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FACTORS AFFECTING *AGROBACTERIUM*-MEDIATED GENETIC TRANSFORMATION OF MARINE MICROALGA, *NANNOCHLOROPSIS* SP.

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Abstract: The optimisation condition of an *Agrobacterium tumefaciens*-mediated transformation method for the marine microalgae *Nannochloropsis* sp. (UMT-M3) is reported using a binary vector harbouring the green fluorescent protein and β-glucuronidase (*gfp-gusA*) as reporter genes and a hygromycin phosphotransferase (*hpt*) as a selection marker. For an efficient *Agrobacterium* mediated genetic transformation of marine microalgae *Nannochloropsis* sp., parameters, namely pre-culture duration, inoculum density, co-cultivation temperature, co-cultivation duration, pH of co-cultivation medium and the concentration of acetosyringone (AS) were evaluated in this study by monitoring β-glucuronidase (*gusA*) expression two days post-infection. Results showed that 5 days of pre-culture, 3 days of co-cultivation at 24°C, medium pH 5.5, bacterial density of OD_{600} =1.0, and 50 μM AS are optimal in the transformation of *Nannochloropsis* sp. The use of combined optimised parameters resulted in an average transformation frequency of 24.5%. The transgenic nature of transformants was confirmed by polymerase chain reaction with the *gfp-gusA* and *hpt*-specific primers on colonies resistant to 18 mg L^{-1} hygromycin. The developed transformation method will enable manipulation of important biochemical pathways in *Nannochloropsis* sp. and facilitate the genetic improvement of this commercially-important microalga.

KEYWORDS: *Agrobacterium*, β-glucuronidase, genetic transformation, microalgae, *Nannochloropsis,* optimization, acetosyringone

Introduction

Nannochloropsis, a unicellular marine alga (class, Eustigmatophyceae) is widely used in many mariculture systems as a sole or partial feed for rotifer production and enrichment, and to create a green water effect in fish larvae tanks (Lubzens *et al*., 1995). It is considered one of the most promising photoautotrophic producers of eicosapentaenoic acid (EPA) and has been the most common alga used in many mariculture hatcheries in Europe since the late 1980s. This species has replaced the commonly-used algal species such as *Isochrysis galbana* and *Monochrysis* sp., due to its ability in contributing a high level of EPA to the food web (Lubzens *et al*., 1995).

Recently, metabolic engineering of microalgae has gained a great deal of interest in the production of valuable molecules such as pigments, pharmaceutical products and biofuels (Rosenberg *et al*., 2008). Over the years, only a few species of microalgae have been genetically modified. The successfully-transformed algal species includes *Chlamydomonas reinhardtii* (Kumar *et al.*, 2004), *Chlorella* (Cha *et al*., 2011), *Volvox* (Jakobiak *et al*., 2004), *Haematococcus* (Steinbrenner & Sandmann, 2006), *Nannochloropsis oculata* (Li & Tsai, 2009) and *Dunaliella salina* (Geng *et al*., 2004). The common method of microalgae transformation involves direct-gene transfer methods, such as electroporation, agitation with glass beads and particle bombardment. The *Agrobacterium*-mediated transformation method offers several advantages over direct-gene transfer methods including its feasibility to transfer big size expression cassettes, low copy of transgene integration and rearrangement, preferential integration into transcriptionally-active regions and its simplicity. To date and to our knowledge, only two species of microalgae,

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namely *Chlamydomonas reinhardtii* (Kumar *et al*., 2004) and *Heamatococcus pluvialis* (Kathiresan *et al*., 2009) are being transformed using *Agrobacterium*. Unlike *C. reinhardtii* and *H. pluvialis*, *Nannochloropsis* is non-flagellated, does not store starch and has been reported to have a multilayered and thick cellulosic cell wall (Rodolfi *et al*., 2003) which may pose difficulty for efficient direct-gene transfer methods (Li & Tsai, 2009). Such morphological difference may also affect efficiency of *Agrobacterium*-mediated transformation of *Nannochloropsis*. Li and Tsai (2009) have reported successful transformation of *Nannochloropsis* using electroporation; but the use of *Agrobacterium*-mediated transformation method eliminates the need of protoplast.

In this study, the establishment of an *Agrobacterium*-mediated genetic transformation method for *Nannochloropsis* sp. and the optimisation of bacterial density, temperature, pre-culture, co-cultivation duration and acetosyringone concentration for successful transformation of this microalga are reported. Research findings will be useful in genetic manipulation to be accomplished on this microalga in the future.

Materials and Methods

Nannochloropsis culture conditions

A local strain of marine *Nannochloropsis* sp. (UMT-M3) was maintained as axenic cultures in liquid and solid freshwater culture medium (FCM) containing Bold's Basal Medium (BBM) major salts (Nichols & Bold, 1965) and supplemented with F medium trace metals and vitamins (Guillard, 1975). Cultures were grown in an environmental test chamber (Sanyo) under continuous fluorescent light at a photon flux density of ~ 40µmol m^{-2} s^{-1} at 25°C and manually agitated daily.

Agrobacterium strain and vector

The binary vector pCAMBIA1304 (http://www.cambia.org) containing a *gfp:gusA* fusion reporter and a selectable marker for hygromycin B resistance driven by the CaMV 35S promoter was used in transformation (Figure 1). The binary vector was mobilised into *Agrobacterium*

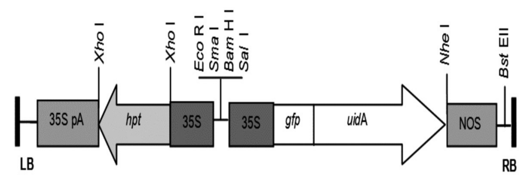

Figure 1. Schematic representation of the T-DNA map of pCAMBIA1304. 35S: CaMV35S promoter; 35S pA: CaMV35S poly(A); NOS: nopaline synthase terminator; *hpt*: hygromycin phosphotransferase gene; *gfp*: green fluorescent protein gene; *uid*A: β-glucuronidase gene; LB: left border and RB: right border.

strain LBA4404 by using the Micropulser™ electroporator (Biorad, Hercules, USA) according to manufacturer's protocol and maintained at –80°C in 25% (v/v) glycerol.

Antibiotic sensitivity test

The effect of the antibiotic cefotaxime on the growth of *A. tumefaciens* was tested by inoculating 200 µl of *Agrobacterium* culture (OD_{600}=1.0) in 5 ml Luria Broth supplemented with different concentrations of cefotaxime (0, 50, 100, 150, 200, 300, 400 and 500 mg L^{-1}) and the growth of *Agrobacterium* in each concentration was determined spectrophotometrically at OD_{600} after 2 days. The effect of the antibiotic cefotaxime on the growth of *Nannochloropsis* was tested by plating a serially-diluted microalgae culture on solid media supplemented with different concentrations of cefotaxime (0, 100, 200, 300, 400 and 500 mg L^{-1}). The agar plates were incubated in the dark for 2 days at 25°C prior to exposure to light and the number of surviving colonies that produced less than 100 colonies was counted in duplicates after 14 days. The sensitivity of *Nannochloropsis* towards hygromycin was determined by plating 1×10^6 cells on solid FCM supplemented with 500 mg L^{-1} cefotaxime and varying concentrations of hygromycin (6, 8, 10, 12, 14, 16, 18, 20, 23 and 26 mg L^{-1}) and incubated for 2 days in the dark at 25°C before exposure to light and the number of surviving colonies was accessed after 20 days. Each treatment was tested in triplicate.

General transformation procedure

Single colony of *A. tumefaciens* initiated from a frozen stock was used to inoculate 10 ml of Luria Broth supplemented with 5 mM glucose, 100 mg L^{-1} streptomycin and 50 mg L^{-1} kanamycin and grown overnight at 27°C with a continuous shaking at 200 rpm in the dark. Five millilitres of this overnight culture was used to inoculate 50 ml of the same medium and grown in the dark at 27°C with shaking at 200 rpm until OD_{600} = 0.8-1.2. The bacterial culture was harvested by centrifugation and washed once with induction medium (FCM + 100 µM acetosyringone, pH 5.6) and diluted to a final density of OD_{600}= 0.5. Prior to co-cultivation, a total of 5 × 10^6 *Nannochloropsis* cells from a log phase culture ($OD_{600=}$ 0.5-1.0) was pre-cultured for 5 days on FCM solidified with 1.2% (w/v) bacto-agar (Oxoid) at 25°C and harvested with induction medium on the day of co-cultivation. The algal cell pellet was mixed with 200 µl of the bacterial suspension and plated on induction medium solidified with 1.2% (w/v) bacto-agar. Co-cultivation was performed for 3 days at 25°C in the dark. Following co-cultivation, cells were harvested with FCM supplemented with 500 mg L^{-1} cefotaxime (Phytotech) in a total volume of 7 ml and incubated in the dark at 25°C for 2 days to suppress and eliminate *Agrobacterium* before the cells were subjected to GUS histochemical assay and visualisation of GFP.

Following confirmation of reporter expression by GUS histochemical staining, remaining microalgal cells were harvested by centrifugation at 5000 rpm for 5 minutes, washed once with fresh liquid media containing 500 mg L^{-1} cefotaxime, resuspended in liquid FCM containing 500 mg L^{-1} cefotaxime and plated on solid FCM media containing 1.2% (w/v) agar, 18 mg L^{-1} hygromycin B and 500 mg L^{-1} cefotaxime. Plates were incubated at 25°C in the dark for 2 days before exposure to light. Single colonies that appeared within 20 days were randomly selected and propagated in liquid media containing 500 mg l^{-1} cefotaxime but lacking hygromycin and used for molecular analysis. Detection of contaminating *Agrobacterium* was performed by plating microalgae cells on LB agar plates without any antibiotics.

GUS histochemical assay and visualisation of GFP

GUS histochemical assay was performed as described by Jefferson *et al*. (1987) with minor modification. Following 2 days of incubation in medium containing 500 mg l^{-1} cefotaxime, 1 ml of algal culture was harvested by centrifugation at 6500 rpm and washed once with sterile deionised water and twice with sterile 0.1M $NaPO_4$ buffer (pH 7.0). The algal cell pellet was resuspended in 500 µl GUS histochemical assay buffer consisting 2mM X-gluc, 0.1M phosphate buffer (pH 7.0), 1 mM $K_3Fe(CN)_6$, 10 mM Na_2EDTA, and 0.1% Triton X-100, and incubated overnight at 37°C. Following GUS assay, chlorophyll was cleared by incubation for 1–2 h in 70% (v/v) ethanol and cells were resuspended in 40 % (v/v) glycerol. Visualisation of *gfp* was performed using a fluorescent microscope (Leica DM Ire2, Wetzlar, Germany) with a bandpass excitation filter of 450–490 nm and a 515 nm dichromatic mirror with a long- pass filter at 510 nm. Images were captured and processed with ACD pro-3 imaging software (ACD systems, Canada).

Optimisation of transformation parameters and statistical analysis

The effects of the following factors were accessed during the process of optimisation: bacterial concentration (0.2, 0.4, 0.5, 0.6, 0.8 and 1.0 at spectral absorbance of OD_{600}); co-cultivation temperature (20, 22, 24, 25, 26, 28 and 30°C); acetosyringone concentration (50, 100, 150, 200 and 300 µM); pre-culture duration (0, 1, 2, 3, 4 and 5 days) pH of co-cultivation medium (pH 5.0, 5.2, 5.5, 5.6, 5.8 and 6.0); and length of co-cultivation (1, 2, 3, 4 and 5 days). One parameter would first be varied while other parameters were kept constant based on the findings of the preliminary test. All of the parameters were optimised by screening for transient GUS expression. Following GUS histochemical assay, cells were cleared of chlorophyll with 70% (v/v) ethanol, visualised under a light microscope (Leica, Wetzlar, Germany) and counted in a haemacytometer chamber. Cells which were sectored/spotted or fully stained blue were recorded as transformed.

Transformation efficiency was calculated as the number of GUS-positive cells/total number of cells counted × 100%. All experiments were conducted in 3 replicates and repeated 3 times, and cells were counted 3 times with a minimum of 200 cells per count.

Statistical significance was contrasted using Tukey's HSD test using SPSS 12.0 (Gaur & Gaur, 2006). Following optimisation, the best variable for each parameter was combined and tested again by using the pCAMBIA1304 vector. The statistical significance between optimised and un-optimised parameters was contrasted by using an independent t-test.

Molecular analysis of transformants

Total genomic DNA was extracted from approximately 40 ml of cells from a log-phase culture using the SDS-potassium acetate method (Tai & Tanksley, 1990). Cells were lysed in 0.5 ml of extraction buffer (0.1M Tris-Cl [pH8.0], 0.05M EDTA [pH8.0], 0.5 M NaCl, 2% SDS and 1% β-mercaptoethanol) for 1 hour at 65°C, followed by addition of 3 µl of RNAse A and incubation at 37°C for 30 min then 170 µl of 5M KoAC was added to the sample and incubated for 20 min on ice, and subsequently extracted with an equal volume of chloroform:isoamyl-alcohol (24:1). The aqueous layer was collected and the DNA was precipitated by adding an equal volume of isopropanol and subsequently washed with 70% ethanol.

PCR analysis was carried out in a 25 µl reaction containing 150 ng DNA, 1.25 mM dNTP, 2 mM $MgCl_2$, 1.25 µM of each primer and 1 U *Taq* DNA polymerase (Geneaid). The primers used to amplify a 686 bp fragment of the *hpt* gene were Hpt-F: 5'–ATATACGCCCGGAGTCGTGGCG-AT–3' and Hpt-R: 5'–CAGCTTCGATGTAG GAGGGCGTGG–3'; while primers GG-F: 5'–TGCCTGAGGGATACGTGC–3' and GG-R: 5'–ACTTCGCGCTGATACCAGACG–3' were used to amplify a 676 bp fragment of the *gfp-gusA* gene fusion. Amplification was carried out in a thermal cycler (Eppendorf) with the following conditions for the *hpt* gene: Initial denaturation at 94°C for 3 min, and 35 cycles of 94°C for 30s and 72°C for 1 min 10s followed by 1 cycle at 72°C for 7 min, while the following conditions were used for amplification of the *gfp-gusA* gene fusion: 94°C for 3 min followed by 35 cycles of 94°C for 30s, 65°C for 30 s, 72°C for 40 s and 1 cycle at 72°C for 7 min. Amplified products were separated on 1% agarose. The amplified PCR products were cloned in pGEM-T vector (Promega) and sequenced to verify the identity of both *hpt* (686 bp) and *gfp-gusA* (676 bp) gene fragments. The nucleotide sequence obtained was aligned with the sequence of pCAMBIA1304 (GenBank Accession No: AF234300) by using ClustalW2 (http://www.ebi.ac.uk/Tools/ clustalw2/).

Results

Antibiotics sensitivity test

The growth of *A. tumefaciens* was inhibited at all concentrations of cefotaxime used (Figure 2A) while the growth of *Nannochloropsis* was found to be uninhibited in cefotaxime-supplemented media of up to 500 mg L^{-1} (Figure 2B). Therefore, the highest concentration of cefotaxime (500 mg L^{-1}) was used in subsequent experiments to ensure thorough elimination of *A. tumefaciens* after co-cultivation. The lowest concentration of hygromycin which completely inhibited the growth of *Nannochloropsis* was 18 mg L^{-1} (Figure 2C) and this concentration was used for subsequent selection of transformants.

***Agrobacterium*-mediated transformation**

A preliminary experiment was conducted in order to establish a suitable *Agrobacterium* transformation method for *Nannochloropsis* sp. before more extensive optimisation experiments were carried out. Physical and biological parameters known to influence the efficacy of *Agrobacterium* T-DNA transfer tested in the preliminary experiment were pre-culture duration (0, 3, 4 and 5 days), pH of co-cultivation media (pH5.2, 5.6 and 6.0), co-cultivation temperature (22, 25 and 28°C), co-cultivation period (1, 2 and 3 days) and type of co-cultivation media (liquid and solid). The acetosyringone concentration and bacterial density were fixed at 100 µM and OD_{600}=0.5 respectively (Kumar *et al*., 2004). The percentage of GUS-positive cells were roughly estimated under a light microscope.

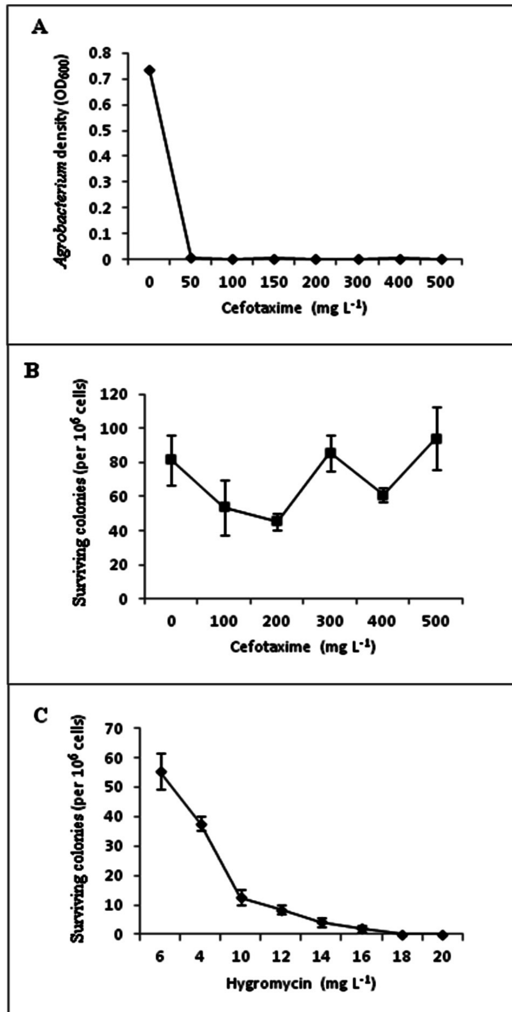

Figure 2. Antibiotic sensitivity test; the effect of cefotaxime on the viability of *Agrobacterium*; spectral absorbance was measured after 2 days of incubation (A), the effect of cefotaxime on the viability of *Nannochloropsis*; number of surviving colonies were counted after 2 weeks and the effect of hygromycin on the viability of *Nannochloropsis*; number of surviving colonies were counted after 20 days (C).

Results from the preliminary experiment showed that GUS-positive cells were only obtained when co-cultivation was performed on solid medium, while no GUS- positive cells were obtained from co-cultivation in liquid medium. The importance of pre-culture of cells on solid media prior to co-cultivation was also noted in the study. It was observed that 5 days of

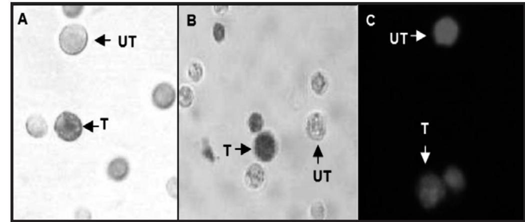

Figure 3: Transformation of *Nannochloropsis* sp. with pCAMBIA1304 caring the *gfp-gusA* fusion reporter genes. (A) GUS histochemical assay of transformed cells without ethanol wash and (B) washed with 70% ethanol. (C) Detection of GFP in transformed cells. T and UT represent transformed and untransformed cells respectively.

pre-culture produced a higher number of GUS-expressing cells than 3 or 4 days. The absence of pre-culture on solid media resulted in very poor GUS expression, despite the fact that cells were obtained from a log-phase algal culture (OD_{600} = 0.5–1.0). In subsequent experiments, 5 days of pre-culture was used. Co-cultivation performed on media with pH 5.6 was found to produce the most GUS-positive cells compared to at pH 5.2 and 5.8 and was used in subsequent experiments. Temperature is another important physical parameter in *Agrobacterium*-mediated transformation of plants. It was observed that co-cultivation at 25°C produced the highest GUS-expressing cells compared to at 22°C and 28°C and was used in subsequent experiments. It was also observed that 3 days of co-cultivation was found to give the highest number of GUS-positive cells in initial experiments compared to 2 and 4 days and was used to optimise other parameters. The expression pattern of GUS in cells after co-cultivation varies; cells either exhibit strong intense blue colouration or weak/ sectored colouration, which may possibly be due to improper penetration of GUS substrate (Kathiresan *et al*., 2009). However, no GUS expression was detected in non-transformed cells, thus cells that were stained regardless of the pattern of GUS expression were assumed to be of transgenic nature (Figure 3A and B). GFP expression was observed as green-yellowish fluorescence, while non-transformed cells only exhibit red chlorophyll autofluorescence (Figure 3C).

Thus, results from preliminary experiment showed that 5 days of pre-culture on solid media, 3 days of co-cultivation, co-cultivation temperature of 25°C and co-cultivation media with pH5.6 produced the most GUS-positive cells. These variables together with AS concentration of 100 mM and bacterial density of OD_{600}=0.5 were fixed and used in subsequent optimisation experiments.

Optimisation of transformation parameters

Effect of pre-culture

A dramatic increase was observed in the frequency of algae cells expressing GUS when cells were pre-cultured on solid media compared to cells which were used directly from a liquid culture. Cells that were not pre-cultured exhibited very low competence for transformation, producing very few GUS-positive cells (0.27%) to none. The highest ($p<0.05$) frequency of cells expressing GUS was observed in cells pre-cultured for 5 days, which produced 16.1% blue cells (Figure 4A). Cell competence for transformation was greatly increased after 1 day of pre-culture by almost 26 fold from 0.27% to 7.1% GUS-expressing cells. There was no significant difference ($p>0.05$) between 2, 3 and 4 days of pre-culture.

Effect of Agrobacterium density

The ratio between *Agrobacterium* and host cell density is an important factor that influences T-DNA transfer frequency; low concentration of *Agrobacterium* may reduce the efficacy of T-DNA transfer, while high concentration of bacteria may affect the viability of plant cells. In this study, it was found that there was an increase in the percentage of GUS-positive cells when an increase in the bacterial density from OD_{600} = 0.2 (6.0%) to 1.0 (20.3%) used (Figure 4B). The highest ($p<0.05$) percentage of GUS- expressing cells was obtained at OD_{600}=1 (8.4×10^{7} cfu mL^{-1}). There was no significant difference ($p>0.05$) between OD_{600}=0.5 and 0.6 (Figure 4B).

Effect of co-cultivation medium pH

It was observed that pH 5.5–5.6 produced a higher ($p<0.05$) percentage of GUS- expressing cells at 11.1% to 12.5% compared to other pH in the range of 5.0 to 6.0. Above or below pH 5.5–5.6, there was a significant decrease ($p<0.05$) in the percentage of GUS-expressing cells. The percentage of GUS-positive cells was the lowest at pH 5.0, producing 2.1% of GUS-xpressing cells, while there was no significant difference ($p>0.05$) between pH 5.2 and 6.0 (Figure 4C).

Effect of co-cultivation period

A co-cultivation period of 3, 4 and 5 days produced between 11.6% to 13.3% of GUS-positive cells, which was significantly higher ($p<0.05$) than 1 and 2 days of co-cultivation (Figure 4D). Increasing the duration of co-cultivation from1 day to 3 days caused an increase in the percentage of GUS-positive cells by almost 7 fold in *Nannochloropsis*. The lowest percentage of GUS-expressing cells was observed after 1 day of co-cultivation, which was 1.9%.

Effect of co-cultivation temperature

Co-cultivation performed at 24°C and 25°C produced a higher ($p<0.05$) percentage of GUS-expressing cells between 12.0% to 14.5% compared to other temperatures in the range of 20°C to 30°C. Interestingly, transformation frequency was decreased when the co-cultivation temperature below 24°C or above 25°C (Figure 4E). The lowest percentage of GUS-positive cells was observed at 20°C, which produced an average of 0.3% GUS- expressing cells, while there was no difference ($p>0.05$) between 22°C and 28°C.

Effect of acetosyringone (AS) concentration

In the absence of AS, T-DNA transfer was still feasible although the percentage of GUS-expressing cells was very low (1.7%). The addition of 50 μM AS caused an almost 8 fold increase in the percentage of GUS-positive cells compared to 0 μM. The percentage of GUS-expressing cells at 50 μM and 100 μM was between 13.1-13.5% and was significantly higher ($p<0.05$) than other concentrations tested in this study. Generally it appears that high concentrations of AS do not necessarily result in higher transformation rates, while high concentrations of this phenolic compound above 50-100 μM caused a reduction in transformation frequency (Figure 4F).

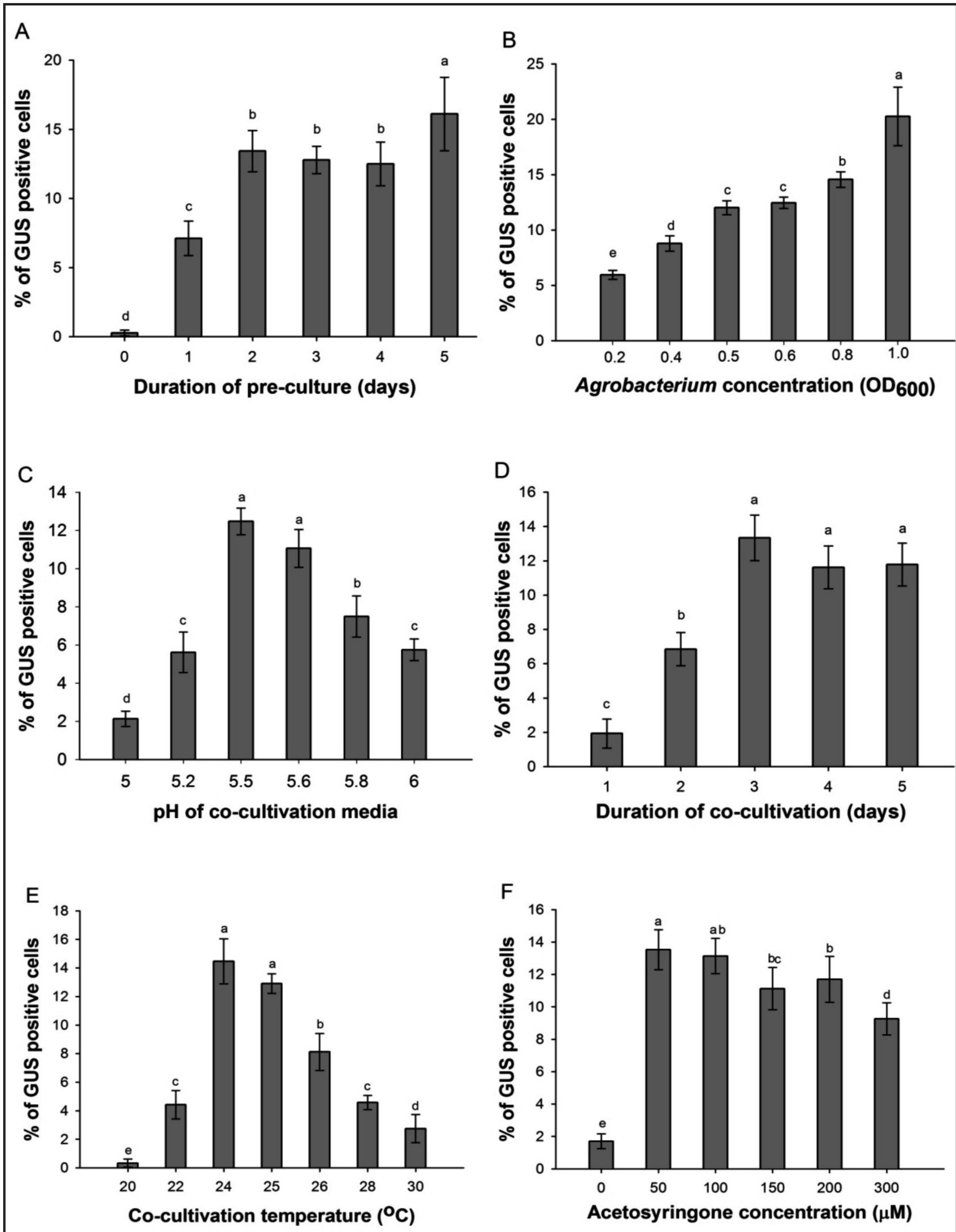

Figure 4: The effects of different *Agrobacterium*-mediated transformation parameters on transformation of *Nannochloropsis* sp. (A) Duration of pre-culture; (B) *Agrobacterium* density; (C) pH of co-cultivation media; (D) Duration of co-cultivation; (E) Co-cultivation temperature and (F) Acetosyringone concentration. Error bars correspond to standard deviation ($n = 9$). Different letters indicate values are significantly different ($p \leq 0.05$).

Comparison of un-optimised and optimised protocols

The optimised protocol consisted of 5 days of pre-culture on solid media (pH6.6-6.8), co-cultivation for 3 days with *Agrobacterium* at a density of OD_{600} = 1.0 at 24°C on solid media at pH 5.5 and supplemented with 50µM of AS (Table 1). Transformation with un-optimised parameters produced 12.5% of GUS- positive cells while transformation using the combination of all optimal parameters (Table 1) resulted in an increase of GUS expression by almost 2 fold ($p<0.05$) to 24.5%.

Table 1: Summary of the un-optimised and optimised genetic transformation parameters values used in *Nannochloropsis sp.*

Transformation Parameter	Un-optimised value	Optimised value
Agrobacterium density (OD_{600})	0.5[b]	1.0
Co-cultivation pH	5.6[a]	5.5-5.6
Pre-culture duration (days)	5[a]	5
Co-cultivation duration (days)	3[a]	3-5
Co-cultivation temperature (°C)	25[a]	24-25
Acetosyringone concentration (µM)	100[b]	50, 100

[a] Indicates un-optimised value obtained from preliminary experiment;
[b] Indicates value taken from Kumar *et al*. (2004) on *Chlamydomonas reinhardtii*

Molecular analysis of transformants

DNA extracted from several independent hygromycin-resistant colonies produced specific predicted PCR products of 686 bp for the *hpt* gene (Figure 5A) and 676 bp for the *gfp-gusA* gene (Figure 5B) using gene-specific primers respectively whereas no amplification was detected in non-infected (control) cells with either primer pairs. The identities of the amplified products were confirmed positive by DNA sequencing, while the possibility of contaminating *Agrobacterium* was ruled out by growing cultures on rich media. Out of 15 putative colonies, five independent cell lines (which was approximately 33% of total resistant colonies screened) were confirmed positive for both *hpt* and *gfp-gusA* gene (Figure 5). The presence of both specific amplification products in these transgenic lines indicates the successful transfer of both the marker and reporter genes as a single T-DNA into the *Nannochloropsis* genome. Since the hygromycin-resistant colonies were form from a single transformed microalga cell that had gone through many cycles of cell division, the presence of both *gusA-gfp* and *hpt* gene fragments in 33% of hygromycin-resistant colonies further suggests the integration of the genes into the genome and subsequent inheritence into new cells of the transgenic lines.

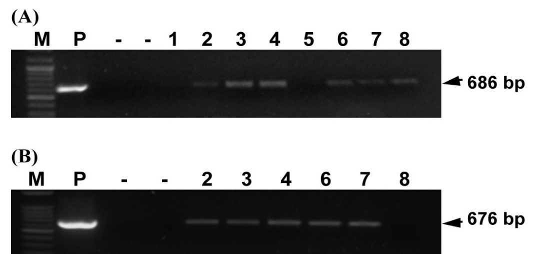

Figure 5: PCR analysis of putative *Nannochloropsis* transformants transformed with pCAMBIA1304. (A) Amplification of the 687 bp fragment of the *hpt* gene. (B) Amplification of the *gfp-gusA* gene from putative transformants which were positive for *hpt* gene. P: positive control;-:wild-type *Nannochloropsis*; 1-8: putative transformants and M: 100bp molecular weight marker.

Discussion

Explant pre-culture is known to induce rapid cell division, and competence, thus providing a large population of competent cells as targets for transformation. It was observed that pre-culture of microalgae cells on solid media prior to co-cultivation was of importance for successful transformation. Kumar *et al*. (2004) and Kathiresan *et al*. (2009) in their work on *Agrobacterium*-mediated transformation of *Chlamydomonas* and *Heamatococcus* respectively also reported a secondary culture period on solid media (2 days for *Chamydomonas* and 5–7 days for *Heamatococcus*) prior to co-cultivation; however the effects of different durations of pre-culture were not reported. It may be noted here in this study together with the

findings of Kumar *et al*. (2004) and Kathiresan *et al*. (2009) that pre-culture of microalgae on solid media is important, if not necessary, for successful transformation of microalgae in general. For plants, the positive effects of pre-culture has been extensively reported, and depending on the plant species and explant type used as targets for *Agrobacterium* infection, the optimal duration of pre-culture varied from 1–2 days to as long as 14 days (Chakrabarthy *et al*., 2002; Shrawat *et al*., 2007).

The optimal *Agrobacterium* density (OD_{600}=1.0) for transformation of *Nannochloropsis* was similar with several reports on *Agrobacterium*-mediated transformation of plants (Shrawat *et al*., 2007). In addition, this concentration has been utilised in routine transformation experiments (Kishimoto *et al*., 2002). Although several reports noted the overgrowth of *Agrobacterium* and explants mortality with high concentrations of *Agrobacterium* (Chakrabarty *et al*., 2002), the overgrowth of bacteria was not visually evident in this study, and such high density of bacteria did not adversely affect transformation frequency. This could probably be attributed to the type of medium in which co-cultivation was performed, given that when the co-cultivation medium was substituted with rich media (LB media, pH 5.6), GUS expression was very low to none, bacterial growth was visible whilst the algal cell suspension turned brownish after co-cultivation, signifying a decrease in cell viability (data not shown).

An acidic pH is generally considered to be suitable for transformation as an acidic pH may induce the *Agrobacterium* virulence genes (Stachel *et al*., 1985), while the optimal pH depends on the explant and the strain of *Agrobacterium* used, as the pH requirements for optimal *vir* gene induction are different for different *Agrobacterium* strains (Turk *et al*., 1991). It was found that pH of 5.5-5.6 is suitable for transformation of *Nannochloropsis* since there was no statistical difference between pH5.5 and 5.6 (Figure 4C). A pH value of 5.6 was reported to be optimal for transformation of grasspea (Barik *et al*., 2005) whereas in transformation of rice calli (Ali *et al*., 2007), pH 5.5 was preferred.

Co-cultivation duration of 2–7 days is generally considered suitable in *Agrobacterium*-mediated transformation (Mondal *et al*., 2001), and the optimum duration of co-cultivation is species and explant specific depending on the medium used for co-cultivation (Barik *et al*., 2005). In recent reports on the *Agrobacterium*-mediated transformation of green microalgae, 2 days of co-cultivation was used (Kumar *et al*., 2004; Kathiresan *et al*., 2009). Our findings, however, indicate that 3 days of co-cultivation was better for *Nannochloropsis* (Fig. 4D). Results reflect the findings of Kumar *et al*. (2009) that 3 days co-cultivation was optimal for transformation of mint and *Pinus*, respectively.

The data obtained in this study suggests that temperature is an important factor that affects *Agrobacterium* T-DNA delivery in *Nannochloropsis*. It was proposed the T-DNA transfer machinery which is coded by the VirB-VirD4 system worked better at lower temperatures (Fullner & Nester, 1996), and temperatures greater than 32°C are inhibitory to *Agrobacterium vir* gene expression (Jin *et al*., 1993). Although several reports have noted the importance of co-cultivation at low temperatures of 21°C (Sunilkumar & Rathore, 2001) and 22°C (Chakrabarty *et al*., 2002), it was observed that the highest percentage of GUS-expressing cells was obtained at 24°C (14.5%) and 25°C (12.9%) and in contrast, temperatures lower than 24°C drastically reduced GUS expression to 4.4% at 22°C and by almost 44 fold to 0.33% at 20°C (Figure 4E). Likewise, increasing the temperature to 26, 28 and 30°C resulted in a drop in GUS expression. It was found that the optimal temperature for transformation of *Nannochloropsis* is higher from those used in transformation of *Chlamydomonas* (Kumar *et al*., 2004) and *Heamatococcus* (Kathiresan *et al*., 2009). Our observation was similar in the transformation of *Alstroemeria* (Kim *et al*., 2007) where 24℃ was reported to be optimal. It is suggested that there may be an optimal temperature at which the plant cell is most receptive or susceptible to *Agrobacterium* infection (Shrawat *et al*., 2007), and optimal temperature for transformation may also vary in different *Agrobacterium* strains.

Plant phenolic compounds, such as AS, stimulate bacterial attachment and could increase transformation efficiency as they enhance *vir* gene function during transformation (Kumar *et al*., 2004; Shrawat *et al*., 2007; Cha *et al*., 2011). It has been reported that *Agrobacterium* infection to *Chlamydomonas* (Kumar *et al*., 2004) and *Heamatococcus* (Kathiresan *et al*., 2009) was feasible without the inclusion of AS. Our findings demonstrated that the percentage of GUS-expressing cells was very low (1.7%) in absence of AS and increased drastically to 13.5% in the presence of 50 µM AS (Figure 4F). Interestingly, increasing the concentration of AS from 50 to 300 µM resulted in a reduction of transformation frequency. This trend is similar in other transformation systems, where the increase of AS concentration did not further increase transformation frequency, and may even be detrimental in transformation (Chakrabarty *et al*., 2002), and it was also noted that AS exhibited bacteriostatic effects on *Agrobacterium* (Sheng and Citovsky, 1996). Nonetheless, in other plants, a high concentration (> 300 µM) is favoured (Shrawat *et al*., 2007). The optimal concentration of AS thus depends on the plant species and may be due to the nature of the explant, duration of co-cultivation and competence of target tissue (Shrawat *et al*., 2007).

Although several independent PCR positive transformants were obtained, the failure of many hygromycin-resistant colonies to produce any amplification products for either primer pairs indicate the incidence of 'escapes' through antibiotic selection while the hygromycin-resistant colonies that were confirmed positive by PCR could be maintained and subcultured on hygromycin-supplemented media.

Conclusion

In summary, a simple and optimised method for *Agrobacterium*-mediated transformation of *Nannochloropsis* sp was successfully developed. This finding will be useful in genetic manipulation of this commercially-important microalga which could be used in algal production of economically-important novel compounds.

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