THE INFLUENCE OF ABSCISIC ACID ON THE INDUCTION OF ANTIOXIDANT ENZYMES DURING SALT STRESS

D.R. Gossett, B. Bellaire and S.W. Banks
M.C. Lucas, A. Manchandia and E.P. Millhollon
Louisiana State University-Shreveport
Shreveport, LA
Louisiana State University Agriculture Center
Red River Research Station,
Bossier City, LA

Abstract

Callus tissue from a NaCl-tolerant cell line was transferred to culture tubes containing 150 mM NaCl (NaCl-tolerant control) and media containing 150 mM NaCl + 0.2 µM fluridone. Following a 2-hour preincubation, the tubes containing 150 mM NaCl and the tubes containing 150 mM NaCl + 0.2 µM fluridone were amended with NaCl to a final concentration of 250 mM NaCl, 0.1 mg/L ABA, 0.1 µM paraquat, or 0.01% H_2O_2. The callus tissue 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. All treatments except hydrogen peroxide and hydrogen peroxide plus fluridone induced significant increases in AP activity above the control activity levels within 1 hour after treatment. Pretreatment with fluridone failed to suppress the increase in AP activity in all treatments except when used with hydrogen peroxide which did not induce an increase AP activity when used alone. Except for fluridone and hydrogen peroxide plus fluridone, all treatments resulted in significant increases in GR activity above the control levels within one hour after treatment. In contrast to AP, pretreatment with fluridone suppressed or significantly delayed the increase in GR activity in all but the paraquat treated tissues. These data suggests that the stress-induced increases in the activities of GR and AP result from different signaling mechanisms. AP activity appears to be rapidly induced by almost any stress metabolite completely independent of ABA, while stress-induced increases in GR activity are generally slower and partially, at least in the case of NaCl stress, associated with ABA concentrations.

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; Poole 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), 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 non-halophytic 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 substance or substances responsible for signaling the upregulation of the antioxidant defense system also remains a major question. It has been suggested that ABA may confer a degree of tolerance to environmental stress (McKersie and Leshem, 1994), and an increase in ABA in vegetative tissues is often associated with increases in stress-induced gene expression (Chandler and Robertson, 1994). It has been demonstrated that ABA levels increase during salt stress (Hale and Orcutt, 1987; Schnapp *et al*., 1990). ABA has been shown to positively enhance the catalase *Cat1* transcript in maize (Williamson and Scandalios, 1992), and Galvez *et al*. (1993) 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 preliminary studies in our laboratory, ABA treatment resulted in increases in SOD, peroxidase, GR, and AP activities in a NaCl-tolerant cotton cell line (Gossett *et al*., 1997). Hence, the increase in antioxidant enzyme activity observed in the NaCl-tolerant cell line may have been due to enhanced gene induction by elevated levels of ABA. It remains to be determined whether the NaCl-induced increases in antioxidant activity is directly or indirectly related to ABA concentrations. The chemical, fluridone, has been shown to be an inhibitor of ABA-precursor synthesis (Fong *et al*., 1983). This experiment was designed to use this inhibitor to help provide additional information about the involvement of ABA in the antioxidant signaling process.

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 + 0.2 µM fluridone. Each culture tube was then connected to an aerator and allowed to preincubate for 2 hours. Following preincubation, the tubes containing 150 mM NaCl and the

Reprinted from the *Proceedings of the Beltwide Cotton Conference*
Volume 2:1396-1399 (1998)
National Cotton Council, Memphis TN

tubes containing 150 mM NaCl + 0.2 µM fluridone were amended with NaCl to a final concentration of 250 mM NaCl, 0.1 mg/L ABA, 0.1 µM paraquat, or 0.01% H_2O_2. The paraquat was used to generate superoxide. The callus tissue was harvested at 30 min., 1 hr, 2 hr, 4 hr, and 8 hr intervals and stored at -70°C 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.* (1994b). 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

AP activity is shown is Table 1. All treatments except hydrogen peroxide and hydrogen peroxide plus fluridone induced significant increases above the control activity levels. The NaCl treatment resulted in a significant increase above the control level within 1 hour, while the fluridone, ABA, ABA plus fluridone, paraquat, paraquat plus fluridone, and NaCl plus fluridone treatments exhibited significant increases within 30 minutes. The elevation in AP activity was apparent throughout the 8 hour treatment period. The increases observed with NaCl, ABA, and the superoxide generated by paraquat were similar to those noted in previous experiments (Gossett *et al.*, 1997). Pretreatment with fluridone failed to suppress the increase in AP activity in all treatments except when used with hydrogen peroxide which did not induce an increase AP activity when used alone. Since fluridone inhibits ABA synthesis, this suggests that ABA may not be involved in the induction of stress-related increases in AP activity. In fact, treatment with fluridone alone resulted in significant increases in AP activity within 30 minutes. Fluridone is not a specific inhibitor of ABA synthesis. Rather, it inhibits the synthesis of the terpene precursors of ABA. Hence, this data suggests that inhibition of terpene synthesis is sufficient to induce a stress response in AP activity.

Table 2 shows GR activity. Except for fluridone and hydrogen peroxide plus fluridone, all treatments resulted in significant increases above the control levels. Increases were observed after 30 minutes with paraquat, paraquat plus fluridone, and hydrogen peroxide, and increases occurred within 1 hour with ABA and NaCl treatments. As reported previously (Gossett *et al.*, 1997), GR activities tend to return to control levels within 8 hours in all treatments except with the paraquat generated superoxide. In contrast to AP, pretreatment with fluridone suppressed or significantly delayed the increase in GR activity in all but the paraquat treated tissues. Fluridone treatment by itself did not cause an increase in GR activity and completely suppressed the short-lived hydrogen peroxide-induced response. The ABA and NaCl-induced increases in GR activity required 4 hours when pretreated with fluridone rather than the 1 hour observed without fluridone pretreatment. These data suggest that ABA may be somehow associated with the NaCl-induced increase in GR activity.

While AP and GR are both important enzymes in the ascorbate and glutathione cycle, the data from this study suggests that the stress-induced increases in the activities of these enzymes are the results of different signaling mechanisms. AP activity appears to be rapidly induced by almost any stress metabolite completely independent of ABA, while stress-induced increases in GR activity are generally slower and partially, at least in the case of NaCl stress, associated with ABA concentrations. Increases in ABA levels appears to be a general response to a number of environmental stress conditions. However, there are cases in which genes regulated by exogenous ABA are not markedly induced by NaCl (Chandler and Robertson, 1994) and cases where genes that are induced by stress are not responsive to exogenous ABA (Yamaguchi-Shinokazi and Shinokazi, 1994). There is considerable evidence that superoxide (Doke, *et al.*, 1994) and perhaps hydrogen peroxide (Scandalios, 1994) serve as signal transduction molecules for stress-induced cellular responses. Superoxide is generated under most types of environmental stress, including NaCl stress (Hernandez *et al.*, 1994), and Singha and Choudhuri (1990) have shown that hydrogen peroxide and superoxide may play an important role in the mechanism of salt-injury in *Vigna catjang* and *Oryza sativa* leaves. Data from the present study suggest that both superoxide and ABA may serve as signal transduction molecules for the upregulation of antioxidant enzymes. The paraquat-generated superoxide resulted in rapid increases in both AP and GR activities, and these increases were not suppressed by fluridone. Hence, superoxide appears to be acting directly as the signal transduction molecule. On the other hand, fluridone significantly delayed the GR response to NaCl stress and completely suppressed the hydrogen peroxided-induced increase in GR activity. It may well be that in NaCl stress, the excess Na^+ and/or Cl^- results in electron leakage and production of hydrogen peroxide which then serves as the signal transduction molecule for the induction of stress metabolites including ABA. The elevated ABA levels could then result in the enhanced induction of the GR encoding genes. Additional research is necessary to provide further insight as to the specific relationship between NaCl stress and the antioxidant response.

Acknowledgments

We wish to thank Cotton Incorporated (91-723), The Louisiana Education Quality Support Fund (LEQSF-1994-95-ENH-TR-21), and the National Science Foundation (USE-9250130) for support of this research.

References

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

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

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.

Doke, N., Y. Miura, Y. Sanchez, 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, FL, pp 177-198.

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.

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

Gossett, D.R., S.W. Banks, M.C. Lucas, E.P. Millhollon. 1997. Induction of Antioxidant Enzyme Activity in Cotton. Proc. Beltwide Cotton Prod. Res. Conf. National Cotton Council. Memphis, TN 1374-1376.

Gossett, D.R., S.W. Banks, E.P. Millhollon, 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.

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

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, F.Sevilla. 1994. Salt stress-induced changes in superoxide dismutase isozymes in leaves and mesophyll protoplasts from *Vigna unguiculata* L. New Phytol 126:37-44.

Mandamanchi, N.R. and R.G. Alscher. 1991. Metabolic bases for differences in sensitivity of two pea cultivars to sulfur dioxide. Plant Physiol 97:88-93.

McKersie, B.D. and Y.Y. Leshem. 1994. *Stress and Stress Coping in Cultured Plants*. Kluwer Academic Press, Boston, pp 51-53.

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

Polle, A and H. Rennenberg. 1994. Photooxidative stress in trees. *In* CH Foyer, PM Mullineaux, eds, *Causes of Photooxidative Stress and Amelioration of Defense Systems in Plants* CRC Press. Boca Raton, FL, pp 199-218.

Scandalios, J.G. 1994. Regulation and properties of plant catalases. *In* CH Foyer, PM Mullineaux, eds, *Causes of Photooxidative Stress and Amelioration of Defense Systems in Plants*, CRC Press. Boca Raton, FL, pp 275-316.

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

Schnapp, S., R. Bresson, P. Hasegawa. 1990. Carbon use efficiency of NaCl adapted tobacco cells. Plant Physiol 93:384-388.

Singha, S. and M.A. Choudhuri. 1990. Effect of salinity (NaCl) stress on H_2O_2 metabolism in *Vigna* and *Oryza* seedlings. Biochemi Physiol Pflanzen 186:69-74.

Spychalla, J.P. and S.L.Desborough. 1990. Superoxide dismutase, catalase, and alpha-tocopherol content of stored potato tubers. Plant Physiol 94:1214-1218.

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

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. Proc Natl 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.

Yamaguchi-Shinokazi, K. And K. Shinokazi. 1994. A novel *cis*-acting element in an Arabidopsis gene is involved in responsiveness to drought, low-temperature, or high-salt stress. Plant Cell 6:251-264.

Table 1. Ascorbate Peroxidase activity (units/g fresh weight ±SE) in control callus tissue (C) and callus tissue treated for 0, 0.5, 1, 2, 4, and 8 hrs with 0.2 µM fluridone (F), 0.1 mg/L ABA (A), 0.1 mg/L ABA + 0.2 µM fluridone (A+F), 0.1 µM paraquat (P), 0.1 µM paraquat + 0.2 µM fluridone (P+F), 0.01% hydrogen peroxide (H), 0.01% hydrogen peroxide + 0.2 µM fluridone (H+F), 250 mM NaCl (N), or 250 mM NaCl + 0.2 µM fluridone (N+F)

	Time					
Treatment	0	0.5 h	1 hr	2 hr	4 hr	8 hr
C	217±8	323±31	187±29	272±41	300±33	129±20
F	217±8	1013±52	498±13	345±32	317±34	289±33
A	217±8	856±46	405±26	601±5	1265±89	747±9
A + F	217±8	610±34	661±14	1061±78	1070±31	1192±37
P	217±8	813±12	482±13	757±27	728±24	776±59
P + F	217±8	1049±8	931±43	1020±46	1027±29	841±69
H	217±8	136±28	308±28	282±15	166±26	180±32
H + F	217±8	476±44	164±15	159±18	125±38	171±36
N	217±8	404±47	667±17	663±43	290±39	305±23
N + F	217±8	605±46	755±30	1089±40	815±21	540±13

Table 2. Glutathione Reductase activity (units/g fresh weight ±SE) in control callus tissue (C) and callus tissue treated for 0, 0.5, 1, 2, 4, and 8 hrs with 0.2 µM fluridone (F), 0.1 mg/L ABA (A), 0.1 mg/L ABA + 0.2 µM fluridone (A+F), 0.1 µM paraquat (P), 0.1 µM paraquat + 0.2 µM fluridone (P+F), 0.01% hydrogen peroxide (H), 0.01% hydrogen peroxide + 0.2 µM fluridone (H+F), 250 mM NaCl (N), or 250 mM NaCl + 0.2 µM fluridone (N+F)

	Time					
Treatment	0	0.5 h	1 hr	2 hr	4 hr	8 hr
C	31±2	41±4	177±5	106±5	59±6	69±4
F	31±2	41±5	130±1	72±8	72±9	47±4
A	31±2	28±4	287±6	215±9	151±8	68±3
A+ F	31±2	32±4	120±8	136±3	131±8	87±7
P	31±2	74±7	332±12	218±15	245±12	263±3
P + F	31±2	82±1	291±22	257±4	229±24	67±7
H	31±2	77±6	372±13	139±1	48±4	59±10
H + F	31±2	28±3	132±6	92±1	63±5	50±3
N	31±2	58±6	441±24	275±8	362±17	103±1
N + F	31±2	25±3	194±12	140±21	189±1	165±10