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ORIGINAL RESEARCH

External K^+ Deficiency Inhibits Photosynthetic Activity Through Superoxide Anion Production in Protoplasts Isolated from the Thallus of *Ulva pertusa*

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Abstract To investigate the effects of potassium on photosynthetic activity in the green alga *Ulva pertusa*, we enzymatically isolated protoplasts from thallus samples. Photochemical quenching showed that protoplasts were capable of electron transport by photosystem II (PS II) during illumination. This quenching was dependent on external pH, with a reduced electron transport rate at pH >6.8 and less ability to use HCO_3^- under alkaline conditions. In the presence of external Na^+, K^+ enhanced PS II quantum yield, indicating a functional role for K^+ during photosynthesis. That yield was enhanced in a [K^+]-dependent manner, with maximum activity at 100 mM. However, potassium alone did not maintain photochemical activity, and its addition supported photosynthetic O_2 evolution only in the presence of Na^+. A deficiency of K^+ led to the production of superoxide anions. Because of that generation, activities of superoxide dismutase and ascorbate peroxidase, two key enzymes involved in scavenging reactive oxygen species in the water–water cycle, also increased during such stress. These results strongly suggest that a series of ROS-scavenging systems are initiated in *Ulva* chloroplasts in response to K^+ deficiency and that enzyme activities might protect algal cell photosynthesis.

Keywords Chlorophyll *a* fluorescence · External pH · K^+ · Na^+ · Photosystem II · Protoplast · Superoxide anion · *Ulva pertusa*

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The ubiquitous genus *Ulva* has been included in numerous physiological studies of marine macroalgae. Species within that genus show suppressed photorespiration because of their O_2-insensitive photosynthetic rates (Beer and Israel 1986) and low CO_2 compensation points (Colman 1984; Maberly 1990). Nonetheless, some species feature C_3 photosynthesis (Beer and Israel 1986), and HCO_3^- uptake may be the basis for a system that internally concentrates inorganic carbon (Beer and Israel 1990). Most organisms examined to date can use CO_2 or HCO_3^-, or both, but species-unique preferences also have been observed (Espie and Kandasamy 1992; Rotatore et al. 1995; Raven 1997; Matsuda et al. 2001; Chen and Gao 2004; Kaplan et al. 2006).

In several species of higher terrestrial plants, potassium is the major driving ion for osmotic water uptake. Thus, K^+-dependent uptake occurs during the growth of *Helianthus*

annuus hypocotyls (McNeil 1976), *Avena sativa* coleoptiles (Stevenson and Cleland 1981), and *Phaseolus vulgaris* leaves (Blum et al. 1992). A similar pattern has been found in the marine red algae *Porphyra umbilicalis* (Lüning 1992) and *Porphyra leucosticte* (Lüning et al. 1997), where uptakes peak at 60 to 90 min after the onset of light. In contrast, the onset of darkness leads to a contraction of the thallus and, therefore, a short-term negative growth rate in both species. Rapid thallus expansion after light-onset could be due to a light-dependent mechanism that increases cell osmotic pressure and the uptake of water by non-dividing but still-growing cells (Lüning 1992; Lüning et al. 1997).

Although plant growth can occur without photosynthesis (van Volkenburgh and Cleland 1990), photosynthesis is necessary for light-driven active transport that is mediated by primary pumps, and for sustained long-term growth of green plants. A reasonable hypothesis for partially reversible thallus expansion after light-onset in *P. leucosticte* is that osmotic water uptake (Lüning 1992) is driven by K^+ fluxes (Escassi et al. 2002). However, Rees (1984) has reported that photosynthetic O_2 evolution in the marine diatom *Phaeodactylum tricornutum* relies on the presence of Na^+ and has suggested that sodium increases inorganic carbon uptake by facilitating HCO_3^- usage. Chen et al. (2006) have presented evidence for K^+-dependent HCO_3^- transport in this alga. However, when *P. tricornutum* is cultured at a pH of 7.2 to 8.0, photosynthetic affinities for HCO_3^- are not affected by potassium. The manner in which K^+ regulates photosynthesis remains unclear, and intensive physiological and biochemical studies are required. Thus, researchers wishing to investigate the unique cellular, or physiological and biochemical, attributes of algal cells must first devise methods by which these attributes may be assessed, either on a single-cell basis and/or in preparations of purified protoplasts. Furthermore, techniques must be developed for the large-scale isolation of protoplasts from *Ulva* species.

In this study, we investigated how photosynthetic activity is influenced by K^+, with or without Na^+, in protoplasts from the green macroalga *Ulva pertusa* by assaying changes in chlorophyll *a* fluorescence. Experiments focused on the relationship between K^+ deficiency stress and the production of superoxide anions. We also established a method for isolating numerous physiologically competent, highly purified protoplasts from thallus tissues.

Materials and Methods

Plant Materials

Samples of *U. pertusa* were collected from the intertidal Songjeong shore of Kyungnam, South Korea (35.1°N, 129.11°E). They were cultivated for up to 3 days in an aerated artificial seawater medium that was prepared by dissolving commercial sea salts (Coralife; Energy Savers, CA, USA) in deionized water to a concentration of 3.5%. Prior to our experiments, culture conditions included 15± 1 C, white light at 60 μmol $m^{-2}s^{-1}$ (FL400; Kum-Ho, Seoul, South Korea), and a 16-h photoperiod. To minimize errors due to seasonal changes, all tests were conducted with materials collected at the same time of year.

Preparation of Protoplasts

Thallus tissues were thoroughly rinsed in deionized water, excised, and minced with a razor blade in a solution of 0.5 M sorbitol, 1 mM CaC_2, and 0.4 M NaCl. After passing through a 200-μm nylon mesh, they were rinsed in that sorbitol solution to remove cell debris and digested in an enzyme solution containing 1.5% cellulase Onozuka RS (Yakult Pharmaceutical Industry Co., Ltd., Tokyo, Japan), 0.5% macroenzyme (Yakult), 0.15% pectolyase Y-23 (Seishin Pharmaceutical Industry Co., Ltd., Tokyo, Japan), 0.5 M sorbitol, 100 mM KCl, 0.4 M NaCl, 1 mM $CaCl_2$, 0.5% BSA, and 1 μmol mL^{-1} of β-mercaptoethanol (pH 5.6). The samples were agitated by shaking (40 rpm) under darkness for 12 h at 15 C to 18 C, as previously described (Goh et al. 1995), albeit with minor modifications. Briefly, optimal times for protoplast release were assessed by microscopic examination of tissues during digestion. Those released protoplasts were passed through a 25-μm mesh and collected via centrifugation (110 g, 7 min, 4 C). They were rinsed three times in 0.5 M sorbitol, 0.4 M NaCl, 100 mM KCl, and 1 mM $CaCl_2$. The isolated protoplasts were then stored in the dark, on ice, in 0.5 M sorbitol and 1 mM $CaC1_2$, with or without 0.4 M NaCl and 100 mM KCl. To analyze the effects of Na^+ and K^+ on photosynthesis, these enzymatically released protoplasts were rinsed three times in 0.5 M sorbitol and 1 mM Ca-gluconate and were then incubated in combinations of NaCl, Na-gluconate, K-gluconate, or gluconate alone.

Assessment of Protoplast Yields and Sizes

Protoplast yields were estimated by counting cell numbers in a constant volume taken from our preparations. Diameters were monitored under a light microscope with an Infinity video camera (Lumenera Corp., Ottawa, Canada) and were measured by the I'MEASURE program (http://www.imagepro.co.kr).

Chlorophyll Fluorescence Analysis

Chlorophyll fluorescence was assessed with an IMAGING-PAM Chlorophyll Fluorometer (Heinz Walz GmbH, Effeltrich, Germany), as described previously (Goh et al.

1999; Schreiber et al. 2007). This instrument was operated using a Pentium II PC and WinControl software (Walz). For the thallus tissues (0.5 mm diam.), fluorescence was induced in artificial seawater made by dissolving commercial sea salt in deionized water to a concentration of 3.5%. We applied our previous methods (Goh et al. 1999) to the isolated cells (11 μg of chlorophyll per sample), with some modifications. The reaction mixtures (0.2 mL) consisted of 0.14 mM Mes/Tris (pH 6.1), 0.5 M sorbitol, 0.4 M NaCl, 100 mM KCl, 1 mM $CaCl_2$, and 2 mM $NaHCO_3$. Stock solutions of $NaHCO_3$ were prepared immediately before use. Quantum yield of the photochemical energy converted in Photosystem II was estimated by the empirical fluorescence parameters of $(F_m' - F)/F_m' = \Delta F/F_m'$ (Genty et al. 1989). The apparent relative electron transport rate (ETR) was calculated as $\Delta F/F_m$' X PPFD X c, where PPFD is the photosynthetic photon flux density of the incident active radiation and constant c corresponds to the absorption factor for these measurements (Schreiber et al. 1994). All experiments were performed at room temperature (RT).

Measurement of Ribulose-1,5-Bisphosphate Carboxylase/Oxygenase Activity

Protoplasts were ground to powder with liquid N_2 in a chilled mortar and pestle with 1% (*w*/*v*) insoluble polyvinylpyrrolidone (PVP). They were then homogenized with 1 mL of cooled 50 mM Tris–HCl (pH 7.5), 1 mM EDTA, 10 mM $MgCl_2$, 12% (*v*/*v*) glycerol, 0.1% (*v*/*v*) β-mercaptoethanol, and 1% (*w*/*v*) PVP-40 (soluble PVP) at 4 C. The supernatant was used to determine the activity of ribulose-1,5-bisphosphate carboxylase/oxygenase (rubisco; EC4.1.1.39), which was assayed according to method of Sawada et al. (2003), with minor modifications. Assays were performed at 30 C in 50 mM HEPES-KOH (pH 8.0), 1 mM EDTA, 20 mM $MgCl_2$, 2.5 mM DTT, 10 mM $NaHCO_3$, 5 mM ATP, 0.15 mM NADH, 5 mM creatine phosphate, 0.6 mM RuBP, 10 units of phosphocreatine kinase, 10 units of glyceraldehyde-3-phosphate dehydrogenase, and 10 units of phosphoglycerate kinase.

Measurement of $O_2^{\cdot -}$

Superoxide anion ($O_2^{\cdot -}$) production was measured by monitoring nitrate formation from hydroxylamine in the presence of $O_2^{\cdot -}$ as described by Elstner and Heupel (1976). Protoplasts were homogenized with liquid N_2 in a chilled pestle and mortar with 1 mL of 65 mM potassium phosphate (pH 7.8). The homogenate was centrifuged at 5,000 g for 10 min at 4 C. Potassium phosphate (0.45 mL; pH 7.8) and 10 mM hydroxylamine hydrochloride (0.05 mL) were added to the supernatant. This mixture was incubated at 25 C for 20 min. Afterward, 0.5 mL of the mixture was injected into a solution (0.5 mL) of 17 mM α-naphthaleneamine at 25 C for 20 min. The mixture was shaken and centrifuged at 1,500 g for 5 min. Absorbance of the pink aqueous phase was then measured at 530 nm.

Enzyme Activity Assays

Protoplasts were homogenized in 1 mL of 50 mM potassium phosphate (pH 7.0) and 1% (*v*/*v*) Triton X-100. The homogenate was centrifuged at 12,000 g for 20 min at 4 C and the supernatant was immediately used for enzyme assays. Superoxide dismutase (SOD; EC 1.15.1.1) activity was measured by the photochemical method with nitro-blue tetrazolium (NBT) according to Beauchamp and Fridovich (1971) and was expressed as units per milligram of protein. One unit of SOD activity was defined as the amount of enzyme required to effect 50% inhibition in the rate of NBT reduction at A_{560}. Ascorbate peroxidase (APX; EC 1.11.1.11) was extracted in 1 mL of 50 mM potassium phosphate (pH 7.8), 1 mM ascorbic acid, 1 mM EDTA, and 2% (*v*/*v*) PVP, added immediately prior to use. APX activity was determined according to the method of Nakano and Asada (1981) using an assay mixture (1 mL) containing 50 mM potassium phosphate (pH 7.0), 0.5 mM ascorbic acid, 0.2 mM H_2O_2, and 50 μl of enzyme extract. Oxidation of ascorbate was initiated by the addition of H_2O_2 and the decrease at A_{290} was then monitored. Activity was expressed as micromole of ascorbate that was oxidized per minute per milligram of protein.

Determination of Photosynthetic O_2 Evolution

Oxygen evolution was assessed with an Oxygraph system (Hansatech, King's Lynn, UK), which evaluates closed gas exchange (Choi et al. 2002). Net rates were determined as previously described (Choi et al. 2002). Actinic light was provided through neutral density filters, using a 150-W quartz-halogen slide projector. Protoplasts (40 μg of chlorophyll) were resuspended at RT in a 1.5-mL solution containing 0.14 mM Mes/Tris (pH 6.1), 1 mM $CaCl_2$, 0.5 M sorbitol, and 2 mM $NaHCO_3$. To determine the effects of sodium and potassium ions on photosynthetic O_2 evolution from the cells, Na^+ and K^+ were supplied as 0.4 M Na-gluconate (or Na-free gluconate) and 100 mM K-gluconate (or K-free gluconate).

Chlorophyll Concentration and Protein Assays

Chlorophyll content was photometrically determined by the method of Arnon (1949). Protein levels were measured using an assay developed by Bradford (1976).

Results

Preparation of Large Quantities of Protoplasts

Protoplasts were enzymatically isolated from the thalli of *U. pertusa*. Most were spherical and contained intact chloroplasts (Fig. 1a). The mean diameter was 15.9± 0.9 μm (n=96; F test=0.01). Inspection from several fields of view showed that protoplast yields averaged 3.6×10^6 cells per thallus (550 μg of protein per gram fresh weight). From these cell preparations, red chlorophyll auto-fluorescence was observed only in protoplasts within the same focal plane (Fig. 1b). Typical preparations contained ≤0.046% broken cell fragments. Pigment analysis of these preparations indicated a Chl *a*:*b* ratio of approximately 1.75:1.00 (data not shown). This is similar to that reported for *U. pertusa* by Yamazaki et al. (2005), who showed that *Ulva* species have lower ratios than the 3:1 found with terrestrial C_3 plants. The smaller ratios in algal cells imply that their two photosystems have larger antennae than do terrestrial plants. Our results demonstrated that the cells were viable and were sufficient for further physiological and biochemical studies.

Effects of External pH on Photosynthetic Activity in Protoplasts

Photosynthetic activity was influenced by pH in isolated protoplasts (Table 1). Although when within the range of 5.5 to 6.1, photochemical quenching of Chl *a* fluorescence was largely unaffected, values for that parameter decreased at pH levels ≥6.8. This inhibition of activity under alkaline conditions was reflected in our calculations of effective PS II quantum yield. For example, the electron transport rate was diminished within 3 min after the actinic light was switched on. At the end of each illumination period, the peak ETR at pH ≥6.8 was inhibited by approximately 17% relative to its reading at pH 6.1, declining rapidly thereafter. This suggested that cells could take up both CO_2 and HCO_3^-, but they had a decreased ability to utilize the latter under alkaline conditions. In further experiments that included the extracellular carbonic anhydrase (CA) inhibitor acetazolamide (AZ), ETRs were not affected by 100 μM AZ at either pH 6.1 or 8.8 when the values were determined 4.92 min after the actinic light was switched on (Table 2). When both NaCl and KCl were present, ETR values at pH 8.8 decreased approximately 37.7% compared with those measured at pH 6.1. These data indicated that rates were affected by NaCl as well as KCl under both alkaline and acidic conditions. Thus, for algae that can assimilate only free CO_2 and have a low affinity for HCO_3^-, such pH changes may affect photosynthesis by limiting the rate of inorganic carbon uptake (Dromgoole 1978). Our results demonstrated that photosynthesis was enhanced in those cells when the CO_2 concentration was elevated by a prior drop in pH.

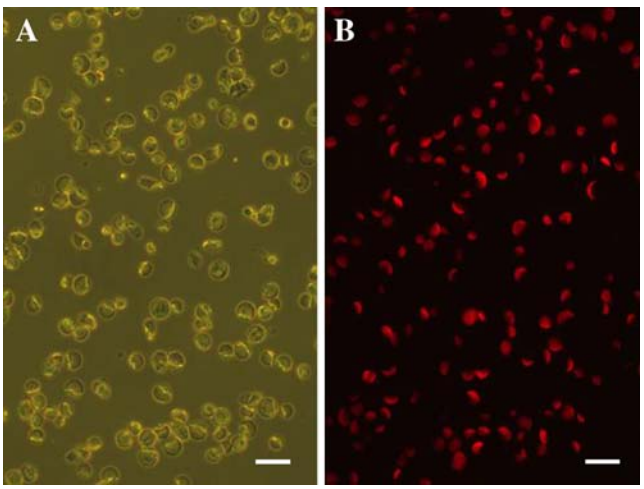

Fig. 1 Protoplasts isolated from thallus of *Ulva pertusa*. **a** Light micrograph of protoplasts (approx. 16 μm diam.) that were enzymatically isolated and suspended in 0.5 M sorbitol, 100 mM KCl, 1 mM $CaCl_2$, and 0.4 M NaCl. **b** Chlorophyll auto-fluorescence. *Bars* are 34 μm

Table 1 Photosynthetic activity[a] of *Ulva* protoplasts at different external pHs

	Media pH			
	6.1	6.8	7.8	8.8
Activity[b]	11.35±0.12	7.88±0.55	2.34±0.38	1.21±0.22

[a] At end of each illumination period, saturating pulse was applied to assess effective quantum yield from which electron transport rate was calculated. Protoplasts were resuspended in a 1-mL solution containing 0.14 mM Mes (pH 6.1 and 6.8) or 0.14 mM Hepes (pH 7.8 and 8.8), plus 0.4 M NaCl, 100 mM KCl, 1 mM $CaCl_2$, 0.5 M sorbitol, 0.4 M NaCl, and 2 mM $NaHCO_3$. Other experimental conditions are as described for Fig. 2

[b] Expressed as means ± SE (n=8) of ETR obtained at 4.92 min after actinic light was switched on

Quenching Analysis in Protoplasts Treated with External Na^+ or K^+

PS II quantum yield was evaluated in the absence or presence of NaCl and KCl in the medium (Fig. 2). Photochemical quenching is defined by the kinetics of time-dependent reductions in yield values when external NaCl is lacking. Here, when protoplasts were prepared with a solution containing 0.4 M NaCl, quantum yield was enhanced over time. The addition of KCl also raised these values. However, yields did not occur when the medium contained no NaCl. If external KCl was present in combination with NaCl in the medium, quantum yields

Table 2 Effects of Na^+ and K^+ deficiency stresses on photosynthetic electron transport rates in *Ulva* protoplasts[a] incubated at pH 6.1 or 8.8

	pH 6.1		pH 8.8	
	−AZ	+AZ	−AZ	+AZ
+NaCl + KCl	11.35±0.12	2.12±0.71	8.24±0.80	1.92±0.62
+NaCl-KCl	7.88±0.55	1.84±0.69	6.94±0.30	1.86±0.86
−NaCl + KCl	2.34±0.38	0.58±0.32	1.56±0.26	0.53±0.25
−NaCl-KCl	1.21±0.22	0.32±0.08	1.28±0.30	0.43±0.17

[a] Protoplasts (40 μg chlorophyll) were resuspended in a 1-mL solution containing 0.14 mM Mes (pH 6.1) or 0.14 mM Hepes (pH 8.8), plus 1 mM $CaCl_2$, 0.5 M sorbitol, and 2 mM $NaHCO_3$. They were held at room temperature for 20 min in presence or absence of acetazolamide (AZ). Media concentrations of Na^+ and K^+ were 0.4 M and 100 mM, respectively. Data are expressed as means ± SE (n=9) All other experimental conditions are as described for Fig. 2

were quite high, i.e., nearly 0.45 from 4 min after the actinic light had been switched on. These results strongly supported our hypothesis that NaCl is necessary for the photochemical activity of chloroplasts and that KCl enhances this activity.

To further determine the effects of external K^+ on photochemical activity, we assessed quantum yield in the presence or absence of [K^+] (as K-gluconate; Cl^- free) from cells prepared in 0.5 M sorbitol and 1 mM Ca-gluconate (Cl^- free), and when 0.4 M NaCl was added to the medium (Fig. 3a, b). Potassium increased that yield by approximately 52% in the presence of NaCl but gluconate alone had no affect (Fig. 3a). In the absence of K^+ but not Na^+, we noted a low F_v/F_m value of 0.65±0.01 (data not shown). Thus, K^+ directly affected PS II activity. Furthermore, Cl^- influenced activity in our algal cells. Other halides were tested for their stimulatory effect (Fig. 3b). Na^+ alone induced an F_v/F_m value of approximately 0.25. Although the addition of Cl^- increased that value to approximately 0.5, other halides had no significant effects, thereby implying that the chlorine ion influences PS II activity.

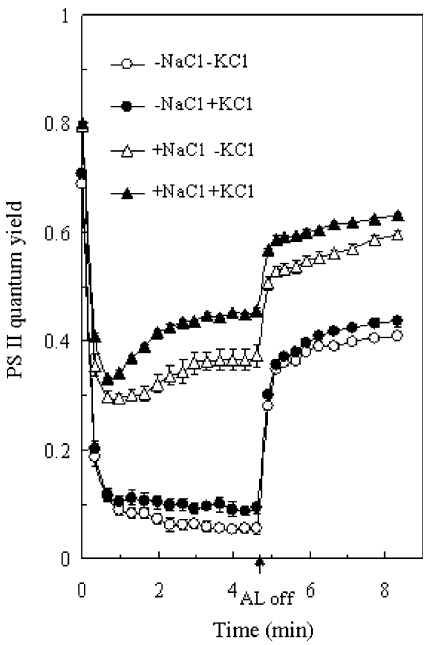

Fig. 2 Effects of NaCl and KCl on PS II quantum yield in *Ulva* protoplasts that were prepared in 0.5 M sorbitol and 1 mM $CaCl_2$ in presence or absence of 0.4 M NaCl. Protoplasts (11 μg chlorophyll) were added to 0.2-mL reaction mixtures containing 0.14 mM Mes/Tris (pH 6.1), 0.5 M sorbitol, 1 mM $CaCl_2$, and 2 mM $NaHCO_3$ in absence or presence of NaCl (0.4 M) and KCl (100 mM). Stock solution of $NaHCO_3$ was prepared immediately before use and pH was adjusted with HCl or KOH. Measuring light (ML) intensity of photosynthetically active radiation was 0.8 μmol $m^{-2}s^{-1}$. Saturation light (SL) was 2,700 μmol $m^{-2}s^{-1}$. Photochemical quenchings (F_m') were repeatedly induced in actinic light (AL; 55 μmol $m^{-2}s^{-1}$) at 20-s intervals, 40 s after SL was switched on. Values are expressed as means ± SE (n=7)

To further determine the effects of external potassium on photochemical activity, we assessed PS II activity in the presence of increasing [K^+] (as K-gluconate) from cells prepared in 0.5 M sorbitol and 1 mM Ca-gluconate and in the presence or absence of 0.4 M Na-gluconate (Cl^- free; Fig. 4a, b). When its concentration was >25 mM, K^+-enhanced quantum yield to an F_v/F_m value of 0.5. In the absence of Na^+, all tested K^+ concentrations manifested low yields, suggesting that photosynthesis was not occurring. Levels of PS II activity were below an F_v/F_m value of 0.25 and were not affected by the addition of 100 mM [K^+]. Thus, we could conclude that K^+ alone did not improve photosynthesis, but that it did effect an enhancement in that process when external Na^+ was present. These results indicated that K^+ plays a functional role in photosynthesis.

O_2 Evolution by Protoplasts in the Absence or Presence of External Na^+ or K^+

When photosynthetic O_2 evolution was measured in the presence or absence of Na^+ and K^+ (Fig. 5), the data suggested that K^+-dependent photochemical activity was occurring within the *Ulva* protoplasts. The rate of that evolution was reduced dramatically by light treatment in the absence of Na^+. When K^+ was lacking, Na^+ stimulated the rate of recovery for evolution from −37.7 (±1.9) to

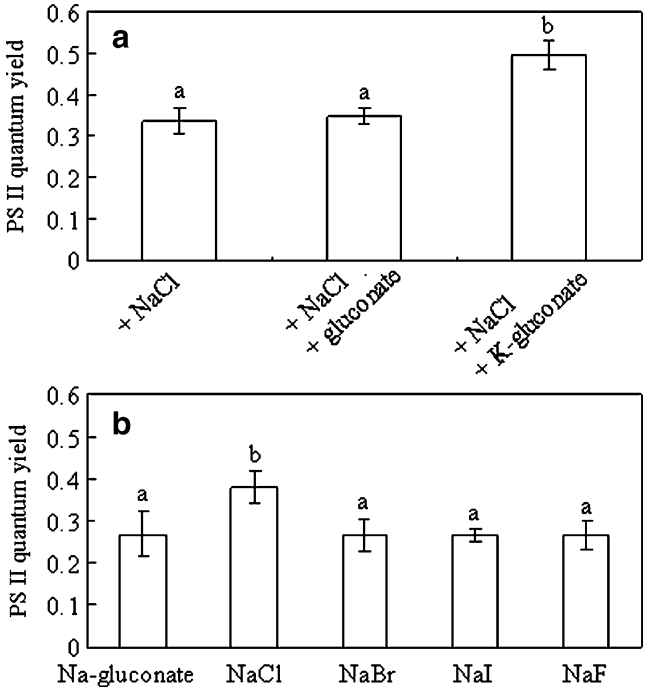

Fig. 3 Effects of K^+ and Cl^- on PS II in *Ulva* protoplasts. **a** Quantum yield in absence or presence of K^+ when NaCl was present externally. Protoplasts were added to 0.2-mL reaction mixtures containing 0.14 mM Mes/Tris (pH 6.1), 0.5 M sorbitol, 1 mM Ca-gluconate (Cl^- free), and 0.4 M NaCl in presence of K^+ (100 mM K-gluconate) or K-free gluconate. **b** Quantum yield in absence or presence of Cl^-. Sodium halide salts (0.4 M) were added to protoplast reaction mixtures (0.2 mL) containing 0.14 mM Mes/Tris (pH 6.1) and 0.5 M sorbitol in presence of Ca-gluconate (1 mM) instead of $CaCl_2$. At end of each illumination period, saturating pulse was applied to assess effective quantum yield. Values are expressed as means ± SE (n=5) measured at 4.92 min after actinic light was switched on. *Letters* indicate statistically significant differences by t test ($P \leq 0.01$ for **a**, 0.05 for **b**). All other experimental conditions are as described for Fig. 2

149.2 (±5.4) nmol of O_2 per minute per microgram of chlorophyll. In the presence of Na^+, the addition of K^+ profoundly enhanced this activity. Therefore, both Na^+ and K^+ appreciably amplified the evolution of photosynthetic O_2.

K^+ Deficiency Stress Affects the Rate of Superoxide Anion ($O_2^{\cdot -}$) Generation in Protoplasts

We showed here that photosynthetic activity in *Ulva* protoplasts was regulated by K^+ in the medium (Figs. 3, 4, and 5), although rubisco activity was not significantly altered (Table 3). This reduction in photosynthesis may have been directly related to ROS-scavenging because K^+ is the major driving ion for osmotic water uptake. Generation of superoxide anions was measured in protoplasts incubated with different combinations of Na^+ and K^+ in the medium (Table 3), and similar rates were found among all tested concentrations of protoplasts (Fig. 6a). When the medium contained no K^+, $O_2^{\cdot -}$ generation was significantly increased, by approximately 40%, compared with the response when potassium was present along with sodium. However, such an increase was not observed when the media concentration of K^+ was >25 mM (Fig. 6b). Increases in $O_2^{\cdot -}$ generation and subsequent redox imbalances can be managed if one controls $O_2^{\cdot -}$ metabolism by altering scavenging rates through the production of antioxidant enzymes. Both SOD and APX are key enzymes involved in ROS-scavenging. Therefore, we compared their activities during a 20-min incubation with combinations of Na^+ and K^+ in the medium under light. The ratio of NBT reduction in the absence of SOD to that in the presence of SOD (S_0/S) was proportional to the SOD concentration (Fig. 7a). APX activity decreased about 50% after 20 min of illumination, declining to just 10% of its initial value after 30 min. An approximately 23% to 29% increase in SOD and APX activities was observed in the absence of

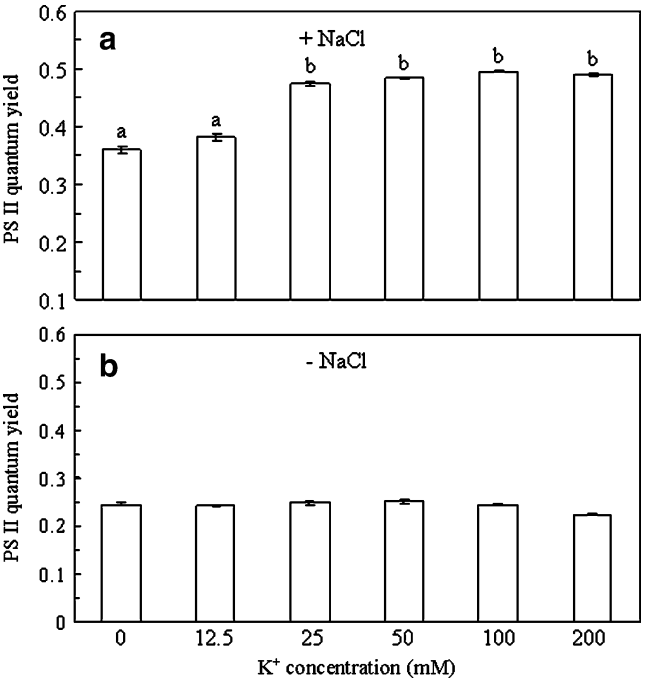

Fig. 4 Effect of K^+ concentration on PS II quantum yield. **a** Protoplasts were prepared in solution of 0.5 M sorbitol and 1 mM Ca-gluconate (Cl^- free Ca^{2+}), with NaCl (0.4 M) added at wash step. **b** Protoplasts were prepared in solution of 0.5 M sorbitol and 1 mM $CaCl_2$ without Na-gluconate. K^+ ions were added as K-gluconate at indicated concentrations. Gluconate was used for both K- and Na-free media. Protoplasts were added to 0.2-mL reaction mixtures containing 0.14 mM Mes/Tris (pH 6.1), 0.5 M sorbitol, and 1 mM $CaCl_2$ with varying K^+ concentrations, in presence or absence of 0.4 M Na^+. At the end of each illumination period, saturating pulse was applied to assess effective quantum yield. Values are expressed as means ± SE (n=6) measured at 4.92 min after actinic light was switched on. *Letters* indicate statistically significant differences by t test ($P \leq 0.01$). All other experimental conditions are as described for Fig. 2

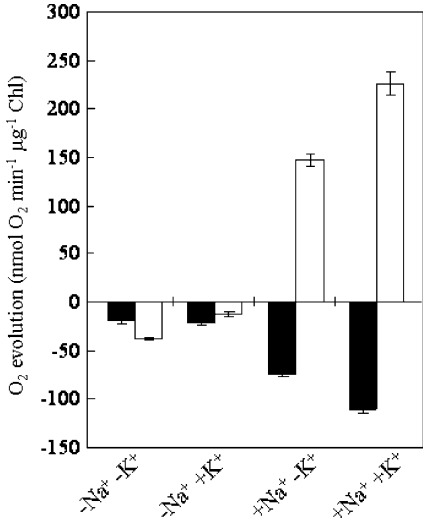

Fig. 5 Rates of photosynthetic O_2 evolution in *Ulva* protoplasts that were prepared in 0.5 M sorbitol, with 1 mM $CaCl_2$ at wash step. Na^+ (0.4 M Na-gluconate) or K^+ (100 mM K-gluconate) was added to reaction mixtures, as indicated. Gluconate was used for both Na- and K-free media. Actinic light (542 μmol $m^{-2}s^{-1}$) was applied 4 min after protoplasts were dark-adapted. Protoplasts (40 μg chlorophyll) were incubated in dark at RT in 2 mL of medium. Oxygen evolution was polarographically measured as function of time. Histograms show net oxygen evolution or uptake under actinic light or darkness. Data are expressed as means ± SE (n=3). Ratio of Chl:protein was 0.71:1.00±0.07 (n=3). *Black* and *white boxes* indicate dark and light responses, respectively

Na^+ in the medium. As $O_2^{\cdot-}$ generation increased when K^+ was lacking, a concomitant rise in SOD and APX activities was observed regardless of whether Na^+ was present. These results indicated that such increases in enzyme activities might eliminate the harmful oxygen species produced in the water–water cycle of *Ulva* chloroplasts in response to a potassium deficiency.

Discussion

To investigate the effects of stress from K^+ deficiency on photosynthesis by the green alga *U. pertusa*, we enzymatically isolated protoplasts with high photosynthetic capacities from thallus tissues. In those protoplasts, the electron transport rate was light-dependent, becoming saturated at 270 μmol $m^{-2}s^{-1}$ and exhibiting half-saturation at 69 μmol $m^{-2}s^{-1}$ (data not shown). ETR values were high at pH 6.1. This inhibition of PS II under alkaline conditions demonstrated that these cells have decreased ability to utilize HCO_3^-. ETRs were significantly inhibited by the addition of 100 μM AZ to the medium under both alkaline and acidic conditions, implying that CA-mediated dehydration followed by free CO_2 uptake is active at pH 6.1 and that an anion exchanger probably mediates the direct uptake of HCO_3^- at pH 8.8 (Axelson et al. 2000). These findings suggest that algal cells assimilate both free CO_2 and HCO_3^-, depending on the external pH of the medium. The marine diatom, *P. tricornutum*, can affect the uptake of both CO_2 and HCO_3^- as photosynthetic substrates, and CO_2:HCO_3^- uptake ratios are diminished with decreasing CO_2 concentration in the medium (Burkhardt et al. 2001; Matsuda et al. 2001). Therefore, HCO_3^- should be the predominant carbon source when *P. tricornutum* cells are being acclimated to high alkalinity (Chen et al. 2006; Friedlander 2007). When that species is cultured at a pH of 7.2 or 8.0, photosynthetic affinities to HCO_3^- are unaffected by K^+. This suggests that K^+-dependent HCO_3^- transport is induced when *P. tricornutum* is exposed to a more alkaline pH. In contrast, our results showed that PS II quantum yield declined under such conditions. Consistent with this finding, photosynthetic rates of *Ulva* species are reduced at alkaline pH (Menendez et al. 2001), indicating their decreased ability to utilize HCO_3^-. Contradictory evidence has been presented regarding the carbon sources for other algae, e.g., Gelidiales sp. (Friedlander 2007). During some measurements, KCl is added to the medium, enabling the possibility of K^+-dependent HCO_3^- uptake in marine algae (Chen et al. 2006). In our experiments with different pH values, more acidic conditions enhanced photosynthetic activity when the medium contained potassium, suggesting the predominant utilization of free CO_2 for photosynthesis in this alga.

We assessed the manner in which K^+ participates in photosynthesis within *Ulva*. These algal protoplasts showed profound ETR activities in a solution of 0.5 M sorbitol and 0.4 M NaCl. The time-dependent quenching of Chl *a* fluorescence was apparently heightened in the presence of Na^+ (data not shown) and this quenching activated PS II quantum yield. Elevated Na^+ concentrations also severely and adversely affect the photosynthetic apparatus in cyanobacteria (van Thor et al. 2000; Lu and Vonshak 2002; Pogoryelov et al. 2003). Significant inhibition of photosynthesis as a result of high [Na^+] also appears to be associated with the PS II complex; saline stress reduces PS II activity in cyanobacteria (Zhao and Brand 1989) and inhibits quantum yields in *Ulva lactuca* (Xia et al. 2004). Consistent with these findings, our results showed that ETRs increase at higher Na^+ concentrations, but are reversed at concentrations >0.5 M NaCl (data not shown). Sodium uptake is probably involved in the bioenergetics of F-type ATPase activity (Ramani et al. 2006). Therefore, these results demonstrate that Na^+ is required for photochemical activity in this algal species.

Both Na^+ and K^+ enhance quantum yields over time. The chlorine ion might also serve as a ligand for Mn in the PS II-catalyzed oxidation of water (Popelková and Yocum 2007). Because this Cl^- ligation may be essential for the oxygen-evolving complex of PS II in those cells, we

Table 3 Effects of Na^+ and K^+ deficiency stresses on rubisco activity, $O_2^{\cdot-}$ generation and SOD and APX activities in *Ulva* protoplasts[a]

	Rubisco activity ($\mu molm^{-2}s^{-1}$)	$O_2^{\cdot-}$ generation rate ($nmolmg^{-1}$ protein min^{-1})	SOD activity (units mg^{-1} protein)	APX activity ($\mu molmg$ protein$^{-1}min^{-1}$)
+Na+K	42.13±2.69	1.68±0.10[a]	23.36±0.54[a]	12.86±0.83[a]
+Na-K	41.36±2.95	2.37±0.12[b]	31.10±0.22[b]	16.04±0.66[b]
−Na+K	44.34±1.98	3.20±0.18[a]	24.52±1.13[a]	13.45±1.31[a]
−Na-K	40.61±2.86	3.77±0.15[b]	30.84±0.70[b]	17.81±1.18[b]

[a] Protoplasts (40 μg chlorophyll) were resuspended for 20 min in a 1-mL solution containing 0.14 mM Mes/Tris (pH 6.1), 1 mM $CaCl_2$, 0.5 M sorbitol, and 2 mM $NaHCO_3$, under red light (600 μmol m^{-2} s^{-1}) after dark-adaptation (10 min) at room temperature. During adaptation, Na^+ and K^+ were supplied to media as 0.4 M Na-gluconate (or Na-free gluconate) and 100 mM K-gluconate (or K-free gluconate). Values represent means ± SE (n=4); letters indicate statistically significant differences by t test ($P \leq 0.05$). All other experimental conditions are as described for Fig. 6.

investigated the effect of Cl^- on photochemical activity in protoplasts. Here, chlorine was the only halide that had a significant effect on that complex, thereby indicating that this activity is not regulated by additional factors, such as Cl^- ion concentrations.

To determine the effects of external K^+, we assessed PS II photochemical activity in the presence of increasing [K^+] (as K-gluconate) in cells incubated in the presence or absence of 0.4 M NaCl. When the medium was supplemented with Na^+, PS II activity was stimulated by the addition of up to 100 mM K^+, in a concentration-dependent manner. When Na^+ was lacking, however, such activity was low, regardless of the [K^+], implying that photosynthesis is inhibited in the absence of Na^+. When photosynthetic O_2 evolution was measured, we found that supplemental K^+ profoundly enhanced this activity in the presence of Na^+. Therefore, the addition of both Na^+ and K^+ appreciably amplifies O_2 evolution.

In plant adaptations to salinity, Na^+/K^+ ratios also affect responses to CO_2 assimilation (Ramani et al. 2006) and PS II

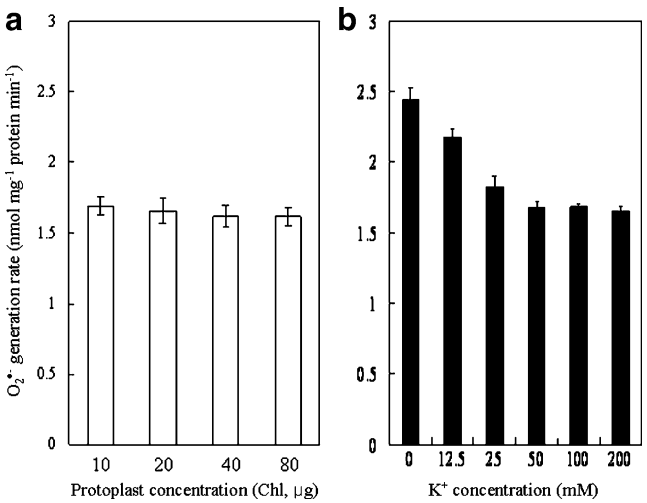

Fig. 6 Effect of K^+ concentration on $O_2^{\cdot-}$ generation in *Ulva* protoplasts. Protoplasts (40 μg chlorophyll) were illuminated for 20 min with red light (600 μmol $m^{-2}s^{-1}$) after dark-adaptation (10 min) at RT. **a** Rate of $O_2^{\cdot-}$ generation based on protoplast concentration. Protoplasts were resuspended in a 1-mL solution containing 0.14 mM Mes/Tris (pH 6.1), 0.4 M Na-gluconate, 100 mM K^+-gluconate, 1 mM $CaCl_2$, 0.5 M sorbitol, and 2 mM $NaHCO_3$. **b** Rate of $O_2^{\cdot-}$ generation based on K^+ concentration. Protoplasts were resuspended in a 1-mL solution containing 0.14 mM Mes/Tris (pH 6.1), 0.4 M Na-gluconate, 1 mM $CaCl_2$, 0.5 M sorbitol, and 2 mM $NaHCO_3$. K^+ was added at indicated concentrations. Values represent means ± SE (n=3)

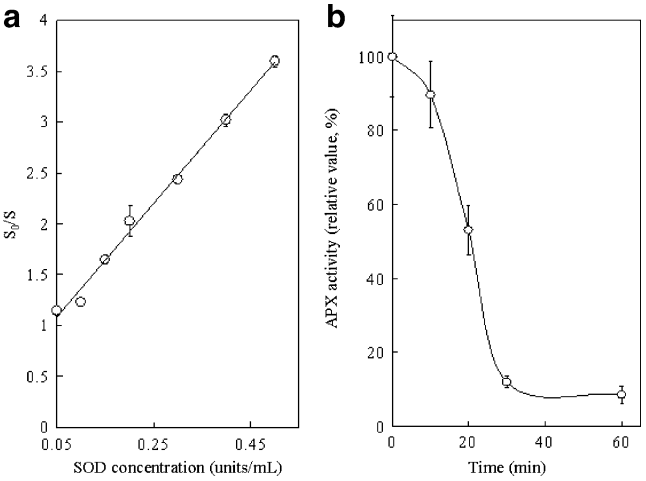

Fig. 7 SOD and APX activities in *Ulva* protoplasts. **a** NBT method used to determine ratio of NBT reduction in absence of SOD to that in presence of SOD (S_0/S). Calibration curve was obtained by least-squares regression analysis. Protoplasts were illuminated at 600 μmol $m^{-2}s^{-1}$ of red light. **b** APX activity. Protoplasts (40 μg chlorophyll) were resuspended in a 1-mL solution containing 0.14 mM Mes/Tris (pH 6.1), 0.4 M Na-gluconate, 100 mM K-gluconate, 1 mM $CaCl_2$, 0.5 M sorbitol, and 2 mM $NaHCO_3$ for 20 min under red light (600 μmol m^{-2} s^{-1}) after dark-adaptation (10 min) at RT. After reaction, protoplasts were immediately frozen in liquid nitrogen for isolating chloroplasts via centrifugation for 15 s. Activities are expressed as percentage of activity at Time zero (12.7±0.8 μmol mg per protein per minute). Values represent means ± SE (n=3)

quantum yield (Naidoo and Kift 2006). However, to avoid the toxic effects of salt on photosynthesis, Na^+ and Cl^- are thought to be stored in compartments, e.g., the vacuoles, as is the case with terrestrial plants (James et al. 2006). For several species, K^+ is considered a macronutrient for growth and metabolism, playing an important role in photosynthesis, enzyme activation, and protein synthesis, as well as being the major driving ion for osmotic water uptake. Periodic oscillations in the short-term growth rate of *P. leucosticte* (Rhodophyta) occur in response to potassium fluxes, which control osmotic pressure and, eventually, relative cell volumes (Escassi et al. 2002). Therefore, an internal K^+ concentration is needed in that species during cell expansion to protect osmotic pressure against the diluting effect of water uptake. Such osmotic stress induces ROS production in the unicellular green alga *Micrasterias* (Darehshouri and Lütz-Meindl 2010). Many algae and cyanobacteria actually excrete H_2O_2 and related scavenging enzymes that are resistant to oxidative inactivation by H_2O_2 (Badger et al. 2000). To determine whether *Ulva* protoplasts also produce active O_2 species under K^+ deficiency stress, we measured the rate of superoxide anion generation from cells in the presence of various combinations of external Na^+ and K^+. Although rubisco activity was unchanged, $O_2^{\cdot -}$ generation in the absence of external K^+ was significantly increased, by approximately 41%, compared with the rate calculated in the presence of both K^+ and Na^+. Furthermore, we observed this trend when $[K^+]$ was <25 mM. Concomitantly, SOD and APX activities increased under the K^+ deficiency stress. Photo-reduction of O_2 to $O_2^{\cdot -}$ is the limiting step in the water–water cycle, through which those two antioxidant enzymes support electron flow from PS II to PS I (Asada 1999). Our results suggest that the enzymes might eliminate harmful oxygen species produced by *Ulva* chloroplasts under that stress.

In conclusion, a K^+ deficiency in algal cells induces oxidative stress and leads to the production of $O_2^{\cdot -}$, which inhibits photosynthetic activity in *Ulva* protoplasts. The water–water cycle might eliminate those harmful oxygen species and protect the chloroplasts during this stress.

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