

# High Salt Stress in Coupled and Uncoupled Thylakoid Membranes: A Comparative Study

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**Abstract**—The effect of high salt concentration on photosystem II (PS II) electron transport rates and chlorophyll *a* fluorescence induction kinetics was investigated in coupled and uncoupled spinach thylakoid membranes. With increase in salt concentration, the rates of electron transport mediated by PS II and the  $F_v/F_m$  ratio were affected more in uncoupled thylakoids as compared to coupled thylakoid membranes. The uncoupled thylakoid membranes seemed to behave like coupled thylakoid membranes at high NaCl concentration (~1 M). On increasing the salt concentration, the uncoupler was found to be less effective and  $\text{Na}^+$  probably worked as a coupling enhancer or uncoupling suppressor. We suggest that positive charge of  $\text{Na}^+$  mimics the function of positive charge of  $\text{H}^+$  in the thylakoid lumen in causing coupled state. The function of NaCl (monovalent cation) could be carried out by even lower concentration of  $\text{Ca}^{2+}$  (divalent cation) or  $\text{Al}^{3+}$  (trivalent cation). We conclude that this function of NaCl as coupling enhancer is not specific, and in general a positive charge is required for causing coupling in uncoupled thylakoid membranes.

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**Key words:** high salt stress, uncoupler, PS II and PS I, electron transport, thylakoid membranes

High salt stress is a major factor limiting crop productivity. Reduction in plant growth and thereby reduced crop yield due to salt stress is often associated with a decrease in photosynthesis. Photosynthesis is the process by which green plants, eukaryotic algae, cyanobacteria, and certain other prokaryotes convert light energy to chemical energy. It is comprised of two reactions, i.e. a light and a dark reaction. The primary components of the light reaction are photosystem I (PS I) and photosystem II (PS II). PS II is a multisubunit integral membrane protein complex used by higher plants and cyanobacteria to catalyze water oxidation and plastoquinone reduction [1]. The central core of PS II is composed of a heterodimer of D1 and D2 proteins, the two chlorophyll (Chl) proteins CP43 and CP47, Cyt  $b_{559}$ , and a water oxidation complex or oxygen evolving complex at the lumenal side consisting

of four Mn atoms per reaction center (RC), a 33 kDa manganese stabilizing extrinsic protein, a 17 kDa extrinsic protein, and a 24 kDa accessory protein [2]. PS I is predominantly found in non-appressed region of thylakoid membrane. PS I acts as a light-driven plastocyanine:ferredoxin oxidoreductase enzyme [3] consisting mainly of RC and light harvesting complex I [4].

The main function of PS II is to drive electron transfer from the water oxidizing manganese cluster at the lumenal side to plastoquinone at the stromal side of the thylakoid membrane leading to formation of a transmembrane proton gradient [5]. The positive charge produced by PS II oxidizes water to molecular oxygen, while the negative charge reduces plastoquinone to plastoquinol. Four protons from two molecules of water are released in the lumen of the thylakoid membrane. Plastoquinol also releases protons into the thylakoid lumen during the transport of an electron to the Cyt  $b_6f$  complex. The proton gradient formed across the thylakoid membrane by the electron transport is used to synthesize ATP.

PS II is more susceptible to stresses like pH, temperature, drought, nutrient deficiency, radiation, osmotic stress, and high salt stress as compared to PS I [6]. The high salt stress leads to a series of changes in biosynthetic

*Abbreviations:* Chl, chlorophyll; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DCPIP, 2,6-dichlorophenol indophenol; ET, electron transport flux;  $F_m$ , maximum fluorescence;  $F_v$ , variable fluorescence; PS I (II), photosystem I (II);  $Q_A$  ( $Q_B$ ), the primary (secondary) quinone acceptor; RC, reaction center; TR, photon trapping index.

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functions including photosynthesis, photorespiration, and amino acid and carbohydrate synthesis [7-9]. Salt stress inhibits PS II activity in higher plants [10]. In *Synechococcus* cells, 0.5 M NaCl inactivates both PS II and PS I mediated electron transport rates due to change in K/Na ratio [11]. Salt stress also decreases the efficiency of PSII ( $\Phi_{PSII}$ ), the efficiency of excitation energy captured by open PS II reaction centers ( $F_v'/F_m'$ ), and the coefficients of photochemical quenching, but causes an increase in non-photochemical quenching [12]. Increase in sodium can cause ion toxicity and membrane instability due to calcium displacement [13].

The rate of electron transport is coupled to proton translocation in the thylakoid lumen. Higher concentration of protons in the thylakoid lumen decreases the electron transport rates. Whether this decrease is due to the decrease in luminal pH [14] or due to the accumulation of positive charge in the thylakoid lumen is yet to be resolved. Uncoupling is generally due to the removal of a proton from the thylakoid lumen dissipating the proton gradient and resulting in a large increase in electron transport rates of PS II [15]. In the present work, we have determined the effect of high salt stress in coupled and uncoupled thylakoid membranes in order to understand the role of sodium ions and other cations as coupling enhancer.

## MATERIALS AND METHODS

**Preparation of thylakoid membranes.** Thylakoid membranes were prepared from market spinach (*Spinacia oleracea*) following the method described in [16]. All steps were performed at temperature between 0 to 4°C in dim green light. Thylakoid membranes were stored in 50% glycerol in liquid nitrogen until further use. The chlorophyll concentration was estimated as described in [17].

**Salt treatment.** Stored thylakoid membranes were thawed slowly at 0-4°C and washed with medium (50 mM Mops-NaOH, pH 7.5) to give osmotic shock. They were then suspended in 0.33 M sucrose and 50 mM Mops-NaOH (pH 7.5). The suspended thylakoid membranes were then incubated in the dark for 15 min in various concentration of salt (NaCl), i.e. starting from 50, 100, 200, 400, 600, 800, and 1000 mM. Such salt-treated thylakoid membranes were used to measure various parameters.

**PS II mediated electron transport rates.** PS II mediated electron transport rates were measured in terms of  $\mu$ moles of dye reduced/mg Chl per hour by measuring photoreduction of the dye 2,6-dichlorophenol indophenol (DCPIP) ( $H_2O \rightarrow DCPIP$ ) spectrophotometrically (Perkin-Elmer Bio 10 spectrophotometer (USA)) at 605 nm. The reaction mixture was composed of 0.33 M sucrose, 50 mM Mops-NaOH, pH 7.5, 35  $\mu$ M DCPIP, and thylakoid suspension equivalent to 10  $\mu$ g Chl/ml. The

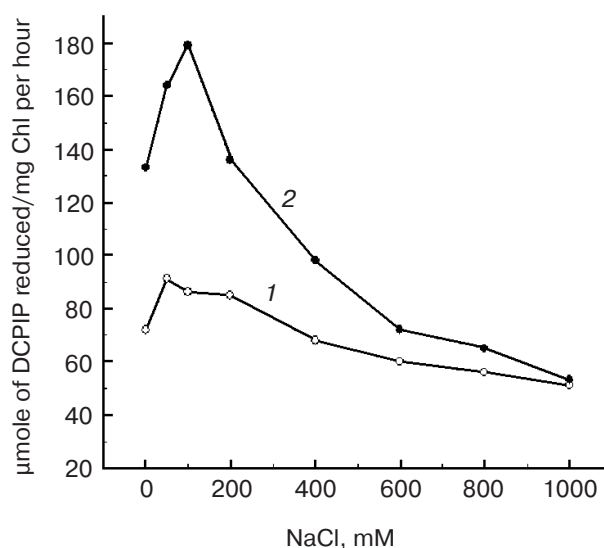
reaction mixture for uncoupled thylakoid membranes consisted of the same composition as above along with 10 mM  $NH_4Cl$ .

**Fluorescence.** The chlorophyll *a* (Chl *a*) fluorescence induction kinetics was measured using a plant efficiency analyzer (PEA; Hansatech, UK). The reaction mixture consisted of 0.33 M sucrose, 50 mM Mops-NaOH, pH 7.5, and thylakoid suspension equivalent to 10  $\mu$ g Chl/ml.

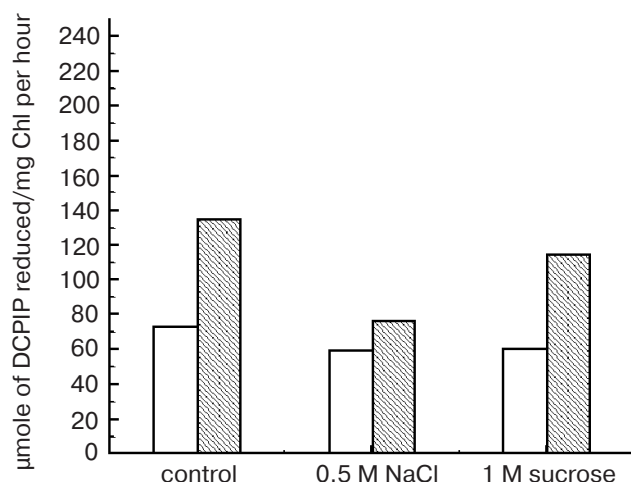
## RESULTS AND DISCUSSION

The PS II mediated electron transport rates were measured in coupled and uncoupled thylakoid membranes. The concentration response for uncoupler ( $NH_4Cl$ ) on PS II and PS I mediated electron transfer was studied and the optimum concentration was found to be 10 mM (data not shown). The function of uncoupler is removal of protons from the thylakoid lumen, which delinks electron transport from phosphorylation, which significantly increases the rate of electron transport [18].

Increase in salt concentration caused a significant decrease in the rates of PS II mediated electron transfer in uncoupled thylakoid membranes (Fig. 1), while a very small decrease was observed in case of coupled thylakoid membranes. Increasing the salt concentration from 50 to 100 mM NaCl increased the PS II electron transport rates ( $H_2O \rightarrow DCPIP$ ). The majority of studies on regulation of photosynthetic processes by cations have been made using 100-200 mM monovalent cation and 5-20 mM divalent cation. At salt concentration up to 200 mM



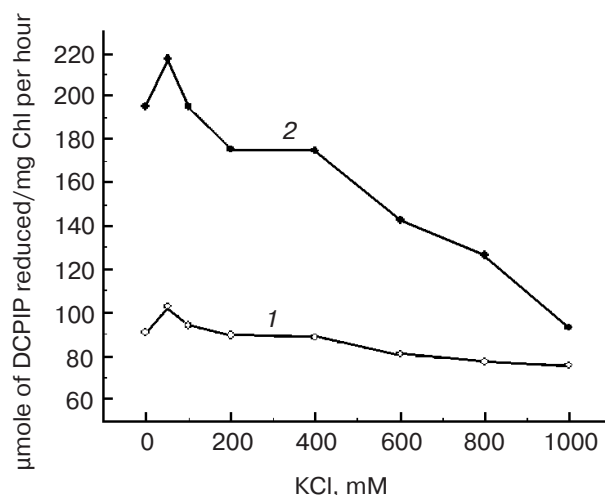
**Fig. 1.** Effect of high salt (NaCl) concentration on Hill activity in coupled (1) and uncoupled (2) thylakoid membranes (10 mM  $NH_4Cl$  was used as the uncoupler). Reaction mixture as described in "Materials and Methods".



**Fig. 2.** Effects of 1 M sucrose and 0.5 M NaCl on Hill activity in coupled (open bars) and uncoupled (filled bars) thylakoid membranes (10 mM  $\text{NH}_4\text{Cl}$  was used as the uncoupler). Reaction mixture as described in "Materials and Methods".

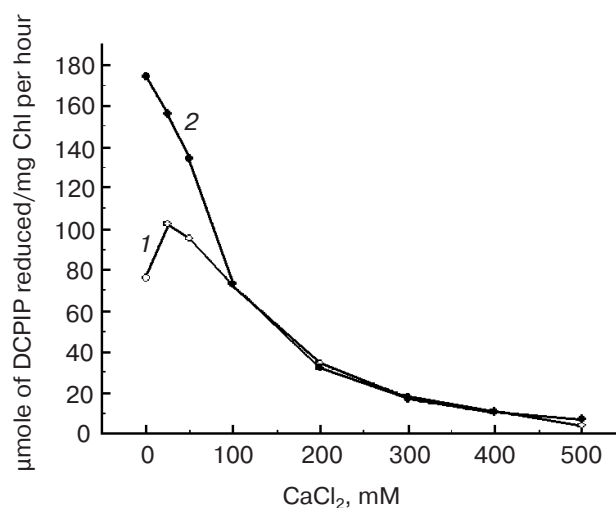
(monovalent cation), cation effects were shown to be predominant [19, 20]. They increase PS II rates by reorganizing the net charge on the thylakoid membrane. However, as a result of charge imbalance, the PS II electron transport rates start decreasing on further increase in NaCl concentration ( $>200$  mM) and at 1 M NaCl electron transport rate in coupled and uncoupled thylakoid membranes were almost equal ( $\sim 50$  units) indicating that at high NaCl concentration uncoupled thylakoid membranes apparently behave like coupled membranes. The coupled thylakoid membranes were found to be more salt tolerant as compared to uncoupled thylakoid membrane. The effect of uncoupler diminished in the presence of increasing NaCl concentration. In other words, the high NaCl concentration seems to cause an apparent coupling of the uncoupled thylakoid membranes.

Salt stress involves osmotic and ionic components. High salt can show its effects in two ways: either by changing the osmoticum of the medium or by changing the number of ionic species in the medium. In order to differentiate between osmotic and ionic effects caused by high salt, we carried out experiments with 1 M sucrose. Osmotically, 1 M sucrose should behave as 0.5 M NaCl. As shown in Fig. 2, the effects shown by 1 M sucrose on PS II rates are different from the effects shown by 0.5 M NaCl both in coupled and uncoupled thylakoid membranes, i.e. 1 M sucrose causes the same degree of inhibition ( $\sim 16\%$ ) both in coupled and uncoupled thylakoid membranes while 0.5 M NaCl causes  $\sim 16\%$  inhibition in coupled thylakoid membranes and  $\sim 47\%$  inhibition in uncoupled thylakoid membranes. This suggests that the effects of NaCl, which we observed in Fig. 1, are mostly because of the ionic effects and not due to change in osmoticum.

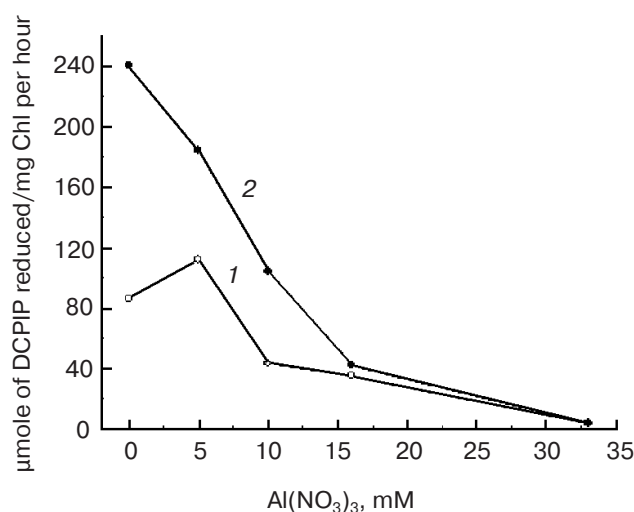


**Fig. 3.** Effect of potassium chloride on Hill activity in coupled (1) and uncoupled (2) thylakoid membranes (10 mM  $\text{NH}_4\text{Cl}$  used as the uncoupler). Reaction mixture as described in "Materials and Methods".

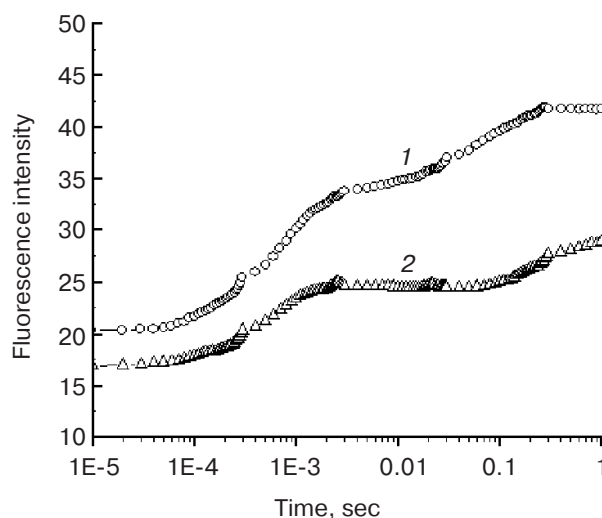
It was argued that if positive charge is responsible for carrying out the coupling action, divalent and trivalent cations should show similar behavior, and that too at lower concentrations than monovalent ions. Experiments were performed with monovalent cation  $\text{K}^+$  ( $\text{KCl}$ ), divalent cation  $\text{Ca}^{2+}$  ( $\text{CaCl}_2$ ), and trivalent cation  $\text{Al}^{3+}$  ( $\text{Al}(\text{NO}_3)_3$ ). The results (Figs. 3-5) show that the uncoupled thylakoids start appearing to behave as coupled thylakoids at  $\text{Ca}^{2+}$  concentration ( $\sim 100$  mM), while in the case of  $\text{Al}^{3+}$  the effect is observed at much lower concentration ( $\sim 15$  mM). This shows that positive charge is required for the coupling of thylakoids and as the charge



**Fig. 4.** Effect of calcium chloride on Hill activity in coupled (1) and uncoupled (2) thylakoid membranes (10 mM  $\text{NH}_4\text{Cl}$  used as the uncoupler). Reaction mixture as described in "Materials and Methods".



**Fig. 5.** Effect of aluminum nitrate on Hill activity in coupled (1) and uncoupled (2) thylakoid membranes (10 mM  $\text{NH}_4\text{Cl}$  used as the uncoupler). Reaction mixture as described in "Materials and Methods".



**Fig. 6.** Fluorescence induction curves of uncoupled thylakoid membranes: 1) control; 2) 600 mM  $\text{NaCl}$  treatment (10 mM  $\text{NH}_4\text{Cl}$  used as an uncoupler). Reaction mixture as described in "Materials and Methods".

density on cation increased, the coupling effect could be seen even at lower concentration of the cation.

#### Chlorophyll *a* fluorescence induction kinetics.

Decrease in PS II electron transport rates in coupled and uncoupled thylakoid membranes in response to high salt stress is supported by Chl *a* fluorescence induction kinetic measurements. A representative fluorescence induction curve is shown in Fig. 6. In this study, we measured minimal level of fluorescence  $F_o$ , the maximal level  $F_m$ , and the fluorescence of variable yield  $F_v$  ( $F_v = F_m - F_o$ ). The initial Chl fluorescence at level "O" ( $F_o$ ) reflects the minimal fluorescence yield when all molecules of  $Q_A$  (primary quinone acceptor) are in the oxidized state.  $F_m$  represents the level when all  $Q_A$  is in the reduced state, and it can be achieved in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU). These

measurements provide information about alteration in PS II, i.e. the decrease in quantum yield of PS II, efficiency of electron transport from  $Q_A^-$  to  $Q_B$  (secondary quinone acceptor), etc. Increase in salt concentration caused decrease in the  $F_v/F_m$  ratio in uncoupled thylakoid membranes thus indicating a significant decrease in the quantum yield of PS II [21, 22]. In coupled thylakoid membranes the  $F_v/F_m$  ratio began to decrease only above 0.6 M  $\text{NaCl}$  concentration, while in uncoupled thylakoid membranes the decrease in the  $F_v/F_m$  ratio was observed from 0.4 M  $\text{NaCl}$  treatment (table). Most of the photons (excitation energy) absorbed by antennae pigments are transferred to the RC as trapping index (TR). In the RCs, the excitation energy is converted to redox energy by reducing  $Q_A$  to  $Q_A^-$ , which is then oxidized to  $Q_A$ , thus leading to an electron transport flux (ET) that

Effect of high salt stress on  $F_v/F_m$  ratio in coupled and uncoupled thylakoid membranes (mean value  $\pm$  S.D.; values in parenthesis show normalized values)

NaCl (mM)	Coupled thylakoid membranes		Uncoupled thylakoid membranes	
	$F_v/F_m$	ET/TR	$F_v/F_m$	ET/TR
Control	$0.59 \pm 0.01$ (100)	$0.50 \pm 0.02$ (100)	$0.54 \pm 0.04$ (100)	$0.48 \pm 0.01$ (100)
100	$0.63 \pm 0.02$ (107)	$0.53 \pm 0.01$ (106)	$0.61 \pm 0.04$ (111)	$0.52 \pm 0.01$ (108)
200	$0.61 \pm 0.01$ (103)	$0.50 \pm 0.01$ (100)	$0.57 \pm 0.03$ (99)	$0.47 \pm 0.01$ (198)
400	$0.61 \pm 0.03$ (103)	$0.50 \pm 0.01$ (100)	$0.50 \pm 0.10$ (91)	$0.43 \pm 0.01$ (90)
600	$0.56 \pm 0.04$ (95)	$0.47 \pm 0.01$ (94)	$0.41 \pm 0.08$ (75)	$0.37 \pm 0.01$ (77)
800	$0.42 \pm 0.04$ (72)	$0.38 \pm 0.01$ (76)	$0.33 \pm 0.05$ (60)	$0.30 \pm 0.01$ (63)
1000	$0.37 \pm 0.09$ (63)	$0.29 \pm 0.01$ (58)	$0.32 \pm 0.10$ (58)	$0.25 \pm 0.01$ (52)

maintains the metabolic reactions of the photosynthetic apparatus [10].

In order to study salt-induced inhibition on the acceptor side of PS II from the fluorescence induction curves, we measured ET per PS II RC and TR. The ratio ET/TR indicates the efficiency with which a trapped exciton can move an electron into the electron transport chain further than  $Q_A^-$  [10]. The reoxidation capacity of  $Q_A^-$  was derived from the fluorescence induction curve. In coupled thylakoid membrane there was no change in ET/TR ( $\Psi$ ) up to 0.6 M NaCl, while in uncoupled thylakoid membranes the value of ET/TR ( $\Psi$ ) was almost constant in samples treated with 0.4 M NaCl and then decreased with increasing NaCl concentration (1 M). A decrease in  $Q_A^-$  reoxidation capacity in uncoupled thylakoid membranes shows that salt stress affects the acceptor side of PS II, i.e. electron transfer from  $Q_A^-$  to  $Q_B$  is restricted, which is not significant in coupled thylakoid membrane, again pointing out that removal of  $H^+$  has resulted in the expression of salt stress. The above observations suggest that the effects of uncoupler were lost in the presence of high concentration of NaCl as well as with other cations of higher valences. Probably high  $Na^+$  or high cation concentration causes apparent coupling in uncoupled thylakoid membranes by providing positive charge. This seems logical if we assume that  $Na^+$  or other cations are fulfilling the requirement of positive charge which otherwise was provided by  $H^+$ . The results delineate the effects of positive charge and of pH in the electron transport chain.

It is well known that in coupled thylakoid membranes the rate of electron transport is slowed down due to proton gradient, which has two components: one of positive charge on the proton and the other of change in pH because of the proton. The proton transport in the thylakoid lumen makes it more acidic as well as increases the number of positive charges in the lumen.  $Na^+$  seems to fulfill the requirement of positive charge but not of pH. The charge density of  $Ca^{2+}$  and  $Al^{3+}$  is much higher than  $Na^+$ , and these cations inhibited the rates of electron transport at much lower concentrations. This led us to propose that inhibition in rate of electron transport is due to ionic charge.

Based on our results we propose a role of positive ions in enhancing the coupled state (coupling enhancer) in uncoupled thylakoid membranes by counteracting the effect of uncoupler (uncoupler suppressor). Thus it seems that it is the charge density in the thylakoid membrane that partly controls the electron transport rates of PS II and PS I.

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