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Involvement of Abscisic Acid-Dependent and – Independent Pathways in the Upregulation of Antioxidant Enzyme Activity During NaCl Stress in Cotton Callus Tissue

BARBARA A. BELLAIRE, JOHN CARMODY, JOHN BRAUD, DALTON R. GOSSETT*, STEPHEN W. BANKS, M. CRAN LUCAS and TOLVERT E. FOWLER

Department of Biological Sciences, Louisiana State University-Shreveport, One University Place Shreveport LA 71115, USA

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The role of abscisic acid (ABA) in the signal transduction pathway associated with NaCl-induced up-regulation of antioxidant enzyme activity was examined in a NaCl-tolerant cotton callus cell line treated with NaCl, ABA, paraquat, or H_2O_2 in the presence and absence or fluridone, an inhibitor of terpene, and therefore, ABA synthesis. Treatment with NaCl resulted in a rapid increase (within 30 minutes) in the ABA levels of the callus tissue, and the NaCl, ABA, and paraquat treatments induced rapid increases in the activities of superoxide dismutase, catalase, peroxidase, and glutathione reductase. Pre-treatment with fluridone significantly suppressed the NaCl-induced increases, but only slightly delayed the increases in tissue subjected to exogenous ABA treatment. This implies that ABA is involved in the signal transduction pathway associated with the NaCl-induced up-regulation of these antioxidant enzymes. Pre-treatment with fluridone had no effect on the paraquat-induced increases, suggesting that these enzymes can also be up-regulated by a pathway other than the one mediated by ABA. Both the NaCl and paraquat treatments produced significant increases in the superoxide levels within the callus, but the increase resulting from the paraquat treatment was significantly higher than the increase resulting from the NaCl treatment. These data suggest that NaCl stress results in the production of reactive oxygen intermediates (ROI) which signals the induction of an ABA-dependent signaling pathway. The production of very high levels of ROI, such as those that occur with paraquat treatment or perhaps during periods of prolonged or extreme stress, may induce an ABA-independent signaling pathway.

Keywords: Abscisic Acid; Antioxidant Enzymes; Cotton; Reactive Oxygen Intermediates; Salt Stress; Superoxide

INTRODUCTION

All plants, in their natural environments, will experience some level of environmental stress which may be the major limiting factor in their productivity[1]. When plants are exposed to environmental stress, the production of reactive oxygen intermediates (ROI), such as singlet oxygen, the superoxide ($O_2^{\cdot-}$) radical, H_2O_2, and the hydroxyl ($^{\cdot}OH$) radical may exceed the quenching activity of the antioxidant system. When this

* Correspondence should be sent to: Dalton R. Gossett, Department of Biological Sciences, Louisiana State University, One University Place, Shreveport, LA 71115, USA. Phone: 318–797–5244, Fax: 318–797–5222, email: dgossett@pilot.lsus.edu

occurs, oxidative stress can seriously disrupt normal metabolism through oxidative damage to lipids, proteins, and nucleic acids[2]. By necessity, plants possess a number of antioxidants for protection against the potentially cytotoxic ROI, and the resistance to environmental stress may depend, at least partially, on the inhibition of ROI production or an increase in the antioxidant levels. 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 withstand stress conditions[3–15].

Data compiled from on-going research in our laboratory indicates that salt stress also elicits an oxidative response in cotton[10–12]. NaCl stress induced the upregulation of superoxide dismutase (SOD), catalase, peroxidases, ascorbate peroxidase (APX), glutathione reductase (GR), and glutathione S-transfersases (GSTs) activities in leaves and/or callus tissue of a more salt-tolerant cultivar and the callus tissue of a NaCl-tolerant cell line. Antioxidants have also been shown to play a role in resistance to salt stress in peas[9,16,17] and rye grass[18]. Recent studies using α-amanitin, an inhibitor of RNA polymerase II, have indicated that the NaCl-induced increases in antioxidant enzyme activity in cotton callus tissue is transcriptionally regulated, proceeding via *de novo* synthesis of poly(A)$^+$RNA[19], but little information is available on signal transduction sequences or the molecular mechanisms associated with this upregulation. The available evidence suggests that the production of ROI may serve as a general alarm to signal for the possible modification of metabolism and gene expression[6], but the specific substance or substances responsible for signaling the upregulation of the antioxidant defense system have yet to be defined[7].

Superoxide is generated under most types of environmental stress, including NaCl stress[9], and there is evidence that superoxide may serve as a signal transduction molecule for stress-induced cellular responses[20]. Intracellular

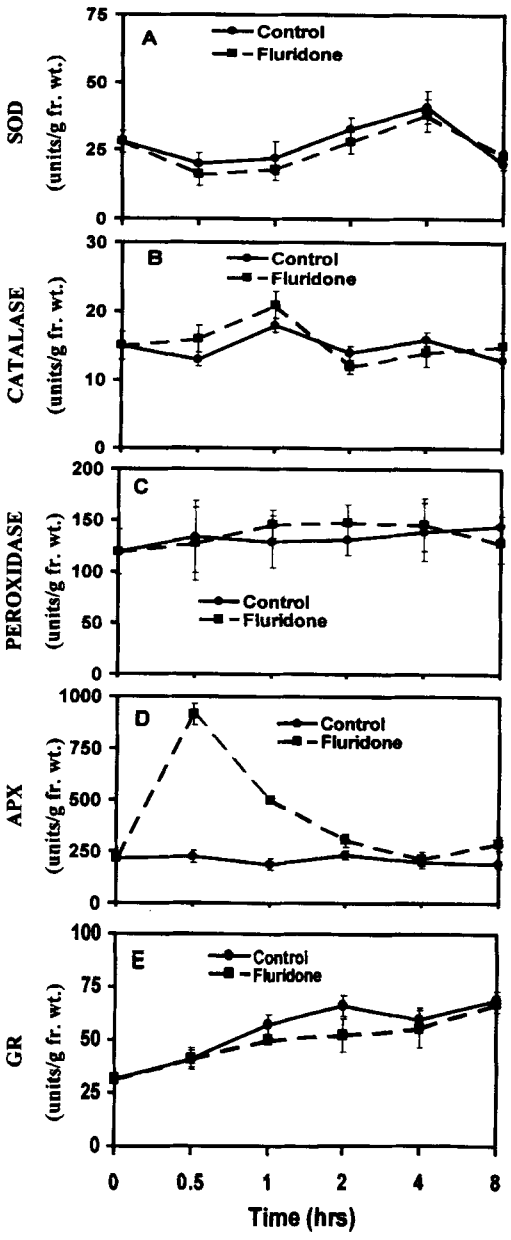

FIGURE 1 SOD (A), catalase (B), peroxidase (C), APX (D), and GR (E) activities (units/g fresh weight ±SE) measured at 0, 0.5, 1, 2, 4, and 8 hours in NaCl-tolerant cotton callus tissue incubated with 150 mM NaCl in the presence and absence (controls) of fluridone

H_2O_2 concentrations also increase under a variety of stress conditions[7,21]. Singha and Choudhuri[22] have shown that H_2O_2 and the superoxide radical may play an important role in the mechanism of salt-injury in *Vigna catjang* and *Oryza sativa* leaves. Hence, H_2O_2 may act as both an intracellular and systemic signal for the induction of a subset of defense genes[7,15]. It has also been suggested that ABA may be an essential mediator in triggering the responses to adverse environmental stimuli in plants[23,24]. It has been demonstrated that ABA levels increase during salt stress[25,26], and an increase of ABA in vegetative tissues is often associated with increases in stress-induced gene expression[27,28]. ABA has also been shown to enhance SOD activity in maize and tobacco[29,30], APX activity in pea[31], and the catalase *Cat1* transcript in maize[32].

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[33,34].

MATERIALS AND METHODS

Growth and Maintenance of Callus Tissue

Control and NaCl-adapted callus tissue derived from the cotton cultivar Coker 312, was generated according to the method of Trolinder and Goodin[35] as modified by Gossett *et al.*[11]. The control callus cell line was grown at 0 mM NaCl. The NaCl-adapted cell line was selected by progressively growing portions of the callus on media with increasing concentrations of NaCl until callus of the selected cell line grew as well on 150 mM NaCl amended media as control callus grown on control media[12]. The callus tissue was maintained by sub-culturing every 4 to 6 weeks. Previous studies[12,19] 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.

Chemicals and Reagents

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[36] supplemented with Gamborg's vitamins[37], 0.75 mg/L $MgCl_2$, and 30 g/L glucose, and 150 mM NaCl adjusted to a pH of 5.8[35].

Time Course Studies

Approximately 4 g of NaCl-tolerant callus 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. Each culture tube was

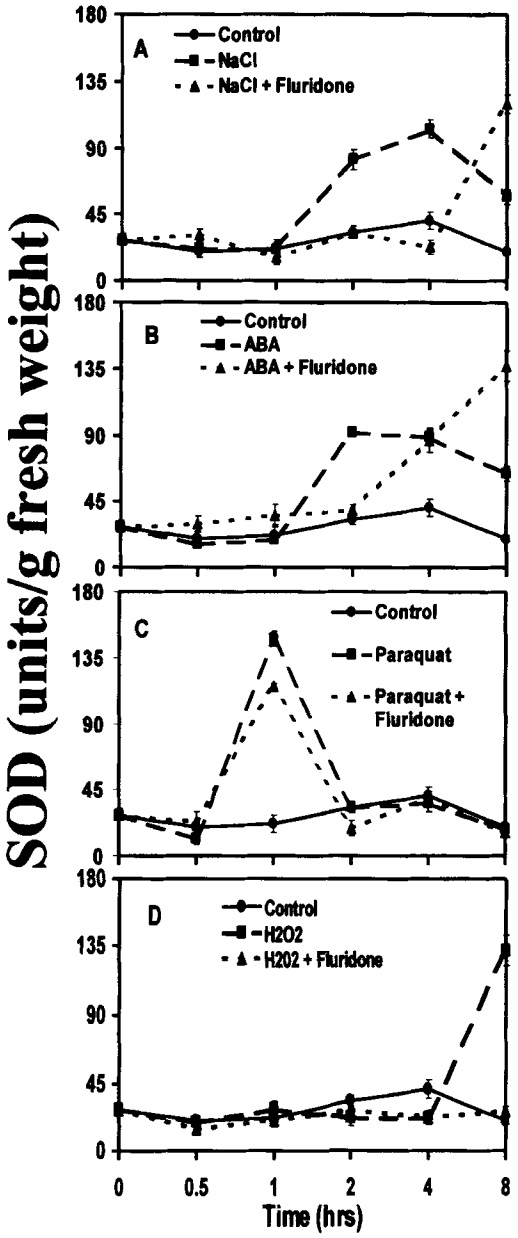

FIGURE 2 SOD activity (units/g fresh weight ±SE) measured at 0, 0.5, 1, 2, 4, and 8 hours in NaCl-tolerant cotton callus tissue controls or after treatment with (A) 250 mM NaCl only and 250 mM NaCl following a 2 hr pre-treatment with 0.2 μM fluridone, (B) 5 μM ABA only and 5 μM ABA following a 2 hr pre-treatment with 0.2 μM fluridone, (C) 0.1 μM paraquat only and 0.1 μM paraquat following a 2 hr pre-treatment with 0.2 μM fluridone, and (D) 10 mM H_2O_2 only and 10 mM H_2O_2 following a 2 hr pre-treatment with 0.2 μM fluridone

then connected to an aerator and pre-incubated for two hours. 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[12]. 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°C for subsequent antioxidant enzyme analyses. 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.

Protein Extraction

Samples were prepared for SOD, catalase, peroxidase, GR and AP analyses according to the method of Anderson *et al.*[38] as modified by Gossett *et al.*[11] 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 × 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.*[38]. 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.

Antioxidant Enzyme Determinations

Catalase activity was determined by monitoring the disappearance of H_2O_2 at 240 nm according

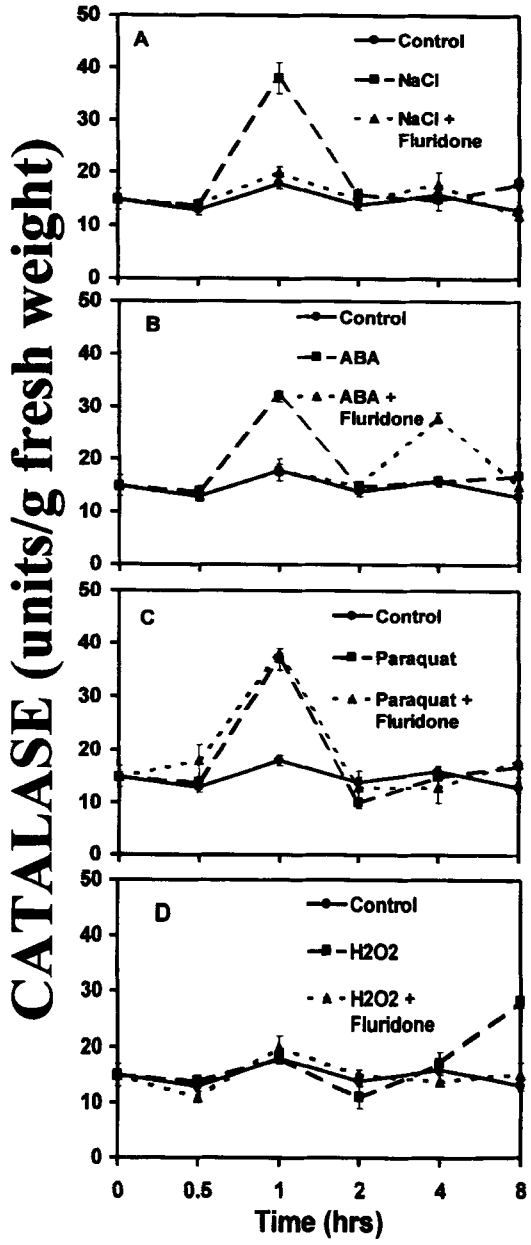

FIGURE 3 Catalase activity (units/g fresh weight ±SE) measured at 0, 0.5, 1, 2, 4, and 8 hours in NaCl-tolerant cotton callus tissue controls or after treatment with (A) 250 mM NaCl only and 250 mM NaCl following a 2 hr pre-treatment with 0.2 μM fluridone, (B) 5 μM ABA only and 5 μM ABA following a 2 hr pre-treatment with 0.2 μM fluridone, (C) 0.1 μM paraquat only and 0.1 μM paraquat following a 2 hr pre-treatment with 0.2 μM fluridone, and (D) 10 mM H_2O_2 only and 10 mM H_2O_2 following a 2 hr pre-treatment with 0.2 μM fluridone

to the method of Beers and Sizer[39]. 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[40]. 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.*[38]. 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[41]. GR activity was determined by monitoring the glutathione-dependent oxidation of NADPH at 340 nm after the method of Schaedle and Bassham[42]. One unit of enzyme for catalase, peroxidase and APX was defined as the amount necessary to decompose 1 μmole of substrate/min at 25°C. One unit of SOD was defined as the amount of enzyme necessary to inhibit the reduction of cytochrome C by 50%. One unit of GR was defined as the amount of enzyme required to reduce 1 nmole of substrate/min at 25°C.

ABA Extraction and Analysis

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 × g for 10 min at 4°C, the supernatant was removed and filtered through a 0.45 μm ultrafilter. The ABA concentration was determined by the HPLC method of Kling and Perkins[43]. 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

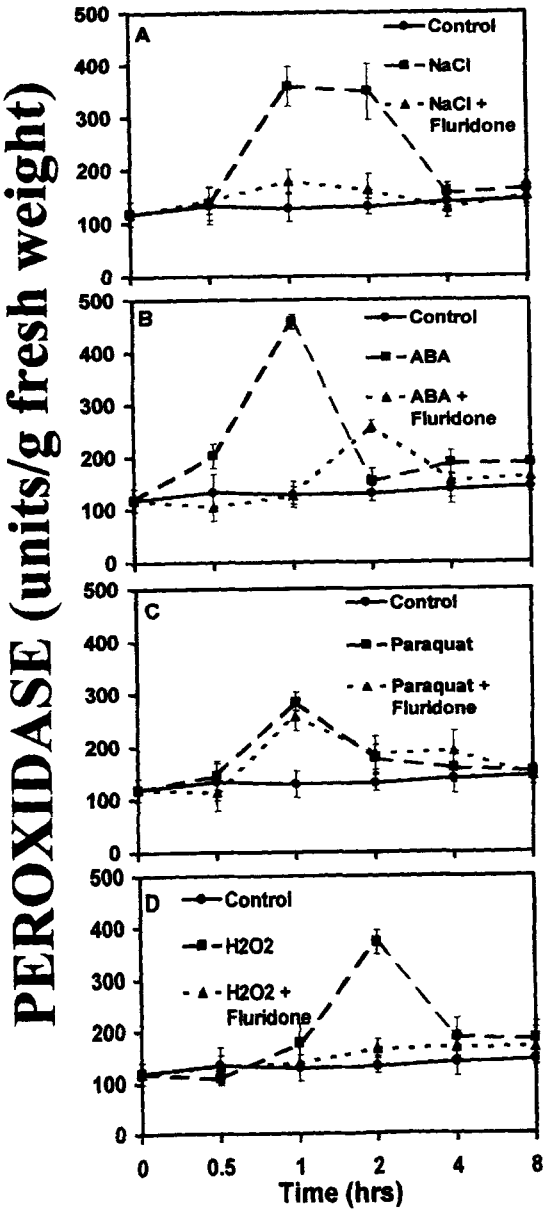

FIGURE 4 Peroxidase activity (units/g fresh weight ±SE) measured at 0, 0.5, 1, 2, 4, and 8 hours in NaCl-tolerant cotton callus tissue controls or after treatment with (A) 250 mM NaCl only and 250 mM NaCl following a 2 hr pre-treatment with 0.2 μM fluridone, (B) 5 μM ABA only and 5 μM ABA following a 2 hr pre-treatment with 0.2 μM fluridone, (C) 0.1 μM paraquat only and 0.1 μM paraquat following a 2 hr pre-treatment with 0.2 μM fluridone, and (D) 10 mM H_2O_2 only and 10 mM H_2O_2 following a 2 hr pre-treatment with 0.2 μM fluridone

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.

Measurement of 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 × 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.

Data Analysis

All experiments were repeated twice, and all data points are based on a mean of the measure-

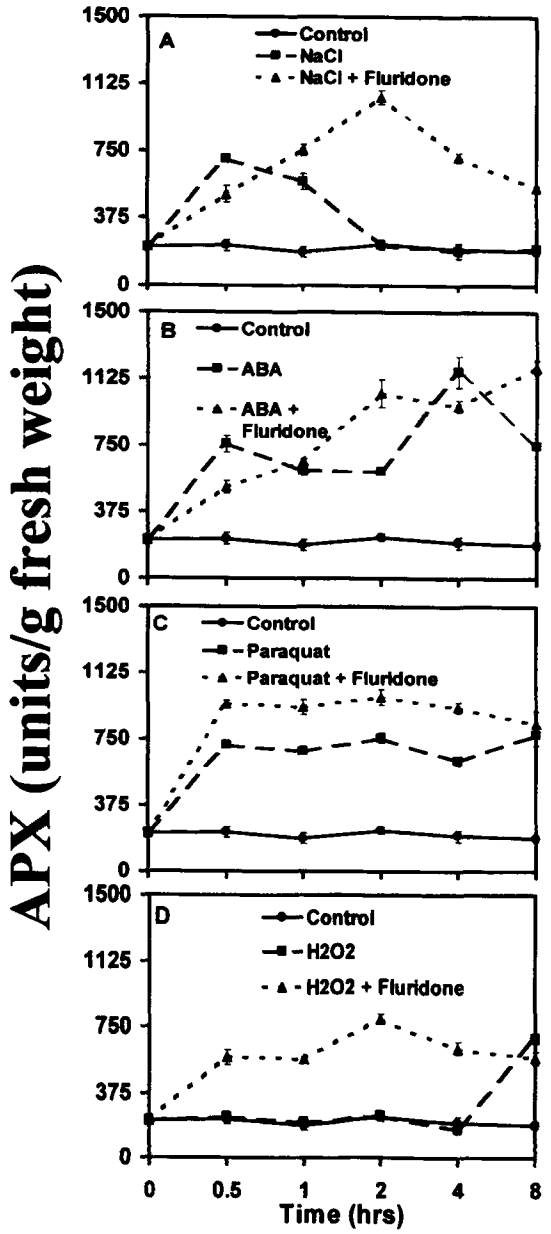

FIGURE 5 APX activity (units/g fresh weight ±SE) measured at 0, 0.5, 1, 2, 4, and 8 hours in NaCl-tolerant cotton callus tissue controls or after treatment with (A) 250 mM NaCl only and 250 mM NaCl following a 2 hr pre-treatment with 0.2 μM fluridone, (B) 5 μM ABA only and 5 μM ABA following a 2 hr pre-treatment with 0.2 μM fluridone, (C) 0.1 μM paraquat only and 0.1 μM paraquat following a 2 hr pre-treatment with 0.2 μM fluridone, and (D) 10 mM H_2O_2 only and 10 mM H_2O_2 following a 2 hr pre-treatment with 0.2 μM fluridone

ments 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

The responses of SOD, catalase, peroxidase, APX, and GR to treatment with fluridone alone are shown in Figure 1. Fluridone treatment resulted in a 4-fold transient increase in APX activity above control values (Figure 1D), but did not produce significant increases in SOD (Figure 1A), catalase (Figure 1B), peroxidase (Figure 1C), or GR (Figure 1E) activities.

Significant increases in SOD activity were observed 2 hours after treatment with NaCl (Figure 2A) or ABA (Figure 2B). Pre-treatment with fluridone delayed the response in both treatments, but the delay time was much shorter in the ABA-treated tissue. In the fluridone pre-treated tissue that received NaCl, a significant increase in SOD activity was not observed until the 8 hour time point (Figure 2A), while a significant increase was observed at 4 hours in the tissue treated with exogenous ABA (Figure 2B). Treatment with paraquat (Figure 2C) resulted in a significant transient increase in SOD activity 1 hour after treatment, and the increase was not delayed in the presence of fluridone (Figure 2C). The hydrogen peroxide treatment did not cause a significant increase in SOD activity until 8 hours after treatment, and no increase was observed within 8 hours in the fluridone pre-treated callus tissue (Figure 2D).

The activities of the enzymes which break down H_2O_2 are shown in Figures 3 and 4. Significant increases in catalase activity (Figure 3) were observed with all treatments. Catalase activity increased transiently within 1 hour after the NaCl (Figure 3A), ABA (Figure 3B), and paraquat (Figure 3C) treatments and at 8 hours after the H_2O_2 (Figure 3D) treatment. Pre-treatment with fluridone completely suppressed the

increase in catalase activity in the NaCl (Figure 3A) and H_2O_2 (Figure 3D) treatments. While the fluridone pre-treatment delayed the catalase increase until 4 hours after the ABA treatment (Figure 3B), it did not suppress it. Pre-treatment with fluridone had no effect on the increase observed with the paraquat treatment (Figure 3C). Peroxidase activity increased significantly 1 hour after the NaCl (Figure 4A), ABA (Figure 4B), and paraquat (Figure 4C) treatments and 2 hours after the H_2O_2 treatment (Figure 4D). Pre-treatment with fluridone completely suppressed the increase in peroxidase activity in the NaCl (Figure 4A) and H_2O_2 (Figure 4D) treatments. In the presence of fluridone, the ABA-induced increase was somewhat lower and delayed until 2 hours, but was not suppressed (Figure 4B). The increase in peroxidase activity resulting from the paraquat treatment was not affected by pre-treatment with fluridone (Figure 4C).

The activities of APX and GR, two enzymes associated with the ascorbate-glutathione cycle, are presented in Figures 5 and 6, respectively. NaCl resulted in a significant increase in APX activity 30 minutes (Figure 5A) after treatment. Similar results were observed with the ABA (Figure 5B) and paraquat (Figure 5C) treatments. Hydrogen peroxide treatment did not produce a significant change in APX activity until 8 hours (Figure 5D). Pre-treatment with fluridone failed to suppress the increase in APX activity in any of the treatments (Figures 5A-5D). APX activity increased significantly at 30 minutes in all four treatments following the pre-treatment with fluridone, but this was not surprising since treatment with fluridone alone resulted in significant increases in this enzymes's activity in 30 minutes (Figure 1D).

GR activity increased significantly 1 hour after treatment with NaCl (Figure 6A) and ABA (Figure 6B) and by 30 minutes after the paraquat treatment (Figure 6C). A significant increase in GR activity was observed 2 hours after treatment with H_2O_2 (Figure 6D). Pre-treatment with flu-

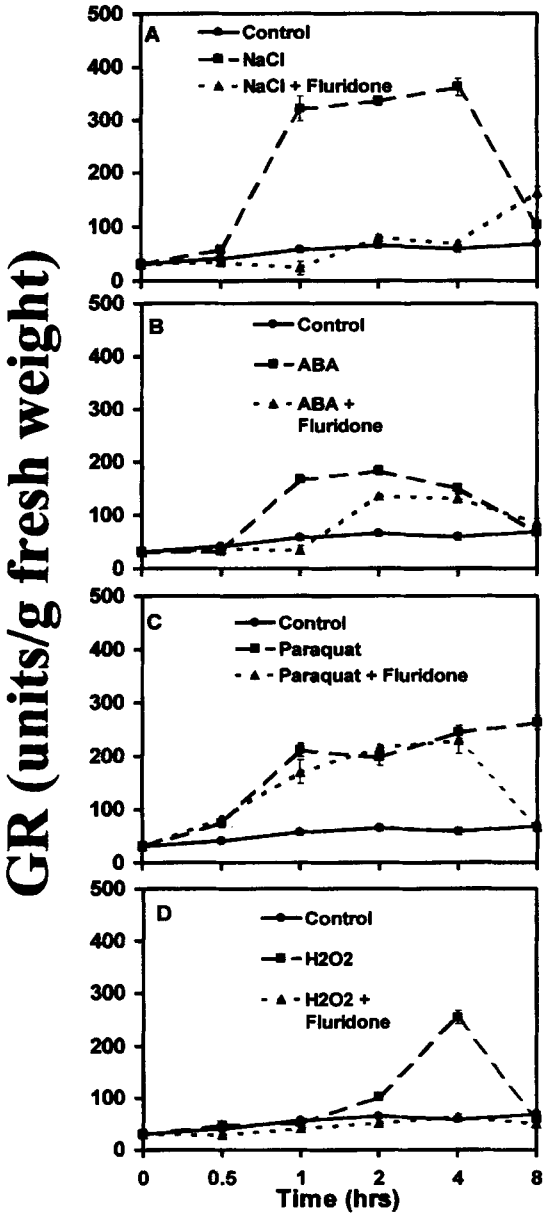

FIGURE 6 GR activity (units/g fresh weight ±SE) measured at 0, 0.5, 1, 2, 4, and 8 hours in NaCl-tolerant cotton callus tissue controls or after treatment with (A) 250 mM NaCl only and 250 mM NaCl following a 2 hr pre-treatment with 0.2 μM fluridone, (B) 5 μM ABA only and 5 μM ABA following a 2 hr pre-treatment with 0.2 μM fluridone, (C) 0.1 μM paraquat only and 0.1 μM paraquat following a 2 hr pre-treatment with 0.2 μM fluridone, and (D) 10 mM H_2O_2 only and 10 mM H_2O_2 following a 2 hr pre-treatment with 0.2 μM fluridone

ridone completely suppressed the increase in GR activity with the H_2O_2 treatment (Figure 6D) and significantly delayed the NaCl- induced increase until 8 hours (Figure 6A). 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 (Figure 6B), Fluridone failed to suppress the increase in GR activity in the paraquat treated callus until 8 hours after treatment. (Figure 6C).

Changes in ABA concentration are shown if Figure 7. When subjected to NaCl stress, ABA levels increased 2-fold within 30 min. after NaCl treatment (Figure 7A) and returned to control levels within 4 hrs. Pre-treatment with fluridone completely suppressed the NaCl-induced increase in ABA concentrations (Figure 7B).

Both the NaCl and paraquat treatments resulted in significant rapid increases in endogenous superoxide levels (Figure 8). The paraquat generated increase in superoxide (Figure 8A) was significantly higher than the increase resulting from the NaCl treatment (Figure 8B).

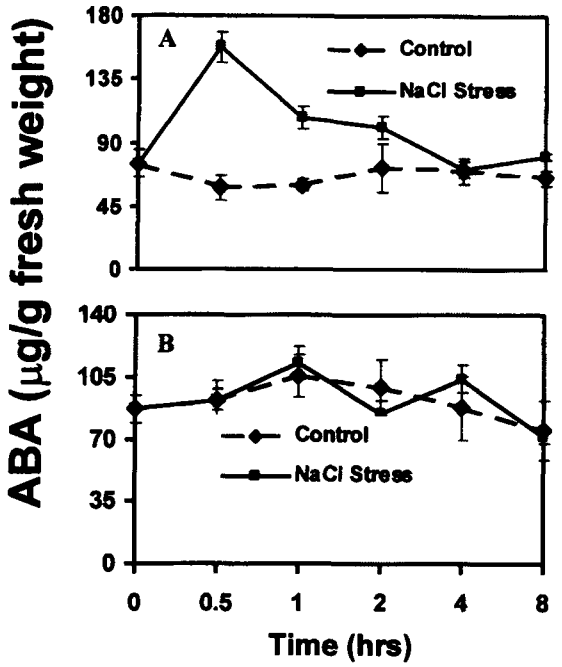

FIGURE 7 ABA concentrations (µg/g fresh weight ±SE) measured in NaCl-tolerant cotton callus tissue at 0, 0.5, 1, 2, 4, and 8 hours in (A) controls and tissue subjected to NaCl stress with 250 mM NaCl and (B) controls and tissue subjected to NaCl stress with 250 mM NaCl following a 2 hr pre-treatment with 0.2 µM fluridone

DISCUSSION

In the callus tissue subjected to NaCl stress, the time between the application of stress and the observed increase in catalytic activity among the five enzymes ranged from 30 minutes for AP to 2 hours for SOD (Figures 2A – 6A). While these increases in catalytic activity may appear to be too rapid to originate at the gene level, other studies with *Arabidopsis*[44,45] and tobacco[46] have shown that in plants, transcription of oxidative stress-induced genes can occur very early after the onset of stress. Sharma and Davis[44] reported 3- to 26-fold increases in GST, phenylalanine ammonia-lyase, and peroxidase mRNA levels in *Arabidopsis* at the first time point (3 hours) after exposure to ozone stress. These investigators did not measure mRNA levels at an earlier time point, but it can be inferred that an increase in the transcription of the genes encoding these mRNAs occurred well before the 3 hour time point. Richards *et al.*[45] have shown that the transcripts for some oxidative stress-induces genes increase as early as 15 minutes after exposure of *Arabidopsis* seedlings and roots to Al^{3+} stress. Significant increases in the relative induction of GST and peroxidase were observed within 30 minutes in dark-grown seedlings and roots subjected to Al^{3+} stress. RNA transcripts have not been measured in our laboratory; however, in a previous study with cotton, Manchandia *et al.*[19] showed that pretreatment with 100 mg/L α-amanitin, a concentration which specifically inhibits RNA polymerase II, completely suppressed the NaCl-induced increases in antioxidant enzyme activity of the five enzymes used in the present study. This strongly suggests that the NaCl-induced up-reg-

ulation of antioxidant catalytic activity in cotton callus tissue is transcriptionally regulated, proceeding via a *de novo* synthesis of poly(A)$^+$RNA. While the preceding information supports a transcriptionally regulated model for the NaCl-induced increases in antioxidant enzyme activity, the rapid increases in catalytic activity suggest that other mechanisms, such as the inactivation of transcription or translation inhibitors, might be involved; however, it is difficult to envision how such a mechanism could explain the results of the study with the RNA polymerase II inhibitor, α-amanitin[19]. Even though the specific mechanisms have not been completely resolved, the data presented in this paper demonstrates that both ABA-dependent and an ABA-independent pathways are in the NaCl-induced upregulation of antioxidant catalytic activity, and future research will focus on the mechanisms operating at the gene level. It should also be noted that the current study was performed on NaCl-acclimated callus tissue, and in a previous study[19], 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[19].

The activities of all of the enzymes either returned to the pre-treatment level or decreased significantly by the 8-hour time point (Figures 2A – 6A). 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

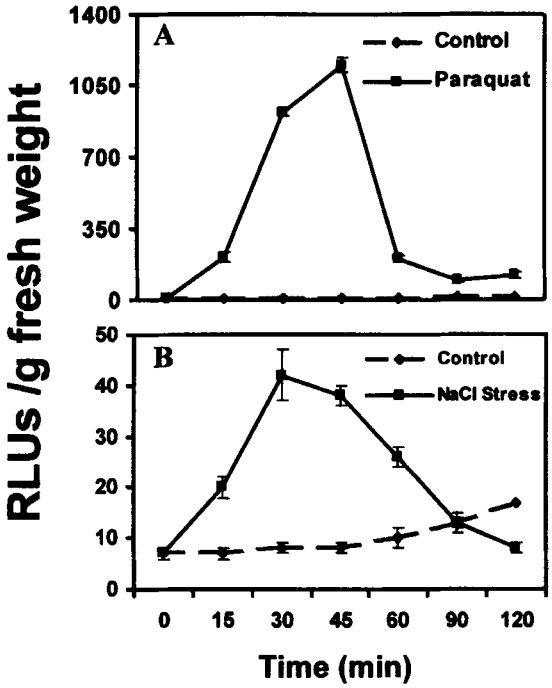

FIGURE 8 RLUs / g fresh weight (±SE) measured in NaCl-tolerant cotton callus tissue at 0, 15, 30, 45, 60, 90, and 120 minutes in (A) controls and tissue treated with 0.2 μM paraquat and (B) controls and tissue subjected to NaCl stress with 250 mM NaCl

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[47] may be invoked. The transient nature and the variation in time of the antioxidant response have been discussed in depth elsewhere[19].

NaCl stress induced early increases (within 2 hours) in the activities of SOD (Figure 2A), catalase (Figure 3A), peroxidase (Figure 4A), and GR (Figure 6A), and identical responses were observed in the callus tissue treated with exogenous ABA (Figures 2B, 3B, 4B, 6B). Pre-treatment with fluridone completely suppressed, in the case of catalase (Figure 3A) and peroxidase (Figure 4A), or significantly delayed, in the case of SOD (Figure 2A) and GR (Figure 6A), the

NaCl-induced increases in enzyme activity. NaCl stress also induced a rapid, transient increase in the ABA concentration (Figure 7A). The increase in ABA concentration occurred prior to the increases observed in SOD, catalase, peroxidase, and GR activities, 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 (Figures 2B, 3B, 4B, 6B), the increases in the activities of these four enzymes, though slightly delayed, were not suppressed.

Results from the paraquat treated tissue suggest that SOD, catalase, peroxidase, APX, and GR activities can also be up-regulated by a pathway other than the one mediated by ABA. Paraquat increased the activities of SOD, catalase, peroxidase, APX, and GR (Figures 2C – 6C) in a manner that closely resembled the increases observed with the NaCl (Figures 2A – 6A) and ABA (Figures 2B – 6B) treatments. In the case of SOD (Figure 2C) and GR (Figure 6C), these increases were observed slightly earlier with the paraquat 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 (Figures 2C – 6C). Since paraquat is known to induce the endogenous production of superoxide[48], this molecule or some other ROI may be involved in a signaling cascade that is apparently independent of ABA.

Shinozaki and Yamaguchi-Shinozaki[28,49] 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, however, is unclear.

NaCl stress in cotton callus tissue does produce a rapid increase in superoxide (Figure 8B). Superoxide can be converted to H_2O_2 through both enzymatic and non-enzymatic reactions. The potential role of H_2O_2 in the signaling cascade has been discussed in detail elsewhere[7,15,50]. Treatment with H_2O_2 has also been shown to increase cytosolic Ca^{2+} in tobacco[51], but the authors concluded that while their data supported a redox control of cytosolic Ca^{2+}, it did not support a hypothesis of a direct H_2O_2 effect. In the human immune system, H_2O_2 has been implicated in the activation of the multisubunit transcription factor NF-κB, but H_2O_2 alone was unable to activate either purified NF-κB or that contained in a cytosolic fraction, and a radical scavenger and metal chelators blocked NF-κB activation by H_2O_2, suggesting that some ROI such as superoxide might be involved in the H_2O_2-induced activation of NF-κB[52]. Data from the present study indicates that a similar scenario occurs in cotton callus tissue. The activities of SOD, catalase, peroxidase, APX, and GR all increased when H_2O_2 was applied to the callus tissue (Figures 2D – 6D); however, these increases were seen much later than the increases observed with the NaCl (Figures 2A – 6A), ABA (Figures 2B – 6B) and paraquat (Figures 2C – 6C) treatments. This difference in timing argues against H_2O_2 serving as the initial signal molecule in the NaCl-induced up-regulation of these enzymes in cotton callus tissue. Also, pre-treatment with fluridone completely suppressed the H_2O_2-induced increases in SOD, catalase, peroxidase, and GR activities (Figures 2D, 3D, 4D, 6D) during the 8 hour time course. Hence, it would appear, at least in cotton callus tissue, that H_2O_2 is converted to some other ROI which, perhaps through direct or indirect reaction with ABA precursors, somehow signals an increase in the ABA concentration.

It has been suggested that superoxide may serve directly as a signal transduction molecule

in both plant[20] and animal[52] systems. The fact that pre-treatment with fluridone had no effect on the rapid superoxide-induced responses suggests that in cotton callus tissue, superoxide or some other ROI was involved in signaling the up-regulation of antioxidant enzymes in an ABA-independent pathway. Evidence of the stress-related activation of ABA-independent signal transduction pathways has been presented by Shinozaki and Yamguchi-Shinozaki[28] and Medina *et al.*[53].

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 (Figure 8), 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 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.

It is difficult to envision how the NaCl-induced increase in APX activity fits into either scenario. The ABA-independent pathway activated by high levels of ROI does not appear to be involved, since the NaCl treatment resulted in an increase in APX activity (Figure 5A) under presumably low to moderate levels of ROI. Yet, the increase does not appear to be related to ABA concentrations, since fluridone failed to suppress any of the observed increases in APX activity with NaCl or any of the other treatments (Figures 5A, 5B, 5C, 5D). On the other hand, ABA treatment did induce an increase in APX activity, suggesting that an ABA-mediated pathway could be involved. The fact that treatment with fluridone alone caused a significant increase in APX activity within 30 minutes (Figure 1D) further clouds the issue and suggests that inhibition of terpene synthesis is sufficient to induce a stress response in APX activity. Furthermore, all of the stressing agents except H_2O_2 induced increases in APX activity earlier than the putative ABA-mediated increases in the other enzymes. In other studies, we have shown that a variety of other chemicals including KCl, sucrose, and $NaNO_3$ also induce rapid increases in APX activity[54]. These data indicate that an increase in APX activity is a rapid response to almost any type of stress. Perhaps there is a second ABA independent pathway in which low to moderate levels of ROI induce a rapid increase in APX catalytic activity. Increases in APX activity may have been regulated by some post-transcriptional mechanism as has been suggested for salt-stressed *Raphanus sativus* plants[55], but studies with α-amanitin indicate that in NaCl-stressed cotton callus tissue, APX activity increases are transcriptionally regulated, proceeding via a *de novo* synthesis of poly(A)$^+$RNA[19].

In conclusion, 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 activities of SOD, catalase, peroxidase, and GR and why fluridone either significantly delayed or completely suppressed the NaCl-induced increases in the activities of these enzymes. 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. The NaCl-induced increase APX activity may occur as the result of a second ABA-independent pathway. Although a number of possibilities have been presented, many questions regarding the specific details of these pathways remain unanswered, and additional research is needed to determine the exact sequence of events that occur between the perception of stress and the up-regulation of enzyme activity.

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References

[1] R. D. Allen (1995) Dissection of oxidative stress tolerance using transgenic plants. *Plant Physiology*, **107**, 1049–1054.

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

[3] R. S. Dhindsa and W. Matowe (1981) Drought tolerance in two mosses: correlated with enzymatic defense against lipid peroxidation. *Journal Experimental Botany*, **32**, 79–91.

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

[5] L. S. Monk and H. V. Davies (1989) Antioxidant status of the potato tuber and Ca^{2+} deficiency as a physiological stress. *Physiologia Plantarum*, **75**, 411–416.

[6] C. H. Foyer, P. Descourviers, and K. J. Kunert (1994) Protection against oxygen radicals: an important defense mechanism in transgenic plants. *Plant Cell Environment*, **17**, 507–523.

[7] C. H. Foyer, H. Lopez-Delgado, J. F. Dat, and I. M. Scott (1997) Hydrogen peroxide- and glutathione-associated mechanisms of acclimatory stress tolerance and signaling. *Physiologia Plantarum*, **100**, 241–254.

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

[9] J. A. Hernandez, F.J. Corpas, M. Gomez, L. A. del Rio, and F. Sevilla (1994) Salt stress-induced changes in superoxide dismutase isozymes in leaves and mesophyll protoplasts from *Vigna unguiculata* L. *New Phytology*, **126**, 37–44.

[10] D. R. Gossett, E. P. Millhollon, and M. C. Lucas (1994). Antioxidant response to NaCl stress in salt-tolerant and salt-sensitive cultivars of cotton. *Crop Science*, **34**, 706–714.

[11] D. R. Gossett, E. P. Millhollon, M. C. Lucas, S. W. Banks, and M. M. Marney (1994) The effects of NaCl on antioxidant enzyme activities in callus tissue of salt-tolerant and salt-sensitive cultivars of cotton. *Plant Cell Reports*, **13**, 498–503.

[12] D. R. Gossett, 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 Physiology*, **112**, 803–809.

[13] V. Jagtap 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 Physiology*, **145**, 195–197.

[14] J. Zhang 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 Physiology, **149**, 489–493.

[15] J. F. Dat, H. Lopez-Delgado H, 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 Physiology*, **116**, 1351–1357.

[16] F. J. Corpas 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 Physiology*, **141**, 160–165.

[17] E. Olmos, 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 Physiology*, **144**, 594–598.

[18] P. Streb and J. Feierabend (1996) Oxidative stress responses accompanying photoinactivation of catalase in NaCl-treated rye leaves. *Botanica Acta*, **109**, 125–132.

[19] A. M. Manchandia, 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 Radical Research*, **30**, 429–438.

[20] N. Doke 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 *Causes of Photooxidative Stress and Amelioration of Defense Systems in Plants* (eds. CH Foyer, PM Mullineaux). CRC, Boca Raton, pp. 177–198.

[21] T. Okuda, 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 Physiology*, **97**, 1265–1267.

[22] S. Singha and M. A. Choudhuri (1990) Effect of salinity (NaCl) stress on H_2O_2 metabolism in *Vigna* and *Oryza* seedlings. *Biochemia Physiologia Pflanzen*, **186**, 69–74.

[23] P. M. Chandler and M. Robertson (1994) Gene expression regulated by abscisic acid and its relation to stress tolerance. *Annual Review of Plant Physiol and Plant Molecular Biology*, **45**, 113–141.

[24] J. Leung and J. Giraudat (1998) Abscisic acid signal transduction. *Annual Review of Plant Physiology and Plant Molecular Biology*, **49**, 199–222.

[25] M. G. Hale and D. M. Orcutt (1987) *The Physiology of Plants Under Stress*. John Wiley and Sons, New York, pp 171–182.

[26] A. F. Galvez, 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 Physiology*, **103**, 257–265.

[27] J. Ingram and D. Bartels (1996) The molecular basis of dehydration tolerance in plants. Annual Review of Plant Physiology and Plant Molecular Biology, **47**, 377–403.

[28] K. Shinozaki and K. Yamaguchi-Shinozaki (1997) Gene expression and signal transduction in water-stress response. *Plant Physiology*, **115**, 327–334.

[29] D. Zhu and J. G. Scandalios (1994) Differential accumulation of manganese-superoxide dismutase transcripts in maize in response to abscisic acid and high osmoticum. *Plant Physiology*, **106**, 173–178.

[30] J. Kurepa, 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 Physiology*, **38**, 463–470.

[31] R. Mittler and B.A. Zalinkas (1993) Detection of ascorbate peroxidase activity in native gels by inhibition of the ascorbate dependent reduction of nitroblue tetrazolium. *Annals of Biochemistry*, **212**, 540–546.

[32] J. D. Williamson 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. *Proceedings of the National Academy of Science USA*, **89**, 8842–8851.

[33] F. Fong, 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 Physiology*, **73**, 899–901.

[34] Y. I. Toshihito, T. Endo, and S. Satoh (1998) Restoration of seed germination at supraoptimal temperatures by fluridone, an inhibitor of abscisic acid biosynthesis. *Plant Cell Physiology*, **39**, 307–312.

[35] N. L. Trolinder and J. R. Goodin (1987) Somatic embryogenesis and plant regeneration in *Gossypium hirsutum*L. *Plant Cell Reports*, **6**, 231–234.

[36] T. Murashige and F. Skoog (1962) A revised medium for rapid growth and bioassays with tobacco tissue culure. *Plant Physiology*, **15**, 473–497.

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

[38] J. V. Anderson, B. I. Chevone J. L. Hess (1992) Seasonal variation in the antioxidant system of eastern white pine needles. *Plant Physiology*, **98**, 501–508.

[39] R. R. Beers and I. Sizer (1952) A spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. *Journal of Biological Chemistry*, **195**, 133.

[40] R. S. Nickel and B. A. Cunningham (1969) Improved peroxidase assay method using leuco 2,3',6 trichloroindophenol and application to comparative measurements of peroxidase catalysis. *Annals of Biochemistry*, **27**, 292–299.

[41] H. J. Forman and I. Fridovich (1973) Superoxide dismutase: A comparison of rate constants. *Archives of Biochemistry and Biophysics*, **158**, 396–400.

[42] M. Schaedle and J.A. Bassham (1977) Chloroplast glutathione reductase. *Plant Physiology*, **59**, 1011–1012.

[43] G. J. Kling and L. M. Perkins (1987) Improved method for determination of indole 3-acetic acid in plant tissue. *Journal of Chromatography*, **407**, 377–383.

[44] Y. K. Sharma and K. R. Davis (1994) Ozone-induced expression of stress-related genes in *Arabidopsis thaliana*. *Plant Physiology*, **105**, 1089–1096.

[45] K. R. Richards, E. J. Schott, Y.K. Sharma, K. R. Davis, and R. C. Gardner (1998) Aluminum induces oxidative stress genes in *Arabidopsis thaliana*. *Plant Physiology*, **116**, .09–418.

[46] H. Willekens, W. Van Camp, M. Van Montagu, D. Inze, C. Langebartels, and H. Sanderman Jr. (1994) Ozone, sulfur dioxide, and ultraviolet B have similar effects on mRNA accumulation of antioxidant genes in *Nicotiana plumaginafolia L. Plant Physiology*, **106**, 1007–1014.

[47] F. Fougere, 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. Physiology*, **96**, 1228–1236.

[48] C. F. Babbs J. A., Pham, R. C. Coolbaugh (1989) Lethal hydroxyl radical production in paraquat-treated plants. *Plant Physiology*, **90**, 1267–1272.

[49] K. Shinozaki and K. Yamaguchi-Shinozaki (1996) Molecular responses to drought and cold stress. *Current Opinions in Biotechnology*, **7**, 161–167.

[50] T. K. Prasad, M.D. Anderson, B. A. Martin, and C. R. Stewart (1994) Evidence for chilling-induced oxidative stress in maize seedlings and a regulatory role for hydrogen peroxide. *Plant Cell*, **6**, 65–74.

[51] A. H. Price, A. Taylor, S. J. Ripley, A. Griffiths, A. J. Trewavas, M. R. Knight (1994) Oxidative signals in tobacco increase cytosolic calcium. *Plant Cell*, **6**, 1301–1310.

[52] R. Schreck, P. Rieber, and P. A. Baeuerle (1991) Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-κB transcription factor and HIV-1. *The EMBO Journal*, **10**, 2247–2258.

[53] J. Medina, M. Barques, J. Terol, M. Perez-Alonso, and J. Salinas (1999) The *Arabidopsis CBF* gene family is composed of three genes encoding AP2 domain-containing proteins whose expression is regulated by low temper-

ature but not by abscisic acid or dehydration. *Plant Physiology*, **119**, 463–469.

[54] D. R. Gossett, B. A. Bellarie, A. M. Manchanda, S. W. Banks, and M. C. Lucas (1999) Specific ion effects on the upregulation of antioxidant enzyme activity in cotton callus tissue. *Proceedings of the Beltwide Cotton Production and Research Conference*. pp. 540–542.

[55] F. Lopez, G. Vansuyt, F. Casse-Delbart, and P. Fourcroy (1996) Ascorbate peroxidase activity, not mRNA level, is enhanced in salt-stressed *Raphanus sativus* plants. *Plant Physiology*, **97**, 13–20.