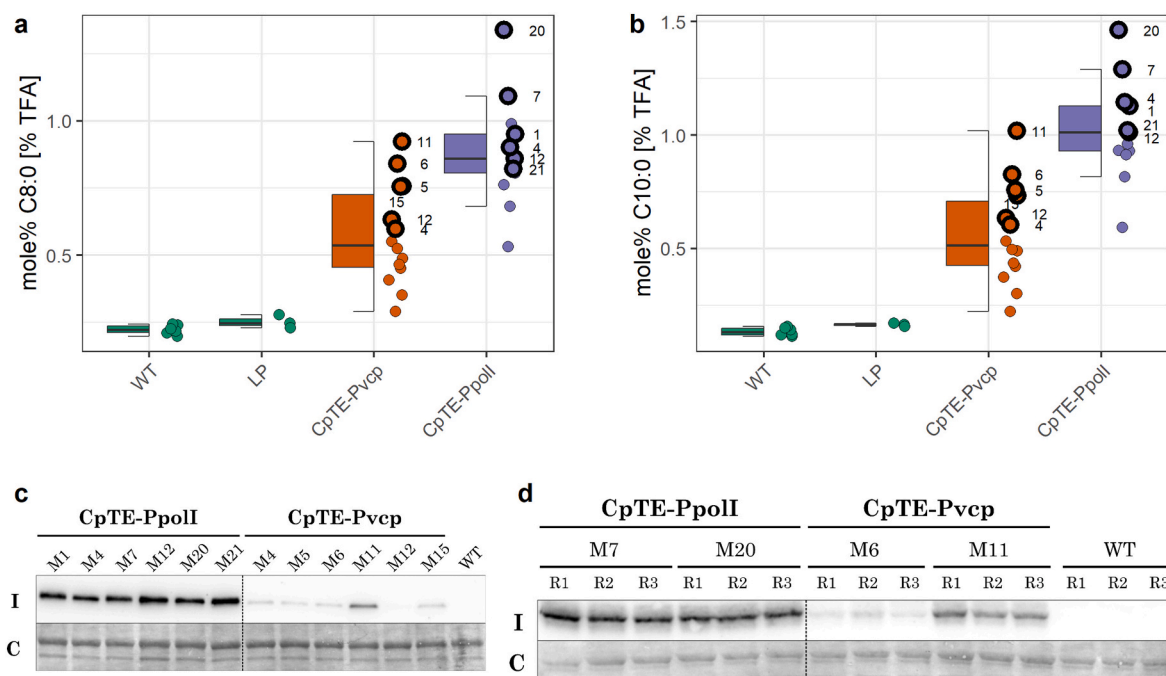


Fig. 2. Screening of mutants for MCFA contents and gene expression. **(a–b)** Mole fractions of C8:0 **(a)** or C10:0 **(b)** per TFA for mutants that were selected by genotyping PCR (Fig. 1c). MCFA contents of 13 (CpTE-PpolI) and 14 (CpTE-Pvcp) mutants ($N = 1$), the wild type (WT, $N = 6$) and the landing pad strain (LP, $N = 3$) are shown as a box-and-whiskers plot, juxtaposed with the values of individual cultures. The upper and lower hinges of boxes extend to the third and first quartiles, respectively. Whiskers extend to the maximum and minimum values within an additional 1.5 interquartile range. CpTE-Pvcp mutants had increased amounts of C8:0 and C10:0 per TFA, compared to the wild type (WT) and the landing pad strain (LP). CpTE-PpolI mutants had higher amounts of both MCFAs compared to both controls and further compared to the average contents of CpTE-Pvcp mutants. Mutants that were selected for Western blot analysis are highlighted and labeled. **(c)** Western blot analysis for six mutants per construct, selected based on MCFA screening. An immunodetection signal (I) was observed at the expected size (38 kDa), and this signal was highly consistent among different CpTE-PpolI mutants, and considerably stronger compared to CpTE-Pvcp mutants. The RuBisCO-band of the Coomassie-stained membrane (C) is shown as loading control. **(d)** Western blot for the two best MCFA producers per construct with biological triplicates (R1–3) confirmed the results shown in (c).

2.4. Lipid analysis for selected mutants

We analyzed MCFA contents in different lipid classes for selected mutants during exponential growth and after exposure to N-depletion stress (Fig. 4). Cultures were harvested after 4 days of cultivation in N-replete media (N+) or after 2 days of cultivation in N-depleted media (N−). Lipids were separated into neutral lipids (NL) and polar lipids (PL) and analyzed by gas chromatography. Interestingly, C8:0 and C10:0 accumulated in NLs of mutants under N+ conditions, whereas MCFA contents in PLs were unchanged compared to the wild type (Fig. 4a). Under N− conditions, MCFAs were substantially enriched in NLs, and further increased by 55% (C8:0) and 58% (C10:0) in PLs of CpTE-PpolI mutants. Whereas C8:0 was undetectable in NLs of the wild type, CpTE-Pvcp mutants on average accumulated 1.68 and 1.32% per NLs under N+ and N− conditions, respectively. In CpTE-PpolI mutants, average C8:0 contents per NLs were 2.84 and 1.97%, for N+ and N− conditions, respectively, increases of 69 and 49% compared with average contents of CpTE-Pvcp mutants. C10:0 accounted for 0.26 and 0.07% of NLs in the wild type under N+ and N− conditions, respectively. For N+ conditions, these values were increased by >9 fold and >14 fold for CpTE-Pvcp and CpTE-PpolI mutants, respectively. Under N− conditions, increases were >17 fold and >29 fold for CpTE-Pvcp and CpTE-PpolI, respectively. The highest fraction of C10:0 in NLs was found for CpTE-PpolI mutants grown under N+ conditions, accounting for 4.15%.

Quantification of total NL and PL contents per dry cell weight (DCW) revealed that CpTE overexpression had a marked effect on NL contents (Fig. 5a,c). NL contents of mutant strains were up to 50 and 51% decreased in N+ and N− cultures, respectively, compared to the wild type. PL contents were similar for all strains (Fig. 5b,d).

We further analyzed the profile of individual FA species present in

the different lipid classes and in total fatty acids (TFA) for the selected strains (Figs. 6, S1). Compared with the wild type, mutant strains showed differences in FA composition, which were more pronounced in NLs, rather than PLs. Changes were more pronounced for N− (Figs. 6, S2b) compared to N+ (Figs. S1, S2a) cultures. Along with the increases in C8:0 and C10:0, the abundance of C12:0 was increased in NLs of CpTE mutants, which is consistent with a substrate preference of CpTE for C12:0, compared to longer chain FAs [49,51]. The total fraction of saturated fatty acids (SFA) per NLs was instead decreased in CpTE mutants, with a prominent reduction in C14:0, C16:0 and C18:0. C16:0 accounts for the bulk of NLs (almost 50% under N− conditions) in wild type *N. oceanica* (Fig. S2). Early chain termination of FA synthesis at the level of MCFAs, catalyzed by CpTE, reduces the size of the substrate pool available for synthesis of LCFAs, which explains decreased levels of C14:0, C16:0 and C18:0 in CpTE mutants. In contrast to contents of long chain SFAs and long chain MUFAs, long chain polyunsaturated fatty acid (LC-PUFA, i.e. C16:2, C18:2, C20:4, C20:5) contents DCW^{−1} were comparable in all strains (Fig. 6).

We also performed a correlation analysis (Fig. 7). We found a strong correlation between C8:0 and C10:0 fractions in NLs and NL content DCW^{−1} for cultures exposed to N-depletion (Figs. 7a, S3a). No correlation was found for these MCFA fractions in PLs and PL contents DCW^{−1} (Figs. 7b, S3b). Fractions of C8:0 and C10:0 in NLs were strongly positively correlated with fractions of C16:1, C16:2, C20:4 and C20:5, and negatively correlated with fractions of C14:0, C16:0, and C18:0 (Fig. 7c). In PLs, fractions of C8:0, C10:0 and C16:1 were positively correlated with each other, and negatively with the fraction of C14:0 (Fig. 7d).

Positive correlations between different FA species suggest that their abundance may be modulated via shared regulatory mechanisms. It is known that C16:0, C16:1, C14:0 and C18:1 constitute the bulk of FAs

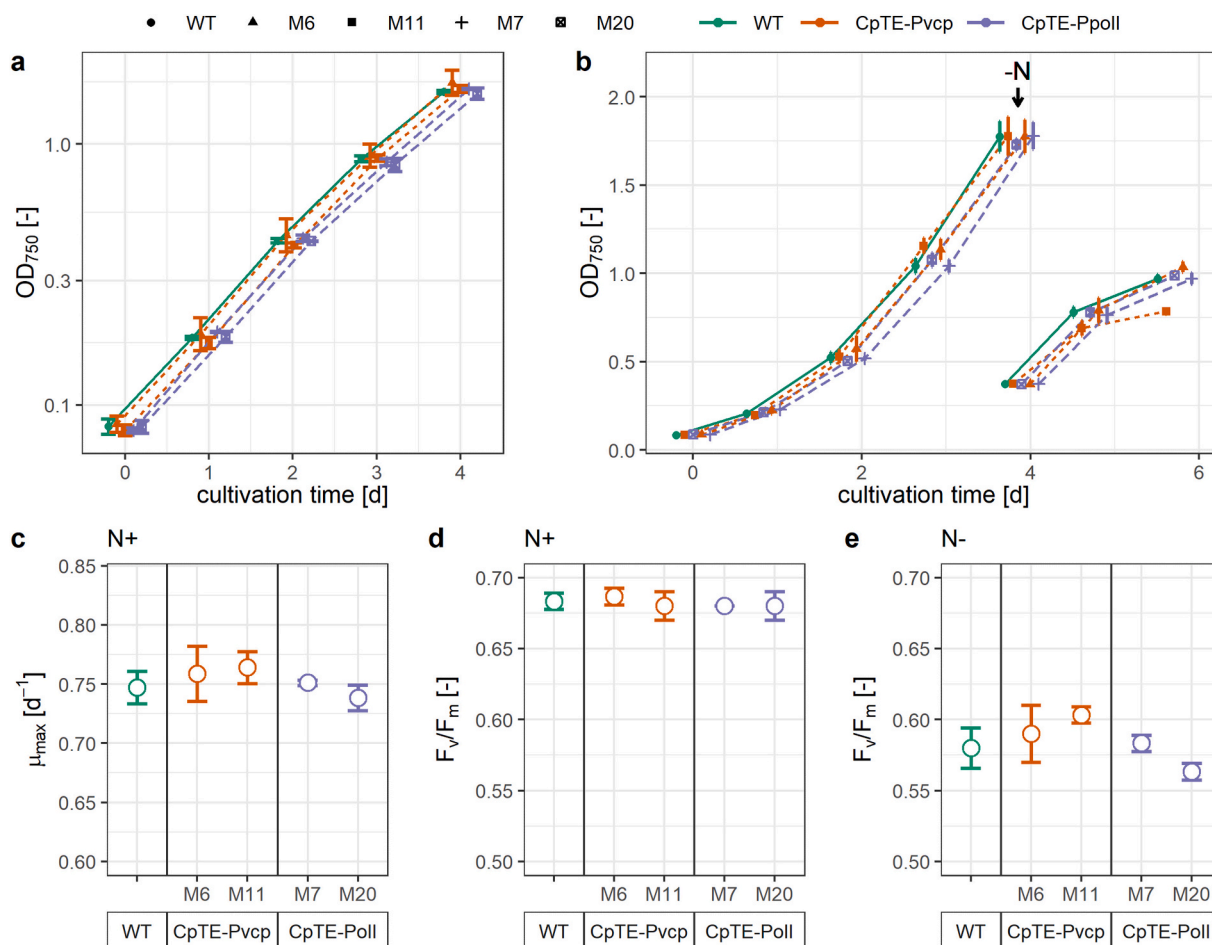


Fig. 3. Physiologic characterization of selected CpTE mutants. **(a–b)** Growth curves of the best performers for both constructs, compared to the wild type, under N-replete conditions (a) and during a 2-stage batch cultivation, consisting of an N-replete and N-depletion stage (b). To induce N-starvation (b), cultures were washed after 4 days of cultivation in N-replete media, and diluted in N-free media. Mutant cultures grew similar to the wild type. Points are horizontally dodged to improve visualization. **(c)** Maximum specific growth rates of selected strains, calculated using the data shown in (a). Statistical analysis (2-way ANOVA) revealed no significant difference between strains. **(d–e)** Maximum efficiency of photosystem II photochemistry after 4 days of N-replete cultivation (N+, d) and after 2 days of N-starvation (N–, e). Despite a significant ANOVA for data shown in (e), no significant difference was found by post hoc analysis (Tukey's HSD, $\alpha = 0.05$) for any mutant compared with the wild type. (a–e) Data shown are based on N = 3 biological replicates.

found in storage TAGs of *N. oceanica*, while PUFAs are enriched in membrane lipids of *Nannochloropsis* [42]. Specifically, the final product of LCFA desaturation in this genus, C20:5, is found in the thylakoid membrane lipid MGDG [56]. Sustained LC-PUFA synthesis (Fig. 6) under conditions of reduced TFA accumulation (Fig. 5a) in CpTE mutants suggests prioritization of LC-PUFA synthesis compared to other LCFAs. This is in agreement with decreased fractions of LC-PUFA synthesis precursors, namely C16:0, C18:0 and C18:1 [57,58], in NLs of these strains (Fig. S2b), and an enrichment of LCFAs that do not serve as substrate in LC-PUFA synthesis, such as C16:1 [56].

3. Discussion

Heterologous expression of a TE gene from *C. palustris* in *N. oceanica* resulted in the accumulation of C8:0 and C10:0 MCFAs, which are naturally present only in trace amounts in this microalga. Despite this, MCFA contents in *N. oceanica* mutants (C8:0 and C10:0 combined up to 3.6% TFA⁻¹) were low compared with natural MCFA-producing plants like coconut (>10% TFA⁻¹) or *Cuphea* (>90% TFA⁻¹), indicating room for improvement. Interestingly, we observed changes in the overall FA profiles of mutant strains, together with a decrease in NL contents. The reason for the decreased NL contents of CpTE mutants (Fig. 5a) requires further investigation. It is widely accepted that MCFA accumulation can

have negative physiological effects on microorganisms [59–61]. Toxicity of MCFAs may be related to their ability to accumulate in cell membranes, which increases membrane fluidity and decreases membrane integrity [60]. Genetically engineered MCFA-producing strains of *E. coli*, e.g., displayed up to 85% reduction in viability and a loss of inner membrane integrity [62]. Further, *Saccharomyces cerevisiae* shows a substantial accumulation of reactive oxygen species and almost complete growth inhibition when media is supplemented with C8:0 or C10:0 [61]. Transgenic *A. thaliana* plants, expressing an MCFA-biased TE, accumulated MCFAs in the membrane lipid phosphatidylcholine, and they showed severe chlorosis and cell death [33,63]. In our experiments, photosynthetic efficiency of CpTE mutants was unaffected, which may suggest that toxicity of MCFAs is not necessarily related to membrane integrity in *Nannochloropsis*. Future studies could help to confirm and better understand MCFA toxicity in this microalga, for instance by quantifying levels of reactive oxygen species and lipid peroxidation, which are commonly used biomarkers for stress in photosynthetic organisms. To prevent intracellular MCFA accumulation, organisms have developed coping mechanisms including downregulation of FA synthesis [64], activation of FA beta-oxidation [61], and active MCFA secretion via membrane transporters [65]. In *S. cerevisiae*, C8:0 is actively exported by ATP binding cassette proteins such as Pdr12 or Tpo1 [61]. Using BLAST analysis we identified several ATP binding cassette

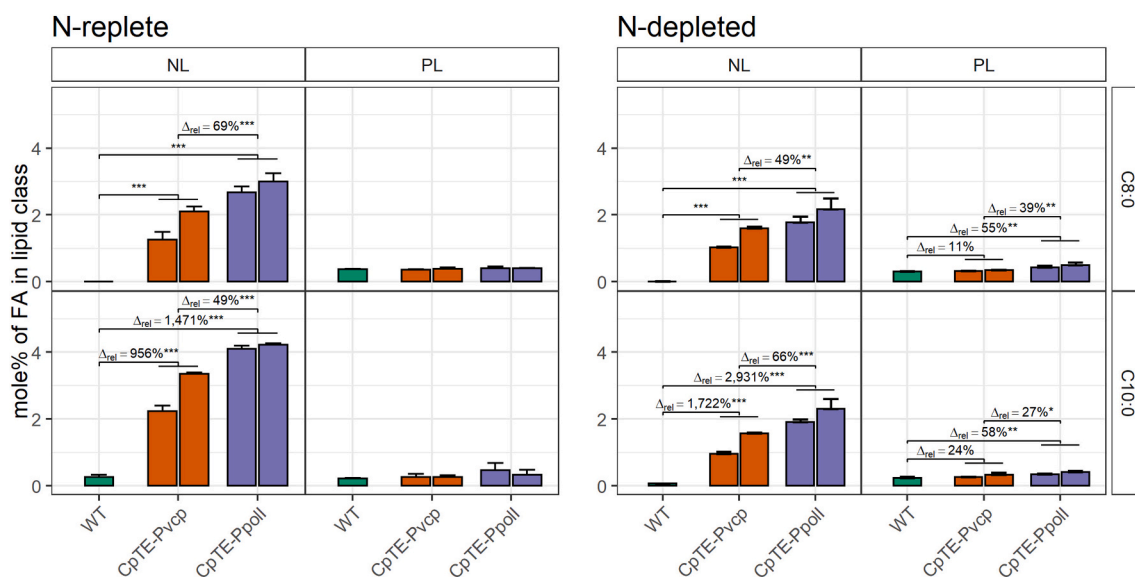


Fig. 4. MCFA content analysis for selected CpTE mutants. Contents of C8:0 and C10:0 are normalized to the total fatty acid (TFA) content in each lipid class. Cultures were harvested either after 4 days of N-replete cultivation (N+) or after 2 days of N-depletion (N-), and total lipids were extracted and separated into neutral lipids (NL) and polar lipids (PL) prior to analysis by gas chromatography. Relative differences (Δ_{rel}) between the average contents of the two best-performing mutants per construct and the wild type are indicated (N = 3) together with significance levels. (*): $p < 0.05$; (**): $p < 0.01$; (***): $p < 0.001$.

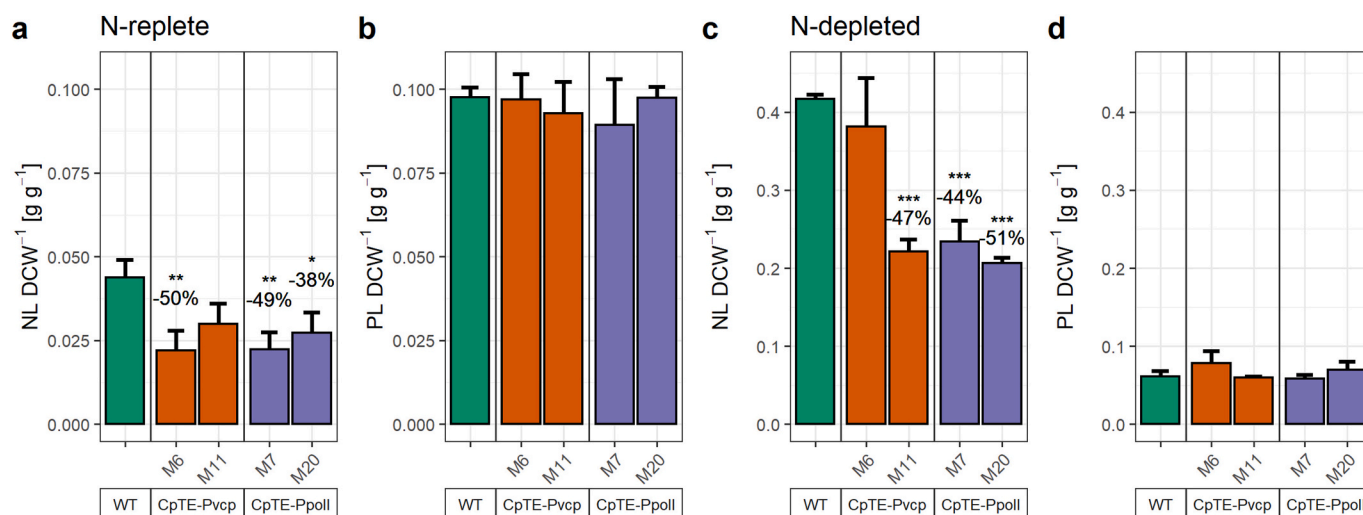


Fig. 5. Lipid content analysis for selected CpTE mutants. (a–d) NL (a,c) and PL (b,d) contents per dry cell weight (DCW) are shown for cultures harvested during N+ (a,b) and N- (c,d) conditions. NL contents of at least three different mutants were substantially decreased for both, N+ and N- conditions. PL contents were similar for all strains. Relative differences are indicated together with significance niveau for statistically significant differences, assessed by ANOVA and Tukey's HSD test. (*): $p < 0.05$; (**): $p < 0.01$; (***): $p < 0.001$.

proteins in *N. oceanica* that may be potential orthologues of Pdr12 such as NO20G00560 (34.11% identity and 53.68% positives on 557/1511 amino acids, 37% query coverage) and NO25G00490 (24.18% identity and 43.14% positives on 1282/1511 amino acids, 37% query coverage), indicating that *Nannochloropsis* may be able to export MCFAs. Despite this, we did not find any extracellular MCFAs in cultures of CpTE-expressing transformants (Fig. S4), suggesting that *N. oceanica* does not secrete MCFAs at detectable levels.

Increased MCFA contents in CpTE mutants may trigger metabolic response mechanisms that prevent the buildup of unusual FAs, in order to circumvent toxic effects. It was previously observed that transgenic expression of a C14-biased TE in the green microalga *Dunaliella tertiolecta* induced transcriptional upregulation of C14-specific beta-oxidation genes, downregulation of FA synthesis and adjustment of central carbon metabolism to limit the supply of FA synthesis precursors [66]. In their

study, Lin and co-workers observed that increased C14:0 synthesis did not affect growth performance, but NL accumulation of transgenic *D. tertiolecta* strains was substantially reduced [66], matching our observations for CpTE mutants. Moreover, transgenic *Brassica napus* plants, expressing a C12-biased TE, were able to synthesize, but not accumulate C12:0 in seeds, likely due to a regulatory response that increased levels of C12-specific beta-oxidation activity, resulting in a futile cycle of MCFA synthesis and breakdown [31,67]. In *Cuphea* seeds, MCFAs are naturally present up to 90 mol% of TAGs [25], which may limit the accumulation of MCFAs in membranes. In agreement with this, a chlorosis phenotype observed in transgenic *Nicotiana benthamiana* plants overexpressing an MCFA-biased TE, was alleviated by co-expression of an MCFA-biased diacylglycerol acyltransferase from palm kernel, which redirected MCFA flux from membrane lipids to TAGs [63]. Similarly, transgenic lines of *A. thaliana* producing unusual hydroxylated FAs

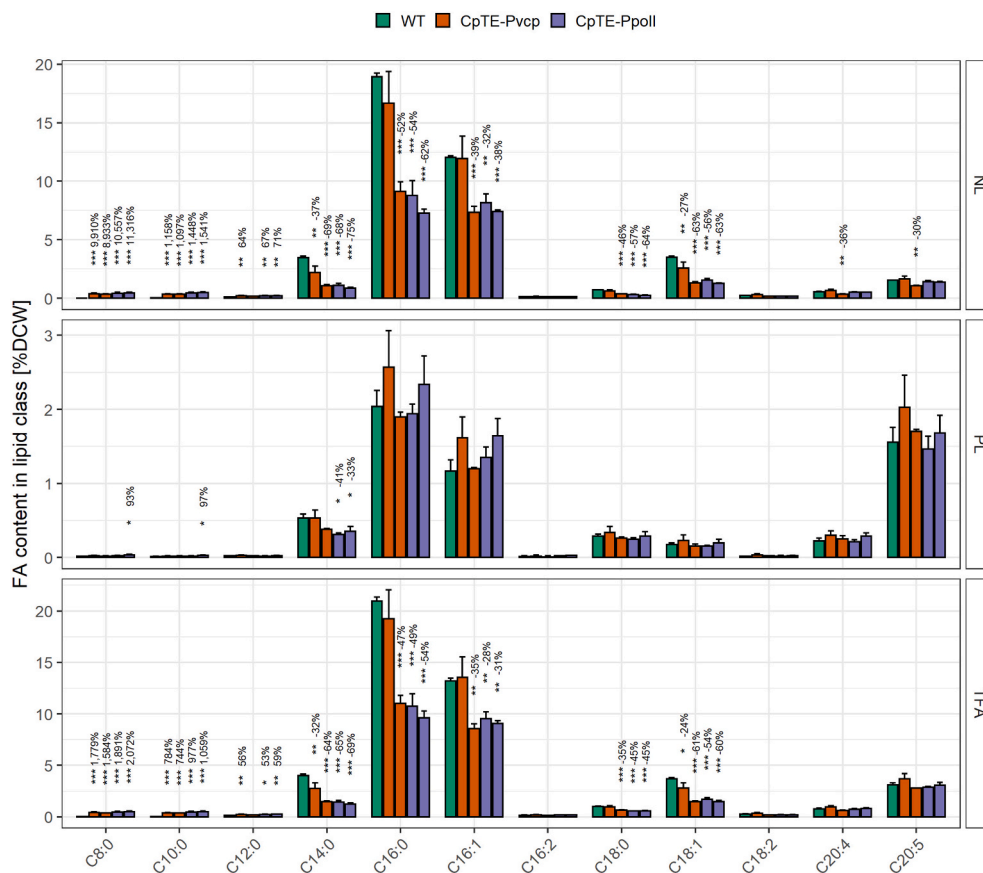


Fig. 6. FA contents in different lipid classes of CpTE mutants and wild type grown under N– conditions. Individual FA contents present in either NLs, PLs, or TFAs are normalized to the DCW. Changes are indicated relative to the wild type for significantly different samples. Significance levels, calculated using Tukey's HSD test, are indicated by asterisks. (*): $p < 0.05$; (**): $p < 0.01$; (***): $p < 0.001$.

showed a ~50% decrease in lipid content compared to the wild type, but this phenotype was alleviated by co-expression of acyltransferases that improved hydroxylated FA flux to TAGs [64]. Moreover, the expression of an MCFA-biased diacylglycerol acyltransferase from *Cuphea pulcherrima* in *S. cerevisiae* prevented lipotoxicity of exogenous C8:0 and C10:0 [34]. Consequently, specialized acyltransferases that facilitate efficient sequestration of MCFAs in TAGs may be the key to improve MCFA accumulation [68]. Therefore, a promising strategy to further increase MCFA contents in *N. oceanica* might be co-expression of CpTE together with MCFA-biased acyltransferase enzymes from *Cuphea* spp. (e.g. CpuDGAT1 from *Cuphea avigera*), to improve the microalga's ability to channel MCFAs into storage lipids.

Downregulation of genes involved in beta-oxidation, such as MCFA-specific acyl-CoA oxidase genes, could be another strategy to enhance MCFA content. In this context, disruption of an MCFA-biased acyl-CoA oxidase was recently shown to increase MCFA contents in the fungus *Mucor circinelloides*, when an MCFA-biased TE was expressed at the same time [69]. MCFA synthesis may be improved by increasing the rates of the corresponding condensation reactions of FA synthesis, which is catalyzed by 3-ketoacyl-ACP synthase. Analogously, efficient MCFA production in *Cuphea* is achieved not only through the MCFA-biased TE, but moreover by a specialized 3-ketoacyl-ACP synthase (KAS IV) gene that acts preferentially on C4-C10-ACPs [32,70]. Co-expression of a KAS IV gene from *Cuphea hookeriana* together with an MCFA-specific TE strongly enhanced MCFA production in *B. napus* seeds compared to TE expression alone [32], and similar results were obtained for the microalga *D. tertiolecta* [40]. In the long run, it may be possible to turn *N. oceanica* into a viable MCFA overproduction platform using a combination of strategies, including redirection of MCFA flux to NLs, expression of MCFA-specific KAS IV proteins, and down-regulation of

MCFA-specific beta-oxidation. It might further be possible to improve MCFA contents by applying genetic engineering strategies that improve overall lipid production, which was demonstrated for *Nannochloropsis* in several studies [71–73]. Another interesting strategy for improved MCFA production would be engineering of a reversal of beta-oxidation, which has thus far only been achieved in bacteria and yeast [74–76]. Establishing a reversed beta-oxidation pathway in the *Nannochloropsis* chloroplast could uncouple MCFA production from the endogenous FA synthesis machinery, which might allow improved product formation, with limited metabolic burden. In the short term, investigating the transcriptomic response of *N. oceanica* CpTE mutants to increased levels of MCFAs, using e.g. RNA sequencing technology, in combination with detailed lipidomic analyses, may improve our understanding of regulatory mechanisms involved in MCFA metabolism of *Nannochloropsis*.

4. Conclusion

In this study we successfully engineered *N. oceanica* strains to heterologously express a thioesterase enzyme gene from *C. palustris* using two different expression systems. We showed that the engineered strains were able to accumulate MCFAs that are normally present in only trace amounts in the wild type. When the heterologous gene was expressed under the control of a strong Pol II promoter, mutant strains accumulated up to 1.6 and 1.57% of C8:0 and C10:0, respectively, in NLs. When using a novel Pol I expression system, contents were increased up to 2.16 and 2.3% for C8:0 and C10:0, respectively. We also observed that MCFA overproduction was negatively correlated with NL accumulation, suggesting a metabolic response of *N. oceanica* to increased MCFA production. Lipid profile analysis showed that PUFA contents per biomass were similar to those of the wild type, whereas SFAs and MUFAs were

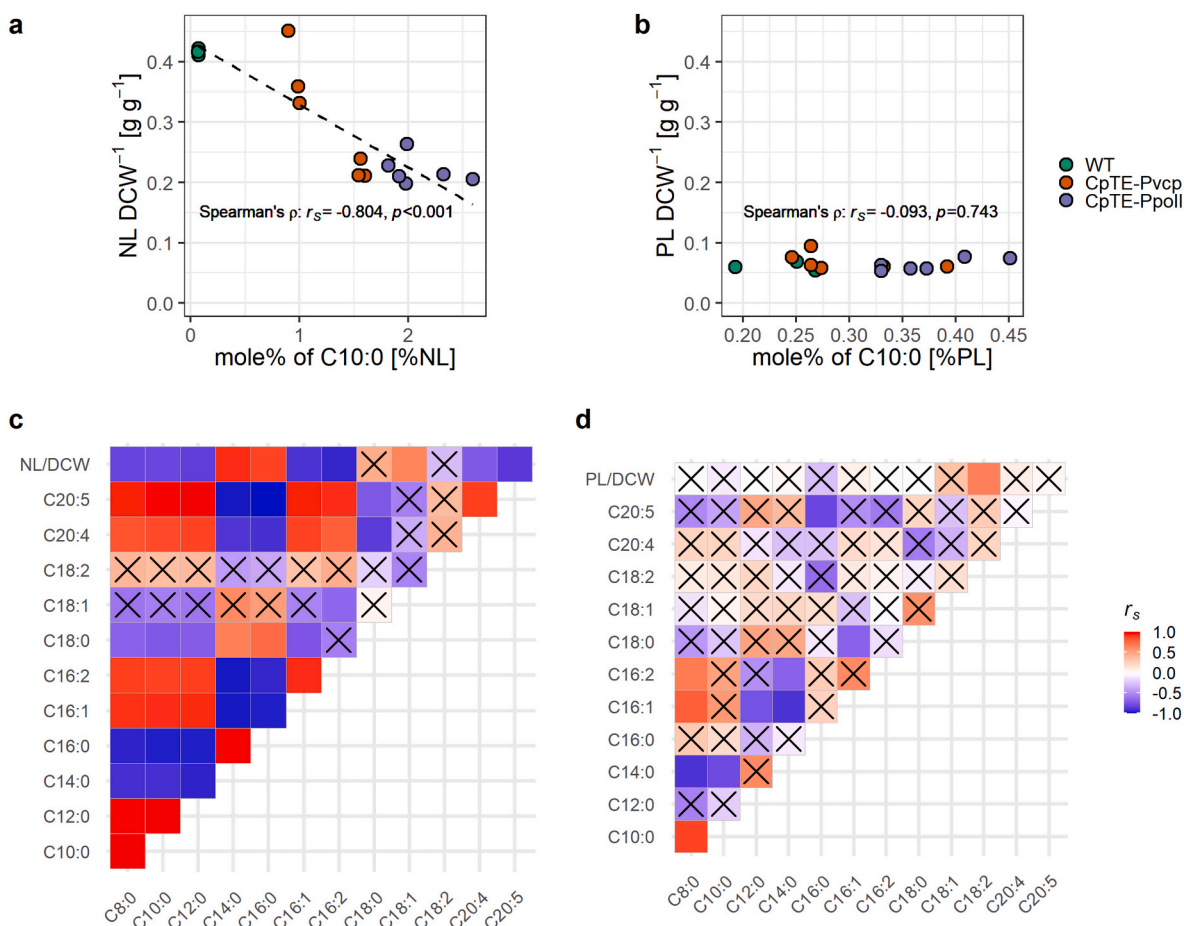


Fig. 7. Correlation analysis for FAs and lipid contents. **(a–b)** Correlation analysis for fractions of C10:0 per lipid class, and lipid contents per DCW for cultures grown under N⁻ conditions. The NL (a) or PL (b) contents of all analyzed cultures are plotted against the fraction of C10:0 in the respective lipid class. Strong negative association was found between NL content and the fraction of C10:0 per NLs, whereas no association was seen for PLs. Similar results were obtained for correlation analysis between lipid contents and fractions of C8:0 (Fig. S3). **(c–d)** Visualization of Spearman correlation matrices for FA contents per TFAs in NLs (c) and PLs (d). Non-significant ($p > 0.01$) correlations are crossed. The correlogram in (c) reveals two clusters of strongly associated FAs in NLs. Fractions of C8:0, C10:0, C12:0, C16:1, C16:2, C20:4 and C20:5 per TFAs in the NL fraction are positively correlated with each other, and negatively correlated with fractions of C14:0, C16:0, C18:0, and with NL content DCW⁻¹. In PLs (d), C8:0, C10:0, C16:1 and C16:2 are positively correlated with each other, and negatively correlated with C14:0.

reduced. Future work could focus on the elucidation of the underlying metabolic mechanisms and on channeling of MCFAs into storage lipids in order to reduce potentially toxic effects of MCFA accumulation in membrane lipids.

5. Materials and methods

5.1. Microalgal strains and cultivation

N. oceanica IMET1 was a kind gift of prof. Jian Xu (Qingdao Institute for Bioenergy and Bioprocess Technology, Chinese Academy of Sciences). Cultivation was done in artificial seawater (ASW, 419.23 mM NaCl, 22.53 mM Na₂SO₄, 5.42 mM CaCl₂, 4.88 mM K₂SO₄, 48.21 mM MgCl₂ and 20 mM HEPES, pH 8), which was supplemented with 2 ml l⁻¹ Nutribloom plus (Necton, Portugal), at 25 °C with a 16:8 h diurnal light cycle. N-replete cultivation was done in an HT Multitron Pro (Infors Benelux, Netherlands) orbital shaker, operated at 150 μmol m⁻² s⁻¹ light intensity, 120 rpm shaking frequency and 0.2% CO₂-enriched air.

Cultivation on solid medium was done by supplementing ASW with 1% (w/v) of agar prior to autoclaving. For selection of antibiotic-resistant transformants, media was supplemented with either 5 μg ml⁻¹ Zeocin, or 100 μg ml⁻¹ Blasticidin S, in addition to 2 ml l⁻¹ of nutribloom plus. Microalgal transformant plates were incubated at an illumination intensity of 60–80 μmol m⁻² s⁻¹ at 25 °C in ambient air.

5.2. Assembly of transformation constructs

The Zeocin resistance gene was cloned between the *N. oceanica* IMET1 elongation factor promoter and Cauliflower mosaic virus 35S terminator using Gibson assembly. The elongation factor promoter was amplified from genomic DNA, *zeo*^R was amplified from pNIM21 [73], and the CaMV35S terminator was amplified from pUC-AcolBPAT [77]. Genomic DNA was extracted as previously described [73]. The truncated CpTE gene was codon-harmonized for *N. oceanica* [78], and synthesized as a gene fragment at Integrated DNA Technologies (USA). The gene was cloned between the endogenous VCP1 promoter and α -tubulin terminator, which were amplified from pNIM21, and assembled together with the *zeo*^R cassette to form pCpTE-Pvcp. For assembly of pCpTE-Ppoll, the CpTE gene was inserted into the previously reported construct pCS-EC-VHH between a Pol I promoter, artificial IRES and the α -tubulin terminator [52]. All plasmids were assembled using either Gibson Assembly technique (NEBuilder HiFi DNA Assembly Master Mix, NEB #E2621) according to manufacturer instructions, or by *Bsa*I-mediated cloning (Thermo Fisher Scientific #FD0293). PCRs for cloning and amplification of linear expression constructs from plasmid templates were carried out using Q5 DNA polymerase (NEB #M0492). Phire DNA polymerase (Thermo Fisher Scientific #F160) was used for genotyping PCRs.

5.3. Transformation of *N. oceanica*

N. oceanica IMET1 was transformed using electroporation as described previously [79]. EC-CpTE-Pvcp was used to transform wild type *N. oceanica*, whereas EC-CpTE-PpolI was used to transform the landing pad strain LP-tdTomato, which harbors the tdTomato gene at the genomic safe harbor locus. This strain was generated in a previous study [52]. Briefly, microalgae were cultivated at $150 \mu\text{mol m}^{-2} \text{s}^{-1}$ illumination for 2–3 days, harvested during mid-exponential growth stage ($2500 \times g$, 5 min, 4°C) and pellets were washed thrice with 375 mM sorbitol (4°C), before resuspending at $2.5 \times 10^9 \text{ cells ml}^{-1}$. 200 μl of the suspension was mixed with 1–2 μg of expression construct in pre-chilled 2 mm electroporation cuvettes, and pulsed using a Bio-Rad GenePulser II (exponential pulse decay protocol; 11 kV cm^{-1} ; 50 μF ; 600 Ω). Cells were immediately transferred to 5 ml of media, recovered for 24 h at dim light, and plated on solid media containing an antibiotic agent. After 4–5 weeks, colonies were selected either randomly (EC-CpTE-Pvcp), or for loss of on-plate tdTomato fluorescence. Selected colonies were transferred to liquid media containing antibiotic, and cultivated for 2 weeks, before streaking cultures on fresh agar plates and proceeding with genotyping.

5.4. On-plate fluorescence screening

On-plate fluorescence of tdTomato was visualized using PathoScreen (PhenoVation Life Sciences, The Netherlands) fluorescence imager. The RFP channel was used together with the dedicated Data Analysis (Version 5.4.7 beta-64b) software (PhenoVation Life Sciences, The Netherlands) to identify colonies without tdTomato fluorescence.

5.5. Physiological and biochemical characterization of transformants

For physiological and biochemical characterization, microalgal cultures were inoculated from agar plates and cultivated for 2 weeks in liquid media at $150 \mu\text{mol m}^{-2} \text{s}^{-1}$ light intensity. Subsequently, cultures were set to an OD_{750} of 0.1 and incubated for an additional 3 days before inoculation of experimental cultures. For all experiments under N-replete conditions, cultures were inoculated to an OD_{750} of 0.085 and incubated for 4 days at $150 \mu\text{mol m}^{-2} \text{s}^{-1}$ before harvesting for biochemical characterization. For N-depleted experiments, pre-cultures were prepared as above at $150 \mu\text{mol m}^{-2} \text{s}^{-1}$. Then, experimental cultures were inoculated to an OD_{750} of 0.085 and incubated for 4 days in an Algem HT24 screening photobioreactor (Algenuity, UK), which was placed inside the same HT Multitron Pro shaker, using 0.2% CO_2 -enriched air and light intensity of $600 \mu\text{mol m}^{-2} \text{s}^{-1}$. After 4 days, cultures were harvested, pellets were washed with ASW, and new cultures were inoculated to an OD_{750} of 0.37 in nitrogen-free media. After 2 days at $600 \mu\text{mol m}^{-2} \text{s}^{-1}$, cultures were harvested and processed for biochemical characterization. In all experiments, samples for physiological characterization were taken 1.5 h before dusk. Maximum specific growth rates were calculated by daily OD_{750} measurements and linear regression of logarithmically-transformed OD values. Cellular bio-volume was measured using a Beckman Coulter Multisizer 3 (Beckman Coulter Inc., USA), equipped with a 50 μm orifice.

5.6. Photosynthetic performance assessment

The maximum efficiency of photosystem II photochemistry was quantified on dark-adapted samples by in vivo chlorophyll fluorescence analysis using an AquaPen AP 100-C (Photon System Instruments, Czech Republic) fluorometer, according to the manufacturer's instructions.

5.7. Quantification of neutral and polar lipids via GC-FID

Neutral and polar lipid content and FA profiles were determined as described previously [73]. Briefly, 650 μl of microalgal suspension was

freeze-dried in a lysing matrix (#116914050-CF, MP Biomedicals), subjected to mechanical cell disruption and lipid extraction using chloroform:methanol (1:1.25) containing either one or two internal standards. For quantification of TFA profile, tripentadecanoin (T4257; Sigma-Aldrich) was added at a known concentration. For quantification of FA profiles per lipid class, tripentadecanoin and 1,2-dipentadecanoyl-sn-glycero-3-phospho-(1'-rac-glycerol) (#840446P, Avanti Polar Lipids Inc.) were added at known concentrations. For TFA determination, lipids were directly resuspended in H_2SO_4 -containing MeOH after evaporation of the extraction solvent using N_2 gas. For FA profile and content determination per lipid class, polar and apolar lipids were separated with Sep-Pak Vac silica cartridges (6 cc, 1000 mg; Waters). Then, NLs were eluted with hexane:diethylether (7:1 v/v), and PLs were eluted with methanol:acetone:hexane (2:2:1 v/v). Solvents were evaporated using N_2 gas, before adding H_2SO_4 -containing MeOH. FAs were transesterified for 1 h at 100°C , extracted with hexane, and analyzed by gas chromatography (GC-FID). FA quantification was based on the relative response of individual FAs compared with the peak area of the internal standards. For lipid content quantification per biomass, the DCW of microalgal suspensions was determined as described elsewhere [80].

FA content of cell-free supernatants was assessed as follows. Cultures were harvested at an OD_{750} of ~ 3 by centrifugation ($4255 \times g$, 5 min), and supernatants were filtered (0.22 μm). FAs were extracted from cell-free supernatants using chloroform containing tripentadecanoin as an internal standard (2 min vortexing, 3 h rotation). After centrifugation ($2500 \times g$, 5 min), the chloroform layer was transferred to a fresh tube and dried under N_2 . FAs were transesterified, hexane-extracted and analyzed by GC-FID as described above. Cultivation media that was supplemented with decanoic acid (Merck #C1875) at $100 \mu\text{g ml}^{-1}$ was used as a control for the extraction process.

5.8. Assessment of MCFA secretion

For MCFA secretion experiments, pre-cultures were pelleted, washed, and inoculated to new flasks at an OD_{750} of 1 in ASW supplemented with 6 ml l^{-1} Nutribloom plus. Cultures were incubated at $150 \mu\text{mol m}^{-2} \text{s}^{-1}$ for 3 days, pelleted ($4200 \times g$, 10 min, 4°C) and the supernatant was filtered (0.22 μm). Media that was supplemented with 100 mg l^{-1} decanoic acid (C1875, Merck) served as a control. 5 ml of supernatant were mixed with 1 ml of extraction solvent (60 mg l^{-1} tripentadecanoin in chloroform), vortexed for 1 min and mixed for 2.5 h on a test tube rotator. After addition of 2.5 ml chloroform, 800 μl methanol, and 1 ml Tris-NaCl (50 mM Tris, 1 NaCl, pH 7.5), samples were vortexed again (1 min) and mixed for an additional 60 min. After phase separation ($2500 \times g$, 10 min, 15°C), the bottom phase was transferred to fresh tubes and dried under N_2 gas. The samples were then transesterified with methanol, extracted with hexane, and analyzed by GC-FID as described previously [73].

5.9. Western blot

Western blotting was carried out as described previously [52] with the following modifications. Soluble protein was extracted from $\sim 3 \times 10^9$ *N. oceanica* cells harvested during exponential growth phase. Pellets were resuspended in 400 μl 0.075 mM Tris buffer (pH 8) and the suspension was bead beat thrice for 20 s at 2500 rpm, with 120 s pauses, in a Lysing Matrix E (#116914500, MP Biomedicals) on a Precellys 24 homogenizer (Bertin Technologies). Tubes were frozen twice at -20°C for 90 min and thawed at 20°C , and pelleted ($15,000 \times g$, 5 min). The supernatants containing proteins were transferred to fresh tubes, and the protein content was quantified by modified Lowry procedure (DC Protein Assay, Biorad #5000116) using a BSA calibration standard. 45 μg of soluble protein was mixed with 5 $\times$ Laemmli reagent and boiled at 95°C for 5 min, before separation by SDS-PAGE on either 8–16% or 12% TGX protein gels (Biorad), using TGS as running buffer for 40–50 min at 200

V. Proteins were blotted onto PVDF membranes (Thermo Fisher Scientific #PB5310) by Power Blotter XL system (Thermo Fisher Scientific #PB0013). Then, membranes were blocked using TBS-T containing 1% (w/v) skim milk powder (Biorad #170-6404), prior to incubation with anti myc antibody (500× diluted, BioXcell #RT0263) for 2 h at 22 °C, and overnight at 4 °C. After three washes with TBS-T, membranes were incubated with HRP-conjugated secondary antibody (2000× diluted, Thermo Fisher Scientific #A10551) for 2 h, and washed three more times before protein detection. Chemiluminescence detection was carried out on an iBright CL1500 Imaging system (Thermo Fisher Scientific #A44114), using clarity Western ECL substrate (Biorad #170-5061). After immunodetection, membranes were stained with Coomassie R-250 (Biorad #161-0436) and destained with several changes of destaining solution (7% v/v acetic acid, 50% v/v methanol), followed by H₂O, to confirm equal blotting efficiencies across samples.

5.10. Bioinformatical protein analysis

The gene model of CpTE was analyzed by querying the conserved domain database of the NCBI [81]. Identification of potential orthologues of *S. cerevisiae* Pdr12 in the *N. oceanica* genome was done by BLASTp analysis, using the *N. oceanica* IMET1v2 reference genome and NanDeSyn database [82], with an E value cutoff of 1×10^{-60} .

5.11. Statistical data treatment

All data processing and statistical tests were done using R statistical software [83]. Significant main effects were tested for by two-way ANOVA, followed by Tukey's HSD test ($\alpha = 0.05$) for comparison of individual groups. Statistical tests including Spearman correlations were carried out using the R stats package. Correlation matrices were visualized using the R corrplot package [84].

6. Oligonucleotides and gene fragments

Oligonucleotides and gene fragments synthesized for this study are shown in Tables S1 and S2, respectively.

Data availability

All data produced in this study are presented in the article or supplementary material.

CRedit author contribution statement

CS: Conceptualization, Methodology, Investigation, Formal analysis, Writing - original draft, Writing - review & editing. AK: Methodology, Investigation. HB: Conceptualization, Investigation. MH: Conceptualization, Methodology. RHW: Project administration, Supervision, Writing - review & editing, Funding acquisition. MJB: Project administration, Supervision, Writing - review & editing. SD: Conceptualization, Project administration, Supervision, Writing - review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Statement of informed consent, human/animal rights

No conflicts, informed consent, or human or animal rights are applicable to this study.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.algal.2022.102665>.

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