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The Influence of α-Amanitin on the NaCl-induced Up-regulation of Antioxidant Enzyme Activity in Cotton Callus Tissue

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Liquid suspensions of cotton callus tissue from a NaCl-sensitive cell line and a NaCl-tolerant cell line were subjected to the following treatments: (a) 0 and 150 mM NaCl, respectively (controls); (b) 75 and 250 mM NaCl, respectively; (c) 100 ng ml^{-1} α-amanitin; or (d) pre-treatment for 2 h with 100 ng ml^{-1} α-amanitin followed by the respective NaCl treatments. The callus tissue was harvested at 0, 0.5, 1, 2, 4, and 8 h and analyzed for antioxidant enzyme activity. In the NaCl-tolerant callus, the 250 mM NaCl treatment resulted in transient 2- to 4-fold increases above the control levels in the activities of ascorbate peroxidase, catalase, glutathione reductase, and peroxidase within 1 h after treatment, while superoxide dismutase activity increased 4-fold within 4 h. This rapid increase suggests that the up-regulation of antioxidant capacity is an early response to NaCl stress and perhaps provides protection against oxidative damage until other acclimating mechanisms can be invoked. In the control callus, peroxidase activity remained unchanged, and significant increases in the other enzymes were not observed until 8 h after treatment with 75 mM NaCl. Pre-treatment with α-amanitin prior to the NaCl treatment completely inhibited the NaCl-induced increase in the activities of all five enzymes in both cell lines. This data supports the conclusion that the NaCl-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. In addition, the results suggest that it is not only the up-regulation of antioxidant activity that bestows a degree of tolerance to environmental stress, but also the speed with which this response occurs.

Keywords: α-Amanitin, salt stress, oxidative stress, cotton, antioxidant enzymes, transcription

INTRODUCTION

Environmental stress often results in oxidative damage,[1–10] and plants with high levels of antioxidants, either constitutive or induced, have been reported to have greater resistance to oxidative damage.[1,3–6,11,12] While the mechanism which imparts salt tolerance to non-halophytic plants

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has not been completely defined, an up-regulation of antioxidant enzyme activity appears to be part of the cascade of events that result in the adaptation to high saline levels. In previous studies with cotton, leaves from an NaCl-tolerant cultivar contained significantly higher NaCl-induced levels of general peroxidase (PER) and glutathione reductase (GR) than did leaves from a more NaCl-sensitive cultivar,[13] and callus tissue from the NaCl-tolerant cultivar[14] as well as from a salt-tolerant cell line[15] showed significant increases above control values in superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), PER, glutathione S-transferase (GST) and GR activities when subjected to NaCl stress. Olmos *et al.*[16] noted the induction of several antioxidant enzymes in a salt-tolerant cell line of *Pisum sativum*, and Hernandez *et al.*[17,18] demonstrated that salt treatments decrease Mn-SOD activity in mitochondria isolated from salt-sensitive peas but induce this isozyme's activity in NaCl-tolerant plants. Lopez *et al.*[19] reported that APX activity increased in salt-stressed *Raphanus sativus*.

Very little information is available on the biochemical process responsible for the NaCl-induced up-regulation of antioxidant activity. Even though Lopez *et al.*[19] measured an increase in APX activity, but not mRNA level, in salt-stressed radish plants, 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. The present research is an effort to test this hypothesis by using the fungal toxin, α-amanitin, a specific inhibitor of poly $(A)^+$ RNA synthesis. α-Amanitin is an octapeptide [cyclic(L-asparaginyl-4-hydroxy-L-propyl-(R)-4,5-dihydroxy-L-isoleucyl-6-hydroxy-2-mercapto-L-tryptophylglycyl-L-isoleucylglycyl-L-cysteinyl) cyclic (4-8)-sulphide-(R)-S-oxide] which occurs in the Death Cap fungus, *Amanita phalloides*.[20] The effects of this toxin on transcription have been well characterized *in vitro*.[21,22] RNA polymerase I synthesizes the 18S and 28S rRNAs; however, this enzyme is not inhibited by α-amanitin at any concentration tested. RNA polymerase III, which synthesizes tRNA and 5S rRNA, is inhibited by α-amanitin concentrations of 1000–10,000 ng mL^{-1}. However, RNA polymerase II, which synthesizes poly $(A)^+$ RNAs, is inhibited by α-amanitin at concentrations between 100 and 1000 ng mL^{-1}. The mechanism of inhibition is also known. α-Amanitin binds to RNA polymerase II in such a way as to prevent the elongation step of transcription.[23] In view of these findings, 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^{-1}.

METHODS AND MATERIALS

Plant Tissue Callus tissue for the cotton cultivar Coker 312 was generated according to the method of Trolinder and Goodin.[24] A NaCl-tolerant Coker 312 cell line acclimated to grow on media containing 150 mM NaCl was developed according to the method outlined by Gossett *et al.*[15]

Chemicals and Reagents All chemicals were reagent grade and purchased from Sigma Chemical Company (St. Louis, MO). All experiments were conducted in sterile liquid media consisting of MS salts[25] supplemented with Gamborg's vitamins,[26] 0.75 mg L^{-1} $MgCl_2$, and 30 g L^{-1} glucose adjusted to a pH of 5.8.[24] Media for the NaCl-tolerant callus tissue also contained 150 mM NaCl. A stock α-amanitin solution at a concentration of 1 mg mL^{-1} was prepared in DMSO and filter sterilized through a 0.2 μm Acrodisk filter. A 2.5 μL aliquit of the α-amanitin stock solution was added to 25 mL of media to give final concentration of 100 ng mL^{-1}. A stock solution of NaCl was prepared at a concentration of 2.51 M and sterilized with an autoclave. One mL of this stock solution was added to 24 mL of the media containing 150 mM NaCl to give a final concentration of 250 mM

NaCl, and 0.75 mL of this stock solution was added to 24.25 mL of the control media to give a final concentration of 75 mM NaCl.

Experimental Procedure At the beginning of each experiment, approximately 4 g of callus tissue from the NaCl-tolerant cell line was transferred to each of either a series of culture tubes containing 24 mL of media amended with 150 mM NaCl (salt-tolerant control) or a series of culture tubes amended with 150 mM NaCl + 100 ng mL^{-1} α-amanitin. Each culture tube was then connected to an aerator and allowed to pre-incubate for 2 h. Following pre-incubation, the tubes containing 150 mM NaCl or the tubes containing 150 mM NaCl + 100 ng mL^{-1} α-amanitin were amended with NaCl to a final concentration of 250 mM NaCl. In a parallel series of experiments, callus tissue from a control cell line was transferred to each of either a series of culture tubes containing 24.25 mL of media amended with 0 mM NaCl (salt-sensitive control) or a series of culture tubes amended with 0 mM NaCl + 100 ng mL^{-1} α-amanitin. Each culture tube was then connected to an aerator and allowed to pre-incubate for 2 h. Following pre-incubation, the tubes containing 0 mM NaCl or the tubes containing 0 mM NaCl + 100 ng mL^{-1} α-amanitin were adjusted to a final concentration of 75 mM NaCl. In both experiments, the callus tissue was harvested at 30 min, 1, 2, 4, and 8 h intervals and stored at −70°C for subsequent antioxidant analyses. *Caution*: α-Amanitin is a highly toxic compound that requires special handling. Suitable protective clothing, gloves, and eye/face protection should be worn, and aqueous solution should be treated with an equal volume of 5 M NaOH for 30 min before disposal.

Protein Extraction Samples were prepared for APX, CAT, GR, PER, and SOD analyses by the method outlined by Anderson *et al.*[27] as modified by Gossett *et al.*[14]

Enzyme Assays CAT activity was determined by monitoring the disappearance of H_2O_2 according to the method of Beers and Sizer.[28] Total SOD activity 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 as outlined by Forman and Fridovich.[29] GR activity was determined by monitoring the glutathione-dependent oxidation of NADPH as described by Schaedle and Bassham.[30] PER activity was measured by monitoring the H_2O_2-dependent oxidation of reduced 2,3′,6-trichloroindophenol according to the method of Nickel and Cunningham.[31] APX activity was assayed by monitoring the ascorbic acid-dependent reduction of H_2O_2 as described by Anderson *et al.*[27] For CAT, PER, and APX, one unit of enzyme was defined as the amount necessary to decompose 1 μmol of substrate/min at 25°C. One unit of GR was defined as the amount of enzyme required to reduce 1 nmol 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%. Data points are based on a mean of a minimum of three replicates. All data were subjected to one way analysis of variance, and significance was determined at the 99% confidence limits.

RESULTS

Enzyme activities for the control callus are presented in Figures 1–5. APX (Figure 1) doubled within 8 h after treatment with NaCl. With the 75 mM NaCl treatment, CAT activity (Figure 2) showed a significant 2-fold increase within 8 h, and within the same time period, a 5-fold increase above control levels in SOD activity (Figure 3) was observed. GR activity (Figure 4) increased almost 3-fold above control level within 4 h when treated with 75 mM NaCl and returned to control level within 8 h. NaCl treatment failed to induce a significant increase in PER (Figure 5) activity within 8 h. Treatment with α-amanitin alone did not change the activity of any of the enzymes. Pre-treatment with α-amanitin prior to the NaCl

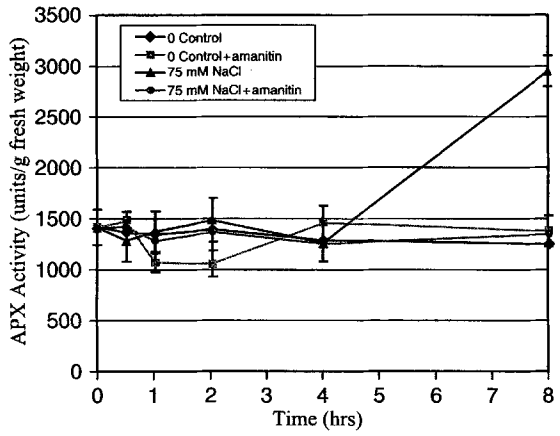

FIGURE 1 APX activity (units/g fresh weight ± SE) in NaCl-sensitive cotton callus tissue harvested 0, 0.5, 1, 2, 4, and 8 h after being subjected to the following treatments: (a) 0 mM NaCl (0 control); (b) 100 ng mL^{-1} α-amanitin (0 control + amanitin); (c) 75 mM NaCl (75 mM NaCl); or (d) a 2 h pre-treatment with 100 ng mL^{-1} α-amanitin followed by treatment with 75 mM NaCl (75 mM NaCl + amanitin).

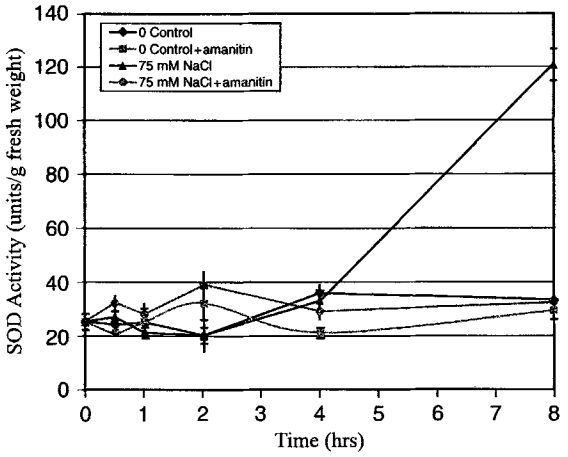

FIGURE 3 SOD activity (units/g fresh weight ± SE) in NaCl-sensitive cotton callus tissue harvested 0, 0.5, 1, 2, 4, and 8 h after being subjected to the following treatments: (a) 0 mM NaCl (0 control); (b) 100 ng mL^{-1} α-amanitin (0 control + amanitin); (c) 75 mM NaCl (75 mM NaCl); or (d) a 2 h pre-treatment with 100 ng mL^{-1} α-amanitin followed by treatment with 75 mM NaCl (75 mM NaCl + amanitin).

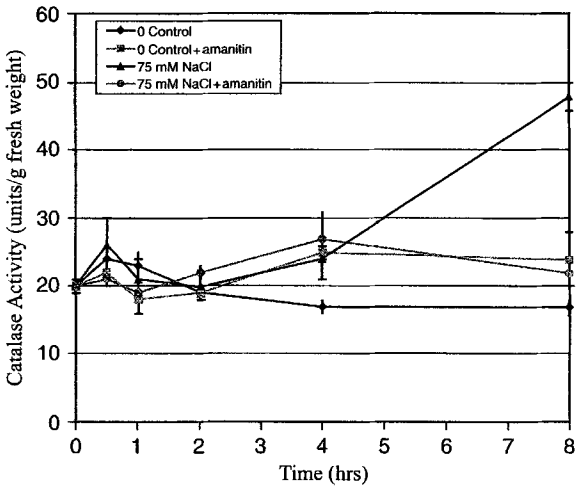

FIGURE 2 Catalase activity (units/g fresh weight ± SE) in NaCl-sensitive cotton callus tissue harvested 0, 0.5, 1, 2, 4, and 8 h after being subjected to the following treatments: (a) 0 mM NaCl (0 control); (b) 100 ng mL^{-1} α-amanitin (0 control + amanitin); (c) 75 mM NaCl (75 mM NaCl); or (d) a 2 h pre-treatment with 100 ng mL^{-1} α-amanitin followed by treatment with 75 mM NaCl (75 mM NaCl + amanitin).

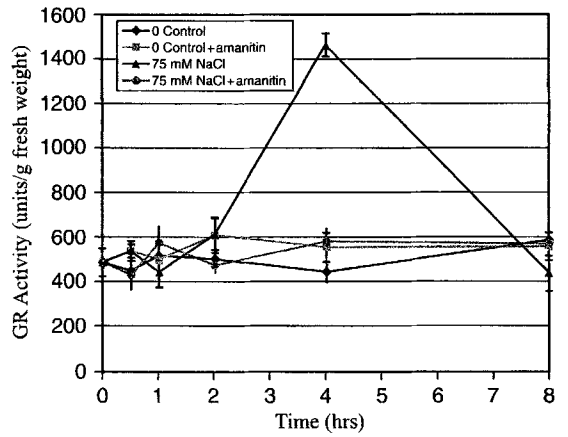

FIGURE 4 GR activity (units/g fresh weight ± SE) in NaCl-sensitive cotton callus tissue harvested 0, 0.5, 1, 2, 4, and 8 h after being subjected to the following treatments: (a) 0 mM NaCl (0 control); (b) 100 ng mL^{-1} α-amanitin (0 control + amanitin); (c) 75 mM NaCl (75 mM NaCl); or (d) a 2 h pre-treatment with 100 ng mL^{-1} α-amanitin followed by treatment with 75 mM NaCl (75 mM NaCl + amanitin).

treatment completely inhibited the NaCl-induced increase in APX, CAT, SOD, and GR activities.

In the NaCl-acclimated callus tissue, the addition of NaCl to a final concentration of 250 mM resulted in significant increases above control values for all five enzymes (Figures 6–10), and these increases occurred much earlier than in the control callus tissue. Treatment with 250 mM

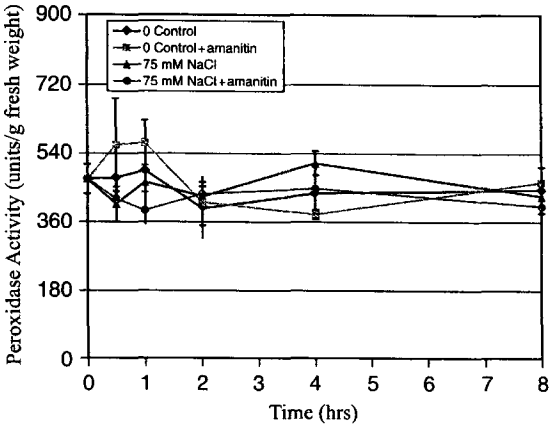

FIGURE 5 Peroxidase activity (units/g fresh weight ± SE) in NaCl-sensitive cotton callus tissue harvested 0, 0.5, 1, 2, 4, and 8 h after being subjected to the following treatments: (a) 0 mM NaCl (0 control); (b) 100 ng mL^{-1} α-amanitin (0 control + amanitin); (c) 75 mM NaCl (75 mM NaCl); or (d) a 2 h pre-treatment with 100 ng mL^{-1} α-amanitin followed by treatment with 75 mM NaCl (75 mM NaCl + amanitin).

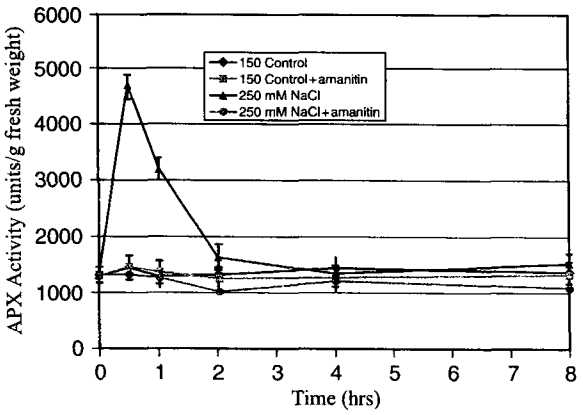

FIGURE 6 APX activity (units/g fresh weight ± SE) in NaCl-tolerant cotton callus tissue harvested 0, 0.5, 1, 2, 4, and 8 h after being subjected to the following treatments: (a) 150 mM NaCl (150 control); (b) 100 ng mL^{-1} α-amanitin (150 control + amanitin); (c) 250 mM NaCl (250 mM NaCl); or (d) a 2 h pre-treatment with 100 ng mL^{-1} α-amanitin followed by treatment with 250 mM NaCl (250 mM NaCl + amanitin).

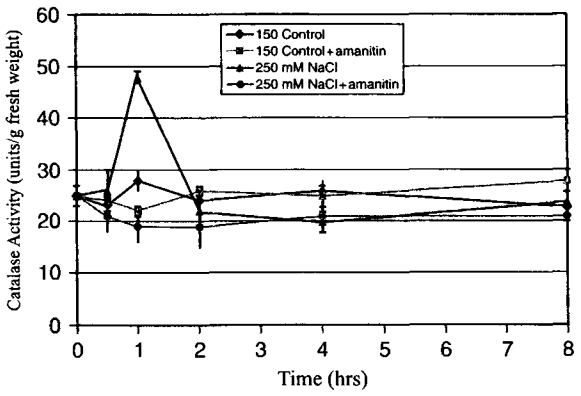

FIGURE 7 Catalase activity (units/g fresh weight ± SE) in NaCl-tolerant cotton callus tissue harvested 0, 0.5, 1, 2, 4, and 8 h after being subjected to the following treatments: (a) 150 mM NaCl (150 control); (b) 100 ng mL^{-1} α-amanitin (150 control + amanitin); (c) 250 mM NaCl (250 mM NaCl); or (d) a 2 h pre-treatment with 100 ng mL^{-1} α-amanitin followed by treatment with 250 mM NaCl (250 mM NaCl + amanitin).

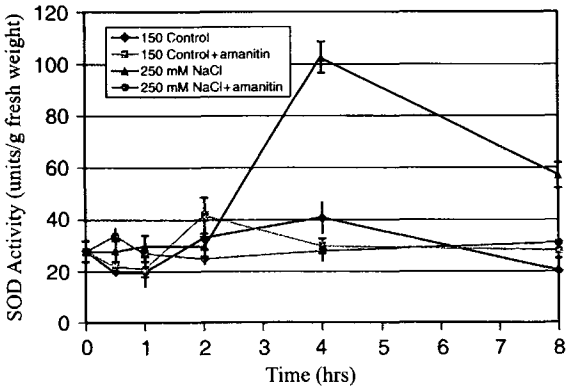

FIGURE 8 SOD activity (units/g fresh weight ± SE) in NaCl-tolerant cotton callus tissue harvested 0, 0.5, 1, 2, 4, and 8 h after being subjected to the following treatments: (a) 150 mM NaCl (150 control); (b) 100 ng mL^{-1} α-amanitin (150 control + amanitin); (c) 250 mM NaCl (250 mM NaCl); or (d) a 2 h pre-treatment with 100 ng mL^{-1} α-amanitin followed by treatment with 250 mM NaCl (250 mM NaCl + amanitin).

NaCl resulted in a 4-fold increase above control levels in APX activity (Figure 6) within 30 min and this activity returned to control levels within 2 h. CAT activity (Figure 7) exhibited a 2-fold increase above control level within 1 h and returned to the control level within 2 h when the tissue was treated with the high NaCl concentration. The 250 mM NaCl treatment resulted in a 4-fold increase above control levels in SOD activity (Figure 8) within 4 h. Within 8 h, SOD activity had decreased to about one-half of the value observed at 4 h, but the activity continued to remain significantly higher than the control level. GR activity (Figure 9) also increased significantly

above control values within 1 h after treatment with 250 mM NaCl. Within 4 h, GR activity was approximately 3.5 times that observed in the control callus, and within 8 h, GR activity had returned to the pre-treatment values. A 2-fold increase above the control level in PER activity (Figure 10) was also observed within 1 h after treatment with the high NaCl concentration. PER activity returned to the control level within 4 h after treatment. As with the control callus tissue, treatment with α-amanitin by itself did not change the activity of any of the enzymes, and pre-treatment with α-amanitin prior to the NaCl treatment completely inhibited the NaCl-induced increase in the activities of all five enzymes.

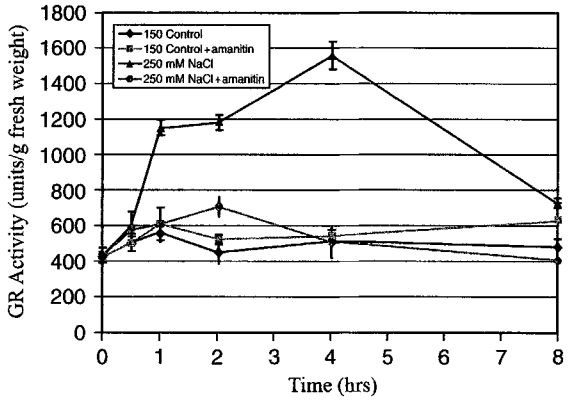

FIGURE 9 GR activity (units/g fresh weight ± SE) in NaCl-tolerant cotton callus tissue harvested 0, 0.5, 1, 2, 4, and 8 h after being subjected to the following treatments: (a) 150 mM NaCl (150 control); (b) 100 ng mL^{-1} α-amanitin (150 control + amanitin); (c) 250 mM NaCl (250 mM NaCl); or (d) a 2 h pre-treatment with 100 ng mL^{-1} α-amanitin followed by treatment with 250 mM NaCl (250 mM NaCl + amanitin).

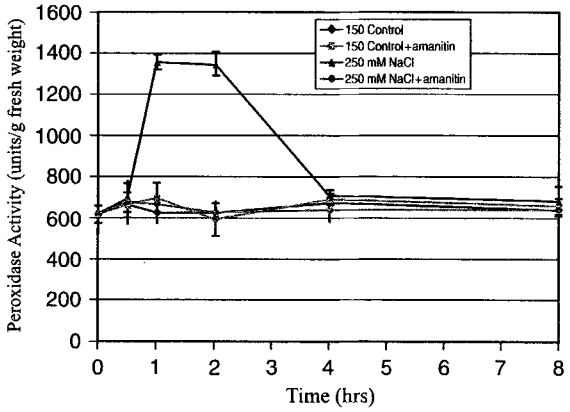

FIGURE 10 Peroxidase activity (units/g fresh weight ± SE) in NaCl-tolerant cotton callus tissue harvested 0, 0.5, 1, 2, 4, and 8 h after being subjected to the following treatments: (a) 150 mM NaCl (150 control), (b) 100 ng mL^{-1} α-amanitin (150 control + amanitin); (c) 250 mM NaCl (250 mM NaCl); or (d) a 2 h pre-treatment with 100 ng mL^{-1} α-amanitin followed by treatment with 250 mM NaCl (250 mM NaCl + amanitin).

DISCUSSION

In each case, significant increases in enzyme activity were observed within 1–4 h in the NaCl-tolerant callus tissue where at least 4–8 h were required for increases to occur in the control tissue. The process associated with this more rapid increase is unknown, but 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 callus, up-regulate its antioxidant defense system more rapidly once the stress had been perceived, or a combination of both. Numerous studies have shown that the tolerance to environmentally induced oxidative stress is correlated with the up-regulation of antioxidant enzyme activity, but very few of these studies have utilized short-term (0–8 h) time-course experiments to compare the stress-induced antioxidant response in tolerant versus non-tolerant plants. The results from our studies suggest that it is not only the up-regulation of antioxidant activity that bestows a degree of tolerance to environmental stress, but also the speed in which this response occurs.

While NaCl stress resulted in an increase in all five of the enzymes in the NaCl-tolerant callus and four of the enzymes in the control callus, there were differences in the degree of response and time for induction among the different enzymes. The degree of the response ranged from a 2-fold increase in the activities of CAT, SOD, and PER to 4-fold increases for APX and GR. The time ranged from as little as 0.5 min for APX in the NaCl-tolerant callus to 8 h for APX, CAT, and SOD in the

control callus. Similar results have been reported in *Arabidopsis*[32,33] and tobacco.[34] These studies showed that oxidative stress genes were variable in both their level of response and time of induction after treatment with different stress-inducing agents. The significance of this spatial difference in the up-regulation in antioxidant enzyme activity is currently unresolved, but in previous studies with cotton leaves and callus tissue,[13,14] it was suggested that the more NaCl-tolerant cultivars had a higher capacity to decompose H_2O_2 and a more active ascorbate-glutathione cycle. Hence, it may be more than coincidence that the activities of two of the enzymes which decompose H_2O_2 (CAT and PER) and two of the enzymes associated with the ascorbate-glutathione cycle (APX and GR) were up-regulated significantly earlier in the NaCl-tolerant callus tissue.

The NaCl-induced increases in antioxidant enzyme activity were also transient. Except for SOD, the activities of all of the enzymes returned to the pre-treatment level within 8 h in the NaCl-tolerant callus. In time-course experiments where enzyme activities were monitored in callus tissue subjected to NaCl stress for longer periods of time (data not shown), neither SOD activity in the NaCl-tolerant callus nor the activity of any of the enzymes in the control callus tissue were significantly different from the pre-treatment values after 16 h. The transient nature of the antioxidant response has been observed in other studies. Richards *et al.*[33] have shown that the excess Al induced transient transcripts of several oxidative stress genes over an 8 h period in *Arabidopsis*. The fact that APX, CAT, GR, and PER activities increased significantly within 1 h and returned to pre-treatment levels within 8 h in the NaCl-tolerant callus suggest that oxidative stress and the antioxidant response to that stress occurs very early after exposure to excessive levels of NaCl. Perhaps Na^+, Cl^-, or the combination of Na^+ and Cl^- ions immediately disrupt electron flow resulting in the rapid formation of reactive oxygen species. On the other hand, callus tissue may respond to NaCl stress via the rapid production of reactive oxygen species through a respiratory burst as has been observed in other plants in the defense against fungal infection.[35,36] In either case, a rapid increase in antioxidant activity would be necessary for tolerance to the stress. It may well be the rapid up-regulation of the antioxidant enzymes in the NaCl-tolerant callus tissue provides the initial defense against cellular damage from the oxidative burst that results from the perceived stress. By the time the tissue has acclimated to the oxidative burst, other adaptive mechanisms such as the accumulation of proline[37] or other low molecular weight organic compounds[38] that may serve as osmoprotectants[39,40] have been invoked, and antioxidant enzymes activities return to more normal levels.

The results of this study 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[21] and other higher plants.[41–43] α-Amanitin at concentrations of 0.10–1.0 μg mL^{-1} specifically inhibited poly(A)$^+$RNA synthesis when added to germinating wheat embryos, whereas higher concentrations of 10–100 μg mL^{-1} were required to inhibit 5S rRNA and tRNA synthesis.[41] On the other hand, the addition of α-amanitin to germinating wheat embryos did not inhibit protein synthesis or polysome formation,[44] nor was *in vitro* translation of mRNA inhibited by α-amanitin in a wheat germ cell-free translation system.[45] Transcription was blocked within 15 min after addition of α-amanitin in carrot suspension cultures.[42] α-Amanitin at concentrations of 0.05–5.0 μg mL^{-1} blocked transcription of RNA associated with fiber development in ovule culture.[21] 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.[46–50] Yamaguchi-Shinozaki and Shinozaki[51] have identified a *cis*-acting element responsible for the induction of an *Arabidopsis* gene involved in responsiveness to drought, low-temperature, and NaCl stress. In addition, it has been shown that cytosolic and chloroplastic Cu, Zn SOD transcripts increase when tomatoes are mechanically wounded or treated with sublethal doses of paraquat which produces large quantities of superoxide,[52,53] and Scandalios[54] has shown that the catalase CAT1 mRNA levels increase substantially in the presence of abscisic acid. It has been suggested that ABA may confer a degree of tolerance to environmental stress,[55] and an increase in ABA in vegetative tissues is often associated with increases in stress-induced gene expression.[56] It has been demonstrated that ABA levels increase during salt stress.[57,58] ABA has been shown to positively enhance the catalase *Cat1* transcript in maize,[59] and Galvez *et al.*[60] have shown that ABA is the likely inducer for the increased transcription of eleven mRNAs associated with the synthesis of early salt-stress induced proteins in *Lophopyrum elongatum*.

In conclusion, the results from this study provide the basis for the construction of a possible scenario of events that occur when cotton callus tissue is subjected to NaCl stress. High NaCl concentrations results in the immediate production of reactive oxygen species either through the disruption of electron flow or a respiratory burst. In the NaCl-tolerant callus tissue, some signal transduction molecule, perhaps $O_2^{-\bullet}$, ABA, or some other currently undefined metabolite, rapidly induces the transcription of genes which encode for antioxidant enzymes. The increase in antioxidant enzyme activity provides protection against oxidative damage to cellular components until other acclimation mechanisms such as the increased production of osmoprotectants can be activated. Once acclimation is achieved, antioxidant activity returns to pre-stress levels. The more NaCl-sensitive tissue lacks the ability to rapidly mobilize its antioxidant defense system. Additional research, particularly in the area of signal transduction, is necessary to verify this scenario.

Acknowledgments

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