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Biphasic ROS accumulation and programmed cell death in a cyanobacterium exposed to salinity (NaCl and Na_2SO_4)

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ABSTRACT

High salinity increases antioxidative activities in plants; however, their significance for overall plant salt tolerance remains to be established. This work provided *in vivo* evidence of salinity induced biphasic reactive oxygen species (ROS) accumulation evoking oxidative stress in the cyanobacterium *Anabaena fertilissima*. First, a transient increase in ROS (intense and short-lived) was observed within 5 min of salt exposure, which peaked within 15 min and reached basal level by 2 h. This was followed by a second relatively long-lived and low magnitude ROS accumulation that started at 4 h of salt stress, attained its maximal at 6 h, followed by a gradual decline but did not attain the basal level by the end of experimentation (12 h). Phase I ROS accumulation timing corresponded to the reaction of cyanobacterial cells to the salt stress, while altered photosynthetic and respiratory parameters corresponded with the phase II ROS generation. Relatively lower magnitude of ROS generation during phase II may be attributed to the rapid activation of robust antioxidative systems in cyanobacteria. Consequently, ROS generation lead to the activation of programmed cell death (PCD) undergoing various apoptotic stages such as externalization of phosphatidylserine, DNA laddering and loss of plasma membrane integrity. *A. fertilissima* exposed to salt in the presence of SO_4^- was relatively better equipped to deal with salt stress.

1. Introduction

Salinity is among the detrimental factors that influence the metabolic activities, growth, yield and survival of the organisms. Crop plants are generally less tolerant to salinity and as a result, salinity is one of the primary causes of yield loss and threat to global food supply [1]. Salinity causes osmotic stress, which results in water deficit in cells, followed by ionic and nutritional imbalances [2]. Salinity induced-increased antioxidative activity has been correlated with salt tolerance in plants [3], although Munns and Tester [4] have contradicted the role of reactive oxygen species (ROS) scavenging pathways in salinity tolerance. These authors have emphasized that the differences in salinity tolerance of the plants are due to genotypic differences. These differences alter the rate of CO_2 fixation and the processes that avoid photoinhibition for which plants have abundant capacity. Ascorbate peroxidase deficient *Arabidopsis* mutants were more tolerant to salt stress demonstrating the redundancy of pathways for ROS regulation and protection [5].

Since O_2 can undergo univalent reductions, ROS happen to be the usual byproducts of the leakage of electrons onto O_2 in photosynthetic organisms. GSH (γ-glutamyl-cystinyl-glycine) is usually present at high concentrations [6] in the cells, and is a strong reductant. It serves as a defense against ROS together with other antioxidants [7]. Sulfur depletion in the ambient environment causes a considerable reduction of glutathione [8].

In the present investigation, we used heterocystous *Anabaena fertilissima* belonging to cyanobacteria, which are responsible for oxygenation of atmosphere and ocean, being the first organisms to confront with oxygen and its byproduct ROS. Cyanobacteria share the thylakoid membrane and plastoquinone pool between photosynthetic and respiratory electron transport [9], thus differ from eukaryotic plants. Majority of the information that we have about the oxidative stress in the photosynthetic organisms, comes from studies on higher plants and the studies on ROS generation in cyanobacteria have just begun. Cyanobacteria are indeed the key to develop a comprehension of evolutionary history of ROS generation and defense mechanisms. It seems obvious that the salt induced ROS formation and the defense mechanisms in cyanobacteria may differ from eukaryotic plants.

Reports are available on the relationship between oxidative stress and PCD induction in higher plants, and few recent reports in cyanobacteria [10–16]. Salinity induced *in vivo* ROS production and the resultant oxidative damage to cyanobacteria is not yet

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substantiated. Present work demonstrates salinity induced *in vivo* biphasic ROS production in the cyanobacterium *A. fertilissima*. The study employ the fluorometric probe, 2′,7′-dichlorofluorescin diacetate (DCFH-DA), leading to apoptotic-like PCD supported by the biochemical markers for early (externalization of phosphatidylserine) and late (DNA fragmentation and cell viability) apoptotic activities.

2. Materials and methods

2.1. Organism, growth conditions and salt treatment

Anabaena fertilissima used in the present investigation is a filamentous N_2-fixing sodic soil cyanobacterium. The organism was routinely grown in combined nitrogen-free Allen–Arnon (AA) medium [17]. An average illumination of 70 μE m^2 s^1 was provided with daylight and white fluorescent lamps for 14 h a day in a culture room maintained at 28 ± 1 °C. Salt stress was imposed by incubating the exponentially growing cells in fresh nutrient solution containing either 250 mM NaCl or a mixture of NaCl + Na_2SO_4 (125 mM, each) under standard growth conditions. Samples were withdrawn at different time intervals and used for analyses. Growth was monitored by recording the optical density of the cyanobacterial cultures at 750 nm, daily in a spectrophotometer (Spectronic 20).

2.2. Determination of total chlorophyll, photosynthetic electron transport chain activity, photosynthetic O_2 evolution and respiration

For total chlorophyll determination, cyanobacterial cultures (5 ml) were washed by centrifugation at 10,000 × *g* for 10 min. The pellets obtained were suspended in 80% acetone (2.5 ml), mixed well and kept at 4 °C overnight in a refrigerator for pigment extraction. The process was repeated for complete extraction of chlorophyll. After centrifugation, the volume was made to 5 ml and absorbance of the clear supernatant was measured at 663 and 645 nm, and chlorophyll mg·ml^{-1} was calculated [18].

$$\text{Chlorophyll suspension}(\text{mg}\cdot\text{ml}^{-1}) = (20.2 \times A_{645} + 8.02 \times A_{663}) \times \frac{\text{Total amount of cell suspension}}{1000 \times \text{original volume of cell}}$$

Photosynthetic O_2 evolution, electron transport activities and respiration were determined using polarographic oxygen electrode (Digital oxygen system, model 10, Rank Brother, UK). Temperature of the reaction vessel was maintained at 28 °C, and illumination was provided at 70 μE m^2 s^1 using a projector lamp [19]. Photosynthesis was determined by recording the total O_2 evolution in light. Respiration was determined in dark and subtracting the non-specific oxygen consumption (in presence of 2 μM antimycin A) from the total oxygen consumption [20].

For the determination of photosynthetic electron transport activities, three basic analyses were used [21,22]. Activity of PSI was determined as O_2-consumption in the presence of DCMU after the addition of ascorbate, 2,6-dichlorophenol indophenol and methyl viologen. PS II was measured as O_2-evolution in the presence of p-benzoquinone, and the whole chain (PS I and PS II dependent) activity in the presence of potassium ferricyanide. All the experiments were performed in triplicate.

2.3. Detection of ROS production

For *in vivo* detection and quantification of ROS, cell-permeable non-fluorescent probe, 2′,7′-dichlorofluorescin diacetate (DCFH-DA; Sigma-Aldrich, Cat # D6883) was used [23]. After entering the cell it is hydrolyzed by cellular esterases to 2′,7′-dichlorodihydrofluorescein (DCFH), which upon oxidation by ROS turns into the highly fluorescent polar derivative 2′,7′-dichlorofluorescein (DCF). Cyanobacterial cells exposed to salt for different time were withdrawn, quickly pelleted and DCFH-DA was added to a final concentration of 5 μM. After an incubation of 45 min in the dark at room temperature, samples were washed thoroughly with phosphate buffered saline (PBS), 50 mM, pH 7.0 and subjected to fluorescence microscopic analysis (Nikon eclipse Ni fluorescence microscope processed by NIS-Elements (BR) software). Each experiment was repeated three times.

2.4. Annexin V-FITC staining

Detection of apoptotic cells was done using the Annexin V-FITC Apoptosis Detection Kit (Sigma-Aldrich Cat # 041M4087). The procedure consists of the binding of Annexin V-FITC to phosphatidylserine in the membrane of cells. During the early stages of apoptosis phosphatidylserine present on the internal part of the plasma membrane in living cells is translocated to the external portion of the membrane [24], and becomes available to bind with the highly fluorescent Annexin V-FITC. That offers a rapid detection method for studying the externalization of phosphatidylserine, an indicator of early stages of apoptosis. Cyanobacterial cells exposed to salt for different time intervals were suspended in 20 μl of 1 × binding buffer for 4 min with gentle mixing to avoid clumping of the filaments. That is followed by the addition of 10 μl of Annexin V- FITC and incubation in the dark at room temperature for 30 min. The cells were washed thoroughly with distilled water and subjected to fluorescence microscopy.

2.5. Vitality staining with 4,6-diamidino-2-phenylindole (DAPI)

To examine the vitality of *A. fertilissima* cells, a highly sensitive DNA stain, 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI; Sigma-Aldrich Cat # D9542) was employed. The dye, when excited by an ultraviolet light (359 nm) binds to the minor groove of A/T-rich double-stranded DNA and forms a stable complex (one molecule of dye for every 3 base pairs), which fluoresces blue and can be detected with maximum emission at 461 nm. The dye unable to permeate the membrane of live cells, readily diffuses into the dying cells due to the loss of plasma membrane integrity [25], hence is used for dead cell staining. DAPI, 50 μg was dissolved in 500 μl of PBS to make 100 × stock solution. The stock solution was diluted to 1:100 with 1 × PBS to make 1 μg·ml^{-1} staining solution. Cyanobacterial cells were rinsed with PBS and stained with 1 × DAPI for an hour in the dark [25]. Stained cells were washed with PBS, briefly rinsed with double distilled water and subjected to fluorescence microscopic analysis.

2.6. Genomic DNA isolation

Cells of *A. fertilissima* exposed to salt stress for different time were harvested and subjected to DNA extraction. Cyanobacterial cells were washed with TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0), suspended in 567 μl of the same buffer containing 50 mg·ml^{-1} lysozyme and incubated at 37 °C. After 30 min, 30 μl of 10% SDS and 10 μl of 20 mg·ml^{-1} proteinase K were added and the mixture was incubated for 2 h at 37 °C. Precipitation of proteins and polysaccharides was done using 80 μl of CTAB (Cetyl trimethyl ammonium bromide) buffer (10% CTAB, 0.7 M NaCl) and 100 μl of 5 M NaCl. Samples were gently mixed by inversion and incubated for 10 min at 65 °C. Separation of nucleic acid was done by a phenol: chloroform: isoamyl alcohol (25:24:1) solution. DNA was finally recovered by precipitation using isopropanol and 3 M sodium acetate, pH 5.0 (6:1). Samples were centrifuged at 15,000 rpm for 5 min at 4 °C. The DNA pellets obtained were washed with cold 70% ethanol, centrifuged at 15,000 rpm for 5 min at 4 °C and dried before suspending in 100 μl TE Buffer. Equal amounts of DNA were separated on a 1.2% agarose-gel and stained with ethidium bromide.

2.7. Glutathione assay

Reduced (GSH) and oxidized (GSSG) glutathione were measured using 5′-dithio-bis-(2-nitrobenzoic acid)/GSSG reductase recycling