รายงานวิจัยฉบับสมบูรณ์

โครงการ

การผลิตกรดไขมันอิ่มตัวสูงชนิด DHA ในมอส *Physcomitrella patens* โดยยีนที่ควบคุมเอนไซม์ Δ^4-desaturase จากสาหร่ายทะเล *Isochrysis galbana* CCMP1312

Production docosahexaeoic acid (DHA) in the moss *Physcomitrella patens* by the marine algae *Isochrysis galbana* CCMP1312 Δ^4-desaturase

โดย

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พฤษภาคม 2556

สัญญาเลขที่ MRG5380182

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และมหาวิทยาลัยสงขลานครินทร์

(ความเห็นในรายงายวิจัยนี้เป็นของผู้วิจัย สกอ. และ สกว.ไม่จำเป็นต้องเห็นด้วยเสมอไป)

บทคัดย่อ

รหัสโครงการ : MRG5380182

ชื่อโครงการ : การผลิตกรดไขมันอิ่มตัวสูงชนิด DHA ในมอส *Physcomitrella patens* โดยยีนที่ควบคุมเอนไซม์ Δ^4-desaturase จากสาหร่ายทะเล *Isochrysis galbana* CCMP1312

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บทคัดย่อ :

กรดไขมันไม่อิ่มตัวสูงสายยาวเป็นกรดไขมันที่มีความสำคัญและพบทั่วไปในอาหาร ปัจจุบันแหล่งของกรดไขมันไม่อิ่มตัวสูงเหล่านี้ได้จากปลาและสาหร่ายทะเล แต่เนื่องจากปัญหาต้นสูงในการผลิต รวมทั้งปริมาณของวัตถุดิบที่มีอยู่อย่างจำกัด ทำให้เกิดปัญหาไม่เพียงพอต่อความต้องการและการบริโภคในปัจจุบัน ดังนั้นการค้นคว้าวิจัยเพื่อพัฒนาแหล่งผลิตอย่างต่อเนื่องของกรดไขมันไม่อิ่มสูงนี้จึงมีความสำคัญเพื่อตอบสนองความต้องการบริโภคดังกล่าว สำหรับงานวิจัยนี้ได้ประยุกต์ใช้เทคนิคทางพันธุวิศวกรรมเพื่อกระตุ้นให้มอส *Physcomitrella patens* สามารถสร้างกรดไขมันไม่อิ่มตัวสูงที่มีความยาว 22 คาร์บอน ได้แก่ กรดโดโคซะเตตระอีโนอิก (กรดอะดรีนิก) และกรดโอเมก้า-3 โดโคซะเพนตะอีโนอิก โดยอาศัยการทำงานของยีน Δ^5-elongase จากสาหร่ายทะเล *Pavlova* sp. ร่วมกับการเติมน้ำมันพืชในอาหารเพาะเลี้ยง พบว่ามอสมีการสะสมของกรดโดโคซะเตตระอีโนอิก (กรดอะดรีนิก) และกรดโอเมก้า-3 โดโคซะเพนตะอีโนอิก ได้เท่ากับ 24.3 และ 11.7 มิลลิกรัมต่อลิตรอาหาร คิดเป็น 2.3 และ 1.1 เปอร์เซ็นต์ของกรดไขมันรวม ตามลำดับ งานวิจัยนี้จึงเป็นผลงานชิ้นแรกที่สามารถกระตุ้นให้มอสสร้างกรดโอเมก้า-3 โดโคซะเพนตะอีโนอิกซึ่งเป็นสารต้นต้นในการสร้างกรดโดโคซะเฮกซะอีโนอิกต่อไป นอกจากนี้ยังพบว่าการทำงานของเอนไซม์ดังกล่าวจะสูงขึ้นเมื่อยีนเชื่อมต่ออยู่กับ green fluorescence protein (GFP) การศึกษานี้จึงยืนยันได้ว่าการประยุกต์กระบวนการตัดต่อยีนหรือเทคนิคทางพันธุวิศวกรรมร่วมกับการเพิ่มแหล่งอาหาร สามารถเปลี่ยนแปลงวิถีชีวสังเคราะห์ของกรดไขมันในพืชชั้นต่ำที่ไม่มีเมล็ด (เช่น มอส) โดยผลิตกรดไขมันไม่อิ่มตัวสูงตามต้องการได้

คำหลัก : มอสฟิสโคมิทรีลา พาเทนส์ • สาหร่ายไอโสไครซิส กาลบานา • สาหร่ายแพบโลวา •เดลต้าโฟร์-ดีแซททูเรส • เดลต้าไฟว์-อีลองเกส • กรดไขมันไม่อิ่มตัวสูงสายยาว • การเติมน้ำมันพืช

Abstract

Project Code : MRG5380182

Project Title : Production docosahexaeoic acid (DHA) in the moss *Physcomitrella patens* by the marine algae *Isochrysis galbana* CCMP1312 Δ^4-desaturase

Investigator :

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Project Period : 3 years

Abstract:

Long chain (C≥20) polyunsaturated fatty acids (LC-PUFAs) represent important components of the human diet. Currently, the predominant sources of these fatty acids are marine fish and algal oils, but high production costs and diminishing feedstock, limit their supply and usage. A more regular sustainable source of these compounds is urgently required and therefore research is being conducted to develop a sustainable, land-based production system. This work describes the metabolic engineering of an artificial pathway that activates the production of C_{22}-PUFAs, adrenic acid (ADA) and ω-3 docosapentaenoic acid (DPA) in *Physcomitrella patens* using a gene from a marine algae *Pavlova* sp. encoding Δ^5-elongase and vegetable oil supplementation. The accumulation of ADA and ω-3 DPA were dramatically increased to 24.3 and 11.7 mg L^{-1} and accounted for 2.3 and 1.1% of total fatty acids, respectively. This is the first report on producing ω-3 DPA, DHA precursor, in *P. patens*. The obtained results prove that this enzyme appears to be more active when fused to a green fluorescence protein (GFP) reporter gene. These finding reveal that the modification of the fatty acid biosynthetic pathway by genetic manipulation and nutritional supplementation, to produce specific PUFAs in a non-seed lower plant, is a promising technique.

Keywords : *Physcomitrella patens* · *Isochrysis galbana* · *Pavlova* sp. · Δ^4-desaturase · Δ^5-elongase · C_{22} polyunsaturated fatty acid · oil supplementation

CONTENTS

LIST OF FIGURES

Figure **Page**

LIST OF FIGURES (CONTINUED)

Figure **Page**

Part II : Metabolic engineering and oil supplementation of *Physcomitrella patens* for activation of C_{22} polyunsaturated fatty acid (adrenic and ω-3 DPA) production

LIST OF TABLES

Table **Page**

Executive summary

Long-chain (C≥20) polyunsaturated fatty acids (LC-PUFAs) including the omega-3 (ω-3) eicosapentaenoic acid (EPA, 20:5 $\Delta^{5,8,11,14,17}$) and docosahexaenoic acid (DHA, 22:6 $\Delta^{4,7,10,13,16,19}$) are essential nutrients for a healthy diet and adequate human development. Several studies have indicated that deficiencies in these fatty acids increase the risk of cardiovascular disease, hypertension, inflammatory diseases, rheumatoid arthritis and neuropsychiatric disorders including dementia and depression.

DHA, an ω-3 PUFA found in fish and certain algae, makes up 60% of the fatty acid in human neuronal cell membranes, and is particularly concentrated in synaptic membranes and in myelin sheaths. DHA is essential for prenatal brain development as well as normal healthy brain functioning. Supplemental DHA has also been shown to have a protective effect against cognitive decline or Alzheimer's disease which may involve triacylglycerols (TAG) lowering. Furthermore, DHA has been recognized as a nutrient for prevention of human cancer and heart disease.

Adrenic acid (ADA, 22:4 $\Delta^{7,10,13,16}$), an ω-6 PUFA, is the third most abundant fatty acid in the brain and it is particularly enriched in myelin lipid. Rapid accumulation of ADA in brain, comparable to ARA accretion levels, occurs during the early post-natal period of brain growth spurt in human infants. Human milk contains a small amount (~3 mg/dl) of ADA. While the specific functions of ADA are not yet clear, it is suggested to play an important role in myelination in neural tissues, its abnormality being implicated in the pathogenesis of Alzheimer's disease, and evidence from *in vitro* studies indicates that it serves as a substrate for dihomoeicosanoid formation tissues. In addition, ADA is an abundant fatty acid in the vasculature that causes endothelium-dependent relaxation in bovine coronary arteries. ADA has also been found to significantly enhance tissue factor

(TF) activity of thrombin-stimulated endothelial cells, and it is therefore a potential prothrombotic agent.

Humans, particularly infants, are unable to synthesize DHA and other LC-PUFAs to any great extent and must therefore obtain them through their diet. The human consumption of LC-PUFAs is steadily increasing, but the production of fish oil, the main source of LC-PUFAs for human consumption, is declining. Generally, the cost of the commercial processing, refining, and stabilizing the fish oils is very high, and the decline in production due to over-fishing continues to drive up the cost of fish oils. The recent finding of toxic compounds in fish oil has further raised safety concerns on the consumption of fish oils.

Increasing demand has therefore raised an interest in obtaining these PUFAs from alternative sources that are more economical and sustainable. Plant oils could be a sustainable alternative source of fatty acids. However, plants do not synthesize LC-PUFAs owing to the lack of Δ^6- and Δ^5-position-specific desaturase and the absence of an elongase. Plant oils are therefore rich in C_{18}-PUFAs, linoleic acid (LA, 18:2 $\Delta^{9,12}$) and α-linolenic acid (ALA, 18:3 $\Delta^{9,12,15}$), but not in LC-PUFAs, EPA and DHA. An attractive possibility is to genetically engineer plants to produce LC-PUFAs.

In algae, DHA can be synthesized from EPA in a two-step process that involves: (a) the elongation of EPA by Δ^5-elongase to generate ω-3 docosapentaenoic acid (ω-3 DPA): and (b) the desaturation of ω-3 DPA by a Δ^4-desaturase to generate DHA (Fig. 1). A number of elongases have been identified from mammals, marine and fresh water teleost fish that can recognize and lengthen multiple chain-length PUFAs, such as C_{18}-, C_{20}- and C_{22}-PUFAs. Recently, elongases specific for C_{20}-PUFAs have been identified from algae and demonstrated to function in the production of DHA. In addition, the moss *Physcomitrella patens* produces several PUFAs, especially arachidonic acid (ARA, 20:4

$\Delta^{5,8,11,14}$) and EPA which are C_{20}-PUFAs required for the synthesis of the LC-PUFAs, docosatetraenoic acid or adrenic acid (ADA) and ω-3 DPA, respectively by a Δ^5-elongase. Although ADA is a minor fatty acid in the brain, it is suggested to be an important component for myelination of neural tissue and also serves as an eicosanoid precursor in tissue. Moreover, ω-3 DPA is a precursor of DHA which has attracted much interest due to its pharmaceutical potential.

Development of transgenic oil plants capable of producing C_{22}-PUFAs, ADA and ω-3 DPA is therefore an attractive route to improve commercial-scale DHA production. The present study demonstrates the production of C_{22}-PUFAs, ADA and ω-3 DPA, ω-6 DPA and DHA from C_{20}-PUFAs, ARA and EPA, respectively in *P. patens* by heterologous expression of marine algae *Isochrysis galbana* Δ^4-desaturase and *Pavlova* sp. Δ^5-elongase and the optimization of culture medium for high C_{22} PUFA production by vegetable oil supplementation.

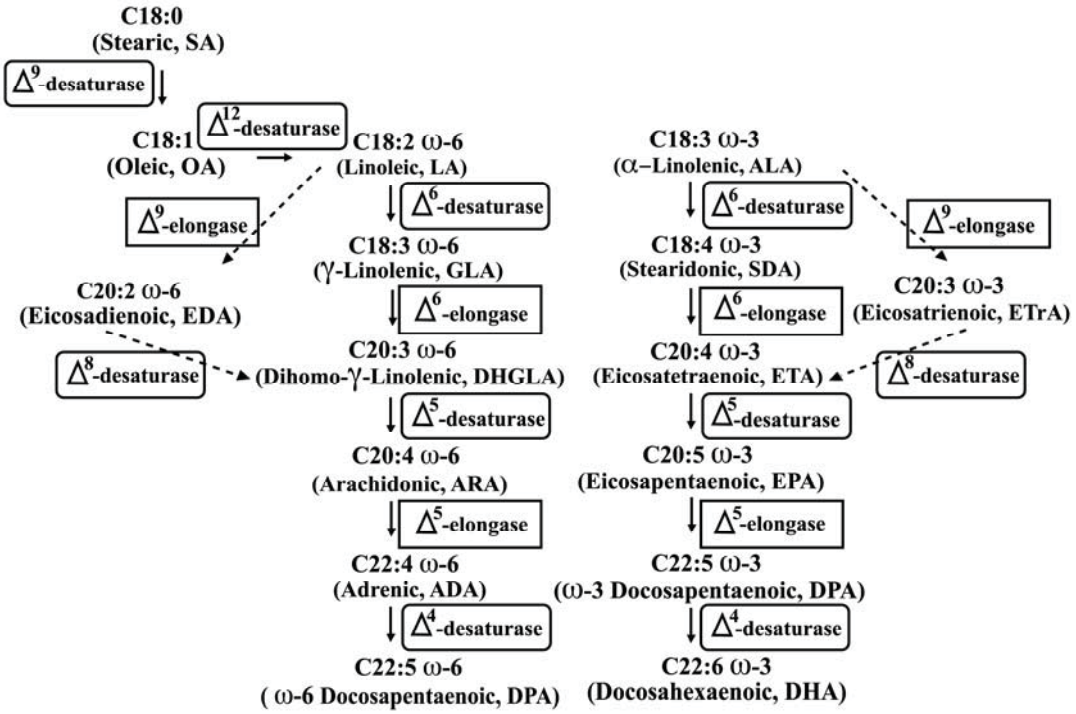

Fig. 1 A simplified scheme of the polyunsaturated fatty acid (PUFA) biosynthesis pathway in non-seed eukaryotes (Pereira et al., 2003).

Objectives

1. To clone Δ^4-desaturase from the algae *Isochrysis galbana* CCMP1312
2. To functional express of the algae *Isochrysis galbana* CCMP1312 Δ^4-desaturase gene in *Physcomitrella patens*
3. To produce novel C_{22} polyunsaturated fatty acids in *Physcomitrella patens*
4. To optimize the culture conditions for high production of novel C_{22} polyunsaturated fatty acids in *Physcomitrella patens*

Part I : Production of C_{22} polyunsaturated fatty acids (ω-6 DPA and DHA) in *Physcomitrella patens* by the marine *Isochrysis galbana* Δ^4-desaturase

Methods

1. Isolation and cloning of the marine algae *Isochrysis galbana* CCMP1312 Δ^4-desaturase gene

1.1 RNA extraction

Approximately 20 mg fresh weight of the *Isochrysis galbana* CCMP1312 was ground to a fine powder under liquid nitrogen using a pre-cooled mortar and pestle. Total RNA was extracted from *Isochrysis galbana* CCMP1312 using the RNeasy Plant Mini Kit (Qiagen) according to the manufacturer's instructions manufacturer's instructions.

1.2 cDNA synthesis

Five μl of total RNA was reversed transcribed to cDNA with the SuperScript™ III Reverse Transcriptase (Invitrogen). One μl of RACE 32 primer and 10 mM dNTPs were added to 5 μl of total RNA in a 14 μl volume reaction, incubated at 65°C for 5 min and placed on ice. The cDNA synthesis mix containing 4 μl of 5x first strand buffer, 1 μl of 0.1 M DTT and 15 units/μl SuperScript™ III Reverse Transcriptase were then added to the previous reaction on ice. After incubation at 4°C for 10 min, followed by 60 min at 50°C, the reaction was terminated by incubating at 75°C for 15 min. Finally, the cDNA diluted with TE buffer in a total volumn of 100 μl was then used as a template for PCR amplification with primers.

1.3 PCR-based cloning

Primers were synthesized based on NCBI sequence data (Pereira et al., 2004). The forward primer was IgDES4-FOR1, 5'- ATG TGC AAC GCG GCG C -3' and the reverse primer was IgDES4-REV1, 5'-TTA GTC CGC CTT GAG CGT GTC G-3'. The PCRs were carried out in a

total volume of 50 µl containing 2 µl of 10 µM each primers, 10x PCR buffer and 10 mM dNTPs, 1 µl of cDNA as a template, 1.5 µl of 50 mM $MgCl_2$, and 0.5 µl of 5 U/µl *Taq* DNA Polymerase (Invitrogen). After initial denaturation at 94°C for 4 min, amplification was performed in 35 cycles of 1 min at 94°C, 0.5 min at 49°C and 2.5 min at 72°C, followed by a final extension at 72°C for another 10 min.

Amplification products were fractionated on 1.0% agrarose gels and aliquot 1 µl was directly ligated into pCR®2.1-TOPO® vector (Invitrogen) (Fig. 1) in a total volume of 6 µl at room temperature for 60 min. Three µl of the ligation reaction was then transformed into One Shot®TOP10 Chemically Competent *E. coli* cells (Invitrogen) and cultured on solid LB medium containing 100 µg mL^{-1} ampicillin, surface spread by 50 µl of 100 mM IPTG and 50 mg/mL^{-1} X-Gal.

Plasmids DNA were purified from transformed *E. coli* culture by High-Speed Plasmid Mini Kit (Geneaid). The resulting plasmid were verified by digestion with *Eco*RI for 60 min and analyzed by 1.0% agarose gel electrophoresis and 2 µl of interested plasmid DNA was sequenced with M13 Forward and Reverse by using an automated sequenced, resulting in the plasmid IgDES4.

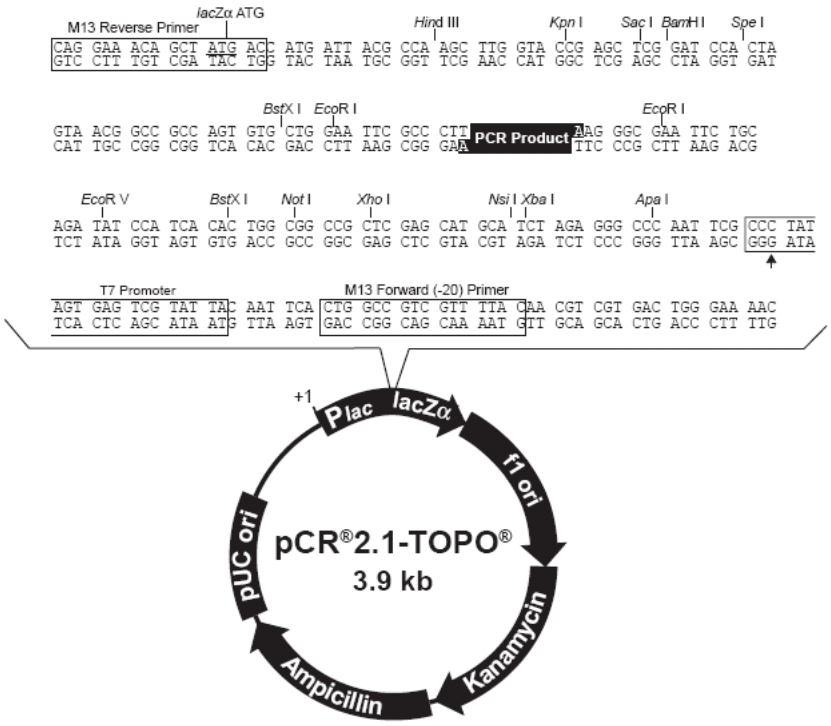

Fig. 1 Map of cloning vector pCR® 2.1-TOPO® (Invitrogen).

2. Construction of expression vector carrying *Isochrysis galbana* Δ^4-desaturase and *Pavlova* sp. Δ^5-elongase

The *Isochrysis galbana* Δ^4-desaturase and *Pavlova* sp. Δ^5-elongase (from our previous TRF research project) were cloned into a plant destination vector containing ubiquitin promoter and hygromycin resistance cassette using Multisite Gateway® Pro 2-Fragment Recombination (Invitrogen) (Fig. 2).

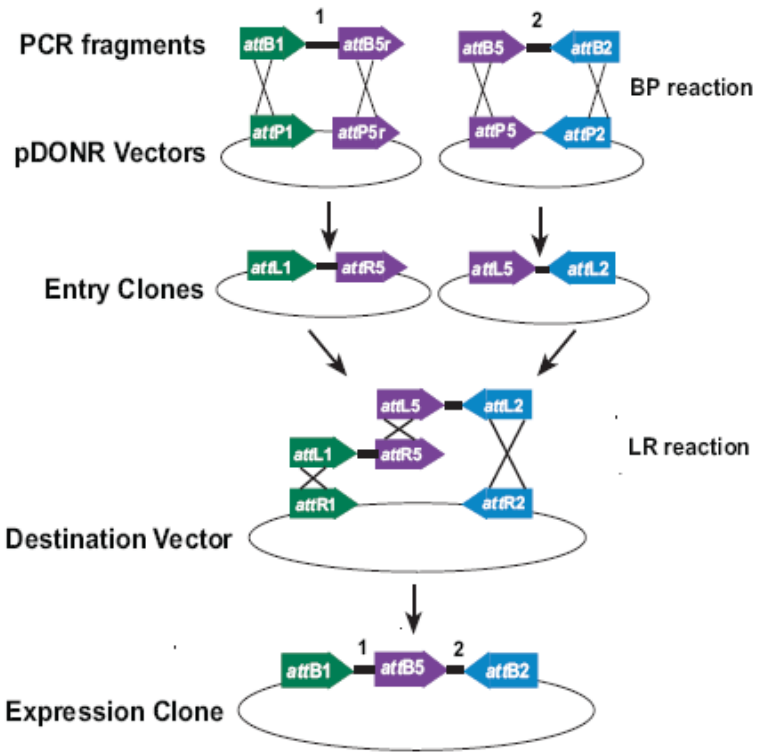

Fig. 2 Multisite Gateway® Pro 2-Fragment Recombination (Invitrogen).

2.1 Construction of entry vector

The primers of attB1-PsELO5-FOR1, 5'- GGG GAC AAG TTT GTA CAA AAA AGC AGG CTG CAT GAT GTT GGC CGC AGG CTA T -3' and attB5r-PsELO5-REV1, 5'- GGG GAC AAC TTT TGT ATA CAA AGT TGT GTT ACT CCG CCT TGA CCG CCT T -3' were used for PCR amplification of the plasmid PsELO5 (from our previous TRF research project) with *Taq* DNA polymerase (Invitrogen). Similarly, the primers of attB5-IgDES4-FOR1, 5'- GGG GAC AAC TTT GTA TAC AAA AGT TGC TAT GTG CAA CGC GGC GCA GGT C -3' and attB2-IgDES4-REV1, 5'- GGG GAC CAC TTT GTA CAA GAA AGC TGG GTA CTT AGT CCG CCT TGA GCG TGT C -3' were used for PCR amplification of the plasmid IgDES4.

PCR was carried out in a total volume of 50 µl. Each reaction contains 2 µl of 10 µM of each primers and 10xPCR buffer, 2 µl of 10 mM dNTPs, 1.5 µl of 50 mM $MgCl_2$, 1 µl of 1/50 diluted plasmids as templates, and 0.5 µl of 5 U/µl *Taq* DNA Polymerase. The thermocycling conditions were as follows; initial denaturation for 4 min at 94°C, followed

by 28 cycles of 1 min at 94°C, 0.5 min at 49°C, 2.5 min at 72°C, and terminated by 10 min final extension at 72°C.

Each 1 μl of corresponding amplification products, *att*B1-PsELO5-*att*B5r and *att*B5-IgDES4-*att*B2, were cloned into pDONR™ 221 P1-P5r and pDONR™ 221 P5-P2 vectors (Invitrogen) (Fig. 3), respectively using BP Clonase™ II enzyme mix (Invitrogen) and transformed into One Shot® Mach1™-T1R Chemically Competent *E. coli* cells (Invitrogen). The transformants were cultured on LB medium containing 50 μg mL^{-1} kanamycin. Plasmids DNA were purified from transformed *E. coli* culture by High-Speed Plasmid Mini Kit (Geneaid) and sequenced with M13 Forward (-20) and M13 Reverse primers, yielding the plasmids *att*L1-PsELO5-*att*R5 and *att*L5-IgDES4-*att*L2, respectively.

Fig. 3 pDONR™ 221 P1-P5r (A) and pDONR™ 221 P5-P2 (B) vectors (Invitrogen).

2.2 Construction of expression vector

This reaction recombined PsELO5 and IgDES4 ORFs from the plasmids *att*L1-PsELO5-*att*R5 and *att*L5-IgDES4-*att*L2, respectively (2 μl each), into *att*R1 and *att*R2 sites of Gateway® plant destination vector, pTUbiGate (1 μl) (Fig. 4) in a total volume of 8 μl solution containing 2 μl of TE buffer (pH 8.0), and 2 ul of Gateway® LR Clonase™ II Plus enzyme mix (Invitrogen) at 25°C for 16 hr. The mixtures were further incubated with 1 μl of Proteinase K at 37°C for 10 min. An aliquot of 3 μl was introduced into One

Shot®Mach1™-T1ᴿ Chemically Competent *E. coli* cells (Invitrogen). The transformants were cultured on LB medium containing 100 μg mL^{-1} ampicillin. Plasmids DNA were further purified from transformed *E. coli* cultures by High-Speed Plasmid Mini Kid (Geneaid). The plasmids carrying *Pavlova* sp. Δ^5-elongase and *Isochrysis galbana* Δ^4-desaturase were verified by PCR amplification with two primer pairs (PsELO5-FOR1: 5'-ATG ATG TTG GCC GCA G-3' and PsELO5-REV1: 5'- TTA CTC CGC CTT GAC CG-3'; IgDES4-FOR1, 5'- ATG TGC AAC GCG GCG C -3' and IgDES4-REV1, 5'-TTA GTC CGC CTT GAG CGT GTC G-3', respectively) and digestion with *Swa*I for 60 min. Then DNA fragments were analyzed by 1.0% agarose gel electrophoresis, resulting in the over-expression vector named pTUbiGate-PsELO5-IgDES4.

The selected clone was then cultivated in 300 ml of selective liquid media before plasmid isolation by Geneaid Plasmid Maxi Kit (Geneaid).

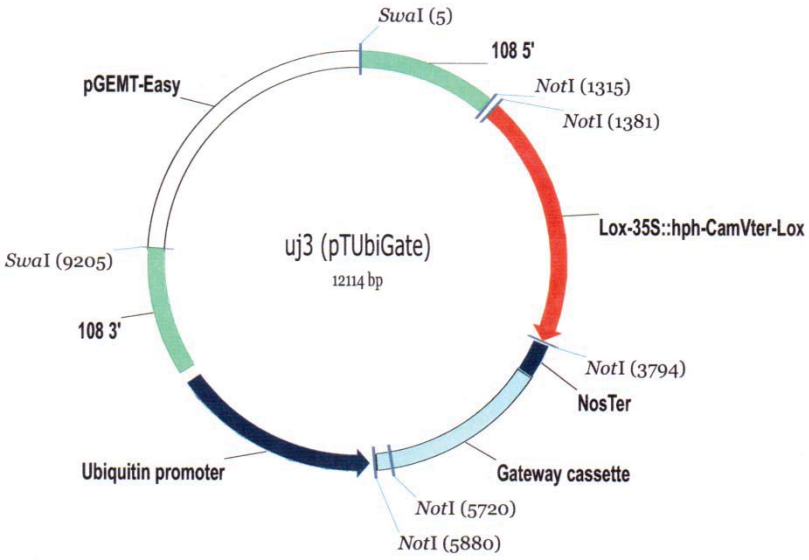

Fig. 4 pTUbiGate destination vector.

3. Protoplast transformation and regeneration

3.1 Protoplast isolation

Protoplasts were isolated from 2 plates of 14-day-old protonemal wild-type cultures by digestion with 20 ml of 0.5% Driselase enzyme suspension dissolved in 8% mannitol for 60 min. The digested moss material was successively passed through sieve with a mesh and carefully washed in 20 ml of 8% mannitol twice. Subsequently, the protoplast pellet was resuspended in 10 ml of CaPW and estimated protoplast density using a haemocytometer.

3.2 Polyethylene-glycol (PEG)-mediated transformation of protoplast and *P. patens* regeneration for transgenic plant expressions

The over-expression vector (pTUbiGate-PsELO5-IgDES4) driven by ubiquitin promoter and nos terminator with hygromycin resistance (Hyg^r) selection cassette was digested with *Swa*I at 37°C for 5 h. The linear DNA fragments were precipitated before transformation.

Fifteen micrograms of each linearized DNA were transformed into $5x10^5$ protoplasts of *P. patens* by PEG-method (Schaefer *et al*., 1991). After protoplast transformation, the regenerating protoplasts were grown on protoplast regeneration medium for 3 weeks and then they were transferred to the solid BCD medium containing hygromycin (25 $\mu g\ mL^{-1}$) and further cultivated for 3 weeks, followed by 3 weeks release period on medium without antibiotic and a second selective medium for a further 3 weeks. The growing plants that still survived on this selection were defined as stable transformants.

4. Phenotype analysis of *P. patens* by GC

To obtain enough *P. patens* tissues for fatty acid analysis by GC, all 22 transgenic lines were expanded in the liquid BCD medium containing 5 mM ammonium-tartrate for

2 months. Total fatty acids extracted from dried cell samples (50 mg) were then transmethylated with methanol containing 2.5% H_2SO_4 in methanol at 85°C for 30 min. Fatty acid methyl esters (FAMEs) were then extracted in heptane, the organic layer was evaporated to dryness by nitrogen gas, and the residue was dissolved with heptane before gas chromatography (GC) (Kaewsuwan *et al*., 2006). GC analysis of FAMEs was conducted using an HP 6890N Series gas chromatograph equipped with 0.25 mm x 30 m x 0.25 μm HP-INNOWax capillary columm and a flame ionization detector with helium as carrier gas. The initial column temperature was 185°C (0.5 min) and was ramped at a rate of 3.5°C min^{-1} to 235°C (14.3 min), followed by maintaining at 235°C for 5.2 min. FAMEs were identified by comparison with retention times of authentic standards (Nu-Chek Prep, Elysian, MN, USA). The amounts of fatty acids were estimated from peak areas compared with calibration standards. The corresponding fatty acids were further characterized by gas chromatography-mass chromatography (GC-MS) using the HP 6890N Series operating at an ionization voltage of 70 eV with a scan range of 50-500 Da.

Results and Discussion

1. Isolation and cloning of the marine algae *Isochrysis galbana* CCMP1312 Δ^4-desaturase gene

The *I. galbana* has received attention because of its ability to produce several very-long chain PUFAs, including ARA (20:4 $\Delta^{5,8,11,14}$), EPA (20:5 $\Delta^{5,8,11,14,17}$) and DHA (22:6 $\Delta^{4,7,10,13,16,19}$) (Pereira et al., 2004). We were therefore interested in the production of C_{22}-PUFAs substrate for further ω-6 DPA and DHA production. To identify a gene coding for Δ^4-desaturase involved C_{22}-PUFA biosynthesis, cDNA from reverse-transcribed mRNA from tissue of *I. galbana* was amplified by PCR method. An amplification product containing the expected length (approximately 1302 bp indicated by arrow) (Fig. 5) was

cloned into pCR®2.1-TOPO® vector (Invitrogen) (Fig. 1) and verified by digested with *EcoR*I. After screening the three plasmids (P1-3), only P2 released the approximately 1300 bp sized gene of interest (I) from the approximately 4,000 bp sized of vector (V). Clone No. P2 was then selected for sequencing with both directions with M13 Forward and Reverse primers, yielding the plasmid IgDES4.

The open reading frame of the IgDES4 cDNA contains 1,302 bp and encodes a protein of 433 amino acids with a calculated molecular mass of 48.2 kDa (Fig. 6). Comparative sequence analysis with other desaturases revealed that IgDES4 (NCBI accession No. AAV33631.1) shared 31% identity with $\Delta^{5/6}$-desaturase of *Perkinsus marinus* (NCBI accession No. XP 002765314.1), Δ^{5}-desaturase of *Perkinsus marinus* (NCBI accession No. ABF58685.1) and Δ^{6}-desaturase of *Ostreocuccus lucimarinus* (NCBI accession No. DAA34893.1). Similar to other PUFA desaturases, this protein contained three conserved histidine motifs (boxed in Fig. 6) that are known to be essential for the enzymatic activity of membrane-bound desaturases (Shanklin et al., 1994) and presented of a heme binding region characterized by the HPGG motif toward the N terminus (Lederer, 1994).

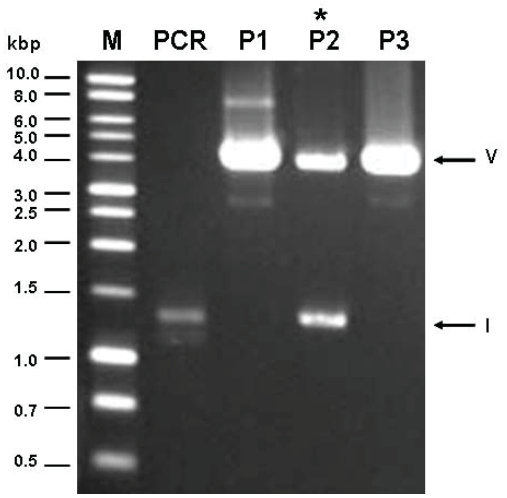

Fig. 5 PCR amplification product from cDNA of *I. galbana* with IgDES4-FOR1 and IgDES4-REV1 primers analyzed on 1% agarose gel electrophoresis. The DNA sizes in kbp are indicated on the left. Arrows indicated the expected size.

M : 1 kbp DNA Ladder (Promega)

PCR : PCR amplification product

P1-3 : Plasmid DNA from clone No. 1-3

V : Approximately 4,000 bp sized pCR® 2.1-TOPO vector (Invitrogen)

I : Approximately 1300 bp sized gene of interested

* : Sequenced clone

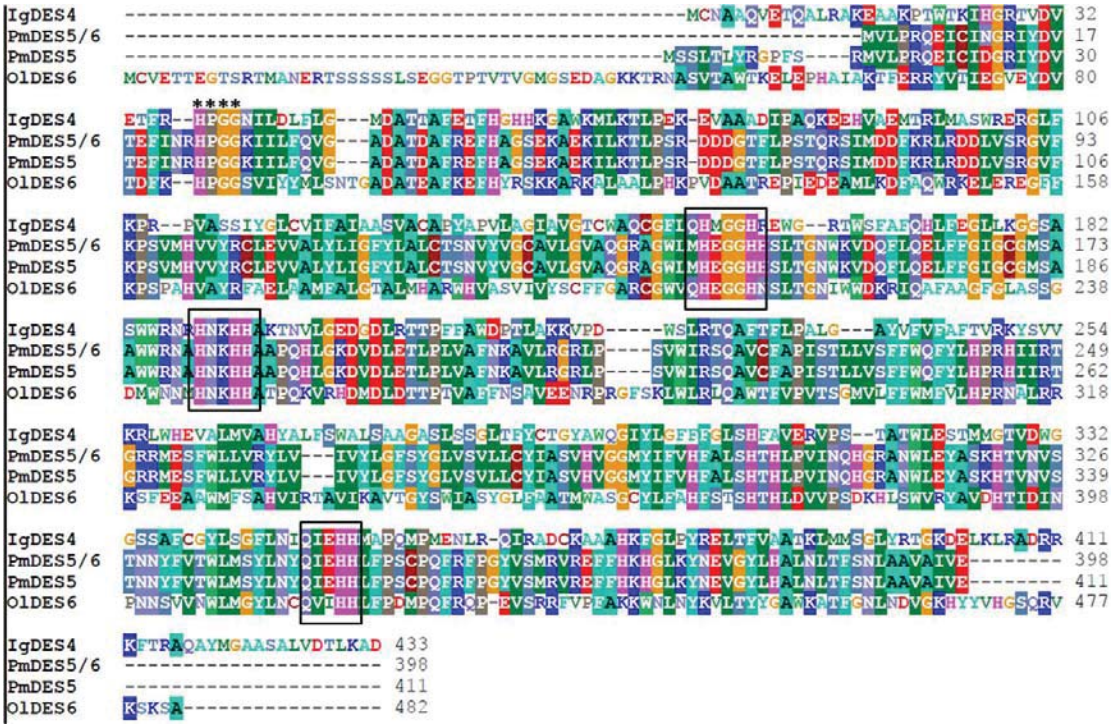

Fig. 6 Comparison of the amino acid sequence of IgDES4 with other Δ^4-, Δ^5-, Δ^6- PUFA desaturases. Identical or conserved residues are shaded. The conserved histidine boxes are boxed. GenBank Accession Numbers of the sequences are AAV33631.1 (IgDES4), XP_002765314 (PmDES5/6), ABF58685.1 (PmDES5) and DAA34893.1 (OlDES6).

2. Construction of expression vector carrying *Isochrysis galbana* Δ^4-desaturase and *Pavlova* sp. Δ^5-elongase

In this approach, PCR was used to amplify the plasmids PsELO5 and IgDES4 with specific primers and the corresponding amplification products, *att*B1-PsELO5-*att*B5r and *att*B5-IgDES4-*att*B2, were facilitated directional incorporation into pDONR^{TM} 221 P1-P5r and pDONR^{TM} 221 P5-P2 vectors (Invitrogen) (Fig. 3), respectively. After sequencing, we found the two resulting recombination plasmids contained PsELO5 and IgDES4 flanked by *att*L1/*att*R5 and *att*L5/*att*L2 sites recombination sequences. Gateway-compatible plant destination vector was used for protein over-expression of PsELO5 and IgDES4 in *P. patens*. The two entry vectors were then coordinately recombined with *att*R sites of desired destination vector, pTUbiGate (Fig. 4) and verified by PCR amplification with three primer pairs (Fig. 7). The primers PsELO5-FOR1/PsELO5-REV1 and IgDES4-FOR1/IgDES4-REV1 amplified approximately 800 and 1300 bp DNA fragments corresponded to the sizes of PvELO5 and IgDES4, respectively (Fig. 7, Lanes 1-2). Amplification with the primers PsELO5-FOR1 and IgDES4-REV1 demonstrated approximately 2,100 bp DNA fragments that were the expected sizes of PvELO5 and IgDES4 combination (Fig. 7, Lane 3). Moreover, digestion with *Swa*I released the approximately 2,914 bp sized DNA fragments from 11,336 bp sized of the vector backbone included by PvELO5 and IgDES4 (Fig. 7, Lane 4). This indicates the successful construction of the over-expression vector, pTUbiGate-PsELO5-IgDES4 carrying *Isochrysis galbana* Δ^4-desaturase (IgDES4) and *Pavlova* sp. Δ^5-elongase (PvELO5) driven by ubiquitin promoter and nos terminator with hygromycin resistance selection cassette, for further transformation into moss protoplasts.

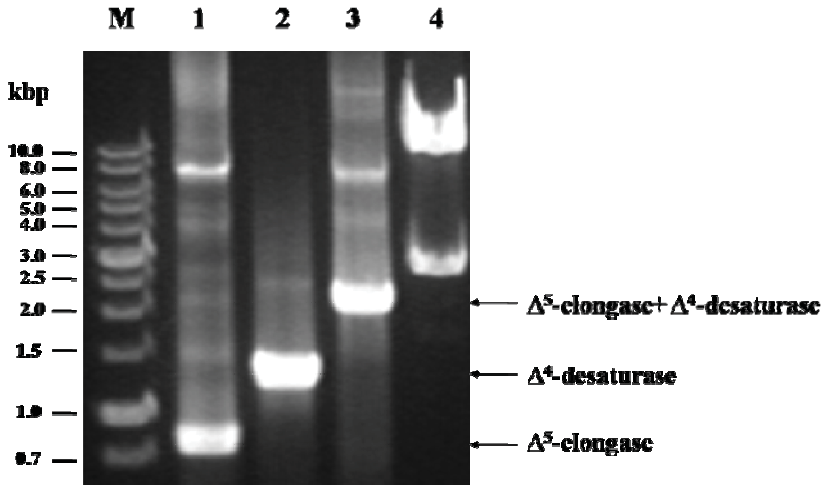

Fig. 7 PCR amplification with different primer pairs and *Swa*I digestion of the plant expression vector (pTUbiGate-PsELO5-IgDES4) analyzed on 1% agarose gel electrophoresis. The DNA sizes in kbp are indicated on the left. Arrows indicated the expected size.

M : 1 kbp DNA Ladder (Promega)

1 : PCR amplification product (PsELO5-FOR1/PsELO5-REV1)

2 : PCR amplification product (IgDES4-FOR1/IgDES4-REV1)

3 : PCR amplification product (PsELO5-FOR1/IgDES4-REV1)

4 : *Swa*I digestion

3. Protoplast transformation and regeneration

After protoplasts transformation, strongly growing plants that still survived on the selection medium were defined as stable transformants. Wild type cannot grow on a selection medium whereas only stable transformants can survive on solid BCD medium containing hygromycin. In the current study, PEG-mediated direct gene transfer of protoplasts with linear DNA fragment was utilized. After protoplasts transformation the linear plasmid (15 μg) into *P. patens* protoplasts (5×10^5), they subsequently grew up on

the regeneration medium and partly died after transfer to the selective BCD medium (Fig. 8). However, strongly growing plants that still survived on the second round of selective medium were defined as stable transformants. At this stage, wild type cannot grow on a selection medium, whereas 22 stable transformants can survive on solid BCD medium containing hygromycin.

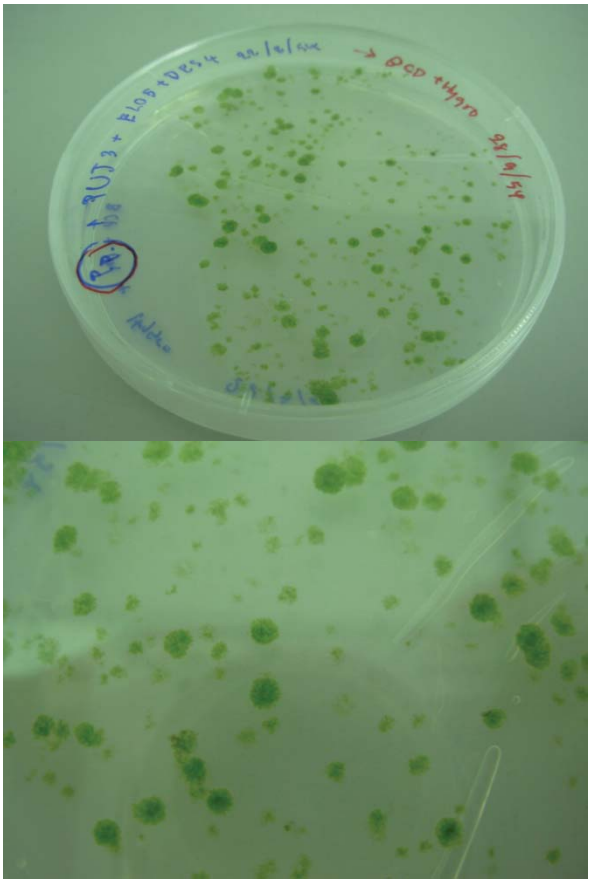

Fig. 8 Transgenic *P. patens* protoplasts (6 weeks) on solid BCD medium containing 25 μg mL^{-1} hygromycin.

4. Phenotype analysis of *P. patens* by GC

To produce ω-6 DPA and DHA, C_{22} PUFAs, in *P. patens*, it is necessary to express the Δ^5-elongase and Δ^4-desaturase genes. Generally, various promoters are used to facilitate gene expression in plants. The CaMV 35S promoter is highly active in most of the transgenic plants. However, a tandemly duplicated CaMV 35S promoter showed a 6-fold higher expression level of activity in *P. patens* than the normal CaMV 35S promoter (Horstmann et al., 2004), whereas other stronger promoters such as complete rice actin (*Act*1) (Horstmann et al., 2004), wheat early-methionine labled (EM) (Knight et al., 1995), and maize ubiquitin promoters (Bezanilla et al., 2003) have been suggested for higher level gene expression in *P. patens*. In the present study, *P. patens* was transformed with the construct that contains PsELO5 and IgDES4 cDNAs (Pereira et al., 2004) under the control of ubiquitin promoter. After cultivation all 22 transgenic lines in the liquid BCD medium for 2 months and analyzed the fatty acid production by GC, only 2 lines (P1 and P2) produced the peaks at 14.9 and 17.7 min identical to the retention times of standard ω-6 DPA and DHA, respectively (Fig. 9), whereas the control wild type gave negative results. This may indicate that our two transgenic *P. patens* produced ω-6 DPA and DHA, the expected fatty acid products of PsELO5 and IgDES4. Unfortunately, after several rounds cultivation on the free and selective BCD medium, those two transgenic lines subsequently died on the selective medium, whereas still grew well on the normal medium (Fig. 10). This suggests that our two proposed stable transgenic lines are now changed to unstable and therefore not suitable for further optimization the culture conditions for high production of novel C_{22} polyunsaturated fatty acids in *P. patens*.

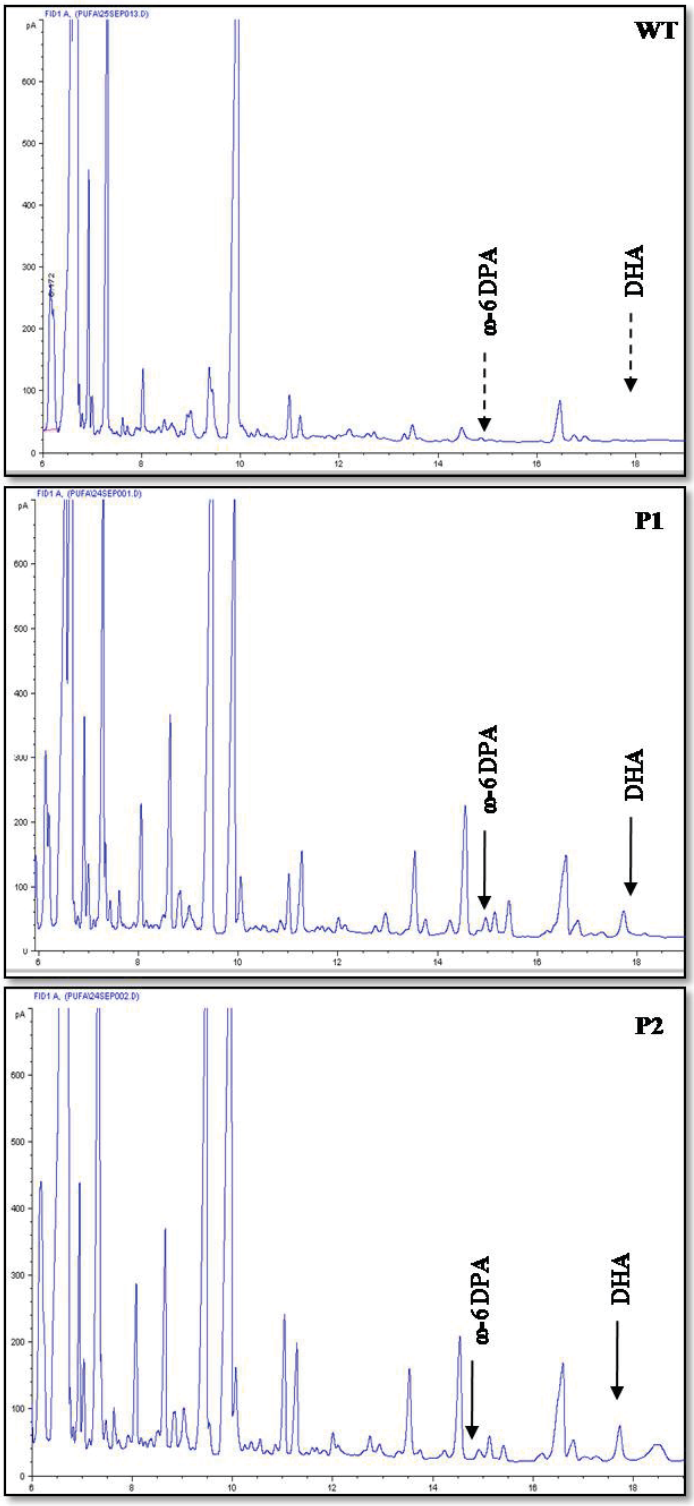

Fig. 9. Fatty acid profiles of *P. patens* wild type (WT) and the transgenic lines (P1 and P2). The FAMEs of the total lipids were analyzed by GC. The chromatograms WT, P1, and P2 show the FAMEs of the gametophytes grown in liquid BCD medium for 2 months. ω-6

DPA, ω-6 docosapentaenonic acid; DHA, docosahexaenoic acid. The additional peaks which correspond to the retention time of ω-6 DPA and DHA are indicated by arrows.

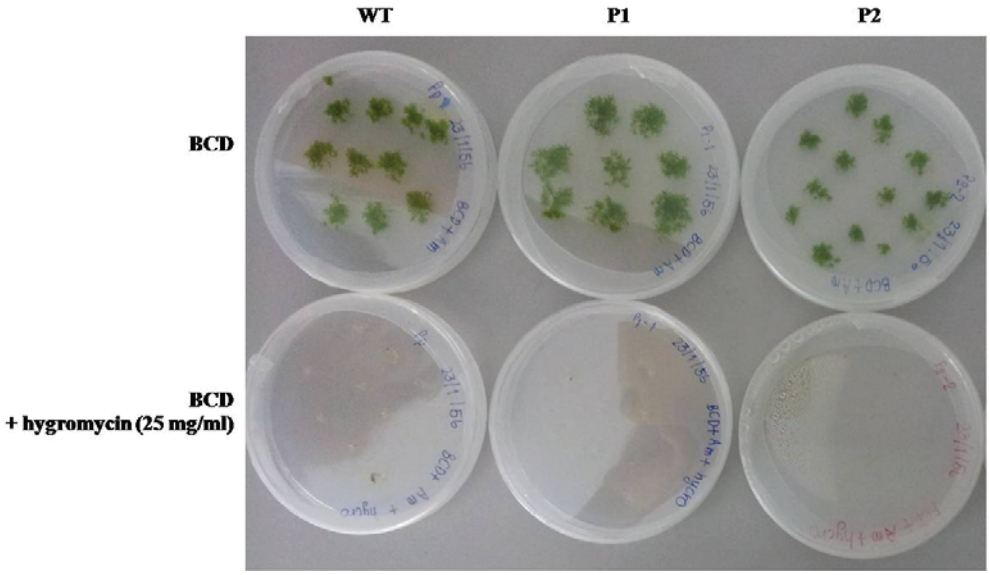

Fig. 10 Transgenic (P1 and P2) and wide-type (WT) *P. patens* grew on solid BCD free and containing 25 μg mL^{-1} hygromycin after several round cultivation.

Conclusions

The experiments described here demonstrate successful isolation of *Isochrysis galbana* Δ^4-desaturase and construction of the expression vector carrying both *Isochrysis galbana* Δ^4-desaturase and *Pavlova* sp. Δ^5-elongase under the control of strong ubiquitin promoter. Even C_{22} PUFAs, ω-6 DPA and DHA were produced in transgenic *P. patens*, whereas long time cultivation of the two transgenic lines for several rounds on normal and selective BCD media resulted in changing them into unstable transformants. Therefore our obtained two transgenic *P. patens* could not be considered as a potential alternative source of this important fatty acid. Further study is still required to be carried out for providing the permanently stable transgenic *P. patens* lines.

Part II : Metabolic engineering and oil supplementation of *Physcomitrella patens* for activation of C_{22} polyunsaturated fatty acid (adrenic and ω-3 DPA) production

Methods

1. Plant materials and culture conditions

Pavlova sp. CCMP459 was purchased from Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP, West Boothbay Harbor, ME, USA). The Gransden strain of *P. patens* (Ashton and Cove, 1977) was used throughout the studies. Wild type and transgenic mosses were grown in liquid BCD basal medium supplemented with 5 mM ammonium tartrate and cultured at 25°C under continuous light provided by fluorescent tubes (Knight et al., 2002; Cove et al., 2009) unless indicated otherwise.

2. Vector construction and expression in *P. patens*

Primers were designed based on the NCBI sequence data of *Pavlova* elongase accession no. AY630573 (Pereira et al., 2004). PCR was conducted using cDNA synthesized from total RNA of *Pavlova* sp. CCMP459 as a template in a master mix that included TaKaRa Ex Taq (TaKaRa Bio, Shiga, Japan). For the generation of *P. patens* over-expressing *PsELO5* expression cassette in which the coding region of *Pavlova* sp. Δ^5-elongase gene was firstly incorporated into the pCR®8/GW/TOPO® entry vector (Invitrogen) and further recombined with desired Gateway® plant destination vector, pMDC43 (ABRC) (Columbus, OH, USA) (Curtis and Grossniklaus, 2003). The chosen destination construct has a tandemly duplicated CaMV 35S promoter together with Hyg^r and GFP6 regions. Finally, this resulted in the generation of the recombinant plasmid pMDC43-*PsELO5* carrying a gene *PsELO5* fused to C-terminus of GFP6, driven by a tandemly duplicated CaMV 35S promoter and nos terminator, and contained the Hyg^r gene as a selection marker (Fig. 1a).

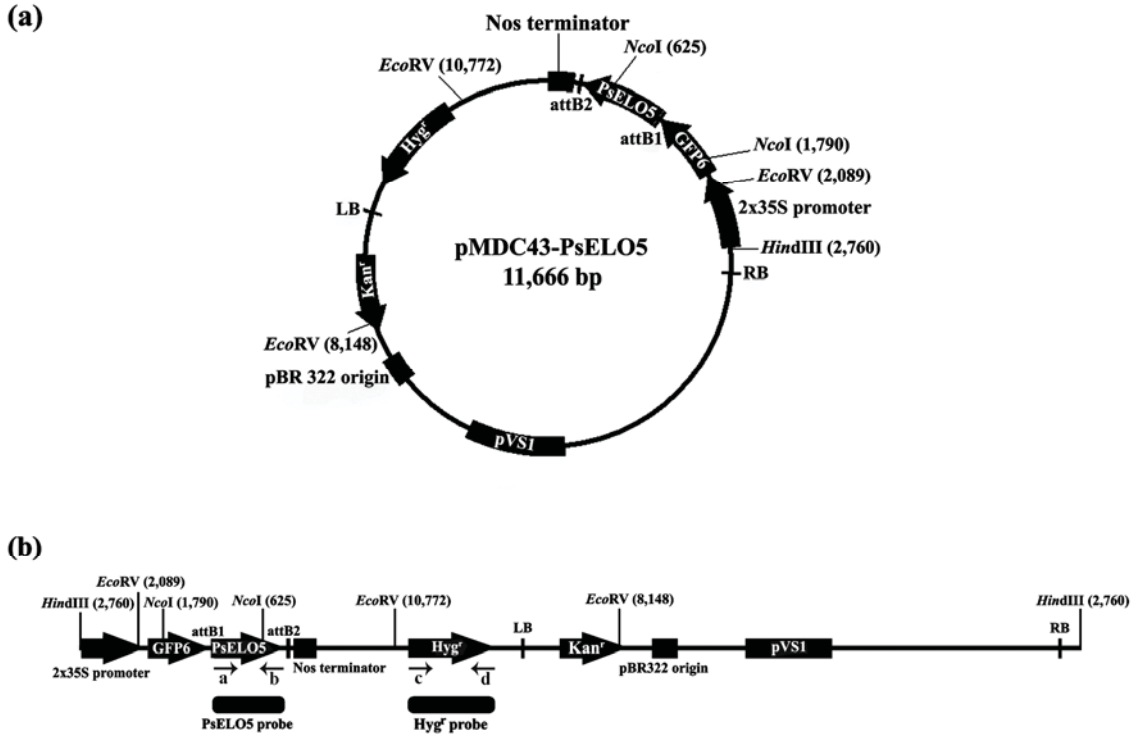

Fig. 1 Structure of the pMDC43-*PsELO5* (a). Its linear fragment used for *P. patens* transformation was digested out with *Hin*dIII (b). *PsELO5* cDNA and Hyg^r coding region are 834 and 1026 bp, respectively. The lettered arrows indicate the binding sites of primers used for PCR analysis. The localization of *PsELO5* or Hyg^r probes and the restriction enzyme sites used for Southern blotting are marked with the blocks below the sequences and indicated in the parentheses, respectively.

3. Transformation and screening of transgenic *P. patens* plants

To induce expression, the recombinant plasmid pMDC43-*PsELO5* was subsequently digested with *Hin*dIII, and the linear fragment (Fig. 1b) was precipitated and used for transformation into protoplast by PEG-mediated direct gene transfer (Cove et al., 2009). The stable transformants were screened with successive rounds of selection on

BCD media containing hygromycin (25 μg mL^{-1}), interspersed with culture on antibiotic-free medium (Knight et al., 2002).

4. Starter inoculum preparation

Protonemata tissue (14-day-old, 1 g) was aseptically blended in 250 mL sterile shake-flasks containing 100 mL of optimized liquid BCD medium (Kaewsuwan et al., 2010) with a homogenizer (OMNI TH_Q, USA) at a speed of 30,000 rpm for 1 min and cultivated in a growth room at 25 °C in an orbital shaker (NewBrunswick Scientific Innova 2100, Champaign, IL, USA) set at 125 rpm under continuous light provided by fluorescent tubes (9,000 lux). After 14 days of growth, the cultured tissues were used to inoculate the liquid medium at a 4% (w/v) level for all subsequent experiments.

5. Effects of oil supplementation

Oil supplementation to improve C_{22}-PUFA production in transgenic *P. patens* (N15, N64, N70 and N77) was carried out based on the previous reported optimized liquid BCD medium containing 22.06 g L^{-1} of sucrose, 1.00 g L^{-1} of KNO_3 and 2.35 g L^{-1} of glutamate (Kaewsuwan et al., 2010). The basal medium supplemented with 0.2% (v/v) each of oils including linseed oil, soybean oil, sunflower oil, corn oil and palm oil was used for oil amendment test. Tween80 at 0.25% (w/v) was used as an emulsifier.

The cultivation of the transgenic *P. patens* was performed by inoculation of 14-day-old protonemata (4% w/v) in 250 mL sterile shake-flasks containing the total volume of 100 mL optimized liquid BCD with oil supplementation. The liquid BCD basal medium (Knight et al., 2004) was used as the control experiment. After 14 days of cultivation in a growth room at 25 °C in an orbital shaker (NewBrunswick Scientific Innova 2100, Champaign, IL, USA) set at 125 rpm under continuous light provided by fluorescent tubes

(9,000 lux), the dry cell weight (DCW) and production of PUFAs in the cells were estimated in triplicates.

6. Dry Cell Weight Determination (Biomass)

For DCW determination, cell samples were harvested from the shake-flasks by filtration through a sieve (100 μm), washed three times with 100 mL of 0.25% (w/v) Tween80 followed by distilled water. The fresh cells were then freeze dried overnight to a constant weight.

7. Lipid extraction and PUFA composition analysis

Lipids extracted from 14-day-old protonemata of moss samples were analyzed by gas chromatography (GC) of their methyl esters. Briefly, lipids of moss tissues from individual wild type and transgenic *P. patens* plants were transmethylated with 2.5% sulfuric acid in methanol at 85°C for 30 min. Fatty acid methyl esters (FAMEs) were then extracted in heptanes (Kaewsuwan et al., 2006). GC analysis of FAMEs was conducted using an Agilent 6890N (USA) equipped with an HP-INNOWax capillary column (0.25 mm × 30 m × 0.25 μM), a flame ionization detector, using helium as the carrier gas. An aliquot (2 μl) of each sample extract was injected onto the GC column using the injector in the split mode. The initial column temperature was 185 °C (0.5 min) and was increased at a rate of 3.5 °C.min^{-1} to 235 °C (14.3 min), and then maintained at 235 °C for 1.0 min. Fatty acids were identified by comparison with the retention times of standards. The amounts of fatty acids were estimated from the peak areas extrapolated with the calibration curves of known fatty acid standards. The corresponding fatty acids were further verified with the same condition by gas chromatography-mass spectrometry (GC-

MS) using the HP 6890N Series operating at an ionization voltage of 70 eV with a scan range of 50-500 Da.

8. Molecular analysis

DNA integration events were verified by PCR and Southern blotting. Genomic DNA of wild type and transgenic plants was extracted and analyzed by PCR experiments with two primer pairs, the first derived from the start and stop regions of *PsELO5* (Pereira et al., 2004) and the second derived from the hygromycin-resistance coding region (Hyg^r) of the selection cassette (Fig. 1b and Table 1).

Table 1 Primers used for PCR amplification.

Primers	Sequence (5' to 3')
For *Pavlova* sp. Δ^5-elongase (*PsELO5*) gene	
Forward (A)	5'-ATG ATG TTG GCC GCA G -3'
Reverse (B)	5'-TTA CTC CGC CTT GAC CG -3'
For hygromycin-resistance (Hyg^r) gene	
Forward (C)	5'-ATG AAA AAG CCT GAA CTA CCG-3'
Reverse (D)	5'-CTA TTT CTT TGC CCT CGG A-3'

For Southern blot analysis, 1 μg aliquots of genomic DNA of wild type and transgenic plants (N15 and 64) were digested with *Eco*RV or *Nco*I, separated on 0.6% (w/v) Seakem LE agarose gel (Cambrex Bio Science Rockland, Rockland, ME, USA). The DNA was then transferred to a Biodyne B positively charged 0.45 nylon membrane (Pall Life Sciences, Ann Arbor, MI, USA) and hybridized with Dig-labeled probe of the full length of *PsELO5* or a probe for the hygromycin coding region (Hyg^r) of selection cassette at 40°C overnight. Probes synthesis was performed with a PCR Dig Probe Synthesis Kit

(Roche Applied Sciences, Mennheim, Germany) according to the manufacturer's instructions. Following hybridization, the membrane was washed at high stringency (0.1×saline sodium citrate, SSC and 0.1% sodium dodecyl sulfate, SDS) at 65°C. Detection was accomplished with a chemiluminescent substrate (CSPD, Roche Applied Sciences, Mennheim, Germany) and exposed to CL-XPosure film (Thermo Scientific, Inc., Rockford, IL, USA).

Results and Discussion

1. Expression of *PsELO5* in *P. patens*

In the present study, the different construct with the previous study (Kaewsuwan et al., 2010) containing *PsELO5* fused to the C-terminus of green fluorescence protein (GFP6) (Fig. 2) was introduced into *P. patens* protoplasts via polyethylene glycol (PEG)-mediated DNA uptake (Cove et al, 2009). Eighty five stable hygromycin-resistant transgenic lines were obtained after selection on a medium supplemented with hygromycin (25 μg mL^{-1}). GC analysis of FAMEs of all transgenic lines showed that they contained only one additional fatty acid (retention time, RT = 14.7 min) compared to the wild type (Fig. 2a). This was identified by co-migration and spiking with a known standard ADA and by the mass spectrometry (MS) fragmentation patterns (Fig. 3a). It was therefore considered to be ADA, an ω-6 elongation product of the *PsELO5*. Further estimation of ADA quantity from the calibration ADA standard revealed that the four pMDC43-*PsELO5* transgenics with the highest ADA production lines (N15, N64, N70 and N77) obviously produced 8.1 to 16.6 times higher levels of ADA compared to the previously reported transgenic line (C6) containing the pMDC32-*PsELO5* construct (Kaewsuwan et al., 2010), while no ADA was detected in the control wild type (Fig. 2a, 4a, Table 2). However, an

ω-3 DPA was not detected from any transgenic lines cultivated in BCD media (Fig. 2a and 4b, Table 2).

Expression of the fusion construct in *P. patens* confirmed that ADA, an ω-6 C_{22}-PUFA, was produced from the native ARA substrate, whereas ω-3 DPA production was not obtained from the endogenous EPA substrate. The Δ^5-elongation conversion efficiency in *P. patens* for ω-6 PUFAs was higher than for ω-3 PUFAs, probably due to lesser amount of EPA substrate in *P. patens*. Recently, the cotton Δ^{12}-desaturase (FAD2-4)- and marine microalgae *Pavlova viridis* C_{20}-elongase- GFP fusion polypeptides appeared to be functionally expressed in transgenic *Arabidopsis* plants and *E. coli*, respectively, and were shown to localized in the cytoplasmic membrane (Niu et al., 2009; Zhang et al., 2009). Our results also showed that when *PsELO5* was attached to the C-terminal of GFP6, *PsELO5* function was stronger than with a non-fusion protein. This evidence suggests that the chemeric elongase produced has higher activity, better membrane integration, or greater stability. Similarly, the functional expression of the human KDEL receptor in *Lactococus lastis* has been improved more than ten times by using GFP fusion to the C-terminus (Drew et al., 2001). Whereas a fusion gene between GFP and *COP1* (Constitutively Photomorphognic 1) exhibited biological activities identical to the native protein, as shown by genetic complementation of a lethal cop1 allele in transgenic *Arabidopsis* (Von Arnim et al,, 1998). Nevertheless, these provide the evidences that GFP does not degrade the function of fusion protein. Furthermore, GFP as a reporter gene under regulatory control of 35S promoter, was proved to reduce or avoid the gene silencing in transgenic soybean (El-Shemy et al., 2008). Thus the GFP gene in a vector system may play a useful role for transgenic evaluation and avoid gene silencing in transformed plants.

2. Effects of oil supplementation

Although only ADA, an ω-6 C_{22}-PUFA was initially produced in the transgenic lines, we further manipulated ω-3 DPA, an ω-3 C_{22}-PUFA production in *P. patens* by vegetable oil supplementation.

Vegetable oils contain various fatty acids that serve as precursors of long chain fatty acid (LC-FA) biosynthesis and supplementation with 1% oil has been reported to improve biomass and PUFA production in *Moretierella alpina* (Jang et al., 2005). We therefore grew the four highest ADA-producting lines (N15, N64, N70 and N77) in the previously optimized medium (liquid BCD medium containing 22.06 g L^{-1} of sucrose, 1.00 g L^{-1} of KNO_3 and 2.35 g L^{-1} glutamate (Kaewsuwan et al., 2010), supplemented with a 1.0% vegetable oil mixture comprising linseed oil, soybean oil, sunflower oil, corn oil, and palm oil (0.2% of each oil). An additional peak (RT = 15.7 min) was detected which was identical to the retention time of authentic ω-3 DPA (Fig. 2b). This compound also showed a molecular ion of 344 m/z, which is the expected molecular ion of methyl ester of ω-3 DPA as well as a fragmentation pattern identical to that of the authentic ω-3 DPA methyl ester (Fig. 3b). We therefore concluded that we have successfully produced ω-3 DPA in *P. patens* in this study.

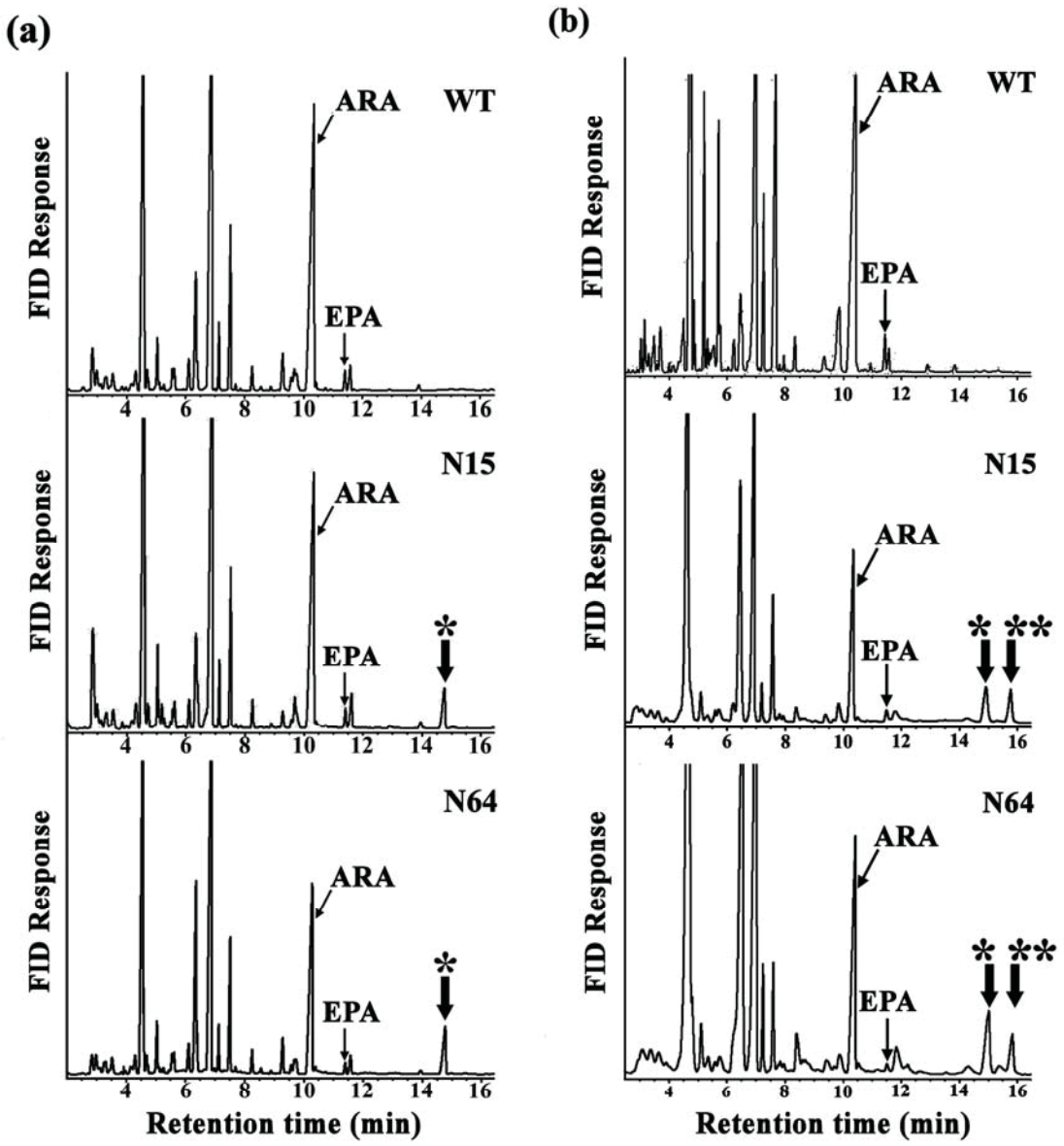

Fig. 2 Gas chromatographic analysis of fatty acid methyl esters (FAMEs) from 14-day-old protonemata *P. patens* wild type (WT) and transgenic lines (N15 and N64). The protonemata is grown in basal liquid BCD medium (a), in previously optimized liquid BCD medium containing 22.06 g L^{-1} of sucrose, 1.00 g L^{-1} of KNO_3 and 2.35 g L^{-1} glutamate and supplemented with a total of 1.0% vegetable oils including linseed oil, soybean oil, sunflower oil, corn oil, and palm oil (b). ARA, arachidonic acid; EPA, eicosapenataenoic acid. The additional peaks which correspond to the retention time of ADA and ω-3 DPA are indicated by single and double asterisks, respectively.

Table 2 Biomass and polyunsaturated fatty acid (PUFA) production from the wild type (WT) and transgenic *P. patens* expressing *PsELO5* grown for 14 days.

Line	Biomass[a] (g L^{-1})	Biomass[b] (g L^{-1})	ω-3 PUFA production (mg L^{-1})					
			ALA[a]	ALA[b]	EPA[a]	EPA[b]	ω-3 DPA[a]	ω-3 DPA[b]
WT[c]	5.53 ±0.95	12.43 ±0.85	9.71 ±1.75	39.60 ±3.75	1.29 ±0.22	7.71 ±0.41	0.00 ±0.00	0.00 ±0.00
C6[c]	4.83 ±0.45	9.60 ±0.63	8.67 ±1.22	13.94 ±1.84	1.22 ±0.35	0.96 ±0.15	0.00 ±0.00	6.35 ±0.43
N15	5.43± 0.45	9.37± 0.52	13.53 ± 2.04	20.48 ± 0.94	1.60 ±0.18	1.83 ±0.08	0.00 ±0.00	10.27 ±0.32
N64	6.00 ±0.38	9.03 ±0.98	13.35 ±1.85	15.36 ±0.87	2.07 ±0.25	1.45 ±0.07	0.00 ±0.00	11.75 ±0.41
N70	4.97 ±0.66	9.33 ±0.73	14.05 ±2.65	16.20 ±2.73	1.84 ±0.11	1.04 ±0.05	0.00 ±0.00	7.60 ±0.21
N77	5.03 ±0.61	9.70 ±0.57	9.55 ±1.38	16.03 ±3.08	1.69 ±0.20	0.92 ±0.12	0.00 ±0.00	8.56 ±0.18

Line	ω-6 PUFA production (mg L^{-1})											
	LA[a]	LA[b]	GLA[a]	GLA[b]	EDA[a]	EDA[b]	DHGLA[a]	DHGLA[b]	ARA[a]	ARA[b]	ADA[a]	ADA[b]
WT[c]	45.58 ±8.94	210.68 ±20.75	2.96 ± 0.54	11.96 ±0.87	2.50 ±0.75	10.74 ±1.28	1.27 ±0.37	11.85 ±0.87	42.89 ±5.85	196.88 ±13.96	0.00 ±0.00	0.00 ±0.00
C6[c]	41.33 ±8.48	88.68 ±8.39	2.88 ±0.33	9.09 ±1.55	2.05 ±0.66	0.90 ±0.14	1.45 ±0.22	2.64 ±0.32	37.25 ±5.33	46.85 ±4.85	0.42 ±0.08	11.18 ±0.75
N15	63.24 ±6.77	70.35 ±12.95	4.68 ±0.48	5.06 ±0.74	5.80 ±0.70	2.17 ±0.37	1.90 ±0.34	5.86 ±0.54	46.08 ±6.52	53.56 ±3.86	4.47 ±0.89	13.31 ±0.84
N64	64.36 ±5.04	144.90± 19.76	3.85 ±0.37	10.86 ±1.56	2.52 ±0.54	0.73 ±0.12	4.35 ±0.54	5.21 ±0.24	64.17 ±7.07	73.41 ±4.73	6.97 ±0.74	24.31 ±0.43
N70	69.49 ±7.84	86.98 ±16.85	5.67 ±0.28	13.36 ±1.37	3.10 ±0.61	0.61 ±0.08	4.25 ±0.61	5.27 ±0.11	50.53 ±5.85	60.27 ±2.79	3.57 ±0.68	12.59 ±0.63
N77	53.22 ±5.83	111.51± 15.96	3.62 ±0.32	14.29 ±1.83	2.52 ±0.44	0.28 ±0.04	4.78 ±0.53	4.83 ±0.77	55.00 ±6.22	59.83 ±6.40	3.40 ±0.55	16.23 ±0.73

LA, linoleic acid; GLA, γ-linolenic acid; EDA, eicosadienoic acid; DHGLA, di-homo-γ-linolenic acid; ARA, arachidonic acid; ADA, adrenic acid; ALA, α-linolenic acid, EPA, eicosapenatenoic acid; ω-3 DPA, docosapentaenoic acid. [a] PUFA production from *P. patens* grown for 14 days in basal liquid BCD medium (Knight et al., 2002). [b] PUFA production from *P. patens* grown for 14 days in optimized liquid BCD medium medium (Kaewsuwan et al., 2010) supplemented with a total of 1.0% vegetable oils including linseed oil, soybean oil, sunflower oil, corn oil, and palm oil. [a,c] Data from Kaewsuwan *et al*., 2010.

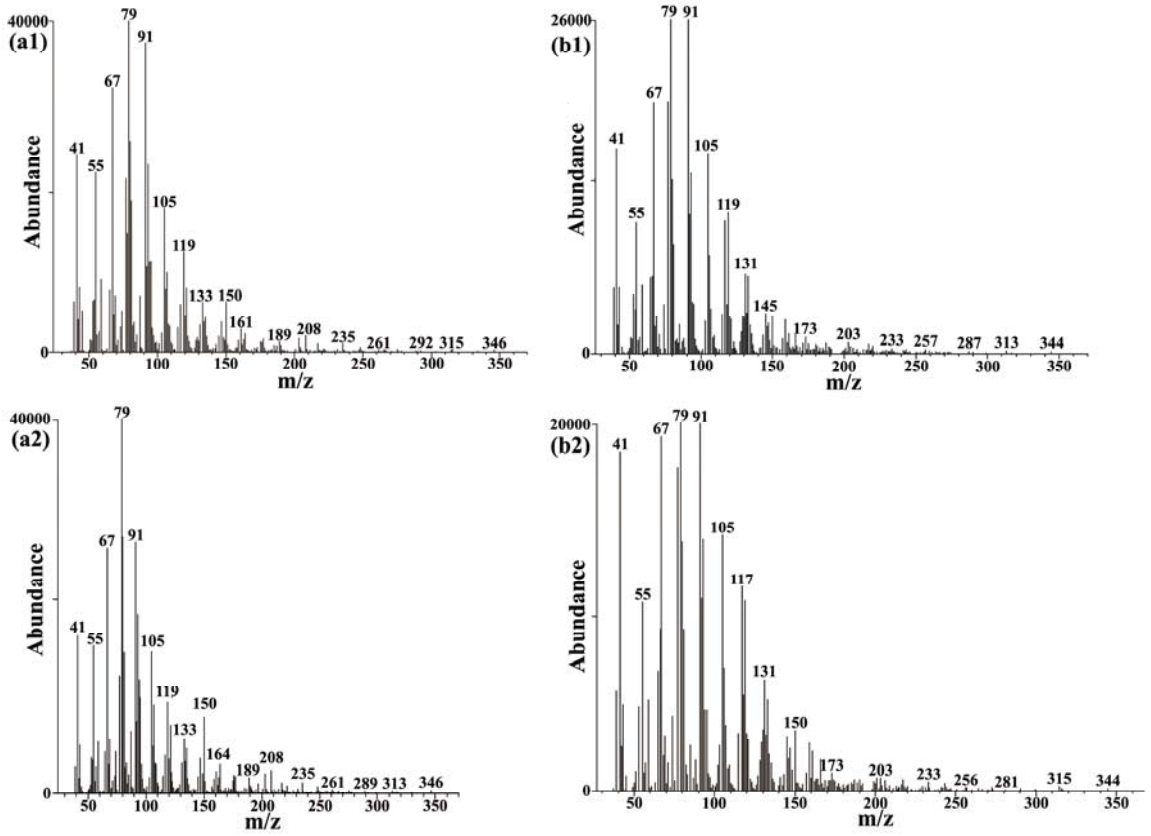

Fig. 3. Gas chromatographic-mass spectrometry analysis of methyl ester derivatives of the novel peaks identified in transgenic *P. patens*. Comparisons are shown of the mass spectra of the novel peaks from Figure 3, single asterisk (a1), double asterisks (b1) and authentic ADA (a2), ω-3 DPA (b2) standards, respectively.

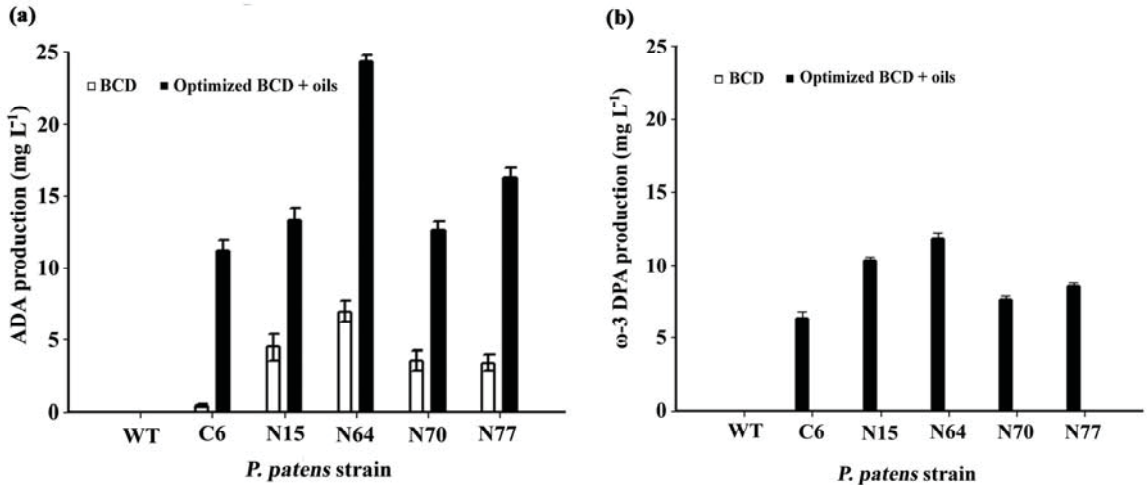

Fig. 4 Comparisons of ADA (a) and ω-3 DPA (b) production from 14-day-old protonemata *P. patens* wild type (WT) and transgenic lines (C6, N15, N64, N70 and N64) grown in basal liquid BCD medium, and in previously optimized liquid BCD medium containing 22.06 g L^{-1} of sucrose, 1.00 g L^{-1} of KNO_3 and 2.35 g L^{-1} glutamate and supplemented with a total of 1.0% vegetable oils including linseed oil, soybean oil, sunflower oil, corn oil, and palm oil. ADA, adrenic acid; ω-3 DPA, docosapentaenoic acid.

The effect of the vegetable oil supplement on the biomass and PUFA production of transgenic *P. patens* plants compared with the wild type is illustrated in Table 2. Supplementation with a total of 1.0% vegetable oils increased biomass 2.24 fold and PUFAs 4.04-9.03 fold, compared to the amounts detected in the wild type in the previously optimized liquid BCD medium without oil supplement (Kaesuwan et al., 2010). In the same way, the addition of oils enhanced biomass and PUFAs in transgenic *P. patens* (N15, N64, N70 and N77) especially C_{22}-PUFAs, ADA and ω-3 DPA (Fig. 4a and 4b, Table 2), whereas eicosadienoic acid (EDA, 20:2 $\Delta^{11,14}$) decreased in those transgenic lines. Vegetable oils generally contain high levels of C_{18}-fatty acid, precursors for LC-FA biosynthesis. For example, LA is the major fatty acid in soybean oil, corn oil and

sunflower oil (56-85%), α-linolenic acid (ALA) is the major fatty acid in linseed oil (58%) (Jang et al., 2005), and oleic acid (OA) is rich in palm oil (30-45%) (Mhanhmad et al., 2011). Therefore the addition of oils allowed moss to convert C_{18}-fatty acid substrates to LC-PUFAs. Shinmen *et al*. (1989) showed that olive oil and soybean oil addition increased the accumulation of ARA in *Mortierella* fungi (Shinmen et al., 1989) and supplementation of oils stimulates cell growth, EPA, DHA and total PUFAs production in *M. alpina* (Jang et al., 2005). The decrease in EDA levels observed in the transgenic *P. patens* was probably due to the presence of *PsELO5*-encoded elongase.

Therefore, metabolic engineering with *PsELO5*-GFP6 fusion together with oil supplementation successfully activated both ω-6 and ω-3 C_{22}-elongation products, ADA and ω-3 DPA, in transgenic lines from 11.2 to 24.3 and 6.3 to 11.7 mg L^{-1}, respectively. These results indicate the maximum accumulation of ADA (2.3% of total fatty acids) and ω-3 DPA (1.1% of total fatty acids) in transgenic line N64 (Table 3), 2-11 times higher than other similar studies performed by heterologous expression of multiple PUFA synthesizing genes in higher plants (0.1-1.2% of total fatty acids) (Tables 3 and 4) (Robert et al., 2005; Petrie et al., 2010a; Petrie et al., 2010b). This is the first report on producing an ω-3 DPA, DHA precursor, in non-seed lower plant.

Table 3 Total fatty acid and new C_{22} PUFA production from the wild type (WT) and transgenic *P. patens* expressing *PsELO5* grown for 14 days.

Line	FA production (mg L^{-1})						Total FA production (mg L^{-1})		C_{22} PUFA (% of total FAs)			
	SA[a]	SA[b]	MUFA[a]	MUFA[b]	PUFA[a]	PUFA[b]	Total[a]	Total[b]	ADA[a]	ADA[b]	ω-3 DPA[a]	ω-3 DPA[b]
WT[c]	86.45 ±11.71	386.27± 13.73	15.80 ±1.93	135.08 ±5.34	106.13± 26.62	489.69± 36.13	208.38 ±37.93	1011.04 ±393.11	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00	0.00 ±0.00
C6[c]	82.22 ±14.08	349.83± 22.39	9.79 ±1.52	241.39 ±20.03	94.96 ±19.55	180.83± 33.47	186.97 ±15.96	772.05 ±33.04	0.22 ±0.03	1.46 ±0.33	0.00 ±0.00	0.82 ±0.16
N15	83.22 ±11.60	590.67± 12.81	13.57 ±1.54	184.85 ±4.48	141.51± 30.52	183.17± 38.83	238.30 ±17.45	958.70 ±22.87	1.87 ±0.30	1.39 ±0.23	0.00 ±0.00	1.07 ±0.17
N64	88.16 ±10.90	465.43± 22.99	17.14 ±2.21	284.43 ±39.24	161.71± 13.73	288.06± 56.86	267.01 ±0.88	1037.91 ±60.88	2.61 ±0.31	**2.33 ±0.32**	0.00 ±0.00	**1.13 ±0.22**
N70	92.04 ±10.31	322.67± 10.43	9.76 ±1.80	253.90 ±16.90	152.57± 28.92	204.27± 50.38	254.37 ±20.34	780.84 ±52.98	1.39 ±0.16	1.62 ±0.29	0.00 ±0.00	0.97 ±0.15
N77	80.22 ±19.17	544.48± 15.01	11.33 ±2.37	289.24 ±16.26	133.78± 4.25	232.73± 22.52	225.34 ±16.5	1066.45 ±15.95	1.51 ±0.07	1.52 ±0.26	0.00 ±0.00	0.80 ±0.10

SA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid; ADA, adrenic acid; ω-3 DPA, docosapentaenoic acid. [a] PUFA production from *P. patens* grown for 14 days in basal liquid BCD medium (Knight et al., 2002). [b] PUFA production from *P. patens* grown for 14 days in optimized liquid BCD medium medium (Kaewsuwan et al., 2010) supplemented with a total of 1.0% vegetable oils including linseed oil, soybean oil, sunflower oil, corn oil, and palm oil. [a,c] Data from Kaewsuwan *et al*., 2010.

Table 4 Examples of polyunsaturated fatty acid (PUFA) production in transgenic plants.

Target gene	Plant host	New fatty acid production (% of total fatty acid)	Reference
Danto rerto Δ^5/Δ^6-desaturase, *Caenorhabditis elegans* Δ^6-elongase, *Pavlova salina* Δ^5-elongase and *P. salina* Δ^4-desaturase	*Arabidopsis thaliana*	GLA (0.4%), SDA (1.5%), DHGLA (1.5%), ETA (0.8%), ARA (1.0%), EPA (2.4%), ω-3 DPA (0.1%), DHA (0.5%)	(Robert et al., 2005)
Micromonas pusilla Δ^6-desaturase, *Pyramimonas cordata* Δ^6-elongase[a] and *Pavlova salina* Δ^5-desaturase	*Nicotiana benthamiana*	GLA (2.1%), ARA (0.6%), SDA (1.5%), ETA (0.6%), EPA (10.7%), ω-3 DPA (0.3%)	(Petrie et al., 2010b)
Echium plantagineum Δ^6-desaturase, *Pyramimonas cordata* Δ^6-elongase[a] and *Pavlova salina* Δ^5-desaturase	*Nicotiana benthamiana*	GLA (4.4%), ARA (1.3%), SDA (2.4%), ETA (0.1%), EPA (3.4%), ω-3 DPA (0.2%)	(Petrie et al., 2010b)
Ostreococcus tauri Δ^6-desaturase, *Pyramimonas cordata* Δ^6-elongase[a] and *Pavlova salina* Δ^5-desaturase	*Nicotiana benthamiana*	GLA (5.8%), ARA (1.2%), SDA (1.1%), ETA (0.4%), EPA (9.6%), ω-3 DPA (0.2%)	(Petrie et al., 2010b)
Pavlova salina Δ^9-elongase, Δ^8-desaturase, Δ^5-desaturase and Δ^5-elongase and Δ^4-desaturase	*Nicotiana benthamiana*	EDA (1.7%), DHGLA (0.5%), ARA (2.4%), ADA (1.2%), ETrA (1.5%), ETA (0.2%), EPA (1.2%), ω-3 DPA (0.6%), DHA (0.7%)	(Petrie et al., 2010a
Pavlova sp. Δ^5-elongase	*Physcomitrella patens*	ADA (2.3%), ω-3 DPA (1.1%)	Current study

SDA, stearidonic acid; GLA, γ-linolenic acid, ARA, arachidonic acid; ETA, eicosatetraenoic acid; EPA, eicosapentaenoic acid; ω-3 DPA, docosapentaenoic acid; EDA, eicodienoic acid; DHGLA, di-homo- γ-linolenic acid; adrenic acid, ADA; ETrA, eicosatrienoic acid; DHA, docosahexaenoic acid. [a] *P. cordata* Δ^6-elongase displays some Δ^5-elogase activity.

3. Molecular analysis

All transformants were morphologically indistinguishable from wild- type plants. Lines N15 and N64 were identified as having higher ω-3 DPA and were therefore chosen for molecular analysis of the transgene. The specific integration of pMDC43-*PsELO5* DNA into *P. patens* genome was analyzed by PCR with two different primer pairs (Fig. 1b and Table 2). For the transgenic lines (N15 and N64), primer pair A/B amplified a fragment of 0.8 kilobase pairs (kbp), which corresponds to *PsELO5* coding sequence, while primer pair C/D, amplified a fragment of 1.0 kbp corresponded to Hyg^r coding region. Neither primer pairs gave a product in the wild type (Fig. 5).

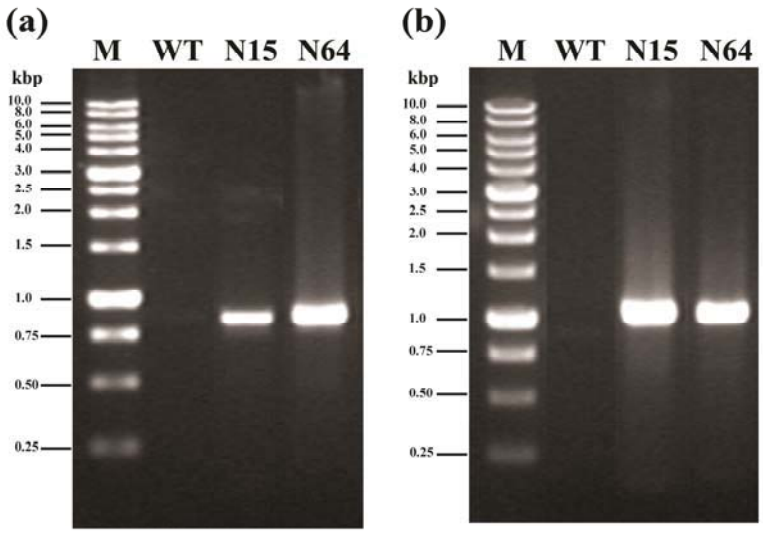

Fig. 5 Verification of *PsELO5* (a) and hygromycin resistance (Hyg^r) (b) genes by PCR amplification of *P. patens* wild type (WT) and transgenic lines (N15 and N64). The DNA sizes in kbp are indicated on the left.

To analyze the integration patterns of transgenes, 14-day-old protonemata of transgenic lines (N15 and N64) and wild type were assessed by Southern blot analysis using probes for the *PsELO5* and Hyg^r genes (Fig. 6). The results showed that hybridization of DNA from the transgenic line N15 digested with *Eco*RV and probed with

the *PsELO5* and Hyg^{r} probes detected strong signals of 2.9 (a) and 2.6 (b) kbp, respectively, consistent with the predicted sizes of the relevant fragments resulting from *Eco*RV digestion of pMDC43-*PsELO5*, whereas weak signals of similar sized DNA were obtained from transgenic line N64. This indicates that line N64 has fewer copies of the transgene than line N15. Digestion with *Nco*I which cuts at 625 bp site within *PsELO5* cDNA, produced a high intensity band of 1.1 kbp (*) in transgenic line N15 when hybridized with *PsELO5* probe, while a lower intensity band was detected in line N64. An additional fragment of 10.5 kbp (**) which is the predicted size of fragments generated by *Nco*I from multiple tandemely repeated copies of pMDC43-*PsELO5*, hybridized with either *PsELO5* or Hyg^{r} probes. However, hybridization with these probes from transgenic line 15 produced the several additional DNA fragments, whereas only one of the flanking sequences was detected in line N64 (arrowed). From these results we suggest that line N64 contains only a single copy of the expression construct in, whereas there are either multiple copies with rearrangements or several insertion sites of the construct in line N15.

Therefore both single copy and multiple copies of plasmid DNA containing the full length of *PsELO5* gene were not only integrated into the transgenic *P. patens* genome and stably inherited to its culture, but also led to successful functional expression. The effects of transgenic copy number on the level of gene expression are known to be complex. Though it was anticipated that the increase of transgene copy number would increase expression level (Dai et al., 2001; El-Shemy et al., 2007), it is now known that gene co-suppression phenomena frequently occur in transgenic plants with repeated transgene or an unusual structure such as inverted repeats (Vaucheret et al., 1998). Moreover, an increase copy number of transgene can correlate with an increased risk of silencing (Lessard et al., 2002). Our transgenic line with single copy number (N64)

improved C_{22}-PUFA production rather more than the line with multiple copies of transgene (N15), suggesting that lower copy number may permit potentially greater gene expression since multiple copy number integration could inhibit trangene expression and even lead to transgene silencing in transgenic plants (Stam et al., 1997). However, we did not control the chromosomal insertion site and so the differences may result from position effects. Future work will investigate this.

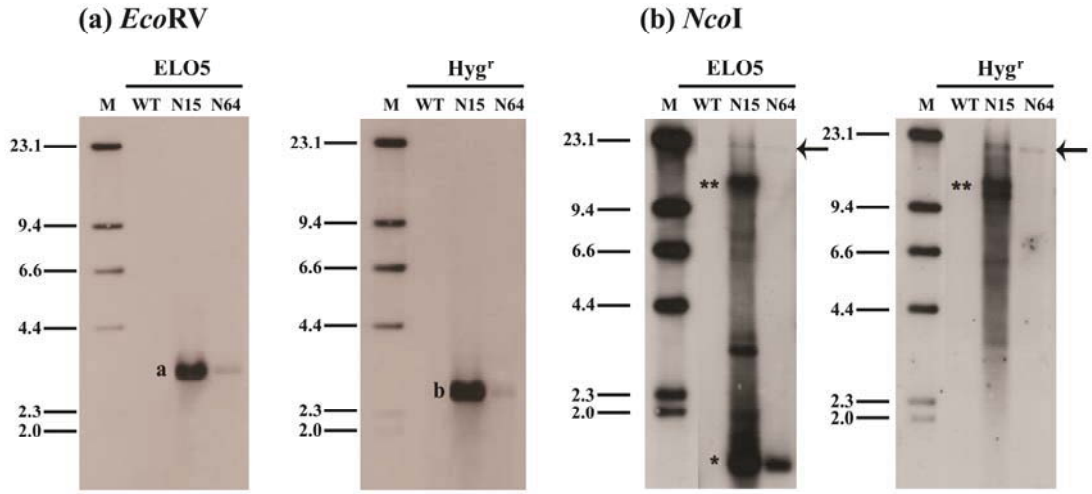

Fig. 6 Southern blotting of *P. patens* wild type (WT) and transgenic lines (N15 and N64). Genomic DNA from wild type and transgenic lines was digested with *Eco*RV (a) and *Nco*I (b) and hybridized with *PsELO5* (ELO5) and hygromycin resistance (Hyg^r) probes. The DNA sizes in kbp are indicated on the left. The letters and asterisks indicate signals predicted. Arrows indicate one of the flanking sequences detected.

Conclusions

The efficient biosynthesis of C_{22} LC-PUFAs in transgenic *P. patens* plants has now been conclusively demonstrated, using reverse-engineering and nutritional supplementation approaches. Conversion of fatty acid precursors from vegetable oils to high-value oil may prove to be an economically viable option or the industry.

P. patens is therefore as an alternative sustainable source of DHA precursor for human consumption. We are confident that the results obtained will be useful in the engineering of stably-transformed genes in land plant. This heterologous expression system has not only realized the possibility of producing the important nutritional compounds in transgenic plants, but also provided a new experiment tool with which to better investigate plant lipid metabolism.

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Acknowledgements

The authors wish to thank the Office of the Higher Education Commission and the Thailand Research Fund (TRF) (MRG5380182) for financial support. We also thank the Marine Natural Products Research Unit and Faculty of Pharmaceutical Sciences for providing laboratories facilities.

Outputs (MRG5380182)

Research Article

Chodok P, Cove DJ, Quatrano RS, Kanjana-Opas A, Kaewsuwan S. Metabolic engineering and oil supplementation of *Physcomitrella patens* for activation of C_{22} polyunsaturated fatty acid production. J Am Oil Chem Soc 2012; 89(3):465-476. *(Corresponding author, Web of Science®, impact factor 1.773)*

Proceeding (Abstract)

Chodok P, Kaewsuwan S. Activation of docosahexaenoic acid (DHA) precursor in the moss *Physcomitrella patens* by marine algae *Pavlova* sp. Δ^5-elongase and vegetable oil supplements. The 13th Symposium on Marine Natural Products (MaNaPro XIII), October 17-22, 2010, Phuket, Thailand.

Proceeding (Abstract, MRG5080203)

Kaewsuwan S, Bunyapraphatsara N, Cove DJ, Quatrano RS, Chodok P. High level production of adrenic acid (ADA) in transgenic moss *Physcomitrella patens*. Society for Experimental Biology Annual Main Meeting 2010. June 30-July 3, 2010. Prague, Czech Republic.

Kaewsuwan S, Chodok P, Kanjana-Opas A. Evaluation of culture conditions for the production of polyunsaturated fatty acids (PUFAs) by *Physcomitrella patens* using the Plackett-Burman statistical design protocols. The Asian Congress on Biotechnology (ACB-2011: Biotechnology for Better Life), May 11-15, 2011, Shanghai, China.

J Am Oil Chem Soc (2012) 89:465–476
DOI 10.1007/s11746-011-1927-4

ORIGINAL PAPER

Metabolic Engineering and Oil Supplementation of *Physcomitrella patens* for Activation of C_{22} Polyunsaturated Fatty Acid Production

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Received: 3 March 2011 / Revised: 9 July 2011 / Accepted: 28 July 2011 / Published online: 14 September 2011

Abstract Long chain (C ≥ 20) polyunsaturated fatty acids (LC-PUFAs) represent important components of the human diet. Currently, the predominant sources of these fatty acids are marine fish and algal oils, but high production costs and diminishing feedstock, limit their supply and usage. A more regular sustainable source of these compounds is urgently required and therefore research is being conducted to develop a sustainable, land-based production system. This work describes the metabolic engineering of an artificial pathway that activates the production of C_{22}-PUFAs, docosatetraenoic acid or adrenic acid (ADA) and n-3 docosapentaenoic acid (DPA) in *Physcomitrella patens* using a gene from a marine algae *Pavlova* sp. encoding Δ^5-elongase and vegetable oil supplementation. The accumulation of ADA and ω-3 DPA were dramatically increased to 24.3 and 11.7 mg L^{-1} and accounted for 2.3 and 1.1% of total fatty acids, respectively. This is the first report on producing n-3 DPA, DHA precursor, in *P. patens*. The obtained results prove that this enzyme appears to be more active when fused to a green fluorescence protein reporter gene. These finding reveal that the modification of the fatty acid biosynthetic pathway by genetic manipulation and nutritional supplementation, to produce specific PUFAs in a non-seed lower plant, is a promising technique.

Keywords *Physcomitrella patens* · *Pavlova* sp. · Elongase · C_{22} polyunsaturated fatty acids · Oil supplementation

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Introduction

Long-chain (C ≥ 20) polyunsaturated fatty acids (LC-PUFAs) including the omega-3 (n-3) eicosapentaenoic acid (EPA, 20:5 $\Delta^{5,8,11,14,17}$) and docosahexaenoic acid (DHA, 22:6 $\Delta^{4,7,10,13,16,19}$) are essential nutrients for a healthy diet and adequate human development. Several studies have indicated that deficiencies in these fatty acids increase the risk of cardiovascular disease [1], hypertension [2], inflammatory diseases, rheumatoid arthritis [3] and neuropsychiatric disorders including dementia and depression [4].

DHA, an ω-3 PUFA found in fish and certain algae, makes up 60% of the fatty acid in human neuronal cell membranes, and is particularly concentrated in synaptic membranes [5] and in myelin sheaths [6]. DHA is essential for prenatal brain development as well as normal healthy brain functioning. Supplemental DHA has also been shown to have a protective effect against cognitive decline or Alzheimer's disease which may involve triacylglycerols (TAG) lowering [7]. Furthermore, DHA has been recognized as a nutrient for prevention of human cancer and heart disease [8].

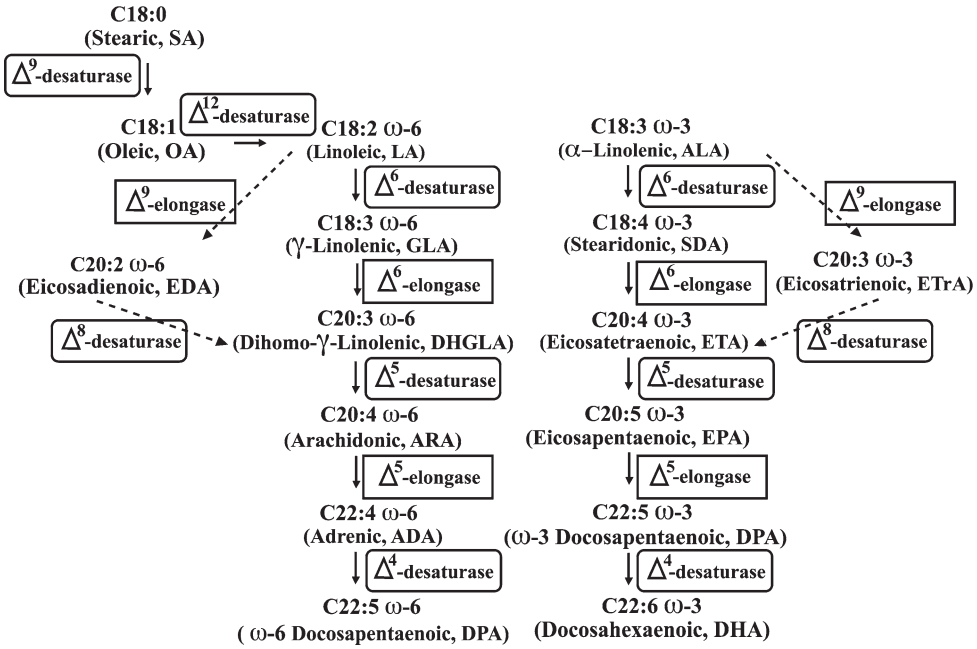

Fig. 1 A simplified scheme of the polyunsaturated fatty acid (PUFA) biosynthesis pathway in non-seed eukaryotes

Humans, particularly infants, are unable to synthesize DHA and other LC-PUFAs to any great extent and must therefore obtain them through their diet. The human consumption of LC-PUFAs is steadily increasing, but the production of fish oil, the main source of LC-PUFAs for human consumption, is declining [9]. Generally, the cost of the commercial processing, refining, and stabilizing the fish oils is very high, and the decline in production due to overfishing continues to drive up the cost of fish oils [10]. The recent finding of toxic compounds in fish oil has further raised safety concerns on the consumption of fish oils [11].

Increasing demand has therefore raised an interest in obtaining these LC-PUFAs from alternative sources that are more economical and sustainable. Plant oils could be a sustainable alternative source of fatty acids. However, plants do not synthesize LC-PUFAs owing to the lack of Δ^6- and Δ^5-position-specific desaturase and the absence of an elongase. Plant oils are therefore rich in C_{18}-PUFAs, linoleic acid (LA, 18:2 $\Delta^{9,12}$) and α-linolenic acid (ALA, 18:3 $\Delta^{9,12,15}$), but not in LC-PUFAs, EPA and DHA [12]. An attractive possibility is to genetically engineer plants to produce LC-PUFAs.

In algae, DHA can be synthesized from EPA in a two-step process that involves: (a) the elongation of EPA by Δ^5-elongase to generate ω-3 docosapentaenoic acid (ω-3 DPA): and (b) the desaturation of ω-3 DPA by a Δ^4-desaturase to generate DHA (Fig. 1) [13]. A number of elongases have been identified from mammals, marine and fresh water teleost fish that can recognize and lengthen multiple chain-length PUFAs, such as C_{18}-, C_{20}- and C_{22}-PUFAs [14–16]. Recently, elongases specific for C_{20}-PUFAs have been identified from algae and demonstrated to function in the production of DHA [17, 18]. In addition, the moss *Physcomitrella patens* produces several PUFAs, especially arachidonic acid (ARA, 20:4 $\Delta^{5,8,11,14}$) and EPA [19, 20] which are C_{20}-PUFAs required for the synthesis of the LC-PUFAs, docosatetraenoic acid or adrenic acid (ADA) and ω-3 DPA, respectively by a Δ^5-elongase [13]. Although ADA is a minor fatty acid in the brain, it is suggested to be an important component for myelination of neural tissue [21] and also serves as an eicosanoid precursor in tissue [22]. Moreover, ω-3 DPA is a precursor of DHA which has attracted much interest due to its pharmaceutical potential.

Development of transgenic oil plants capable of producing C_{22}-PUFAs, ADA and ω-3 DPA is therefore an attractive route to improve commercial-scale DHA production. The present study demonstrates the primary step for the production of C_{22}-PUFAs, ADA and ω-3 DPA, from C_{20}-PUFAs, ARA and EPA, respectively in *P. patens* by heterologous expression of marine algae *Pavlova* sp. Δ^5-elongase and vegetable oil supplementation.

Experimental Procedures

Materials

Restriction enzymes and polymerases were obtained from TaKaRa Bio (Shiga, Japan). DNA modifying enzymes were purchased from Promega (Madison, MI, USA). All other chemicals used were reagent grade from Sigma (St. Louis, MO, USA). Cloning vectors and plant gateway destination vectors were obtained from Invitrogen

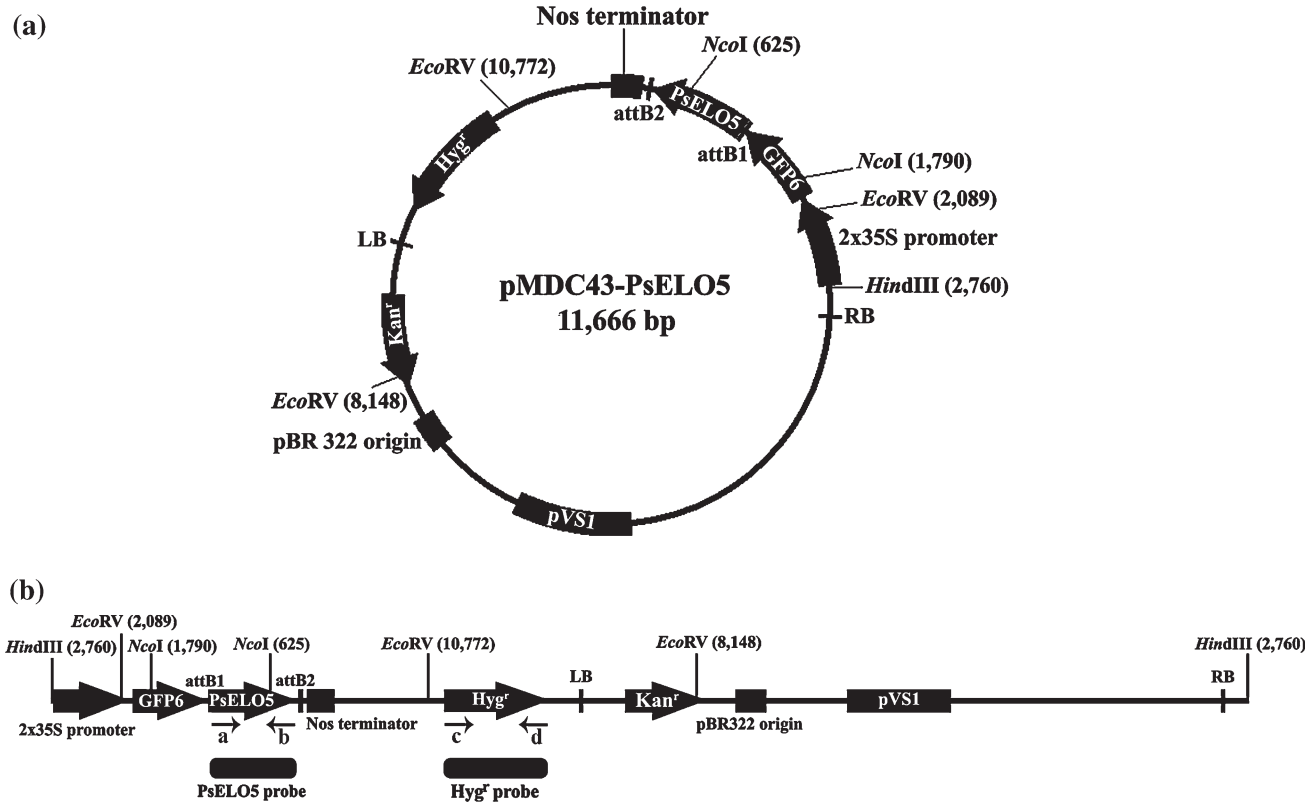

Fig. 2 Structure of the pMDC43-*PsELO5* (**a**). Its linear fragment used for *P. patens* transformation was digested out with *Hin*dIII (**b**). *PsELO5* cDNA and Hyg^r coding region are 834 and 1,026 bp, respectively. The *lettered arrows* indicate the binding sites of primers used for PCR analysis. The localization of *PsELO5* or Hyg^r probes and the restriction enzyme sites used for Southern blotting are marked with the blocks below the sequences and indicated in the parentheses, respectively

(Carlsbad, CA, USA) and Arabidopsis Biological Resource Center (ABRC) (Column, OH, USA), respectively. Fatty acids were purchased from Nu-Check-Prep (Elysian, MN, USA).

Plant Materials and Culture Conditions

Pavlova sp. CCMP459 was purchased from Provasoli-Guillard National Center for Culture of Marine Phytoplankton (CCMP, West Boothbay Harbor, ME, USA). The Gransden strain of *P. patens* [23] was used throughout the studies. Wild type and transgenic mosses were grown in liquid BCD basal medium supplemented with 5 mM ammonium tartrate and cultured at 25 °C under continuous light provided by fluorescent tubes [24, 25] unless indicated otherwise.

Vector Construction and Expression in *P. patens*

Primers were designed based on the NCBI sequence data of *Pavlova* elongase accession no. AY630573 [18]. PCR was conducted using cDNA synthesized from total RNA of *Pavlova* sp. CCMP459 as a template in a master mix that included TaKaRa Ex Taq (TaKaRa Bio, Shiga, Japan). For the generation of *P. patens* over-expressing *PsELO5* expression cassette in which the coding region of *Pavlova* sp. Δ^5-elongase gene was firstly incorporated into the pCR®8/GW/TOPO® entry vector (Invitrogen) and further recombined with desired Gateway® plant destination vector, pMDC43 (ABRC) (Columbus, OH, USA) [26]. The chosen destination construct has a tandemly duplicated CaMV 35S promoter together with Hyg^r and GFP6 regions. Finally, this resulted in the generation of the recombinant plasmid pMDC43-*PsELO5* carrying a gene *PsELO5* fused to C-terminus of GFP6, driven by a tandemly duplicated CaMV 35S promoter and nos terminator, and contained the Hyg^r gene as a selection marker (Fig. 2a).

Transformation and Screening of Transgenic *P. patens* Plants

To induce expression, the recombinant plasmid pMDC43-*PsELO5* was subsequently digested with *Hin*dIII, and the linear fragment (Fig. 2b) was precipitated and used for transformation into protoplast by PEG-mediated direct

gene transfer [25]. The stable transformants were screened with successive rounds of selection on BCD media containing hygromycin (25 μg mL^{-1}), interspersed with culture on antibiotic-free medium [24].

Starter Inoculum Preparation

Protonemata tissue (14 day-old, 1 g) was aseptically blended in 250-mL sterile shake-flasks containing 100 mL of optimized liquid BCD medium [27] with a homogenizer (OMNI TH_Q, USA) at a speed of 30,000 rpm for 1 min and cultivated in a growth room at 25 °C in an orbital shaker (New Brunswick Scientific Innova 2100, Champaign, IL, USA) set at 125 rpm under continuous light provided by fluorescent tubes (9,000 lux). After 14 days of growth, the cultured tissues were used to inoculate the liquid medium at a 4% (w/v) level for all subsequent experiments.

Effects of Oil Supplementation

Oil supplementation to improve C_{22}-PUFA production in transgenic *P. patens* (N15, N64, N70 and N77) was carried out based on the previous reported optimized liquid BCD medium containing 22.06 g L^{-1} of sucrose, 1.00 g L^{-1} of KNO_3 and 2.35 g L^{-1} of glutamate [27]. The basal medium supplemented with 0.2% (v/v) each of oils including linseed oil, soybean oil, sunflower oil, corn oil and palm oil was used for oil amendment test. Tween 80 at 0.25% (w/v) was used as an emulsifier.

The cultivation of the transgenic *P. patens* was performed by inoculation of 14 day-old protonemata (4% w/v) in 250 mL sterile shake-flasks containing the total volume of 100 mL optimized liquid BCD with oil supplementation. The liquid BCD basal medium [24] was used as the control experiment. After 14 days of cultivation in a growth room at 25 °C in an orbital shaker (New Brunswick Scientific Innova 2100, Champaign, IL, USA) set at 125 rpm under continuous light provided by fluorescent tubes (9,000 lux), the dry cell weight (DCW) and production of PUFAs in the cells were estimated in triplicate.

Dry Cell Weight Determination (Biomass)

For DCW determination, cell samples were harvested from the shake-flasks by filtration through a sieve (100 μm), washed three times with 100 mL of 0.25% (w/v) Tween 80 followed by distilled water. The fresh cells were then freeze dried overnight to a constant weight.

Lipid Extraction and PUFA Composition Analysis

Lipids extracted from 14 day-old protonemata of moss samples were analyzed by gas chromatography (GC) of their methyl esters. Briefly, lipids of moss tissues from individual wild type and transgenic *P. patens* plants were transmethylated with 2.5% sulfuric acid in methanol at 85 °C for 30 min. Fatty acid methyl esters (FAMEs) were then extracted in heptanes [19]. GC analysis of FAMEs was conducted using an Agilent 6890N (USA) equipped with an HP-INNOWax capillary column (0.25 mm × 30 m × 0.25 μM), a flame ionization detector, using helium as the carrier gas. An aliquot (2 μl) of each sample extract was injected onto the GC column using the injector in the split mode. The initial column temperature was 185 °C (0.5 min) and was increased at a rate of 3.5 °C min^{-1} to 235 °C (14.3 min), and then maintained at 235 °C for 1.0 min. Fatty acids were identified by comparison with the retention times of standards. The amounts of fatty acids were estimated from the peak areas extrapolated with the calibration curves of known fatty acid standards. The corresponding fatty acids were further verified with the same condition by gas chromatography–mass spectrometry (GC–MS) using the HP 6890N Series operating at an ionization voltage of 70 eV with a scan range of 50–500 Da.

Molecular Analysis

DNA integration events were verified by PCR and Southern blotting. Genomic DNA of wild type and transgenic plants was extracted and analyzed by PCR experiments with two primer pairs, the first derived from the start and stop regions of *PsELO5* [18] and the second derived from the hygromycin-resistance coding region (Hyg^r) of the selection cassette (Fig. 2b; Table 1).

For Southern blot analysis, 1-μg aliquots of genomic DNA of wild type and transgenic plants (N15 and 64) were digested with *Eco*RV or *Nco*I, separated on 0.6% (w/v) Seakem LE agarose gel (Cambrex Bio Science Rockland, Rockland, ME, USA). The DNA was then transferred to a Biodyne B positively charged 0.45 nylon membrane (Pall Life Sciences, Ann Arbor, MI, USA) and hybridized with Dig-labeled probe of the full length of *PsELO5* or a probe for the hygromycin coding region (Hyg^r) of selection cassette at 40 °C overnight. Probe synthesis was performed with a PCR Dig Probe Synthesis

Table 1 Primers used for PCR amplification

Primers	Sequence (5′ to 3′)
For *Pavlova* sp. Δ^5-elongase (*PsELO5*) gene	
Forward (A)	5′-ATG ATG TTG GCC GCA G-3′
Reverse (B)	5′-TTA CTC CGC CTT GAC CG-3′
For hygromycin-resistance (Hyg^r) gene	
Forward (C)	5′-ATG AAA AAG CCT GAA CTA CCG-3′
Reverse (D)	5′-CTA TTT CTT TGC CCT CGG A-3′

Kit (Roche Applied Sciences, Mannheim, Germany) according to the manufacturer's instructions. Following hybridization, the membrane was washed at high stringency (0.1× saline sodium citrate, SSC and 0.1% sodium dodecyl sulfate, SDS) at 65 °C. Detection was accomplished with a chemiluminescent substrate (CSPD, Roche Applied Sciences, Mannheim, Germany) and exposed to CL-XPosure film (Thermo Scientific, Inc., Rockford, IL, USA).

Results and Discussion

Expression of *PsELO5* in *P. patens*

In the present study, the different construct with the previous study [27] containing *PsELO5* fused to the C-terminus of green fluorescence protein (GFP6) (Fig. 2) was introduced into *P. patens* protoplasts via polyethylene glycol (PEG)-mediated DNA uptake [25]. Eighty-five stable hygromycin-resistant transgenic lines were obtained after selection on a medium supplemented with hygromycin (25 μg mL^{-1}). GC analysis of FAMEs of all transgenic lines showed that they contained only one additional fatty acid (retention time, RT = 14.7 min) compared to the wild type (Fig. 3a). This was identified by co-migration and spiking with a known standard ADA and by the mass spectrometry (MS) fragmentation patterns (Fig. 4a). It was therefore considered to be ADA, an ω-6 elongation product of the *PsELO5*. Further estimation of ADA quantity from the calibration ADA standard revealed that the four pMDC43-*PsELO5* transgenics with the highest ADA production lines (N15, N64, N70 and N77) obviously produced 8.1–16.6 times higher levels of ADA compared to the previously reported transgenic line (C6) containing the pMDC32-*PsELO5* construct [27], while no ADA was detected in the control wild type (Fig. 3a, 5a; Table 2). However, an n-3 DPA was not detected from any transgenic lines cultivated in BCD media (Fig. 3a, 5b; Table 2).

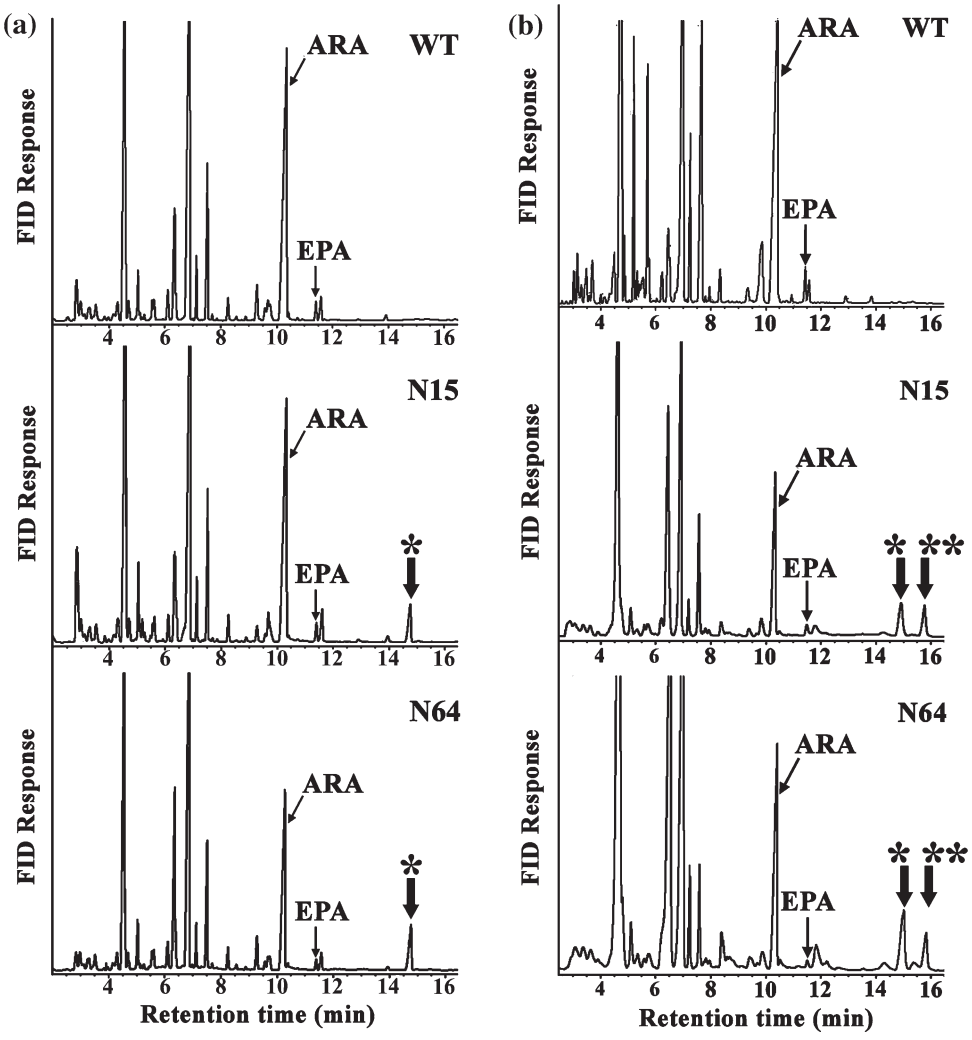

Fig. 3 Gas chromatographic analysis of fatty acid methyl esters (FAMEs) from 14 day-old protonemata *P. patens* wild type (WT) and transgenic lines (N15 and N64). The protonemata is grown in basal liquid BCD medium (**a**), in previously optimized liquid BCD medium containing 22.06 g L^{-1} of sucrose, 1.00 g L^{-1} of KNO_3 and 2.35 g L^{-1} glutamate and supplemented with a total of 1.0% vegetable oils including linseed oil, soybean oil, sunflower oil, corn oil, and palm oil (**b**). *ARA* arachidonic acid, *EPA* eicosapentaenoic acid. The additional peaks which correspond to the retention time of ADA and ω-3 DPA are indicated by *single asterisk* and *double asterisks*, respectively

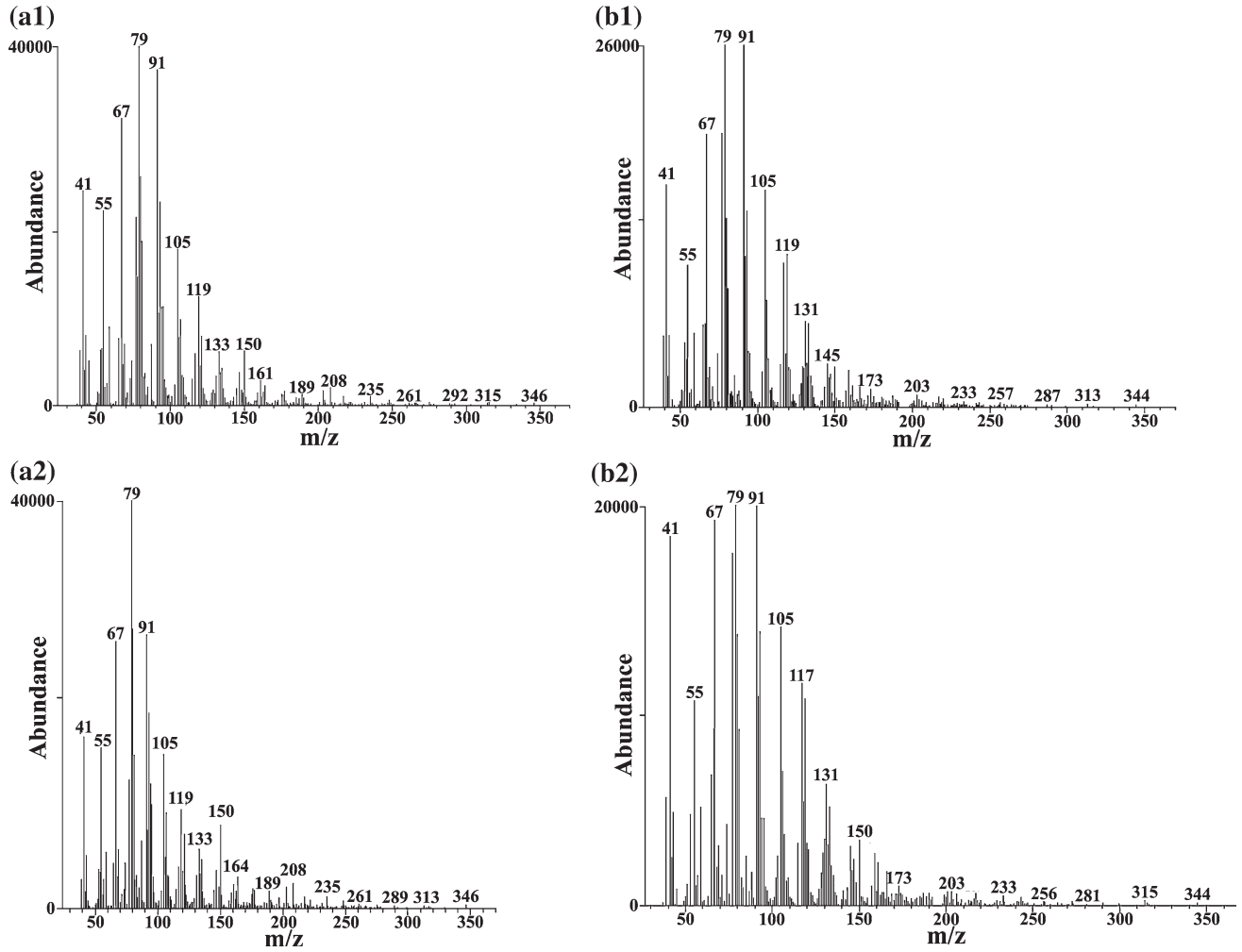

Fig. 4 Gas chromatographic–mass spectrometry analysis of methyl ester derivatives of the novel peaks identified in transgenic *P. patens*. Comparisons are shown of the mass spectra of the novel peaks from Fig. 3, *single asterisk* (**a1**), *double asterisks* (**b1**) and authentic ADA (**a2**), ω-3 DPA (**b2**) standards, respectively

Expression of the fusion construct in *P. patens* confirmed that ADA, an ω-6 C_{22}-PUFA, was produced from the native ARA substrate, whereas ω-3 DPA production was not obtained from the endogenous EPA substrate. The Δ^5-elongation conversion efficiency in *P. patens* for ω-6 PUFAs was higher than for ω-3 PUFAs, probably due to lesser amount of EPA substrate in *P. patens*. Recently, the cotton Δ^{12}-desaturase (FAD2-4)- and marine microalgae *Pavlova viridis* C20-elongase- GFP fusion polypeptides appeared to be functionally expressed in transgenic *Arabidopsis* plants and *E. coli*, respectively, and were shown to localized in the cytoplasmic membrane [28, 29]. Our results also showed that when *PsELO5* was attached to the C-terminal of GFP6, *PsELO5* function was stronger than with a non-fusion protein. This evidence suggests that the chimeric elongase produced has higher activity, better membrane integration, or greater stability. Similarly, the functional expression of the human KDEL receptor in *Lactococus lastis* has been improved more than ten times by using GFP fusion to the C-terminus [30]. Whereas a fusion gene between GFP and *COP1* (Constitutively Photomorphogenic 1) exhibited biological activities identical to the native protein, as shown by genetic complementation of a lethal cop1 allele in transgenic *Arabidopsis* [31]. Nevertheless, these provide the evidences that GFP does not degrade the function of fusion protein. Furthermore, GFP as a reporter gene under regulatory control of 35S promoter, was proved to reduce or avoid the gene silencing in transgenic soybean [32]. Thus the GFP gene in a vector system may play a useful role for transgenic evaluation and avoid gene silencing in transformed plants.

Effects of Oil Supplementation

Although only ADA, an ω-6 C_{22}-PUFA was initially produced in the transgenic lines, we further manipulated ω-3

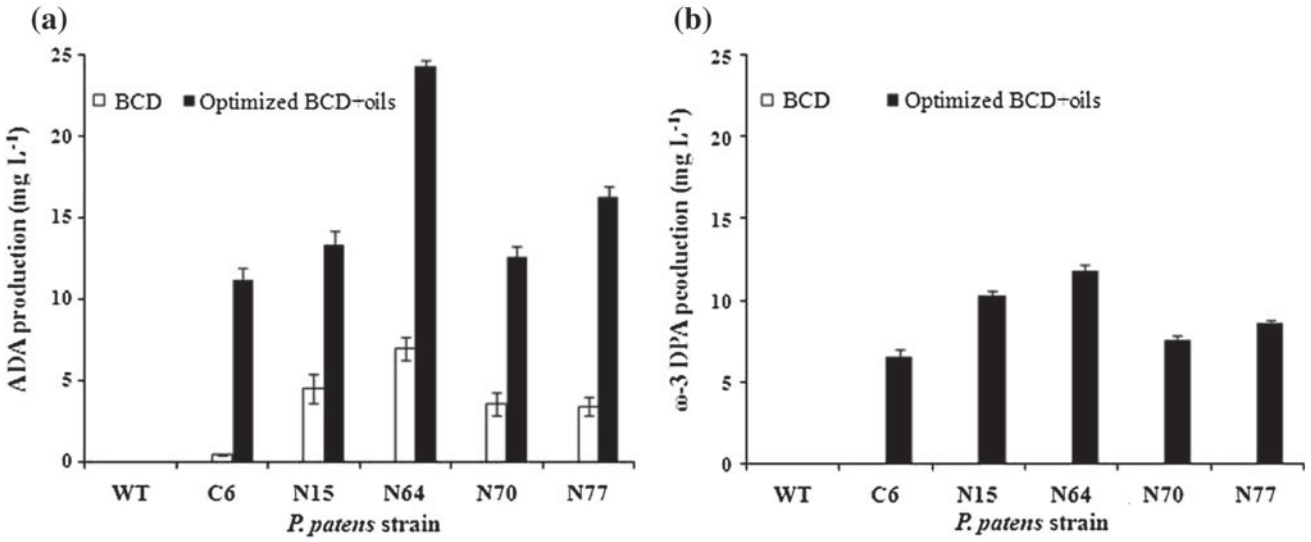

Fig. 5 Comparisons of ADA (**a**) and ω-3 DPA (**b**) production from 14 day-old protonemata *P. patens* wild type (WT) and transgenic lines (C6, N15, N64, N70 and N64) grown in basal liquid BCD medium, and in previously optimized liquid BCD medium containing 22.06 g L^{-1} of sucrose, 1.00 g L^{-1} of KNO3 and 2.35 g L^{-1} glutamate and supplemented with a total of 1.0% vegetable oils including linseed oil, soybean oil, sunflower oil, corn oil, and palm oil. *ADA* adrenic acid; ω-3 *DPA* docosapentaenoic acid

DPA, an ω-3 C_{22}-PUFA production in *P. patens* by vegetable oil supplementation.

Vegetable oils contain various fatty acids that serve as precursors of long chain fatty acid (LC-FA) biosynthesis and supplementation with 1% oil has been reported to improve biomass and PUFA production in *Mortierella alpina* [33]. We therefore grew the four highest ADA-producing lines (N15, N64, N70 and N77) in the previously optimized medium (liquid BCD medium containing 22.06 g L^{-1} of sucrose, 1.00 g L^{-1} of KNO_3 and 2.35 g L^{-1} glutamate [27], supplemented with a 1.0% vegetable oil mixture comprising linseed oil, soybean oil, sunflower oil, corn oil, and palm oil (0.2% of each oil). An additional peak (RT = 15.7 min) was detected which was identical to the retention time of authentic ω-3 DPA (Fig. 3b). This compound also showed a molecular ion of 344 *m/z*, which is the expected molecular ion of methyl ester of ω-3 DPA as well as a fragmentation pattern identical to that of the authentic ω-3 DPA methyl ester (Fig. 4b). We therefore concluded that we have successfully produced ω-3 DPA in *P. patens* in this study.

The effect of the vegetable oil supplement on the biomass and PUFA production of transgenic *P. patens* plants compared with the wild type is illustrated in Table 2. Supplementation with a total of 1.0% vegetable oils increased biomass 2.24-fold and PUFAs 4.04–9.03-fold, compared to the amounts detected in the wild type in the previously optimized liquid BCD medium without oil supplement [27]. In the same way, the addition of oils enhanced biomass and PUFAs in transgenic *P. patens* (N15, N64, N70 and N77) especially C_{22}-PUFAs, ADA and ω-3 DPA (Fig. 5a, b; Table 2), whereas eicosadienoic acid (EDA, 20:2 $\Delta^{11,14}$) decreased in those transgenic lines. Vegetable oils generally contain high levels of C_{18}-fatty acid, precursors for LC-FA biosynthesis. For example, LA is the major fatty acid in soybean oil, corn oil and sunflower oil (56–85%), α-linolenic acid (ALA) is the major fatty acid in linseed oil (58%) [33], and oleic acid (OA) is rich in palm oil (30–45%) [34]. Therefore the addition of oils allowed moss to convert C_{18}-fatty acid substrates to LC-PUFAs. Shinmen et al. [35] showed that olive oil and soybean oil addition increased the accumulation of ARA in *Mortierella* fungi and supplementation of oils stimulates cell growth, EPA, DHA and total PUFA production in *M. alpina* [33]. The decrease in EDA levels observed in the transgenic *P. patens* was probably due to the presence of *PsELO5*-encoded elongase.

Therefore, metabolic engineering with *PsELO5*-GFP6 fusion together with oil supplementation successfully activated both ω-6 and ω-3 C_{22}-elongation products, ADA and ω-3 DPA, in transgenic lines from 11.2 to 24.3 and 6.3 to 11.7 mg L^{-1}, respectively. These results indicate the maximum accumulation of ADA (2.3% of total fatty acids) and ω-3 DPA (1.1% of total fatty acids) in transgenic line N64 (Table 3), 2–11 times higher than other similar studies performed by heterologous expression of multiple PUFA synthesizing genes in higher plants (0.1–1.2% of total fatty acids) (Tables 3, 4) [36–38]. This is the first report on producing an ω-3 DPA, DHA precursor, in non-seed lower plant.

Table 2 Biomass and polyunsaturated fatty acid (PUFA) production from the wild type (WT) and transgenic *P. patens* expressing *PsELO5* grown for 14 days

Line	Biomass[a] (g L^{-1})	Biomass[b] (g L^{-1})	ω-3 PUFA production (mg L^{-1})					
			ALA[a]	ALA[b]	EPA[a]	EPA[b]	ω-3 DPA[a]	ω-3 DPA[b]
WT[c]	5.53 ± 0.95	12.43 ± 0.85	9.71 ± 1.75	39.60 ± 3.75	1.29 ± 0.22	7.71 ± 0.41	0.00 ± 0.00	0.00 ± 0.00
C6[c]	4.83 ± 0.45	9.60 ± 0.63	8.67 ± 1.22	13.94 ± 1.84	1.22 ± 0.35	0.96 ± 0.15	0.00 ± 0.00	6.35 ± 0.43
N15	5.43 ± 0.45	9.37 ± 0.52	13.53 ± 2.04	20.48 ± 0.94	1.60 ± 0.18	1.83 ± 0.08	0.00 ± 0.00	10.27 ± 0.32
N64	6.00 ± 0.38	9.03 ± 0.98	13.35 ± 1.85	15.36 ± 0.87	2.07 ± 0.25	1.45 ± 0.07	0.00 ± 0.00	11.75 ± 0.41
N70	4.97 ± 0.66	9.33 ± 0.73	14.05 ± 2.65	16.20 ± 2.73	1.84 ± 0.11	1.04 ± 0.05	0.00 ± 0.00	7.60 ± 0.21
N77	5.03 ± 0.61	9.70 ± 0.57	9.55 ± 1.38	16.03 ± 3.08	1.69 ± 0.20	0.92 ± 0.12	0.00 ± 0.00	8.56 ± 0.18

Line	ω-6 PUFA production (mg L^{-1})											
	LA[a]	LA[b]	GLA[a]	GLA[b]	EDA[a]	EDA[b]	DHGLA[a]	DHGLA[b]	ARA[a]	ARA[b]	ADA[a]	ADA[b]
WT[c]	45.58 ± 8.94	210.68 ± 20.75	2.96 ± 0.54	11.96 ± 0.87	2.50 ± 0.75	10.74 ± 1.28	1.27 ± 0.37	11.85 ± 0.87	42.89 ± 5.85	196.88 ± 13.96	0.00 ± 0.00	0.00 ± 0.00
C6[c]	41.33 ± 8.48	88.68 ± 8.39	2.88 ± 0.33	9.09 ± 1.55	2.05 ± 0.66	0.90 ± 0.14	1.45 ± 0.22	2.64 ± 0.32	37.25 ± 5.33	46.85 ± 4.85	0.42 ± 0.08	11.18 ± 0.75
N15	63.24 ± 6.77	70.35 ± 12.95	4.68 ± 0.48	5.06 ± 0.74	5.80 ± 0.70	2.17 ± 0.37	1.90 ± 0.34	5.86 ± 0.54	46.08 ± 6.52	53.56 ± 3.86	4.47 ± 0.89	13.31 ± 0.84
N64	64.36 ± 5.04	144.90 ± 19.76	3.85 ± 0.37	10.86 ± 1.56	2.52 ± 0.54	0.73 ± 0.12	4.35 ± 0.54	5.21 ± 0.24	64.17 ± 7.07	73.41 ± 4.73	6.97 ± 0.74	24.31 ± 0.43
N70	69.49 ± 7.84	86.98 ± 16.85	5.67 ± 0.28	13.36 ± 1.37	3.10 ± 0.61	0.61 ± 0.08	4.25 ± 0.61	5.27 ± 0.11	50.53 ± 5.85	60.27 ± 2.79	3.57 ± 0.68	12.59 ± 0.63
N77	53.22 ± 5.83	111.51 ± 15.96	3.62 ± 0.32	14.29 ± 1.83	2.52 ± 0.44	0.28 ± 0.04	4.78 ± 0.53	4.83 ± 0.77	55.00 ± 6.22	59.83 ± 6.40	3.40 ± 0.55	16.23 ± 0.73

LA linoleic acid, *GLA* γ-linolenic acid, *EDA* eicosadienoic acid, *DHGLA* di-homo-γ-linolenic acid, *ARA* arachidonic acid, *ADA* adrenic acid, *ALA* α-linolenic acid, *EPA* eicosapentaenoic acid, *ω-3 DPA* docosapentaenoic acid

[a] PUFA production from *P. patens* grown for 14 days in basal liquid BCD medium [24]

[b] PUFA production from *P. patens* grown for 14 days in optimized liquid BCD medium [27] supplemented with a total of 1.0% vegetable oils including linseed oil, soybean oil, sunflower oil, corn oil, and palm oil

[a,c] Data from Kaewsuwan et al. [27]

Molecular Analysis

All transformants were morphologically indistinguishable from wild- type plants. Lines N15 and N64 were identified as having higher ω-3 DPA and were therefore chosen for molecular analysis of the transgene. The specific integration of pMDC43-*PsELO5* DNA into *P. patens* genome was analyzed by PCR with two different primer pairs (Fig. 2b; Table 3). For the transgenic lines (N15 and N64), primer pair A/B amplified a fragment of 0.8 kilobase pairs (kbp), which corresponds to *PsELO5* coding sequence, while primer pair C/D, amplified a fragment of 1.0 kbp corresponded to Hyg^r coding region. Neither primer pairs gave a product in the wild type (Fig. 6).

To analyze the integration patterns of transgenes, 14 day-old protonemata of transgenic lines (N15 and N64) and wild type were assessed by Southern blot analysis using probes for the *PsELO5* and Hyg^r genes (Fig. 7). The results showed that hybridization of DNA from the transgenic line N15 digested with *Eco*RV and probed with the *PsELO5* and Hyg^r probes detected strong signals of 2.9 (a) and 2.6 (b) kbp, respectively, consistent with the predicted sizes of the relevant fragments resulting from *Eco*RV digestion of pMDC43-*PsELO5*, whereas weak signals of similar sized DNA were obtained from transgenic line N64. This indicates that line N64 has fewer copies of the transgene than line N15. Digestion with *Nco*I which cuts at 625 bp site within *PsELO5* cDNA, produced a high intensity band of 1.1 kbp (*) in transgenic line N15 when hybridized with *PsELO5* probe, while a lower intensity band was detected in line N64. An additional fragment of 10.5 kbp (**) which is the predicted size of fragments generated by *Nco*I from multiple tandemly repeated copies of pMDC43-*PsELO5*, hybridized with either *PsELO5* or Hyg^r probes. However, hybridization with these probes from transgenic line 15 produced the several additional DNA fragments, whereas only one of the flanking sequences was detected in line N64 (arrowed). From these results we suggest that line N64 contains only a single copy of the expression construct in, whereas there are either multiple copies with rearrangements or several insertion sites of the construct in line N15.

Therefore both single copy and multiple copies of plasmid DNA containing the full length of *PsELO5* gene were not only integrated into the transgenic *P. patens* genome and stably inherited to its culture, but also led to successful functional expression. The effects of transgenic copy number on the level of gene expression are known to be complex. Though it was anticipated that the increase of transgene copy number would increase expression level [39, 40], it is now known that gene co-suppression phenomena frequently occur in transgenic plants with repeated transgene or an unusual structure such as inverted repeats

Table 3 Total fatty acid and new C_{22} PUFA production from the wild type (WT) and transgenic *P. patens* expressing *PsELO5* grown for 14 days

Line	FA production (mg L^{-1})					Total FA production (mg L^{-1})		C_{22} PUFA (% of total FAs)			
	SA[a]	SA[b]	MUFA[a]	MUFA[b]	PUFA[a]	PUFA[b]	Total[a]	Total[b]	ADA[a]	ADA[b]	ω-3 DPA[a]	ω-3 DPA[b]
WT[c]	86.45 ± 11.71	386.27 ± 13.73	15.80 ± 1.93	135.08 ± 5.34	106.13 ± 26.62	489.69 ± 36.13	208.38 ± 37.93	1011.04 ± 393.11	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
C6[c]	82.22 ± 14.08	349.83 ± 22.39	9.79 ± 1.52	241.39 ± 20.03	94.96 ± 19.55	180.83 ± 33.47	186.97 ± 15.96	772.05 ± 33.04	0.22 ± 0.03	1.46 ± 0.33	0.00 ± 0.00	0.82 ± 0.16
N15	83.22 ± 11.60	590.67 ± 12.81	13.57 ± 1.54	184.85 ± 4.48	141.51 ± 30.52	183.17 ± 38.83	238.30 ± 17.45	958.70 ± 22.87	1.87 ± 0.30	1.39 ± 0.23	0.00 ± 0.00	1.07 ± 0.17
N64	88.16 ± 10.90	465.43 ± 22.99	17.14 ± 2.21	284.43 ± 39.24	161.71 ± 13.73	288.06 ± 56.86	267.01 ± 0.88	1037.91 ± 60.88	2.61 ± 0.31	**2.33 ± 0.32**	0.00 ± 0.00	**1.13 ± 0.22**
N70	92.04 ± 10.31	322.67 ± 10.43	9.76 ± 1.80	253.90 ± 16.90	152.57 ± 28.92	204.27 ± 50.38	254.37 ± 20.34	780.84 ± 52.98	1.39 ± 0.16	1.62 ± 0.29	0.00 ± 0.00	0.97 ± 0.15
N77	80.22 ± 19.17	544.48 ± 15.01	11.33 ± 2.37	289.24 ± 16.26	133.78 ± 4.25	232.73 ± 22.52	225.34 ± 16.5	1066.45 ± 15.95	1.51 ± 0.07	1.52 ± 0.26	0.00 ± 0.00	0.80 ± 0.10

SA saturated fatty acid, *MUFA* monounsaturated fatty acid, *PUFA* polyunsaturated fatty acid, *ADA* adrenic acid, *ω-3 DPA* docosapentaenoic acid

[a] PUFA production from *P. patens* grown for 14 days in basal liquid BCD medium [24]

[b] PUFA production from *P. patens* grown for 14 days in optimized liquid BCD medium [27] supplemented with a total of 1.0% vegetable oils including linseed oil, soybean oil, sunflower oil, corn oil, and palm oil

[a,c] Data from Kaewsuwan et al. [27]

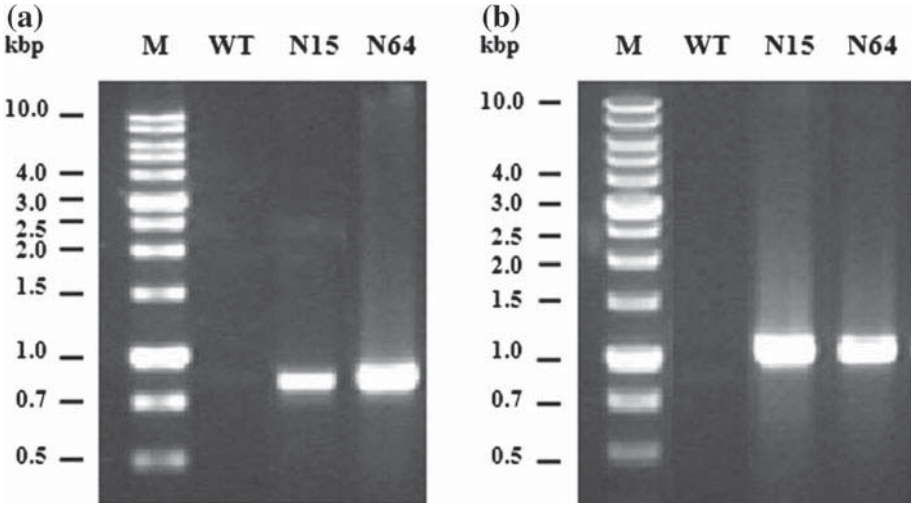

Fig. 6 Verification of *PsELO5* (**a**) and hygromycin resistance (Hyg^r) (**b**) genes by PCR amplification of *P. patens* wild type (WT) and transgenic lines (N15 and N64). The DNA sizes in kbp are indicated on the *left*

Table 4 Examples of polyunsaturated fatty acid (PUFA) production in transgenic plants

Target gene	Plant host	New fatty acid production (% of total fatty acid)	Reference
Danio rerio Δ^5/Δ^6-desaturase, *Caenorhabditis elegans* Δ^6-elongase, *Pavlova salina* Δ^5-elongase and *P. salina* Δ^4-desaturase	*Arabidopsis thaliana*	GLA (0.4%), SDA (1.5%), DHGLA (1.5%), ETA (0.8%), ARA (1.0%), EPA (2.4%), ω-3 DPA (0.1%), DHA (0.5%)	[36]
Micromonas pusilla Δ^6-desaturase, *Pyramimonas cordata* Δ^6-elongase[a] and *Pavlova salina* Δ^5-desaturase	*Nicotiana benthamiana*	GLA (2.1%), ARA (0.6%), SDA (1.5%), ETA (0.6%), EPA (10.7%), ω-3 DPA (0.3%)	[37]
Echium plantagineum Δ^6-desaturase, *Pyramimonas cordata* Δ^6-elongase[a] and *Pavlova salina* Δ^5-desaturase	*Nicotiana benthamiana*	GLA (4.4%), ARA (1.3%), SDA (2.4%), ETA (0.1%), EPA (3.4%), ω-3 DPA (0.2%)	[37]
Ostreococcus tauri Δ^6-desaturase, *Pyramimonas cordata* Δ^6-elongase[a] and *Pavlova salina* Δ^5-desaturase	*Nicotiana benthamiana*	GLA (5.8%), ARA (1.2%), SDA (1.1%), ETA (0.4%), EPA (9.6%), ω-3 DPA (0.2%)	[37]
Pavlova salina Δ^9-elongase, Δ^8-desaturase, Δ^5-desaturase and Δ^5-elongase and Δ^4-desaturase	*Nicotiana benthamiana*	EDA (1.7%), DHGLA (0.5%), ARA (2.4%), ADA (1.2%), ETrA (1.5%), ETA (0.2%), EPA (1.2%), ω-3 DPA (0.6%), DHA (0.7%)	[38]
Pavlova sp. Δ^5-elongase	*Physcomitrella patens*	ADA (2.3%), ω-3 DPA (1.1%)	Current study

SDA stearidonic acid, *GLA* γ-linolenic acid, *ARA* arachidonic acid, *ETA* eicosatetraenoic acid, *EPA* eicosapentaenoic acid, *ω-3 DPA* docosapentaenoic acid, *EDA* eicosadienoic acid, *DHGLA* di-homo-γ-linolenic acid, *ADA* adrenic acid, *ETrA* eicosatrienoic acid, *DHA* docosahexaenoic acid

[a] *P. cordata* Δ^6-elongase displays some Δ^5-elongase activity

[41]. Moreover, an increased copy number of transgene can correlate with an increased risk of silencing [42]. Our transgenic line with single copy number (N64) improved C_{22}-PUFA production rather more than the line with multiple copies of transgene (N15), suggesting that lower copy number may permit potentially greater gene expression since multiple copy number integration could inhibit transgene expression and even lead to transgene silencing in transgenic plants [43]. However, we did not control the chromosomal insertion site and so the differences may result from position effects. Future work will investigate this.

Conclusions

The efficient biosynthesis of C_{22} LC-PUFAs in transgenic *P. patens* plants has now been conclusively demonstrated, using reverse-engineering and nutritional supplementation approaches. Conversion of fatty acid precursors from vegetable oils to high-value oil may prove to be an economically viable option for the industry.

P. patens is therefore as an alternative sustainable source of DHA precursor for human consumption. We are confident that the results obtained will be useful in the engineering of stably transformed genes in land plants.

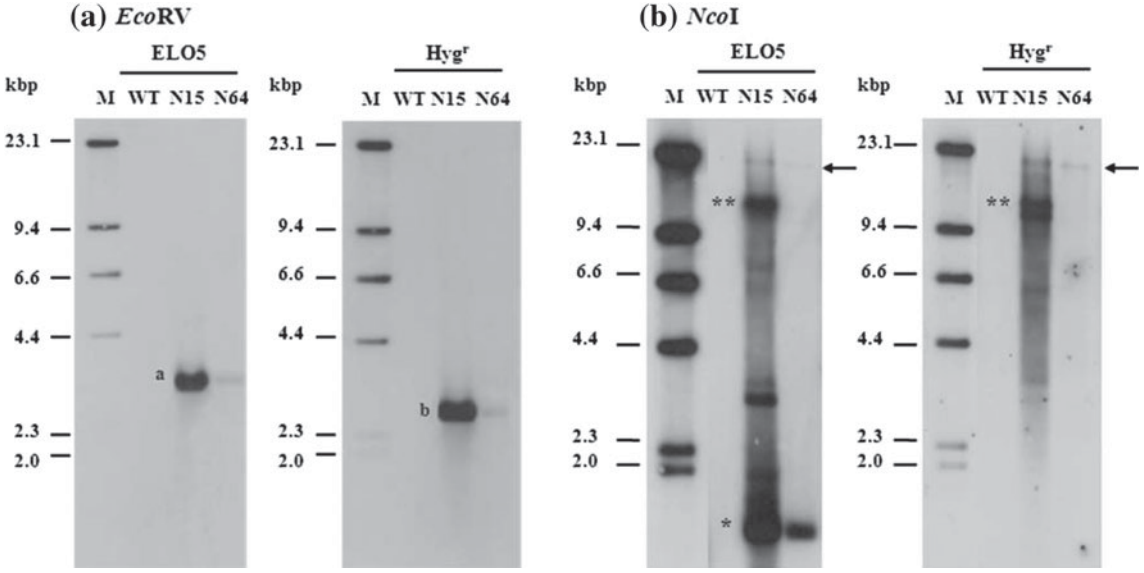

Fig. 7 Southern blotting of *P. patens* wild type (WT) and transgenic lines (N15 and N64). Genomic DNA from wild type and transgenic lines was digested with *Eco*RV (**a**) and *Nco*I (**b**) and hybridized with *PsELO5* (ELO5) and hygromycin resistance (Hyg^r) probes. The DNA sizes in kbp are indicated on the *left*. The *letters* and *asterisks* indicate signals predicted. *Arrows* indicate one of the flanking sequences detected

This heterologous expression system has not only realized the possibility of producing the important nutritional compounds in transgenic plants, but also provided a new experimental tool with which to better investigate plant lipid metabolism.

Acknowledgments We gratefully acknowledge Pierre-Francois Perroud, our colleague in the Quatrano laboratory for technical assistance. This work was supported by the research fund from the Thailand Research Fund (TRF, MRG5380182), the International Foundation for Science (IFS, Grant No.F/4618-1), the Higher Education Research Promotion and National Research University Project of Thailand, Office of the Higher Education Commission (PHA5405395) and the Marine Natural Products Research Unit (MNP) at the Faculty of Pharmaceutical Sciences, Prince of Songkla University (PSU), Thailand.

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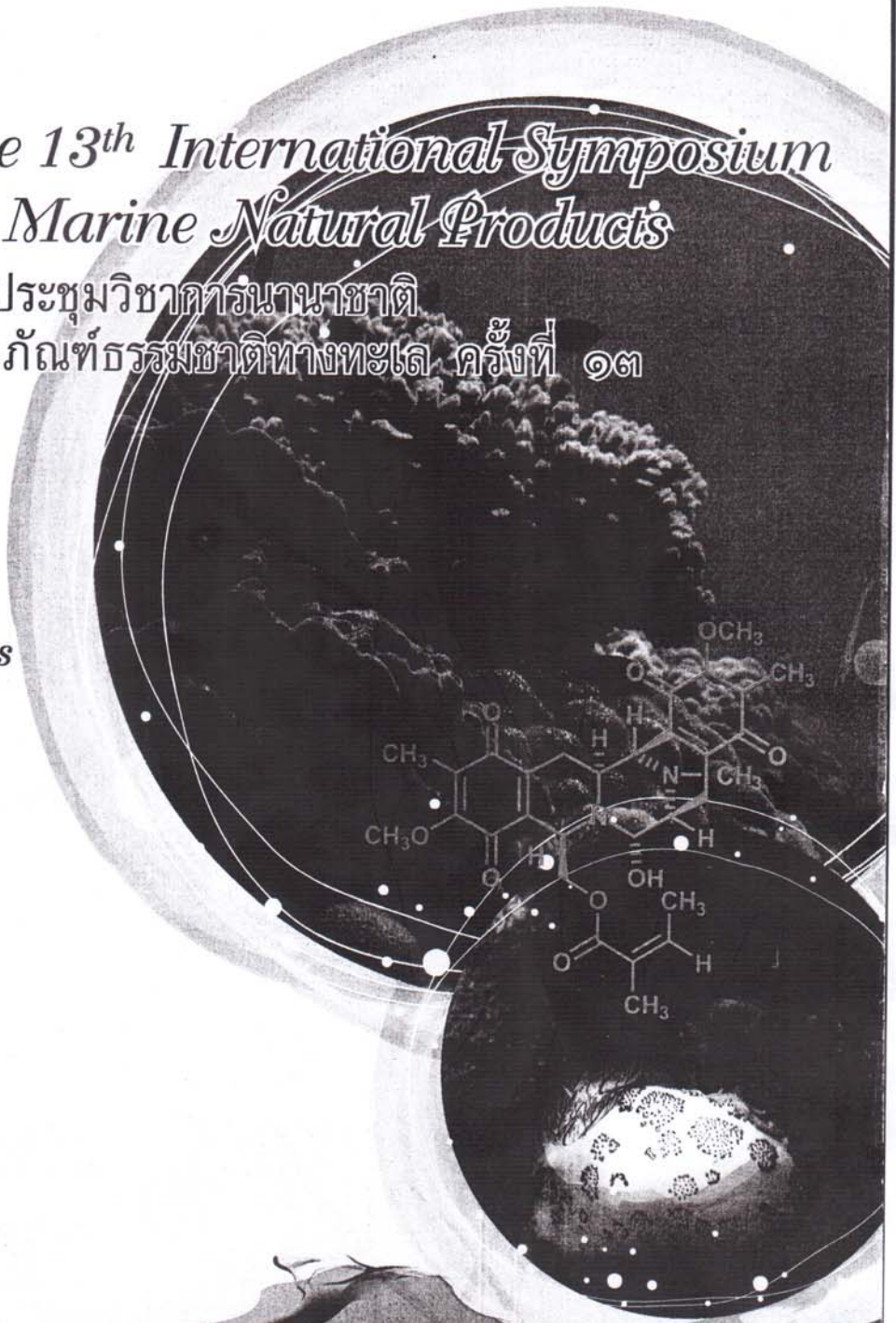

Programme and Abstracts

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PP-22

ACTIVATION OF DOCOSAHEXAENOIC ACID (DHA) PRECURSOR IN THE MOSS *PHYSCOMITRELLA PATENS* BY MARINE ALGAE *PAVLOVA* SP. Δ^5-ELONGASE AND VEGETABLE OIL SUPPLEMENTS

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Docosahexaenoic acid acid (DHA), an ω-3 C_{22} polyunsaturated fatty acid (PUFA), has attracted much interest due to its pharmaceutical potential. It is synthesized from eicosapentaenoc acid (EPA), an ω -3 C_{20} PUFA via a series of reactions catalyzed by Δ^5-elongase and Δ^4-desaturase enzymes. Genetic engineering the plants to produce foreign fatty acids is therefore much interested for establishment the sustainable and economical alternative sources. In this study, a marine algae *Pavlova* sp. Δ^5-elongase involved in the biosynthesis of C_{22} PUFAs was targeted to enable production of DHA precursor in the moss *Physcomitrella patens* containing endogenous EPA. Heterologous expression of this gene under the control of a tandemly duplicately 35S promoter together with vegetable oil supplements resulted in the production of 24.31 mg/l of adrenic acid (ADA), an ω -6 C_{22} PUFA, and 11.75 mg/l of docosapentaenoic acid (DPA), aṇ ω -3 C_{22} PUFA , in transgenic *P. patens*. The latter fatty acid is a remarkable DHA precursor. In addition, PCR and southern blotting experiments also showed that a marine algae *Pavlova* sp. Δ^5-elongase was transferred into *P. patens* genome. Hence it was successfully activation of DHA precursor in *P. patens* for further DHA production.

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pointed out by environmental analysis, are selected with intention of addressing the main issues, and attempting to delineate some peculiar policies and market intervention for overcoming the actual nodal points.

Email Address for correspondence: leksinaj@libero.it
Poster Session 17:00 – 19:00 Friday 2nd July 2010

P4.22

Vectors for genetically modified crop development

Sofia Moriam (Khulna University)

Until 1950s scientist were using conventional breeding system for modification of crop species. Although the scientists were successful enough for modification of crops but the problem is for developing a variety it nearly takes 10-12 years. Another problem is that the rapidly growing population in the world. The conventional breeding systems were not enough to meet the needs of this vast population of the world. That's why scientist started to seek for a better option that could meet the needs of this vast population in a better way. After conducting many research scientists first develop transgenic plant in 1983 by using a cloning vector named Ti plasmid. After the development of Ti plasmid vector scientist has develop lots of cloning vector besides Ti plasmid viz. pUC vector, binary and superbinary vector, co-integrated vector, yeast plasmid vector, BACs, YACs, phages, cosmids, transposon etc, for production of GM crops. This review will discuss about all the vectors that are being using to date .Their techniques of manipulation, feasibility of using them as a vector. Because although proponents of genetic engineering emphasize the benefits of more efficient production processes, improved resistance of plants and animals to diseases, higher nutritional quality and better palatability of human food, and reduced dependence on non-renewable resources, such applications are also giving rise to serious concerns about the ethical issues. That's why this review also discusses about the problem associated with the use of cloning vector for growing GM crops.

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Poster Session 17:00 – 19:00 Friday 2nd July 2010

P4.23

Observations on the traditional phytotherapy among the inhabitants of Raipur Upazila in Lakshmipur district, Bangladesh

Md. Ariful Haque Mollik (Peoples Integrated Alliance), A.B.M. Abul Hossain (Peoples Integrated Alliance), Romeo McField (Peoples Integrated Alliance), A.K.M. Fahmidul Haque (SHACO-Health Education Society), Israt Jahan Mukti (Biogene Life Care), Md. Ikhtiar Zahid (North South University)

The rural population of Bangladesh relies primarily on traditional healers for cure of various ailments. The traditional healers in turn possess considerable knowledge of the medicinal properties of plants. Each traditional healer, although following general Ayurvedic principles of medicine, also has his own formulations, which may thus vary between various regions of the country. We accordingly conducted an ethnomedicinal survey of traditional healers in the Raipur Upazila, Lakshmipur district, Bangladesh to collect information on plants used by them for cure of various diseases. Interviews were conducted with the help of a semi-structured questionnaire, and plant samples as pointed out by the traditional healers were photographed and individual specimens identified at the Bangladesh National Herbarium. A total of seventy-two plants distributed into forty-nine families were observed to be used by the traditional healers. Various plant parts were used in the medicinal formulations. Leafs formed the part of the plant most frequently used; however, a number of formulations also made use of roots, barks, flowers, and seeds. Cumulatively, the plants were used to treat a diverse variety of ailments including edema, leucorrhea, syphilis, gonorrhea, dermatitis, alopecia, burns, wounds, coughs, fevers, leprosy, rheumatoid arthritis, impotency, irregular menstruation, tumor, cancer, hepatic disorders, cardiovascular disorders, eye diseases, earache, toothache, indigestion, parasitic infections, constipation, diabetes, malaria, tuberculosis, tetanus, paralysis, and snake-bites. Our survey indicated that patients demonstrated a large degree of satisfaction in treatment with plants. The plants, therefore, possess considerable potential for scientific studies, which may lead to discovery of novel pharmacological compounds.

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Poster Session 17:00 – 19:00 Friday 2nd July 2010

P4.24

High level production of adrenic acid (ADA) in transgenic moss *Physcomitrella patens*

Songsri - Kaewsuwan (Prince of Songkla University), Nantavan Bunyapraphatsara (Mahidol University), David J Cove (Washington University in St. Louis), Ralph S Quatrano (Washington University in St. Louis), Pichit Chodok (Prince of Songkla University)

Adrenic acid (ADA), an ω-6 polyunsaturated fatty acid (PUFA), has attracted much interest due to its pharmaceutical potential. Exploiting the wealth of information currently available on *in planta* oil biosynthesis, and coupling this information with the tool

of genetic engineering, it is now feasible to deliberately alter fatty acid biosynthetic pathways to generate unique oils in commodity crops. In this study, a Δ^5-elongase gene from the algae *Pavlova* sp. related to the biosynthesis of C_{22} PUFAs was targeted to enable production of ADA in the moss *Physcomitrella patens*. Heterologous expression of this gene was under the control of a tandemly duplicate 35S promoter. It was established that ADA (0.42 mg/l) was synthesized in *P. patens* from endogenous arachidonic acid (ARA) via the expressed *Pavlova* sp. Δ^5-elongase in the moss. In an attempt to maximize ADA production, medium optimization was effected by the response surface methodology (RSM), resulting in a significant elevation of ADA (4.51 mg/l) production under optimum conditions. To the best of our knowledge, this is the first study describing the expression of a PUFA synthesizing enzyme in non-seed lower plant without supplying the exogenous fatty acid.

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Poster Session 17:00 – 19:00 Friday 2nd July 2010

P4.25

Brownfield or greenfield? An ecological assessment of a vulnerable greenfield site

Stuart Norris (University of Plymouth), John N Bull (University of Plymouth), Stuart D Lane (University of Plymouth)

There is current debate over redevelopment of sports facilities for industrial development. This study examines a former playing field complex adjoining the River Tamar - a Special Area of Conservation - in Devon. The site lies beside an existing County Wildlife Site (CWS) but is a suggested location for a waste incinerator. The investigation aimed to clarify whether the area meets established CWS standards and its biodiversity value. To determine current ecological status of the area, comprehensive vegetation and seed bank surveys were undertaken alongside collection of environmental data. Results suggested that a significant, previously unsurveyed, part of the site could meet CWS criteria and should be included in the existing CWS. The remainder of the site does not currently reach required criteria but should be included in the CWS as its seed bank could allow restoration of the area to a characteristic vegetative community assemblage through minor ecological management. Although some areas of the site are currently in a degraded state, as a whole it provides a biodiversity refuge on the outskirts of the Plymouth and buffers the adjacent Tamar Valley Area of Outstanding Natural Beauty, Special Protection Area and Special Area of Conservation from the impacts of development. Development of this environmentally sensitive area for waste management would undoubtedly impact on local biodiversity and conservation value. Sites that are taken out of use can easily be considered of low biodiversity value, but this study confirms that every site needs full consideration of its environmental merits.

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Poster Session 17:00 – 19:00 Friday 2nd July 2010

P4.26

Leaf anatomical characteristics of *Acer negundo L. and A. pseudoplatanus L.* grown in air polluted urban conditions

Marina P Macukanovic-Jocic (Faculty of agriculture), Branka B Stevanovi263 (Institute of Botany and Botanical Garden ''Jevremovac'' Faculty of biology)

Ecoanatomical investigations of the introduced boxelder (*Acer negundo L.*) and autochtonous sycamore (*A. pseudoplatanus L.*), commonly grown along the streets and parks in Belgrade urban environment, were carried out. The investigations were concerned with the leaves structure injuries of both species analyzed from the downtown localities and from the vicinity of Belgrade respectively, during the vegetation season, in order to establish their vitality and ability to survive in polluted urban areas.
Numerous irregular marginal and interveinal necrotic and chlorotic blotches were evident on boxelder expanded leaves on both localities.With regard to anatomical leaf structure, epidermis showed injury simptoms due to cell death, layers of mesophyll were disordered, and many cells of spongy, more rarely of palisade parenchyma, were either injured or their shape and content changed. At the beginning of summer injury symptoms on the epidermal cells and deformations of mesophyll tissue cells were evident on sycamore leaves in the air polluted city localities.
A. pseudoplatanus exhibited considerable resistance to synergistic effects of air pollutants and general climatic and microclimatic conditions in relation to *A. negundo*. In contempt of injury symptoms appearing already on the young leaves, sycamore retained the unaltered ability of renewal, unlike *A. negundo* which is less tolerant on unfavourable urban environmental stresses, reflected in obvious thinning and decrement of tree crown.

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Poster Session 17:00 – 19:00 Friday 2nd July 2010

P4.27

Identification and expression analysis of CBF/DREB1 and COR15a in dehydration stress resistant mutants of cauliflower (*Brassica oleraceae* v. *botrytis*)

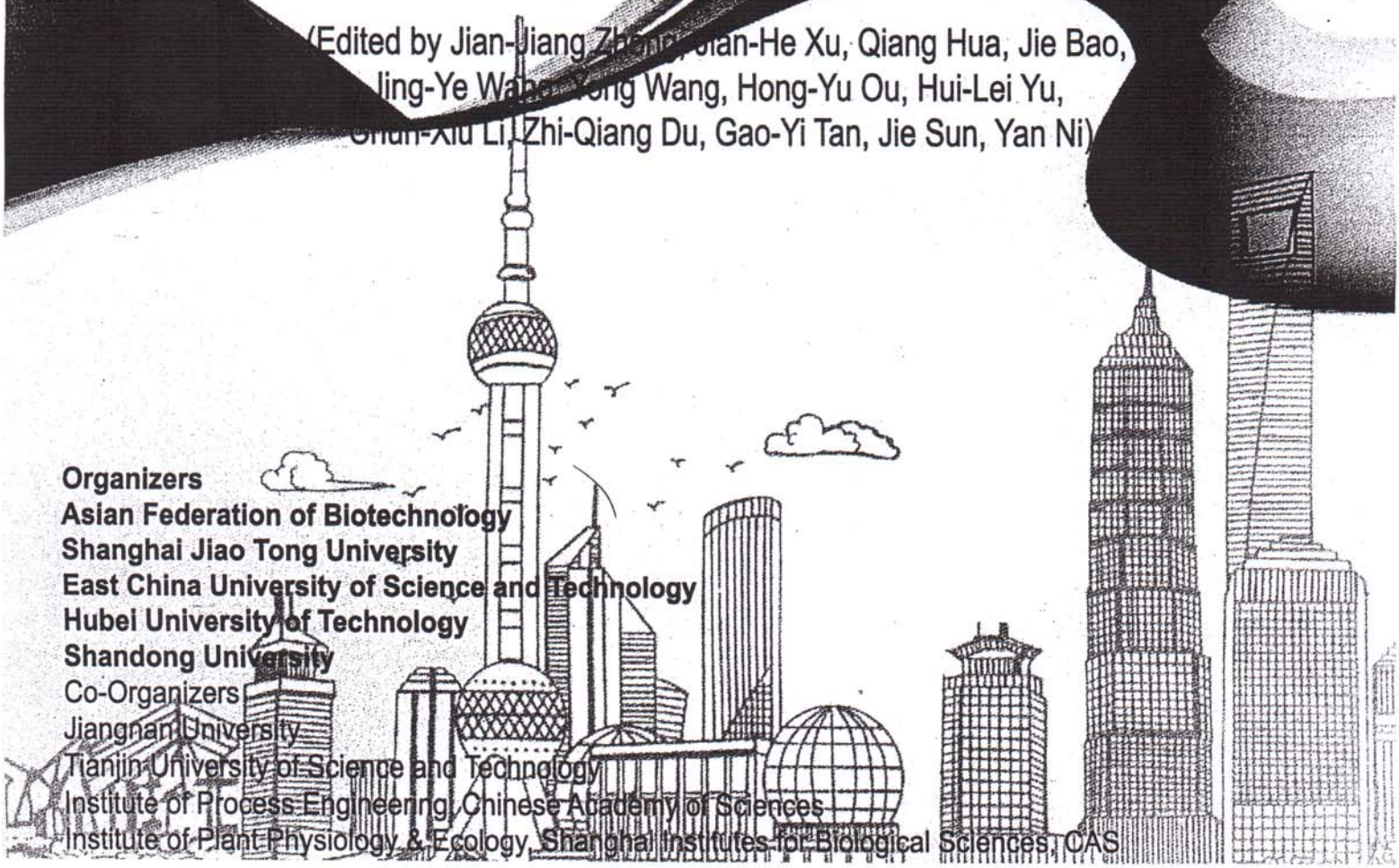
ACB-2011

Asian Congress on Biotechnology

May 11-15, 2011 • Shanghai

Book of Abstracts

(Edited by Jian-Jiang Zhong, Jian-He Xu, Qiang Hua, Jie Bao,
Ling-Ye Wang, Dong Wang, Hong-Yu Ou, Hui-Lei Yu,
Chun-Xiu Li, Zhi-Qiang Du, Gao-Yi Tan, Jie Sun, Yan Ni)



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Pregnancy gingivitis is a common disease for pregnant women and it is not suitable for treating with chemical medicine. *Prevotella intermedia* (*P. intermedia*) is the main gram-negative anaerobe associated with hormone-induced pregnancy gingivitis. The objective of this study was to prepare specific egg yolk immunoglobulin (IgY) against pregnancy gingivitis -causing *P. intermedia* and evaluate its activity *in vitro*. Laying hens were immunized with formaldehyde killed *P. intermedia*. IgY was isolated and purified from egg yolks by water dilution, salt precipitations and ultrafiltration. Enzyme-linked immunosorbent assay indicated that the titer of specific IgY reached to peak value (6400) between 40-day to 90-day after the first immunization. Growth inhibition of specific IgY at various concentrations (0.625 mg/ml, 1.25 mg/ml, 2.5 mg/ml, 5 mg/ml, 10 mg/ml, 20 mg/ml) to *P. intermedia* was tested in CDC liquid culture medium using tinidazole (1.5 mg/ml) as a positive control. Specific IgY inhibited the growth of *P. intermedia* at a dose-dependent manner. Inhibition activity of 20 mg/ml IgY was similar to that of tinidazole which inhibited the growth of bacterial completely. This result indicates the potential of specific IgY to be an alternative therapy for pregnancy gingivitis or other periodontal diseases correlating with *P. intermedia*.

I1012

Therapeutic Potential of Specific IgY to Lipopolysaccharide-induced Endotoxemia in a Murine Model

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Lipopolysaccharide (LPS) from the outer membrane of Gram-negative bacteria is a major cause of endotoxemia, a serious clinical problem with high mortality. The objective of this study was to estimate the efficacy of specific egg yolk immunoglobulin (IgY) to LPS induced endotoxemia in a murine model. IgY obtained from yolk of hens immunized with formaldehyde killed *E. coli* O111 showed high binding activity to LPS by enzyme-linked immunosorbent assay (ELISA). Endotoxemia was induced in mice by intraperitoneal injection of LPS at a dosage of 20 mg/kg for measuring survival percentage and at a dosage of 10mg/kg for cytokines measure. The survival percentages of mice treated with 200 mg/kg specificIgY was 70%, same to that of mice treated 5 mg/kg Dexamethasone (Dex) while that of normal saline (NS) treatment group was zero within 7 days. Specific IgY could significantly ($p < 0.05$) decrease tumor necrosis factor-α (TNF-α) level and increase IL-10 level in the serum of endotoxemia mice. The effect of specific IgY on decreasing TNF-α was similar with that of Dex, but that on increasing interleukin-10 (IL-10) was stronger than Dex. H&E-stained sections indicated IgY could attenuate the damnify in lung and liver of endotoxemia mice. These results showed the therapeutic potential of specific IgY to lipopolysaccharide-induced endotoxemia in murine model.

I1104

Evaluation of Culture Conditions for the Production of Polyunsaturated Fatty Acids (PUFAs) by *Physcomitrella patens* Using the Plackett-Burman Statistical Design Protocols

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Identification of the parameters that had significant effects on polyunsturated ftt acids (PUFAs) and biomass production by the moss *Physcomitrella patens* was performed using nine culture variables (temperature, agitation speedm pH, sucrose, di-ammonium tartrate, $CaCl_2.2H_2O$, $MgSO_4.7H_2O$, KH_2PO_4 and KNO_3) with the statistical design technique of the Plackett-Burman. Statistical analysis revealed that two physical variables (pH and temperature) had significant effects on the production of both biomass and PUFAs (linoleic acid, LA; gamma-linolenic acid, GLA; alpha-linolenic acid, ALA; eicosadienoic acid, EDA; di-homo-gamma-linolenic acid, DHGLA; arachidonic acid, ARA; eicosapentaenoic acid, EPA). Three nutritional variables (sucrose, $CaCl_2$ and $MgSO_4$) had an influence only on the production of some of the PUFAs. Of the two levels used, higher concentrations of sucrose has a positive effect on LA, ARA and EPA production, whereas higher concentrations of the metal ions ($CaCl_2$ and $MeSO_4$) had a negative effect only on ARA and EPA production. After the adjustment by multiple linear regression, it can be concluded that pH, temperature, sucrose, $CaCl_2$ amd $MgSO_4$ were the most significant parameters for the growth and PUFA production of *P. patens*.

I1135

Quantitative Comparison of Bone Growth Behavior in Different Type Particulate for Dental Application

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