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Metabolic engineering toward enhanced LC-PUFA biosynthesis in *Nannochloropsis oceanica*: Overexpression of endogenous Δ12 desaturase driven by stress-inducible promoter leads to enhanced deposition of polyunsaturated fatty acids in TAG

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ABSTRACT

Nannochloropsis oceanica is an important source for omega-3 long-chain polyunsaturated fatty acids (LC-PUFA) such as eicosapentaenoic acid (EPA, 20:5 $n-3$), and a potent candidate for biofuel production, due to its outstanding capability for rapid induction of triacylglycerol (TAG) overproduction. In contrast to membrane lipids, TAG of *N. oceanica* is poor in the valuable LC-PUFA, EPA. We identified, cloned and characterized a *N. oceanica* microsomal-like Δ12-desaturase (*NoD12*) mediating the committing step of LC-PUFA biosynthesis by converting oleic acid (18:1 $n-9$) to linoleic acid (LA,18:2 $n-6$). We generated strains of *N. oceanica* overexpressing *NoD12* under the control of the stress-inducible endogenous lipid droplet surface protein (LDSP) promoter, resulting in robust expression under nitrogen starvation conditions. The overexpression of *NoD12* significantly altered fatty acid composition of total lipids and of individual lipid classes, such as a drastic increase in 18:2 proportion in phosphatidylcholine and in TAG was observed under nitrogen starvation. Some LA was converted further toward LC-PUFA resulting in a substantial increase in arachidonic acid (20:4 $n-6$) in TAG. Our data demonstrate the feasibility of metabolic engineering to increase LC-PUFA content in the biotechnologically important microalga using native genes and promoters, and provide novel insights into the regulation of LC-PUFA flux to TAG under nitrogen starvation.

1. Introduction

Planktonic heterokont microalgae of the genus *Nannochloropsis* (Eustigmatophyceae [1]), represented by several members [2–7], belong to the phylum Stramenopiles, together with other taxa of nonphotosynthetic protists and algae, such as diatoms and brown algae [2,8] (http://tolweb.org/Eukaryotes/3). These algae are assumed to have a common evolutionary origin in the secondary endosymbiosis of red algae with a heterotrophic eukaryote [8,9]. Due to these evolutionary events, the plastids of Stramenopile algae are surrounded by four membranes [10,11].

Nannochloropsis species inhabit a wide range of marine and freshwater ecosystems, indicating their diversity [3,5,12] and their osmotic adaptability, as recently confirmed by laboratory studies [13,14]. Different species of *Nannochloropsis* are grown outdoors in ponds and photobioreactors for aquaculture, and are also considered promising for mass cultivation for biofuel production. This is due to their ability to accumulate storage triacylglycerols (TAGs) to over half of their dry weight under conditions of nitrogen (N) starvation [13,15,16]. Given its industrial potential, significant effort has been invested in developing this alga into a model oleaginous microalga. Several research groups have sequenced the genome of different *Nannochloropsis* species [7,17–19]. The genomic data have been integrated with transcriptome [7,17,19–22], proteome [23], and lipidome [20] data for a better understanding of the cellular mechanisms underlying the *Nannochloropsis* response to nitrogen starvation and oil accumulation. Furthermore, efficient nuclear transformation has been established using high-voltage electroporation

Abbreviations: LC-PUFA, long-chain polyunsaturated fatty acid; OA, oleic acid; LA, linoleic acid; AA, arachidonic acid; EPA, eicosapentaenoic acid; TAG, triacylglycerol; CoA, coenzyme A; PC, phosphatidylcholine; PE, phosphatidylethanolamine; DGTS, diacylglyceroltrimethylhomoserine; *NoD12*, *Nannochloropsis oceanica* microsomal Δ12-desaturase; LDSP, lipid droplet surface protein.

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[7,18,24]. The availability of genomic data along with efficient transformation protocols provides a promising approach for detailed gene functional analysis and genetic engineering of *Nannochloropsis* species. Further development of homologous recombination-based gene transformation has also been recently reported in *N. oceanica* [24], and may potentially provide a molecular tool for further research into understanding fundamental metabolic and cellular processes.

A biotechnologically important cellular process in *Nannochloropsis* is the biosynthesis of long-chain polyunsaturated fatty acids (LC-PUFA). LC-PUFA, such as arachidonic acid (AA, 20:4 $n-6$), eicosapentaenoic acid (EPA, 20:5 $n-3$), and docosahexaenoic acid (DHA, 22:6 $n-3$) are essential constituents of human nutrition, with key roles in growth and development of infants and in maintaining mental and cardiovascular health in adults [25–30]. The depletion of wild fish stocks, which are the main human dietary source of EPA and DHA [31] and essential components in aquaculture feed, stresses the need for alternative, sustainable sources of LC-PUFA [32–34]. Marine phytoplankton microalgae, such as *Nannochloropsis*, are primary producers of the ω-3 (omega-3) LC-PUFA in the aquatic food chain. Both marine and freshwater *Nannochloropsis* species produce high concentrations of EPA predominantly as a component of the chloroplast membrane galactolipids [35–37]. Due to their high LC-PUFA content, various species of *Nannochloropsis* are widely used in aquaculture [38–40] and hold promise as an efficient EPA source for human nutrition.

A set of genes encoding membrane-bound endoplasmic reticulum (ER)-localized enzymes involved in LC-PUFA biosynthesis in *N. oceanica* has been annotated, namely putative Δ12-, Δ6-, Δ5- and ω3-desaturases and Δ6-PUFA elongase [7] (Fig. 1).

Comparative analysis of lipid and fatty acid (FA) profiles under nutrient-replete and nitrogen-starved conditions has indicated that nitrogen scarcity, and possibly other stresses, lead to arrest of LC-PUFA biosynthesis at the level of oleic acid (OA, $18{:}1^{\Delta 9}$; $n-9$) [41]. LC-PUFA contents decrease under nitrogen starvation in line with a concomitant reduction in thylakoid membrane lipids, which are the primary LC-PUFA reservoir in *Nannochloropsis* cells [37,41]. This is accompanied by accumulation of storage lipids TAG enriched in the *de novo*-synthesized in chloroplast saturated or monounsaturated C16 FA (16:0 and 16:1) as well as OA.

The committing step of LC-PUFA biosynthesis is conversion of OA to linoleic acid (LA, $18{:}2^{\Delta 9,12}$, $n-6$), mediated by a Δ12-desaturase. In photosynthetic cells, LA can be produced by two Δ12-desaturase isoforms in two different cellular compartments, the plastid and the ER [42]. The microsomal Δ12-fatty acid desaturase (FAD2) located in the ER uses predominantly phosphatidylcholine (PC) as a substrate, and the plastidial Δ12-fatty acid desaturase (FAD6), located in the chloroplast, uses primarily glycoglycerolipids as substrates [43,44]. In some microalgae, the extraplastidial betaine lipids are deemed to be another lipid substrate for microsomal Δ12-desaturase [45]. Numerous Δ12-desaturase genes have been characterized from higher plants [46–49], animals [50], insects [51], and fungi [52], and from several microalgae and microalgae-like organisms [53–57]. To the best of our knowledge, none of the algal Δ12-desaturases have been overexpressed in their own host.

In order to conduct metabolic engineering in *Nannochloropsis* for improved LC-PUFA content under nitrogen starvation conditions, we identified, cloned and then overexpressed the endogenous Δ12-desaturase coding gene (cDNA), designated *NoD12*. This study provides first evidence of successful stress-induced overexpression of an endogenous desaturase in *N. oceanica* and paves the way for the overexpression of additional homologous genes as a means of modifying the LC-PUFA content in TAG of *N. oceanica*.

2. Materials and methods

2.1. Strain and culture conditions

Nannochloropsis oceanica strain CCMP1779 (axenic culture) was kindly provided by Prof. Christoph Benning (Michigan State University, USA) and is available from the Provasoli-Guillard National Center for Culture of Marine Phytoplankton (https://ncma.bigelow.org/).

Algal cultures (100 mL) were grown in 1/2 Reef Salt Enriched (RSE) liquid media in sterilized 250-mL glass Erlenmeyer flasks, in incubator shakers (New Brunswick Scientific, USA, Model Innova 42) with CO_2-enriched atmosphere at 200 mL min^{-1}, under constant illumination (75 μmol photons m^{-2} s^{-1}), at a constant temperature of 25 °C. 1/2 RSE medium composition was: 17 g L^{-1} Reef Salt (Seachem TM, Madison, GA), 2 g L^{-1} KNO_3, 70 mg L^{-1} KH_2PO_4, 5.74 g L^{-1} $MgSO_4 \cdot 7H_2O$, 1 mL L^{-1} A5 + Co microelement solution (0.222 g L^{-1} $ZnSO_4 \cdot 7H_2O$, 0.079 g L^{-1} $CuSO_4 \cdot 5H_2O$, 0.39 g L^{-1} $Na_2MoO_4 \cdot 2H_2O$, 2.86 g L^{-1} H_3BO_3, 1.81 g L^{-1} $MnCl_2 \cdot 4H_2O$, and 0.05 g L^{-1} $Co(NO_3)_2 \cdot 6H_2O$) and 1 mL L^{-1} Fe–citrate mixture (9 g L^{-1} ferric citrate and 9 g L^{-1} citric acid).

Growth was quantified based on volumetric content of chlorophyll *a* (Chl *a*), and biomass dry weight (DW), as described in [14]. Before the start of the experiment, the cultures were subjected to three cycles of dilution to 25 mg L^{-1} Chl *a* and 6 days of nitrogen replete growth. At the onset of each experiment, three independent biological samples were replenished with fresh medium and grown for 6 days, starting from 20 mg L^{-1} Chl *a* and attaining ~75 mg L^{-1} Chl *a* and biomass content of about ~0.8 g L^{-1}. At this time point samples were collected for the early stationary growth phase. The cells were harvested by

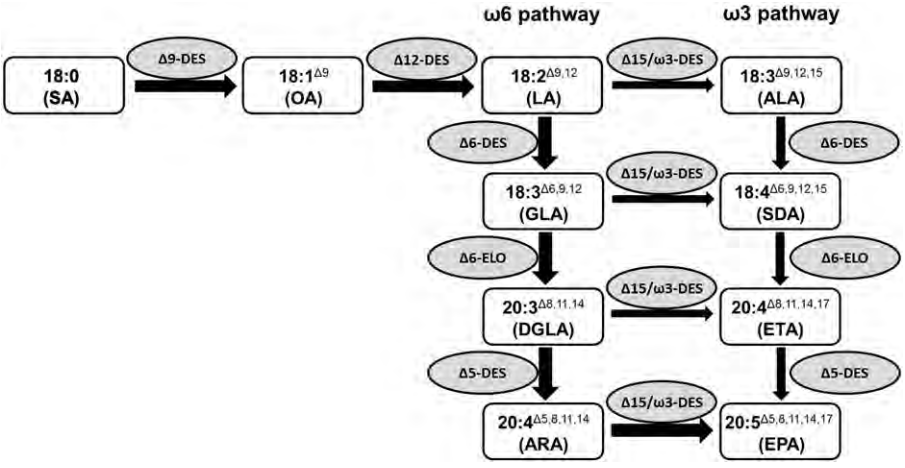

Fig. 1. LC-PUFA biosynthesis pathway. Proposed pathway of desaturation and elongation of fatty acyl chains in *Nannochloropsis*. Several desaturases and one elongase are tentatively identified and functionally annotated based on their predicted amino acid sequences (reproduced from [7]). Wide arrows indicate the predominant pathway.

centrifugation at 12,000 *g* for 5 min, flash-frozen in liquid N_2, and stored at −80 °C. For sampling in the stationary growth phase, the cultures were grown for 6 more days to reach ~ 100 mg L^{-1} Chl *a*, and harvested in the same manner. For nitrogen starvation, the cells in the early stationary growth phase were collected by a low speed centrifugation (1200 *g* for 10 min), washed twice with 1/2 RSE medium lacking nitrogen (1/2 RSE–N), and resuspended in this medium to the initial Chl *a* concentration of 15 mg L^{-1}. The cells were then grown under nitrogen starvation conditions as described above for 5 days, and were harvested at ~10 mg L^{-1} Chl *a*; biomass content ~1.2 g L^{-1} by the above procedure. At least three independent biological samples were collected and analyzed for each line.

2.2. DNA isolation

Genomic DNA was extracted from algal cells, following the modified cetyltrimethyl ammonium bromide (CTAB)-based protocol [58]. CTAB buffer (2% CTAB, 1.4 M NaCl, 0.2% ß-mercaptoethanol, 20 mM EDTA, 100 mM Tris–HCl, pH 8.0) preheated to 60 °C was added to cell pellets ground in liquid N_2. After 30 min incubation at 60 °C, samples were extracted twice by chloroform-isoamyl alcohol (24:1, v/v). Cold isopropanol was added to the upper aqueous phase, and the DNA was precipitated by centrifugation (12,000 *g*). The pellet was washed in 70% ethanol and resuspended in PCR-grade water. The concentration and purity of the DNA was determined by Nano-Drop (Thermo-Scientific, Logan, UT).

2.3. Isolation of RNA and cDNA synthesis

The cells were harvested by centrifugation at 12,000 *g* for 5 min, flash-frozen in liquid nitrogen, and stored at −80 °C until further use. Total RNA was isolated from 10 mg dry weight biomass using the SV Total RNA Isolation system protocol (Promega, Madison, WI). cDNA was prepared from 1 μg of total RNA-template with the Verso cDNA kit (Thermo Fisher Scientific, Epson, UK).

2.4. Cloning procedures

PCR products were generated using PrimeSTAR HS PCR PreMix (Takara Bio, Tokyo, Japan) and a TProfessional Thermocycler (Biometra, Goettingen, Germany). The sequences of all primers used are given in Appendix Table A.1. PCR products were gel-purified by AccuPrep Gel Purification Kit (Bioneer, Korea) and cloned into pJET1.2 with the CloneJET PCR cloning kit (Thermo Scientific, USA). The products were then sequenced (ABI PRISM 3100 Genetic Analyzer). An In-Fusion HD Cloning Kit (Clontech, Mountain View, CA) was used for the directional cloning of DNA fragments into target vectors, according to the manufacturer's instructions.

2.5. Nuclear transformation by electroporation

Nuclear transformation was performed using the linearized pSelect100-*NoD12* construct and the high-voltage (11,000 V/cm) electroporation method described by [7]. Construct design was based on the pSelect100 backbone which was kindly provided by Prof. Christoph Benning (Michigan State University).

The pSelect100 construct is composed of the endogenous lipid droplet surface protein (LDSP) promoter [59], which drives transcription of the *aphVII* gene (conferring resistance to hygromycin B) and the 35S CaMV terminator. An open reading frame (ORF) coding for the putative *NoD12* was cloned into the pSelect100 expression vector by substitution (In-Fusion HD Cloning Kit, Clontech, USA) of the *aphVII* gene with the *NoD12* ORF, resulting in a cassette containing the LDSP promoter, *NoD12* ORF and 35S terminator. This cassette, flanked by NotI and BamHI restriction sites, was inserted by ligation into the multiple cloning site of the original pSelect100 and was designated pSelect100-*NoD12* construct (Fig. 2).

2.6. Functional characterization in the yeast Saccharomyces cerevisiae

Saccharomyces cerevisiae strain BY742 (relevant genotype: MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0) was obtained from EUROSCARF (University of Frankfurt). Before transformation, yeast cells were cultivated in 1% (w/v) yeast extract, 2% (w/v) peptone and 2% (w/v) glucose (YPG medium) at 30 °C.

The ORF encoding the putative Δ12-desaturase was amplified and cloned into the pYES2 vector (Invitrogen, Carlsbad, USA) with the Δ12-YES2 primer pair (Appendix Table A.1). The forward primer contained a yeast translation consensus sequence (boldfaced) followed by ATG. The construct was used to transform *S. cerevisiae* strain BY742 by the PEG/lithium acetate method [60]. The yeast cells harboring the empty pYES2 vector were used as controls. Transformants were selected by uracil auxotrophy on yeast synthetic medium (YSM) lacking uracil (Invitrogen). For functional expression, a minimal selection medium containing 2% (w/v) raffinose was inoculated with the Δ12-desaturase transformants and incubated at 30 °C for 24 h in a shaker. YSM (20 mL) was inoculated with raffinose-grown cultures to an OD_{600} of 0.2. Expression was induced by adding galactose to a final concentration of 2% (w/v), and cultures were grown at 24 °C for an additional 48 h. Cells were harvested by centrifugation (1200 *g* for 5 min), freeze-dried, and used for FA analysis.

2.7. Quantitative real-time PCR (qRT-PCR)

qRT-PCR analysis was carried out in optical 96-well plates using the CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad, Hercules, CA). qRT-PCR primer pairs (Appendix Table A.1) were designed for *NoD12* and the housekeeping gene *NcAct* (actin). Each reaction contained 5 μL of iTaq™ Universal SYBR® Green Supermix (Bio-Rad), 20 ng cDNA, and 280 nM of each gene-specific primer pair to a final volume of 10 μL. Further serial dilutions of the cDNAs were prepared,

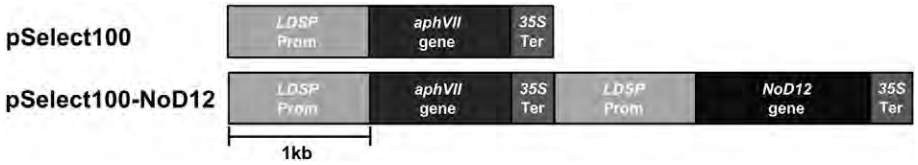

Fig. 2. Transformation constructs. Linearized constructs used for *N. oceanica* CCMP1779 transformation. The pSelect100 construct vector [7] harbors the endogenous lipid droplet surface protein promoter (LDSP Prom) driving transcription of the *aphVII* gene (conferring resistance to hygromycin B) and the 35S CaMV terminator (35S Ter). The pSelect100–*NoD12* construct is based on pSelect100 with an added sequence composed of the LDSP promoter, *NoD12* cDNA ORF (*NoD12* gene) and 35S terminator.

and qRT-PCR was performed with each primer pair to generate a standard curve and to estimate PCR efficiency. PCR cycling conditions consisted of an initial polymerase activation step at 95 °C for 30 s followed by 40 cycles at 95 °C for 5 s and 60 °C for 30 s, and a final melting step at 65–95 °C. Results were analyzed using the $2^{-\Delta\Delta Ct}$ method, a function of the CFX Manager Software v3.0, using the relative expression value of the housekeeping gene as the calibrator. The experiment was performed twice, with two biological replicates and three technical replicates for each sample.

2.8. Lipid extraction and analyses

Lyophilized biomass samples (50 mg) of *N. oceanica* were stirred with0.2 mL dimethyl sulfoxide (DMSO) for 10 min at 80 °C in the presence of argon. Lipids were further extracted and separated into individual classes by thin-layer chromatography (TLC) according to [45]. Neutral and polar lipids were resolved into individual classes by TLC (Silica Gel 60, 10 × 10 cm, 0.25 mm thickness, Merck, Darmstadt, Germany). Polar lipids were separated by two-dimensional TLC using a solvent system of chloroform:methanol:water (65:25:4, v/v/v) for the first direction and of chloroform:methanol:1-isobutylamine: conc. ammonia (65:35:0.5:5, v/v/v/v) for the second direction. Neutral lipids were resolved with petroleum ether:diethyl ether:conc. acetic acid (70:30:1, v/v/v). Spots corresponding to individual lipids were scraped off the plates, and FA profile and content were determined gas chromatography (GC) following direct transmethylation.

2.9. GC analysis of FA composition and content

Direct transmethylation was performed by incubating freeze-dried biomass or individual lipids in dry methanol containing 2% (v/v) H_2SO_4 at 80 °C for 1.5 h under argon atmosphere with continuous stirring. Heptadecanoic acid (C17:0; Fluka, Buchs, Switzerland) was added as an internal standard. FA methyl esters (FAME) were quantified on a Trace GC Ultra (Thermo, Milan, Italy) equipped with a flame ionization detector and a programmed-temperature vaporizing injector as previously described [14].

2.10. Southern blot

Southern blot hybridization was performed using the North2South® Chemiluminescent Hybridization and Detection Kit (Thermo Scientific) according to the manufacturer's instructions. Briefly, genomic DNA (5 μg) was digested with BglII (NEB) endonuclease, desalinated by EtOH precipitated and separated by electrophoresis on a 0.7% agarose (Seakem, Lonza) gel. After depurination with 250 mM HCl, incubation in denaturation solution (0.5 M NaOH, 1.5 M NaCl) and neutralization with 0.5 M Tris–HCl pH 7.5, 1.5 M NaCl, the gel was equilibrated with 20× SSC (saline-sodium citrate). After overnight capillary transfer of DNA from the gel to a Magnagraph nylon membrane (GE Water & Process Technologies, USA) in 20× SSC buffer, the membrane was rinsed with 2× SSC and baked for 2 h at 80 °C. The membrane was blocked, hybridized and washed, and bands were detected exactly according to the kit manual. Chemiluminescent detection was performed using a Microchemi camera (Bioimaging System, Israel). The probe was synthesized by PCR using primers: 94_Nanno_D12_Ex2_Fr (5′GCTCCCTACGGC GATATCCTC3′) and Δ12 des RP (5′CTATGCCCGCTGCTTGTAGAATACC3′) with plasmid DNA as the template and biotinylated dUTP (Thermo Scientific) added to a final concentration of 50 nM. Efficiency of probe-labeling was verified by electrophoresis on an agarose gel and comparing molecular weights of labeled and unlabeled PCR products.

2.11. Statistical analysis

Student's t test for significance with a confidence interval of 95% was applied using the JMP (http://www.jmp.com) statistical software.

3. Results

3.1. Identification of a putative microsomal Δ12-desaturase from N. oceanica

Using the *N. oceanica* CCMP1779 genomic data, available in the genome browsers of the Benning group at Michigan State University (//benning-linux.bch.msu.edu/cgi-bin/gb2/gbrowse/Nannochloropsis_oceanica_CCMP1779_v1/) and the National Center for Biotechnology Information (NCBI), we identified the 1576-bp genomic sequence of the putative microsomal Δ12-desaturase gene (designated *NoD12*) containing an ORF of 1317 bp encoding a polypeptide of 438 amino acids. The deduced polypeptide sequence contained a Δ12-FADS-like conserved domain similar to FAD2 in *Arabidopsis* (Appendix Fig. A.1.). Multiple protein-sequence alignment of the deduced *NoD12* with related microsomal Δ12-desaturases is shown in Fig. 3A. The deduced protein showed high sequence similarity to previously characterized Δ12-desaturases from various organisms, including the green microalgae *Chlamydomonas reinhardtii* (47% identity; GenBank: ACF98526.1; [53]) and *Chlorella vulgaris* (44% identity; GenBank: ACF98528.1; [61]), and *Arabidopsis thaliana* (45% identity; GenBank: AAA32782.1; [62]). Analysis of the deduced amino acid sequence of this gene revealed the presence of the three conserved His-rich motifs essential for desaturase activity [63,64]: the HXXXH motif (HECGH, 162–166), the HXXHH motif (HGKHH, 198–202), and the HXXHH motif (HVCHH, 375–379), at the carboxyl terminus (Fig. 3A). Moreover, in silico analysis of the putative *NoD12* sequence predicted five transmembrane domains (http://www.cbs.dtu.dk/services/TMHMM/) along with an ER subcellular-localization prediction (Plant-mPLoc; [65,66]). Phylogenetic analysis using more distantly related algal and plant plastidic and ER Δ12-desaturases showed that *NoD12* is more similar to the ER-localized desaturases from algae and plants than to the plastidic desaturases, confirming its affiliation to the microsomal Δ12-desaturases (Fig. 3B).

To amplify the 1317-bp *NoD12* ORF, PCR primers (Appendix Table A.1) and reactions were designed using the CCMP1779 genome data and functional gene annotation [7]. The ORF encoding the putative *NoD12* was isolated from cDNA templates which were synthesized from RNA extracted from an exponentially growing culture of *N. oceanica* CCMP1779.

3.2. Functional expression of the putative N. oceanica microsomal Δ12-desaturase (NoD12) in the yeast transformation system

To elucidate the function of the putative *NoD12*, we expressed the cloned gene in *S. cerevisiae*. The amplified ORF was cloned into the yeast expression vector pYES2 to give a construct designated pYNoD12. *S. cerevisiae* strain BY742 was transformed with either pYNoD12 or the empty vector pYES2. The FA profiles of the transformed yeast were evaluated by GC analysis. The yeast cells transformed with the empty vector pYES2 showed the FA composition typical for *S. cerevisiae* (16:0, $16:1^{\Delta 9}$, 18:0, and $18:1^{\Delta 9}$) (Fig. 4A). Expression of pYNoD12 resulted in the appearance of an additional peak, which was identified as LA ($18:2^{\Delta 9,12}$). The production of LA in the yeast cells transformed with pYNoD12 was associated with a concomitant decrease in OA ($18:1^{\Delta 9}$), as expected for a substrate–product relationship (Fig. 4B).

3.3. Overexpression of the endogenous microsomal Δ12-desaturase (NoD12) in N. oceanica CCMP1779

We used the nuclear transformation system developed by Vieler et al. [7] to create transgenic *N. oceanica* overexpressing *NoD12*. A cassette containing the LDSP promoter, *NoD12* ORF and 35S terminator was inserted into the multiple cloning site of the original pSelect100 and was designated pSelect100–*NoD12* (Fig. 2). The pSelect100–*NoD12* construct was used for the transformation of *N. oceanica* CCMP1779 via electroporation at a field strength of ~11,000 V cm^{-1}

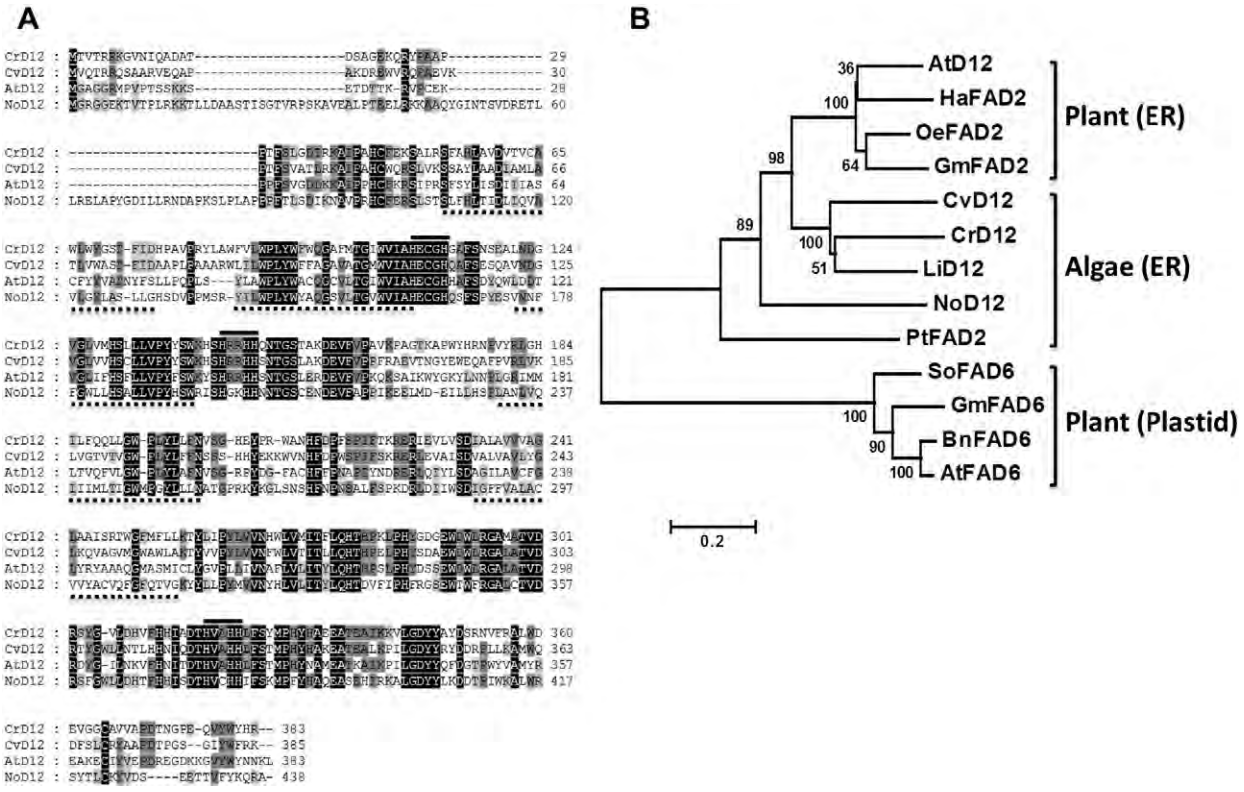

Fig. 3. Microsomal Δ12-desaturases protein alignment and phylogeny. (A) Sequence comparison of the deduced amino acid sequence of the *N. oceanica* CCMP1779 microsomal Δ12-desaturase (*NoD12*) and those of known and putative microsomal Δ12-desaturases in *A. thaliana* (AtD12; GenBank: ACF98526), *C. reinhardtii* (CrD12; GenBank: ACF98526) and *C. vulgaris* (CvD12; GenBank: ACF98528). For alignment, the ClustalW program (EMBL-EBI; Larkin 2007) was used (gap opening 10, gap extension 0.2). The conserved amino acids are white on a black background. The three conserved His motifs are indicated by overlying solid lines and the potential transmembrane domains are marked with dashed underlining. (B) Phylogenetic tree of *N. oceanica* microsomal Δ12-desaturase and some other functionally characterized Δ12-FA desaturases (FAD). The tree was constructed by the neighbor-joining method using MEGA5 software [67]. The protein sequences (with GenBank accession nos.) used for the analysis were: *N. oceanica* (*NoD12*: NannoCCMP1779_10636-mRNA-1), *C. reinhardtii* (CrD12: ACF98526), *C. vulgaris* (CvD12: ACF98528), *P. tricornutum* (PtFAD2: AAO23564), *Lobosphaera incisa* (LiD12: ADB81954), *A. thaliana* (AtD12: AAA32782), *Olea europaea* (OeFAD2: AAW63040), *Helianthus annuus* (HaFAD2: AAL68981), *Glycine max* (GmFAD2: BAD89861), *Spinacia oleracea* (SoFAD6: CAA55121), *Glycine max* (GmFAD6: AAA50158), *Brassica napus* (BnFAD6: AAA50157), *A. thaliana* (AtFAD6: AAA92800). The numbers indicate bootstrap values.

[7,24] and resulted in independent hygromycin B-resistant (50 μg mL^{-1} hygromycin B) colonies at an efficiency of 0.5–1 × 10^{-6} (no. of colonies generated per electroporated cell using 3 μg DNA).

One month after transformation, 30 resistant colonies were picked from the selection plates and transferred to fresh hygromycin B (50 μg mL^{-1}) plates or liquid medium. Genomic DNA was isolated from these strains and the presence of the transgene cassette was verified by genomic PCR using two independent sets of primers (Fig. 5A and B). The first set amplified a transformant-unique 447-bp genetic fragment which encloses a short part of the *NoD12* carboxyl terminus and the 35S terminator. This PCR-amplified fragment was detected in the three presented transformants (C21, C22 and C23), but not in the wild-type strain of *N. oceanica* (Fig. 5A). The second set of primers was designed to include an intron, resulting in two different-sized products, which enabled distinguishing between the endogenous genomic DNA fragment (537 bp) and the cDNA fragment (278 bp) introduced by genetic engineering. pSelect100–*NoD12*-transgenic strains C21, C22 and C23 showed both PCR fragments and, as expected, the engineered cDNA fragment could not be detected in the wild type (Fig. 5B). To confirm stable integration, the selected lines were grown for 3 months with continuous redilution and selection on antibiotic-containing medium. The transformed strains maintained their resistance to hygromycin B for more than 6 months in such continued cultivation in liquid medium (data not shown). These three *NoD12*-transformed strains were also subjected to Southern blot analysis (Appendix Fig. A.2). All transformants contained at least one additional copy of the transformed gene.

The engineered strains C21, C22 and C23, and the wild-type strain were further analyzed by qRT-PCR to determine *NoD12* expression levels (Fig. 5C,D). Expression levels of the transgene were quantified in the early stationary growth stage (Fig. 5C), and after 5 days of nitrogen starvation (Fig. 5D). In the early stationary growth stage, all transgenic strains showed two- to threefold higher levels of *NoD12* expression compared to the wild type without showing any negative effect on growth (Appendix Fig. A.3). Under conditions of nitrogen starvation, the transgenic strains showed 11 to 12-fold higher levels of *NoD12* expression compared to the wild type (Fig. 5D); notably none of the growth parameters were significantly affected and all lines gained similar biomass (1.2 g L^{-1} on average; not shown). This elevated transgene expression under nitrogen starvation is in agreement with the LDSP promoter driving higher expression levels under these conditions [59]. The highest levels of transcript accumulation were observed in strains C23 and C22, which were selected for further lipid characterization.

3.4. FA profiling of wild-type and transgenic N. oceanica overexpressing Δ12-desaturase under conditions of different N availability

Under nitrogen starvation, besides a profound increase in 16:0 and 16:1 and decrease in EPA, the FA profile of wild-type *N. oceanica* featured relatively high levels of OA, while the other C18 PUFA precursors for AA and EPA biosynthesis were present at very low levels [14]. Transcriptional downregulation of *NoD12* during stress is a plausible explanation for this observation. We therefore studied the importance

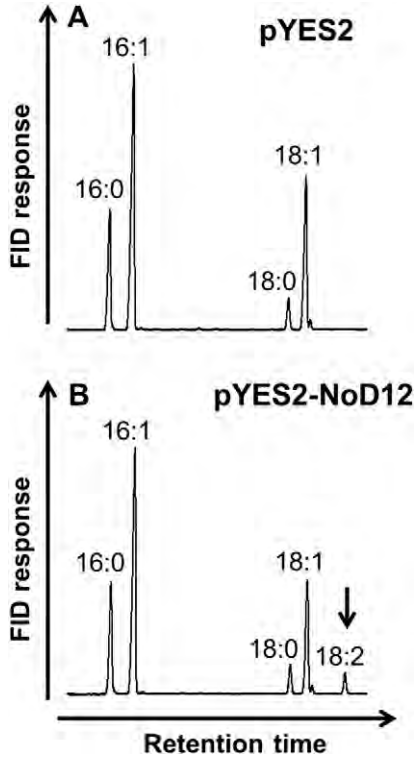

Fig. 4. Functional characterization of *NoD12* in *S. cerevisiae*. Functional characterization of *N. oceanica* microsomal Δ12-desaturase (*NoD12*) by heterologous expression in yeast *S. cerevisiae* and GC-FID analysis of fatty acid composition. *NoD12* was expressed in yeast cells (BY742) under the control of the galactose-inducible GAL promoter present in the pYES2 expression vector (pYES2-*NoD12*; B), or as a control, yeast cells transformed with the empty pYES2 vector (A). The production of LA ($18{:}2^{\Delta 9,12}$) in transgenic yeast expressing *NoD12* is apparent (solid arrow).

of *NoD12* in PUFA biosynthesis by its overexpression under stress conditions, to determine whether the desaturation step performed by the *NoD12* enzyme is in fact rate limiting for channeling OA into LC-PUFA biosynthesis and whether it can be altered by overexpression. We compared the FA composition and content of the wild type and two *NoD12*-overexpressing strains (C22, C23) of *N. oceanica* in different growth phases and under different nitrogen availabilities. OA accumulated to lower percentages in the FA profile of the *NoD12*-overexpressing strains (C22, C23) compared to the wild type under all conditions examined (Fig. 6, Table 1). Remarkably, under nitrogen starvation, the percentage of OA out of total FA was significantly lower in the transgenic lines (~4%) relative to the wild type (~11%) (Table 1). Consequently, the engineered strains displayed four times higher LA percentage than the wild type under N starvation. The proportion of LA, the immediate Δ12-desaturase product, increased most conspicuously in the stationary phase and in the nitrogen-starved cultures in line with using the stress-activated LDSP promoter and formation of lipid droplets under these conditions [59]. Notably, downstream in the LC-PUFA-biosynthesis pathway, AA (20:4 $n-6$) also increased by 50%–75% in *NoD12*-overexpressing strains compared to the wild type under nitrogen starvation and stationary growth conditions.

3.5. Fatty acid composition of the individual lipid classes in wild-type and transgenic N. oceanica overexpressing Δ12-desaturase under nitrogen starvation

To determine whether *NoD12* overexpression leads to altered FA composition in specific lipid classes, we analyzed FA composition in the individual lipid classes in wild-type *N. oceanica* and in one of the *NoD12*-overexpressing strains (C22). This analysis was performed with the N-starved cultures (5 days), which demonstrated the most significant differences in FA composition upon *NoD12* overexpression. The difference in the relative percentage of OA was most conspicuous in PC, comprising 40.5% and 4.2% of TFA, in the wild type and the C22 strain, respectively. Conversely, LA increased most significantly in PC of C22, reaching 41.6%, as compared to 9% in the wild type strain, supporting that this phospholipid is a major site for Δ12-desaturation [63,68]. Other extraplastidial lipids, albeit to a lesser degree, also showed substantial reductions in OA percentage: in phosphatidylethanolamine (PE), from 9.3% to 2.1%, in diacylglyceroltrimethylhomoserine (DGTS), from 4.4% to 1.1%, and in phosphatidylinositol (PI), from 12.7% to 8.2%, in C22 compared to the wild type, with corresponding increases in LA contents in those lipids in the engineered strain.

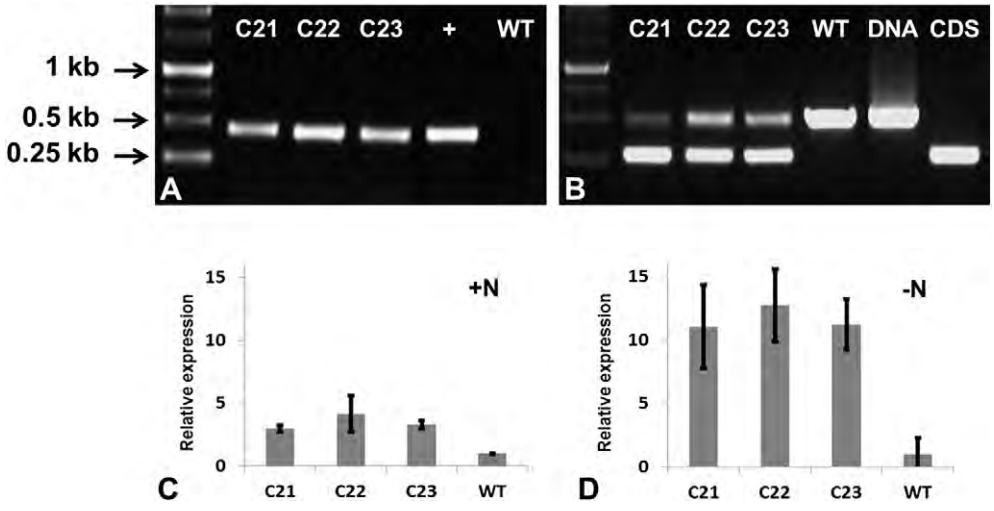

Fig. 5. Overexpression of the endogenous *NoD12* in *N. oceanica*. (A) Genomic PCR of transgenic strains C21, C22, C23, pSelect100-*NoD12* (+) and the wild type (WT). The expected PCR product is a 447-bp fragment which encloses a short part of the *NoD12* C terminus and the 35S terminator. (B) Genomic PCR of transgenic strains C21, C22, C23, the WT, genomic *NoD12* (DNA) and pSelect100-*NoD12* (CDS). In the transgenic strains, there were two expected PCR products: endogenous DNA fragment (537 bp) and the transformed cDNA fragment (278 bp). (C, D) Quantitative RT-PCR analysis of *NoD12* expression in transgenic strains C21, C22, C23 and the WT. The experiment was performed using three independent transgenic strains and values are the average of two independent experiments in which early stationary growth stage (panel C; +N) and nitrogen starved (panel D; −N) cultures were analyzed. Fold-change values are relative to the *Act2* reference gene and WT expression was set to 1.

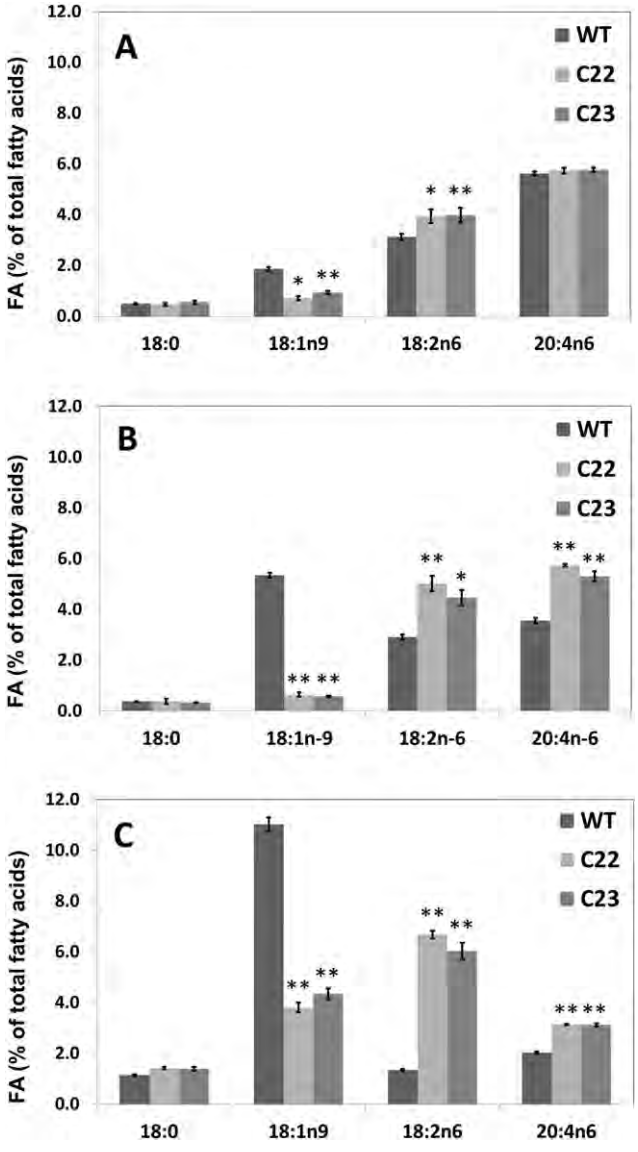

Fig. 6. Fatty acid profiling of transgenic *N. oceanica* overexpressing *NoD12*. Percentage of selected fatty acids in *N. oceanica* wild-type (WT) and transgenic *NoD12*-overexpressing *N. oceanica* strains (C22 and C23) in the early stationary growth phase (A), stationary growth phase (B), and after 5 days nitrogen starvation (C). Values are the average of three separate biological samples and two technical replicates for each. Error values show ± standard deviation (±SD). One way t-test was performed (JMP); * and ** indicate significant differences between WT and the transgenic line (C22, C23) at 95% confidence level (**$p < 0.001$, *$p < 0.01$).

Remarkably, an apparent change was also evident in TAG, with OA decreasing from 10.9 to 4%, and LA increasing from 1 to 5.6%, in the WT and line C22, respectively. A similar trend was documented in TAG of C23. Notably, the transgenic strains C22 and C23 also accumulated slightly higher absolute levels of AA and EPA in their TAG (Table 2, Appendix Fig. A.4.). The 18:3 $n-6$ and 20:3 $n-6$ fatty acids (intermediates in the LC-PUFA biosynthesis) were found at very low levels in most lipid classes, except in PC, where 18:3 $n-6$ constituted approximately 1% in the WT and in the transgene, and in PE, where 20:3 $n-6$ constituted 1.4% in the WT and 2.6% in the transgene. Remarkably, a conspicuous rise in the proportion of AA (20:4 $n-6$) was determined in PE as well as in DGTS of the transgene line as compared to the WT, indicating that overexpression of *NoD12* exerted a strong effect on conversion of 18:1 via the entire ω-6 pathway toward AA.

4. Discussion

The microalga *Nannochloropsis* has high biotechnological value, with applications in aquaculture and significant potential for LC-PUFA or biofuel production. The recent publication of several *Nannochloropsis* species' genomes and transformation protocols has enabled metabolic engineering of these species with the aim of increasing their market success and economic exploitation potential [69]. Here we present the first approach for metabolic engineering of the PUFA biosynthesis pathway in *N. oceanica* by stable integration and stress-induced expression of an endogenous Δ12-desaturase, leading to enhanced production of $n-6$ PUFA, LA and AA, under nitrogen starvation and their incorporation into the algal TAG fraction. A recent successful approach for LC-PUFA metabolic engineering in microalgae, using heterologous microalgal elongase and desaturase, was demonstrated in *Phaeodactylum tricornutum*, whereby the co-expression of an acyl-CoA-dependent Δ6-desaturase with the Δ5-elongase from the picoalga *Ostreococcus tauri* led to significant DHA production at the expense of EPA [70].

However, heterologous genes introduced into the nuclear genome, can often be poorly expressed in recombinant microalgae, due to epigenetic suppression, lack of promoter or enhancer elements, or poor translation of foreign mRNA lacking the codon bias found in the transformed algae [71–73]. The use of endogenous genes and promoters allows taking advantage of species-specific factors, such as codon usage, localization signals and regulatory promoter motifs for higher expression levels and transformation success [59,72,73]. The availability of efficient nuclear transformation technologies along with genomic data and a stress-inducible promoter provide a promising approach for the development of *N. oceanica* into a robust model for oleaginous microalga [7,24]. Those techniques now permit detailed gene-function analysis

Table 1
Major fatty acid composition (% of total fatty acids) and content of *N. oceanica* strains: wild type (WT) and two *NoD12*-overexpressing strains (C22, C23) in the early stationary growth phase (A), stationary growth phase (B) and after 5 days of nitrogen starvation(C).

Growth phase	Strain	Fatty acid composition (% of total fatty acids)[a]							
		14:0	16:0	16:1	18:1$n-9$	18:2$n-6$	20:4$n-6$	20:5$n-3$	TFA (% DW)
A	WT	6.5 ± 0.2	21.0 ± 0.3	25.6 ± 0.2	1.9 ± 0.1	3.1 ± 0.1	5.6 ± 0.1	30.3 ± 0.4	10.9 ± 0.8
	C22	6.7 ± 0.1	21.1 ± 0.3	23.5 ± 0.2	0.7 ± 0.1*	4.0 ± 0.3*	5.8 ± 0.1	30.8 ± 0.3	12.5 ± 0.4
	C23	6.6 ± 0.2	20.8 ± 0.3	23.6 ± 0.5	0.9 ± 0.1**	4.0 ± 0.3**	5.8 ± 0.1	29.7 ± 0.3	10.2 ± 0.8
B	WT	7.0 ± 0.1	21.4 ± 0.1	29.9 ± 0.2	5.3 ± 0.2	2.9 ± 0.1	3.5 ± 0.1	25.4 ± 0.1	25.5 ± 0.7
	C22	7.1 ± 0.1	21.8 ± 0.1	28.9 ± 0.1	0.6 ± 0.1**	5.0 ± 0.1**	5.7 ± 0.1**	26.6 ± 0.1	22.4 ± 0.7
	C23	7.2 ± 0.1	22.0 ± 0.2	28.8 ± 0.6	0.6 ± 0.1**	4.5 ± 0.1*	5.3 ± 0.2**	27.1 ± 0.3	22.0 ± 0.8
C	WT	5.7 ± 0.2	40.3 ± 0.5	30.0 ± 0.4	11.0 ± 0.3	1.4 ± 0.1	2.0 ± 0.1	6.6 ± 0.5	28.5 ± 1.2
	C22	5.9 ± 0.1	42.0 ± 0.1	28.6 ± 0.4	3.8 ± 0.2**	6.7 ± 0.1**	3.1 ± 0.1**	6.0 ± 0.1	30.6 ± 2.4
	C23	5.9 ± 0.2	42.0 ± 0.5	28.9 ± 0.4	4.3 ± 0.2**	6.0 ± 0.3**	3.1 ± 0.1**	6.0 ± 0.2	31.0 ± 4.2

[a] Values are the average of three separate biological samples and two technical replicates for each. Error values show ± standard deviation (±SD). One way t-test was performed (JMP); * and ** indicate significant differences between WT and the transgenic line (C22, C23) at 95% confidence level (**$p < 0.001$, *$p < 0.01$).

Table 2
Fatty acid composition and cell dry weight content of individual lipid classes of wild-type (WT) and *NoD12*-overexpressing (strain C22) *N. oceanica* after 5 days of nitrogen starvation.

Lipid class	Strain	Fatty acid composition of individual lipid classes (% of total fatty acid)[a]								Lipid cont.
		14:0	16:0	16:1	18:0	18:1*n* − 9	18:2*n* − 6	20:4*n* − 6	20:5*n* − 3	μg mg^{-1} DW
MGDG	WT	±12.90.8	15.1 ± 1.1	8.4 ± 0.4	1.2 ± 0.6	1.2 ± 0.3	0.8 ± 0.1	3.0 ± 0.3	55.5 ± 1.1	6.2
	C22	13.6 ± 0.2	16.2 ± 1.5	8.6 ± 0.7	1.1 ± 0.4	0.9 ± 0.3	1.6 ± 0.1	3.6 ± 0.2	53.2 ± 2.3	5.5
DGDG	WT	5.5 ± 0.4	30.8 ± 0.8	19.7 ± 0.5	1.6 ± 0.8	1.0 ± 0.1	1.1 ± 0.1	0.9 ± 0.1	38.0 ± 0.8	3.1
	C22	6.0 ± 0.2	30.7 ± 1	19.1 ± 0.2	1.5 ± 0.4	0.8 ± 0.1	2.3 ± 0.2	1.0 ± 0.0	36.7 ± 1.1	3.1
SQ	WT	4.7 ± 0.1	49.5 ± 2.3	35.1 ± 2.3	2.3 ± 1.5	1.5 ± 1.2	1.5 ± 1.2	1.2 ± 0.9	2.7 ± 1.4	1.4
	C22	4.9 ± 0.4	48.1 ± 2	34.8 ± 1.7	2.9 ± 1.4	1.2 ± 0.6	2.4 ± 1.3	0.9 ± 0.2	1.9 ± 0.1	1.3
PG	WT	2.4 ± 0.4	39.0 ± 3.1	10.6 ± 1.6	4.2 ± 1.8	2.5 ± 0.6	1.3 ± 0.3	4.9 ± 1.8	26.4 ± 3.0	3.6
	C22	2.8 ± 0.5	40.3 ± 3.5	12.8 ± 1.6	3.9 ± 1.6	2.7 ± 1.2	2.3 ± 0.4*	3.0 ± 1.1	27.0 ± 5.0	3.4
DGTS	WT	7.0 ± 0.4	19.2 ± 1.6	11.8 ± 0.9	2.0 ± 0.7	4.4 ± 0.3	1.6 ± 0.1	5.8 ± 0.6a	46.0 ± 2.2	1.2
	C22	7.9 ± 0.2	20.5 ± 0.7	10.5 ± 0.3	2.0 ± 0.3	1.1 ± 0.1**	6.8 ± 0.1**	8.9 ± 0.4a**	38.9 ± 1.3	1.3
PC	WT	1.2 ± 0.5	16.0 ± 2.2	18.5 ± 0.7	1.5 ± 1.1	40.5 ± 1.3	9.0 ± 0.5	7.1 ± 0.6	4.1 ± 0.7	2.5
	C22	1.2 ± 0.1	20.6 ± 0.2	12.8 ± 0.4	1.5 ± 0.4	4.2 ± 0.5**	41.6 ± 0.8**	8.6 ± 0.2	4.2 ± 0.4	1.8
PE	WT	1.7 ± 0.8	11.5 ± 4.5	4.7 ± 1.4	3.7 ± 1.5	9.3 ± 0.8	2.7 ± 0.2	40.2 ± 4.9	17.7 ± 2.4	1.5
	C22	1.8 ± 0.5	11.3 ± 1.6	6.4 ± 0.6	3.8 ± 1.1	2.1 ± 0.6**	8.0 ± 0.4**	45.3 ± 1.7**	15.3 ± 1.2	1.6
PI + PA	WT	5.1 ± 0.5	45.3 ± 1.6	28.2 ± 2.1	3.2 ± 1.5	13.4 ± 1.0	1.2 ± 0.7	0.7 ± 0.4	1.2 ± 0.8	3.5
	C22	5.0 ± 0.8	41.9 ± 1.8	30.1 ± 0.9	3.2 ± 1.7	8.2 ± 1.0**	5.1 ± 0.7	1.0 ± 0.2	2.7 ± 0.6	3.7
TAG	WT	5.8 ± 0.2	43.6 ± 0.6	32.1 ± 1.0	1.3 ± 0.1	10.9 ± 0.2	1.0 ± 0.1	1.0 ± 0.2	2.6 ± 0.2**	254.8
	C22	6.0 ± 0.2	44.8 ± 0.7	30.6 ± 0.7	1.5 ± 0.1	4.0 ± 0.3**	5.6 ± 0.3**	2.3 ± 0.1**	3.2 ± 0.2**	260.0

[a] Values are the average of three separate biological samples. Error values show ± standard deviation (±SD); * and ** indicate a significant differences between WT and the transgenic line C22 at 95% confidence level, (**p < 0.001, *p < 0.01).

and metabolic engineering of biosynthetic pathways, as exemplified here.

Flux regulation in the LC-PUFA pathway in microalgae has not been fully characterized. It is evident from comparisons of the FA profiles of nitrogen-starved vs. nitrogen-replete cultures of *Nannochloropsis* that LC-PUFA synthesis is arrested at OA under conditions of nitrogen starvation. The major C16 saturated and monounsaturated fatty acids as well as OA accumulate in the augmented TAG fraction with a commensurate decrease in the share of EPA-enriched membrane lipids [41]. This observation indicates that expression of the enzymes involved in LC-PUFA biosynthesis is downregulated by nitrogen depletion, as corroborated by transcriptomic studies [20]. The relatively high amounts of OA found in the total cellular lipids of the nitrogen-starved cultures indicate that Δ12 desaturation of OA to LA is the first and probably committing step that is strongly negatively affected by transcriptional downregulation. Taken this into account, we cloned, characterized and overexpressed the Δ12-desaturase from *N. oceanica* under control of the *LSDP* promoter.

Numerous FAD2 (Δ12-desaturase) genes from microalgae have been characterized [53–56], however, to the best of our knowledge, none of them has been overexpressed in eukaryotic algae as an endogenous gene under an endogenous promoter, besides the purposes of cellular localization [54]. The microsomal Δ12-desaturase enzyme associated with ER-localized LA biosynthesis has not been previously functionally characterized in *Nannochloropsis*. Similar to the previously characterized membrane-bound desaturases, Δ12-desaturase of *N. oceanica* contains three His boxes that most likely coordinate the di-iron center of the active site, where substrate oxidation occurs and are critical for desaturase activity [63,64,74]. The deduced protein coding by *NoD12* exhibits highest sequence homology to the microsomal-like FA desaturase of the brown alga *Ectocarpus siliculosus* (57%) and to the putative FA desaturases of a number of *Phytophthora* species (56%), all of which belong to the heterokonts (Stramenopiles) in the kingdom Chromalveolata [8,75]. Notably, it also exhibits high homology to microsomal Δ12 desaturases from the green algae and higher plants (approximately 45% identity). Interestingly, the microsomal Δ12-desaturases of the diatom *P. tricornutum* showed lower homology, only 33% identity, to *NoD12*, even though both organisms belong to the same evolutionary group of Stramenopiles [8]. Recent genome-wide analyses have identified a large number of green algal genes that were probably transferred to some chromalveolates [8,76]; such knowledge derived from genome-wide analyses may provide a better understanding of the evolution of Stramenopiles.

We first confirmed the functionality of the protein encoded by the cloned *NoD12* gene i.e. conversion of OA to LA by expression in *S. cerevisiae* that lacks PUFA. For this reason, a *S. cerevisiae* expression system is often employed for functional characterization of enzymes engaged in PUFA biosynthesis [43,55,77]. Functional characterization of *NoD12* in *S. cerevisiae* showed that this enzyme converted endogenous yeast OA to LA but did not accept palmitoleic acid (16:1 *n* − 7) as a substrate. This has also been observed, for example, with microsomal Δ12-desaturases from the filamentous fungus *Rhizopus arrhizus* [78] and olive (*Olea europaea*) [46]. However, with Δ12-desaturases of some other higher plants this characteristic preference for OA was not observed, and 16:1 also served as a substrate for this enzyme [48,79,80]. This difference in acyl group specificity of FAD2 remains to be explained.

By using the transformation method described in [7], we obtained stable transformant colonies of *N. oceanica* at high efficiency; the transgene lines showed significantly higher expression of *NoD12* than the wild type, a difference that was most pronounced under nitrogen starvation. This is in agreement with characterization of LDSP which demonstrated higher abundance under nitrogen-starvation conditions in correlation with TAG content [59].

Further, we compared the FA composition and content of the wild-type and *NoD12*-overexpressing *N. oceanica* strains to test whether overexpression of *NoD12* driven by the stress-activated promoter can overcome the metabolic bottleneck at the OA-desaturation level for persistent LC-PUFA biosynthesis in *N. oceanica* under nitrogen starvation conditions. All of the *NoD12*-overexpressing lines indeed produced more LA and less OA than the wild type, and this difference was most dramatic under nitrogen starvation. We also found that the downstream product of the ω-6 LC-PUFA biosynthesis pathway, AA, accumulated to 50–75% higher concentration in the *NoD12*-overexpressing strains compared to the wild type under nitrogen starvation and in the stationary growth phase, though this increase was significantly less than that of LA. The predominant accumulation of LA in the FA profile of the engineered strains is in agreement with suppression of the consecutive steps converting LA to C20 LC-PUFA

under nitrogen starvation, preventing a robust flow of LA toward AA and EPA.

For a better understanding of the consequences of enhanced *NoD12* activity at the individual lipid level, we profiled the FA composition in the individual lipid classes in the wild-type and C22-transgenic *N. oceanica* strains under nitrogen starvation conditions, where we found the highest conversion of OA to LA in the TFA analysis. OA accumulated to the highest percentage in PC in the wild type, concurrently with a sharp increase in LA in PC of the transgenic strain C22. This phenotype confirms that this phospholipid is indeed a major site for Δ12-desaturation in *Nannochloropsis* [68]. This phenomenon was also significant, but to a lesser extent, in other extraplastidial membrane lipids. The accumulation of OA in PC under nitrogen starvation allow suggesting that the newly synthesized OA enters the ER-localized pathway of *de novo* TAG assembly via PC in line with the recently proposed role of this lipid as a primary acceptor of 18:1 exported from the chloroplast [81]. Furthermore, the contribution of an acyl-exchange reaction at the *sn*-2 position of PC mediated by lyso-PC acyltransferase to the FA flux through this phospholipid has been previously inferred in *Nannochloropsis* sp. [68]. In addition, an acyl-editing mechanism involving PC has been suggested to operate in *N. oceanica* strain IMET1 under conditions of nitrogen starvation, as indicated by substantial alterations in the composition of PC molecular species in the course of starvation [20]. The PC molecular species dominated with those enriched in OA, whereas those PC species with a higher degree of unsaturation decreased [20]. This is in line with our data of the wild-type FA composition of PC, featuring a high percentage of OA and commensurately decreased percentage of LA. Based on the general preference of the *sn*-2 position of PC for C18 FA [63] and the changes in the FA composition of PC in the transgenic lines featuring a high percentage of LA, we can assume that molecular species harboring LA in the *sn*-2 position become the dominant molecular species (e.g. 16:0/18:2, 16:1/18:2). Admittedly, while our GC-FID analysis provided strong evidence to alterations in the acyl group composition in membrane and storage lipids, LC-MS-based approaches would reveal further details on molecular species composition and stereo-specific distribution of acyl groups.

To summarize, we speculate that the observed increases in LA and AA content in the transgenic TAG result from the lipid-remodeling process and consequent enrichment of the acyl-coenzyme A (CoA) pool with LA-CoA, derived from PC, and to a lesser extent with AA-CoA, derived from other polar lipids, presumably from PE as a major site of AA synthesis. As shown in the present work, it is significant and encouraging that upregulation of only one step in the LC-PUFA-biosynthesis pathway leads to a substantial increase in $n-6$

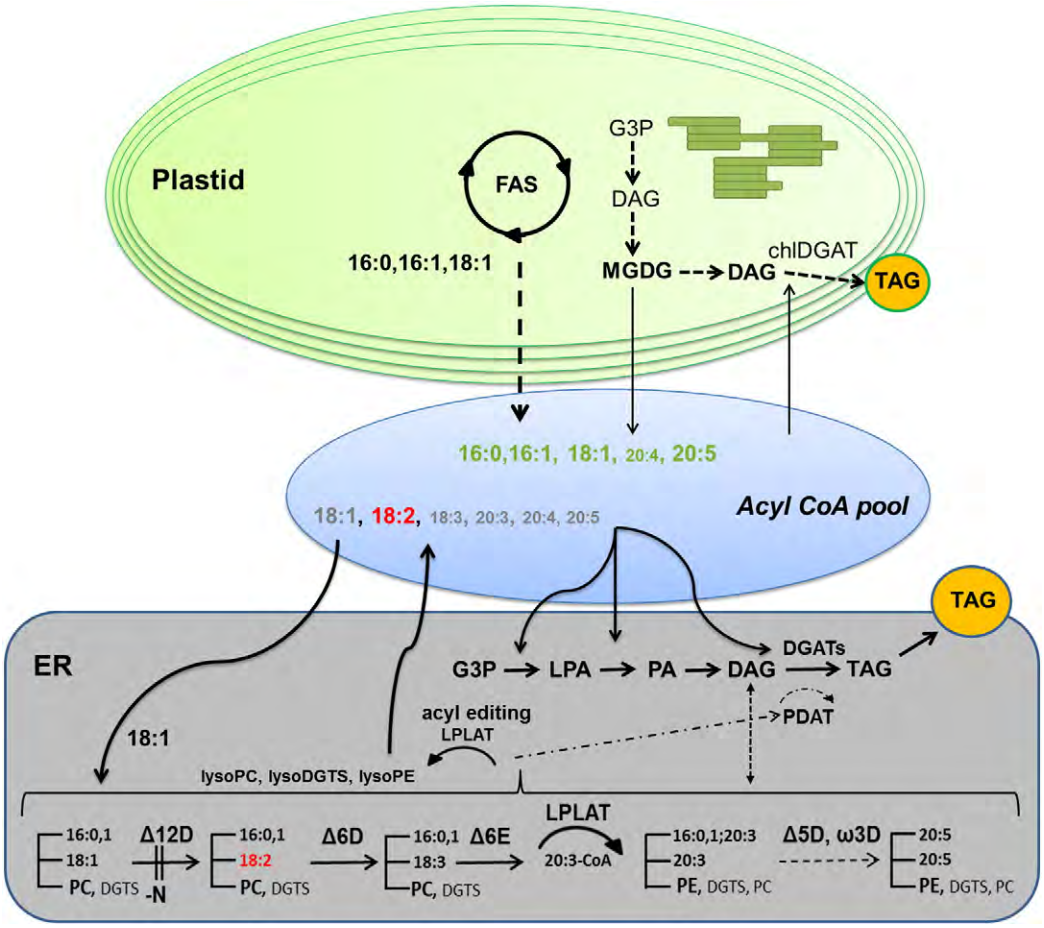

Fig. 7. Simplified scheme of PUFA supply for TAG assembly in *N. oceanica*. Simplified hypothesized scheme of the different routes of acyl group supply for TAG assembly, and convergence of the microsomal LC-PUFA-biosynthesis pathway (based on [20,68,70,81] and the data obtained in the present work). The acyl groups' compartmentalization in the *N. oceanica* cell is speculated with an emphasis on the fate of 18:2, the product of *NoD12* (marked in red). Acyl groups synthesized *de novo* in the chloroplast or cleaved from chloroplast lipids under nitrogen starvation are shown in green. Acyl groups released as a result of extraplastidial lipid remodeling or lipolytic activities in the ER are shown in gray. Abbreviations: G3P, glycerol-3-phosphate; DAG, diacylglycerol; TAG, triacylglycerol; MGDG, monogalactosyldiacylglycerol; FAS, fatty acid synthase; CoA, coenzyme A; LPA, lysophosphatidic acid; PA, phosphatidic acid; PC, phosphatidylcholine; DGTS, diacylglyceroltrimethylhomoserine; DGAT, diacylglycerol acyltransferase; PDAT, phospholipid:diacylglycerol acyltransferase; LPLAT, lyso polar lipid acyltransferase (hypothetical).

PUFA in the TAG fraction. A known example of the oleaginous microalga that is effectively funneling LC-PUFA into TAG under nitrogen starvation conditions is the green microalga *Lobosphaera* (formerly *Parietochloris*) *incisa* [55]. The unique feature of this microalga is upregulation of the entire AA biosynthesis pathway upon transfer to nitrogen starvation thereby providing sufficient pool of LC-PUFA for incorporation to TAG [55,82].

Based on the significant alterations in the PC of engineered strains driven by the *LDSP* promoter under N starvation, we assume that it will be possible to achieve adequate LC-PUFA biosynthesis and consequent deposition in TAG by similar modulation of additional enzymatic steps. A significant role in this process is posited for the efficient polar lipid remodeling, involving extraplastidial acyltransferases and lipases that may enable the transfer and the exchange of intermediates of LC-PUFA biosynthesis between different polar lipids [68,70], as well as their deposition in TAG upon transfer to nitrogen starvation. The complexity and differential cellular compartmentalization of the insufficiently characterized terminal reactions of TAG assembly in *Nannochloropsis* is noteworthy in this respect [20]. Gaining additional insights into this process would provide additional cues for genetic engineering of LC-PUFA deposition in TAG of *Nannochloropsis*. As hypothesized in Fig. 7, cooperative action of desaturases and Δ6 PUFA-elongase, governed by their increased transcription driven by the stress-induced promoter can lead to elevated LC-PUFA levels in TAG assembled under nitrogen starvation. This approach requires cloning, characterization and expression of additional genes by means similar to those presented in this work, namely by preventing their downregulation under nitrogen starvation, as well as fine-tuning of cultivation conditions.

5. Conclusions

In this work, we report the cloning and functional characterization of an *N. oceanica* microsomal Δ12-desaturase (*NoD12*) by heterologous and homologous overexpression. We demonstrate that i) this gene codes an enzyme responsible for the lipid-linked synthesis of LA, predominantly in PC and to a lesser extent in other extraplastidial lipids; ii) expression of *NoD12* driven by LDSP results in conversion of OA to LA which, in *N. oceanica*, is partly funneled through the ω-6 pathway toward AA under stationary growth and N-starvation conditions; iii) in the *NoD12*-overexpressing strains, LA and AA are then partly deposited in TAG. This study provides the first evidence of successful overexpression of an endogenous microsomal Δ12-desaturase in eustigmatophyte microalgae, and demonstrates the possibility of overexpressing homologous genes as a means of modifying the LC-PUFA-biosynthesis pathway in *N. oceanica*.

Conflict of interest

Authors declare no conflict of interest.

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Appendix. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.algal.2015.05.003.

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