Plant Physiol. (1984) 531–535
0032-0889/84/76/0531/05$01.00/0

Properties of Thylakoid Membranes of the Mangroves, *Avicennia germinans* and *Avicennia marina*, and the Sugar Beet, *Beta vulgaris*, Grown under Different Salinity Conditions[1]

Received for publication December 14, 1983 and in revised form April 20, 1984

MARILYN C. BALL*[2], SCOTT E. TAYLOR, AND NORMAN TERRY
Department of Plant and Soil Biology, University of California, Berkeley, California 94720

ABSTRACT

Properties of thylakoids isolated from leaves of three salt tolerant species, *Avicennia germinans* L., *Avicennia marina* var *resinifera*, and *Beta vulgaris* L., were not affected by the salinity in which the plants were grown. With increase in the growth salinity from 50 to 500 millimolar NaCl, there were no major effects on the per chlorophyll concentrations of lipids or proteins, or on the rates of uncoupled electron transport per chlorophyll mediated by either the whole chain or the partial reactions of photosystems I and II. Responses of the partial and whole chain reactions to variation in the sorbitol and NaCl concentrations in the assay media were independent of the salinity experienced during leaf growth and not substantially different from those of a salt-sensitive species, *Cucurbita sativus* L. Uncoupled rates of electron flow from water to *p*-benzoquinone mediated by photosystem II were insensitive to the NaCl concentration unless thylakoids were rendered Cl^- deficient by treatment with uncoupler under alkaline conditions. Loss of 65% to 85% of the photosystem II activity in these Cl^--deficient thylakoids was restored by addition of 10 to 20 millimolar Cl^-.

Inorganic ions play an important role in the regulation of photosynthetic processes (3). There is some evidence, albeit equivocal, that chloroplasts of salt-tolerant plants may be a site of high salt accumulation, with the levels of Na^+, K^+, and Cl^- reported to be as much as 3 to 5 times greater than those in chloroplasts of salt-sensitive species (9, 11; but see also 8, 15, 16). Thus, the photosynthetic membranes of salt-tolerant species may have to function *in vivo* at high ionic strengths in addition to low water potentials. Differences would therefore be expected in the properties of photosynthetic membranes if the ionic environment of the thylakoids should differ between salt-tolerant and salt-sensitive species to the extent indicated in the literature. Indeed, the Cl^- requirement for PSII has been reported to be two orders of magnitude greater in salt-tolerant than in salt-sensitive plants (4, 5), suggesting that the photosynthetic membranes may be a site of adaptation to high salinities. The present study examines the composition and functional attributes of thylakoids isolated from three salt-tolerant species, *Avicennia germinans*, *Avicennia marina*, and *Beta vulgaris*, grown at a range of salinities, and compares them with those of a salt-sensitive species, *Cucurbita sativus*.

MATERIALS AND METHODS

Plant Material. Propagules of *Avicennia germinans* L. and *Avicennia marina* var *resinifera* were collected from trees at Ormond Beach, FL, and San Diego, CA, respectively. These propagules and seeds of *Beta vulgaris* L. (variety number F58-554-H1) were germinated under greenhouse conditions on sand beds irrigated with tap water. The mangroves were cultivated in this way until they reached a four leaf postcotyledonary phase of development (approximately 4 weeks) and the sugar beets were grown for 2 weeks before the seedlings were transferred to 15-L containers for hydroponic culture in half-Hoagland solution. The plants were grown a further 2 weeks following transplantation before the salinities were adjusted by stepwise increments of 50 mM NaCl every 2nd d to give three final concentrations of 50, 250, and 500 mM NaCl. All measurements were made on fully expanded leaves grown entirely under these final conditions.

Thylakoid Isolation. All preparations were made from a composite of three leaves. Leaves were washed in distilled H_2O, the midvein removed, and sliced by hand into strips 1 to 2 mm wide under ice-cold extraction buffer containing 50 mM Tricine-NaOH (pH 7.8), 10 mM NaCl, 5 mM $MgCl_2$, 0.1% BSA, 5 mM DTT, 1% PVP-40 and either 400 or 1000 mM sorbitol depending on whether the leaves were from plants grown at 50 and 250 mM NaCl or 500 mM NaCl, respectively. The slices were ground by hand with a mortar and pestle and the resulting slurry was filtered through two layers of Miracloth before centrifuging at 480*g* for 2 min. The pellet, which typically contained 50% intact chloroplasts according to the ferricyanide test (12), was resuspended in 10 ml wash buffer which contained 50 mM Tricine-NaOH (pH 7.8), 10 mM NaCl, 5 mM $MgCl_2$ and either 100 or 400 mM sorbitol for preparations from plants grown at 50 and 250 or 500 mM NaCl, respectively. This lowering of the sorbitol concentration was sufficient to lyse the chloroplasts. The osmotic strength was adjusted by addition of sorbitol to give a final concentration of 400 mM, then centrifuged for 2 min at 3000*g*. The pellet was resuspended in wash buffer containing 400 mM sorbitol and stored on ice in the dark.

Chloride-depleted thylakoids from leaves of plants grown with 250 mM NaCl were prepared according to the recommendations of Theg and Homann (19). The leaves were divided down the midrib into two sections so that the effects of two different treatments could be compared with thylakoids isolated from the same leaves. In one group, the uncoupler, 5 mM $(NH_4)_2SO_4$, was included in the isolation and washing buffers. The thylakoid isolation buffer contained 50 mM Hepes (pH 8.1), 5 mM $MgSO_4$, 1% PVP-40, 0.1% BSA, 5 mM DTT, and 400 mM sorbitol. The

[1] Supported by the University of California and the Kearney Foundation for Soil Science.

[2] Current address: Department of Biogeography and Geomorphology, Research School of Pacific Studies, G.P.O. 4, Australian National University, Canberra, A.C.T. 2601 Australia.

pellet was resuspended in buffer containing 50 mM Hepes (pH 8.1), 5 mM $MgSO_4$, and 100 mM sorbitol. The sorbitol concentration was raised to 400 mM before centrifugation and this washing procedure was repeated twice.

Thylakoid Composition. The concentration of Chl in leaf discs and in aliquots of the thylakoid preparations was determined according to Arnon (1).

Lipids were extracted from isolated thylakoids according to Roughan and Batt (17). Lipids were separated by TLC with acetone:benzene:water of 91:30:8 (14) and bands localized by exposure to I_2 (vapor). Galactolipid levels were determined colorimetrically as described in Roughan and Batt (17).

Thylakoid protein levels were determined according to Markwell *et al.* (13) except that the amount of SDS was reduced to 0.25%. Thylakoid proteins were analyzed by LiDS/PAGE[3] using a modification of the system developed by Delepelaire and Chua (6). Proteins were extracted with LiDS and β-mercaptoethanol (Chl:LiDS, 1:180, final concentration 2% β-mercaptoethanol in Tris-Cl, pH 6.8), heated to 80°C for 3 min to ensure denaturation, and electrophoresed on 7.5% to 15% polyacrylamide gradient gels at 4°C. Gels were stained according to Fairbanks *et al.* (7).

Thylakoid Function. Effects of variation in either sorbitol or NaCl concentration on uncoupled rates of electron transport were assayed in a water-jacketed cuvette (total volume, 3 ml) maintained at 25°C. Oxygen consumption or evolution was measured polarographically with a Rank oxygen electrode equipped with a strip chart recorder. The cuvette was illuminated by a quartz-iodide slide projector lamp, which provided an incident quantum flux density of 1000 $\mu E\ m^{-2}s^{-1}$. Thylakoids were incubated in the assay solution for 30 s in the dark before assays were commenced.

Whole chain-mediated electron flow from water to MV was determined by monitoring oxygen uptake. The buffer contained 30 mM $Na_4P_2O_7$ (pH 8.0), 5 mM $MgCl_2$, 10 mM NaCl, 0.2 mM MV, and 2.5 mM NH_4Cl as uncoupler.

PSI-mediated electron flow, from ascorbate plus DCIP to MV was determined by monitoring oxygen uptake. The buffer was the same as that used in the assay for whole chain activity plus 1 mM Na-ascorbate and 0.1 mM DCIP.

PSII-mediated electron flow from water to pBQ was determined by measuring oxygen evolution. The buffer contained 50 mM Hepes (pH 7.6), 5 mM $MgCl_2$, 10 mM NaCl, 1 mM EDTA, 0.25 mM pBQ, and 2.5 mM NH_4Cl as uncoupler.

Chloride-deficient thylakoids were assayed for PSII activity in buffer containing 50 mM Hepes (pH 8.1), 5 mM $MgSO_4$, and 5 mM $(NH_4)_2SO_4$, as an uncoupler. The NaCl concentration was varied from 0 to 100 mM and the corresponding concentrations of sorbitol ranged from 400 to 200 mM, respectively, to maintain a constant osmotic strength. Thylakoids were incubated in the assay solutions for 5 min in the dark at room temperature before assays were commenced.

RESULTS

Composition. The Chl concentration in intact leaves of the three salt-tolerant species was differentially influenced by increase in the growth salinity from 50 to 500 mM NaCl (Table I). There was a significant decrease ($P < 0.05$) in the concentration of Chl per leaf area with increase in the salinity in which the two mangrove species were grown, with *A. marina* being more sensitive to salinity treatment than *A. germinans*. In contrast, the Chl content of leaves of *B. vulgaris* was not significantly affected ($P < 0.05$) by growth under different saline conditions.

Table I. *Concentration of Chl in Leaves of Salt-Tolerant Species Grown in Nutrient Solution Containing NaCl*

Values are mean ± SD, $n = 8$. Only those pairs of points marked by the same letter within a species are significantly different (*t* test, $P < 0.05$).

Species	Growth Salinity	Chl/Leaf Area	Chl *a*/Chl *b*
	mM NaCl	*μmol m⁻²*	*mol mol⁻¹*
A. germinans	50	674 ± 77b	3.5 ± 0.4
	250	718 ± 104a	3.6 ± 0.2
	500	546 ± 39a,b	3.8 ± 0.4
A. marina	50	811 ± 109a,b	3.1 ± 0.2
	250	496 ± 82a	3.5 ± 0.3
	500	405 ± 167b	4.1 ± 0.8
B. vulgaris	50	519 ± 59	3.6 ± 0.5
	250	559 ± 38	3.3 ± 0.2
	500	553 ± 74	3.6 ± 0.5

Table II. *Effect of Growth Salinity on Thylakoid Composition*

All values are mean ± SD, $n = 4$ except *, $n = 3$. None of the pairs of points within a species are significantly different (*t* test, $P > 0.05$).

Species	Growth Salinity	Protein/ Chl	Galactolipid/ Chl	MGDG/ DGDG
	mM NaCl	*g mmol⁻¹*	*mol mol⁻¹*	
A. germinans	50	1.5 ± 0.6	1.4 ± 0.5	2.2 ± 0.2
	250	1.1 ± 0.7*	2.1 ± 0.5*	1.6 ± 0.2*
	500	1.3 ± 0.7*	2.5 ± 0.7	1.8 ± 0.3
A. marina	50	1.1 ± 0.1	2.0 ± 0.6	1.5 ± 0.4
	250	1.1 ± 0.2*	1.6 ± 0.6	1.7 ± 0.5
	500	1.4 ± 0.1*	1.9 ± 0.7	1.6 ± 0.3
B. vulgaris	50	0.9 ± 0.1*	1.9 ± 0.2*	1.8 ± 0.2*
	250	1.0 ± 0.1*	2.3 ± 0.8*	2.4 ± 0.3*
	500	1.0 ± 0.1	1.6 ± 0.5	2.2 ± 0.7

Several lines of evidence indicate that the growth salinity had little apparent effect on the composition of thylakoids isolated from the leaves of salt-tolerant plants. First, the ratios of protein/Chl, galactolipid/Chl, and MGDG/DGDG in isolated thylakoids were relatively constant (Table II). Second, examination of thylakoid polypeptides by LiDS-PAGE showed differences in the polypeptides between species, but not between salinity treatments within a species (Fig. 1). This contention was supported by densitometer scans of these Coomassie-blue stained gels. However, there was some evidence of a response to increased salinity in the 'a' and 'b' polypeptides (*i.e.* those at 58 and 14 kD, respectively) isolated from the *Avicennia* species. The mol wt of these bands suggests that they were subunits of ribulose-1,5 bisphosphate carboxylase- oxygenase. Finally, the rates of uncoupled photosynthetic electron transport per Chl (Table III) were not substantially affected by the salinity in which the plants were grown. These rates of electron transport were measured during the winter and are as much as 2 times lower than similar measurements made in the summer (data not shown).

Functioning. The influence of the growth salinity on functional attributes of the thylakoid membranes was examined by measuring rates of uncoupled electron transport *in vitro* in relation to the sorbitol and NaCl concentration in the assay media. Rates of uncoupled electron transport from ascorbate and DCIP to MV mediated by PSI were insensitive to variation in the sorbitol

[3] Abbreviations: LiDS-PAGE, lithium dodecyl sulfate-polyacrylamide gel electrophoresis; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DCIP, dichloroindophenol; DGDG, digalactosyldiglyceride; MGDG, monogalactosyldiglyceride; MV, methyl viologen; pBQ, *p*-benzoquinone.

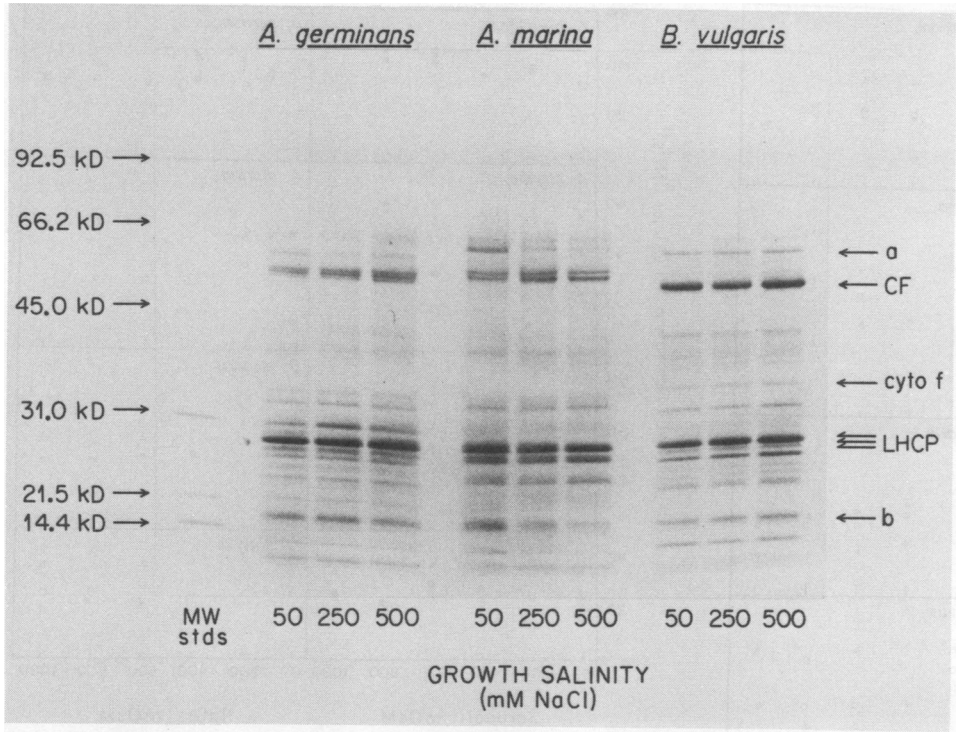

FIG. 1. LiDS-PAGE analysis of polypeptides extracted from isolated thylakoids of *A. germinans*, *A. marina*, and *B. vulgaris* grown at different salinities. a,b: subunits of ribulose,1-5 bisphosphate carboxylase-oxygenase; CF: coupling factor; cyto f: Cyt *f* (*c* type); LHCP: light-harvesting Chl-protein complex.

Table III. *Rates of Uncoupled Photosynthetic Electron Transport by Thylakoids Isolated from Leaves of A. germinans, A. marina, and B. vulgaris Grown in Different Salinity Conditions*

Values are mean ± SD, $n = 3$. The assay media had a total osmotic strength of 500 mOsM, obtained by buffer containing 10 mM NaCl and 400 mM sorbitol. Only those pairs of points marked by the same letter within a species are significantly different (t test, $P < 0.05$).

Species	Growth Salinity	Oxygen Exchange Rate		
		Ascorbate + DCIP → MV ($O_2\downarrow$)	$H_2O \rightarrow$ pBQ ($O_2\uparrow$)	$H_2O \rightarrow$ MV ($O_2\downarrow$)
	mM NaCl	*mmol O_2 mol^{-1} Chl s^{-1}*		
A. germinans	50	76.7 ± 9.0	37.8 ± 3.8	14.6 ± 3.5a
	250	66.5 ± 7.6	39.3 ± 2.9	17.4 ± 3.5
	500	90.4 ± 31.5	48.0 ± 9.9	24.2 ± 6.2a
A. marina	50	68.1 ± 8.5	30.5 ± 1.2a	15.1 ± 0.7
	250	75.4 ± 7.8	43.7 ± 5.4a	15.8 ± 1.7
	500	74.9 ± 14.7	33.1 ± 7.1	11.8 ± 3.3
B. vulgaris	50	91.2 ± 7.8a	64.9 ± 15.4	31.2 ± 4.7a
	250	113.5 ± 10.9a,b	57.0 ± 8.1	32.2 ± 2.6b
	500	77.2 ± 10.9b	49.5 ± 6.4	23.9 ± 2.3a,b

concentration from 0 to 1000 mM, but were diminished approximately 30% with increase in NaCl concentration from 0 to 500 mM (Fig. 2). Variation in the concentration of sorbitol and NaCl had no substantial effects on the rates of uncoupled electron transport mediated by PSII from water to pBQ, an uncharged, lipophilic electron acceptor (Fig. 3). Rates of uncoupled electron transport from water to MV mediated by PSI + PSII were enhanced by increase in the sorbitol concentration from 0 to 600 mM, with higher concentrations having little additional effect on electron transport activity in thylakoids of the salt-tolerant species (Fig. 4). Rates of whole chain electron transport by thylakoids of *Cucurbita sativus* were not as sensitive to variation in the sorbitol concentration. Increase in the NaCl concentration over the same range of osmotic strengths had little effect on rates of uncoupled electron transport by the whole chain system in either salt-tolerant or salt-sensitive species (Fig. 4). These data show that the responses of PSI (Fig. 2), PSII (Fig. 3), and PSI + PSII (Fig. 4) to variation in sorbitol or NaCl were independent of the salinity in which *A. germinans*, *A. marina*, and *B. vulgaris* were grown (see also Table III) and were not substantially different from those of a salt-sensitive species, *C. sativus*.

Chloride Dependency of PSII. The relative insensitivity of PSII to the NaCl concentration in the assay medium (Fig. 3) was unexpected in view of recent work (4, 5) which proposed that thylakoids isolated from salt-tolerant species may require as much as 500 mM Cl^- for normal PSII activity. We investigated this further. Thylakoids which were isolated from *A. germinans*, *A. marina*, and *B. vulgaris*, grown with 250 mM NaCl and washed repeatedly in Cl^--free buffer showed no loss of PSII activity and no response to the addition of Cl^- to the assay medium (Fig. 5). However, PSII activity was depressed approximately 65% and 85% in thylakoids of the two mangrove species and in those of *B. vulgaris*, respectively, isolated and washed under alkaline conditions in the presence of uncoupler, 5 mM $(NH_4)_2SO_4$. The PSII activity of these Cl^--deficient thylakoids was substantially restored by addition of 10 to 20 mM Cl^- to the assay mixture (Fig. 5). Similar results were obtained with thylakoids from plants grown with either 50 or 500 mM NaCl (data not shown).

DISCUSSION

The present study shows that thylakoid membranes do not undergo adaptive changes during growth of salt-tolerant higher plant species under saline conditions. This conclusion is supported by three lines of evidence. First, a 10-fold increase in the growth salinity from 50 to 500 mM NaCl had little apparent effect on the composition of photosynthetic membranes (Fig. 1; Tables I, II, and III). Second, there is no convincing evidence that the photosynthetic membranes of salt-tolerant species function maximally at the low water potentials which characterize the leaves of plants grown under saline conditions (Figs. 2–4) consistent with previous studies (18, 20). Finally, there is no convincing evidence in the present or previous studies (20) that

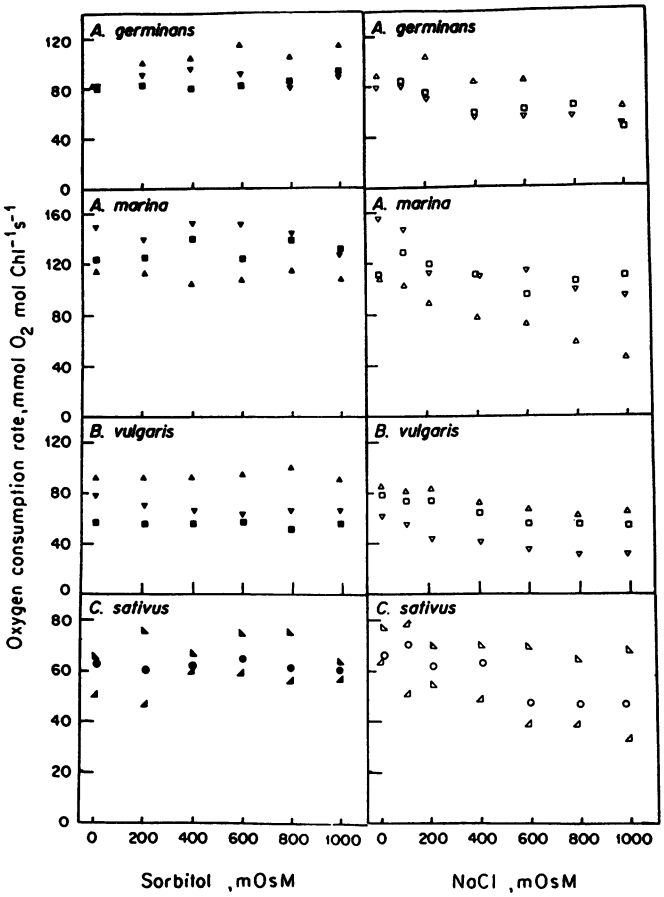

FIG. 2. Effect of sorbitol and NaCl on rates of uncoupled electron transport mediated by PSI from ascorbate plus DCIP to MV. Thylakoids were isolated from leaves of *A. germinans*, *A. marina*, and *B. vulgaris* grown in nutrient solution containing 50 (■, □), 250 (▲, △), and 500 (▼, ▽) mM NaCl and from *C. sativus* grown without NaCl, each different symbol denoting an independent thylakoid preparation.

the photosynthetic membranes of salt-tolerant species function maximally at higher NaCl concentrations than those of a salt-sensitive species (Figs. 2–4). These results are consistent with recent reports that the ionic composition of chloroplasts does not differ between salt-tolerant and salt-sensitive species (7, 15, 16).

In contrast, high concentrations of NaCl have been reported to stimulate both PSI and PSII in *A. marina* and other salt-tolerant species, whereas these partial reactions were inhibited by increasing levels of NaCl in *C. sativus* (4, 5). Further, the Cl^- requirement for functioning of PSII (10) has been reported to increase by two orders of magnitude to as much as 500 mM Cl^- in thylakoids of salt-tolerant species, including *A. marina* (4, 5). However, the results of these studies were obtained with thylakoids which could not mediate electron flow from water to MV and showed DBMIB-insensitive reduction of ferricyanide (4), clearly indicating that the membranes were fragmented. This could account for the difference between these results and those of the present study.

The present study shows that PSII activity as indicated by pBQ dependent oxygen evolution, is insensitive to brief exposures to different NaCl concentrations (Fig. 3) even after repeated washings in Cl^- free media (Fig. 5). As the thylakoids are permeable to NaCl (2), the more than 20 min required for the preparation of these washed thylakoids would be sufficient to remove all but the membrane bound Cl^-. The latter apparently is very tightly bound, but the membranes can be rendered Cl^--deficient by

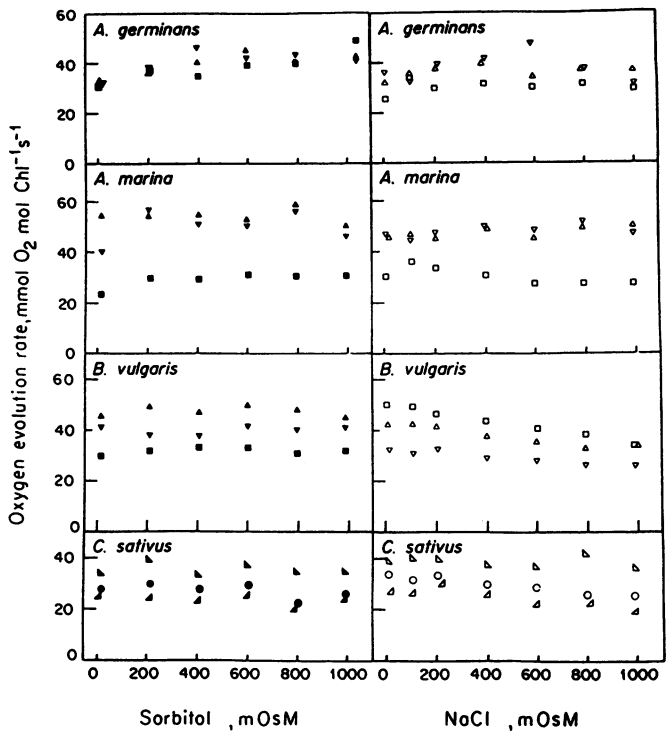

FIG. 3. Effect of sorbitol and NaCl on rates of uncoupled electron transport mediated by PSII from water to pBQ. Symbols as in Figure 2.

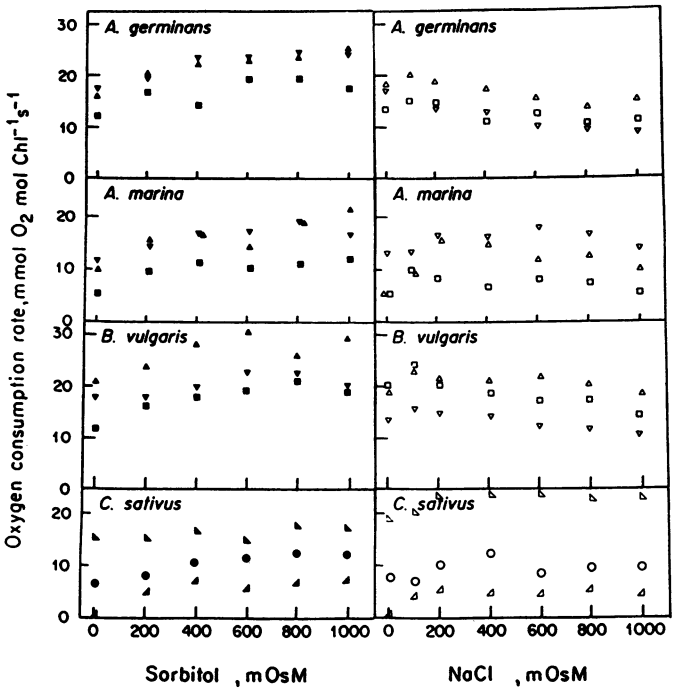

FIG. 4. Effect of sorbitol and NaCl on rates of uncoupled electron transport mediated by the whole chain (PSI plus PSII) from water to MV. Symbols as in Figure 2.

treatment with uncoupler under alkaline conditions (19). The resulting loss of from 65% to 85% of the PSII activity could be restored by the addition of only 10 to 20 mM NaCl (Fig. 5) consistent with recent findings of Theg and Homann using spinach (19). Of course, it is uncertain how much functional Cl^- remained in the washed membranes, and thus an estimate of the Cl^- requirement can not be made from these data. Nevertheless, it is clear that the Cl^- responses did not differ substantially from

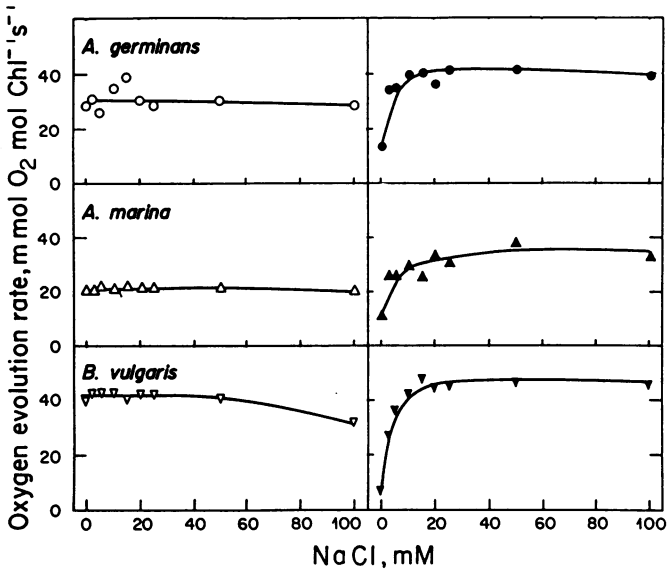

FIG. 5. Effect of Cl^- concentration on rates of uncoupled electron transport mediated by PSII from water to pBQ. Thylakoids were isolated in Cl^--free buffer with (●, ▲, ▼) or without (○, △, ▽) uncoupler, 5 mM $(NH_4)_2SO_4$, as described in "Materials and Methods."

those of similarly treated salt-sensitive plants.

In conclusion, the present study shows that the concentration of Chl per leaf area may be influenced by the salinity in which salt-tolerant species are grown. However, variation in salinity does not induce changes in the attributes of the photosynthetic membranes of salt-tolerant species which would distinguish them from those of salt-sensitive species or which would identify them as sites of adaptation to high internal salt concentrations.

Note Added in Proof. Bertil Andersson *et al.* (1984 FEBS Lett 168: 113–117) have elegantly shown that the requirement of as much as 500 mM Cl^- for maximum rates of photosynthetic O_2 evolution in thylakoids isolated from *A. marina* according to Critchley (4, 5) was due to the loss of the 23 kD polypeptide during the isolation procedure.

Acknowledgments—It is a pleasure to thank Mr. Cliff Carlson for plant culture, Drs. A. A. Benson and Howard Sleeper for donation of the *Avicennia marina* propagules, and Drs. Jan Anderson and Ivan Ryrie for their thoughtful comments on the manuscript.

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