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Metabolic engineering of fatty acid biosynthesis in *Chlorella vulgaris* using an endogenous omega-3 fatty acid desaturase gene with its promoter

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A B S T R A C T

This study demonstrated the overexpression of omega-3 fatty acid desaturase (*ω-3 FAD*) gene under the control of its endogenous promoter and to elucidate its effect on fatty acid biosynthesis pathway in transgenic *Chlorella vulgaris*. Two stable transgenic lines (Ch-TL1 and Ch-TL2) were selected and cultured under nitrate-replete and nitrate-deficient BBM culture conditions, respectively to determine the growth, biomass production, total oil content, fatty acid composition and the expression level of four fatty acid biosynthetic genes of the transgenic *C. vulgaris*. Results revealed that both transgenic lines and wild-type displayed similar growth patterns. Higher biomass production and total oil content were recorded for both transgenic lines that were cultured under nitrate-deficient condition. The *ω-3 FAD* gene expression was consistently up-regulated in Ch-TL2 in all culture conditions which led to higher α-linolenic acid (C18:3n3) content. Similarly, the expression of *β-ketoacyl ACP synthase I* (*KAS I*), *stearoyl-ACP desaturase* (*SAD*) and *omega-6 desaturase* (*ω-6 FAD*) genes were also up-regulated in Ch-TL2. However, the accumulation of palmitic (C16:0), stearic (C18:0), oleic (C18:1) and linoleic (C18:2) acids were found to be differentially regulated at either transcriptional or post-transcriptional levels. PCR-base genome walking of Ch-TL2 genomic DNA successfully elucidated the integration sites and flanking regions of the inserted vector cassette. These findings provide valuable insight that could pave way for further genetic improvement of the *C. vulgaris* for various downstream applications.

1. Introduction

To date, the edible oil is vegetable-based that was derived from different plant sources such as soybean, rapeseed, palm, corn, coconut and olive oils. However, these oil producing crops are only able to synthesize polyunsaturated fatty acids (PUFAs) with carbon chain-length of up to C18, which includes linoleic (LA; C18:2) and α-linolenic (ALA; C18:3n3) acids. The incapability of higher plants to synthesize PUFAs with chain-length beyond C18, in particular the eicosapentaenoic (EPA; C20:5n3) and docosahexaenoic (DHA; C22:6n3) acids is attributed to the lack of specific endogenous elongases and desaturases that targets fatty acid with carbon chain-length C18 and longer [1]. Research studies clearly demonstrated that both EPA and DHA possess significant therapeutic value in clinical and epidemiological studies [2]. The omega-3 long-chain PUFAs (LC-PUFAs) are essential elements for normal composition of sperm, retina and brain lipids as well for optimal maturation of visual and cortical function in pre-term infants. LC-PUFAs also stimulate growth and protect the development of cardiovascular health [3]. Besides that, it also serves to prevent diseases like rheumatoid arthritis and ulcerative colitis [4], atopic dermatitis and psoriasis [5] as well as hinder hypertension by lowering the blood pressure [6]. In addition, both EPA and DHA were also observed to decrease the size and number of tumors [7] and reported to lower the risk of breast cancer in women [8]. Currently, fish oil is the major source for the commercial production of PUFAs. The various health and therapeutic potentials of EPA and DHA on human have escalated the exploitation of marine fish oils to meet the ever-increasing demand of rising human population, which is expected to reach nine billion by 2050 [1,9]. Over-exploitation of marine resources has caused great concern on the issues of food security, marine biodiversity and ecosystem protection [1]. The quality of fish oil is dependent on several factors such as the fish species, climate, geographical location of catching sites, the consumed fish food quality and the risk of contamination by heavy metals and other industrial wastes [1]. The application of fish oil PUFAs in foods, such as in infant formulas, or for pharmaceutical uses, may have some shortcomings due to risk of aforementioned contamination as well as the fishy smell and unpleasant taste that the fish oil carries. Furthermore, purification of PUFAs from

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low-grade crude fish oil is expensive and relatively difficult due to its complex mixture of fatty acids with varying lengths and degrees of unsaturation [10].

Microalgae constitute a vast group of photoautotropic organisms which is well known for their capability to produce various natural products such as pigments, proteins, lipids, enzymes, sugars, amino acids, vitamins and unusual bioactive compounds [10]. In recent years, the presence of high lipid content in diverse groups of microalgae has been a great impetus for intensive research that can hopefully fulfil the increasing demand from edible and non-edible oil industries [9]. Oleaginous microalgae with > 20% of lipid content are well recognized as potential candidates to produce single cell oil for biodiesel conversion [11]. LC-PUFA producing species from groups such as Cryptophyta, Haptophyta, Heterokontophyta and Dinophyta are potential candidates as an alternative to EPA and DHA production from marine fish source [1,12]. The use of microalgae as a source for LC-PUFAs is promising due to the fast growing rate, ease of production and abundance of low cost raw materials for growth such as sunlight, water, carbon dioxide and nutrients [12]. Microalgae from the group Chlorophyta, such as *Chlorella* is one of key player for large-scale commercial production for various products in pharmaceutical and nutraceutical industries [13,14]. In addition, various *Chlorella* strains are also regarded as attractive for biodiesel production due to their high lipid content and favorable fatty acid composition which is rich in saturated (e.g., palmitic acid; C16:0) and monounsaturated (e.g., oleic acid; C18:1) fatty acids [15].

Despite of the existing technological advancements, the major drawback for *Chlorella* strains is their incapability to produce EPA and DHA. The main PUFA produced by Chlorophyta such as *C. vulgaris*, *Chlorella sorokiniana* and *Chlorella zofingiensis* are C18:2 and C18:3n3 which are very similar to major oilseeds crops [16,17]. The *C. vulgaris* strain UMT-M1 used in this research was reported to produce higher amount of C18:2 (> 20%) as compared to C18:3n3 (< 7%) in several different culture conditions [18–20]. In order to enhance PUFA production, it is essential to characterize genes encoding key enzymes that are directly involved in the biosynthesis of PUFA, in particular the desaturases and elongases. The ω-3 fatty acid desaturase (ω-3 FAD) enzyme takes part in the desaturation reaction of C18:2 to C18:3n3. The C18:3n3 is the precursor for the synthesis of EPA and DHA [21]. The relatively low accumulation of C18:3n3 in the species could be a hindrance that limit further effort to overexpress relevant downstream desaturases and elongases for EPA and DHA productions. Therefore, this study aimed to reconstitute the fatty acid biosynthesis pathway in *C. vulgaris* strain UMT-M1 by overexpressing the ω-*3 FAD* gene driven by its own endogenous promoter in the effort to increase the C18:3n3 content.

2. Materials and methods

2.1. Materials and culture conditions

Chlorella vulgaris strain UMT-M1 [22] used in this study was obtained from the microalgae stock culture at Institute of Marine Biotechnology, Universiti Malaysia Terengganu, Malaysia. A single colony of the microalga strain was picked from the stock agar plate and subcultured into liquid Bold's Basal Medium (BBM) [23]. The microalga culture was placed under continuous illumination of T5 fluorescent light (Philips) at 23–25 °C. A portion of the culture was replaced with fresh medium every month to maintain cell growth.

Agrobacterium tumefaciens strain LBA4404 harbouring the expression vector pO3DPro-VF3 was initiated from frozen glycerol stock into Luria-Bertani (LB) broth supplemented with rifampicin (50 μg mL^{-1}) and kanamycin (50 μg mL^{-1}) and then cultivated at 27.5 °C together with agitation at 200 rpm in dark. After 48 h, the bacteria culture was employed for transformation according to previous study [22].

2.2. Construction of the transformation vector pO3DPro-VF3

Fragments of the full-length ω-*3 FAD* gene cDNA (Genbank Acc. No: EU100100) and its promoter (KX100035) were inserted into the multiple cloning site of the binary vector pCAMBIA 1304 (www.cambia.org) at the *Sma*I and *Xba*I restriction enzyme sites (Supplementary Fig. S1). Firstly, the full-length ω-*3 FAD* gene cDNA (1957 bp) and promoter (1836 bp) fragments were amplified using primer pairs O3D-5F/O3D-3R and O3D-VF3/O3D-VR1 (Supplementary Table S1), respectively. The gene-specific forward primer, O3D-5F was designed at the 5′-UTR by replacing three single nucleotides of the wild-type sequence to create a *Bam*H1 restriction site in the middle of the primer. The gene-specific reverse primer, O3D-3R was designed at the end of 3′-UTR with an anchored *Xba*I restriction site. On the other hand, the promoter-specific forward primer, O3D-VF3 was anchored with *Dra*I restriction site. While the promoter-specific reverse primer, O3D-VR1 was the reverse complement of the O3D-5F, which also bare the same *Bam*HI restriction site. The *Bam*HI restriction site served as joining site for both promoter and full-length cDNA fragment of the ω-*3-FAD*. PCR reactions were carried out in a total volume of 25 μL containing: 2.5 μL of 10× *Pfu* Buffer, 0.5 μL of dNTPs mix (10 mM), 1.0 μL each of forward (10 μM) and reverse (10 μM) primers, 0.5 μL of diluted plasmid DNA (containing promoter/cDNA fragments), 0.25 μL of *Pfu* DNA polymerase (2.5 U μL^{-1}) from Fermentas, and sterilized deionized water to the final volume of 25 μL. PCR amplifications were performed with initial denaturation at 95 °C for 3 min, followed by a 40 cycles of denaturation at 95 °C for 30 s; annealing at 65 °C for 30 s, extension at 72 °C for 2 min and a final extension at 72 °C for 7 min.

Secondly, the full-length ω-*3 FAD* gene cDNA was double-digested with *Bam*HI/*Xba*I and ligated with *Bam*HI/*Xba*I double-digested pCAMBIA 1304 vector to generate recombinant plasmid pO3D-FL. Finally, the *Dra*I/*Bam*HI double-digested promoter fragment was ligated with *Sma*I/*Bam*HI double-digested pO3D-FL plasmid to generate the final expression vector pO3DPro-VF3. All restriction enzymes and T4 DNA ligase used were purchased from Fermentas and the procedures were as recommended by the manufacturer. The correct orientation of the promoter and cDNA at the *Bam*HI joining site was confirmed by DNA sequencing (First BASE Laboratories, Malaysia) using gene-specific primer, O3D-PR4 (Table S1).

The expression vector was then mobilized into *A. tumefaciens* strain LBA4404 by using the Micropulser™ electroporator (Biorad) as described by manufacturer and the recombinant clone was kept at − 80 °C in 25% (v/v) glycerol for storage.

2.3. Transformation of C. vulgaris with modified Agrobacterium-mediated method

Agrobacterium-mediated transformation of *C. vulgaris* was carried out as previously described [22] with slight modifications. A total of 5 × 10^6 *C. vulgaris* cells from log phase (OD_{600} = 0.5–1.0) culture was plated on BBM agar plate and pre-cultured for 5 days at 25 °C. Prior to co-cultivation, the pre-cultured cells were harvested and treated with cellulase (Sigma-Aldrich) as previously described [24]. Briefly, the microalgal cell pellet was resuspended in 0.5 mL of 4% (w/v) cellulase solution and incubated at 30 °C in dark for 14 h with gentle shaking. The mixture was harvested by centrifugation at 5000 rpm for 5 min. The cell pellet was washed twice with 1.5 mL of Induction Medium (BBM, pH 5.6 and 200 μM cinnamic acid). Finally, the cell pellet was resuspended with 50 μL Induction Medium and ready for co-cultivation.

A. tumefaciens harbouring the pO3DPro-VF3 expression vector was prepared from frozen glycerol stock as previously described [22]. Induction medium containing 200 μM cinnamic acid was used in this experiment [25]. After co-cultivation, the cells were harvested into 5 mL of BBM containing 500 μg mL^{-1} cefotaxime. The mixture was incubated at 25 °C for 2 days in dark to eliminate contaminating *Agrobacterium*. To check if there is any contaminating *Agrobacterium*, the

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