Effects of NaCl stress on the structure, pigment complex composition, and photosynthetic activity of mangrove *Bruguiera parviflora* chloroplasts

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Abstract

Exposure of two-month-old seedlings of *Bruguiera parviflora* to NaCl stress (0 to 400 mM) for 45 d under hydroponic culture caused notable disorganisation of the thylakoid structure of chloroplasts in NaCl-treated leaves as revealed from transmission electron microscopy. The absorption spectra of treated and control thylakoid samples were similar having a red peak at 680 nm and Soret peaks at 439 and 471 nm in the blue region of the spectrum. The spectra of treated samples differed from control samples by gradual decrease in absorbance of 100, 200, and 400 mM NaCl treated samples at 471 and 439 nm, which could be due to scattering of radiation in these samples. Thus, absorption characteristics of thylakoid membranes indicated no major alterations in the structural integrity of the photosynthetic membranes during salt stress in *B. parviflora*. Analysis of pigment protein complexes of thylakoids on non-denaturing gel showed that CP1 complex consisting of photosystem (PS) 1 reaction centre decreased marginally by 19 % and the CP47 constituting the core antenna of PS2 declined significantly by 30 % in 400 mM NaCl treated samples in respect to control. This decrease in structural core antenna might cause inefficient photon harvesting capacity. However, CP43 content did not alter. An increase in CP2/CP1 ratio from 3.2 in control to 4.0 in 400 mM NaCl treated samples indicated significant structural changes in the thylakoids of salt treated plants. Haem staining of thylakoids revealed significant losses in cytochrome (Cyt) f and Cyt b_6 contents by NaCl stress. However, Cyt b_{559} content remained nearly constant in both control and NaCl treated samples. SDS-PAGE of thylakoid proteins showed that the intensity of many of Coomassie stained polypeptide bands ranging from 15-22 and 28-66 kDa regions decreased significantly in NaCl treated samples as compared to control. Electron transport activity of thylakoids, measured in terms of DCPIP photoreduction, was 22 % lower in 400 mM NaCl treated plants than in the control ones. Hence, NaCl induces oxidative stress in chloroplasts causing structural alterations in thylakoids. These structural alterations might be responsible for declined efficiency of photosystems and reduced electron transport activity.

Additional key words: electron transport; hydroponic plants; light-harvesting complexes; pigment-protein complexes; photosystems; polypeptides; salt stress; thylakoids.

Introduction

Salt stress negatively impacts agricultural yield throughout the world by limiting plant productivity. High salinity causes both hyperionic and hyperosmotic stress effects, and the consequences of these can be plant death (Hasegawa *et al.* 2000). The detrimental effects of high concentrations of salt on plants can be observed at

the whole plant level as the death of plants and/or decrease in productivity. Reductions in plant growth due to salt stress are often associated with decreases in photosynthetic activities such as the electron transport.

Mangroves are constituent plants of tropical intertidal forest community. They include woody trees and shrubs, which flourish in inhospitable zone between land and sea along tropical coastlines. Mangroves are taxonomically diverse; true mangroves include about 54 species in 20 genera belonging to 16 families (Das *et al.* 1997). The most striking feature of mangroves is their ability to tolerate NaCl at sea water level (~500 mM). Mangroves are divided into two distinct groups on the basis of their salt management strategies. One is "secretors" having salt glands or salt hairs for excretion of excess salt and the other is "non-secretors" lacking such morphological features. *B. parviflora* is included in the latter group that grows in a range of salinities from brackish water up to two times the sea water level. Although non-secretors including *B. parviflora* excluded 99 % of the salt in surrounding sea water by ultra-filtration (Scholander 1968), a high content of sodium in xylem sap of *Bruguiera gymnorrhiza* was detected (Takemura *et al.* 2000). Due to high salt concentrations in the growth medium halophytes such as mangroves are confronted with the problem of maintaining turgor pressure and protecting their metabolism from high inorganic ion concentration (Flowers *et al.* 1977). This is accomplished by the sequestration of inorganic ions within the vacuole and/or by the synthesis of inorganic compounds (Hasegawa *et al.* 2000). Mangroves have developed physiological adaptations to their environment such as salt glands, leaf succulence, and ultra-filtration by roots (Tomlinson 1986). Further, the possible function of the hypocotyl as an additional filter to retain salt from the shoot is considered for the mangrove *B. gymnorrhiza* (Werner and Stelzer 1990). *B. parviflora,* in particular, is not provided with salt glands, but it keeps the xylem sap essentially free of NaCl by ultra-filtration in the membranes of root cells (Scholander 1968, Mallery and Teas 1984). There was increased accumulation of sugars, proline, and polyphenols as compatible solutes in leaves of *B. parviflora* to regulate water potential (Parida *et al.* 2002). We also noticed higher contents of Na^+ and Cl^- in leaves of salt stressed plants as compared to control (Parida *et al.*, unpublished). These evidences suggest that leaf cells experience salt and osmotic stress when cultured in saline media. Salt stress induced alterations in structure and functions of chloroplasts or thylakoid membranes have been examined in various crops (Hernández *et al.* 1995, Delphine *et al.* 1998, Mitsuya *et al.* 2000), cells of cyanobacteria (Allakhverdiev *et al.* 2000a), and in a facultative halophyte (Adams *et al.* 1992), but there are scanty reports on mangroves (Kao and Tsai 1999). In the present study, we examined the effects of NaCl stress on chloroplast ultrastructure, pigment-protein complexes, thylakoid polypeptides, and electron transport activity in a true mangrove *B. parviflora*.

Materials and methods

Plants: Propagules of *Bruguiera parviflora* (Roxb.) Wt. & Arn. *ex* Griff. were collected from Bhitarkanika mangrove forest of Orissa, latitude 20°4′ to 20°8′ north and longitude 86°45′ to 87°50′ east, India. Seedlings were raised in greenhouse with non-saline and non-brackish water under photosynthetically active radiation (PAR) of 1 220–1 236 μmol m^{-2} s^{-1}. Two-month-old healthy seedlings were selected for hydroponic culture in Hoagland's nutrient medium, pH 6.0 (Hoagland and Arnon 1940) salinised at four levels of NaCl (100, 200, and 400 mM). The culture nutrient medium was continuously aerated with an air bubbler. Hydroponic cultures were maintained in a culture room under a 14 h photoperiod at 300 μmol m^{-2} s^{-1}, 22±2 °C, and 80 % relative humidity (RH). The culture medium was changed with fresh medium every 7 d. Fourth pair of leaves from the top of the shoot was harvested after 45 d of NaCl treatment for isolation of thylakoids.

Thylakoid membranes were isolated from leaves of control and NaCl treated plants according to the procedure of Nakatani and Barber (1977). Fresh leaves (10 g) from control and NaCl treated plants were homogenised in chilled isolation buffer containing 0.4 M sorbitol, 15 mM tricine (pH 7.8), and 5 mM $MgCl_2$ (buffer A), in a tissue homogeniser (*Ultra Turrax-T_{25}, Ika*, Germany). The homogenates were filtered through four layers of mira cloth (*Calbiochem*, Switzerland), and centrifuged at 3 000×*g* for 5 min at 4 °C. The supernatants and most of the loose pellets were discarded. Rest of the pellets was washed in buffer containing 10 mM tricine (pH 7.8), 10 mM NaCl, and 5 mM $MgCl_2$ (buffer B). The pellets consisting of thylakoid membranes were suspended in a buffer consisting of 0.1 M sorbitol, 10 mM tricine (pH 7.8), 10 mM NaCl, and 5 mM $MgCl_2$ (buffer C) to a final chlorophyll (Chl) concentration of 1 kg m^{-3}. 30 % glycerol was included in buffer C when thylakoid membranes were stored at −85 °C for further use.

Intact chloroplasts from control and 400 mM NaCl stressed plants were isolated following the procedure of Robinson (1982) with minor modifications. About 30 g of leaves were ground in a tissue homogeniser (*Ultra Turrax-T25, Ika*, Germany) in a buffer containing 50 mM HEPES-KOH (pH 7.8), 2 mM iso-ascorbate, and 0.1 % BSA (buffer 1). The bray was squeezed through two layers of mira cloth and the filtrate was centrifuged at 1 200×g for 3 min. The pellet was re-suspended in buffer

2 containing 18 cm^3 of 330 mM sorbitol, 2 mM EDTA, 1 mM MgCl$_2$, 1 mM MnCl$_2$, 50 mM HEPES-KOH (pH 7.6), and 0.2 % BSA (*Sigma*, Fraction V), and then loaded over 14 cm^3 of the same medium (buffer 2) plus 40 % (v/v) *Percoll*, then the tubes were centrifuged at 2 000×g for 3 min. Broken chloroplasts formed a band at the top of the *Percoll* layer, whereas intact chloroplasts were pelleted at the bottom. The supernatant was discarded and the pellet of intact chloroplasts was resuspended in buffer 2. Immediately after isolation, the intact chloroplasts were fixed in 2.5 % glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4, and were used for transmission electron microscopy.

Transmission electron microscopy (TEM): In order to study gross structural changes in the shape and thylakoid arrangement of chloroplasts upon NaCl treatment, intact leaves and chloroplasts were processed for TEM studies. The sample was pre-fixed in 2.5 % (v/v) glutaraldehyde in 0.1 M sodium phosphate buffer, pH 7.4 at 4 °C. The samples were then washed 2–3 times in phosphate buffer and post-fixed in 2 % osmium tetroxide in the same buffer for 1 h in ice bath in a fume hood. The samples were washed 5-6 times with phosphate buffer and dehydrated in graded series of ethanol solution (15, 80, and 90 %, two changes for 10 min each). The samples were then embedded in araldite: thin sections (0.1 μm) were cut and mounted on copper grids. The sections were stained with both uranyl acetate (saturated solution in 60 % ethanol) and lead citrate (2 % in 0.2 M NaOH) and examined under transmission electron microscope model *CM12 Philips* operated at 30 kV.

Absorption spectra of thylakoids at room temperature were recorded in a UV-visible spectrophotometer (*Jasco V-530*, Japan) at room temperature. Isolated thylakoids equivalent to 7 g(Chl) m^{-3} were suspended in buffer C and spectra were recorded from 360-750 nm at room temperature with a slow scan speed. The necessary adjustments for scattering were made at 750 nm and the spectra were overlapped for comparative analysis to ascertain the difference in absorption characteristics, if any, among control and NaCl treated samples.

Chl and protein contents in isolated thylakoids: Chl contents in thylakoid suspension were estimated using 80 % acetone following the equation of Lichtenthaler (1987). Protein content in thylakoid suspension was estimated following the procedure of Lowry *et al*. (1951).

Resolution of thylakoid polypeptides by urea, SDS-PAGE: Samples for urea-SDS denaturing polyacrylamide gel electrophoresis (PAGE) were prepared by mixing the pelleted thylakoid membranes in sample buffer (1 cm^3 sample buffer per mg Chl) containing 6 M urea, 2 % SDS (m/v), 2.5 % 2-mercaptoethanol (m/v), 62.5 mM Tris-HCl (pH 6.8), and 20 % glycerol (m/v). The mixture was

incubated for 20-30 min at room temperature with intermittent shaking. The sample was then centrifuged at high speed for 2 min prior to loading. Thylakoids (equivalent to 20 μg Chl) were loaded per lane.

Electrophoresis was carried out using *Bio-Rad Protein II* electrophoresis system. Gels were made according to Laemmli (1970) with some modifications. A 12.5 % separating gel containing 2 M urea, 0.1 % m/v SDS, 375 mM Tris-HCl, pH 8.8, and 400 cm^3 m^{-3} TEMED was used for resolving the polypeptides. A 4 % stacking gel containing 2 M urea, 0.1 % m/v SDS, 125 mM Tris-HCl, pH 6.8, and 500 cm^3 m^{-3} TEMED was used to concentrate (stack) the polypeptides. Electrophoresis running buffer consisted of 25 mM Tris, 192 mM glycine, and 0.1 % SDS, pH 8.3. Gels were run at a constant 35 mA for 4 h using *Bio-Rad*, *Protein II xi* electrophoresis system.

After separating the polypeptides on the gel, they were detected by staining with 0.25 % Coomassie Brilliant Blue R-250 (*Sigma*) in 50 % methanol and 10 % acetic acid for 2 h, and de-stained with 50 % methanol and 10 % acetic acid until the background was clear. The gel was photographed and scanned using a densitometer (*GS-710, Bio-Rad*, USA) and analysed with *Quantity one* software from *Bio-Rad*.

Resolution of pigment-protein complexes of thylakoids (green gel): Different pigment-protein complexes of thylakoid membranes were analysed on non-denaturing polyacrylamide gels (PAGE) following the procedure of Delepelaire and Chua (1979) with minor modifications. A 15 % acrylamide gel was used for separation of pigment-protein complexes. Both the separating (15 %) and stacking gel (5 %) did not contain detergent. Electro-phoresis running buffer consisted of 0.1 % LDS instead of 0.1 % SDS, as in Laemmli (1970). Both the separating and stacking gels contained the cross-linker N,N-methylene-bis-acrylamide at concentration of 0.33 %, as against 2.6-2.8 % in the Laemmli system.

Samples were prepared by mixing thylakoids with equal volume of 2× sample buffer containing 125 mM Tris-HCl (pH 6.8), 20 % (m/v) glycerol, and 4 % (m/v) LDS (LDS : Chl, 25 : 1). The above mixture was incubated for 25-30 min at 4 °C. Thylakoids equivalent to 10 μg Chl were loaded per lane. These unstained green gels were run at 4 °C at a constant voltage of 100 V for 1.5 h in dark using *Bio-Rad mini protein II* electrophoresis system. Immediately after electrophoresis the gel was photographed and scanned. The pigment-protein complexes were labelled according to Delepelaire and Chua (1979).

Identification of haem proteins in the thylakoid polypeptide patterns: Electrophoresis of the control and NaCl treated thylakoids was performed according to the procedure of Delepelaire and Chua (1979) with minor modifications. The pigment-protein complexes were

resolved on 15 % polyacrylamide gel with LDS as the detergent and gels run at 4 °C in dark. Staining for haem associated peroxidase activity was carried out according to the procedure of Høyer-Hensen (1980). Immediately after electrophoresis was completed, the gels were immersed in solution composed of 3 parts of 6.3 mM TMBZ (in methanol) and 7 parts of 0.25 M Na-acetate, pH 5.0, and kept in dark at room temperature with gentle shaking for 2 h. Hydrogen peroxide was then added to a final concentration of 30 mM. Blue coloured haem bands were detected after 5-10 min, which intensified for subsequent 1 h. The gels were then washed with a solution of 3 parts of iso-propanol and 7 parts of 0.25 M sodium acetate, pH 5.0, to remove excess TMBZ. Washing procedure was repeated 2-3 times and the gels were photographed, scanned using a densitometer (GS-710, *Bio-Rad*, USA), and analysed with *Quantity one* software from *Bio-Rad*.

Electron transport rate: The PS2 mediated electron transport activity in terms of DCPIP photoreduction was measured in thylakoids isolated from control and NaCl treated plants following the procedure of Prasad *et al*. (1991). The assay mixture (total volume 3 cm^3) consisted of: 50 mM HEPES-NaOH buffer (pH 7), 10 mM NaCl,

2 mM $MgCl_2$, and 50 µM DCPIP. In each assay thylakoid membranes equivalent to 5 g(Chl) m^{-3} were used. Irradiation-induced reduction of DCPIP by thylakoid membranes was determined at 25 °C by monitoring changes in A_{590} usin a spectrophotometer. The rate of loss of absorption was converted to rate of reduction of DCPIP by using the extinction coefficient for DCPIP as 16 mM^{-1} cm^{-1} at 590 nm (Allen and Holmes 1986). The PS2 mediated electron transport activity from DPC to DCPIP was measured in the same assay mixture containing 0.5 mM DPC. A pH profile of the rate of DCPIP photoreduction in thylakoids isolated from control and NaCl stressed plants was established by measuring the rates at pH 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, and 8.5. The assay buffer consisted of 50 mM MES (for pH 5.5-6.5), 50 mM HEPES (for pH 7.0-7.5), or 50 mM Tricine (for pH 8.0-8.5). The PS2 mediated electron transport activity in terms of DCPIP reduction was also measured in loosely coupled and NH_4Cl mediated uncoupled thylakoids. The concentration of NH_4Cl was 5 mM. Effects of electron transport inhibitors on rate of DCPIP photoreduction in thylakoids from control and NaCl stressed plants were determined by measuring the rate in assay mixture containing 5-15 µM DCMU.

Results

Changes in ultrastructure of chloroplasts: The cell structure looked generally similar in both control and 400 mM NaCl treated leaves (Fig. 1*A,B*) with the organelles towards periphery and a large vacuole in the centre. However, salt treated sample showed a smaller number of more compressed chloroplasts per cell section. The major effect of NaCl was on degree of membrane appression (Fig. 1*C,D*). Control leaves showed distinct granal and stromal thylakoid arrangement and clear stroma matrix whereas the NaCl treated (400 mM) leaves exhibited elongated and compressed chloroplasts with homogeneous membrane distribution and significant loss of clear stromal matrix, thereby indicating a probable loss in stacking of the thylakoid membranes into grana. The numbers of plastoglobuli were increased in salt stressed chloroplasts. The overall shape of chloroplasts also changed slightly from elliptical to elongated and the envelope membrane in the NaCl treated samples appeared to be ruptured. However, further experiments with higher NaCl treatments are needed, since the micrographs in the present investigation suggest that 400 mM NaCl might be a transition concentration where the thylakoid organisation begins to disrupt. NaCl stress induces reduction in grana stacking (Hernández *et al*. 1995). Our experiments on composition and structural analysis of photosynthetic membranes under NaCl stress indicated no great alteration. In higher plants and algae, thylakoid stacking represents the functional integrity of the photosystems. Elec-

tron micrographs of sections of control and 400 mM NaCl treated leaves indicated loss of distinct granal and stromal regions in treated samples.

Changes in spectral characteristics of thylakoid membranes (Fig. 2): Apparently no spectral shifts were observed in NaCl (100, 200, and 400 mM) treated samples when compared to control thylakoids. The absorption spectra of treated and control thylakoid samples were similar having a red peak at 680 nm, Soret peaks at 439 nm, and a hump at 471 nm in the blue region of the spectrum. The spectra of treated samples differed from control samples by gradual decrease in absorbance in 100, 200, and 400 mM NaCl treated samples, respectively, as compared to the control at 471 and 439 nm, which could be due to scattering of radiation in these samples. Hence, absorption characteristics of thylakoid membranes indicated no major alterations in the structural integrity of the photosynthetic membranes during salt stress in *B. parviflora*.

Changes in Chl *a*/*b* and protein/Chl ratios of thylakoids: Protein/Chl ratio of thylakoids was 16 % higher in thylakoids isolated from 400 mM NaCl treated plants in respect to control (Table 1). Chl *a*/*b* ratio decreased from 2.25 in control thylakoids to 2.02 in 400 mM NaCl treated thylakoids (Table 1).
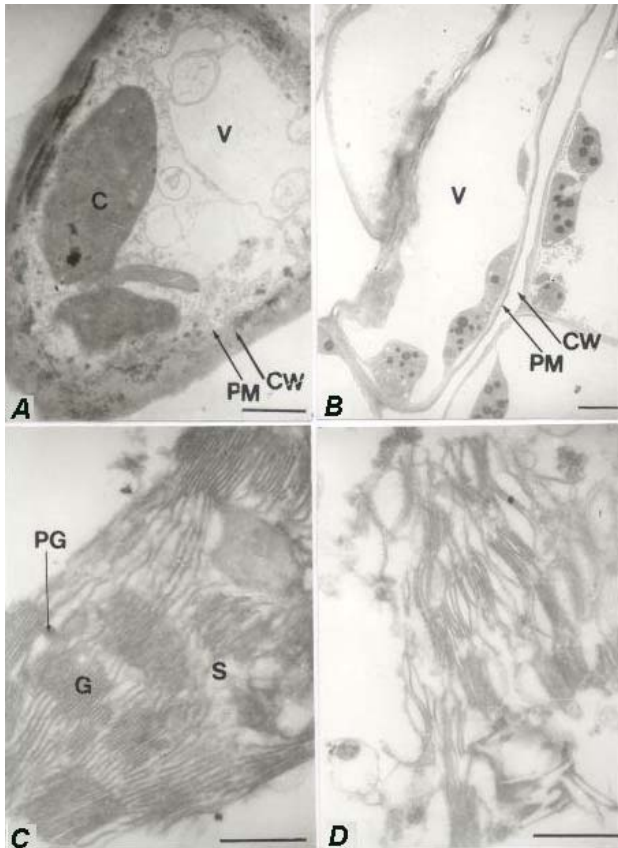
Fig. 1. Transmission electron micrographs of whole mesophyll cells (*A, B*) (bar = 1 μm) and chloroplasts (*C, D*) (bar = 0.5 μm) of control leaves (*A, C*) and leaves of 400 mM NaCl treated plants (*B, D*). C = chloroplast; CW = cell wall; EM = envelope membranes; G = granum; PG = plastoglobuli; PM = plasma membrane; S = stroma; V = vacuole.

Table 1. Effects of NaCl [mM] on chlorophyll (Chl) *a/b* and protein/Chl ratios in thylakoids of *B. parviflora* isolated after 45 d of NaCl treatment. Means±S.E. (*n* = 6).

NaCl	Chl *a/b*	protein/Chl
0	2.25±0.08	9.92±0.54
100	2.27±0.03	8.85±0.43
200	2.11±0.05	10.29±0.38
400	2.02±0.05	11.53±0.52

Polypeptide profile of the thylakoids as analysed by urea-SDS PAGE: The thylakoid membranes isolated from control and NaCl treated leaves were solubilised in SDS-sample buffer containing 6 M urea and the polypeptides were resolved on 12 % polyacrylamide gel. Polypeptide profile in control and treated samples consisted of 22-25 polypeptides and was mostly identical (Fig. 3*A*). However, the intensity of many Coomassie stained polypeptide bands ranging from 15-22 and

28-66 kDa regions showed significant decrease in NaCl treated samples as compared to control thylakoid sample.

Analysis of pigment-protein complexes (green gels): Six pigment-protein complexes were resolved in the thylakoids isolated from *B. parviflora* leaves by the modified procedure of Delepelaire and Chua (1979). Pigment-protein complex profile of thylakoids (Fig. 3*B*) showed certain alterations in the salt stressed samples when compared to control. Both the gel and the densitometric scan indicated a very minor or no alteration in the free pigment zone in control and treated samples. The uppermost band of pigment-protein (CP1a) did not change due to salt stress; this may be linked to PS1 complex. As quantified from the densitometric scan, CP1 complex, consisting of PS1 reaction centre decreased marginally by 19 % and the CP47 declined significantly
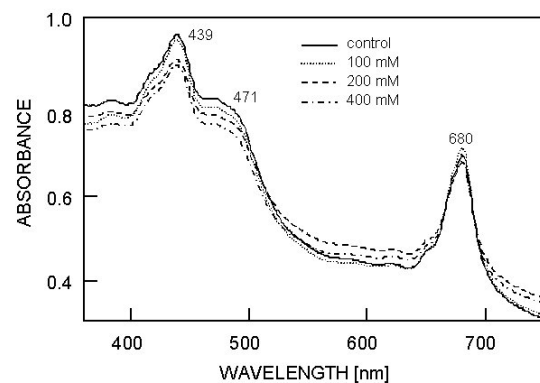

Fig. 2. Room temperature absorption spectra of thylakoids isolated from control and 100, 200, and 400 mM NaCl treated plants. Thylakoids equivalent to 7 g(Chl) m^{-3} were suspended in buffer C and spectra were recorded from 750 to 360 nm in a UV-visible spectrophotometer.
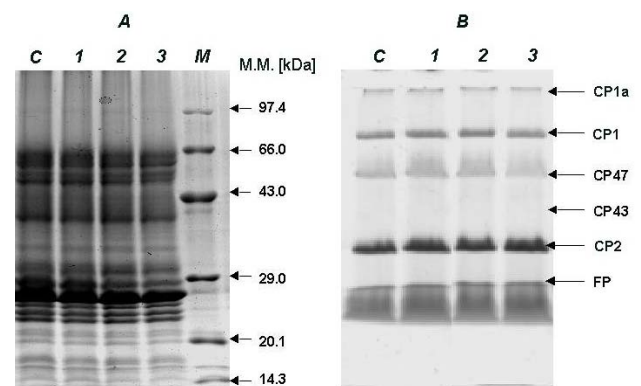

Fig. 3. Effects of NaCl stress on thylakoid polypeptides as analysed on SDS-PAGE (*A*) and on pigment-protein complexes of thylakoids as analysed by green gel (*B*). Lanes *C, 1, 2,* and *3* represent thylakoids isolated from control, 100, 200, and 400 mM NaCl treated plants, respectively, and lane *M* represents molecular mass markers.

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by 30 % in 400 mM NaCl treated samples in respect to control. The CP43 content, however, did not show any alteration. A concomitant increase in CP2 complex (by 18 %) was also observed (Fig. 3*B*).
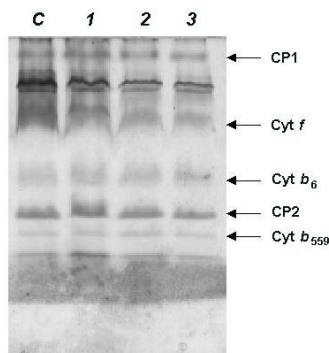

Fig. 4. Effects of NaCl stress on cytochrome complexes of thylakoids as analysed by haem staining. Lanes *C*, *1*, *2*, and *3* represent thylakoids isolated from control, 100, 200, and 400 mM NaCl treated plants, respectively.

Identification of haem groups in the thylakoid protein profile: The TMBZ-H_2O_2 staining procedure was performed to localise haem groups and show the contents of haem containing proteins in the thylakoids. Three prominent haem containing protein bands of apparent molecular masses 35 kDa (Cyt f), 20 kDa (Cyt b_6), and 14 kDa (Cyt b_{559}) were detected in both control and NaCl treated samples. The contents of Cyt f and Cyt b_6 decreased in NaCl treated samples in respect to control. However, contents of Cyt b_{559} remained nearly constant in control as well as NaCl treated samples (Fig. 4).

Changes in electron transport activity: No significant changes in rate of DCPIP photoreduction was observed in thylakoids isolated from NaCl stressed plants up to 15 d of treatment in respect to control (Table 2). After 30 d of treatment, the rate of DCPIP photoreduction decreased marginally in NaCl stressed plants as compared to control (8.6 and 15.3 % in 200 and 400 mM NaCl treated plants, respectively). After 45 d of treatment, a significant decrease in DCPIP reduction was found in thylakoids of NaCl stressed plants in respect to control (13.2 and 22.4 % in 200 and 400 mM NaCl treated plants, respectively) (Table 2). Exogenous electron donor (DPC) could not restore NaCl induced inhibition of DCPIP photoreduction in a concentration dependent manner (Table 2).

Table 2. Effects of NaCl [mM] on photosystem 2 (PS2) mediated electron transport activity [mmol(DCPIP) kg^{-1}(Chl) s^{-1}] in *B. parviflora* as monitored in terms of DCPIP photoreduction. The rate was measured at different days after NaCl treatment in a reaction mixture at pH 7.0 as $H_2O \rightarrow$ DCPIP without any uncoupler and inhibitor or as DPC$\rightarrow$DCPIP. Means±S.E. (*n* = 6).

	NaCl	0 d	15 d	30 d	45 d
$H_2O \rightarrow$ DCPIP	0	20.27±0.69	19.76±0.36	19.96±0.56	19.63±0.47
	100	20.27±0.75	20.86±0.42	20.93±0.36	21.06±0.42
	200	20.27±0.56	19.59±0.42	18.23±0.47	17.02±0.28
	400	20.27±0.64	18.96±0.56	16.89±0.28	15.23±0.69
DPC$\rightarrow$DCPIP	0	31.50±0.75	31.01±0.38	30.99±0.28	30.71±0.50
	100	31.64±0.56	31.53±0.50	32.09±0.64	31.84±0.69
	200	31.27±0.56	30.75±0.42	29.65±0.69	25.96±0.64
	400	31.33±0.42	29.12±0.56	23.84±0.67	21.85±0.47

Table 3. Effects of electron transport inhibitor (DCMU) on rate of DCPIP photoreduction [mmol(DCPIP) kg^{-1}(Chl) s^{-1}] measured in thylakoids isolated from control and NaCl treated plants after 45 d of treatment in a reaction mixture at pH 7.0. Means±S.E. (*n* = 6).

NaCl [mM]	DCMU [μM] 0	5	10
0	19.76±0.69	2.05±0.22	1.92±0.14
100	21.03±0.64	2.42±0.17	1.86±0.06
200	17.07±0.78	1.87±0.22	1.68±0.14
400	15.23±0.39	1.60±0.17	1.49±0.14

Optimum pH for reduction of DCPIP was 6.5 for loosely coupled thylakoids and 7.0 for uncoupled thylakoids in this plant (Fig. 5*A*). Optimum pH for uncoupled DCPIP assay in control thylakoids was 7.0 and that in NaCl treated thylakoids was 6.5 (Fig. 5*B*). Using 5–10 μM DCMU as inhibitor, we observed a reduction in electron transport rate by 90–91 % in the reaction mixture containing thylakoids with 10 μM DCMU in respect to the reaction mixture containing thylakoids without DCMU (Table 3). The % inhibition of electron transport activity by DCMU remained unaffected by NaCl stress.

Discussion

The thylakoid membranes were partially de-stacked and the numbers of plastoglobuli were increased in chloroplasts of NaCl treated plants. These results are in agreements with the report of Hernández *et al.* (1995), although other authors did not see profuse de-stacking of the membranes (Bruns and Hecht-Buchholz 1990, Hernández *et al.* 1995, Khavari-Nejad and Mostofi 1998, Mitsuya *et al.* 2000). Our results suggest that 400 mM NaCl represents mild salt stress for *B. parviflora* resulting in transition stage of normal chloroplast to an altered one. Nazarenko and Serebryakova (1990) reported an increase in the number and size of chloroplast plastoglobuli in *Euglena* cells under salinity. Plastoglobuli are ubiquitous in both chloroplasts and chromoplasts, and play a probable role in salt tolerance in plants (Burguess 1985).
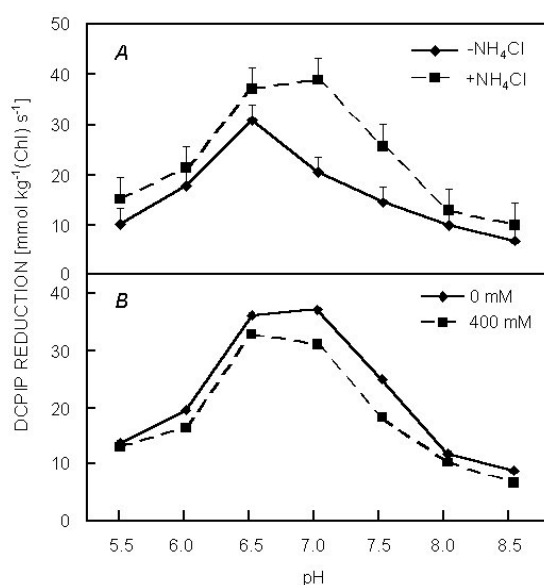

Fig. 5. pH profile of coupled and uncoupled electron transport activity (*A*) and effects of 400 mM NaCl on the profile (*B*) in *B. parviflora* as monitored in terms of DCPIP photoreduction.

PS2, PS1, Cyt b_6/f, and ATPase complexes of thylakoid membranes consist approximately of 24, 23, 5, and 9 polypeptides, respectively (Staehelin and van der Staay 1996). When thylakoid membranes are solubilised and resolved on polyacrylamide gel, protein bands having molecular mass of 3.3–83 kDa can be seen. Many of these proteins have been identified and characterised in several plants (spinach, pea). Molecular masses of proteins of the above-mentioned four multi-protein complexes of the thylakoid membranes that function in the electron transfer from water to NADP and also bind the pigment molecules are known. However, the roles of some of the polypeptides that can be resolved on the polyacrylamide gels are still not known. It is, therefore, possible to identify the onset and extent of loss in the

specific proteins of these five thylakoid complexes during salt stress. SDS-PAGE of thylakoids revealed that the intensity of the many polypeptide bands ranging from 15–23 and 28–66 kDa regions significantly decreased in NaCl treated samples as compared to control thylakoid sample. This suggests that target site of salt stress may be the dissociation of certain thylakoid polypeptide. This is in consistence with the observed increase in protein/Chl ratio by NaCl stress. Our result also supports the finding of Murata *et al.* (1992) on the dissociation of the 23-kDa polypeptide extrinsically bound to PS2, which plays major role in oxygen evolution.

At low temperature, most of the pigments remain attached to the proteins. Thus mild detergent treatment and subsequent gel electrophoresis can resolve pigment-protein complexes. Fig. 4 depicts different pigment-protein complexes separated on a non-denaturing polyacrylamide gel and the complexes were labelled according to Delepelaire and Chua (1979). This system resolves the higher plant thylakoid membranes into 4–5 distinct pigment-protein complexes in addition to a free pigment zone (FP). The CP1 complex consists of PS1 and molecules of Chl *a*. CP2 complex contains both Chl *a* and *b* and is derived from the light-harvesting complex (LHC2) of PS2. Two more complexes, CP3 and CP4, contain only Chl *a* and no Chl *b*. Each of these two complexes consists of one molecule of β-carotene, four to five molecules of Chl *a*, and a polypeptide in 45–50 kDa range. Therefore, these complexes may correspond to CP47 and CP43 that constitute the core antenna of PS2. Analysis of pigment-protein complexes by green gel showed a significant decline in CP47 complex in 400 mM NaCl treated samples as compared to control. Since CP47 constitutes the core antenna of PS2 (besides CP43), a decrease in its content would lead to decrease in structural core antenna and thus to an inefficient photon harvesting capacity. Such a loss in CP47 can also be expected because of decrease in Chl *a/b* ratio since CP47 contains only Chl *a* and no Chl *b* (Green 1988). CP2/CP1 ratio in control samples was 3.2 whereas 400 mM NaCl treated samples recorded a ratio of 4.0. An increase in CP2/CP1 ratio indicates an increase in the size of LHC2 suggesting significant structural changes in the thylakoids of salt treated plants, which is similar to shade type adaptation (Leong and Anderson 1984, De la Torre and Burkey 1990, Vani 1998). However, the nature of the increase in LHC2 (CP2) has not been studied.

Analysis of thylakoids by haem staining showed significant lower contents of Cyt *f* and Cyt b_6 in NaCl treated samples than in the control. This suggests that salt stress may accelerate the breakdown of thylakoid components that includes lower steady state contents of Cyt *f* and Cyt b_6. Our results supported earlier reports of Harding *et al.* (1990) who observed a decrease in steady

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state content of Cyt *f* in wheat plants after one week of mild heat stress (35 °C). They were attributed to accelerated senescence due to heat stress, because electron transport activities other than PS2 became thermolabile and injury clearly depended on senescence pattern. Content of Cyt b_6/f complexes decreases prior to decrease in other thylakoid components in senescing oat and Cyt b_6/f complex content limits the intersystem electron transport in barley during senescence (Holloway *et al.* 1983). We also noticed a loss in Cyt *f* and Cyt b_6 contents. We suggest that salt induced alterations shown in our study are due to NaCl induced acceleration of senescence since significant loss in Chl and the breakdown of intersystem thylakoid components were observed.

As thylakoid stacking represents functional integrity of photosystems and optimal energy harvest and distribution, we suggest that 400 mM treatment for 45 d causes major structural disarrangement within the photosynthetic membranes, disturbing the normal functioning of thylakoid membranes in energy capture and utilisation. The NaCl stress induced decline in PS2 activity monitored in terms of DCPIP photoreduction might be due to this disarrangement in thylakoids. NaCl induced loss in PS2 electron transport activity has already been reported (Wignarajah and Baker 1981, Kuwabara and Murata 1982, Miyao and Murata 1984, Murata *et al.* 1992, Hernández *et al.* 1995, Tiwari *et al.* 1997, Allakhverdiev *et al.* 2000a,b). The decrease in photochemical activity may be due to dissociation of certain thylakoid polypeptides or to partial impairing of energy transfer in the Chl/PS1 protein complex (Öquist *et al.* 1980, Valcke and van Poucke 1983). Na^+ content in leaves may also affect the stability of PS2 functions (Muranaka *et al.* 2002).

In order to define sites of action of NaCl on PS2, we studied the effects of artificial electron donor on inhibition of DCPIP photoreduction using 0.5 mM DPC. The NaCl induced inhibition of DCPIP photoreduction could not be restored by exogenous electron donors, suggesting that inhibition of electron transport be either due to inactivation of the PS2 reaction centre or to a change in membrane structure of the photosynthetic apparatus. These results are in agreement with previous reports of the inhibitory effects of nickel and zinc on barley chloro-

plasts (Tripathy and Mohanty 1980, Tripathy *et al.* 1981) and chromium and lead on *Nostoc* cells (Prasad *et al.* 1991). In order to ascertain if the NaCl stress induced loss in PS2 mediated electron transport activity is associated with the change in characteristics of electron carriers in the electron transport chain, the PS2 mediated electron transport activity was measured at different pH of the assay buffer (pH 5.5–8.5). The pH optimum for PS2 photochemical reaction was similar for untreated and for NaCl treated samples (Fig. 5*B*). The extent of inhibition by NaCl stress remained independent of the pH of the assay medium. Hence the exposure of *B. parviflora* seedlings to NaCl stress did not alter the characteristics of the electron carriers of PS2.

The measurement of electron transport activity in loosely coupled and NH_4Cl mediated uncoupled thylakoids showed that the addition of uncoupler enhanced the rate of electron transport activity significantly both in control and NaCl stressed samples (Fig. 5*A*). Our results are in good agreement with those of Bansal *et al.* (1991) who observed a similar enhancement in electron transport activity in wheat chloroplasts using NH_4Cl and gramicidine-S as uncouplers.

DCMU (diuron) is an inhibitor of electron transport, which acts at the reducing side of PS2, although DCMU at high concentration may also act on the oxidising side of PS2 (Vani *et al.* 2001). DCMU supported Hill activity was measured at various concentrations of DCMU (0–15 μM). Table 3 shows that the extent of inhibition of electron transport activity by DCMU was similar (~90 %) for both control and NaCl stressed samples.

In summary, our experiments on structure and functional analyses of photosynthetic membranes in *B. parviflora* under NaCl stress for 45 d suggest that NaCl imposed ionic and oxidative stress on chloroplasts caused no significant alterations in composition of the intrinsic thylakoid proteins. Nevertheless, it made structural alterations in thylakoid membranes that induce significant loss in functional ability of thylakoid membranes such as (*1*) the ability to capture and utilise photon energy efficiently, (*2*) to perform photochemical electron transport, and (*3*) intersystem electron transport between the two photosystems.

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