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DCMU-resistance mutation confers resistance to high salt stress in the red microalga *Porphyridium* sp. (Rhodophyta)

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The response of photosynthesis to salinity stress in wild-type and DCMU-resistant (strain DC-2) *Porphyridium* sp. was investigated. Both wild-type and DC-2 were able to acclimate and establish a new steady state of growth when exposed to salt stress, but growth rate of the DC-2 mutant strain was 1.25-fold higher than growth rate of wild-type cells. Following establishment of a new steady state under the salt stress conditions, the maximal irradiance-saturated photosynthetic capacity (P_{max}), light saturation (I_k) and the photosynthetic efficiency (α) were 1.5-, 1.8- and 1.1-fold, respectively, higher in the DC-2 mutant cells than wild-type. Furthermore, salt-stressed DC-2 mutant cells exhibited 1.6-fold higher maximal efficiency of PS II (Fv/Fm) after 200 min exposure and 3-fold higher level of D1 protein after 24 h exposure to salt than wild-type cells. Exposing the salt-stressed cells to high photon flux density (3000 $\mu mol\, m^{-2}\, s^{-1}$) for 1 h resulted in a higher resistance of mutant cells than wild-type to light stress, as reflected in 1.3-fold decline in the Fv/Fm. This finding corresponds well with the levels of the D1 protein measured in the cells. These observations indicate that the D1 protein of the PS II apparatus in the DC-2 mutant was more resistant to stress. However, under non-stressed (light and salt) conditions, no differences were observed between wild-type and the DC-2 mutant cells for all the parameters studied. The DC-2 mutant responded better than wild-type to environmental stresses, namely salinity and light due to the modification induced in PS II.

Key words: D1 protein, DCMU-resistant mutant, maximal photochemical efficiency (Fv/Fm), photoinhibition, photosynthesis, photosystem II, *Porphyridium* sp., red microalga, salt stress, variable chlorophyll fluorescence

Introduction

The response of photosynthesis to environmental changes is well documented for a range of species (reviewed in Baker, 1996; Powles, 1984). It has been reported that one of the primary sites of damage due to environmental stresses on photosynthesis is located in the reaction center of Photosystem II (PS II) (Powles, 1984; Baker, 1991; Aro *et al.*, 1993; Melis, 1999; Andersson & Aro, 2001; Netondo *et al.*, 2004).

Several studies in higher plants have shown that salt stress inhibits PS II activity (Mishra *et al.*, 1991; Masojidek & Hall, 1992; Belkhodja *et al.*, 1994; Everard *et al.*, 1994; Singh & Dubey, 1995; Tiwari *et al.*, 1997; Kao *et al.*, 2003; Parida *et al.*, 2003), whereas others have indicated that salt stress does not affect PS II (Robinson *et al.*, 1983; Brugnoli & Bjorkman, 1992; Morales *et al.*, 1992) and even increases PS II activity (Smillie & Nott, 1982). Salt stress very often occurs in combination with light stress under natural environment. It exerts two stress effects on living cells, namely an increase in the osmotic pressure and the concentration of inorganic ions (Karandashova & Elanskaya, 2005) and apparently enhances the photoinhibition of PS II induced by exposure to high light intensity (Neale & Melis, 1989; Sharma & Hall, 1992; Allakhverdiev *et al.*, 2002). The effect of salt stress has been examined in various salt-sensitive and salt-tolerant plants, but the mechanism by which salt stress enhances the photodamage of PS II still remains unclear.

DCMU is a herbicide that binds to the Q_B site in the D1 protein of PS II and hence inhibits PS II electron transport and oxygen evolution. Thus, a DCMU-resistant mutant might serve as a useful tool to study the impact of PS II modification on the response of photosynthetic organisms to an environmental stress.

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It has been reported that resistance to DCMU is often acquired by an alteration of the herbicide-binding site (D1 protein) on the thylakoid membranes, which results in a reduced affinity of the herbicide with the binding site (Galloway & Mets, 1984; Erickson *et al*., 1989; Galloway & Mets, 1989). DCMU-resistant mutants of higher plants and green algae were shown to involve single amino-acid substitutions in the D1 protein (Hirschberg & McIntosh, 1983; Hirschberg *et al*., 1987; Erickson *et al*., 1989; Mengistu *et al*., 2000, 2005). Such mutants usually present additional pleiotropic effects, such as an increased sensitivity to strong illumination (Sundby *et al*., 1993; Alfonso *et al*., 1996), tolerance to irradiance (Singh & Singh, 1997), tolerance to heat stress (Alfonso *et al*., 2001) and tolerance to salt stress (Singh & Kshatriya, 2002).

In this study, we have used a stable DCMU-resistant mutant of *Porphyridium* sp. (DC-2) isolated by Sivan & Arad (1995). We compared the response of wild-type and mutant cells to salinity and light stress in order to evaluate whether or not the mutant cells, which had acquired resistance to DCMU, adapted differently to multiple stress.

Materials and methods

Algal strains and growth conditions

Porphyridium sp. (UTEX 637) cells, obtained from University of Texas Algal Culture Collection and DC-2 mutant (DCMU-resistant mutant) cells previously isolated by Sivan & Arad (1995), were grown in artificial sea water (ASW) according to Jones *et al*. (1963). In 250-ml Erlenmeyer flasks 100 ml of culture was incubated in a rotary shaker at 25°C under continuous white light intensity of 150 $\mu mol\, m^{-2}\, s^{-1}$ in 1% CO_2. Illumination was supplied continuously from above by cool-white fluorescent lamps. The initial cell concentration in culture was adjusted to 2×10^6 cells ml^{-1} with the aid of a haemacytometer.

Salinity stress

Exponentially-grown cells were harvested and resuspended in a 100 ml fresh medium containing additional 1.0 M NaCl in 250 ml flasks. This concentration of NaCl was chosen after preliminary studies, which showed that 1.0 M additional NaCl was the maximum concentration of salt within which wild-type cells can grow. Cell densities of the cultures were 4×10^6 cells ml^{-1} and they were incubated at 150 $\mu mol\, m^{-2}\, s^{-1}$ photon flux density (PFD), at 25°C.

Photosynthetic activity

Oxygen evolution. Cultures were harvested by centrifugation and resuspended to a final concentration of 5.0 μg chl ml^{-1} in fresh ASW supplemented with 0.02 M sodium bicarbonate. The photosynthetic activity was assayed by measuring the rate of oxygen evolution using a Clark-type O_2 electrode in a thermo-regulated double-jacket cylindrical glass vessel (internal diameter 1.5 cm). Illumination was provided by a slide projector lamp, at an intensity of 150 $\mu mol\, m^{-2}\, s^{-1}$ and the temperature was maintained constant at 25°C. For the photosynthesis–irradiance (P–I) curves, light intensities were changed by inserting different neutral density filter between the cells and light source. The P–I curve graph was created from the rate of photosynthesis measured at various light intensities and subsequently values for P–I parameters were calculated.

Photoinhibition experiments. Cells were harvested by centrifugation and resuspended in fresh ASW supplemented with 0.02 M sodium bicarbonate. Cells were diluted to a final concentration of 25 μg chl ml^{-1} and 10 ml of resuspended culture was placed in a thermo-regulated double-jacket cylindrical glass vessel (internal diameter 1.5 cm) at constant temperature of 25°C and then illuminated at high PFD of 3000 $\mu mol\, m^{-2}\, s^{-1}$ by a high intensity halogen lamp (1000 W). Plastic glass chamber with 0.01% copper sulfate solution was placed between the vessel and lamp, as a heat filter. Samples were drawn at time intervals of 10, 20, 30, 45 and 60 min and examined for their photosynthetic activity, namely oxygen evolution rate and maximal PS II photochemical efficiency (Fv/Fm). The extent of photoinhibition was determined by calculating the rate of photosynthetic activities expressed as percent of a control measured immediately before photoinhibitory treatment.

Chlorophyll fluorescence measurement. Chlorophyll fluorescence was measured by a portable fluorometer (Plant Efficiency Analyzer, Hansatech, UK). Cells were diluted to a final concentration of 20.0 μg chl ml^{-1} with fresh ASW supplemented with 0.02 M sodium bicarbonate. Cells were kept in transparent tubes and incubated in darkness for 5 min to allow for full dark adaptation. Excitation of chlorophyll was done by exposing the culture to red light. Parameters of Fo and Fm were measured. Fv/Fm was calculated as (Fm–Fo)/Fm (Torzillo & Vonshak, 1994).

Electrophoresis

Sample preparation. Control and salt-stressed cells of wild-type and DC-2 mutant strains were grown at various light intensities of 70, 150 and 300 $\mu mol\, m^{-2}\, s^{-1}$. Cells were harvested by centrifugation and washed twice with 50 mM pH 7.0 sodium phosphate buffer and resuspended in the same buffer. Cells were mechanically disrupted by three cycles in the French press at 21 000 psi at 4°C. The cell lysate was centrifuged at 3000 rpm ($1,500 \times g$ for 10 min), and the supernatant was stored at 4°C for further use.

Protein assay. Cells were disrupted in a French press as described above. Total protein and chlorophyll were assayed. Total protein was estimated using a Lowry procedure (Lowry *et al*., 1951) with Bovine serum albumin as the reference standard (0–80 μg/ml).

SDS–PAGE. The total protein from *Porphyridium* sp. were denatured in Laemmli sample buffer by heating at 70°C for 5 min. Samples were loaded in equal amounts of protein in each well of SDS–PAGE (Laemmli, 1970). The stacking gel contained 5% polyacrylamide and the separating gel contained 12% polyacrylamide.

Western-blot analysis. Proteins were resolved by SDS–PAGE as described above and transferred onto a nitrocellulose membrane (Bio-Rad tank transfer system). The membrane was blocked and probed with anti-D1 antiserum, and followed by anti-rabbit secondary antibody conjugated with Horse radish peroxidase. The antigen–antibody complexes were detected by colour assay.

Results

Effect of elevated NaCl concentration

Growth. Cells of wild-type and the DCMU-resistant mutant (DC-2) of *Porphyridium* sp. grown in the artificial sea water (ASW) medium containing 27 g l^{-1} (0.466 M) NaCl. Cell cultures of 4×10^6 cells ml^{-1} were incubated in rotary shaker at 25°C under continuous white light intensity of 150 µmol m^{-2} s^{-1} in 1% CO_2. The cells were then exposed to an additional 1.0 M NaCl. *Porphyridium* sp. cells exposed to the high salinity stress were able to acclimate and establish a new steady state of growth as depicted in Fig. 1. While no difference in the growth rate between wild-type and DC-2 mutant cells was observed under control conditions, the growth of both strains was significantly reduced under the additional 1.0 M NaCl (Fig. 1). However, the growth of DC-2 mutants was less inhibited than that of wild-type cells. The specific growth rate of stressed wild-type culture was 0.16 day^{-1} as compared to 0.20 day^{-1} of the DC-2 mutant culture, indicating that the mutant cells tolerate high salinity better than wild-type cells.

P–I curve. Comparison of the light response curves (P–I) of wild-type and the DC-2 mutant cells may provide additional information on the nature of the induced mutation and indicate if the improved ability of the mutant cells to cope with salt stress is correlated with the modification in the photosynthetic apparatus.

We followed the P–I curve of wild-type and DC-2 mutant *Porphyridium* sp. cells grown at steady state under optimal or at the high salinity conditions (Fig. 2). The different parameters were calculated and are summarized in Table 1. No significant difference between wild-type and mutant cells was observed when grown under control conditions. However, significant changes in the light-saturating (Ik), maximal irradiance-saturated photosynthetic capacity (Pmax) and photosynthetic efficiency (α) were observed between wild-type and mutant cells. The decline in all the parameters was higher in wild-type than DC-2 mutant cells. In salt-stressed cultures, the maximal irradiance-saturated photosynthetic capacity (Pmax) of wild-type cells was lowered by 42% as compared with 10% in the DC-2 mutant. Ik and α values were also lowered in the salt-stressed wild-type cultures by 66% and 24%, respectively, whereas in the DC-2 mutant the decrease was only 42% and 8%. This observation showed that the DC-2 mutant tolerates high salinity stress and maintains a higher level of activity than wild-type cells in all measured photosynthetic parameters after acclimation to salt stress.

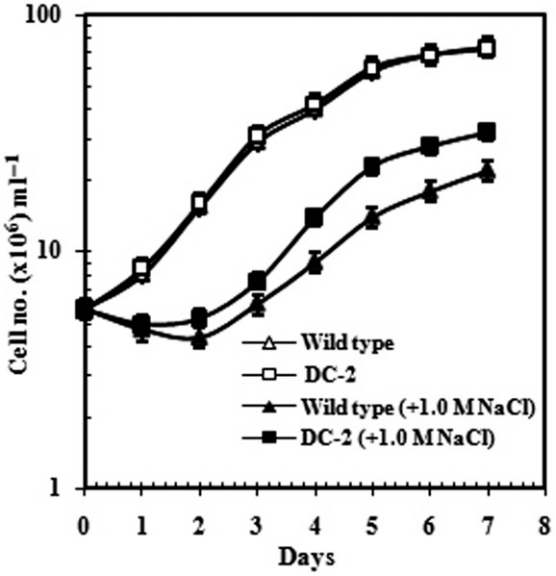

Fig. 1. Growth of *Porphyridium*. sp. wild-type and DC-2 mutant cells in standard growth medium or in media containing an additional 1.0 M NaCl. Cells were grown at 25°C under light intensity of 150 µmol µmol m^{-2} s^{-1} in regular artificial sea water (ASW) (0.46 M NaCl) and ASW containing 1.0 M additional NaCl (1.46 M NaCl). Growth was estimated by increase in number of cells. Values are means ± SE ($n = 4$).

Maximal photochemical efficiency of photosystem II (Fv/Fm)

To further characterize the nature of the induced mutation and its correlation with the ability of cells to acclimate to salt stress, the changes in the maximal photochemical efficiency of PS II (Fv/Fm) were followed during the initial stages of exposure to high salinity. Upon exposure to additional 1.0 M NaCl, an immediate decrease in Fv/Fm in both cultures was observed (Fig. 3). After 15 min of exposure, the reduction was 25% in wild-type cells and 12% in DC-2 mutant cells. After 200 min of exposure, the decline in wild-type cells was 46% while the decline in DC-2 cells was only 28%, demonstrating that the mutant cells acquired a capacity to withstand a higher resistance to salt stress.

Changes in the level of the D1 protein

In many photosynthetic organisms the degree of damage induced in PSII by environmental stress is associated with a decreased level of the D1 protein (Baker, 1991). Thus, monitoring changes in the level of the D1 protein as a result of exposure to salt stress may provide another means for understanding the connection between the ability of the strains to tolerate salinity stress and the modification induced in PSII.

Wild-type and the mutant cells of *Porphyridium*. sp. were exposed to salt stress and the level of D1 protein was monitored at different time intervals of exposure. Exposing the cells to an additional 1.0 M NaCl resulted in a decreased level of the D1 protein with increase in the exposure time (Fig. 4, Lanes 1–4). Analysis of band intensity of the immunoblot of the D1 protein in wild-type cells indicates a reduction of 17% after 6 h of exposure followed by a decline of 43% and 75% after 12 and 24 h of exposure, respectively. On the other hand, the level of D1 protein in salt-stressed DC-2 mutant cells dropped only 1% after 6 h exposure followed by a decline of 6% and 25% after 12 and 24 h, respectively (Fig. 4, Lanes 5–8). The lower level of D1 protein induced by the high salt stress in wild-type cells further supports the hypothesis that the induced mutation results in a modification in PSII enabling the mutant cells to tolerate the high salinity stress more effectively.

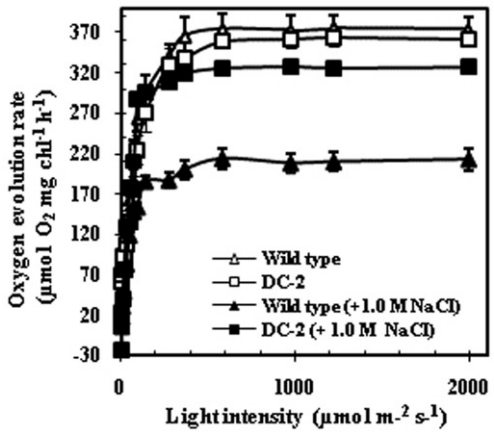

Fig. 2. Photosynthesis–irradiance (P–I) curves of wild-type and DC-2 mutant *Porphyridium*. sp. cells grown at a regular growth medium (0.46 M NaCl) or in media containing an additional 1.0 M NaCl. Cells grown at log phase were harvested and resuspended to a final chlorophyll concentration of 5 µg ml^{-1} in fresh corresponding medium. Values are means ± SE ($n = 3$).

Response to high light intensity and salt stress

Photoinhibition is considered to be the result of light absorbed by the photosynthetic apparatus in excess of its ability to utilize it in the

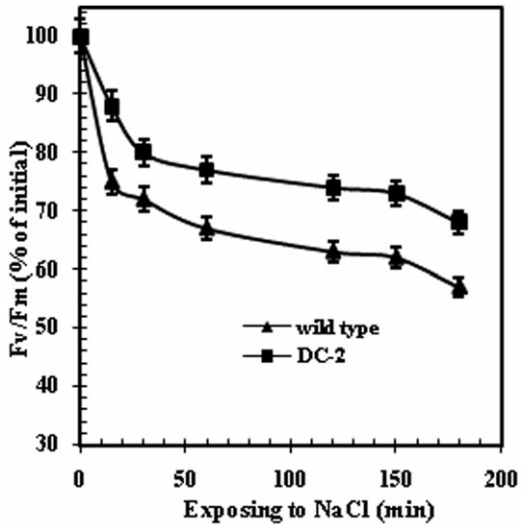

Fig. 3. Changes in the maximal photochemical efficiency of photosystem II (Fv/Fm) of *Porphyridium* sp. cells of wild-type and DC-2 mutant exposed to an additional 1.0 M NaCl. Cells grown at optimal conditions were harvested and resuspended in fresh medium containing 1.0 M additional NaCl (1.46 M NaCl). Samples were withdrawn at various time intervals and dark-adapted for 5 min. Fv/Fm was recorded as described in Materials and methods. Maximum activity (100%) was 0.755 and 0.770 in the wild-type and DC-2 mutant, respectively. Values are means ± SE ($n = 3$).

Table 1. Photosynthesis–irradiance (P–I) parameters of DC-2 mutant and wild-type *Porphyridium* sp. cells grown under control conditions or with 1.0 M additional NaCl. Logarithmic phase grown cells were harvested and resuspended in fresh medium to a constant chlorophyll concentration of 5 µg ml^{-1}. Values are mean ± SE ($n = 3$).

	Wild-type		DC-2 mutant	
Photosynthetic parameters	Control	+1.0 M NaCl	Control	+1.0 NaCl
Maximal irradiance-saturated Photosynthetic capacity (P_{max})	375 ± 10	210 ± 8	361 ± 12	325 ± 10
Light saturation (I_k)	150 ± 6	50 ± 7	160 ± 5	90 ± 4
Photosynthetic efficiency (α)	2.5 ± 0.08	1.9 ± 0.05	2.28 ± 0.06	2.11 ± 0.08

Notes: Logarithmic phase grown cells were harvested and resuspended in fresh medium to a constant chlorophyll concentration of 5 µg ml^{-1}. Values are mean ± SE ($n = 3$). Units used are P_{max}: µmol O_2mg chl^{-1} h^{-1}, I_k: µmol photon m^{-2} s^{-1} and α: µmol O_2mg chl^{-1} h^{-1} / µmol photon m^{-2} s^{-1}.

photochemical process (Powles, 1984; Aro *et al*., 1993; Long *et al*., 1994; Keren & Ohad, 1998). Other environmental stress such as salinity or temperature, modify the ability of the cells to utilize the absorbed solar energy and thus may also induce a photoinhibitory stress. The primary site of damage or down-regulation is considered to be in PSII (Neale & Melis, 1989; Komenda & Masojidek, 1995). It was interesting to evaluate the synergistic effect of high light and salinity stresses in the DC-2 mutant. Thus, we monitored the changes in Fv/Fm as an indication of the changes induced in the maximal photochemical efficiency of PS II induced by exposing the salt-stressed culture to high photon flux densities (HPFD) of 3000 μmol m^{-2} s^{-1}. As depicted in Fig. 5, exposing the cells to HPFD resulted in a decrease in Fv/Fm in both wild-type and DC-2 mutant cells. It is worth noting that the decline in wild-type cells was faster than that of the mutant cells. Similarly, exposure of salt-stressed wild-type and mutant cultures to HPFD resulted in a higher level of decline as compared with non-stressed cultures, and yet the decline was significantly higher in wild-type than DC-2 mutant cells (Fig. 6). After 1 h exposure the decrease was 72% in wild-type and only 56% in DC-2 mutants. These observations revealed that salt stress DC-2 mutant also tolerate high light intensity.

Discussion

The objective of the current study was to further characterize a DCMU-resistant mutant of the unicellular marine red alga *Porphyridium* sp. (DC-2) previously characterized by Sivan & Arad (1995). It was hypothesized that the mutation providing resistance to DCMU is a result of a modification in the PSII apparatus of the alga. Since PSII is considered to be a major site for inactivation or modification induced as a result of exposure to environmental stress (Gilmour *et al*., 1984; Baker, 1991; Barber, 1995; Endo *et al*., 1995), we have suggested that the response of the mutant to salinity and light stress would also be modified. Furthermore, we examined whether the induced DCMU-resistance mutation enables the algal cells to cope better with the indicated stresses. It was suggested that the effect of the salt stress on the PSII machinery is mainly through reduced repair of the photodamaged PSII via suppression of the activities of the transcription and translation machinery (Allakhverdiev *et al*., 2002; Nishiyama *et al*., 2005). Thus the ability of photoautotrophic cells to acclimate to an environmental stress will be reflected in the ability to down regulate its PSII machinery and establish a new steady

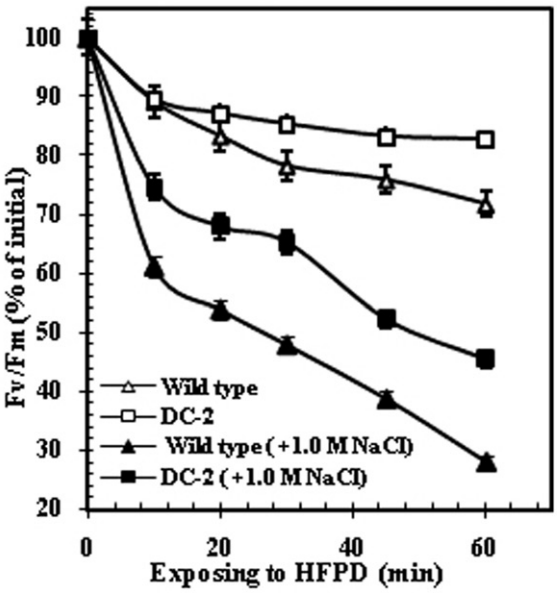

Fig. 5. Changes in the maximal photochemical efficiency of photosystem II (Fv/Fm) of the wild-type and DC-2 mutant *Porphyridium* sp. cells exposed to HPFD of 3,000 μmol m^{-2} s^{-1} grown in either regular growth medium (0.46 M NaCl) or in an additional 1.0 M NaCl. Cells were harvested and resuspended to a final chlorophyll concentration of 25 μg ml^{-1} in fresh medium according to the indicated growth conditions and then exposed to HPFD of 3000 μmol m^{-2} s^{-1}. Samples were withdrawn at various time intervals and diluted with fresh medium to a concentration of 20 μg ml^{-1} chlorophyll, dark-adapted for 5 min. Fv/Fm was recorded at as described in Materials and methods. Maximum activity (100%) was 0.725 and 0.740 in the wild-type and the DC-2 mutant grown in regular growth medium, and 0.695 and 0.630 in the wild-type and the DC-2 mutant grown in an additional 1.0 M NaCl, respectively. Values are means ± SE ($n = 3$).

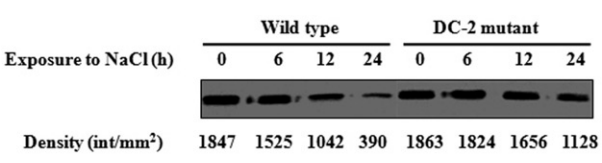

Fig. 4. Western-blot analysis of the D1 protein extracted from culture exposed to 1.0 M additional NaCl at various time intervals (0, 6, 12 and 24 h) and probed with anti-D1 antiserum. The density of protein bands shown below the picture was measured by BioRad's Quantity One v4.6.3 software.

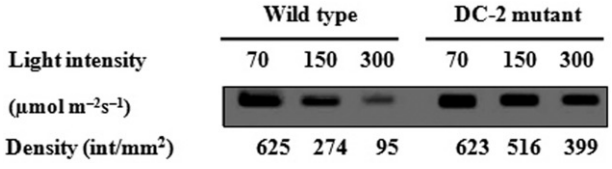

Fig. 6. Western-blot analysis of the D1 protein extracted from culture grown in artificial sea water supplemented with 1.0 M NaCl under 70, 150 and 300 μmol m^{-2} s^{-1} and probed with anti–D1 antiserum. The density of protein bands shown below the picture was measured by BioRad's Quantity One v4.6.3 software.

state of growth. It was shown that the *Porphyridium* sp. cells are capable of acclimating to salinity stress as reflected in their ability to establish a new logarithmic growth phase after a short lag period resulting from the exposure to the elevated salinity stress (Fig. 1). Both wild-type and mutant cells had a reduced growth rate under the salt-stress conditions. However, the DC-2 mutant was more tolerant than wild-type, suggesting that the induced mutation in the DC-2 cells may provide an adaptive advantage to cells exposed to salt stress. In addition, higher value of the P–I curve parameters of DC-2 than wild-type in high-salt concentration (Table 1) showed that the acclimation process to the high salinity involves modifications in the photosynthetic apparatus (Fig. 2).

Indeed, if the induced mutation results in a modification in PSII, this would explain the ability of the cells to maintain a higher level of photosynthetic activity and to utilize the absorbed energy in a more efficient way in comparison to wild-type. One would expect that exposing the cells to the salinity stress would be reflected in the changes in the maximal photochemical efficiency of PSII as reflected in the measurement of Fv/Fm (Fig. 3). Exposing the algal cells to the elevated salt concentration resulted in a decline in the D1 protein. This finding is in line with the well-documented role of the D1 protein in down-regulating the activity of PSII (Smith *et al*., 1990; Harrison *et al*., 1992; Shipton & Barber, 1994; Komenda & Masojidek, 1995; Zeng & Vonshak, 1998; Sudhir *et al*., 2005). The decrease in the level of the protein in response to high salt was much higher in wild-type cells. These differences provide further support to our hypothesis that the induced modification in the photosynthetic apparatus of the DC-2 mutant may be associated with its ability to withstand and tolerate higher level of salt concentration better than wild-type.

Exposing photosynthetic cells to light energy in excess of their ability to utilize the absorbed energy in the photochemical reactions may result in a reduction in activity of PSII defined as 'down-regulation' or photoinhibition. Thus, exposing cells to HPFD and monitoring the changes in Fv/Fm levels may indicate the ability of cells to withstand environmental stress (Baker & Horton, 1987). The mutation induced the ability to resist DCMU resulted in a modification in PSII, which, in turn, produced a more robust PSII apparatus enabling the PSII system to cope better with environmental stress known to affect PSII (Fig. 4). The mutant cells were not only more resistant to a light stress but also when the cells were exposed to a combined stresses of light and salt, the difference in the level of inhibiting the maximal photochemical efficiency of PSII is much more pronounced indicating that mutant cells are much more resistant than wild-type cells. The effect of the combined stresses on the level of the D1 protein strongly support the hypothesis that the randomly induced mutation that resulted in cell resistant to DCMU–an inhibitor of PSII activity–indeed affected the PSII apparatus in such a way that provides the algal mutant cell with a better tolerance to light and salinity stress. It may be interesting to evaluate to what extent this mutant acquired a broad resistance to other environmental or nutritional stress, such as temperature stress or exposure to heavy metals, which are known to effect the PSII apparatus.

Mass culturing of algal cells is mainly limited by the photosynthetic activity of the algal cells. Although few studies have been reported in which high photosynthetic efficiencies were attained, the overall productivity of most of the production systems are far from reaching the theoretical photosynthetic efficiency and in the many cases the main limitation is imposed by environmental stress, such as light, salinity, and temperature. Research focusing on strains in which their PSII apparatus has been modified may provide useful information in developing strains that can tolerate environmental stress and thus be more productive.

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