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Overexpression of Cotton Glutathione S-Transferase (GST) cDNA and Increase of Low Temperature and Salt Tolerance in Plants

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Abstract

Cotton Glutathione S-Transferase(GST: EC 2.5.1.18) was cloned and *Gh-5* cDNA was overexpressed in tobacco (*Nicotiana tabacum*) plants. The transformation of cotton *GST* in tobacco plant was confirmed by northern blot analysis. Type I and Type II transcript patterns were identified in *Gh-5* transgenic tobacco plants. Type I transcripts was only discussed in this paper. Glutathione and 1-chloro-2,4-dinitrobenzene (CDNB) were used as the substrates, and the activity of GST in the type I transgenic plants was about 2.5-fold higher than the non-expressers and wild type tobacco plants. The expression of cotton GST in tobacco plants proved that Gh-5 could be translated into functional protein. Type I transgenic plants produced functional GST in the cells. Type I showed higher GST specific activity than Type II in the transgenic plants. Control and transgenic seedlings were grown in the growth chamber and under the light at 15℃, and the effects of cotton GST in the seedlings was evaluated. The growth rate of Gh-5 overexpressors was better than the control and non-transgenic tobacco plants. Salinity tolerance was also analyzed on the seeds of transgenic plants. Seeds of Gh-5 overexpressors and the wild type tobacco seedlings were germinated and grown at 0, 50, 100, 150, and 200 mM NaCl solution. Gh-5 transgenic seedlings showed higher growth rate over control seedlings at both 50 and 100 mM NaCl solution. But at 0, 150, and 200 mM NaCl concentration, the difference in growth rate was not detected.

Introduction

The most well known role of plant GST is detoxification of nucleophilic xenobiotic compounds by conjugating GSH with the substrates (Ishikawa, 1992; Sandermann, 1992). They catalyze the conjugation of reduced glutathione with reactive electrophilic and hydrophobic molecules. However, the multiplicity of GST isoforms in most organisms indicates that these enzymes have a wide range of substrates. Multiple forms of GST are often found in a single tissue or cell type (Mannervik and Danielson, 1988). The multiple isozymes are thought to have evolved to accommodate exposure to diverse substrates and ligands (Listowsky, 1993). Thus, various classes of GSTs possess overlapping but distinctive binding and substrate specificity (Mozer et al., 1983).

The classification of plant GSTs depends on the amino acid sequence identity and conservation of exon:intron replacement (Droog et al., 1995; Marrs, 1996). Pemble and Taylor (1992) suggested that class-Theta GSTs are representative of the ancient progenitor GST gene that may have a prokaryotic origin. Amino acid sequences of Theta-class GSTs are highly conserved in rat, *Drosophila, Zea mays* and *Methylobacterium* (Pemble and Taylor, 1992). Although plants apparently do not contain Alpha-, Mu- and Pi- class GSTs, the Theta-class is conserved (Pemble and Taylor, 1992). Although the enzymes in these classes are thought to be homologous, only six residues are conserved in all of the known GST sequences. These highly conserved residues are expected to be important for the structure

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and/or function of the enzymes.

According to phylogenetic trees of plant GSTs trees, almost all plant GSTs belongs to the theta class. Plant GSTs were further classified into four subgroups, according to the amino acid sequence identity and conservation of intron:exon placement. Droog et al. (1995) cataloged plant GST genes into three types, i.e., Type I, Type II, and Type III, and Marrs (1996) added an unclassfied subgroup.

Pathogenic bacteria, virus, and fungi are attacking plants and insects, nematodes and other animals are feeding plants always. Plants have developed an substantial method to defend themselves from these attacks by producing secondary metabolites. Secondary plant metabolites are being produced by many plants that are products of specialized biosynthetic pathways (Marrs, 1996). Plants produce antimicrobial chemicals known as phytoalexins to fight pathogens, such as viruses, bacteria and fungi. Most phytoalexins are phenolic phenylpropanoides that are products of the shikimic acid pathway, although some are isoprenoid compounds and a few are polyacetylenes (Salisbury and Ross, 1992). Elicitors produced by activity of microbes, trigger synthesis of phytoalexins in the plants and these compounds are much more toxic to fungi than to bacteria and viruses (Saliberury and Ross, 1992).

Elimination of toxic chemicals from the cells is important for the survival of an individual organism. In animals, glutathione-conjugates of xenobiotics as well as endogenous substrates such as leukotriens are actively eliminated or secreted from the cell by ATP-dependent glutathione s-conjugate transport, found in the liver, kidneys, and other organ (Ishikawa, 1992). Toxic chemicals in the plant are generally stored in the vacuole or apoplast because plants do not have any excretion system. Xenobiotics which are tagged by glutathione are transported into the vacuoles by Mg-dependent ATP transporter. The inactive, water-soluble conjugates of both synthetic and natural chemicals in the cytosol can be secreted into the apoplast via an exocytosis (Sanderman, 1992). They may become associated with cell wall components such as pectin, hemicellulose, or lignins as an insoluble conjugate termed a "bound residue" (Sandermann, 1994).

Reactive oxygen species (ROIs) can be induced by environmental stress, such as, high light intensity, heavy metal, ozone, pathogen attack, wounding (Allen, 1995; Tenhaken et al., 1995; Bartling et al., 1993). Plant GSTs protect plants from oxidative damage triggered by ROIs to lipid bilayers, DNA and proteins (Sandermann, 1994). In mammals, leukocytes and other phagocytic cells fight microbes, such as bacteria, and virus-infected cells by destroying them with nitrogen oxide, superoxide hypochlorite, and hydrogen peroxide, a mutagenic oxidizing agent. These oxidants help to protect animals from immediate death from infection. These oxidants can also cause oxidative damage to DNA. DNA damage can be a starting point to the carcinogenic processes. Plants are similar mechanisms to defend themselves from pathogen attack.

Materials and Methods

Construction of chimeric GST sense constructs

Gh-5 cDNA fragment was isolated from cotton using polymerase chain reaction (PCR), cloned into the pCR 2.1 vector, and used to develope the construction of sense Gh-5 gene. restriction enzymes *Nco* I and *Sac* I were used to clone Gh-5 cDNA fragment. The digested Nco I/Sac Ifragment was ligated into the *Nco* I/*Sac* I sites of the pRTL 2 expression vector (kindly provided by Dr. James Carrington, Dept. of Biology, Texas A&M Univ., Figure 1). That vector carries an enhanced CaMV 35S promoter, a tobacco etch virus ribosomal binding site, and a 35S-terminator polyadenylation signal. The Gh-5 gene cassette was excised as a *Sph* I fragment and ligated into the binary plant transformation vector pCGN 1578 to create pCGN-Gh-5. The method of van Haute et al. (1983) was hired to transform pCGN-Gh-5 plasmid into *Agrobacterium* strain EHA 101.

Tissue culture medium

Inoculation plates (Roxas et al., 1997) were used and the ingredients are 1 bag of Murashige and Skoog Basal Salt, 30 g sucrose, 1 mL 1000X vitamin B-5, 10 L 10 mg/mL

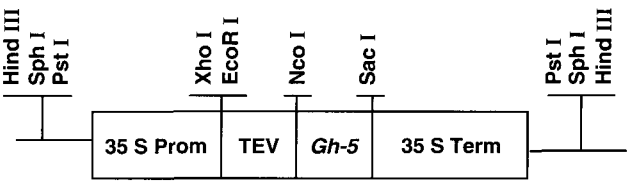

Figure 1. The development of the sense *Gh-5* construct. Cloned cDNA fragment was digested with *Nco* I/*Sac* I and ligated into the *Nco* I/*Sac* I sites of the pRTL 2 expression vector. This vector carries an enhanced CaMV 35S promoter, a tobacco etch virus ribosomal binding site, and a 35S-terminator polyadenylation signal. The Gh-5 gene cassette ligated into the binary plant transformation vector pCGN 1578 to create pCGN-Gh-5. The pCGN-Gh-5 plasmid was transformed into *Agrobacterium* strain EHA 101.

NAA, 200 L 5 mg/mL BA, pH was 7.0 and adjust volume to 1 L, 2.00 g Phytagel was add and autoclave. Plates for shoot and callus formation were made using same as inoculation plates except for the addition of antibiotics as follows: 50 ug/mL Kanamycin, 300 ug/mL Cefotaxime, and 300 ug/mL Carbenicillin. Plates for shoot and callus formation were prepared same as shoot and callus formation plates without NAA or BA. The concentrations of the antibiotics are the same.

Agrobacterium-mediated plant transformation

The sense Gh-5 gene construct in pCGN 1578 was mobilized into *A. tumefaciens* strain EHA 101 by direct transformation. Putative transformant colonies were grown in liquid culture with 100 g/mL gentamycin and 50 g/mL kanamycin. Plasmids were recovered, and analyzed by restriction analysis and Southern blotting. Two ml cultures of positive EHA 101 clones were used for inoculation of tobacco leaf disks. Inoculation of tobacco (*Nicotiana tabacum* cv. Xanthi) leaf disks was performed following the protocol of Horsch et al. (1985). Fully expanded, healthy leaves from about two month old tobacco plants were collected, disinfected in 14% chlorox for 5 min and washed 5 times with sterile water. A number 7 cork borer was used to make leaf disk punches 1.5 cm in diameter. The leaf disks were immersed for 5 min in the *Agrobacterium* solution that contains the chimeric gene construct of interest, blotted on sterile 3M paper and placed upside down on MSA nutrient plates for 2 days in the dark and at room temperature to allow infection to occur. They were then transferred to MSB plates containing hormones and antibiotics that promote growth of callus and shoot formation. Shoots that formed from the calluses were cut and transferred to MSC plates for root formation. The regenerated plantlets were transferred into Magenta boxes with sterile potting soil and slowly acclimated prior to potting in 1 gallon containers in the greenhouse.

Northern blot analysis

Isolated total RNA (Chomczynski and Sacchi, 1987) (30 μg) from transgenic tobacco plants was electrophoresed on 1.0% agarose gels containing 2.2 M formaldehyde and transferred to the nitrocellouse. The filter was prehybridized for 1-2 hours at 42℃ in prehybridization solution (50% (v/v) formamide, 10X Denhardts reagent (1X Denhardts: 0.02% PVP, 0.02% Ficoll, and 0.02% BSA), 1.0% SDS, and 6X SSPE (1X SSPE: 0.18M NaCl, 10mM NaH_2PO_4, pH 7.7, 1 mM EDTA). Hybridization was performed in the same prehybridization solution and the probe. The probe was labeled with [α-^{32}P] dCTP using a random primer DNA labeling kit (United State Biochemical Co., USA). After overnight hybridization, the filters were washed, exposed to the X-ray film at 80℃ with an intensifying screen and developed (Sambrook et al., 1989).

GST enzyme assay for transgenic plants

The procedure of Mozer et al. (1983) was used to measure the activities of GST enzyme from the transgenic tobacco and control tobacco plants. The model substrate 1-chloro-2,4-dinitrobenzene (CDNB) and reduced glutathione were used to evaluate GST activity by determining kinetic parameters. The reaction mixture contains final concentrations of 10mM reduced GSH and 1mM CDNB, which is dissolved in 2.5% ethanol, and potassium-phosphate buffer (0.1M, pH 6.5). GST activity was calculated by measuring the absorbance at 340 nm and an extinction coefficient of 9.6 mM/cm (Habig and Jakoby, 1981).

Analysis of transgenic seedlings

Roxas et al. (1997) reported that overexpression of glutathione S-transferase/glutathione peroxidase enhances the growth of transgenic tobacco seedlings during stress. When cotton GST cDNA was expressed in *E. coli*, GST gene produced functional enzyme (not published) as did tobacco GST cDNA (NT107).

To find whether increased levels of cotton GST can affect the tolerance of plants to abiotic stress, such as low temperature, heavy metals, and salt conditions. Seeds of Gh-5 transgenic tobacco plants were treated in various stress conditions, low temperature, heavy metals, and salt stress, and analyzed the effects of overexpressed cotton GST gene. Mature seeds from six independent lines of transgenic Gh-5 overexpressing plants and Xanthi were sown on Petri dishes. Filter papers, nutrient media, or soils were used to supply nutrients. Growth chambers were adjusted at 15℃, and the Petri dishes were incubated under the light. For the low temperature, nutrient media was used to nourish the seedlings because it took long time. Sodium chloride solutions were prepared with different concentrations of 0, 50, 100, 150, 200 mM. In salt experiment the standard solution was diluted in distilled H_2O. Filter papers were layed into petridish and each concentration of solutions were added. Only distilled H_2O was added to parallel control. Each treatment was repeated three times. The seeds of transgenic plants were placed in a growth chamber at 25℃ in the dark.

Results and Discussion

Expression of Gh-5 in transgenic tobacco plants

The pCGN-Gh-5 gene constructs were introduced into tobacco using *Agrobacterium*-mediated transformation, and analyzed to determine whether Gh-5 could be overexpressed in transgenic plants. Three independently transformed Gh-5 tobacco plants that expressed the Gh-5 construct were analyzed (CGST2, CGST9, and CGST11). Northern blot analyses were hired to initially identify overexpressed transgenic tobacco plants. ^{32}P-labeled full length Gh-5 cDNA probes were prepared and used to detect the successful constructs. ^{32}P-labeled full length Gh-5 cDNA probes hybridized with three lanes of RNA blots and represent to tobacco plants that contain the Gh-5 gene construct. The autoradiogram indicated that six of Gh-5 transgenic tobacco plants had high steady-state levels of Gh-5 mRNA. These include CGST2, CGST9, and CGST11 and selected for assay. No hybridization was detected in wild type non-transgenic tobacco plants (Figure 2).

Cotton GST activity in transgenic tobacco plants

The transformation efficiency of Gh-5 was about 6%. Levels of total GST specific activity was assayed in transgenic plants. Using CDNB and reduced glutathione as substrates, spectrophotometric assay of Type I GST-expressing transgenic tobacco plants enhanced approximately 2.5-fold increase in GST specific activity in leaf extracts over control non-expressers plants (Figure 3). These GST specific activities were similar to the transgenic tobacco plants which were transformed with tobacco GST NT107 (Roxas et al., 1997). Also these results were similar to the GST activities of transgenic tomato plants which were transformed with tobacco GST NT107 (Data not shown). The GST specific activities were very high in *E. coli*, which were both transformed with cotton and tobacco GST cDNAs, Gh-5 or NT107. But GST activities were not over 3-fold both in tobacco and tomato plants, which were transformed with cotton and tobacco GST cDNAs. GST specific activity in Type I CGST plants was somewhat higher than in Type II (Data not shown) plants. Type II transgenic tobacco plants increased approximately 1.5 times higher than control plants in GST specific enzyme activity and the size of transcript was larger than Type I. The results from this assay confirmed that Gh-5 transgenes encode an active GST in transgenic tobacco plants.

There are plentiful reports on the protective role of plant GST (Allen, 1995; Tenhaken et al., 1995; Bartling et al., 1993; Sandermann, 1994). Roxas et al., (1997) reported that overexpression of glutathione S-transferase enhances the growth and germination of transgenic tobacco seedlings during low temperature stress. They also reported that stress tolerance during thermal and salt stress in transgenic tobacco seedlings that overexpress glutathione S-transferase/glutathione peroxidase (Roxas et al., 2000). Pathogen resistance of cotton GST cDNA was studied in transgenic *Scrophularia buergeriana* Misrule (Kang et al., 2002). Under the exposure of transgenic tobacco plants to low temperature and salt stress, these results clearly

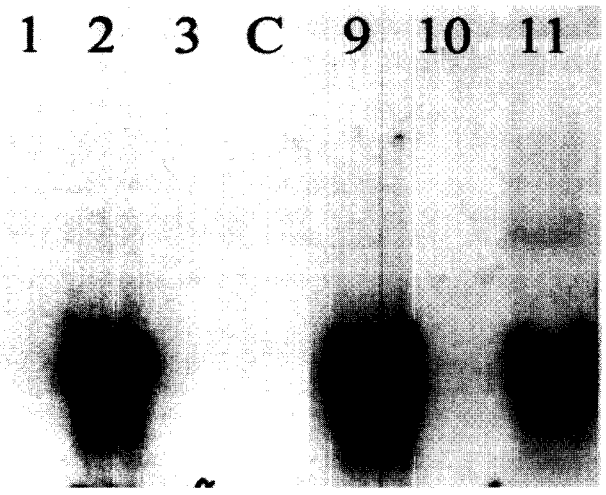

Figure 2. GST transcripts. Expression of cotton GST gene in transgenic *Nicotiana tabacum* plants. Northern blot of lines of CGST2, CGST9 and CGST11 which can be classified as Type I. Only Type I CGST lines were discussed in this paper. Isolated total RNA (30 μg) from the leaves of the transgenic tobacco plants, non-expressor, and wild type plant was separated, blotted onto nitrocellulose, and hybridized with a ^{32}P-radiolabeled Gh-5 cDNA probe. The autoradiograms for GST resulted from 3-day film exposure. Lanes 1, 3, and 10 are non-expressors. "C" means wild type control Xanthi.

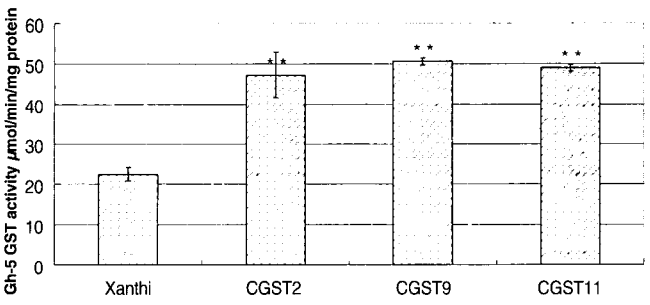

Figure 3. Spectrophotometric GST assay of Gh-5 transgenic tobacco plants using CDNB and reduced glutathione as substrates. In Gh-5 GST specific activity, Type I GST showed about 2.5-fold GST specific activity.

explain that transgenic tobacco seedlings that overexpress cotton GST grow faster than wild type seedlings. Plant GST has the dual functions of activity as Glutathione S-transferase and Glutathione peroxidase in model plant, *Arabidopsis thaliana*, and in the plant cell may be involved in the removal of the products of lipid peroxidation (Bartling et al., 1993). Also the possible increase of protection of Gh-5 transgenic tobacco seedlings from low temperature was induced from glutathione peroxidase (GPX) activity, according to previous published data (Roxas et al., 1997; 2000). They reported that transgenic tobacco seedlings overexpressing a cDNA encoding an enzyme with both glutathione S-transferase (GST) and glutathione peroxidase (GPX) activity had GST- and GPX-specific activities approximately two-fold higher than wild-type seedlings (Roxas et al., 1997). In the other publication, increase of a tobacco glutathione S-transferase with dual activity of glutathione peroxidase (GST/GPX) in transgenic tobacco protected seedling growth under low temperature and salt stress (Roxas et al., 2000). Two published articles (Roxas et al., 1997; 2000) showed that transgenic tobacco seedlings overexpressing tobacco GST/GPX can be protected from several environmental stress factors. In cotton plants, Gh-5 transcripts were only detected in the fast growing tissues such as cotton fiber or hypercotyls, and 2,4-D induced the GST in cotton leaves (Kang, 1998). Takahashi et al. (1992) had suggested that tobacco GST is related to the induction of proliferative activity in differentiated and nondividing mesophyll protoplasts, and found that in the presence of 2,4-D a significant increase of GST activity was detected in the tobacco mesophyll protoplast cells. In Gh-5, the increase of seedling growth of Gh-5 transgenic tobacco plants may induced from unknown activity of cotton GST or glutathione peroxidase (GPX) activity under the low temperature or salt stress conditions. Their function in metabolism is not clear, although plant GSTs are known as detoxification of toxic compounds by conjugating glutathione with electrophilic xenobiotics and protection of plants from environmental stress. The other roles of GST are glutathione-dependent isomerization and detoxification of toxic organic hydroperoxides by stress or normal metabolism in plants, but GSTs might possibly have non-catalytic function as phytochemical carriers. (Edwards, 2000).

Abiotic stressors, such as low temperature, heavy metals, and salinity, were added to the seedlings or seeds to evaluate the effects of overexpression of cotton GST. The effects of cotton GST in the seedlings was evaluated by growing the control and transgenic seedlings at 15°C in the growth chamber in the light. Overexpressors were grown well compared to the control plants (non-expressors) (Figure 4). To test for tolerance to salinity, seeds of Gh-5 overexpressors and the wild type Xanthi seedlings were grown in solutions of 0, 50, 100, 150 and 200 mM NaCl. The growth pattern was similar in both transgenic and

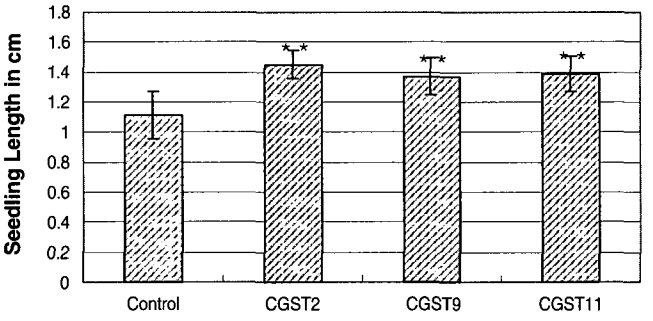

Figure 4. Low temperature tolerance of seedlings from control Xanthi and GST transgenic tobacco. Seedlings from control and transgenic plants were grown at 15°C for three weeks and measured the length. Error bars indicate standard deviations.
**Denotes significant difference at $P < 0.01$.

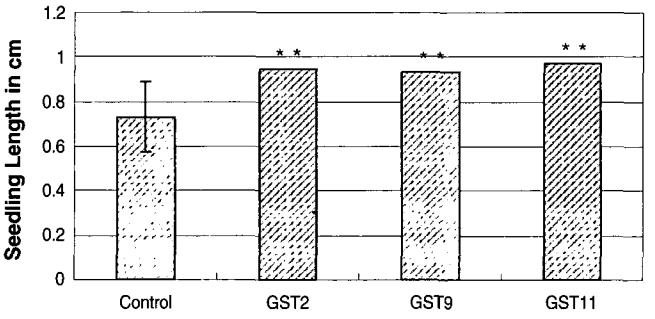

Figure 5. Salt tolerance of seedlings from transgenic tobacco seedling. Seedlings were grown at the 50 mM NaCl solution. The growth rate was significantly different with control and transgenic seedlings. Error bars indicate standard deviations.
**Means significant at $P < 0.01$.

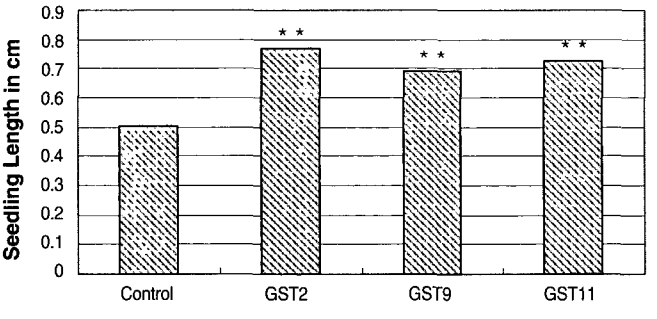

Figure 6. Salt tolerance of seedlings from transgenic tobacco seedlings. Seedlings were grown at 100 mM NaCl solution. The growth rate was significantly different with control and transgenic seedlings. Error bars indicate standard deviations.
**Means significant at $P < 0.01$.

non-expressor seedlings at control (0 mM NaCl). At 50 and 100 mM NaCl, Gh-5 transgenic seedlings showed higher growth rate over control seedlings (Figure 5, 6). But at 150 and 200 mM NaCl concentration for six days incubation, the difference in growth rate was not detected. The conclusion from salt experiment is that Gh-5 gene in transgenic tobacco transgenic seedlings may provide protection from mild and moderate salt concentration, but cannot protect the seedlings from higher concentration of salt at 150 and 200 mM.

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