INDUCTION OF ANTIOXIDANT ENZYME ACTIVITY IN COTTON

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Abstract

Callus tissue from a control and an NaCl-tolerant cell line was transferred to culture tubes containing media amended with 0 NaCl (NaCl-sensitive control) or 150 mM NaCl (NaCl-tolerant control) and media amended with 250 mM NaCl, 0.1 mg/L ABA, 0.01% H_2O_2, or 2.5 µM xanthine and 0.05 units of xanthine oxidase. The callus tissue was harvested at 30 min, 1 hr, 2 hr, 4 hr, and 8 hr intervals and analyzed for antioxidant enzyme activity. The NaCl-acclimated callus (NaCl-tolerant cell line) initiated an upregulation of antioxidant activity much earlier than the control callus. While NaCl, ABA, H_2O_2, and $O_2^{-\cdot}$ all initiated upregulation of superoxide dismutase (SOD), ascorbate peroxidase (AP), glutathione reductase (GR), and peroxidase in the NaCl-tolerant cell line, the increases resulting from ABA and $O_2^{-\cdot}$ either closely matched or preceded the increases observed with the NaCl treatment. Increase in enzyme activity resulting from H_2O_2 treatment were generally much later than those observed with the other treatments. These data suggest a possible mechanism whereby in NaCl stress, the excess Na^+ and/or Cl^- results in electron leakage and production of $O_2^{-\cdot}$ which serves as the signal molecule for the induction of stress metabolites including ABA. The elevated ABA levels may then result in the enhanced induction of antioxidant enzyme encoding genes.

Introduction

When plants are exposed to environmental stress, an imbalance between the production of reactive O_2 metabolites and the quenching activity of antioxidants often results in oxidative damage (Asada, 1994; Krause, 1994). 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 with salt tolerance in cotton (Gossett, *et al*., 1994a, 1994b), the relationship between antioxidant enzymes and NaCl tolerance was examined in leaves and callus tissue from putative NaCl-tolerant and NaCl-sensitive cultivars. Leaves from the NaCl-tolerant cultivars contained significantly greater constitutive levels of catalase and NaCl-induced levels of peroxidase and glutathione reductase (GR). In response to salt stress, callus from the salt-tolerant cultivar showed significant increases above control values in SOD, catalase, AP, and GR activities. An additional study involving a comparison of antioxidant activities in a control and a salt-tolerant cell line from the same cultivar (Coker 312) showed that antioxidant enzyme activity was 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 remains a major question. Hydrogen peroxide, superoxide, abscisic acid (ABA), or NaCl may be responsible for signal induction, and this experiment was designed to help provide information as to which of these compounds may be involved in the 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 culture tubes containing media amended with 150 mM NaCl (NaCl-tolerant control) and media amended with 250 mM NaCl, 0.1 mg/L ABA, 0.01% H_2O_2, or 2.5 µM xanthine and 0.05 units of xanthine oxidase. The xanthine/xanthine oxidase system was used to generate superoxide. Approximately 4 g of control callus tissue was also transferred to culture tubes containing media without NaCl (NaCl-sensitive control) and media amended with 150 mM NaCl, 0.1 mg/L ABA, 0.01% H_2O_2, or 2.5 µM xanthine and 0.05 units of xanthine oxidase. Each culture tube was then connected to an aerator. 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*. (1994b). Catalase activity was determined by monitoring the disappearance of H_2O_2 according to the method of Beers and Sizer (1952). Total SOD activity was measured by determining the amount of enzyme required to produce 50% inhibition of the reduction of cytochrome C

Reprinted from the *Proceedings of the Beltwide Cotton Conference*
Volume 2:1374-1377 (1997)
National Cotton Council, Memphis TN

by superoxide generated by xanthine oxidase as outlined by Forman and Fridovich (1973). Glutathione reductase (GR) activity was determined by monitoring the glutathione-dependent oxidation of NADPH as described by Schaedle and Bassham (1977). Peroxidase 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 (1969). Ascorbate peroxidase (AP) activity was assayed by monitoring the ascorbic acid-dependent reduction of H_2O_2 as described by Anderson *et al.* (1992). For catalase and peroxidase, one unit of enzyme was defined as the amount necessary to decompose 1 µmole of substrate/min at 25ºC. One unit of GR or AP was defined as the amount of enzyme required to reduce 1 nmole of substrate/min at 25ºC. 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.

Results and Discussion

Antioxidant enzyme activities for the NaCl-tolerant and control callus tissue are presented in Tables 1-5. In general, the NaCl-acclimated callus (NaCl-tolerant cell line) initiated an upregulation of antioxidant activity much earlier than the control callus. SOD activity (Table 1) does not change significantly in the control callus under any of the treatments, but the ABA, H_2O_2, and $O_2^{\cdot-}$ treatments resulted in significant increases in SOD activity in the NaCl-tolerant cell line. ABA and $O_2^{\cdot-}$ initiated the increase in 2 hrs, while the increase by H_2O_2 was not observed until after 8 hrs. In both the ABA and $O_2^{\cdot-}$ treated callus, the SOD activity returned to control levels after 8 hrs. Catalase activity (Table 2) did not change significantly in either cell line under any of the treatments, but there was a tendency for catalase activity to decrease in the control cell line. Peroxidase activity (Table 3) also remained somewhat constant under all treatment conditions in the control callus, but NaCl, ABA, and $O_2^{\cdot-}$ induced increases in peroxidase activity in the NaCl-tolerant cell line. The NaCl-induced increase was observable after 2 hrs and peaked after 4 hrs, while the $O_2^{\cdot-}$-induced increase peaked after 1 hr. The ABA induced increase was observible after 1 hr and continued to increase through 8 hrs. Both NaCl and ABA produced noticeable increases in GR activity (Table 4) after 2 hrs in the NaCl-tolerant cell line and after 4 hrs in the control callus. Superoxide initiated an increase in GR activity in 4 hrs in the control callus and in 8 hrs in the NaCl-tolerant cell line. Increases in GR activity was observed after 8 hrs in both cell lines treated with H_2O_2. Only NaCl produced an increase in AP activity (Table 5) in the control callus, and this increase was not observed until the 8 hr measurement. The NaCl, ABA, and $O_2^{\cdot-}$ treatments all resulted in increases in AP activity within 2 hrs in the NaCl-tolerant cell line.

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 the present study, ABA treatment resulted in increases in SOD, peroxidase, GR, and AP activities in the NaCl-tolerant cell line. 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 increase in ABA is directly or indirectly related to NaCl stress. The Na^+ and/or Cl^- could possibly be directly responsible for elevating ABA concentrations which, in turn, could be responsible for the enhanced induction of antioxidant genes. However, increases in ABA levels appears to be a general response to a number of environmental stress conditions. 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 $O_2^{\cdot-}$ (Doke, *et al*., 1994) and perhaps H_2O_2 (Scandalios, 1994) serve as signal induction 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 H_2O_2 and $O_2^{\cdot-}$ may play an important role in the mechanism of salt-injury in *Vigna catjang* and *Oryza sativa* leaves. In the present study, H_2O_2 did increase the activities of SOD, GR, and AP, but in most cases, the increases came much later than those observed for NaCl, ABA, or $O_2^{\cdot-}$. On the other hand, $O_2^{\cdot-}$ increased the activities of SOD, peroxidase, GR, and AP at about the same time as the increases observed for the NaCl or ABA treatments. It may well be that in NaCl stress, the excess Na^+ and/or Cl^- results in electron leakage and production of $O_2^{\cdot-}$ which serves as the signal molecule for the induction of stress metabolites including ABA. The elevated ABA levels could then result in the enhanced induction of antioxidant enzyme 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.

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Table 1. Superoxide dismutase (SOD) activity (units/g fresh weight) in a salt-tolerant (ST) cell line and a control (C) cell line treated for 0, 0.5, 1, 2, 4, and 8 hrs with NaCl (150 mM for the control cell line and 250 mM for the salt-tolerant cell line), 0.1 mg/L ABA, 0.01% H_2O_2 , or 2.5 µM xanthine and0.05 units of xanthine oxidase

Time	NaCl		ABA		H_2O_2		$O_2^{-\cdot}$	
	ST	C	ST	C	ST	C	ST	C
0 hr	14.6	9.8	14.6	9.8	14.6	9.8	14.6	9.8
0.5 hr	10.2	7.6	18.9	7.3	13.6	8.7	19.6	5.8
1 hr	10.1	6.3	23.4	2.2	18.5	10.1	38.2	12.9
2 hr	10.4	14.5	33.3	4.5	15.1	13.2	42.7	11.4
4 hr	14.0	12.0	42.6	3.7	12.3	4.1	13.9	3.7
8 hr	12.8	5.9	12.8	8.7	39.0	3.8	10.1	3.5

Table 2. Catalase activity (units/g fresh weight) in a salt-tolerant (ST) cell line and a control (C) cell line treated for 0, 0.5, 1, 2, 4, and 8 hrs with NaCl (150 mM for the control cell line and 250 mM for the salt-tolerant cell line), 0.1 mg/L ABA, 0.01% H_2O_2 , or 2.5 µM xanthine and 0.05 units of xanthine oxidase

Time	NaCl		ABA		H_2O_2		$O_2^{-\cdot}$	
	ST	C	ST	C	ST	C	ST	C
0 hr	12.9	15.2	12.9	15.2	12.9	15.2	12.9	15.2
0.5 hr	9.1	7.8	11.6	11.0	14.9	8.0	13.3	9.0
1 hr	10.1	8.2	8.6	6.7	11.3	10.4	14.1	6.7
2 hr	10.7	6.6	12.8	3.1	9.4	14.2	8.4	5.3
4 hr	12.3	7.0	9.0	3.5	10.3	3.5	3.0	6.2
8 hr	5.3	8.7	7.5	5.2	14.7	5.8	3.6	2.6

Table 3. Peroxidase activity (units/g fresh weight) in a salt-tolerant (ST) cell line and a control (C) cell line treated for 0, 0.5, 1, 2, 4, and 8 hrs with NaCl (150 mM for the control cell line and 250 mM for the salt-tolerant cell line), 0.1 mg/L ABA, 0.01% H_2O_2 , or 2.5 µM xanthine and 0.05 units of xanthine oxidase

Time	NaCl		ABA		H_2O_2		$O_2^{-\cdot}$	
	ST	C	ST	C	ST	C	ST	C
0 hr	241	316	241	316	241	318	241	316
0.5 hr	261	442	312	278	283	144	278	297
1 hr	264	350	325	159	234	192	571	103
2 hr	317	228	423	174	191	270	537	182
4 hr	530	211	491	193	84	211	230	128
8 hr	548	247	889	199	89	93	200	89

Table 4. Glutathione reductase activity (units/g fresh weight) in a salt-tolerant (ST) cell line and a control (C) cell line treated for 0, 0.5, 1, 2, 4, and 8 hrs with NaCl (150 mM for the control cell line and 250 mM for the salt-tolerant cell line), 0.1 mg/L ABA, 0.01% H_2O_2 , or 2.5 µM xanthine and 0.05 units of xanthine oxidase

Time	NaCl		ABA		H_2O_2		$O_2^{-\cdot}$	
	ST	C	ST	C	ST	C	ST	C
0 hr	41	72	41	72	41	72	41	72
0.5 hr	61	50	55	57	29	52	51	58
1 hr	57	84	43	87	32	58	46	51
2 hr	85	78	124	96	88	43	51	46
4 hr	151	158	217	136	64	63	49	226
8 hr	101	172	271	131	132	130	293	232

Table 5. Ascorbate Peroxidase (AP) activity (units/g fresh weight) in a salt-tolerant (ST) cell line and a control (C) cell line treated for 0, 0.5, 1, 2, 4, and 8 hrs with NaCl (150 mM for the control cell line and 250 mM for the salt-tolerant cell line), 0.1 mg/L ABA, 0.01% H_2O_2, or 2.5 µM xanthine and 0.05 units of xanthine oxidase

Time	NaCl		ABA		H_2O_2		$O_2^{-\cdot}$	
	ST	C	ST	C	ST	C	ST	C
0 hr	552	310	552	310	552	310	552	310
0.5 hr	611	220	714	284	173	124	755	87
1 hr	523	269	455	251	387	94	631	273
2 hr	910	354	1393	399	273	160	1032	281
4 hr	640	310	1086	301	212	138	607	384
8 hr	475	746	580	312	1093	69	529	264