COTTON CALLUS TISSUE TRANSFORMED WITH GLUTATHIONE REDUCTASE cDNA DERIVED FROM *PISUM SATIVUM* L. EXHIBIT INCREASED GLUTATHIONE REDUCTASE ACTIVITY

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Abstract

Detailed investigations of the activity profile of antioxidant enzymes in salt stressed cotton plants, cotton callus and ovule culture has focused attention on the possible role of these enzymes in the mechanism of salt tolerance in non-halophytes. The critical role of glutathione reductase in the antioxidant enzyme system in plants has prompted us to advance the hypothesis that an increase in the transcription of glutathione reductase may up-regulate the ascorbate-glutathione cycle and confer an enhanced ability of transformed tissue to tolerate higher levels of salt. In order to test this hypothesis we have genetically transformed cotton callus with a cDNA encoding glutathione reductase derived from pea plants. Transformed calli show an increase in glutathione reductase activity of between 128-330% over controls. We are currently examining the growth performance of this tissue on salt amended growth media.

Introduction

During normal aerobic metabolism activated oxygen species such as $O2^{\cdot -}$ (superoxide), H_2O_2 (hydrogen peroxide) and $OH^{\cdot}$ (the hydroxyl radical) are produced through a series of univalent reductions of ground state oxygen. Electron leakage from electron transport chains in the mitochondria and photosystems I and II in the chloroplast (Asada 1994, Asada *et al.,* 1994) can react with O_2 to produce the superoxide ($O_2^{\cdot -}$) radical shown in Figure 1. (Mehler, 1951; Halliwell and Gutteridge, 1985). Superoxide dismutase is a major scavenger of $O_2^{\cdot -}$, producing hydrogen peroxide (H_2O_2). Catalase, general peroxidases and ascorbate peroxidase (AP) catalyze the breakdown of H_2O_2 (Chen and Asada 1989). The ascorbate-glutathione cycle acts by the enzymatic conversion of ascorbate (ASA) to monodehydroascorbate radicals (MDA). These are then reduced to dehydroascorbate (DASA) by NADPH-dependent monodehydroascorbate radical reductase (Hossain *et al.* 1984). Dehydroascorbate is reduced back to ascorbate by dehydroascorbate reductase (Polle *et al.,* 1992). The resulting oxidized form of glutathione (GSSG) is, in turn, converted back to the reduced form (GSH) by NADPH-dependent glutathione reductase (Foyer *et al.,* 1991). When plants are subjected to physiological stress such as drought, temperature extremes, herbicide treatment or mineral deficiency, the production of reactive oxygen species exceeds the capacity of the scavenging reactions of the antioxidant system. This leads to cellular oxidative damage to lipids (Fridovich, 1986; Liebler *et al,* 1986; Price and Hendry 987; Wise and Naylor, 987), proteins (Halliwell and Gutteridge 1985; Kim *et al.,* 1985; Davies, 1987), and nucleic acids (Fridovich, 1986; Imlay and Linn 1988). Plants with higher constitutive, or inducible antioxidant enzyme levels have been reported to have greater resistance to oxidative damage (Dhindsa and Matowe, 1981; Harper and Harvey, 1987; Wise and Naylor 1987; Monk and Davis 1989; Spychalla and Desborough 1990). Cotton is classified as a salt-tolerant plant (Ayers and Wescot, 1977), and a comparison between the cultivars shows varietal differences in the levels of salt tolerance (Lauchi *et al.,* 1981; Gossett *et al.,* 1992, 1994a). In cotton, high salt concentrations have been shown to reduce yield (Fowler 1986) as well as growth, stomatal conductance, stomatal resistance and transpiration rates (Gossett, 1991). Previous studies carried out with salt- stressed whole plants (Gossett *et al.,* 1992), callus tissue (Gossett *et al.,*1994a,b), and ovule cultures (Banks *et al.,* 1997) has revealed a significant increase in the activity of antioxidant enzymes in the more salt-tolerant tissues compared to controls. In addition, it is now known that the increases in antioxidant enzyme activities are transcriptionaly regulated (Banks *et al.,* 1998; Manchandia *et al.,* 1999). Collectively, this work has monitored the activities of catalase, peroxidase, ascorbate peroxidase, and glutathione reductase in a control and a salt-tolerant cell line. The NaCl-tolerant cultivars and cell line had higher antioxidant enzyme activities as well as a significantly lower ascorbate/oxidized ascorbate ratio and a significantly higher GSH/GSSG ratio, (Gossett *et al.,*1994a,b). These results indicate that elevation in the activity of the ascorbate-glutathione cycle probably confers a degree of salt tolerance. The critical role of glutathione reductase in the ascorbate-glutathione cycle (Figure 1) prompted us to advance the hypothesis that an increase in the transcription of the glutathione reductase genes may up-regulate the ascorbate-glutathione cycle and thus confer an enhanced ability of transformed tissue to tolerate higher levels of salt. In order to test this hypothesis we have genetically transformed cotton with a cDNA encoding glutathione reductase under the control of a constitutive promoter.

Materials and Methods

Construction of pSGR1

A cDNA encoding the enzyme glutathione reductase was isolated originally as a lgt11 clone, from pea plants (*Pisum sativum* L.) by Creissen *et al.* (1991). The full length *Bam* H1 fragment was ligated into pBluescript II SK (+) to make the plasmid, pGR 201. pGR 201 was generously supplied to us by Dr. Gary Creissen of the John Innes Institute, Norwich, U.K. The 2KB glutathione reductase cDNA was

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excised from pGR 201 using *Bam* H1 and ligated into the *Bam* H1 site within the T-region of the *Agrobacterium* binary vector, pBI 121 (Clontech™ Laboratories Inc.) adjacent to the constitutive promoter, CaMV 35S to construct the plasmid pSGR1 (Figure 2). Ligation mixtures were used to transform electrocompetent cultures of *E.coli* strain HB101 (Biorad) using kanamycin sulfate as the selectable marker at 50mg ml^{-1} according to the technique described by Jacobs *et al.* (1990). Digestion of pSGR1 with *Hind* III revealed a 2.610 KB fragment. This confirmed the ligation of the 2KB GR cDNA into pBI 121 in the sense orientation. Had the GR cDNA been ligated into pBI 121 in the antisense orientation, a 1.090 KB fragment would have been generated upon digestion with *Hind* III (Figure 2). pSGR1 was subsequently extracted from *E.coli* HB101 using the technique described by Maniatis *et al.* (1982a), electroporated into *Agrobacterium tumefaciens* strain EHA 105 using the technique described by Mersereau (1990) and the transformed cells, EHA 105:pSGR1 identified by selection on modified LB-agar media amended with kanamycin at 50mg g^{-1}(Maniatis *et al.*, 1982b; Banks *et al.* 1993).

Cotton Transformation Experiments

Surface sterilized seeds (Trolinder and Goodin, 1987) of cotton, (Coker 312) were germinated on Stewart's media (Stewart and Hsu, 1977) and grown for *ca.* eight days at 26°C under a 16 hour light:8hour dark cycle. Hypocotyl segments (*ca.* 8mm) were incubated for three days at 27°C also under a 16 hour light:8hour dark cycle on pre-culture media containing on a per liter basis, 4.4g MS salts with Gamborg's vitamins, 0.75mg $MgCl_2$, 30g glucose and 2g phytogel (Trolinder and Goodin 1987), 0.1mg 2,4-D and 0.5mg kinetin were filter sterilized and added to the media after autoclaving. *Agrobacterium* strains EHA 105 (control) and EHA 105:pSGR1 (experimental) were grown for 40 hours at 30°C to a titer of approximately 10^7cells ml^{-1} in modified LB media. Hypocotyl segments were inoculated by bathing them in the *Agrobacterium*-LB broth for one minute, blotting them dry to remove excess bacteria, and returning them to the pre-culture plates for three days in the dark at 27°C. The hypocotyl segments were then transferred to callus initiation media containing the ingredients of the pre-culture plates and in addition filter sterilized amoxicillin (1000mg ml^{-1}) and potassium clavulanate (500mg ml^{-1}) *i.e.,* Augmentin™, (Beecham Laboratories, Bristol, TN), to kill the *Agrobacterium*. In addition to these antibiotics, callus initiation media used for the hypocotyl segments exposed to EHA 105:pSGR 1 were amended with 50mg ml^{-1} kanamycin for selection of transformed calli. Calli were transferred to fresh media monthly. Hypocotyls exposed to EHA 105 (control) did not give rise to callus tissue when incubated on callus initiation media containing kanamycin. Death of the *Agrobacterium* on both control and experimental plates was confirmed by crushing and smearing 30-day old callus onto LB-agar plates. No bacterial colonies were observed.

Glutathione Reductase Assays

Callus samples were prepared for glutathione reductase assays according to the method of Foster and Hess (1980). One g of frozen callus tissue was homogenized in 1 ml of an ice-cold solution containing 100mM Tris (pH 7.0), 10mM D-isoascorbic acid, 2% PVP-10, 0.1mM EDTA. 0.2% Triton X-100 and 100ml of antifoam A emulsion. The homogenate was then centrifuged at 15,000g for 2 min at 4°C. One ml of the supernatant was centrifuge-desalted at 4°C through a 10ml bed of Sephadex G50-300 (Helmerhorst and Stokes 1980). Glutathione reductase activity was determined by monitoring the glutathione-dependent oxidation of NADPH at 340nm in a reaction mixture containing 950ml of 0.15mM NADPH, 0.5 mM GSSG and 3mM $MgCl_2$ in 50mM Tris (pH 7.5) and 50ml of extract (Schaedle and Bassham, 1977). One unit of GR is defined as the amount of enzyme required to reduce 1 nmole of substrate min^{-1} at 25°C.

Results and Discussion

Glutathione reductase activity was recorded from four separate cultures of control callus (*i.e.,* exposed to EHA 105 and generated on initiation media lacking 50mg g ml^{-1} kanamycin) and four separate cultures of experimental callus (*i.e.,* exposed to EHA 105:pSGR1 and generated on initiation media amended with 50mg g ml^{-1} kanamycin) (Table 1). The GR activity for control callus yielded an average of 95 enzyme units g fresh weight $callus^{-1}$,whereas GR activity from experimental samples ranged from 122-314 units g fresh weight $callus^{-1}$. These results reveal an increase in GR activity in transformed callus ranging from 128-330% when compared to the control average. The variation in GR activity observed in transformed tissue may be explained by the phenomenon known as genetic position effect (Lewin, 1997). The insertion of T-DNA in to the host plant genome is known to be a random event. The T-DNA carrying the GR cDNA may insert into an area of the plant genome that is actively transcribed. Such an event would lead to a higher level of overall transcription of the T-region than if the insertion into the plant genome occurs in a an area of relatively low transcription. Moreover, not all areas of the T-region are transcribed equally after insertion into the plant genome (Dhaese *et al.,* 1983; Depicker *et al.,* 1883, Bevan *et al.,* 1982). We are currently examining the ability of the transformed callus to grow on salt amended media.

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Table 1. Glutathione Reductase Activity from Transformed and Control Callus.

Sample #	1	2	3	4
Control Callus: Glutathione Reductase Activity g fresh weight callus^{-1}	103	83	83	112
Experimental Callus: Glutathione Reductase Activity g fresh weight callus $^{-1}$	122	186	314	239

One unit of glutathione reductase is defined as the amount of enzyme required to reduce 1 nmole of substrate min^{-1} at 25^{o}C.

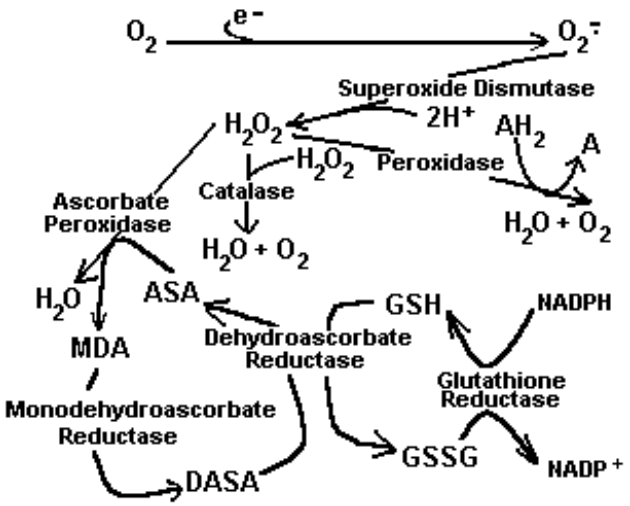

Figure 1. Relationships Among Antioxidant Enzymes in Plants

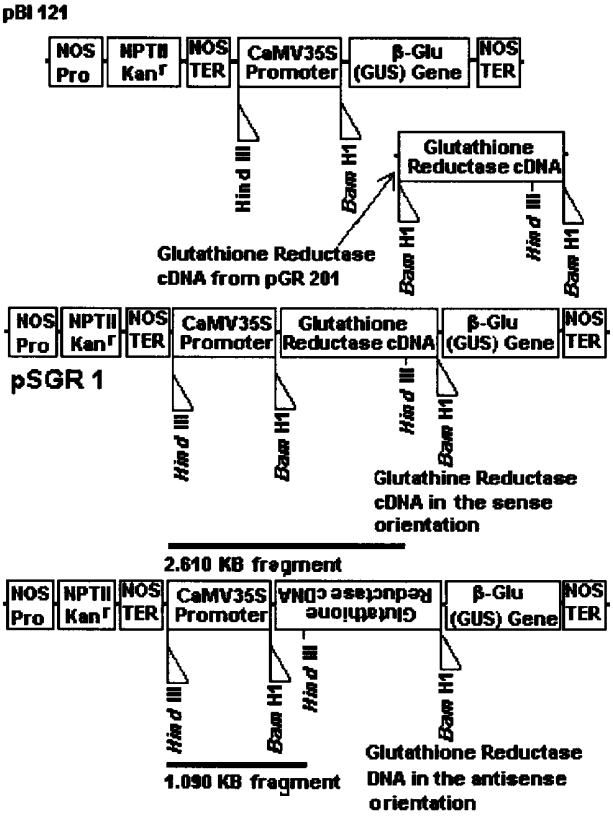

Figure 2. Construction of Plasmid pSGR1