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Effects of disrupted omega-3 desaturase gene construct on fatty acid composition and expression of four fatty acid biosynthetic genes in transgenic *Chlorella vulgaris*

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ABSTRACT

This study demonstrated the genetic modification of *Chlorella vulgaris* by introducing a copy of disrupted omega-3 desaturase (*ω-3 FAD*) gene into its genome to study the effect on fatty acid biosynthesis pathway. Results revealed that the expression of endogenous *ω-3 FAD* gene in transgenic lines (C28 and C30) was temporarily suppressed during the early stage of sub-culture and then reactivated subsequently after six months of alternate sub-culturing. Furthermore, the endogenous *ω-3 FAD* expression was found to be upregulated to > 3-fold without any notable changes in C18:3n3 composition when cultured under nitrate-deficient medium. Interestingly, a significant shift in fatty acid saturation profile towards the production of higher C16:0 was observed along with a reduction in C18:1 composition in transgenic lines. In addition, PCR walking experiment confirmed that the entire T-DNA region and vector backbone were co-transferred and randomly integrated into the host genome. These findings provide valuable insight in understanding the regulation of fatty acid biosynthesis pathway in *C. vulgaris* and could pave the way for further genetic improvement of the species for various downstream applications.

1. Introduction

Genetic engineering of lipid metabolism in microalgae was first reported in 1996, where an extra copy of *ACCase* gene was transformed into *Cyclotella cryptica*, but failed to enhance overall lipid production of the transgenic line [1]. However, several recent studies have successfully reported an improved lipid and fatty acids accumulation through genetic engineering approaches in several microalgae species, such as *Nannochloropsis salina* [2], *Nannochloropsis oceanica* [3] and *Chlamydomonas reinhardtii* [4]. The overall development in lipid production from these genetically modified strains could boost their feasibility for biofuel production.

Our previous study has identified *C. vulgaris* strain UMT-M1 as a potential candidate for lipid production [5]. Under several culture conditions, this microalgae strain is capable of producing over 20% (of cell dry weight) total oil content, in which the major fatty acid composition are palmitic (C16:0), stearic (C18:0), oleic (C18:1), linoleic (C18:2) and α-linolenic (C18:3n3) acids [5–7]. In addition to that, the genes encoding for key enzymes that are responsible for the fatty acid biosynthesis pathway had been isolated and these includes the genes that encode for β-ketoacyl-ACP synthase I (GenBank accession no. EU590913), stearoyl-ACP desaturase (GenBank accession no. KJ561355), omega-6 desaturase (GenBank accession no. KJ561356) and omega-3 desaturase (GenBank accession no. EU100100 and KX100035). The isolation of these genes facilitate us to investigate the regulatory mechanisms of fatty acid biosynthesis pathway through genetic engineering approach, which in turn favors the production of saturated fatty acid (SFA) which serves as biodiesel precursor.

In the past, different species of *Chlorella* were transformed with various transformation methods, in which the commonly used methods include electroporation [8,9], glass-bead method [10] and *Agrobacterium*-mediated transformation [11,12]. Physical transformation such as electroporation and glass-bead methods that are efficient only on cell-wall deficient or wild type strains that are pre-treated with enzyme or chemical prior to transformation, thus resulting in low efficiencies of the stable transformation and loss of transgene expression in transformants [9,13]. On the other hand, *Agrobacterium*-mediated transformation is a natural-occurring mechanism that involves complex biological processes via regulation of different virulence genes to efficiently transfer and integrate the T-DNA region into host organisms

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[14]. Therefore, the simplicity and the efficiency of *Agrobacterium*-mediated transformation in foreign gene transfer and integration is considered as one of the most preferred transformation methods for *C. vulgaris* and other microalgae species.

With the aim to increase the production of SFA in *C. vulgaris* to favor biodiesel production, the reactions involve further desaturation of SFA and lead to the production of polyunsaturated fatty acids (PUFAs) are sought to be disrupted. In order to gain insight into the regulation of PUFAs biosynthesis, the conversion of C18:2 to C18:3n3 by the enzyme omega-3 desaturase (ω-3 FAD) is of particular interest, owing to the role of C18:3n3 as a precursor for the synthesis of very long-chain essential fatty acids, such as eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids [15].

Therefore, in this current study, we introduced a copy of disrupted *ω-3 FAD* gene into *C. vulgaris* (strain UMT-M1) through *Agrobacterium*-mediated transformation. The transgene effects at genomic and phenotypic levels were assessed in the selected transgenic lines in comparison to the wild-type strain. The variations observed in fatty acid biosynthesis pathway after the transgene expression will certainly be beneficial in understanding the overall regulation of the pathway and contribute positively to the genetic engineering of the biofuel producing-strain.

2. Materials and methods

2.1. Construction of pCAMBIA 1304-dO3D vector cassette

The ~4.8 kb disrupted *ω-3 FAD* gene vector cassette was constructed by replacing a 2141 bp endogenous *ω-3 FAD* gene-promoter fragment with *hptII* gene which conferred resistance to hygromycin B. It was constructed by sequential cloning of PCR-amplified DNA fragments into the T-DNA region (between *Xho*I and *Xba*I sites) of pCAMBIA 1304 vector (www.cambia.org). Restriction site-anchored primers (Supplementary Table S1) were designed based on the *ω-3 FAD* gene sequence (GenBank accession number: KX100035) to amplify the left flank (~1.5 kb) and right flank (~1.1 kb) fragments from genomic DNA of *C. vulgaris*. The hygromycin resistance gene (*hptII*) including its CaMV 35S promoter (~2.2 kb) was amplified from pCAMBIA 1304 vector. The PCR reaction contained 1 × buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTP mix, 1 μL of template (~50 ng), 0.4 μM of each forward and reverse primers, 0.4 U GoTaq DNA polymerase (Promega) and sterile double-distilled water (ddH_2O) to final volume of 25 μL. The PCR conditions were as follows: initial denaturation 94 °C for 3 min; 35 cycles of 94 °C for 60 s, 65 °C for 60 s, 72 °C for 2 min; and final extension at 72 °C for 5 min. The gene orientations of left frank, *hpt* gene and right flank were confirmed by DNA sequencing. The recombinant vector pCAMBIA1304-dO3D was PCR-amplified and cloned into *E. coli* strain *DH5α* followed by transformation into *Agrobacterium tumefaciens* strain LBA4404 as described in previous study [16].

2.2. C. vulgaris and A. tumefaciens culture

The *C. vulgaris* strain UMT-M1 [11] was obtained from microalgae stock culture at Universiti Malaysia Terengganu, Malaysia. A single colony from the stock agar plate was inoculated into 100 mL of Bold's Basal medium (BBM) [16,17]. Microalgae culture was continuously agitated in shaker at 120 rpm and illuminated with T5 fluorescent light (Philips) at 23–25 °C. A 50 mL of culture was replenished by the same quantity of fresh medium every 2–3 weeks to maintain cell growth.

A. tumefaciens strain LBA4404 harboring vector pCAMBIA1304-dO3D was inoculated into LB broth supplemented with rifampicin (50 μg mL^{-1}) and kanamycin (50 μg mL^{-1}) and cultivated at 27.5 °C together with agitation at 200 rpm in dark. After 48 h, 1 mL of culture was inoculated into 20 mL selective LB medium under the same condition as described above until OD_{600} reading reached 0.5. A total of 200 μL recombinant *A. tumefaciens* cell culture was washed with BBM containing 50 μg mL^{-1} acetosyringone once and the cells were used for co-cultivation as described in previous study [16].

2.3. Agrobacterium-mediated transformation of C. vulgaris

The *Agrobacterium*-mediated transformation approach was modified from previous studies [11,16]. A day prior to co-cultivation, a total of 5×10^7 *C. vulgaris* cells from a log-phase culture (OD_{600} = 0.5–1.0) was harvested by centrifugation at 13,000 rpm and resuspended into BBM broth containing lysozyme (500 μg mL^{-1}). The mixture was incubated overnight at 27 °C in dark with agitation at 130 rpm. The cells were then pelleted by centrifugation at 6000 rpm for 2 min and rinsed with ddH_2O, resuspended into BBM containing 0.2% w/v cellulose and 0.2% w/v pectinase followed by incubation for 2 h at 27 °C in dark with agitation at 130 rpm. The enzyme-treated *C. vulgaris* cells were pelleted by centrifugation at 6000 rpm for 2 min, rinsed twice with sterile ddH_2O and resuspended with remaining supernatant. The enzymes-treated *C. vulgaris* cells were co-cultivated with 200 μL of *Agrobacterium* cells on BBM solid medium containing 50 μg mL^{-1} acetosyringone at 27 °C for 3 days in dark as described in previous study [16]. After co-cultivation, cell mixture was harvested from BBM agar plate and transferred into 5 mL BBM broth containing 500 μg mL^{-1} of cefotaxime, followed by incubation at 27 °C for 2 days in dark. Following that, transformed cells were pelleted with centrifugation and the cell pellet was spread on fresh BBM agar and allowed for 7 days incubation at 27 °C under light for cell recovery. Then, cells were harvested again and spread on selective BBM agar plate supplemented with 20 μg mL^{-1} of hygromycin. Approximately after 2 weeks, 100 resistant colonies from primary hygromycin (20 μg mL^{-1}) selection plates were randomly selected and grown on non-selective BBM agar plates for a week. The colonies were then sub-cultured onto hygromycin selection plates for one week. This alternate selection steps were repeated for three cycles to ensure that only stable transformants were selected for further experiments.

2.4. Confirmation of stable transformants by PCR and detection of transgene transcript by RT-PCR

The remaining 16 hygromycin-resistant colonies that were obtained after the initial three cycles of alternate hygromycin selection (Section 2.3) were subjected to PCR confirmation. A loopful of fresh cells was resuspended into 0.2 mL microcentrifuge tube containing 10 μL of sterile ddH_2O and incubated in mini thermal cycler (Biorad) at 100 °C for 20 min. Tube was then centrifuged at 14,000 rpm for 1 min and the aqueous layer was collected as DNA template for PCR. In order to confirm the presence of transgenes in the genomic DNA, three pairs of primers (Supplementary Table S2) were designed to amplify three internal transgene regions (JSLF, HPT9 and JSRF) within the construct (Fig. 1). The PCR was carried out in 50 μL reaction mixture containing 1 × Ranger reaction buffer, 0.4 μM each forward and reverse primers, 0.2 mM dNTP mix, 4 U Ranger DNA polymerase (Bioline), 5 μL of DNA template and sterile ddH_2O to final volume of 50 μL. Wild type (WT) *C. vulgaris* genomic DNA was used as negative control. PCR was initiated at 94 °C for 3 min, followed by 35 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s, and a final extension at 72 °C for 5 min. The PCR product was analyzed by electrophoresis and subsequently purified by Wizard® SV gel and PCR Clean-up System (Promega) followed by DNA sequencing validation.

For the detection of transgenes expression, two transgenic colonies (C28 and C30) were selected as all the three transgene fragments (JSLF, HPT9 and JSRF) were present in these transgenic colonies. The cells were picked and spread on fresh non-selective agar plate to obtain a layer of fresh cells. Total RNA were extracted from harvested cells using GF-1 Total RNA Extraction Kit (Vivantis) according to manufacturer's protocol and treated with DNase I (Thermo Scientific). RevertAid reverse transcriptase (Thermo Scientific) was used to synthesize first