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Establishment of an efficient genetic transformation method in *Dunaliella tertiolecta* mediated by *Agrobacterium tumefaciens*

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ABSTRACT

Microalgae are photosynthetic microorganisms widely used for the production of highly valued compounds, and recently they have been shown to be promising as a system for the heterologous expression of proteins. Several transformation methods have been successfully developed, from which the *Agrobacterium tumefaciens*-mediated method remains the most promising. However, microalgae transformation efficiency by *A. tumefaciens* is shown to vary depending on several transformation conditions.

The present study aimed to establish an efficient genetic transformation system in the green microalgae *Dunaliella tertiolecta* using the *A. tumefaciens* method. The parameters assessed were the infection medium, the concentration of the *A. tumefaciens* and co-culture time. As a preliminary screening, the expression of the *gusA* gene and the viability of transformed cells were evaluated and used to calculate a novel parameter called Transformation Efficiency Index (TEI). The statistical analysis of TEI values showed five treatments with the highest *gusA* gene expression. To ensure stable transformation, transformed colonies were cultured on selective medium using hygromycin B and the DNA of resistant colonies were extracted after five subcultures and molecularly analyzed by PCR. Results revealed that treatments which use solid infection medium, *A. tumefaciens* OD₆₀₀ = 0.5 and co-culture times of 72 h exhibited the highest percentage of stable *gusA* expression. Overall, this study established an efficient, optimized *A. tumefaciens*-mediated genetic transformation of *D. tertiolecta*, which represents a relatively easy procedure with no expensive equipment required. This simple and efficient protocol opens the possibility for further genetic manipulation of this commercially-important microalgae for biotechnological applications.

1. Introduction

Microalgae are photosynthetic unicellular microorganisms particularly prominent in the biotechnology industry due to their utility in the production of valuable compounds with diverse applications in food, cosmetics, and pharmaceuticals (Gong et al., 2011; Norzagaray-Valenzuela et al., 2017). Recently, researchers and entrepreneurs have begun to realise the value of microalgae for use in genetic engineering as a manufacturing platform for the heterologous expression of proteins. The favourable characteristics of microalgae, such as the relatively short multiplication rate, the ability of being grown in an enclosed photobioreactor protecting the environment from the flow of

transgenes, and the relatively low cost of culture and maintenance position this transformation system as an eminently viable alternative to the most commonly used expression platforms, including plants and bacteria (Walker et al., 2005; Barzegari et al., 2010; Specht et al., 2010; Gong et al., 2011).

Moreover, green microalgae are used for industrial purposes and are classified as GRAS (Generally Regarded as Safe); this indicates that these microalgae products can be directly used as a powder or freeze-dried, with a little or no extra processing purification methods prior to industrial use, highlighting the feasibility of producing microalgae-derived highly-valued compounds (Walker et al., 2005).

Green microalgae species belonging to the genus *Dunaliella* are a

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good target of genetic transformation, as their lack of a rigid cell wall, and due to its halotolerance properties, the risk of contamination during the manufacturing is reduced, facilitating the transformation process (Borowitzka and Siva, 2007).

Despite recent advances in microalgae genetic engineering, genomic manipulation of the *Dunaliella* genus has been limited to a few species, including *D. salina* and *D. bardawil*, whilst the use of *D. tertiolecta* has received less attention. *D. tertiolecta* has been shown to synthesize bioactive compounds such as β -carotene, phenolic compounds and polysaccharides, all with significant commercial value (Borowitzka, 2013). Additionally, *D. tertiolecta* proteins and its protein hydrolysates have shown antioxidant and antiaging potential (Raja et al., 2008; Norzagaray-Valenzuela et al., 2017). Moreover *D. tertiolecta*, as marine microalgae, can be cultured on a large scale using readily available seawater. With regards to genetic engineering purposes, *D. tertiolecta* can be cultured in open photobioreactors associated with a low cost and without the risk of gene escaping (Barzegari et al., 2010); therefore, it would be of high interest to develop genetic engineering approaches to enhance the potential commercial exploitation of this microalga.

Several methods have successfully been used for the genetic transformation of *Dunaliella* species. For example, *D. salina* and *D. bardawil* have been transformed by electroporation (Geng et al., 2003), micro-projectile bombardment (Tan et al., 2005), glass beads (Feng et al., 2009) and *Agrobacterium tumefaciens* (Anila et al., 2011; Simon et al., 2016). Among these transformation methods, the *A. tumefaciens*-mediated transformation system represents one of the most promising methods for stable integration of foreign genes into host cells (Zhang et al., 2014). The methodology exploits *A. tumefaciens* bacterial pathogenicity through its capacity to transfer foreign genes using the T-DNA region from its tumor inducing plasmid (Ti plasmid) to a host genome (Gelvin, 2000).

The effectiveness of *Agrobacterium*-mediated genetic transformation of microalgae is reported to be modulated by several factors. For instance, the *Agrobacterium* strain and its concentration (OD₆₀₀), the co-culture time, infection medium, pH, temperature, and microalgae species have been suggested as key variables for the success of microalgae transformation (Cha et al., 2011; Úbeda-Mínguez et al., 2015; Srinivasan and Gothandam, 2016). Thus, it is of vital importance to find the most effective parameter conditions for a particular microalgae transformation.

Therefore, this study aimed to find an optimized genetic transformation method for *Dunaliella tertiolecta* using the *A. tumefaciens* system by optimizing the following transformation conditions: the infection medium (liquid and solid), *Agrobacterium* concentration (OD₆₀₀ = 0.5, 1.0 and 1.5) and co-culture times (24, 48 and 72 h).

2. Materials and methods

2.1. Microalgal strain and culture conditions

D. tertiolecta (strain DUT2) was obtained from the Centro de Investigación Científica y Educación Superior de Ensenada (CICESE), culture collection in Baja California, México. *D. tertiolecta* was cultured in F/2 medium (Guillard and Ryther, 1962), and used for the analysis of cell growth and viability kinetics. For transformation and selection experiments solid and liquid TAP medium (Tris-Acetate-Phosphate) (Harris, 1989) were used, adjusted to contain a final concentration of 0.2 M NaCl. For solid medium 1.5% agar was added.

2.2. Plasmid and bacterial strains

The binary vector pCambia1304 (GenBank accession: AF234300) containing a modified β -glucuronidase (*gusA*) gene, and the hygromycin phosphotransferase II (*hpt* II) gene, both regulated by the constitutive promoter CaMV35S, was used for genetic transformation. *Agrobacterium tumefaciens* strain EHA105 containing the binary vector

was used for genetic transformation.

2.3. Cell growth and viability kinetics

D. tertiolecta cultures were established using 1×10^6 cells mL⁻¹ as inoculum in a total volume of 300 mL of F/2 medium (Guillard and Ryther, 1962). Cultures were maintained at 24 °C, with 1% CO₂, and continuous light (irradiance of 120–130 μ mol photons m⁻² s⁻¹). Cell density was recorded every 24 h for 18 days by direct counting using a hemacytometer of 0.0025 mm² (Optik Labor™, Milan, Geneva, CH). Parameters of growth efficiency such as specific growth rate (μ) and doubling time (*dt*) were determined as described by Godoy-Hernandez and Vázquez-Flota (2006). Both parameters were calculated with the equations Eqs. (1) and (2), respectively.

$$\mu = (\ln x - \ln x_0)/t \quad (1)$$

$$dt = \ln 2/\mu \quad (2)$$

where μ was used for specific growth rate; *dt* for doubling time; *x*₀ for the initial cell density; and *x* for the cell density at time *t*.

Cell viability kinetics was determined as reported by Richardson and Fedoroff (2001) with the number of viable cells by dye exclusion. Briefly, 0.2 mL of 0.1% trypan blue dye was added to 0.8 mL of microalgae culture, the suspension was homogenized and then counted using a hemacytometer of 0.0025 mm² (Optik Labor™, Milan, Geneva, CH). Cells stained blue were considered as non-viable. Viability percentages were obtained by expressing the number of viable cells as a percentage of total cells.

2.4. Antibiotic sensitivity assay

The sensitivity of *D. tertiolecta* to the antibiotic Hygromycin B (Hyg B) was monitored in solid 0.2 M TAP medium. Approximately 1.5×10^6 cells were spread onto agar in 90 mm Petri dishes containing different concentrations of the antibiotic ranging from 70 to 110 mg L⁻¹, and incubated under continuous light at an irradiance of 120–130 μ mol photons m⁻² s⁻¹ at 25 °C. Microalgae growth was monitored at daily intervals for 21 days. The minimum inhibitory concentration of Hyg B is determined by the concentration in which no colony growth can be observed. When cells were plated on solid TAP medium containing 110 mg L⁻¹ hygromycin B, no growth was observed (Fig. 1). Therefore, this concentration was taken as the minimum inhibitory concentration of antibiotic, and was used for the selection medium.

2.5. Agrobacterium-mediated transformation procedure

The optimization of the genetic transformation system of *D. tertiolecta* using *A. tumefaciens* method was achieved by varying the infection medium (liquid and solid), *Agrobacterium* concentration (OD₆₀₀ of 0.5, 1.0, and 1.5), and co-culture time (24, 48 and 72 h). These three parameters resulted in 18 possible different treatment combinations (Table 1).

Agrobacterium was inoculated into 10 mL of Luria Bertani medium in the presence of selective antibiotics (50 mg L⁻¹ kanamycin and 80 mg L⁻¹ gentamycin), and grown overnight in a rotary shaker at 200 rpm and 28 °C in the dark until reach an OD₆₀₀ = 0.5, 1.0 and 1.5. For virulence (*vir*) gene induction, the bacterial suspension was supplemented with 100 μ M acetosyringone and incubated for 4 h at 28 °C. Prior to co-cultivation, an aliquot of 1.5×10^6 of *D. tertiolecta* cells (taken at the day in which the highest cell viability was recorded) was pre-cultured on solid TAP medium at 25 °C for 10 days, with continuous light, allowing a confluent layer of cells to be formed. *D. tertiolecta* cells were then infected using both a solid and a liquid infection medium. For solid infection, 200 μ L of induced *A. tumefaciens* were added onto microalgae culture plate; for liquid infection, microalgae cells were harvested by centrifugation, resuspended in 2 mL of TAP medium, and

Fig. 1. Effect of varying hygromycin B concentration on the growth of *D. tertiolecta* in solid medium; a, Cells growth without antibiotic, used as a control; b–f, cells growth at 70, 80, 90, 100 and 110 mg L^{−1} Hyg-B, respectively. Each image is a schematic representation of three replicates.

Table 1

Optimization of critical parameters in the *Agrobacterium tumefaciens*-mediated transformation method.

Treatment (Tx)	Infection medium	<i>Agrobacterium</i> optical density (OD ₆₀₀)	Co-culture time (hours)
1	Liquid	0.5	24
2	Liquid	0.5	48
3	Liquid	0.5	72
4	Liquid	1.0	24
5	Liquid	1.0	48
6	Liquid	1.0	72
7	Liquid	1.5	24
8	Liquid	1.5	48
9	Liquid	1.5	72
10	Solid	0.5	24
11	Solid	0.5	48
12	Solid	0.5	72
13	Solid	1.0	24
14	Solid	1.0	48
15	Solid	1.0	72
16	Solid	1.5	24
17	Solid	1.5	48
18	Solid	1.5	72

mixed with 200 μ L of induced *A. tumefaciens*. Then, both solid and liquid treatments were incubated at 25 °C for 30 min in a rotary shaker at 200 rpm. After infection, cells from the liquid infection step were spread plated onto solid TAP medium. Plates from both treatments were further incubated at 25 °C at the three different co-culture times (24, 48 and 72 h) in dark. After co-cultivation, cells were harvested and washed three times with TAP medium supplemented with 100 mg L^{−1} Timentin, in order to eliminate bacterial residue, and recovered through centrifugation. Washed cells were resuspended in TAP medium and the suspension was divided into two parts; one was used to determine the percentage (%) of cell viability; the other one, was used to obtain the percentage (%) of GUS positive cells calculated through the biochemical GUS assay. Both percentages, viability and GUS positive cells, were used to calculate a novel parameter called Transformation

Efficiency Index (TEI), according to:

$$\text{TEI} = \% \text{ cell viability (0.5)} + \% \text{ of GUS positive cells (0.5)} \quad (3)$$

2.6. Hygromycin B-resistance selection

TEI was used for screening the transient expression of *gusA* in the 18 independent experiments. In order to select the combination exhibiting the highest stable transformation efficiency, treatments presenting the highest TEI values were selected for Hyg B-resistance. After the co-culture period, cells were collected and washed with TAP medium supplemented with 100 mg L^{−1} Timentin, recovered through centrifugation, resuspended in liquid TAP medium, and plated onto selection medium (TAP supplemented with 100 mg L^{−1} Timentin and Hyg B at the minimum inhibitory concentration). Hyg-B resistant colonies were recorded and the transformation frequency was calculated as the ratio between the number of Hyg B-resistant colonies among the initial number of *Agrobacterium*-infected cells.

The Hyg B-resistant colonies were grown independently in liquid TAP medium supplemented with Hyg B, at the minimum inhibitory concentration and 100 mg L^{−1} Timentin in order to ensure the elimination of bacterial residue, at 25 °C, with continuous light during 3 days to ensure the selection of only transformed cells; this procedure was repeated five times, then were separated into two aliquots, one was used to perform the biochemical GUS assay and the other used to confirm by molecular analysis the stable T-DNA integration by molecular analysis (Fig. 2).

Single plate of *D. tertiolecta* without *A. tumefaciens* infection (Wild Type, WT), was taken as a negative control. Experiments were performed in triplicate.

2.7. Biochemical GUS assay

Detection of the reporter gene expression was carried out through the biochemical GUS assay according to Jefferson (1987). *D. tertiolecta* colonies were collected by centrifugation and resuspended in a staining solution containing the substrate X-Gluc [5-bromo-4-chloro-3-indolyl b-

Fig. 2. Schematic illustration of the optimized genetic transformation method in *Dunaliella tertiolecta* mediated by *Agrobacterium tumefaciens*.

D-glucuronide (GUSS-1KT; Sigma, St. Louis, MO)], and incubated overnight in the dark at 37 °C. After incubation, cells were rinsed 3 times with 70% ethanol to remove chlorophyll, and *gusA* expression was visualized as indigo-blue spots using a digital microscope (Primo Star ZEISS, Germany). Total cells were counted using a hemacytometer of 0.0025 mm² (Optik Labor™); cells showing blue staining color were considered as GUS positive cells. The percentage of GUS positive cells was obtained by calculating the difference between the number of blue cells and the number of total cells, and was expressed as a percentage.

2.8. Analysis of T-DNA integration and transgene expression

In order to confirm the stable integration of the *gusA* gene into the genome, molecular analysis was performed by the isolation of genomic DNA from the putative transformed cells, this procedure was carried out after five subcultures of the single colonies in selective medium. Approximately 10 mL of *Dunaliella* culture was centrifuged at 3500 rpm for 5 min, then mixed with 100 µL of Plant DNAzol reagent (Invitrogen™, Carlsbad, CA) according to the manufacturer's

instructions. The quantity and quality of the isolated DNA was analyzed using a Nanodrop Spectrophotometer (ThermoFisher™). Good quality isolated DNA (abs 260 nm/230 nm > 1.80) was subjected to PCR analysis to detect the presence of the *gusA* gene. The following primers were used to amplify an internal fragment of the *gusA* gene with a predicted fragment size of 982-bp: forward 5'-CCGTGGTGACGCATGT CGCGCAAGAC-3' and reverse 5'-CGGCGTGGATACGTTAGCCGGGCTG CACTC-3'. PCR was carried out in a 20 µL final reaction volume, containing 0.2 mM of dNTPs, 0.2 mM of each primer, 3 U Taq polymerase, 1.5 mM of MgCl₂, and 200 ng of purified sample DNA (Zhang et al., 1998). The reaction mixtures were subjected to the following amplification conditions: 2 min at 94 °C, 35 cycles of 1 min at 94 °C, 2 min at 58 °C, 2 min at 72 °C, and finally 5 min at 72 °C (Cruz-Méndivil et al., 2011). Plasmid pCambia1304 was used as positive control and DNA from *D. tertiolecta* wild type as negative control. The amplified fragments were separated by electrophoresis on 1.2% (w/v) agarose gels, stained with ethidium bromide, and fluorescence visualized under ultraviolet light (Fig. 6). Images were captured in a Molecular Imager Chemi-Doc™ XRS System (Bio-Rad, Hercules, CA).

Taking into account the molecular analysis, the percentage of transformation efficiency was calculated as the difference between the number of PCR positive colonies and the number of total screened colonies.

2.9. Statistical analysis

Experiments were conducted in a randomized design, using three replicates per treatment condition. The TEI values were analyzed using a multifactorial analysis of variance and means were compared using the Fisher's least significant difference test ($p < .05$) using Statgraphics Centurion XV software (Statpoint Technologies, USA).

3. Results

3.1. Cell growth and viability kinetics

D. tertiolecta cell growth and viability kinetics are shown in Fig. 3. A typical sigmoidal behavior of microalgae growth was observed. Cell growth reached the highest cellular density at day 14 ($2.3 \pm 0.05 \times 10^6$ cells mL⁻¹). This kinetic revealed a specific growth rate (μ) of 0.167 day⁻¹ and doubling time (dt) of 4.14 day. The highest percentage of cell viability was observed at day 4 (86%), so this day was chosen to perform the genetic transformation procedures.

Fig. 3. Comparison of cell growth and viability kinetics. Each point is the average of three replicates. Error bars indicate standard deviation.

Fig. 4. Biochemical assay of *gusA* expression. a) *D. tertiolecta* non-infected cells used as negative control; b) and c), *Agrobacterium*-infected *Dunaliella* cells from solid and liquid infection medium, respectively. Bars represent 20 μm .

3.2. Genetic transformation

The effect of three different critical transformation conditions on the transformation efficiency was studied, including the infection medium (solid and liquid medium), concentration of the *A. tumefaciens* ($\text{OD}_{600} = 0.5, 1.0$, and 1.5), and the co-culture time (24, 48 and 72 h). These three conditions lead to 18 independent experiments, as shown Table 1.

3.3. Transformation efficiency index (TEI)

Agrobacterium-infected *D. tertiolecta* cells cultured using the 18 independent experiments were analyzed using the biochemical GUS assay, performed immediately after termination of co-culture. Transformed cells were stained blue, whilst untransformed cells did not retain any staining (Fig. 4). The Transformation Efficiency Index (TEI) was evaluated in the 18 independent experiments, through assessment of the percentage of GUS-positive cells and the percentage of cell viability. Overall, the 18 analyzed treatments showed a relatively high TEI (> 65), as shown in Fig. 5. Statistical analysis presented five different treatments with the highest TEI: tx 12 (solid infection medium, $\text{OD}_{600} = 0.5$, and 72 h of co-culture), tx 14 (solid infection medium, $\text{OD}_{600} = 1.0$, and 48 h of co-culture), tx 15 (solid infection medium, $\text{OD}_{600} = 1.0$, and 72 h of co-culture), tx 17 (solid infection medium, $\text{OD}_{600} = 1.5$, and 48 h of co-culture) and tx 18 (solid infection medium, $\text{OD}_{600} = 1.5$, and 72 h of co-culture), with no significant difference

between them.

3.4. Stable transformation frequency

In order to find the best combination showing the highest stable transformation efficiency, the transformation procedure was repeated for the five treatments with the highest TEI values. Hyg B-resistant colonies became visible after 3-weeks incubation in selection medium. These colonies were recorded and used to calculate the stable transformation frequencies, showing results of 928×10^{-6} , 288×10^{-6} , 388×10^{-6} , 229×10^{-6} and 212×10^{-6} , for tx 12, tx 14, tx 15, tx 17, and tx 18, respectively (Table 2).

3.5. Stable transformation efficiency

To confirm the presence of the transgene in the genome of Hyg B-resistant colonies, genomic DNA was extracted and PCR was performed using specific primers to amplify a fragment corresponding to the *gusA* gene. A 982-bp amplicon was obtained from PCR of Hyg B-resistant cells, which was the same as that of the positive control, the vector pCambia 1304. This amplicon was not observed in genomic DNA isolated from untransformed (WT) cells (Fig. 6). PCR screening of Hyg B-resistant colonies is shown in Table 3. The tx 12 (solid infection medium, $\text{OD}_{600} = 0.5$, and 72 h of co-culture) showed the highest percentage of transformation efficiency ($\sim 20\%$); meanwhile, tx 14, tx 15, tx 17, and tx 18 showed percentages of 3.13, 8.7, 3.23 and 2.86%,

Fig. 5. Transitory Efficiency Index of different treatments tested for transformation of *D. tertiolecta*. TEI values are a result of combining the percentage of cell viability and the efficiency of the *gusA* gene biochemical assay. Each value is the mean of three independent experiments. Errors bars indicate the LSD value (3.88).

Table 2
Transformation frequencies in *Agrobacterium*-mediated transformation of *D. tertiolecta*.

Treatment	Cells plated ($\times 10^6$)	Number of Hyg-B resistant colonies	Transformation Frequency ($\times 10^6$ cel $\pm$ SD)
tx 12 (solid infection medium, OD ₆₀₀ = 0.5, 72 h)	1.5	1392	928 $\pm$ 105.0 ^c
tx 14 (solid infection medium, OD ₆₀₀ = 1.0, 48 h)	1.5	432	288 $\pm$ 23.2 ^{ab}
tx 15 (solid infection medium, OD ₆₀₀ = 1.0, 72 h)	1.5	581	388 $\pm$ 63.6 ^b
tx 17 (solid infection medium, OD ₆₀₀ = 1.5, 48 h)	1.5	344	229 $\pm$ 40.3 ^{ab}
tx 18 (solid infection medium, OD ₆₀₀ = 1.5, 72 h)	1.5	317	212 $\pm$ 66.2 ^a

Data represent the mean of three determinations $\pm$ standard deviation. Different letters denote significant differences by Fisher test ($p < .05$).

respectively. Additionally, PCR-positive *D. tertiolecta* cells were inspected by the biochemical GUS assay. Results revealed most of the stably transformed cells successfully express the *gusA* gene, and only a minor portion of transformed cells did not show the blue staining characteristic of β -glucuronidase expression, as visualized under a microscope (Fig. 7).

4. Discussion

In this study we describe, for the first time, the establishment of an efficient genetic transformation system in *D. tertiolecta* using an *A. tumefaciens*-mediated protocol. To do this, key transformation conditions: such as the infection medium, concentration of *A. tumefaciens* and co-culture time were optimized. Following a preliminary screening, 5 out of the 18 evaluated independent experiments were further assessed due

to their promising transformation efficiency, as indicated by the Transitory Efficiency Index (TEI). These five treatments were analyzed molecularly to reveal the optimal conditions for stable transformation. Results demonstrate that using a solid infection medium, an OD₆₀₀ = 0.5, and a 72 h co-culture time represent the best combination, having the highest stable transformation efficiency.

In the last decade, there has been an increased interest in microalgae as a promising source of bioactive molecules. Their utility and potential as a source of recombinant proteins with pharmaceutical and biotechnological applications is due to the ability to genetically modify any number of specific metabolic pathways to obtain desired compounds (Song et al., 2008; Specht et al., 2010). Thus, different methods are reported for the successful genetic transformation of microalgae including electroporation, microprojectile bombardment, glass beads, and the *A. tumefaciens*-mediated method (Walker et al., 2005; Qin et al., 2012).

Fig. 6. PCR molecular analysis in putatively transformed *D. tertiolecta* cells. Each lane represents an individual experiment, with the treatment depicted above the appropriate lane(s). WT; genomic DNA of untransformed microalgae cells used as a negative control, M; molecular weight marker, e; empty lane, C+; pCAMBIA1304 plasmid used as positive control. Arrows indicate expected fragment size of 982-bp.

Table 3
Stable transformation efficiency in *Dunaliella tertiolecta* cells.

Treatment no.	No. analyzed colonies	<i>gusA</i> (PCR +)	% transformation efficiency
tx 12 (solid infection medium, OD ₆₀₀ = 0.5, 72 h)	26	5	19.23
tx 14 (solid infection medium, OD ₆₀₀ = 1.0, 48 h)	32	1	3.13
tx 15 (solid infection medium, OD ₆₀₀ = 1.0, 72 h)	23	2	8.70
tx 17 (solid infection medium, OD ₆₀₀ = 1.5, 48 h)	31	1	3.23
tx 18 (solid infection medium, OD ₆₀₀ = 1.5, 72 h)	35	1	2.86

Fig. 7. Biochemical *GUS* assay of transgenic *D. tertiolecta* cells. Microscopic view: a, untransformed control *D. tertiolecta* (Wt); b, c and d, transgenic *D. tertiolecta* cells showing *GUS* expression. Bars represent 20 μ m.

The *A. tumefaciens* method has multiple advantages; it is a relatively easy way to deliver DNA into the host cells; it copes well with reasonably large T-DNA molecules; there is a low incidence of transgene integration and rearrangement; and it does not require special equipment (Gelvin, 2000; Cha et al., 2012). Therefore, this method has been widely used to transform different microalgae species, namely *Tetraselmis chuii* (Úbeda-Mínguez et al., 2015), *Chlorella vulgaris* (Cha et al., 2012), and *Chlamydomonas reinhardtii* (Kumar et al., 2004; Pratheesh et al., 2014). The successful genetic transformation of microalgae by *A. tumefaciens* is influenced by numerous transformation conditions (Cha et al., 2012).

Before beginning the transformation process, cell growth and viability characteristics of *D. tertiolecta* were analyzed. These results suggested day 4 to be the time period with the highest cell viability and a cell density close to 1×10^6 cells (Fig. 3); thus, this day was selected for downstream transformation experiments. Indeed, a high cell viability ensures proliferation and a higher resistance in the transformation process (Pratheesh et al., 2014). Moreover, cell kinetic parameters, specific growth rate ($\mu = 0.167 \text{ day}^{-1}$) and doubling time ($dt = 4.14 \text{ day}$), were found to be better than the reported previously by Arun and Singh (2013), who showed values of $\mu = 0.13 \text{ day}^{-1}$ and $dt = 5.8 \text{ day}$ for the same *D. tertiolecta*. Higher values of μ indicates better ability of an organism to grow under a given set of environmental conditions; while, lower dt values indicate shorter time (days) required for cells to divide.

To determine the minimum inhibitory concentration of Hyg B, different concentrations were tested, and 110 mg L^{-1} was found to completely inhibit *D. tertiolecta* growth (Fig. 1). This concentration was used for subsequent selection of transformants. Hygromycin B is an aminoglycoside antibiotic produced by *Streptomyces hygroscopicus* that potentially inhibits spontaneous reverse translocation of tRNAs and mRNA on the ribosome causing a disruption in polypeptide synthesis (Cabañas et al., 1978; Borovinskaya et al., 2008). Hyg B has a large action spectrum in both prokaryotic and eukaryotic cells, and has been shown as an effective selectable antibiotic in a wide diversity of transgenic cellular cultures, such as tobacco callus (Santos-Ballardo et al., 2013), tomato (Cruz-Mendivil et al., 2011; Germán-Báez et al., 2014), rice callus (Tran and Sanan-Mishra, 2015), and some microalgae (Anila et al., 2011; Pratheesh et al., 2012).

In microalgae, the minimum inhibitory concentration of Hyg B for transgenic selection is somewhat controversial. Higher concentrations than those reported for freshwater microalgae such as *Chlamydomonas reinhardtii*, 10 mg L^{-1} (Kumar et al., 2004; Pratheesh et al., 2012); *Haematococcus pluvialis*, 10 mg L^{-1} (Kathiresan et al., 2009); and *Chlorella vulgaris*, 20 mg L^{-1} (Cha et al., 2012), were found in the present study. One potential reason for this is that freshwater microalgae can grow without salt in the medium, and salt concentration is associated with a reduced or lack of antibiotic effectiveness (Allnutt et al., 2000). Indeed, Prasad et al. (2014) and Úbeda-Mínguez et al. (2015) reported no selectable effect of hygromycin B in marine

microalgae (Prasad et al., 2014; Úbeda-Mínguez et al., 2015); meanwhile, Anila et al. (2011) showed an antibiotic effectiveness in *Dunaliella bardawil* when the salt concentration in the medium was decreased. Preliminary experiments, by adjusting the salt molarity (0.1, 0.15, 0.2 and 0.5 M), showed 0.2 M as the concentration that allows the grow of *D. tertiolecta* microalgae and *A. tumefaciens* together, and at the same time to maintain the antibiotic effectiveness (data not shown).

The TEI equation assesses the biochemical activity of the *gusA* product and cell viability to measure successful transformation of *D. tertiolecta*. Transformation efficiency was analyzed by monitoring the transient expression of the reporter *gusA* gene in the 18 different selected treatments (Table 1). This reporter gene is commonly used as a gene fusion marker for the analysis of transient gene expression in transformed cells, as it utilizes the substrate 5-bromo-4-chloro-3-indolyl glucuronide [X-Gluc, (Jefferson et al., 1987)], the product of which can be detected directly under a microscope (Jefferson, 1987; Naleway, 1992). The difference in intensity of the blue color in the transformed cells is indicative of differencing expression levels of the *gusA* gene; sectorized/spotted blue cells observed could be explained as a result of difference in X-Gluc penetration in the cells. This phenomenon has been previously reported in microalgae (Kathiresan et al., 2009; Cha et al., 2012). No intrinsic GUS activity was detected (Fig. 4a).

The second component of the transformation efficiency equation assesses cell viability, something often overlooked, despite the importance of having cells in better conditions to support possible damage caused by *Agrobacterium* virulence (Gupta et al., 2017). The basis of a successful genetic transformation is to promote the attachment between the *Agrobacterium* and the host cells to enable insertion of foreign T-DNA molecule while maintaining the cell viability for long term (Berrios et al., 2015; Gupta et al., 2017). By giving both cell viability and the percentage (%) of GUS positive cells equal weighting (50:50), within the TEI equation (Eq. (3)), we regard both parameters with equal importance, based on the literature.

All analyzed treatments had a relatively good TEI with values > 65, indicating the suitability of *A. tumefaciens*-mediated transformation method to perform the correct insertion of their T-DNA into the host microalgae cell (Fig. 4). Statistical analysis of TEI values revealed tx 12, 14, 15, 17 and 18 to have the highest TEI with values around 85 (Fig. 5). Interestingly, all five treatments found presenting the highest TEI corresponded to solid infection medium. This increased TEI is likely due to the microalgae remaining immotile during the infection procedure, allowing more bacteria-microalgae interaction, thus facilitating the transformation process (Cheng et al., 2011).

The concentration of *A. tumefaciens* in a particular host cell is an important factor which could influence on the transference of T-DNA (Kumar et al., 2004; Cha et al., 2012). A synergy between a quantity of bacteria sufficient for an effective T-DNA transfer whilst retaining adequate cell viability is essential. The three different concentrations of *A. tumefaciens* (OD₆₀₀ = 0.5, 1.0 and 1.5) used in this study were demonstrated to be effective for the genetic transformation (Fig. 5), however their efficiency varies depending on combination with the other variables. Co-culture times of 48 and 72 h were demonstrated to be more effective and result in a higher TEI; however, prolonged co-culture time, for instance longer than 72 h, is associated with an overgrowth of bacteria, potentially affecting microalgae viability (Hu et al., 2006; Kathiresan et al., 2009; Cha et al., 2012). Again, factors such as the *Agrobacterium* species, the host cell and the co-culture medium may further influence optimal culture time (Kumar et al., 2004; Kathiresan et al., 2009; Prasad et al., 2014).

The TEI parameter allows for an assessment of *gusA* expression in a rapid manner from a number of different treatments. It is intended to serve as an initial screening step, a high-throughput method to identify promising optimal conditions. In order to ensure the transgene was indeed integrated into the genome, it is then necessary to evaluate the stable expression by repeating the transformation process using the five best treatments, and then performing the molecular and biochemical

analyses.

High transformation frequencies were observed in all five analyzed treatments (Table 2). These transformation frequencies were similar to those reported by Kumar et al. (2004) in *Chlamydomonas reinhardtii* ($355 \pm 0.20 \times 10^{-6}$), and higher than those reported by Úbeda-Mínguez et al. (2015), who optimized different transformation conditions such as pH, *Agrobacterium* concentration and co-culture time in *Tetraselmis chuii*, reporting a frequency average of $150 \pm 0.93 \times 10^{-6}$. In the present study, the tx 12 (solid infection medium, OD₆₀₀ = 0.5 and 72 h of co-culture) exhibited the highest transformation frequency (Table 2). Tx 12 was higher than the reported by Pratheesh et al. (2014), in *Chlamydomonas reinhardtii*, with a transformation frequency of $523 \pm 10.2 \times 10^{-6}$ using the conditions of OD₆₀₀ = 0.5 and 48 h of co-culture indicating an improvement of the transformation by the increase in co-culture time.

Molecular analysis was performed in Hyg B-resistant colonies (Fig. 6 a-c). PCR was used to amplify the *gusA* gene (Fig. 6a-c). The results showed tx 12 to have the highest transformation efficiency (~20%); and treatments tx 14, 15, 17 and 18 to have transformation efficiency percentages ranging between 2 and 9% (Table 2). This result is in agreement with several studies describing a co-culture time of 72 h to result in the highest transformation efficiency in several microalgae. Cha et al. (2011) optimized the transformation procedure for *Chlorella vulgaris*, suggesting co-culture times between 72 and 96 h; meanwhile, Úbeda-Mínguez et al. (2015) reported 72 h for *Tetraselmis chuii*. In both studies, the optimized the *Agrobacterium* concentration was found at OD₆₀₀ = 1.0, however an *Agrobacterium* concentration of OD₆₀₀ = 0.5 was shown by Simon et al. (2016) to be optimal for *D. salina* infection, indicating the necessity of optimizing transformation conditions for each particular microalgae species. The possibility of *Agrobacterium* contamination was ruled out by maintaining transformed cells in selective medium containing the gram negative antibiotic Timentin, which is commonly used to eliminate this bacterium.

Results from the biochemical GUS assay of the stably transformed cells reveal that some cells did not present the blue staining that is characteristic of transformed cells, indicative of a lack of *gusA* gene expression. Several factors are associated with the different gene expression pattern observed, including the efficiency of the promotor, mutagenic effects of position on integration events (randomly insertion in the nuclear genome), and the epigenetic-derived transgene silencing, as previously reported by several authors (Louis and Miller, 1998; Mayfield and Franklin, 2005; Cha et al., 2012; Doron et al., 2016).

5. Conclusions

An optimized method for genetic transformation of the green microalgae *Dunaliella tertiolecta* by *Agrobacterium tumefaciens* has been developed. The novel parameter TEI resulted a suitable tool to evaluate the transformation efficiency of a high number of treatments in a short period of time, and the efficiency data thrown by this parameter was subsequently confirmed at a molecular level. This study highlights the relevance of using the *A. tumefaciens* mediated-transformation method, which represents a relatively easy procedure with no expensive equipment required or any special cell preparation. This easy, simple and efficient protocol opens the possibility for further genetic manipulation of this commercially-important microalgae for biotechnological applications.

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