DEVELOPMENT OF *AGROBACTERIUM*-MEDIATED TRANSFORMATION METHOD FOR MARINE MICROALGAE
(*Chlorella* sp. and *Nannochloropsis* sp.)

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(*Chlorella* sp. and *Nannochloropsis* sp.)

WILLY YEE

Thesis Submitted in Fulfillment of the Requirement for the Degree of Master of Science in the Faculty of Science and Technology Universiti Malaysia Terengganu

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DEVELOPMENT OF *AGROBACTERIUM*-MEDIATED TRANSFORMATION METHOD FOR MARINE MICROALGAE
(*Chlorella* sp and *Nannochloropsis* sp.)

WILLY YEE

FEBRUARY 2011

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An important step in the development of an efficient *Agrobacterium*-mediated transformation system is the establishment of optimal conditions for efficient T-DNA transfer. Parameters known to influence the efficacy of *Agrobacterium tumefaciens* T-DNA transfer such as pre-culture of algal cells, inoculum density, co-cultivation temperature, co-cultivation duration, pH of co-cultivation medium and the concentration of acetosyringone were evaluated by monitoring transient GUS expression two days post-infection. *A. tumefaciens* strain LBA4404 harboring the binary vector pCAMBIA1304 containing the *mgfp5-gus* fusion reporter and a hygromycin selectable marker driven by the CaMV 35S promoter was used for transformation. The expression of GUS and GFP was detected after algae cells were co-cultivated with *Agrobacterium.* The optimized parameters for *Chlorella* sp. were 2 days of pre-culture, 3 days of co-cultivation at 24°C in pH 5.5 media, bacterial density of OD_{600} =1.0, and 150 μM acetosyringone whereas for *Nannochloropsis* sp. the optimal parameters were similar with *Chlorella* sp. with the exception of 5 days of pre-culture and 50 μM acetosyringone. The combined optimized parameters resulted in an average transient transformation frequency of 24.95% for *Chlorella* sp. and 24.55% for *Nannochloropsis* sp. which was 3-folds and 2 folds higher compared to the un-optimized protocol. The combined optimal parameters were used to transfer two fatty acid constructs; p35SAP-1304 and pPROKASII-1304 into both species of microalgae. Microalgae transformed with both the fatty acid constructs showed GUS expression, however the transformation frequency was lower than that obtained with pCAMBIA1304. Resistant colonies were obtained following selection

on solid media supplemented with 18 μg/mL and 20μg/mL hygromycin B for *Nannochloropsis* sp. and *Chlorella* sp. respectively. Resistant colonies randomly picked and propagated in non-selective media were used for molecular analysis. The transgenic nature of the transformants were confirmed by positive polymerase chain reaction with the *gus* and *hpt* specific primers while the identity of the PCR amplicons were confirmed by DNA sequencing. Out of 30 resistant colonies of *Chlorella*, 9 were confirmed positive by PCR, while for *Nannochloropsis*, 5 out of 15 resistant colonies were positive. Two PCR positive colonies were recovered from a total of 18 resistant colonies of *Nannochloropsis* transformed with p35SAP-1304, whereas none were recovered for *Chlorella*. Transformation using the pPROKASII-1304 construct did not yield any PCR positive transformants for both microalgae. After 2-3 generations of subculture, the *hpt* gene fragment could still be detected from the transformants, suggesting the integration of T-DNA into the genome and subsequent inheritance to the next generation. The possibility of *Agrobacterium* contamination was ruled out by growing the algal culture on rich media. This study reveals the possibility of genetic manipulation of *Chlorella* and *Nannochloropsis* using the *Agrobacterium*-mediated transformation method. The developed transformation method will facilitate the genetic improvement of these commercially important algae.

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PEMBANGUNAN KAEDAH TRANSFORMASI PERANTARAKAN *AGROBACTERIUM* UNTUK MIKROALGA MARIN (*Chlorella* sp. dan *Nannochloropsis* sp.)

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FEBUARI 2010

Pengerusi : **Dr. Cha Thye San**

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Salah satu langkah penting dalam pembangunan sistem transformasi *Agrobacterium* yang efisyen adalah pembangunan keadaan optimal untuk translokasi T-DNA yang cekap. Parameter yang diketahui mempengaruhi kecekapan translokasi T-DNA *Agrobacterium tumefaciens* ke alga marin *Chlorella* sp. dan *Nannochloropsis* sp. seperti pra-kultur sel alga, kepadatan inokulum, suhu ko-kultur, tempoh ko-kultur, pH media ko-kultur dan kepekatan acetosyringone telah dinilai dengan memantau expresi transien *gus* dua hari selepas infeksi. *A. tumefaciens* strain LBA4404 yang mengandungi vektor binari pCAMBIA1304 yang membawa gen pelapor gandingan *mgfp5-gus* dan penanda pemilihan hygromycin yang dikawal oleh promoter CaMV35S digunakan untuk transformasi. Pengekspresan gen *gus* dan *gfp* telah dikesan selepas pengkulturan bersama dengan *Agrobacterium*. Parameter optimum untuk *Chlorella* sp. adalah 2 hari pra-kultur, 3 hari pengkulturan bersama pada 24°C-25°C dalam media dengan pH 5.5-5.6, kepadatan bakteria OD_{600} =1.0 dan 150 µM atau 300 µM acetosyringone manakala untuk *Nannochloropsis* sp., parameter optimum adalah sama dengan *Chlorella* sp. dengan pengecualian 5 hari bagi pra-kultur dan 50 µM-100 µM acetosyringone. Frekuensi transformasi meningkat kepada 24.95% untuk *Chlorella* sp. dan 24.55% untuk *Nannochloropsis* sp. apabila parameter-parameter optimum digabungkan. Kombinasi parameter optimum digunakan untuk transformasi kedua-dua konstruk p35SAP-1304 dan pPROKASII-1304 ke kedua dua spesies mikroalga. Mikroalga yang di transformasi dengan kedua-dua konstruk asid lemak menunjukkan expresi gen *gus*, walau bagaimanapun, frekuensi transformasi adalah lebih rendah berbanding pCAMBIA1304. Koloni

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rintang diperoleh selepas pemilihan dengan hygromycin dalam media pejal pada kepekatan 18 µg/mL and 20µg/mL untuk *Nannochloropsis* sp. dan *Chlorella* sp. masing-masing. Koloni rintang yang dipilih secara rawak dan di kultur dalam media tanpa antibiotik digunakan untuk analisis PCR. Sifat transgenik transforman didapati positif dengan kaedah PCR menggunakan primer spesifik kepada gen *gus* dan *hpt*, manakala identiti produk PCR dikenalpasti dengan penjujukan DNA. Sembilan daripada 30 koloni didapati positif untuk *Chlorella* manakala 5 daripada 15 koloni untuk *Nannochloropsis* didapati positif dengan PCR. Dua koloni positif *Nannochloropsis* yang ditransformasi dengan konstruk p35SAP-1304 diperolehi daripada 18 koloni rintang, manakala tiada yang diperoleh daripada *Chlorella*. Transformasi menggunakan konstruk pPROKASII-1304 tidak menghasilkan transforman yang positif untuk PCR. Selepas 2-3 generasi subkultur, fragmen gen *hpt* masih boleh diamplifikasi daripada transforman. Ini menunjukkan bahawa T-DNA telah diintegrasi dalam genom dan diwariskan ke generasi berikutnya. Kemungkinan kontaminasi dengan *Agrobacterium* didapati negatif apabila alga dikultur dalam media pengkayaan. Keputusan yang diperoleh dalam kajian ini menunjukkan bahawa manipulasi genetik *Chlorella* dan *Nannochloropsis* menggunakan *Agrobacterium* berjaya dilakukan. Kaedah transformasi yang diperoleh dalam kajian ini akan memudahkan usaha manipulasi genetik kedua-dua alga yang mempunyai nilai komersil ini.