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Subcellular adaptation to salinity and irradiance in *Dunaliella salina*

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Abstract

Dunaliella salina V-63 was cultivated in different concentrations of NaCl (0.5, 1.0, 2.5, 3.0, or 4.0 M) and at two irradiances (170 or 220 μmol $m^{-2}s^{-1}$). Concentration-dependent suppression of growth was observed above 1 M NaCl, and elevated salinity induced formation of salt-containing vacuoles. However, the changes in the chloroplast ultrastructure following changes in salinity and irradiance (increase of invaginations and protuberances, numerous grana with low number of thylakoids, less number of starch grains, *etc.*) appeared to be of primary importance.

Aditional key words: cell ultrastructure, chloroplast, wall-less alga, halotolerance, long-term stress.

Introduction

The importance of studies investigating salt-induced damage to plant cells and halotolerance in general is highlighted by the ever-increasing areas of land affected by salinity and the fact that most plants are sensitive to salt stress. The salt-tolerant, wall-less unicellular, green alga *Dunaliella salina* is a convenient model (Cowan *et al.* 1992, Dondini *et al.* 2000, Giordano *et al.* 2000). In addition, the marine alga is intensively cultured to yield valuable commercial products, including glycerol and β-carotene (Ginzburg 1987, Borowitzka and Borowitzka 1988). It responds to osmotic stress by regulating the flux of carbon between the synthesis of starch in the chloroplasts, and glycerol (osmoticum) in the cytoplasm (Degani *et al.* 1985). Although the physiological mechanisms by which this algae tolerates salt stress are known, the only ultrastructural study on long-term effect of salt-stress was done in *D. bioculata* (Berube *et al.* 1999). In contrast to *D. bioculata*, *D. salina* can accumulate carotenoids in stress conditions (Cowan *et al.* 1992).

The aim of this study was to elucidate the adaptive ultrastructural changes of *D. salina* cells cultivated under salt stress and differential irradiances. The ultrastructural changes were evaluated taking into account respective changes in growth of the alga.

Materials and methods

Dunaliella salina strain V-63 was isolated from the Black Sea salt-works near the town of Bourgas. The algae were cultivated at 28 ± 2 °C, bubbling with air with elevated CO_2 concentration (1 %). The cultures were supplemented by different NaCl concentrations (0.5, 1.0, 2.5, 3.0, or 4.0 M), and grown under irradiance of 170 or 220 μmol $m^{-2}s^{-1}$. Control algae were cultivated on synthetic media (Eddy 1956) containing 1 M NaCl at irradiance of 170 μmol $m^{-2}s^{-1}$.

For electron microscopy, 3.24 cm^3 concentrated glutaraldehyde (25 %, EM grade) was added to 20 cm^3 sample of cell culture to give a final concentration of 3.56 %. The cells were fixed at room temperature for 1 h, rinsed and postfixed for 1 h by adding OsO_4 (1 % final concentration). The samples were subsequently centrifuged, dehydrated in acetone series, and embedded in low-viscosity resin (Spurr 1969). 25 - 30 cells per variant were analysed. Microphotographs were taken using a *JEM-100 B (JEOL*, Kyoto, Japan) electron microscope.

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Results and discussion

Growth of the cells: Light microscopic observations showed that at 0.5 M NaCl approximately 30 % of the cells were destroyed. Maximum growth of *D. salina* V-63 was obtained in medium containing 1 M NaCl and at irradiance of 170 μmol $m^{-2}s^{-1}$. Clear concentration-dependent suppression of growth was observed above 1 M NaCl. At 4 M NaCl the algal yield was 5 % of the material maintained under optimal salt concentrations (1 M NaCl). Nevertheless, the viable cells, though reduced in numbers, still retained their integrity.

Ultrastructure of the cell compartments: When grown under control conditions (1 M NaCl, irradiance of 170 μmol $m^{-2}s^{-1}$) the cells contained a well-developed photosynthetic apparatus (Fig. 1*A*). The chloroplast in the cell was band-shaped with tightly bound thylakoids typically situated in parallel to the longitudinal axis of the chloroplast and the plasmalemma. The most prominent feature of the chloroplast was the large pyrenoid usually surrounded by a variable number of small starch grains. Isolated groups of thylakoids penetrate into the pyrenoid body and terminate blindly. Mitochondria were near both end-sides of the chloroplasts. Typically there was a large nucleus with well-defined inner and outer membranes of the nuclear envelope and nuclear pores. Plastoglobules were scattered throughout the chloroplast along with a few small starch grains. Occasionally, a few Golgi apparatuses and relatively small globules of cytoplasmic lipids were observed throughout the cytoplasm. Several neighbouring vacuoles containing osmiophilic inclusions with irregular forms occupied the large area of the cell.

The cultivation of the algae under elevated salinity (Fig. 1*B,C*) resulted in an increase in number and relative area of the cell vacuoles, which includes formless, angular inclusions. Similar vacuoles have been observed previously by Bohem and Sprey (1979) who report an increase in numbers of heavy osmiophilic formless crystalloid inclusions. Using energy dispersive X-ray analysis and laser microprobe analysis, they were able to show that these inclusions contain high content of silicon, phosphorus and sulfur. The authors suggest that the presence of these formations may be a direct result of culturing the *Dunaliella* cells on agar medium. The present experiments show that the cells, not cultivated on agar, were also able to prevent high salt concentration within the cytoplasm by the formation of salt-containing vacuoles. Frequently, the compartmentation was followed by the expulsion of vacuolar content (Fig. 1*A,C*). These vacuoles were rarely visible in algae grown in optimum salinity and high irradiance (Fig. 1*D,E*). The isolation of redundant salts from the cytoplasm and its organelles may be an important mechanism for the alga survival. However, the main structural peculiarities of the cells in prolonged salt-stress are chloroplast-related changes (Fig. 1*B,C,D,E*).

The chloroplast is the largest cell compartment in *D. salina* and its plasticity allows the increase of invaginations and protuberances during stress conditions. Usually, the remaining cellular organelles resemble chloroplast inclusions in stressed algae (Fig. 1*B,C,D*). The serial slides identify that they are only inserted in cup-shaped invaginations of the chloroplast. There, the organelles are surrounded by some cytoplasm, but isolated to some extent from the major part of the cytoplasm. Therefore, the increased plasticity of the chloroplast during conditions of stress facilitates contact between the other organelles via the chloroplast body. We also suppose, the internal chloroplast system of membranes may well act as a skeleton for wall-less algal cells. This hypothesis is strengthened by the especially clear set-like development of the system at conditions of high irradiance and prolonged combined stresses, *i.e.* high irradiance and salinity. Grana-like formations are numerous although formed by stacking low number (2 - 3) thylakoids (Fig. 1*C,D*). At light saturation, only dark-running enzyme reactions could limit the photosynthetic rate. The balance is achieved by the development of low number thylakoids inside the grana. Two types of starch grains were clearly distinguished in the chloroplasts – firstly those situated between thylakoids (stromal starch grains), and secondly those connected with the pyrenoid. The two types of grains differed in electron density and presumably in chemical composition. During cultivation under high irradiance and low salinity the starch grains took up the largest part of the cell volume (Fig. 1*D*). The large starch grains were numerous and almost uniformly distributed all over the chloroplast, divided by thylakoid sets. In high irradiance-cultivated algae the inter-thylakoidal space was able to accumulate osmiophilic substances. The number and relative volume of starch inclusions decreased in conditions of combined stress (Fig. 1*C*). Some starch grains or their usually empty sheaths were scattered evenly into the stroma, separated by thylakoids. The concomitant pyrenoid starch grains were rarely visible during these conditions with numbers reflecting those observed during the low irradiance (Fig. 1*A,B*).

The synthesis of glycerol after degradation of starch is the mechanism that maintains the pressure potential of the cell (Degani *et al.* 1985, Ginzburg 1987, Goyal *et al.* 1987, Borowitzka and Borowitzka 1988). The lack of starch grains in the chloroplasts at relatively low irradiance indicates an almost full turnover of starch into glycerol (Fig. 1*A,B*). At high irradiance and relatively low salinity only a part of synthesised starch sufficed to maintain the needs for glycerol synthesis. These cells were full of starch grains (Fig. 1*D,E*). During combined stress (Fig. 1*C*), large proportions of synthesised starch were used for glycerol synthesis and the starch grains was smaller than those in cells stressed only by high

irradiance. Small number actively functioning Golgi stacks were observed even in control conditions. Golgi usually appears to be rather unfunctional in stressed cells (Fig. 1*E*). In addition, their number in *Dunaliella salina* cells was not higher in conditions of higher salinity, as distinct from that shown for *D. bioculata* (Berube *et al.* 1999). Mitochondria in *D. salina* cells were usually with reduced internal membrane system. The functional importance of mitochondria visibly increased in conditions of high irradiance, when well-developed cristae that fill the mitochondrial body were visible (Fig. 1*E*).

The cultivation of *D. bioculata* at salt stress led to a reduced pyrenoid area. In *D. salina* cultivated at irradiance of 220 μmol $m^{-2}s^{-1}$ enhanced pyrenoid osmiophility (Fig. 1*C*,*D*) was observed. Pyrenoid is a protein complex in the chloroplast stroma of eukaryotic algae. It is believed that the pyrenoid might function as a reservoir of ribulose biphosphate carboxylase/oxygenase (Rubisco),

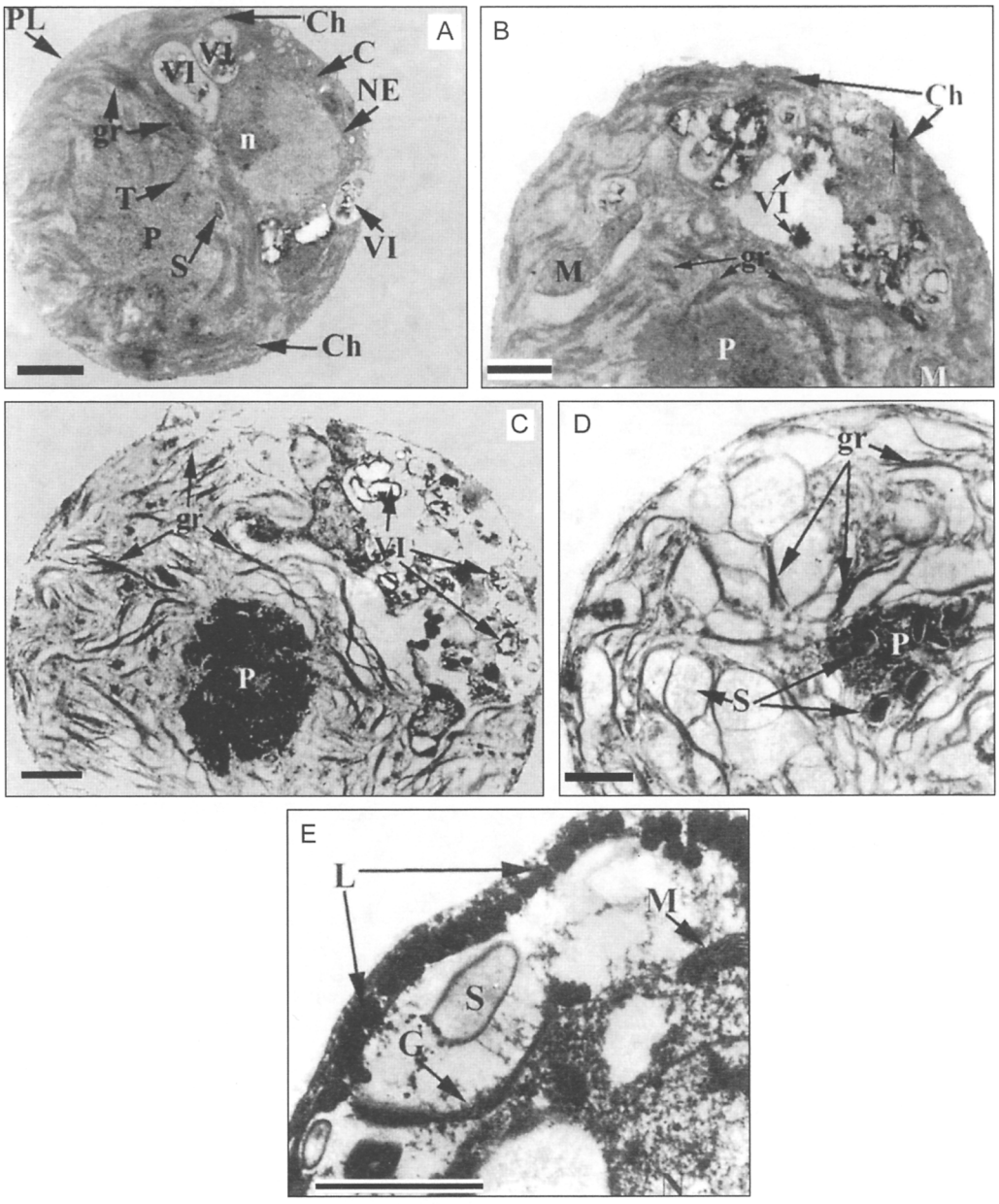

Fig. 1. Electron micrograph of a *Dunaliella salina* V-63 cells: *A* - cell grown in 1 M NaCl and at irradiance of 170 μmol $m^{-2}s^{-1}$; *B* - cell grown in 2.5 M NaCl and at 170 μmol $m^{-2}s^{-1}$; *C* - cell grown in 2.5 M NaCl and at 220 μmol $m^{-2}s^{-1}$; *D*, *E* - cells grown in 1 M NaCl and at 220 μmol $m^{-2}s^{-1}$. C - cytoplasm, Ch - chloroplast, G - Golgi apparatus, gr - grana, L - lipid droplet, M - mitochondrion, N - nucleus, n - nucleolus, NE - inner and outer membranes of the nuclear envelope, P - pyrenoid, PL - plasmalemma, S - starch grain, T - thylakoid, V - vacuole, VI - vacuolar inclusions. *Bars* represent 1 μm.

as a specific metabolic compartment that runs efficient carbon dioxide fixation and starch metabolism (Kuchitsu *et al.* 1988, McCay *et al.* 1991), or that concentrates CO_2 in the vicinity of pyrenoid (Badger and Price 1994). Terrestrial plants adapted to high irradiance are known to contain more Rubisco in their chloroplasts. Recently, Douce and Neuberger (1999) show that the photorespiration has a protective function during stress by acting against the possible damages caused by free radicals in the green leaf. In light of this statement the enhancement of pyrenoid osmiophility shown for the alga may reflect the enhancement of photorespiration rate, at least during exposure to stress and when the rate of carboxylation was relatively depressed. The observed cultivation-dependant changes in the pyrenoid morphology indicate that *D. salina* may be successfully used in more detailed experiments in order to establish a link between the pyrenoid and processes of photosynthesis and photorespiration.

The ultrastructural observations show that the cultivation of cells at irradiance of 220 μmol m^{-2} s^{-1} is accompanied by compartmentation of lipids and carotenoids into osmiophilic globules. The droplets of different in size and osmiophility are dispersed in the cytoplasm (Fig. 1*D*,*E*) or form rosaries situated immediately below the plasmalemma, thereby forming a protective screen against the light, well visible at high magnification (Fig. 1*E*). The high retention of carotenoids could have a physiological significance by protecting chlorophyll molecules from damage by free oxygen radicals, formed preferentially during stress and senescence (Young and Britton 1990, Čatský *et al.* 1995). The picture shown here explains another mechanism of the protecting effect of the carotenoids and the physiological meaning of the observed great accumulation of carotenoids in *D. salina* at similar conditions of cultivation (Ben-Amotz and Avron 1983).

Changes in Golgi apparatus as well in endoplasmatic reticulum during prolonged salt stress are pointed as a leading factor for cell preservation for *D. bioculata* (Berube *et al.* 1999). As distinct from these results, subcellular changes in *D. salina* following changes in salinity and irradiance appear to be secondary results of primary adaptive changes in the chloroplast structure and function.

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