Metabolic Engineering Communications 11 (2020) e00142

Contents lists available at ScienceDirect

Metabolic Engineering Communications

journal homepage: www.elsevier.com/locate/mec

Improving lipid productivity by engineering a control-knob gene in the oleaginous microalga *Nannochloropsis oceanica*

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ARTICLE INFO

Keywords:
1-Deoxy-D-xylulose-5-phosphate synthase
Lipid biosynthesis
Metabolic engineering
Oleaginous microalga
Nannochloropsis oceanica

ABSTRACT

Nannochloropsis spp. are promising industrial microalgae for scalable oil production and the lipid production can be boosted by nutrient starvation and high irradiance. However, these stimuli halt growth, thereby decreasing overall productivity. In this study, we created transgenic *N. oceanica* where *AtDXS* gene encoding 1-deoxy-D-xylulose 5-phosphate synthase (DXS) derived from *Arabidopsis thaliana* was overexpressed *in vivo*. Compared with the wild type (WT), engineered *Nannochloropsis* showed a higher CO_2 absorption capacity and produced more biomass, lipids, and carbohydrates with more robust growth in either preferred conditions or various stressed conditions (low light, high light, nitrogen starvation, and trace element depletion). Specifically, relative to the WT, lipid production increased by ~68.6% in nitrogen depletion (~1.08 g L^{-1}) and ~110.6% in high light (~1.15 g L^{-1}) in the transgenic strains. As for neutral lipid (triacylglycerol, TAG), the engineered strains produced ~93.2% more in nitrogen depletion (~0.77 g L^{-1}) and ~148.6% more in high light (~0.80 g L^{-1}) than the WT. These values exceed available records in engineered industrial microalgae. Therefore, engineering control-knob genes could modify multiple biological processes simultaneously and enable efficient carbon partitioning to lipid biosynthesis with elevated biomass productivity. It could be further exploited for simultaneous enhancement of growth property and oil productivity in more industrial microalgae.

1. Introduction

Environmental challenges such as global warming, air pollution and resource depletion have increased the need for a shift away from fossil fuels towards renewable energy. Microalga-based biochemical factories are regarded as an ideal way of sequestering carbon dioxide and producing versatile molecules ranging from therapeutic proteins to biofuels (Hu et al., 2008). CO_2 is captured and incorporated into biomass through microalgal photosynthesis, at an efficiency much higher than that of land plants (Wobbe et al., 2016). However, few natural strains exhibit the traits required in feedstock for sustainable and scalable biofuel production (Scott et al., 2010). Genetic engineering of microalgal species offers a viable means of optimizing crucial processes (Gimpel et al., 2013); however, conventional genetic engineering strategies for enhancing the production of specific metabolites rely on modifying individual genes that encode components of a metabolic pathway. These studies have achieved mixed success in microalgae; in some cases, the abundance or composition of the targeted metabolite can remain largely unchanged. An alternative strategy is engineering metabolic or regulatory nodes, which could modify multiple components of a metabolic pathway simultaneously, for instance by engineering regulators such as transcription factors (Bajhaiya et al., 2017).

In higher plants, isoprenoids are derived via either the methylerythritol phosphate (MEP) or mevalonate (MVA) pathways. Most algae (e.g., *Nannochloropsis* spp.) lack the MVA pathway and rely solely on the MEP pathway localized in chloroplasts for isoprenoid production (Lu et al., 2014a). Enzyme 1-deoxy-D-xylulose 5-phosphate synthase (DXS) catalyzes the rate-limiting step of the MEP pathway, catalyzing the formation of 1-deoxy-D-xylulose 5-phosphate (DXP) via condensation of D-glyceraldehyde 3-phosphate (D-GAP) and pyruvate (Brammer et al., 2011). DXP is then converted into isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) (Withers and Keasling, 2007). IPP and DMAPP are universal precursors for the biosynthesis of a wide range of isoprenoid

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https://doi.org/10.1016/j.mec.2020.e00142
Received 29 May 2020; Received in revised form 6 August 2020; Accepted 19 August 2020

metabolites, such as chlorophylls (Chls), sterols, carotenoids, and a number of phytohormones, which determine cellular properties critical for growth or the production of valuable chemicals (Cazzonelli and Pogson, 2010; Paetzold et al., 2010; Wright et al., 2014). Members of these diverse group participate in a wide variety of biological processes such as photosynthesis, respiration, growth, cell cycle control, and stress tolerance (Estevez et al., 2001), which are relevant to lipid productivity. For example, photosynthesis determines efficiency of CO_2 fixation, thereby the carbon precursor for lipid biosynthesis while growth capacity and stress adaptation determine the overall biomass production under lipid-biosynthesizing-favored conditions (e.g., nitrogen depletion and high light). Therefore, DXS could serve as a control-knob gene of which the manipulation would lead to an increased biosynthesis of valuable chemicals (e.g., lipid, carotenoids, terpenoids) and more robust growth or stress tolerance. DXS genes have been isolated in various organisms (e.g., *Arabidopsis thaliana* (Wright et al., 2014), *Aquilaria* (Xu et al., 2014) and microalga *Botryococcus braunii* (Matsushima et al., 2012)) and engineered to enhance stress resistance in poplars (Wei et al., 2019a), improve the production of terpenoids in *Arabidopsis* (Estevez et al., 2001; Wright et al., 2014), tobacco (Wu et al., 2006), bacteria *Escherichia coli* (Martin et al., 2003), and *Bacillus subtilis* (Xue and Ahring, 2011), and perturb life cycle in potato (Morris et al., 2006). Manipulation of DXS thus offers great opportunities to improve the overall stress tolerance and productivity of value-added chemicals (Chang and Keasling, 2006; Roberts, 2007). However, DXS engineering has been very rarely reported so far in microalgae (Eilers et al., 2016), particularly the perturbation on stress tolerance and lipid productivity in industrial microalgae.

Nannochloropsis spp. are a genus of unicellular photosynthetic microalgae belonging to the heterokonts. These algae are of industrial interest because they grow rapidly and can synthesize large amounts of triacylglycerol (TAG) and high-value polyunsaturated fatty acids (FAs; for example, eicosapentaenoic acid) (Xin et al., 2017). Moreover, they are excellent research models for microalgal systems and synthetic biology, due to their small genome size, simple gene structure (Carpinelli et al., 2014; Vieler et al., 2012), and demonstrated genetic tools, such as gene overexpression (Eric et al., 2018; Kang et al., 2015a; Kaye et al., 2015; Koh et al., 2018), random insertional mutagenesis (Perin et al., 2015), chloroplast genome engineering (Gan et al., 2017), RNAi-based targeted gene knockdown (Ma et al., 2017; Wei et al., 2017), and CRISPR/Cas9-mediated genome editing (Ajjawi et al., 2017; Poliner et al., 2018; Wang et al., 2016). Investigations are accumulating on *Nannochloropsis* spp. regarding to cellular mechanisms of stress-induced TAG synthesis (Li et al., 2014), carbon partitioning (Alboresi et al., 2016; Wei et al., 2019b), sterol metabolism (Lu et al., 2014b), phytohormone function (Lu et al., 2014a; Lu and Xu, 2015) and transcriptional-factor regulation (Kang et al., 2015b; Kwon et al., 2018). Therefore, *Nannochloropsis* spp. can be a premium chassis for sustainable supply of various bioresources for human beings.

Assuming, as indicated by the preceding evidence, that DXS plays gateway role which could bypass a trade-off between production and growth, targeting the industrial oleaginous microalga *Nannochloropsis oceanica*, we create transgenic *N. oceanica* where AtDXS gene encoding 1-deoxy-D-xylulose 5-phosphate synthase derived from *A. thaliana* is integrated into the algal genome. The results demonstrate that engineering a single gateway gene enables efficient carbon partitioning to lipid biosynthesis with boosted environmental tolerance and biomass productivity, which could be further exploited for simultaneous enhancement of growth property and oil productivity in more industrial microalgae.

2. Materials and methods

2.1. Growth conditions

N. oceanica was maintained in the dim light on solid modified f/2 medium and inoculated into fresh medium and cultivated in the organism's preferred physiological conditions (enriched f/2, 25 °C, and 50 μmol·photons·m^{-2}·s^{-1} light) (Lu et al., 2014a). In otherwise environmental conditions, light intensity and medium recipe were set as indicated. Nitrogen deprivation was imposed as previously described (Li et al., 2014).

2.2. Transformation

The AtDXS expression vector was designed as described in the Results section. A codon-optimized version of the AtDXS was inserted into the overexpression vector pMEM01. Vectors were constructed as previous described (Xin et al., 2017). Transformation was conducted by using electroporation as described in our early publications (Wang et al., 2016). Transformants were selected on f/2 agar plate containing 2.5 μg ml^{-1} zeosin.

2.3. Screening and identification of AtDXS expression microalgae

Colonies appeared after approximately three weeks and were typically transferred after 25 days. Individually picked colonies were transferred into liquid culture containing 2.5 μg mL^{-1} zeosin until the culture turned into green color. Total RNA isolation and reverse transcriptional PCR analysis were carried out as previously described (Cui et al., 2018). The Pvcp-AtDXS fragments were amplified by a pair of gene-specific primers VCP-F and AtDXS-R (Table S1). The plasmid pMEM01-AtDXS was used as positive template.

2.4. Phenotyping

Microalgae were cultured routinely in 50 mL conical flasks with 20 mL fresh medium. Growth was monitored by measuring cell number, turbidity (OD_{750}), or dry weight (DW) at indicated intervals as earlier description (Gan et al., 2017). Log-phase cells were collected for oxygen evolution rate measurement using a Clark-type Liquid-Phase Oxygen Measurement System (Chlorolab-2, Hansatech Ltd, UK) (Dall'Osto et al., 2019).

2.5. Metabolic analysis

Microalgae were cultured in column photobioreactor in 100 mL fresh medium bubbling with ambient air. Cells were harvested at the indicated time via centrifugation at 4 °C and 5000 g min^{-1} for 5 min, freeze-dried, and used for lipid analysis. Lipid extraction and TLC analysis of the neutral lipids were performed as described by Yoon (Cui et al., 2018; Xin et al., 2017). For TLC analysis, a mixture of normal hexane/diethyl ether/acetic acid (70/30/1 by volume) was used as the mobile phase for TAG analysis. TAG was detected by iodine vapor and 50% sulfuric acid. The carbohydrate content was measured using the phenol-sulfuric acid method (Dubois et al., 1956). Chl contents were analyzed according to a previous study (Li et al., 2019). In brief, 1 mL algal culture was centrifuged (12,000 *g* for 3 min) and the supernatant was disposed. Cell pellets were resuspended in 1 mL methanol, bead beat with glass beads for 1 min twice, and left in the dark at 60 °C for 15 min. Then the mixture was centrifuged at 12,000 *g* for 15 min to remove cellular debris and the supernatant was used to determine the pigment contents by measuring absorbance at 440 nm, 644 nm, 662 nm, 665 nm, and 750 nm.

2.6. Transcriptional analysis

Mid-logarithmic phase algal cells were collected and washed three times with axenic seawater. Equal numbers of cells were re-inoculated: (i) in nitrogen replete medium (N-replete condition, or N+) and nitrogen-deprived medium (N-depleted condition, or N-) with 50 μmol·photons·m^{-2}·s^{-1} light intensity; (ii) grown under constant light intensities 50 and 200 μmol·photons·m^{-2}·s^{-1}. Cell aliquots were collected for RNA isolation after being transferred to the designated conditions for 24, 48, and 72 h. Three biological replicates of algal cultures were established under each of the above conditions, respectively. To isolate total RNA, cells were harvested by centrifugation at 6000 rpm

for 5 min, frozen in liquid nitrogen, and stored at −80 °C. Total RNA was extracted using an Eastep® Super Total RNA Extraction Kit (Promega, Shanghai). The $2^{-\Delta\Delta CT}$ method was used to quantify relative changes in transcript levels from the qPCR data. Levels of the transcripts under individual treatment at each time point were firstly normalized to actin expression levels. Then the obtained values of each gene were normalized to the values in the control treatments at the corresponding time point. Values are means and standard errors obtained from three experiments. Primers are listed in Table S1.

2.7. Statistical analysis

To ensure reproducibility, the experiments were all performed with at least three biological replicates. Each of the values presented corresponds to a mean ± SD. Statistical analyses were performed using the SPSS statistical package.

3. Results

3.1. Generate transgenic Nannochloropsis oceanica

DXS is the first and committed enzyme of the MEP pathway (Fig. 1a). AtDXS (AT4G15560) was employed and codon-optimized based on the codon frequency in *N. oceanica* (Wang et al., 2014) (Supplementary Dataset 1). The AtDXS vector harbors a full-length *AtDXS* gene and a codon-optimized zeosin resistance (*eBle*) gene, each of which is driven by an endogenous promoter and terminator (Fig. 1b). Specifically, the *AtDXS* gene is driven by the violaxanthin/chlorophyll *a* binding protein promoter (Pvcp) and terminated by the α-tubulin termination (Tα-tub) region. Expression of the *eBle* gene is driven by the β-tubulin promoter (Pβ-tub) and terminated by the violaxanthin/chlorophyll *a* binding protein terminator (Tvcp). The proper *in vivo* functioning of each of these regulatory elements in the algal cells was validated individually. Transcription and translation of the *eBle* gene were verified by selecting transformants on f/2 plates supplemented with 2.5 μg mL^{-1} zeosin. The transcription and mRNA stability of the *AtDXS* gene were validated via reverse transcriptase PCR, which confirmed a significant level of transcription in algal transformants (Fig. 1c). For the transgenic lines, PCR products with a length of 1612 bp were confirmed by sequencing.

3.2. Growth feature of transgenic microalgae under different conditions

In the organism's preferred physiological conditions, proliferation of the transgenic strains was faster than wild-type cells (Fig. S1a). Among all the selected transgenic lines, the cell number was approximately 17.8% more at the maximal than that of the WT at the end of detection (Fig. S1a). Among the transgenic lines, the ones exhibiting fastest growth were selected for further investigations and nominated as AtDXSoe1, AtDXSoe2, and AtDXSoe3 (Fig. 2a). We next examined the growth of the microalgal cells under stress conditions (low light, high light, nitrogen depletion, and trace-element depletion). WT and transgenic cells were cultivated at two different light intensities: low light (LL; 10 μmol·photons·m^{-2}·s^{-1}) and high light (HL; 200 μmol·photons·m^{-2}·s^{-1}). Regardless of light intensities, transgenic strains grew faster than WT (Fig. S1b). Specifically, at the end of the measurement period, the biomass of AtDXSoe3 were ~80.7% and ~171.0% higher than that of WT in LL and HL conditions, respectively (Fig. 2b).

Nitrogen depletion is practically employed to boost lipid accumulation in microalgae. Following nitrogen depletion, compared with the WT, the growth rate of the three transformants was higher (Fig. S1c), which eventually produced 5.0%, 3.3%, and 19.7% more biomass, respectively (Fig. 2b). Trace elements (TEs) starvation occasionally encounters in outdoor microalgal cultivation. Compared to WT, the growth of the transgenic strains was slightly faster than WT in TE-depleted conditions (Fig. S1d). All three transgenic lines (AtDXSoe3 in particular; 36.3% higher than the WT) produced more biomass than WT at the end of detection under such conditions (Fig. 2b). The AtDXS overexpression, particularly AtDXSoe3, therefore appears to grow more robust than WT

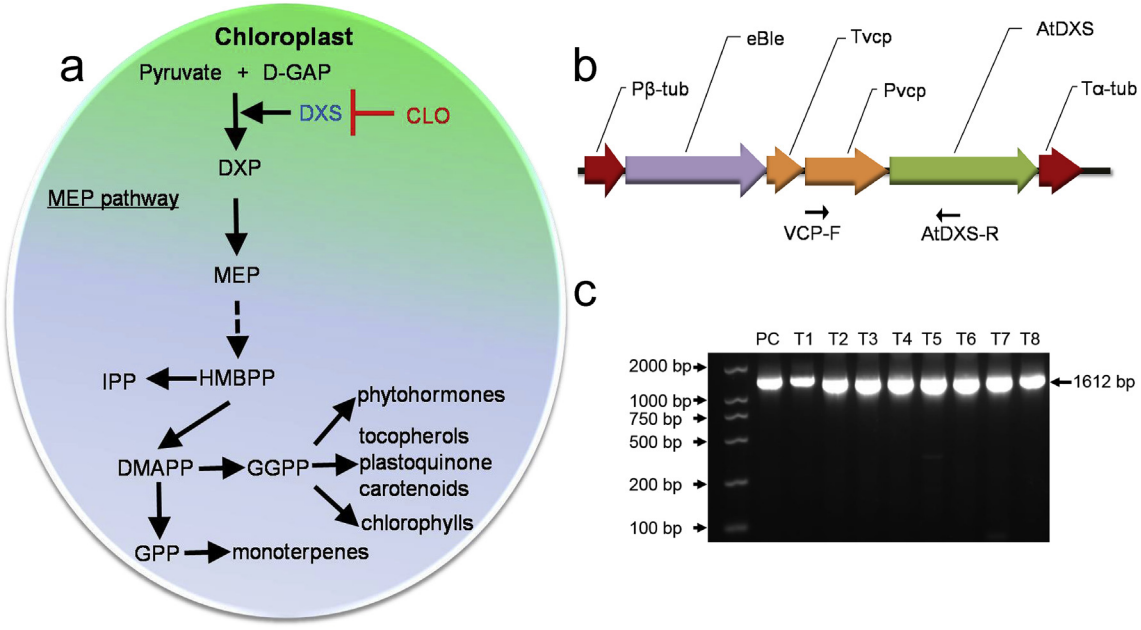

Fig. 1. Overexpression of AtDXS gene in *N. oceanica*. (a) Postulated isoprenoid biosynthesis pathway in *N. oceanica*, and sites of action of inhibitor clomazone (CLO). Enzyme abbreviations: DXS, 1-deoxy-D-xylulose 5-phosphate synthase. Abbreviations for metabolites: GAP, D-glyceraldehyde 3-phosphate; DXP, 1-deoxy-D-xylylose 5-phosphate; MEP, methyl erythritol phoshphate; HMBPP, hydroxymethylbutenyl4-diphosphate; DMAPP, dimethylallyl diphosphate; IPP, isopenenyl pyrophosphate; GPP, geranyl diphosphat; GGPP, geranylgeranyl diphosphate. Abbreviations for inhibitors: CLO, clomazone. (b) The core region of the overexpression vector pMEM01-AtDXS. Abbreviations: Pβ-tub, β-tubulin promoter; *eBle*, codon-optimized zeosin resistance gene; Tvcp, violaxanthin/chlorophyll *a* binding protein terminator; Pvcp, violaxanthin/chlorophyll *a* binding protein promoter; *AtDXS*, codon-optimized gene encoding 1-deoxy-D-xylulose 5-phosphate synthase derived from *A. thaliana*; Tα-tub, α-tubulin termination region. Arrows indicate the primers used for validation PCR (VCP–F and AtDXS-R). (c) PCR amplification of the genomic DNA of *Nannochloropsis* transformants. Arrow indicates the PCR product with expected size of 1612 bp. Abbreviations: PC, positive control; T1-T10: transformants.

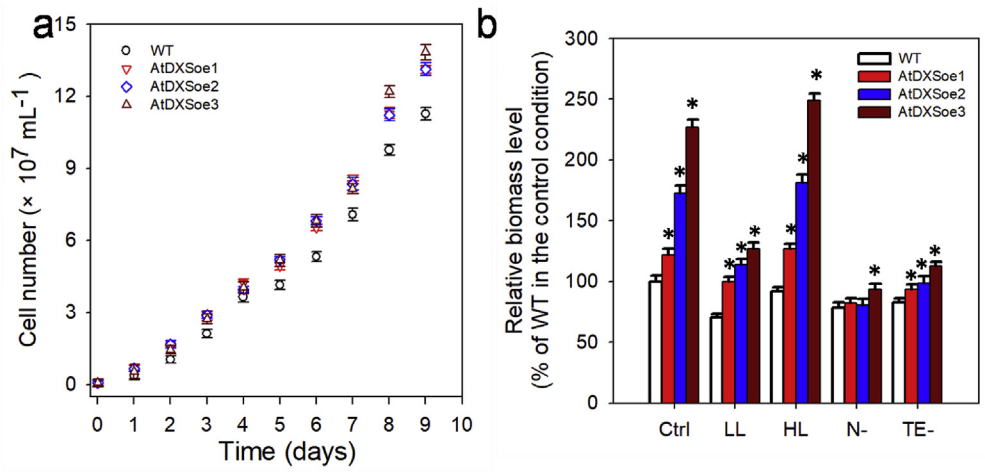

Fig. 2. Growth behavior of wild-type *N. oceanic* (WT) and the AtDXS overexpression strains (AtDXSoe). (a) Growth curve of the WT and the AtDXSoes. Algal cells were cultured in enriched f/2 medium in 25 °C, and 50 μmol·photons·m^{-2}·s^{-1} light. (b) Relative biomass of the WT and the AtDXSoes. All values were normalized to the dry weight of WT cultured in the control conditions (set as 100%). Abbreviations: Ctrl, preferred physiological conditions (enriched f/2, 25 °C, and 50 μmol·photons·m^{-2}·s^{-1} light intensity); LL, 10 μmol·photons·m^{-2}·s^{-1} light; HL, 200 μmol·photons·m^{-2}·s^{-1} light; N-, nitrogen depleted conditions; TE, trace-element free conditions. Data are presented as means ± SDs (n = 4). Asterisks (*) indicate statistically significant differences between the WT and the transformants in designated conditions (*P* values ≤ 0.05).

across various environmental and nutritional conditions. Therefore, we selected AtDXSoe3 for further investigations.

3.3. *AtDXS overexpression attenuates clomazone (CLO) inhibition*

Pharmaceutical azole drug CLO highly specifically inhibits DXS (Fig. 1a). Chl contents per cell decreased in both WT and AtDXSoe3 following CLO administration presumably by limiting IPP supplies to Chl biosynthesis pathway (Fig. 3a). Nevertheless, AtDXSoe3 with CLO inhibition showed higher levels of Chl than WT (Fig. 3a). In particular, after CLO application for four days, AtDXSoe3 cells produced ~10.9% more Chl than the WT. While the decrease of Chl led to a concomitant decrease in growth of both WT and AtDXSoe3, the cell numbers of former decreased (~42.6%) more dramatically than AtDXSoe3 (~6.3%) at the end of detection (Fig. 3b).

3.4. *AtDXS overexpression strains have a higher CO_2 absorption capacity*

To probe the physiological mechanisms underpinning the robustness of growth of AtDXSoe3, the light-saturation curve of photosynthesis was measured under *in vivo* conditions (Fig. 4). In general, AtDXSoe3 showed a higher O_2 evolution rates than the WT. Specifically, the photosynthetic rates at 200 μmol·photons·m^{-2} s^{-1} were 1.6 μmol O_2·mg^{-1} Chl·min^{-1} for AtDXSoe3, which was 60% greater than that of the WT (1.0 μmol O_2·mg^{-1} Chl·min^{-1}). Within a light intensity range from 0 to 400 μmol·photons·m^{-2}·s^{-1}, photosynthetic activity increased as a function of irradiance. The increase was linear for AtDXSoe3 and WT, specifically in the region between 0 and 100 μmol·photons·m^{-2}·s^{-1} light intensity (Fig. 4). The slope of these linear regressions was measured to be 0.33 and 0.17 in relative units for AtDXSoe3 and WT, respectively. Photosynthetic activity in either WT or AtDXSoe3 saturated at approximately 500 μmol·photons·m^{-2}·s^{-1}. Higher light intensities did not bring about any further increase in the rate of photosynthesis (Fig. 4). Combined with the higher Chl contents in the transgenic cells, it would explain their higher overall CO_2 absorption capacity which potentially contributes to the more robust growth of engineered cells than the WT.

3.5. *Production of sugars and their derivatives (SDs) in transgenic microalgae*

SDs includes simple sugars, oligosaccharides, polysaccharides, and their derivatives, such as the methyl ethers with free or potentially free reducing groups (Dubois et al., 1956). AtDXSoe3 produced 59.8% more SDs than WT in 10 μmol·photons·m^{-2}·s^{-1} (LL in Fig. 5). As the light intensity increased, SDs of WT peaked at 50 μmol·photons·m^{-2}·s^{-1} (Ctrl in Fig. 5) while subtle difference was observed between 50 and 200 μmol·photons·m^{-2}·s^{-1} (HL in Fig. 5). In contrast, the SD contents of AtDXSoe3 increased as the intensities rose from 50 to 200 μmol·photons·m^{-2}·s^{-1} (Fig. 5). At the end of the measurement period, the SD contents of AtDXSoe3 was ~89.2% higher than that of WT in 200 μmol·photons·m^{-2}·s^{-1} (Fig. 5). In nitrogen-depleted conditions, levels of SDs in AtDXSoe3 was ~75.0% more than the WT while the difference

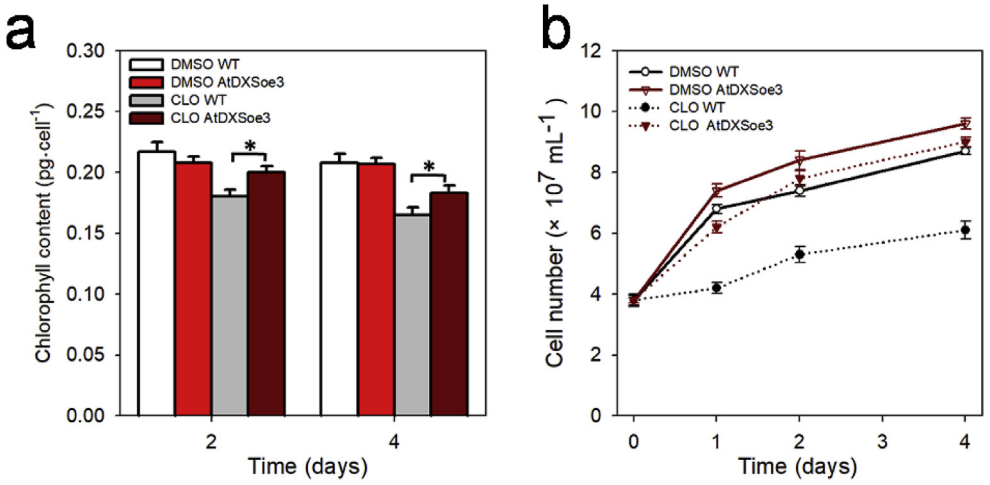

Fig. 3. Growth performance of wild-type *N. oceanic* (WT) and the AtDXS overexpression strains (AtDXSoe) following inhibitor clomazone (CLO) administration. (a) Chlorophyll contents of WT and the AtDXSoe following CLO administration. (b) Growth curve of the WT and the AtDXSoe following CLO administration. Algal cells with an identical starting concentration (4 × 10^7 cells·mL^{-1}) were applied with CLO or equal amount of DMSO (mock controls) and cultured in enriched f/2 medium in 25 °C, and 50 μmol·photons·m^{-2}·s^{-1} light. Data are presented as means ± SDs (n = 4). Asterisks (*) indicate statistically significant differences between the WT and the transformants in designated conditions (*P* values ≤ 0.05).

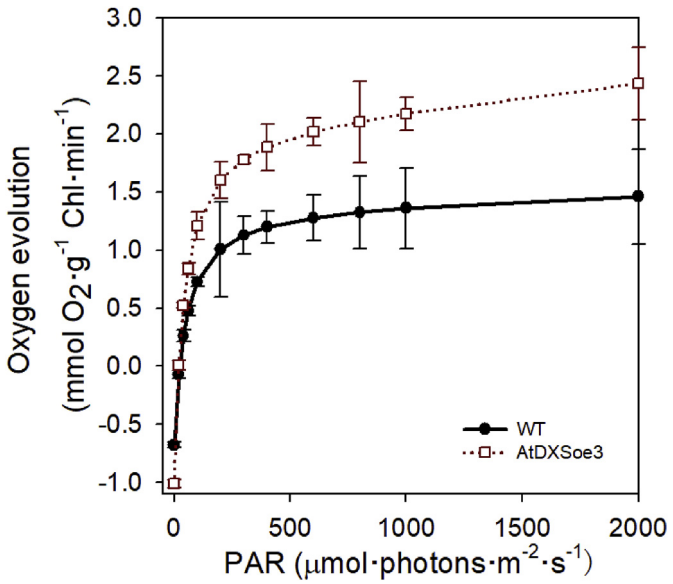

Fig. 4. O_2 evolution of wild-type *N. oceanic* (WT; closed circles) and the AtDXS overexpression lines (AtDXSoe; open squares). Data are presented as means ± SDs (n = 3).

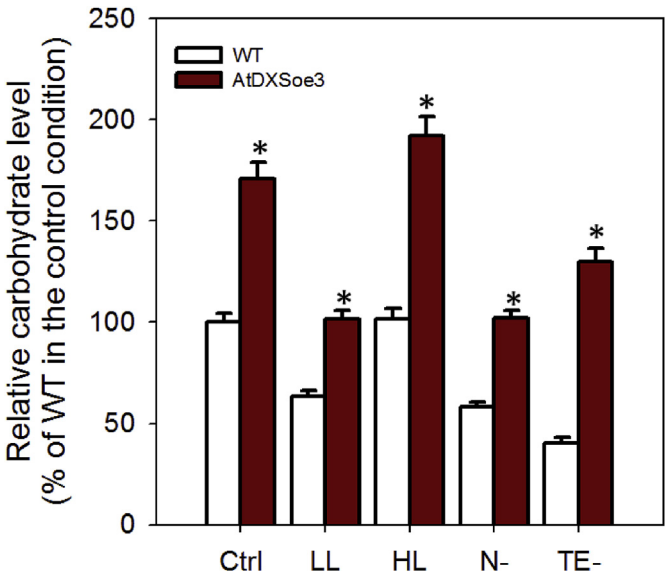

Fig. 5. Relative carbohydrate levels of wild-type *N. oceanic* (WT) and the AtDXS overexpression lines (AtDXSoe) in different conditions. All values were normalized to the carbohydrate content of WT in control conditions (set as 100%). Abbreviations: Ctrl, preferred physiological conditions (enriched f/2, 25 °C, and 50 μmol·photons·m^{-2}·s^{-1} light intensity); LL, 10 μmol·photons·m^{-2}·s^{-1} light; HL, 200 μmol·photons·m^{-2}·s^{-1} light; N-, nitrogen depleted conditions; TE, trace-element free conditions. Data are presented as means ± SDs (n = 4). Asterisks (*) indicate statistically significant differences between the WT and the transformants in designated conditions (*P* values ≤ 0.05).

was further increased to ~223.6% when algal cells were cultured in TE-deprived conditions (Fig. 5).

3.6. Lipid productivity of transgenic microalgae

While stress conditions, such as low light, high light, nitrogen depletion, and trace-element depletion, are occasionally encountered in batch culture of microalgae, lipid production in the many microalgae, including *Nannochloropsis* sp. are generally maximized by nutrient starvation (Ajjawi et al., 2017; Li et al., 2014) or high light (Alboresi et al., 2016). Thus, nitrogen depletion and high light are practically employed to boost lipid accumulation in microalgae. To best display the lipid production capacity of AtDXSoe3, we further investigated the difference in lipid productivity between WT and AtDXSoe3 in nitrogen-depleted and high-light conditions. AtDXSoe3 accumulated higher levels of total lipids (TLs; Fig. 6a) and neutral lipid (TAG, the main source for biodiesel; Fig. 6b) than the WT in both nitrogen-depleted and high-light conditions. For example, upon high irradiance, the TLs (79.9 ± 3.0% of DW; Fig. 6a) and TAG (55.6 ± 1.9% of DW; Fig. 6b) in AtDXSoe3 increased by ~65.3% and ~95.1% relative to the WT. On the other hand, following nitrogen depletion, the TL (70% of DW; Fig. 6a) and TAG (50.1% of DW; Fig. 6b) contents in AtDXSoe3 were 42.3% and 62.9% higher than that of the WT, respectively. Meanwhile, the DW of AtDXSoe3 (1.54 g L^{-1}) was 119% that of WT in nitrogen depletion conditions, which translated to a total lipid production of 1.08 g L^{-1} and a TAG productivity of 0.77 g L^{-1} in AtDXSoe3, with 68.8% and 93.2% increases compared with that of WT. As for high light conditions, the discrepancy increased to 110.6% for TLs and 148.6% for TAG, where production of 1.15 g L^{-1} (TLs) and 0.80 g L^{-1} (TAG) were obtained in AtDXSoe3 (Fig. 6c). These values are competitive with records in oil production in engineered microalgae, such as *Phaeodactylum tricornutum* (TLs, 57.8% of DW) (Xue et al., 2015) or *N. gaditana* (TLs, 40–55% of DW in mutant in nutrient-replete conditions) (Ajjawi et al., 2017). Therefore, AtDXSoe3 exhibits considerable advantages as a feedstock for the production of lipids and TAG which can be exploited as value-added chemicals for nutrient supplements or biofuels.

4. Discussion and conclusions

Isoprenoids are a group of biologically active molecules number in the tens of thousands. Members of this diverse group participate in a wide variety of biological processes such as photosynthesis, respiration, growth, cell cycle control, plant defense, chloroplast biogenesis, and adaptation to environmental conditions (Estevez et al., 2001). Specific examples include photosynthetic pigments (Chls and carotenoids), structural components of membranes (phytosterols), a side chain of the electron transporter (plastiquinone), and antimicrobial agents (phytoalexins in plants) (Lu et al., 2014b).

Here we show that the photosynthetic capacity, growth, stress tolerance, and lipid biosynthesis in *Nannochloropsis* are simultaneously improved by expressing an exogenous *DXS* gene. While the rationale behind remain to be illuminated in details, we saw a down-regulation of the transcript of endogenous *Nannochloropsis DXS* gene in high-light conditions (*unpublished data*) and nitrogen-depleted (Li et al., 2014). We speculate that the overexpression of *AtDXS* compensates the decreased transcript levels of endogenous *DXS* gene in *N. oceanica* at least under high-light or nitrogen-depleted conditions.

Meanwhile, comparison between transcripts in AtDXSoe3 and WT showed different abundance of *N. oceanica dxs* and genes encoding committed enzymes involved in photosynthesis (ribulose bisphosphate carboxylase large subunit, RBCL), isoprene biosynthesis (isopentenyl pyrophosphate, IPP), sterol biosynthesis (squalene synthase, SQS), carotenoid biosynthesis (phytoene synthase, PSY), neutral lipid (diacylglycerol acyltransferase, DGAT1A and DGAT2A), and phytohormone biosynthesis (zeaxanthin epoxidase ZEP and 9-*cis*-epoxycarotenoid dioxygenase NCED in abscisic acid biosynthesis; isopentenyltransferase IPT in cytokinin biosynthesis; *ent*-kaurene oxidase KO and *ent*-kaurenoic acid oxidase KAO in gibberellin biosynthesis) . For example, under high-light conditions, transcripts of RBCL and ZEP were lower whereas IPP, SQS, PSY, DGAT2A, IPT, KO, KAO were higher in AtDXSoe3 than WT . Following nitrogen starvation, DXS was moderately downregulated whereas PSY, HEMA, DGAT1A, NCED, IPT, KO, and KAO were upregulated in AtDXSoe3 relative to WT at least in one time point . IPP and RBCL showed transient downregulation, with its transcript levels peaking at 48 h or 72 h following the onset of nitrogen depletion . Downregulation of ZEP occurred rapidly within the 24 h upon nitrogen starvation, returned

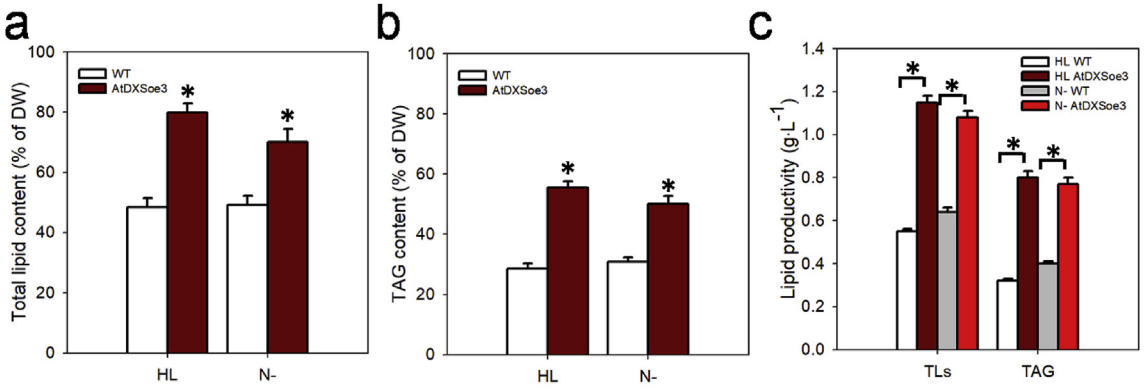

Fig. 6. Lipid production of the WT and the AtDXSoes in nitrogen depleted or high-light conditions. (a) Total lipid contents; (c) TAG contents; (c) Lipid productivities. Abbreviations: HL, 200 μmol·photons·m^{-2}·s^{-1} light; N-, nitrogen depleted conditions; TLs, total lipids; TAG, triacylglycerol. Data are presented as means ± SDs (n = 4). Asterisks (*) indicate statistically significant differences between the WT and the transformants in designated conditions (*P* values ≤ 0.05).

to a moderately increased level at 48 h and declined thereafter . Photosynthesis and the rates of biosynthesis of isoprene, sterol, neutral lipid TAG, and phytohormones are thus regulated in AtDXSoe3 compared with WT as part of the response to nitrogen depletion and high light. This strongly suggests that overexpression of AtDXS perturbs at least photosynthesis and the biosynthesis of isoprene, sterols, lipids, and phytohormones in *N. oceanica*. Meanwhile, we saw increased levels of Chls in the engineered microalgal cells. It may suggest that AtDXS overexpression in *Nannochloropsis* could lead to an elevated activity of the MEP pathway and increased production of IPP and DMAPP which serve as common precursors for the biosynthesis of various downstream isoprenoid derivatives. A higher activity of the MEP pathway might contribute to higher Chl contents in AtDXSoe than WT. This hypothesis is supported by the observation that the AtDXS overexpression lines, relative to WT, demonstrate alleviated inhibition in Chl accumulation following the supplementation of pharmaceutical azole drug CLO, which inhibits DXS activity. Moreover, isoprenoids are precursors for many signaling molecules that regulate growth and development in plants, such as plant hormones (abscisic acid, gibberellins, cytokinins, and brassinosteroids) (Lu et al., 2014a). These hormones regulate crucial and economically relevant processes such as development, dormancy, germination, vegetative growth, and stress responses in flowering plants and emerging evidence supports an early origin and broad functions of the hormone systems in microalgae (Lu and Xu, 2015). Therefore, an elevation of DXS activity may in turn contribute as a switch-on control regulating biological processes such as photosynthesis (through pigment biosynthesis), electron transport (perturbing on plastiquinone biosynthesis), or many other processes (e.g., stress tolerance; through the regulation of hormones). These effects could jointly lead to improved growth, stress tolerance, and favored carbon allocation towards lipid biosynthesis.

In summary, a high lipid production strain *Nannochloropsis* AtDXSoe3 was generated by overexpressing *Arabidopsis* DXS gene. Assessment of growth and production performance in different environmental and nutritional conditions (low light, high light, nitrogen starvation, and trace element depletion) pinpoints the stable and robust characteristics of AtDXSoe3. These results have important implications for the viable development of trait-improved industrial strains with simultaneously improved lipid biosynthesis and biomass productivity by engineering a single 'control knob' gene.

Declaration of competing interests

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.

Acknowledgements

We are grateful to the anonymous reviewers for their valuable improvement to this manuscript. This manuscript was supported by the Natural Science Foundation of China (32060061), the National Key Research and Development Program of China (2016YFB0601001), the Basic and Applied Basic Research Programs for the Talents of Hainan Province (grant no. 2019RC033), the Project of Innovation & Development of Marine Economy (grant no. HHCL201803), the Major Program of Shandong Province Natural Science Foundation (ZR2018ZB0208), the Chinese Government Scholarship (grant no. CSC201807565004), the Project of State Key Laboratory of Marine Resource Utilization in South China Sea (grant no. 2018004), theFoundation of Hainan University (grant no. KYQD1561), and the Project of Collaborative Innovation of Tianjin University and Hainan University (grant no. HDTDU201903).

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.mec.2020.e00142.

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Author contributions

Y.L. conceived and designed the study. F.L. and X.S performed the experimental work. X.H. analyzed the data; Y.L. and X.H. wrote the manuscript. All authors read and approved the final manuscript.

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