CHAPTER III

Glucose induced hygromycin sensitivity and phenolic profile of D. bardawil during Agrobacterium mediated transformation
Summary

The successful transformation of *Dunaliella* by *Agrobacterium tumefaciens* described in Chapter II also brings forward two significant observations; the increased tolerance of *Dunaliella* to various antibiotics in high NaCl containing medium and a phenol free co-cultivation unlike other *Agrobacterium* transformation systems. Thus the present chapter, in continuation with Chapter II, focuses on two important observations during *Agrobacterium* mediated transformation in *Dunaliella*. Chapter III is presented in two different sections. The first part deals with the effect of glucose as a possible activator of H\(^+\)-ATPase. Addition of glucose at 10 mM concentration in the selection medium increased the sensitivity of *Dunaliella* to hygromycin. A decrease in hygromycin tolerance level from 100 to 25 mgL\(^{-1}\) was noted for HRC. Control cells were sensitive to hygromycin levels as low as 12 mgL\(^{-1}\). H\(^+\)-ATPase activation by glucose was confirmed by medium acidification and H\(^+\)-ATPase assay. The H\(^+\)-ATPase assay values indicated maximum activity of 18 µmol Pi /hour/mg protein at 10 mM concentration of glucose. Vanadate at 10 µM and DES at 15 µM completely inhibited the activation by glucose which confirmed that H\(^+\)-ATPase is involved in medium acidification and that it is activated by glucose. There was a slight increase in the transformation frequency of treatments with 10 mM glucose. The transformants were confirmed with PCR amplification of *hpt* from genomic DNA. The second part of chapter III attempted to characterise the phenolic compounds in *Dunaliella* which may act as vir gene inducers during *Agrobacterium* mediated gene transfer in *Dunaliella*. Phenolic compounds present in cell free medium of *D. bardawil* were analysed using HPLC and HPTLC methods. Phenolic compounds identified in cell free medium at basic pH were acetosyringone, vanillic acid, vanillin and proto-catechuic acid. Acetosyringone was observed as the major phenolic compound among the above. The compounds were further confirmed with mass spectrometry (ESI negative mode). The identified phenolic compounds were checked for their transforming efficiency by incorporating them at 100 µM concentration in the co-cultivation medium. A maximum cfu of 95±13 per 10\(^6\) cells plated was obtained by addition of vanillin in the co-cultivation medium. The observations of the present study may thus increase the potential of *Agrobacterium* mediated gene transfer methodology for genetic manipulation in this alga.
3.1 Glucose induced activation of H⁺-ATPase and its role in hygromycin resistance

3.1.1 Introduction

The mechanisms that enable *Dunaliella* to tolerate extreme saline conditions are well investigated in recent times. The alga is able to survive in saturated salt solutions by maintaining relatively lower intracellular sodium concentrations (Pick, 1986). Previous studies have revealed factors like intracellular glycerol content, plasma membrane carbonic anhydrase, and iron transferring protein contributing for salt tolerance in this alga (Ben-Amotz and Avron et al, 1973; Fisher et al, 1996; Fisher et al, 1997). Thus *Dunaliella* possess a very sturdy mechanism for recovery from osmotic shocks even under extreme stressful conditions.

Although there are mechanisms which enable *Dunaliella* to tolerate high salinity, it also leads to lowered sensitiveness of the alga towards many antibiotics. There are previous reports pointing out the ineffectiveness of commercial antibiotics in the high salt growth medium of *Dunaliella* (Allnutt et al, 2000). The ineffectiveness of many commercially available antibiotics in killing *Dunaliella* has already been described in Chapter II. The requirement of higher concentration of selection antibiotic hygromycin even at a NaCl concentration of 0.2 M is less economic and may be considered as a disadvantage of the standardised transformation procedure. Under these circumstances, further investigation was directed towards unwinding the mechanism possibly behind the antibiotic resistance of *Dunaliella* and to increase its hygromycin sensitivity. Studies pertaining to drug transport mechanism in this alga have not been detailed out till now.

The interrelation between ATPase activity and hygromycin B sensitivity was studied extensively in yeast by David et al (1989). The *pma1* mutants obtained as a result of UV radiation were selected by their resistance to hygromycin. The mutant H⁺-ATPases were found to have altered membrane potential formation and kinetic defects that significantly altered Km value & Vmax. They also suggested that resistance to hygromycin B is closely correlated with a depolarization of cellular membrane potential. Hygromycin B uptake in yeast is thus coupled to the
electrochemical proton gradient and the reduced driving force could substantially limit uptake and confer cellular resistance to the antibiotic in pmal mutants.

Plasma membrane (PM) P-type ATPases play a central role in the growth and development of the plant systems which includes nutrient ion uptake, regulation of intracellular pH etc. through creation and maintenance of electrochemical proton gradients (Morsomme et al, 2000). In addition, this membrane protein plays a major role in many physiological processes, including salt tolerance, intracellular pH regulation, stomatal opening and cell elongation. So regulation of the expression and activity of the PM H⁺-ATPase may represent an important cellular mechanism for salt tolerance (Kerkeb et al, 2001; Chen et al, 2010). In yeast, H⁺-ATPase plays a major role in providing tolerance to toxic cations including sodium and hygromycin (Goossens et al, 2000). Activation of the plasma membrane ATPase is also suggested to trigger osmoregulation in Dunaliella (Oren-Shamir et al, 1989).

In S. cerevisiae, it has been demonstrated that addition of glucose or related sugars to derepressed cells causes rapid activation of plasma membrane H⁺-ATPase (Serrano, 1983). Internal alkalinization and external acidification apparently resulting from H⁺-ATPase activity are associated with growth induction of yeast cells in glucose containing media. Acidification of the external medium is due to metabolism of the sugar. When the extracellular pH is lower than the cytoplasmic pH, passive proton movement tends to acidify the cytoplasm. However, due to plasma membrane H⁺-ATPase activity, the internal pH of yeast cells is maintained between 6.0 and 7.5 even when great variations of extracellular pH occur (Serrano, 1985).

In yeast, the H⁺-ATPase activity is regulated at the plasma membrane depending on the physiological state of the cells, increasing during glucose metabolism (Serrano, 1983) and by acidification of the growth medium (Eraso and Gancedo, 1987). ATPase activation by glucose metabolism results from a combined effect on the $K_m$, $V_{max}$, pH optimum and vanadate (and by analogy, phosphate) binding site of the enzyme activity. Since the activation persists upon plasma membrane isolation, it seems to be the result of covalent modification. Phosphorylation of yeast H⁺-ATPase has been demonstrated by several groups (Yanagita et al, 1987) and the result suggests that dephosphorylation leads to
deactivation of the enzyme (Kolarov et al, 1988). This suggests that H\(^+\)-ATPase activation after glucose addition might be the result of a phosphorylation process, possibly catalysed by cAMP-dependent protein kinase.

Similar effect by glucose has been reported in other organisms too. Activation of the plasma membrane H\(^+\)-ATPase through addition of glucose and other sugars to derepressed cells of the fungus *Fusarium oxysporum* var. *Zini* was observed by Brandao et al (1992). Sugar-induced plasma membrane H\(^+\)-ATPase activation in *Fusarium* also resembles activation of the enzyme in *Saccharomyces* in other respects. The parallel inhibition of H\(^+\)-ATPase activation and increased cAMP levels appears to confirm the existence of a causal relationship between the two phenomena.

The present investigation is in continuation of the observation that low salt content in the medium makes *Dunaliella* sensitive to hygromycin B, which is otherwise ineffective in its growth. The study also aims to find the possible activation of H\(^+\)-ATPase of *Dunaliella* by glucose to increase its hygromycin sensitivity of *Dunaliella*.

### 3.1.2 Materials and Methods

#### 3.1.2.1 *D. bardawil* culture and maintenance

*D. bardawil* culture, maintenance of the culture and growth conditions were followed as in the section 2.3.1, 2.3.1 and 2.3.2 of chapter II. Glass wares and plastic wares used in the experiments is same as described in section 2.2.2, 2.2.3 and 2.2.4 of chapter II.

#### 3.1.2.2 Sensitivity of *D. bardawil* to glucose and to hygromycin in the presence of glucose

*D. bardawil* is an obligate autotroph. So initial studies were carried out to find the level of glucose in the medium that *D. bardawil* can withstand during its growth. Glucose at various concentrations (5, 10, 20, 30, 50, 100, 150 and 200 mM) was incorporated in solid TAP medium to check the growth of *D. bardawil*. As concentrations of glucose increased over 20 mM, growth of *Dunaliella* decreased and therefore 10 mM glucose was used in further experiments.
Various concentrations of hygromycin (10-80 mg L\(^{-1}\)) concentrations were made in the selection medium with 5 mM, 10 mM and 15 mM glucose and Dunaliella cells were grown on these plates (1.5x10\(^{5}\) cells) for a period of 30 days.

3.1.2.3 Glucose induced medium acidification in *D. bardawil*

Control Dunaliella cells grown in MAS100 for one week were pelleted by centrifugation and were washed twice with milliQ water containing 0.5 M sorbitol. Initial pH was adjusted to 7.2 with 0.01 M HCl. Once a stable pH base line was established, the cells were resuspended in the same and glucose at various concentrations (5, 10 and 15 mM) was added to 50 ml of culture. The change in pH was recorded after 10 minutes using a rapidly responding pH meter (Eutech pH tutor, Malaysia).

The external acidification due to glucose addition was further confirmed under the effect of SOV (sodium ortho vanadate) and DES (diethyl stilbesterol) (Sigma – Aldrich, USA). As the vanadate inhibition is competitive with phosphate, and also the active site of ATPase is within the cell, it is necessary to prestarve the algae for phosphate prior to treatment for maximal inhibition (Oren-Shamir et al, 1989). For the treatment with SOV, *D. bardawil* cells were allowed to grow in growth medium containing lowered phosphate (20 µM) for one day and were pelleted and resuspended as described above. SOV and DES (5, 10 and 15 µM) were added to the suspended cells. After 5 minutes, initial pH was noted and 10 mM glucose was added. The final pH was measured after 10 minutes.

3.1.2.4 H\(^+\)-ATPase assay for *D. bardawil* cells grown at different glucose concentrations

A set of *D. bardawil* cultures in 50 ml volume was grown in liquid TAP medium containing 0.1 M NaCl for a week. Glucose at different concentrations (5, 10 and 15 mM) was added to different flasks. A control was maintained without glucose addition. Cells were harvested by centrifugation after 2 hours and plasma lemma-enriched membrane fractions were prepared from the pelleted cells following the procedure of Larissa et al (2005). The pellet was washed twice with 20 mL of 1.25 M glycerol, 5 mM Tris–HCl, pH 7.5, 2 mM MgSO\(_4\), 10 mM KCl, and 0.5 mM EGTA (Buffer A). The following steps were carried out at 4\(^{0}\) C. The washed cells were
exposed to hypo osmotic shock in 400 mL of 5 mM Tris–MES, pH 7.5, 1 mM DTT, 0.5 mM K$_2$S$_2$O$_5$, 0.5 mM EGTA, and 1 mM PMSF (Buffer B). Cell suspension was slowly stirred with a magnetic rod for 5 min. After 5 min incubation, MgSO$_4$ was added at 1 mM final concentration and unbroken cells, chloroplasts and cell debris were removed by centrifugation of the cell suspension at 700g for 15 min. The supernatant was collected and centrifuged at 15000g for 20 min. The pellet obtained was re-suspended in 5 mL of 0.4 M sucrose, 5 mM BTP-MES, pH 7.5, 1 mM MgSO$_4$, 1 mM DTT, 0.5 mM EGTA, and 1 mM PMSF (Buffer C) and re-centrifuged at 3000g for 15 min to remove residual intact organelles and large cell debris. The supernatant was collected and layered on discontinuous (30/38%) sucrose gradient prepared with buffer C that contained additionally 20 mM MgSO$_4$. Following centrifugation at 112000g for 30 min, a band containing plasma membranes was formed at the 30/38% sucrose interface. The PM from the interface was collected, diluted to 25 mL with Buffer C and centrifuged at 15000g for 20 min. The pellet was re-suspended in a small volume of Buffer C. The PM vesicles were frozen in liquid nitrogen and stored at -20°C for further use.

H$^+$-ATPase activity was determined by measuring the release of Pi from ATP according to the method of Madhu et al (2001). 10µl of microsomal membrane fraction was added to incubation buffer containing 50 mM Mes-tris, pH 6.5, 5 mM MgSO$_4$, 50mM KNO$_3$, 5 mM sodium azide, 0.2 mM ammonium molybdate with 3 mM ATP as substrate and with and without 0.2 mM diethylstilbestrol, a specific inhibitor of H$^+$-ATPase. The reaction mixture was incubated at 37 °C for 30 min and 150 µl of phosphate reagent containing 0.5% sodium dodecyl sulphate, 0.5% ammonium molybdate, 2% (v/v) sulphuric acid and 1.2% ascorbic acid was added. The reaction was carried out in a microtiter plate. After 5–10 min, the absorbance was taken at 620 nm in an ELISA reader (Dynatech, MR5000). The H$^+$-ATPase was measured as the difference between the activities in presence and absence of diethylstilbestrol and the activity was expressed as µmol Pi released mg$^{-1}$ protein h$^{-1}$. Total protein content was estimated by the method of Bradford (Bradford, 1976) using BSA as the standard.
3.1.2.5 Agrobacterium mediated transformation of D. bardawil and selection in glucose containing medium

*Dunaliella* was transformed using *Agrobacterium* using the procedure described in section 2.2.8 of chapter II. The cells after co-cultivation were plated on selection medium with only hygromycin (80-200 mgL\(^{-1}\)) and with 10 mM glucose and hygromycin (10-200 mgL\(^{-1}\)). The plates were incubated for a period of 8 weeks for calculating transformation frequency. For treatments without glucose, hygromycin concentrations in the range of 10-80 mgL\(^{-1}\) was not tried as the standardized selection concentration was 100 mgL\(^{-1}\) (Section 2.3.4, Chapter II). Each plate was maintained in triplicates.

3.1.2.6 Hygromycin sensitivity of control and transformed *D. bardawil* in the presence of glucose and SOV

Various concentrations of hygromycin (50-300 mgL\(^{-1}\)) were made in selection medium with 10 mM glucose and wild and transformed *Dunaliella* cells were grown on these plates (1.5x10\(^5\) cells). The effect of various hygromycin and NaCl concentrations at constant glucose concentration of 10 mM was also investigated.

Similarly, SOV at various concentrations (5-15 µM) were also added to the selection medium at two different hygromycin concentrations (50 and 100 mgL\(^{-1}\)). As addition of SOV increases the requirement of hygromycin, the experiment was carried out in wild *D. bardawil* cells only. Colonies were counted after a period of 30 days.

3.1.2.7 Confirmation of transformation

The selected colonies after transformation from both selection plates (with and without glucose) were grown in MAS100 for a period of 20 days. GFP expression in the transformants was carried out using the procedure followed in section 2.3.10 of Chapter II.

Genomic DNA was isolated from the transformants and PCR was carried for *hpt* gene following the procedure described in section 2.3.11 of chapter II.
3.1.3 Results

3.1.3.1 Sensitivity of *D. bardawil* to glucose and to hygromycin in the presence of glucose

The sensitivity of *Dunaliella* towards various glucose concentrations in STAP was determined. The growth was determined on the basis of appearance of cfu after a period of 20 days. The results are presented in Figure 3.1. At higher glucose (20 mM and above) concentrations cells survived for three days and later got bleached. cfu is the average of three replicates.

![Figure 3.1: Sensitivity of *D. bardawil* to various glucose concentrations in STAP medium](image)

Even though growth of *Dunaliella* was observed in 20 mM glucose, cell death occurred after 25 days. So 10 mM glucose concentration was proved to be optimum for *D. bardawil* growth and was selected for further studies. The effect of glucose concentrations (5 mM, 10 mM and 15 mM) on hygromycin sensitivity showed that at 10 mM glucose concentration, 20 mgL\(^{-1}\) hygromycin completely inhibited the growth of the cells (Figure 3.2). However, at 10 mgL\(^{-1}\) hygromycin, only slight growth of cells was observed. Although higher glucose concentration of 15 mM was also effective in killing *Dunaliella* at 20 mgL\(^{-1}\) hygromycin, the cells on control plate (without hygromycin and with 15 mM glucose) eventually got bleached after a period of 25 days.

3.1.3.2 Glucose induced medium acidification

The initial pH was maintained at 7.2 for all experiments. Maximum acidification of 0.8 units was observed for 10 mM glucose. Concentration of 5 mM
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and 15 mM gave the minimum value of 6.9 and 6.7 respectively (Figure 3.3). Vanadate at 10 µM and DES at 15 µM completely inhibited the activation by glucose as revealed through pH values (data not shown).

Figure 3.2: Effect of various glucose concentrations on Dunaliella growth at different hygromycin concentrations (C- Control without hygromycin addition).

Figure 3.3: Glucose induced medium acidification in D. bardawil.
3.1.3.3 H⁺-ATPase assay for control *D. bardawil* grown at different glucose concentrations

The H⁺-ATPase assay values indicated maximum activity of 18 µmol Pi/hour/mg proteins at 10 mM concentration of glucose. Control and cultures with 5mM glucose had almost similar values indicating insufficient glucose concentration. Glucose concentration of 15 mM had higher values initially which decreased after 1.5 hours (Figure 3.4).

![Figure 3.4](image)

**Figure 3.4**: Effect of glucose on plasma membrane H⁺ATPase activity in *D. bardawil*.

3.1.3.4 Selection of transformants in the presence of glucose

The selection of the transformants was carried out in selection medium with and without 10 mM glucose and both with varying hygromycin concentrations. Transformation frequency remained almost same for treatments with and without glucose. Selection of transformants was carried out at 25 mgL⁻¹ hygromycin concentration in the presence of 10 mM glucose and at 100 mgL⁻¹ in the absence of glucose in the selection medium. The transformation efficiency of *D. bardawil* cells when selected in the presence and absence of glucose was found to be 45.0 ± 3 cfu per 10⁶ cells and 48.0 ± 4 cfu per 10⁶ cells respectively(Table 3.1).
### Table 3.1: Transformation frequency in selection medium with and without 10 mM glucose. Values are average of three replicates

<table>
<thead>
<tr>
<th>Hygromycin (mgL⁻¹)</th>
<th>Without* Glucose (cfu)</th>
<th>With 10 mM Glucose (cfu)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>NT</td>
<td>89</td>
</tr>
<tr>
<td>20</td>
<td>NT</td>
<td>43</td>
</tr>
<tr>
<td>40</td>
<td>NT</td>
<td>51</td>
</tr>
<tr>
<td>50</td>
<td>NT</td>
<td>50</td>
</tr>
<tr>
<td>80</td>
<td>NT</td>
<td>58</td>
</tr>
<tr>
<td>100</td>
<td>48</td>
<td>67</td>
</tr>
<tr>
<td>150</td>
<td>47</td>
<td>59</td>
</tr>
<tr>
<td>200</td>
<td>38</td>
<td>48</td>
</tr>
</tbody>
</table>

* cfu count is the average of 3 replicates;
NT- combinations which are not tried

### 3.1.3.5 Hygromycin sensitivity of control and transformed *D. bardawil* in the presence of SOV and glucose

**(A) SOV**
SOV is a proven inhibitor of the H⁺-ATPase. Addition of SOV in the medium resulted in an increase in the hygromycin resistance of the algae. The effect was more at higher NaCl concentrations. More than 200 colonies were observed at 0.6 M NaCl (Table 3.2).

**(B) Glucose**
The effect of various hygromycin and NaCl concentrations at constant glucose concentration of 10 mM was investigated. As previously discussed in Chapter 2, higher NaCl concentrations increased the resistance of *D. bardawil* towards hygromycin (Figure 3.5). The growth of wild and HRC of *D. bardawil* was monitored in selection medium containing 10 mM glucose. A decrease in hygromycin tolerance level was observed for HRC (30 mgL⁻¹). Wild cells were sensitive to hygromycin levels as low as 12 mgL⁻¹ (Figure 3.6).
Table 3.2: Effect of SOV on hygromycin resistance of *D. bardawil* under various NaCl concentrations. Cfu values are average ± standard deviation

<table>
<thead>
<tr>
<th>NaCl(M)</th>
<th>SOV(mM)</th>
<th>Hygromycin(mg/L)</th>
<th>cfu</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.2</td>
<td>0</td>
<td>50</td>
<td>49±2</td>
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<tr>
<td></td>
<td></td>
<td>100</td>
<td>0±3</td>
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<tr>
<td></td>
<td>5</td>
<td>50</td>
<td>58±4</td>
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<tr>
<td></td>
<td></td>
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<td>50</td>
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<tr>
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<td>100</td>
<td>23±2</td>
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<td>15</td>
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<tr>
<td>0.4</td>
<td>0</td>
<td>50</td>
<td>68±4</td>
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<td></td>
<td></td>
<td>100</td>
<td>23±5</td>
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<tr>
<td></td>
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<td>50</td>
<td>76±4</td>
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<tr>
<td></td>
<td></td>
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<td>&gt;200</td>
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<tr>
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<td>0</td>
<td>50</td>
<td>193</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100</td>
<td>&gt;200</td>
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<td>&gt;200</td>
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<td>&gt;200</td>
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<td></td>
<td></td>
<td>100</td>
<td>&gt;200</td>
</tr>
</tbody>
</table>

**Figure 3.5**: Effect of hygromycin on *D. bardawil* growth under different NaCl concentrations at constant glucose concentration (10 mM).
Figure 3.6: Comparative colony count of control and transformed *D. bardawil* cells on selection medium with 10 mM glucose and varying hygromycin concentration

3.1.3.6 Confirmation of transformation

GFP expression obtained for transformants selected in the presence and absence of glucose did not show any difference. PCR was carried out for the presence of *hpt* gene (Figure 3.7). Amplification of gene fragment (400 bp) was observed for both transformants.

Figure 3.7: Amplification of *hpt* from genomic DNA of transformants Lane 1: Wild Lane 2-4: transformants selected without glucose; Lane 5-7: transformants selected with 10 mM glucose; Lane 8: pCAMBIA 1304 Lane M: 3 kb marker
3.1.4 Discussion

During the course of genetic transformation studies in *Dunaliella*, the resistance of this alga to many of the commonly available antibiotics was noted. However, by reducing the NaCl concentration in the medium, the algae were found to be sensitive to hygromycin. Allnutt et al, (2000) has reported that high salt can significantly reduce the efficacy of many antibiotics and herbicides in the growth media of *Dunaliella*. It may be assumed that high amount of NaCl in the medium either degrades or prevents the action of antibiotics. The initial experiments were carried out to see the relation between NaCl and hygromycin requirement. An inverse relationship was noticed between NaCl and hygromycin in the medium. The NaCl concentration of 0.2 M was selected for experiments and the hygromycin required for selection in this medium was optimised as 100 mgL\(^{-1}\) which was high when compared to similar systems. Thus studies were pursued to reduce hygromycin requirement for selection in *Dunaliella*.

David et al (1989) has reported that the *pmaI* mutants of yeast have a defective H\(^+\)-ATPase with altered kinetic properties and they are resistant to the antibiotic hygromycin whereas the wild cells were susceptible to the same. The defective mutant *pmaI* has altered membrane potential formation which in turn leads to hygromycin resistance. It may be assumed that activation of H\(^+\)-ATPase by the addition of reported activators leads to polarization of the membrane potential and thereby increases the susceptibility of *Dunaliella* cells to hygromycin. In the present study, the interrelationship between the activation of H\(^+\)-ATPase through glucose and its subsequent effect in hygromycin sensitivity of *Dunaliella* is discussed.

The H\(^+\)-ATPase from plasma membranes in plants is regulated at different levels by multiple internal and external factors. A remarkable characteristic of H\(^+\)-ATPase is that it is activated in the presence of glucose through transcriptional and post-transcriptional mechanisms that increase the level of ATPase activity in yeast cells (Serrano, 1983; Sychrova and Kotyk, 1985; Capieaux et al, 1989; Eraso and Portillo, 1994). Phosphorylation involved in yeast H\(^+\)-ATPase activation has been demonstrated by several groups (McDonough and Mahler, 1982; Yanagita et al, 1987) and dephosphorylation leads to deactivation of the enzyme (Kolarov et al, 1988). In
yeast, other than glucose, ethanol (Monteiro et al, 1994), environmental stresses such as high temperature (Viegas et al, 1995), organic acids (Viegas and Sa-Correia, 1991; Viegas et al, 1998) and deprivation of nitrogen source (Benito et al, 1992) have also been reported to stimulate activity of yeast H^+-ATPase. Sugar-induced plasma membrane H^+-ATPase activation has been previously reported in the fungus Fusarium in addition to Yeast (Brandao et al, 1992). No such reports on H^+-ATPase activation by glucose are available in microalga. However in Dunaliella acidophila Sekler et al (1994) have reported that the plasma membrane ATPases can be activated by trypsin treatment.

Studies on intact cells of D. tertiolecta proved that the cells assimilate glucose at very low rates due to impermeabilty of the cell membrane (Kwon et al, 1971). Although enzymes for glucose metabolism are present in Dunaliella, majority of the carbon incorporated during photosynthesis is coming from CO_2 than from added glucose. Based on these reports, the work was carried out with lowered glucose levels of 5 to 20 mM. Glucose concentrations above 10 mM were unable to sustain algal growth on agar plates containing 0.2 M NaCl. However, Kwon et al (1971) has reported that D. tertiolecta cells are able to survive on growth medium containing 1% glucose in light and CO_2 provided conditions. In the present study, it was observed that at higher glucose concentrations cells were viable till day 6 after which they got bleached. Barbara et al (1996) has reported that glucose uptake by D. parva remains same irrespective of the NaCl concentrations in the growth medium. The study also proves that there exists a saturation point for glucose uptake in D. parva after which no uptake was possible even if the growth medium has higher concentration of glucose.

The involvement of glucose in ATPase activation in the present study was further confirmed through H^+-ATPase assay and medium acidification experiments. The addition of glucose to starved yeast cells leads to a several-fold increase in H^+-ATPase activity, linked to internal alkalinisation and external acidification (Serrano, 1983; Brandao et al, 1992). The results of the present study are also in accordance with the previous works where H^+-ATPase activity and consequent medium acidification was observed with the addition of glucose. Acidification of external medium is due to sugar metabolism. As explained previously the saturation of
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Dunaliella cells with 10 mM glucose may be the reason for similar values which were obtained for 10 mM and 15 mM glucose concentrations during ATPase assay and medium acidification experiments.

Whereas activation of the H⁺-ATPase causes a hyperpolarization of the plasma membrane and extracellular acidification, its inhibition leads to rapid membrane depolarization and alkalinization of the cell exterior. Glucose is a reported activator of H⁺-ATPase while sodium ortho vanadate (SOV) and diethylstilbestrol (DES) inhibit the same. Vanadate was shown to inhibit the plasma membrane ATPase by forming a stable vanadate complex that locks the enzyme in one confirmation (Macara, 1980). DES is a non competitive inhibitor of plasma membrane ATPase which affects both ATP hydrolysis and proton pumping activity (Martin & Flatmark, 1988). Existence of vanadate and DES sensitive H⁺-ATPase in plasma membrane preparations from Dunaliella has been demonstrated (Gilmour, 1985). The complete inhibition of glucose activation in the medium acidification experiment was observed by the addition of 10 µM SOV and 15 µM DES which are specific inhibitors of plasma membrane ATPase. This was also evident from the increased resistance of Dunaliella cells in the presence of SOV in the selection medium. These results confirmed the role of ATPase in hygromycin transport in Dunaliella. The use of SOV and DES in specific inhibition of plasma membrane ATPase of Dunaliella was also reported by Weiss et al (1989).

The decrease in hygromycin concentration from 100 mgL⁻¹ to 25 mgL⁻¹ during selection of HRC through the addition of 10mM glucose in the selection medium ensured proper selection with efficient and economic use of hygromycin. The stability of the transformants obtained by this modified procedure was also ensured by comparing the transformation frequency and by checking trans-gene integration. The transformation frequency remained the same with the addition of glucose in the selection medium and transformants obtained were having stable integration as evident by GFP expression and hpt amplification. Therefore, the present modified transformation method is an improvement over the previously standardized procedure with respect to low level of hygromycin used in the selection medium. However, the effect of glucose on other antibiotics is yet to be studied.
D. bardawil, despite its frequent use as a commercially important microalga, is far less studied with respect to signal transduction of the major metabolic events than some other micro-organisms of biotechnological importance, such as yeast. The present study shows the possible involvement of H\(^{+}\)-ATPase in hygromycin transport in Dunaliella and also pH gradient created as the primary event in the cascade of events eventually leading to hygromycin transport mechanism. Thus the activation of ATPase is directly or indirectly linked to hygromycin transport in Dunaliella. However the role of membrane potential as an alternate to pH gradient in drug transport is not excluded due to low values of \(\Delta p\text{H}\). This requires further investigation. Although the mechanism of hygromycin transport is not clear, the present study may be a starting point in further attempts to unravel this phenomenon.
3.2 Identification of phenolic compounds in *Dunaliella* that promotes *Agrobacterium* mediated gene transfer

3.2.1 Introduction

Among the transformation methods used to introduce foreign genes into host plants, *Agrobacterium* mediated genetic transformation remains the most efficient method for the production of transgenic plants due to its wide host range, inexpensive procedure, ability to produce single or fewer copies of integrated transgene with little rearrangement, and enables the transfer of large segments of DNA up to 150 kb into the host genome (Tzfira and Citovsky, 2006). However, the efficiency of this method in transforming different species of plants is highly dependent on the plant genotype, *Agrobacterium* strain being used, plasmid vectors, extent of virulence (*vir*) gene induction, co-cultivation period, temperature and medium composition (Opabode, 2006). The capacity for gene transfer led to the development of *A. tumefaciens* as a powerful tool to introduce foreign genes into plant system by suitably engineering the Ti plasmid and also as an important model system, at the forefront in understanding how pathogens recognize hosts and deliver macromolecules into target cells, resulting in disease.

The commitment by the *Agrobacterium* to the virulence-inducing processes is carefully regulated and occurs only when a competent host is recognized and available. The first step in the T-DNA transfer process is chemical recognition of host and activation of virulence gene expression. Specifically, a portion of the Ti plasmid, the transferred DNA (T-DNA), delineated by 23 base pair (bp) border repeats, is transferred into the plant cells, integrated into the nuclear DNA, and expressed (Figure 3.8). A variety of signals from the host responsible for the activation of the T-DNA transfer process have been defined. These are phenols, aldose monosaccharides, low pH, and low PO₄ (Brencic and Winans, 2005; Palmer et al, 2004). The diversity of plant phenolic compounds has been proposed to afford a rich source of developmental signals or chemo-attractants in the establishment of specific symbiotic interactions between microbes and plants. A commonality, therefore, of both the phenol and sugar signaling is the diversity of plant compounds recognised. This likely plays a role in achieving the broad host range exhibited by *Agrobacterium*. 
Figure 3.8: General model of Agrobacterium-mediated transformation of a plant cell  
(Adapted from McCullen et al, 2006)

A diverse set of synthetic and naturally occurring phenols, derived from the phenylpropanoid pathway in plants, is active vir gene inducers (Palmer et al, 2004). Several important structural features of the active molecules have been identified.

1. Aromatic hydroxyl is essential.
2. Monomethoxy derivatives are more active than those lacking methoxy substitutions on the phenol ring, but dimethoxy derivatives invariably are the most active of these three classes.
3. The potential capacity of the group para to the aromatic hydroxyl to hydrogen bond (e.g., with a polar or acidic group of the receptor) generally is associated with higher activities, and chirality at this carbon center is critical for inducing activity (McCullen et al, 2006)

Acetosyringone is generally considered to be a wound-induced metabolite in plants, which more than 10 years ago was shown to trigger virulence genes in the wound pathogen Agrobacterium tumefaciens (Baker et al, 2002, 2005)(Figure 3.9 A). Acetosyringone can be viewed as vinylogous carboxylic acid where ionization of the phenol is stabilized by resonance delocalization through the carbonyl oxygen. Under assay conditions, the phenol of acetosyringone is protonated, but in the binding site,
an acidic residue protonates the carbonyl oxygen, greatly increasing acidity of the phenol. This activated phenol, probably bound in a relatively hydrophobic pocket, could protonate a basic site on the receptor surface and induces a conformational change (Figure 3.9 B) that would set up the proposed phosphorylation cascade involved in Vir G activation (Jin et al, 1990; Hess et al, 1991).

![Figure 3.9](image)

**Figure 3.9(A)** Structure of acetosyringone (B) Proposed mechanism for phenolic induction of vir expression. Protonation at a basic site (B) on the phenolic receptor, presumably VirA, induces indicated conformational change for Vir G activation (Adapted from Jin et al, 1990).

Acetosyringone, and likely other extracellular phenolics, may have bioactive characteristics that can influence plant–bacterial pathogenesis. The phenols, which are absolutely required for vir gene activation, may be especially important in this regard. The capacity of the vir inducing system to recognize diverse phenols allows the expression of the virulence machinery across a broad range of host plants and, conceivably, different cell types within the plant.

As evident from the results of Chapter II, *Agrobacterium* mediated genetic transformation in *Dunaliella* does not require the presence of acetosyringone. Co-cultivation of *Dunaliella* in the absence of acetosyringone consistently produced reporter gene expression in hygromycin resistant cells with transformation frequency almost similar to that with acetosyringone. Similar observation was also reported during *Agrobacterium* mediated gene transformation in other Chlorophycean
members, *C. reinhardtii* (Kumar et al, 2004) and *H. pluvialis* (Kathiresan et al, 2009). The ability of *Chlamydomonas* cells to induce *Agrobacterium vir* gene and bacterial attachment, which are essential for T-DNA transfer, was investigated by Kumar and Rajam (2007). The cell free medium, containing the possibly secreted molecules and the cell extracts, were used to study vir gene induction in *Agrobacterium* strain harbouring a VirE: LacZ fusion vector and analyzing them for β-galactosidase activity. The results of the study indicated that *Chlamydomonas* and the related algal species may be in the natural host range of *Agrobacterium*.

Microalgae like *Chlamydomonas* have been reported to secrete several small molecules, including phenolics into the culture medium. It is likely that some of these molecules are capable of inducing the *Agrobacterium vir* genes, explaining the transformation as well as vir gene induction. So an attempt was made to identify the phenolic compounds present in either cell free medium or cell extract of *D. bardawil* which may induce vir gene so as to facilitate gene transfer without acetosyringone.

### 3.2.2 Materials and methods

#### 3.2.2.1 Bacterial and algal culture maintenance

*D. bardawil* culture, maintenance of the culture and growth conditions were followed as in the section 2.3.1, 2.3.1 and 2.3.2 of chapter II. *A. tumefaciens* strain EHA 101, transformed with the binary vector pCAMBIA 1304 using the procedure described by Hofgen et al (1988), was used for the co-cultivation experiments. The transformed *Agrobacterium* was maintained in LB medium containing 100 mgL\(^{-1}\) kanamycin and 50 mgL\(^{-1}\) rifampicin. Glass wares and plastic wares used in the experiments were same as described in section 2.2.2, 2.2.3 and 2.2.4 of chapter II.

#### 3.2.2.2 Co-cultivation of *D. bardawil* and *Agrobacterium* and phenolic extraction

*D. bardawil* culture was freshly inoculated to liquid medium from stock culture on slants. The culture was grown for a period of 10 days and the log phase culture was harvested and resuspended in liquid TAP medium so that the final cells density is 1.5 x 10\(^6\) cells ml\(^{-1}\). Single colony of *A. tumefaciens* culture harbouring pCAMBIA 1304 was also freshly inoculated in LB medium and was grown overnight at 200 rpm at 28\(^0\)C. The culture was reinoculated on the next day to 50 ml and incubated till the culture reached optimum OD (A\(_{600}\) 0.5). The cells were harvested and resuspended in
2 ml LTAP medium. *D. bardawil* culture (100 ml) was co-cultivated with freshly grown culture of *A. tumefaciens* (200 µl) in LTAP medium for 48 hours. A control was also maintained without the addition of *Agrobacterium*. After 48 hours the culture was centrifuged at an initial speed of 1000 rpm. The algal cells were separated from the supernatant (referred as algal cell pellet-ACP). The remaining supernatant was again centrifuged at a speed of 5000 rpm and the bacterial cells were separated (referred as bacterial cell pellet- BCP) and supernatant as cell free medium (CFM).

Total phenolics were extracted from ACP, BCP and CFM. The CFM extract was divided into two parts. pH of one part was adjusted to 4 with 0.1 N HCl. The pH of the other part was checked using a pH meter and was found to be alkaline (9.4). *Dunaliella* culture usually maintains an alkaline pH in the medium during its growth. Total phenolics from ACP and BCP were extracted using ethyl acetate by disrupting the cells in mortar and pestle and by sonication respectively. After disruption the content was centrifuged and solvent layer was separated and dried under nitrogen. Phenolics from acidic and basic CFM were also extracted with ethyl acetate. Ethyl acetate (20 ml) was added to cell free medium (100 ml), mixed thoroughly and the solvent layer with phenolics was collected using a separating funnel. The extraction step was repeated. The solvent phases from two steps were pooled and was dried using a rotovapour. The phenolics were then redissolved in known volume of methanol.

### 3.2.2.3 Estimation of total phenolics

Total phenolics from all samples were initially determined using Folin–Ciocalteu reagent (Osawa and Namiki, 1981) using gallic acid as the standard. The absorbance was measured at 750 nm using the spectrophotometer Shimadzu 160A. The total phenolics content was estimated from standard graph of gallic acid. The experiment was carried out in triplicates.

### 3.2.2.4 HPLC analysis

The separation and chromatographic analysis of phenolic compounds were preformed in Shimadzu HPLC equipped with a UV /Vis detector, using a reverse phase C-18 column (Supelco, 25 cm x 4.6 mm) at a flow rate of 1 ml min⁻¹. The mobile phase consisted of: solvent A, 0.5% acetic acid; solvent B methanol, and the gradient
program applied was: 0 min, 90% A; 26.15 min, 60% A; 30 min, 90% A. Detection was carried out at 290 nm, and their identification and quantification was achieved by injecting known amounts of standards.

### 3.2.2.5 HPTLC analysis
The phenolic extracts along with standards of acetosyringone, vanillin, vanillic acid protocatechuic acid (Sigma- Aldrich, USA) were analysed by HPTLC. A Camag HPTLC system equipped with an automatic TLC applicator (Linomat IV applicator) and TLC scanner (CAMAG Scanner III CATS (4.06), Switzerland) was used for experiment. Detection was carried out at 290 nm. Stationary phase used was Precoated Silica gel F Plates (MERCK) and mobile phase was a mixture of Acetic acid: Dioxan: Benzene (4:25:90).

### 3.2.2.6 Mass spectral analysis of the phenolics extracts
The phenolic extracts were also analysed for mass spectra in a Q-tof Ultima (UK) mass spectrometer in ESI negative mode. Direct injection of samples and standards were carried out to obtain the mass spectrum.

### 3.2.2.7 Transformation efficiency analysis
Approximately 1.5 x 10^6 cells of *D. bardawil* were plated on TAP medium with 0.2 M NaCl. The major phenolic compound identified through HPLC and MS were checked for their transforming efficiency by incorporating them at 100 µM concentration in the co-cultivation medium. Cells were co-cultivated with *A. tumefaciens* for 48 hours. A control was maintained with STAP medium alone. Following co-cultivation period, cells were washed with medium containing 500 mgL\(^{-1}\) cefotaxime and 300 mgL\(^{-1}\) potassium clavulanate and plated on STAP medium containing 100 mgL\(^{-1}\) hygromycin. Colonies grown for 8 weeks were counted to calculate transformation efficiency. Each treatment was maintained in triplicates.

### 3.2.3 Results
#### 3.2.3.1 Estimation of phenolics
The extracted phenolics were quantified using standard procedure using Folin-Ciocalteau reagent. The phenolic content was almost absent in BCP and ACP contained 0.03 µg/50 mg wet biomass. Acidified CFM showed low phenoile content
(0.5 mg/100 ml cell free medium) while untreated (basic) cell free medium contained high content of 1.2 mg in 100 ml of cell free medium.

3.2.3.2 Qualitative analysis of phenolic extracts using HPTLC

HPTLC analysis of phenolic extract and standards showed the presence of acetosyringone, vanillin, vanillic acid and protocatechuic acid. The acidic CFM extract showed low concentration of the compounds along with absence of acetosyringone (Figure 3.10 & 3.11).

![Figure 3.10: HPTLC profile of phenolic extracts from cell free medium of control and co-cultivated cells of D. bardawil. Lane 1: Vanillin; Lane 2: Vanillic acid; Lane 3: Protocatechuic acid; Lane 4: Acetosyringone; Lane 5: Control; Lane 6: CFM acidic; Lane 7: CFM basic](image)

3.2.3.3 HPLC analysis of phenolic extract

HPLC analysis of phenolic extracts from ACP and BCP showed that both were having phenolic content in trace amounts which was difficult to be detected in HPLC. Phenolics identified in CFM (basic) are PCA, V, VA and AS out of which AS was observed as the major one (Figure 3.12). When compared to CFM (basic), the phenolic content of CFM (acidic) was much lower and also acetosyringone and protocatechuic acid were not detected in the later. The maximum AS content of 2.567±0.5 µg/100 mL cell free medium was observed in CFM (basic) (Table 3.3). Control and co-cultivated cell free medium showed similar phenolic profile without much variation in the concentration of compounds. PCA and AS was not extracted into solvent phase from cell free medium at acidic pH.
Figure 3.11: Chromatogram of HPTLC analysis of phenolic extract

Figure 3.12: (A) HPLC profile of phenolic extracts from CFM (basic) of 48 hours co-cultivated cells *D. bardawil*. (PCA- Protocatechuic acid; V- Vanillin; VA - Vanillic acid and AS- Acetosyringone) (B) HPLC profile showing retention time of phenolics standards
Table 3.3: Concentration of phenolic compounds in CFM

<table>
<thead>
<tr>
<th>CFM type</th>
<th>Phenolic compound (µg/100 ml)*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PCA</td>
</tr>
<tr>
<td>Basic control</td>
<td>0.0883±0.003</td>
</tr>
<tr>
<td>Acidic control</td>
<td>0</td>
</tr>
<tr>
<td>Basic co-cultivated</td>
<td>0.1004±0.005</td>
</tr>
<tr>
<td>Acidic co-cultivated</td>
<td>0</td>
</tr>
</tbody>
</table>

*Values are mean ± standard deviation; (PCA- Protocatechuic acid; V- Vanillin; VA - Vanillic acid and AS- Acetosyringone)

3.2.3.4 Mass Spectral analysis of phenolic extract

A mass spectrum of the phenolic extract was obtained in ESI negative mode. Mass of each compound was calculated as [M-H]. The spectrum of phenolic extract from CFM (basic) with mass of four phenolic compounds is represented in figure 3.13.

![D 48 TOF MS ES-](image)

Figure 3.13: LCMS (ESI negative) of phenolics extracted from cell free medium of co-cultivated *D. bardawil* (CFM-basic). Arrow indicating mass of compounds [M-H]. (PCA- Protocatechuic acid; V- Vanillin; VA - Vanillic acid and AS- Acetosyringone)

3.2.3.5 Transformation efficiency analysis

The four major phenolic compounds identified were checked for their transforming efficiency by incorporating them at 100 µM concentration in the co-cultivation
medium and incubating the algae and *Agrobacterium* for 48 hours. A maximum cfu count of 95±8 per 10^6 cells was obtained by addition of vanillin in the co-cultivation medium. Addition of acetylsyringone gave 47±5 cfu per 10^6 cells while protocatechuic and vanillic acid values were 32±4 cfu per 10^6 cells and 35±4 cfu per 10^6 cells respectively. The cfu of control without the addition of any phenolic compound is 43±5 cfu per 10^6 cells (Table 3.4).

**Table 3.4:** Transformation frequency in *D. bardawil* after co-cultivating with 100 µM of identified phenolic compounds.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of colonies (cfu)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>43±5</td>
</tr>
<tr>
<td>Vanillic acid</td>
<td>35±4</td>
</tr>
<tr>
<td>Vanillin</td>
<td>95±8</td>
</tr>
<tr>
<td>Protocatechuic Acid</td>
<td>32±4</td>
</tr>
<tr>
<td>Acetylsyringone</td>
<td>47±5</td>
</tr>
</tbody>
</table>

*Values are mean ± standard deviation. Cfus is per 10^6 cells plated

### 3.2.4 Discussion

Host recognition by *Agrobacterium* is the first and important step resulting in T-DNA transfer. A diverse set of synthetic and naturally occurring phenols, derived from the phenylpropanoid pathway in plants, are considered as active signals in initiating this first step. The type and concentration of phenolics in the surroundings govern the interactions of *Agrobacterium* with their host plants. In the case of *Agrobacterium*, a variety of phenolic compounds are known to induce *vir* gene expression. Acetylsyringone and hydroxyacetylsyringone exuded from plant wounds are potent chemo-attractants even at very low concentrations (Escobar and Dandekar, 2003). The limitation of host range remains a significant problem in the use of this organism as a vector for genetic engineering in plants. Although ubiquitous in higher plants, phenols with varying substitution patterns can be specific for particular genera or species (Dixon et al., 2002).

Phenols are an important group of natural products with antioxidant and other biological activities. Yet there is insufficient information either on individual phenols and hydroxybenzaldehydes in algae or about their physiological function in the human organism. The enzyme L-phenylalanine ammonia-lyase was found for the first
time in cyanobacteria (*Anabaena variabilis* and *Nostoc punctiforme*) and characterized using X-ray crystallography (Moffitt et al., 2007). According to previously published results (Onofrejova et al., 2010), a group of 9 phenolic acids (protocatechuic, *p*-hydroxybenzoic, 2,3-dihydroxybenzoic, vanillic, syringic, caffeic, *p*-coumaric, salicylic, and chlorogenic acids) were found in freshwater cyanobacteria and algae. Benzaldehyde and selected volatile organic compounds were previously analyzed in pentane extracts prepared from cyanobacterium *Oscillatoria perornata* (Tellez et al., 2001). The phenols especially cinnamic and *p*-coumaric acids are crucial precursors in the synthesis of different bioactive phenols (usually polyphenols) in photosynthetic organisms.

Not only phenolic acids but also derivatives of benzaldehydes like *p*-hydroxybenzaldehyde, 3,4-dihydroxybenzaldehyde, vanillin were identified in phenolic extracts of fresh water algae *Anabaena doliolum* and *Spongiochloris spongiosa* and from two macroalgae *Porphyra tenera* and *Undaria pinnatifida* (Onofrejova et al., 2010). In addition, *p*-hydroxybenzaldehyde was found in the red alga *Corallina pilulifera* (Yuan et al., 2006) and bromoaldehydes like 3-bromo-4, 5-dihydroxybenzaldehyde and 3, 5-dibromo-4-hydroxybenzaldehyde have been analyzed in the red alga *Polysiphonia urceolata* (Li et al., 2008). It was proposed that aldehydes (or their halogen derivatives) are natural antioxidant constituents of Cyanobacteria and algae (Li et al., 2008).

On the basis of previous reports and the result of present study, it is possible that similar phenylalanine dependent biosynthesis (Boudet, 2007) of simple phenols occurs in cyanobacteria and algae. Phenols are *in vivo* synthesized in lower concentration levels in cyanobacteria than in the case of freshwater algae (Onofrejova et al., 2010). The higher amount of phenols in algae when compared to cyanobacteria is probably due to the fact that algae (eukaryotic organisms) are more evolutionary advanced organisms than cyanobacteria (prokaryotic organisms). It was also suggested by Onofrejova et al (2010) that algae probably have more developed phenol-based metabolic pathways than cyanobacteria. The antioxidant activities of algae extracts containing phenolic compounds were recently studied by several authors (Chang et al., 1993; Onofrejova et al., 2010). These compounds may play an important role in algal cell defence against abiotic and biotic stress.
The exact pathway for acetosyringone biosynthesis in plants is not known, although the ring substitution pattern suggests that acetosyringone is derived from chain shortening of a dimethylated hydroxycinnamic acid derivative such as sinapic acid. Acetosyringone may in fact be released from the lignin fraction, in which it has been proposed to exist as an 8-O-4-linked unit with a ketone group at C7 (Chesson et al, 1997), by alkaline hydrolysis. The exact origin of the ketone group in acetosyringone or its putative precursor is; however, not known. It may be also proposed that through chain shortening, sinapic acid can be converted into syringic acid. From syringic acid via the formation of syringaldehyde, acetosyringone is synthesized. The presence of other phenolic compounds like vanillin, vanillic acid and proto catechuic acid identified in the extracts is also in favour of the possible presence of phenylpropanoid pathway in Dunaliella and other green micro algae.

The use of acetosyringone in Agrobacterium mediated gene transfer into plant hosts has been favored for the past few decades. The influence of other phenolic compounds and their effectiveness in Agrobacterium mediated plant transformation systems has been less investigated. Cha et al (2011) reported the efficacy of three phenolic compounds (cinnamic acid, vanillin and coumarin) on Agrobacterium-mediated transformation of the unicellular green alga Nannochloropsis spp. and the result was compared to that of acetosyringone. They have also reported that the presence of methoxy group in the phenolic compounds may not be necessary for Agrobacterium vir gene induction and receptor binding as suggested by previous studies.

Some of the phenolic compounds identified in the present study are previously reported as vir gene inducers. Ferulic, vanillic and protocatechuic acids, vanillin and hydroxybenzoic acid induce the transcriptional locus of the vir loci in Agrobacterium (Melchers et al, 1989). A specific structure of phenolic molecules is essential for vir gene induction. The aromatic hydroxyl group, together with several other structural features, is absolutely essential. For acetophenones, a syringyl nucleus is more effective at vir induction (Spencer and Towers, 1988). The methyl esters of ferulic, syringic, and sinapic acids exhibited significantly greater activity than the corresponding free acids. This may be the reason for high activity of acetosyringone with two methyl groups. It may be also proposed that synergistic or cumulative effect
of different phenolic compounds, results in high transformation efficiency (Sharma and Kuhad, 2010).

Similar to Dunaliella, Agrobacterium infection of the unicellular algae C. reinhardtii (Kumar et al, 2004) and H. pluvialis (Kathiresan et al, 2009) was feasible without the inclusion of acetylansyngone. The transformation frequencies remained same without the addition of acetylsyringone in a phenolic free medium suggesting that significant amounts of endogenous vir gene inducers were released by this species during the process of infection. A similar study which was carried out in C. reinhardtii by Kumar and Rajam (2007) also reported the presence of active vir gene inducers in the cell free medium of algal cells. However, the particular compound which acted as the vir inducer was not identified in their study. The present study was mainly focused on the identification of phenolic compounds in the cell free medium of Dunaliella that can act as an inducing agent during Agrobacterium mediated transformation.

From the results of the present investigation, four major phenolic compounds viz: acetylansyngone, vanillin, vanillic acid and protocatechuic acid were identified in the cell free medium of Dunaliella cells. It was also observed that co-cultivation with Agrobacterium did not change the phenolic profile of the algae. In order to identify the particular phenolic compound which acts as the main inducer, the respective compounds in pure form was incorporated into the selection medium at 100 µM concentration. Vanillin was showing higher transformation frequency with that of control and acetylansyngone (Table 3.4). 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. Also addition of NaCl is reported to have strong inhibitory effect on vir induction (Melchers et al, 1989).

Evaluation of other potential phenolic compounds that may be more potent than acetylansyngone is needed for their application in future algal genetic transformation studies. The presence of natural vir gene inducers in Dunaliella with improved transformation efficiency, selection with lowered levels of hygromycin and stable integration with Agrobacterium mediated transformation procedure further strengthen the use of this unicellular algae for cell factory applications.