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SALT TOLERANCE IN COTTON CALLUS TISSUE TRANSFORMED WITH ASCORBATE PEROXIDASE, GLUTATHIONE REDUCTASE, AND SUPEROXIDE DISMUTASE

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Abstract

Exposure to high levels of NaCl has been shown to induce an oxidative stress in cotton (*Gossypium hirsutum*) callus tissue. In addition, it has been demonstrated that cotton cell lines which are capable of up-regulating their antioxidant enzyme activities are more tolerant to NaCl stress than cell lines which do not increase the activities of these enzymes. This experiment was designed to determine if cotton callus tissue which had been genetically transformed with cDNA which encodes for antioxidant enzymes was more tolerant to NaCl stress than a NaCl-sensitive cell line and a NaCl-tolerant cell line selected to grow on elevated NaCl levels. Callus from a NaCl-sensitive cell line, a NaCl-tolerant cell line manually selected to grow at higher NaCl concentrations, a transgenic cell line transformed with glutathione reductase (GR+), a transgenic cell line transformed with ascorbate peroxidase (APX+), and a transgenic cell line transformed with superoxide dismutase (SOD+) was grown on media amended with 0, 150 mM NaCl, or 250 mM NaCl. After 28 days, the callus was harvested, weighed, and analyzed for catalase, peroxidase, GR, APX, and SOD activities. The growth studies showed that the transgenic cell lines grew better at both the 0 and the 150 mM NaCl levels than did either the NaCl-sensitive or the NaCl-tolerant cell lines. At the 250 mM NaCl level, the transgenic cell lines were more tolerant than the NaCl-sensitive cell line which died before the end of 28 days; however, the NaCl-tolerant cell line exhibited significantly more NaCl tolerance than any of the transgenics. An analysis of the antioxidant enzymes revealed that while each of the transgenics over expressed its respective antioxidant enzyme, the activities of the other enzymes remained relatively low, and in some cases the activity was actually lower than the activity expressed by the NaCl-sensitive cell line. On the other hand, the NaCl-tolerant cell line expressed significantly higher activities of all five antioxidant enzymes when grown on high NaCl than did the NaCl-sensitive cell line. These data suggest that in order to achieve a significant level of NaCl tolerance, the activities of several of the antioxidant enzyme must be up-regulated simultaneously.

Introduction

All aerobic organisms, and photosynthetic organisms in particular, continually exposed to oxidative stress. This stress often leads to release of free radicals such as reactive oxygen species (ROS) due to the production of superoxide and subsequent enzymatic products associated with the metabolism of oxygen. In all aerobic organisms, electron leakage from the mitochondria (and chloroplast in the case of plants) is a regular occurrence during normal metabolic activity. These electrons can react with molecular oxygen and result in the production of superoxide. When a stressor such as NaCl or a herbicide causes electrons to be leaked at a rate greater than the plants antioxidant machinery can handle, oxidative stress occurs. Oxidative stress results from a wide variety of biotic and abiotic factors. Abiotic stresses include NaCl, temperature extremes, drought, physical damage (farm equipment), heavy metals, radiation, pesticides and flooding, all of which can lead to decreased crop yield. Since free radicals can be produced in almost every known biological system, there must be a way to combat their overproduction during stress. The antioxidant enzyme defense system and free radical scavengers serve as the cellular defense system against the deleterious actions of free radicals (Sies, 1991). Plants with increased levels of antioxidant enzymes have been reported to have greater resistance to oxidative damage (Dhindsa and Matowe, 1981; Harper and Harvey, 1978; Monk and Davies, 1989; Spychalla and Desborough, 1990; Wise and Naylor, 1987). Thus, plants with increased antioxidant enzyme activity are better equipped to handle environmental stress. Antioxidant defense systems are composed of both enzymatic and non-enzymatic components. Molecules such as isoflavonoids, flavones, isoflavonones, α-tocopherol (vitamin E), ascorbic acid (vitamin C), RSH antioxidants and glutathione comprise the nonenzymatic antioxidants. Enzymatic sources of antioxidants include superoxide dismutase (SOD), catalase (CAT), general peroxidases (PER), ascorbate peroxidase (APX), and glutathione reductase (GR), as well as several other antioxidant enzymes. SOD is an enzyme which breaks down $O_2^{\bullet-}$ by combining it with H^+ to form H_2O_2. Catalase catalyzes the breakdown of hydrogen peroxide into water and molecular oxygen and is not found in the chloroplast (Chen and Asada, 1989). General peroxidases also catalyze the breakdown of hydrogen peroxide to H_2O and require a reducing agent (Chang *et al*., 1984). When salt or drought stress or a combination of the two occur, the activities of superoxide dismutase (SOD), ascorbate peroxidase (APX), glutathione reductase (GR), and monode-

hydroascorbate reductase (MDHAR) have been shown to increase in plants (Schoner and Krause, 1990; Smirnoff, 1995; Gossett *et al*., 1994, 1996; Grace and Logan, 1996; Logan *et al*., 1998a,1998b).

NaCl stress has recently gained interest in the study of environmental stress on non-halophytic plants. Data compiled in our laboratory (Gossett *et al*., 1994a, 1994b, 1996, 2001; Manchandai *et al*., 1999; Bellaire *et al*., 2000; Banks *et al*., 2001) has indicated that salt stress elicits an oxidative response in cotton, and cotton cultivars with elevated levels of antioxidant enzymes, either constitutive or induced, have been shown to exhibit greater tolerance to NaCl stress. These data suggest that plants genetically transformed to over express antioxidant enzyme activity could possibly exhibit a greater degree of NaCl tolerance. While working in Randy Allen's laboratory, Paxton Payton used the *Agrobacterium* mediated transfer method to successfully transform cotton with GR, APX, and SOD. Drs. Payton and Allen kindly supplied our laboratory with seeds from each of these transgenic lines. The objective of this experiment was to determine if callus tissue generated form these transgenic lines exhibited NaCl tolerance as compared to a NaCl-sensitive cell line and a NaCl-tolerant cell line which had been selected to grow at high NaCl concentrations.

Materials and Methods

Callus Tissue

Callus tissue was generated from sterile hypocotyls of Coker 312 by the method described by Trolinder and Goodin (1987). The callus tissue was maintained on T1 media containing MS salts (Murashige and Skoog, 1962) supplemented with Gamborg's vitamins (Gamborg, 1978), 0.75 mg/L $MgCl_2$, 0.1 mg/L 2,4-D, 0.1 mg/L kinitin, 2 g/L phytogel, and 30 g/L glucose adjusted to a pH of 5.8 (Trolinder and Goodin, 1987). Callus tissue generated in this manner served as the NaCl-sensitive controls. A NaCl-tolerant cell line, selected as described by Gossett *et al.* (1996) and maintained on T1 amended with 150 mM NaCl, served as the NaCl-tolerant control. Callus tissue transformed with glutathione reductase (GR+), ascorbate peroxidase (APX+), or superoxide disumutase (SOD+) was generated from transgenic seed produced by Paxton Payton in Randy Allen's laboratory was generated and maintained in the same manner described for the NaCl-sensitive callus with the exception of the addition of kanamycin to the Stuart's media used to germinate the seed. Transgenics were selected for callus culture based on their positive kanamycin resistance as indicated by the growth of roots into the germination media.

At the beginning of each experiment, approximately 0.5 g of each of the cell line (i.e NaCl-sensitive, NaCl-tolerant, GR+, APX, or SOD+ was transferred to media amended with either 0, 150, or 250 mM NaCl. After 28 days, the callus was removed from the media, weighed, and frozen at -70^0 C for subsequent enzyme analyses. NaCl tolerance was assessed on the basis of the fresh weight measurements.

Antioxidant Enzyme Assays

Samples were prepared for SOD, catalase, peroxidase, GR and APX analyses according to the method of Anderson *et al.* (1992) as modified by Gossett *et al.* (1996). Samples were prepared by homogenizing 1 g of frozen leaf or 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^{0}C at 10,000 X 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.* (1992). A portion of the eluate was analyzed immediately for catalase activity, and the remainder was stored at -70^{o}C for subsequent analysis of SOD, GR, APX, and peroxidase activities. Catalase activity was determined by monitoring the disappearance of H_2O_2 at 240 nm according to the method of Beers and Sizer (1952). 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 (1969). 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*. (1992). 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 (1973). GR activity was determined by monitoring the glutathione-dependent oxidation of NADPH at 340 nm after the method of Schaedle and Bassham (1997). One unit of catalase was defined as the amount of enzyme to decompose 1 µmole of substrate/min at 25^0 C, and one unit of peroxidase, APX and GR was defined as the amount of enzyme required to reduce 1 nmole of substrate/min at 25^{0}C. One unit of SOD was defined as the amount of enzyme necessary to inhibit the reduction of Cyt c by 50%. All data points are based on a mean of the measurements 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 and Discussion

The results of the growth studies are presented in Table 1. All of the cell lines grew well on media with no NaCl, and in fact, the transgenic cell lines had significantly higher fresh weights than either the NaCl-sensitive cell line or the NaCl-tolerant cell line. When grown on media amended with 150 mM NaCl, the NaCl-sensitive cell line grew very poorly and appeared to be almost dead. The NaCl-tolerant and transgenic cell lines performed very well on the 150 mM NaCl media, and the SOD+ cell

line actually produced a significantly higher fresh weight than the other three cell lines. The NaCl-sensitive cell line did not survive on the media amended with 250 mM NaCl, and the GR+, APX+, and SOD+ cell lines exhibited significant decreases in fresh weight (67%, 85%, and 81% respectively) when grown at this level of NaCl. On the other hand, there was no significant difference in growth when the NaCl-tolerant cell line was grown on 250 mM NaCl as compared to the growth on either the 0 or the 150 mM NaCl media.

The results of the enzyme analyses are shown in Tables 2-6. Catalase activity is presented in Table 2. In the NaCl-sensitive controls, catalase activity remained unchanged when the callus was grown at 150 mM NaCl, while in the NaCl-tolerant callus, catalase activity increased two-fold when grown at the higher NaCl levels. The transgenic callus tissue transformed with GR was the only transgenic line to exhibit catalase activity as high as the controls at the 0 NaCl level, and the activity of this enzyme decreased significantly when gown on media amended with high concentrations of NaCl. Catalase activity in both the APX+ and GR+ cell lines was significantly below either of the controls when grown on 0 NaCl, and the activity remained low when the callus was grown at 150 and 250 mM NaCl. Peroxidase activity is shown in Table 3. Callus from the transgenic APX+ cell line had a significantly higher peroxidase activity at the 0 NaCl level than the other four cell lines; however peroxidase activity decreased with each increment of additional NaCl, and at the 250 mM NaCl level, peroxidase activity was significantly lower in the APX+ cell line than in the other cell lines. Among the other four cell lines, there was no difference in peroxidase activity at the 0 NaCl level. Peroxidase activity in the GR+ and SOD+ cell lines remained at the control level when these cell lines were grown on media with elevated NaCl concentrations. Both the NaCl-sensitive and NaCl-tolerant cell lines exhibited significant increases in peroxidase activity when grown at the 150 mM NaCl level. The NaCl-tolerant callus was the only cell line to exhibit a significant increase in peroxidase activity above the control level when grown at 250 mM NaCl.

The NaCl-tolerant and transgenic cell lines all had significantly higher GR activity than the NaCl-sensitive cell line when grown in the absence of NaCl, and the GR+ cell line exhibited the highest GR activity (Table 4). GR activity remained moderately high in the NaCl-tolerant cell line when it was grown at the 150 mM NaCl level and increased almost two-fold when grown on media amended with the highest NaCl concentration. In the GR+ cell line, GR activity remained very high when grown at both of the higher NaCl levels. Peroxidase activity in the APX+ cell line remained moderately high when grown at either 150 or 250 mM NaCl, while in the SOD+ cell line, peroxidase activity remained moderately high at the 150 mM NaCl level but decreased significantly when this cell line was gown on media amended with 250 mM NaCl. APX activity is presented in Table 5. The NaCl-tolerant and APX+ cell lines exhibited significantly higher APX activity than the other three cell lines when grown on media with 0 NaCl. APX activity did not change in the NaCl-sensitive cell line when the callus was grown at 150 mM NaCl. When grown at either 150 or 250 mM NaCl, APX activity remained high in the NaCl-tolerant cell line. The activity of this enzyme also remained high in APX+ cell line when grown at 150 mM NaCl, but APX decreased significantly when this cell line was grown on media amended with 250 mM NaCl. APX activity remained relatively constant regardless of the NaCl level in callus from the GR+ and SOD+ cell lines. When grown on 0 NaCl, SOD activity (Table 6) was the highest in the callus from the SOD+ cell line, but SOD activity was also significantly higher in the NaCl-tolerant cell line than in the NaCl-sensitive or the other two transgenic cell lines. SOD activity increased with each additional increment of NaCl in the NaCl-tolerant cell line, and it remained relatively high in SOD+ callus when this cell line was grown at the higher NaCl levels. In callus tissue from the NaCl-sensitive, GR+, and APX+ cell lines, SOD remained at the control level when grown on media amended with higher NaCl concentrations. At the 250 mM NaCl level, the NaCl-tolerant callus tissue exhibited a significantly higher SOD activity than even the SOD+ transgenic cell line.

In previous studies (Gossett *et al*., 1996; Tanaka, 1994), it was hypothesized that GR might be the rate limiting enzyme in defense in against active O_2 toxicity at moderate levels of NaCl stress (150 mM). This might help explain why callus from the NaCl-tolerant and transgenic cell lines exhibited tolerance at the 150 mM NaCl treatment, while callus from the NaCl-sensitive cell line grew very poorly. At the 150 mM NaCl treatment, GR activity in the callus from the NaCl-sensitive cell line did not change from its already low control level, but all of the moderately NaCl-tolerant cell lines expressed moderately high levels of GR activity when grown on media amended with 150 mM NaCl. Hence, it would appear that elevated GR activity plus an increase in either APX or SOD is sufficient to enhance NaCl tolerance at the 150 mM NaCl level. Gossett and co-workers (Gossett *et al.*, 2002) also proposed that as levels of environmental stress increase, there is also an increase in the level of oxidative stress. At the higher level of oxidative stress, it may require the up-regulation of additional antioxidant enzyme activity in order to enhance tolerance. The callus from the NaCl-tolerant cell was the only tissue which showed any significant tolerance when grown on media amended with 250 mM NaCl, and it was the only tissue to express significantly higher activities of all five antioxidant enzymes when grown on this high NaCl treatment. Thus it would appear that cotton callus tolerance to NaCl at concentrations as high as 250 mM requires the up-regulation of the activity of several antioxidant enzymes.

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Table 1. Fresh weight (g ±SE) in NaCl-sensitive controls, NaCl-tolerant controls, callus transformed with glutathione reductase (GR+), callus transformed with ascorbate peroxidase (APX+), and callus transformed with superoxide dismutase (SOD+) grown for 28 days on media amended with 0, 150, or 250 mM NaCl . a, b, etc denotes significant differences between NaCl treatments and [1, 2, etc.] denotes significant difference between callus tissues.

	NaCl Treatment (mM)		
Callus Tissue	**0**	**150**	**250**
NaCl-sensitive Control	6.9±0.4a[1]	1.4±0.2b[1]	-
NaCl-tolerant Control	4.9±0.4a[1]	6.0±0.2a[2]	5.0±0.8a[2]
GR+	8.9±0.2a[2]	8.1±0.2a[2]	1.9±0.2b[1]
APX+	8.7±0.3a[2]	9.5±0.3a[3]	1.4±0.1b[1]
SOD+	13.7±1.1a[3]	11.8±0.7a[3]	2.2±0.1b[1]

Table 2. Catalase activity (units/g fresh weight ±SE) in NaCl-sensitive controls, NaCl-tolerant controls, callus transformed with glutathione reductase (GR+), callus transformed with ascorbate peroxidase (APX+), and callus transformed with superoxide dismutase (SOD+) grown for 28 days on media amended with 0, 150, or 250 mM NaCl. a, b, etc. denotes significant differences between NaCl treatments and [1, 2, etc] denotes significant difference between callus tissues.

	NaCl Treatment (mM)		
Callus Tissue	**0**	**150**	**250**
NaCl-sensitive Control	8.2±0.7a[2]	6.6±0.4a[2]	-
NaCl-tolerant Control	7.0±0.7a[2]	15.4±1.1b[3]	18.5±2.3b[3]
GR+	7.6±0.5b[2]	3.1±0.5a[1]	1.6±0.2a[1]
APX+	2.3±0.5a[1]	1.4±0.1a[1]	5.0±0.5b[2]
SOD+	2.6±0.3a[1]	2.2±0.3a[1]	3.6±0.1a[1]

Table 3. Peroxidase activity (units/g fresh weight ±SE) in NaCl-sensitive controls, NaCl-tolerant controls, callus transformed with glutathione reductase (GR+), callus transformed with ascorbate peroxidase (APX+), and callus transformed with superoxide dismutase (SOD+) grown for 28 days on media amended with 0, 150, or 250 mM NaCl. a, b, etc. denotes significant differences between NaCl treatments and [1, 2, etc.] denotes significant difference between callus tissues.

	NaCl Treatment (mM)		
Callus Tissue	**0**	**150**	**250**
NaCl-sensitive Control	459±8a[1]	2000±79b[2]	-
NaCl-tolerant Control	962±59a[2]	1686±89b[2]	1936±85b[3]
GR+	388±80a[1]	675±48a[1]	454±57a[2]
APX+	1041±64a[2]	506±28b[1]	226±48c[1]
SOD+	332±37a[1]	342±28a[1]	503±69a[2]

Table 4. Glutathione Reductase (GR) activity (units/g fresh weight ±SE) in NaCl-sensitive controls, NaCl-tolerant controls, callus transformed with glutathione reductase (GR+), callus transformed with ascorbate peroxidase (APX+), and callus transformed with superoxide dismutase (SOD+) grown for 28 days on media amended with 0, 150, or 250 mM NaCl. a, b, etc. denotes significant differences between NaCl treatments and [1, 2, etc.] denotes significant difference between callus tissues.

	NaCl Treatment (mM)		
Callus Tissue	**0**	**150**	**250**
NaCl-sensitive Control	35±3a[1]	36±4a[1]	-
NaCl-tolerant Control	158±12a[2]	132±12a[2]	231±10b[3]
GR+	290±15a[2]	291±22a[3]	261±21a[3]
APX+	138±17a[1]	108±8a[1]	111±11a[2]
SOD+	122±5b[1]	108±5b[1]	59±5a[1]

Table 5. Ascorbate Peroxidase (APX) activity (units/g fresh weight ±SE) in NaCl-sensitive controls, NaCl-tolerant controls, callus transformed with glutathione reductase (GR+), callus transformed with ascorbate peroxidase (APX+), and callus transformed with superoxide dismutase (SOD+) grown for 28 days on media amended with 0, 150, or 250 mM NaCl. a, b, etc. denotes significant differences between NaCl treatments and [1, 2, etc.] denotes significant difference between callus tissues.

	NaCl Treatment (mM)		
Callus Tissue	**0**	**150**	**250**
NaCl-sensitive Control	154±6a[1]	138±8a[1]	-
NaCl-tolerant Control	282±23a[2]	300±15a[2]	386±16a[3]
GR+	128±6a[1]	125±16a[1]	90±13a[1]
APX+	381±7b[3]	401±9b[3]	171±14a[2]
SOD+	149±17a[1]	179±17a[1]	153±18a[1,2]

Table 6. Superoxide Dismutase (SOD) activity (units/g fresh weight ±SE) in NaCl-sensitive controls, NaCl-tolerant controls, callus transformed with glutathione reductase (GR+), callus transformed with ascorbate peroxidase (APX+), and callus transformed with superoxide dismutase (SOD+) grown for 28 days on media amended with 0, 150, or 250 mM NaCl. a, b, etc. denotes significant differences between NaCl treatments and [1, 2, etc.] denotes significant difference between callus tissues.

	NaCl Treatment (mM)		
Callus Tissue	**0**	**150**	**250**
NaCl-sensitive Control	27.9±1.9a[1]	29.9±0.4[1]	-
NaCl-tolerant Control	74.5±4.2a[2]	156.3±4.7b[2]	241.5±6.0c[3]
GR+	33.0±4.6a[1]	32.9±3.8a[1]	35.5±5.4a[1]
APX+	35.1±4.9a[1]	37.0±5.4a[1]	31.1±4.5a[1]
SOD+	144.5±4.9a[3]	146.5±6.7a[2]	150.0±9.4a[2]