NaCl-INDUCED DNA DAMAGE IN SALT-SENSITIVE CALLUS VERSUS SALT-TOLERANT CALLUS

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Abstract

Physiological stress induced by environmental factors results in an up-regulation in the antioxidant defense mechanism in cotton. In this study, DNA damage was determined by analyzing 8-oxoguanine which is a strategic marker on DNA caused by Reactive Oxygen Species (ROS) such as superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical (•OH). During oxidative stress, abasic sites such as 8-oxoguanine are produced primarily by •OH which correlate to DNA damage. If ROS production exceeds antioxidant processing capability, stress occurs. Superoxide levels have been shown to be higher in NaCl-sensitive callus as compared to NaCl-acclimated callus. Under normal physiological conditions endogenous ROS can produce around 200,000 base lesions per cell per day. The NaCl-acclimated cotton callus (150mM NaCl) was found to have a nine-fold less basal DNA damage level compared to the control (0mM NaCl) cotton callus. These observations are synchronous with previous studies which demonstrated an up-regulation of antioxidant enzymes in NaCl-tolerant callus.

Introduction

All aerobic species are constantly exposed to background levels of ionizing radiation and physiological stressors such as drought, heat and cold stress, and salinity. Oxidative stress occurs when reactive oxidative species (ROS) build up beyond the ability of cellular defenses to eliminate them (Aruoma and Halliwell 1995). ROS's produced by a series univalent reductions of ground state oxygen include superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), and hydroxyl radical (•OH). These activated oxygen species can seriously disrupt normal metabolism through oxidative damage to lipids (Fridovitch 1986; Wise and Naylor 1987), protein (Halliwell and Guteridge 1985; Davies 1987) and nucleic acids (Fridovitch 1986; Imlay and Linn 1988).

Few studies on DNA damage in plants have been performed compared to numerous studies in mammalian models. A number of clinical conditions in which oxygen free radicals are thought to be involved include Parkinson's disease, atherosclerosis, rheumatoid arthritis, and ageing (Aruoma and Halliwell 1995). DNA damage in all aerobic systems is thought to occur by two mechanisms: increased intracellular Ca^{++} release which can fragment DNA by activating Ca^{++}-dependent endonucleases (Orrenius *et al.* 1989; Farber 1990) and "site-specific" reactions in which metal ions such as Fe^{+3} sitting on non-histone regions of the DNA become reduced by the •OH to form abasic sites as suggested by Mello-Filho *et al*. (1984). These two mechanisms are not mutually exclusive. There are twenty different possible base mutations when free radical attack occurs (Aruoma *et al.* 1989), but 8-oxoguanine is the most common adjunct used to quanitify DNA damage. This hallmark base became recognized for its high affinity to bind to avidin and streptavidin (Struthers *et al*. 1998). Previous studies done on 8-oxoguanine detection were performed by high performance liquid chromatography coupled with electrochemical detection (HPLC-ED) in which measurements often give unsatisfactory results (Shigenaga *et al.* 1990). The technique demonstrated in this current experiment utilizes detection of DNA damage by levels of enzyme activity through horseradish peroxidase (HRP) coupled to streptavidin which binds to the 8-oxoguanine. The HRP processes a substrate that is read at 650nm and correlates to the amount of damage present (Asaeda *et al*. 1998).

Materials and Methods

Growth and Harvest of Callus Tissue

The cotton callus used in the experiments was generated from the Coker 312 cotton cultivar according to the methods of Trolinder and Goodin (1987). These salt-sensitive callus cultivars were either maintained or selected for NaCl-tolerance by intermittently increasing salt concentrations to mimic agricultural conditions until 150mM NaCl growth was achieved (Gossett *et al.* 1996). The callus lines were always kept in a sterile environment at room temperature. The resulting callus tissue was subcultured every four to six weeks.

Detection of DNA Damage

Approximately 4 g of control or NaCl-tolerant callus was weighed out and placed in a 50 mL tube containing 25 mL of previously defined growth medium. This callus solution was then bubbled for 2 hr and treated with the respective solutions of paraquat (0.2µM) or NaCl. The control (0mM NaCl) callus was treated with 150mM NaCl and the NaCl-tolerant (150mM NaCl) was treated with 250mM NaCl. The concoction was then aerated for 4 hr total with harvest points correlated with time 0, 0,5, 1,2, and 4 hr. These samples were then frozen at -70°C for subsequent analyses.

The DNA extraction occurred by placing 1 g of the callus tissue in a 40 mL conical vial with 3 mL of DNAzol™ES which is a guanidine detergent based extraction solution. The tubes were inverted 8-10 times and incubated at room temperature for 5 minutes. 3 mL of chloroform was then added to remove proteins and polysaccarides, and the tubes were again incubated for 5 min. The solution was spun at 12,000xg for 10 min. 2 mL of the top layer was then transferred to a 15 mL Corex tube and 4 mL of 100% EtOH was added to extract the total genomic DNA. This solution was spun again at 5000xg for 4 min. The supernatant was decanted and the pellet was redissolved in 0.2 mL of 10mM EDTA and washed with a DNAzol™ES/EtOH made 1.5:1 and spun again at 5000xg for 4 min at room temperature. The supernatant was then poured off and the DNA pellet was resuspended in 100 µL of TE buffer. The isolated DNA was then treated with ARP (aldehyde reacting product) according to the guidelines by Dojindo's DNA Damage Detection Kit. Ten µL (0.1 µg) of isolated plant genomic DNA was treated with 10 µL of ARP and incubated at 37°C for 1 hr. This reaction solution was then mixed with 380 µL of TE buffer and placed in a filtration tube to be spun at 2500xg for 15 min. The ARP-treated DNA was then resuspended with 400 µL of TE. 90 µL of this solution was then diluted with 310 µL of TE buffer. 60 µL of this diluted ARP-DNA was placed on a 96-well cell culture plate and fixed with 100 µL of DNA-binding solution. Standard damaged DNA samples were also fixed to the plate (see standard curve, Fig. 1). Once incubated overnight, the solution was discarded from each well and 250 µL of washing buffer was used to wash the wells five times. 150 µL of diluted HRP-streptavidin solution was added to each well which binds to the biotinylated DNA abasic site. This solution was then allowed to incubate for 1 hr at 37°C and relinquished from each well. 100 µL of substrate solution was placed in each well and incubated at 37°C for 1 hr. All samples including standards were read at 650 nm spectrophotometrically and duplicated three times. All time course samples were also duplicated three times.

Results and Discussion

Under normal circumstances in plants, the major source of free radicals in cells is electron leakage from electron transport chain in the mitochondria and chloroplasts (Tuteja *et al.* 2001). Until now, DNA damage has never been quantified in NaCl-stressed cotton callus. Using ARP, the amount of abasic sites such as 8-oxodeoxyguanosine and 8-oxoguanine can be determined to quantify the amount of DNA Damage. The hypothesis being tested is whether NaCl-sensitive cotton callus contains more damage than NaCl-tolerant callus. It has been previously shown that NaCl-tolerant cultivars contain higher levels of antioxidant enzyme activity than NaCl-sensitive cultivars (Gossett *et al.* 1994; Gossett *et al*. 1996). Also, a curiosity existed on the amount of damaged DNA in cotton callus when stressed with NaCl and paraquat. Paraquat is a known superoxide producer (Harper and Harvey 1978) which quickly produces •OH, believed to be the main player in oxidative DNA damage (Aruoma and Halliwell 1995).

The NaCl-sensitive (0mM NaCl) cotton callus exhibited a much higher basal level of DNA damage (10-fold higher) than the NaCl-tolerant (150mM NaCl) cotton callus (Fig. 2). This is in agreement with the previous findings of Gossett *et al* that NaCl-tolerant callus has a greater amount of antioxidant enzyme activity level than NaCl-sensitive callus (Gossett *et al.* 1994; Gossett *et al.* 1996). This higher level of antioxidant enzyme activity is believed to be a prophylactic mechanism of DNA damage protection. These discoveries are also consistent with the higher superoxide levels, which is one culprit behind •OH production, present in NaCl-sensitive callus vs. lower levels in NaCl-acclimated callus (Bellaire *et al.* 2000).

The time course experiments also demonstrated an increased amount of DNA damage in NaCl-sensitive callus as compared to NaCl-acclimated callus which remained unchanged through 4 hr (Fig. 3). This again suggests that the NaCl-tolerant cotton callus has acquired the ability to prevent DNA damage resulting from NaCl stress. Cotton callus stressed with NaCl and paraquat are not represented graphically but preliminary results show increased damage in both.

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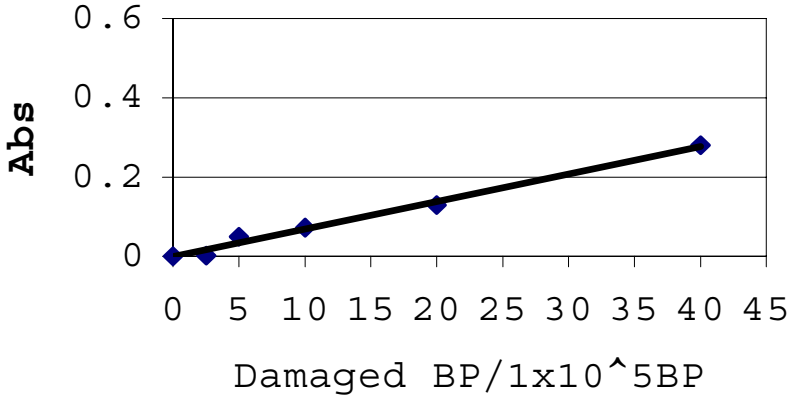

Figure 1. Standard curve made from absorbances of ARP standards used to directly correlate DNA damage in experiments. Units in damaged base pairs per 100,000 normal base pairs. Note the direct linear relationship.

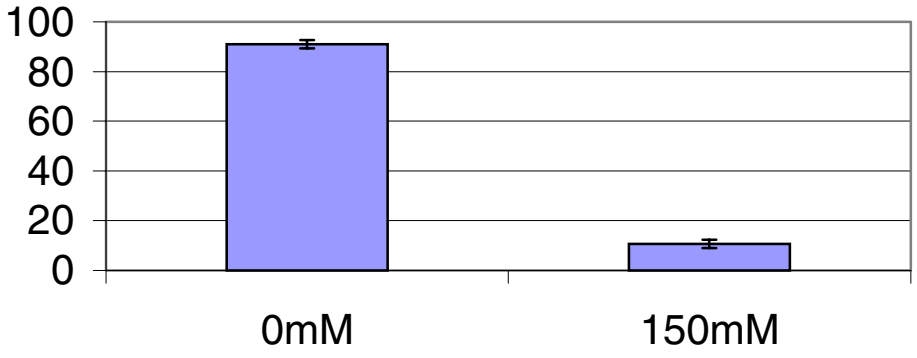

Figure 2. Basal DNA damage compared between control (0mM NaCl) cotton callus and salt-tolerant (150mM NaCl) cotton callus.

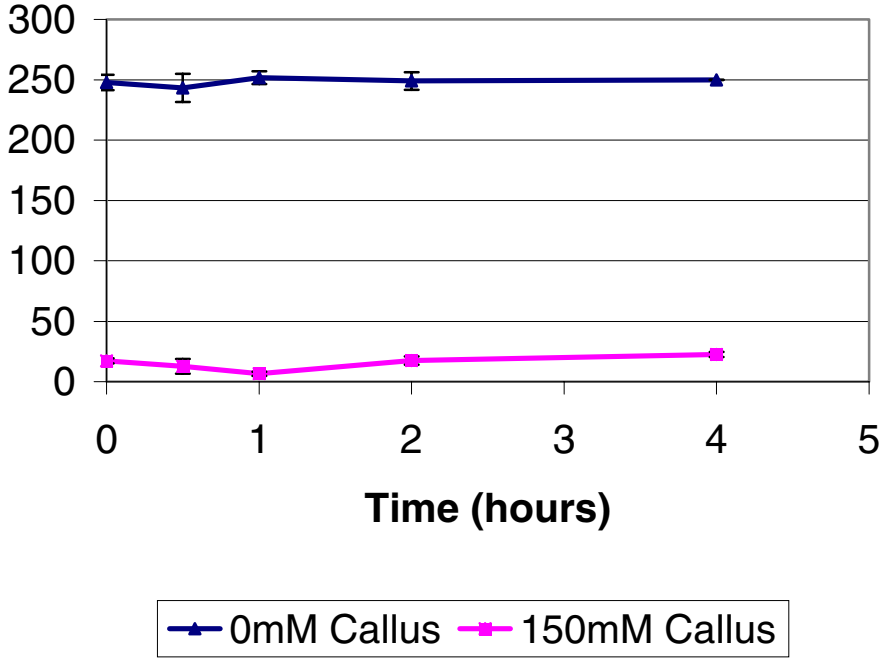

Figure 3. Time course experiment involving control (0mM NaCl) callus and salt-tolerant (150mM NaCl) callus.