Changes of soluble proteins in leaf and thylakoid exposed in high saline condition of a mangrove taxa *Bruguiera gymnorrhiza*

Bishnupriya Behera¹, Anath Bandhu Das¹ and Prasanna Mohanty²

¹Cytogenetics and Mangrove Research Laboratory, Regional Plant Resource Centre, Nayapalli, Bhubaneswar - 751015, Orissa, India.

²INSA Honorary Scientist, Regional Plant Resource Centre, Nayapalli, Bhubaneswar - 751015, Orissa, India

ABSTRACT

One-year-old seedlings of *Bruguiera gymnorrhiza* (L) Savingay were exposed to 500 mM NaCl for 6d under hydroponic culture condition to characterize the changes in leaf and thylakoid protein profiles in response to short-term salt exposures. Significant changes in leaf dry mass, chlorophylls and soluble leaf proteins were observed in short term of salt exposures, as it happens under tidal situations in nature. Chlorophyll a/b ratio showed decrease of light harvesting efficiency in salt treatment. Total soluble proteins in leaves were extracted from control and NaCl-treated plants at 2d intervals and were analyzed by SDS-PAGE. Intensity of several protein bands of different molecular mass of leaf protein profile ranging from 10 to 86 kDa (10, 16, 23, 33, 37, 42, 44, 50 and 86 kDa) were decreased due to high salt treatment. Out of these, 16, 23 and 33 kDa protein bands decreased dramatically from 1-3 fold but recovered in 7d growth, except the 33 kDa band. SDS-PAGE profile of thylakoid protein revealed that both number and the intensity of several protein bands got altered by salt concentration. However, 33 kDa protein band of thylakoid reappeared in recovery that might not be of the same characteristics with same molecular mass as shown in total leaf protein profile. The numbers of major bands found in SDS-PAGE were reduced when analyzed in urea-SDS-PAGE to minimize protein aggregations by high salt. It was noted that 47 kDa disappeared while some proteins of apparent molecular mass like 23 kDa, 33 kDa, 37 kDa and 50 kDa degraded to minor bands. Partial restoration of protein bands occurred when the salt-treated plants were brought back to initial growth condition. These results clearly demonstrate that short term high salt concentration could cause major alterations to photosynthetic apparatus of a true non salt-secreting tree mangrove *Bruguiera gymnorrhiza* and adapted against fluctuation of salinity by altering leaf protein pool relatively more than the thylakoid proteins. [Physiol. Mol. Biol. Plants 2009; 15(1) : 53-59] E-mail : a_b_das@hotmail.com, photosis@rediffmail.com

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Abbreviations : kDa=kilo Dalton, SDS=Sodium dodecyl sulfate, PAGE=Polyacrylamide gel electrophoresis, MW=Molecular weight, Tris=Hydroxymethyl amino methane, TEMED=Tetra ethylene methylene diamine, Tricine=N-[tris-(hydroxymethyl)-methyl] glycine, PMSF=Phenylmethanesulphonyl fluoride.

INTRODUCTION

Mangrove forests form a unique community in tropical coastal regions including tidal lowlands and act as coastal guard to combat natural calamities. They are considered to be ecologically essential components in protecting adjacent land from tidal waves and storm erosions and even from tsunami (Hogarth, 1999).

Bruguiera gymnorrhiza, popularly called, as “Burma mangrove” is a prominent board leaved mangrove species in Indian mangrove forests. It belongs to the family Rhizophoraceae and is considered as a true non-secreting salt resistant tree mangrove. This mangrove vegetation is mainly seen at the deltaic regions of Mahanadi river and Bhitarkanika in the coastal Orissa, which is the second largest mangrove formation in India, covering around 211 sq. km. It thrives under a broad range of intertidal conditions, having fluctuations in salinity level from near fresh water to full strength sea water and is also known to tolerate a range of flooding condition and varied soil types (Selvam *et al.*, 2002).

Correspondence and reprint requests : Anath Bandhu Das
Telephone: 00-91-674-2557925; Fax: 00-91-674-2550274

Effect of salt stress on plants is complex but largely it imposes a water deficit condition because of osmotic effects on a wide variety of metabolic activities (Greenway and Munns, 1980; Cheeseman, 1988). To avoid this adverse condition, the mangroves synthesize osmotically active metabolites, specific salt-inducible proteins and regulate water fluxes and support scavenging oxygen radicals and chaperons (Jitesh *et al.*, 2006). Soluble protein contents of leaves are usually reduced in response to salinity (Gadallah, 1999; Wang and Nil, 2000; Parida *et al.*, 2002). Agastiana *et al.* (2000) have reported that soluble leaf proteins were increased at low salinity level and decreased at high salinity in mulberry, a glycophyte. It has also been reported that high salinity could decrease several proteins of different molecular weights, such as, 17, 23, 32, 33 and 34 kDa in *B. parviflora* (Parida *et al.*, 2004). Recently, we have shown that among *Bruiguiera* species, *B. gymnorrhiza* remained tolerant to quite high short-term salt shock (Behera and Das 2008). In this communication, we report the detailed changes in leaf and thylakoid proteins profiles upon short-term high salt exposure and reappeared protein bands during recovery from salt stress in *B. gymnorrhiza*. One of the most salt-sensitive proteins of thylakoid is the 33 kDa manganese stabilized protein in the oxygen evolving complex of PS-II (Sugihara *et al.*, 2000). Our protein profiling analysis showed that besides this protein, other proteins and pigments are affected and altered by changes in salt concentration due to tidal effects.

MATERIALS AND METHODS

Plant material and growth conditions

Propagules of *B. gymnorrhiza* were collected from Bhitarkanika mangrove forest of Orissa, India (latitude 20°4' to 20°8' N and longitude 86°45' to 87°50' E). Seedlings were raised for one year in the experimental nursery (1:1, sand:clay) under non-saline condition and were exposed to daylight with photosynthetic active radiance PAR of 1220-1236 ($\mu\text{mole m}^{-2}\text{s}^{-1}$) before testing to hydroponic culture medium. One-year-old healthy seedlings of uniform size were selected for hydroponic culture in Hoagland's nutrients medium (Hoagland and Arnon, 1940) supplemented with 500 mM of NaCl and a pH of 5.8. Plants were directly exposed to 500 mM NaCl with out any prior gradual acclimatization in different salt concentrations following the procedure of Parida *et al.* (2005). The hydroponic cultures were maintained in a growth chamber under a photoperiod of 14h at light intensities of $300 \mu\text{mole m}^{-2}\text{s}^{-1}$ at $22 \pm 2^\circ\text{C}$

temperature and 80 % relative humidity (RH). The cultures were aerated continuously with air bubblers as mentioned earlier by Parida *et al.* (2002). The nutrient solution was replaced with freshly prepared solution at 2d intervals. The fourth pair of leaves from the apex of the growing shoots was harvested after 0, 2, 4, 6d of the treatment from control and salt treated (500 mM NaCl) plants for the isolation of total soluble leaf and thylakoid proteins. These salt-treated plants were transferred to fresh Hoagland's medium (without NaCl) for 7 more days for recovery.

Extraction and estimation of total leaf protein

Leaf tissues (0.5 g) were homogenized with 3 ml of a buffer containing 50 mM Tris (hydroxymethyl amino methane) and glycine, (pH 8.3), 0.5 M sucrose, 50 mM EDTA, 0.1 M KCl, 2 mM PMSF and 0.1 % (v/v) 2-mercaptoethanol in a chilled pestle and mortar at 4°C . The homogenate was centrifuged in a refrigerated centrifuge (Sigma, 2K15, Germany) at 14,000 g for 10 min. Protein concentration in the supernatant samples were estimated according to the method of Bradford (1976) and were stored in small aliquots at -85°C for SDS-PAGE analysis.

Analysis of leaf protein profile by SDS-PAGE

Supernatant samples (40 μg protein) were mixed with equal volumes of solubilizing buffer (62.5 mM Tris-HCl, pH 6.8, 20 % (w/v) glycerol, 2 % (w/v) SDS, 5 % (v/v) 2-mercaptoethanol and 0.01 % Bromophenol blue) and heated for 4 min at 95°C and cooled on ice before loading on 12.5 % SDS polyacrylamide slab gels.

Separating gels (12.5 %) containing 375 mM Tris-HCl, pH 8.8, 0.1 % (w/w) SDS, 0.05 % (w/w) ammonium persulfate and $0.4 \mu\text{l.ml}^{-1}$ TEMED was used for resolving the polypeptides, whereas a 4 % stacking gel containing 125 mM Tris-HCl, pH 6.8, 0.1 % (w/v) SDS, 0.5 % (w/v) ammonium persulfate and $0.5 \mu\text{l.ml}^{-1}$ TEMED was used according to Laemmli, (1970). The electrophoresis running buffer consisted of 25 mM Tris, 192 mM glycine and 0.1 % SDS, pH 8.3. Electrophoresis was accomplished at 35 mA for 4h using a Bio-RAD, protein II XI electrophoresis system. After separating the polypeptides on gel, they were detected by staining with 0.25 % Coomassie Brilliant Blue R-250 (Sigma) dissolved in 50 % methanol and 10 % acetic acid for overnight on a shaker rotated at 80-100 rpm. De-staining of gel was made in methanol : acetic acid : distilled water (5:1:4) until the background was cleared and then fixed in 7 % acetic acid. The protein molecular weight

marker from Bangalore Genei, India was used. The gel was photographed and scanned using a densitometer (GS-710, Bio-Rad, USA) and analyzed in Quantity One software from Bio-Rad.

Extraction and estimation of thylakoid proteins

Thylakoid membranes were isolated from the leaves of control and 500 mM NaCl treated *B. gymnorrhiza* plants according to the procedure of Nakatani and Barber, (1977). Fresh leaves (5 g) from control and NaCl-treated plants were homogenized in chilled isolation buffer containing 0.4 M sorbitol, 15 mM tricine (pH 7.8), and 5 mM MgCl₂ (buffer A), in a tissue homogeniser (Ultra Turax-T₂₅, 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 and the rest of the pellets were washed in a buffer containing 10 mM tricine (pH 7.8), 10 mM NaCl and 5 mM MgCl₂ (buffer B) in order to remove all other soluble cellular proteins, so that the contamination of other proteins were totally minimized. The pellets consisting of thylakoid membranes were suspended in a buffer containing 0.1 M sorbitol, 10 mM tricine (pH 7.8), 10 mM NaCl and 5 mM MgCl₂ (buffer C) to a final chlorophyll (*Chl*) concentration of 1 Kg m⁻². 30 % glycerol was included in buffer C when thylakoid membranes were stored at -85 °C for further use. Chlorophyll contents in thylakoid suspension were estimated using 80 % acetone, following the equation of Lichtenthaler (1987). Estimation of protein content in thylakoid suspension was made following the procedure of Lowry *et al.* (1951).

Resolution of thylakoid polypeptides by SDS-PAGE

Thylakoid membrane samples were mixed with equal volumes of solubilizing buffer (62.5 mM Tris-HCl, pH 6.8, 20 % (w/v) glycerol, 2 % (w/v) SDS, 5 % (v/v) 2-mercaptoethanol and 0.01 % bromophenol blue), heated for 4 min at 95 °C and cooled on ice before loading on 12.5 % polyacrylamide slab gels as described earlier for leaf protein profile analysis.

Urea-SDS-PAGE protein profiling of thylakoid protein

Samples of urea-SDS denaturing polyacrylamide gel electrophoresis (PAGE) were prepared by mixing the pelleted thylakoid membranes in a sample buffer (1 ml sample buffer per mg *Chl*) containing 6 M urea, 2 % SDS (w/v), 2.5 % 2-mercaptoethanol (w/v), 62.5 mM Tris-HCl (pH 6.8) and 20 % glycerol (v/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 XI electrophoresis system (Bio-Rad, USA). Gels were made according to Laemmli, (1970) with some modifications. A 12.5% separating gel containing 2 M urea, 0.1 % (w/v) SDS, 375 mM Tris-HCl (pH 8.8) and 4 µl/ml TEMED was used. Electrophoresis running buffer used consisted of 25 mM Tris, 192 mM glycine and 0.1 % SDS (pH 8.3). The gels were run at a constant current of 35 mA for 4 h. 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 and destained until the background was clear. The gel was photographed and scanned using a densitometer (GS-710, Bio-Rad, USA) and analyzed in Quantity One software from Bio-Rad.

Statistical analysis

Statistical analysis of the results was carried out according to Duncan's multiple range test. Data were subjected to a two-way ANOVA and the LSD at $p=0.01$ was determined following the method of Sokal and Rohlf (1995).

RESULTS AND DISCUSSION

Plant growth, photosynthetic pigments and protein contents

Water potentials and osmotic potentials of the plant become negative with increasing salinity (Morales *et al.*, 1998; Hernandez *et al.*, 2000; Khan *et al.* 2000, Nandy (Datta) and Ghose, 2003; Nandy (Datta) *et al.*, 2005) and tissues lose water to external medium in high salt solution. We measured the fresh weight and dry weight of the leaves to estimate the relative extent of water loss due to salt shock. In control plant samples, the relative water content of leaves decreased marginally (3.5 %) after 6d where as it was significantly decreased by nearly 6 % when treated with NaCl (Table 1). In the control sample, there was no significant change found in both dry and fresh weight of leaves. In treated plants, however, there was about 36.4 % increase in fresh weight and 49.7 % increase in dry weight. This extra 13.3 % increase in dry mass could not be due to an increase in biomass but very likely due to accumulation of salt in leaf tissues. This is in line with the results of Takemura *et al.*, (2000), who detected high level of sodium in the

Table 1. Relative changes in leaf water content on different days of 500 mM NaCl treatment (*n=3)

Days of treatment	Fresh weight of plant (g/leaf $\pm$ SE*)	Dry weight of plant (g/leaf $\pm$ SE*)	Relative water content (%)
0 (Control)	1.362 $\pm$ 0.08	0.456 $\pm$ 0.03	83.65
6 (Control)	1.363 $\pm$ 0.05	0.481 $\pm$ 0.02	80.18
0 (Salt treated)	1.307 $\pm$ 0.11	0.448 $\pm$ 0.03	82.04
6 (Salt treated)	1.783 $\pm$ 0.36	0.671 $\pm$ 0.13	76.00

xylem sap of *Bruguiera gymnorrhiza*. Total *Chl* and thylakoid protein to *Chl* ratio was enhanced on the 2nd day of salt treatment, while no change was noticed in control. However, the pigment contents and *Chl* a/b ratio decreased after 4d and continued till 6d of salt exposure (Table 2), suggesting major changes in light harvesting abilities of photosynthetic apparatus by the short term high salt shock and its rapid recovery upon removal of high salt (Kathiresan and Moorthy 1994).

Relative changes of leaf and thylakoid proteins were depicted in Figure 1. The total soluble leaf proteins showed rapid fluctuation, depending upon the duration of NaCl exposure in relation to thylakoid proteins (Fig. 1). A close look at the results reflects that the leaf protein : thylakoid protein ratio decreased from 1.50 to 0.62 on 6d of NaCl treatment, which is largely due to change in the leaf protein content. On recovery, this ratio increased from 0.62 to 1.66 and this increase was mostly due to the increase in leaf soluble protein. This suggests that the recovery of soluble protein occurred more rapidly in cytosol than the thylakoid.

Fig. 1. Changes in total soluble leaf and thylakoid protein contents in response to 500 mM NaCl treatment. R=7d recovery (see materials and methods).

Like the chlorophylls, the total soluble leaf protein content decreased as a result of NaCl treatment and again no noticeable changes were observed in 6d untreated controls (Fig. 1). The protein content increased noticeably on 2d after salt treatment compared to control, which subsequently declined. The abrupt increase in soluble protein on 2d resulting in high protein to *Chl* ratio might be the outcome of active transcription mechanism in leaf upon high salt treatment which gradually reduced with increasing duration of salt exposure. The loss of protein by high salt suggests that salt treatment induced large losses in protein than in *Chl*. Loss of soluble protein due to salt stress have been reported earlier by us in mangroves (Parida *et al* 2002, 2004, 2005) and others (Nandy (Datta) *et al.* 2005). The changes in the quantity of protein by salt stress and in the soluble protein profile in leaves have been reviewed recently by Das *et al.* (2006). The protein content, however, decreased after 4d of treatment and it continued up to 6d of salt treatment and then showed

Table 2. Effect of 500 mM NaCl treatment on progressive changes in leaf *Chl* content, a/b ratio, protein content and protein/*Chl* ratios in the thylakoids of *B. gymnorrhiza*

Days of Exposure	Total <i>Chl</i> mg/g f.wt.	<i>Chl</i> a mg/g f.wt.	<i>Chl</i> b mg/g f.wt.	<i>Chl</i> a/b mg/g f. wt.	Protein mg/g f. wt.	Protein/ <i>Chl</i> ratio
0	0.288	0.206	0.082	2.510 $\pm$ 0.003	0.600	2.083
2	0.332	0.239	0.093	2.569 $\pm$ 0.020	1.141	3.436
4	0.348	0.255	0.093	2.742 $\pm$ 0.010	1.000	2.873
6	0.179	0.116	0.063	1.841 $\pm$ 0.036	0.276	0.837
+7d Recovery	0.259	0.189	0.070	2.700 $\pm$ 0.029	0.534	0.501

partial recovery from the loss after 7d, when plants were returned to normal Hoagland's medium to recheck recovery from the salt shock. Finally, the plants survived and were re-established in nursery after recovery (data not shown).

Changes in soluble leaf protein SDS-PAGE profile

Earlier studies suggested that salt exposures not only reduce leaf protein contents, but also altered the protein profiles when assayed by SDS-PAGE. We thus analyzed the progressive changes of leaf soluble protein profiles in *B. gymnorrhiza* with and without high salt exposures (Fig. 2). SDS PAGE was run in samples exposed to 500 mM NaCl for 0, 2, 4 and 6 d and then again after 7d of transfer to normal medium. In control samples, under our experimental conditions, there were some 9 to 10 clearly visible protein bands and their number remained unchanged for 6d of experiment. In salt-treated leaf samples, the number of bands after 4d were reduced to 8. The intensity of several protein bands like 10, 16, 23, 33, 37, 42, 44, 50, 49 and 86 kDa decreased as a result of NaCl treatment (Fig. 2). The average density of several protein bands increased on 2d and then decreased up to 6d of salt exposure. Analysis of relative changes in band intensities revealed distinct differential decrease

Fig. 2. Changes in PAGE profile of leaf soluble protein on different days due to high NaCl treatment. M=Molecular weight marker of protein [205kDa=Myosin, Rabbit Muscle; 97.4kDa=Phosphorylase b; 66.0 kDa=Bovine Serum Albumin; 43.0 kDa=Ovalbumin; 29.0 kDa=Carbonic Anhydrase; 20.1 kDa=Soyabean Trypsin Inhibitor; 14.3 kDa=Lysozyme; 6.5kDa Aprotinin; 3.0kDa=Insulin (a and b chains)]. 0dT=0day of treatment, 2dT=2days of treatment, 4dT=4days of treatment, 6dT=6 days of treatment, 7dR=7days of recovery.

upon salt exposures (Table 3). Minor protein bands of apparent molecular mass, 16, 42 and 44 kDa were completely absent after 6d of treatment in 500 mM NaCl, but interestingly it reappeared after 7d of transfer to material to normal Hoagland's medium used for recovery (Fig. 2). The disappearance and reappearance of some leaf proteins due to high salt concentrations seemed to be a strategy for salt tolerance in mangroves (Parida and Das, 2005, Mitra *et al.*, 2006). Furthermore, a major 33 kDa protein band reduced significantly in 6d salt treatment which could not reappear or reappeared very slowly (Fig. 2). The nature and localization of these proteins would be of interest and importance to elucidate salt adaptation mechanism in tree mangrove.

Thylakoid polypeptide profiles analyzed by SDS-PAGE and urea-SDS PAGE

Since very significant loss in *Chl* content and alterations in *Chl* a/b ratio were observed by high salt treatment, thylakoid protein profiles in both SDS and urea SDS PAGE (Figs. 3 and 4) were also analyzed that showed the changes in SDS PAGE profile in control and treated thylakoids of plants exposed to 0, 2, 4 and 6d and in recovering plants after removal from high salt to normal situation (Fig. 3). The intensity of protein bands of apparent molecular mass of 10, 16, 23, 33, 37, 50 and also of 86 kDa decreased as a result of NaCl exposure. No significant changes were found in the number of protein bands by salt treatment. Very interestingly, it

Fig. 3. Changes in PAGE profile of leaf thylakoid proteins leaf on different days due to high NaCl treatment. M=Molecular weight marker of protein, 0dT=0day of treatment, 2dT=2days of treatment, 4dT=4days of treatment, 6dT=6 days of treatment, 7dR=7 days of recovery.

Table 3. Relative changes in average density of leaf protein bands in response to 500 mM NaCl treatment.

Treatments	Protein bands with relative density								
	90kDa	50kDa	44kDa	42kDa	37kDa	33kDa	23kDa	16kDa	10kDa
Control	1	1	1	1	1	1	1	1	1
2d	1.35	1.06	1.56	1.14	0.67	1.32	1.35	0.887	1.02
4d	1.18	0.87	1.40	0.88	0.44	0.82	0.84	0.61	0.74
6d	0.97	0.71	1.15	0.77	-	0.85	0.73	0.35	0.467
7d Recovery	1.27	0.71	1.28	0.91	0.72	0.51	0.99	0.59	0.55

was noted that the 33kDa thylakoid protein band reappeared prominently unlike the leaf 33 kDa protein band. Thus, it is suggested that although the molecular mass of 33 kDa protein band of leaf and thylakoids protein band are the same but are not of same functional characteristics (Figs. 2 and 3). It appears that some rapidly turning over cytosolic proteins are indicative of salinity levels in mangroves than the thylakoid proteins. The earlier reported 33 kDa protein of the light harvesting complex of PS II by Sugihara *et al.* (2000) in *B. gymnorrhiza* may not be the same as we observed in this experiment. Further analysis of light harvesting complexes is required for elucidating changes in pigment protein complexes by salt shock.

The thylakoid membranes isolated from control and NaCl-treated leaves were mixed in SDS-sampling buffer containing 6 M urea to minimize aggregations of proteins. The intensity of many protein bands ranging from 10 to 86 kDa showed extensive decrease in NaCl-treated samples as compared to the control thylakoid sample. The progressive decrease in protein bands due to increase in duration of salt exposure suggested that the target site of salt stress could be the degradation of certain specific thylakoid proteins. The nature of the dissociations of complexes and proteolytic degradation of proteins need to be investigated for high salt shock-induced changes in light harvesting complexes of chloroplasts.

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Fig. 4. Changes in urea-SDS PAGE profile of thylakoid protein on different days due to high NaCl treatment of *B. gymnorrhiza*. M= Molecular weight marker of protein, 0dT=0day of treatment, 2dT=2days of treatment, 4dT=4days of treatment, 6dT=6 days of treatment, 7dR= 7days of recovery

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