CHAPTER II

*Agrobacterium* mediated genetic transformation of *Dunaliella bardawil* using selectable marker genes
Summary

*Dunaliella bardawil*, a unicellular green microalga is known for its halotolerant nature and β-carotene production. However, a stable transformation technique is lacking in this organism. In the present study, *Agrobacterium tumefaciens* mediated stable transformation protocol was standardised for *D. bardawil*. Preliminary studies like, sensitivity of algae for different antibiotics, media selection for the co-cultivation for both algae and the bacteria were carried out. Various selection antibiotics tested against *D. bardawil* proved to be insensitive even at higher concentrations in growth medium. The sensitivity studies showed that the *D. bardawil* was able to tolerate cefotaxime and potassium clavulanate upto 1000 mgL\(^{-1}\) and 1500 mgL\(^{-1}\) while minimum concentration of these antibiotics that arrested growth of *A. tumefaciens* was determined to be 500 mgL\(^{-1}\) and 300 mgL\(^{-1}\) respectively. Co-cultivation and selection was carried out in solid TAP medium containing 0.2 M NaCl. The selection antibiotic, hygromycin was found to be effective at a concentration of 100 mgL\(^{-1}\) for complete killing of *D. bardawil* cells. Hygromycin sensitivity of *D. bardawil* was inversely proportional to the NaCl concentration in the medium. The transformation frequency observed with the addition of 100 µM acetosyringone was found to be 42.0 ± 3 per 10\(^6\) cells plated. Addition of acetosyringone during co-cultivation has no effect on transformation frequency. Transformation was confirmed by GUS, GFP expression, PCR for *hpt* gene, southern and western blotting. The site of integration of trans-gene in the transformants were analysed using adaptor ligated genome walking and the sequences showed that the gene is integrated in non-coding region. Pigment and growth profile of transformants were similar to that of wild *D. bardawil*. The transformants obtained through the standardised protocol were found to be stable even in the absence of selection antibiotic in the growth medium for a period of more than 3 years. Molecular characterization of *Dunaliella* spp. used in the present study using 18S rDNA showed that the alga belongs to the variety *Dunaliella salina* var teod, a hyper β-carotene accumulating strain of *Dunaliella salina*. The stable transformation procedure standardised may be thus used for its genetic manipulation and its better utilisation in molecular farming techniques.
2.1 Introduction

Transformation may be defined as “the introduction of exogenous genes into plant cells, tissues or organs employing direct or indirect means developed by molecular and cellular biology” (Birch, 1997). Progress in genetic engineering has been spectacular since the first successful plant transformation in the early 1980s. Application of molecular techniques resulted in the generation of numerous transgenic species with commercially important genes enabling pharmaceutical, nutraceutical, and agronomic improvement, easier processing and other alternative uses. Nuclear transformation of various microalgal species is now routine as there has been interest in the “mining” of algal species for novel pharmaceuticals (Skulberg, 2000). Transgenic microalgae may be exploited as cell factories for the production of valuable recombinant products such as vaccines, specialty oils, and novel carotenoids (Leon-Banares et al, 2004). Presently the genetic transformation of various algal species have been reported for *Chlamydomonas reinhardtii* (Kumar et al, 2004), *Chlorella* sp (Maruyama et al, 1994; Dawson et al, 1997), *Spirulina* sp (Toyomizu et al, 2001), Volvox carteri (Schiedlmeier et al, 1994), *Synechocystis* (Dzelzkalns and Bogorad, 1986), *Haematococcus pluvialis* (Teng et al, 2002; Steimbrenner and Sandman, 2006) etc using different transformation methods like electroporation, particle bombardment, glass beads, silicon carbide, cell wall-deficient mutants or protoplasts (Kindle, 1990; Lumbreras et al, 1998; Dawson et al, 1997). But *Agrobacterium* mediated genetic transfer which is a highly efficient, stable method of genetic transformation was followed only for *Chlamydomonas reinhardtii* (Kumar et al, 2004) and *Haematococcus pluvialis* (Kathiresan et al, 2009). So far no other microalgae have been attempted using this method probably due to the incompatibility between the hosts for its adherence, induction of *vir* genes and integration of T-DNA.

Genetic transformation, mediated by *Agrobacterium tumefaciens*, has become the most used method for the introduction of foreign genes into plant cells and the subsequent regeneration of transgenic plants. *A. tumefaciens* is a gram-negative pathogenic soil bacterium, causing crown gall disease in a wide range of plants by transferring the T-DNA from its tumour inducing plasmid (Ti plasmid) to the genome of plants. The trans-kingdom gene transfer is initiated by the activity of Ti plasmid.
encoded virulence \((\text{vir})\) genes in response to low molecular weight phenolic compounds like acetosyringone (Gelvin, 2000) that are released from the wound region of plants. The molecular machinery needed for T-DNA generation and transport into the host cell comprises proteins that are encoded by the bacterial chromosomal virulence \((\text{chv})\) genes and encoded by the Ti-plasmid virulence \((\text{vir})\) genes.

Every stable transformation process demands the simultaneous occurrence of two independent biological events; stable insertion of the trans-gene into the plant genome and regeneration of those cells in which it integrated, producing a non-chimeric transgenic. This makes it a constraint for higher transformation efficiency. The ease of the procedure, the transfer of relatively large segment of DNA (up to 150 kb) with little rearrangements, preferential insertion of T-DNA into potentially transcribed regions and the integration of single copy of the transgene(s) into plant chromosomes (Kumria et al, 2001) have made this a fast method for gene transfer to a large number of plant species including dicots, monocots and fungi (De Groot et al, 1998).

*Dunaliella* spp. has been so far transformed using variety of methods including particle bombardment (Tan et al, 2005), electroporation (Geng et al, 2003; Walker et al, 2005c; Degui et al, 2002; Sun et al, 2005) and glass bead (Jin et al, 2001) systems. These gene transfer techniques have been associated with low transformation frequency and unstable transgene integration. One constraint faced by previous workers was that many commonly used selection antibiotics such as kanamycin, hygromycin and spectinomycin were unable to inhibit growth of *D. salina* even at a concentration of 1200 mgL\(^{-1}\) (Tan et al, 2005). Allnutt et al (2000) pointed out that high salt concentration in the growth medium reduced the efficiency of antibiotics. *D. bardawil* is therefore lacking a reliable and robust transformation technique till now. The importance of *D. bardawil* as a perfect tool for genetic engineering made the development of a stable transformation technique in this alga imperative. Two microalgae belonging to chlorophyceae viz, *Chlamydomonas reinhardtii* (Kumar et al, 2004) and *Haematococcus pluvialis* (Kathiresan et al, 2009) have been efficiently transformed using *Agrobacterium*. The successful
transformation results in the above algae made it possible for adapting the same strategy for *Dunaliella* transformation.

*D. salina* and *D. salina/bardawil* are the only reported β-carotene hyperproducer species of the genus (10% w/w), that grows in salt saturated lagoons (Ben Amotz et al, 1991). However, despite the excellent and comprehensive work on taxonomy, controversy still exists about identification of *D. salina/bardawil* as different species of *D. salina* (Borowitzka et al, 2007). Even today, differentiation among halophilic and carotenogenic *Dunaliella* species in both green and red stages is difficult and time consuming. Molecular identification provides a useful tool to distinguish inter and intra-specific morphologically similar species (Gomez et al, 2004) and mixed populations. Species specific oligonucleotides could be useful to identify either species from culture collections or from natural environments (Ki et al, 2007). Slight phylogenetic and taxonomic differences in *Dunaliella* species can conceal profound differences in their potential for production of metabolites such as carotenoids. However, intron-sizing method can be efficiently used to characterise each hyper producer species with an exclusive 18S rDNA fingerprint profile (Olmos et al, 2009). Conserved and variable regions of 16S–18S rDNA sequences have been used as targets for primer-directed DNA amplification by polymerase chain reaction (PCR) for the identification of micro-organisms (Jayarao et al, 1991). Taxonomic characterization is important to identify and differentiate species; however, differentiation between subspecies, ecotypes or similar species with taxonomy tools is a difficult and time consuming task and sometimes impossible. For this reason, the 18S rDNA intron-sizing method provides a novel and powerful DNA-fingerprinting methodology to accomplish a specific, rapid and sensitive identification of carotenogenic *Dunaliella* species.

Hence the present chapter describes the molecular characterisation of *D. bardawil* using 18S rDNA and development of a stable transformation technique developed for the alga using *A. tumefaciens*. 

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**Agrobacterium** mediated genetic transformation | CHAPTER II
2.2 Materials

2.2.1 Algal strain

*Dunaliella bardawil* strain V-101 was obtained from Centre for Advanced Studies in Botany, University of Madras, Chennai.

2.2.2 Glass wares:

All the glasswares, such as conical flasks, test tubes, culture tubes, measuring cylinders, pipettes etc., used for the experiments, were from Vensil Ltd., Mumbai or Borosil Glass works Limited, Mumbai, India.

2.2.3 Plastic wares

The microcentrifuge tubes, microtips and screwcap centrifuge tubes were from Tarsons Products Pvt. Ltd. Kolkata. The polyethylene (LDPE) bags and tubes were procured from local market.

2.2.4 Chemicals

All the media chemicals used for the experiments were analytical grade, obtained from companies- HiMedia Laboratories Pvt. Ltd.,-Mumbai; Sisco Research Laboratories Pvt. Ltd.,-Mumbai; Ranbaxy Fine Chemicals Ltd.,-New Delhi; Loba Chemie Pvt. Ltd.,-Mumbai; and Qualigens Fine Chemicals- Mumbai. HPLC grade solvents were obtained from Qualigens, Mumbai. Authentic standards and fine chemicals such as astaxanthin, β-carotene and lutein were obtained from Sigma-Aldrich Chemicals - USA. Antibiotics used were obtained from HiMedia Laboratories Pvt. Ltd.,-Mumbai and Ducheffa-The Netherlands. Molecular biology chemicals and enzymes were obtained from Fermentas International Inc., Burlington - Canada, Bangalore Genei - Bangalore, Sigma-Aldrich Chemicals–USA. Different kits used for molecular analysis were obtained from Sigma-Aldrich Chemicals - USA (Gene elute Genomic DNA isolation kit); Qiagen, GmpH-Germany (Qiagen PCR purification kit and Qiagen Gel elution kit from); Ambion Inc, Texas-USA (Brightstar psoralen-Biotin nonisotopic Labelling kit, RNA aqueous kit). Commercial nitrogen gas cylinders were procured from Vinayaka Agencies - Mysore. All oligo synthesis and sequencing work services were taken from Bioserve biotechnologies, Hyderabad.
2.3 Methodology

2.3.1 Maintenance of Dunaliella bardawil cultures

The axenic cultures of *D. bardawil* were maintained in AS-100 media (Vonshak, 1986) with modification. The Tris buffer (1gL⁻¹) used in AS-100 medium was replaced with NaHCO₃ (2.0 gL⁻¹). In this medium the concentration of total salts were 64.49 gL⁻¹ of which 58.44 g was NaCl. This modified medium is referred to as MAS100 in the experiments. Chemical composition of the MAS100 medium is presented in Table 2.1.

**Table 2.1 Chemical compositions of MAS100 medium**

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>(gL⁻¹)</th>
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</thead>
<tbody>
<tr>
<td>MgSO₄</td>
<td>2.4</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.05</td>
</tr>
<tr>
<td>KCl</td>
<td>0.6</td>
</tr>
<tr>
<td>NaNO₃</td>
<td>1.0</td>
</tr>
<tr>
<td>NaCl</td>
<td>50.0</td>
</tr>
<tr>
<td>Na₂HCO₃</td>
<td>2.0</td>
</tr>
<tr>
<td>Trace metal solution</td>
<td></td>
</tr>
<tr>
<td>H₃BO₃</td>
<td>3.426 gL⁻¹</td>
</tr>
<tr>
<td>CoCl₂.6H₂O</td>
<td>1.215 mgL⁻¹</td>
</tr>
<tr>
<td>MnCl₂.4H₂O</td>
<td>0.432 mgL⁻¹</td>
</tr>
<tr>
<td>ZnCl₂</td>
<td>31.5 mgL⁻¹</td>
</tr>
<tr>
<td>Conc.H₂SO₄</td>
<td>1.0 mL</td>
</tr>
<tr>
<td>(NH₄)₆Mo₇O₂.4H₂O</td>
<td>31.19 mgL⁻¹</td>
</tr>
<tr>
<td>Chelated Iron solution</td>
<td></td>
</tr>
<tr>
<td>(10g of Na₂EDTA dissolved in 500 mL of hot water + 0.81g of FeCl₃.6H₂O in 500mL of 0.1N HCl, mix and make up to 1L)</td>
<td>3 mL</td>
</tr>
</tbody>
</table>

The pH was adjusted to 7.2-7.5 using a pH meter (Cyber Scan 510, Oakton, USA) prior to autoclaving at 121ºC, for 20min. The solid medium was prepared by gelling with 1.5% (w/v) tissue culture grade agar (Hi-media, Mumbai, India), in test tubes and in petriplates, each containing 15 mL of the medium. The tubes and flasks were closed with cotton plugs. The slants, petriplates and flasks were inoculated with
D. bardawil cultures under aseptic conditions in laminar airflow cabinet (Airflow control systems, Bangalore, India). D. bardawil cultures were maintained in MAS100 liquid and solid medium in Erlenmeyer flasks and on agar slants respectively under 16 hours light at an irradiance of 18.75-25.0 μmol m⁻² s⁻¹ (1.5-2 KLux) at 25±1°C (Figure 2.1). The cultures were shaken manually once a day. Light intensities were measured using a lux meter (TES 1332, Digital lux meter, Taiwan). The cultures were sub cultured once in every 30 days with D. bardawil cultures of ~10⁶ cells mL⁻¹ as inoculum.

**Figure 2.1** D. bardawil cultures maintained in (A) flasks, (B) slants and (C) petri plates in MAS100 medium. (D) Microscopic pictures of D. bardawil in growth phase under 100 x magnification

### 2.3.2 Growth of *Dunaliella* in MAS 100 medium

The suspension cultures of D. bardawil were prepared from the axenic cultures maintained in slants. Erlenmeyer flasks (150 mL) in triplicates containing 50 mL MAS100 media were inoculated with the algae from the fully grown slants. These cultures were incubated at conditions mentioned in section 1.3.1 for a period of 30 days. The growth of D. bardawil was monitored by the following parameters.

**A. Cell count**

Growth of D. bardawil was measured in terms of cell number. The cells were counted using haemocytometer after fixing the cells by adding dilute hydrochloric acid (0.1N HCl). Cell count was expressed as number of cells mL⁻¹ culture. Growth was also measured in terms of optical density (OD) at 590 nm.
B. Dry weight (DW)
The dry weight of algae was determined after centrifugation of the culture at 3000 rpm for 10 min. Algal cells, after centrifugation, were washed with distilled water three to four times to remove salt and other media chemicals and dried in a hot air oven (Sanyo, Electric Biomedical Co., Ltd, Japan) at 60 ± 2°C till a constant weight was attained on a glass petridish. Biomass was expressed as gram per liter of culture.

2.3.3 Standardisation of MAS100 medium for optimum growth and carotenoid production
The concentration of main constituents of MAS100 medium viz NaCl, NaHCO₃, K₂HPO₄ and KNO₃ was optimised for growth and carotenoid content. The range of concentrations selected were NaCl (25-55 mgL⁻¹), NaHCO₃ (0.69-2.0 mgL⁻¹), K₂HPO₄ (0-0.25 mgL⁻¹), KNO₃ (0-0.75 mgL⁻¹). The experiment was carried out in triplicates and the cells were incubated at 25±1°C under the light intensity of 18.75 μmol m⁻² s⁻¹. Culture was shaken manually once a day. After 30 days cells were harvested by centrifuging at 3000 rpm for 5 minutes. Dry weight was taken as mention in 1.3.2 and the pigments were analysed through HPLC using the Shimadzu LC-10AT liquid chromatograph instrument using reverse phase C18 column (Supelco, 25 cm × 4.6 mm). Gradient solvent system consisting of acetone (10%) and 90% (v/v) methanol at a flow rate of 1.25 ml minute⁻¹ was used. The separated carotenoids were identified by comparing retention times and spectra against known standards. The peaks were integrated by Class VP version 6.14 SP1 software (Shimadzu, Singapore) at 445nm to quantify carotenoids. The peaks were also integrated at 645nm to detect chlorophylls. Standard β-carotene and lutein were purchased from Sigma-Aldrich (St.Louis, MO, USA).

2.3.4 Standardization of co-cultivation medium
The requirements for optimum growth of Dunaliella and Agrobacterium were different. So it was necessary to standardise a co-cultivation medium which sustain the growth of both. Tris Acetate Phosphate medium (TAP) had been successfully used during transformation of both C. reinhardtii (Kumar et al, 2004) and H. pluvialis (Kathiresan et al, 2009). In the present study, TAP medium (Harris, 1989) was
modified to contain sodium chloride at 0.2 M so that it supports the growth of *Dunaliella* and *Agrobacterium* (Table 2.2).

**Table 2.2** Constituents of TAP medium

<table>
<thead>
<tr>
<th>Constituents</th>
<th>g/100mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>NH₄Cl</td>
<td>0.400</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>0.100</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>0.108</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.540</td>
</tr>
<tr>
<td>Tris</td>
<td>2.400</td>
</tr>
<tr>
<td>Glacial acetic acid</td>
<td>1 ml</td>
</tr>
<tr>
<td>Hunters trace elements</td>
<td>1 ml</td>
</tr>
</tbody>
</table>

Hunters trace elements (g/100 mL): EDTA Disodium: 50.00; ZnSO₄·7H₂O: 22.00; H₃BO₃:11.00; MnCl₂·4H₂O: 5.06; FeSO₄·7H₂O: 4.99; CoCl₂·6H₂O: 1.61; CuSO₄: 1.57; (NH₄)₆MoO₂₄·4H₂O: 1.10

pH of the medium used was 6.8. Solid and liquid TAP medium used in the transformation experiment contained 0.2 M and 0.1 M NaCl respectively.

Different co-cultivation media were tested for the growth of both alga and bacterium. The solid co-cultivation medium viz., MAS100 medium, MAS100 + ¼ strength of LB (Luria Bertani) medium, MAS100 + ½ strength of LB medium, MAS100 medium with 0.2 M NaCl and TAP (Tris Acetate Phosphate) medium with 0.2 M NaCl alone were tested. Plates were prepared using solid medium in petri plates. The alga and bacterium were separately plated in petri plates containing these co-cultivation media and growth was monitored at weekly intervals.

### 2.3.5 Plasmid construct and bacterial strains

pCAMBIA1304 harbouring the *hpt* gene driven by the CaMV 35S promoter, and *GFP (gfp):uidA* fusion as a reporter gene under the control of the CaMV 35S promoter (Figure 2.2 A & B), is a widely used binary vector for *Agrobacterium*-mediated transformation in microalgae (Kumar et al, 2004; Kathiresan et al, 2009).
Agrobacterium meditated genetic transformation

*Agrobacterium* strain EHA101 (ICRISAT, Hyderabad) was transformed using pCAMBIA 1304 using the procedure described by Hofgen et al (1988). *E. coli* strain DH5α (NBRP, Japan) was used for maintenance of the plasmid. Both strains were maintained in LB containing 100 mgL⁻¹ kanamycin.

![Figure 2.2](image)

Figure 2.2 (A) Vector map of pCAMBIA 1304 (B) Linear map of T-DNA portion of pCAMBIA 1304 with restriction sites

### 2.3.6 Sensitivity of *D. bardawil* to different antibiotics

*D. bardawil* was tested for its sensitivity towards different antibiotics in liquid MAS100 medium. Initially the alga was tested for antibiotics like cefotaxime, potassium clavulanate at the concentration of 250, 500, 1000 and 2000 mgL⁻¹ in MAS100 medium. The sensitivity of alga to various selection antibiotics (kanamycin, hygromycin, ampicillin, and chloramphenicol) ranging from 50-1000 mgL⁻¹ was also monitored.
2.3.7 Effect of salt concentration on growth and antibiotic sensitivity

Experiments were carried out to determine the growth of *D. bardawil* and its sensitivity to hygromycin in solid TAP medium containing different concentrations of NaCl (0.1-1.0 M) and hygromycin (50-700 mgL\(^{-1}\)). Approximately 1.5 x 10\(^6\) cells of *D. bardawil* were plated onto 90 mm petri plates and incubated under 16 hours light at an irradiance of 18.75 ± 2.5 \(\mu\)mol m\(^{-2}\)s\(^{-1}\) at 25 ± 1°C. The number of algal colonies were counted after a period of 20 days.

*A. tumefaciens* cells (OD at \(A_{600}\)-0.5) were plated onto TAP medium containing various NaCl concentrations (0.1 to 1.0 M) and growth was categorized subjectively as high, medium and low after two days of incubation.

2.3.8 Transformation procedure

The cultures of *D. bardawil* were transferred every second day into MAS100 medium gradually reducing the concentrations of NaCl until the cells were adapted to 0.2 M NaCl. The cells were then transferred to TAP medium containing 0.2 M NaCl. Approximately 1.5 x 10\(^6\) cells of *D. bardawil* were plated onto solid TAP medium. Cells were grown in the presence of 100 \(\mu\)M &200 \(\mu\)M acetosyringone and in its absence and were incubated under 16 hours light with an irradiance of 18.75 \(\mu\)mol photons m\(^{-2}\) s\(^{-1}\) at 25 ± 1°C for 6 days until a lawn of cells was formed. Five replicates were maintained for each treatment. An overnight-grown culture of *A. tumefaciens* harbouring pCAMBIA 1304 plasmid in liquid LB broth (10 ml) was allowed to grow for 4 hours (\(A_{600}\)-0.5) in 50 ml of liquid LB. The culture was pelleted and resuspended in 10 ml of liquid TAP medium. A suspension (200 \(\mu\)l) of *A. tumefaciens* was plated over the lawn of *D. bardawil* cells and incubated under the same culture conditions for 48 hours. Following co-cultivation, cells were scraped, suspended in liquid TAP and harvested by centrifugation at 1500 rpm for 2 minutes. The cells were then washed three times with liquid TAP containing 500 mgL\(^{-1}\) cefotaxime and 300 mgL\(^{-1}\) potassium clavulanate and pelleted by centrifugation at 3500 g for 2 minutes. The pelleted cells were resuspended in liquid TAP and plated onto solid TAP medium containing 100 mgL\(^{-1}\) hygromycin. After four weeks the colonies became visible. The cells surviving 8 weeks of growth on hygromycin were suspended again in liquid TAP and plated onto solid TAP containing 100 mgL\(^{-1}\) hygromycin. This was carried out to ensure selection of transformed cells only. The
colonies (cfu) appearing later were counted to calculate the transformation frequency. The schematic representation of transformation procedure followed is given in Figure 2.3.

2.3.9 Stability and maintenance of hygromycin resistant cells

The hygromycin resistant cells (HRC) of *D. bardawil* grown on selection medium for 8 weeks were transferred every two days into liquid TAP medium with a progressive increase in the concentration of NaCl to adapt the cells to a concentration of 1.0 M. Eventually cells were grown only in MAS100 as liquid TAP did not support vigorous algal growth. No hygromycin was used when salt concentration was increased. Hygromycin resistant cells were also maintained in solid TAP medium with 100 mgL\(^{-1}\) hygromycin.

In order to study the hygromycin tolerance of the cells in solid and liquid TAP media, both HRC and control cells, maintained in MAS100 medium, were taken to liquid TAP through gradual decrease of NaCl concentration. Once the cells were adapted to 0.2 M NaCl, they were exposed to different hygromycin concentrations (50-700 mgL\(^{-1}\)) in solid and liquid TAP media. The growth was evaluated by counting the number of colony forming units (cfu) and surviving cells respectively after a period of 20 days. Control and transformed cells at an inoculation density of 100x10\(^4\) cells mL\(^{-1}\) were also inoculated in liquid TAP medium at a hygromycin concentration of 600 mgL\(^{-1}\). Cell count was taken at every 3 days intervals for a period of 25 days in order to study hygromycin tolerance of both.

HRC were maintained in MAS100 liquid medium with regular subculturing. The ability of HRC to retain hygromycin resistance has been periodically monitored by plating these and control cells on selection medium. Data for three transformant lines (T1, T2 and T3) are presented.
2.3.10 Reporter gene expression in hygromycin resistant cells

A. GFP expression

Chlorophyll was partially removed using solvents to facilitate GFP expression in HRC. Control cells were also treated similarly. HRC and control cells of *D. bardawil*
were collected after centrifugation at 3000 rpm for 5 min. In order to partially remove chlorophyll, cells were incubated in tetrahydrofuran and methanol (1:1) for 20 min. The cells were then washed several times with distilled water and were observed for fluorescence under an excitation filter (BP460-490C) and barrier filter (BA520IF) of a fluorescent microscope (OLYMPUS CKX41, Olympus Optical Co. Ltd., Tokyo, Japan) at an excitation wavelength of 488 nm.

B. GUS expression
Hygromycin-resistant and control cells of *D. bardawil* were harvested and centrifuged at 3500 g for 5 min, incubated in 0.5 M sorbitol containing pectinase (0.2%) and cellulase (0.1%) enzymes at 37°C for 4 hours and then washed with 0.5 M sorbitol several times. GUS (β-glucuronidase) in these cells was visualized using a GUS staining kit (Sigma-Aldrich, USA) according to the manufacturer’s instructions. The cells were then incubated for 2 days at 37°C, followed by destaining with tetrahydrofuran and methanol (1:1) twice and subsequent washing with sterile water six times. The cells were observed under a light microscope (OLYMPUS BX40 F4, Olympus Optical Co. Ltd., Tokyo, Japan).

2.3.11 Analysis of transgene integration and expression
A. PCR for *hpt* gene in the transformants
Hygromycin-resistant cells (T1, T2 and T3) and control cells were harvested by centrifugation at 3000 rpm and genomic DNA was isolated using the Gene Elute-Plant Genomic extraction kit (Sigma- Aldrich, USA). PCR primers HPTF (5’-CAACCAAGCTCTGATAGAGT-3’) and HPTR (5’-TGGCGTGGATTTCATATGCGC -3’) were used to amplify a part of the *hpt* gene (400 bp) present in the T-DNA region using genomic DNA and cDNA as templates in a thermocycler (Eppendorf Thermal cycler, Germany). PCR conditions included 5 min of initial denaturation at 94°C and 35 cycles of 1 min denaturation at 94°C, 1 min annealing at 58°C, 1 min extension at 72°C. The final extension was at 72°C for 10 min. PCR products were separated on agarose gels (1.2%), stained with ethidium bromide and documented (Chemidoc XRS, Biorad, Germany).
B. RT-PCR for hpt gene
Total RNA from control cells and HRC was extracted using the RNAqueous® kit (Ambion, TX, USA) and DNase treated using TURBO DNase (Ambion, TX, USA) according to the instructor’s manual. cDNA was synthesized from 1.5 µg of total RNA in a 20 µl final volume, using AMV reverse transcriptase (Sigma-Aldrich, USA) and oligo-dT (18-mer) primer (Ambion, TX, USA).

C. Southern blot of transformants against hpt probe
The PCR amplicon (400 bp) which is a portion of the hpt gene, amplified using the primers HPTF and HPTR, was purified using PCR purification kits (Himedia, Mumbai) and labelled using a Psoralen biotin labelling kit (Ambion, TX, USA). 40 µl of genomic DNA (30 µg) of three transformed (T1, T2 and T3) and one non-transformed (C) cell lines were digested for 2 hours with SacII and Ndel (MBI, Fermentas, Lithuania) as described by Sambrook et al (1989). The vector pCAMBIA1304 was also digested with the same enzymes. The products of restriction digestion were separated on a 0.8% agarose gel prior to transfer to a nitrocellulose membrane (Hi Bond, Amersham, USA). The hybridization was carried out at 58ºC for the transferred nylon membrane and the blot was visualized using the BioDetect Kit (Ambion, Texas) using BCIP-NBT (Banglore genei, Bangalore) as the substrate.

D. Western blot of transformants against GFP antibody
Total cell protein was extracted from control and HRC D. bardawil cell lines using a modified protocol (Walker et al, 2005c). Exponentially growing cells of control and HRC D. bardawil were harvested by centrifuging at 3000 rpm for 5 minutes and washed three times with distilled water. The pellet was resuspended in 100 µl of extraction buffer containing 0.15 M NaCl, 50 mM Tris (pH 8) and 1% Triton X and was disrupted through sonication followed by centrifugation at 5000 rpm for 15 minutes. The supernatant, which contained proteins, was quantified using the Lowry method (Lowry et al, 1951). Total proteins (40 µg) from T1, T2, T3 and control cells were separated on SDS-PAGE. The protein was transferred to a nitrocellulose membrane using a Trans-Blot semi-dry blotting apparatus (Biorad, Germany) and was blocked with gelatine (2%) for 2 hours. The membrane was incubated with primary anti-GFP antibody (Sigma-Aldrich, USA) (1: 1000) for 2 hours and was detected using secondary antibody, rabbit anti-mouse alkaline phosphatase conjugate (1:1000)
using BCIP-NBT as the substrate. All chemicals were obtained from Merck Bioscience, Bangalore.

2.3.12 Detection of \( A. \) \( t \)umefaciens\) contamination in the transformants

**A. PCR for \( npt \)II gene**

The presence of \( Agrobacterium \) in the HRC (T1, T2 & T3) was checked using PCR for the \( npt \)II gene fragment which lies outside the T-DNA region of pCAMBIA1304. This gene confers resistance to the antibiotic kanamycin. Amplification was performed in a thermocycler (Eppendorf Thermal cycler, Germany) using the primers KanF (5’GCAGAAGGCAATGTCATACC-3’) and KanR (5’-AGGCTCTTTTCA CTCCATCG-3’) for genomic DNA of HRC (T1, T2 & T3). pCAMBIA 1304 was used as a positive control. PCR conditions were as above, except that the annealing temperature was for one minute at 55°C. The PCR products were visualized as above.

**B. Scanning Electron Microscopy**

Co-cultivated algal cells, HRC and control algal cells were harvested and centrifuged at 3000 rpm for 5 minutes. Pellet was washed with distilled water several times and was later fixed with 2% gluteraldehyde in 0.5 M sorbitol for 2 hours. Cells were then washed with 0.5 M sorbitol several times and passed through alcohol series for dehydration and were suspended overnight in absolute alcohol. The cells were coated with gold using a sputter coater and examined using scanning electron microscope (Leo Electron Microscopy Ltd., (now Carl Zeiss) Cambridge, UK. Jeol JSM-35C).

2.3.13 Identification of the site of integration of T-DNA in the transformants

The site of integration of the transgene was determined using a modified PCR based Genome walking method (Cottage et al, 2001). DNA library was made by digesting the genomic DNA using \( Dra \) I (Fermentas, USA) for one hour at 37°C. The enzyme used was selected because it has six-base recognition sites and generate blunt ends. A special adaptor is ligated to the ends of DNA fragments generated by digestion of genomic DNA separately using T4 DNA ligase (Fermentas, USA) at 4°C overnight.

Following adaptor ligation, a small amount of the DNA is used as a template for primary PCR using adaptor primer (ASP1) and gene specific primer (WHG). The PCR program was initial denaturation of five minutes at 94°C and step of 35 cycles
consisting the denaturation of one minute at 94\(^{\circ}\)C, annealing of one minute at 54\(^{\circ}\)C and final extension of one minute at 72\(^{\circ}\)C followed by a final extension of ten minutes at 72\(^{\circ}\)C. The primary PCR product was separated on 1% agarose gel stained with ethidium bromide. A secondary PCR reaction was conducted with 1\(\mu\)l of a 100-fold dilution of the primary PCR using adaptor primer ASP2 and the nested non overlapping gene-specific primer. The tertiary PCR was also carried out in the same way using the next nested internal primers. The PCR products were separated on 1% agarose gel stained with ethidium bromide and was documented. The PCR fragments above 500 bp were selected and reamplified and purified using Qiagen PCR purification kit. The purified product was further ligated to T-tail Cloning vector pTZ R/T (Fermentas, Germany). The clone was confirmed by restriction digestion and was sequenced. The obtained sequence was subjected for blast analysis in NCBI BLAST and JGI genome blast. The method is depicted in figure 2.4. Primer sequences and adaptor sequences are listed in table 2.3.

![Diagram](image)

**Figure 2.4:** Analysis of T-DNA insertion sites by PCR-walking (ASP1, ASP2: Adaptor specific primers; WPA, WHYG: gene specific primers)
Table 2.3: List of Primers used for genome walking

| Adapter long arm:              | 5’CTAATACGACTCACTATAGGGCTCGAGCGGCCGC-3’        |
| Adapter short arm:             | 5’- ACCTGCCC -NH2-3’                            |
| Adapter specific primers:      | ASP1 :  5’- GGATCCTAATACGACTCACTATAGGGGC-3’     |
|                               | ASP2 :  5’- AATAGGGCTCGAGCGGC-3’                |
| Gene Specific Primers:         | WPA 3:  5’-AGGTTTCGCTCATGTGTTG-3’               |
|                               | WHG 6:  5’-ACTCGCCGATAGTGAAACC-3’               |
|                               | WPA 4:  5’-GATAAGGGAAATAGGGTTCC-3’              |
|                               | WHYG 7: 5’-TCGCATTGGTCTTGACCAA-3’               |
|                               | WHYG 5: 5’-AGCACTCGTCCGAGGGCAAA-3’              |

2.3.14 Growth measurement and pigment analysis from transformed *D. bardawil*

2.3.14.1 Growth measurement
Growth was estimated by cell count as described previously in section 2.3.3.

2.3.14.2 Pigment analysis using spectrophotometry
For pigment analysis, an aliquot of culture was harvested by centrifugation at 3000 g for 10 min and was freeze-dried. A known quantity of biomass was extracted with 100% acetone in a mortar and pestle. The extraction was repeated till the pellet became colourless. The extracts were centrifuged at 5000 rpm for 5 min and the supernatants were pooled. Aliquot of extract is flushed with N$_2$ gas and stored at -20ºC for spectrophotometric and HPLC analysis. All operations were carried out under dim light.

The acetone extracts absorbance was recorded at 470, 480, 645 and 661.5 nm using spectrophotometer (Shimadzu 160A, Singapore). Chlorophyll and total carotenoid contents were calculated using Lichtenthaler (1987) equations.

\[
\text{Chlorophyll a - Ca (µgml}^{-1} ) = 11.24 \text{ OD661.5} - 2.04 \text{ OD645} \\
\text{Chlorophyll b - Cb (µgml}^{-1} ) = 20.13 \text{ OD645} - 4.19 \text{ OD661.5} \\
\text{Total Chlorophyll - Ca+b (µgml}^{-1} ) = 7.05 \text{ OD661.5} + 18.09 \text{ OD645}
\]
\[
\text{Total carotenoid (µgml}^{-1}) = \frac{1000 \times \text{OD}_{470} - (1.9 \text{Ca} + 63.14 \text{Cb})}{214}
\]

\[
\text{Total carotenoid (mgg}^{-1}) = \frac{(\text{Total carotenoid (µgml}^{-1}) \times \text{Volume of extract} \times \text{Dilution factor})}{\text{Biomass taken for extraction (mg)}}
\]

2.3.14.3 High Performance Liquid Chromatograph (HPLC) of pigments

*D. bardawil* extracts from both control and transformed cells were subjected to HPLC analysis in Shimadzu LC-10AT liquid chromatograph instrument as described in section 2.3.3.

2.3.15 Molecular characterisation of *D. bardawil* using 18S rDNA

Genomic DNA from *D. bardawil* strain was used as the template for the experiments. Conserved primers M1, M2 and M3 and species specific primers DB and DS were designed based on the reports by Olmos et al (2009). Oligonucleotides M1, M2 and M3 were designed from the conserved sequences of the 5’ and 3’ termini of the 18S rDNAs and used to amplify the full sequence of all strains. Specific primers (DS and DB) were designed from the first intron of both strains. PCR was carried out with M1, M2, M3, DB and DS as primers in Biorad thermocycler (Biorad, Germany).

PCR conditions included 5 min of initial denaturation at 94°C and 35 cycles of 1 min denaturation at 94°C, 1 min annealing at 60°C, 1 min extension at 72°C. The final extension was at 72°C for 10 min. PCR products were separated on agarose gels (1.2%), stained with ethidium bromide and documented (Chemidoc XRS, Biorad, Germany). Primer sequences used in the experiment are listed below:

M1- 5’-CGGGATCCGTAGTCATA TGCTTGTCCTC-3’
M2- 5’-CG GAATTCCTTCTGCAGGTTCACC-3’
M3- 5’-GGAATTCCGG AAACCTTGTTACGAC-3’
DS- 5’-GCAGGAGAGCTAATAGGA-3’
DB- 5’-GGGAGTCTTTTCCACCT-3’
2.4 Results

2.4.1 Growth of *D. bardawil* in MAS100 medium

*D. bardawil* was grown in MAS100 medium for evaluating its growth profile in terms of OD, cell number and biomass. During the growth phase, a maximum of 900 ± 5 mgL\(^{-1}\) biomass (dry weight) was obtained on 30\(^{th}\) day with a cell count of 10x10\(^6\) cells ml\(^{-1}\) (Figure 2.5).

The effects of the major media constituents of MAS100 medium were studied on growth and caroteneid production in *Dunaliella*. The optimum conditions showed that maximum biomass of 1.7 gL\(^{-1}\) was obtained at the concentration of sodium chloride 37.53 gL\(^{-1}\), sodium bicarbonate 1.05 gL\(^{-1}\), potassium nitrate 0.55 gL\(^{-1}\) and dipotassium hydrogen phosphate 0.14 gL\(^{-1}\). The pigments mainly lutein and β-carotene were analysed for all the combinations in which a maximum lutein content of 3.36% was obtained in the above combination of medium constituents (Anila et al, 2011b). However β-carotene production was observed to be 0.8% in the same combination.

![Figure 2.5. Growth of *Dunaliella bardawil* in 150 mL flasks under temperature 20-22\(^0\)C and 18.75-25.0 \(\mu\)mol m\(^{-2}\) s\(^{-1}\) light intensity. The parameters studied for the growth include cell count ( — ), optical density ( — ) and the total biomass weight ( — ).](image-url)
2.4.2 Standardisation of co-cultivation medium

Initial studies were focused on standardisation of co-cultivation medium that would allow the simultaneous growth of both *D. bardawil* and *Agrobacterium*. *D. bardawil* and *Agrobacterium* were inoculated separately in different co-cultivation media. Among the co-cultivation media tested, MAS100 + ½ strength of LB and MAS100 medium + ¼ strength of LB media favoured profuse growth of *Agrobacterium* (full mat like appearance of bacteria was observed in plates within 24 hours of inoculation) but limited growth for the alga (visible growth of the alga was not observed in the plates even after one week of inoculation) (Table 2.4). MAS100 medium favoured profuse growth of alga (mat like appearances of algal colonies were observed after one week of inoculation) but not *Agrobacterium* (growth was not observed in the plates even after 48 hours of inoculation). TAP medium containing 0.2 M NaCl favoured optimum growth of both the alga and *Agrobacterium* and it was therefore selected as the media for co-cultivation and selection in the transformation studies.

**Table 2.4:** Growth of both *D. bardawil* and *A. tumefaciens* in different co-cultivation media

<table>
<thead>
<tr>
<th>Medium</th>
<th>Growth*</th>
<th><em>D. bardawil</em></th>
<th><em>Agrobacterium</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>MAS100 medium</td>
<td>Profuse growth</td>
<td>No growth</td>
<td></td>
</tr>
<tr>
<td>MAS100 + ¼ strength of LB</td>
<td>Limited growth</td>
<td>Profuse growth</td>
<td></td>
</tr>
<tr>
<td>MAS100 + ½ strength of LB</td>
<td>Limited growth</td>
<td>Profuse growth</td>
<td></td>
</tr>
<tr>
<td>MAS100 + 0.2 M NaCl</td>
<td>Good growth</td>
<td>Limited growth</td>
<td></td>
</tr>
<tr>
<td>TAP</td>
<td>No growth</td>
<td>Profuse growth</td>
<td></td>
</tr>
<tr>
<td>TAP + 0.2 M NaCl</td>
<td>Good growth</td>
<td>Good growth</td>
<td></td>
</tr>
</tbody>
</table>

*Limited growth*  
- Visible growth of the alga was not observed in the plates even after one week of inoculation  
- Minute small colonies of bacteria after 24 hours

*Good growth*  
- Single, enormous minute colonies of alga was observed after one week of inoculation and growth of bacteria was observed like a thin slimy layer on the medium after 2 days of inoculation

*Profuse growth*  
- Full mat like appearance of bacteria was observed in the plates within 24 hours of inoculation and alga after one week of inoculation

*No growth*  
- Growth of the bacteria was not observed in the plates even after 48 hours of inoculation
2.4.3 Sensitivity of *D. bardawil* to different antibiotics

Different antibiotics such as ampicillin, kanamycin, hygromycin and chloramphenicol which were tested against *D. bardawil* proved to be insensitive in MAS100 medium even in higher concentrations of 1000 mgL⁻¹. Investigation into the effect of cefotaxime and potassium clavulanate on the growth of *D. bardawil* revealed that the alga was tolerant to 1000 mgL⁻¹ and 1500 mgL⁻¹ of antibiotics respectively while minimum concentration of these antibiotics that arrested growth of *A. tumefaciens* was determined to be 500 mgL⁻¹ and 300 mgL⁻¹ respectively (data not shown).

2.4.4 Effect of salt concentration on growth and antibiotic sensitivity of *D. bardawil* and Agrobacterium

In order to adapt the halotolerant alga *D. bardawil* to low salt, the cells grown in MAS100 were gradually adapted to low salt TAP medium containing 0.1 M NaCl. The growth of the algae in liquid TAP medium containing different concentrations of salt was first investigated. It was observed that algal cells were viable only in the range of 0.1 M to 1.0 M NaCl. Growth profile of *D. bardawil* was also checked in liquid media of MAS100, MAS100 + 0.2 M NaCl and LTAP for a period of 25 days. *D. bardawil* was found to have better growth profile in MAS100 with 1 M NaCl when compared to MAS100 with 0.2 M NaCl or LTAP media (Figure 2.6).

![Figure 2.6: Growth profile of *D. bardawil* in MAS100, MAS100+ 0.2 M NaCl and LTAP (Liquid TAP medium with 0.1 M NaCl)](image)

Growth of *D. bardawil* in solid TAP medium containing different NaCl and hygromycin concentration was monitored. It was observed that cells did not grow in
solid medium containing salt concentrations lower than 0.2 M NaCl. As the NaCl concentration increases in the medium, hygromycin sensitivity of \textit{D. bardawil} cells decreases. At higher NaCl concentration, more hygromycin is required to kill \textit{D. bardawil} cells. Solid TAP medium which contains 0.2 M NaCl and 75 mgL\(^{-1}\) hygromycin enabled complete killing of control \textit{D. bardawil}. The lowest concentration of NaCl which allowed healthy growth of \textit{D. bardawil} and subsequent selection of transformants with 100 mgL\(^{-1}\) hygromycin was found to be 0.2 M (Figure 2.7). Therefore STAP with 100 mgL\(^{-1}\) hygromycin was chosen as the selection medium.

Growth of \textit{A. tumefaciens} on solid TAP medium containing different concentrations of NaCl was tested. \textit{Agrobacterium} was unable to grow on solid TAP medium containing NaCl at concentrations higher than 0.5 M. However NaCl at 0.2 M concentration allowed the growth of \textit{D. bardawil} with high sensitivity to low levels of hygromycin. The transformation of \textit{D. bardawil} was carried out using the standardised procedure and different stages of transformation are presented in Figure 2.8.

\subsection*{2.4.5 Transformation frequency}

The number of colonies which were observed on the selection plates after 8 weeks of incubation was counted for transformation frequency. The transformation frequency of \textit{D. bardawil} co-cultivated in STAP medium containing 100 µM and 200 µM acetosyringone was found to be 42.0 ± 3 and 39.0 ± 3 per 10\(^6\) cells plated respectively whereas in acetosyringone free medium the transformation frequency was 41.0 ± 4 per 10\(^6\) cells. This indicates that addition of acetosyringone during co cultivation has no effect on transformation frequency in \textit{D. bardawil} (Table 2.5). The selected colonies were grown and maintained in MAS100 medium without hygromycin.
Figure 2.7: Combined effects of hygromycin and NaCl on the growth of control *D. bardawil* cells in solid TAP medium

Figure 2.8: Different stages of the *D. bardawil* transformation (A) TAP+0.2 M NaCl before co-cultivation (B) Co-cultivation with *Agrobacterium* (C) Small minute colonies of *D. bardawil* after 2 week of selection (D) Minute single cell colony of *D. bardawil* after 4 weeks of co-cultivation – a closer view (E) Clear single cell colony of transformed *D. bardawil* after 8 weeks of selection (F) Wild *D. bardawil* cells on selection medium after 2 weeks of selection.
Table 2.5: Transformation frequency* of hygromycin resistant cells

<table>
<thead>
<tr>
<th>Sl. No</th>
<th>Treatments for co-cultivation medium</th>
<th>No. of Cells plated</th>
<th>No. of hygromycin resistant colonies</th>
<th>Transformation frequency (per10^6 cells ) (Mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>TAP</td>
<td>1.5x 10^6</td>
<td>62</td>
<td>41± 4</td>
</tr>
<tr>
<td>2</td>
<td>TAP +100 µM AS</td>
<td>1.5x 10^6</td>
<td>64</td>
<td>42± 3</td>
</tr>
<tr>
<td>3</td>
<td>TAP+ 200 µM AS</td>
<td>1.5x 10^6</td>
<td>59</td>
<td>39± 3</td>
</tr>
</tbody>
</table>

*Five replicates were maintained for each treatment; AS- Acetosyringone

2.4.6 Reporter gene expression

Wild and transformed *D. bardawil* (HRC) cells were analysed for GUS and GFP expression studies (Figure 2.9). Hygromycin resistant cells emitted bright greenish yellow fluorescence (A) characteristic of GFP. The greenish yellow fluorescence was completely absent in control cells treated similarly. However, auto fluorescence (red colour) was observed for control cells, possibly due to traces of chlorophyll present (B), whereas GFP fluorescence was predominant in HRC. Fluorescence was uniformly distributed in HRC. Gus activity was detected in hygromycin resistant cells after incubation for 12 hours with substrate (C) while control cells responded negatively (D).

2.4.7 Stability analysis of the transformed *D. bardawil*

Control cells and HRC grown in LTAP without hygromycin were transferred into liquid and solid TAP media containing varying concentrations of hygromycin. Control cells growing in solid TAP were killed at 100 mgL^-1 of hygromycin while HRC were able to survive in hygromycin concentration as high as 250 mgL^-1 (Table 2.6). HRC and control cells growing in liquid TAP medium were able to tolerate concentration of hygromycin up to 900 mgL^-1 and 500 mgL^-1 respectively. Hygromycin resistant cells grown in MAS100 even after a period of 18 months retained their ability to grow on hygromycin (data not shown). The growth profile of wild and transformed cells at 600 mgL^-1 hygromycin showed a gradual decrease in the cell density and eventual death of control cells (Figure 2.10).
Figure 2.9: Detection of GUS and GFP expression observed in HRC (T1) and control
*D. bardawil* cells (A) Hygromycin resistant *D. bardawil* cells. Bright greenish yellow
fluorescence indicates expression of the *gfp* gene and (B) Control cells emitting slight
red fluorescence characteristic of chlorophyll due to traces of chlorophyll.
(C) Transformed *D. bardawil* stained blue due to activity of GUS. Inset: Single HRC
(1000 x). (D) Control *D. bardawil* cells in which blue colour is absent. (E) and (F)
shows the microscopic images (100x) of HRC in bright field and fluorescence field
respectively.

Figure 2.10: Growth profile of wild and transformed cells in liquid TAP medium
containing 600 mgL$^{-1}$ hygromycin
**Table 2.6:** Effect of varying hygromycin concentration on the growth of control cells and HRC in solid and liquid TAP media

<table>
<thead>
<tr>
<th>Hygromycin (mg/L)</th>
<th>Solid TAP medium (Cfu)</th>
<th>Liquid TAP medium (x 10^5 cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Transformed</td>
</tr>
<tr>
<td>0</td>
<td>218</td>
<td>215</td>
</tr>
<tr>
<td>50</td>
<td>124</td>
<td>201</td>
</tr>
<tr>
<td>75</td>
<td>34</td>
<td>188</td>
</tr>
<tr>
<td>100</td>
<td>-</td>
<td>124</td>
</tr>
<tr>
<td>150</td>
<td>-</td>
<td>112</td>
</tr>
<tr>
<td>200</td>
<td>-</td>
<td>85</td>
</tr>
<tr>
<td>250</td>
<td>-</td>
<td>13</td>
</tr>
<tr>
<td>300</td>
<td>-</td>
<td>-</td>
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<tr>
<td>400</td>
<td>NT</td>
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<td>500</td>
<td>NT</td>
<td>NT</td>
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<tr>
<td>600</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>700</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>900</td>
<td>NT</td>
<td>NT</td>
</tr>
<tr>
<td>1000</td>
<td>NT</td>
<td>NT</td>
</tr>
</tbody>
</table>

*Initial inoculum plated is 1.5x 10^6 cells and 1.5x10^7 cells for solid and liquid media respectively; Cfu and cell count is average of three replicates; “-” indicates absence of viable cells and NT indicates treatment which are not tried.

**2.4.8 Trans-gene integration and analysis**

A 400 bp fragment was amplified from the DNA of the T1, T2 and T3 transformants (Lane 2-4) with HPTF and HPTR primers whereas no amplification was observed with DNA (Lane 1) from control cells. The size of the amplicon obtained in PCR and (Lane 2-4) & RT-PCR (Lane 5-7) from HRC with primers specific for *hpt* was the same as that obtained when the vector pCambia 1304 was used as template in PCR (Lane 7) (Figure 2.11).
**Figure 2.11**: PCR amplification of a fragment of the *hpt* gene from genomic DNA and cDNA from HRC (T1) and control *D. bardawil* cells using HPTF and HPTR primers. Lane 1, Genomic DNA from control cells; Lane 2, genomic DNA T1; Lane 3, genomic DNA T2; Lane 4, genomic DNA T3; Lane 5, cDNA T1; Lane 6, cDNA T2; Lane 7, cDNA T3; Lane 8, pCAMBIA 1304; Lane M, 3 Kb marker. 400 bp amplicon is seen in all lanes except for first lane which is genomic DNA from control cells.

A Southern blot of plasmid DNA, DNA from T1, T2 and T3 and control cells digested with *NdeI* and *SacII* and probed with *hpt* gene fragment is depicted in Fig 2.12A. *NdeI* positioned in the *hpt* of T-DNA region (10,034) and *SacII* positioned after the left border of the T DNA region (8,903) would be expected to release a fragment which is 1.136 kb in size as per the sequence of pCAMBIA 1304 that contains the probe region (Lane C). Fragments of different sizes greater than 1.136 kb were observed in lane T1, T2 and T3 when digested DNA from three transformant was probed. No band was observed in the lane containing DNA of control cells digested with same enzymes (Lane W). Southern blot and corresponding gel is presented as figure 2.12 A & a.

Immunoblot analysis of the transformants (Figure 2.12 B) revealed the presence of GUS-GFP fusion protein (Lane T1, T2 and T3) of size 90 kDa while no protein was detected in the control cells (Lane W). Western blot and corresponding gel is presented as figure 2.12 B & b.
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**Figure 2.12:** (A) Southern blot analysis of HRC (T1, T2 and T3) and control cells. Lane C, Control vector pCAMBIA 1304 digested with *Nde*I and *Sac*II (band size ~1.136 kb); Lanes T1, T2 and T3, genomic DNA from transformants T1, T2 and T3 digested with *Nde*I and *Sac*II; Lane W, control DNA digested with *Nde*I and *Sac*II (no band detected). The 1.129 kb fragment observed in lane C includes the vector pCAMBIA 1304 region hybridized with the probe and was visualized in the Southern blot. The bands observed in lanes T1 (~4.5 kb), T2 (~4.5, 5 Kb) and T3 (~5 kb, 6 kb) are larger than in lane C (a) Corresponding agarose gel

(B) Immunoblot analysis of total protein extracted from HRC (T1, T2 and T3) and control *D. bardawil* cells. The expected size of the GUS/GFP protein is about 90 kDa which may be seen in lanes T1, T2 and T3 which contain total proteins extracted from respective HRC lines. Lane W is total protein extracted from control *D. bardawil* cells where no band was observed (b) Corresponding SDS-PAGE gel

1.4.9 Detection of *Agrobacterium* contamination

A 400 bp amplicon was amplified from plasmid pCAMBIA 1304 using kanamycin specific primers (KanF and KanR). PCR using genomic DNA isolated from HRC as template gave no amplification with the above primers (Figure 2.13). This shows absence of *Agrobacterium* in *D. bardawil* transformants.

Scanning electron microscopy of wild and transformed cells of *D. bardawil* revealed the absence of *Agrobacterium* in the algal cells (Figure 2.14 A & C). Co-cultivated cells (Figure 2.14 B) showed the presence of *Agrobacterium* adhering to the cells.
Figure 2.13 PCR amplification of a fragment of the \textit{nptII} gene from genomic DNA and cDNA from HRC and control \textit{D. bardawil} cells using KanF and KanR primers. Lane C: pCAMBIA 1304; Lanes T1, T2 & T3 transformants and Lane M: 3 Kb marker.

Figure 2.14: Scanning Electron Microscopic photographs of (A) wild, (B) co-cultivated and (C) transformed \textit{D. bardawil} cells.

2.4.10 Identification of site of integration by transgene walking

Three transformants lines (T1, T2 and T3) were selected for transgene walking. Three fragments of approximate size 500–700 bp were cloned and sequenced from the three transformants (Figure 2.15). The sequences did not show any similarity in NCBI and JGI (\textit{Chlamydomonas} and other chlorophycean members genome) blast revealing that the flanking sequences were integrated into the non coding regions. The sequences that were apparently derived across the T-DNA left border and in the \textit{Dunaliella} genome were A/T rich and showed repeated stop codons on ORF analysis using EXPASY. The sequences revealed that, 3-45 bases were missing from the T-DNA left.
border. The vector sequences were removed and three sequences with sizes 403 bp, 408 bp and 753 bp were submitted to NCBI (Accession no. HQ224501- HQ224503; sequences are provided in appendix).

![Figure 2.15: Adaptor ligated walking for finding trans-gene integration site: Genomic DNA digested with different restriction enzymes (Lanes 1-4); Secondary PCR (Lane 5) and tertiary PCR (Lane 6 &7); M is 10 kb marker; Ligated plasmid pTZ R/T with the PCR product (Lane L) and control pTZ (Lane C)](image_url)

2.4.11 Growth measurement and pigment analysis from transformed *D. bardawil*

2.4.11.1 Growth measurement

Growth profile of transformants followed the same pattern of wild *D. bardawil* (Figure 2.16). The maximum cell count was reached on 30th day for both wild and transformed *D. bardawil*.

2.4.11.2 Pigment analysis using spectrophotometer

Total carotenoid and chlorophyll content in wild and transformed *D. bardawil* cells after 30 days of growth in MAS100 medium was estimated using a spectrophotometer. Total chlorophyll content in control and transformed cells were found to be 342 ± 12 µgmg⁻¹ dry biomass and 334 ± 9 µgmg⁻¹ dry biomass respectively on 30th day. Carotenoid content was 123± 8 µgmg⁻¹ and 105± 9 µgmg⁻¹ dry weight respectively for control and transformed cells (Figure 2.17).
Figure 2.16: Growth profile of control and transformed *D. bardawil* cells grown in MAS100 medium

Figure 2.17: Total chlorophyll and carotenoid content in control and transformed *D. bardawil* cells grown in MAS100 medium (Car C: carotenoids in control; Car T: carotenoids in transformant; Chl C: Chlorophyll in control; Chl T: Chlorophyll in transformant)

2.4.11.3 Pigment analysis using HPLC

The HPLC profile showed similar pattern for control and transformed *D. bardawil* (Figure 2.18). No specific or new peak was observed in the HPLC profile of transformed cells.
2.4.12 Molecular characterisation of *D. bardawil* using 18S rDNA

PCR amplification using conserved and species specific primers were carried for *D. bardawil* (Figure 2.19). An amplicon with size 2.1 kb was obtained with specific primers M1 and M2 (Lane 2), while the amplification with DS-M2 gave the amplification of 700bp. Species specific primer DB-M2 did not provide any amplification.

![Figure 2.19: PCR amplification of 18S rDNA from genomic DNA of *D. bardawil* using conserved and species specific primers. Lane 1: DB-M2; Lane 2: M1-M2; Lane 3: DS-M2 and Lane M: 3 kb marker](image)

1.5 Discussion

Preliminary studies on the sensitivity of *D. bardawil* to different antibiotics revealed the insensitivity of antibiotics upto 1000 mgL\(^{-1}\) in growth media of *D. bardawil*. This was also reported by Tan et al (2005) who observed that even higher concentrations of different antibiotics like streptomycin, hygromycin, Kanamycin etc. were unable to
inhibit the growth of *D. salina*. Allnutt et al (2000) had reported that high salt can significantly reduce the efficacy of many antibiotics and herbicides in the growth media of *Dunaliella*.

It is imperative that for developing an efficient transformation system, an effective selection system is necessary. TAP medium has been previously used in *Agrobacterium* mediated transformation of *Haematococcus pluvialis* (Kathiresan et al, 2009) and *Chlamydomonas reinhardtii* (Kumar et al, 2004). Moreover it contained lower levels of salts (4.0 g L\(^{-1}\)) than MAS100 (55.5 g L\(^{-1}\)). In order to test the tolerance of *D. bardawil* to varying concentrations of hygromycin and NaCl, cells grown in MAS100 were transferred to TAP medium. TAP medium containing 0.2 M NaCl supported adequate growth of *D. bardawil* while sparse or negligible growth occurred at NaCl concentration below 0.2 M. Similar observation was also made by Ginzburg et al (1987) where the minimum NaCl concentration required for *Dunaliella* growth was found to be 0.2 M. The gradual adaptation of *Dunaliella* to increasing and decreasing NaCl concentrations in the media was carried out during transformation. Previous reports are available in which it has been stated that sudden changes in NaCl concentration brought cell breakage and death. This may be ascribed to virtual loss of water from the cell interior as the *Dunaliella* cells are surrounded only by a cell membrane, the elasticity of which is limited (Ginzburg et al, 1987). The NaCl concentration was lowered to 0.2 M in the co cultivation of *Dunaliella* as high NaCl in the growth medium does not favour *Agrobacterium* growth. This was also supported by the fact that the addition of NaCl had a strong inhibitory effect on vir induction (Melchers et al, 1989).

The concentration of four MAS100 medium constituents viz K\(_2\)HPO\(_4\), NaHCO\(_3\), NaCl and KNO\(_3\) was optimised for increased biomass and carotenoids production in *Dunaliella*. The alga was found to produce more lutein than β-carotene under the standardised conditions. This may be possibly due to more phosphate content in the medium which promote more vegetative growth than β-carotene accumulation. As *Dunaliella* produces more β-carotene under high light conditions, the low intensity of light provided during the experiment may also be a factor for low β-carotene content.
Agrobacterium mediated genetic transformation

It was obvious from the experiments that *D. bardawil* was able to tolerate high levels of hygromycin when grown in higher salt concentration than at lower salt concentrations. The insensitiveness of *D. bardawil* to various antibiotics could be ascribed to the higher salt content in its growth media as indicated by the results of the present study. Increasing concentration of NaCl beyond 0.2 M attenuated the growth of the *A. tumefaciens* while accelerating growth of the alga. This provided an alternative for use of antibiotics to check the growth of *A. tumefaciens* after co-cultivation. However, in order to increase the transformation efficiency and to reduce the growth of *A. tumefaciens*, a mixture of cefotaxime and potassium clavulanate was used as described in previous studies (Kumar et al, 2006).

In *Dunaliella* spp., different transformation methodologies have been reported such as glass bead (Jin et al, 2001), electroporation (Walker et al, 2005c; Geng et al, 2004; Degui et al, 2002; Sun et al, 2005) and particle bombardment (Tan et al, 2005, Guo-Zhong et al, 2005) to express foreign genes. Stable integration of these genes has been reported only by Geng et al (2003) and Walker et al (2005c). However, Coll (2006) reported the rapid loss of transformation phenotype during long term culturing of transformants. *A. tumefaciens* mediated transformation has been reported to overcome the major limitations of other transformation procedures, providing stable integration at lower copy number, potentially leading to fewer problems with transgene co-suppression and instability (Hansen et al, 1997). Although *A. tumefaciens* mediated transformation has been reported for green alga *C. reinhardtii* (Kumar et al, 2004) and *H. pluvialis* (Kathiresan et al, 2009) there have been no reports of such studies in any *Dunaliella* spp. The stability of the transgene integration in the present study is supported by southern and western blot experiments which have been carried out in 15 to 18 months old transformant cell lines grown in hygromycin free medium.

*vir* Genes are known to be activated by phenolics such as acetosyringone derived from plant cell wall. In the present study acetosyringone did not appear to make any difference in the number of HRC obtained, which is in agreement with the earlier report by Kathiresan et al (2009). It has been reported that acetosyringone significantly increases the transformation frequency in *C. reinhardtii* (Kumar et al; 2004). However, later it was reported (Kumar and Rajam, 2007) that *vir* gene expression can be induced by *C. reinhardtii* cell extract without the requirement of
The present study results indicate that *D. bardawil* may also be in the natural host range of *A. tumefaciens*.

Although in the present study the transformation frequency obtained (41.0 ± 4 cfu per 10⁶ cells) is not higher than those reported for other methods *viz* 83 cfu per 10⁶ cells in glass bead transformation (Jin et al 2001), 1900 cfu per 10⁶ cells (Degui et al, 2002) and 150 cfu per 10⁶ cells (Geng et al, 2003) by electroporation, the transformants obtained are found to be stable for almost four years. This is evident from amplification of *hpt* gene from the genomic DNA of hygromycin resistant cells taken regularly at two months intervals (data not shown). It is reported that when transgenic algal clones are not maintained under selection conditions, expression of the exogenous gene might be suppressed. This gene silencing has been attributed to a variety of epigenetic mechanisms similar to those observed in plants and other eukaryotic cells, and is thought to be related to the control of development and to the response of a cell to viruses, transposable elements, or transgenes (Wu-Scharf et al, 2000). In the present study, stable expression of *hpt* was observed even after continuous maintenance in hygromycin free medium.

*D. bardawil* was found to differ in its sensitivity towards hygromycin when grown in LTAP and STAP media. Control *D. bardawil* cells were able to tolerate only 75 mgL⁻¹ hygromycin when grown in solid TAP, while the level of tolerance was 500 mgL⁻¹ in liquid TAP. A similar observation has been made earlier where chloramphenicol sensitivity of *D. bardawil* was reported at 60 mgL⁻¹ in solid Johnson’s medium (Geng et al, 2003) while Tan et al (2005) observed the same at 400 mgL⁻¹ in Johnson’s liquid medium. The salt pumps which enable the survival of alga in high saline environment may also be responsible for the antibiotic resistance of this alga particularly in liquid media.

The incubation of alga with *A. tumefaciens* was carried out at 22⁰C which is reported as the optimum temperature for other plant species (Dillen et al, 1997). A close association between *A. tumefaciens* and *D. bardawil* was revealed through scanning electron microscopy of co-cultivated cells (Figure 2.14 B) following the method of Kathiresan et al (2009). It is also interesting to note in case of *D. bardawil* that the gradual increase in salinity of the medium from 0.2 to 1.0 M proved as an
efficient method for the elimination of *A. tumefaciens* after co-cultivation. This method for the removal of *Agrobacterium* after co-cultivation has not been used before. The possibility of *Agrobacterium* contamination in high NaCl (1.0 M) containing growth medium that supported growth of the putative transformants was ruled out with the use of PCR for the *nptII* gene which confers kanamycin resistance. The absence of *Agrobacterium* on the cell surface of *D. bardawil* was checked through SEM of the transformants (Figure 2.14 C) which was also used by Kathiresan et al (2009). Both the methods confirmed the absence of *Agrobacterium* in HRC.

Integration of the *hpt* gene in the genome was confirmed through PCR, RT-PCR and expression of *gus* and *gfp* genes. The fluorescence of GFP was of high intensity, uniform and clear while the staining for GUS activity was faint and not uniform. The reason for poor GUS staining may be due to low permeability of substrate. Expression of *gfp* gene has been previously reported in transformed *C. reinhardtii* (Kumar et al, 2004) and *H. pluvialis* (Kathiresan et al, 2009). Identification of site of integration of T-DNA in the transformants through genome walking method has revealed the integration in non coding regions of the genomic DNA of the transformants. The same technique was also used by Siebert et al (1995) to walk upstream from 5'-end coding regions of the human tissue-type plasminogen activator gene. DNA transfer in *Agrobacterium* mediated transformation is initiated by virD2-mediated cleavage within the 25-bp right border sequence. T-DNA integrates into the genome by illegitimate recombination (Zupan et al, 2000) via unknown mechanism. Characterization of a limited number of T-DNA insertions into genes showed an apparently even repartition along *Arabidopsis thaliana* chromosomes with no preferential integration into a specific gene structure (Azpiroz-Leehan and Feldmann, 1997). Small, linearized plasmids tend to be inserted into the nuclear genome preferentially through their ends, causing deletions (5–20 kb) and/or rearrangements at the integration site. The deletion of variable length in the host DNA after T-DNA integration was also reported by Brunaud et al (2002) like in the present study. They also have proposed that integration of T-DNA usually prefer T-rich region in the host genome. As in higher plants, most nuclear transformations observed in chlorophytes, diatoms and dinoflagellates appear to involve random integration into the genome of one to several copies of the introduced DNA, depending on the DNA
concentration used (Kindle, 1998). Nuclear homologous recombination has also been observed in *Chlamydomonas*, but at much lower frequency (Kindle, 1998).

*Ndel* is having four cutting sites at 245, 4526, 7015 & 10035 and *SacII* with single cut at 8906 which may be deduced from nucleotide sequence of pCAMBIA 1304. This will give fragments of approximate sizes 4.3 kb, 1.2 kb, 2.6 kb & two fragments of 1.9 kb when pCAMBIA 1304 is digested with the *SacII* and *Ndel*. The fragment of 1.129 kb which includes a portion of the hygromycin gene, left border and a portion beyond the left border from pCAMBIA 1304 hybridized with the probe and was visualized in the southern blot (Figure 2.12 A & a; Lane C). The signals observed in the lanes T1 (~ 4.5 kb), T2 (~ 4.5 & 5 Kb) and T3 (~ 5 kb & 6 kb) in the blot are of approximate sizes greater than the plasmid band which indicates the transgene integration into the genomic DNA. This indicates the integration of the hygromycin gene into the genome of the transformant. The presence of multiple bands reactive to the probe in lanes T2 and T3 is diagnostic of multiple T-DNA copy integration into genomic DNA. In plants and yeasts, homologies have been observed between T-DNA borders and pre integration sites (Tzfira et al, 2004, 2006). However, there are no such reports in algae. Immunoblot analysis clearly indicated the expression of GUS-GFP reporter gene in the transformants. A 90 kDa protein has been previously reported in the immunoblot analysis of transformed *C. reinhardtii* for the GUS-GFP fusion protein using the GUS antibody (Kumar et al, 2004). Transformation did not cause either morphological or physiological changes in *Dunaliella* other than reporter and marker gene expression. This was also evident from the similar growth pattern and pigment profile of both control and transformed *D. bardawil* cells.

Within the subgenus *Dunaliella*, the taxonomy at the species level is obscure, and some species described as separate entities may be found eventually to be polymorphic forms of only one taxon (Preisig, 1992). It is well established in the literature that morphological (cell shape and size) and structural attributes (presence or absence of stigma, refractile granules, chloroplast size and shape) cannot be used consistently for species differentiation in unicellular green algae. The intron-sizing method provides a novel and very powerful DNA-fingerprinting technique to accomplish a specific, rapid and sensitive identification of carotenogenic *Dunaliella*
species. Molecular characterisation of several *Dunaliella* spp. using 18S rDNA has been previously reported by Olmos et al (2000, 2009). A more recent classification of *Dunaliella* species using molecular markers indicated that, most likely, fewer species of *Dunaliella* exist than those established previously based on morphological and physiological attributes (Gonzalez et al, 2009). Overall, systematic molecular data (Olmos et al, 2000, 2009; Cifuentes et al, 2001; Gonzalez et al, 2001, 2009; Gomez and González, 2004; Raja et al, 2007; Hejazi et al, 2010) should be extended and be used in combination with morphological and physiological markers to re-evaluate species classification. The present result revealed that the *Dunaliella* spp. selected for the study belongs to a β-carotene hyper producer strain of *D. salina*. *D. salina* strains with the fingerprinting profile of MA1–MA2 = ~2100 bp and DS-MA2 = ~700 bp as obtained in the present study, are distributed worldwide, grow on hypersaline environments and their β-carotene hyper production capacity is well conserved. In order to facilitate identification, Olmos et al (2009) suggested a sub-classification calling these strains as "*D. salina var Teod*". All hyper producer species are known to carry I or II introns within the 18S rDNA gene. *D. salina var Teod* has one intron in the 18S rDNA.

The present study has shown stable transgene integration in *D. bardawil* even after continuous culturing in hygromycin free medium and these cells are free from *A. tumefaciens*. This is the first report for the successful transformation of *D. bardawil* using *A. tumefaciens* (Anila et al, 2011a). The simplicity of the method along with stability of transformants even in the absence of selection pressure will enable the use of this microalga as an efficient system for production of novel proteins and value added compounds.