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A ROLE FOR SUPEROXIDE IN THE NaCl-INDUCED UP-REGULATION OF ANTIOXIDANT ENZYME ACTIVITY

Stephen W. Banks, Rocky W. Fowler, Dalton R. Gossett, and M. Cran Lucas
Department of Biological Sciences
Louisiana State University-Shreveport
Shreveport, LA

Abstract

Physiological stress induced by environmental factors results in an up-regulation of the antioxidant defense system and an increase in tolerance towards salt stress in cotton (*Gossypium hirsutum*). The mechanism which specifies salt-tolerance to a non-halophyte plant is not well understood. NaCl-induced up-regulation of antioxidant enzymes is thought to be imparted through a signal transduction cascade initiated through superoxide ($O_2^{\bullet}$). This hypothesis was tested by using time-course studies in which antioxidant enzyme activity was measured in callus tissue from a NaCl-tolerant cell line subjected to NaCl and paraquat stress in the presence and absence of a reactive oxygen species (ROS) scavenger, N-acetyl L-cysteine (NAC). An increase in superoxide anion production was observed in both NaCl- and paraquat-stressed callus tissue, and NAC effectively scavenged ROS produced during either NaCl stress or paraquat stress in the NaCl-tolerant callus tissue. Pre-treatment with NAC also effectively inhibited the NaCl-induced up-regulation of catalase (CAT), general peroxidase (PER), ascorbate peroxidase (APX), and glutathione reductase (GR) during NaCl and paraquat stress. This NAC-driven consumption of ROS production strongly suggests that ROS do play a role in the NaCl-induced up-regulation of the stress response in cotton.

Introduction

Photosynthetic organisms producing oxygen are continually exposed to stress. Stress often leads to release of free radicals such as reactive oxygen species (ROS) due to the production of superoxide ($O_2^{\bullet}$) and subsequent enzymatic products associated with the metabolism of oxygen. In all aerobic organisms, electron leakage from the mitochondria (and chloroplast in the case of plants) is a regular occurrence during normal metabolic activity. These electrons can react with molecular oxygen and result in the production of superoxide. When a stressor such as NaCl or a herbicide causes electrons to be leaked at a rate greater than the plants antioxidant machinery can handle, oxidative stress occurs. Oxidative stress results from a wide variety of biotic and abiotic factors. Abiotic stresses include NaCl, temperature extremes, drought, physical damage (farm equipment), heavy metals, radiation, pesticides and flooding, all of which can lead to decreased crop yield. Since free radicals can be produced in almost every known biological system, there must be a way to combat their overproduction during stress. The antioxidant enzyme defense system and free radical scavengers serve as the cellular defense system against the deleterious actions of free radicals (Sies, 1991). Plants with increased levels of antioxidant enzymes have been reported to have greater resistance to oxidative damage (Dhindsa and Matowe, 1981; Harper and Harvey, 1978; Monk and Davies, 1989; Spychalla and Desborough, 1990; Wise and Naylor, 1987). Thus, plants with increased antioxidant enzyme activity are better equipped to handle environmental stress. Antioxidant defense systems are composed of both enzymatic and non-enzymatic components. Molecules such as isoflavonoids, flavones, α-tocopherol (vitamin E), ascorbic acid (vitamin C), RSH antioxidants and glutathione comprise the non-enzymatic antioxidants. Enzymatic sources of antioxidants include superoxide dismutase (SOD), catalase (CAT), general peroxidases (PER), ascorbate peroxidase (APX), and glutathione reductase (GR), as well as several other antioxidant enzymes. SOD is an enzyme which breaks down $O_2^{\bullet}$ by combining it with H^+ to form H_2O_2. Catalase catalyzes the breakdown of hydrogen peroxide into water and molecular oxygen and is not found in the chloroplast (Chen and Asada, 1989). General peroxidases also catalyze the breakdown of hydrogen peroxide to H_2O and require a reducing agent (Chang *et al.*, 1984). When salt or drought stress or a combination of the two occur, the activities of superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione reductase (GR), and monodehydroascorbate reductase (MDHAR) have been shown to increase in plants (Schoner and Krause, 1990; Smirnoff, 1995; Gossett *et al.*, 1994, 1996; Grace and Logan, 1996; Logan *et al.*, 1998a,1998b).

NaCl stress has recently gained interest in the study of environmental stress on non-halophytic plants. Data compiled by Gossett *et al.* (1994a; 1994b; 1996) has indicated that salt stress elicits an oxidative response in cotton, and cotton cultivars with elevated levels of antioxidant enzymes, either constitutive or induced, have been shown to exhibit greater tolerance to NaCl stress. To date, the mechanisms which impart salt tolerance have remained elusive (Gossett *et al.*, 1996). A recent mechanism proposed by Gossett and coworkers includes both an abscisic acid (ABA)-dependent and an ABA-independent pathway. This mechanism suggests that ROS play a crucial role in the NaCl-induced up-regulation of antioxidant enzyme activity and to stress tolerance. It has been shown that N-acetyl L-cysteine (NAC) scavenges reactive oxygen species. NAC is an aminothiol which has been used in clinical practice since the 1960's (Ziment, 1986; Flanngan, 1987; De Vries and De Flora, 1993; Van Zandwijk, 1995). NAC acts as a nucleophile itself as well as a scavenger of reactive oxygen species by increasing glutathione concentrations in the cell (De Flora *et al.*, 1991; Bonanomi and Gazzaniga, 1980). Initially, this study was designed to measure the capacity of NAC to scavenge the NaCl-stress generated ROS. Subsequently, the ability of NaCl-

stress to induce an up-regulation of antioxidant enzyme activity in callus tissue pretreated with NAC was determined in order to provide evidence of the role of ROS in the signal transduction pathway associated with the NaCl-induced up-regulation of antioxidant enzyme activity.

Materials and Methods

Callus Tissue

Callus tissue was generated from sterile hypocotyls of Coker 312 by the method described by Trolinder and Goodin (1987). The callus tissue was maintained on T1 media containing MS salts (Murashige and Skoog, 1962) supplemented with Gamborg's vitamins (Gamborg, 1978), 0.75 mg/L $MgCl_2$, 0.1 mg/L 2,4-D, 0.1 mg/L kinitin, 2 g/L phytogel, and 30 g/L glucose, and 150 mM NaCl adjusted to a pH of 5.8 (Trolinder and Goodin, 1987). For the time-course studies with a NaCl-tolerant cell line, a NaCl-tolerant cell line was selected as described by Gossett *et al.* (1996) and maintained on T1 amended with 150 mM NaCl.

Measurement of Superoxide Production

Approximately 2.5 g of NaCl-tolerant callus tissue was placed into aerated 50 mL tube containing 15 mL of the previously defined growth media. Samples were either left untreated as controls or stressed with 250 mM NaCl following a 2 hr equilibration period in the presence or absence of 20 mM N-acetyl L-cysteine (NAC) added 1 hr prior to the addition of NaCl to scavenge superoxide. As positive controls, other samples were treated with 0.2 µM paraquat following a 2 hr equilibration period in the presence or absence of 20 mM NAC added 1 hr prior to the addition of paraquat. This sub-lethal level of paraquat was used to generate superoxide endogenously (Gossett *et al*., 1996). After two hours, the superoxide content produced by the callus was analyzed by a Turner Design TD 20/20 Luminometer by pipetting 100 µL of media which bathed the callus into a pre-weighed luminometer tube. All steps prior to centrifugation were kept at 4°C. The tube was then centrifuged at 1500 x g for 2 minutes and the supernatant was discarded. The tube was then reweighed to nearest 0.001 g to account for the amount of callus tissue transferred. The assay explained below follows the instructions given by Stratagene's Lumimax Superoxide Anion Detection Kit. 5 µL of 4 mM luminol, 5 µL of 5 mM enhancer, and 190 µL of SOA assay medium were added to each tube. The tubes containing the callus and detection solution were shaken and exactly 30 seconds later, a 30 second reading on the luminometer was recorded. These data points were replicated 4 times for accuracy.

Time Course Studies

Approximately 4 g of NaCl-tolerant callus tissue was placed into aerated tubes with the previously described growth media amended with 150 mM salt and subjected to the following treatments: 250 mM NaCl, 250 mM NaCl + 20 mM NAC, 0.2 µM paraquat, 0.2 µM paraquat + 20 mM NAC, controls or control + 20 mM NAC. Again, the NaCl or paraquat was added after the 2 hr pre-incubation period with 20 mM NAC being added 1 hr prior to the addition of NaCl or paraquat. In all the experiments, the callus tissue was harvested 0, 0.5, 1, 2, and 4 hr intervals, filtered through Miracloth (Calbiochem), and stored in 10 mL Petri dishes at -70°C for subsequent antioxidant enzyme activity determination.

Antioxidant Enzyme Assays

Samples were prepared for SOD, catalase, peroxidase, GR and APX analyses according to the method of Anderson *et al.* (1992) as modified by Gossett *et al.* (1996). Samples were prepared by homogenizing 1 g of frozen leaf or callus tissue, 0.25 g of insoluble polyvinylpyrrolidone (PVP), and one drop of antifoam A emulsion in 2.5 mL of ice cold 50 mM Pipes buffer (pH 6.8), containing 6mM cysteine hydrochloride, 10 mM D-isoascorbate, 1 mM EDTA, 1% PVP-10, and 0.3% (v/v) Triton X-100. The homogenate was centrifuged for 20 min at 4^0C at 10,000 X g. Following centrifugation, 1 mL of the supernatant was centrifuge-desalted through a 10 mL bed of Sephadex G-25 according to the procedure outlined by Anderson *et al.* (1992). A portion of the eluate was analyzed immediately for catalase activity, and the remainder was stored at -70^oC for subsequent analysis of SOD, GR, APX, and peroxidase activities. Catalase activity was determined by monitoring the disappearance of H_2O_2 at 240 nm according to the method of Beers and Sizer (1952). Peroxidase activity was measured by monitoring at 675 nm the H_2O_2-dependent oxidation of reduced 2,3',6-trichloroindophenol after the method of Nickel and Cunningham (1969). APX activity was assayed at 265 nm by monitoring the ascorbic acid-dependent reduction of H_2O_2 by the method described by Anderson *et al*. (1992). Total SOD activity was assayed at 550 nm and was measured by determining the amount of enzyme required to produce 50% inhibition of the reduction of cytochrome c by superoxide generated by xanthine oxidase according to the method of Forman and Fridovich (1973). GR activity was determined by monitoring the glutathione-dependent oxidation of NADPH at 340 nm after the method of Schaedle and Bassham (1997). One unit of catalase and peroxidase was defined as the amount of enzyme to decompose 1 µmole of substrate/min at 25^0 C, and one unit of APX and GR was defined as the amount of enzyme required to reduce 1 nmole of substrate/min at 25^0 C. All data points are based on a mean of the measurements taken from a minimum of four tissue samples. All data were subjected to a one-way analysis of variance, and significance was determined at the 95% confidence limits.

Results and Discussion

The main purpose of these superoxide anion detection studies was to investigate the possible role of superoxide produced during NaCl stress and the effect of NAC which acts as a reactive oxygen species scavenger. It is evident from Table 1 that NaCl stress and paraquat significantly increased superoxide production in the salt-tolerant callus. The increase in superoxide levels was small, yet significant in the NaCl-stressed tissue as compared to the paraquat-stressed callus. In either case, it is apparent that NAC has the ability to scavenge superoxide. While the superoxide level increased significantly in the NaCl-stressed tissue, superoxide levels were below the detectable limit in the NaCl-stressed tissue pre-treated with NAC. NAC also effectively eliminated superoxide when a known superoxide inducer such as paraquat was used to stress the cotton callus.

Since it was shown that N-acetyl-L-cysteine (NAC) effectively scavenged the superoxide generated during NaCl and paraquat stress, it was used in a series of experiments to investigate the potential role of reactive oxygen species in the signal transduction pathway associated with the NaCl-induced up-regulation of antioxidant activity. Table 2 shows the catalase activity in the callus tissue subjected to NaCl and paraquat stress in the presence and absence of NAC. Catalase activity increased significantly within 2 hr in the NaCl-stressed callus and within one hour in the paraquat treatment, but remained relatively constant throughout the 4 hr time period in the control and control + NAC treatments. Pretreatment with NAC resulted in an inhibition of the increase in catalase activity in both the NaCl and paraquat treated callus. Peroxidase activity is shown in Table 3. Peroxidase activity remained relatively constant in the controls but increased significantly when the callus was subjected to either NaCl or paraquat stress. Peroxidase activity decreased in the controls treated with NAC and actually decreased, rather than increased, in NaCl- and paraquat-treated samples when subjected to a pretreatment with NAC.

Table 4 shows the APX activity in the callus tissue treated with NaCl and paraquat in the presence or absence of NAC. Both NaCl and paraquat stress resulted in a significant up-regulation of APX activity within 2 hr, while APX activity remained relatively constant in the controls. APX activity also decreased in the controls as well as the NaCl- and paraquat-treated tissue when subjected to the NAC pretreatment. GR activity is shown in Table 5. Again, the NaCl and paraquat treatments resulted in significant increases in GR activity within 2 hr, but the activity in the control and the control + NAC samples remained relatively constant. Pretreatment with NAC inhibited both the NaCl- and paraquat-induced increases in GR activity.

In previous time-course studies, (Bellaire *et al.*, 2000), the NaCl-tolerant cell line was subjected to treatment with additional NaCl, ABA, paraquat, and H_2O_2 following a 2 hr equilibration period or following a 2 hr pre-incubation period with fluridone, an inhibitor of ABA synthesis. APX, catalase, peroxidase, and GR activities increased significantly within 1 -2 hours when treated with NaCl, ABA, and paraquat and after 4-8 hours with the H_2O_2 treatment. Pre-treatment with fluridone completely suppressed or significantly delayed the increase in catalase, peroxidase, and GR activities in all but the paraquat treated tissues. When ABA concentrations were measured in a time course experiment following the application of NaCl stress, ABA levels increased 2-fold within 30 minutes after NaCl treatment, and fluridone pretreatment suppressed this increase (Bellaire *et al.*, 2000). These results showed that ABA concentrations increased prior to the observed increases in catalase, peroxidase, and GR activities. Since the paraquat-generated superoxide increases in the activities of these enzymes were not suppressed by fluridone, superoxide may directly serve as a signal transduction molecule for the up-regulation of antioxidant enzymes in an ABA-independent pathway. The activation of the ABA-independent pathway appears to be regulated by the concentration of the ROS. In a study using the luminol-enhanced assay for superoxide anion, both paraquat and NaCl treatments significantly increased the superoxide concentration in the NaCl-tolerant cotton callus tissue (Bellaire *et al.*, 2000), but the paraquat-generated increase was several fold higher than the increase generated by NaCl stress. Fluridone suppressed the NaCl- and H_2O_2-induced increases in enzyme activity and had no effect on the increases induced by paraquat. Hence, it may have been that under low or moderate levels of ROS, as would be produced by NaCl stress, the ABA-dependent signaling pathway was invoked, but under very high levels of ROS, as would be generated by paraquat, an ABA-independent pathway was induced.

Other research supports the hypothesis that the salt-induced up-regulation of antioxidant enzyme activity in cotton callus tissue is transcriptionally regulated, proceeding via a *de novo* synthesis of poly(A)$^+$RNA and is not due to the translation of existing transcripts or the mobilization of existing enzyme pools (Manchandia *et al.*, 1999). Recent experimentation with the calcium channel the blockers $LaCl_3$, ruthenium red, and TMB-8 (Banks *et al.*, 2001) and the protein kinase inhibitors staurosporine and K-252a (Gossett *et al.*, 2001) has shown that both calcium and protein kinases are involved in the NaCl-induced up-regulation of antioxidant activity in cotton callus tissue. Based on this previous work as well as the work of Foyer *et al.* (1997), Gossett and coworkers have proposed the following as part of the hypothetical signal transduction pathway connecting stress perception to activation of genes involved in stress tolerance: Exposure to stress leads to the activation of plasma membrane-associated NAD(P)H-dependent superoxide synthase. The activation of this enzyme results in the production of the superoxide radical followed by the production of H_2O_2 by apoplastic SOD. The increased concentration of these oxidants may lead to an increase in ABA levels which, in turn, may affect the level of cytosolic calcium. This change in calcium flux may then activates protein kinases responsible for the reversible protein phosphorylations (Jaiwal *et al.*, 1997; Leung and Giraudat, 1998) that induce genes that encode for the antioxidant genes involved in stress tolerance.

A major component of this hypothetical signal transduction pathway is the initial production of ROS in response to NaCl stress and the subsequent putative role of these ROS in triggering the signal cascade that eventually leads to the up-regulation of antioxidant enzyme activity. The generation of ROS has been proposed as a central trigger for defense metabolism following exposure to both abiotic and biotic stresses (Doke *et al.*, 1994; Foyer *et al.*, 1997; Shinozaki and Yamaguchi-Shinozaki, 1997). Superoxide is generated under most types of environmental stress, including NaCl stress (Hernandez *et al*., 1994; Bellaire *et al*, 2000). Stress situations exacerbate H_2O_2 production in the apoplast (Foyer *et al.*, 1997). Exposure to stress causes activation of the plasma membrane-associated NADPH-dependent superoxide synthase leading to the production of superoxide which is then converted to H_2O_2 by apoplastic SOD (Doke *et al.*, 1994). Singha and Choudhuri (1990) have shown that the superoxide radical and H_2O_2 may play an important role in the mechanism of salt-injury in *Vigna catjang* and *Oryza sativa* leaves. Hence, H_2O_2, which has been shown to generate an increase in cytosolic Ca^{2+} (Price *et al*., 1994; Prasad *et al.*, 1994) and which is known as a relatively stable and diffusible metabolite, may act as a second messenger (both as an intracellular and systemic signal) for the induction of a subset of the battery of defense genes (Levine *et al.*, 1994; Foyer *et al*., 1997; Dat *et al*., 1998). The work presented here provides additional evidence that ROS are indeed produced as a result of NaCl stress. More importantly, it shows that when the ROS are scavenged by NAC, the NaCl-induced up-regulation of antioxidant enzyme activity fails to occur. This strongly supports the hypothesis that ROS play a significant role in triggering the NaCl-induced signal cascade that leads to the up-regulation of antioxidant enzyme activity.

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Table 1. Relative Light Units (RLU)/g fresh weight ±SE in NaCl-tolerant callus tissue measured 2 hrs after the following treatments: Controls, Controls following a 1-hr pretreatment with 20 mM NAC (Control + NAC), 250 mM NaCl, 250 mM NaCl following a 1-hr pretreatment with 20 mM NAC (NaCl + NAC), 0.2 µM Paraquat, or 0.2 µM Paraquat following a 1-hr pretreatment with 20 mM NAC (Paraquat +NAC).

	Treatment					
	Control	**Control + NAC**	**NaCl**	**NaCl + NAC**	**Paraquat**	**Paraquat + NAC**
RLU	40±3	0*	65±5*	0*	881±70*	0*

* denotes a significant increase or decrease.

Table 2. Catalase activity (units/g fresh weight ±SE) in NaCl-tolerant callus tissue measured at 0, 0.5, 1, 2, and 4 hours after being subjected to the following treatments: Control, pretreatment with 20 mM NAC (NAC), 250 mM NaCl (NaCl), 250 mM NaCl following pretreatment with 20 mM NAC (NaCl + NAC), 0.2 µM Paraquat (Paraquat), or 0.2 µM Paraquat following pretreatment with 20 mM NAC (Paraquat + NAC).

	Time (hrs)				
Treatment	**0**	**0.5**	**1**	**2**	**4**
Control	28±3	31±2	37±2	29±5	32±3
Control + NAC	33±3	33±4	34±7	34±4	36±6
NaCl	35±4	34±3	39±2	70±4*	53±7
NaCl + NAC	41±4	33±5	25±3	24±3	21±4
Paraquat	31±1	42±2	67±6*	66±6*	49±6
Paraquat + NAC	32±3	35±2	37±3	43±5*	40±4

* denotes a significant increase or decrease.

Table 3. Peroxidase activity (units/g fresh weight ±SE) in NaCl-tolerant callus tissue measured at 0, 0.5, 1, 2, and 4 hours after being subjected to the following treatments: Control, pretreatment with 20 mM NAC (NAC), 250 mM NaCl (NaCl), 250 mM NaCl following pretreatment with 20 mM NAC (NaCl + NAC), 0.2 µM Paraquat (Paraquat), or 0.2 µM Paraquat following pretreatment with 20 mM NAC (Paraquat + NAC).

	Time (hrs)				
Treatment	**0**	**0.5**	**1**	**2**	**4**
Control	204±3	218±11	218±16	202±8	214±11
Control + NAC	54±6*	34±7*	35±4*	41±2*	28±3*
NaCl	203±13	209±13	312±4*	299±8*	480±22*
NaCl + NAC	36±13*	35±4*	29±5*	25±2*	35±8*
Paraquat	195±12	201±8	317±15*	370±8*	405±8*
Paraquat + NAC	111±9*	70±3*	31±8*	26±1*	23±2*

* denotes a significant increase or decrease.

Table 4. APX activity (units/g fresh weight ±SE) in NaCl-tolerant callus tissue measured at 0, 0.5, 1, 2, and 4 hours after being subjected to the following treatments: Control, pretreatment with 20 mM NAC (NAC), 250 mM NaCl (NaCl), 250 mM NaCl following pretreatment with 20 mM NAC (NaCl + NAC), 0.2 µM Paraquat (Paraquat), or 0.2 µM Paraquat following pretreatment with 20 mM NAC (Paraquat + NAC).

	Time (hrs)				
Treatment	**0**	**0.5**	**1**	**2**	**4**
Control	767±14	765±24	788±8	798±6	939±11
Control + NAC	384±6*	189±2*	77±13*	112±8*	144±28*
NaCl	756±8	810±47	747±48	1399±30*	1386±41*
NaCl + NAC	385±32*	390±33*	152±1*	165±5*	136±16*
Paraquat	721±25	782±38	899±51	1408±16*	1425±26*
Paraquat + NAC	379±19*	163±16*	85±12*	102±10*	232±16*

* denotes a significant increase or decrease.

Table 5. GR activity (units/g fresh weight ±SE) in NaCl-tolerant callus tissue measured at 0, 0.5, 1, 2, and 4 hours after being subjected to the following treatments: Control, 20 mM NAC (NAC), 250 mM NaCl (NaCl), 250 mM NaCl following pretreatment with 20 mM NAC (NaCl + NAC), 0.2 µM Paraquat (Paraquat), or 0.2 µM Paraquat following pretreatment with 20 mM NAC (Paraquat + NAC).

	Time (hrs)				
Treatment	**0**	**0.5**	**1**	**2**	**4**
Control	53±1	56±1	56±2	55±1	55±1
Control + NAC	55±2	53±1	55±1	53±2	52±1
NaCl	55±1	55±1	56±1	95±2*	95±1*
NaCl + NAC	54±1	54±1	41±1*	43±1*	32±1*
Paraquat	54±1	55±1	68±2*	99±3*	94±1*
Paraquat + NAC	51±1	53±1	51±1	53±1	51±1

* denotes a significant increase or decrease.