A TENTATIVE SIGNAL TRANSDUCTION PATHWAY FOR THE NaCl-INDUCED UPREGULATION OF ANTIOXIDANT ENZYME ACTIVITY IN COTTON CALLUS TISSUE

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

Abstract

A variety of pharmacological agents (i.e. agonists and inhibitors) were used in time course studies to examine the signal transduction pathway associated with the NaCl-induced upregulation of antioxidant enzyme activity was examined. NaCl stress was shown to initiate an increase in superoxide levels, and the more NaCl tolerant callus tissue showed significant increases in antioxidant enzyme activity. Studies with fluridone, an inhibitor of ABA synthesis, suggested that both ABA-dependent and ABA-independent signaling pathways are operative. Studies with calcium channel blockers, a calcium ionophore, and protein kinase inhibitors indicate that both the mobilization of calcium and the activation of protein kinases are involved in the signal transduction pathway. An inhibitor of phospholipase C blocked the NaCl-induced upregulation of antioxidant enzyme activity, while the addition of phosphatidylinositol, myoinositol 1,4,5-triphosphate (IP_3), and diacylgycerol resulted in an increase in antioxidant enzyme activity, suggesting that phosphoinositides also play a role in the signal transduction pathway. A tentative signal transduction pathway for the NaCl-induced upregulation of antioxidant enzyme activity is provided.

Introduction

Environmental stress such heat, cold, drought, mineral deficiency, ion toxicity, fungal infection, and insect damage induces as cascade of plant responses. One of the earliest responses to environmental stress is the upregulation of the antioxidant enzyme system. When plants are subjected to stress, the production of reactive oxygen intermediates (ROI), such as the superoxide ($O_2^{\cdot-}$) radical, hydrogen peroxide (H_2O_2), and the hydroxyl radical ($^{\cdot}OH$) may surpass the quenching activity of the antioxidant system. As a result, oxidate stress can seriously disrupt normal metabolism through oxidative damage to lipids, proteins, and nucleic acids (Scandalios, 1990). By necessity, plants possess a number of antioxidants for protection against the potentially cytotoxic ROI, and the resistance to environmental stress may depend, as least partially, on the inhibition of ROI production or an increase in the antioxidant levels. The major plant antioxidants include the free radical scavengers ascorbate, glutathione, α-tocopherol, and carotenes and the antioxidant enzymes ascorbate peroxidase (APX), catalase, dehydroascorbate reductase (DHAR), glutathione reductase (GR), glutathione S-transferase (GST), monodehydroascorbate radical reductase (MDHAR), peroxidase, and superoxide dismutase (SOD). Numerous investigators have shown that plants with high levels of antioxidants, either constitutive or induced, are more resistant to damage by ROI and are better adapted to tolerate stress conditions (Dhindsa and Matowe, 1981; Wise and Naylor, 1987; Monk and Davies, 1989; Dodge, 1994; Foyer et al, 1994; Jagtap and Bhargawa, 1995; Zhang and Kirkam, 1996; Foyer et al., 1997; Dat et al., 1998).

Data compiled from on-going research in our laboratory indicates that NaCl stress also elicits an oxidative response in cotton (Gossett et al., 1994 a, 1994b, 1996; Manchandia et al., 1999; Bellaire et al., 2000). NaCl stress induced the upregulation of APX, catalase, GR, GST, peroxidase, and SOD activities in leaves and/or callus tissue of a more salt-tolerant cultivar and the callus tissue of a NaCl-tolerant cell line derived from a NaCl-sensitive cultivar (Table 1). Antioxidants have also been shown to play a role in resistance to salt stress in peas (Corpas et al., 1993; Olmos, 1994; Hernandez et al., 1993, 1995, 1999, 2000, 2001; Gomez, 1999), rye (Streb and Feieranbed, 1996), *Lycopersicon pennelii* (Shalata and Tal, 1998), and citrus (Gueta-Dahan et al., 1997). While there is significant evidence that NaCl stress induces an increase in antioxidant enzyme activity, the mechanism or mechanisms responsible for this upregulation are still unclear. The origin and development of signal perception and transduction are considered to operate as adaptive responses to changes in environmental conditions and are, therefore, basic to an understanding of the functioning of plants in such environments. However, little information is available in plants on the signaling mechanisms that underlie the orchestration of successful antioxidant responses by the coordination of the expression of the genes which are involved in the production of antioxidants and the specific substance(s) responsible for signaling upregulation of the antioxidant defense system (Alscher et al., 1997; Foyer et al., 1997). Characterization of the putative signal transduction molecules is one of the obligatory steps towards a better understanding of the oxidative stress response in plants (Foyer et al., 1994).The available evidence suggests that the production of ROI may be used as a general alarm for the possible modification of metabolism and gene expression under stress (Foyer et al., 1994). Both intracellular and systemic signaling systems, some of which may involve calcium protein kinases, phosphoinositides, and phytohormones such as ABA, appear to

be present. The major objective of this research was to determine if and how molecules such as superoxide, hydrogen peroxide, ABA, calcium, protein kinases, and phosphoinositides might be involved in salt stress perception and signal transduction.

Methods and Materials

The signal transduction pathway associated with the NaCl-induced upregulation of antioxidant enzyme activity was examined by the use of a variety of pharmacological agents (i.e. agonists and inhibitors) in time course studies. All chemicals, except fluridone, were reagent grade and purchased from Sigma Chemical Company (St. Louis, MO). Fluridone was purchased from Chem Service, Inc., West Chester, PA. All experiments were conducted in sterile liquid media consisting of MS salts(Murashige and Skoog, 1962) supplemented with Gamborg's vitamins (Gamborg, 1978), 0.75 mg/L $MgCl_2$, and 30 g/L glucose, and 150 mM NaCl adjusted to a pH of 5.8 (Trolinder and Goodin, 1987). Approximately 4 g of callus tissue (either NaCl-sensitive or NaCl-tolerant) was transferred to a series of culture tubes and suspended in the appropriate media (amended with either 0 NaCl or 150 mM NaCl, respectively) or a series of culture tubes containing the appropriate media plus the various inhibitors. Each culture tube was then connected to an aerator and pre-incubated for two hours. Following pre-incubation, the culture tubes were subjected to treatment with the various agonists. No treatments were added to the culture tubes used as the controls. The callus tissue was harvested at 0.5, 1-, 2-, 4, and 8-hour intervals and stored at -70^0C for subsequent antioxidant enzyme analyses.

Samples were prepared for SOD, catalase, peroxidase, GR and AP 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 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°C 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 eluent was analyzed immediately for catalase activity, and the remainder was stored at -70°C 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 (1977). Since the results for all five of the enzymes were similar, only the data for GR will be presented in this paper. One unit of GR was defined as the amount of enzyme required to reduce 1 nmole of substrate/min at 25°C.

ABA extraction was performed by homogenizing approximately 1 g of frozen tissue in 3 mL of 0.1 N acetic acid. After centrifugation at 10,000 X g for 10 min at 4°C, the supernatant was removed and filtered through a 45 µm ultrafilter. The ABA concentration was determined by the HPLC method of Kling and Perkins (1987). Separation was accomplished using a 3.9 X 150 mm Nova-Pak C_{18} oxtadecyl reverse-phase column (Waters #86344) protected by a Nova-Pak C_{18} pre-column insert (Waters #15220) and a gradient solvent system delivered at a flow rate of 1 mL per minute. At the beginning of each run, the solvent system consisted of 100% solvent A (0.1 N acetic acid, pH 2.8) and 0% solvent B (0.1 N acetic acid in 95% aqueous ethanol, pH 4.2). The gradient was changed linearly until it reached a ratio of 0% solvent A and 100% solvent B, 20 minutes into the run. The gradient was then immediately shifted back to 100% solvent A and 0% solvent B and allowed to run for 5 minutes before the next injection. ABA content was determined by measuring the absorbance of the ABA peak at 254 nm. Peaks were calculated using the Waters Millineum software and a standard curve prepared from ABA standards in concentrations ranging from 20 to 200 µg/mL. All ABA data is expressed in µg/g fresh weight.

In order to measure superoxide levels, approximately 2.5 g of callus tissue was weighed and suspended in 10 mL of the previously defined growth media. This callus solution was then placed in a 50 mL tube and aerated for 1 hr period prior to treatment with either 0.2 µM paraquat or 250 mM NaCl. At time points of 0, 15, 30, 45, 60, 90, and 120 minutes after treatment, 100 µL of the cellular suspension were pipetted out and transferred to a pre-weighed luminometer tube. All steps prior to centrifugation were kept at 4°C. This tube was centrifuged for 2 min. at 1,500 X g, and the supernatant was discarded. The tube was reweighed to the nearest 0.0001 g to measure the amount of callus tissue transferred. The assay protocol followed the instructions in Stratagene's LumiMax™ Superoxide Anion Detection kit (Stratogene, La Jolla, CA). Five µL of 4.0 mM luminol, 5.0 µL of 5.0 mM enhancer, and 190 µL of SOA assay medium were added to each tube. The tubes containing the callus and detection solution were slightly shaken and exactly 30 seconds later, a 30 second reading on a TD-20/20 luminometer (Turner Designs, Sunnyvale, CA) was recorded. Results are expressed as relative light units (RLUs)/g tissue, and each data point represents the mean of 4 replications.

All experiments were repeated twice, and 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

Direct Evidence of Superoxide Generation During Salt Stress in Cotton

Based on the fact that NaCl stress resulted in an upregulation of antioxidant enzyme activity, it was inferred that NaCl stress must be contributing to oxidative stress in whole plants or callus tissue To test the hypothesis that NaCl produces oxidative stress in cotton, we have used a reagent, luminol, to directly measure the relative concentrations of superoxide radicals in control and NaCl stressed cotton callus. Previous studies have indicated that the superoxide anion is produced in plants under salt stress (Hernandez et al., 1993; Hernandez et al., 1994). Superoxide anions, until now, have not been directly measured in NaCl stressed cotton callus. To test the hypothesis that NaCl treatment produces oxidative stress, the relative levels of the superoxide anion in control callus and salt-adapted callus was measured after NaCl and paraquat treatment. Paraquat is known to induce superoxide production *in vivo* (Harper and Harvey, 1978).

Under control conditions the NaCl-sensitive (0 mM NaCl) callus was observed to have much higher superoxide levels than NaCl-tolerant (150 mM NaCl) callus with as much as a 20-fold difference observed between the two (Table 2). Both the NaCl-sensitive and NaCl-tolerant callus tissue stressed with paraquat showed significant increases in superoxide concentration at 30 min. and 45 min., but the superoxide levels dropped faster in the 150 mM NaCl adapted callus (Table 2). The 0 mM NaCl control callus continued to produce higher levels of O_2^- for up to 60 minutes. When both callus lines were stressed with 150 mM and 250 mM NaCl respectively, there was a significant increase in superoxide levels in the 0 mM NaCl callus at 30-45 minutes (Table 2). During the same time frame the 150 mM NaCl adapted callus tissue showed no significant change in superoxide concentration (Table 2).

There was a dramatic difference in the control levels of superoxide in the NaCl-sensitive and NaCl-tolerant callus (Table 2). Both NaCl and paraquat induced increases in the levels of the superoxide anion in cotton callus tissue. The differences between the NaCl-sensitive and NaCl-tolerant callus were even more dramatic under stress conditions. The lower levels of superoxide in NaCl-adapted cotton was most likely due to the enhanced oxidative stress response machinery in this cell line. There were also differences in the speed at which the superoxide production returned to near control levels when the NaCl-tolerant cell line was stressed with paraquat versus the rate at which superoxide levels declined in the NaCl-sensitive cell line. The most striking difference between the two cell lines occurred under NaCl stress. The NaCl-induced superoxide generation in the NaCl-adapted cell line was minuscule in comparison with NaCl-induced superoxide in the NaCl-sensitive cell line. This suggests that the NaCl-adapted cotton callus had acquired the ability to prevent NaCl-induced oxidative stress. This conclusion is supported by numerous studies involving salt stress, H_2O_2 stress, osmotic stress, and studies involving differential regulation of SOD in sensitive cultivars versus stress tolerant cultivars (Matters and Scandalios, 1986; Tsang et al., 1991; Gossett et al., 1994b; Gossett et al., 1996; Guan and Scandalios, 1998; Manchandia et al., 1999).

Evidence of Both an ABA-Dependent and an ABA-Independent Signaling Pathway

Superoxide is generated under most types of environmental stress, including NaCl stress (Hernandez et al., 1994; Bellaire et al., 2000), and there is evidence that superoxide may serve as a signal transduction molecule for stress-induced cellular responses (Doke et al., 1994). Intracellular H_2O_2 concentrations also increase under a variety of stress conditions (Okuda et al., 1991; Foyer et al., 1997). Hence, H_2O_2 may act as both an intracellular and systemic signal for the induction of a subset of defense genes (Foyer et al., 1997; Dat et al., 1998). It has also been suggested that ABA may be an essential mediator in triggering the responses to adverse environmental stimuli in plants (Chandler and Robertson, 1994; Leung and Giraudt, 1998). It has been demonstrated that ABA levels increase during salt stress (Hale and Orcutt, 1987; Galvez et al., 1993), and an increase of ABA in vegetative tissues is often associated with increases in stress-induced gene expression (Ingram and Bartels, 1996; Shinozaki and Yamaguchi-Shinozaki, 1997). ABA has also been shown to enhance SOD activity in maize and tobacco (Zhu and Scandalios, 1994; Kurepa et al., 1997), APX activity in pea (Mittler and Zalinkas, 1993), and the catalase *Cat1* transcript in maize (Williamson and Scandalios, 1992). Since ABA, superoxide, and H_2O_2 have all been implicated in the various signal transduction pathways, the objective of this study was to determine if there are ABA-dependent signal transduction pathways and/or ABA-independent signal transduction pathways involving superoxide and/or H_2O_2 associated with the up-regulation of antioxidant enzyme activity during salt stress in cotton callus tissue. This objective was addressed by performing a series of time-course experiments in which ABA concentrations and/or antioxidant enzyme activity was measured over an 8-hour period in a NaCl-tolerant cell line treated with NaCl, H_2O_2, sub-lethal doses of paraquat to generate endogenous superoxide, or ABA and in a NaCl-tolerant cell line subjected to the same treatments after a 2-hour pre-incubation period with fluridone [1-methyl-3-phenyl-5-(3-trifluoromethyl)phenyl)-4(1H)-pyridinone]. Fluridone inhibits the action of phytoene desaturase, an essential enzyme for the conversion of phytoene to lycopene in the terpene biosynthesis pathway. Since

lycopene is a precursor for ABA as well as carotenoids and xanthophylls, fluridone has been routinely used as an effective inhibitor of ABA synthesis (Fong et al., 1983; Toshihito et al., 1998).

Previous studies (Gossett et al., 1996; Manchandia et al., 1999) have shown that during NaCl stress, antioxidant enzyme activities increase dramatically in the NaCl-tolerant cell line, while the activities of these same enzymes remain virtually unchanged in the NaCl-sensitive callus tissue; therefore, only the NaCl-tolerant callus was used in the following experiments. Callus tissue was transferred to a series of culture tubes and suspended in media amended with 150 mM NaCl or a series of culture tubes containing 150 mM NaCl + 0.2 µM fluridone. Following pre-incubation, the culture tubes were subjected to one of the following treatments: 250 mM NaCl, 5 µM (±)-*cis,trans*-ABA, 10 mM H_2O_2, or 0.1 µM paraquat (methyl viologen). This sub-lethal level of paraquat was used to generate superoxide endogenously. No treatments were added to the culture tubes used as the controls. ABA concentrations were determined in the callus tissue which had been treated with 250 mM NaCl only and 250 mM NaCl following a pre-treatment with 0.2 µM fluridone.

GR activity increased significantly 1 hour after treatment with NaCl and ABA and by 30 minutes after the paraquat treatment (Table 3). A significant increase in GR activity was observed 2 hours after treatment with H_2O_2. Pre-treatment with fluridone completely suppressed the increase in GR activity with the H_2O_2 treatment and significantly delayed and reduced the NaCl- induced increase until 8 hours. Fluridone also delayed the ABA-induced increase in GR activity, but this delay was only from the 1 hour to the 2 hour time point. Fluridone failed to suppress the increase in GR activity in the paraquat treated callus until 8 hours after treatment. Changes in ABA concentration are shown in Table 4. When subjected to NaCl stress, ABA levels increased 2-fold within 30 min. after NaCl treatment and returned to control levels within 4 hrs. Pre-treatment with fluridone completely suppressed the NaCl-induced increase in ABA concentrations.

In other studies (Manchandia et al., 1999), it was shown that the upregulation of antioxidant enzyme activity occurred much earlier and to a higher degree in the NaCl-acclimated tissue than in control tissue. This resulted in the conclusion that while the process associated with this more rapid increase is unknown, it appears that the NaCl-acclimated tissue has developed a mechanism whereby it can recognize the onset of oxidative stress much earlier than the control tissue, up-regulate its antioxidant defense system more rapidly once the stress has been perceived, or a combination of both. The activity of GR as well as the other enzymes either returned to the pre-treatment level or decreased significantly by the 8-hour time point (Table 3). This transient increase in antioxidant activity suggests that oxidative stress and the antioxidant response to that stress occur very early after exposure to excessive levels of NaCl. The quick up-regulation of the antioxidant enzymes in the NaCl-tolerant callus may provide the initial defense against cellular damage from the oxidative burst that results from the perceived stress, and once the tissue has neutralized the impact of the oxidative burst, other adaptive mechanisms such as the accumulation of low molecular weight osmoprotectants (Fougere et al., 1991) may be invoked.

NaCl stress induced an early increase (within 1 hour) in the activity of GR, and an identical responses was observed in the callus tissue treated with exogenous ABA (Table 3). Pre-treatment with fluridone significantly delayed and dramatically reduced the NaCl-induced increase in enzyme activity. NaCl stress also induced a rapid, transient increase in the ABA concentration (Table 4). The increase in ABA concentration occurred prior to the increase GR activity or the activities of the other enzymes, and pre-treatment with fluridone suppressed the NaCl-induced increase in the ABA concentration. These data suggest that ABA is involved in the signal transduction pathway associated with the NaCl-induced upregulation of these antioxidant enzymes. The probable involvement of ABA is further substantiated by the fact that in the fluridone pre-treated tissue subjected to exogenous ABA treatment, the increases in the activity of GR, though slightly delayed, was not suppressed. Results from the paraquat treated tissue suggest that GR activity can also be upregulated by a pathway other than the one mediated by ABA. Paraquat increased the activities of GR in a manner that closely resembled the increase observed with the NaCl treatment. Even more significant, however, is the fact that pre-treatment with fluridone did not delay any of the increases in enzymatic activity caused by paraquat. Since paraquat is known to induce the endogenous production of superoxide (Babbs et al., 1989), this molecule or some other ROI may be involved in a signaling cascade that is apparently independent of ABA.

Shinozaki and Yamaguchi-Shinozaki (1996, 1997) have shown that under dehydration conditions, two ABA-dependent and two ABA-independent independent signal pathways function in the activation of stress-inducible genes. The results from the present study suggest that the up-regulation of antioxidant enzyme activity is also mediated by ABA-dependent and ABA-independent pathways. The specific roles of the various components in either of the pathways is, however, is unclear. The activation of this ABA-independent pathway appears to be regulated by the concentration of the ROI. In the 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 (Table 2), 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 be that under low or moderate levels of ROI, as would be produced by NaCl stress, the ABA-dependent

signaling pathway was invoked, but under very high levels of ROI, as would be generated by paraquat, an ABA-independent pathway was induced.

The origin and development of signal perception and transduction pathways are considered to operate as adaptive responses to changes in environmental conditions and are therefore basic to an understanding of the functioning of plants in such environments. The results of this study provide the basis for the construction of a possible scenario of events that occur when NaCl-tolerant cotton callus tissue is subjected to NaCl stress. It appears that the excess Na^+ and/or Cl^- results in electron leakage and the production of ROI, at least one of which then serves as a transduction molecule to signal the induction of an ABA-dependent signaling pathway. This ABA-dependent pathway could explain why either NaCl or ABA treatment alone could induce an up-regulation in the activity of GR and why fluridone either significantly delayed or completely suppressed the NaCl-induced increases in the activities of this enzyme. The production of high levels of ROI, as would occur with paraquat treatment or perhaps during periods of prolonged or extreme stress, may induce an ABA-independent signaling pathway. This could explain why fluridone failed to suppress or delay the paraquat-induced increases in the activities of these enzymes. It is highly probable that both pathways are activated at high ROI concentrations.

Transcriptional Regulation of NaCl-Induced Upregulation of Antioxidant Enzyme Activity

We have generally assumed that the increased antioxidant activity in salt-stressed cotton callus tissue was due to an increase in the transcription of the genes encoding these enzymes. This was an effort to test this hypothesis by using the fungal toxin, α-amanitin, a specific inhibitor of poly $(A)^+$ RNA synthesis. The effects of this toxin on transcription have been well characterized *in vitro* (Triplet, 1998; Weiland and Faulstich, 1978). RNA polymerase II, which synthesizes poly $(A)^+$ RNA's, is inhibited by α-amanitin at concentrations between 100 and 1000 ng/mL. Hence, it is possible to specifically inhibit the transcription of any genes that might be up-regulated during NaCl stress by using α-amanitin at a concentration of 100 ng/mL. In these experiments, callus tissue from a control (NaCl-sensitive) or NaCl-tolerant cell line was transferred to a series of culture tubes containing media amended with 0 NaCl (control) or 150 mM NaCl (salt-tolerant) or a series of culture tubes amended with either 0 NaCl or 150 mM NaCl + 100 ng/mL α-amanitin. Following pre-incubation, the tubes containing 150 mM NaCl or the tubes containing 150 mM NaCl +100 ng/mL α-amanitin were amended with NaCl to a final concentration of 250 mM NaCl. The tubes containing 0 mM NaCl or the tubes containing 0 mM NaCl +100 ng/mL α-amanitin were adjusted to a final concentration of 75 mM NaCl.

In the control callus, GR activity increased nearly 3-fold within 4 hr when treated with 75 mM NaCl, while the in NaCl-tolerant callus tissue, GR activity increased approximately 3-fold within 1 hr after treatment 250 mM NaCl (Table 5). In both the control and NaCl-tolerant callus tissue, pre-treatment with α-amanitin completely inhibited the NaCl-induced upregulation of GR activity (Table 5) and the other antioxidant enzymes (data not shown). Theses results show that α-amanitin inhibits the salt-induced increase in the activities of all the antioxidant enzymes studied in cotton callus tissue. The *in vivo* effects of α-amanitin on RNA synthesis have been demonstrated in cotton (Triplett, 1998) other higher plants (Jendrisak, 1980; Pitto *et al.*, 1983; Kiozuka *et al.*, 1995). This information supports the conclusion 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. This conclusion is in agreement with other stress-related studies. An increase in the transcription of genes involved in the synthesis of phytoalexins, lignins, chitins, and other forms of stress metabolites has been reported (Kuhn et al., 1984; Edwards et al., 1985; Smith and Banks, 1986; Ramagopal, 1987; Hurkman et al., 1989).

Involvement of Ca^{2+} and Protein Kinases in the NaCl-Induced Upregulation of Antioxidant Enzyme Activity

Very little is known about the activation of second messengers in plants. In animal systems, inositol triphosphate (IP_3) is involved in the release of Ca^{2+} from intracellular stores into the cytosol, and Ca^{2+} has been well documented as a second messenger. It has been suggested that phosphoinositide may play a similar role in plant cells (Shinozaki and Yamaguchi-Shinozaki, 1997). Experimental evidence has implicated the likely involvement of cytosolic calcium (Lynch et al., 1989; Jaiwal et al., 1997) and phosphoinositide turnover (Shinozaki and Yamaguchi-Shinozaki, 1996, 1997; Jaiwal et al., 1997) in the salt stress signaling cascade. Since ABA (McAinsh et al., 1990), NaCl (Lynch et al., 1989), and ROI (Price et al., 1994) have all been shown to generate an increase in cytosolic Ca^{2+} in plants, IP_3 and Ca^{2+} are possible candidates as second messengers in the salt stress response in plants. Phosphorylation processes are believed to have important roles in various signal transduction cascades, and various protein kinases are thought to function in phosphorylation processes associated with signal transduction pathways (Shinozaki and Yamaguch-Shinozaki, 1996). Ca^{2+}-dependent protein kinases and phosphatases have been inferred as potential downstream elements in ABA signaling (Leung and Giraudat, 1998), and several mRNAs that encode protein kinases have been shown to be induced by ABA and salt stress (Holappa and Walker-Simmons, 1995; Hwang and Goodman, 1995; Hong et al., 1997). Pardo, et al., (1998) suggested that the Ca^{2+}- and calmodulin-dependent protein phosphatase, calcineurin, signal pathway regulates determinants of salt tolerance for stress adaptation. Several studies have shown an increase in protein kinase activity in response to external stimuli. These studies include tomato tissue culture cells exposed to elicitor (Felix et al., 1991), soybean exposed to phytopthora infection (Feller, 1989), and tomato exposed to citrus exocortis viroid infection (Vera and Conejero,

1990). Such protein kinases are activated in response to stress stimuli such as wounding or elicitors. These studies have proven a link between protein phosphorylation and the activation of defense responses in plants. Ohto and Nakamura (1995) affirmed that protein kinases can play an important function in the regulation of gene expression by phosphorylating transcription factors that regulate the expression of target genes. Calcium dependent protein kinase (CDPK) is the prototype for a family of calcium regulated protein kinases in which kinase activity is regulated by direct binding of calcium (Harmon et al., 1987). In plant plasma membranes, a predominant protein kinase activity is stimulated by calcium (Schaller and Sussman, 1987; Ladror and Zielinski, 1989; Klimczak and Hind, 1990). Both the zucchini plasma membrane protein kinases (Verhey et al., 1993) and the soybean CDPKs (Harmon et al., 1987; Putnam-Evans et al., 1990) have been shown to require calcium for autophosphorylation. CDPKs have been shown to be induced by cold, drought, and ABA (McAinsh et al., 1992; Braam, 1992; Assmann, 1993; Knight et al., 1997) Sheen (1966) demonstrated that two related CDPKs induced a stress and ABA responsive promoter.

The purpose of this study was to determine whether calcium and protein kinases are involved in the NaCl-induced up-regulation of antioxidant enzyme activity by examining the effects of two protein kinases inhibitors (staurosporine and K252a), three calcium channel blockers (lanthanum chloride, ruthenium red, and TMB-8), and a calcium ionophore (A23187) on the NaCl stress-induced response. Staurosporine and K252a have been shown to markedly inhibit protein kinases in both animals (Tamaoki et al., 1985) and plants (Ohto and Nakamura, 1995; Yan et al., 1997). Price et al. (1994) showed that 1mM lanthanum chloride decreased cytoplasmic Ca^{2+} by approximately one-half, and for total abolition, the amount of lanthanum chloride had to be increased to 10 mM. For ruthenium red, 20 mM resulted in total abolition of cytoplasmic Ca^{2+}. In animal cells, TMB-8 has been shown to inhibit the activity of protein kinase C in a dose-dependent manner, and TMB-8 is frequently used to assess the involvement of intracellular calcium pools in a variety of cellular processes (Kojima et al., 1985). The inhibitory effect of TMB-8 on calcium flux has been proven to be reversed by the effect of calcium ionophores such as A23187 (Kojima et al., 1985). NaCl-tolerant cotton callus tissue was transferred to culture tubes amended with either 150 mM NaCl or 150 mM NaCl plus one of the following: 2 µM staurosporine, 2 µM K252a, 20 mM ruthenium red, 10 mM lanthanum chloride, 5 µM TMB-8, 100 nM A23187, or 5 µM TMB-8 + 100 nM A23187. After the pre-incubation period, the tubes were left untreated as controls or amended to a final concentration of 250 mM NaCl. The callus tissue was harvested at 0, 30 min, 1 hr, 2 hr, 4 hr, and 8 hr intervals for the staurosporine, K252a, lanthanum chloride, and ruthenium red treatments. Since previous studies (Bellaire *et al.*, 2000) as well as these studies have shown a rapid increase in antioxidant enzyme activities that level off at or before the 2-hour period, the TMB-8, A23187, and TMB-8 + A23187 treatments were harvested at 0, 1, and 2 hours.

The results for GR are shown in Table 6. GR activity did not change significantly throughout the treatment period in the controls and the controls pre-treated with staurosporine, K252a, lanthanum chloride, ruthenium red, or TMB-8. The NaCl treatment resulted in a significant increase in GR activity within 1 hour after treatment. Staurosporine pre-treatment significantly diminished the GR response and K252a, lanthanum chloride, ruthenium red, and TMB-8 completely abolished the NaCl-induced increase in GR activity. Pre-treatment with A23187 resulted in an increase in GR activity within 1 hour in the controls, and pre-treatment with TMB-8 + A23187 also resulted in an increase in GR activity within 1 hour in the NaCl-stressed callus. These results strongly suggests that calcium and protein kinases are involved in the signal transduction pathway for the NaCl-induced up-regulation of antioxidant enzyme activities in cotton callus tissue. Moreover, the results from the experiments using TMB-8 and A23187, in which A23187 abolished the inhibitory effect of TMB-8 on the increase in antioxidant activity, suggest that calcium may play a second messenger role by activating protein kinases in the signal transduction pathway.

Phosphoinosiditides May Be Involved in the NaCl-Induced Upregulation of Antioxidant Enzyme Activity

Phosphoinositides are membrane phospholipids that play many roles in cellular processes. It has been suggested that salinity and hyperosmotic stress may activate a phosphoinositide signaling systems (Pical *et al.*, 1999). Phosphoinositides can be hydrolyzed into the signaling molecules myoinositol 1,4,5 triphosphate (IP_3)and diacylglycerol (DAG), which trigger calcium release from intracellular stores and activate protein kinase C (PKC), respectively (Berridge, 1993). IP_3- binding channels have been identified in plants (Allen *et al.*, 1995), and more importantly, delivery of IP_3 to plant cells has been found to cause the release of intracellular calcium (Alexandre *et al.*, 1990; Franklin-Tong *et al.*, 1996). It has also been recently reported that there is a correlation between synthesis of phosphatidylinositol (PI), IP_3 production, and calcium mobilization (DeWald *et al.*, 2001). These studies indicate that phosphoinositide-derived second messengers are involved in calcium signaling, which has been shown to alter gene expression and plant adaptation to salinity and osmotic stress (Knight *et al.* 1997). Phospholipase C (PLC), a G-protein-mediated phospholipase found in the plasma membrane, yields phosphatidylinositol 4,5 biphosphate, which is cleaved to yield IP_3 and DAG. U-73122 is an inhibitor of phospholipase C (PLC). Phospholipase D (PLD) is activated by abscisic acid and is most likely mediated by G-protein (Ritchie and Gilroy, 2000). PLD acts upon membrane phospholipids to produce phosphatidic acid (PtdOH), which may trigger the ABA response signal transduction cascade (Ritchie and Gilroy, 1998). Phosphatidic acid is thought to function as an ionophore that allows Ca^{2+} to move freely across the plasma membrane. Increases in PtdOH can also amplify the PLC signaling pathway through second messengers. DAG can also contribute to the levels of PtdOH through a reaction catalyzed by DAG kinase. The purpose of

this study was to examine the possible involvement of phospholipid signaling in the NaCl-induced upregulation of antioxidant enzyme activity in cotton callus.

NaCl-tolerant callus tissue was transferred to culture tubes amended with 150 mM NaCl and subjected to following treatments: (a) Controls- pre-incubated for 2 h and left untreated, (b) 250 NaCl- pre-incubated for 2 h and then amended with NaCl to a final concentration of 250 mM at time point zero, (c) ABA- pre-incubated for 2 h and then treated with 20 µM ABA at time point zero, (d) ABA + 1 butanol (But)- pre-incubated for 2 h with 0.1% 1-butanol and then treated with 20 µM ABA at time point zero, (e) Control + 1 But - pre-incubated with 0.1% 1-butanol for 2 h with no further treatment at time point zero, (f) 250 NaCl + 1 But - pre-incubated with 0.1% 1-butanol for 2 h and then amended with NaCl to a final concentration of 250 mM at time point zero, (g) Control + U73122 - pre-incubated with 1 µM U73122 (1-(6-[([17β]-3-methoxyestra-1,3,5[10]-trien-17-yl)-amino]hexyl)-1H-pyrrole-2,5,-dione) (Sigma # U6756) for 2 h with no further treatment at time point zero, (h) 250 NaCl + U73122 - pre-incubated with 1 µM U73122 for 2 h and then amended with NaCl to a final concentration of 250 mM at time point zero, (i) Control + phosphatidylinositol (PI) - treated with 35 µM PI (1,2-diacyl-sn-glycero-3-phospho-(1-D-myo-inositol)) (Sigma # P2517) and harvested after 2 h (j) 250 NaCl + PI - treated with NaCl at a final concentration of 250 mM and 35 µM PI and harvested after 2 h, (k) Control + IP_3 - treated with NaCl at a final concentration of 250 mM and 3 µM IP3 (D-myoinositol 1 ,4,5-triphosphate) (Sigma # I7012) for 2 h with no further treatment at time point zero, (l) 250 NaCl + IP3 - treated with NaCl at a final concentration of 250 mM and 3 µM IP3 and harvested after 2 h, (m) Control + diacylgycerol (DAG) - treated with 50 µM DAG (1-stearoyl-2-arachidonyl-sn-glycerol(C18:0/C20:4, [cis,cis,cis,cis]-5,8,11,14)) (Sigma # S6389) and harvested after 2 h, (n) 250 NaCl + DAG - treated with NaCl at a final concentration of 250 mM and 50 µM DAG and harvested after 2 h. The callus tissue was harvested at 0, 0.5, 1, 2, and 4 intervals in treatments (a) through (h) and only at the 2 h time point in treatments (i) through (m).

Table 7 shows the GR activity measured in the different treatments. While GR activity in the NaCl-tolerant control showed a slight, but significant, increase at 4 hours, treatment with NaCl (250 mM) resulted in a significant increase in GR activity within 1 hour. Pretreatment with 1-butanol not only failed to inhibit the increase in GR in the callus tissue subjected to NaCl treatment, but instead resulted in a significant increase above the NaCl induced level. Pretreatment of the NaCl-tolerant controls with 1-butanol also resulted in a significant increase in GR activity at the 2 hour time point. As with the treatment with NaCl, treatment of the NaCl-tolerant controls with ABA showed a significant increase in GR activity at 1 hour. Pre-treatment of the NaCl-tolerant controls with 1-butanol prior to receiving the ABA treatment produced a significant increase rather than an inhibition in GR activity at 1 hour . Pretreatment with U73122 significantly decreased GR activity at 2 and 4 hours in the NaCl-tolerant controls. While U73122 pretreatment of the NaCl-treated callus did not inhibit the NaCl induced increase, it did reduce the magnitude of the response and delayed the increase in GR activity until the 2-hour time point. Treatment with DAG, IP_3, and PI resulted in significant increases in GR activity within 2 hours after receiving the respective treatments.

Since pretreatment of both control and NaCl stressed cotton callus with 1-Butanol actually resulted in an increase rather than a decrease in GR activity, it would appear that phospholipase D has a very limited, and perhaps no, role the NaCl-induced up-regulation of GR activity. Pretreatment with 1-Butanol also failed to inhibit the ABA-induced response, suggesting that ABA is involved in the up-regulation of GR as has been previously shown, and that the signal pathway does not involve PLD. Perhaps it could be argued that the concentration of 1-Butanol used in this study, 1%, was not sufficient to cause inhibition, or the inhibitor was not taken up by the callus tissue. While either of these arguments is possible, it should be noted that 1% 1-Butanol is sufficient to inhibit other plant responses such as the ABA-induced closure of the stomata when applied to the leaf surface (Ritchie and Gilroy, 2000). Pretreatment with U73122 decreased and delayed the upregulation of GR activity in both the control and NaCl-treated cotton callus tissue, while pretreatment of the control and NaCl-stressed callus with either PI, IP3, or DAG resulted in rapid increases in GR activity. These findings would suggest that phospholipase C may be involved in the NaCl-induced up-regulation of glutathione reductase in cotton callus tissue. Inhibition of PLC resulted in significant delays and/or reductions in the NaCl-induced increases in the activities all four enzymes and pretreatment with the enzymatic products of PLC caused significant rapid increases in antioxidant activity, even in the control tissue. While phosphoinositide signaling pathways have not been well characterized in plants, recent studies with *Arabidopsis* have also suggested a role for PLC in response to NaCl stress (DeWald et al., 2001). These researchers showed that plant cells rapidly increase phosphatidylinositol 4,5-bisphosphate synthesis and accumulation of its hydrolytic product, IP_3 in response to NaCl stress. Furthermore, they demonstrated that the NaCl-induced accumulation of IP_3 was dramatically reduced when the plants were pretreated with the inhibitor U73122. In animals, IP_3 is associated with calcium mobilization, and DeWald *et al.* (2001) showed not only that NaCl stress increases calcium mobilization, but also that inhibition of IP_3 accumulation with U73122 inhibited the mobilization of calcium.

The results of the studies in our laboratory have shown that inhibition of ABA synthesis (Bellaire et al, 2000), calcium mobilization (Banks et al., 2001), and protein kinase activity (Gossett et al., 2001) all inhibit the NaCl-induced up-regulation of antioxidant enzyme activity. The results of the phosphoinositide study, along with those of DeWald et al. (2001), allow the inclusion of phosphoinositides in the following possible signal transduction pathway: NaCl stress results in the production of ROMs such as superoxide (Banks et

al., 1999). As the ROMs accumulate, an ABA synthesis pathway is activated. ABA levels increase, and perhaps through a G-protein mediated path, activate PLC. PLC then hydrolyzes phosphatidylinositol to IP_3 and DAG which in turn mobilize calcium and activate protein kinases and phosphatases that phosphorylate the transcription factors necessary for the initiation of transcription of the antioxidant encoding genes. While this is only one hypothetical pathway and there are still components such as the G-protein, which have not been demonstrated, it does offer a possible explanation for the data presented in this paper.

Acknowledgements

This research was supported by Cotton Incorporated and the Louisiana Board of Regents Support Fund.

References

Alexandre, J., J.P. Lassalles, and R.T. Kado. 1990. Opening of Ca^{2+} channels in isolated red beet root vacuole membrane by inositol 1,4,5 triphosphate. Nature. 343:567-570.

Allen, G.J., S.R. Muir, and D. Sanders. 1995. Release of Ca^{2+} from individual plant vacuoles by both $InsP_3$ and cyclic ADP-ribose. Science. 268:735-737.

Anderson, J.V., B.I. Chevone, and J.L. Hess. 1992. Seasonal variation in the antioxidant system of eastern white pine needles. Plant Physiol. 98:501-508.

Assmann, S.M. 1993. Signal transduction in guard cells. Annu. Rev. Cell Biol. 9:345

Alscher, R.G., J.L. Donahue, and C.L. Cramer. 1997. Reactive oxygen species and antioxidants: realtionships in green cells. Physiol. Plant. 100:224-230.

Babbs, C.F., J.A.. Pham, R.C. Coolbaugh. 1989. Lethal hydroxyl radical production in paraquat-treated plants. Plant Physiol. 90:1267-1272.

Banks, S.W., J.A.H. Braud, M.C. Lucas, and D.R. Gossett. 1999. Superoxide levels in NaCl-stressed cotton callus. Proc. Beltwide Cotton Prod. Res. Conf. National Cotton Council. Memphis, TN. 540-541.

Banks, S.W., T. Heck, D.R. Gossett, and M.C. Lucas. 2001. Involvement of calcium in the NaCl-induced upregulation of antioxidant enzyme activity in cotton callus. Proc. Beltwide Cotton Prod. Res. Conf. National Cotton Council. Memphis, TN. 452-456.

Beeridge, M.J. 1993. Inositol triphosphate and calcium signaling. Nature 361:315-325.

Beers, R.R. and I. Sizer. 1952. A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. J.Biol. Chem. 195:133.

Bellaire, B.A., J.C. Carmody, J.H.A. Braud, D.R. Gossett, S.W. Banks, M.C. Lucas, and T.E. Fowler. 2000. Involvement of abscisic acid-dependent and -independent pathways in the upregulation of antioxidant enzyme activity during NaCl stress in cotton callus tissue. Free Rad. Res. 33:531-545.

Braam, J. 1992. Regulated expression of the calmodulin-related TCH genes in cultured *Arabidopsis* cells: induction by calcium and heat shock. Proc. Natl. Acad. Sci. USA 89:3213.

Chandler, P.M. and M. Robertson. 1994. Gene expression regulated by abscisic acid and its relation to stress tolerance. Ann. Rev.Plant Physiol. Plant Mol. Biol. 45:113-141.

Corpas, F.J., M. Gomez, J.A. Hernandez, L.A. del Rio, and F. Sevilla. 1993. Metabolism of activated oxygen in peroxisomes from two *Pisum sativum* L. cutivars with different sensitivity to sodium chloride. Journal of Plant Physiol. 141:160-165.

Dat, J.F., H. Lopez-Delgado, C.H. Foyer, and I..M. Scott. 1998. Parallel changes in H_2O_2 and catalase during thermotolerance induced by salicylic acid on heat acclimation in mustard seedlings. Plant Physio. 116:1351-1357.

DeWald, D.B., J. Torabinejad, C.A. Jones, J.C. Shope, A.M. Cangelosi, J.E. Thompson, G.D. Prestwich, and H. Hama. 2001. Rapid accumulation of phosphatidylinositol 4,5-bisphosphate and inositol 1,4,5-triphosphate correlates with calcium mobilization in salt-stressed *Arabidopsis*. Plant Physiol. 126:759-769.

Dhindsa, R.S. and W. Matowe (1981) Drought tolerance in two mosses: correlated with enzymatic defense against lipid peroxidation. J. Exp. Bot. 32:79-91.

Dodge, A. 1994. Herbicide action and effects on detoxification processes. In C. H. Foyer and P. M. Mullineaux (eds.) *Causes of Photooxidative Stress and Amelioration of Defense Systems in Plants*. CRC, Boca Raton, pp 219-236.

Doke, N., Y. Miura, L.M. Sanchez, and K. Kawakita. 1994. Involvement of superoxide in signal transduction: Responses to attack by pathogens, physical and chemical shocks, and UV irradiation. In CH Foyer, PM Mullineaux (eds.) *Causes of Photooxidative Stress and Amelioration of Defense Systems in Plants*. CRC, Boca Raton, pp. 177-198.

Edwards, K., C.L. Cramer, G.P. Bolwell, R.A. Dixon, W. Schuch, C.J. Lamb. 1985. Rapid transient induction of phenylalanine-ammonia lyase mRNA in elicitor-treated bean cells Proc. Nat Ac Sci. 82:6731-6735.

Felix, G., D. G. Grosskoft, M. Regenass, and T. Boller. 1991. Rapid changes in protein phosphorylation are involved in the transduction of elicitor signals in plant cells. Proc. Natl. Acad. Sci. USA 88:8831-8834.

Feller, K. Characterization of a protein kinase from soybean seedlings. Study of the variations of calcium-regulated kinase activity during infection with the incompatible race 1 and the compatible race 3 *Phytopthora megasperma* F. Sp. Glycinea by in vitro phosphorylation of calf thymus histone H1. Plant Sci. 60:67-75.

Fong, F., J.D. Smith, and D.E. Koehler. 1983. Early events in maize seed development: 1-methyl-5(3-[trifluoromethyl]phenyl)-4-(1H)-pyridinone induction in vivipary. Plant Physiol. 73:899-901.

Forman, H.J. and I. Fridovich. 1973. Superoxide dismutase: A comparison of rate constants. Arch. Biochem. Biophys. 158:396-400.

Fougere, F., D. Le Rudulier, and J.G. Streeter. 1991. Effects of salt stress on amino acid, organic acid and carbohydrate composition of roots, bacteroides, and cytosol of alfalfa (*Medicago sativa* L). Plant Physiol. 96:1228-1236.

Foyer, C.H., P. Descourviers, and K. J. Kunert (1994) Protection against oxygen radicals: an important defense mechanism in transgenic plants. Plant Cell Environ. 17:507-523.

Foyer, C.H., H. Lopez-Delgado, J.F. Dat, and I.M. Scott. 1997. Hydrogen peroxide- and glutathione-associated mechanisms of acclimatory stress tolerance and signaling. Physiol. Plant. 100: 241-254.

Franklin-Tong, V.E., B.K. Drobak, A.C. Allen, P.A.C. Watkins, and A.J. Trewavas. 1996. Growth of pollen tubes of *Papaver rheoas* is regulated by a slow-moving calcium wave propagated by inositol 1,4,5-triphosphate. Plant Cell. 8:1305-1321.

Galvez, A.F., P.J. Gulick, and J. Dvorak. 1993. Characterization of the early stages of genetic salt stress response in salt-tolerant *Lyphopyrum elongatum*, salt-sensitive wheat, and their amphiploids. Plant Physiol. 103:257-265.

Gamborg, O.L. 1978. *Plant Tissue Culture* (ed. T. A. Thorpe) Academic Press, Orlando, FL, p.36.

Gomez, J.M., J.A. Hernandez, A. Jimenez, L.A. del Rio, and F. Sevilla. 1999. Differential response to antioxidant enzymes of chloroplasts and mitochondria to long-term NaCl stress of pea plants. Free Rad. Res. 31:11-18.

Gossett, D.R., E.P. Millhollon, and M.C. Lucas. 1994a. Antioxidant response to NaCl stress in salt-tolerant and salt-sensitive cultivars of cotton. Crop Sci. 34:706-714.

Gossett, D.R.. E.P. Millhollon, M.C. Lucas, S.W. Banks, and M.M. Marney, 1994b. The effects of NaCl on antioxidant enzyme activities in callus tissue of salt-tolerant and salt-sensitive cultivars of cotton. Plant Cell Rep. 13:498-503.

Gossett, D.R., S.W. Banks, E.P. Millhollon, and M.C. Lucas. 1996. Antioxidant response to NaCl in a control and an NaCl-tolerant cell line grown in the presence of paraquat, buthionine sulfoximine, and exogenous glutathione. Plant Physiol. 112:803-809.

Gossett, D.R., C. Orellana, S.W. Banks, and M.C. Lucas. 2001. Involvement of protein kinases in the NaCl-induced upregulation of antioxidant enzyme activity in cotton callus. Proc. Beltwide Cotton Prod. Res. Conf. National Cotton Council. Memphis, TN. 448-452.

Guan, L. and J.G. Scandalios. 1998. Two structurally similar maize cytosolic dismutase genes sod4 and sod4a respond differently to abscisic acid and high osmoticum. Plant Physiol. 117:217-224.

Gueta-Dahan, Y., Z Yaniv, B.A. Zilinskas, and G. Ben-Hayyim. 1997. Salt and oxidative stress: similar and specific responses and their relation to salt tolerance in *Citrus*. Planta. 203:460-469.

Hale, M.G. and D. M. Orcutt. 1987. *The Physiology of Plants Under Stress*. John Wiley and Sons, New York, pp 171-182.

Harmon, A. C., C. L. Putnam-Evans, and M. Cormier. 1987. A calcium-dependent but calmodulin-independent protein kinase from soybean. Plant Physiol. 83: 830-837.

Harper, D.B. and B.M.R. Harvey. 1978. Mechanisms of paraquat tolerance in perennial ryegrass: II. Role of superoxide dismutase, catalase, and peroxidase. Plant Cell Environ. 1:211-215.

Hernandez, J.A., F.J. Corpas, M. Gomez, L.A. del Rio, and F. Sevilla. 1993. Salt-induced oxidateive stress mediated by activated oxygen species in pea leaf mitochondria. Physiol. Plant. 89:103-110.

Hernandez, J.A., L.A. del Rio, and F. Sevilla. 1994. Salt-induced changes in superoxide dismutase isozymes in leaves and mesophyll protoplasts from *Vignia unguiculata* (L.) Walp. New Phytol. 126:37-44.

Hernandez, J.A., E. Olmos, F.J. Corpas, F. Sevilla, and L.A. del Rio. 1995. Salt-induced oxidative stress in chloroplasts of pea plants. Plant Sci. 105:151-167.

Hernandez, J.A, A Campillo, A. Jimenez, J.J. Alarcon, and F. Sevilla. 1999. Response of antioxidant systems and leaf water relations to NaCl stress in pea plants. New Phytol. 141:241-251.

Hernandez, J.A., A. Jimenez, P.M. Mullineaux, and F. Sevilla. 2000. Tolerance of pea (*Pisum sativum* L.) To long-term salt stress is associated with induction of antioxidant defences. Plant Cell Environ. 23:853-862.

Hernandez, J.A., M.A. Ferrer, A. Jimenez, A. Ros-Barcelo, and F. Sevilla. 2001. Antioxidant systems and $O_2^{.-}$ /H_2O_2 production in the apoplast of *Pisum sativum* L. leaves: its relation with NaCl-induced necrotic lesions in minor veins. Plant Physiol. 127:817-831.

Holappa, L.D. and M.K. Walker-Simmons. 1995. The wheat abscisic acid-responsive protein kinase mRNA, PKABA, is up-regulated by dehydration, cold treatment, and osmotic stress. Plant Physiol. 108:1203-1210.

Hong, S.W., J.H. Jon, J.M. Kwak, and H.G. Nam. 1997. Identification of a receptor-like protein kinase gene rapidly induced by abscisic acid, dehydration, high salt, and cold treatments in *Arabidopsis thaliana*. Plant Physiol. 113:1203-1211.

Hurkman, W.J., C.S. Fornia, and C.K. Tanaka. 1989. A comparison of the effects of salt on polypeptides and translatable mRNAs in roots of a salt-tolerant and salt-sensitive cultivar of barley. Plant Physiol. 90: 1444-1456.

Hwang, I.W. and H.M. Goodman. 1995. An *Arabidopsis thaliana* root-specific kinase homology is induced by dehydration, ABA, and NaCl. Plant J. 8:37-45.

Ingram. I. and D. Bartels. 1996. The molecular basis of dehydration tolerance in plants. Ann. Rev. Plant Physiol. Plant Mol. Biol. 47:377-403.

Jagtap, V. and S. Bhargawa. 1995. Variation in the antioxidant metabolism of drought tolerant and drought susceptible varieties of *Sorghum bicolor* (L.) Moench. exposed to high light, low water, and high temperature stress. Journal of Plant Physiol. 145:195-197.

Jaiwal, P.K., R.P. Singh, and A. Gulati. 1997. Perception of salt signals by higher plants In: Jaiwal, P.K., R.P. Singh, and A. Gulati (eds.) *Strategies for Improving Salt Tolerance*. Oxford and IBH Pub. Co. Pvt. Ltd. New Delhi. pp. 41-53.

Jendrisak, J. 1980. The use of α-amanitin to inhibit in vivo RNA synthesis and germination in wheat embryos. J. Biol. Chem. 255:8529-8533.

Klimczak, L. J. and G. Hind. 1990. Biochemical similarities between soluble and membrane-bound protein kinases of barley. Plant Physiol. 92:919-923.

Kling, G.J. and L.M. Perkins. 1987. Improved method for determination of indole 3-acetic acid in plant tissue. J.Chromatography. 407:377-383.

Knight, H., A. J. Trewavas and M. R Knight. 1997. Calcium signaling in *Arabidopsis thaliana* responding to drought and salinity. Plant J. 12(5):1067-1078.

Koizuka, N., Y. Tanaka, and Y. Morohashi. 1995. Expression of alpha-amylase in response to wounding in mung bean. Planta 195:530-534.

Kojima, I., K. Kojima, and H. Rasmussen. 1985. Mechanism of inhibitory action of TMB-8 {8-(NN-diethylamino)octyl 3,4,5-trimethoxybenzoate} on aldosterone secretion in adrenal glomerulosa cells. Biochem. J. 232:87-92.

Kuhn, D. N., J. Chappell, A. Boudet, K. Hahlbrock. 1984. Induction of phenylalanine ammonia-lyase and 4-coumarate: CoA ligase mRNAs in cultured plant cells by UV light or fungal elicitor. Proc. Nat Ac Sci. 81:1102-1106.

Kurepa, J., D. Herouart, M.Van Montagu, and D. Inze. 1997. Differential expression of CuZn- and Fe-superoxide dismutase genes of tobacco during development, oxidative stress, and hormonal treatment. Plant Cellular Physiol. 38:463-470.

Ladror, U. S. and R. E. Zielinski. 1989. Protein kinase activities in tonoplast and plasmolemma membranes from corn roots. Plant Physiol. 89:151-158.

Leung, J. and J.Giraudat. 1998. Abscisic acid signal transduction. Ann. Rev. Plant Physiol. Plant Mol. Biol.49:199-222.

Lynch, J. V.S. Polito, A. Lauchli. 1989. Salinity stress increases cytoplasmic Ca^{2+} activity in maize root protoplasts. Plant Physiol. 90:127112-76.

McAinsh, M.R., C. Brownlee, and A.M. Hetherington. 1990. Abscisic acid-induced elevation of guard cell cytosolic Ca^{2+} precedes stomatal closure. Nature. 343:186-189.

McAinsh, M.R., C. Brownlee, and A.M. Hetherington. 1992. Visualizing changes in cytosolic free Ca^{2+} during the response of stomatal guard cells to abscisic acid. Plant Cell 4:1113.

Manchandia, A.M., S.W. Banks, D.R . Gossett, B.A. Bellaire, M.C.Lucas, and E.P. Millhollon. 1999. The influence of α-amanitin on the NaCl-induced up-regulation of antioxidant enzyme activity in cotton callus tissue. Free Rad. Res. 30:429-438.

Matters, G.L. and J.G. Scandalios. 1986. Effect of the free radical generating herbicide paraquat on the expression of superoxide dismutase (Sod) genes in maize. Biochem. Biophys. Acta. 882:29-38.

Mittler, R. and B. A. Zalinkas. 1993. Detection of ascorbate peroxidase activity in native gels by inhibition of the ascorbate dependent reduction of nitroblue tetrazolium. Annals o1f Biochem. 212: 540-546.

Monk, L.S. and H.V. Davies. 1989. Antioxidant status of the potato tuber and Ca^{2+} deficiency as a physiological stress. Physiol. Plant. 75:411-416.

Murashige, T. and F. Skoog. 1962. A revised medium for rapid growth and bioassays with tobacco tissue culure. Plant Physiol. 15:473-497.

Olmos, E., J.A. Hernandez, F. Sevilla, and E. Hellin. 1994. Induction of several antioxidant enzymes in the selection of a salt-tolerant cell line of *Pisum sativum*. Journal of Plant Physiol. 144:594-598.

Nickel, R.S. and B.A. Cunningham. 1969. Improved peroxidase assay method using leuco 2,3',6-trichloroindophenol and application to comparative measurements of peroxidase catalysis. Annals Biochem. 27:292-299.

Okuda, T., Y. Matsuda, A. Yamanaka, and S. Sagisaka. 1991. Abrupt increase in the level of hydrogen peroxide in leaves of winter wheat caused by cold treatment. Plant Physiol. 97:1265-1267.

Ohto, M. and K. Nakamura. 1995. Sugar-induced increase of calcium-dependent protein kinases associated with plasma membrane in leaf tissues of tobacco. Plant Physiol. 109:973-981.

Pardo, J.M., M.P. Reddy, S. Yang, A. Maggio, G.H. Hue, T. Matsumoto, M.A. Coca, H. Koiwa, D.J. Yun, A.A. Watad, R.A. Bressan, and P.M. Hasegawa. 1998. Stress signaling through Ca^{2+}/calmodulin-dependent phosphate calcineurin mediates stress adaption in plants. Proc. Natl. Acad. Sci. USA. 95:9681-9686.

Pical, C., T. Westergren, S.K. Dove, C. Larsson, and M. Sommarin. 1999. Salinity and hyperosmotic stress induce rapid increases in phosphatidylinositol 4,5 bisphosphate, diacylglycerol phosphate, and phosphatidylcholine in *Arabidopsis thaliana* cells. J. Biol. Chem. 274:38232-38240.

Pitto, L., F. LoSchiavo, and G. Giulano. 1983. Analysis of the heat-shock protein pattern during somatic embryogenesis of carrot. Plant Mol. Biol. 2:231-237.

Price, A.H., A. Taylor, S.J. Ripley, A. Griffiths, A.J. Trewavas, and M.R. Knight. 1994. Oxidative signals in tobacco increase cytosolic calcium. Plant Cell. 6:1301-1310.

Putnam-Evans, C. L., A. C. Harmon, and M. J. Cormier. 1990. Purification and characterization of a novel calcium-dependent protein kinase from soybean. Biochemistry 29:2488-2495.

Ramagopal, S. 1987. Salinity stress induced tissue specific proteins in barley. Plant Physiol. 84:324-331.

Ritchie, S. and S. Gilroy. 2000. Abscisic acid stimulation of phospholipase D in the barley aleurone is G-protein mediated and localized to the plasma membrane. Plant Physiol. 124:693-702.

Scandalios, J.G. 1990. Response of plant antioxidant defense genes to environmental stress. In J.G. Scandalios and T.F.R. Wright (eds.) *Genetics: Volume 28: Genomic Responses to Environmental Stress* Academic Press, San Diego, pp. 1-41.

Schaedle, M. and J. A. Bassham. 1977. Chloroplast glutathione reductase. Plant Physiol. 59:1011-1012.

Schaller, G. E. and M. R. Sussman. 1987. Kinase-mediated phosphorylation of the oat plasma membrane H^+-ATPase. In C. Leaver, H. Sze (ed.) *Plant Membranes: Structure, Function, Biogenesis.* Alan R. Liss, New York. pp. 419-429.

Shalata, A. and M. Tal. 1998. The effect o f salt stress on lipid peroxidation and antioxidants in the leaf of the cultivated tomato and its wild salt-tolerant relative *Lycopersicon pennellii*. Physiol. Plant. 104:169-174.

Sheen, J. 1996. Ca^{2+} -dependent protein kinases and stress signal transduction in plants. Science 274: 1900-1902.

Shinozaki, K. and K.Yamaguchi-Shinozaki. 1996. Molecular responses to drought and cold stress. Current Opinions in Biotechnology. 7:161-167.

Shinozaki, K. and K. Yamaguchi-Shinozaki. 1997. Gene expression and signal transduction in water-stress response. Plant Physiol. 115:327-334.

Smith, D.A. and S.W. Banks. 1986. Biosynthesis elicitation and biological activity of isoflavonoid phytoalexins. Phytochem. 25:979-995.

Streb, P. and J. Feierabend. 1996. Oxidative stress responses accompanying photoinactivation of catalase in NaCl-treated rye leaves. Botanica Acta, 109:125-132.

Tomaoki, T., H. Nomoto, I. Takahashi, Y. Kato, M. Morimoto, and F. Tomita. 1986. Staurosporine, a potent inhibitor of phospholipid/calcium dependent protein kinase. Biochem. Biophys. Res. Cummun. 135:397-402.

Toshihito, Y.I., T. Endo, and S. Satoh. 1998. Restoration of seed germination at supraoptimal temperatures by fluridone, an inhibitor of abscisic acid biosynthesis. Plant Cell Physiol. 39:307-312.

Triplett, B. Stage-specific inhibition of cotton fiber development by adding α-amanitin to ovule cultures. In Vitro Cell. Dev. Biol. Plant. 34:27-33.

Trolinder, N.L. and J.R. Goodin .1987. Somatic embryogenesis and plant regeneration in *Gossypium hirsutum* L. Plant Cell Rep. 6:231-234.

Tsang, E.W., C. Bowler, D. Herouart, W. Van Camp, R. Genetello, M. Van Montague, and D. Inze. 1991. Differential regulation of superoxide dismutase in plants exposed to environmental stress. Plant Cell. 3:783-792.

Vera, P. and V. Conejero. 1990. Citrus exocortis viroid alters the in vitro pattern of protein phosphorylation in plants. Science 223:167-169.

Verhey, S. D., J. C. Gaiser, and T. L. Lomax. 1993. Protein kinases in zucchini. Plant Physiol. 103:413-419.

Weiland, T. and H. Faulstich. 1978. Amatoxins, phallotoxins, phallolysin, and antamanide: the biologically active components of poisonous *Amanita* mushrooms. Crit. Rev Biochem. 5:185-260.

Williamson, J.D. and J.G. Scandalios. 1992. Differential development response of the maize catalases to abscisic acid: Vp1 transcript activator is not required for ABA-regulated *Cat1* expression. Proceed. Nat. Acad. Sci. USA. 89:8842-8851.

Wise, R.R. and A.W. Naylor (1987) Chilling-enhanced photooxidation: Evidence for the role of singlet oxygen and endogenous antioxidants. Plant Physiol. 83:278-282.

Yan, W. L., J. Kuzma, and E. Marechal. 1997. Abscisic acid signaling through cyclic ADP-ribose in plants. Science 278:2126-2131.

Zhang, J. and M.B. Kirkham. 1996. Lipid peroxidation in sorghum and sunflower seedlings as affected by ascorbic acid, benzoic acid, and propyl gallate. Journal of Plant Physiol. 149:489-493.

Zhu, D. and J. G. Scandalios. 1994. Differential accumulation of manganese-superoxide dismutase transcripts in maize in response to abscisic acid and high osmoticum. Plant Physiol. 106:173-178.

Table 1. APX, catalase, GR, peroxidase, and SOD activities (units/g fresh weight ±SE) in the relatively NaCl-tolerant Acala 1517-88 and relatively NaCl-sensitive Deltapine 50 leaves (AC-88L and DP-50L, respectively), Acala 1517-88 and Deltapine 50 callus tissue (AC-88C and DP-50C, respectively), and a Coker 312 derived salt-sensitive cell line (SS-Cell Line) subjected to either 0 NaCl (Control) or 150 mM NaCl (Stressed) and a Coker 312 derived NaCl-tolerant cell line (ST-Cell Line) subjected to 150 mM NaCl (Control) and 250 mM NaCl (Stressed).

	Enzyme				
Tissue	**APX**	**Catalase**	**GR**	**Peroxidase**	**SOD**
AC-88(L)					
Control	225±6	51±6	77±4	74±3	57±10
Stressed	210±4	39±9	162±3	127±5	122±9
DP-50 (L)					
Control	295±21	18±3	101±8	120±6	127±15
Stressed	195±15	11±1	98±6	71±4	121±13
AC-88(C)					
Control	148±9	21±3	86±6	165±14	35±3
Stressed	351±12	47±4	377±17	346±18	97±7
DP-50(C)					
Control	152±5	16±1	127±12	351±23	43±5
Stressed	158±6	17±2	129±9	361±18	32±4
SS-Cell Line					
Control	308±46	9±1	30±5	300±79	20±6
Stressed	253±27	9±1	50±6	310±57	22±3
ST-Cell Line					
Control	187±29	14±1	57±5	129±25	33±4
Stressed	578±43	28±2	321±24	358±38	82±7

Table 2. Superoxide generated relative light units (RLU)/g fresh weight ±SE) over a 2-hour period in NaCl-sensitive (Control) and NaCl-tolerant (NaCl-Tol) callus tissue, control and NaCl-tolerant callus tissue treated with 0.2 µM paraquat (Control + P and NaCl-Tol + P), and control and NaCl-tolerant callus tissue subjected to NaCl stress, 150 mM NaCl and 250 mM NaCl respectively (Control + NaCl and NaCl-Tol + NaCl.

	Time (min.)						
Treatment	**0**	**15**	**30**	**45**	**60**	**90**	**120**
Control	580±21	615±25	691±18	470±45	830±62	300±10	562±23
NaCl-Tol	7±	7±1	8±1	8±1	10±1	13±2	17±1
Control + P	580±21	550±75	1725±108	1685±40	1410±92	600±30	1052±53
NaCl-Tol + P	7±1	210±21	915±13	1153±37	205±15	96±11	123±15
Control + NaCl	580±21	1665±95	1952±208	1106±55	743±56	681±20	954±32
NaCl-Tol + NaCl	7±1	20±2	42±5	38±2	21±2	13±1	8±1

Table 3. GR activity (units/g fresh weigth ±SE) measured at 0, 0.5, 1,2,4, and 8 hours in NaCl-tolerant callus tissue Controls or after treatment with 0.2 µM Fluridone only, 250 mM NaCl only or 250 mM NaCl following a 2-hr pre-treatment with 0.2 µM Fluridone (NaCl + Fluridone), 0..5 µM ABA only or 0.5 µM ABA following a 2-hr pre-treatment with 0.2 µM Fluridone (ABA + Fluridone), 0.1 µM Paraquat only or 0.1 µM paraquat following a 2-hr pre-treatment with 0.2 µM Fluridone (Paraquat + Fluridone), and 10 mM H_2O_2 only or 10 mM H_2O_2 following a 2-hr pre-treatment with 0.2 µM Fluridone (H_2O_2 + Fluridone).

	Time (hrs)					
Treatment	**0**	**0.5**	**1**	**2**	**4**	**8**
Control	31±2	41±4	57±5	66±5	59±6	69±4
Fluridone	31±2	41±5	50±1	52±8	52±9	67±5
NaCl	31±2	58±6	321±24	235±8	362±17	103±1
NaCl + fluridone	31±2	25±3	74±12	100±21	69±1	165±10
ABA	31±2	28±4	167±6	185±9	151±8	68±3
ABA + fluridone	31±2	32±4	20±8	136±3	131±8	87±7
Paraquat	31±2	74±7	212±12	178±15	245±12	263±3
Paraquat + fluridone	31±2	82±1	171±22	217±4	229±24	67±7
H_2O_2	31±2	47±6	52±3	102±6	254±13	59±10
H_2O_2+ fluridone	31±2	28±3	12±6	52±1	63±5	50±3

Table 4. ABA concentration (µg/g fresh weigth ±SE) measured at 0, 0.5, 1,2,4, and 8 hours in NaCl-tolerant callus tissue controls, controls following a 2-hr pre-treatment with 0.2 µM fluridone, NaCl-tolerant callus tissue subjected to NaCl stress with 250 mM NaCl, and NaCl-tolerant callus tissue subjected to NaCl stress with 250 mM NaCl following a 2-hr pre-treatment with 0.2 µM fluridone.

	Time (hrs)					
Treatment	**0**	**0.5**		**2**	**4**	**8**
Control	75±10	58±9	60±4	72±17	70±9	65±5
Control + Fluridone	87±8	92±110	106±12	99±16	88±18	75±17
NaCl Stress	75±10	158±11	108±8	101±8	71±6	80±2
NaCl Stress+ fluridone	87±8	92±6	113±9	84±8	104±8	71±3

Table 5. 3.GR activity (units/g fresh weigth ±SE) measured at 0, 0.5, 1,2,4, and 8 hours in NaCl-sensitive and NaCl-tolerant callus tissue with no treatment (Controls) or after treatment with 100 ng/mL α-amanitin (Control +AM), 75 mM NaCl (NaCl Stress in NaCl-Sensitive Callus), 250 mM NaCl (NaCl Stress in NaCl-tolerant Callus), 75 mM NaCl following pretreatment with 100 ng/mL α-amanitin (NaCl Stress + AM in NaCl-Sensitive Callus)), or 250 mM NaCl following pre-treatment with 100 ng/mL α-amanitin (NaCl Stress + AM in NaCl-tolerant Callus).

	Time (hrs)					
Treatment	**0**	**0.5**	**1**	**2**	**4**	**8**
NaCl-Sensitive Callus						
Control	38±4	42±4	37±6	46±5	39±6	49±3
Control + AM	38±4	39±5	40±5	52±8	45±8	37±6
NaCl Stress	38±4	48±6	49±4	55±7	165±11	53±5
NaCl Stress + AM	38±4	35±3	34±2	43±4	49±3	55±6
NaCl-Tolerant Callus						
Control	51±3	58±5	67±5	65±5	51±1	58±2
Control + AM	51±3	62±6	60±7	56±4	61±3	57±5
NaCl Stress	51±3	64±7	196±15	218±17	65±9	63±4
NaCl Stress + AM	51±3	58±3	52±5	52±2	61±4	52±2

Table 6. GR activity (units/g fresh weight ±SE) in salt-tolerant callus tissue treated for 0, 0.5, 1, 2, 4, or 8 hrs with 150 mM NaCl (Control), Controls + 2 μM staurosporine , Controls + 2 μM K252a, Controls + 10 mM Lanthanum Chloride, Controls + 20 mM Ruthenium Red, Controls + 5 μM TMB-8, Controls + 100 nM A23187, 250 mM NaCl (NaCl Stress), 250 mM NaCl (NaCl Stress) + 2 μM staurosporine, 250 mM NaCl (NaCl Stress) + 2 μM K252a, 250 mM NaCl (NaCl Stress) + 10 mM Lanthanum Chloride, 250 mM NaCl (NaCl Stress) + 20 mM Ruthenium Red, 250 mM NaCl (NaCl Stress) + 5 μM TMB-8, and 250 mM NaCl (NaCl Stress) + 5 μM TMB-8 + 100 nM A23187.

	Time					
Treatment	**0**	**0.5 h**	**1 hr**	**2 hr**	**4 hr**	**8 hr**
Control	31±2	41±4	57±5	56±5	59±6	49±4
Staurosporine	40±4	41±7	44±4	41±5	44±4	29±4
K252a	32±5	29±4	29±4	28±3	36±5	33±2
Lanthanum Chloride	49±7	42±4	43±5	54±7	31±4	35±3
Ruthenium Red	35±4	23±3	30±4	28±3	58±6	40±3
TMB8	30±4		23±6	28±5		
A23187	32±5		47±2	52±3		
NaCl Stress	44±4	49±4	140±4	116±4	34±6	35±5
NaCl Stress + Staurosporine	47±5	42±3	86±7	40±3	40±3	40±9
NaCl Stress + K252a	52±6	57±5	31±1	18±2	18±3	15±1
NaCl Stress + Lanthanum Chloride	44±5	33±5	29±5	32±6	25±4	23±1
NaCl Stress + Ruthenium Red	41±4	48±5	45±5	29±7	36±2	15±1
NaCl Stress + TMB8	41±4		35±5	27±4		
NaCl Stress + TMB8 + A23187	44±5		76±6	224±7		

Table 7. GR activity (units/g fresh weight ±SE) in salt-tolerant callus tissue treated for 0, 0.5, 1, 2, 4, or 8 hrs with 150 mM NaCl (Controls), Controls + 20 μM ABA , Controls + 0.1% 1-Butanol, Controls + 20 μM ABA + 0.1% 1-Butanol, Controls + 1 μM U73122, Controls + 35 μM Phosphatidylinositol (PI), Controls + 3 μM Myoinositol 1,4,5 Triphosphate (IP_3), Controls + 50 μM Diacyglycerol (DAG), 250 mM NaCl (NaCl Stress), 250 mM NaCl (NaCl Stress) + 0.1% 1-Butanol, 250 mM NaCl (NaCl Stress) + 1 μM U73122 , 250 mM NaCl (NaCl Stress) + 35 μM Phosphatidylinositol (PI) , 250 mM NaCl (NaCl Stress) + 3 μM Myoinositol 1,4,5 Triphosphate (IP_3), and 250 mM NaCl (NaCl Stress) + 50 μM Diacylglycerol (DAG).

	Time				
Treatment	**0**	**0.5 h**	**1 hr**	**2 hr**	**4 hr**
Control	46±3	63±6	48±9	44±4	78±9
ABA	44±6	55±5	86±6	45±4	55±7
1-Butanol	51±8	66±4	71±7	239±21	93±8
ABA + 1-Butanol	44±6	49±5	269±17	72±5	25±3
U73122	42±4	31±3	32±2	24±12	17±3
PI	43±3		189±17		
IP_3	42±4		131±8		
DAG	41±4		177±5		
NaCl Stress	47±2	79±5	147±12	161±8	220±13
1-Butanol	50±5	56±6	256±24	96±7	99±4
U73122	42±6	40±3	44±13	139±7	45±2
PI	44±5		153±5		
IP_3	41±4		139±9		
DAG	43±4		143±9		