FUNCTIONAL CHARACTERIZATION OF OMEGA-3 FATTY ACID DESATURASE (*ω-3 FAD*) GENE INVOLVED IN THE FATTY ACID BIOSYNTHESIS PATHWAY IN *CHLORELLA VULGARIS* (STRAIN UMT-M1)

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ABSTRACT

Abstract of thesis presented to the Senate of Universiti Malaysia Terengganu in fulfillment of the requirement for the degree of Doctor of Philosophy

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A large number of microalgae have been studied in term of lipid class and fatty acids composition as microalgae such as *Chlorella vulgaris* have great potential sources of polyunsaturated fatty acids (PUFAs). Genetic engineering of the fatty acid biosynthesis pathway has been applied to improve PUFAs production of microalgae. Thus, it is essential to identify the genes coding for the key enzymes that contribute to fatty acid synthesis and accumulation. One of the enzyme involved in desaturation is omega-3 fatty acid desaturase (*ω-3 FAD*) enzyme which takes part in conversion of linoleic acid (LA, C18:2) to alpha linolenic acid (ALA, C18:3n3). ALA is the precursor for the synthesis of other essential fatty acids such as eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA). In this study, the promoter of *ω-3 FAD* gene was successfully isolated from genomic DNA of *C. vulgaris* (strain UMT-M1) using PCR-Genome Walking method. From five GenomeWalker libraries (*Dra*I, *Eco*RV, *Pvu*II, *Sma*I and *Stu*I), *Stu*I managed to produce higher putative fragment with approximately 2.3 kb. Alignment analysis revealed that from the 2.3 kb fragment, only 170 bp sequence contained 100 % of homology regions with the full-length *ω-3 FAD* cDNA sequence isolated from the same species and the remaining sequence of the fragment (2186 bp) upstream from the *ω-3 FAD* putative transcription start site (+1) was undoubtedly a fragment of *ω-3 FAD* gene promoter

(O3D-Pro). Sequence analysis of promoter region using the online PlantCARE and PLACE program demonstrated the presence of potential functional elements in the promoter region such as basic element in eukaryote's promoter gene (TATA-box, CAAT-box and GC-box), several light responsive elements, DNA binding with one finger (Dof), G/A-hibrid/ bZIP, abscisic acid responsive element (ABRE), CAT Box, CGTCA-motif, ERE, GARE-motif, MBS, GC-motif, silencer-element (SBF-1) and AT-rich region as enhancer-element. Other than that, the full-length endogenous *ω-3 FAD* gene was also successfully isolated from genomic DNA of *C. vulgaris* which contain seven exons and six introns. The four vector cassettes, pO3DPro-VF1, pO3DPro-VF2, pO3DPro-VF3 and pO3DPro-VF4 were successfully constructed and mobilized into *C. vulgaris* (strain UMT-M1) using improved *Agrobacterium*-mediated transformation method using cellulase enzyme. Treating cells with cellulase before co-cultivation steps in transformation showed a dramatic increment in transformation efficiency with approximately 82 ± 1.69 % of GUS positive cells as compared to non-cellulase treatment which produce only 21 ± 1.18 % GUS positive cells. Thus, the inclusion of cellulase enzymatic treatment to degrade the *C. vulgaris* cell wall layer successfully aids the simultaneous *Agrobacterium*-mediated transformation. The highest GUS positive cells were observed for pO3DPro-VF3 vector cassette with approximately 88.5 ± 0.52 % as compared to other vector cassettes. After three cycles (90 days) of alternate hygromycin selection, four out of 30 stable transgenic lines harboring the pO3DPro-VF3 vector cassette were randomly selected for verification with PCR technique. The four transgenic lines produced PCR products of specific size for *hpt* gene (687 bp), *gfp-gus*A gene (676 bp), *ω-3 FAD* gene (302 bp) and Hpt-GG fragments (7372 bp). The overexpression of pO3DPro-VF3 vector cassette in transgenic *C. vulgaris* line especially Ch-TL2 successfully increased the total SFAs, total PUFAs, C18:3n3 production and *ω-3 FAD* gene expression in both growth phases (mid exponential and early stationary) and under nitrate-deficient condition as compared to wild-type *C. vulgaris*. The potential transgenic line (Ch-TL2) was further investigated with genome integration analysis and the presence of unknown sequences at both borders of pCAMBIA1304 binary vector confirmed the integration of T-DNA into the genome of *C. vulgaris*. Thus, from this study, it is possible to manipulate genetically the fatty acid biosynthesis pathway by overexpressing the *ω-3 FAD* gene driven by O3D-Pro

endogenous promoter (pO3DPro-VF3 vector cassette) in microalgae or other plants in order to achieve both high lipid and high PUFAs under different stressors for industrial production.

ABSTRAK

Abstrak tesis yang dikemukakan kepada Senat Universiti Malaysia Terengganu sebagai memenuhi keperluan untuk ijazah Doktor Falsafah

PENCIRIAN KEFUNGSIAN GEN ASID LEMAK OMEGA-3 DESATURASE (*ω-3 FAD*) YANG TERLIBAT DI DALAM TAPAK JALAN BIOSINTESIS ASID LEMAK DALAM *CHLORELLA VULGARIS* (STRAIN UMT-M1)

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Sebilangan besar mikroalga telah dikaji dari segi kelas lipid dan komposisi asid lemak kerana mikroalga seperti *Chlorella vulgaris* mempunyai potensi besar sumber asid lemak poli tak tepu (PUFA). Kejuruteraan genetik tapak jalan biosintesis asid lemak telah digunakan untuk meningkatkan pengeluaran PUFA dari mikroalga. Oleh itu, ia adalah penting untuk mengenalpasti gen pengekodan bagi enzim utama yang menyumbang kepada sintesis dan pengumpulan asid lemak. Salah satu enzim yang terlibat dalam desaturasi adalah enzim omega-3 fatty acid desaturase (*ω-3 FAD*) yang terlibat dalam penukaran asid linoleik (LA, C18: 2) kepada asid alfa linolenik (ALA, C18: 3n3). ALA adalah pelopor untuk sintesis asid lemak penting yang lain seperti asid eikosapentaenoik (EPA) dan asid dokosahexaenoik (DHA). Dalam kajian ini, promoter gen *ω-3 FAD* telah berjaya dipencilkan daripada DNA genomik *C. vulgaris* (strain UMT-M1) menggunakan kaedah PCR-Genome Walking. Daripada lima perpustakaan GenomeWalker (*Dra*I, *Eco*RV, *Pvu*II, *Sma*I and *Stu*I), *Stu*I berjaya menghasilkan serpihan jangkaan yang tertinggi dengan anggaran 2.3 kb. Analisis penjajaran mendedahkan bahawa daripada serpihan 2.3 kb, hanya 170 bp urutan mengandungi 100 % daripada kawasan homologi dengan urutan penuh panjang *ω-3 FAD* cDNA yang dipencilkan daripada spesies yang sama dan baki urutan daripada serpihan (2186 bp) hulu daripada *ω-3 FAD* tapak transkripsi permulaan jangkaan (+1) sudah pasti serpihan promoter gen *ω-3 FAD* (O3D-Pro).

Analisis urutan bagi kawasan promoter dengan menggunakan program PlantCARE dan PLACE dalam talian itu menunjukkan kehadiran unsur-unsur fungsian yang berpotensi di kawasan promoter seperti elemen asas dalam promoter gen eukariot (kotak-TATA, kotak-CAAT dan kotak-GC), beberapa elemen responsif cahaya, DNA pengikat dengan satu jari (Dof), G/A-hibrid/ bZIP, elemen responsif asid absisik (ABRE), kotak-CAT, CGTCA-motif, ERE, GARE-motif, MBS, GC-motif, elemen-penyenyap (SBF-1) dan rantau kaya-AT sebagai elemen-penambah. Selain daripada itu, urutan penuh dalaman gen *ω-3 FAD* juga telah berjaya dipencilkan daripada DNA genomik *C. vulgaris* yang mengandungi tujuh ekson dan enam intron. Empat kaset vektor, pO3DPro-VF1, pO3DPro-VF2, pO3DPro-VF3 dan pO3DPro-VF4 telah berjaya dikonstruk dan dipindahkan ke dalam *C. vulgaris* (strain UMT-M1) dengan menggunakan kaedah transformasi *Agrobacterium*-mediated yang lebih baik dengan menggunakan enzim selulase. Rawatan sel-sel dengan selulase sebelum langkah-langkah ko-kultur dalam transformasi menunjukkan peningkatan dramatik dalam kecekapan transformasi dengan anggaran 82 ± 1.69 % daripada sel-sel positif GUS berbanding dengan tanpa rawatan dengan selulase yang hanya menghasilkan 21 ± 1.18 % sel-sel positif GUS. Oleh itu, kemasukan rawatan enzim selulase untuk memecahkan lapisan dinding sel *C. vulgaris* berjaya membantu transformasi *Agrobacterium*-mediated. Sel-sel positif GUS yang paling tinggi telah diperhatikan bagi kaset vektor pO3DPro-VF3 dengan anggaran 88.5 ± 0.52 % berbanding kaset vektor yang lain. Selepas tiga kitaran (90 hari) pemilihan hygromycin secara alternatif, empat daripada 30 titisan transgenik stabil yang mengandungi kaset vektor pO3DPro-VF3 telah dipilih secara rawak untuk pengesahan dengan teknik PCR. Empat titisan transgenik menghasilkan produk PCR bersaiz tertentu untuk gen *hpt* (687 bp), gen *gfp-gus*A (676 bp), gen *ω-3 FAD* (302 bp) dan serpihan Hpt-GG (7372 bp). Pengekspresan berlebihan kaset vektor pO3DPro-VF3 dalam titisan transgenik *C. vulgaris* terutama Ch-TL2 berjaya meningkatkan jumlah SFA, jumlah PUFA, pengeluaran C18: 3n3 dan ekspresi gen *ω-3 FAD* dalam kedua-dua fasa pertumbuhan (pertengahan eksponen dan awal pegun) dan di bawah keadaan kekurangan nitrat berbanding dengan *C. vulgaris* jenis liar. Potensi titisan transgenik (Ch-TL2) telah dikaji lanjut dengan analisis integrasi genom dan kehadiran urutan yang tidak diketahui di kedua-dua sempadan vektor binari pCAMBIA1304 mengesahkan integrasi T-DNA ke dalam genom *C. vulgaris*. Oleh itu, dari kajian ini,

ia adalah mungkin untuk memanipulasi secara genetik tapak jalan biosintesis asid lemak dengan pengekspresan berlebihan gen *ω-3 FAD* yang dipandu oleh promoter dalaman O3D-Pro (kaset vektor pO3DPro-VF3) dalam mikroalga atau tumbuhan lain untuk mencapai kedua-dua lipid dan PUFA yang tinggi di bawah tekanan yang berbeza untuk pengeluaran perindustrian.