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Increase of Salt and Low Temperature Tolerance by Overexpressing Glutathione S-Transferase (GST) Gene

Won Hee Kang* · Jun Chol Kim[1] · Il Seop Kim

Division of Applied Plant Science, College of Agriculture and Life Sciences, Kangwon National University Chunchon, 200-701, Korea

[1]Department of Biology, College of Natural Sciences, Kangwon National University, Chunchon, 200-701, Korea

Abstract

Cotton Glutathione S-Transferase (GST: EC 2.5.1.18) was cloned and overexpressed in tobacco (*Nicotiana tabacum*) plants. Northern blot analysis confirmed the successful transformation of cotton gst gene in tobacco plant. Type I and Type II transcript patterns were identified in transgenic tobacco plants and only Type I transcripts were discussed in this paper. The activity of GST in the type II transgenic plants was about 1.5-fold higher than those of the wild type and non-expresser by using 1-chloro-2,4-dinitrobenzene (CDNB) and reduced glutathione as the substrate. The expression of cotton GST in tobacco plants proved that Gh-5 could be translated into functional protein. Type II transgenic plants produced functional GST in the cells. 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). To test for tolerance to salinity, seeds of Gh-5 overexpressors and the wild type Xanthi seedlings were grown at 0, 50, 100, 150, and 200 mM NaCl solution. Gh-5 transgenic seedlings showed higher growth rate over control seedlings on 50 and 100 mM NaCl solution. There was no difference in growth rate at 150 and 200 mM NaCl concentration.

Key words: Glutathione S-transferases (GST), low temperature, overexpession, salinity

*Corresponding author

Introduction

Glutathione S-transferases (GST: EC 2.5.1.18) are super families of enzymes that conjugate reduced glutathione to a wide variety of compounds that are lipophilic and have an electrophilic center (Mannervik and Danielson, 1988; Pickett and Lu, 1989). Multiple GST isozymes are present in most plants. GSTs catalyze the phase II conjugation of reduced glutathione (GSH) with electrophilic compounds. This reaction yields a GSH conjugate that is often inactive, water-soluble, and is usually less toxic than the parent compound (Droog et al., 1993).

In plants, GSTs play a role in the detoxification of herbicidal compounds. Recently, a group of GSTs have been reported to be associated with various stress responses in plants including pathogen attack (Dudder, 1991) and heavy metal toxicity (Hagen, 1988). They are also involved in the synthesis of secondary products such as anthocyanins and cinnamic acid (Marrs et al., 1995) and are reported to modulate the activity and uptake of auxins from membranes, and trafficking auxins to receptors (Bilang and Stum, 1995; Jones, 1994).

GSTs have been implicated in protection from oxidative damage. Bartling et al. (1993) reported the cloning of a GST from *Arabidopsis thaliana* that also exhibited glutathione peroxidase (GPX: EC 1.11.1.9) activity. In animal cells, GPX plays a key role in cellular detoxification by catalyzing the reaction of GSH with hydrogen peroxide and other organic hydroperoxides (Arrick and Nathan, 1984; Roxas et al., 1997).

Plants are under almost constant attack by pathogenic bacteria, virus, fungi, and feeding by insects, nematodes and other animals. Therefore, plants have evolved an substantial arsenal to defend themselves from these pests.

Many plants produce defensive compounds called secondary plant metabolites that are products of specialized biosynthetic pathways (Ames et al., 1990; Ryan and Jagendorf, 1995; Eisner and Meinwald, 1995). Some of the enzymes involved in the biosynthesis of these compounds. Cytochrome P450s, UDP glucosyltransferases, and GSTs are the same enzymes of xenobiotic metabolism (Sandermann, 1992). Recent evidence suggests that naturally synthesized plant metabolites are recognized, transported, and metabolized in similar ways as herbicides and other xenobiotics.

In plants, copper catalyzes the oxidation of cellular thiols and GSH, and copper results in the production of free radicals and subsequent lipid peroxidation (De Vos et al., 1992). In animals, metals and oxygen radicals also play a role in lipid peroxidation (Halliwell and Gutteridge, 1984). Heavy metals such as cadmium, mercury, nickel, and lead, cause plants to synthesize phytochelatin (PC), thiol-rich peptides $(glu\text{-}cys)_n$-gly that are synthesized directly from glutathione (glu-cys-gly) and whose function is to sequester and detoxify excess metal ions (Rauser, 1990; Steffen, 1990).

Materials and Methods

Construction of Chimeric GST Sense Constructs

The sense Gh-5 gene construct was developed by isolating the cDNA fragment from the pCR 2.1 vector. The 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 (kindly provided by Dr. James Carrington, Dept. of Biology, Texas A&M Univ.). 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 pCGN-Gh-5 plasmid was transformed into *Agrobacterium* strain EHA 101 (van Haute et al., 1983).

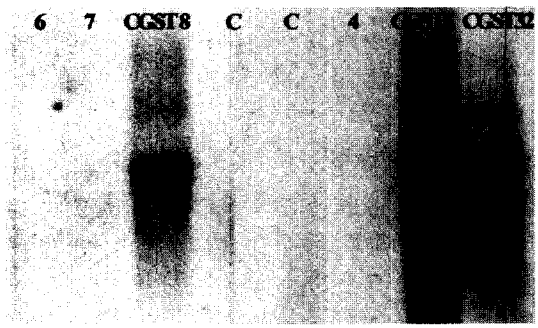

Fig. 1. Northern blot of lines of CGST5, CGST8 and CGST32 which were classified as Type II. Isolated total RNA (30 μg) from the transgenic tobacco plants and wild type plant was separated, blotted onto nitrocellulose, and hybridized with a ^{32}P-labeled Gh-5 cDNA. C; wild type Xanthi tobacco plants. Lane 6, 7, and 4 are nonexpressors. The size of thick band in CGST5, CGST8 and CGST32 is about 0.9 kb.

Agrobacterium-mediated Plant Transformation

The sense Gh-5 gene construct in pCGN 1578 was mobilized into *A. tumefaciens* strain EHA 101 by direct transformation. A single colony was inoculated into 50 ml of MG/L media with 50 g/ml kanamycin. The culture was grown overnight at 30°C in a shaking incubator (250 rpm) and the overnight culture was added into new 50 ml MG/L media without antibiotics and grown for 3 h. Cells were collected by centrifugation for 10 min at 12,000 rpm and resuspended in 2 ml of fresh MG/L media. Approximately 1 g of the purified plasmid that contains a specific chimeric gene construct was mixed with 200 μL of the pelleted cells. The mixture was frozen immediately in liquid nitrogen, thawed at 37°C for 5 min, transferred to 1 ml of MG/L and grown for 2 h at 30°C in a shaking incubator. The culture was plated onto MG/L plates containing 100 g/ml gentamycin and 50 g/ml kanamycine, and grown at 30°C. Putative transformant colonies were grown in liquid culture with 100 g/ml gentamycin and

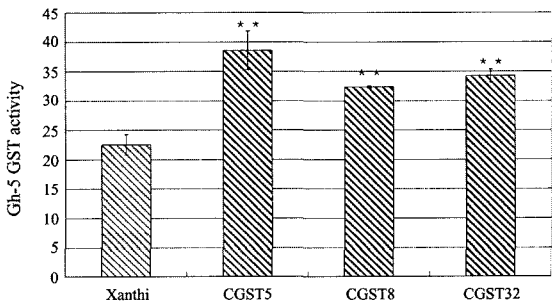

Fig. 2. Spectrophotometric GST assay of Gh-5 transgenic tobacco plants using CDNB as a substrate. In the Gh-5 GST activity, GST transcripts showed about 2.5- and 1.5-fold GST specific activity, respectively. Unit is μmol/min/mg protein.

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).

Northern Blot Analysis

Isolated total RNA (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°C in hybridization solution. The solution contained 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: 0.18 M NaCl, 10 mM 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., Cleveland, OH). After overnight hybridization, the filters were washed, exposed to the X-ray film at 80 with an intensifying screen and developed.

GST Enzyme Assay for Transgenic Plants

Activities of GST enzyme from the transgenic tobacco and control tobacco plants were evaluated by determining kinetic parameters with respect to the model substrate 1-chloro-2,4-dinitrobenzene (CDNB) according to the procedure of Mozer et al (1983). The reaction mixture contains final concentrations of 10 mM GSH and 1 mM CDNB, which is dissolved in 2.5% ethanol, and potassium-phosphate buffer (0.1 M, 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

To determine whether increased levels of GST can affect the tolerance of plants to abiotic stress, the growth of seedlings was analyzed under various stress conditions such as low temperature and salt stress. Mature seeds from three independent lines of transgenic Gh-5 overexpressing plants and Xanthi as control plants were harvested and sown on petri dishes lined with filter papers. For low temperature treatment, the Petri dishes were incubated in growth chambers at 15°C. For the salt treatment, seeds were treated with different concentrations of NaCl, i.e., 0, 50, 100, 150, 200 mM, and incubated at 25°C in the dark. Seeds were also used for the successful transformation of cotton gst gene into tobacco plants by testing seedlings on the medium which are containing antibiotic kanamycine (50 μg/ml) (Fig. 6).

Result and Discussion

To determine whether Gh-5 could be overexpressed in transgenic plants, the pCGN-Gh-5 gene constructs were introduced into tobacco using *Agrobacterium*-mediated transformation. Independently transformed Gh-5 tobacco plants that expressed the Gh-5 construct were analyzed. Transgenic tobacco plants that Northern blot analysis using a ^{32}P-labeled full length Gh-5 cDNA probes were initially identified overexpressed constructs (Fig. 1). Three T_0 tobacco plants that contain the Gh-5 gene construct were selected for assay. These include CGST5, CGST8, and CGST32. A Northern blot of the Gh-5 expressors and non-expressors was hybridized to a ^{32}P-labelled full length Gh-5 cDNA. The autoradiogram indicated that three of Gh-5 transgenic tobacco plants had high steady-state levels of Gh-5 mRNA. No hybridization was detected in wild type non-transgenic and wild type tobacco plants (Fig. 1). These were designated Type II. Levels of total GST specific activity was assayed in transgenic plants. Using CDNB and reduced glutathione as substrates, spectrophotometric assay of Type II GST-expressing transgenic tobacco plants enhanced approximately 1.5-fold increase in activity in leaf extracts over control and non-expressers plants (Fig. 2). The results from this assay confirmed that Gh-5 transgenes encode an active GST in transgenic tobacco plants. Evaluation about the effects of overexpression of cotton GST was performed on the several environmental stressors, such as low temperature and salinity. 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) (Fig. 3). To test for tolerance to salinity, seeds of Gh-5 overexpressors and the wild type Xanthi seedlings were grown in solutions of 0,

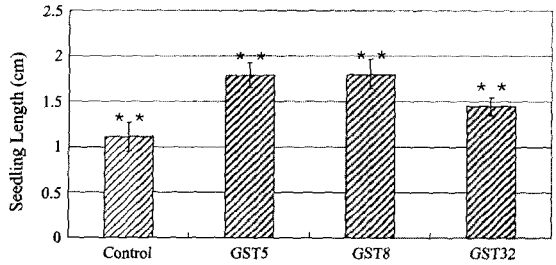

Fig. 3. Seedling length of Gh-5 transgenic tobacco at 15°C. Error bars indicate S.E. **denotes significant difference at P<0.01.

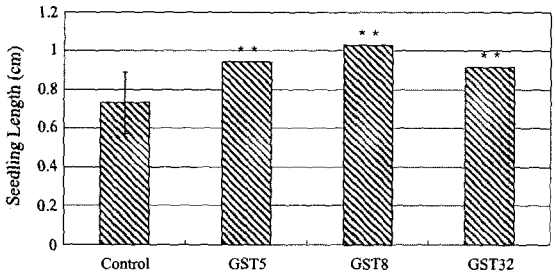

Fig. 4. Seedling growth in the 50 mM NaCl solution. The growth rate was significantly different with control and transgenic seedlings. Error bar indicates S.E. **means significant at P<0.01.

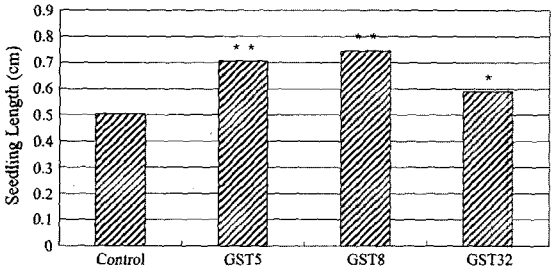

Fig. 5. Seedling growth of *Nicotiana tabacum* at 100 mM NaCl. * and ** mean significant at P<0.05 and P<0.01, respectively.

50, 100, 150 and 200 mM NaCl. The growth pattern was similar in both transgenic and non-expressor seedlings at control (water). At 50 and 100 mM NaCl, Gh-5 transgenic seedlings showed higher growth rate over control seedlings (Fig. 4 and 5). But at 150 and 200 mM NaCl concentration for six days incubation, the difference in growth rate was not detected. From the result of the salt experiment, 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.

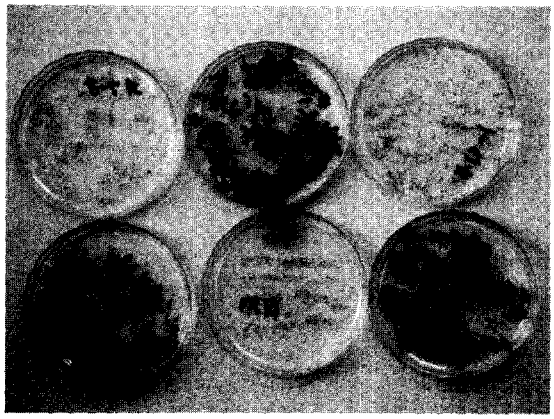

Fig. 6. Kanamycine selection of T_0 seeds. Seedlings from transgenic plants of *Nicotiana tabacum* are growing without death, but non-expressor seedlings are white because of death.

As a result, Northern blot analysis confirmed the successful transformation of cotton gst gene in tobacco plant. Two types of transcript patterns were identified in transgenic tobacco plants. The activity of GST in the Type II transgenic plants was about two-fold activity higher than those of the wild type and non-expressers by using CDNB as the substrate. The expression of cotton GST in tobacco plants proved that Gh-5 could be translated into functional protein. Overexpression of cotton Gh-5 GST gene resulted in increased GST activity in transgenic tobacco plants. Type II Gh-5 transgenic plants produced functional GST in the cells. Transgenic seedlings of Type II GST performed faster growth rate than Type I at low temperature, though GST specific activities were low than Type I transcripts. Type II transcripts of cotton gst gene can be expressed in tobacco plants and can protect transgenic plants from low temperature and mild salty stress conditions.

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염분과 저온에 대한 내성증진을 위한 GST 유전자의 과발현

강원희 · 김준철[1] · 김일섭

강원대학교 농생명대학 원예학부, [1]강원대학교 자연과학대학 생물학전공

적 요

목화의 Glutathione S-Transferase(GST) cDNA를 cloning한 뒤 담배식물체에서 과발현시킨 뒤 유전자의 기능을 분석하였다. Northern blot 분석으로 목화의 GST 유전자가 성공적으로 담배식물체의 염색체에 도입된 것을 확인하였다. Type I과 Type II의 전사체들이 인지되었고 이 보고에서는 Type II 전사체들의 역할을 기술하였다. Type II 전사체들을 발현하는 형질전환 식물체들은 야생형 또는 비형질전환체와 비교하였을 때 약 1.5배 이상의 GST 효소활성을 나타내었다. GST 효소의 활성은 1-chloro-2,4-dinitrobenzene (CDNB)와 글루타치온을 기질로 사용하여 측정하였다. 담배식물체에서 목화 GST cDNA의 과발현은 이 유전자가 기능을 갖는 단백질로 번역이 될 수가 있다는 것을 보여준다. 형질전환된 담배 유묘를 저온(15°C)과 광이 있는 상태에서 키워 GST유전자의 역할에 대한 기능을 시험하였다. GST 유전자의 형질 전환체들은 대조구의 유묘들과 비교하여 보았을 때 성장이 좋았다. 소금에 대한 내성 시험에서도 효과를 보였다. 0, 50, 100, 150, and 200 mM NaCl 농도에서 생장시험을 하였다. 50, 100 mM NaCl 농도에서 GST 형질전환 유묘들은 성장이 대조구에 비하여 유의성을 보였으나 0, 150, 그리고 200 mM의 소금농도에서는 성장의 차이를 보이지 않았다.

주제어 : 저온, 염분, Glutathione S-transferases(GST), 과발현, 내성