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# ROLES OF GLUTATHIONE S-TRANSFEREASE IN MAIZE (*Zea mays* L.) UNDER COLD STRESS

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## ABSTRACT

Glutathione S-transferase (GST) activities involved in antioxidant defense and methylglyoxal detoxification were investigated in the seedlings of a Bangladeshi maize variety, BARI hybrid maize-7, to understand the protecting mechanism under cold stress condition. The activities of glutathione S-transferase (GST) increased, while the activities of catalase (CAT) decreased with the duration of stress. The western blot analysis of the dominant GST revealed that it significantly accumulated during the stress period. The continual increase in $H_2O_2$ contents along with reduced redox state and activities suggested their roles in maintaining the glutathione homeostasis. The accumulation of GST with the content of $H_2O_2$ suggested its detoxification roles for organic hydroperoxides during chilling stress. Considering all, glutathione S-transferase (GST) enzymes showed protective role in maize from oxidative damages under Chilling condition.

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## INTRODUCTION

Cold stress adversely affects plant growth and development. Cold stress is the collective form of Chilling and freezing stresses. Generally chilling stress occurs in between 2 to 10$^{o}$C temperature. But, some tropical species, rice and sugarcane are highly sensitive to chilling and show injury symptoms up to 15$^{o}$C (Thomashow, 1999). In freezing stress, ice forms with in plant tissues. Chilling injury is one of the serious problems during germination and early seedling growth in many plant species including maize and rice (Bedi and Basra, 1993). Visible symptoms of chilling damage depend on the species, plant age and the duration of exposure. Seedlings showed wilting, reduced leaf expansion and chlorosis upon exposure to chilling stress in rice (Yoshida et al., 1996). Due to aberrant metabolism, toxic metabolites and reactive oxygen species (ROS) accumulates in the injured cells (Farooq et al., 2009). Chilling stress results accelerated senescence and ultimately the plant death (Sharma et al., 2005).

In recent years, Rangpur, Rajshahi and most part of Dhaka divisions has been experiencing chilling temperatures during winter season for at least 30 to 40 days. During this time, farmers sow maize seeds or the early varieties are in seedling stage. Therefore, chilling stress will be a major headache for farmers to cultivate maize. On the other hand, these areas are major producer of maize. BARI Hybrid Maize-7 (BMH-7) is a good yielder and has stress tolerance capability. Therefore, the farmers are using the variety for more production. In this view, this study was designed to investigate the biomolecular responses of some glutathione dependent enzyme in adaptive response of BMH 7 under chilling in seedling sage in green house condition.

## MATERIALS AND METHODS

### Plant material

Maize (Zea mays L.) was managed in greenhouse condition as plant material. Bangladesh Agricultural Research Institute (BARI) released hybrid maize variety BARI Hybrid Maize-7 (BMH-7), has stress tolerance capability, and was taken into consideration.

### Stress treatment

Maize seedlings were grown in pot in greenhouse condition. After reaching two leaf stages, the seedlings were ready for stress treatment. The plants were kept at 4$^{0}$C temperature to induce chilling stress.

### Separation and purification of GSTs from crude enzyme solution of maize seedlings

Crude enzyme was extracted by homogenizing 80 g of fresh maize seedlings (except green part) in an equal volume of 25 mMTris-HCl buffer (pH 8.5), which contained 1 mM EDTA and 1% (w/v) ascorbate, with a Waring lender. The homogenate was squeezed through two layers of nylon cloth and centrifuged at 11500×g for 10 minutes, and the supernatant was used as a crude enzyme solution. Proteins were precipitated by ammonium sulfate at 65% saturation of the crude enzyme solution and centrifuged at 11500×g for 10 minutes. The proteins were dialyzed against 10 mM Tris-HCl buffer (pH 8) containing 0.01% (w/v) β-mercaptoethanol and 1 mM EDTA (buffer A) overnight to completely remove low molecular inhibitors. The dialyzate was applied to a column (1.77 cm i.d. × 20 cm) of DEAEcellulose (DE-52; Whatman, U.K.) that had been equilibrated with buffer A and eluted with a linear gradient of 0 to 0.2 M KCl in 600 ml of buffer A. The high active fractions of the highest GST peak were pooled and used to detect their inhibitory substances present in maize seedlings extract.

The pooled GST solution was directly applied on affinity column (0.76 cm i.d. × 4.0 cm) of S-hexylglutathioneagarose (Sigma, St. Louis, MO) that had been equilibrated with 10 mMTris-HCl buffer (pH 8.0) containing 0.01% (v/v) β-mercaptoethanol (buffer B). The column was washed with buffer B containing 0.2 M KCl and eluted with buffer B containing 1.5 mM S-hexylglutathione. The high active protein fractions eluted with S-hexylglutathione were combined and dialyzed against buffer B and the dialyzate was used as the purified GST.

**Production of polyclonal antibodies against GST**

A rabbit (weighing about 2.5 kg) received subcutaneous injections of a 0.5 mg of purified GST protein in Freund's complete adjuvant at several sites. After two weeks, the rabbit was given a first booster injection of 0.5 mg of the purified GST protein in incomplete adjuvant, and then a second booster injection of 0.5 mg of the purified protein in incomplete adjuvant was given two weeks after the first booster injection. Blood was taken from the ear vein one week after the second booster injection.

**SDS-PAGE and Western blotting**

SDS-PAGE was done in 12.5% (w/v) gel containing 0.1% (w/v) SDS by the method of Laemmli (1970). The gel was stained with silver. Western blotting was done on the basis of the Amersham ECL detection system.

## Protein quantification

Protein was estimated following the method of Bradford (1976).

**Preparation of soluble protein extracts**

The plant materials were homogenized with proportional volumes of 25 mMTris–HCl buffer (pH 8.0) containing 1 mM EDTA, 1% (w/v) ascorbate and 10% (w/v) glycerol with mortar pestle. The homogenate was centrifuged at 11500 x g for 15 min, and the supernatant was used as the crude enzyme solution. All procedures were performed at 0-4°C. For H2O2 assay, Fresh seedling samples were extracted by homogenizing in 6X volume of 50 mM K-P buffer (pH 6.5). The homogenate was centrifuged at 11500 x g for 15 min, and 2.5 ml supernatant was used for further centrifugation at 11500 x g for 15 min with 833 µl reaction mixture containing 5ml $H_2SO_4$ and 15 µl TiCl4. The 2nd supernatant was used for the assay of $H_2O_2$ spectrophotometrically.

**Enzyme assay**

Glutathione S-transferase (GST, EC: 2.5.1.18) activity was assayed following the methods of Rohman et al. (2010) spectrophotometrically (UV-1800, Shimadzu, Japan). The activity was calculated using the extinction coefficient of 9.6 mM-1Cm-1.

**Measurement of $H_2O_2$**

$H_2O_2$ was assayed according to the method described by Yu et al. (2000). $H_2O_2$ was extracted by homogenizing 0.5 g of leaf samples with 3 ml of 50 mM K-phosphate buffer pH (6.5) at 40C. The homogenate was centrifuged at 11,500g for 15 min. Three ml of supernatant was mixed with 1 ml of 0.1% $TiCl_4$ in 20% $H_2SO_4$ (v/v), and the mixture was then centrifuged at 11,500g for 12 min at room temperature. The optical absorption of the supernatant was measured spectrophotometrically at 410 nm to determine the $H_2O_2$ content ( = 0.28 $\mu M^{-1}$ $Cm^{-1}$) and expressed as µmol $g^{-1}$ fresh weight.

**SDS-PAGE and Western Blotting to check GST accumulation**

To check the accumulation of GSTa SDS-PAGE was done in 12.5% (w/v) gel containing 0.1% (w/v) SDS by the method of Laemmli (1970) followed by western blotting.

# RESULTS AND DISCUSSION

**Purification of dominant GST from maize seedlings for polyclonal antibody production**

To purify the dominant GST for polyclonal antibody production, crude enzyme solution was extracted from maize seedlings. The crude protein was precipitated by 65% $(NH_4)_2SO_4$ and dialyzed overnight in Buffer A (mentioned in Materials and Methods). The dialyzed protein was applied to a DEAE (DE-52, UK) cellulose column chromatography. A 750 ml gradient solution (0-0.4 MKCl) was passed through the column. Total 170 fractions (each contains 5 ml) were collected and activity ($A_{340}$) and absorbance ($A_{280}$) were measured. It was found that 3 GST peaks were eluted at 91.67, 162.50 and 234.04 mMKCl of the gradient solution (Fig.1).

Among the GSTs, peak eluted at 91.67 mMKCl contained more than 92.2% of total activity and it was termed as dominant GST, GSTa (Fig. 1) and other two peaks eluted at 162.50 and 234.04 mMKCl termed as minor GSTs, GSTb and GSTc. The dominant GST was further purified by subsequent application of S-hexylglutathione-agarose.

Total 1.5 mg purified dominant GST protein was prepared for production of polyclonal antibody. As basal dose, 0.5 mg protein was injected subcutaneously into a rabbit in complete adjuvant. After two weeks a booster dose was injected in incomplete adjuvant. Again after one week an income dose was injected and after one week blood was collected from rabbit and centrifuged at 3000xg and serum was used as antibody. To test the specificity of the antibody, the cross reactivity the high active GST fractions of each peak (Fig.1) was tested by western blotting. Fraction 87, 88 and 90 (dominant GST) reacted with the antibody and produced thick bands, but 127, 128, 129, 155,156 and 157 number fraction (minor GSTs) did not react with the antibody.

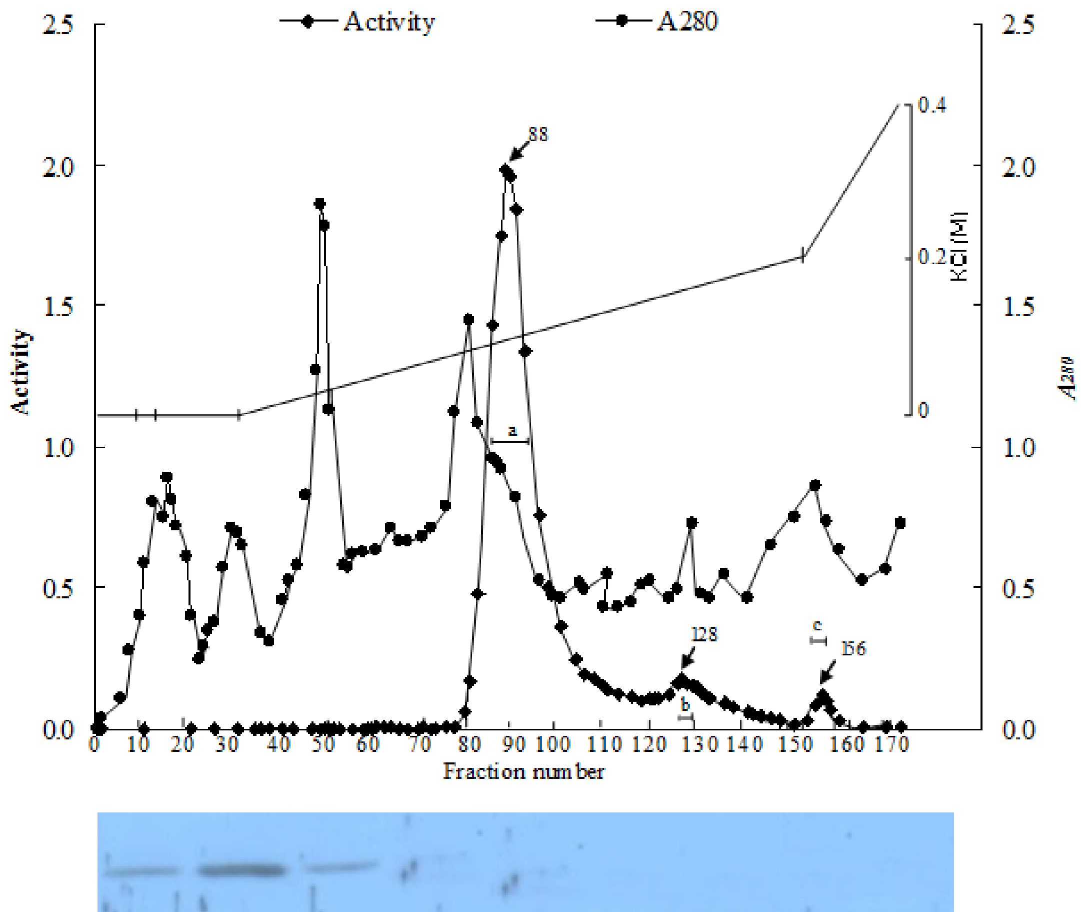


**Figure 1.** A typical column chromatography of DEAE-cellulose of soluble proteins prepared from 78 g maize seedlings (except green part). For each fraction, absorbance at 280 nm (●) and GST activity toward CDNB (♦) were determined. Activity is expressed as µmol $min^{-1}$ $ml^{-1}$. Bars indicate the high active peak fractions of three maize GSTs. The fractions under the bar of GSTa peak were pooled for subsequent purification. The curve shows the gradient solution of KCl (0-0.4 mM). The plate shows the cross reactivity of antibody with active fractions of the GST peaks.

This result suggested that the antibody is highly specific to GST against which it was developed.

**Glutathione S-transferease (GST) under chilling stress**

Expression of maize GST activities was observed to increase under chilling stress at 10 days of stress treatment. Chilling treatment at 10 day showed maximum GST activity (202.19 nmol/min/mg protein) followed by 7 day (138.61 nmol/min/mg protein) and 4 day (106.45 nmol/min/mg protein) of chilling treatment. Minimum GST activity was recorded in controlled plants (99.18 nmol/min/mg protein). The GST activity of untreated and 4 day treated plants was statistically similar (Fig. 2).

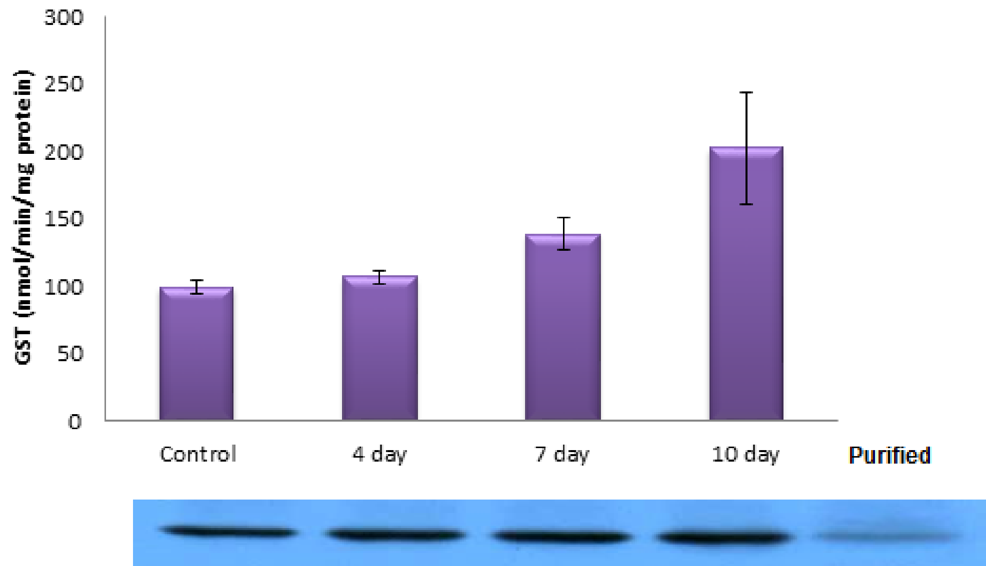


**Figure 2.** Activities and expressions of maize GSTs at different duration of chilling stress. Bar graph shows the GST activities towards CDNB in soluble protein extracts. Western blotting results show the expressions of GSTa at different duration of chilling stress treatment. Each lane contained 45 µg of the protein.

Western blotting of the GST protein extracts from different duration of chilling treatment was done to recognize the accumulation of GSTa. The expression of GSTa accumulation was detected at all conditions but the levels of GST accumulation varied greatly among different conditions (Fig. 2). Chilling stress caused significant increase in accumulation of GSTa compared to control. The highest expression was found at 10 day followed by 7 day, 4 day of chilling treated seedlings.

$H_2O_2$ increased continuously with duration of chilling treatments. Plants of 10 day chilling treatment showed highest $H_2O_2$ activity (11.52 µmol/g FW) followed by 7 day (11.32 µmol/g FW) chilling treatment. Plants of 4 day chilling treatment (7.10 µmol/g FW) and untreated plants showed the lowest (4.49 µmol/g FW) $H_2O_2$ contents (Fig. 3).

The CAT activity was found to be highest at 4 day (192.31 µmol/min/mg protein) which decreased successively at 7 day (84.35 µmol/min/mg protein) and 10 day (78.07 µmol/min/mg protein) of chilling treatment. Untreated plants showed lowest (57.84 µmol/min/mg protein) CAT activity (Fig. 3).

The chilling tolerance is a complex phenomenon, which entails an array of physiological and biochemical processes at whole plant, organ, cell and subcellular levels. These processes are reduced water loss by stomatal resistance, enhanced water uptake with the development of prolific root systems and synthesis and accumulation of osmolytes (Farooq et al., 2008a & b, 2009). Chilling stress is associated with increased oxidative stress due to enhanced accumulation of ROS, particularly $O_2^-$ and $H_2O_2$ in chloroplasts , mitochondria , and peroxisomes. Therefore, the induction of antioxidant enzyme activities is a general adaptation strategy which plants use to overcome oxidative stresses (Foyer and Noctor, 2003) and the up-regulation of GST activity over time would be indicating the adaptation of plants to chilling stress. In this study, maximum induction of GST over control was at 10 day stress treatment (103.85%) followed by 7 day (39.75%) and 4 day (7.32%) stress treatment.

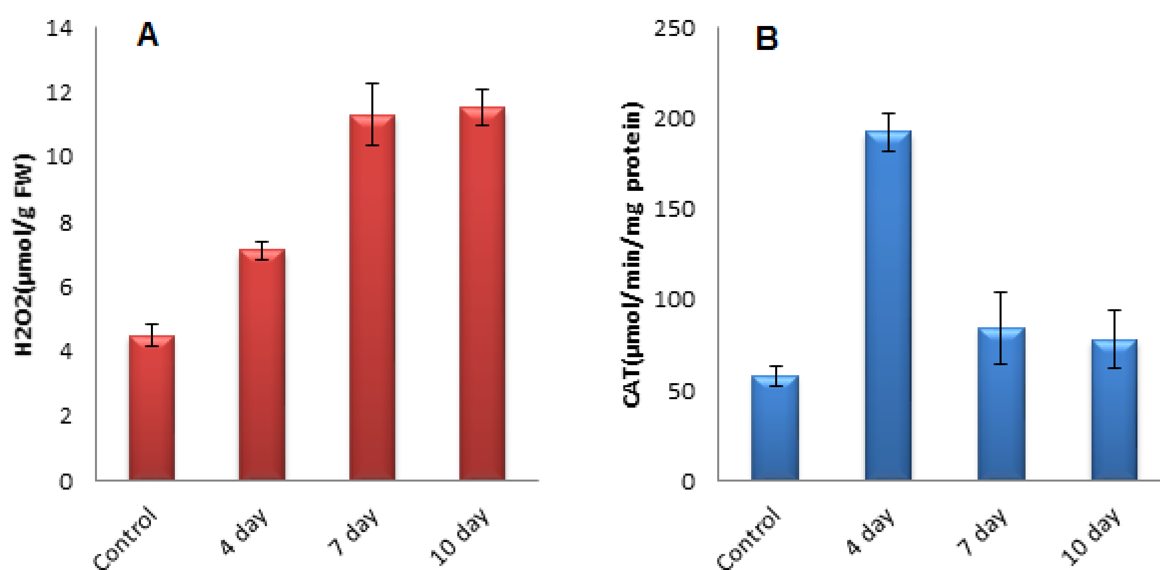


**Figure 3.** $H_2O_2$ contents (A) and catalase activities (B) in maize seedlings under different duration of chilling stress condition.

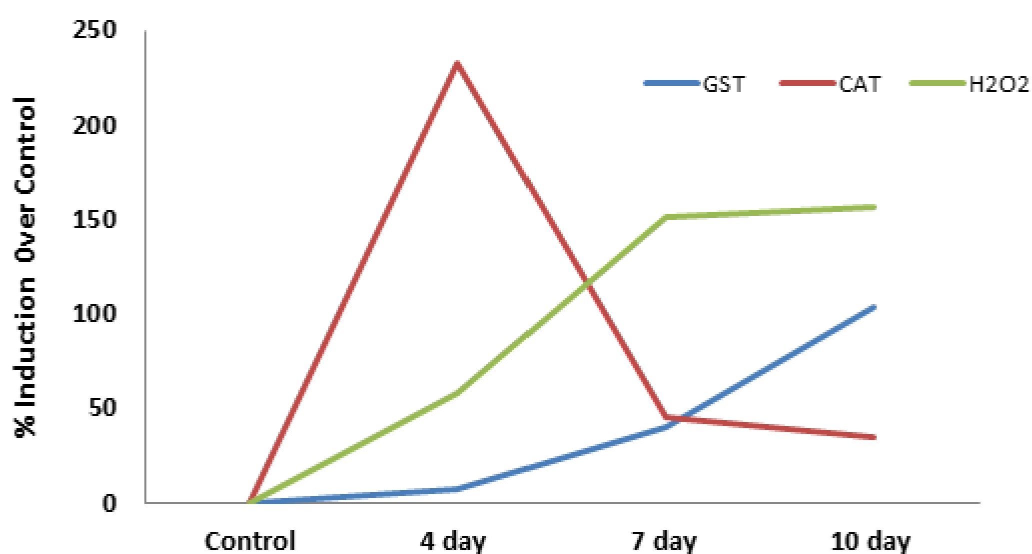


**Figure 4.** Induction rate of GST, CAT and $H_2O_2$ over control at different duration of cold stress treatment.

The induction of $H_2O_2$ over control was maximum at 10 day (156.46%) followed by 7 day (151.69%) and 4 day (58.1%) of stress treatment (Fig. 4). This result supported by the study of Lee and Lee (2000) as they found increased SOD activity in cucumber under chilling stress. Spontaneous dismutation and SOD decomposes ROS to produce $H_2O_2$. Therefore, the over production of $H_2O_2$ is supposedly due to the increased activity of spontaneous dismutation and SOD. However, GST usually not found to induce in all plant species.

The activity level and Western blotting of GSTa in maize under chilling stress suggested their protective role of maize under cold. The catalase activity showed the decreasing trend over time (Fig. 4). The induction of CAT over control was maximum at 4 day (232.48%) followed by 7 day (45.82%) and 10 day (34.98%) of stress treatment. Lee and Lee (2000) supported the result as they also found that under chilling stress CAT activity decreased in cucumber over time. The reduction of CAT activity is supposedly due to the inhibition of enzyme synthesis or change in the assembly of enzyme subunits under chilling stress condition.

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