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Up-regulation of onion bulb glutathione *S*-transferases (GSTs) by abiotic stresses: A comparative study between two differently sensitive GSTs to their physiological inhibitors

[1,2]M. M. Rohman*, [2]M. S. Uddin and [1]M. Fujita

[1]Department of Applied Biological Science, Faculty of Agriculture, Kagawa University, Miki-cho, Kita-gun, Kagawa 761-0795, Japan
[2]Plant Breeding Division, Bangladesh Agricultural Research Institute, Gazipur, Bangladesh

***Corresponding author: motiar_1@yahoo.com**

Abstract

Among the five GST isoforms (designated as GSTa and GSTb as minor, and GSTc, GSTd and GSTe as dominant GSTs) in onion bulb, GSTc, a phi-type GST, is highly sensitive to the inhibitions its physiological inhibitors. On the other hand, GSTe is less sensitive to such inhibition. In this study, GSTe was purified and its antibody was developed in rabbit antiserum to investigate its stress responses in comparison to those of GSTc. The distributions of GSTe and GSTc were also investigated in healthy onion plants. GSTe was purified 93-fold with a recovery of 3.5%. The purified enzyme showed single band with a molecular mass of 27 kDa in sodium dodecylsulfate-polyacrylamide gel electrophoresis. The expression levels of GSTe and GSTc were greatly varied in indifferent organs. Accumulation level of GSTe was abundant in onion bulb followed by callus, root and mature bulb, but not detected in young leaves. On the other hand, expression of GSTc was detected in all organs, where, the levels were abundant in root and bulb. However, expression level was higher in mature organs than young ones. Stress responses of GSTe and GSTc were examined in onion calli, where the levels of GST accumulation were also different by external stimuli. The level of GSTe was induced significantly in both saline and heavy metal treated calli. However, induction of GSTe was weak in osmotic stress and not detectable in low temperature stress. At the same time, level of increased accumulation of GSTc was detected under all stresses, where osmotic stress (PEG) efficiently induced the expression level. Though these two onion bulb GSTs responded differently to stresses, this study implicated that they have important role in stress combating.

Keywords: Onion GSTs, comparison, up-regulation, abiotic stress

Abbreviations: CDNB_1-Chloro-2,4-dinitrobenzene; DEAE_Diethylaminoethyl; EDTA_Ethylenediaminetetraacetic acid; GST_Glutathione *S*-transferase; i.d._Internal diameter; h_Hour; g_Gram; kg_Kilogram; kDa_Kilo Dalton; HM_ Heavy metal; min_Minute; PEG_Polyethyleneglycol; ROS_Reactive oxygen species; 2,4-D_2,4-Dichlorophenoxy acetic acid

Introduction

Glutathione *S*-transferases (GSTs; E.C. 2.5.1.18) are a family of enzymes which catalyze the conjugation of reduced glutathione (GSH, a tripeptide *γ*-Glu-Cys-Gly) to a variety of electrophilic compounds to direct them to specific sites both intra- and extra-cellularly. In plant, GSTs, originally characterized by their ability to modify xenobiotics covalently by glutathionation, are rapidly induced by their substrates, particularly plant herbicides (Marrs et al., 1995; Aharoni and Vorst, 2002; Dean et al., 2005). There has been increasing evidence indicating that GSTs may be implicated in many other physiological processes. These include stress responses (Anderson and Davis, 2004; Ezaki et al., 2004), transportation of endogenous substrates (Mueller et al., 2000), reduction of organic hydrogen peroxides and isomerization of specific metabolites (Edwards et al., 2000), binding of auxin (Bilang et al., 1993; Zettl et al., 1994), cytokinin (Gonneau et al., 1998), and UV light dependent signal transduction (Loyall et al., 2000). A wide range of biotic and abiotic factors induce plant GST expression. These include herbicides, heavy metal, pathogen attack, ethylene, ozone, plant growth factor auxin, salicylic acid and hydrogen peroxide (Marrs, 1996). Environmental stresses like osmotic stress (Galle et al., 2008), low temperature (Boot et al., 1993), saline stress (Sappl et al., 2004) and cadmium (Marrs and Walbot, 1997) also induce GST expression in plant. Therefore, GSTs are thought to play vital roles in the diversity of stress physiologies through some other functions as well as GST-mediated detoxification. A protective role of these enzymes is suggested in the enzymatic up-regulation of plant GSTs as being one of the important parameters under environmental conditions for cellular survival.

Onion bulb exhibits higher GST activity compared

to other vegetable crops (Hossain et al., 2007). Mature onion bulbs and culture cells have therefore been used in GST research in our laboratory. Onion bulb GSTs have five component GSTs (designated as GSTa and GSTb as minor, and GSTc, GSTd and GSTe as dominant GSTs), and among the dominant GSTs, GSTc, a phi-type GST, is highly sensitive to the inhibition of its physiological inhibitors, quercetin-4'-glucoside and quercetin-3,4'-diglucoside (Rohman et al., 2009a,b). However, GSTe was less sensitive to such inhibitoion. Therefore, GSTc and GSTe might also response differently to abiotic stress. Moreover, flavonoid related proteins have limited information to induce under environmental stresses. *Bz2*, which is flavonoid carrier GST protein in maize, has been reported to be up-regulated by abiotic stresses (Marrs and Walbot, 1997). Therefore, flavonoid sensitive onion GST, GSTc might also be up-regulated under environmental stress. In this study, we compared the stress responsibilities of GSTe and GSTc to abiotic stresses, including NaCl, cold temperature (4ºC), PEG and heavy metal ($CdCl_{2)}$. For these purposes, GSTe was purified and its antibody was developed in rabbit blood serum and this antibody was used to detect the expression of GSTe in soluble protein extracts of different organ of onion plants and calli. Antibody of GSTc (Rohman et al., 2009a) was used to recognize the expression of GSTc. In this report, we also described the expression pattern of GSTe as well as GSTc in healthy onion plants.

Materials and methods

Plant materials

Mature bulb (*Allium cepa* L.,), variety "Senshyu Yellow" was used as source of GSTe. Expressions of GSTe and GSTc were examined in onion seed callus (prepared from same variety) and different parts of onion plant.

Induction of callus and stress treatments

Callus was induced from onion seed cultured on Murashige and Skoog (MS) (Murashige and Skoog, 1962) solid medium that contained 4.5 µM 2,4-D and 0.5 µM kinetin at 25ºC. Propagated calli, after 3 subcultures, were used for various stress treatments. One gram (g) of calli was then transferred to the same medium that contained 200 mM NaCl for salinity stress, 1 mM $CdCl_2$ for heavy metal stress and 10% PEG for osmotic stress, and incubated at 25ºC in the dark for 96 h. For low temperature stress, callus was incubated at 4ºC in the dark. Callus transferred to MS medium in the dark at 25ºC was used as control.

Extraction of soluble protein

Callus or organs were extracted by homogenizing in an equal volume of 25 mM Tris-HCl buffer (pH 8.5), which contained 1 mM ethylenediaminetetraacetic acid (EDTA) and 1% (w/v) ascorbate, with a Waring blender. The homogenate was squeezed through two layers of nylon cloth and centrifuged at 11,500×*g* for 10 minutes, and supernatant was used as soluble protein solution for enzyme purification and western blotting analysis.

DEAE-cellulose chromatography

Proteins were precipitated by ammonium sulfate at 65% saturation from the supernatant and centrifuged at 11,500×*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 (crude enzyme solution) was applied to a column (1.77 cm i.d. × 20 cm) of DEAE-cellulose (DE-52, Whatman, UK) 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 eluted peak at around 157 mM KCl was collected for further purification.

Hydroxyapatite chromatography

The pooled sample of GSTe, separated by DEAE-cellulose column chromatography, was applied on a hydroxyapatite column (1.5 cm i.d. × 5.5 cm) that had been equilibrated with buffer A. The column was eluted with a 300 ml linear gradient of potassium phosphate buffer (0-20 mM; pH 7.0) in buffer A. The high active fraction (5 ml) was found to elute which was collected and further purified by affinity chromatography.

Affinity chromatography

The collected sample was applied to a column (0.76 cm i.d. × 4.0 cm) of *S*-hexylglutathione-agarose (Sigma, St. Louis, MO) that had been equilibrated with 10 mM Tris-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 1.2 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 GSTe.

Production of polyclonal antibodies against GSTe

A rabbit (weighing about 3 kg) received subcutaneous injections of a 0.5 mg of purified GSTe 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 GSTe 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.

Enzyme assay *and protein quantification*

GST activity was determined spectrophotometrically

by the method of Fujita and Hossain (2003). The reaction mixture contained 100 mM potassium phosphate buffer (pH 6.5), 1.5 mM reduced glutathione (GSH), 1 mM 1-chloro-2,4-dinitrobenzene (CDNB), and inhibitor and enzyme solutions in a final volume of 0.7 ml. The enzyme reaction was initiated by the addition of CDNB, and absorbance (A_{340}) was monitored at 25˚C for 1 min. Protein was estimated following the method of Bradford (1976) and Lowry et al. (1951).

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.

Results and discussion

Onion bulb GSTs, GSTa, GSTb, GSTc, GSTd and GSTe elute at 43, 65, 106, 117 and 157 mM KCl, respectively, of a DEAE-cellulose chromatography (Rohman et al., 2009a,b). Among the dominant GSTs, GSTc and GSTe are differently sensitive to the inhibition of their physiological inhibitors (Rohman et al., 2009a,b). Therefore, these two GSTs might also be different in responses to abiotic stresses. To examine their responses, first, GSTe was purified DEAE-cellulose, hydroxyapatite, and affinity column chromatography. The soluble protein fraction prepared from 150 g fresh onion bulb tissue was applied on DEAE-cellulose column chromatography and eluted with a liner gradient of KCl (0-0.2 M) (Fig. 1). The GST activity was measured towards model substrate CDNB. The fractions corresponding to the high GSTe active peak at around 157 mM KCl were combined as the GSTe pool. The GSTe pool was subjected to subsequent purification steps.

The high active GSTe pool was applied to a hydroxyapatite column (1.5 cm i.d. × 5.5 cm) and eluted with a linear gradient of potassium-phosphate (K-P) buffer (0-20 mM). A high active GST fraction (fraction number 14) (Fig. 2) was found which was subjected to purify on an affinity column.

The active fraction was put on a column of *S*-hexylglutathione-agarose to complete the purification. In the purification, the final product was purified 93 fold with a yield of 3.5%. The specific activity of purified GSTe was 72295 nmol min^{-1} ml^{-1} protein towards CDNB. The purity and molecular mass of the preparation was examined by sodium dodecyl-sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The silver staining of the purified GSTe indicated that final product was highly purified and migrated as a single band on SDS-PAGE with an apparent molecular mass of 27 kDa (Fig. 3).

To recognize the expression of GSTe in stressed organ as well as in healthy plant and callus, its antibody was developed in rabbit antiserum against the purified enzyme. To test the specificity of the antibody,

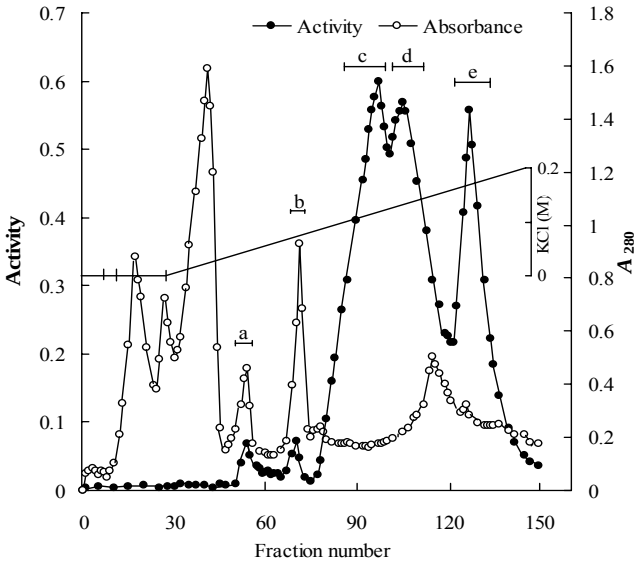

Fig 1. A typical column chromatography of DEAE-cellulose of soluble proteins prepared from 150 g onion bulb tissues. For each fraction, absorbance at 280 nm (●) and GST activity toward CDNB (o) were determined. Activity is expressed as μmol min^{-1} ml^{-1}. Bars indicate the high active peak fractions of five onion bulb GSTs. The fractions under the bar of GSTe peak were pooled for subsequent purification. The curve shows the KCl gradient.

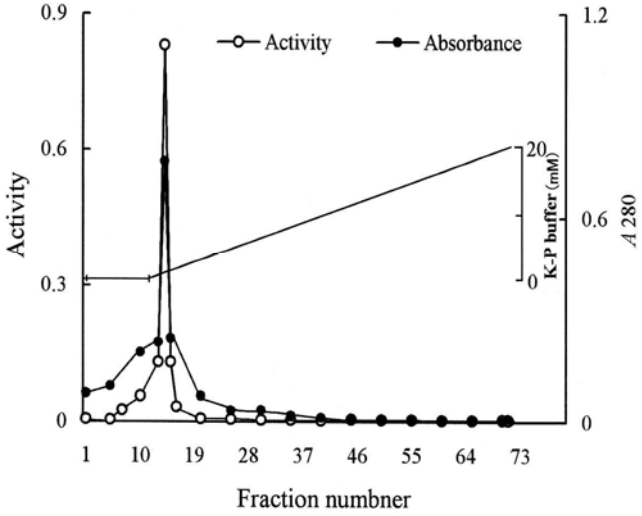

Fig 2. Elution profile of GSTe from hydroxyapatite column chromatography. The line indicates the linear gradient of potassium-phosphate (K-P) buffer. For each fraction, absorbance at 280 nm (●) and GST activity toward CDNB (o) were determined. Activity is expressed as μmol min^{-1} ml^{-1}.

the cross reactivity of each peak fraction of all the isoforms of onion bulb GSTs (Fig. 1) was tested by Western blotting. The antibody reacted only with the protein of peak fraction of GSTe (Fig. 4). Therefore, this antiserum was used as antibody of GSTe.

To investigate the abundance of GST expressions in different plant parts and callus, soluble proteins were extracted and GST activities of the soluble protein extracts were measured towards model substrate

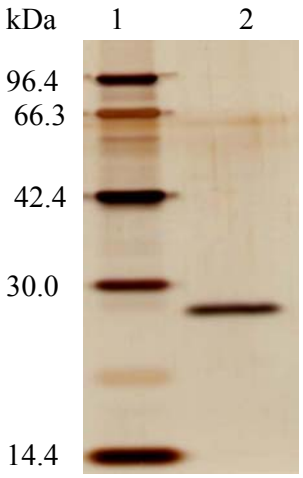

Fig 3. SDS-PAGE of purified GSTe from onion bulb. GSTe, finally purified by chromatography on *S*-hexyl-glutathione-agarose, was submitted to SDS-PAGE. The gel was stained with silver. 1, molecular marker; 2, purified GSTe.

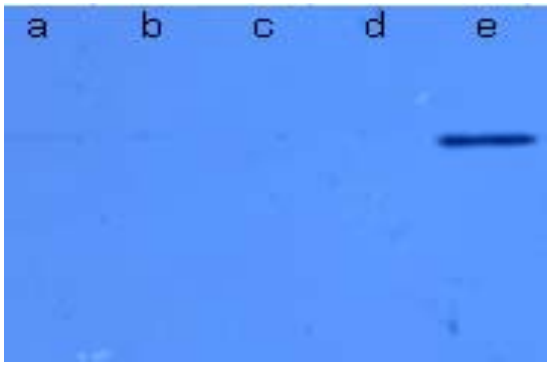

Fig 4. Cross reactivities of anti-GSTe antiserum against the peak fraction of five GSTs (corresponding to Fig. 1) from onion bulb GSTs separated by DEAE-cellulose chromatography. A 14 µl sample from each peak was applied on SDS-PAGE. a, GSTa; b, GSTb; c, GSTc; d, GSTd and e, GSTe. The antiserum detected only GSTe.

CDNB. Root showed the highest GST activity (747 nmol min^{-1} ml^{-1} protien) followed by mature bulb (662 nmol min^{-1} ml^{-1} protein), callus (378 nmol min^{-1} ml^{-1} protein) and young bulb (374 nmol min^{-1} ml^{-1} protein) (Fig. 5). Young and mature leaves showed lower GST activity (17 and 66 nmol min^{-1} ml^{-1} protein, respectively). However, the GST activity was higher in mature organs compared to young ones. To recognize the accumulation of GSTe and GSTc, Western blottings of the GST protein extracts from different organs were done and it was found that levels of GST accumulation varied greatly among different parts, where the expression of GSTc accumulation was detected in all the organs but GSTe was not detectable in young leaves. Accumulation of GSTe was highest in mature onion bulb followed by callus, young bulb, root and mature leaves. On the other hand, root accumulated the highest level of GSTc followed by mature bulb, callus,

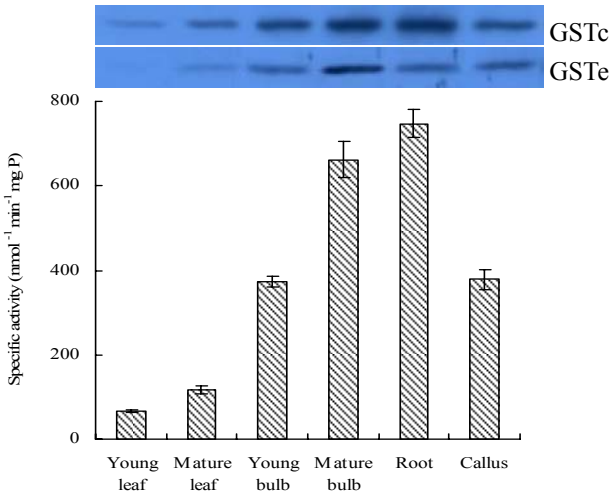

Fig 5. Activities and expressions of onion GSTs in different parts of onion plant and callus. Bar graph shows the GST activities towards CDNB in soluble protein extracts from young leaves, mature leaves, young bulb, mature bulb, root and callus. Western blotting results show the expressions of GSTe and GSTc in different organs of onion plants and callus. Each lane contained 30 µg of the protein. Results were obtained from three independent experiments, and typical Western blotting results is shown from one experiment.

young bulb and mature leaves. However, expression level of GSTc in young leaves was least abundant. GST expression may vary with gene member in tissues or spatially and temporally during plant growth and development. In *Arabidopsis*, one GST homolog, At103-1a, has been shown to express constitutively in the green part of the plant but the expression is detectable only in roots (van der Kop et al., 1996). However, expression of another GST gene, At103-1b, can be detected in both green plant part and root. Several GST isoforms (*At*GSTF2, *At*GSTF8 and *At*GSTU19) specifically expressed during the formation of early root epidermis of *Arabidopsis* have also been isolated (Mang et al., 2004). Nevertheless, results of the present study show that GST expression can be detected in all onion organs tested but the accumulation level varies spatially, with most abundant level accumulating in the roots and bulbs. In leaves, level of GST accumulate preferentially in the older than younger tissues, indicating that GST expression is temporally regulated. The biological significance of preferential accumulation of GST level in roots is not clear. It is speculated that the presence of GSTs in roots at high levels may be associated with plant defense against stress in the growing environment (Gong et al., 2005).

Abiotic stresses reduce the productivity of agricultural crops and lead to oxidative damage (Bowler et al., 1992). Plants employ several defense mechanisms to combat oxidative stress (Price et al., 1994). Among the defense mechanisms, enzymatic defense is the most important one. In enzymatic defense, the importance of GSTs in intracellular redox

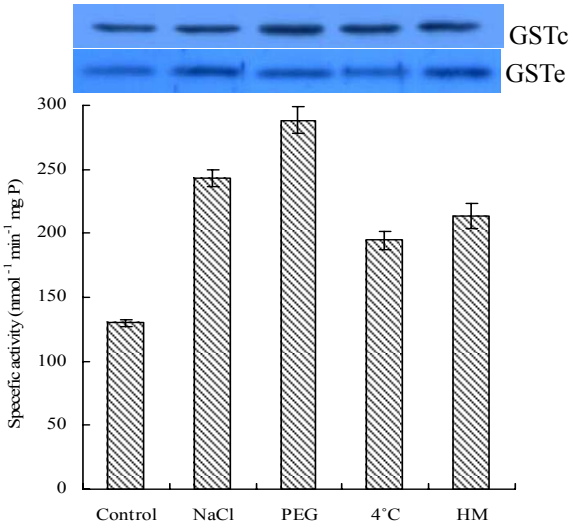

Fig 6. Effects of various stresses on the activities and expressions of GSTs in onion calli. Bar graph shows the GST activities towards CDNB in soluble protein extracts under stresses: salinity (NaCl, 200mM), osmotic (PEG, 10%), low temperature (4ºC) and heavy metal (HM; $CdCl_2$, 1 mM). The Western blotting results showed the induction of expression level of GSTe and GSTc under the stresses. Each lane contained 20 µg of the protein. The experiment was repeated three times and typical western bolt results are shown from one experiment.

state has been reported by several research groups (Pinkus et al., 1996; Roxas et al., 1997; Polidoros and Scandalios 1999; Dixon et al., 2002). To compare the physiological responses of the two GSTs, the expressions of GSTe and GSTc were investigated in soluble protein extracts of onion calli under salinity (NaCl), osmotic (PEG), low temperature (4˚C) and heavy metal ($CdCl_2$) stresses. All of the stresses caused significant increase in GST activity in soluble extracts towards CDNB within a short period (96 h) treatment. Osmotic stress sharply increased (2.2-fold) the GST activity compared to non-treated callus, which was followed by salinity (1.8-fold), heavy metal (1.6-fold) and low temperature (1.5-fold) stresses (Fig. 6). Therefore, the soluble extracts were subjected to Western blotting to observe the level of accumulation of GSTe and GSTc proteins. It was observed that different stresses affected the accumulation level of GSTe and GSTc differently. Saline and heavy metal stresses induced the accumulation of GSTe expression, while PEG induced very weakly. However, expression GSTe was not found to be induced in low temperature. On the other hand, level of GSTc was found to induce in all the stresses, where the osmotic stress accumulated its expression most abundantly indicating its important contribution to increased GST activity in onion callus. The level of increased accumulation in the both GSTs under saline and cadmium stresses revealed that both GSTs are responsible for increasing GST activity under these stresses. However, these results suggested that GSTe and GSTc are differently responsive to different stresses. So, it is possible that the roles of theses two GSTs in onion are different. Inducibility of different GSTs in particular organ can be different in a given stress. Three pumpkin GSTs, *Cm*GSTU1, *Cm*GSTU2 and *Cm*GSTF1 were reported to be differently responsive to different stresses (Fujita and Hossain, 2003). Boot et al. (1993) worked with tobacco GST gene pCNT103, -107, -114, and –115 and reported that pCNT107 mRNA showed increased accumulation in response to heat and cold stress. Kiyosue et al. (1993) reported the induction of two *Arabidopsis GST* genes by dehydration stress.

Plant stresses usually accompanied by increased reactive oxygen species (ROS) production (Holmberg and Bulow, 1998). PEG (10%), which effectively caused GSTc accumulation through imposing osmotic stress in onion callus cells, can also provoke other adverse effect by limiting oxygen movement (Verslues et al. 1998; Moons, 2003) and/or other toxic effects (Plouta and Federman, 1985). Induction of GSTs by PEG was reported in wheat (Galle et al., 2008), rice by Moons (2003). An aspect of salinity stress in plants is the stress induced production of ROS causing oxidative damage to different cellular components including membrane lipids, proteins and nucleic acids (Haliwell and Gutteridge, 1986). Low temperature may trigger oxidative stress through accumulation of hydrogen peroxide in cells, resulting in lipid peroxidation in membranes, which are the primary sites for freeze injury in plants (Steponkus, 1990; Anderson and Davis, 2004). Cadmium produces oxidative stress possibly by generating free radicals and ROS. Many plant GSTs are reported in mitigating the oxidative stress by improving redox state in cell (Dixon et al., 2002; Misra and Gupta, 2006; Jogeswar et al., 2006)). Therefore, though the inducible responses of GSTe and GSTc to stresses were different, this study implicated that they have important role in stress tolerance in onion. However, the molecular characterization of GSTe is of timely essence.

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