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Antioxidant Response to NaCl Stress in a Control and an NaCl-Tolerant Cotton Cell Line Grown in the Presence of Paraquat, Buthionine Sulfoximine, and Exogenous Glutathione[1]

Dalton R. Gossett*, Stephen W. Banks, Eddie P. Millhollon, and M. Cran Lucas

Department of Biological Sciences, Louisiana State University–Shreveport, One University Place, Shreveport, Louisiana 71115 (D.R.G., S.W.B, M.C.L.); and Louisiana State University Agricultural Center, Red River Research Station, Highway 71, Bossier City, Louisiana 71113 (E.P.M.)

A cotton (*Gossypium hirsutum* L.) control and NaCl-tolerant cell line (cv Coker 312) were grown on media with or without NaCl in the presence or absence of paraquat, buthionine sulfoximine, and oxidized glutathione. On medium with 150 mM NaCl the NaCl-tolerant cell line exhibited no reduction in growth, whereas a 96% reduction was observed in the control line. The NaCl-tolerant cell line that was grown on 150 mM NaCl exhibited significantly greater catalase (341%), peroxidase (319%), glutathione reductase (287%), ascorbate peroxidase (450%), γ-glutamylcysteine synthetase (224%), and glutathione *S*-transferase (500%) activities than the intolerant control. The NaCl-tolerant cell line had a significantly lower dehydroascorbic acid/ascorbic acid ratio. Paraquat reduced growth by 20 and 53.7%, respectively, in the NaCl-tolerant and control cell line. The NaCl-tolerant cell line also showed a slight tolerance to buthionine sulfoximine. In the buthionine sulfoximine experiments reduced glutathione restored growth in both cell lines, whereas oxidized glutathione restored growth only in the NaCl-tolerant cell line. These data indicate that the NaCl-tolerant cell line exhibited a cross-tolerance to a variety of stress variables and had a more active ascorbate-glutathione cycle.

When plants are subjected to environmental stress, the balance between the production of reactive O_2 species and the quenching activity of antioxidants is upset, often resulting in oxidative damage (Harper and Harvey, 1978; Dhindsa and Matowe, 1981; Rabinowitch and Fridovich, 1983; Wise and Naylor, 1987; Monk and Davies, 1989; Spychalla and Desborough, 1990; Cakmak and Marschner, 1992; Polle et al., 1992; Asada, 1994; Krause, 1994). Plants with high levels of antioxidants, either constitutive or induced, have been reported to have a greater resistance to this oxidative damage (Harper and Harvey, 1978; Dhindsa and Matowe, 1981; Wise and Naylor, 1987; Monk and Davies, 1989; Spychalla and Desborough, 1990; Mandamanchi and Alscher, 1991; Polle and Rennenberg, 1994).

The mechanism that imparts NaCl tolerance to nonhalophytic plants has eluded definition. However, resistance to oxidative stress has been implicated in several studies involving NaCl stress. Singha and Choudhuri (1990) have shown that hydrogen peroxide and $O_2^{\cdot-}$ may play an important role in the mechanism of NaCl injury in *Vigna catjang* and *Oryza sativa* leaves, and Hernandez et al. (1993, 1994) have demonstrated that NaCl treatments decrease Mn-SOD activity in mitochondria isolated from NaCl-sensitive peas but induce this isozyme's activity in NaCl-tolerant plants. In previous studies with cotton (*Gossypium hirsutum* L.; 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 GR. In response to NaCl stress callus from the NaCl-tolerant cultivar showed significant increases above control values in SOD, catalase, AP, and GR activities. In contrast, callus tissue from the NaCl-sensitive cultivar showed no difference from the nonstressed levels in the activity of these enzymes. Although these studies suggested a strong correlation between the antioxidant levels and NaCl tolerance, the data were collected from different cultivars. Therefore, it is unclear whether the observed antioxidant responses were due to differences in NaCl tolerance or to cultivar variation. In this study an NaCl-tolerant cell line was selected from a single, relatively NaCl-sensitive cv Coker 312, and the antioxidant activity of this NaCl-tolerant cell line was compared with the antioxidant activity in parental control callus. In addition, the NaCl-tolerant and control cell lines were examined for cross-tolerance to paraquat, a bipyridinium herbicide that generates massive oxidative damage within the plant. Since GSH plays a pivotal role in stress tolerance and adaption to environmental stress (Meister, 1983; Alscher,

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* Corresponding author; e-mail dgossett@pilot.lsus.edu; fax 1–318–797–5230.

Abbreviations: AP, ascorbate peroxidase; AsA, reduced ascorbic acid; BSO, buthionine sulfoximine; DAsA, dehydroascorbic acid; GGCS, γ-glutamylcysteine synthetase; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; GST, glutathione *S*-transferase; SOD, superoxide dismutase.

1989; Sen Gupta et al., 1991; May and Leaver, 1993; Creissen et al., 1994), both cell lines were also grown in the presence of BSO, a potent inhibitor of GSH synthesis (Griffith and Meister, 1979), and exogenous GSH and GSSG to help further elucidate the role of GR in the development of NaCl stress in cotton.

MATERIALS AND METHODS

Callus Preparation

Callus tissue for cotton (*Gossypium hirsutum* L.) cv Coker 312 was generated according to the method of Trolinder and Goodin (1987). The resulting callus tissue was subcultured every 4 to 6 weeks. An NaCl-tolerant cell line was selected by progressively growing a portion of the callus on media with increasing concentrations of NaCl until callus growth of the selected cell line grew on medium with 150 mM NaCl, as well as the control callus on medium without NaCl.

At the beginning of each experiment, approximately 500 mg of callus tissue from the NaCl-tolerant cell line was transferred to media with 150 mM NaCl (NaCl-tolerant control) and 150 mM NaCl plus 0.1 μM paraquat, or 50 μM BSO, or 100 mM GSH, or 100 mM GSSG, or 50 μM BSO and 100 mM GSH, or 50 μM BSO and 100 mM GSSG. Approximately 500 mg of control callus tissue was also transferred to medium without NaCl (0 mM NaCl control) and medium without NaCl plus the previously mentioned amendments. In addition, approximately 500 mg of callus tissue from the NaCl-tolerant cell line was transferred to medium without NaCl, and approximately 500 mg of control callus tissue was transferred to medium with 150 mM NaCl. After 42 d, the callus tissue was harvested, weighed, and stored at −70°C for subsequent antioxidant analyses.

Antioxidant Extraction

Samples were prepared for catalase, peroxidase, GR, and GGCS analyses by the method described by Foster and Hess (1980) as modified by Gossett et al. (1994b). AP and ascorbate were extracted according to the method of Anderson et al. (1992). Samples were prepared for GST analysis according to the method of Mozer et al. (1983). Glutathione, Cys, and cystine were extracted by the method of Reed et al. (1982).

Antioxidant Enzymes

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 Cyt *c* by superoxide generated by xanthine oxidase, as described by Forman and Fridovich (1973). 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). AP activity was assayed by monitoring the ascorbic acid-dependent reduction of H_2O_2, as described by Anderson et al. (1992). GGCS activity was measured by monitoring the oxidation of NADH, according to the method of Seelig and Meister (1985). GST activity was assayed according to the method of Habig et al. (1974). For catalase, peroxidase, GGCS, and AP, 1 unit of enzyme was defined as the amount necessary to decompose 1 μmol substrate/min at 25°C; 1 unit of GR and GST was defined as the amount of enzyme required to reduce 1 nmol substrate/min at 25°C; and 1 unit of SOD was defined as the amount of enzyme necessary to inhibit the reduction of Cyt *c* by 50%. Data are reported as means from a minimum of four replicates. All data were subjected to an unpaired Student's *t* test, and significance was determined at the 95% confidence limits.

Antioxidant Scavengers

Ascorbate was determined by HPLC using a 3.9- × 150-mm Nova-Pak C_{18} octadecyl reverse-phase column (Waters no. 86344) protected by a Nova-Pak C_{18} precolumn insert (Waters no. 15220), according to the method of Lee et al. (1984) as modified by Anderson et al. (1992). For each sample, an aliquot of the extract was incubated overnight with 30 mM DTT to reduce DAsA to AsA. DAsA values were determined by subtracting AsA values (samples minus DTT) from the total ascorbate values (samples plus DTT). GSH, GSSG, Cys, and cystine concentrations were determined by HPLC using a 4.1- × 250-mm Versa-Pack amine column (no. 28142, Alltech Associates, Deerfield, IL) protected by a Bond-Pak amine precolumn insert (Waters no. 26260), according to the method of Reed et al. (1982). Total glutathione values were determined from the sum of the GSH and GSSG values. For all HPLC analyses, peaks were calculated using Waters' Millennium software.

RESULTS

When grown at either 0 or 150 mM NaCl, the cell line selected to grow on a 150 mM NaCl medium (NaCl-tolerant) exhibited no significant difference in growth when compared with the control cell line grown at 0 mM NaCl. However, growth of the control line was reduced by 94% when subcultured directly onto the 150 mM NaCl

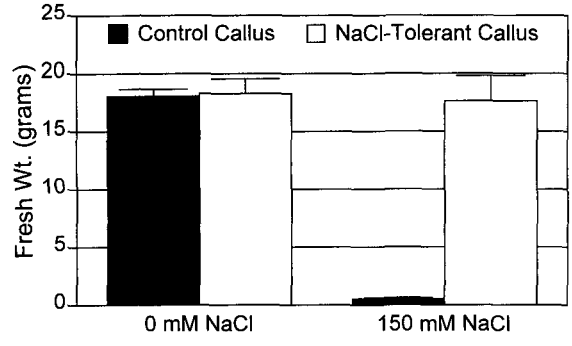

Figure 1. Fresh weight (Wt.; g, mean ± SE) for control callus and NaCl-tolerant callus tissue grown on media containing 0 and 150 mM NaCl.

medium (Fig. 1). Growth of both cell lines was inhibited significantly on medium with 0.1 μM paraquat (Fig. 2). However, the 53.8% reduction in the control cell line was significantly greater than the 20.5% reduction in the NaCl-tolerant cell line. No significant difference in growth was observed when either the control or the NaCl-tolerant cell line was grown on medium with 100 mM GSH or 50 μM BSO plus 100 mM GSH (Fig. 2). On medium with 100 mM GSSG, growth of the control cell line was significantly reduced compared with its control, whereas growth of the NaCl-tolerant cell line did not change significantly. Medium amended with 50 μM BSO resulted in significant growth reductions in both cell lines. However, the reduction in the control cell line was significantly greater than the reduction observed in the NaCl-tolerant cell line. On medium with 50 μM BSO plus 100 mM GSSG, both cell lines showed significant growth reductions compared with their respective controls, but the reduction in the control cell line was significantly greater than the reduction exhibited by the NaCl-tolerant cell line.

Antioxidant enzyme activities for the control and the NaCl-tolerant cell lines grown at 0 and 150 mM NaCl are presented in Figure 3. The nonsignificant difference in SOD activity indicates that both cell lines exhibited similar dismutating capacities; however, there were significant differences in the enzymes that decompose H_2O_2. In the NaCl-tolerant cell line grown on 150 mM NaCl, catalase and peroxidase activities were significantly greater than in the control callus grown at 0 mM NaCl. Activities of the ascorbate-glutathione cycle enzymes AP and GR were also greater in the NaCl-tolerant cell line. GR and AP activities were 3- to 4-fold greater in the NaCl-tolerant cell line grown on 150 mM NaCl than in the control cell line grown on 0 mM NaCl. In the callus tissue only the peripheral cells may have responded to the treatment, which might not have been fully administered to the central portion of the callus; therefore, the signal may have been diluted and the responses to the various treatments may have been underestimated.

The effect of paraquat, BSO, and exogenous reduced and oxidized forms of glutathione on GR activity is shown in Figure 4. Although GR activity increased significantly in

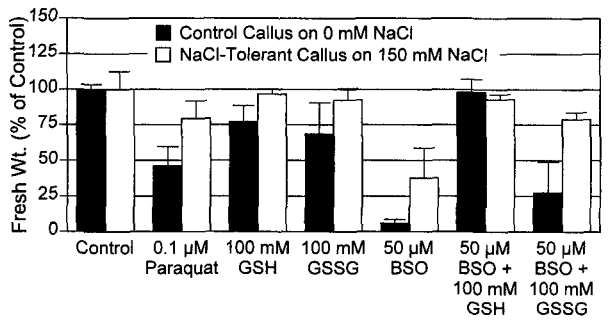

Figure 2. Fresh weight (Fresh Wt.; mean percentage of control ± SE) for control callus grown on medium without NaCl and NaCl-tolerant callus grown on media containing 150 mM NaCl with 0.1 μM paraquat, 100 mM GSH, 100 mM GSSG, 50 μM BSO, 50 μM BSO plus 100 mM GSH, or 50 μM BSO plus 100 mM GSSG.

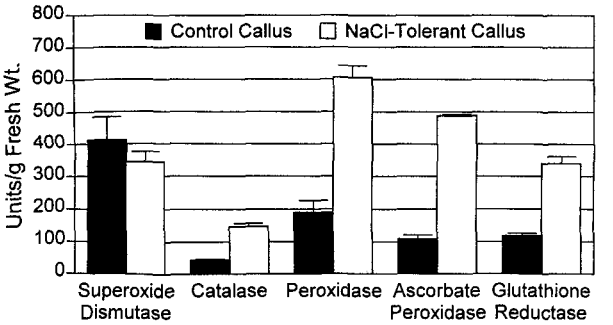

Figure 3. Antioxidant enzyme activity (mean ± SE) of control callus grown on 0 mM NaCl and NaCl-tolerant callus grown on 150 mM NaCl. Fresh Wt., Fresh weight.

both cell lines in response to medium with 0.1 μM paraquat, the increase was much greater in the NaCl-tolerant cell line and resulted in this cell line exhibiting activity almost 3 times that of the control cell line. The addition of BSO resulted in a significant increase in GR activity in the NaCl-tolerant callus, but it inhibited growth in the control callus such that there was insufficient tissue for GR analysis. Compared with their respective controls, GR activity increased significantly in both the control and the NaCl-tolerant callus when grown on medium containing 100 mM GSH, 100 mM GSSG, and 50 μM BSO plus 100 mM GSH. In each case the GR activity of the NaCl-tolerant callus was significantly greater than the control callus. On medium containing 50 μM BSO and 100 mM GSSG, GR activity of the control callus decreased significantly when compared with callus grown on the control medium, whereas GR activity of the NaCl-tolerant callus increased significantly compared with its control.

Total ascorbate (AsA plus DAsA) and AsA concentrations were significantly greater in the NaCl-tolerant cell line when grown at the 150 mM NaCl level than in the control cell line grown on the 0 mM NaCl medium (Fig. 5). Because of the increase in the AsA concentration, the DAsA/AsA ratio was significantly lower in the NaCl-

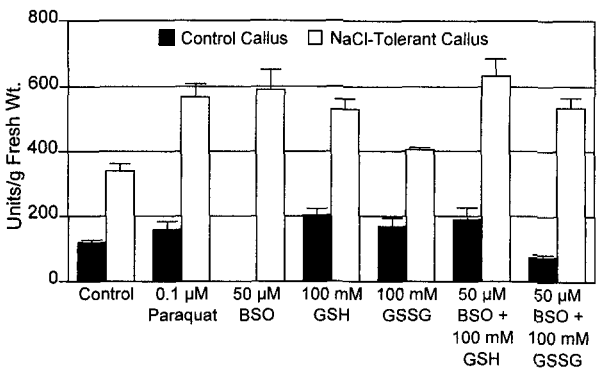

Figure 4. GR activity (mean ± SE) for control callus grown on medium with 0 mM NaCl and NaCl-tolerant callus grown on medium containing 150 mM NaCl (control) or the same medium with 0.1 μM paraquat, 100 mM GSH, 100 mM GSSG, 50 μM BSO, 50 μM BSO plus 100 mM GSH, or 50 μM BSO plus 100 mM GSSG. Fresh Wt., Fresh weight.

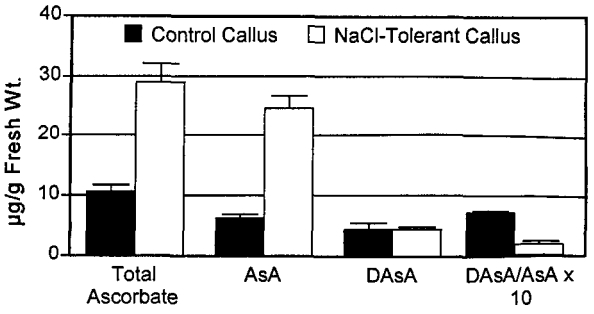

Figure 5. Total ascorbate, AsA, DAsA, and DAsA/AsA × 10 (mean ± SE) for control callus grown on medium containing 0 mM NaCl and NaCl-tolerant callus grown on medium containing 150 mM NaCl. Fresh Wt., Fresh weight.

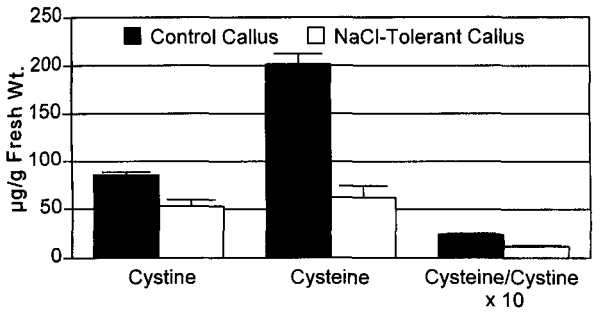

Figure 7. Cystine, Cys, and Cys/cystine × 10 (mean ± SE) for control callus grown on medium containing 0 mM NaCl and NaCl-tolerant callus grown on medium containing 150 mM NaCl. Fresh Wt., Fresh weight.

tolerant callus tissue grown at 150 mM NaCl, indicating that a much greater percentage of the total ascorbate was in the reduced form (84% AsA as compared with 58% AsA in the control callus grown on 0 mM NaCl).

Total glutathione (GSH plus GSSG), GSH, and GSSG were all significantly lower in the NaCl-tolerant cell line grown on 150 mM NaCl than in the control cell line grown at 0 mM NaCl (Fig. 6). The significantly greater ratio of GSH to GSSG in the NaCl-tolerant callus grown on 150 mM NaCl medium indicates that, compared with the control callus grown on 0 mM NaCl, more of the glutathione was in the reduced form. Cys and cystine levels were significantly less in the NaCl-tolerant callus grown on 150 mM NaCl when compared with the control callus grown on 0 mM NaCl (Fig. 7). In the control callus tissue the Cys:cystine ratio was approximately 2:1, while the ratio was approximately 1:1 in the NaCl-tolerant cell line.

The GGCS and GST activities for the control and NaCl-tolerant cell lines are shown in Figure 8. When compared with the control callus grown on 0 mM NaCl, GGCS and GST activities were 2- to 5-fold greater in the NaCl-tolerant cell line grown on 150 mM NaCl.

When the NaCl-tolerant cell line was grown on medium without NaCl, GR activity did not differ significantly from the activity observed in the 150 mM NaCl control (Fig. 9). Both catalase and AP activities decreased significantly from the values observed in the 150 mM control but remained significantly higher than the activities in the control callus. When grown on control medium, peroxidase activity in the NaCl-tolerant cell line decreased to the level observed in the control callus. SOD activity did not differ significantly in the 0 and 150 mM NaCl controls, and it did not change when the NaCl-tolerant cell line was grown on medium without NaCl.

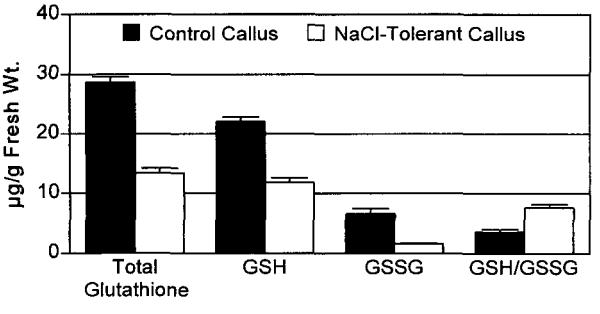

Figure 6. Total glutathione, GSH, GSSG, and GSH/GSSG (mean ± SE) for control callus grown on medium containing 0 mM NaCl and NaCl-tolerant callus grown on medium containing 150 mM NaCl. Fresh Wt., Fresh weight.

DISCUSSION

Since no reduction in growth occurred in the NaCl-tolerant cell line when grown on 150 mM NaCl, and growth of the control cell line was reduced by 94%, we concluded that the NaCl-tolerant cell line had achieved tolerance to NaCl stress. This NaCl tolerance was accompanied by a significant increase in antioxidant activity. Although both cell lines had similar dismutating capacities, catalase and peroxidase activities were significantly greater in the NaCl-tolerant cell line. The relationship between NaCl stress and increases in catalase and/or peroxidase activity was also observed in NaCl-tolerant as compared with NaCl-sensitive cultivars of cotton (Gossett et al., 1994a, 1994b).

The NaCl-tolerant cell line had a much greater percentage of the total ascorbate present in the reduced form; comparable DAsA/AsA ratios were also observed in NaCl-tolerant cotton cultivars (Gossett et al., 1994a). An increase in the rate of ascorbate oxidation can result from elevated AP activity (Nakano and Asada, 1981), and AP activity was 4 times higher in the NaCl-tolerant cell line than in the control cell line. Ascorbate can also be oxidized by $O_2^{\cdot-}$ or the α-chromoxyl radical of oxidized α-tocopherol.

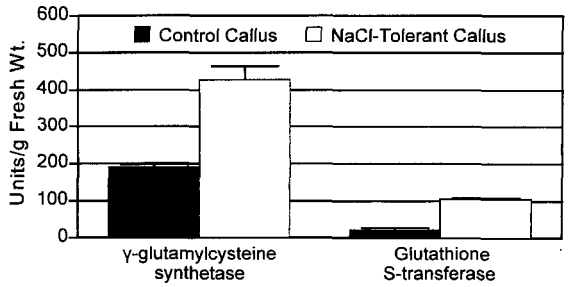

Figure 8. GGCS and GST activities (mean ± SE) for control callus grown on 0 mM NaCl and NaCl-tolerant callus grown on 150 mM NaCl. Fresh Wt., Fresh weight.

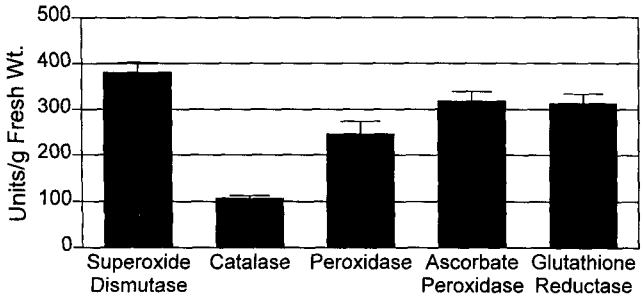

Figure 9. Antioxidant enzyme activity (mean ± SE) of the NaCl-tolerant callus grown on medium without NaCl. Fresh Wt., Fresh weight.

α-Tocopherol was not measured in this study, but in previous studies NaCl-tolerant cultivars of cotton had significantly greater constitutive levels of α-tocopherol than did more NaCl-sensitive cultivars (Gossett et al., 1994a).

Changes in the glutathione pool can account for increased ascorbate reduction (Foyer et al., 1991), and the NaCl-tolerant cell line had a significantly greater GSH/GSSG ratio than the control cell line. Increases in the activities of DAsA reductase (Cakmak and Marschner, 1992) or monodehydroascorbate radical reductase (Arrigoni et al., 1981) could also result in increased ascorbate reduction (Fig. 10), but neither of these enzymes was assayed. Variations in the GSH/GSSG ratio reflect differences in the glutathione turnover rates that are controlled, at least in part, by the activity of GR. Elevated GR activities have been observed in cotton that was subjected to elevated atmospheric O_2 (Foster and Hess, 1980) and NaCl stress (Gossett et al., 1994a). In this study GR activity was significantly greater in the NaCl-tolerant cell line.

GSH plays an important role in stress tolerance, and it has been suggested that in higher plants GR may be a rate-limiting enzyme for defense against active O_2 toxicity (Tanaka, 1994). In addition, it has been shown that the induction of GSH synthesis by an oxidative stimulus probably plays a crucial role in determining the susceptibility of cells to oxidative stress (May and Leaver, 1993), and data from the BSO and exogenous glutathione experiments in this study indicate the importance of maintaining sufficient GSH pools. BSO reduced the growth of the control cell line by 94%, whereas the NaCl-tolerant cell line showed significantly less growth reduction. When medium containing BSO was supplemented with exogenous GSH, growth was restored in both cell lines; however, when GSSG was added to medium with BSO, growth was almost completely restored in only the NaCl-tolerant cell line. This was most likely due to the elevation of GR activity and an increased ability to convert GSSG to GSH. The slight tolerance to BSO in the NaCl-tolerant cell line was likely due to the increased activity of GGCS in this cell line. This increase in activity was further evidenced by the significant differences in the Cys and cystine concentrations.

Cys is a precursor for glutathione synthesis, and the Cys/cystine ratio was much lower in the NaCl-tolerant cell line than in the control callus, suggesting that Cys was being converted to glutathione at a greater rate in the NaCl-tolerant cell line. These data suggest that the capacity to grow on medium with 150 mM NaCl may depend, at least in part, on the ability of the plant or tissue to up-regulate the ascorbate-glutathione cycle (Fig. 10). Therefore, it is possible that during NaCl-induced oxidative stress ascorbate levels (particularly AsA) increase at the upper end of the ascorbate-glutathione cycle, whereas glutathione levels decrease at the lower end, GR activity increases to keep the GSH/GSSG ratio favorable to ascorbate reduction, and GGCS activity is up-regulated to facilitate the synthesis of additional glutathione from Cys (Fig. 10). Further evidence for the NaCl-induced up-regulation of the antioxidant defense system is the apparent cross-tolerance to sublethal doses of paraquat exhibited by the NaCl-tolerant cell line. Paraquat is known to generate strong oxidants, and paraquat tolerance has been correlated with elevated antioxidant activities (Harper and Harvey, 1978); therefore, paraquat resistance was most likely due to the greater antioxidant activities exhibited by the NaCl-tolerant cell line.

It is interesting that GST activity increased significantly in the NaCl-tolerant cell line. Although the primary role of this enzyme has been assigned to the detoxification of xenobiotics (Mozer et al., 1983), it has also been shown to exhibit antioxidant activity (Reddy et al., 1981). Chemically induced increases in GST activity have been reported in both animals and plants (Droog et al., 1995), and Conklin and Last (1995) have shown that treatment with ozone causes a 4-fold increase in GST mRNA in Arabidopsis. It

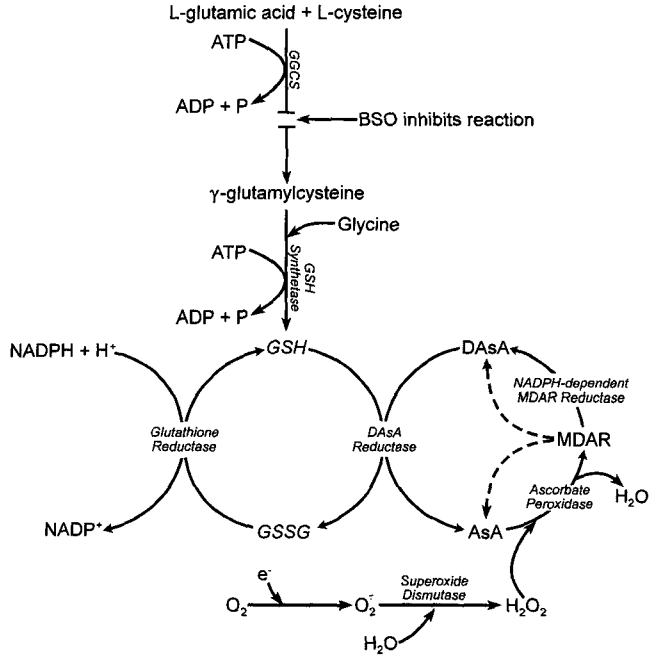

Figure 10. Relationship of GGCS, GSH synthetase, and SOD to the ascorbate-glutathione cycle. Enzymes are printed in italics, and – – – represents nonenzymatic disproportionation. The estimated relative flux rate for the turnover of GSSG to GSH was 3.33 at 0 mM NaCl and 7.38 at 150 mM NaCl. The estimated relative flux rate for the turnover of AsA to DAsA was 1.41 for 0 mM NaCl and 5.57 for 150 mM NaCl. MDAR, Monodehydroascorbate radical.

has been suggested that this enzyme may play an important role in the protection of cellular membranes against lipid peroxidation by converting membrane-bound hydroperoxides and cyclic endoperoxides to less reactive intermediates (Reddy et al., 1981). In previous studies it was shown that NaCl-induced lipid peroxidation occurred at a much lower rate in an NaCl-tolerant cotton cultivar than in an NaCl-sensitive cultivar (Gossett et al., 1994a).

It is not known whether the NaCl-induced increases in antioxidant enzyme activities observed in this study were due to an increased synthesis of the enzymes or an increased activation of constitutive enzyme pools. An increase in the transcription of genes involved in the synthesis of various stress metabolites, including antioxidant enzymes, has been reported (Kuhn et al., 1984; Edwards et al., 1985; Ramagopal, 1987; Perl-Treves and Galun, 1991; Scandalios, 1994), and Yamaguchi-Shinozaki and Shinozaki (1994) have identified a *cis*-acting element responsible for the induction of an Arabidopsis gene involved in responsiveness to drought, low-temperature, and NaCl stress. Therefore, it may well be that in cotton callus acclimation in response to elevated NaCl levels is due, at least in part, to the up-regulation of the genes encoding these antioxidant enzymes. The data from the experiment in which the NaCl-tolerant cell line was grown on 0 mM NaCl control medium suggest that once induced by NaCl stress, AP, GR, and catalase activities remain constitutively high after at least one callus transfer, even when the stress is removed, but that peroxidase activity is transitory and is elevated only in the presence of high NaCl concentrations. Additional research is necessary to provide further insight concerning the specific relationship between NaCl stress and the antioxidant response.

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