**รายงานวิจัยฉบับสมบูรณ์**

**โครงการ**

# การเพิ่มการผลิตเชื้อเพลิงชีวภาพของจุลสาหร่าย *Neochloris oleoabundans* โดยวิธีพันธุวิศวกรรม

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**กุมภาพันธ์ 2562**

สัญญาเลขที่ BRG5780005

**รายงานวิจัยฉบับสมบูรณ์**

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**สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัยและมหาวิทยาลัยมหิดล**

(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกว. และมหาวิทยาลัยมหิดลไม่จำเป็นต้องเห็นด้วยเสมอไป)

## กิตติกรรมประกาศ

โครงการวิจัยนี้ได้รับทุนสนับสนุนจาก ทุนวิจัยองค์ความรู้ใหม่ที่เป็นพื้นฐานต่อการพัฒนา สำนักงานกองทุนสนับสนุนการวิจัย (สกว.) ในระหว่าง วันที่ 31 กรกฎาคม 2557 ถึงวันที่ 30 กรกฎาคม 2560 โดยได้ขอขยายเวลาถึง 28 กุมภาพันธุ์ 2562 ทุนดังกล่าวมีส่วนสนับสนุนการทำวิทยานิพนธ์ของนักศึกษาปริญญาโทสาขาวิชาพันธุศาสตร์ระดับโมเลกุลและพันธุวิศวกรรมศาสตร์ ได้แก่ นางสาวปวีณา กิจรักษา นายอัครพล วัชราวิภาส นางสาวกาญจนาพร อารีย์ นายสิทธิศักดิ์ เกตุขุนทด และนักศึกษาปริญญาเอกในสาขาเดียวกันคือ นางสาวเพกา คล้ายทอง นับว่าได้มีส่วนช่วยเสริมสร้างความเข้มแข็งแก่งานวิจัยอย่างยิ่ง คณะวิจัยขอขอบคุณ สกว. มา ณ. โอกาสนี้ด้วย

ขอขอบคุณ ผู้ช่วยศาสตราจารย์ ดร.กุศล ภูธนะกิจ สถาบันชีววิทยาศาสตร์โมเลกุล มหาวิทยาลัยมหิดล สำหรับพลาสมิด pYES2-FLPAT34

# บทคัดย่อ

เนื่องจากจุลสาหร่ายผลิตลิพิด (lipid) และมีชีวมวล (biomass) สูงกว่าพืชบก จึงมีความเป็นไปได้ที่จะใช้จุลสาหร่ายเป็นแหล่งลิพิดไตรเอซิลกลีเซอรอล (triacylglycerol, TAG) สำหรับผลิตไบโอดีเซล ปัจจุบันการผลิตไบโอดีเซลจากจุลสาหร่ายนั้นมีความเป็นไปได้ในทางเทคนิค แต่ยังไม่คุ้มทุนในเชิงพาณิชย์ การเพิ่มปริมาณ TAG ในจุลสาหร่ายด้วยวิธีทางพันธุวิศวกรรม จะนำไปสู่การพัฒนาการผลิตไบโอดีเซลอย่างมีประสิทธิภาพ วิถีการสังเคราะห์ (biosynthesis pathway) TAG ประกอบด้วยเอนไซม์สำคัญต่าง ๆ เช่น lysophosphatidic acid acyltransferase (LPAAT) และ diacylglycerol acyltransferase (DGAT) จุลสาหร่าย *Neochloris oleoabundans* สามารถสะสมลิพิด 36-54% ของน้ำหนักแห้ง และอาจมี 80% ของลิพิดทั้งหมดเป็น TAG ที่ส่วนใหญ่ประกอบด้วยกรดไขมันอิ่มตัว (saturated fatty acid) ที่มีคาร์บอนประมาณ 16-20 อะตอมซึ่งเหมาะสำหรับการผลิตไบโอดีเซล

คณะวิจัยมีเป้าหมายเพิ่มการสะสมลิพิดใน *N. oleoabundans* ด้วยวิธีทางพันธุวิศวกรรม เพื่อบรรลุเป้าหมายดังกล่าว คณะผู้วิจัยจึงได้พัฒนาระบบการถ่ายโอนยีนเข้าสู่ *N. oleoabundans* พร้อมทั้งได้โคลน (clone) และศึกษาคุณลักษณะของ cDNA ที่สร้างเอนไซม์สำคัญ LPAAT (*NeoLPAAT*) และ DGAT (*NeoDGAT2*) จาก *N. oleoabundans* คณะผู้วิจัยได้ประสบความสำเร็จในการถ่ายโอน *NeoDGAT2* cDNA เข้าสู่นิวเคลียจีโนมของ *N. oleoabundans* ทรานส์ฟอร์แมนท์ (transformant) ที่ได้มีการสะสมลิพิดเพิ่มขึ้นอย่างมีนัยสำคัญเมื่อเปรียบเทียบกับสายพันธุ์ลักษณะปกติ (wild type) อย่างไรก็ดี ความรู้เกี่ยวกับการควบคุมการแสดงออกของยีน *NeoLPAAT* และ *NeoDGAT2* จะมีความสำคัญต่อการเพิ่มการสะสม TAG

โครงการวิจัยนี้จึงมีวัตถุประสงค์คือ เพื่อโคลนและศึกษาคุณลักษณะของลำดับดีเอ็นเอที่ควบคุมการแสดงออกของยีน *NeoLPAAT* และ *NeoDGAT2* เพื่อเพิ่มปริมาณ TAG โดยวิธีเพิ่มการแสดงออกของ *NeoLPAAT* cDNA และวิธีเพิ่มการแสดงออกร่วมกันของ cDNA *NeoLPAAT* และ *NeoDGAT2* ใน *N. oleoabundans* ผลการทดลองพบว่า (i) มีส่วนที่คาดว่าเป็น *cis*-regulatory elements หลายแห่งในส่วน upstream sequences ของยีน *NeoLPAAT* และ *NeoDGAT2* (ii) regulatory sequences ของยีน *NeoLPAAT* และ*NeoDGAT2* ถูกเหนี่ยวนำในสภาวะขาดแคลนไนโตรเจน (iii) การเพิ่มการแสดงออกของ *NeoLPAAT* cDNA ใน *N. oleoabundans* ผลการทดลองพบว่าเมื่อเปรียบเทียบกับสายพันธุ์ลักษณะปกติ ทรานส์ฟอร์แมนท์ B2-LP สามารถเร่งสะสมนิวทรัลลิพิดสูงกว่าถึง 2.5 เท่า มี *NeoLPAAT* transcript สูงกว่า 1.9 เท่า มีปริมาณลิพิดทั้งหมดสูงขึ้น 1.9 เท่า มีปริมาณ TAG เพิ่มขึ้น 2.1 เท่า นอกจากนี้ องค์ประกอบของกรดไขมันในทรานส์ฟอร์แมนท์มีการเปลี่ยนแปลง โดย C18:2 เพิ่มขึ้น 1.8 เท่า (iv) การเพิ่มการแสดงออกร่วมกันของ cDNA *NeoLPAAT* และ *NeoDGAT2* ใน *N. oleoabundans* พบว่าเมื่อเปรียบเทียบกับสายพันธุ์ลักษณะปกติ ทรานส์ฟอร์แมนท์ AR-LD เพิ่มการสะสมนิวทรัลลิพิด มีปริมาณลิพิดทั้งหมดสูงขึ้น 1.6 เท่า มีปริมาณ TAG เพิ่มขึ้น 2.1 เท่า และองค์ประกอบของกรดไขมันในทรานส์ฟอร์แมนท์มีการเปลี่ยนแปลง โดยมี C16:0 เพิ่มขึ้น และ C18:0 ลดลง ดังนั้นปริมาณ TAG ที่เพิ่มขึ้นอยู่ในระดับที่ใกล้เคียงกับทรานส์ฟอร์แมนท์ B2-LP

ผลการทดลองแสดงให้เห็นว่า regulatory sequences ของยีน *NeoLPAAT* และ*NeoDGAT2* ถูกเหนี่ยวนำในสภาวะขาดแคลนไนโตรเจน การเพิ่มปริมาณและการผลิต TAG ใน *N. oleoabundans* โดยเพิ่มการแสดงออกของ *NeoLPAAT* และการเพิ่มการแสดงออกร่วมกันของ *NeoLPAAT* และ *NeoDGAT2* ซึ่งจะเป็นก้าวแรกที่นำไปสู่การทำให้จุลสาหร่ายเป็นแหล่งผลิตไบโอดีเซลในเชิงพาณิชย์

## Abstract

Because microalgae have much higher lipid and biomass productivity compared to terrestrial plants, they are promising sources of lipid triacylglycerol (TAG) for biodiesel production. Currently, biodiesel production from microalgae is technically feasible, but not yet economically viable. Increasing microalgal TAG content via genetic engineering could be a potential approach to improve the efficiency of biodiesel production. The TAG biosynthesis pathway includes key enzymes, lysophosphatidic acid acyltransferase (LPAAT) and diacylglycerol acyltransferase (DGAT). The microalga, *Neochloris oleoabundans*, has been shown to accumulate 36-54% lipids of its cell dry weight. Up to 80% of the total lipids are TAG mainly comprised of the saturated fatty acids containing carbon around 16-20 atoms ideal for biodiesel production.

Our research is aimed to increase the accumulation of lipids in *N. oleoabundans* via genetic engineering. To achieve our goal, the stable gene transfer system of *N. oleoabundans* was developed in our laboratory. The cDNA encoding key enzymes LPAAT (*NeoLPAAT*) and DGAT (*NeoDGAT2*) of *N. oleoabundans* were cloned and characterized. We successfully transferred the *NeoDGAT2* cDNA into the nuclear genome of *N. oleoabundans*. The lipid accumulation of the resulting transformants is significantly higher than that of wild type. How the *NeoLPAAT* and *NeoDGAT2* gene are regulated would be important for increasing TAG accumulation. This study is aimed: to clone and characterize the regulatory sequences of *NeoLPAAT* and *NeoDGAT2* gene; to overexpress the *NeoLPAAT* cDNA; and to co-overexpress the *NeoLPAAT* and *NeoDGAT2* cDNA in *N. oleoabundans*. Results indicated that: (i) there are several predicted *cis*-regulatory elements of the upstream sequences of *NeoLPAAT* and *NeoDGAT2* gene; (ii) the regulatory sequences of *NeoLPAAT* and *NeoDGAT2* gene were induced under N-starvation condition; (iii) overexpression of *NeoLPAAT* cDNA in *N. oleoabundans* transformant B2-LP accelerated neutral lipid accumulation about 2.5-fold higher than in wild type. The *NeoLPAAT* transcript in transformant B2-LP was 2-fold higher than in wild type; total lipid content increased 1.9-fold; TAG content increased 2.1-fold when compared to wild type. The fatty acid composition of the transformant was altered when compared to that of wild type: C18:2 was increased 1.8-fold; (iv) Co-overexpression of *NeoLPAAT* and *NeoDGAT2* cDNA in *N. oleoabundans* transformant AR-LD increased neutral lipid accumulation, total lipid content of transformant AR-LD increased 1.6-fold; TAG content increased 2.1-fold when compared to wild type. The fatty acid composition of transformant AR-LD was altered when compared to that of wild type: C16:0 increased and C18:0 decreased. Thus, the level of increased TAG content in transformant AR-LD is similar to that in transformant B2-LP.

Our results demonstrate that the regulatory sequences of *NeoLPAAT* and *NeoDGAT2* gene were induced under N-starvation condition. The increased TAG content and productivity in *N. oleoabundans* by overexpression of *NeoLPAAT* and co-overexpression of *NeoLPAAT* and *NeoDGAT2* may offer the first step towards making microalgae an economically feasible source for biodiesel.


**Key words:** Biodiesel, Microalgae, Lysophosphatidic acid acyltransferase (LPAAT), Diacylglycerol acyltransferase (DGAT)**,** Triacylglycerol, Lipids

## Executive Summary

**Project title** (Thai): การเพิ่มการผลิตเชื้อเพลิงชีวภาพของจุลสาหร่าย *Neochloris oleoabundans* โดยวิธีพันธุวิศวกรรม

(English): The increased biofuel production of microalga *Neochloris oleoabundans* via genetic engineering

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**Budget for the project**: 2,000,000 Bath

**Duration of the project:** 3 years (July 31, 2014 to July 30, 2017; extended to Feb 28, 2019)

### Rationale

Recent rising oil price and depleting fossil fuel reserve have generated interest in searching alternative and renewable sources. Biodiesel has been considered as an alternative biofuel that can replace petrodiesel. Biodiesel appears to have several favorable environmental properties resulting in no net increase of carbon dioxide and very low sulfur content. It is generally made by transesterification of vegetable oil. The significant economic and environmental impact of using agricultural crops, especially food crops, for examples: palm, soybean, rapeseed and sunflower, as a feedstock for biodiesel raises crucial sustainability issues. Microalgae are a promising alternative source of vegetable oil. They have much higher rates of biomass and oil production than convention crops and can be grown in non-cultivable land and do not replace food crops. Therefore, microalgae are a promising alternative source of lipid for biodiesel production. However, currently, production of biodiesel from microalgae is technically, but not yet economically, feasible. Increasing algal lipid content via genetic engineering could improve microalgae as an economically viable biofuel feedstock.

Due to the limited understanding of microalgae genetics and physiology, lipid metabolism from higher plants and bacteria has been the basis from which the accumulation of triacylglycerol (TAG) in microalgae has been modeled. It has been shown that overexpression of genes involved in TAG assembly (Kennedy pathway) increase lipid content. For examples, overexpression of glycerol-3-phosphate acyltransferase (GPAT, EC: 2.3.1.15), lysophosphatidic acid acyltransferase (LPAAT EC: 2.3.1.51), or diacylglycerol acyltransferase (DGAT EC: 2.3.1.20) all result in significant increases in plant lipid production.

The unicellular fresh-water microalga, *Neochloris oleoabundans* (a taxonomic synonym of *Ettlia oleoabundans*), seems to be one of microalgae having highest lipid content and productivity. It has been shown that *N. oleoabundans* is able to accumulate 36-54% lipids of its cell dry weight under nitrogen starvation condition and its TAG comprises 80% of the total lipids. The TAG of *N. oleoabundans* is mainly comprised of the saturated fatty acids containing carbon around 16-20 atoms ideal for biodiesel production. There is no published data concerning genes encoding LPAAT and DGAT of *N. oleoabundans*. No genomic sequences and molecular genetic techniques are available in *N. oleoabundans*.

Our research is aimed to increase the accumulation of lipids in *N. oleoabundans* via genetic engineering. To achieve our goal, the stable gene transfer system of *N. oleoabundans* has been developed recently in our laboratory. The cDNA encoding key enzymes LPAAT and DGAT2 in TAG biosynthesis pathway of *N. oleoabundans* were cloned and characterized. We successfully transferred the *DGAT2* cDNA under the control of heterologous promoter into the nucleus genome of *N. oleoabundans.* Our preliminary results indicated that the lipid accumulation of the resulting transgenic *N. oleoabundans* occurred at earlier stage of cells and was approximately 50- to 200-fold higher than that of wild type. Co-overexpression of LPAAT might provide more substrate for DGAT and result in increased lipid accumulation.

In this project, we aim to increase the lipid accumulation in *N. oleoabundans* by overexpression of *LPAAT* cDNA alone or together with *DGAT2* cDNA. In addition, the regulatory sequences of *LPAAT* and *DGAT2* gene will be cloned and characterized using green fluorescent protein or its variant as a reporter. The knowledge of *LPAAT* and *DGAT2* gene regulation can be used to increase the lipid accumulation in microalgae.

**Objectives**

This project is aimed to increase the accumulation of lipids in *N. oleoabundans* via genetic engineering.

1). To clone and characterize the regulatory sequence of *NeoLPAAT* gene.

2). To clone and characterize the regulatory sequence of *NeoDGAT2* gene.

3). To increase the lipid accumulation in *N. oleoabundans* by overexpression of *NeoLPAAT* cDNA.

4). To increase the lipid accumulation in *N. oleoabundans* by overexpression of both *NeoLPAAT* and *NeoDGAT2* cDNA.

**Results and Discussion**

1). Investigation of the use of mTq2Cfp as a reporter in *N. oleoabundans*

Plasmid pAR-mTq2 containing mTurquoise2 cyan fluorescent protein gene (*mTq2Cfp*) under the control of *AR* promoter and *Hyg3* gene as a selectable marker was electroporated into *N. oleoabundans* cells to obtain transformant AR-mTq2. Results indicated that *mTq2Cfp* gene can be used as a reporter in *N. oleoabundans.* Introns have been shown to have a positive effect on gene expression in eukaryotes, because their splicing improves and accelerates nuclear mRNA export. To investigate the effect of intron 1 of *rbcS2* gene in *mTq2Cfp* gene expression, plasmids pAR-mTq2-int1 and pAR-mTq2-int2 harboring one and two copies of the intron1, respectively, were constructed and electroporated into the nuclear genome of *N. oleoabundans.* The mTq2Cfp fluorescence activity of the resulting transformant AR-mTq2-int2 was higher than that of AR-mTq2-int1, therefore, mTq2-int2 would be used as a reporter in further experiments.

2). Cloning and characterization of the regulatory sequence of *NeoLPAAT* gene

The regulatory sequence of *NeoLPAAT* gene was identified using genomic walking method, 5’ rapid amplification of genomic ends (5’RAGE). The 2,081-bp upstream sequence (from start codon) of the *NeoLPAAT* gene was obtained. The functional motifs of the upstream sequence were predicted using computer software. To map the regulatory region by deletion analysis, plasmids containing reporter gene *mTq2-int2* under the control of various size of the

*NeoLPAAT* upstream sequence were constructed and transformed into the nuclear genome of *N. oleoabundans*. Results revealed that *NeoLPAAT* regulatory sequence responds to nitrogen starvation and also contains putative MYB element.

3). Cloning and characterization of the regulatory sequence of *NeoDGAT2* gene.

The regulatory sequence of *NeoDGAT2* gene was identified using 5'RAGE. The 2,013 bp of *NeoDGAT2* regulatory sequence was obtained. To map the regulatory region by deletion analysis, plasmids containing reporter gene *mTq2-int2* under the control of various size of the *NeoDGAT2* upstream sequence: UpNeoDGAT2, Δ1, Δ2, Δ3, Δ4 and Δ5 were constructed and transformed into the nuclear genome of *N. oleoabundans*. Results revealed that the regulatory sequence responds to nitrogen starvation. The *NeoDGAT2* regulatory sequence contains the putative nitrogen-starvation responsive element, MYB.

4). Overexpression of *NeoLPAAT* cDNA in *N. oleoabundans*

Plasmids pAR-LPs and pAR-LPo harboring *NeoLPAAT* cDNA under the control of *AR* promoter was electroporated into *N. oleoabundans* cells. The resulting transformant AR-LP clones were screened for neutral lipid accumulation using Nile red fluorescence dye. The AR-LP transformants exhibiting highest fluorescence were selected for further study. Genomic PCR revealed that *NeoLPAAT* cDNA was integrated into the nuclear genome of all the selected transformants. The growth of selected AR-LP transformants was slightly slower than that of wild type. Nile red staining analysis indicated that although the maximum lipid accumulation of AR-LP transformant no. 48 was slightly higher than that of wild type, the lipid accumulation was earlier. Because promoter *AR* is heat inducible, the effect of heat induction on lipid accumulation in transformant AR-LP was investigated. Results indicated that lipid accumulation in heat induction condition was not significantly different from that of without heat induction.

To improve the lipid accumulation, plasmids pB2-LP harboring *NeoLPAAT* cDNA under the control of constitutive promoter β2-tubulin was constructed and electroporated into *N. oleoabundans* cells to obtain transformant B2-LP. The neutral lipid accumulation in transformants B2-LP detected by Nile red staining was accelerated and 2.5-fold higher than in wild type. The *NeoLPAAT* transcript in transformants B2-LP was 2-fold higher than in wild type. In transformants B2-LP, total lipid content increased 1.9-fold and TAG content increased

2.1-fold when compared to wild type. The fatty acid composition of transformant B2-LP was altered when compared to that of wild type; C18:2 was increased 1.8-fold. Thus, Overexpression of *NeoLPAAT* cDNA in *N. oleoabundans* significantly increased TAG content and altered fatty acid composition.

5). Co-overexpression of both *NeoLPAAT* and *NeoDGAT2* cDNA in *N. oleoabundans*

Plasmid pAR-LD and pB2-LD harboring *NeoLPAAT* and *NeoDGAT2* cDNA under the control of promoters AR and β2-tubulin, respectively, were constructed and electroporated into *N. oleoabundans* cells. Screening of neutral lipid using Nile red revealed that transformant AR-LD has higher neutral lipid accumulation than B2-LD, therefore transformant AR-LD was used for further investigation. Neutral lipid accumulation of transformant AR-LD was higher than that of wild type. Total lipid content of transformant AR-LD increased 1.6-fold; TAG content increased 2.1-fold when compared to wild type. The fatty acid composition of transformant AR-LD was altered when compared to that of wild type: C16:0 increased and C18:0 decreased. Thus, the level of increased TAG content in transformant AR-LD is similar to that in transformant B2-LP.

Our results demonstrate that the regulatory sequences of *NeoLPAAT* and *NeoDGAT2* gene were induced under N-starvation condition. The increased TAG content and productivity in *N. oleoabundans* by overexpression of *NeoLPAAT* and co-overexpression of *NeoLPAAT* and *NeoDGAT2* may offer the first step towards making microalgae an economically feasible source for biodiesel.

**Output**

**Presentation at conference:**

1). เสนอผลวิจัยแบบ Oral ในหัวข้อ “Increased lipid production and altered lipid composition in microalga *Neochloris oleoabundans* by overexpression of diacylglycerol acyltransferase 2” ในการประชุมวิชาการเรื่อง 11th International Phycological Congress ระหว่างวันที่ 13-19 สิงหาคม 2560 ณ เมือง Szczecin ประเทศโปแลนด์

โดยมีการตีพิมพ์ Congress abstract ในวารสารวิชาการ Phycologia:

Chungjatupornchai W; Klaitong P and Fa-aroonsawat S. Increased lipid production and altered lipid composition in microalga *Neochloris oleoabundans* by overexpression of diacylglycerol acyltransferase 2. Phycologia (2017) 56 (4) supplement: 32-33.

2). เป็นวิทยากร ในหัวข้อ “Exploring microalgae as fuel” ใน Student Science Training Program Year 2017 วันที่ 4 กรกฎาคม 2560 ณ สถาบันชีววิทยาศาสตร์โมเลกุล มหาวิทยาลัยมหิดล

3). เป็นวิทยากร ในหัวข้อ “Exploring microalgae as fuel” ใน Student Science Training Program Year 2016 วันที่ 14 กรกฎาคม 2559 ณ สถาบันชีววิทยาศาสตร์โมเลกุล มหาวิทยาลัยมหิดล

4). เป็นวิทยากร ในหัวข้อ “Exploring microalgae as fuel” ใน Student Science Training Program Year 2015 วันที่ 25 มิถุนายน 2558 ณ สถาบันชีววิทยาศาสตร์โมเลกุล มหาวิทยาลัยมหิดล

**Publication**

**ผลงานวิจัยที่ตีพิมพ์ในวารสารวิชาการระดับนานาชาติ**

(ระบุทุนวิจัย สกว. ใน Acknowledgements, *=corresponding author)

1). Chungjatupornchai W* and Watcharawipas A (2015) Diacylglycerol acyltransferase type 2 cDNA from the oleaginous microalga *Neochloris oleoabundans*: cloning and functional characterization. J Appl Phycol 27:1499–1507.

2). Chungjatupornchai W*, Kitraksa P and Fa-aroonsawat S. (2016) Stable nuclear transformation of the oleaginous microalga *Neochloris oleoabundans* by electroporation. J Appl Phycol 28:191–199.

3). Klaitong P, Fa-aroonsawat S and Chungjatupornchai W* (2017) Accelerated triacylglycerol production and altered fatty acid composition in oleaginous microalga *Neochloris oleoabundans* by overexpression of diacylglycerol acyltransferase 2. Microb Cell Fact. 16: 61.

4). Chungjatupornchai W*, Areerat K and Fa-aroonsawat S (2019) Increased triacylglycerol production in oleaginous microalga *Neochloris oleoabundans* by overexpression of plastidial lysophosphatidic acid acyltransferase. Microb Cell Fact. 18: 53.

# เนื้อหางานวิจัย

## Introduction to the research problem and its significance

Recent rising oil price and depleting fossil fuel reserve have generated interest in searching alternative and renewable sources. Biodiesel has been considered as an alternative biofuel that can replace petrodiesel. Biodiesel appears to have several favorable environmental properties resulting in no net increase of carbon dioxide and very low sulfur content. It is generally made by transesterification of vegetable oil. The significant economic and environmental impact of using agricultural crops, especially food crops, for examples: palm, soybean, rapeseed and sunflower, as a feedstock for biodiesel raises crucial sustainability issues. Microalgae are a promising alternative source of vegetable oil. They have much higher rates of biomass and oil production than convention crops and can be grown in non-cultivable land and do not replace food crops (Mata *et al.,* 2010). Therefore, microalgae are a promising alternative source of lipid for biodiesel production. However, currently, production of biodiesel from microalgae is technically, but not yet economically, feasible. Increasing algal lipid content via genetic engineering could improve microalgae as an economically viable biofuel feedstock.

Due to the limited understanding of microalgae genetics and physiology, lipid metabolism from higher plants and bacteria has been the basis from which the accumulation of triacylglycerol (TAG) in microalgae has been modeled. It has been shown that overexpression of genes involved in TAG assembly (Kennedy pathway) increase lipid content. For examples, overexpression of glycerol-3-phosphate acyltransferase (GPAT, EC: 2.3.1.15), lysophosphatidic acid acyltransferase (LPAAT EC: 2.3.1.51), or diacylglycerol acyltransferase (DGAT EC: 2.3.1.20) all result in significant increases in plant lipid production (Jain *et al*., 2000; Taylor *et al*., 2002; Lardizabal *et al*., 2008).

The unicellular fresh-water microalga, *Neochloris oleoabundans* (a taxonomic synonym of *Ettlia oleoabundans*) (Deason *et al*., 1991), seems to be one of microalgae having highest lipid content and productivity (Mata *et al*., 2010). It has been shown that *N. oleoabundans* is able to accumulate 36-54% lipids of its cell dry weight under nitrogen starvation condition and its TAG comprises 80% of the total lipids (Tornabene *et al*., 1983). The TAG of *N. oleoabundans* is mainly comprised of the saturated fatty acids containing carbon around 16-20 atoms (Tornabene *et al*., 1983) ideal for biodiesel production. Recently, transcriptomic analysis of *N. oleoabundans*

revealed that under nitrogen limitation, the expression of gene encoding LPAAT is up-regulated but that of gene encoding DGAT is relatively no change (Rismani-Yazdi *et al*., 2012). There is no published data concerning genes encoding LPAAT and DGAT of *N. oleoabundans*. No genomic sequences and molecular genetic techniques are available in *N. oleoabundans*.

Our research is aimed to increase the accumulation of lipids in *N. oleoabundans* via genetic engineering. To achieve our goal, the stable gene transfer system of *N. oleoabundans* has been developed recently in our laboratory (Kitruksa and Chungjatupornchai, unpublished data). The cDNA encoding key enzymes LPAAT and DGAT2 in TAG biosynthesis pathway of *N. oleoabundans* were cloned and characterized (Phienluphon and Pootanakit unpublished data; Watcharawipas and Chungjatupornchai unpublished data, respectively). We successfully transferred the *DGAT2* cDNA under the control of heterologous promoter into the nucleus genome of *N. oleoabundans.* Our preliminary results indicated that the lipid accumulation of the resulting transgenic *N. oleoabundans* occurred at earlier stage of cells and was approximately 50- to 200-fold higher than that of wild type. However, transgenic *N. oleoabundans* seems to bleach faster than wild type (Klaitong and Chungjatupornchai unpublished data). This might be due to the limitation of substrate for DGAT2 enzyme. Therefore, co-overexpression of LPAAT might provide more substrate for DGAT and result in increased lipid accumulation and prolonged cell life.

In this project, we aim to increase the lipid accumulation in *N. oleoabundans* by overexpression of *LPAAT* cDNA alone or together with *DGAT2* cDNA. In addition, the regulatory sequence of *LPAAT* and *DGAT2* gene will be cloned and characterized using green fluorescent protein or its variant as a reporter. The knowledge of *LPAAT* and *DGAT2* gene regulation can be used to increase the lipid accumulation in microalgae.

## Objectives

This project is aimed to increase the accumulation of lipids in *N. oleoabundans* via genetic engineering.

1). To clone and characterize the regulatory sequence of *NeoLPAAT* gene.

2). To clone and characterize the regulatory sequence of *NeoDGAT2* gene.

3). To increase the lipid accumulation in *N. oleoabundans* by overexpression of *NeoLPAAT* cDNA.

4). To increase the lipid accumulation in *N. oleoabundans* by overexpression of both *NeoLPAAT* and *NeoDGAT2* cDNA.

## Results and Discussion

1). Investigation of the use of mTq2Cfp as a reporter in *N. oleoabundans*

Plasmid pAR-mTq2 (Fig.1a), containing *mTq2Cfp* gene under the control of *AR* promoter and *Hyg3* gene as a selectable marker, was constructed and electroporated into *N. oleoabundans* cells. The resulting transformant AR-mTq2 clones selected on BBM agar containing Hygromycin B were screened for CFP activity. Results in Fig. 1b indicated that *mTq2Cfp* gene can be used as a reporter in *N. oleoabundans.*

Introns have been shown to have a positive effect on gene expression in eukaryotes, because their splicing improves and accelerates nuclear mRNA export (Reed and Hurt 2002; Rose and Last 1997). In *C. reinhardtii*, heterologous gene expression can be increased by the presence of intron1 of endogenous *rbcS2* gene (Lumgreras et al. 1998; Berthold et al. 2002). To investigate the effect of intron 1 of *rbcS2* gene in *mTq2Cfp* gene expression, plasmids pAR-mTq2-int1 and pAR-mTq2-int2 harboring one and two copies of the intron1, respectively, were constructed (Fig.1a) and electroporated into the nuclear genome of *N. oleoabundans*. The mTq2Cfp fluorescence activity of the resulting transformant AR-mTq2-int2 was higher than that of AR-mTq2-int1 (Fig.1b), therefore, mTq2-int2 would be used as a reporter in further experiments.

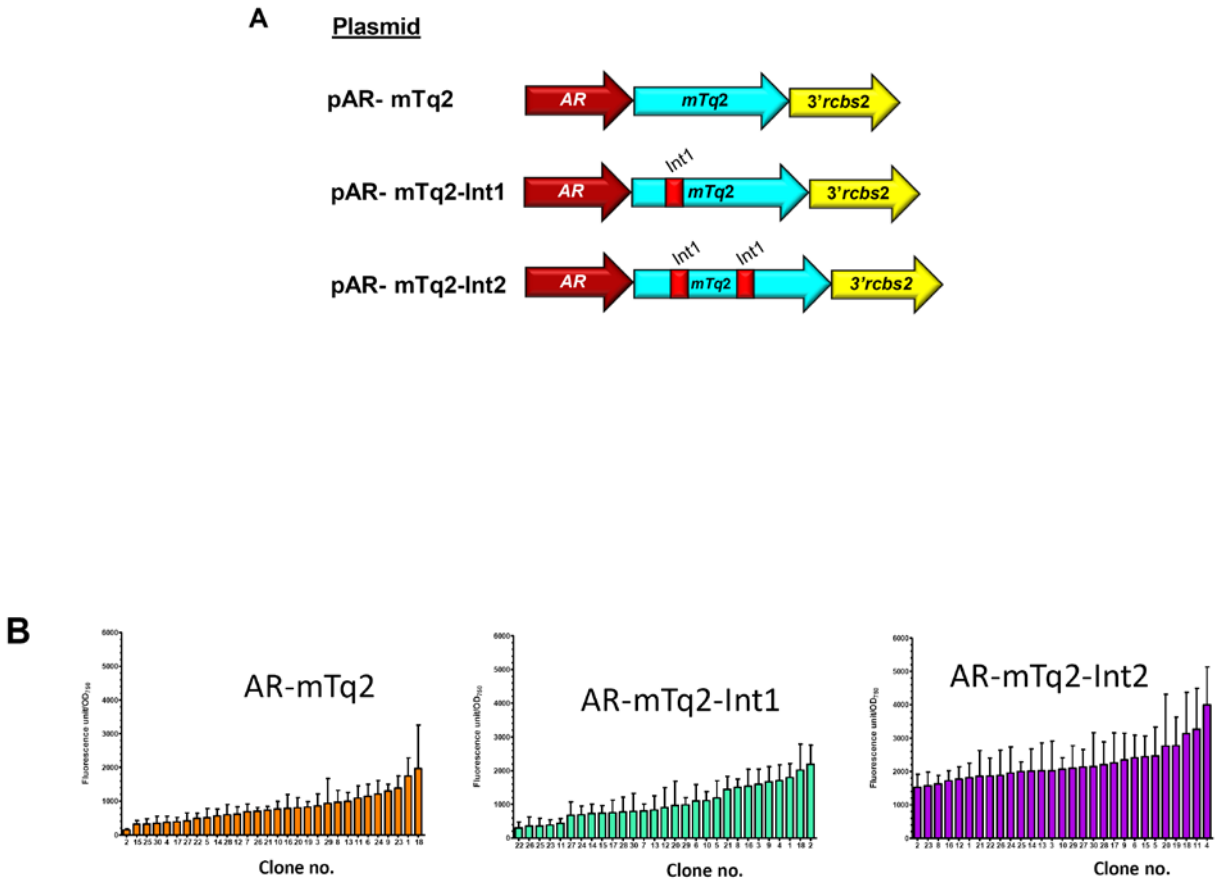



**Fig. 1. CFP fluorescence intensity of *N. oleoabundans* transformants**

(A) Schematic diagram of plasmids harboring *mTq2* reporter gene. *AR*, *HSP70A/RBCS2* hybrid promoter of *C. reinhardtii* (Schroda et al. 2000); Int1, intron 1 of *C. reinhardtii rbcS2* gene; *3'rbcS2*, 3' untranslated region of the *C. reinhardtii* ribulose bisphosphate carboxylase small subunit gene (Fuhrmann et al. 1999). The figure is not drawn to scale.

(B) Plasmids (in A) were electroporated into *N. oleoabundans* to obtain transformants AR-mTq2, AR-mTq2-Int1 and AR-mTq2-Int2. The fluorescence intensities in the transformants were measured using a spectrofluorometer. Values of control without CFP (wild-type strain) were subtracted. Each value and error bar represents the mean of three independent experiments and the standard deviation

## 2. Cloning and characterization of the regulatory sequence of *NeoLPAAT* gene

### 2.1 Cloning of the regulatory sequence of *NeoLPAAT* gene

The regulatory sequence of *NeoLPAAT* gene was identified using specific primers based on *NeoLPAAT* cDNA sequence (Phienluphon and Pootanakit, unpublished data) and genomic walking method, 5' rapid amplification of genomic ends (5'RAGE). The resulting PCR products were cloned into a vector and the DNA sequences were determined using automated DNA sequencing. The 2,081-bp upstream sequence (from start codon) of the *NeoLPAAT* gene was obtained (Fig. 2).

### 2.2 Characterization of the regulatory sequence of *NeoLPAAT* gene

The functional motifs of the upstream sequence were predicted using computer software (Fig. 3 and 4). To map the regulatory region by deletion analysis, plasmids containing reporter gene *mTq2-int2* under the control of various size of the *NeoLPAAT* upstream sequence were constructed (Fig. 5) and transformed into the nuclear genome of *N. oleoabundans*. Results revealed that *NeoLPAAT* regulatory sequence responds to nitrogen starvation (Fig. 6) and also contains putative MYB element.

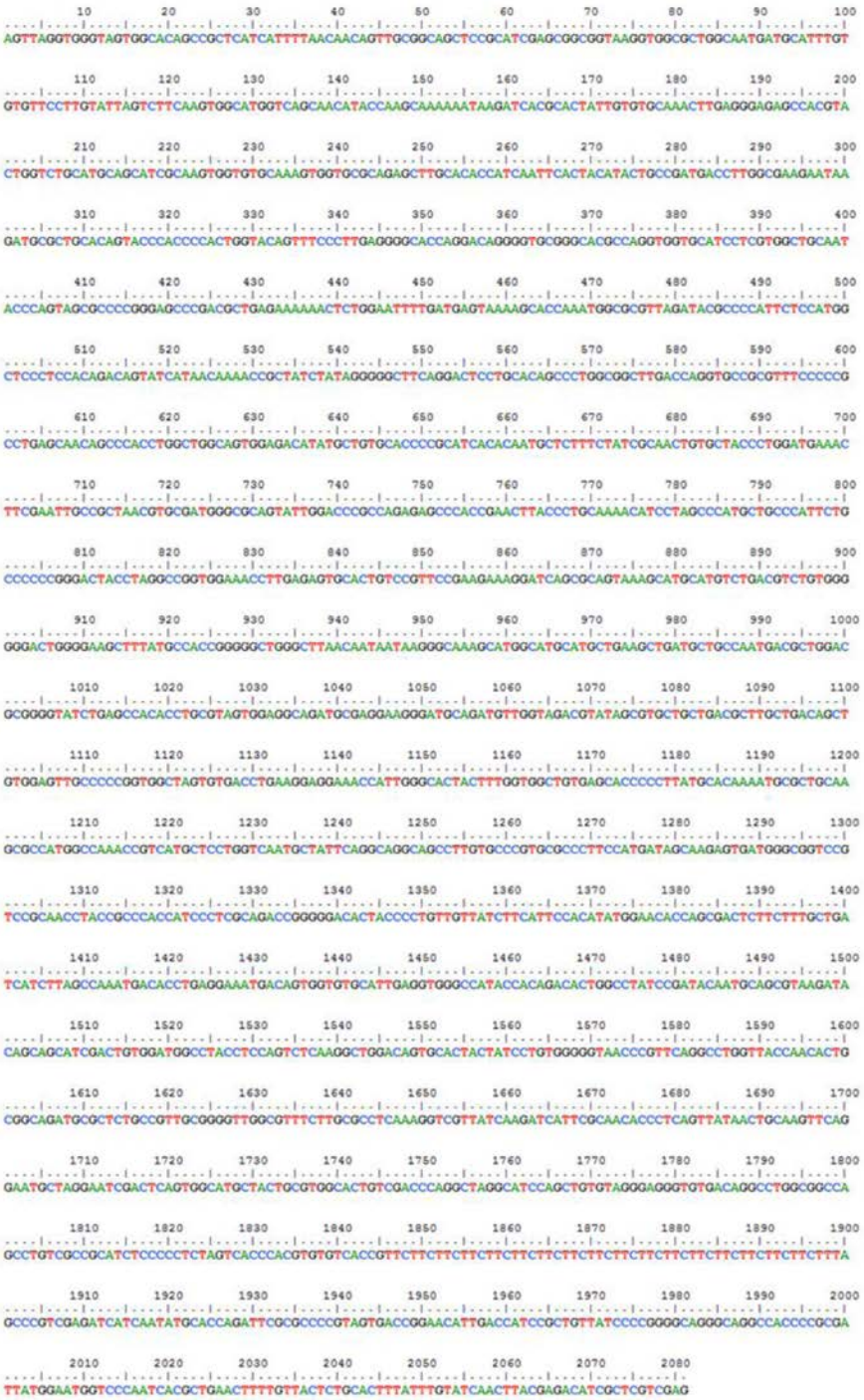


**Fig. 2. The upstream sequence of *NeoLPAAT* gene**

The 2,081-bp upstream sequence (from start codon) of the *NeoLPAAT* gene was obtained using genomic walking method, 5'RAGE.

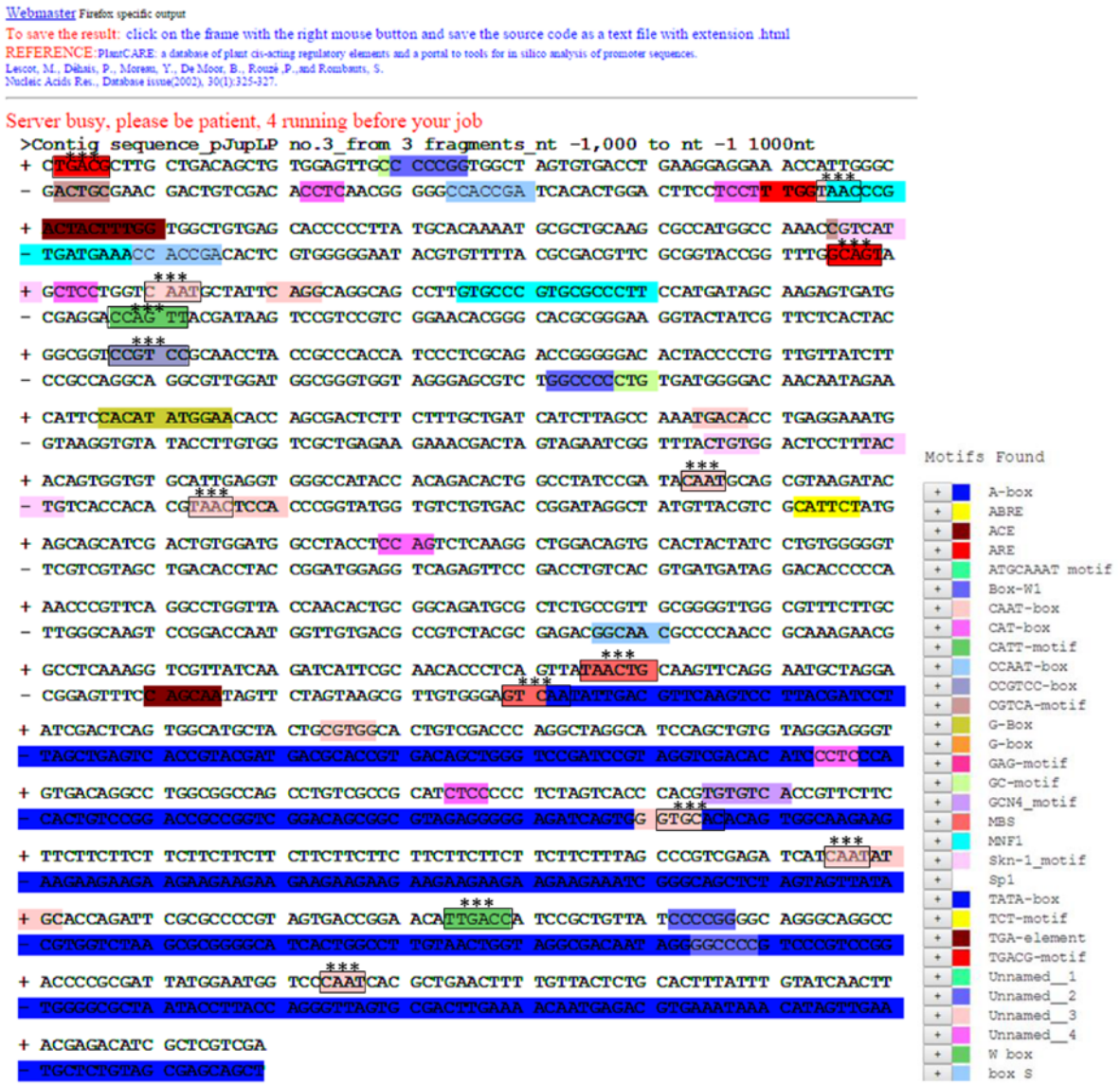



**Fig. 3. Predicted *cis*-regulatory elements in upstream sequence of *NeoLPAAT* gene (nt -1,000 to -1)**

The *cis*-acting regulatory elements: enhancers and repressors were analyzed by accessing PlantCARE database (http://sphinx.rug.ac.be:8008/PlantCARE/). The motifs were indicated. Asterisks indicate the motifs also predicted by PLACE database (http://www.dna.affrc.go.jp/htdocs/PLACE/).

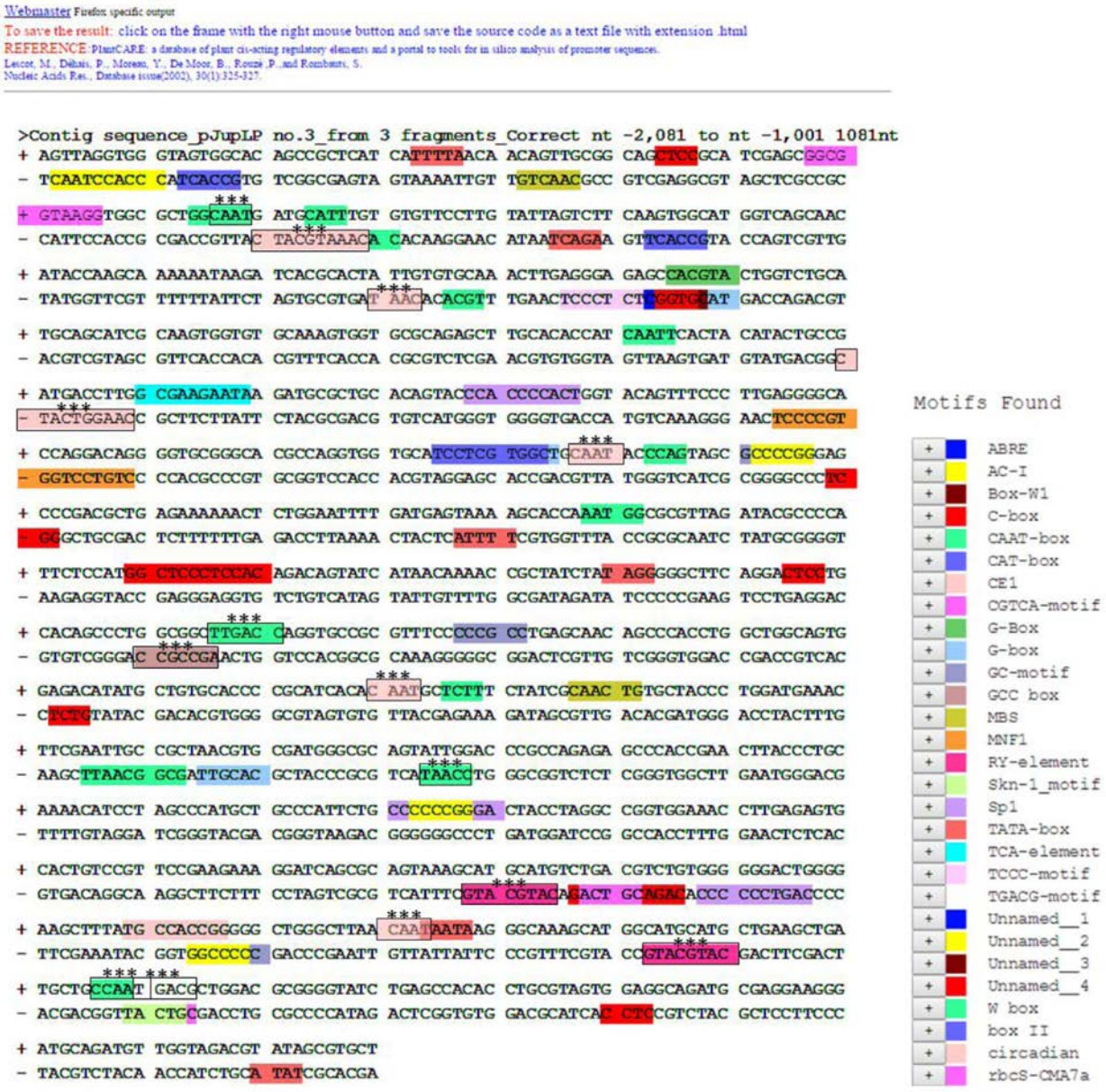



**Fig. 4. Predicted *cis*-regulatory elements in upstream sequence of *NeoLPAAT* gene (nt -2,081 to -1,001)**

The *cis*-acting regulatory elements: enhancers and repressors were analyzed by accessing PlantCARE database (http://sphinx.rug.ac.be:8008/PlantCARE/). The motifs were indicated. Asterisks indicate the motifs also predicted by PLACE database (http://www.dna.affrc.go.jp/htdocs/PLACE/).

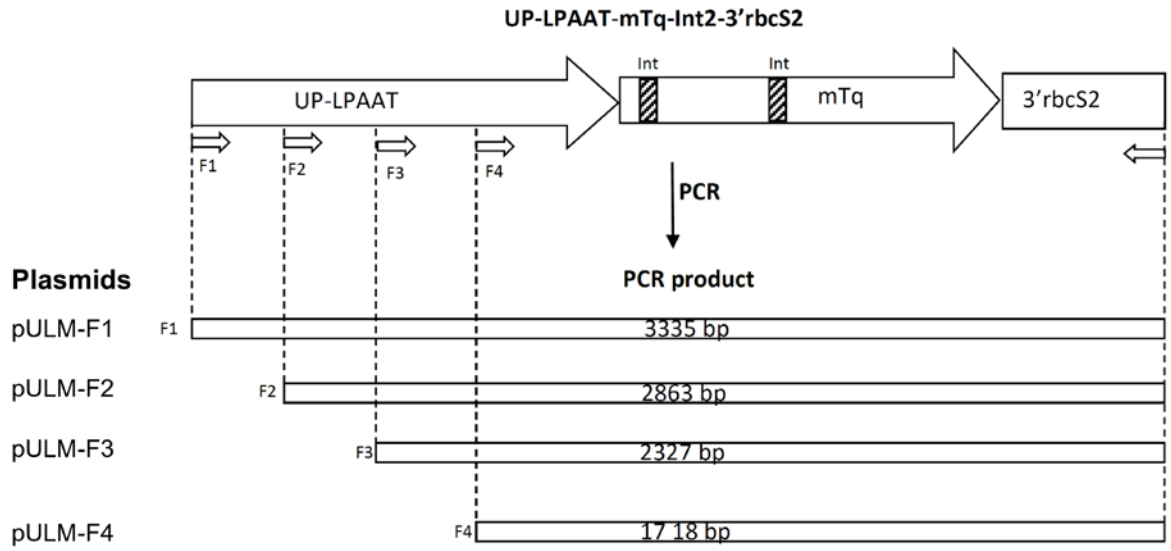



**Fig. 5. Plasmid constructs for deletion analysis of *NeoLPAAT* regulatory sequence**

The plasmids pULM-F1, pULM-F2, pULM-F3 and pULM-F4 containing the reporter gene *mTq2-int2* under the control of various size of *NeoLPAAT* regulatory sequence are indicated.

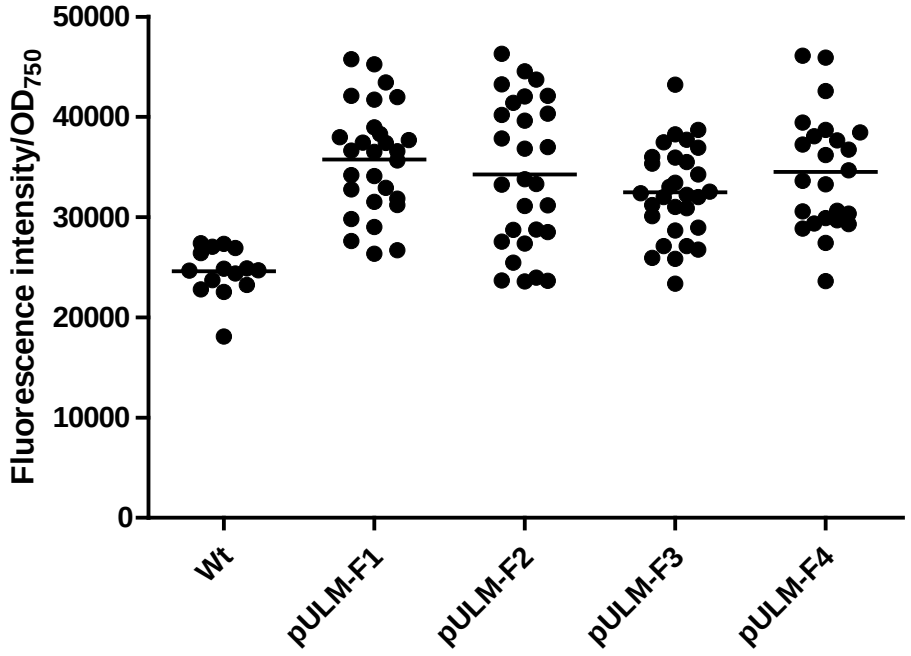



**Fig. 6. Fluorescence intensity of pULM harbouring varied sizes of *NeoLPAAT* regulatory sequence**

The fluorescence intensities of transformant ULM grown under N-starvation conditions was measured. Lines represent mean values. Wild type (Wt) was used as negative control. The experiment was performed at least three independent experiments.

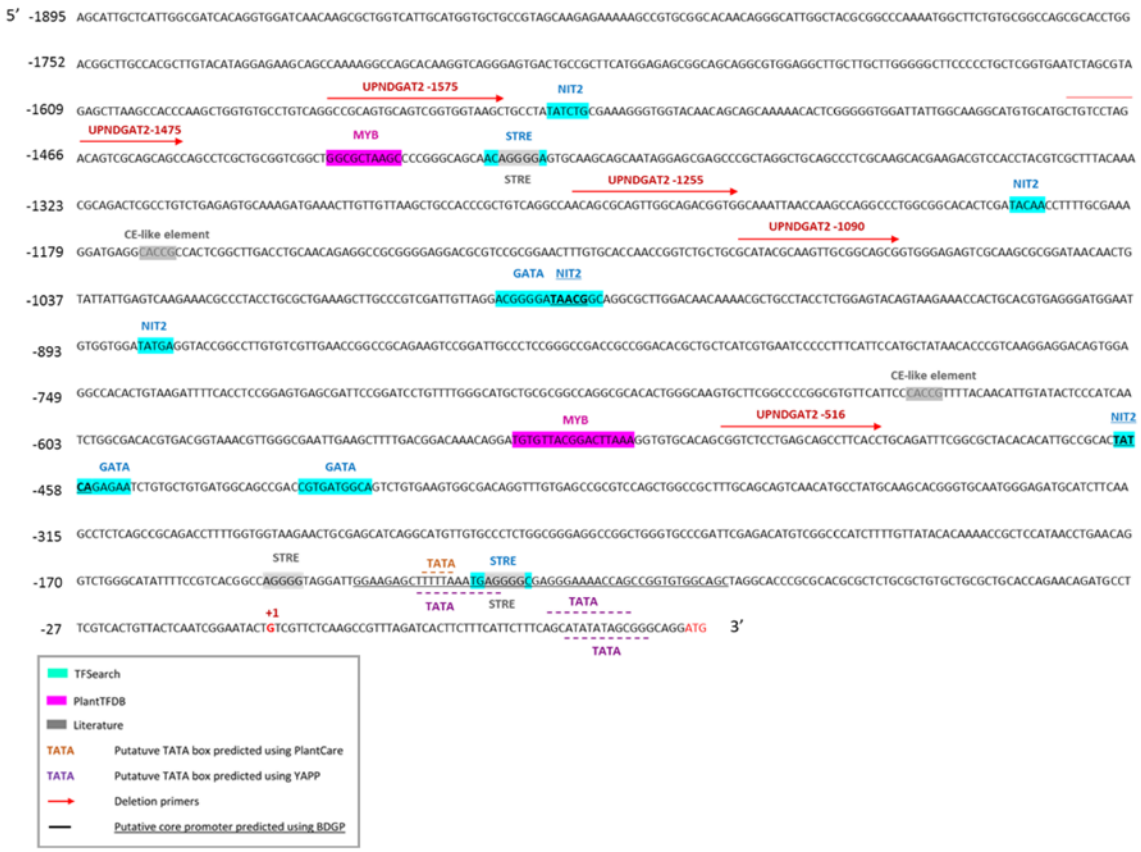
## 3. Cloning and characterization of the regulatory sequence of *NeoDGAT2* gene.

### 3.1 Cloning of the regulatory sequence of *NeoDGAT2* gene.

The regulatory sequence of *NeoDGAT2* gene was identified using specific primers based on *NeoDGAT2* cDNA sequence (Chungjatupornchai and Watcharawipas, 2015) and 5'RAGE. The resulting PCR products were cloned into a vector and the DNA sequences were determined using automated DNA sequencing. The 2,013 bp of *NeoDGAT2* regulatory sequence was obtained (Fig. 7).

### 3.2 Characterization of the regulatory sequence of *DGAT2* gene.

To map the regulatory region by deletion analysis, plasmids containing reporter gene *mTq2-inl2* under the control of various size of the *NeoDGAT2* upstream sequence: UpNeoDGAT2, Δ1, Δ2, Δ3, Δ4 and Δ5 were constructed (Fig. 8) and transformed into the nuclear genome of *N. oleoabundans*. Results revealed that the regulatory sequence responds to nitrogen starvation (Fig.9). The *NeoDGAT2* regulatory sequence contains the putative nitrogen-starvation responsive element, MYB. The MYB related transcription factor ROC40 is the most induced protein on nitrogen-starvation condition; transcription factor ROC40 has a role in nitrogen-starvation induced lipid accumulation (Goncalves et al. 2016). To verify the function of putative MYB on *NeoDGAT2* regulatory sequence, electrophoretic mobility shift assay (EMSA) using purified recombinant protein ROC40 was under investigation.




**Putative cis- regulatory elements involved in N and P starvation and light induction**

| | |
|---|---|
| TATA-box | Core promoter element around -30 of transcription start |
| NIT2 | Binding site of NIT2 regulatory protein in nitrate assimilation pathway. In *C. reinhardtii*, NIT2 has been shown to be up-regulated under –N (Fernandez and Galvan 2008). |
| GATA | Binding site of GATA family transcription factor |
| CE1-like element | Binding site of abscisic acid insensitive 4 (ABI4) transcription factor. In A. thaliana, ABI4 has been shown to be up-regulated under –N and to directly bound to CE1-like element in *A. thaliana DGAT1* promoter and regulate DGAT expression under –N (Yang et al. 2011). |
| STRE | In yeast, STRE (stress response element) has been shown to be a N-starvation responsive element of the promoter of genes inducible by –N (Seymour and Piper 1999). |
| MYB | Binding site of MYB-related transcription factors. MYB-related transcription factors has been shown to have a role in N starvation-induced lipid accumulation in *C. reinhardtii* and binding site of MYB-related transcription factors is present *CrDGTT1* promoter. MYB-related transcription factors has also been shown to up-regulate under –P condition and involved in the induction of P starvation-responsive genes (Rubio et al. 2014). |

**Fig. 7. Predicted *cis*-regulatory elements in upstream sequence of *NeoDGAT2* gene**

The elements were predicted using PlantCare database (http://www.bioinformatics.psb.ugent.be/webtools/plantcare/html/) (Lescot et al. 2002), TFSEARCH (http://www.rwcp.or.jp/lab/pdappl/papia.html) (Akiyama 1995) and YAPP (http://www.bioinformatics.org/yapp/cgi-bin/yapp.cgi). Core promoter region was predicted using BDGP (http://www.fruitfly.org/seq_tools/promoter.html) (Fisher et al. 2012). Transcriptional start site is indicated as +1. Primers used in deletion analysis are indicated as arrow.

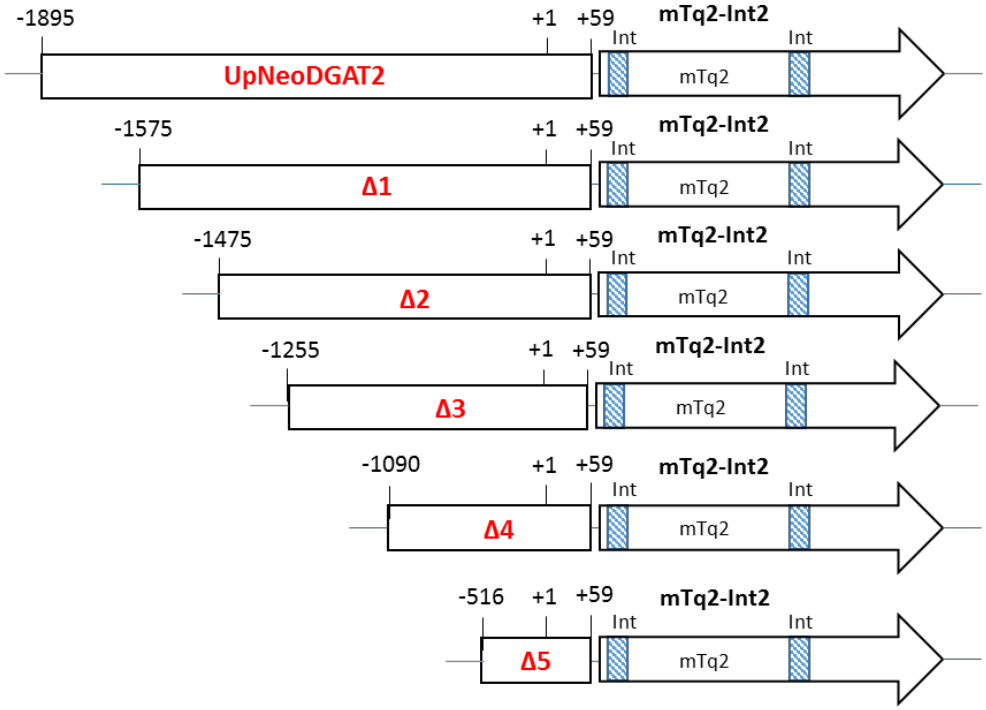



**Fig. 8. Plasmid constructs for deletion analysis of *NeoDGAT2* regulatory sequence**

The upstream sequence of *NeoDGAT2* gene (*UpNeoDGAT2*) and its derivatives: Δ1, Δ2, Δ3, Δ4 and Δ5 located upstream of the reporter gene *mTq2-int2* are indicated. The transcriptional start site (+1) is indicated.

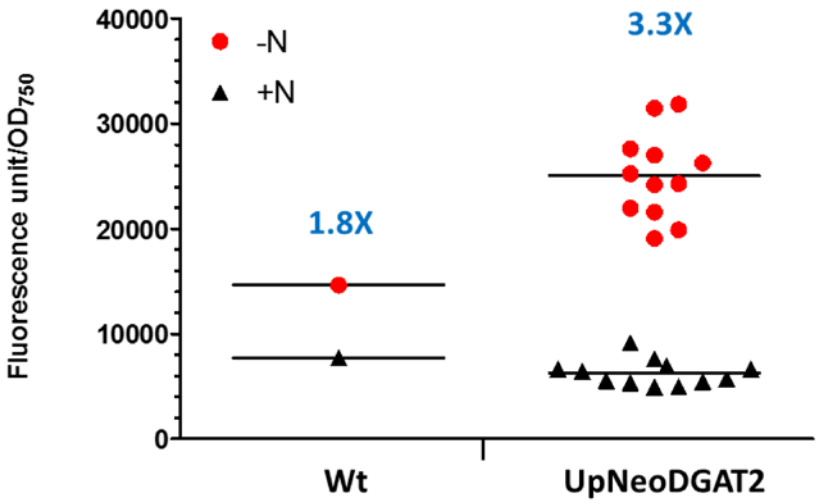



**Fig. 9. The mTq2 activity of transformant UpNeoDGAT2 under N-starvation condition**

The fluorescence intensities of transformant UpNeoDGAT2 grown under N-sufficient and N-starvation conditions was measured. Lines represent mean values. Relative fold inductions are indicated. Wild type (Wt) was used as negative control. The experiment was performed at least three independent experiments.

**4. Overexpression of *NeoLPAAT* cDNA in *N. oleoabundans***

Plasmids pAR-LPs and pAR-LPo harboring *NeoLPAAT* cDNA under the control of *AR* promoter and *Hyg3* gene as a selectable marker (Fig.10), was constructed and electroporated into *N. oleoabundans* cells. The transformant AR-LP clones were selected on BBM agar containing Hygromycin B. The transformants AR-LP were screened for neutral lipid accumulation using Nile red fluorescence dye. The AR-LP transformants exhibiting highest fluorescence were selected for further study. Genomic PCR revealed that *NeoLPAAT* cDNA was integrated into the nuclear genome of all the selected transformants (Fig. 11). The growth of selected AR-LP transformants was slightly slower than that of wild type (Fig. 12). Nile red staining analysis indicated that although the maximum lipid accumulation of AR-LP transformant no. 48 was slightly higher than that of wild type, the lipid accumulation was earlier (Fig. 13). Because promoter *AR* is heat inducible, the effect of heat induction on lipid accumulation in transformant AR-LP was investigated. Results indicated that lipid accumulation in heat induction condition was not significantly different from that of without heat induction (Fig. 14).

To improve the lipid accumulation, plasmids pB2-LP harboring *NeoLPAAT* cDNA under the control of constitutive promoter β2-tubulin was constructed (Fig. 15) and electroporated into *N. oleoabundans* cells to obtain transformant B2-LP. The neutral lipid accumulation in transformants B2-LP detected by Nile red staining was accelerated and 2.5-fold higher than in wild type. The *NeoLPAAT* transcript in transformants B2-LP was 2-fold higher than in wild type. In transformants B2-LP, total lipid content increased 1.9-fold and TAG content increased 2.1-fold when compared to wild type (Fig. 16). The fatty acid composition of transformant B2-LP was altered when compared to that of wild type; C18:2 was increased 1.8-fold (**$P < 0.05$) (Table 1). Thus, Overexpression of *NeoLPAAT* cDNA in *N. oleoabundans* significantly increased TAG content and altered fatty acid composition.

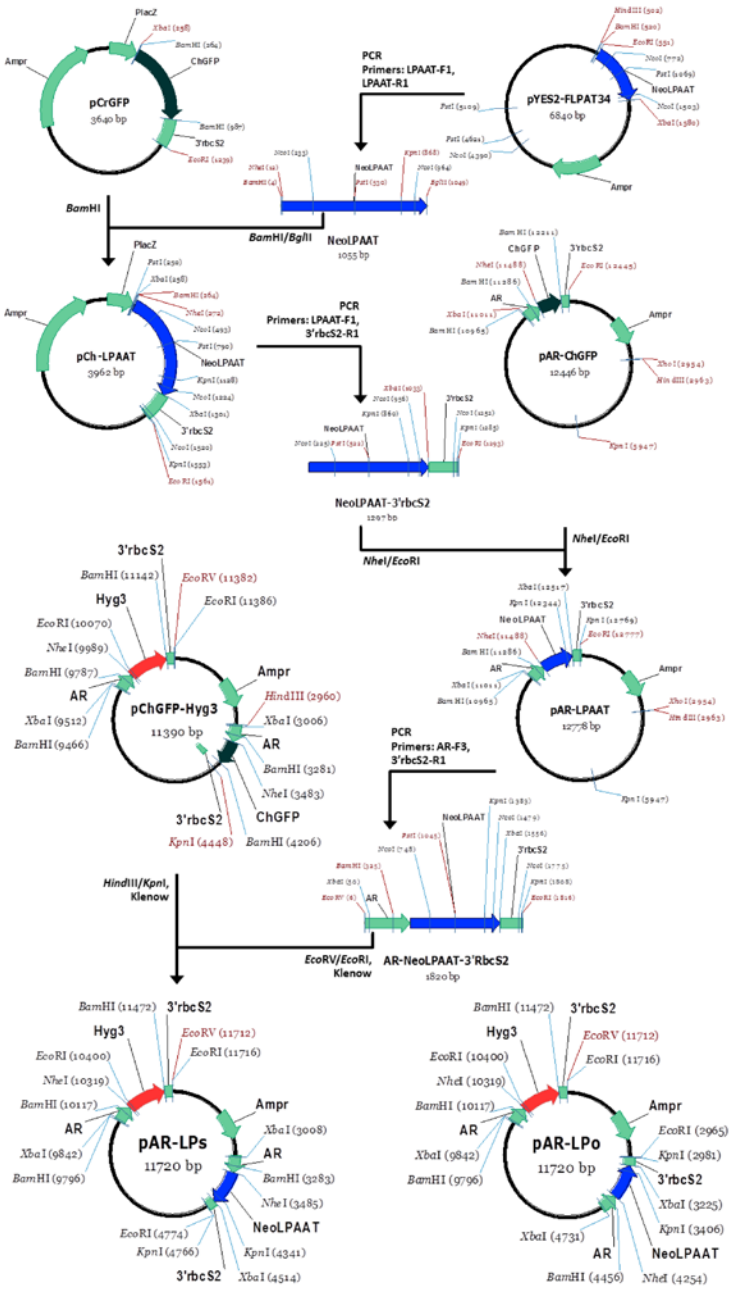



**Fig. 10 Schematic diagram of pAR-LPs and pAR-LPo plasmid construction**

Plasmids pAR-LPs and pAR-LPo harbore hygromycin B-resistant gene *Hyg3* and *NeoLPAAT* cDNA under the control of *AR* promoter in the different orientation. Plasmids pYES-FLPAT34 (Pootanakit and Phienluphon, unpublished data), pCrGFP (Fuhrmann et al. 1999), pAR-ChGFP (Chungjatupornchai et al 2015) and pChGFP-Hyg3 (Chungjatupornchai et al 2015) were indicated.

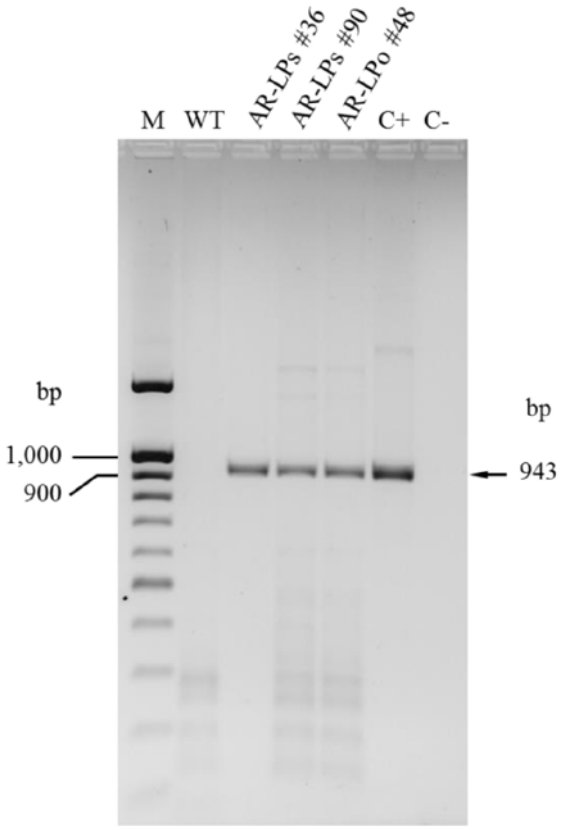



**Fig. 11. Detection of NeoLPAAT cDNA integrated into the nuclear genome of transformant AR-LPs and AR-LPo**

Agarose gel stained with ethidium bromide. Lane M, Lambda DNA digested with PstI; WT, used as negative control PCR product; C+, plasmid pAR-LPs no. 58 used as positive control PCR product; C-, no template (used as negative control PCR reaction); and the transformants are indicated. The PCR product of 943 bp from *NeoLPAAT* cDNA is indicated.

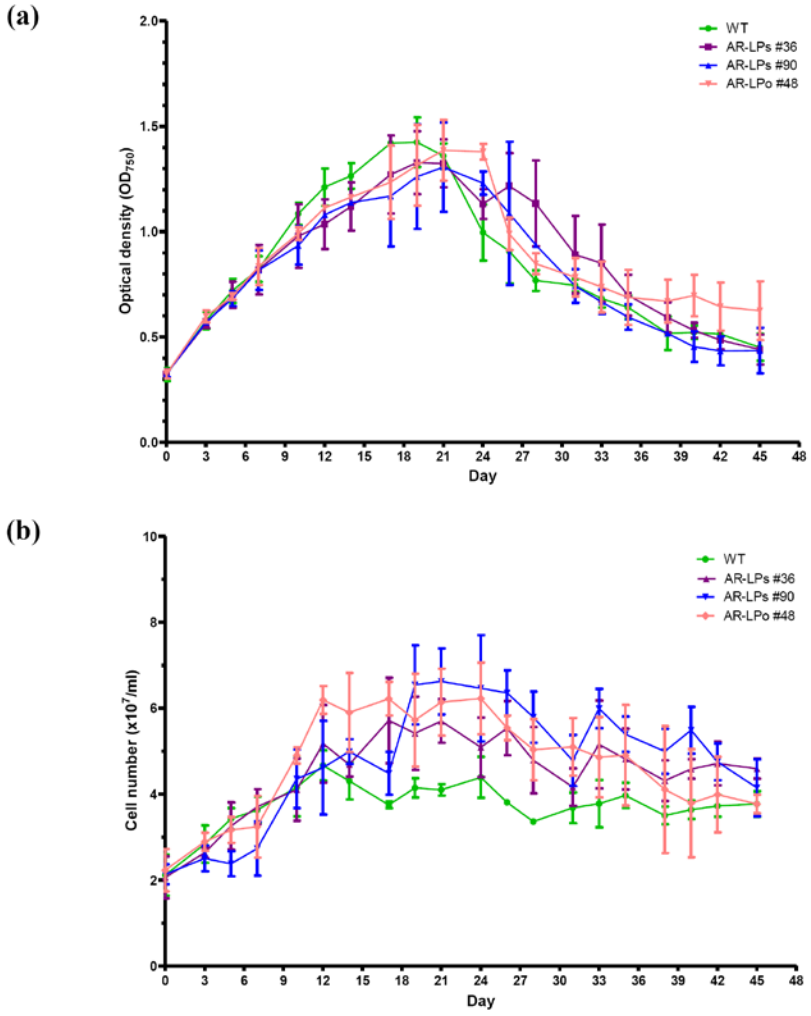



**Fig. 12. Growth of transformants AR-LPs and AR-LPo under basal condition**

Transformants AR-LPs no. 36, AR-LPs no. 90, AR-LPo no. 48 and wild-type were cultured in liquid BBM without hygromycin B. Cell growth was monitored by (a), measuring cell density at $OD_{750}$; and (b), counting cell number using Haemacytometer. Wild-type is represented with green. Transformants AR-LPs no. 36, AR-LPs no. 90 and AR-LPo no. 48 are indicated in the figure. Each value and error bar represents the mean of three independent experiments and the standard deviation.

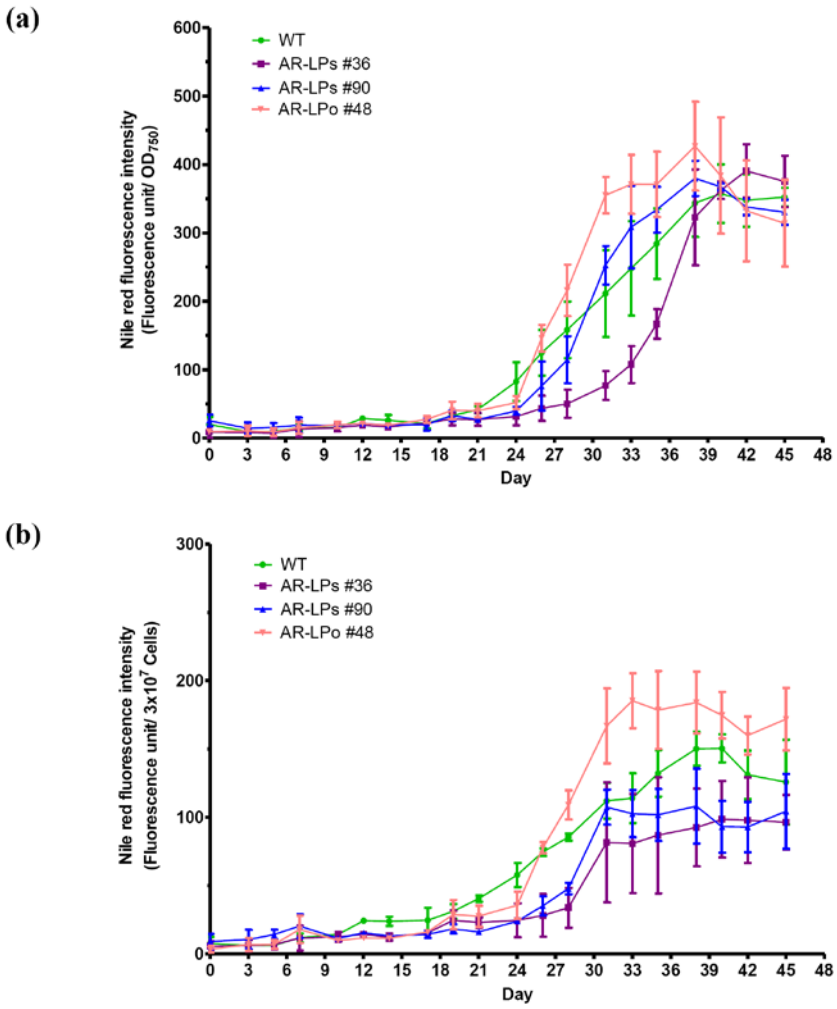



**Fig. 13. Nile Red fluorescence assay of transformants AR-LPs and AR-LPo under basal condition**

Transformants AR-LPs no. 36, AR-LPs no. 90 and AR-LPo no. 48 including wild-type were cultured in liquid BBM without hygromycin B. Neutral lipid accumulation was monitored using Nile Red fluorescence staining. (a) Nile Red fluorescence intensity in 1 $OD_{750}$ of the cells. (b) Nile Red fluorescence intensity in $3x10^7$ cells. Wild-type is represented with green. Transformants AR-LPs no. 36, AR-LPs no. 90 and AR-LPo no. 48 are indicated in the figure. Each value and error bar represents the mean of three independent experiments and the standard deviation.

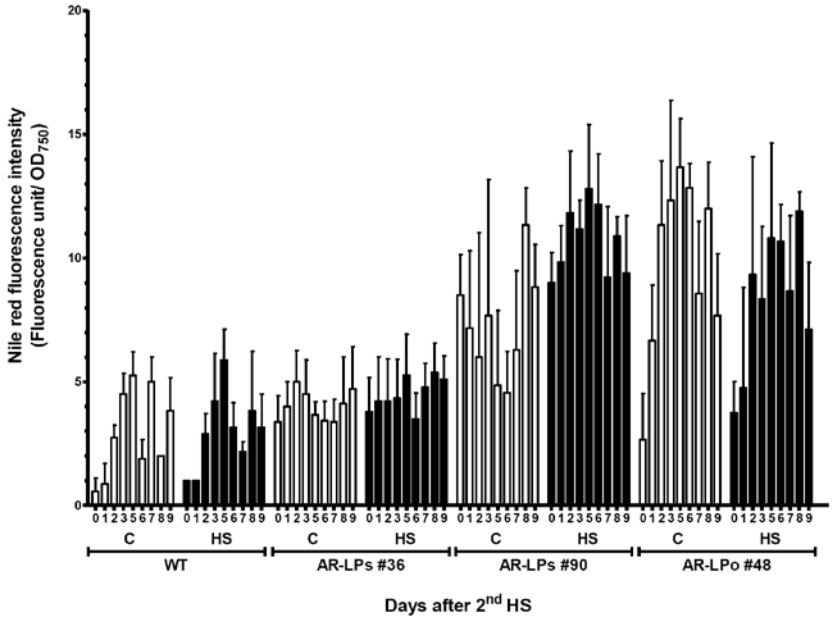



**Fig. 14. Nile Red fluorescence assay of transformants AR-LPs and AR-LPo under heat shock condition**

Transformants AR-LPs no. 36, AR-LPs no. 90 and AR-LPo no. 48 including wild-type were cultured in liquid BBM without hygromycin B. Heat shock (1st HS) was firstly performed for 40 min at 42°C then after 24 hours, heat shock (2nd HS) for 20 min at 42°C was performed. Neutral lipid accumulation was monitored using Nile Red fluorescence staining.

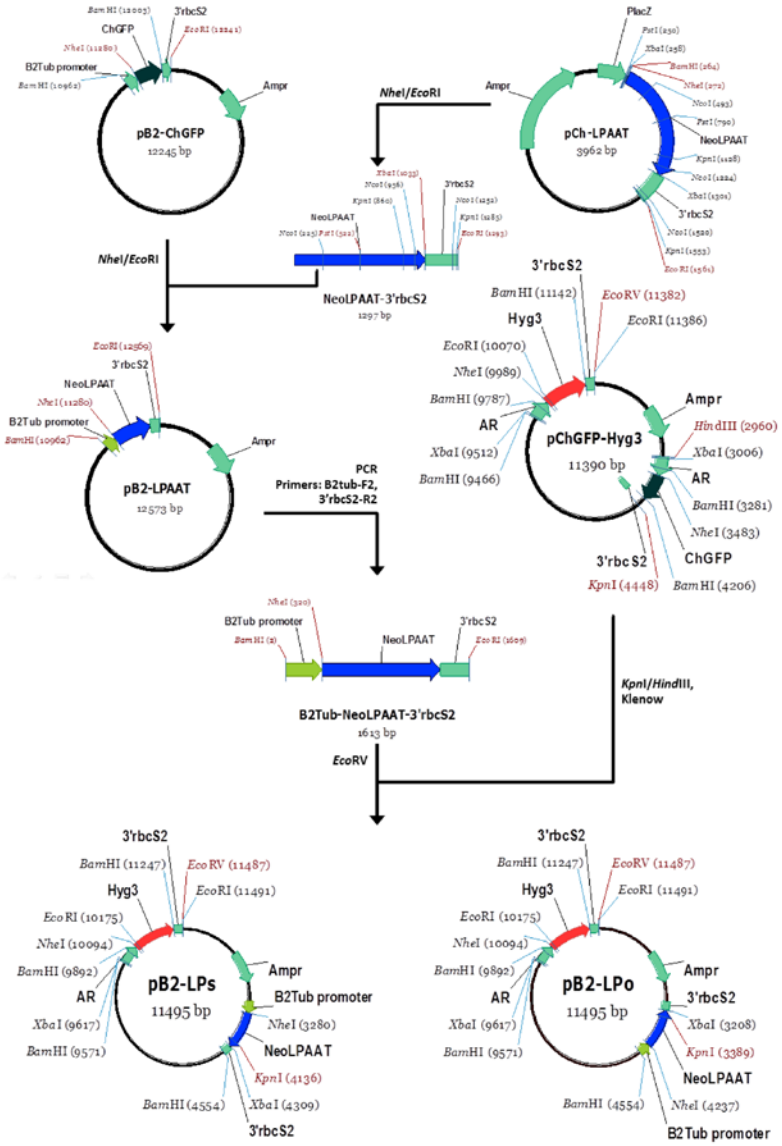



**Fig. 15. Schematic diagram for construction of plasmids pB2-LPs and pB2-LPo**

Plasmids pB2-LPs and pB2-LPo contain *NeoLPAAT* cDNA under the control of β2 – tubulin promoter.

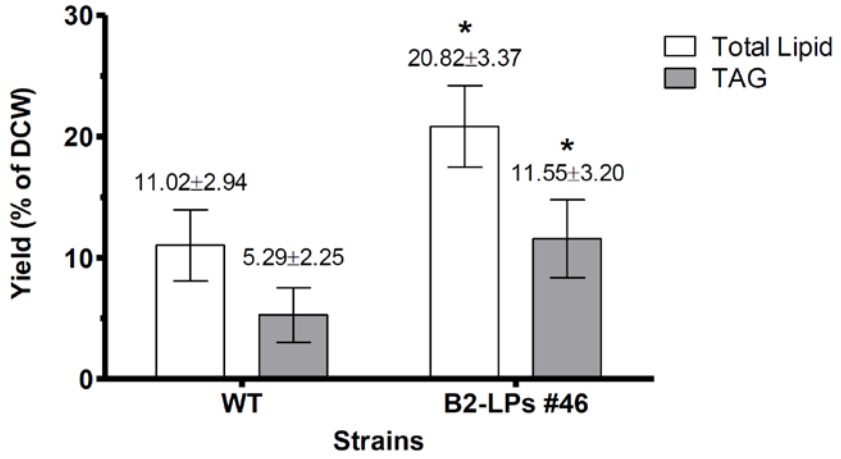



**Fig. 16. Total lipid and TAG content of transformant overexpressing *NeoLPAAT***

Total lipid and TAG content (% of DCW) of transformant B2-LP overexpressing *NeoLPAAT* was significantly increased when compared to that of wild-type (*$P < 0.05$). Each value and error bar represents the mean of three independent experiments and the standard deviation.

**Table 1. Fatty acid composition of TAG from transformant B2-LP overexpressing *NeoLPAAT***

Each value and error bar represents the mean of three independent experiments and the standard deviation.

| Fatty acid identity | Percentage (% of fatty acids) | |
|---|---|---|
| | **Wild-type** | **Transformant B2-LPs no. 46** |
| C 14:0 | 1.32 ± 0.71 | 1.22 ± 0.31 |
| C 14:1 | ND | ND |
| C 15:0 | 0.30 | 0.24 |
| C iso-15:0 | ND | ND |
| C 16:0 | 35.02 ± 3.66 | 28.09 ± 3.35 |
| C 16:1 | 1.03 ± 0.34 | 1.71 ± 0.30 |
| C 16:2 | ND | ND |
| C 17:0 | 0.51 ± 0.04 | 0.53 ± 0.18 |
| C iso-17:0 | ND | ND |
| C 17:1 | 0.48 | 0.48 |
| C 18:0 | 8.81 ± 2.38 | 7.52 ± 0.50 |
| C 18:1 | 43.90 ± 3.39 | 42.88 ± 2.57 |
| C 18:2 | 10.57 ± 4.10 | 18.76 ± 1.75 |
| C 18:3 | 0.72 ± 0.01 | 1.53 ± 0.18 |
| C 19:0 | ND | ND |
| C iso-19:0 | ND | ND |
| C 19:1 | ND | ND |
| C 20:0 | 0.43 | 0.45 |
| C 20:1 | 0.37 | ND |

ND – not detected

**5. Co-overexpression of both *NeoLPAAT* and *NeoDGAT2* cDNA in *N. oleoabundans***

Plasmid pAR-LD and pB2-LD harboring *NeoLPAAT* and *NeoDGAT2* cDNA under the control of promoters AR and β2-tubulin, respectively, were constructed (Fig. 17) and electroporated into *N. oleoabundans* cells. Screening of neutral lipid using Nile red revealed that transformant AR-LD has higher neutral lipid accumulation than B2-LD, therefore transformant AR-LD was used for further investigation. Neutral lipid accumulation of transformant AR-LD was higher than that of wild type (Fig. 18 and 19). Total lipid content of transformant AR-LD increased 1.6-fold; TAG content increased 2.1-fold when compared to wild type (Fig. 20). The fatty acid composition of transformant AR-LD-28 was altered when compared to that of wild type: C16:0 increased and C18:0 decreased (Fig. 21). Thus, the level of increased TAG content in transformant AR-LD is similar to that in transformant B2-LP.

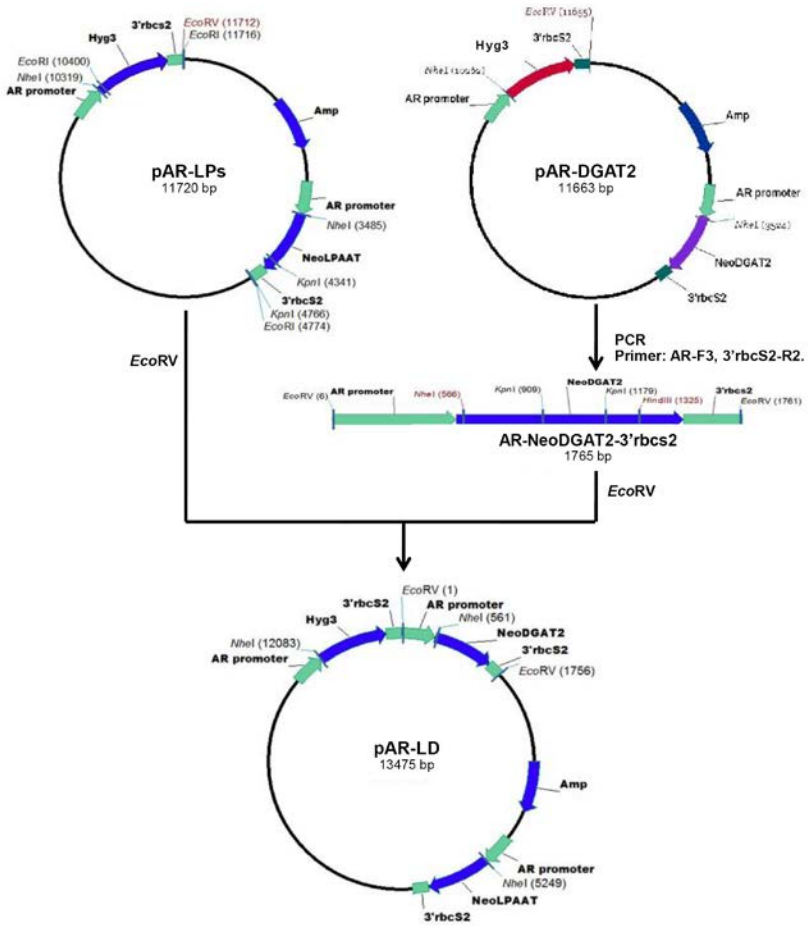



**Fig. 17. Schematic diagram of the pAR-LD construction**

The NeoDGAT2 PCR product containing the *AR* promoter, *NeoDGAT*2 cDNA and 3'*rbcS*2 was amplified from pAR-DGAT2 (Klaithong, unpublished data) template using primers AR-F3 and 3'*rbc*S2-R2. The *EcoR*V digested PCR product was inserted into the corresponding sites of pAR-LPs (Areerat, unpublished data). The plasmid pAR-LPs harbors *NeoLPAAT* cDNA under the control of *AR* promoter, and hygromycin-resistance gene *Hyg3*. Ampicillin-resistance gene (*Amp*) and *Hyg3* gene were used for selection of recombinant *E. coli* clones and transformant *N. oleoabundans*, respectively.

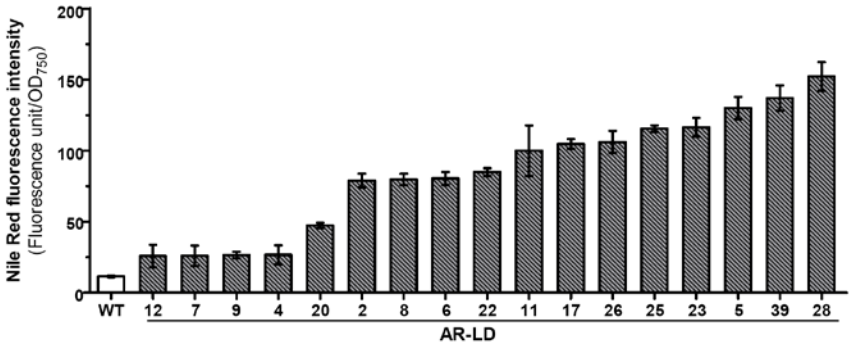



**Fig. 18. Nile Red fluorescence intensity of transformant AR-LD**

Wild-type and transformants AR-LD grown on BBM agar containing hygromycin B for 14 days were stained with the fluorescence dye Nile Red. The Nile Red fluorescence intensity was determined using a spectrofluorometer. Transformant clone number is indicated in the figure. All of transformants revealed significantly increased of Nile Red fluorescence intensity in comparison to wild-type (*P<0.05). Each value and error bar represents the mean of three independent experiments and the standard deviation.

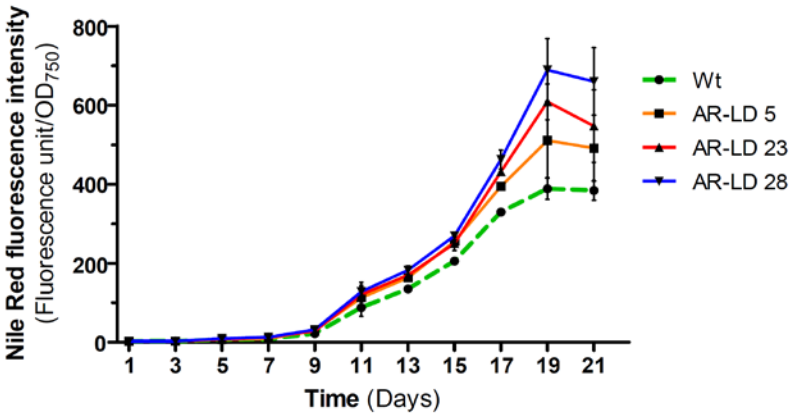



**Fig. 19. Nile Red fluorescence intensity of transformant AR-LD under N-starvation condition**

Each value and error bar represents the mean of three independent experiments and the standard deviation.

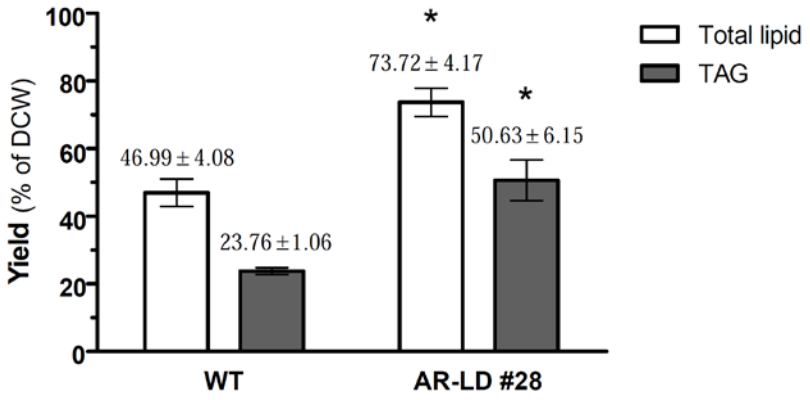



**Fig. 20. Total lipid and TAG of transformant overexpressing both *NeoLPAAT* and *NeoDGAT2* cDNA**

Total lipid and TAG (% of DCW) of transformant AR-LD overexpressing both *NeoLPAAT* and *NeoDGAT2* cDNA was significantly increased when compared to that of wild-type (*$P$ < 0.01). Each value and error bar represents the mean of three independent experiments and the standard deviation.

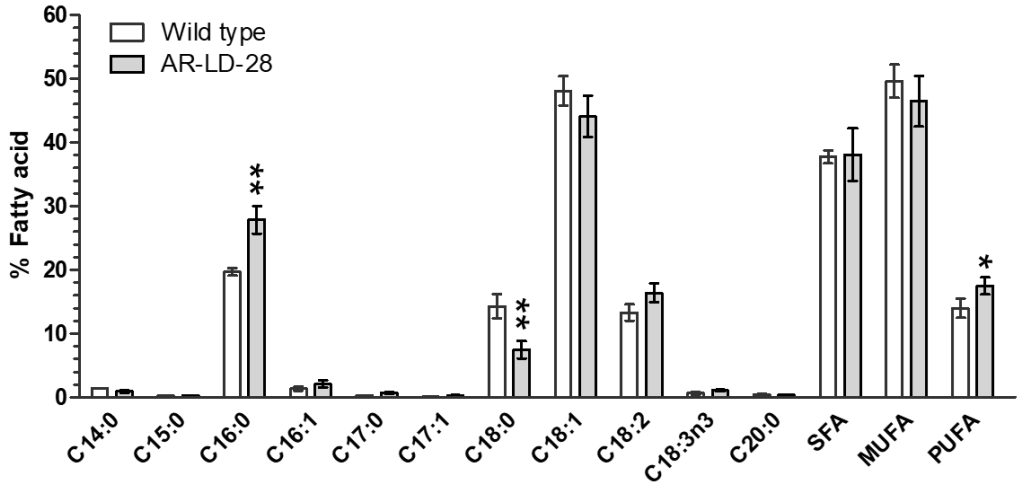



**Fig. 21. Fatty acid composition in transformant AR-LD-28.**

FAME in transformant AR-LD-28 and wild type were analyzed using GC-MS. Fatty acid C16:0 and PUFA increased in transformant AR-LD-28, C18:0 decreased when compared to wild type. SFA, MUFA and PUFA are Saturated, Monounsaturated and Polyunsaturated fatty acids. Each value represents mean ± SD (n=3). Significant difference between transformant AR-LD-28 and wild type is indicated (*$P < 0.05$, **$P < 0.01$, *t* test).

# Output ที่ได้จากโครงการ

## ผลงานวิจัยที่ตีพิมพ์ในวารสารวิชาการระดับนานาชาติ

(ระบุทุนวิจัย สกว. ใน Acknowledgements, *=corresponding author)

1). Chungjatupornchai W* and Watcharawipas A (2015) Diacylglycerol acyltransferase type 2 cDNA from the oleaginous microalga *Neochloris oleoabundans*: cloning and functional characterization. J Appl Phycol 27:1499–1507.

2). Chungjatupornchai W*, Kitraksa P and Fa-aroonsawat S. (2016) Stable nuclear transformation of the oleaginous microalga *Neochloris oleoabundans* by electroporation. J Appl Phycol 28:191–199.

3). Klaitong P, Fa-aroonsawat S and Chungjatupornchai W* (2017) Accelerated triacylglycerol production and altered fatty acid composition in oleaginous microalga *Neochloris oleoabundans* by overexpression of diacylglycerol acyltransferase 2. Microb Cell Fact. 16: 61.

4). Chungjatupornchai W*, Areerat K and Fa-aroonsawat S (2019) Increased triacylglycerol production in oleaginous microalga *Neochloris oleoabundans* by overexpression of plastidial lysophosphatidic acid acyltransferase. Microb Cell Fact. 18: 53.

## รายละเอียดการได้รับเชิญไปเป็นวิทยากร

1). เสนอผลวิจัยแบบ Oral ในหัวข้อ “Increased lipid production and altered lipid composition in microalga *Neochloris oleoabundans* by overexpression of diacylglycerol acyltransferase 2” ในการประชุมวิชาการเรื่อง 11$^{th}$ International Phycological Congress ระหว่างวันที่ 13-19 สิงหาคม 2560 ณ เมือง Szczecin ประเทศโปแลนด์
โดยมีการตีพิมพ์ Congress abstract ในวารสารวิชาการ Phycologia:
Chungjatupornchai W; Klaitong P and Fa-aroonsawat S. Increased lipid production and altered lipid composition in microalga *Neochloris oleoabundans* by overexpression of diacylglycerol acyltransferase 2. Phycologia (2017) 56 (4) supplement: 32-33.

2). เป็นวิทยากร ในหัวข้อ “Exploring microalgae as fuel” ใน Student Science Training Program Year 2017 วันที่ 4 กรกฎาคม 2560 ณ สถาบันชีววิทยาศาสตร์โมเลกุล มหาวิทยาลัยมหิดล

3). เป็นวิทยากร ในหัวข้อ “Exploring microalgae as fuel” ใน Student Science Training Program Year 2016 วันที่ 14 กรกฎาคม 2559 ณ สถาบันชีววิทยาศาสตร์โมเลกุล มหาวิทยาลัยมหิดล

4). เป็นวิทยากร ในหัวข้อ “Exploring microalgae as fuel” ใน Student Science Training Program Year 2015 วันที่ 25 มิถุนายน 2558 ณ สถาบันชีววิทยาศาสตร์โมเลกุล มหาวิทยาลัยมหิดล

# ภาคผนวก

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# INCREASED LIPID PRODUCTION AND ALTERED LIPID COMPOSITION IN MICROALGA *NEOCHLORIS OLEOABUNDANS* BY OVEREXPRESSION OF DIACYLGLYCEROL ACYLTRANSFERASE 2

Biodiesel production from microalgae is technically feasible, but not yet economically viable. Increasing microalgal lipid triacylglycerol (TAG) content via genetic engineering could be a potential approach to improve microalgae as an economically viable biodiesel feedstock. Because genetic manipulation of microalgae requires the accessibility to stable nuclear transformation, we have successfully developed a stable nuclear transformation system of the oleaginous microalga *Neochloris oleoabundans* using electroporation. The cDNA encoding the key enzyme of TAG biosynthesis pathway, diacylglycerol acyltransferase (NeoDGAT2) of *N. oleoabundans*, has been cloned and characterized. In this study, in order to increase TAG accumulation, *NeoDGAT2* expression cassettes was transformed into *N. oleoabundans*. The neutral lipid accumulation in the transformant detected by Nile red staining was accelerated and 1.9-fold higher than in wild type; the lipid bodies in the transformant visualized under fluorescence microscope were also larger. The *NeoDGAT2* transcript was 2-fold higher in the transformant than wild type. Remarkably higher TAG accumulation was found in the transformant than wild type: TAG content increased 1.8- to 3.2-fold up to 46.1±1.6 % dry cell weight and TAG productivity increased 1.6- to 4.3-fold up to 8.9±1.3 mg/L/day, depending on cell culture condition. A significantly altered fatty acid composition was detected in the transformant compared to wild type; the levels of saturated fatty acid C16:0 increased double to 49%, whereas C18:0 was reduced triple to 6%. Long-term stability was observed in the transformants continuously maintained in solid medium over 100 generations in a period of about 4 years. The increasing TAG content in *N. oleoabundans* by overexpression of *NeoDGAT2* may offer the first step towards making microalgae an economically feasible source for biodiesel production.

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ได้เสนอผลวิจัยนี้ แบบ Oral ในการประชุมวิชาการเรื่อง 11th International Phycological Congress ระหว่างวันที่ 13-19 สิงหาคม 2560 ณ เมือง Szczecin ประเทศโปแลนด์ โดยมีการตีพิมพ์ Congress abstract ในวารสารวิชาการ Phycologia:

Chungjatupornchai W; Klaitong P and Fa-aroonsawat S. Increased lipid production and altered lipid composition in microalga *Neochloris oleoabundans* by overexpression of diacylglycerol acyltransferase 2. Phycologia (2017) 56 (4) supplement: 32-33.

J Appl Phycol (2015) 27:1499–1507
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# Diacylglycerol acyltransferase type 2 cDNA from the oleaginous microalga *Neochloris oleoabundans*: cloning and functional characterization

**Wipa Chungjatupornchai · Akaraphol Watcharawipas**

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**Abstract** Currently, biodiesel production from microalgae is technically, but not yet economically, feasible. Increasing microalgal triacylglycerol (TAG) content via genetic engineering could be a potential approach to improve the efficiency of biodiesel production. In all organisms studied so far, diacylglycerol acyltransferase (DGAT) is the primary enzyme of TAG biosynthesis. In this study, we cloned the cDNA encoding DGAT type 2 (NeoDGAT2) from the oleaginous microalga *Neochloris oleoabundans*. The open reading frame of *NeoDGAT2* cDNA was 978 bp encoding a protein of 325 amino acids. Although *N. oleoabundans* has been classified in the class Chlorophyceae, NeoDGAT2 was closely related to Treboxiophycean DGAT2 as it shared 80 % amino acid identity with CvDGAT2-2 of *Chlorella variabilis*, but distantly related to Chlorophycean DGAT2 as it shared only 34 % identity with CrDGAT2A (DGTT4) of *Chlamydomonas reinhardtii*. As suggested by hydropathy analysis, NeoDGAT2 might contain 4 to 5 transmembrane domains. We tested whether *NeoDGAT2* cDNA encoded a protein with DGAT activity by functional complementation assay in yeast *Saccharomyces cerevisiae* mutant H1246. NeoDGAT2 was able to compensate the endogenous DGAT2 activity of mutant H1246 and restore the lipid body formation and TAG synthesis. The successful cloning of cDNA encoding a protein with DGAT activity provides a candidate for genetic manipulation in microalgae to increase TAG content for biodiesel production.

**Keywords** Microalgae · *Neochloris oleoabundans* · Biodiesel · Triacylglycerol biosynthesis · Diacylglycerol acyltransferase (DGAT)

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## Introduction

Biodiesel has been considered as an alternative biofuel that can replace the petrodiesel. Commercial biodiesel is mainly produced from higher plant lipids. Because microalgae have much higher lipid and biomass productivity compared to terrestrial plants, they are promising sources of lipid triacylglycerol (TAG) for biodiesel production (Chisti 2007). However, currently, biodiesel production from microalgae is technically, but not yet economically, feasible. Increasing TAG content of microalgae via genetic engineering could be a potential approach to improve the efficiency of biodiesel production. Therefore, we need to have a better understanding of how TAG biosynthesis is controlled in microalgae. The TAG biosynthesis pathway in microalgae is considered to be most similar to that operating in higher plants (Chen and Smith 2012). TAG can be synthesized by sequential transfer of fatty acyl chains from acyl-CoA through the glycerol 3-phosphate pathway, commonly known as the Kennedy pathway (Kennedy and Weiss 1956). In this pathway, diacylglycerol acyltransferase (DGAT; EC 2.3.1.20) catalyzing the final and committed step has been identified as the rate-limiting enzyme for lipid accumulation in plants (Jako et al. 2001; Lung and Weselake 2006). In all organisms studied so far, DGAT is thought to be the primary enzyme for de novo TAG biosynthesis, while two other acyl-CoA independent TAG synthases: phospholipid/diacylglycerol acyltransferase (PDAT) and diacylglycerol transacylase (DGTA) play a significant role in specialist aspects of TAG biosynthesis (Chen and Smith 2012). Thus, DGAT could be the first candidate gene for genetic manipulation of TAG biosynthesis in microalgae. DGAT is composed of three isozymes: DGAT1 (Cases et al. 1998), DGAT2 (Lardizabal et al. 2001), and DGAT3 (Saha et al. 2006). Recently, most microalgae species have been shown to have one *DGAT1* and multiple *DGAT2* genes (Chen and Smith 2012). Although putative DGAT of

various microalgae have been identified (for further information, see (Chen and Smith 2012)), so far, only DGAT from few microalgae have been functionally characterized, i.e., *Phaeodactylum tricornutum* (Gong et al. 2013; Guiheneuf et al. 2011), *Ostreococcus tauri* (Wagner et al. 2010), and *Chlamydomonas reinhardtii* (Boyle et al. 2012; Miller et al. 2010; Msanne et al. 2012; Hung et al. 2013). However, the DGAT from oleaginous microalgae, such as *Neochloris oleoabundans*, is not defined.

*Neochloris oleoabundans*, a taxonomic synonym of *Ettlia oleoabundans* (Deason et al. 1991), can accumulate 36–54 % lipids of its cell dry weight under nitrogen starvation condition. Besides, 80 % of its total lipids is TAG mainly comprised of the saturated fatty acids containing carbon around 16–20 atoms (Tornabene et al. 1983), ideal for biodiesel production. The knowledge concerning *N. oleoabundans* is very limited; no genomic sequences and no report concerning the full-length coding sequences of genes involved in TAG biosynthesis are available. Recently, transcriptomic analysis of *N. oleoabundans* reveals that under nitrogen limitation, the expression of gene encoding DGAT has relatively no change (Rismani-Yazdi et al. 2012). Because *N. oleoabundans* can accumulate a high amount of TAG, knowledge of *DGAT* gene from *N. oleoabundans* would enable subsequent genetic manipulation of the TAG biosynthesis.

In this study, we report the cloning and characterization of the full-length cDNA sequence of *DGAT2* gene from *N. oleoabundans* (*NeoDGAT2*). We tested whether *NeoDGAT2* cDNA encoded a protein with DGAT activity by functional complementation assay in yeast *Saccharomyces cerevisiae*.

## Materials and methods

### Strains and growth conditions

*Neochloris oleoabundans* strain UTEX 1185, obtained from the Algal Culture Collection at the University of Texas at Austin, was grown in liquid BBM medium (Bischoff and Bold 1963) at 30 °C under constant illumination of 40–54 μmol photons $m^{-2}$ $s^{-1}$. *Escherichia coli* DH5α was grown in LB broth or on agar (Sambrook and Russell 2001) at 37 °C. *Saccharomyces cerevisiae* neutral-lipid deficient quadruple mutant strain H1246 (*Δdga1 Δlro1 Δare1 Δare2*) (Sandager et al. 2002) and wild-type strain SCY62 (Sandager et al. 2002) were grown in liquid or solid (2 % Conda agar) YPD medium (Sambrook and Russell 2001) at 30 °C. Yeast transformants were grown in SD-ura medium (Sambrook and Russell 2001).

### cDNA synthesis

To synthesize cDNA, total RNA was extracted from *N. oleoabundans* grown in BBM broth (Bischoff and Bold 1963) for 15 days using TRI reagent (Molecular Research Center, USA). The cDNA was prepared using total RNA as template, oligo$(dT)_{18}$ primer, and RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Canada).

### Cloning of *NeoDGAT2* cDNA

To clone the full-length *DGAT* cDNA sequence of *N. oleoabundans* (*NeoDGAT*), various reverse transcription (RT)-PCR strategies were performed. Briefly, to identify *NeoDGAT2* partial sequence, consensus degenerate hybrid oligonucleotide primer (CODEHOP)-mediated PCR (Rose et al. 1998) was performed using oligo$(dT)_{18}$-primed cDNA template with primers D2Ca-F1 and D2Db-R2 designed based on two conserved blocks in the multiple sequence alignment of DGAT2 proteins from closely related green microalgae (data not shown). To identify 3′end of *NeoDGAT2* cDNA sequence, 3′rapid amplification of cDNA ends (3′RACE)-PCR was performed using oligo$(dT)_{18}$-primed cDNA template with primer sets: 3R-D2-F1 and Prt, 3R-D2-F2 and PmI for first and semi-nested PCR, respectively. To identify 5′end of *NeoDGAT2* cDNA sequence, 5′rapid amplification of cDNA ends (5′RACE)-PCR was performed using 5R-D2-R3-primed cDNA containing 3′A-tailing as template with primer sets: 5R-D2-R1 and Prt, 5R-D2-R2 and PmI for first and semi-nested PCR, respectively. Primers 3R-D2-F1, 3R-D2-F2, 5R-D2-R3, 5R-D2-R2, and 5R-D2-R1 were designed based on the resulting *NeoDGAT2* partial cDNA sequence. To obtain full-length *NeoDGAT2* cDNA sequence, PCR was performed using oligo$(dT)_{18}$-primed cDNA as template with primers N-DGAT2-F1 and N-DGAT2-R1 designed based on the assembled 5′end and 3′end partial *NeoDGAT2* sequence. The PCR products were inserted into pGEM-T Easy plasmid (Promega, USA) and transformed into *E. coli* DH5α. The resulting plasmid was designated as pG-NeoDGAT2. DNA sequences of PCR products were determined by automated sequence analyzer. Primers used in this study were shown in Table 1.

### *NeoDGAT2* cDNA sequence analysis

To analyze the *NeoDGAT2* cDNA sequence, various methods were performed as follows. The NeoDGAT2 deduced amino acid sequence was aligned with other DGAT2 using CLUSTALW multiple alignment program (Thompson et al. 1994). The phylogenetic tree of NeoDGAT2 was constructed using the neighbor-joining method with MEGA 4 (Tamura et al. 2007). The membrane topology of NeoDGAT2 was

**Table 1** Primers used in this study. The restriction sites KpnI, BamHI, and EcoRI are underlined. The start and stop codon are in bold. The yeast consensus sequence for enhanced translation is in italics

| Primer | Sequence (5′-3′) | Target sequence |
|---|---|---|
| D2Ca-F1 | CCGATCGTGCGACACCTGTGGWSGTGGHTGGG | *DGAT2* |
| D2Db-R2 | CG CCAGTAGCTGTACGCCCGGGAYTGNCCAA | *DGAT2* |
| Oligo(dT)$_{18}$ | TTTTTTTTTTTTTTTTTT | Poly A sequence |
| Prt | CCGGAATTCAAGCTTCTAGAGGATCCTTTTTTTTT TTTTTTT | Poly A sequence |
| PmI | CCGGAATTCAAGCTTCTAGAGGATCC | Adaptor sequence |
| 3R-D2-F1 | ATGGAGCGGGAGAGTGGCAGGG | 3′ end *NeoDGAT2* |
| 3R-D2-F2 | ACGCCACGGCTTTGTCAGGC | 3′ end *NeoDGAT2* |
| 5R-D2-R1 | CTCCCTGCCACTCTCCCGCTCC | 5′ end *NeoDGAT2* |
| 5R-D2-R2 | CCACAGACCTGCCCTTCTTCAGC | 5′ end *NeoDGAT2* |
| 5R-D2-R3 | GACAAAGCCGTGGCGTTTGCG | 5′ end *NeoDGAT2* |
| N-DGAT2-F1 | GGGGTACCCC*ACATA***ATG**GCGGCTCAGCGCGGTTTCG | 5′ end *NeoDGAT2* |
| N-DGAT2-R1 | CGGGATCCCG**TTA**GTAGATGGTAAGACTGGCATGTGG | 3′ end *NeoDGAT2* |
| N-DGAT2-F1B | CGGGATCC*ACATA***ATG**GCGGCTCAGCGCGGTTTCG | 5′ end *NeoDGAT2* |
| N-DGAT2-R1E | CGGAATTC**TTA**GTAGATGGTAAGACTGGCATGTGG | 3′ end *NeoDGAT2* |
| N-DGAT2-R1-6HE | CGGAATTC**TTA**ATGGTGATGGTGATGATG/GTAGAT GGTAAG ACTGGCATGTGG | 6 His/ 3′ end *NeoDGAT2* |

predicted using five common topological softwares: HMMTOP v2.0 [http://www.enzim.hu/hmmtop/index.php] (Tusnády and Simon 2001), TMHMM v2.0 [http://www.cbs.dtu.dk/services/TMHMM/] (Krogh et al. 2001), TopPred 2 [http://www.sbc.su.se/~erikw/toppred2/] (von Heijne 1992), TMpred [http://www.ch.embnet.org/software/TMPRED_form.html] (Hofmann and Stoffel 1993), and SOSUI [http://bp.nuap.nagoya-u.ac.jp/sosui/] (Hirokawa et al. 1998). Hydropathy analysis of NeoDGAT2 was performed using Kyte-Doolittle algorithm (Kyte and Doolittle 1982).

### Construction of plasmids harboring *NeoDGAT* cDNA for expression in *S. cerevisiae*

To construct plasmids for expression of *NeoDGAT* cDNA in yeast, the PCR products containing full-length *NeoDGAT2* cDNA without and with six histidine residues at the C-terminus were amplified using pG-NeoDGAT2 as template with primer sets: N-DGAT2-F1B and N-DGAT2-R1E, N-DGAT2-F1B and N-DGAT2-R1-6HE, respectively. The resulting *Bam*HI/*Eco*RI-digested PCR products were inserted into the corresponding sites of yeast expression vector pYES2 (Invitrogen, USA) to obtain plasmids pY-NeoDGAT2 and pY-NeoDGAT2H, respectively. The plasmids were transformed into *S. cerevisiae* wild-type strain SCY62 and mutant strain H1246 as described (Invitrogen, USA). The expression of *NeoDGAT2* cDNA in yeast was induced by supplementing galactose to 2 % (*w*/*v*).

### Nile red fluorescence assay and microscopy

To estimate the level of neutral lipids, Nile red fluorescence assay was carried out essentially as described (Siloto et al. 2009). In brief, after induction with galactose, *S. cerevisiae* cells were suspended in phosphate buffer saline ($OD_{600}$=0.4) and stained with Nile red at final concentration of 0.04 mg/mL. The cells were incubated in the dark for 5 min. The fluorescence intensity was measured in a 96-well plate using a spectrofluorometer (Beckman Coulter DTX-880, USA) with excitation at 485 nm and emission at 535 nm. Specific fluorescence intensities were normalized by the optical density measured at 600 nm. The lipid bodies in the cells stained with Nile red were visualized under inverted fluorescence microscope (Nikon Eclipse Ti-S, Japan) with excitation at 420–490 nm and emission at 520 nm.

### Lipid extraction and analysis

Total lipids were extracted from yeast cells according to a modified version of Bligh and Dyer method (Bligh and Dyer 1959). The yeast cells suspended in methanol:chloroform (2:1 *v*/*v*) were lysed using 0.5 mm glass beads with vortexing. The cell lysate was obtained by centrifugation. Total lipid was extracted from cell lysate using 50 mM citric acid:chloroform (2:3 *v*/*v*), and chloroform was evaporated using $N_2$ gas. The total lipid was kept at −20 °C until use. To separate lipid classes by thin-layer chromatography (TLC), the total lipid was dissolved in chloroform and applied to Silica gel 60 $F_{254}$ (Merck, USA). Chromatogram was developed in

hexane:diethyl ether:glacial acetic acid (70:30:1 *v*/*v*/*v*). The separated lipid classes were visualized under UV 254 nm.

## Results and discussion

### Predicted sequence features and phylogenetic position of NeoDGAT2

The full-length *NeoDGAT2* cDNA was successfully cloned using various RT-PCR strategies (see "Materials and methods"). The open reading frame of *NeoDGAT2* cDNA was 978 bp encoding a protein of 325 amino acids, NeoDGAT2. The sequence data have been submitted to GenBank database under accession number KJ470774. Comparison of NeoDGAT2 with other DGAT2 from related green microalgae revealed that NeoDGAT2 contained all the six highly conserved motifs that were identified as signature motifs within the DGAT2 subfamily (Cao 2011): motif 1, PH block; motif 2, PR block; motif 3, GGE block; motif 4, RGFA block; motif 5, VPFG block; and motif 6, G block (Fig. 1a). Therefore, the results indicated that indeed the DGAT of *N. oleoabundans* in this study was a member of DGAT2 family. NeoDGAT2 was closely related to Treboxiophycean DGAT2 as it shared 80 and 52 % amino acid identity with CvDGAT2-2 and CvDGAT2-1 of *Chlorella variabilis*, respectively. However, NeoDGAT2 was quite distantly related to Chlorophycean DGAT2 as it shared only 34 % identity with CrDGAT2A (DGTT4) of *C. reinhardtii* and 31 % identity with VcDGAT2A of *Volvox cateri*. In a phylogenetic tree, NeoDGAT2 was grouped in the same clade as CvDGAT2 (Fig. 1b), suggesting that NeoDGAT2 possessed the closest evolutionary relationship with CvDGAT2. However, *N. oleoabundans* has been classified to class Chlorophyceae based on uninucleate cell morphology (Komárek 1989). Classification of *N. oleoabundans* remains to be verified by 18S and 28S rDNA sequence analyses.

Majority of microalgae species have been shown to have at least one *DGAT1* and multiple *DGAT2* genes (Chen and Smith 2012), whether this observation holds true for *N. oleoabundans* remains to be investigated. The *DGAT* transcript of *N. oleoabundans* is no change under nitrogen limitation (Rismani-Yazdi et al. 2012). However, the *DGAT* transcripts of *C. reinhardtii* are expressed differently; transcripts of *DGAT2A* (*DGTT4*), *DGAT2B* (*DGTT1*), and *DGAT2D* (*DGTT3*) are increased by nitrogen starvation, whereas, that of *DGAT2E* (*DGTT2*) is constitutively expressed (Boyle et al. 2012; Msanne et al. 2012).

The membrane topology of NeoDGAT2 was predicted using five common topological softwares: HMMTOP v2.0 (Tusnády and Simon 2001), TMHMM v2.0 (Krogh et al. 2001), TopPred 2 (von Heijne 1992), TMpred (Hofmann and Stoffel 1993), and SOSUI (Hirokawa et al. 1998). All the five algorithms indicated that NeoDGAT2 resided in the endoplasmic reticulum (ER) membrane with the N-terminus oriented toward the cytosolic side (data not shown). The NeoDGAT2 N-terminus lacked the ER signal peptide as analyzed by SignalP 4.1 server (Petersen et al. 2011) (data not shown), suggesting that other segments may play a role in ER membrane association. The exact working location of NeoDGAT2 remains to be verified experimentally. NeoDGAT2 might contain 4 to 5 transmembrane domains as predicted by hydropathy analysis (Fig. 2). Similar results have been reported that the DGAT2 of *S. cerevisiae*, murine, and tung contains 4, 2, and 2 transmembrane domains, respectively, with the N- and C-termini residing in the cytosolic side (Liu et al. 2011; Shockey et al. 2006; Stone et al. 2006).

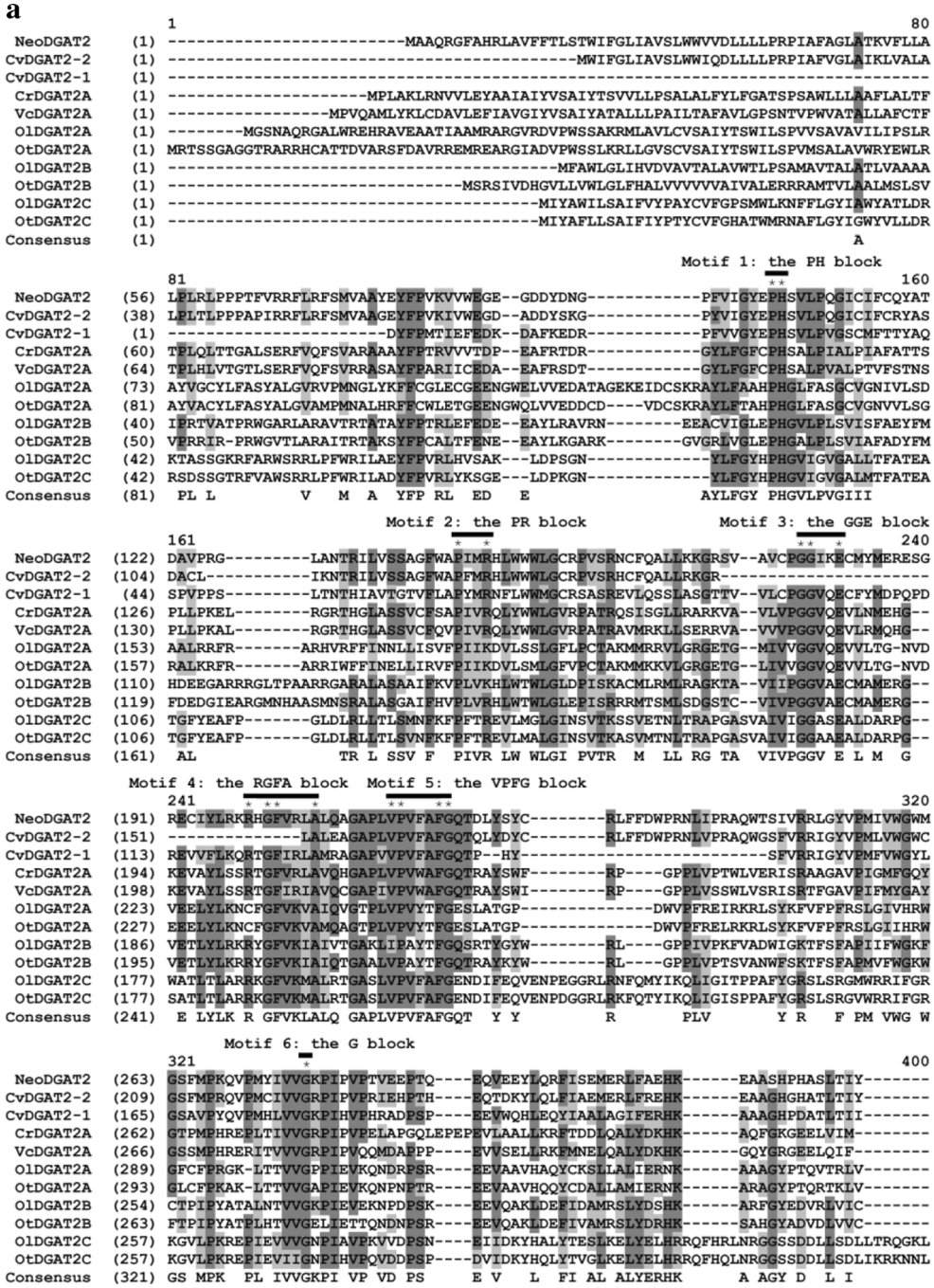
**Fig. 1** Comparison of NeoDGAT2 with other DGAT2 from green microalgae. **a** Sequence alignment of NeoDGAT2. The amino acid sequences were aligned using ClustalW algorithm. The conserved motifs 1, 2, 3, 4, 5, and 6 are indicated. The highly conserved residues in the motif are indicated by *asterisks*. **b** The phylogram of NeoDGAT2. The phylogenetic tree was constructed by neighbor-joining method with MEGA 4. NeoDGAT2 (*N. oleoabundans*, GenBank: KJ470774), CvDGAT2-1 (*Chlorella variabilis*, GenBank: EFN51306), CvDGAT2-2 (*C. variabilis*, GenBank: EFN52435), CrDGAT2A (DGTT4) (*Chlamydomonas reinhardtii,* GenBank: XP001693189), VcDGAT2A (*Volvox carteri*, GenBank: XP002957707), OtDGAT2A (*Ostreococcus tauri*, GenBank: CAL54993), OtDGAT2B (*O. tauri*, GenBank: CAL58088), OtDGAT2C (*O. tauri*, GenBank: CAL56438), OlDGAT2A (*Ostreococcus lucimarinus*, GenBank: XP001419156), OlDGAT2B (*O. lucimarinus*, GenBank: XP001421576), and OlDGAT2C (*O. lucimarinus*, GenBank: XP001421075)

### Functional analysis of NeoDGAT2 in *S. cerevisiae* mutant H1246

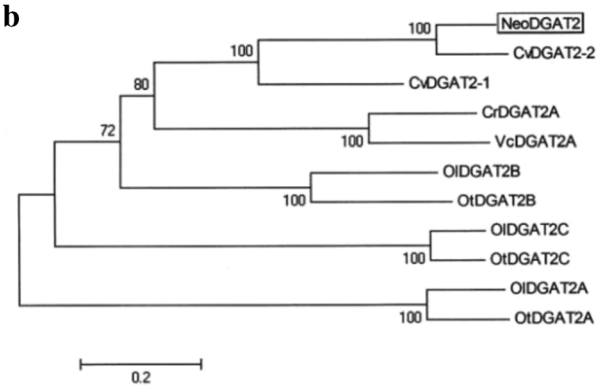
Because majority of microalgal species have been shown to have multiple *DGAT2* genes (Chen and Smith 2012) which may or may not encode the functional protein for TAG biosynthesis (Gong et al. 2013; Hung et al. 2013), it is necessary to verify whether *NeoDGAT2* cDNA encoded a protein with DGAT activity. The biological function of NeoDGAT2 was determined using heterologous complementation assay in *S. cerevisiae* mutant H1246 devoid of TAG and lacking lipid bodies (Sandager et al. 2002). The complementation assay in mutant H1246 has been successfully used to detect the biological function of microalgal *DGAT*, i. e., *OtDGAT2* from *O. tauri* (Wagner et al. 2010), *PtDGAT1* from *P. tricornutum* (Guiheneuf et al. 2011), and *PtDGAT2B* from *P. tricornutum* (Gong et al. 2013).

To express *NeoDGAT2* cDNA in yeast, plasmids pY-NeoDGAT2 and pY-NeoDGAT2H containing *DGAT2* cDNA without and with His at the C-terminal, respectively, were transformed into mutant H1246. Mutant H1246 and wild-type SCY62 harboring vector pYES2 were used as

**a**


Motif 1: the PH block
Motif 2: the PR block
Motif 3: the GGE block
Motif 4: the RGFA block
Motif 5: the VPFG block
Motif 6: the G block

**b**


NeoDGAT2
CvDGAT2-2
CvDGAT2-1
CrDGAT2A
VcDGAT2A
OlDGAT2B
OtDGAT2B
OlDGAT2C
OtDGAT2C
OlDGAT2A
OtDGAT2A
100
100
80
72
100
100
100
100
0.2

negative and positive controls, respectively. Neutral lipids accumulated in the yeast cells were stained with a fluorescent dye, Nile red. Nile red fluorescence assay has been shown to provide a good estimate of TAG accumulation in mutant H1246 expressing a functional heterologous DGAT. Besides, the changes in Nile red fluorescence intensity correlate well with DGAT activity quantified by a standard in vitro radioactive assay (Siloto et al. 2009).

Nile red fluorescence assay was performed to determine the optimal induction time of *NeoDGAT2* cDNA expression in yeast. In Fig. 3, the fluorescence intensities of the mutant H1246 harboring pY-NeoDGAT2 increased from day 1 to reach the maximum at day 4 then decreased at day 5; the fluorescence intensities were higher than those of the corresponding negative control. The fluorescence intensities of positive control from days 1 to 4 were not significantly different then slightly decreased at day 5. Whereas, the fluorescence intensities of negative control from days 1 to 3 were not significantly different, they drastically decreased at days 4 and 5. The results indicated that NeoDGAT2 was able to complement TAG deficient phenotype of mutant H1246, leading to long-term survival in stationary phase, in good agreement with previous report (Sandager et al. 2002). The optimal induction time for fluorescence intensity was at day 4. Therefore, further Nile red fluorescence assay was performed using cells with 4-day induction.

To investigate the effect of His tag at the C-terminal of NeoDGAT2, Nile red fluorescence intensities of yeast cells were compared. Fluorescence intensity of mutant H1246 harboring pY-NeoDGAT2H was about 2- and 4-folds lower than that of pY-NeoDGAT2 and positive control, respectively. The fluorescence intensity of negative control was barely detectable (Fig. 4). The results indicated that C-terminal His tag intended to facilitate the detection of NeoDGAT2 caused the reduction of NeoDGAT2 activity. Therefore, we did not further perform Western Blot analysis. Similar results have been reported that V5 epitope tagged at the C-terminus of *S. cerevisiae* DGAT2 causes the reduction of activity (Liu et al. 2011).

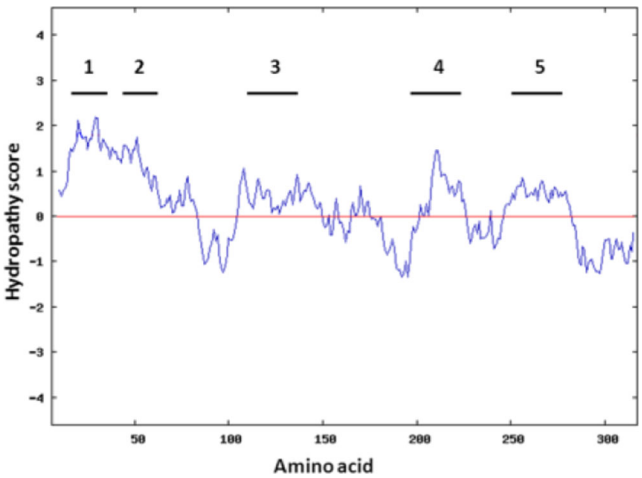



**Fig. 2** Hydropathy plot of NeoDGAT2. Hydropathy analysis of NeoDGAT2 was performed using Kyte-Doolittle algorithm. The predicted transmembrane domains are indicated as *bars*

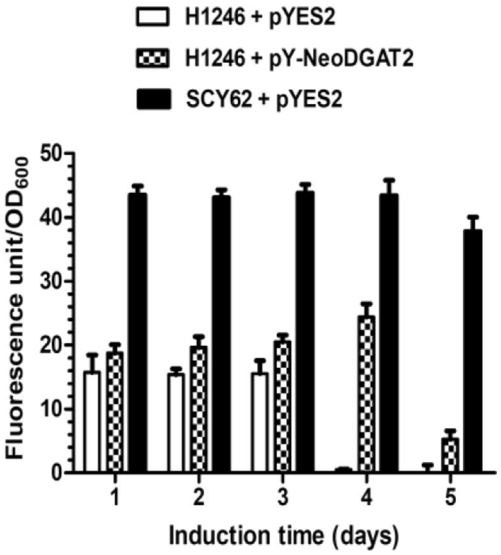



**Fig. 3** Nile red fluorescence assay of *S. cerevisiae* at various induction time courses. Cultures of mutant H1246 harboring plasmid pY-NeoDGAT2 induced with galactose were harvested each day and stained with Nile red. The fluorescence intensities were measured using a spectrofluorometer. The mutant H1246 and wild-type SCY62 harboring the vector pYES2 were used as negative and positive controls, respectively. Each *value* and *error bar* represents the means of three independent experiments and its standard deviation

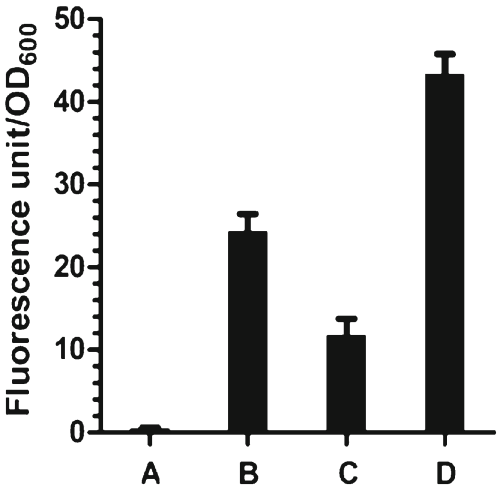



**Fig. 4** Nile red fluorescence assay of mutant H1246 expressing NeoDGAT2. The cultures induced with galactose for 4 days were harvested and stained with Nile red. The fluorescence intensities were measured using a spectrofluorometer. Lanes **a** mutant H1246 harboring vector pYES2 (used as negative control), **b** harboring pY-NeoDGAT2, **c** harboring pY-NeoDGAT2H, and **d** wild-type SCY62 harboring pYES2 (used as positive control). Each *value* and *error bar* represents the means of three independent experiments and its standard deviation

### Lipid body formation in mutant H1246 expressing NeoDGAT2

Because lipid bodies in mutant H1246 can be used as an indicator for TAG formation (Sandager et al. 2002), we determined whether NeoDGAT2 could restore the lipid bodies by visualizing the cells stained with Nile red under fluorescence microscope. Significant accumulation of lipid body formation was detected in mutant H1246 harboring pY-NeoDGAT2 and pY-NeoDGAT2H as well as positive control, but as expected, not detected in negative control (Fig. 5). The results indicated that NeoDGAT2 and NeoDGAT2-His restored lipid body formation and TAG synthesis in mutant H1246.

### TAG accumulation in mutant H1246 expressing NeoDGAT2

We verified whether TAG was restored in mutant H1246 expressing NeoDGAT2 by performing TLC using wild-type SCY62 with well-characterized lipid composition (Sandager et al. 2002) as positive control. The TAG of mutant H1246 harboring pY-NeoDGAT2 and pY-NeoDGAT2H was noticeably detected, although their TAG levels were not significantly different (Fig. 6). The restored TAG accumulation in mutant H1246 was much lower than that in positive control, in good agreement with previous observation (Sandager et al. 2002). The background TAG was barely detected in negative control and might be nonenzymatically produced as reported previously (Sandager et al. 2002; Stobart et al. 1997). The results confirmed that the lipid synthesized by the activity of NeoDGAT2 was TAG.

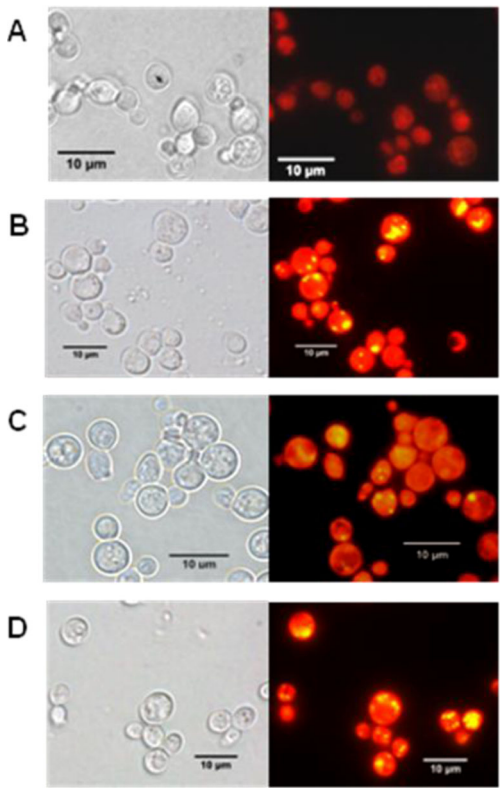



**Fig. 5** Lipid body formation in mutant H1246 expressing NeoDGAT2. The cultures induced with galactose for 4 days were harvested and stained with Nile red. The lipid bodies in the cells were visualized under a bright field microscope (*left panel*) and fluorescence microscope (*right panel*). Mutant H1246 **a** harboring vector pYES2 (used as negative control), **b** harboring pY-NeoDGAT2, **c** harboring pY-NeoDGAT2H, and **d** wild-type SCY62 harboring vector pYES2 (used as positive control)

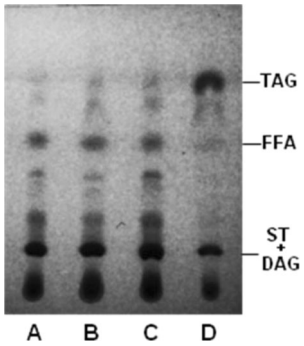



**Fig. 6** TAG accumulation in mutant H1246 expressing NeoDGAT2. Total lipids extracted from the yeast cells were separated using TLC (8 μL in lanes **a**, **b**, and **c**; 3 μL in lane **d**). Lanes **a** total lipids from mutant H1246 harboring vector pYES2 (used as negative control), **b** harboring pY-NeoDGAT2, **c** harboring pY-NeoDGAT2H, and **d** from wild-type SCY62 harboring vector pYES2 (used as positive control). The TAG, FFA (free fatty acid), and ST (sterols) including DAG (diacylglycerol) are indicated

Taken together, results from Nile red fluorescence assay (Fig. 4), fluorescence microscopy (Fig. 5), and TLC (Fig. 6) indicated that NeoDGAT2 was able to compensate the activity of the endogenous yeast DGAT2 and participate in yeast lipid metabolism. Therefore, NeoDGAT2 possesses the DGAT activity in vivo.

TAG biosynthesis in microalgae is believed to occur mainly through the Kennedy pathway. An alternative route known as acyl-CoA independent mechanism might take place in some microalgae since the homologues of gene encoding for PDAT have been found in *Dunaliella tertiolecta* transcriptome (Rismani-Yazdi et al. 2011). However, such homologues have not been discovered in *N. oleoabundans* transcriptome (Rismani-Yazdi et al. 2012), making it unclear whether PDAT contributes to TAG biosynthesis in *N. oleoabundans*. More work needs to be done to identify and characterize genes along TAG biosynthesis pathways as well as the regulatory controls operating on the pathway, if progress is to be made on genetically engineering microalgae to increased biodiesel production.

## Conclusions

We successfully cloned the *NeoDGAT2* cDNA from *N. oleoabundans*. Although *N. oleoabundans* has been

classified to class *Chlorophycea*, NeoDGAT2 had a closer evolutionary relationship with DGAT2 of *Treboxiophycea* than that of *Chlorophycea*. NeoDGAT2 was able to compensate the endogenous DGAT2 activity of yeast and restore the lipid body formation and TAG synthesis. This work also contributes to a growing understanding of DGAT in microalgal TAG biosynthesis. NeoDGAT2, likely to be the primary enzyme for TAG biosynthesis, would be a candidate for genetic manipulation in microalgae to increase TAG content for biodiesel production in our next trial.

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# Stable nuclear transformation of the oleaginous microalga *Neochloris oleoabundans* by electroporation

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**Abstract** Biodiesel from microalgae is technically feasible, but not yet economically viable. A potential approach to improve microalgae as an economically viable biodiesel feedstock is to increase microalgal lipid content via genetic engineering. Genetic manipulation of microalgae requires the accessibility to stable nuclear transformation. In this study, we describe a strategy for developing a stable nuclear transformation system of the oleaginous microalga *Neochloris oleoabundans* using electroporation. The hygromycin B-resistant gene *Hyg3*, which was used as a positively selectable marker, consisted of *aph7*" gene encoding aminoglycoside phosphotransferase of *Streptomyces hygroscopicus* and intron1 of *Chlamydomonas reinhardtii rbcS2* gene, under the control of *C. reinhardtii HSP70A-RBCS2* hybrid promoter. The transformation frequency was $5.2\times10^{-4}$ transformants $mg^{-1}$ DNA. The transformants showed stable hygromycin B-resistant phenotype for at least 6 months in the absence of the antibiotic selection. Co-transformation frequency of unselectable green fluorescent protein gene (*Gfp*) adapted to *C. reinhardtii* codon usage (*ChGfp*) and selectable *Hyg3* gene was $2.6\times10^{-4}$ transformants $mg^{-1}$ DNA; up to 90 % of the transformants exhibited green fluorescent protein (GFP) activity. The *ChGfp* and *Hyg3* gene were integrated into the nuclear genome of *N. oleoabundans*. The GFP fluorescence signal of the transformants under confocal laser scanning microscope was visible. The successful stable nuclear transformation system not only provides a basis for molecular genetics study, but also enables subsequent genetic engineering in the microalga to increase lipid content for biodiesel production. The strategy for developing the stable nuclear transformation system presented in this study may be applicable to other microalgal species without sequenced genome.

**Keywords** Microalgae · *Neochloris oleoabundans* · *Ettlia oleoabundans* · Nuclear transformation · Electroporation · Green fluorescent protein (GFP) · Biodiesel

## Introduction

Biodiesel from microalgae has received much attention in recent years because microalgae have much higher lipid and biomass productivity than oleaginous crops used as feedstock for biodiesel production (Chisti 2007). However, at present, microalgal biodiesel production is technically feasible, but not yet economically viable. Improving microalgae as an economically viable biodiesel feedstock could be achieved by using biotechnological approaches including increasing microalgal lipid content via genetic engineering.

Genetic engineering and genetic manipulation of microalgae require the accessibility to stable nuclear transformation. So far, stable nuclear transformation system have been successfully established in a limited number of microalgal species; for example, in green microalgae (Chlorophyta): *Chlamydomonas*, *Dunaliella*, *Haematococcus*, and *Chlorella*, and in heterokont microalgae: *Phaeodactylum* and *Nannochloropsis* (for review, see Radakovits et al. 2010). Stable nuclear transformation of some microalgal species has been achieved by exposing cell walls to high-intensity electrical field pulses or electroporation (for review, see Qin

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et al. 2012; Coll 2006). Transformant isolation is greatly facilitated by the use of selectable markers including antibiotic resistance. Antibiotic hygromycin B-resistant gene *aph7"*, encoding aminoglycoside phosphotransferase of *Streptomyces hygroscopicus*, has been successfully used in *Chlamydomonas reinhardtii* as a selectable marker (Berthold et al. 2002). The promoter choice for driving the expression of heterologous genes has been explored, for example, *β2-tubulin* (*β2-Tub*) promoter of *C. reinhardtii* (Brunke et al. 1984) has been used to control the expression of *aph7"* selectable marker gene (Berthold et al. 2002). The *HSP70A-RBCS2* (*AR*) hybrid promoter of *C. reinhardtii* (Schroda et al. 2000) has been used to drive nuclear gene expression in closely related green microalgae, i.e., *Volvox carteri* (Hallmann and Wodniok 2006) and *Gonium pectorale* (Lerche and Hallmann 2009). The 35S promoter of cauliflower mosaic virus (*CaMV35S*) (Odell et al. 1985) has been shown to function in microalgae such as *Dunaliella salina* (Tan et al. 2005) and *Chlorella vulgaris* (Chow and Tung 1999). The promoter activities can be analyzed using a reporter, green fluorescent protein (GFP). The *Gfp* gene under control of heterologous promoter is often poorly expressed (Fuhrmann et al. 1999). The difficulties of *Gfp* gene expression have been overcome by adaptation of *Gfp* to the nuclear codon usage of heterologous hosts, for example *ChGfp* gene adapted to *C. reinhardtii* codon usage (Fuhrmann et al. 1999) and *AcGfp* gene adapted to human codon usage (Gurskaya et al. 2003). However, no stable nuclear transformation system has been reported in green microalga *Neochloris oleoabundans*.

*Neochloris oleoabundans*, a taxonomic synonym of *Ettlia oleoabundans* (Deason et al. 1991), is a promising source of lipids for biodiesel production, because under nitrogen starvation condition, it produces lipids 36–54 % of its cell dry weight. Up to 80 % of its total lipids is triacylglycerol (TAG) mainly comprised of the saturated fatty acids containing carbon around 16–20 atoms (Tornabene et al. 1983), ideal for biodiesel production. The knowledge concerning *N. oleoabundans* is very limited; no genomic sequences and no report concerning molecular genetics tools are available. To enable genetic manipulation of TAG biosynthesis and molecular genetics study, we need to develop a stable nuclear transformation system of *N. oleoabundans*.

In this study, we describe a strategy for developing a stable nuclear transformation system of *N. oleoabundans*. We tested whether the *Gfp* reporter gene variants, the heterologous promoters, and the *aph7"* selectable marker gene variants could function in *N. oleoabundans.* Electroporation parameters that induce uptake of the functional heterologous genes into *N. oleoabundans* are defined. We report an application of our system; the *ChGfp* gene was integrated into the nuclear genome and expressed in *N. oleoabundans.*

## Materials and methods

**Strain and growth conditions** *Neochloris oleoabundans* strain UTEX 1185, obtained from the Algal Culture Collection at the University of Texas, was grown in liquid or in solid (1.5 % Difco Bacto agar) Bold's basal medium (BBM) (Bischoff and Bold 1963) at 30 °C under constant illumination of 40–55 μmol photons $m^{-2}$ $s^{-1}$. The antibiotic hygromycin B (HygB) was added at 5 μg $mL^{-1}$ when required.

**Plasmid construction** Plasmids used for electroporation are shown in Fig. 1. To evaluate the efficiency of *Gfp* gene variants, plasmids pAR-AcGFP and pAR-ChGFP were constructed by replacing the *HSP70B* gene of plasmid pCB740 (Schroda et al. 2000) with *AcGfp* gene from pAcGFP1-N1 (Clontech, USA) and *ChGfp* gene from pCrGFP (Fuhrmann et al. 1999), respectively, including the 3'UTR of *3'rbcS2* from pCrGFP (Fuhrmann et al. 1999). To determine the heterologous promoter activities, plasmids pβ2-ChGFP and p35S-ChGFP were constructed by replacing the *AR* promoter of pAR-ChGFP with *β2-Tub* promoter from pHyg3 (Berthold et al. 2002) and *CaMV35S* promoter from pKG-35S (Chungjatupornchai et al. 1999), respectively. To investigate the efficiency of *Aph7"* gene variants, *Aph7"* including intron 1 (*Hyg3*) and *Aph7"* intronless (*Hyg4*), plasmids pAR-Hyg3 and pAR-Hyg4 were constructed by replacing the *HSP70B* gene of plasmid pCB740 (Schroda et al. 2000) with *Hyg3-3'rbcS2* from pHyg3 (Berthold et al. 2002) and *Hyg4-3'rbcS2* from pHyg4 (Berthold et al. 2002), respectively. Plasmid pChGFP-Hyg3 was constructed by replacing the HindIII/KpnI fragment of pAR-Hyg3 with *AR-ChGfp-3'rbcS2* from pAR-ChGFP. Details of plasmid construction and primers are given in supplement S1 and Table S1, respectively.

**Transformation by electroporation** Plasmid DNA was extracted from *Escherichia coli* and purified using Qiagen Plasmid Midi Kit (Qiagen, Germany). Transformation by electroporation was carried out using a modified protocol (Brown et al. 1991) as follows. *N. oleoabundans* cells at the logarithmic phase ($OD_{750}$ 0.4–0.5) were harvested by centrifugation at 1000×*g* for 5 min and resuspended in BBM medium to approximately $3\times10^7$ cells $mL^{-1}$. The cell suspension of 500 μL including 5 μg of plasmid DNA (concentration at 1 μg $\mu L^{-1}$) was transferred into a disposable electroporation cuvette with a 4-mm gap (Bio-Rad Labs., USA). The cell suspension without plasmid DNA was used as negative control. The cells were electroporated using a Gene Pulser (Bio-Rad Labs.) set resistance at 200Ω, capacitance at 25 μF, electric field strength at 1000 V $cm^{-1}$ (unless otherwise indicated), and one time of pulse then incubated on ice for 5 min. The cell suspension was transferred to a new tube and incubated on ice for at least 5 min. The electroporated cells harvested by

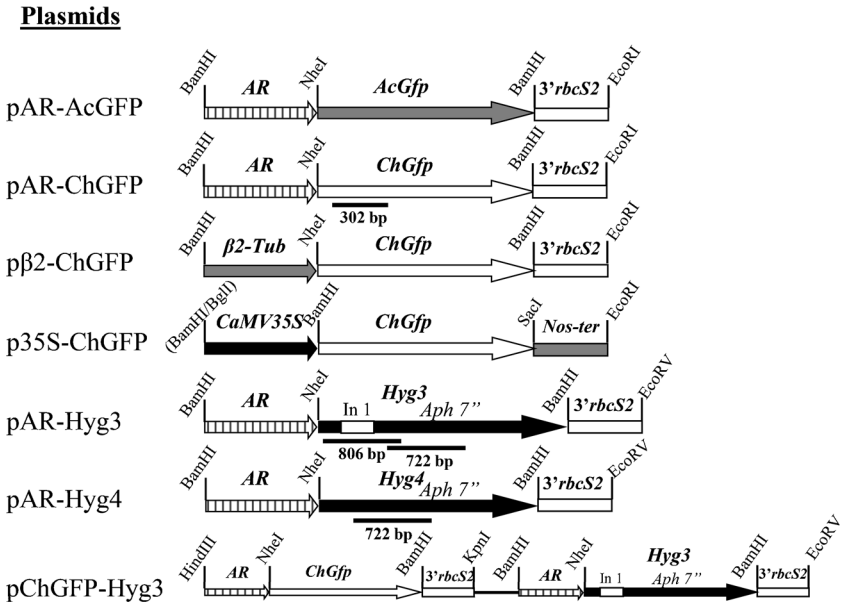


**Fig. 1** Schematic diagram of plasmids used for electroporation in *N. oleoabundans*. *AR*, *HSP70A/RBCS2* hybrid promoter of *C. reinhardtii* (Schroda et al. 2000); *β2-Tub*, *β2-tubulin* promoter of *C. reinhardtii* (Brunke et al. 1984); *CaMV35S*, 35S promoter of cauliflower mosaic virus (Odell et al. 1985); *AcGfp*, *Gfp* gene adapted to human codon usage (Gurskaya et al. 2003); *ChGfp*, *Gfp* gene adapted to *C. reinhardtii* codon usage (Fuhrmann et al. 1999); *Hyg3* and *Hyg4*, *Streptomyces hygroscopicus* aminoglycoside phosphotransferase gene (*aph 7"*) with intron 1 (In1) and intronless, respectively (Berthold et al. 2002); 3'*rbcS2*, 3' untranslated region of the *C. reinhardtii* ribulose bisphosphate carboxylase small subunit gene (Fuhrmann et al. 1999); *Nos-ter*, termination sequence of the nopaline synthase gene (Jefferson et al. 1987). Locations of 302-bp, 806-bp, and 722-bp PCR products amplified using primer sets: ChGFP-F3 and ChGFP-R3; aph7-F3 and aph7-R2; aph7-F4 and aph7-R3, respectively, are indicated. The figure is not drawn to scale

centrifugation were resuspended in 3 mL of BBM medium for GFP transient expression and in 400 μL of BBM medium for stable nuclear transformation. After the first heat induction at 42 °C for 40 min under illumination at 15–20 μmol photons $m^{-2}$ $s^{-1}$, the cells were cultured in an orbital shaker at 150 rpm and 30 °C under continuous illumination for 1 day. Then the second heat induction was performed by incubation at 42 °C for 20 min under illumination. The heat induction treatments were performed in cells electroporated with plasmids harboring heat-inducible *AR* promoter but were omitted in cells electroporated with pβ2-ChGFP and p35S-ChGFP. For GFP transient expression, the total treated cells were used directly for GFP activity assay. For stable nuclear transformation, 200 μL of the treated cells was spread on a BBM agar plate containing HygB at 5 μg $mL^{-1}$ and incubated at 28 °C under continuous light of 25–35 μmol photons $m^{-2}$ $s^{-1}$. Transformants appeared after incubation for 2 weeks.

**Genomic PCR analysis** The heterologous genes integrated into the nuclear genome of *N. oleoabundans* were verified using genomic PCR. The genomic DNA of *N. oleoabundans* was isolated as described (Draper and Scott 1998). To detect the *Hyg3* gene, the 806-bp PCR product was amplified using primers aph7-F3 and aph7-R2. To detect the genes *Hyg3* and *Hyg4*, the 722-bp PCR product was amplified using primers aph7-F4 and aph7-R3. The 302-bp PCR product containing *ChGfp* gene was amplified using primers ChGFP-F3 and ChGFP-R3. Locations of the PCR products are indicated in Fig. 1. Details of primers are given in supplement Table S1.

**GFP activity assay and microscopy** The GFP fluorescence intensity of transformed cells was measured in a 96-well plate using a spectrofluorometer (Beckman coulter, DTX880) with excitation at 485/20 nm and emission at 535 nm. Specific fluorescence intensities were normalized by the optical density measured at 750 nm. The images of GFP fluorescence signal in the transformants were obtained using a confocal laser scanning microscope (CLSM) (Fluoview FV1000, Olympus) with excitation at 488 nm and emission at 507 nm. The chlorophyll autofluorescence was detected using excitation at 652 nm and emission at 668 nm.

## Results and discussion

The important prerequisites for developing a stable nuclear transformation system of *N. oleoabundans* are the availabilities of (i) an appropriate selectable marker gene, (ii) a functional promoter that can drive the expression of the selectable marker gene, and (iii) a reporter that can be used to evaluate the function of the selected promoters. Because knowledge concerning *N. oleoabundans* is very limited, we utilized selectable marker genes, promoters, and reporter genes that have been shown to function in the related microalga *C. reinhardtii*

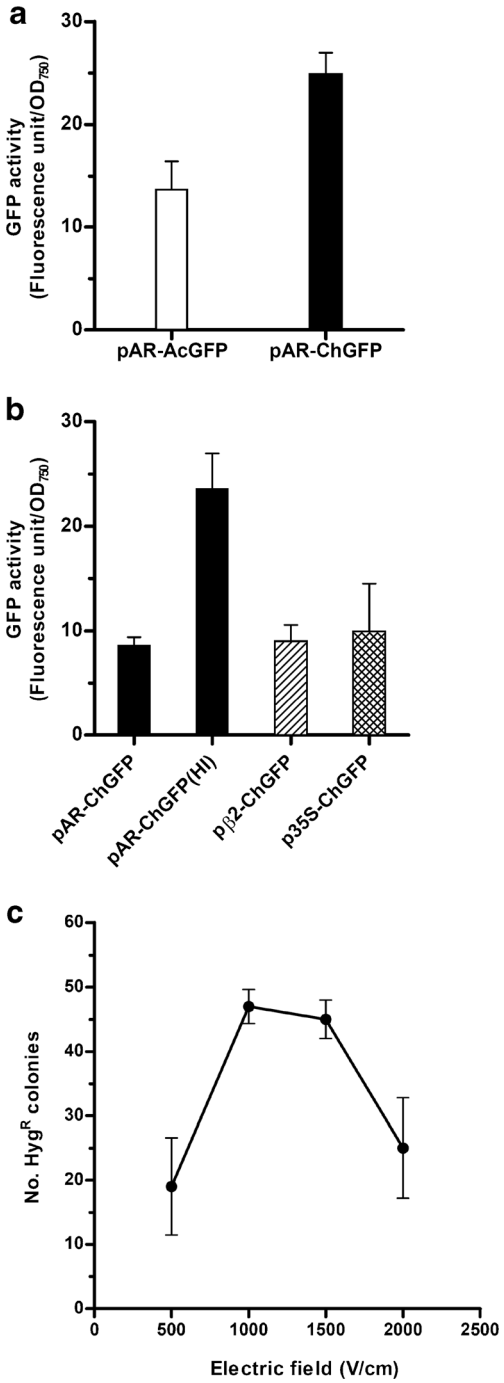


**Fig. 2** Development of stable nuclear transformation system in *N. oleoabundans*. **a** The GFP activity in transient expression. Plasmid pAR-AcGFP and pAR-ChGFP were introduced into *N. oleoabundans* by electroporation followed by heat induction. The GFP fluorescence intensity was determined using spectrofluorometer. Values of control without GFP were subtracted. *Each value and error bar* represents the means of five independent experiments and the standard deviation. **b** Heterologous promoter activities in transient expression. Plasmid pAR-ChGFP, pβ2-ChGFP, and p35S-ChGFP were introduced into *N. oleoabundans* by electroporation. Heat induction (*HI*) was performed in cells electroporated with pAR-ChGFP. The GFP fluorescence intensity was determined using a spectrofluorometer. Values of control without GFP (wild-type strain) were subtracted. *Each value and error bar* represents the means of five independent experiments and the standard deviation. **c** Optimal electric field strength for electroporation. Plasmid pAR-Hyg3 (5 μg) was introduced into *N. oleoabundans* using electroporation with various electric field strengths: 500, 1000, 1500, and 2000 V $cm^{-1}$. Transformant colonies grown on BBM agar containing HygB were counted. *Each value and error bar* represents the means of three independent experiments and the standard deviation

for developing a stable nuclear transformation system of *N. oleoabundans.*

**The efficient GFP reporter in *N. oleoabundans*** GFP has been used as a reporter in many different organisms; however, the *Gfp* gene under the control of heterologous promoters is often poorly expressed (Fuhrmann et al. 1999). Adaptation of *Gfp* gene to the codon usage of target host has been shown to improve the gene expression, i.e., *AcGfp* adapted to human codon usage (Gurskaya et al. 2003) and *ChGfp* adapted to *C. reinhardtii* nuclear codon usage (Fuhrmann et al. 1999).

To compare the *AcGfp* and *ChGfp* gene expression in *N. oleoabundans*, plasmids pAR-AcGFP and pAR-ChGFP (Fig. 1) were introduced into the cells by electroporation. Transient expression revealed that GFP activity of cells electroporated with pAR-ChGFP was 1.82-fold higher than that with pAR-AcGFP (Fig. 2a), indicating that *ChGfp* was more efficient than *AcGfp*. The results suggested that the codon usage of *N. oleoabundans* might be closer to that of the microalga *C. reinhardtii* than that of humans. Therefore, in subsequent experiments, *ChGfp* gene was used as a reporter to evaluate the activities of heterologous promoters.

**The functional heterologous promoters in *N. oleoabundans*** The promoters such as the heat-inducible *AR* promoter of *C. reinhardtii* (Schroda et al. 2000), the strong constitutive *β2-Tub* promoter of *C. reinhardtii* (Brunke et al. 1984), and the constitutive *CaMV35S* promoter of cauliflower mosaic virus (Odell et al. 1985) have been shown to successfully drive the heterologous gene expression in the nucleus of several microalgae (Hallmann and Wodniok 2006; Lerche and Hallmann 2009; Berthold et al. 2002; Tan et al. 2005; Chow and Tung 1999).

To test whether the promoters *AR*, *β2-Tub*, and *CaMV35S* could function in *N. oleoabundans* by using *ChGfp* as a reporter, plasmids pAR-ChGFP, pβ2-ChGFP, and p35S-ChGFP (Fig. 1) were introduced into the cells by electroporation. Transient expression revealed that the GFP activities of promoters *AR* (without heat induction), *β2-Tub*, and *CaMV35S* were not significantly different (Fig. 2b), indicating that these promoters could function in *N. oleoabundans*. The GFP activity of *AR* promoter with heat induction was 2.75-fold higher than without heat induction, indicating that *AR* promoter was

heat inducible and was the most efficient promoter. Therefore, *AR* promoter was subsequently used to drive the expression of selectable marker gene for stable nuclear transformation in *N. oleoabundans*.

**Transformation of *N. oleoabundans* by electroporation** Because antibiotic resistance can be used as selectable markers to facilitate transformant isolation, we tested *N. oleoabundans* sensitivity to antibiotics kanamycin, ampicillin, and hygromycin B (HygB) by culturing $3.1\times10^8$ cells on BBM agar containing various concentrations of the antibiotics. *N. oleoabundans* was resistant to kanamycin and ampicillin at 160 μg $mL^{-1}$, but was sensitive to HygB with minimal inhibitory concentration (MIC) at 5 μg $mL^{-1}$. Therefore, HygB concentration at 5 μg $mL^{-1}$ was used in subsequent experiments for *N. oleoabundans* transformant selection.

The HygB-resistant gene *Hyg3* (*Aph7*" including intron1 of *rbcS2* gene) has been successfully used as a selectable marker in *C. reinhardtii* (Berthold et al. 2002). To test whether *Hyg3* gene could be used as a selectable marker in *N. oleoabundans*, the plasmid pAR-Hyg3 (Fig. 1) was introduced into the cells by electroporation. Electroporation was performed using a single pulse with resistance at 200Ω and capacitance at 25 μF. The optimal electric field strength for electroporation was investigated (Fig. 2c). The number of transformants increased from 500 V $cm^{-1}$ to reach a maximum at 1000 V $cm^{-1}$ then decreased at 1500 and 2000 V $cm^{-1}$. However, the number of transformants generated at 1000 and 1500 V $cm^{-1}$ was not significantly different. Therefore, we used the optimal electric field strength at 1000 V $cm^{-1}$ for the remaining experiments. The transformation frequency in this study (Table 1) was one order of magnitude higher than that of intact cell wall *C. reinhardtii* using almost identical electroporation condition except with two consecutive pulses (Brown et al. 1991). Addition of carrier DNA in the electroporation mixture has been shown to increase transformation frequency of *C. reinhardtii* by one order of magnitude (Shimogawara et al. 1998). Whether the addition of carrier DNA in the electroporation mixture could increase transformation frequency of *N. oleoabundans* remains to be investigated.

**Table 1** Stable nuclear transformation frequency of *N. oleoabundans* using electroporation

| Plasmids | Transformation frequency[a] (transformants $mg^{-1}$ DNA)[b] |
|---|---|
| pAR-Hyg3 | $5.2\times10^{-4}$ |
| pAR-Hyg4 | $2.0\times10^{-4}$ |
| pChGFP-Hyg3 | $3.0\times10^{-4}$ |
| pAR-ChGFP and pAR-Hyg3 | $2.6\times10^{-4}$ |

[a] Transformants were selected on BBM agar containing HygB

[b] Numbers of transformants/recipient cells treated/mg DNA

**The efficiency of *Hyg3* and *Hyg4* gene in *N. oleoabundans*** Introns have been shown to have a positive effect on gene expression in eukaryotes, because their splicing improves and accelerates nuclear mRNA export (Reed and Hurt 2002; Rose and Last 1997). In *C. reinhardtii*, heterologous gene expression can be increased by the presence of intron1 of endogenous *rbcS2* gene (Lumbreras et al. 1998; Berthold et al. 2002). We compared the efficiency of HygB-resistant gene variants, *Hyg3* (*Aph7*" including intron1 of *rbcS2* gene) and *Hyg4* (*Aph7*" intronless), in *N. oleoabundans*. Plasmids pAR-Hyg3 and pAR-Hyg4 (Fig. 1) were electroporated into *N. oleoabundans* to obtain transformants Hyg3 and Hyg4, respectively. The transformation frequency of pAR-Hyg3 ($5.2\times10^{-4}$ transformants $mg^{-1}$ DNA) was 2.6-fold higher than that of pAR-Hyg4 ($2.0\times10^{-4}$ transformants $mg^{-1}$ DNA) (Table 1). Thus, the intron1 of *rbcS2* gene increased transformation frequency in *N. oleoabundans*. The results agreed well with a previous report that in *C. reinhardtii*, the presence of the intron1 of *rbcS2* gene in selectable marker genes *Aph7*" and *ble* increases transformation frequency (Berthold et al. 2002; Lumbreras et al. 1998).

The integration of *Hyg3* and *Hyg4* genes into the nuclear genome of *N. oleoabundans* was verified using genomic PCR. The expected 806-bp and 722-bp PCR products were detected in transformants Hyg3 (Fig. 3a) and Hyg4 (Fig. 3b), respectively, but not in wild type used as negative control. DNA sequencing analysis of the PCR products revealed that they were indeed *Hyg3* and *Hyg4* genes (data not shown). Therefore, *Hyg3* and *Hyg4* genes were successfully integrated into the nuclear genome of *N. oleoabundans*.

Because heterologous gene expression is often hampered by gene silencing of the host system (Schroda 2006), this

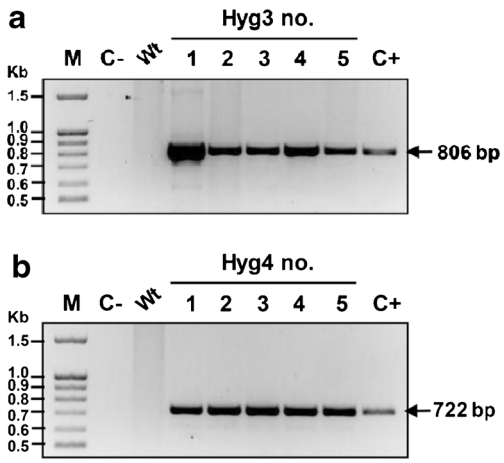


**Fig. 3** Genomic PCR detection of transformants Hyg3 and Hyg4. PCR using genomic DNA of transformants Hyg3 (**a**) and Hyg4 (**b**) were performed with primer sets shown in Fig. 1. *Lanes M*, 100-bp DNA ladder; *C*−, no template (used as negative control); *C*+, plasmid pAR-Hyg3 used as positive control in (**a**) and pAR-Hyg4 in (**b**); and *Wt*, wild-type strain (used as negative control)

phenomenon may have a direct influence on the stability of transformant phenotypes. To test the long-term stability of *Hyg3* and *Hyg4* gene expression without selection, randomly selected transformants Hyg3 and Hyg4 were subcultured on BBM agar in the absence of HygB for at least 6 months, and then they were transferred to BBM agar containing HygB at 5 μg $mL^{-1}$. All the tested transformants showed unimpeded growth, suggesting that they were stable transformants. Thus, the selectable marker genes *Hyg3* and *Hyg4* yield high transformation frequency, sharp selection with no background from non-transformed colonies, and stable transformants in *N. oleoabundans*.

**Transformation and co-transformation of *ChGfp* gene** Because genes of interest are often unselectable, they cannot be used for direct transformation selection. Transformation system that allows co-transformation of unselectable and selectable genes in two separate plasmids is useful and convenient; there is no need to construct a transformation plasmid containing both genes. To test the transformation system in this study, plasmid pChGFP-Hyg3

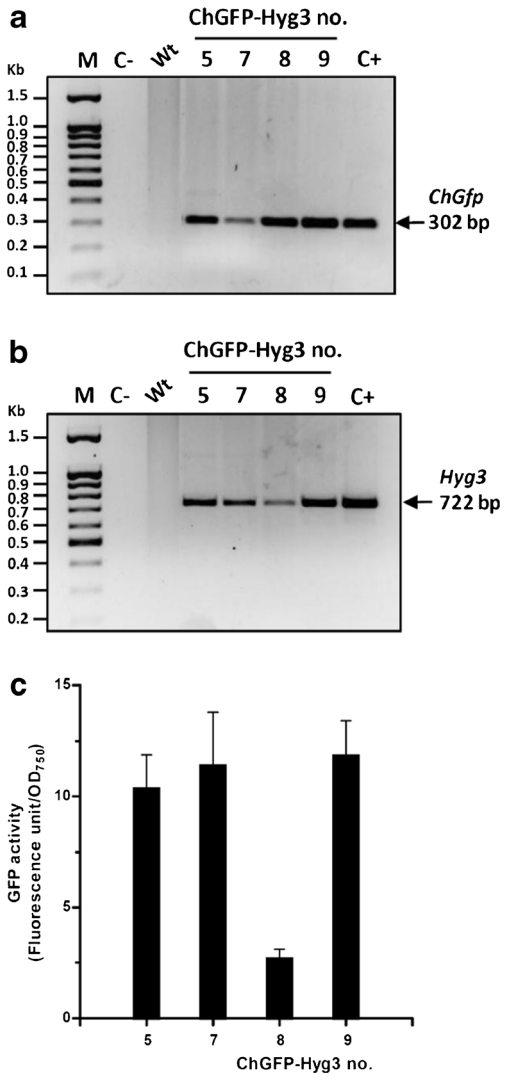


**Fig. 4** Evaluation of transformant ChGFP-Hyg3. **a** *ChGfp* and **b** *Hyg3* PCR detection. Genomic PCR of transformant ChGFP-Hyg3 were performed with primer sets shown in Fig. 1. *Lanes M*, 100-bp DNA ladder; *C*−, no template (used as negative control); *C*+, plasmid pChGFP-Hyg3 used as positive control; and *Wt*, wild-type strain (used as negative control). **c** The GFP activity. The GFP fluorescence intensities in the transformant ChGFP-Hyg3 were measured using a spectrofluorometer. Values of control without GFP (wild-type strain) were subtracted. *Each value and error bar* represents the mean of three independent experiments and the standard deviation

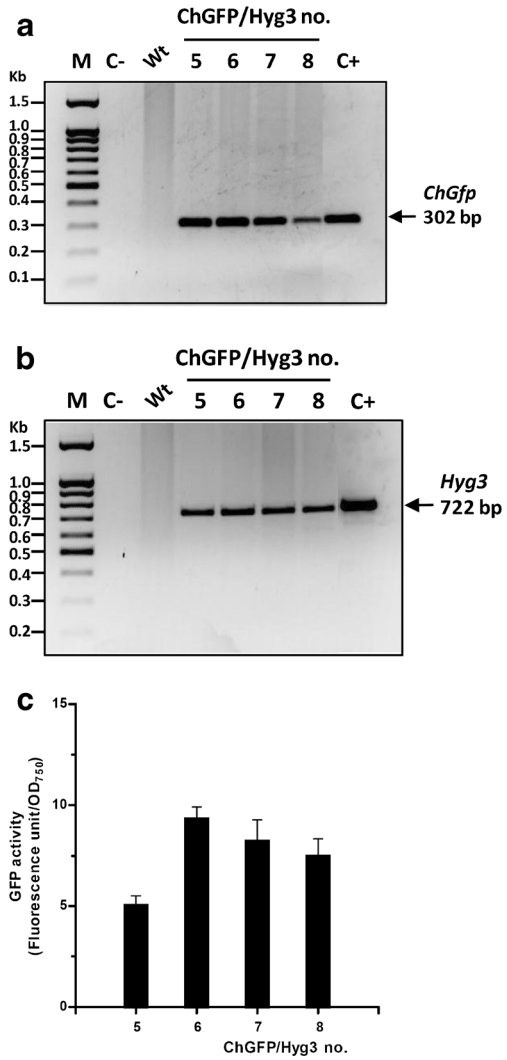


**Fig. 5** Evaluation of transformant ChGFP/Hyg3 from co-transformation. **a** *ChGfp* and **b** *Hyg3* PCR detection. Genomic PCR of transformant ChGFP/Hyg3 were performed with primer sets shown in Fig. 1. *Lanes M*, 100-bp DNA ladder; *C*−, no template (used as negative control); *C*+, plasmid pAR-ChGFP used as positive control in (**a**) and pAR-Hyg3 in (**b**); and *Wt*, wild-type strain (used as negative control). **c** GFP activity. The GFP fluorescence intensities in the transformant ChGFP/Hyg3 were measured using a spectrofluorometer. Values of control without GFP (wild-type strain) were subtracted. *Each value and error bar* represents the mean of three independent experiments and the standard deviation

containing both unselectable gene *ChGfp* and selectable gene *Hyg3* (Fig. 1) was used for transformation to obtain transformant ChGFP-Hyg3, whereas the combination of unselectable plasmid pAR-ChGFP and selectable plasmid pAR-Hyg3 (Fig. 1) was used for co-transformation to obtain transformant ChGFP/Hyg3. The frequency of transformation ($3.0\times10^{-4}$ transformants $mg^{-1}$ DNA) and co-transformation ($2.6\times10^{-4}$ transformants $mg^{-1}$ DNA) were not significantly different; however, the frequency was 1.7- and 2-fold, respectively, lower than that of plasmid pAR-Hyg3 ($5.2\times10^{-4}$ transformants $mg^{-1}$ DNA) (Table 1). Up to 90 % of HygB-resistant colonies from co-transformation expressed GFP activities. Therefore, co-transformation occurs at a high frequency and *Hyg3* gene is an efficient selectable marker. High efficiency of unselectable gene (up to 60 %) in co-transformation with selectable gene has been reported in the diatom *Phaeodactylum tricornutum* (Zhang and Hu 2014).

To verify the presence of *ChGfp* and *Hyg3* genes in the genome, genomic DNA of transformants ChGFP-Hyg3 and ChGFP/Hyg3 were isolated and analyzed by PCR. The expected PCR products of 302 and 722 bp were detected in transformants ChGFP-Hyg3 (Fig. 4a, b) and ChGFP/Hyg3 (Fig. 5a, b), respectively, whereas no PCR product was detected in wild-type strain used as negative control. DNA sequencing analysis revealed that the PCR product of 302 bp contained *ChGfp* gene and 722 bp contained *Hyg3* gene (data not shown). The results indicated that the *ChGfp* and *Hyg3* genes, by transformation and co-transformation, were integrated into the genome. Therefore, co-transformation of unselectable and selectable plasmid was feasible in *N. oleoabundans*.

The copy number of genes integrated into the *N. oleoabundans* genome was investigated using Southern blot analysis; only one expected band of *Hyg3* or *ChGfp* gene was barely detected even when using a high amount of transformant genomic DNA (10 μg) (data not shown), suggesting that at least one copy of the genes was integrated into the transformant genome. Although the results from Southern blot analysis were inconclusive, the results from genomic PCR clearly showed that *Hyg3* and/or *ChGfp* gene was present in the genome (Figs. 3; 4a, b; and 5a, b). In *C. reinhardtii*, transformation by electroporation reveals that only one or a few copies of heterologous DNA are integrated into the nuclear genomes (Brown et al. 1991).

**The *ChGfp* gene expression in *N. oleoabundans*** To determine the GFP expression in *N. oleoabundans*, the fluorescence intensities of whole cells were measured using a spectrofluorometer. The GFP activities of transformants ChGFP-Hyg3 (Fig. 4c) and ChGFP/Hyg3 (Fig. 5c) were detected. Therefore, the *ChGfp* gene was expressed in both transformants. Whether the different levels of GFP activities in the transformant clones (Figs. 4c and 5c) are due to copy number or integration positional effect of the *ChGfp* gene remains to be investigated.

The GFP expression in live transformants ChGFP-Hyg3 and ChGFP/Hyg3 was visualized under confocal laser scanning microscope (CLSM). Very bright GFP fluorescence signal was clearly visible in transformants ChGFP-Hyg3 and ChGFP/Hyg3 but not in wild-type strain used as negative control (Fig. 6). Very little green autofluorescence background, co-localized with chlorophyll red autofluorescence,

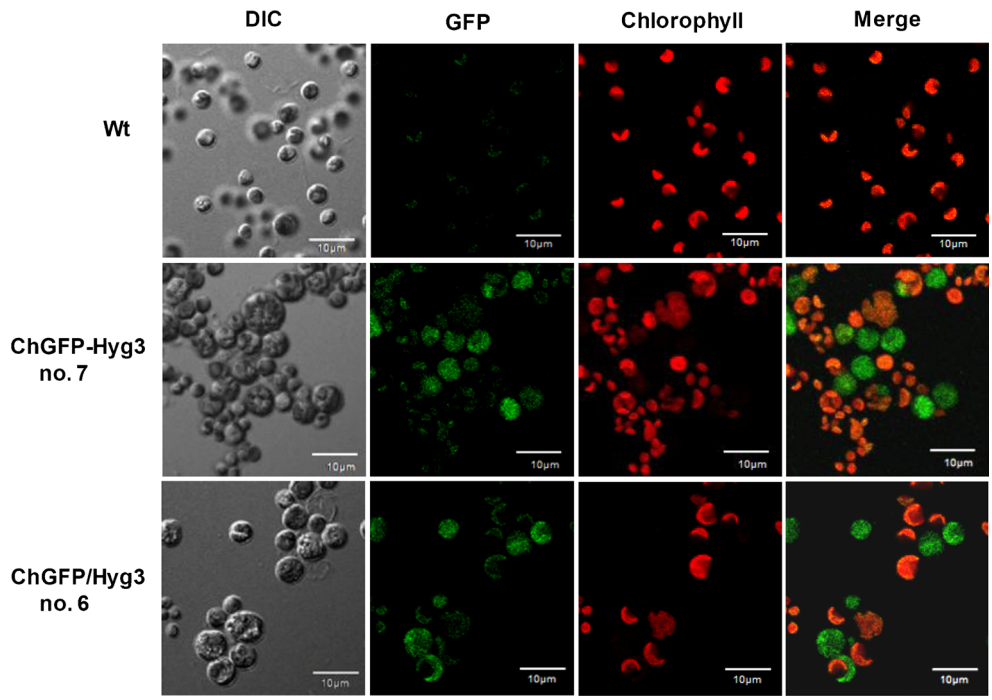


**Fig. 6** CLSM images of transformants expressing GFP. DIC, differential interference contrast; *GFP*, GFP fluorescence (ex: 488 nm; em: 507 nm); *Chlorophyll*, chlorophyll autofluorescence (ex: 652 nm; em: 668 nm); *Merge*, merged image of GFP and Chlorophyll. *N. oleoabundans* wild-type strain (*Wt*) was used as negative control. Transformants ChGFP-Hyg3 and ChGFP/Hyg3 are indicated

was detected in transformants and wild-type strain. The green autofluorescence background might be emitted by carotenoids located in the chloroplast as reported previously in *Dunaliella salina* (Kleinegris et al. 2010).

Taken together, results from transformation frequency (Table 1), integration of *Hyg3* and *Hyg4* gene into the nuclear genome (Fig. 3), evaluation of transformants ChGFP-Hyg3 (Fig. 4) and ChGFP/Hyg3 (Fig. 5), and CLSM images of transformants expressing ChGFP (Fig. 6) indicated that (i) the stable nuclear transformation of *N. oleoabundans* was successfully developed using electroporation; (ii) the unselectable gene *ChGfp* and the selectable marker gene *Hyg3*, by transformation and co-transformation, were integrated into the nuclear genome of *N. oleoabundans*; and (iii) the *ChGfp* gene was expressed in *N. oleoabundans*; under CLSM, GFP fluorescence signal was visible.

Recently, the cDNA encoding the primary enzyme of *N. oleoabundans* TAG biosynthesis, diacylglycerol acyltransferase type 2 (NeoDGAT2), has been cloned (Chungjatupornchai and Watcharawipas 2014). The availability of a transformation system in *N. oleoabundans* would enable overexpression of NeoDGAT2 to increase TAG for biodiesel production.

## Conclusions

The stable nuclear transformation system of *N. oleoabundans* by electroporation was successfully established; co-transformation of unselectable and selectable plasmid was feasible. As an application of the system, *ChGfp* gene was integrated into the nuclear genome and expressed in *N. oleoabundan*. The availability of the stable nuclear transformation system not only provides a basis for molecular genetics study but also enables subsequent genetic manipulation in the microalga to increase TAG for biodiesel production. The strategy for developing the stable nuclear transformation system presented in this study may be applicable to other microalgal species without sequenced genome.

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RESEARCH

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# Accelerated triacylglycerol production and altered fatty acid composition in oleaginous microalga *Neochloris oleoabundans* by overexpression of diacylglycerol acyltransferase 2

Paeka Klaitong, Sirirat Fa-aroonsawat and Wipa Chungjatupornchai*

## Abstract

**Background:** Microalgae are promising sources of lipid triacylglycerol (TAG) for biodiesel production. However, to date, microalgal biodiesel is technically feasible, but not yet economically viable. Increasing TAG content and productivity are important to achieve economic viability of microalgal biodiesel. To increase TAG content, oleaginous microalga *Neochloris oleoabundans* was genetically engineered with an endogenous key enzyme diacylglycerol acyltransferase 2 (NeoDGAT2) responsible for TAG biosynthesis.

**Results:** The integration of *NeoDGAT2* expression cassettes in *N. oleoabundans* transformant was confirmed by PCR. The neutral lipid accumulation in the transformant detected by Nile red staining was accelerated and 1.9-fold higher than in wild type; the lipid bodies in the transformant visualized under fluorescence microscope were also larger. The *NeoDGAT2* transcript was two-fold higher in the transformant than wild type. Remarkably higher lipid accumulation was found in the transformant than wild type: total lipid content increased 1.6-to 2.3-fold up to 74.5 ± 4.0% dry cell weight (DCW) and total lipid productivity increased 1.6- to 3.2-fold up to 14.6 ± 2.0 mg/L/day; while TAG content increased 1.8- to 3.2-fold up to 46.1 ± 1.6% DCW and TAG productivity increased 1.6- to 4.3-fold up to 8.9 ± 1.3 mg/L/day. A significantly altered fatty acid composition was detected in the transformant compared to wild type; the levels of saturated fatty acid C16:0 increased double to 49%, whereas C18:0 was reduced triple to 6%. Long-term stability was observed in the transformant continuously maintained in solid medium over 100 generations in a period of about 4 years.

**Conclusions:** Our results demonstrate the increased TAG content and productivity in *N. oleoabundans* by *NeoDGAT2* overexpression that may offer the first step towards making microalgae an economically feasible source for biodiesel production. The strategy for genetically improved microalga presented in this study can be applied to other microalgal species possessing desired characteristics for industrial biofuel production.

**Keywords:** Biofuel, Biodiesel, Diacylglycerol acyltransferase (DGAT), Microalgae, Lipids, Genetic engineering

## Background

Biofuels are thought to represent a secure, renewable and environmentally safe alternative to fossil fuels. Biodiesel, one of the commonly used biofuels, is predominantly produced from oleaginous plants. Because microalgae have much higher lipid and biomass productivity than terrestrial plants, can utilize saline or wastewater for their growth, and require non-arable land [1], they are promising sources of lipid triacylglycerol (TAG) for biodiesel production. To date, biodiesel production from

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microalgae is technically, but not yet economically, feasible. There are several challenges that need to be overcome before microalgal biodiesel can be economically produced at a commercial scale, one of which is the lack of microalgal strains with high TAG content and biomass [1, 2]. Increasing TAG content in microalgae possessing several desired characteristics could be achieved by targeted genetic engineering of the key genes in TAG biosynthesis pathway, offering the first step towards making microalgae an economically feasible source for biodiesel production [3–5]. To date, the microalgal oleaginous trait has been extensively studied primarily in the model green microalga *Chlamydomonas reinhardtii*, however, high genetic diversity for this trait has been demonstrated in microalgae [6].

The TAG biosynthetic pathway in microalgae is poorly known, however, it is considered to be most similar to that operating in higher plants [7]. In the de novo TAG biosynthetic pathway, diacylglycerol acyltransferase (DGAT; EC 2.3.1.20) catalyzing the final and committed step has been identified as the rate-limiting enzyme for lipid accumulation in plants [8, 9]. DGAT catalyzing the formation of TAG from diacylglycerol and Acyl-CoA is thought to be the key enzyme for de novo TAG biosynthesis in all organisms [7]. DGAT has also been suggested as one of the most promising target genes for genetic engineering to enhance TAG accumulation in microalgae [4]. Most microalgal species have been shown to have DGAT isozymes derived from one *DGAT* type 1 (*DGAT1*) and multiple *DGAT* type 2 (*DGAT2*) genes [7]. The isozymes DGAT1 and DGAT2 of *C. reinhardtii* have been predicted to localize in the chloroplast and endoplasmic reticulum, respectively [10]. DGAT2 has been identified as the potent enzyme in TAG biosynthesis [11–13].

Overexpression of *DGAT2* for enhancing TAG accumulation has been attempted so far in a few microalgal species with varying success. In *C. reinhardtii*, endogenous *DGAT2* overexpression neither boosts TAG accumulation nor alters the fatty acid composition [14], however, enhanced TAG accumulation has been observed when *DGAT2* expressed under a phosphorus-starvation inducible promoter [15]. Heterologous *DGAT2* expression has been shown to enhance neutral lipid accumulation but subsequently encounter gene silencing [16]. Enhanced lipid accumulation also has been observed in *Nannochloropsis oceanica* and *Phaeodactylum tricornutum* overexpressing endogenous *DGAT2* [17, 18], and *Scenedesmus obliquus* expressing heterologous *DGAT2* [19]. The maximum TAG content produced by *DGAT2*-overexpressing microalgae that has been reported so far is 11% of dry cell weight [15–17]. However, *DGAT2* overexpression has not been explored so far in oleaginous microalga *Neochloris oleoabundans*.

*Neochloris oleoabundans*, a taxonomic synonym of *Ettlia oleoabundans* [20], has been demonstrated to be one of the most suitable lipid sources for biodiesel production [21–23]. Under nitrogen starvation condition, *N. oleoabundans* produces 35–54% lipids of dry cell weight; up to 80% of its total lipids is TAG mainly comprised of the saturated fatty acids in the range of 16–20 carbons [24] ideal for biodiesel production. However, the knowledge concerning *N. oleoabundans* is very limited; no genomic sequences are available. To enable genetic manipulation of TAG biosynthesis and molecular genetics study, the cDNA encoding a functional DGAT2 protein of *N. oleoabundans* (*NeoDGAT2*) has been cloned [11] and the stable nuclear transformation system of *N. oleoabundans* has been established [25].

In this study, we tested whether overexpression of an endogenous key enzyme DGAT catalyzing the final step would affect lipid biosynthesis in oleaginous microalga. The *NeoDGAT2* expression cassettes were transformed into *N. oleoabundans*. The *NeoDGAT2*-overexpressing transformant was characterized in detail with regards to growth characteristics, neutral lipid accumulation and lipid bodies in the cells, lipid content and productivity, and fatty acid composition.

## Results

### Selection of *N. oleoabundans* transformants

The unicellular microalga *N. oleoabundans* was transformed with plasmids pAR-DGAT2 and pB2-DGAT2 harboring endogenous diacylglycerol acyltransferase type 2 (*NeoDGAT2*) cDNA under the control of promoters *HSP70-RBCS2* (*AR*) and *β2-tubulin* (*β2-Tub*), respectively (Fig. 1a), via electroporation. The resulting transformants AR-DGAT2 and B2-DGAT2 were selected on hygromycin B-supplemented BBM agar with a transformation frequency of about 90 ± 10 colonies/1.5 × $10^7$ cells. To screen for clones with potential high neutral lipid accumulation, about 25 colonies selected from each plasmid transformation were grown on nitrogen-depleted (BBM-N) agar plates for 3 days and then stained with Nile red, a reagent that yields brilliant fluorescence in a neutral lipid environment [26]. On the basis of high Nile red fluorescence intensity, transformants AR-DGAT2-33, AR-DGAT2-40, B2-DGAT2-8 and B2-DGAT2-9 were selected for subsequent experiments.

### Evaluation of *NeoDGAT2*-expression cassette integration

The integration of *NeoDGAT2* expression cassettes in *N. oleoabundans* was confirmed by genomic PCR using primer pair specific to *NeoDGAT2* coding sequence. The expected amplicon of 517 bp was detected in the four selected transformants AR-DGAT2-33, AR-DGAT2-40, B2-DGAT2-8 and B2-DGAT2-9, but not in

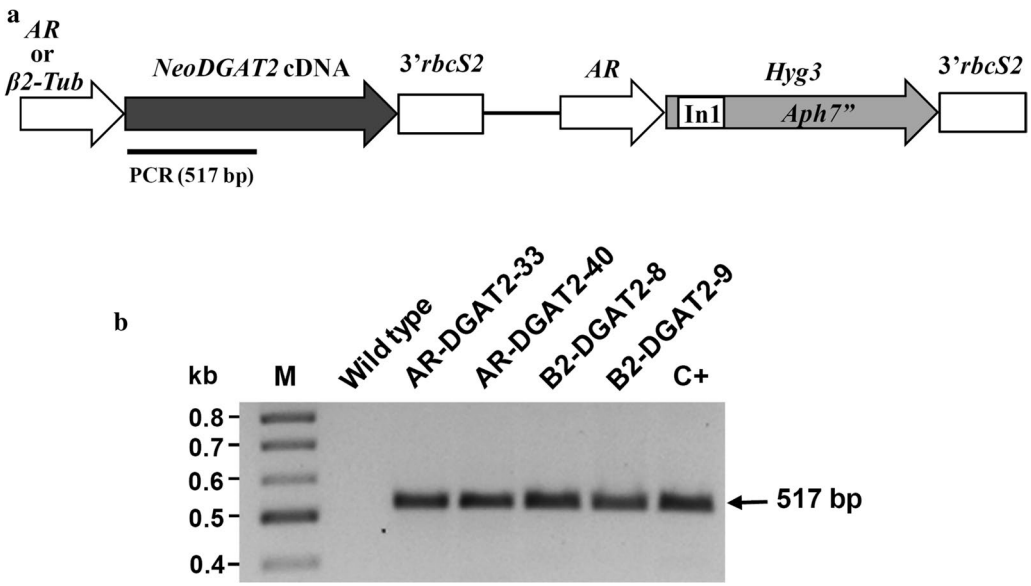


**Fig. 1** Generation of *N. oleoabundans* transformants. **a** Schematic diagram of plasmids pAR-DGAT2 and pB2-DGAT2 used to transform the *N. oleoabundans* cells. The *NeoDGAT2* cDNA (Accession no. GenBank: KJ470774) [11] was expressed by either promoter *AR* [28], or *β2-Tub* [29] and contained *3′rbcS2* [41] at 3′end. *Hyg3* gene used as selectable marker [40]. The 517-bp PCR amplicon, denoted by a *line*. **b** PCR confirmation of *NeoDGAT2*-expression cassette integration in the transformants. Genomic PCR of transformants AR-DGAT2, B2-DGAT2 and wild type was performed with primers specifically bind to *NeoDGAT2* coding sequence. The 517-bp amplicon was detected in the transformants but not in wild type which was used as negative control. *Lanes M*, 100-bp DNA ladder; C+, plasmid pAR-DGAT2 (used as positive control)

wild type (Fig. 1b). The 517-bp amplicon was subjected to DNA sequencing and confirmed to be *NeoDGAT2* coding sequence. Thus, the *NeoDGAT2*-expression cassettes were successfully introduced into the transformants. Partial sequences from genomic walking revealed that the amplicon from the resident *NeoDGAT2* gene including introns was at least 3 kb. Because the PCR condition used in this study was designed to amplify amplicon of about 1 kb, the amplicon from the resident *NeoDGAT2* gene was not amplified. The PCR-positive transformants were further analyzed for growth characteristics.

### Growth of transformants

To evaluate whether *NeoDGAT2* overexpression had any effect on growth characteristics, we analyzed growth curve of the transformants and wild type under N-sufficient growth condition. All of the selected transformants showed overall similar growth curve compared to wild type, while slightly lower growth during the stationary phase (Fig. 2). However, the doubling time during exponential growth of transformants AR-DGAT2-40 (6.8 ± 1.0 days), B2-DGAT2-8 (7.0 ± 1.0 days), and B2-DGAT2-9 (7.3 ± 1.3 days), except AR-DGAT2-33 (9.7 ± 0.9 days), was not significantly different from that of wild type (6.4 ± 0.4 days) at $p < 0.01$. Thus, *NeoDGAT2* overexpression did not have an apparent effect on the growth of transformants AR-DGAT2-40, B2-DGAT2-8, and B2-DGAT2-9.

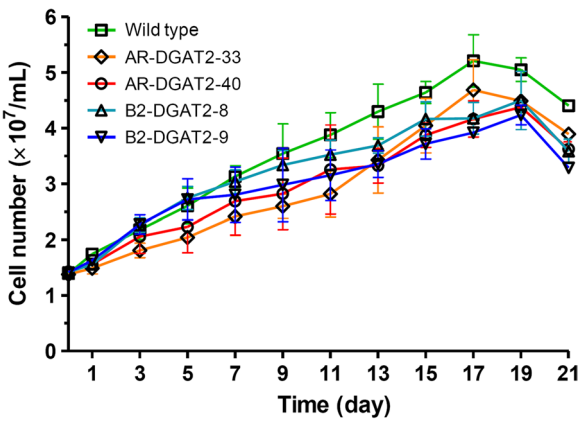


**Fig. 2** Growth curve of *N. oleoabundans* transformants AR-DGAT2 and B2-DGAT2 during N-sufficient growth condition. Each value represents mean ± SD (n = 3)

### Neutral lipid analysis by Nile red staining

*Neochloris oleoabundans*, like many microalgae, accumulates neutral lipids under N-starvation condition [22, 24, 27]. To evaluate the lipid-production potential of the transformants, neutral lipids in the cells cultured

under N-starvation condition I (see "Methods") were stained with fluorescent dye, Nile red. The neutral lipid accumulation in the transformants AR-DGAT2-33, AR-DGAT2-40, B2-DGAT2-8, B2-DGAT2-9 was accelerated and dramatically increased; all transformants were found to reach maximum neutral lipid content (day 25) earlier than wild type (day 35) (Fig. 3a). Among the transformants, AR-DGAT2-40 showed the highest neutral lipid content which increased to 1.9-fold compared to the maximum content in wild type. Therefore, transformant AR-DGAT2-40 was selected for subsequent experiments.

The lipid bodies in transformant AR-DGAT2-40 and wild type were further visualized under fluorescence microscope. Intense golden-color lipid bodies with larger volume were observed in transformant AR-DGAT2-40, whereas, light golden-color lipid bodies with smaller volume in wild type. The red-color background was due to chlorophyll autofluorescence (Fig. 3b). Thus, *NeoDGAT2* overexpression in transformant AR-DGAT2-40 enhanced the accumulation of lipid bodies that was in accordance with the increased neutral lipid content (Fig. 3a).

### Evaluation of *NeoDGAT2* transcript

To examine whether the integrated *NeoDGAT2*-expression cassette in the transformant expressed at transcriptional level, the relative *NeoDGAT2* transcript abundance in cells cultured under N-starvation condition was determined by quantitative real-time PCR (qPCR) using *NeoActin* transcript as a reference. Transformant AR-DGAT2-40 was observed to have *NeoDGAT2* transcript increased twofold compared to wild type (Fig. 4), indicating that the increased transcript was enhanced by *NeoDGAT2* overexpression.

### Lipid productivity analysis

For economically viable to produce biodiesel, microalgal strains should have both high lipid content and enhanced biomass. Lipid analysis in this study was performed in

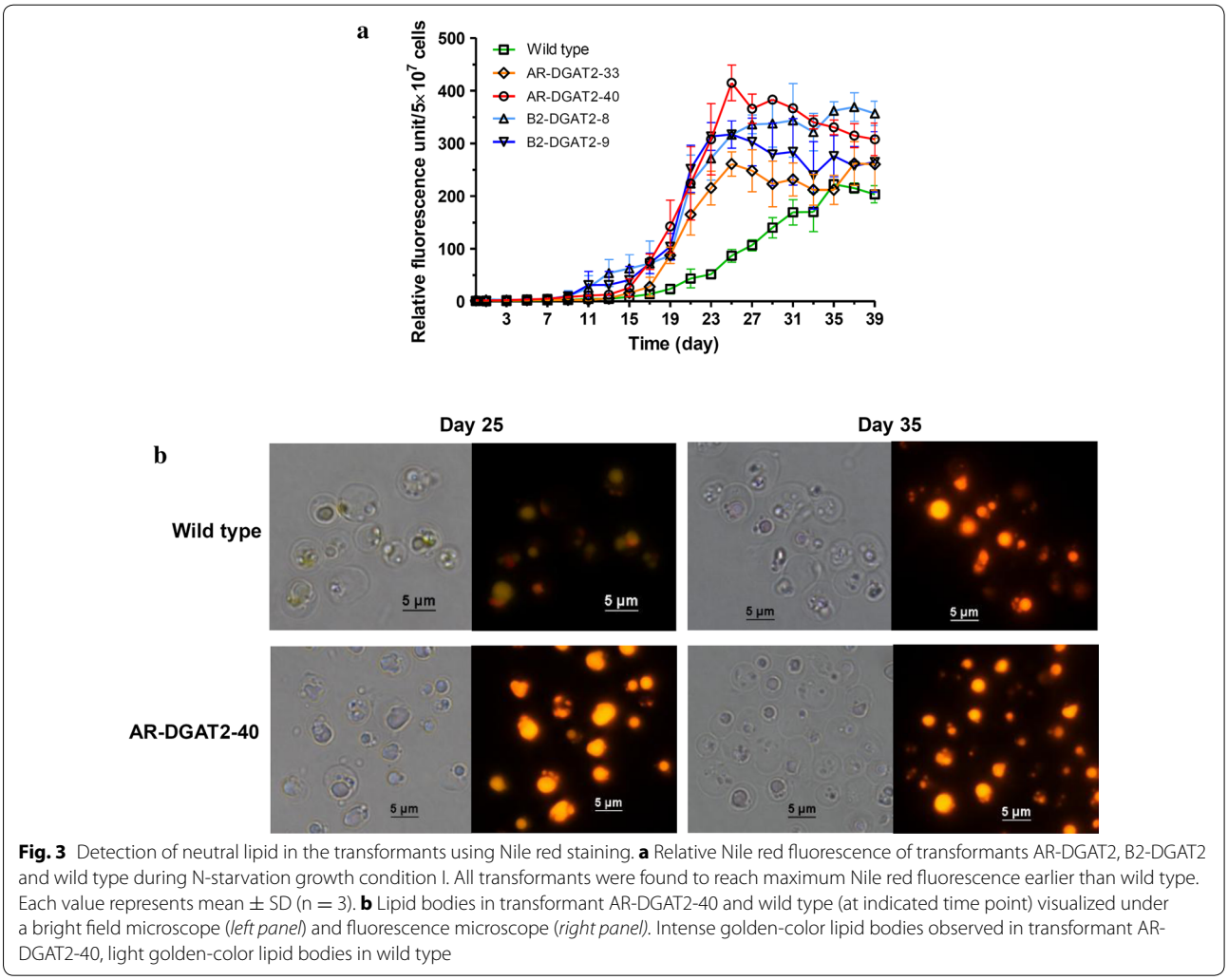


**Fig. 3** Detection of neutral lipid in the transformants using Nile red staining. **a** Relative Nile red fluorescence of transformants AR-DGAT2, B2-DGAT2 and wild type during N-starvation growth condition I. All transformants were found to reach maximum Nile red fluorescence earlier than wild type. Each value represents mean ± SD (n = 3). **b** Lipid bodies in transformant AR-DGAT2-40 and wild type (at indicated time point) visualized under a bright field microscope (*left panel*) and fluorescence microscope (*right panel*). Intense golden-color lipid bodies observed in transformant AR-DGAT2-40, light golden-color lipid bodies in wild type

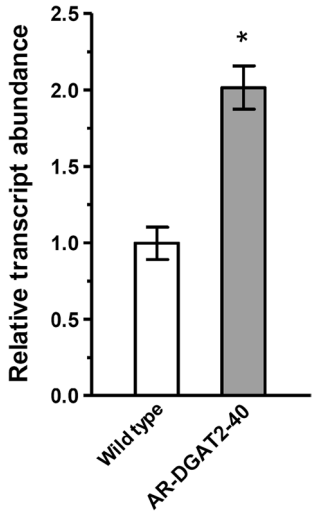


**Fig. 4** Relative *NeoDGAT2* transcript abundance in transformant AR-DGAT2-40. The levels of *NeoDGAT2* transcript during N-starvation growth condition I were determined by quantitative real-time PCR. The values are normalized to the expression level of endogenous *NeoActin*. Each value represents mean ± SD (n = 3). Significant difference between transformant AR-DGAT2-40 and wild type is indicated (*$p < 0.01$, *t* test)

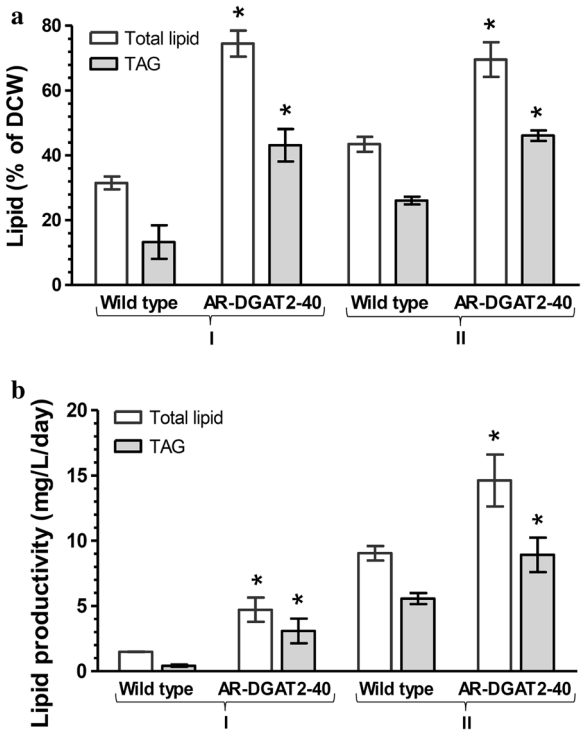


**Fig. 5** Lipid analysis of transformant AR-DGAT2-40. **a** Lipid content and **b** Lipid productivity of wild type and transformant AR-DGAT2-40. Lipids extracted from cells grown under N-starvation condition I and II. N-starvation condition II for increasing cell mass was the same as condition I, except increasing aeration. Each value represents mean ± SD (n = 3). Significant difference between transformant AR-DGAT2-40 and wild type in the same growth condition is indicated (*$p < 0.01$, *t* test)

*N. oleoabundans* cultured under N-starvation condition I and the enhanced cell growth by increasing aeration, N-starvation conditions II. The cells were harvested when they reached maximum neutral lipid accumulation monitored by Nile red staining and the dried cells were determined gravimetrically as dry cell weight (DCW). Biomass of transformant AR-DGAT2-40 under N-starvation condition I and II were 117 ± 12 and 449 ± 57 mg DCW/L, respectively, increasing about 3.8-fold; while that of wild type were 163 ± 21 and 462 ± 27 mg DCW/L, respectively, increasing about 2.8-fold. Under the same growth condition, biomass was not significantly different between the transformant and wild type ($p < 0.05$). Thus, the biomass was enhanced by increasing aeration under N-starvation condition and not affected by *NeoDGAT2* overexpression.

Because TAG is the major component for biodiesel production, the TAG content separated from total lipids using TLC was quantified. Up to 66% of total lipids extracted from transformant AR-DGAT2-40 and wild type were TAG (Fig. 5a). The lipid content and productivity in transformant AR-DGAT2-40 were compared to wild type: under N-starvation condition I, total lipid content in transformant (74.5 ± 4.0% DCW) increased 2.3-fold, TAG content (43.1 ± 5.0% DCW) increased 3.2-fold, total lipid productivity (4.7 ± 0.9 mg/L/day) increased 3.2-fold, TAG productivity (3.1 ± 0.9 mg/L/day) increased 4.3-fold; under N-starvation condition II, total lipid content in transformant (69.6 ± 5.3% DCW) increased 1.6-fold, TAG content (46.1 ± 1.6% DCW) increased 1.8-fold, total lipid productivity (14.6 ± 2.0 mg/L/day) increased 1.6-fold and TAG productivity (8.9 ± 1.3 mg/L/day) increased 1.6-fold (Fig. 5a, b). Therefore, transformant AR-DGAT2-40 was observed to have dramatically increase in lipid accumulation both under N-starvation condition I and II when compared to wild type: the total lipid content and productivity increased 1.6-to 2.3-fold and 1.6- to 3.2-fold, respectively; while the TAG content and productivity increased 1.8- to 3.2-fold and 1.6- to 4.3-fold, respectively (Fig. 5a, b). *NeoDGAT2* overexpression markedly improved TAG content and productivity in the microalga.

### Fatty acid composition analysis

Because fatty acid (FA) composition can impact the quality of the biodiesel, we tested whether *NeoDGAT2* overexpression would have any effect on FA composition. Fatty acid methyl esters (FAME) obtained by transesterification of TAG were analyzed using gas chromatography

equipped with flame ionization detector (GC-FID). FAME in the chain-length range of C11–C23 was determined by comparison with the standard reference. The palmitic acid (C16:0), a saturated fatty acid (SFA) and oleic acid (C18:1), a monounsaturated fatty acid (MUFA), were the most abundant FA in the TAG (Fig. 6). The FA of C16 increased whereas C18 was reduced in transformant AR-DGAT2-40 compared to wild type: C16:0 increased double the amount to 49% from 24% and C16:1 (palmitoleic acid, MUFA) increased to 7% from 0%, whereas C18:0 (stearic acid, SFA) was reduced triple to 6% from 18%, C18:1–26% from 37%, and C18:2 (Linoleic acid, polyunsaturated fatty acid (PUFA)) to 6% from 10%. The overall SFA increased to 58% from 40%, MUFA was not significantly different from the wild type, whereas PUFA was reduced to 8% from 24% (Fig. 6). The C16:0 increased double, suggesting that C16:0-acyl-CoA might be a preferred substrate of NeoDGAT2. A significant alteration of the FA composition in transformant AR-DGAT2-40 was due to *NeoDGAT2* overexpression.

### Long-term stability of transformants

Transformants AR-DGAT2 and B2-DGAT2 were continuously maintained in solid BBM medium by subculturing (every 2 weeks) over 100 generations in a period of about 4 years. The transformants were periodically checked for neutral lipid accumulation by Nile red staining; no loss in higher lipid-accumulation than wild-type trait was observed in all the transformants used in this study, indicating the *NeoDGAT2* overexpression stability.

## Discussion

This study is based on the overexpression of endogenous diacylglycerol acyltransferase type 2 (*NeoDGAT2*) in *N. oleoabundans* to improve triacylglycerol (TAG) accumulation for potential biodiesel production. The important prerequisites for *NeoDGAT2* overexpression in *N. oleoabundans* are the availabilities of: (i) the stable nuclear transformation system [25], (ii) the *NeoDGAT2* cDNA encoding a functional DGAT protein. NeoDGAT2 fused with His tag at the C-terminus for facilitating Western blot analysis has been shown to reduce the NeoDGAT2 activity [11]. NeoDGAT2 without tag was therefore used in this study, and (iii) the functional promoter that can drive the expression of the *NeoDGAT2*. Because no data concerning *N. oleoabundans* endogenous promoter are available, promoters *AR* and *β2-Tub* from *C. reinhardtii* [28, 29] that have been shown to function in *N. oleoabundans* with similar activity [25] were utilized in this study. Among the transformants, AR-DGAT2-40 showed the highest neutral lipid content which increased to 1.9-fold compared to the maximum content in wild type (Fig. 3a). This transformant overexpressing *NeoDGAT2* under *AR* promoter may produce more lipids at high temperature, because *AR* promoter has been shown to be heat inducible in *N. oleoabundans* [25]. However, cells cultured

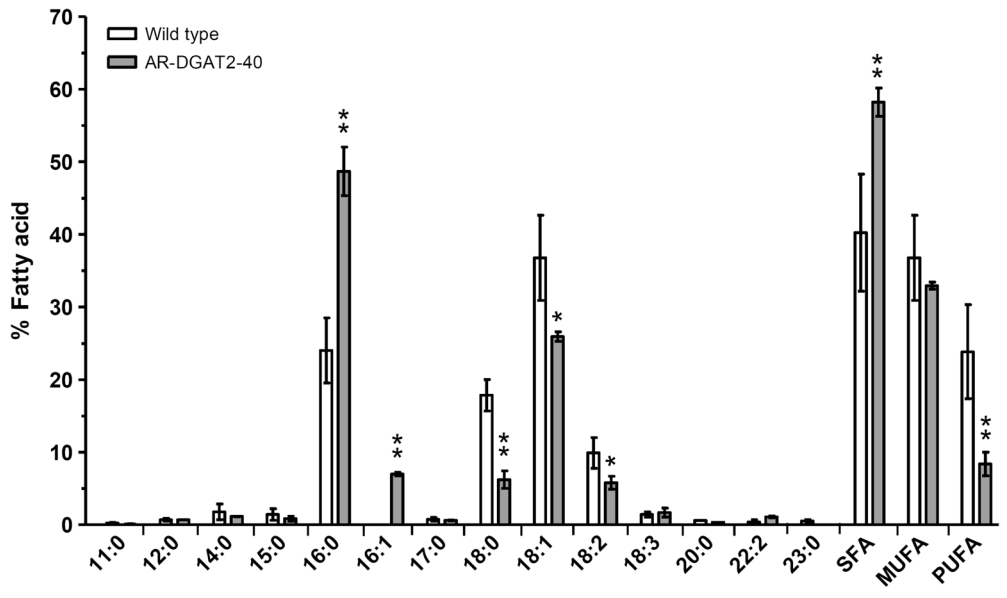


**Fig. 6** Fatty acid composition in transformant AR-DGAT2-40. Fatty acid methyl esters in transformant AR-DGAT2-40 and wild type were analyzed using GC-FID. Fatty acid composition in the predominant region of carbon chain length C16-C18 in transformant AR-DGAT2-40 was altered when compared to wild type: C16:0 and C16:1 increased, whereas C18:0, C18:1 and C18:2 were reduced. SFA, MUFA and PUFA are saturated, monounsaturated and polyunsaturated fatty acids. Each value represents mean ± SD (n = 3). Significant difference between transformant AR-DGAT2-40 and wild type is indicated (**$p < 0.01$, *$p < 0.02$, *t* test)

under N-starvation condition already have poor growth [22], additional stress conditions were omitted in this study.

The lipid bodies with higher fluorescence intensities and larger volume were observed in transformant AR-DGAT2-40 than in wild type (Fig. 3b). The lipid bodies under N starvation in green microalgae has been shown as a results of increased de novo synthesis of TAG [6]. Lipid analysis in this study was performed in cells cultured under N-starvation condition I and the enhanced cell growth by increasing aeration, N-starvation conditions II. Biomass of transformant AR-DGAT2-40 under N-starvation condition II was about 3.8-fold higher than under condition I. Thus, the biomass was enhanced by increasing aeration under N-starvation condition and not affected by *NeoDGAT2* overexpression. The TAG productivity in the transformant could be further improved by optimizing the growth condition to enhance biomass. The TAG content of 46.1 ± 1.6% DCW produced by the transformant in this study (Fig. 5a) was the highest in comparison to those produced by *DGAT2*-overexpressing microalgae reported so far with maximum TAG content of 11% DCW [15–17].

A significant alteration of the FA composition was observed in transformant AR-DGAT2-40 compared to wild type. The C16:0 in the transformant increased double, suggesting that C16:0-acyl-CoA might be a preferred substrate of NeoDGAT2; in agreement with preferred substrate of *C. reinhardttii* DGAT2 [30]. Similar incident of FA composition alteration has been reported previously. Expression of *Brassica DGAT2* in *C. reinhardtii* increases PUFA while reduces SFA [16]. Overexpression of *DGAT2* in *N. oceanica* increases SFA and PUFA whereas decreases MUFA [17]. FA compositions of TAG in *DGAT2*-overexpressing *C. reinhardtii* are different between N- and phosphorus-limited growth conditions [15].

The FA composition of TAG plays an important role in some critical parameters of the biodiesel, such as cetane number [31]. Cetane number is one of the important factors in determining the quality of diesel fuel; the higher number the more easily the fuel will combust in a compression setting [32]. High cetane numbers have been observed for esters of SFA such as C16:0 and C18:0 and MUFA such as C18:1 [33]. However, increased PUFA content unfavorably impacts the cetane number and oxidation stability of the biodiesel [31]. The FA composition of *N. oleoabundans* TAG consists of mainly C18:1 followed by C16:0 and C18:0 [22] (Fig. 6). Linolenic acid (C18:3) proportion was below 12% which meets the requirements of the European Standard EN 14214 for biodiesel production [22, 34] (Fig. 6). In this study, SFA C16:0 of transformant AR-DGAT2-40 increased double to 49%, PUFA was reduced triple to 8% (Fig. 6), thus the altered FA composition will still maintain the good quality of *N. oleoabundans* biodiesel production.

Lipid quality in most of the microalgal species is not good enough to be directly used as a substrate for producing good quality biodiesel [16]. However, *N. oleoabundans* has been demonstrated to have the best performance potential for biodiesel production, i.e. growth characteristics, lipid content, fatty acid composition, acid value and iodine value that meets the requirements of the European Standard EN 14214 for biodiesel production [22, 34]. Therefore, *N. oleoabundans* is a suitable renewable lipid source for biodiesel production.

Silencing of a foreign gene shortly after nuclear integration is not uncommon and often permanent [35, 36]. *C. reinhardtii* has been found to often silence or down regulate the non-required heterologous genes when expressed at high levels; the losses of trait or genetic changes in *C. reinhardtii* cultures have been observed in solid medium [16, 37]. Similar phenomenon of heterologous gene silencing has been observed in *N. oleoabundans*; the green fluorescent protein (GFP) activity in the transformants introduced with *Gfp* gene [25] seemed to diminish when the transformants continuously maintained in solid BBM medium for over a year (Kitraksa and Chungjatupornchai, unpublished observations). To avoid heterologous gene silencing, in the present study, endogenous *NeoDGAT2* was transformed into *N. oleoabundans* for enhancing TAG accumulation. Transformants AR-DGAT2 and B2-DGAT2 were continuously maintained in solid BBM medium for a long time; no loss in higher lipid-accumulation than wild-type trait was observed for over 100 generations in a period of about 4 years, indicating the *NeoDGAT2* overexpression stability. Endogenous *DGAT2* overexpression in *C. reinhardtii* has been shown to neither boost TAG accumulation nor alter the FA composition despite the higher levels of transcripts observed that might be due to unknown negative feedback inhibition [14]. However, such negative feedback inhibition was not observed in *N. oleoabundans*; *NeoDGAT2* overexpression altered FA composition and markedly increased TAG production (Figs. 5, 6).

## Conclusions

We successfully generated *N. oleoabundans* transformant overexpressing NeoDGAT2 with remarkably accelerated and higher TAG content and productivity. The TAG productivity in the transformant could be further improved by optimizing the growth condition to enhance biomass. A significantly altered fatty acid composition was detected in the transformant compared to wild type. Long-term stability was observed in the transformant continuously maintained in solid medium over

100 generations in a period of about 4 years. Thus, the increasing TAG content in *N. oleoabundans,* one of the most suitable lipid sources for biodiesel production, was achieved by targeted genetic engineering of the key enzyme in TAG synthesis pathway, DGAT2. This may offer the first step towards making microalgae an economically feasible source for biodiesel production. The strategy for genetically improved microalga presented in this study can be applied to other microalgal species possessing desired characteristics for industrial biofuel production.

## Methods

### Strain and growth conditions

*Neochloris oleoabundans* strain UTEX 1185, obtained from the Algal Culture Collection at the University of Texas, was cultured in liquid or in solid (1.5% Difco Bacto agar) Bold's basal medium (BBM) [38, 39] at 30 °C under constant illumination of 55–60 μmol photons/$m^2$/s. Cultures in liquid medium were inoculated with cells at starting density of ~1.5 × $10^7$ cells/mL ($OD_{750}$ = 0.3). For cell growth under nitrogen (N)-sufficient condition, cultures were maintained in 500 mL Erlenmeyer flasks containing 150 mL of BBM; the flasks were sealed and shaken at 100 rpm. Cell concentration of the cultures was monitored using spectrophotometer for optical density measurement at 750 nm and using hemocytometer for cell counting. Doubling time of the cells was calculated as described [22] using the formula $t_d = \ln 2/\mu_{max}$, where $\mu_{max}$ is the maximum specific growth rate, calculated as the maximum slope from the plot of ln OD versus culture time. For N-starvation condition I, the cells grown in BBM at exponential phase were harvested, washed and resuspended in 150 mL of BBM without $NaNO_3$ (BBM-N) in 500 mL Erlenmeyer flasks supplied with 12 L/h bubbling-filtered air. N-starvation condition II for enhancing growth was the same as condition I, except cells were resuspended in 200 mL of BBM-N, shaken at 50 rpm and supplied with 50 L/h bubbling-filtered air.

### Construction of transformation vectors

To construct *NeoDGAT2* cDNA (GenBank: KJ470774) under the control of *HSP70-RBCS2* (*AR*) promoter of *C. reinhardtii* [28] and *β2-tubulin* (*β2-Tub*) promoter of *C. reinhardtii* [29], plasmids pAR-DGAT2 and pB2-DGAT2 harboring the gene cassettes *AR-NeoDGAT2-3′rbcS2* and (*β2-Tub)-NeoDGAT2-3′rbcS2*, respectively (Fig. 1a), were constructed by replacing the *AR-ChGfp-3′rbcS2* fragment of pChGFP-Hyg3 [25] with the PCR fragments containing: (i) *AR* promoter from pCB740 [28] or *β2-Tub* promoter from pHyg3 [40], (ii) *NeoDGAT2* cDNA (GenBank: KJ470774) from pGEM-NeoDGAT2 [11], and (iii) 3′UTR of *3′rbcS2* from pCrGFP [41]. Both pAR-DGAT2 and pB2-DGAT2 harbored selectable marker gene *Hyg3* conferring hygromycin B resistance [25, 40].

### Transformation of *N. oleoabundans*

To generate transformants overexpressing DGAT2, plasmids pAR-DGAT2 and pB2-DGAT2 were transformed into the *N. oleoabundans* nuclear genome using electroporation as described [25]. *N. oleoabundans* cells were electroporated using a Gene Pulser (Bio-Rad Labs.) set resistance at 200 Ω, capacitance at 25 μF and electric field strength at 1000 V/cm. The electroporated cells were spread on a BBM agar plate containing 5 μg/mL hygromycin B. The resulting transformants AR-DGAT2 and B2-DGAT2 appeared after incubation for 2 weeks.

### Genomic PCR analysis

The *NeoDGAT2*-expression cassettes integrated into the nuclear genome of *N. oleoabundans* were verified using genomic PCR. The genomic DNA of *N. oleoabundans* was isolated as described [42]. Genomic PCR was performed with primers DGAT2-F2 (CGGGATCCTAGCTAGCATGGCGGCTCAGCGCGGTTTCG) and DGAT2-R2 (CCACAGACCTGCCCTTCTTCAGC) specifically bind to *NeoDGAT2* coding sequence. The touchdown PCR was carried out 11 cycles for the first phase (denature for 10 s at 98 °C, annealing 30 s at 80 °C which was reduced by 1 °C every successive cycle until 70 °C, extension 30 s at 72 °C) and 25 cycles for second phase (denature for 10 s at 98 °C, annealing 30 s at 70 °C, extension 30 s at 72 °C), including initial denaturation for 3 min at 98 °C and final extension for 7 min at 72 °C. PCR product was examined in a 1.5% agarose gel and further confirmed by DNA sequencing analysis. The amplicon of the *NeoDGAT2* coding sequence was 517 bp.

### Nile red fluorescence assay and microscopy

To evaluate the level of neutral lipids, *N. oleoabundans* grown under N-starvation condition I (~1.5 × $10^7$ cells/mL) was stained with fluorescent dye Nile red dissolved in acetone to final concentration of 1 μg/mL and incubated in the dark for 10 min. The fluorescence intensity was measured in a 96-well plate using a spectrofluorometer (Beckman Coulter DTX-880, USA) with excitation at 535 nm and emission at 574 nm. The observed intensities were corrected by subtracting the fluorescence value difference in Nile red stained and unstained cells. Specific fluorescence intensities were normalized by cell numbers. The lipid bodies in the cells stained with Nile red were visualized under an inverted fluorescence microscope (Nikon Eclipse Ti-S, Japan) with excitation at 420–490 nm and emission at 520 nm.

### Quantitative real time PCR analysis

Relative *NeoDGAT2* transcript abundance was quantified using quantitative real-time PCR (qPCR). Total RNA was extracted from cells cultured under N-starvation condition I at stationary phase using TRI Solution (GeneMark, Taiwan). The cDNA was prepared from the total RNA using oligo (dT)18 primer and RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, Canada). The cDNA was amplified by KAPA SYBR FAST qPCR Kit (Kababiosystems, USA) using *NeoDGAT2*-gene specific primers: DGAT-RT-F1 (GGCGACAAAGGTCTTCCTCC) and DGAT-RT-R1 (GGCTCGTATCCGATTACAAAGG) and endogenous *Actin* (*NeoActin*)-gene specific primers: NeoActin-F1 (ACACTGTGCCCATCTATGAGGG) and NeoActin-R1 (CTTGATGTCACGCACGATTTCG). Mastercycler realplex4 and realplex software (Eppendorf, Germany) were used for the analysis. Fold difference of transcript was calculated using the ΔΔCt method. The *NeoDGAT2* transcript level was normalized to *NeoActin* transcript used as a reference.

### Lipid extraction and quantification

Total lipids of *N. oleoabundans* grown under N-starvation condition were extracted based on Bligh and Dyer method [43]. The cell pellet of 30 $OD_{750}$ (≈50 mg) was suspended in chloroform:methanol (2:1, v/v). The cells were lysed using 0.5 mm glass beads with vortexing at 2700 rpm (Vortex Genie2 G560E, Scientific Industries, USA), then chloroform:water (1:1, v/v) was added to the mixture. Chloroform phase was collected and evaporated using nitrogen gas. Total lipids were then determined gravimetrically. TAG was subsequently separated from total lipids by thin-layer chromatography (TLC) using the solvent system hexane:diethyl ether:acetic acid (70:30:1, v/v/v) and glyceryl trioleate (92860 Sigma-Aldrich, USA) as a reference substance. Quantification of TAG stained with iodine was performed using Quantity One 1-D analysis software (Bio-Rad Labs., USA). Dry cell weight (DCW) of a sample was determined gravimetrically after drying *N. oleoabundans* cells. Total lipid and TAG content was calculated as percentage of dry cell weight (% DCW). The lipid productivity was calculated using the formula

$$P_{\mathrm{Lipid}}(\mathrm{mg/L/day}) = [C_{\mathrm{Lipid}}(\mathrm{mg/mg}) \times \mathrm{DCW}\ (\mathrm{mg/L})]/\mathrm{Time}\ (\mathrm{day}),$$

where $C_{\mathrm{Lipid}}$ is lipid content of cells, DCW is dry cell weight, and Time is the cultivation period, as described [21].

### Fatty acid composition analysis

To obtain fatty acid methyl esters (FAME), TAG extracted from TLC was incubated with 5% (v/v) sulfuric acid in methanol at 70 °C for 3 h in the presence of glyceryl trinonadecanoate (91988 Sigma-Aldrich) used as internal standard. FAME analysis was carried out using gas chromatography-flame ionization detector (GC-FID) (7890A GC system, Agilent USA) equipped with Agilent DB-WAX capillary column (30 m × 0.25 mm × 0.25 μm) and helium as the carrier gas. The oven temperature was increased from 50 to 200 °C at a rate of 28.5 °C/min, 200 to 240 °C at a rate of 3.4 °C/min and held at 240 °C for 16 min. Supelco 37-component FAME mix (Supelco 47885-U, Sigma-Aldrich) was used as the external standard to identify retention time for specific FAME. Fatty acid composition was calculated as percentage of the total fatty acids present in the sample, determined from the peak areas.

### Statistical analysis

To determine the statistical differences between wild type (control) and transformant samples, two-tailed student's *t* test was performed using SPSS Base 16.0 software (SPSS, USA).

#### Abbreviations

DCW: dry cell weight; DGAT: diacylglycerol acyltransferase; GC-FID: gas chromatography-flame ionization detector; N: nitrogen; qPCR: quantitative real-time PCR; FA: fatty acids; FAME: fatty acid methyl ester; MUFA: monounsaturated fatty acid; PUFA: polyunsaturated fatty acid; SFA: saturated fatty acid; TAG: triacylglycerol; TLC: thin-layer chromatography.

#### Authors' contributions

PK performed the majority of experimental analysis. SF performed transcription analysis. WC designed the experiments, conceptualized the study and wrote the manuscript. All authors read and approved the final manuscript.

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#### Competing interests

The authors declare that they have no competing interests.

#### Availability of data and supporting materials

The dataset supporting the conclusions of this article is included within the article.

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## Publisher's Note



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# Increased triacylglycerol production in oleaginous microalga *Neochloris oleoabundans* by overexpression of plastidial lysophosphatidic acid acyltransferase

Wipa Chungjatupornchai*, Kanchanaporn Areerat and Sirirat Fa-Aroonsawat

## Abstract

**Background:** Microalgae are promising sources of lipid triacylglycerol (TAG) for sustainable production of natural edible oils and biofuels. Nevertheless, products derived from microalgal TAG are not yet economically feasible; increasing TAG content via targeted genetic engineering of genes in TAG biosynthesis pathway are important to achieve economic viability. To increase TAG content, oleaginous microalga *Neochloris oleoabundans* was genetically engineered with the endogenous enzyme lysophosphatidic acid acyltransferase (NeoLPAAT1) responsible for plastidial TAG biosynthesis

**Results:** NeoLPAAT1 was found to contain all canonical motifs attributed to LPAAT proteins, two hypothetical membrane-spanning domains and a putative chloroplast transit peptide, indicating as a member of plastidial LPAAT type 1 subfamily. The *NeoLPAAT1*-expression cassette integrated in *N. oleoabundans* transformant was confirmed by PCR. The neutral lipid content in the transformant detected by Nile red staining was 1.6-fold higher than in wild type. The *NeoLPAAT1* transcript was twofold higher in the transformant than wild type. Considerably higher lipid quantity was found in the transformant than wild type: total lipid content increased 1.8- to 1.9-fold up to 78.99 ± 1.75% dry cell weight (DCW) and total lipid productivity increased 1.8- to 2.4-fold up to 16.06 ± 2.68 mg/L/day; while TAG content increased 2.1- to 2.2-fold up to 55.40 ± 5.56% DCW and TAG productivity increased 1.9- to 2.8-fold up to 10.67 ± 2.37 mg/L/day. A slightly altered fatty acid composition was detected in the transformant compared to wild type; polyunsaturated fatty acid (C18:2) increased to 19% from 11%. *NeoLPAAT1*-overexpression stability was observed in the transformant continuously maintained in solid medium over 150 generations in a period of about 6 years.

**Conclusions:** Our results demonstrate the considerably increased TAG content and productivity in *N. oleoabundans* by overexpression of plastidial *NeoLPAAT1* that are important for products derived from microalgal TAG to achieve economic viability. Plastidial LPAAT1 can be a candidate for target genetic manipulation to increase TAG content in other microalgal species with desired characteristics for production of natural edible oils and biofuels.

**Keywords:** Biofuels, Lysophosphatidic acid acyltransferase (LPAAT), 1-Acyl-*sn*-glycero-3-phosphate acyltransferase (AGPAT), Microalgae, Lipids, Edible oils

## Background

Microalgae are promising sources of lipid triacylglycerol (TAG) for sustainable production of natural edible oils as an alternative to plant derived oil [1, 2] and biofuels as an environmentally safe alternative to fossil fuels [3, 4]; because they possess short life cycles, perform photosynthesis, require non-arable land and absorb a large amount of $CO_2$. Nevertheless, there are several challenges that need to be overcome before products derived from microalgal TAG can be

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economically produced at a commercial scale, one of which is the lack of microalgal strains with high TAG content [3, 5]. Increasing TAG content in microalgae could be achieved by targeted genetic engineering of genes in TAG biosynthesis pathway [4, 6, 7].

The TAG biosynthesis pathway in microalgae is not yet fully understood but considered to be most similar to that operating in higher plants [8]. In the model plant *Arabidopsis thaliana* and the model microalga *Chlamydomonas reinhardtii*, two sets of homologous enzymes catalyze two distinct and parallel TAG biosynthesis pathways, one in the plastid (prokaryotic pathway) and the other in the endoplasmic reticulum (ER; eukaryotic pathway) [9-13]. The ER pathway has been shown as an important route of TAG biosynthesis, however, increasing evidence suggests that the plastidial pathway also plays an important role in TAG biosynthesis in *C. reinhardtii* [13, 14]. In TAG biosynthesis pathway, lysophosphatidic acid acyltransferase (LPAAT or LPAT; EC 2.3.1.51) also known as 1-acyl-*sn*-glycero-3-phosphate acyltransferase (AGPAT) catalyzes the acylation of the *sn*-2 position of lysophosphatidic acid to generate a key intermediate, phosphatidic acid [15]. *C. reinhardtii* has been shown to possess CrLPAAT2 localized to ER membranes and CrLPAAT1 localized to plastid [11, 16]. Overexpression of *LPAAT1* for enhancing TAG accumulation has been attempted so far in very few microalgal species. *CrLPAAT1* overexpression in *C. reinhardtii* led to > 20% increase in oil content [11] and *LPAAT1* (*AGPAT1*) overexpression in diatom *Phaeodactylum tricornutum* led to increase in lipid content by 1.81-fold [17]. However, no *LPAAT1* overexpression has been reported so far in oleaginous microalga *Neochloris oleoabundans*.

*Neochloris oleoabundans,* a taxonomic synonym of *Ettlia oleoabundans* [18], is a promising source of TAG; because under nitrogen starvation condition, it produces lipids 36–54% of its cell dry weight and up to 80% of its total lipids is TAG [19]. However, the knowledge concerning *N. oleoabundans* is very limited; no genomic sequences are available. To enable targeted genetic manipulation of TAG biosynthesis, the stable nuclear transformation system of *N. oleoabundans* has been established [20] and the cDNA encoding LPAAT1 of *N. oleoabundans* (*NeoLPAAT1*) has been cloned [21].

In this study, we tested whether overexpression of endogenous plastidial *LPAAT1* would affect TAG biosynthesis in oleaginous microalga. The plastidial *LPAAT1* cDNA sequence of *N. oleoabundans* (*NeoLPAAT1*) was characterized. The *NeoLPAAT1*-overexpressing *N. oleoabundans* was generated and characterized with regards to growth and neutral lipid accumulation in the cells, lipid content and productivity, and fatty acid composition.

## Results

### Comparative homologue of NeoLPAAT1

The *LPAAT* cDNA of *N. oleoabundans*, cloned previously (GenBank: MF706164; protein id: AUS8446) [21], was designated as *NeoLAPAAT1* in this study. When compared with the well characterized LPAAT1 of *Chlamydomonas reinhardtii* (CrLPAAT1) [11] and *Arabidopsis thaliana* (AtLPAAT1/AtATS2) [15] using ClustalW program [22], NeoLPAAT1 was found to share high level of amino acid sequence identity to CrLPAAT1 (46.8%) and AtLPAAT1 (37.3%) (Fig. 1a). All four canonical motifs attributed to LPAAT proteins [23]: motif I, NH(X4)D; motif II, GVIFIDR; motif III, EGTR and motif IV, IVPIVM were observed in NeoLPAAT1. Two hypothetical membrane-spanning domains as predicted by TMHMM v2.0 [24] were also detected in NeoLPAAT1 (highlighted with yellow in Fig. 1a). In addition, N-terminus of NeoLPAAT1, as predicted by PredAlgo [25], contained a putative chloroplast transit peptide (~69 amino acids, highlighted with red in Fig. 1a), suggesting that NeoLPAAT1 could be targeted to the chloroplast of *N. oleoabundans*. Therefore, the results indicated that NeoLPAAT1 is a homolog of the plastidial LPAAT1 of *C. reinhardtii* and *A. thaliana.* In the phylogenetic tree constructed using MEGA 7 [26], NeoLPAAT1 sharing 66.0% amino acid identity to *Chlorella variabilis* CvLPAAT1 was grouped in the same clade (Fig. 1b), suggesting that NeoLPAAT1 possessed the closest evolutionary relationship with CvLPAAT1.

### Selection of *N. oleoabundans* transformants

*Neochloris oleoabundans* was electroporated with plasmids pAR-LPAAT and pB2-LPAAT containing *NeoLPAAT1* cDNA under the control of promoters *HSP70-RBCS2* (*AR*) and *β2-tubulin* (*β2-Tub*), respectively (Fig. 2a). These promoters have been shown to function in *N. oleoabundans* [20, 27]. The resulting transformants AR-LPAAT and B2-LPAAT were selected on BBM agar supplemented with hygromycin B. To screen for clones with potential high-neutral-lipid accumulation, about 100 colonies selected from each plasmid transformation were grown on BBM agar plates for 14 days and then stained with Nile red yielding intensely fluorescence in a neutral lipid environment [28]. Transformants AR-LPAAT-48, AR-LPAAT-90, B2-LPAAT-38 and B2-LPAAT-46 found to have high Nile red fluorescence intensity were selected for subsequent experiments.

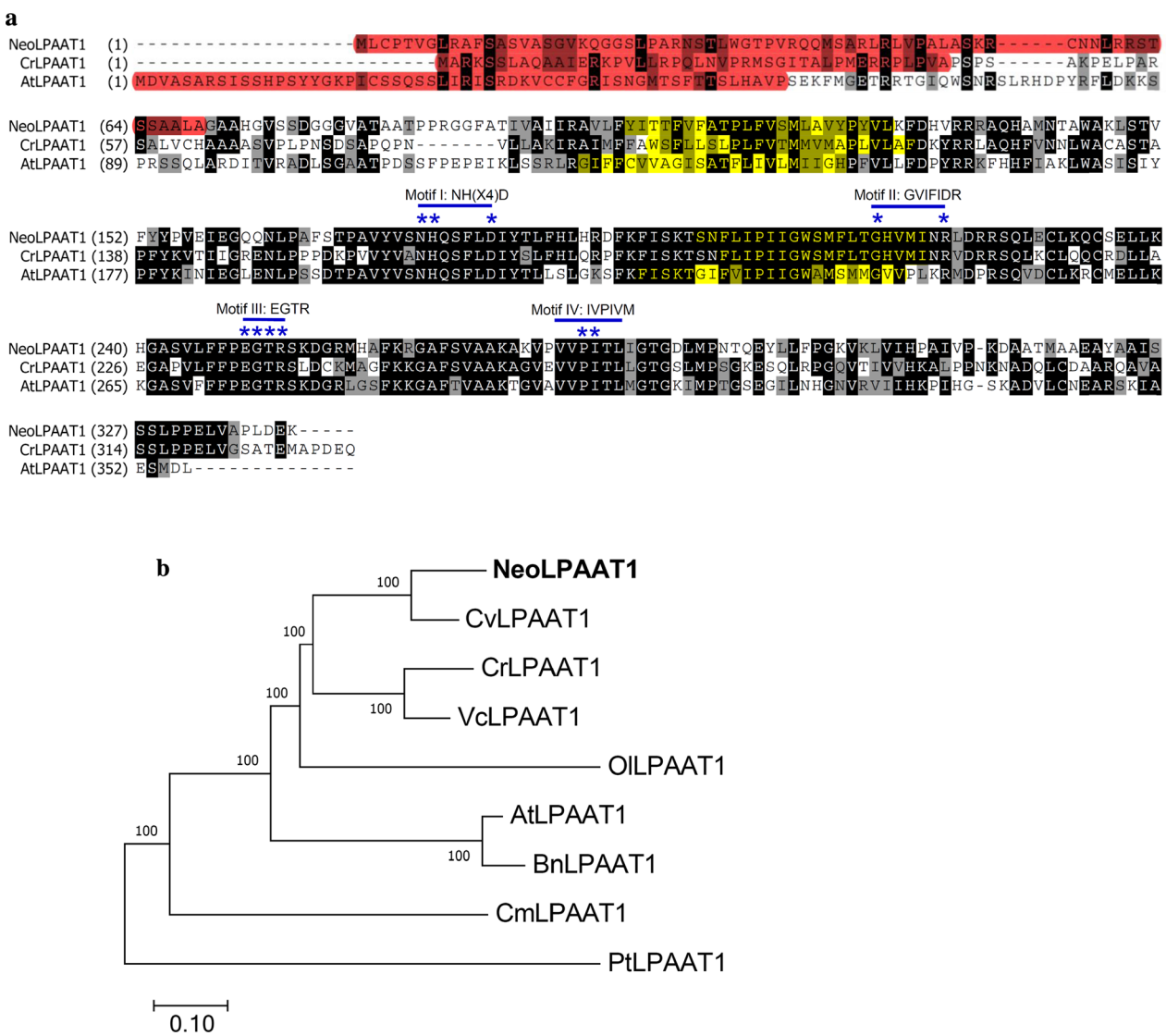


**Fig. 1** Comparison of NeoLPAAT1 with other plastidial LPAAT1. **a** Sequence alignment of CrLPAAT1, AtLPAAT1 (ATS2) and NeoLPAAT1. The amino acid sequences of CrLPAAT1 (*Chlamydomonas reinhardtii*, Uniprot: A8J0J0), AtLPAAT1 (*Arabidopsis thaliana*, Uniprot: Q8GXU8**)** and NeoLPAAT1 (*N. oleoabundans*, GenBank protein id: AUS8446) were aligned using ClustalW algorithm [22]. The chloroplast transit peptides of CrLPAAT1, AtLPAAT1 [15] and NeoLPAAT1 predicted using PredAlgo [25] are highlighted in red. Membrane-spanning domains predicted by TMHMM [24] are highlighted in yellow. Conserved and similar residues are indicated by black and gray boxes, respectively. The conserved motifs I, II, III and IV [23] are indicated. The highly conserved residues in the motif are indicated by asterisks. **b** Phylogenetic analysis of NeoLPAAT1. The phylogenetic tree was constructed using MEGA 7 [26]. The percentage of neighbor-joining boot strap replications is shown above each node. Scale bar indicates 0.1 amino acid substitutions per site. Green algae: NeoLPAAT1 (*N. oleoabundans*, GenBank protein id: AUS8446), CvLPAAT1 (*Chlorella variabilis*, Uniprot: E1ZQN6), CrLPAAT1 (*C. reinhardtii*, Uniprot: A8J0J0), VcLPAAT1 (*Volvox carteri*, Uniprot: D8U1V6) and OlLPAAT1 (*Ostreococcus lucimarinus*, Uniprot: A4S0H0). Red alga: CmLPAAT1 (*Cyanidioschyzon merolae*, Uniprot: M1V4N2). Diatom: PtLPAAT1 (*Phaeodactylum tricornutum*, Uniprot: B7FQL9). Plants: AtLPAAT1 (*A. thaliana*, Uniprot: Q8GXU8**)** and BnLPAAT1 (*Brassica napus*, Uniprot: Q9LLY4)

### Detection of *NeoLPAAT1*-expression cassette integration

The integration of *NeoLPAAT1*-expression cassettes in *N. oleoabundans* was analyzed using genomic PCR with primers specific to *NeoLPAAT1* coding sequence. The expected 943-bp amplicon was detected in the selected transformants: AR-LPAAT-48, AR-LPAAT-90, B2-LPAAT-38 and B2-LPAAT-46, but not in wild type (Fig. 2b). The 943-bp amplicon subjected to DNA sequencing was confirmed to be *NeoLPAAT1* coding sequence. Therefore, the *NeoLPAAT1*-expression cassettes were successfully introduced into the transformants. The amplicon from the resident *NeoLPAAT1* gene

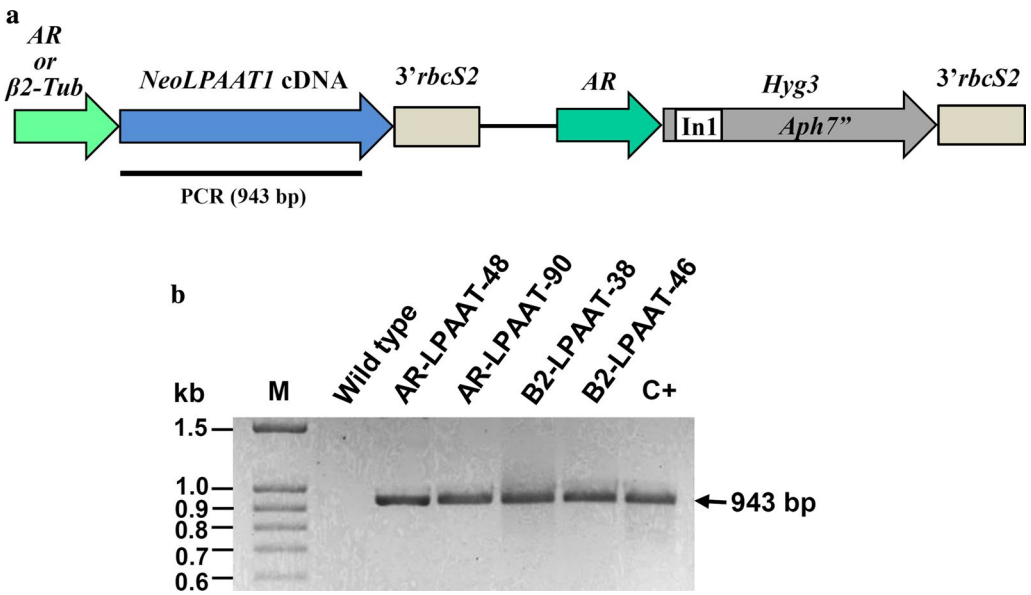


**Fig. 2** Generation of *N. oleoabundans* transformants. **a** Schematic map of plasmids pAR-LPAAT and pB2-LPAAT used for electroporation in *N. oleoabundans*. The *NeoLPAAT1* cDNA (GenBank accession no.: MF706164) [21] was expressed under the control of either promoter *AR* (*HSP70-RBCS2*) [44] or *β2-Tub* (*β2-tubulin*) [45] and harbored *3′rbcS2* [47] at 3′end. *Hyg3* gene, used as selectable marker [20, 46]. The 943-bp PCR amplicon, denoted by a line. **b** Genomic PCR detection of transformants harboring the *NeoLPAAT1*-expression cassettes. Genomic PCR of transformants AR-LPAAT, B2-LPAAT and wild type was performed with primers specifically bind to *NeoLPAAT1* coding sequence. The 943-bp amplicon was detected in the transformants but not in wild type (used as negative control). Lanes M, 100-bp DNA ladder; C+, plasmid pAR-LPAAT (used as positive control)

including introns was 1865 bp. Because one of the two primers used in this PCR was located on two exons, the resident *NeoLPAAT1* gene was not amplified. The PCR-positive transformants were further analyzed for growth characteristics.

#### Growth of the transformants

To evaluate the effect of *NeoLPAAT1* overexpression on growth, we analyzed the growth curve under nitrogen-sufficient (+N) condition of the transformants. All selected transformants showed overall similar growth compared to wild type, whereas slightly lower growth during the stationary phase (Fig. 3a). The doubling time during exponential growth of the transformants (4–5 days) was not significantly different from that of wild type (at $p < 0.02$). Therefore, *NeoLPAAT1* overexpression did not have an apparent effect on growth of the selected transformants AR-LPAAT-48, AR-LPAAT-90, B2-LPAAT-38 and B2-LPAAT-46.

#### Neutral lipids of the transformants

To investigate the potential high-lipid production of the selected transformants, neutral lipids in the cells during +N growth condition were monitored by Nile red staining. The exponential growth of *N. oleoabundans* has been shown to accompany the decrease in N concentration; N-limited growth can stimulate the cells to produce more lipids [29]. The levels of neutral lipid accumulation in transformants AR-LPAAT-48, AR-LPAAT-90, B2-LPAAT-38 and B2-LPAAT-46 were different; they were higher than that in wild type (Fig. 3b). Whether the different levels of neutral lipid accumulation in the transformant clones are due to copy number or integration positional effect of the *NeoLPAAT1*-expression cassettes remains to be investigated. Among the transformants, B2-LPAAT-46 was found to reach maximum neutral lipid content (day 33) earlier than wild type (day 38) and have the highest neutral lipid content which increased to 1.6-fold compared to the maximum content in wild type (Fig. 3b). Therefore, transformant B2-LPAAT-46 was selected for subsequent experiments.

#### Evaluation of *NeoLPAAT1* transcript

The up-regulated LPAAT (AGPAT) transcripts in response to N-starvation has been observed by transcriptomic analysis of *N. oleoabundans* [30]. To evaluate whether the *NeoLPAAT1*-expression cassette integrated in the transformant expressed at transcriptional level, the relative *NeoLPAAT1* transcript abundance in the transformant B2-LPAAT-46 cultured under −N condition was determined by quantitative real-time PCR (qPCR)

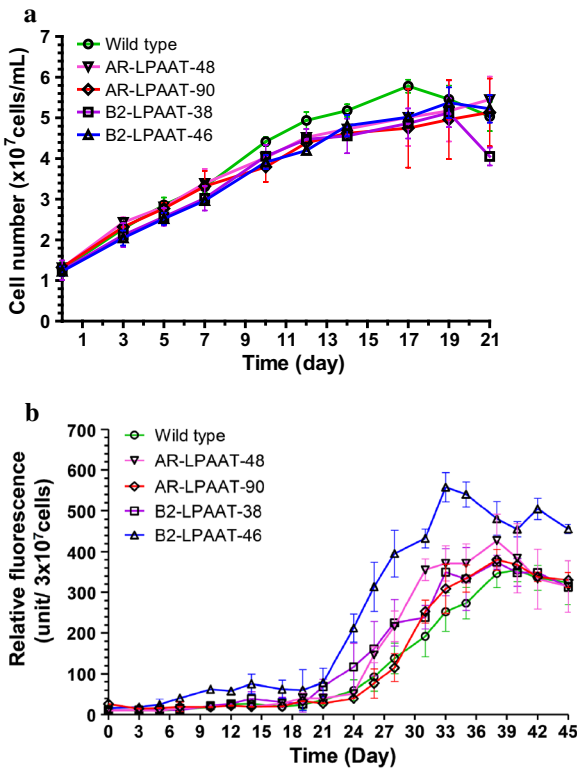


**Fig. 3** Growth and neutral lipid accumulation of transformants AR-LPAAT and B2-LPAAT. **a** Growth curve and **b** Neutral lipid accumulation of the transformants and wild type during +N growth condition. Neutral lipid accumulation was evaluated using Nile red staining. Transformant B2-LPAAT-46 was found to reach maximum Nile red fluorescence earlier and higher than wild type. Each value represents mean ± SD (n = 3)

using *NeoActin* transcript as a reference. Transformant B2-LPAAT-46 was observed to have *NeoLPAAT1* transcript increased twofold compared to wild type (Fig. 4), indicating that the increased transcript was enhanced by overexpression of *NeoLPAAT1*.

### Lipid productivity analysis

Transformant B2-LPAAT-46 and wild type were not significantly different in doubling time under +N growth condition, followed by N-limited growth that stimulated neutral-lipid accumulation at maximum level on day 33 and 38, respectively (Fig. 3a, b). *Neochloris oleoabundans*, like many microalgae, accumulates neutral lipids under N-starvation condition [19, 29, 31]. To accelerate the lipid production, transformant B2-LPAAT-46 and wild type were cultured under −N condition and the neutral-lipid accumulation as monitored by Nile red staining reached maximum level on about day 18 to 20. The dried cells were determined gravimetrically as dry cell weight (DCW). Biomass under −N condition was not significantly different between the transformant B2-LPAAT-46 (461 ± 65 mg DCW/L) and wild type (462 ± 27 mg DCW/L) ($p < 0.05$). Thus, the biomass was not affected by *NeoLPAAT1* overexpression. Lipid analysis was performed in transformant B2-LPAAT-46 and wild type cultured under +N and −N condition with the maximum neutral lipid accumulation monitored by Nile red staining. Because TAG is the major component for natural edible oil and biofuel production, the TAG content separated from total lipids using thin-layer chromatography (TLC) was quantified. Up to 70% of total lipids extracted from transformant B2-LPAAT-46 and wild type were TAG (Fig. 5a). The lipid content and productivity in transformant B2-LPAAT-46 were compared to wild type: under +N condition, total lipid content in transformant (20.8 ± 3.4% DCW) increased 1.9-fold, TAG content (11.55 ± 3.2% DCW) increased 2.2-fold, total lipid productivity (8.61 ± 1.39 mg/L/day) increased 2.4-fold, TAG productivity (4.77 ± 1.32 mg/L/day) increased 2.8-fold; under −N condition, total lipid content in transformant (78.99 ± 1.75% DCW) increased 1.8-fold, TAG content (55.40 ± 5.56% DCW) increased 2.1-fold, total lipid productivity (16.06 ± 2.68 mg/L/day) increased 1.8-fold, TAG productivity (10.67 ± 2.37 mg/L/day) increased 1.9-fold (Fig. 5a, b). Therefore, transformant B2-LPAAT-46 was observed to considerably increase in lipid accumulation when compared to wild type: the total lipid content and productivity increased 1.8- to 1.9-fold and 1.8- to 2.4-fold, respectively; while the TAG content and productivity increased 2.1- to 2.2-fold and 1.9- to 2.8-fold, respectively (Fig. 5a, b). *NeoLPAAT1* overexpression dramatically increased TAG content in the microalga.

### Fatty acid composition analysis

Because fatty acid (FA) composition can affect the quality and cost of natural edible oils and biofuels, we evaluated whether *NeoLPPAT1* overexpression would have any effect on FA composition. TAG was extracted from transformant B2-LPAAT-46 and wild type under +N condition harvested on day 33 and 38, respectively. Fatty acid methyl esters (FAME) obtained by transesterification of TAG were analyzed using GC–MS (Fig. 6). FAME in the chain-length range of C14–C20 was determined by comparison with the standard reference. The palmitic acid (C16:0) and oleic acid (C18:1) were the most abundant FA in the TAG (Fig. 6). The linoleic acid (C18:2) in transformant B2-PLAAT-46 compared to wild type increased to 19% from 11%; the rest is not significantly different. The overall saturated fatty acid and monounsaturated fatty acid were not significantly different from the wild type, whereas polyunsaturated fatty acid was increased to 20% from 11%

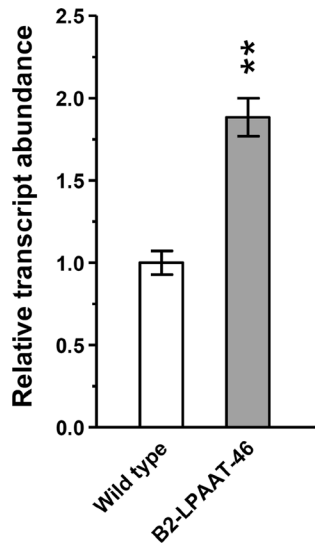


**Fig. 4** Relative *NeoLPAAT1* transcript abundance in transformant B2-LPAAT-46. The levels of *NeoLPAAT1* transcript under −N growth condition were determined by quantitative real-time PCR. The values are normalized to the expression level of endogenous *NeoActin*. Each value represents mean ± SD (n = 3). Significant difference between transformant B2-LPAAT-46 and wild type is indicated (**$p < 0.01$, *t* test)

(Fig. 6). Thus, a slight alteration of the FA composition in transformant B2-LPAAT-46 was due to overexpression of *NeoLPAAT1*.

### Long-term stability of transformants

Transformants AR-LPAAT and B2-LPAAT were subcultured (every 2 weeks) in solid BBM over 150 generations in a period of about 6 years. Neutral lipid accumulation in the transformants were periodically checked by Nile red staining; higher lipid-accumulation than wild-type trait was observed in all transformants used in this study, indicating the *NeoLPAAT1* overexpression stability.

## Discussion

This study is based on the overexpression of endogenous plastidial *NeoLPAAT1* in *N. oleoabundans* to increase TAG accumulation for sustainable production of natural edible oils and biofuels. The plastidial LPAAT of *C. reinhardtii* and the putative plastidial LPAAT of other microalgae with sequenced genomes, including *Volvox carteri, Ostreococcus lucimarinus* and *Coccomyxa subellipsoidea*, belongs to the same subcluster as LPAAT1 from cyanobacteria and plants, suggesting a common origin of the plastidal isoform of LPAAT1 [11, 32]. Similar to the well characterized CrLPAAT1 of the model microalga *C. reinhardtii* [11] and AtLPAAT1 of the model plant *Arabidopsis thaliana* [15], plastidial NeoLPAAT1 was found to contain all four canonical motifs attributed to LPAAT proteins, two hypothetical membrane-spanning domains and a putative chloroplast transit peptide (Fig. 1a). NeoLPAAT1 had the closest evolutionary relationship with CvLPAAT1 of Treboxiophycean *C. variabilis* but was quite distantly related to CrLPAAT1 of Chlorophycean *C. reinhardtii* (Fig. 1b). This result is consistent with previous report that diacylglycerol acyltransferase type 2 (NeoDGAT2) of *N. oleoabundans* has the closest evolutionary relationship with CvDGAT2 of *C. variabilis* but is quite distantly related to CrDGAT2A (DGTT4) of *C. reinhardtii* [33]. However, *N. oleoabundans* has been classified based on uninucleate cell morphology to class Chlorophyceae [34]. Classification of *N. oleoabundans* using 18S and 28S rDNA sequence remains to be verified. *N. oleoabundans* possesses ER-located NeoDGAT2 [33] and plastidial NeoLPAAT1 (in this study), suggesting the existence of two distinct and parallel TAG biosynthesis pathways. Similar incidents of duplicated sets of TAG assembly enzymes have been observed in *C. reinhardtii*: CrLPAAT1 and CrDGAT1 located in plastid [11, 35], and CrLPAAT2 and CrDGAT2 located in ER [16, 36].

Among the *NeoLPAAT1*-overexpressing transformants, B2-LPAAT-46 exhibited the highest neutral lipid content which increased to 1.6-fold compared to the maximum content in wild type (Fig. 3b). The lipid content and productivity were dramatically increased under −N condition when compared to +N condition: in transformant B2-LPAAT-46, total lipid content and productivity increased 3.8- and 1.9-fold, respectively, TAG content and productivity increased 4.8- and 2.2-fold, respectively; in wild type, total lipid content and productivity increased 3.9- and 2.5-fold, respectively, TAG content and productivity increased 4.9- and 3.2-fold, respectively (Fig. 5a, b). The results agreed well with previous report that *N. oleoabundans* begins accumulating lipids with the application of minimal N starvation, just following exhaustion of exogenous N. In addition, *N. oleoabundans* exhibits only a small decrease in growth and drastically higher lipid content under N starvation condition; concurrent growth and lipid accumulation result in higher lipid productivity [37]. Lipid productivities of wild type and transformant B2-LPAAT-46 may further increase when the cells cultured under optimal conditions. The lipid productivities of *N. oleoabundans* have been reported with different experimental conditions resulting in large variations in performance, i.e. total lipid productivities vary from 9 to 134 mg/L/day [27, 29, 37, 38] and TAG productivities vary from 6 to 216 mg/L/day [27, 39], making it difficult to compare the reports with each other. The TAG content of 55.40 ± 5.56% DCW produced

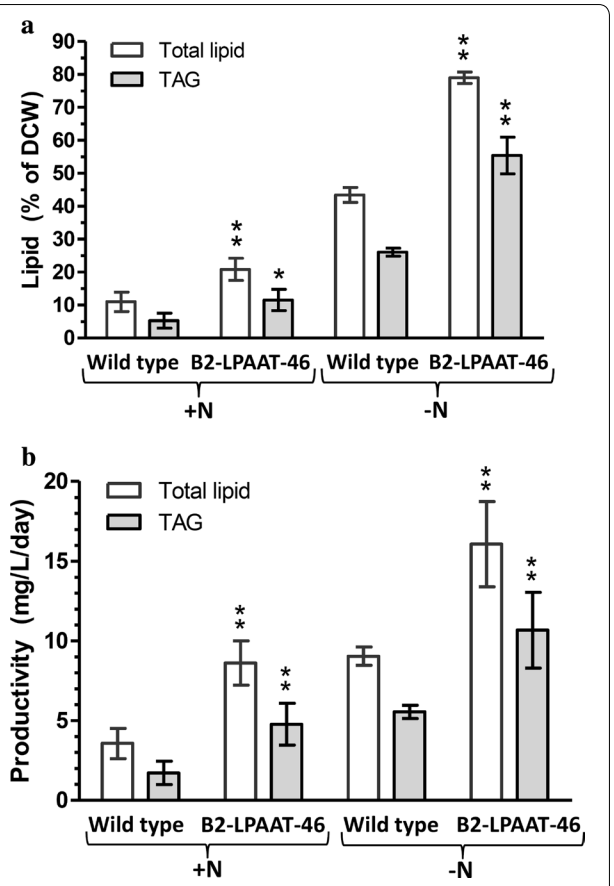


**Fig. 5** Lipid analysis of transformant B2-LPAAT-46. **a** Lipid content and **b** Lipid productivity. Lipids extracted from transformant B2-LPAAT-46 and wild type grown under +N and −N condition. Each value represents mean ± SD (n = 3). Significant difference between transformant B2-LPAAT-46 and wild type in the same growth condition is indicated (*$p < 0.05$, **$p < 0.01$, *t* test)

by the transformant in this study (Fig. 3b) was the highest in comparison to those produced by *LPAAT1*-overexpressing microalgae reported so far [11, 17] and also 20% higher than that (46.1 ± 1.6% DCW) produced by *N. oleoabundans* overexpressing *NeoDGAT2* [27]. The results suggest that the plastidial pathway also plays an important role in TAG biosynthesis in *N. oleoabundans*. The FA composition in transformant B2-LPAAT-46 was slightly altered; the linoleic acid (C18:2) increased to 19% from 11% when compared to wild type (Fig. 6). Whether C18:2-acyl-CoA is a preferred substrate of NeoLPAAT1 remains to be determined.

Silencing or down regulation of the non-required heterologous genes when expressed at high levels has been reported in *C. reinhardtii* [40, 41]. Heterologous gene silencing has also been observed in *N. oleoabundans*; when the transformants introduced with *Gfp* gene [20] continuously maintained in solid BBM medium for over a year, the green fluorescent protein activity seems to diminish [27]. However, overexpression of endogenous *NeoDGAT2* in *N. oleoabundans* has been shown to be stable [27]. Therefore, to avoid heterologous gene silencing, endogenous *NeoLPAAT1* was used in this study. Transformants AR-LPAAT and B2-LPAAT were continuously maintained in solid BBM over 150 generations in a period of about 6 years. All transformants used in this study were periodically checked for neutral lipid accumulation by Nile red staining and found to have higher lipid accumulation than wild-type, indicating the *NeoLPAAT1* overexpression stability.

## Conclusions

We characterized the plastidial NeoLPAAT1 sequence and successfully created *N. oleoabundans* transformant overexpressing NeoLPAAT1 with considerably increased TAG content and productivity. A slightly altered fatty acid composition was detected in the transformant compared to wild type. Stability of *NeoLPAAT1* overexpression was observed in the transformant continuously maintained in solid medium over 150 generations in a period of about 6 years. The considerably increased TAG content and productivity in *N. oleoabundans* by overexpression of *NeoLPAAT1* are important for products derived from microalgal TAG to achieve economic viability. Plastidial LPAAT1 can be a candidate for target genetic manipulation to increase TAG content in other microalgal species with desired characteristics for production of natural edible oils and biofuels.

## Methods

### Strain and growth conditions

*Neochloris oleoabundans* strain UTEX 1185, obtained from the Algal Culture Collection at the University of Texas, was cultured in liquid or in solid (1.5% Difco Bacto agar) Bold's basal medium (BBM) [42, 43] under constant illumination of 55–60 µmol photons/$m^2$/s at 30 °C. Cultures in liquid medium started with cells at density of ~ $1.5 \times 10^7$ cells/mL ($OD_{750} = 0.3$). For cell growth under nitrogen-sufficient (+N) condition, cultures were maintained in 500 mL Erlenmeyer flasks containing 200 mL of BBM; the flasks were sealed and shaken at 100 rpm. Cell density of the cultures was evaluated using hemocytometer for cell counting and spectrophotometer for $OD_{750}$. Doubling time of the cells was calculated as described [29]. For nitrogen-starvation (−N) condition, the cells grown in BBM at exponential phase were harvested, washed and resuspended in 200 mL of BBM without $NaNO_3$ (BBM-N) in 500 mL Erlenmeyer flasks, shaken at 50 rpm and supplied with 50 L/h bubbling-filtered air.

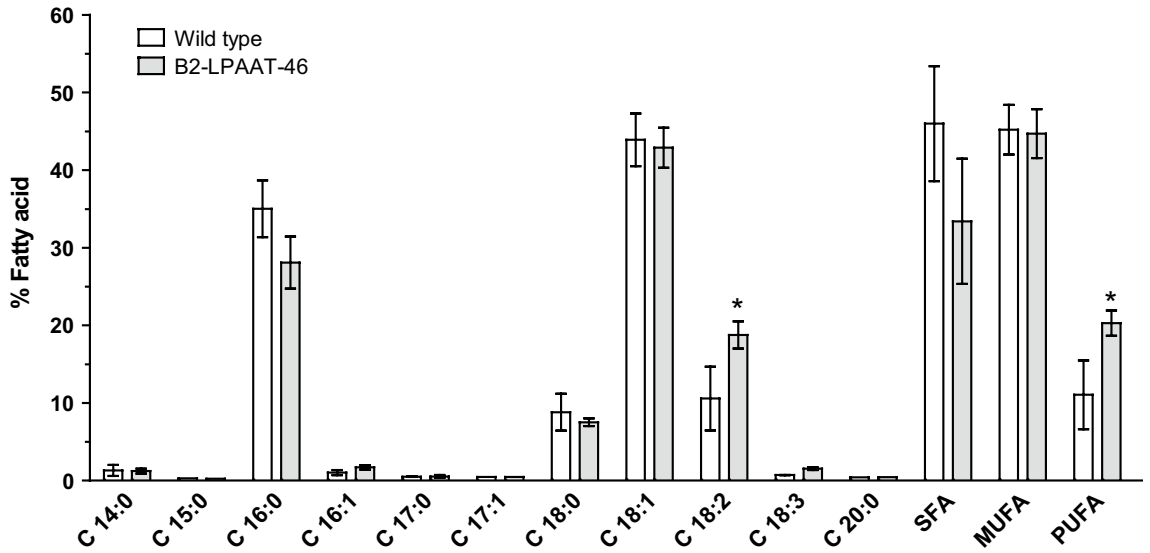


**Fig. 6** Fatty acid composition in transformant B2-LPAAT-46. FAME of transformant B2-LPAAT-46 and wild type were analyzed using GC–MS. Fatty acid C18:2 and PUFA increased in transformant B2-LPAAT-46 when compared to wild type. SFA, MUFA and PUFA are Saturated, Monounsaturated and Polyunsaturated fatty acids. Each value represents mean ± SD (n = 3). Significant difference between transformant B2-LPAAT-46 and wild type is indicated (*$p < 0.05$, *t* test)

### NeoLPAAT1 sequence analysis

The 1,023-bp *LPAAT* cDNA sequence of *N. oleoabundans* encoding 340 amino acids (GenBank: MF706164; protein id: AUS8446) has been cloned previously as plasmid pYES2-FLPAT34 [21]. We designated this protein as NeoLPAAT1. To further analyze the NeoLPAAT1 sequence, various methods were performed as follows. The NeoLPAAT1 was aligned with other LPAAT1 using ClustalW multiple alignment program [22]. The membrane-spanning domains were predicted by TMHMM v2.0 [24]. The chloroplast transit peptide of the NeoLPAAT1 was predicted using PredAlgo [25]. The phylogenetic tree of NeoLPAAT1 was constructed using the neighbor-joining method in MEGA 7 [26].

### Construction of transformation vectors

To construct *NeoLPAAT1* cDNA under the control of promoters *HSP70-RBCS2* (*AR*) [44] and *β2-tubulin* (*β2-Tub*) [45] of *C. reinhardtii*, plasmids pAR-LPAAT and pB2-LPAAT harboring the gene cassettes *AR-NeoLPAAT1-3′rbcS2* and (*β2-Tub)-NeoLPAAT1-3′rbcS2*, respectively (Fig. 2a), were constructed by replacing the *AR-ChGfp-3′rbcS2* fragment of pChGFP-Hyg3 [20] with the PCR fragments containing: (i) *AR* promoter from pCB740 [44] or *β2-Tub* promoter from pHyg3 [46] (ii) *NeoLPAAT1* cDNA (GenBank: MF706164) from pYES2-FLPAT34 [21], and (iii) 3′UTR of *3′rbcS2* from pCrGFP [47]. Both pAR-LPAAT and pB2-LPAAT contained hygromycin B resistance gene *Hyg3* [20, 46] used as a selectable marker.

### Transformation of *N. oleoabundans*

To generate transformants overexpressing *NeoLPAAT1*, plasmids pAR-LPAAT and pB2-LPAAT were transformed into the *N. oleoabundans* nuclear genome using electroporation as described previously [20]. *N. oleoabundans* cells were electroporated using a Gene Pulser (Bio-Rad Labs) set electric field strength at 1000 V/cm, capacitance at 25 μF and resistance at 200 Ω. The electroporated cells were spread on a BBM agar plate containing 5 μg/mL hygromycin B. After incubation for 2 weeks, the resulting transformants AR-LPAAT and B2-LPAAT appeared.

### Genomic PCR analysis

The integration of *NeoLPAAT1*-expression cassettes in the nuclear genome of *N. oleoabundans* were verified using genomic PCR. The genomic DNA of *N. oleoabundans* was isolated as described [48]. PCR was performed with primers specific to *NeoLPAAT1* coding sequence: LPAAT-F1 (CGGGATCCTAGCTAGCATGCTGTGCCC CACTGTCG) and LPAAT-R10 (GATGGCAGGGTG

AATCACGAGTTTAACTTTGCCTGG). The touchdown PCR was carried out 11 cycles for the first phase (denature at 98 °C for 10 s, annealing at 80 °C for 30 s with a 1 °C decrease in every successive cycle, extension at 72 °C for 30 s) and 25 cycles for second phase (denature at 98 °C for 10 s, annealing at 70 °C for 30 s, extension at 72 °C for 30 s, including initial denaturation at 98 °C for 3 min and final extension at 72 °C for 7 min). The PCR product was investigated in a 1.5% agarose gel and further verified by DNA sequencing analysis. The amplicon of the *NeoLPAAT1* coding sequence was 943 bp.

#### Nile red fluorescence assay

To analyze the level of neutral lipids, cells grown under +N condition ($\sim 1.5 \times 10^7$ cells/mL) was stained with fluorescent dye Nile red dissolved in acetone to final concentration of 1 µg/mL and incubated in the dark for 10 min. The fluorescence intensity of the stained cells in a 96-well plate was determined using a spectrofluorometer (Beckman Coulter DTX-880, USA) with excitation at 535 nm and emission at 574 nm. Specific fluorescence intensities were obtained by subtracting the autofluorescence of the unstained cells and normalized by cell numbers.

#### Quantitative real time PCR analysis

Relative *NeoLPAAT1* transcript abundance was quantified using quantitative real-time PCR (qPCR). Total RNA was extracted from cells cultured under −N condition for 1 day using TRI Solution (GeneMark, Taiwan). The cDNA was prepared from the total RNA with oligo (dT)18 primer using RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Scientific, Canada). The cDNA was amplified using KAPA SYBR FAST qPCR Kit (Kababiosystems, USA) with *NeoLPAAT1*-gene specific primers: LPAAT-RT-F1 (GAGCTTCCTGGACATTTA CACGC) and LPAAT-RT-R1 (CAGCTCGCTGCATTG TTTTAGG); and endogenous *Actin* (*NeoActin*)-gene specific primers: NeoActin-F1 (ACACTGTGCCCATCT ATGAGGG) and NeoActin-R1 (CTTGATGTCACGCAC GATTTCG). Quantitative RT-PCR analysis was performed using Mastercycler realplex4 and realplex software (Eppendorf, Germany). The *NeoLPAAT1* transcript level was normalized to a reference, *NeoActin* transcript.

#### Lipid extraction and quantification

Total lipids of *N. oleoabundans* grown under +N and −N condition were extracted based on modified Bligh and Dyer method [49]. The cell pellet of approximately 80 mg (50 $OD_{750}$) was suspended in chloroform:methanol (1:2, v/v). The cells were lysed using vortexing at 2700 rpm (Vortex Genie2 G560E, Scientific Industries, USA) including 0.5 mm glass beads, then chloroform:water (1:1, v/v) was added to the mixture. Chloroform phase was collected and evaporated using nitrogen gas. Total lipids were determined gravimetrically. The TAG was subsequently separated from total lipids using thin-layer chromatography (TLC) with solvent hexane:diethyl ether:acetic acid (70:30:1, v/v/v) and a reference substance, glyceryl trioleate (92860 Sigma-Aldrich, USA). TAG was quantified using iodine staining and Quantity One 1-D analysis software (Bio-Rad Labs., USA). Total lipid and TAG content was calculated as percentage of dry cell weight (% DCW). DCW of a sample was determined gravimetrically after drying the cells. The lipid productivity was calculated using the formula $P_{\text{Lipid}}$ (mg/L/day) = [$C_{\text{Lipid}}$ (mg/mg) × DCW (mg/L)]/ Time (day), where $C_{\text{Lipid}}$ is lipid content of cells, DCW is dry cell weight, and Time is the cultivation period, as described [27, 38].

#### Fatty acid composition analysis

To generate fatty acid methyl esters (FAME) by transesterification, TAG extracted from TLC was incubated with 5% (v/v) sulfuric acid in methanol at 70 °C for 3 h in the presence of an internal standard, glyceryl trinonadecanoate (91988 Sigma-Aldrich). FAME analysis was carried out using GC–MS (Agilent 7890A GC system and Agilent 5975C inert XL MSD with Triple-Axis Detector) equipped with Agilent DB-WAX column (30 m length, 0.25 mm i.d., 0.25 µm film thickness). The oven temperature was increased from 50 to 250 °C at a rate of 3 °C/min, the injector and detector temperature were 240 °C and 250 °C, respectively. Helium was used as the carrier gas at flow rate of 1 mL/min. The mass spectra were analyzed using MSD ChemStation software (version E.02.00.493, Agilent) and compared with the NIST08.L database. Fatty acid composition was calculated as percentage of the total fatty acids present in the sample.

#### Statistical analysis

The statistical differences between wild type (used as control) and transformant samples were analyzed using two-tailed student's *t* test of SPSS Base 16.0 software (SPSS, USA).

#### Abbreviations

LPAAT: lysophosphatidic acid acyltransferase; AGPAT: 1-acyl-*sn*-glycero-3-phosphate acyltransferase; DGAT: diacylglycerol acyltransferase; DCW: dry cell weight; GC–MS: gas chromatography–mass spectrometry; N: nitrogen; qPCR: quantitative real-time PCR; FA: fatty acids; FAME: fatty acid methyl ester; MUFA: monounsaturated fatty acid; PUFA: polyunsaturated fatty acid; SFA: saturated fatty acid; TAG: triacylglycerol; TLC: thin-layer chromatography.

**Authors' contributions**
WC conceived of the study, designed the experiments, analyzed the data, performed the in silico analysis and wrote the manuscript, KA and SF performed the experiments. All authors read and approved the final manuscript.

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**Competing interests**
The authors declare that they have no competing interests.

**Availability of data and supporting materials**
The dataset supporting the conclusions of this article is included within the article.

**Consent for publication**
Not applicable.

**Ethics approval and consent to participate**
Not applicable.

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