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RESEARCH PAPER

Salt stress-induced cell death in the unicellular green alga *Micrasterias denticulata*

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Abstract

Programmed cell death (PCD) is a key element in normal plant growth and development which may also be induced by various abiotic and biotic stress factors including salt stress. In the present study, morphological, biochemical, and physiological responses of the theoretically immortal unicellular freshwater green alga *Micrasterias denticulata* were examined after salt (200 mM NaCl or 200 mM KCl) and osmotic stress induced by iso-osmotic sorbitol. KCl caused morphological changes such as cytoplasmic vacuolization, extreme deformation of mitochondria, and ultrastructural changes of Golgi and ER. However, prolonged salt stress (24 h) led to the degradation of organelles by autophagy, a special form of PCD, both in NaCl- and KCl-treated cells. This was indicated by the enclosure of organelles by ER-derived double membranes. DNA of NaCl- and KCl-stressed cells but not of sorbitol-treated cells showed a ladder-like pattern on agarose gel, which means that the ionic rather than the osmotic component of salt stress leads to the activation of the responsible endonuclease. DNA laddering during salt stress could be abrogated by addition of Zn^{2+}. Neither cytochrome *c* release from mitochondria nor increase in caspase-3-like activity occurred after salt stress. Reactive oxygen species could be detected within 5 min after the onset of salt and osmotic stress. Respiration, photosynthetic activity, and pigment composition indicated an active metabolism which supports programmed rather than necrotic cell death in *Micrasterias* after salt stress.

Key words: Autophagy, green algae, *Micrasterias denticulata*, photosynthesis, programmed cell death, ROS, salt stress, ultrastructure, zinc.

Introduction

Salt stress is known to have severe effects on plant growth and development. As plants are sessile organisms they must cope with changing environmental conditions by adapting to stress situations via various molecular and physiological processes. A high concentration of salt in the soil may lead to three major types of stress, namely ionic, osmotic, and oxidative stress (Zhu, 2002). Ionic stress results from an imbalance in ion homeostasis. In the case of NaCl salinity, Na^+ competes with K^+ for its uptake and therefore results in K^+ deficiency within the cytosol. Because of the physicochemical similarities between the two anionic cations, enzymes with K^+ binding sites can be inhibited by Na^+ (Maathuis and Amtmann, 1999). In addition, high salt in the soil or, in case of aquatic organisms in the surrounding medium, lowers the osmotic potential and therefore leads to a restricted uptake of water. As a consequence, plants produce compatible solutes like proline or glycine betaine to adjust the osmotic potential within the cell. It is also suggested, that compatible solutes act as osmoprotectants to stabilize enzymes during osmotic stress (Schwab and Gaff, 1990; Garcia *et al.*, 1997; Hasegawa *et al.*, 2000). A recent study has shown that free amino acids modulate salt stress-induced K^+ efflux possibly

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Abbreviations: CTAB, cetyl trimethylammonium bromide; ER, endoplasmatic reticulum; FDA, fluorescein diacetate; F_m, maximum fluorescence; F_v, variable fluorescence; H_2DCFDA, 2′,7′-dichlorofluorescein diacetate; HPLC, high performance liquid chromatography; PCD, programmed cell death; PSII, photosystem II; ROS, reactive oxygen species; TAE, tris-acetate-EDTA; TEM, transmission electron microscopy.

by affecting the activity of key membrane transporters (Cuin and Shabala, 2007*a*). Salt stress also causes oxidative stress as reactive oxygen species (ROS) are generated (Zhu, 2001; Lin *et al.*, 2006; Leshem *et al.*, 2007). Various antioxidative enzymes like superoxide dismutase (SOD), ascorbate peroxidase (APX) or catalase are involved in the detoxification of ROS and the avoidance of resulting damage under salt stress (Cavalcanti *et al.*, 2007; Sekmen *et al.*, 2007). Moreover, compatible solutes may also play regulatory roles in mitigating damaging effects caused by oxidative stress (Cuin and Shabala, 2007*b*).

Whereas most studies on salt stress have been performed in higher plants, data on the salt stress responses in green algae are rather limited. In the halotolerant green alga *Dunaliella tertiolecta*, salt stress causes an increase in the intracellular lipid content and glycerol pool (Takagi *et al.*, 2006; Goyal, 2007), and leads to changes in the activity of antioxidative enzymes (Jahnke and White, 2003). An increase in the expression levels of antioxidative enzymes was also detected in the freshwater alga *Chlamydomonas reinhardtii* upon NaCl stress (Yoshida *et al.*, 2004). In the green algae *Chlorella zofingiensis* and *Haematococcus pluvialis*, salt stress induced the production of the secondary carotenoid astaxanthin (Boussiba and Vonshak, 1991; Cordero *et al.*, 1996; Orosa *et al.*, 2001; Pelah *et al.*, 2004). High salinity also decreased photosynthetic efficiency as shown in *Scenedesmus obliquus* (Demetriou *et al.*, 2007). Severe changes in ultrastructure have been reported after chronic salt stress in *Dunaliella bioculata* (Bérubé *et al.*, 1999).

Only recently it has been shown, that high salinity leads to programmed cell death (PCD) in higher plants which could be regarded as a salt adaptation mechanism (Huh *et al.*, 2002). PCD is a crucial event during normal plant growth and development. It plays an important role in the differentiation of tracheary elements (Fukuda, 2000), endosperm development (Young and Gallie, 2000) or leaf senescence (van Doorn and Woltering, 2004). Besides this involvement in normal developmental processes, PCD is also induced by various biotic or abiotic stresses in algae and higher plants such as hypersensitive response during pathogen attack (Greenberg and Yao, 2004), heat (Fan and Xing, 2004; Zuppini *et al.*, 2007), UV-C irradiation (Danon and Gallois, 1998; Moharikar *et al.*, 2006) or upon H_2O_2 induction (Darehshouri *et al.*, 2008).

Many types of PCD with overlapping morphological and physiological hallmarks have been described not only in animals but also in plants which has led to a claim for a detailed classification of cell death events (Kroemer *et al.*, 2005; van Doorn and Woltering, 2005; Reape *et al.*, 2008).

Typical hallmarks of PCD both in plants and animals include the fragmentation of the DNA into discrete fragments of about 180 bp ('DNA laddering'), condensation and shrinkage of the cytoplasm, release of cytochrome *c* from mitochondria, elevation in cytosolic calcium concentration and an activity increase of caspase-like enzymes (Danon *et al.*, 2000; Krishnamurthy *et al.*, 2000; Wood, 2001; Hoeberichts and Woltering, 2002). One form of PCD is autophagy, morphologically defined in transmission electron microscopy (TEM) (Kroemer *et al.*, 2005) and occurring during development and stress responses in plants (Bassham *et al.*, 2006). During autophagy, organelles are degenerated and enclosed by membraneous structures probably derived from the ER (Kroemer *et al.*, 2005; van Doorn and Woltering, 2005; Reape *et al.*, 2008; Uchiyama *et al.*, 2008).

In the present study, biochemical, physiological, and ultrastructural changes were examined during high salinity in the unicellular freshwater green alga *Micrasterias denticulata.* NaCl and KCl were used as salt stress inductors to differentiate between Na^+- and K^+-specific responses. In addition, iso-osmotic sorbitol stress was used as a control to distinguish ionic from osmotic effects. *Micrasterias* as a model organism for our study was chosen for several reasons. First, the algae grow in acidic peat bog ponds with a frequently changing osmolality by evaporation of water during high temperatures or dilution by rain (Meindl *et al.*, 1989) and can therefore shed light on salt acclimation processes in freshwater green algae in general. Second, salt stress may become an increasing threat for *Micrasterias* due to fertilization of the natural habitat by farming or due to salinization by road salt. Many physiological and ultrastructural studies have already been performed in *Micrasterias* which has increased our knowledge about cell differentiation, growth, and physiology in this unicellular green alga (Kiermayer, 1981; Meindl, 1993; Lütz *et al.*, 1997; Weiss *et al.*, 1999; Oertel *et al.*, 2004; Aichinger and Lütz-Meindl, 2005; Darehshouri *et al.*, 2008) and represent the basis for the present investigation.

As a 'non-classical' model organism, studies in *Micrasterias* may help to detect pathways and morphological changes during PCD which would otherwise remain undiscovered when only classical model organisms like *Arabidopsis thaliana* are used (Golstein *et al.*, 2003). PCD hallmarks in *Micrasterias* upon H_2O_2 induction (Darehshouri *et al.*, 2008) have recently been described. It is one of the aims of the present investigation to find out whether salt stress results in a similar response.

Materials and methods

All chemicals were purchased from Sigma-Aldrich (Vienna, Austria) or Roth (Karlsruhe, Germany) unless stated differently.

Cell cultures

The freshwater green alga *Micrasterias denticulata* was grown in liquid Desmidiaceaen medium (Schlösser, 1982) and kept at a 14–10 h light–dark regime at 20 °C. Cultures were subcultured every 4–5 weeks and the algae divide every 3–4 d by mitosis (for details of culture method see Meindl *et al.*, 1989). Cell cultures were used about 3 weeks after subculturing for all experiments unless stated differently.

Salt and osmotic stress treatments

For salt treatments, 200 mM NaCl or 200 mM KCl was added to the culture medium. To discriminate ionic from osmotic effects, sorbitol concentration (339 mM) iso-osmotic to KCl was used. In addition, 300 mM KCl was used for some experiments. Osmolality was measured with a freezing point osmometer (Osmomat 030, Gonotec, Berlin, Germany).

Vitality staining with fluorescein diacetate (FDA)

Cell cultures were treated with 200 mM KCl, 200 mM NaCl or 339 mM sorbitol for 1, 3, 6, 12, 24, and 48 h, respectively. After the treatments, cells were washed in medium and stained with 0.23 mM FDA for 45 min at RT according to the method described by Yamori *et al.* (2005) and adapted for *Micrasterias* (Darehshouri *et al.*, 2008). After washing, cells were viewed in a Zeiss Axiovert 100M inverted microscope equipped with a confocal laser scanner (Zeiss LSM 510, Oberkochen, Germany) using an argon laser with an excitation of 488 nm. Emission was detected with a band-pass filter (505–550 nm). For each experiment 50 cells were analysed per concentration and time point. Each experiment was performed three times in total using independent cell cultures.

Light microscopy

To obtain defined interphase stages, dividing cells were collected and were treated 48 h after completion of cell growth with the respective salt and sorbitol concentration. Cells were exposed to 200 mM KCl, 200 mM NaCl or 339 mM sorbitol for 3 h and 24 h, respectively, and photographed in a light microscope (UNIVAR, Reichert, Vienna, Austria).

TEM analysis

Cells 48 h after mitosis were treated with 200 mM KCl for 3, 20, and 24 h, 300 mM KCl for 24 h, 200 mM NaCl for 3 h and 24 h, and 339 mM sobitol for 3 h, respectively. Cells were fixed by a high pressure freeze-fixation method as described previously (Meindl *et al.*, 1992; Aichinger and Lütz-Meindl, 2005) and were embedded in Agar low viscosity resin (LV Resin, VH1 and VH2 Hardener, and LV Accelerator; Agar Scientific, Essex, Great Britain). Ultrathin sections were placed on Formvar coated copper grids for TEM analysis and viewed in a LEO 912 transmission electron microscope (Zeiss, Oberkochen, Germany) with an in-column energy filter. The TEM was operated with a LaB_6 cathode at 80 kV. A Slow Scan Dual Speed CCD camera TRS Sharpeye (Troendle, Moorenwies, Germany) controlled by iTEM Software (SIS, Soft Image System, Münster, Germany) was used for image acquisition.

Measurement of photosynthetic efficiency

Cell cultures were brought to approximately 4000 cells per ml and were treated with 200 mM KCl, 200 mM NaCl or 339 mM sorbitol for 0.5, 1, 3, 6, 12, and 24 h, respectively. Assay of photosystem II (PSII) activity was performed by fast chlorophyll fluorescence according to Strasser *et al.* (1995), using the Handy-Pea from Hansatech (King's Lynn, England). Five drops of the cell suspension were pipetted on pieces of filter paper fixed in the clips of the Handy-Pea and incubated in darkness for 15 min. Cells were kept moist and controls showed the normal induction curves (Kautsky-effect) and values of the F_v/F_m ratio of approximately 0.78 (see Results). In each experiment, a minimum of seven parallel measurements were taken for controls and treatments. Each experiment was performed three times. For the calculation of mean curves, controls and treatments were compared using the biophysical parameters of primary photochemistry according to the formula given by Strasser *et al.* (1995) and Srivastava *et al.* (1999).

Determination of chloroplast pigment composition

Cultures were treated with 200 mM KCl, 200 mM NaCl or 339 mM sorbitol for 0.5, 1, 3, 6, and 12 h, respectively. Pigments were extracted from 1.5 ml suspension of cells (approximately 2000 cells per ml). Cells were centrifuged and the algae pellet resuspended in 1 ml acetone. The insoluble material was removed by centrifugation and pigment separation was performed on an Agilent 1100 HPLC with diode-array detection and cooled sample compartment, as described by Lütz *et al.* (1997).

Measurement of photosynthesis and respiration

Micrasterias cell cultures (approximately 4000 cells per ml) were treated with 200 mM KCl, 200 mM NaCl or 339 mM sorbitol for 0.5, 1, 3, 6, and 12 h, respectively. Photosynthesis and dark respiration were measured by means of polarographic oxygen determination (Hansatech, King's Lynn, England), as performed in *Micrasterias* in earlier experiments (Lütz *et al.*, 1997; Weiss *et al.*, 1999) at a constant temperature of 22 °C and at an illumination of ~200 μmol photons m^{-2} s^{-1}.

Preparation of cytosolic protein fraction

Micrasterias cells were treated with 200 mM NaCl or 200 mM KCl for 3 h and collected by centrifugation. Cells were resuspended in homogenization buffer (0.4 M mannitol, 20 mM HEPES–KOH pH 7.4, 1 mM EDTA, 0.1% BSA, 0.6% PVPP, 8 mM cystein) and homogenized according to Lombardi *et al.* (2007). The homogenates were centrifuged at 15 000 *g* for 20 min at 4 °C. This centrifugation step was repeated and in some experiments was followed by an additional ultracentrifugation step at 100 000 *g* for 1 h at 4 °C. The supernatant represented the cytosolic fraction. Protein concentration was determined using Roti-Nanoquant (Roth, Karlsruhe, Germany).

Western blot analysis for detection of cytochrome c

For Western blot analysis, equal amounts of protein were loaded on a 15% SDS gel using standard procedures according to Laemmli (1970). The proteins were transferred to a PVDF membrane (Millipore, Bedford, USA), blocked (5% non-fat dry milk with 0.1% Tween 20 in TRIS-buffered saline (TBS)) and incubated over night at 4 °C with a monoclonal antibody against cytochrome *c* (1:500 in TBS+0.1% Tween 20 + 1% BSA; clone 7H8.2C12, BD Biosciences Pharmingen, San Diego, USA). After washing in TBS+0.1% Tween 20 (3×5 min), blots were incubated in a secondary antibody (1:2500 in TBS+0.1% Tween 20 + 1% BSA; anti-mouse IgG HRP conjugated; Promega, Madison, USA), washed again (3×10 min TBS+0.1% Tween 20), and immunolabelled proteins were detected by chemiluminescence using NOWA kit (MoBiTec, Göttingen, Germany).

DNA isolation

Cell cultures were treated with 200 mM KCl, 200 mM NaCl or 339 mM sorbitol for 1, 3, 6, and 12 h. To examine the effect of Zn^{2+}, an inhibitor of Ca^{2+}-dependent endonucleases (Mittler and Lam, 1995; Sugiyama *et al.*, 2000) on DNA laddering, cells were treated with 0.5 mM $ZnSO_4$ for 1 h. 200 mM KCl or 200 mM NaCl were added thereafter for another 6 h. In addition, *Micrasterias* cells were treated for 7 h with 0.5 mM $ZnSO_4$ as a control.

After the treatments, cells were centrifuged at 2755 *g* for 2 min, washed twice with ddH_20 and frozen in liquid nitrogen. Genomic DNA was isolated using CTAB (Murray and Thompson, 1980). In brief, cells were homogenized to a fine powder using a mortar and pestle under liquid nitrogen and thawed in 2× CTAB extraction buffer (2% w/v CTAB, 1.4 M NaCl, 20 mM EDTA, 1% w/v PVP-40, 100 mM TRIS-HCl pH 8.0). RNase A was added and the homogenate was incubated for 1 h at 37 °C. DNA was extracted twice with an equal volume of chloroform:isoamylalcohol (24:1 v/v) and precipitated with 0.6 vols of isopropanol. For visualization of the DNA ladder, equal amounts of DNA (2 μg) were loaded on a 1.5% TAE agarose gel and stained with ethidium bromide.

Caspase-3-like activity

Cell cultures were treated with 200 mM KCl, 200 mM NaCl or 339 mM sorbitol for 0.5 h and 3 h. Caspase-3-like activity was measured as previously described by Darehshouri *et al.* (2008). The ratios of the caspase-3-like activity of treated cells versus control cells (induction factor) were calculated and averaged from three independent experiments.

ROS production

Cells were treated with 200 mM KCl, 200 mM NaCl or 339 mM sorbitol for 5 min, 30 min, and 3 h, respectively. Control and treated cells were stained with 100 μM 2′,7′-dichlorofluorescein diacetate (H_2DCFDA) (Invitrogen, Eugene, USA) for 45 min at room temperature. H_2DCFDA is a cell permeable non-fluorescent dye until the acetate groups are removed by intracellular esterases and oxidation occurs within the cell (Zuppini *et al.*, 2007). Cells were observed in a confocal laser scanning microscope (Zeiss LSM 510, Oberkochen, Germany). Excitation was generated with an argon laser at 488 nm; emitted light was 505–550 nm band-pass filtered. Each experiment was done with 50 cells and repeated three times.

Results

Cell viability after salt stress

FDA staining was used to detect cell viability during salt and osmotic stress in *Micrasterias* cell culture (Fig. 1). As shown in Fig. 1, about 80% of the cells were viable after salt (200 M NaCl or 200 mM KCl), respectively, osmotic stress (339 mM sorbitol) within 6 h treatment. However, differences in cell viability could be detected after 12 h. At this timepoint, about 80% of the cells stained FDA positive during NaCl salinity whereas only 60% or 50% survived the sorbitol or KCl stress, respectively. This situation was similar after 24 h. Cell viability dropped to 40% after 48 h NaCl and sorbitol exposure, whereas only 10% of the cells were alive after KCl treatment.

Morphological and ultrastructural changes in Micrasterias after salt and osmotic stress

Light microscopic investigations have shown that KCl leads to marked foam-like vacuolization in *Micrasterias* after 3 h (Fig. 2B). This was not observed in NaCl (Fig. 2C) or sorbitol treated cells (Fig. 2D). When compared to controls (Fig. 2A) the chloroplast was retracted from the cell cortex but no clear sign of plasmolysis could be detected, neither in NaCl- nor KCl-stressed algae (Fig. 2B, C). However, cells exposed to the iso-osmotic sorbitol treatment showed clear plasmolysis (Fig. 2D). Vacuolization in KCl-treated *Micrasterias* (Fig. 2E) and retraction of the chloroplast in NaCl- or KCl-stressed cells (Fig. 2E, F) as well as plasmolysis after sorbitol exposure (Fig. 2G) became more distinct after 24 h.

In comparison to control cells (Fig. 3A, B), almost all mitochondria showed balloon-shaped membrane protrusions at one pole and had an electron dense matrix after 3 h treatment with 200 mM KCl (Fig. 3D–F, H). The lumen of ER cisternae was swollen (Fig. 3G, I, J, L), ER compartments appeared involute (Fig. 3L) and in several cases began to surround microbodies which seemed to be trapped by the ER (Fig. 3I, J). Golgi bodies were also involute. As no vesicles were pinched off they appeared inactive and the number of their cisternae was reduced (Fig. 3E, G) when compared to controls (Fig. 3B). Multivesicular bodies were also observed after this treatment (Fig. 3F). After treatments with 200 mM and 300 mM KCl for 20 h or 24 h similar ultrastructural changes were observed. Dictyosomal cisternae were still involute and vesicle production at the cisternal rims had completely ceased (Fig. 4A, D, E, H, I). The number of dictyosomes was reduced. Dictyosomes and other organelles

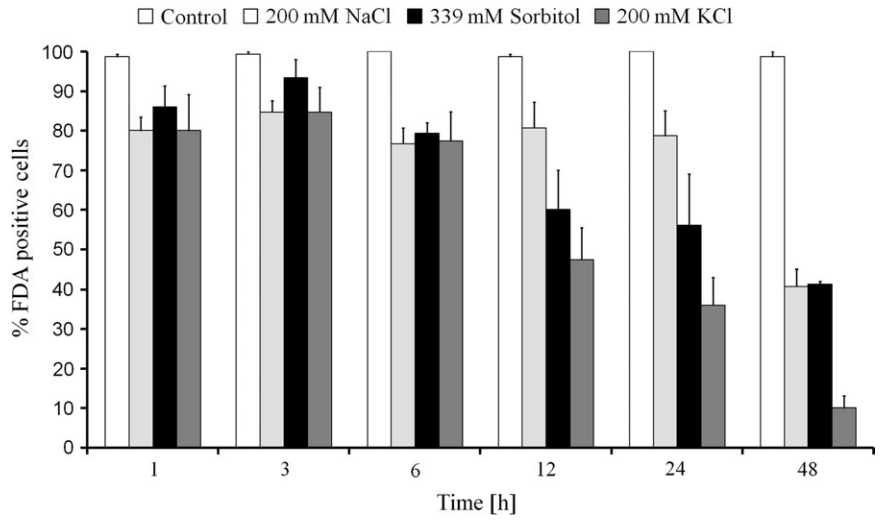

Fig. 1. Percentage of FDA positive *Micrasterias* cells after treatment with 200 mM NaCl, 200 mM KCl or 339 mM sorbitol for 1, 3, 6, 12, 24, and 48 h. Control represents untreated cells. Data are the means of three independent experiments +SE.

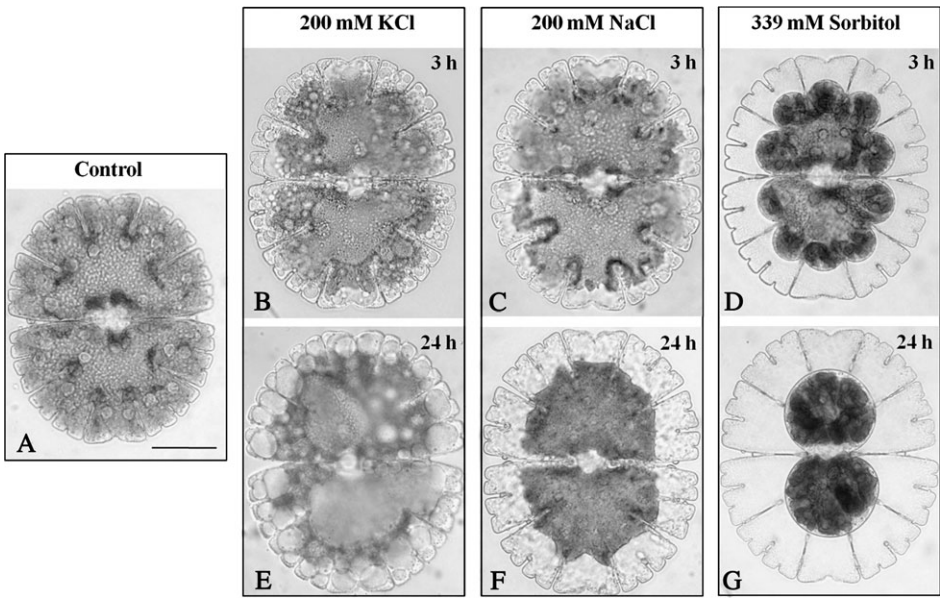

Fig. 2. Light microscopic images of *Micrasterias*. Untreated control (A), 3 h treatment (B–D) and 24 h treatment (E–G) with 200 mM KCl (B, E), 200 mM NaCl (C, F), and 339 mM sorbitol (D, G). Scale bar=50 μm.

were frequently surrounded by swollen ER compartments creating double membrane autophagosomes which indicate autophagy (Fig. 4A, B, F, H, I). Only very few mitochondria showed balloon-shaped membrane protrusions (Fig 4C), but all mitochondria still revealed an electron dense matrix (Fig. 4A, C, G–I). During all KCl treatments, chloroplast thylakoids appeared slightly dilated and the stroma revealed high electron density (Figs 3K, L, 4G, I).

After treatment with 200 mM NaCl for 3 h, mitochondria showed normal shape but an electron dense matrix (Fig. 5A). In contrast to KCl treatment, dictyosomes did not reveal any specific morphological changes (Fig. 5B). After 24 h, ER compartments were swollen and dilated and their number was increased (Fig. 5C). In these cells dictyosomes were disintegrated into numerous small vesicles (Fig. 5C, D). Autophagy by enclosure of organelles such as microbodies by double membranes could be observed here as well (Fig. 5C, E, F). In comparison to control cells, the number of mitochondria was markedly increased (Fig. 5C) and the chloroplast stroma revealed high electron density (Fig. 5C, E).

No ultrastructural alterations were observed after treatment with an iso-osmotic concentration of sorbitol (339 mM) for 3 h (Fig. 3C). None of our experiments induced changes in the number of organelle interactions (Aichinger and Lütz-Meindl, 2005) and the cell wall remained intact (data not shown).

Photosynthetic efficiency (F_v/F_m) after salt and osmotic stress

To determine the effect of salinity and osmotic stress on photosynthesis, the maximum photochemical yield (F_v/F_m)

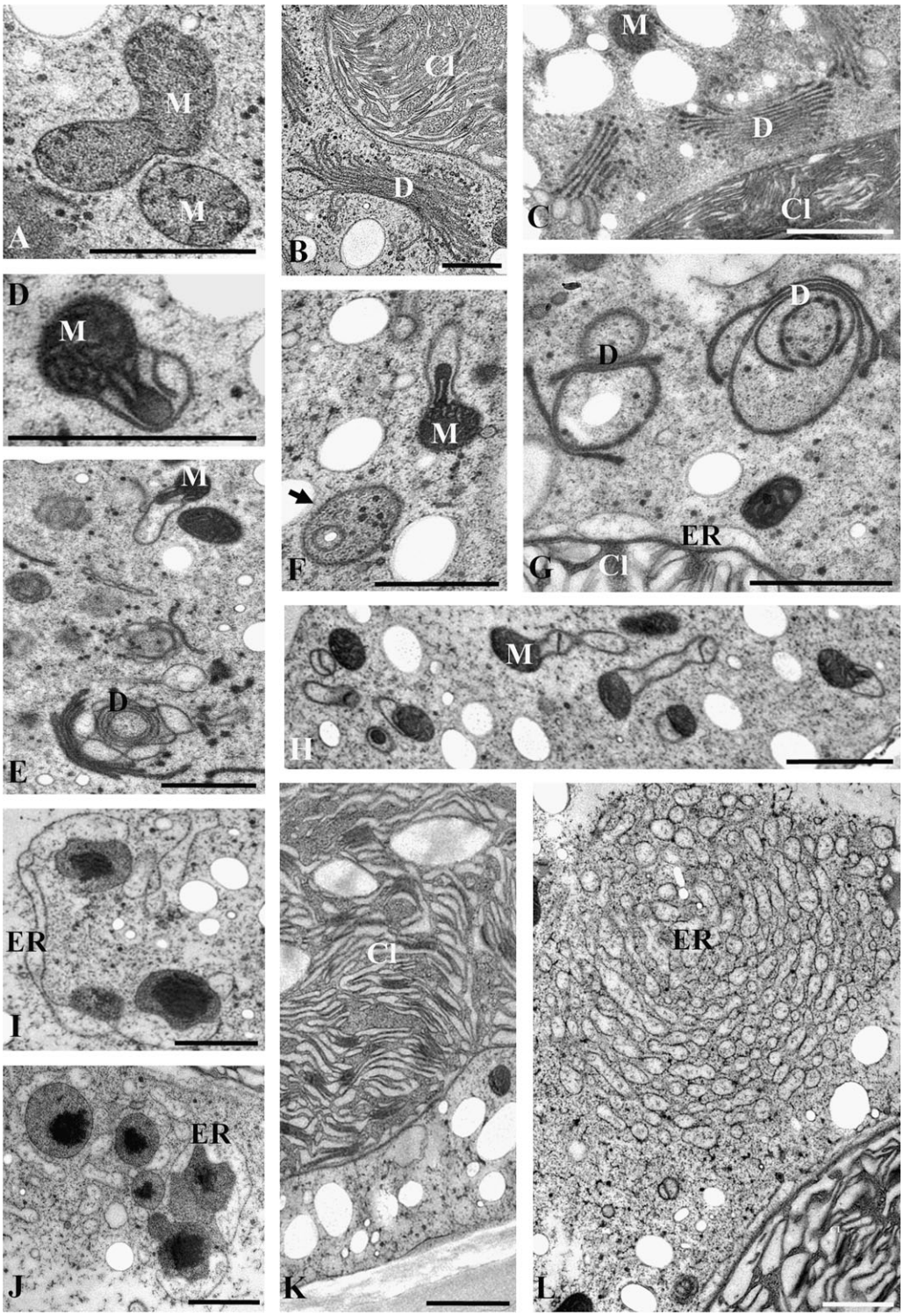

Fig. 3. TEM micrographs showing ultrastructure of control cell (A, B), of cells after treatment with 339 mM sorbitol for 3 h (C) and ultrastructural changes after treatment with 200 mM KCl for 3 h (D–L). (D–F, H) Mitochondria with electron dense matrix and balloon-shaped membrane protrusions, (E, G) involute and inactive dictyosomes with decreased cisternal number, (F) multivesicular body (arrow), (I, J) swollen ER cisternae beginning to surround microbodies, (K) chloroplast with slightly dilated thylakoids and dense matrix, (L) ER compartments involute. Cl, chloroplast; D, dicytosome; M, mitochondrion. Bar=1 μm.

of photosystem II was measured (Fig. 6). Within 6 h treatment, the F_v/F_m values of 200 mM KCl, 200 mM NaCl or 339 mM sorbitol stressed cells, respectively, were in a physiological range of 0.67–0.77, comparable to the control (F_v/F_m=0.78), and typical for *Micrasterias* as described elsewhere (Darehshouri *et al.*, 2008). In contrast to NaCl- or sorbitol-treated *Micrasterias* cells, where the F_v/F_m values remained similar to the 6 h values, a decrease in

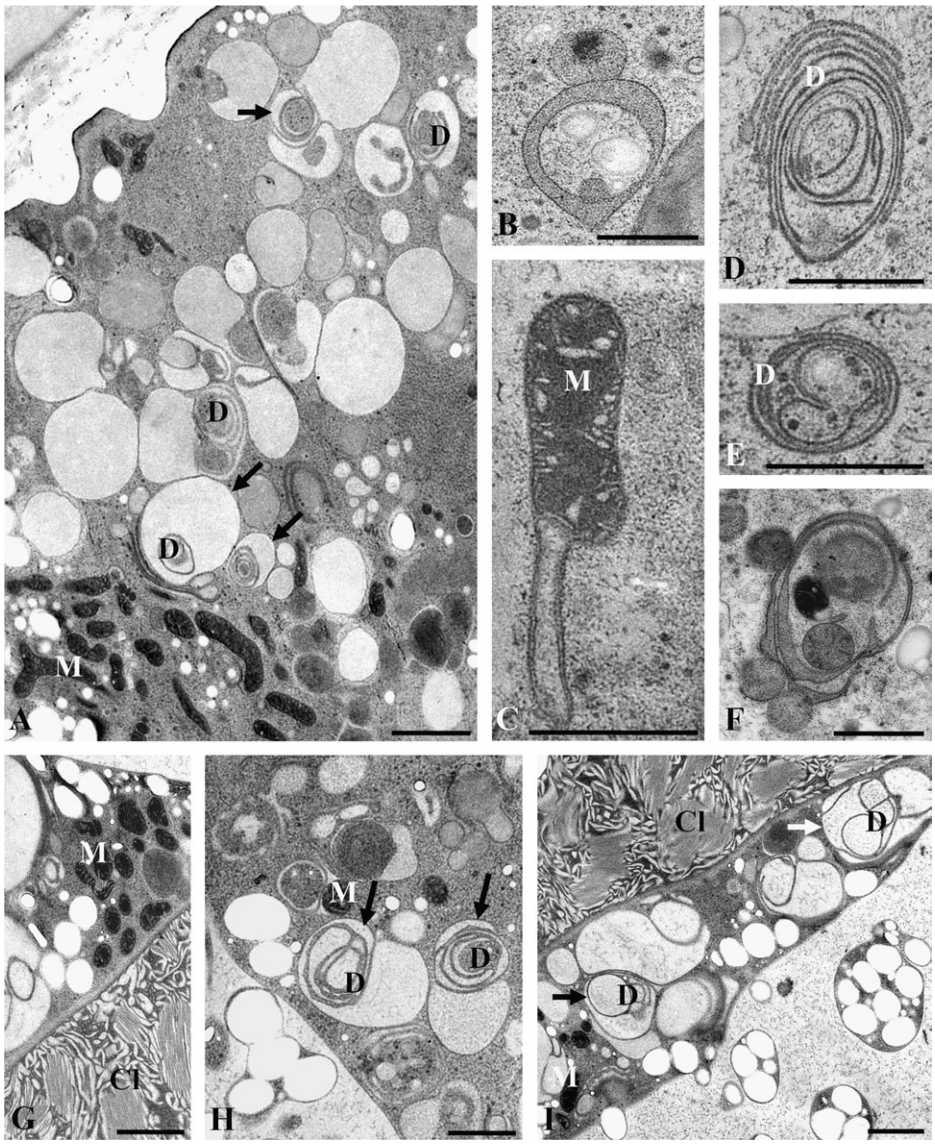

Fig. 4. TEM micrographs showing ultrastructural changes after 24 h treatment with 200 mM KCl (A), with 300 mM KCl (B–F), and 20 h treatment with 200 mM KCl (G–I). (A, H, I) Involute and inactive dictyosomes with decreased number of cisternae surrounded by swollen ER compartments (arrows) indicating autophagy. (A, C, G–I) Mitochondria with electron dense matrix, (G, I) chloroplast reveals dense stroma and slightly dilated thylakoids, (B, F) autophagosomes as indicated by surrounding of organelles with ER cisternae, (C) mitochondrion with balloon-shaped membrane protrusion, (D, E) involute and inactive dictyosomes. Cl, chloroplast; D, dicytosomes; M, mitochondrion. Bar=1 μm.

primary photosynthetic efficiency (F_v/F_m=0.54) was measured in KCl-treated cells after 12 h. After 24 h stress, photosynthetic efficiency decreased in all treatments. The lowest efficiency was measured in KCl-stressed cells (F_v/F_m=0.39), followed by NaCl stressed cells (F_v/F_m=0.51) and a comparably better photosynthetic efficiency in sorbitol treated cells (F_v/F_m=0.66).

Measurements of gross photosynthesis and respiration

Gross photosynthesis and respiration was measured by means of polarographic oxygen determination during salt (200 mM KCl or 200 mM NaCl; Fig. 7A, B) and osmotic (339 mM sorbitol) stress (Fig. 7C). Within 1 h KCl stress, photosynthesis was about 87% of the control. After 3 h a further decrease to 67% was observed. The decrease in photosynthesis continued to 42% of the control after 12 h (Fig. 7A). NaCl had similar effects on photosynthesis, but these were less pronounced. Within the first h almost no decrease was observed and photosynthesis dropped to 64% of the control in average until 12 h (Fig. 7B). Cells treated with sorbitol showed increased photosynthesis, even after 12 h and reached 134% of the control (Fig. 7C).

Respiration was similar in controls and NaCl-treated cells, whereas KCl and sorbitol showed higher respiration when compared to NaCl-stressed *Micrasterias* cells. After 12 h, sorbitol respiration increased more than 2-fold which

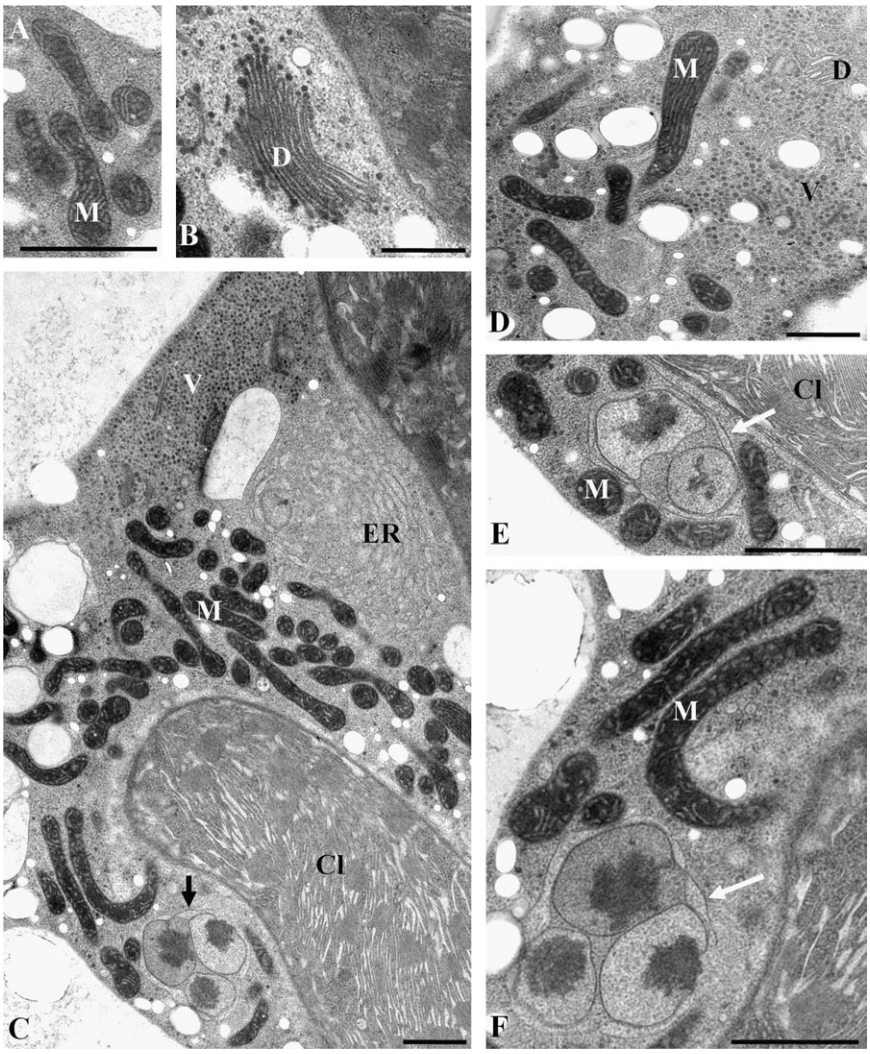

Fig. 5. TEM micrographs showing ultrastructural changes after 3 h treatment with 200 mM NaCl (A, B) and 24 h treatment with 200 mM NaCl (C–F). (A) Mitochondria with electron dense matrix, (B) dictyosome without any specific ultrastructural changes, (C) numerous mitochondria with electron dense matrix, dictyosomes are disintegrated into numerous small vesicles, autophagosome (arrow) including three microbodies, dilated ER compartments, unchanged chloroplast structure but electron dense stroma, (D) dictyosomes disintegrating into numerous small vesicles, mitochondria with electron dense matrix, (E, F) autophagosomes (arrows), mitochondria with electron dense matrix, (F) enlarged detail of (C). Cl, chloroplast; D dictyosome; M, mitochondrion; V, vesicles. Bar=1 μm.

means a reduced net carbon gain compared to shorter treatments.

Pigment composition

HPLC-based analysis was performed to detect changes in pigment composition during salt and osmotic stress. In none of the treatments (200 mM KCl, 200 mM NaCl or 339 mM sorbitol) and time points (0.5, 1, 3, 6, and 12 h) a significant change in pigment amount and composition compared to the control could be detected (data not shown).

DNA laddering in Micrasterias *after salt or osmotic stress*

Agarose gel analysis of genomic DNA isolated from cells treated with 200 mM NaCl, 200 mM KCl or 339 mM sorbitol was used to find out whether salt or osmotic stress induced ladder-like degradation of DNA (Fig. 8A). Both 200 mM NaCl and 200 mM KCl induced DNA laddering within 6 h treatment, whereas in NaCl-treated cells, the DNA ladder could already be detected 1 h after the onset of salt stress. After 12 h, the ladder started to disappear both in NaCl- and KCl-treated cells. The iso-osmotic sorbitol treatment did not lead to DNA laddering within 12 h treatment. No DNA laddering was detected in controls.

Influence of Zn^{2+} on DNA laddering

To study the effect of Zn^{2+}, known to inhibit Ca^{2+}-dependent endonucleases (Mittler and Lam, 1995; Sugiyama *et al.*, 2000), on DNA laddering, cells were treated with 0.5 mM $ZnSO_4$ for 1 h before the application of salt stress (200 mM KCl or 200 mM NaCl). The cells

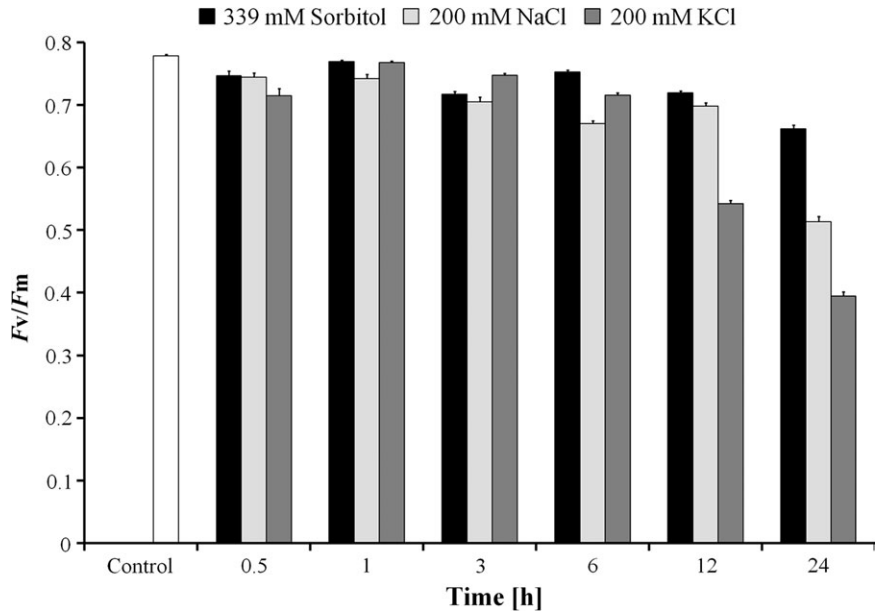

Fig. 6. Photosynthetic efficiency (F_v/F_m) of *Micrasterias* cells treated with 200 mM KCl, 200 mM NaCl or 339 mM sorbitol for 0.5, 1, 3, 6, 12, and 24 h. Control represent untreated *Micrasterias* cells. Data are means of three experiments +SE.

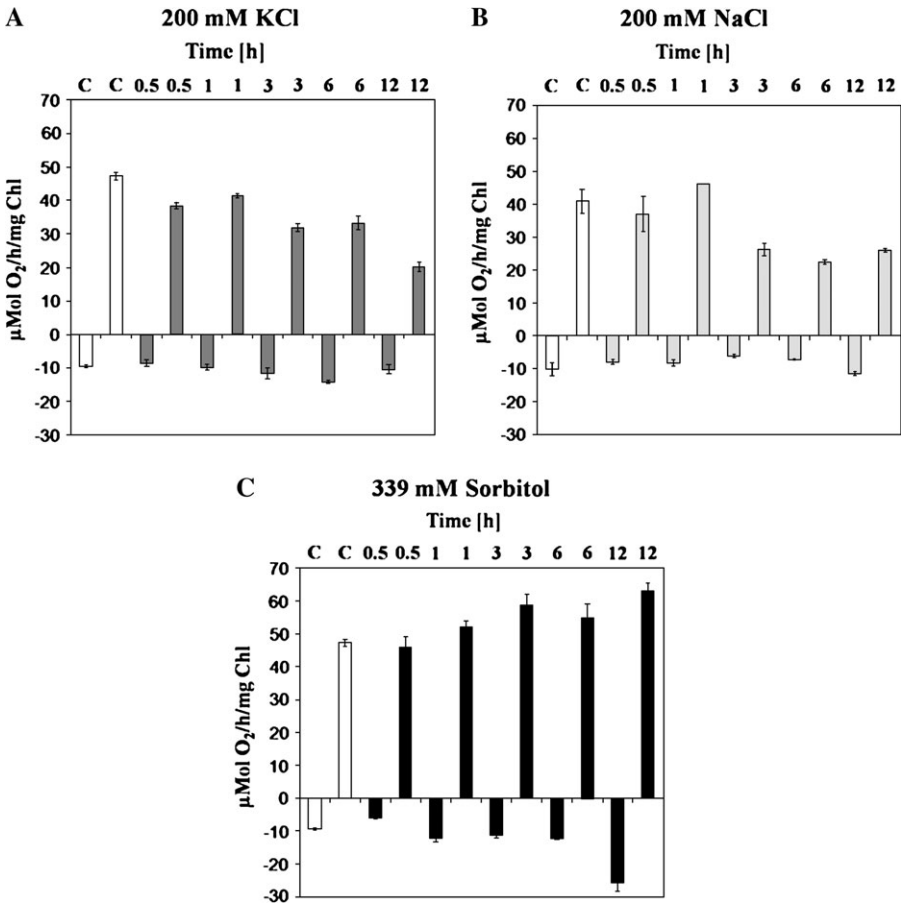

Fig. 7. Changes in photosynthetic O_2 production and in O_2 consumption (dark respiration) by different solutes with time. (A) 200 mM KCl, (B) 200 mM NaCl, (C) 339 mM sorbitol. C, control. Error bar indicates ±SE.

were exposed to salt stress for 6 h because, at this time point, DNA laddering was most prominent both in NaCl- and KCl-treated cells (see also Fig. 8A). As illustrated in Fig. 8B, no DNA laddering was detected in controls (lane 1), Zn^{2+}-treated (lane 2), or in KCl- or NaCl-stressed cells pretreated with $ZnSO_4$ (lane 3 and lane 5, respectively).

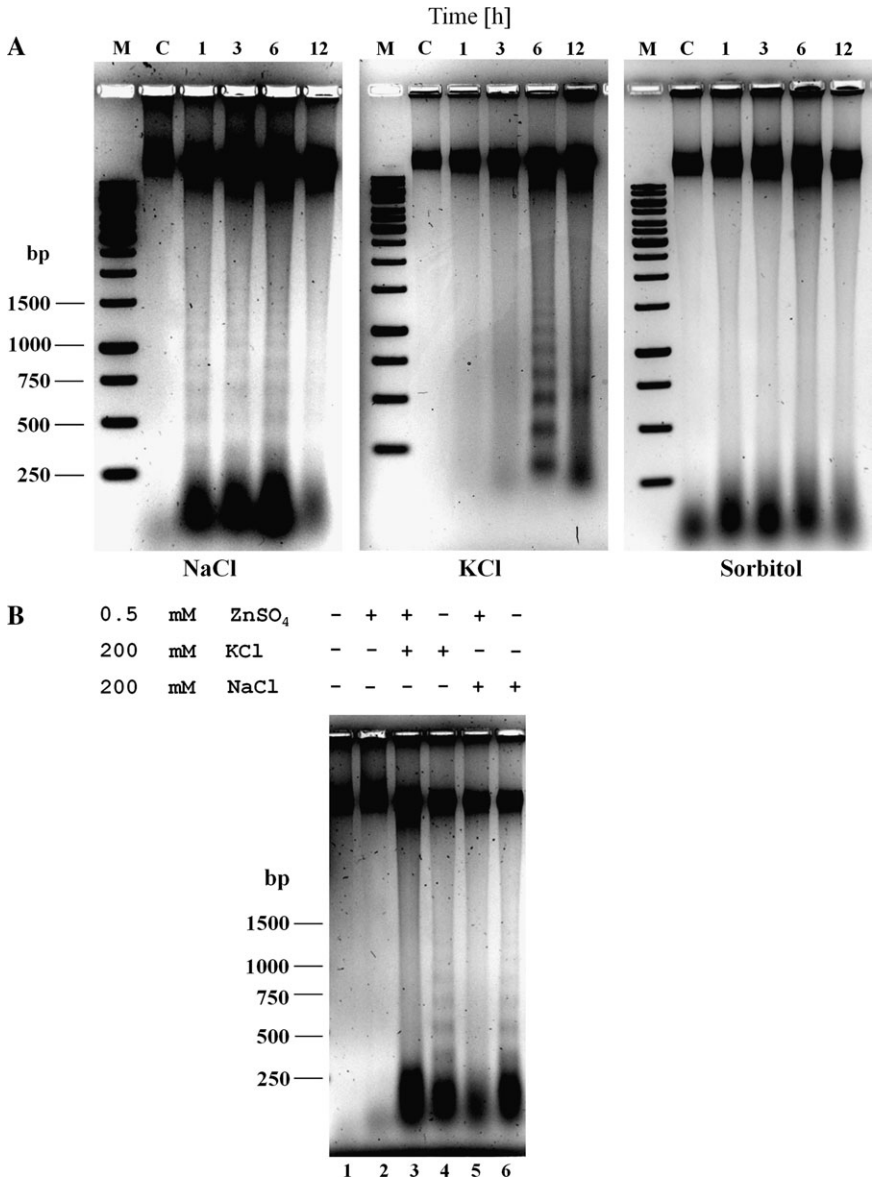

Fig. 8. (A) Time-dependent DNA laddering in *Micrasterias* after treatment with 200 mM NaCl, 200 mM KCl or 339 mM sorbitol. C DNA of untreated control cells. M, DNA marker. (B) Inhibition of DNA laddering by Zn^{2+} in *Micrasterias*. Lanes 1–6: DNA of cells after different treatments. Lane 1, controls; lane 2, 0.5 mM $ZnSO_4$ for 7 h; lanes 3 and 5, pre-treatment with 0.5 mM $ZnSO_4$ for 1 h before the additon of 200 mM KCl or 200 mM NaCl for 6 h, respectively; lanes 4 and 6, 200 mM KCl or 200 mM NaCl alone for 6 h, respectively. 2 μg DNA were loaded in each lane of a 1.5% agarose gel. Images are shown in inverted mode.

DNA laddering could only be detected in *Micrasterias* treated for 6 h with 200 mM KCl (lane 4) or 200 mM NaCl alone (lane 6).

Caspase-3-like activity

Caspase-3-like activity was measured after salt and osmotic stress. After 0.5 h treatment with 200 mM KCl, 200 mM NaCl or 339 mM sorbitol, the activity of caspase-3-like enzyme was decreased when compared to controls (Fig. 9). Caspase-3-like activity was also reduced when compared with the controls after 3 h treatment with 200 mM KCl. However, in the case of 200 mM NaCl and 339 mM sorbitol, activity of this enzyme went back almost to the control level (Fig. 9).

Cytochrome c detection

Western blot anaylsis was performed to detect cytochrome *c* in the cytosol during salt stress. No increase in cytochrome *c* could be detected after 3 h of treatment neither during NaCl nor KCl stress (data not shown).

ROS production

Production of intracellular reactive oxygen species (ROS) was shown by green fluorescence in confocal laser scanning

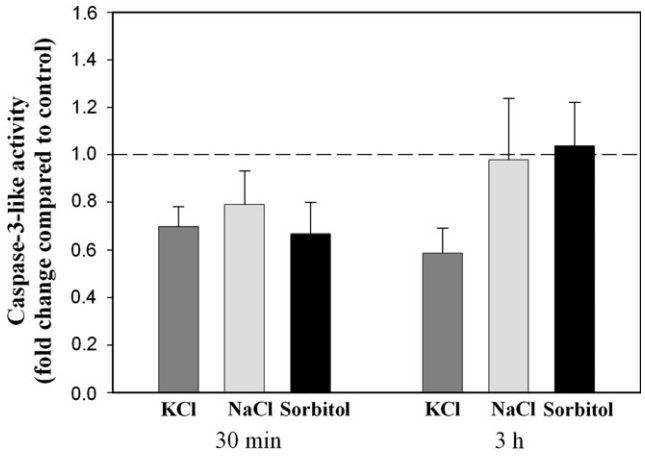

Fig. 9. Effect of salt and osmotic stress (200 mM KCl, 200 mM NaCl or 339 mM sorbitol) on the activity of caspase-3-like enzyme after 30 min and 3 h. Bars represent means of fold change enzyme activity +SE compared to control (set to 1 as indicated by the horizontal dashed line).

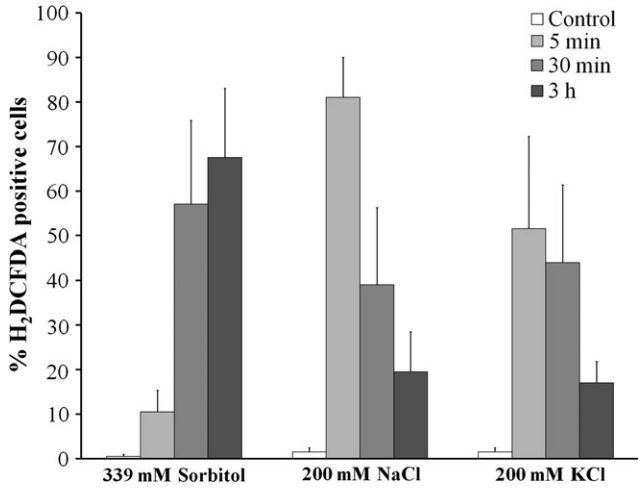

Fig. 10. Intracellular reactive oxygen species (ROS) production in control *Micrasterias* cells and after 5 min, 30 minutes and 3 h treatment with 339 mM sorbitol, 200 mM NaCl or 200 mM KCl. Data are means of four independent experiments +SE.

microscopy. After 5 min treatment with 200 mM NaCl, about 81% of the cells showed ROS production which was decreased dramatically to 39% after 30 min and to about 20% after 3 h. Similar results were obtained by treatment with 200 mM KCl for 5 min when 50% of the cells showed ROS production, which was decreased to 44% after 30 min and to about 17% after 3 h. The course of ROS production was different in sorbitol-treated cells when compared with salt stress. Whereas only 11% of the cells produced ROS after 5 min treatment with 339 mM sorbitol, ROS production increased to 57% after 30 min and to 68% after 3 h (Fig. 10).

Discussion

In the present study, ultrastructural, biochemical, and physiological changes were examined in *Micrasterias* exposed to salt (KCl or NaCl) and osmotic stress induced by iso-osmotic sorbitol concentration.

Light microscopic images show that neither NaCl nor KCl plasmolysed the cells in contrast to sorbitol, suggesting that the salt-stressed cells counterbalance the drop in the osmotic potential of the surrounding medium. Both Na^+ and K^+ have been described to act as osmolytes which are sequestered in the vacuole to maintain turgor pressure in plants during high salinity (Hasegawa *et al.*, 2000). In addition, KCl-treated *Micrasterias* cells show marked foam-like vacuolization of the cytoplasm. Vacuolization was also found in the prokaryotic blue-green alga *Anabaena* sp. (Ning *et al.*, 2002) and in yeast after salt stress (Huh *et al.*, 2002) and has been attributed as a cytological hallmark of PCD therein. Also, other PCD inducers in plants like cadmium led to 'splitting of vacuoles', similar to the KCl-treated *Micrasterias* cells (Kuthanova *et al.*, 2008). In *Micrasterias*, vacuolization occurred, for example, after H_2O_2-induced PCD, although in different appearance (Darehshouri *et al.*, 2008).

To get deeper insight into the morphological changes during salt and osmotic stress, mitochondria, ER, dictyosomes, chloroplasts, and the cell wall were examined in detail by transmission electron microscopy. After short-term treatment with KCl, mitochondria appeared to be the major target of ultrastructural changes, as they showed extreme balloon-shaped membrane protrusions. Similar changes were reported under anoxic stress in *Triticum aestivum* (Virolainen *et al.*, 2002) and in nerve cells during cell death (Muriel *et al.*, 2000). Changes in mitochondrial morphology were also observed during cell death processes in *Arabidopsis* (Scott and Logan, 2008), and have been described as early and specific indicators of cell death therein. Interestingly, the severe ultrastructural changes of mitochondria during KCl treatment in *Micrasterias* were not reflected in decreased respiration. Thus, the morphological changes of the mitochondrial shape may be attributed to K^+-induced osmotic changes within the organelles which do not affect their physiological activity. This is also confirmed by the negative cytochrome *c* leakage test.

The ultrastructural changes induced by salt stress in *Micrasterias* clearly indicate the occurrence of autophagic cell death. The autophagic process starts by the swelling of ER compartments which begin to surround organelles. This leads to double membrane enclosures of organelles as typical for autophagosomes in yeast, plant, and animal cells (Huang and Klionsky, 2002; Reggiori and Klionsky, 2005; Thompson and Vierstra, 2005; Uchiyama *et al.*, 2008). The fate of the autophagosomes in *Micrasterias* is not yet clear. Degradation via lytic compartments would be one possibility, as a previous study by Aichinger and Lütz-Meindl (2005) has demonstrated the occurrence of interactions between different organelles and lytic compartments in this alga.

The formation of double membrane, autophagosome-like bodies was reported during uninfluenced growth of *Dunaliella primolecta* (Eyden, 1975). The presence of autophagic-like

vacuoles was observed in the diatom *Cyclotella meneghiniana* after treatment with chlorinated benzenes and in *Chlamydomonas reinhardtii* exposed to rapamycin (Sicko-Goad *et al.*, 1989; Crespo *et al.*, 2005). As none of these studies indicated an involvement of autophagic processes in cell death, our results provide the first evidence for autophagy during PCD in unicellular algae.

After short- and long-term treatment with KCl, Golgi bodies in *Micrasterias* were involute and inactive. The same phenomenon was also observed in *Micrasterias* cells after H_2O_2 exposure (Darehshouri *et al.*, 2008) where it has been discussed as an additional indicator for PCD. Disintegration of dictyosomes into small vesicles as observed in the present study has also been reported in *Micrasterias* after long-term salt stress by concentrating the nutrient solution in an earlier investigation (Meindl *et al.*, 1989).

Chloroplast structure showed only minor changes. Although the F_v/F_m values were slightly decreased during salt and osmotic stress, they remain in a physiological range at least within the first 6 h of salt stress, when about 80% of the cells are viable. This demonstrates that photosystem II is not damaged. A decrease in gross photosynthesis, measured by oxygen development during NaCl and KCl stress, has to be attributed to changes in enzyme activity or impact on envelope membrane-transport while thylakoids seem to remain unaffected (no treatment effects on pigments). Prolonged salt stress markedly decreased photosynthetic efficiency in *Micrasterias* in a similar way as in the salt-stressed green alga *Scenedesmus* (Demetriou *et al.*, 2007) and during KCl-induced senescence in *Helianthus annuus* L. cv. SH222 (Santos *et al.*, 2001).

Besides the appearance of autophagy, DNA laddering during KCl and NaCl stress also point towards a programmed cell death-like response to salt stress in *Micrasterias*. DNA laddering has been used to diagnose PCD in animals and plants (Wyllie, 1980; Danon *et al.*, 2000; Jiang *et al.*, 2008). Whereas DNA laddering in green algae as a response to various abiotic stresses like heat in *Volvox carteri* and *Chlamydomonas reinhardtii* (Nedelcu, 2006) and UV-C irradiation in *C. reinhardtii* (Moharikar *et al.*, 2006) have been described before, data on salt stress-induced DNA laddering in algae are still missing. To our knowledge, only one publication has shown DNA degradation during KCl-induced PCD in *Anabaena* sp. (Ning *et al.*, 2002). In the present study, it is demonstrated that the ionic rather than the osmotic component of salt stress (NaCl or KCl) causes DNA laddering in *Micrasterias*. This is in accordance with Huh *et al.* (2002) who presented evidence, that salt-induced PCD in plants and yeast is caused by ionic stress. Salt stress-induced DNA laddering as a hallmark of PCD has also been described in barley roots (Katsuhara and Kawasaki, 1996; Katsuhara, 1997), rice root tips (Li *et al.*, 2007*a*, *b*) or tobacco protoplasts (Lin *et al.*, 2005, 2006).

The fact that DNA laddering was already visible 1 h after the onset of NaCl stress and even after a freeze–thaw procedure (Darehshouri *et al.*, 2008) points towards fast activation processes of the responsible endonuclease in *Micrasterias*. This is in agreement with Kuthanova *et al.* (2008) who could detect DNA laddering during a freeze–thaw procedure and after a $CdSO_4$ treatment in tobacco BY-2 cells, which induced PCD. The authors also suggest that '… the same enzymatic apparatus might be involved in realization of the internucleosomal fragmentation during both slow programmed cell death and rapid accidental death', although the precise effectors of DNA cleavage and endonuclease activation remain unknown.

Based on their requirement for different divalent cations, plant endonucleases consist of two classes, namely Zn^{2+}-dependent and Ca^{2+}-dependent endonucleases (Sugiyama *et al.*, 2000). It has also been shown, that Zn^{2+} inhibits Ca^{2+}-dependent endonucleases which are associated not only with apoptosis (Lizard *et al.*, 1997; Torriglia *et al.*, 1997; Yakovlev *et al.*, 2000) but also with plant PCD (Mittler and Lam, 1995; Sugiyama *et al.*, 2000; Jiang *et al.*, 2008). Our results show a clear abrogation of DNA laddering after NaCl and KCl stress by Zn^{2+} supplementation as also reported from tomato protoplasts and tobacco cells during toxin- or hyperthermia-induced PCD, respectively (Wang *et al.*, 1996; Chen *et al.*, 1999). Besides a direct impact on endonucleases, the divalent cation zinc could also affect ion fluxes across the plasma membrane (Demidchik and Tester, 2002, Shabala *et al.*, 2005, 2006) thus diminishing the effect of salt stress in *Micrasterias* as, for example, indicated by abrogation of DNA laddering. In tobacco expression of animal anti-apoptotic CED-9 gene leads to higher salt tolerance by affecting the activities of two different ion channels (Shabala *et al.*, 2007).

An important group of enzymes which participate in PCD of animals are cysteine proteases. These enzymes are involved in different cell activities including DNA degradation processes (Kroemer and Martin, 2005). Although plant genomes do not contain caspase orthologues, there are reported to be up to eight distinct caspase-like activities in plants (Bonneau *et al.*, 2008). An increase in caspase-3-like activity has been found in algal cells such as *Dunaliella tertiolecta* (Segovia *et al.*, 2003) and *Chlorella saccharophila* (Zuppini *et al.*, 2007) during PCD. Enhancement of caspase-3-like activity was also measured in *Micrasterias* after H_2O_2 treatment in a previous study and was abrogated by a specific caspase-3 inhibitor (Darehshouri *et al.*, 2008). By contrast, caspase-3-like activity decreased shortly after the induction of salt and osmotic stress and went back almost to the control level after longer treatment with NaCl and sorbitol, whereas it remained at a lower level during KCl exposure. An increase in caspase-3-like activity seems not to be a general PCD hallmark in *Micrasterias*. Caspase-like independent PCD has also been reported in other plants (Bonneau *et al.*, 2008). A decrease in the caspase-3-like protein amount was observed during apoptotic-like cell death in UV-C treated unicellular cells of *Chlamydomonas reinhardtii* as well (Moharikar *et al.*, 2006). However, the present results do not rule out the possibility of other caspase-like enzymes (VPEs and metacaspases; Bonneau *et al.*, 2008) to be involved in salt stress-induced PCD in *Micrasterias*.

ROS production was induced in *Micrasterias* both after salt (NaCl or KCl) and osmotic stress. However, the different kinetics of ROS production may activate different signals expressed in PCD during salt stress and a general defence response during osmotic stress. Besides their toxic effects on proteins or DNA, some ROS have been reported to act as regulating signals in several cellular defence processes including PCD and they are indispensable elements for salt stress-induced PCD in tobacco protoplasts (Lin *et al.*, 2006).

Although little is known about the signalling cascade during PCD in plants, recent studies have shown that activation of phospholipase C (PLC) and phospholipase D (PLD) is required during camptothecin- and cadmium-induced PCD in tomato suspension cells (Yakimova *et al.*, 2006; Woltering *et al.*, 2007). As PLD and PLC have also been described to be activated during salt stress caused by NaCl or KCl in the unicellular green alga *Chlamydomonas moewusii* (Munnik *et al.*, 2000; Meijer *et al.*, 2002; Arisz *et al.*, 2003) a similar signalling mechanism could play a pivotal role during salt stress-iduced PCD in *Micrasterias*.

In summary, it has been demonstrated that *Micrasterias* shows PCD hallmarks like autophagy, vacuolization, ultrastructural changes, and DNA laddering upon salt stress. As the iso-osmotic sorbitol treatment does not result in these effects, the ionic, rather than the osmotic component of salt stress seems to lead to PCD in *Micrasterias*. The appearance of these changes was accompanied by an active metabolism measured by viability assay, pigment composition, photosynthesis, and respiration pointing towards a programmed cell death and not to a necrotic, accidental cell death event. Our data also reveal that KCl has more pronounced effects on viability and on ultrastructural changes when compared to NaCl. This suggests that *Micrasterias* can cope better with NaCl than with KCl. A possible explanation could be that salt stress in nature is usually caused by NaCl and not by KCl (Ramos *et al.*, 2004). The physiological, biochemical, and ultrastructural changes observed in *Micrasterias* cells during salt stress-induced PCD differ in several features from those described after H_2O_2 induction (see Dahrehshouri *et al.*, 2008). As Morel and Dangle (1997) suggested, the diversity of morphologies during cell death (including PCD) probably reflects different ways in which cells may die. In addition, our results show that different inducers may lead to different cell death pathways in one and the same organism.

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