**THE INFLUENCE OF *a*-AMANITIN ON THE INDUCTION OF ANTIOXIDANT ENZYMES DURING SALT STRESS**

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## Abstract

When callus tissue derived from Coker 312 is exposed to salt treatment an increase in the activity of antioxidant enzymes including ascorbate peroxidase and glutathione reductase is observed. In order to address the question of whether the increase in activity is due to an increase in the transcription of the genes encoding these enzymes or whether it is due to the translation of existing transcripts or possibly to the mobilization of existing enzyme pools, a series of experiments was undertaken using *a*-amanitin, a specific inhibitor of RNA polymerase II. Callus tissue from a salt-tolerant cell line was transferred to aerated culture tubes containing either 150 mM NaCl (NaCl-tolerant control) or media containing 150 mM NaCl + 100 ng $ml^{-1}$ *a*-amanitin. Following a 2-hour pre-incubation, the tubes containing 150 mM NaCl and the tubes containing 150 mM NaCl + 100ng $ml^{-1}$ *a*-amanitin were adjusted to a final concentration of 250 mM NaCl. In a parallel series of experiments, callus tissue from a control cell line (NaCl-sensitive control) was transferred to aerated culture tubes containing either 0 mM NaCl or media containing 0 mM NaCl + 100ng $ml^{-1}$ *a*-amanitin. Following a 2-hour pre-incubation period, the tubes containing 0 mM NaCl and the tubes containing 0 mM NaCl + 100ng $ml^{-1}$ *a*-amanitin were adjusted to a final concentration of 75 mM NaCl. The callus tissue from both experiments was harvested at 30 min, 1 hr, 2 hr, 4 hr, and 8 hr intervals and analyzed for ascorbate reductase (AP) and glutathione reductase (GR) activity. In both sets of experiments a complete suppression of the salt induced increase in the activity of ascorbate peroxidase and glutathione reductase was observed in replicates to which *a*-amanitin was added. These results suggest that the upregulation of the activity of AP and GR in response to salt stress is due to a *de novo* transcription of the genes encoding these two enzymes and is not due to the translation of existing transcripts or mobilization of existing enzyme pools.

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## Introduction

Plants possess a number of antioxidants for protection against the cytotoxic species of activated oxygen, and plants with high levels of antioxidants, either constitutive or induced, are reported to be more resistant to damage by activated oxygen species generated during different environmental stress conditions (Dhindsa and Matowe, 1981; Harper and Harvey, 1978; Wise and Naylor, 1987; Monk and Davies, 1989; Spychalla and Desborough, 1990; Mandamanchi and Alscher, 1991; Polle and Rennenberg, 1994). In previous studies involving a comparison of antioxidant activities in a control and a salt-tolerant cell line from the same cultivar (Coker 312) showed that the activity of ascorbate peroxidase (AP) and glutathione reductase (GR), two antioxidant enzymes in the ascorbate-glutathione cycle, were significantly higher in the salt-tolerant cell line (Gossett *et al.,* 1996).

While the mechanism which imparts salt tolerance to nonhalophytic plants has eluded definition, plant response to salt stress most likely involves a cascade of events. Results from the aforementioned studies suggest that some of these events evoke the antioxidant defense system. The specific sequence of events responsible for signaling the upregulation of the antioxidant defense system also remains a major question. This paper reports upon the results of an investigation into the nature of this upregulation using the fungal toxin, *a*-amanitin, a specific inhibitor of poly $(A)^{+}$RNA synthesis. *a*-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* (Dewick 1997). The effects of this toxin on transcription have been well characterized *in vitro* (Triplett 1998, Weiland and Faulstich, 1978). RNA polymerase I synthesizes the 18S and 28S rRNA's, and this enzyme is not inhibited by *a*-amanitin at any concentration tested. RNA polymerase III which synthesizes tRNA and 5S rRNA is inhibited by *a*-amanitin at concentrations of 1000-10,000 ng $ml^{-1}$. However RNA polymease II, which synthesizes poly$(A)^{+}$RNA's is inhibited by *a*-amanitin at concentrations between 100 and 1000 ng $ml^{-1}$. The mechanism of inhibition is also known. *a*-Amanitin binds to RNA polymerase II in such a way as to prevent the elongation step of transcription (Cochet-Meilhac and Chambon, 1974). In view of these findings it is possible to specifically inhibit RNA polymerase II using *a*-amanitin at a concentration of 100ng $ml^{-1}$.

## Methods and Materials

Callus tissue for the cotton cultivar Coker 312 was generated according to the method of Trolinder and Goodin (1987). A salt-tolerant Coker 312 cell line was developed according to the method outlined by Gossett *et al.* (1996). 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 media amended with 150 mM NaCl (salt-tolerant control) or a series of culture tubes amended with 150 mM NaCl +100ng $ml^{-1}$ *a*-amanitin. Each culture tube was then connected to an aerator and allowed to pre-incubate for 2 hours. Following pre-incubation, the tubes containing 150 mM NaCl or the tubes containing 150 mM NaCl + 100ng $ml^{-1}$ *a*-amanitin were adjusted to final concentration of 250 mM NaCl under sterile conditions. In a parallel series of experiments, callus tissue from a control cell line (NaCl-sensitive control) was transferred to culture tubes containing either 0 mM NaCl or media containing 0 mM NaCl + 100 ng $ml^{-1}$ *a*-amanitin. Following a 2-hour pre-incubation period, the tubes containing 0 mM NaCl and the tubes containing 0 mM NaCl + 100ng $ml^{-1}$ *a*-amanitin were adjusted to a final concentration of 75 mM NaCl. The callus tissue was harvested at 30 min., 1 hr, 2 hr, 4 hr, and 8 hr intervals and stored at -70ºC for subsequent antioxidant analyses.

Samples were prepared for enzyme analyses according to the method of Anderson *et al.* (1992) as modified by Gossett *et al.* (1994). Glutathione reductase (GR) activity was determined by monitoring the glutathione-dependent oxidation of NADPH as described by Schaedle and Bassham (1977). Ascorbate peroxidase (AP) activity was assayed by monitoring the ascorbic acid-dependent reduction of $H_2O_2$ as described by Anderson *et al.* (1992). Data points are based on a mean of a minimum of three replicates.

## Results and Discussion

The results from the analysis of ascorbate peroxidase activity shown in Tables 1 and 2 and the results of the analysis of glutathione reductase activity shown in Tables 3 and 4 reveal a significant increase in the activity of these enzymes when both the callus derived from the salt tolerant cell line (150mM acclimated callus) and the callus derived from the salt-sensitive cell line are salt stressed to final concentrations of 250 mM and 75 mM respectively. These results are consistent with previous data (Gossett *et al.* 1996) and data presented at this meeting (Gossett *et al.*1998). The salt-induced increase in activity observed for both enzymes is totally suppressed, however, in those replicates to which *a*-amanitin was added. Since *a*-amanitin is a specific inhibitor of RNA polymerase II at 100 $ngml^{-1}$, these findings suggest that the upregulation of AP and GR 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.

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Table 1. Ascorbate peroxidase activity for salt-tolerant callus (*i.e.* acclimated to growth on 150mM media).

| Time | 150 control callus | 150 callus + 250 mM NaCl | 150 callus + *a*-AM | 150 callus +*a*-AM +250 mM NaCl |
|---|---|---|---|---|
| 0 | 1321±146 | 1321±146 | 1321±146 | 1321±146 |
| 0.5hr | 1462±172 | 1652±227 | 1460±220 | 1321±93 |
| 1 hr | 1298±197 | 4712±218* | 1380±208 | 1272±146 |
| 2 hr | 1351±186 | 3220±197* | 1270±202 | 1026±46 |
| 4 hr | 1456±206 | 1349±161 | 1310±188 | 1223±200 |
| 8 hr | 1379±163 | 1506±219 | 1353±177 | 1094±122 |

*AP (units/g fresh weight) activity rates over 8 hr time course in salt-tolerant callus tissue.*
** Denotes significant change from control at that time point*
(*a*-AM =*a-amanitin at a final concentration of 100ng ml*$^{-1}$)

Table 2. Ascorbate peroxidase activity for salt-sensitive callus( *i.e.* grown on media with 0mM NaCl)

| Time | control callus | control callus + 75 mM NaCl | control callus + *a*-AM | control callus +*a*-AM 75mM NaCl |
|---|---|---|---|---|
| 0 | 1422±168 | 1422±168 | 1422±168 | 1422±168 |
| 0.5hr | 1377±181 | 1292±201 | 1475±102 | 1425±173 |
| 1 hr | 1351±143 | 1385±197 | 1080-±96 | 1293±149 |
| 2 hr | 1405±156 | 1497±215 | 1068±123 | 1383±192 |
| 4 hr | 1296±97 | 2963±183* | 1426±178 | 1259±162 |
| 8 hr | 1257±131 | 1274±187 | 1382±152 | 1353±177 |

*AP (units/g fresh weight) activity rates over 8 hr time course in control callus tissue.*
** Denotes significant change from control at that time point.*
(*a*-AM =*a-amanitin at a final concentration of 100ng ml*$^{-1}$)

Table 3. Glutathione reductase activity for salt-tolerant callus (*i.e.* acclimated to growth on 150mM media).

| Time | 150 control callus | 150 callus + 250 mM NaCl | 150 callus + a-AM | 150 callus +*a*-AM +250 mM NaCl |
|---|---|---|---|---|
| 0 | 430±37 | 430±37 | 430±37 | 430±37 |
| 0.5hr | 511±38 | 608±65 | 508±52 | 583±80 |
| 1 hr | 563±51 | 1154±39* | 614±90 | 611±94 |
| 2 hr | 457±63 | 1187±43* | 529±25 | 712±56 |
| 4 hr | 521±71 | 1563±77* | 551±37 | 518±90 |
| 8 hr | 493±44 | 736±26 | 636±102 | 416±17 |

*GR (units/g fresh weight) activity rates over 8 hr time course in salt tolerant callus tissue.*
** Denotes significant change from control at that time point*
(*a*-AM =*a-amanitin at a final concentration of 100ng ml*$^{-1}$)

Table 4. Glutathione reductase activity for salt -sensitive callus (*i.e.* grown on media with 0mM NaCl)

| Time | control callus | control callus + 75 mM NaCl | control callus +*a*-AM | control callus +*a*-AM + 75 mM NaCl |
|---|---|---|---|---|
| 0 | 486±63 | 486±63 | 486±63 | 486±63 |
| 0.5hr | 451±54 | 538±44 | 538±31 | 428±63 |
| 1 hr | 519±36 | 443±67 | 498±15 | 572±77 |
| 2 hr | 501±42 | 618±73 | 610±77 | 473±34 |
| 4 hr | 445±49 | 1465±52* | 557±66 | 584±55 |
| 8 hr | 587±52 | 436±80 | 559±63 | 569±44 |

*GR (units/g fresh weight) activity rates over 8 hr time course in control callus tissue.*
** Denotes significant change from control at that time point.*
(*a*-AM =*a-amanitin at a final concentration of 100ng ml*$^{-1}$)