BIOLOGICAL ROLES OF SODIUM IONS, REQUISITE FOR THE PRIMARY PRODUCTION IN THE MARINE ENVIRONMENT

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Abstract

Sodium chloride is a major chaotropic effecter which limits primary productivity of land plants and freshwater photoautotrophs. Mechanisms of salt tolerance in freshwater plants have been well studied and it is well established that plants exclude excess Na^+ from the cytosol to maintain proper internal Na^+ concentrations. On the other hand, little information is available on relationships between photoautotrophs and salt in marine environment, which is now known to be a major fraction of global primary production. In the present study, effects of reduced salt concentration to cell physiology and intracellular localization of salt were investigated using the marine diatom *Phaeodactylum tricornutum*. Our results showed that cells of marine diatom require Na^+ for normal growth and photosynthesis whereas Cl^- acted inhibitory to photosynthesis. Intracellular behavior of these ions was investigated by labeling with specific indicators, Sodium Green and MEQ. It was shown that under salt concentrations below seawater level (<0.5 M), Na^+ and Cl^- were shown to be accumulated at the membrane system surrounding the chloroplast. Most probably, salt is reserved at the matrixes of four layered chloroplast membranes, which is a unique structure observed in secondary symbionts. These data strongly suggest that, in contrast to land plants, primary production by photoautotrophs in the ocean requires Na^+.

Introduction

Sodium ions occur ubiquitously on the earth and function as a chaotropic effecter to higher plants. It is well known that Na^+ inhibits photosystem II (PSII) in land plants and Cl^- counteracts to Na^+ as a neutralizing anion (Greenway and Munns, 1980; Kuwabara and Murata, 1983; Gaxiola at al., 1998; Allakhverdiev et al., 2000). That is, it has been shown that, in freshwater species of cyanobacteria, green algae, and higher plants, NaCl above 200 mM suppressed Na^+/H^+-antiporter and electron transport chain of photosystems and respiration (Blumwald et al., 2000; Allakhverdiev et al., 2005). It is also pointed out that excess influx of Na^+ disturbs membrane potentials which leads to difficulty in uptakes of mineral nutrients (Niu et al., 1995; Hasegawa et al., 2000). On the other hand, Cl^- act as a counter anion which maintains intracellular pH neutral (Gaxiola et al., 1998) and also be required for the water oxidizing activity in the photosystem in plant cells under Na^+ stresses (de Paula et al., 1986). In these plants under Na^+ stresses, biosynthesis of compatible osmolyte such as glycine betaine and expression of Na^+/H^+-antiporters are activated (Erdmann and Hagemann, 2001; Tuteja, 2007). These results indicate that plants acquire salt tolerance by stimulating activities to exclude Na^+ from the cytosol, or neutralizing it intracellularly when they are exposed to high Na^+ concentrations.

Salt tolerance described above is rather a unique feature observed in photoautotrophs which have adapted to land and freshwater environment, but are not widespread beyond many types living organisms. For example, mammal and other animal cells require relatively high concentrations of Na^+ to maintain intracellular homeostasis, and some of archaea grow optimally even under saturating concentrations of Na^+. In these living organisms, high concentrations of Na^+ appear to be required for the optimal expression of housekeeping physiologies such as membrane transport and enzymatic reactions.

Land plants account for overwhelming biomass among photoautotrophs on the earth. Contrary to this, biomass of phytoplanktons living in the hydrosphere accounts for only 1-2% of all biospheres. However, it was pointed out recently that their primary productivity almost competes to that of land plants (Tréguer et al,. 1995; Falkowski et al., 1998). Marine diatoms are the most successful group of marine algae and estimated to be responsible for 20-25% of global primary production (Tréguer et al,. 1995; Falkowski et al., 1998). These photoautotrophs thus actively sediment photosynthetic products and other nutrients very rapidly from the surface to the bottom of the sea, functioning as the biological pump, which plays a vital role in global element cycles.

The question is how these primary producers in marine environment manage or utilize high concentrations of NaCl of nearly 500 mM in seawater. Little is studied for relationship between basic physiology and salts in marine photoautotrophs. Considering the high salt environment of their inhabitation and the fact that plants originally evolved in

seawater and gradually adapted to freshwater environment, it is possible that halophilic physiologies had been inherited in photoautotrophs, which have been remained in marine environment. In the present study, changes in fundamental physiologies such as cell morphology, growth, photosynthesis, NaCl uptake, and gene expressions were profiled under changing salt conditions using the marine diatom *Phaeodactylum tricornutum*.

Materials and Methods

Strains and culture conditions. The marine diatom *P. tricornutum* (UTEX 640) was obtained from the University of Texas culture collection (Austin) and was axenically cultured in artificial seawater supplemented with half strength of Guillard's "F" solution (F/2ASW) (Guillard and Ryther, 1962; Harrison *et al.*, 1980) under atmospheric air and continuous illumination at photon flux density of 70 μmol m^{-2} s^{-1} at 20°C. Cells were harvested and transferred to F/2ASW with modified salinities and salt ingredient and allowed to grow in the same growth conditions as above for 2 weeks in some experiment. Detailed salt conditions of each acclimation experiment will be described in the results and figures.

Measurement of photosynthetic activity. Cells of *P. tricornutum* were cultured in the standard F/2ASW (0.5 M Na^+/0.6 M Cl^-). Cells were harvested, washed, and resuspended in the F/2ASW with modified salinities and salt ingredient. Detailed salt conditions in each measurement will be described in the results and figures. Activities of the maximum rate of net photosynthesis and PSII electron flow were measured in a Clark-type oxygen electrode by illuminating the cell culture at chlorophyll *a* density of 10 μg mL^{-1} with photon flux density of 400 μmol m^{-2} s^{-1} at 20°C in the presence of 5 mM of HCO_3^- at pH 7.0. Before starting the illumination, cells were preincubated for 30 min in the absence or presence of 0.5 mM 1,4-*p*-benzoquinone (BQ). Variable chlorophyll fluorescence was measured with a PAM2001 fluorometer (Waltz). For the PAM assay, cells were dark adapted for 30 min at 20°C and then illuminated by measurement light at level 4, actinic light at level 7, and saturation pulse light at level 10.

Preparation of active thylakoid membrane and measurement of oxygen evolution rates. The cells were harvested, washed, and resuspended in a buffer containing 1 M glycinbetain, 50 mM MES (pH 6.5), 10 mM $MgCl_2$, 5 mM $CaCl_2$. Cell suspension was then frozen in liquid nitrogen and stored at -80 °C. The frozen cells were thawed in water bath at 20 °C under the dark, harvested by centrifuging at 13,000 rpm for 3 min, and resuspended in a measurement buffer containing 0.1 M Tris-HCl (pH7.1) and different concentrations and ingredient of salts. Detailed salt conditions in each measurement will be described in the results and figures. The activity of the PSII in these samples was measured immediately after preparation in a Clark-type oxygen electrode by illuminating under photon flux density of 400 μmol m^{-2} s^{-1} at 20°C in the presence of 0.5 mM BQ.

Intracellular fluorescent ion indicator

treatment. Fluorescent Cl^- indicator 6-Methoxy-N-ethylquinolinium iodide (MEQ; Molecular Probes, Eugene, OR) was prospectively rendered cell-permeant by masking its positively charged nitrogen to create a lipophilic, Cl^--insensitive compound, 6-methoxy-N-ethyl-1,2-dihydroquinoline (dihydro-MEQ) following the manufacture instructions. This dihydro-MEQ and cell permeant type sodium green (Molecular Probes, Eugene, OR) were used as intracellular fluorescent Cl^- indicator and fluorescent Na^+ indicator, respectably. Cells ware treated with sodium green (50 μg/ml with 2% DMSO) and dihydro-MEQ (1 mg/ml) under the dark for 30 min and immediately observed under fluorescent microscopes.

Treatment with modulators for ion channels and ion transports. In some localization experiment for intracellular ions, cells of *P. tricornutum* grown in the normal F/2ASW were treated with modulators described as follows. Cells before fluorescent microscopy assay were treated with each of 1 mM 5-(*N*-ethyl-*N*-isopropyl)-amiloride (EIPA), a blocker for Na^+/H^+ exchanger; 360 μM ouabain (OU), a blocker for Na^+/K^+-ATPase; 60 μM tetradotoxin (TTX), a blocker for voltage-gate Na^+ channel; 30 μM indonyloxyacetic acid-94 (IAA-94), a blocker for Cl^- channel; 0.5 mM disodium-4,4'-diisothiocyanatostilebene-2,2'-disulfonate (DIDS), an inhibitor for anion permeation; 20 μM monensin (Mon), anionophor for Na^+.

Laser-scanning confocal microscopy. Fluorescent microscopy of chlorophyll *a/c* autofluorescence and both ion indicators was carried out with a laser-scanning confocal microscope, LSM 510 META, version 3.0 (Carl Zeiss, Oberkochen, Germany), at the lambda mode. The high-frequency transduction UV/488/543/633 filter was selected as the main dichroic splitter and three laser lines (488, 543, and 633 nm) were activated for the excitation of the specimen. The lambda mode scanning data were taken in a range from 488 to 700 nm. Each specific fluoroimage was extracted by subtracting prescanned fluorescent spectrum information of each material (chlorophyll *a/c*, sodium green or dihydro-MEQ) from the total fluorescent information.

Sectioning Fluorescent Microscopy. A three-dimensional microscopic fluorescent image of chlorophyll *a/c* autofluorescence and intracellular ion indicators were modeled by combination of a sectioning fluorescent microscopy system, DeltaVision (Applied Precision, Issaquah, WA), and softWoRx version 2.5, an image analysis and model-building software (Applied Precision). To acquire the chlorophyll *a/c* autofluorescence images, a CY-5 (640/20) excitation filter and a CY-5 (685/40) emission filter were selected for excitation and emission, respectively. The dihydro-MEQ fluorescent and sodium green images were also taken by setting the DAPI (360/40) excitation filter and the DAPI (457/50) emission filter and the GFP (470/40) excitation filter and the GFP (525/50) emission filter for excitation and emission, respectively.

Results and Discussion

Effects of Na^+ and Cl^- concentrations on cell growth. Effects of 3 different concentrations of NaCl on the growth of *P. tricornutum* cells were tested. Cells cultured in low NaCl medium (0.1 M Na^+/0.2 M Cl^-) and high NaCl medium (1.0 M Na^+/1.1 M Cl^-) exhibited growth rate about 85% that of cells grown in the ordinary F/2ASW containing 0.5 M Na^+ and 0.6 M Cl^-. Furthermore, enrichment of Na^+ alone to a final concentration of 1.0 M by the addition of 0.45 M Na_2SO_4 stimulated the growth to the rate equivalent to that observed in the ordinary F/2ASW. The enrichment of SO_4^{2-} alone to a final concentration of 0.45 M by the addition of 0.45 M Tris-H_2SO_4 showed no stimulation of growth. In contrast, under the condition of high Cl^- and low Na^+ at final concentrations of 1.1 M and 0.1 M respectively, growth of cells was severely retarded. These results strongly suggest cells' preference to Na^+ and inhibitory effect of Cl^- for the optimal growth. It is also shown that cells of *P. tricornutum* are able to acclimate to a wide range of NaCl concentrations.

Cells of *P. tricornutum* showed an acclimation accompanied by a clear morphological change when they were transferred from the ordinary F/2ASW (0.5 M NaCl) to that of reduced NaCl (0.1 M Na^+/0.2 M Cl^-). Most cells cultured in 0.5 M NaCl were fuciform, a floating form, which developed "wings" at the distal tip of the cell. On the other hand, cells acclimated to 0.1 M NaCl lost the wings, transformed to oval form, a benthic form, which clumped together at the bottom of the culture vessel (data not shown). Such transformation was not observed in enriched salt conditions. This morphological alteration presumably reflected how cells perceived each salt condition. It is probable that cells minimize the surface area under low [NaCl] conditions, indicating that, for optimal morphogenesis, this marine diatom prefer significantly high concentrations of NaCl above seawater levels.

Sodium ion also appeared to function to mitigate stresses in *P. tricornutum* cells. Cells transferred to modified F/2ASW containing 0.1 M Na^+, 0.4 M Cl^- and supplementary 0.2 M Li^+ or K^+ were allowed to grow for 1 week. Lithium ion or K^+ severely inhibited cell growth. However, the addition of 0.5 M Na_2SO_4 to modified F/2ASW significantly mitigated these inhibitory effects of Li^+ and K^+ (data not shown). This apparent mitigation was observed more evidently when 1.0 M Na2SO4 was added (data not shown).

Table 1. Photosynthesis under changing Na^+ Cl^- concentrations

Treatment	Na^+	Cl^-	Relative photosynthetic rate
0.5M NaCl	0.5M	0.6M	100%
0.1M NaCl	0.1M	0.2M	71%
1.0M NaCl	1.0M	1.1M	30%
+0.45M Na_2SO_4	1.0M	0.2M	127%
+0.9M cholineCl	0.1M	1.1M	33%

Photosynthetic activities under changing Na+ and Cl- concentrations. The maximum rate of O2 evolution measured under different NaCl concentrations were shown in Table 1 as relative values to that measured in the ordinary F/2ASW. In the low NaCl medium (0.1 M Na+, 0.2 M Cl-), the relative rate of the maximum O2 evolution was approximately 71% that in the ordinary F/2ASW. The rate was also decreased to approximately 30% under high NaCl concentrations (1.0 M Na+, 1.1 M Cl-). Interestingly, the relative rate was stimulated to 127% in the presence of Na2SO4, which is substituted for NaCl in order to enrich Na+ to 1.0 M without elevating Cl- concentration. In contrast, enrichment of Cl- alone to 1.1 M by the addition of 0.9 M choline-Cl resulted in the inhibition of O2 evolution to the rate approximately 33% that in the ordinary F/2ASW. Similar behavior of PSII activities relating to changes in Na+ and Cl- concentrations, which were determined as BQ dependent rate of O2 evolution and variable-chlorophyll fluorescence (Fv/Fm) (data not shown). It was particularly evident that BQ-dependent electron flow and Fv/Fm were stimulated respectively to 129 and 123% in the presence of 0.45 M Na2SO4 but inhibited both in 1.0 M NaCl (PSII activity and Fv/Fm were reduced to 90% and 56%, respectively) and in 0.9 M choline-Cl (to 74% and 79%, respectively). These data indicate that Na+ stimulates PSII whereas Cl- works inhibitory. In freshwater autotrophs, Cl- is an essential element for the oxygen-evolving complex (OEC) of the PSII (Gaxiola et al. 1998; Popelková and Yocum 2007). In the marine diatom P. tricornutum, in contrast, increase in Cl- concentration immediately inhibited net photosynthesis and PSII activities, which was mitigated by the presence of Na+ and substitution of Cl- with SO42- efficiently stimulated photosynthetic activities (Table 1), and these results were consistent with growth responses to salt, indicating that sensitivities of diatom photosystem II to Na+ and Cl- are opposite to those in land plants and freshwater algae.

Intracellular localizations of Na+ and Cl-. Cell of P. tricornutum were treated with specific indicator dyes for Na+ and Cl^-, respectively, Sodium green and MEQ. Fluorescent signals of both Sodium green and MEQ in cells grown in ordinary seawater (0.5 M Na^+, 0.6 M Cl^-) were localized in the area surrounding chloroplast autofluorescence but were not detected clearly in the cytosol (Fig. 1). The similar localizations of Sodium green and MEQ were also observed in cells grown in low NaCl concentration (0.1 M Na^+, 0.2 M Cl^-). In contrast, in cells exposed to high NaCl (1.0 M Na^+, 1.1 M Cl^-), both Sodium green and MEQ signals overlapped to chlorophyll fluorescence, indicated that these indicator dyes localized in the stroma (Fig. 1). The similar localization was observed in cells grown in high Na^+ and low Cl^- (1.0 M Na^+, 0.2 M Cl^-), but the MEQ signal in these cells were trace (Fig. 1).

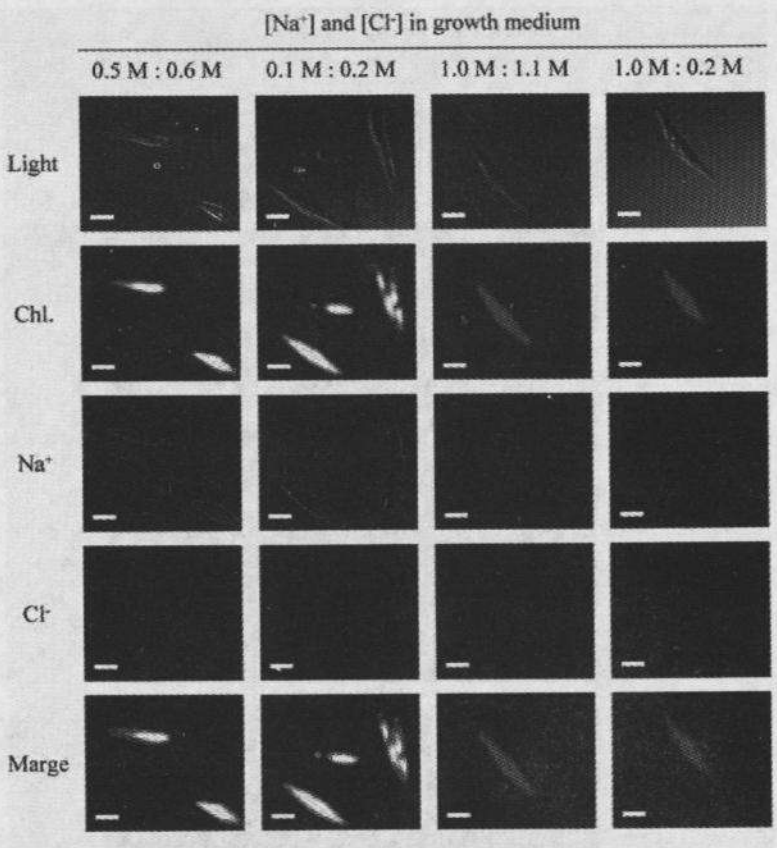

Figure 1. Localization of Na+ and Cl- in cells grown in different concentrations of Na+ and Cl-. Final concentrations of Na+ and Cl- are indicated at the top part of the figure. Light, light image; Chl, chlorophyll autofluorescence; Na+, Sodium green signal; Cl-, MEQ signal; Merge, merged image of Chl, Na+, and Cl-.

Locations of Sodium green and MEQ in cells grown in normal F/2ASW were further characterized using confocal laser (Fig. 2A) and sectioning fluorescent microcopy (Fig. 2B, C). Both Sodium green and MEQ were clearly localized at area surrounding the chloroplast fluorescence (Fig. 2A). Sectioning microscopy of Sodium green and MEQ were deconvoluted and build three dimensional images were polygonized to enhance the surface image of 3-D structure. As a result, chlorophyll fluorescence was localized in a shape presumably reflecting the structures of girdle lamellae, the globe shaped thylakoid membrane in diatom chloroplast, and Sodium green and MEQ were apparently localized surrounding the chlorophyll fluorescence from all directions (Fig. 2B). In the merged images, chlorophyll fluorescence did not appear at the surface, which clearly indicates that NaCl layers surround the chloroplast (Fig. 2B). A cross section of the portion boxed by red line in Figure 2A was constructed with a polygonized deconvolution image (Fig. 2C). The cross section image clearly showed that signals of sodium green and MEQ surrounded the chlorophyll fluorescence as thin layers, which are presumably the parts of the four-layered chloroplast membrane system in secondary symbionts (Fig. 2C).

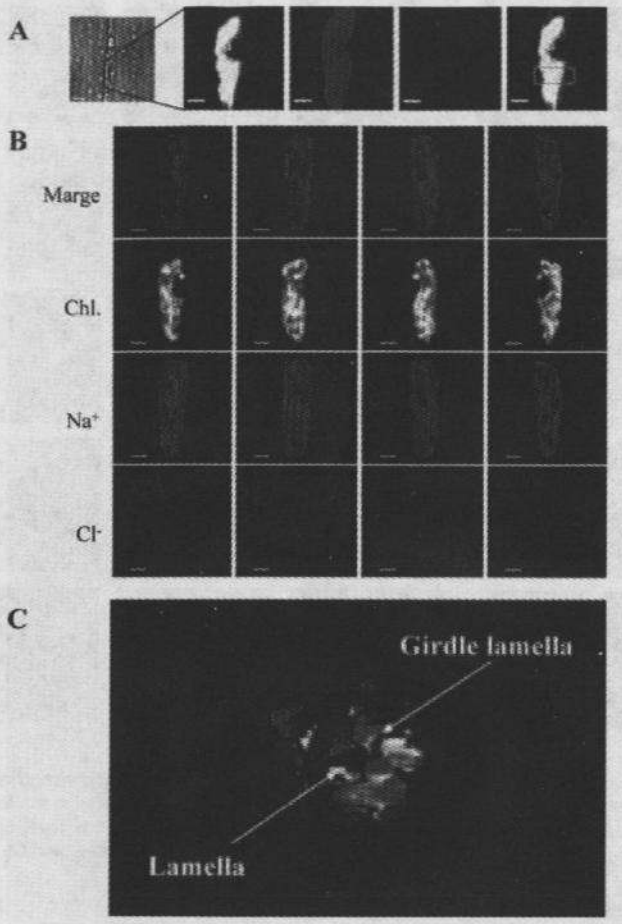

Figure 2. Three-dimensional model analysis of intracellular localization of Na+ and Cl- by sectioning fluorescent microscopy. A, from the left, light image; chlorophyll fluorescent; Sodium green; MEQ; merged image of all three fluorescent images. B, polygonized image of each 3-D fluorescent microgram. Images are rotated every 60 degree. C, Cross section image of the portion indicated in the merged image of A as the red box part. The cross section was rotated 90 degree.

Sodium green and MEQ are the specific indicators for respectively Na^+ and Cl^- and thus their localizations in cells grown in the normal F/2ASW imply that four-layered chloroplast membrane system which specifically occurs in secondary symbionts such as heterokontophyte and haptophyte accumulate Na^+ and Cl^- ions when grown in salt concentrations equal or below the seawater levels. However, accumulations of these ions at such peripheral areas of chloroplast were not observed when cells were exposed to salt a concentration 2 times higher the seawater level and these ions appeared to enter the interior of the chloroplast (Fig. 1). Marine diatom cells grown in salt concentrations below seawater levels probably maintain properly balanced Na^+ and Cl^- concentrations in the stroma by forming concentration gradient using

accumulated ions at the four-layered chloroplast membrane but such system would relax when salt concentration is high. This assumption also accounts for photosynthetic characteristics that the PSII in *P. tricornutum* is activated in the presence of high 0.5 to 1.0 M of Na^+.

Effects of various modulators for localization of ions. Treatment with all modulators except TTX and DIDS caused a severe inhibition of localization of Na^+ and Cl^- at the peripheral area of the chloroplast (Fig. 3). Treatments with EIPA and Mon appeared to **disperse Na^+ and Cl^- ions throughout the cells, whereas OU and IAA-94 treatments seemed to disp**erse ions within the stroma and Sodium green signal appeared to be attenuated in these treatments (Fig. 3). Treatments with TTX and DIDS did not disturb the peripheral chloroplast localizations of Na^+ and Cl^-, but signals of Sodium green and MEQ were apparently attenuated in these treatments (Fig. 3). The mechanisms for the localizations of Na^+ and Cl^- to the four-layered membrane system are not known at present. However, it is well suggested in the present study that some active transport systems participate in formation of concentration gradient of NaCl at peripheral area of the chloroplast, but that channels for Na^+ and anions do not contribute to this localization. Additionally, an inhibitory effect of OU to the localization of ions strongly suggests the participation of Na^+/K^+-ATPase to the accumulation of NaCl at the four-layered membrane system of the chloroplast.

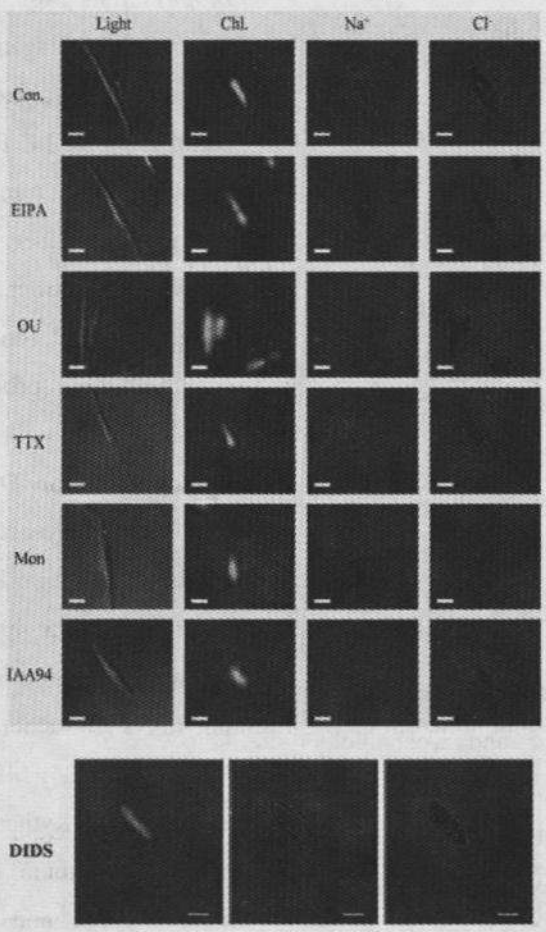

Figure 3. Effect of modulators on Na+ and Cl- localizations in cells grown in the normal F/2ASW. Light image is not shown for treatment with DIDS.

Conclusion

In the present study, basic physiology such as growth and photosynthesis in the marine diatom *P. tricornutum* were shown to exhibit a strong dependency to Na^+ and Cl^- concentrations in the media. Interestingly, all aspects of physiology tested was stimulated in the presence of Na^+ at or above seawater levels, but Cl^- acted oppositely. These responses are sharply contrasting to those in land plants and freshwater algae. These data indicate that marine diatoms possess halophilic nature which requires Na^+ for optimal expressions of housekeeping systems such as PSII. Intracellular flow of Na^+ and Cl^- appeared to be controlled at multiply layered membrane system surrounding the chloroplast as accumulation centre for these ions. Secondary symbionts have developed four-layered plastid membranes and this unique structure of organelle may function, using its layered matrix, as a strong buffer for homeostatic controls of intracellular ions in these organisms.

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