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Stress-induced changes of methylglyoxal level and glyoxalase I activity in pumpkin seedlings and cDNA cloning of glyoxalase I gene

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Abstract

Abiotic stresses cause extensive losses to agricultural production worldwide. In this study, the effects of various abiotic stresses on the upregulation of methylglyoxal levels and glyoxalase I activities in pumpkin seedlings (*Cucurbita maxima* Duch.) were investigated. Most of the stresses caused significant increases in methylglyoxal level and glyoxalase I activity, white light causing the highest induction followed by salinity, chemical, drought, and heavy metal stresses. We showed that accumulation of methylglyoxal in plants under various stressful conditions is a common phenomenon, and methylglyoxal could therefore act as a signal for plants to respond to stress. The stress-induced increases in methylglyoxal level, glyoxalase I activity and *Gly I* transcript found in the present study suggest an important role of glyoxalase I in conferring tolerance to plants under stress conditions and showed that the glyoxalase pathway is the main detoxification pathway of methylglyoxal in plants. The multistress response of glyoxalase I gene indicates its future utility in developing tolerance to various stresses in crop plants. A cDNA encoding glyoxalase I has been isolated, subcloned and nucleotide sequence was determined. The pumpkin glyoxalase I cDNA consists of 975-bp nucleotides encoding a polypeptide of 185 amino acids having a predicted molecular weight of 20,772.14 Da. Based on the number of amino acids, it is categorized as short-type glyoxalase I and the nucleotide sequence of pumpkin glyoxalase I showed significant homology with other known glyoxalase I sequences of plants.

Keywords: abiotic stress; methylglyoxal; glyoxalase I; *Cucurbita maxima*; cDNA cloning.

Abbreviations:
2,4-D_2,4-Dichlorophenoxyacetic acid; ABA_abscisic acid; CAT_catalase; CDNB_1-Chloro-2,4-dinitrobenzene; DHAP_dihydroxyacetone phosphate; EDTA_ethylene diamine tetraacetic acid; GAP_ glyceraldehyde -3-phosphate; Gly I_ glyoxalase I; Gly II_ glyoxalase II; GSH_reduced glutathione; GSSG_glutathione disulphide, GST_glutathione *S*-transferase; IPTG_isopropyl β-D-thiogalactopyranoside; MG_ methylglyoxal; NZY_NZ amine-yeast extract; ROS_ Reactive oxygen species.

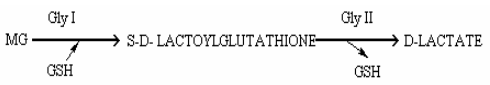
Introduction

Plants are constantly challenged by various biotic and abiotic stresses in nature. Abiotic stresses, such as drought, salinity, cold, high temperature, chemical toxicity, high light intensity and oxidative stresses lead to a series of morphological, physiological, biochemical and molecular changes that adversely affect plant growth and productivity (Wang et al., 2003). As a result, and the course of their evolution, plants have developed numerous unique adaptation and defense mechanisms to help them cope with unavoidable stresses that may be imposed upon them. One such defense mechanism is the development of an enzyme system for protection against potentially toxic effects of xenobiotics and reactive oxygen species generated during environmental stresses. Different environmental stresses of a plant may result in similar responses at the cellular and molecular levels. This

is due to the fact that diverse environmental stresses often activate similar cell signaling pathways (Shinozaki and Yamaguchi-Shinnozaki, 2000; Knight and Knight, 2001; Zhu, 2001) and cellular responses, such as the production of stress proteins, up-regulation of anti-oxidants and accumulation of compatible solutes (Vierling and Kimpel, 1992; Cushman and Bohnert, 2000).

Methylglyoxal (MG), the primary physiological substrate for glyoxalase I (lactoylglutathione lyase; EC 4.4.1.5), is a potent cytotoxic compound produced spontaneously under physiological conditions from the glycolysis and photosynthesis intermediates glyceraldehyde-3-phosphate (GAP) and dihydroxyacetone phosphate (DHAP) (Richard, 1993). Under stress, the rate of glycolysis increases, leading to an imbalance (in upper and lower five reactions) in the pathway. Triose phosphates are very unstable metabolites, and removal of the phosphoryl group by β-elimination from 1, 2-enediolate of these trioses leads to the formation of MG (Richard, 1984, 1993). Therefore, spontaneous production of MG is an unavoidable consequence of the glycolysis pathway during stress. Endogenous production of MG has been reported in microorganisms (yeasts), animals and higher plants (Thornalley, 1990; Yadav et al., 2005a). MG level has been reported to increase during various stresses in animals, mammals, yeast (*Saccharomyces cerevisiae*), and bacterial systems (Cooper, 1984; Abordo et al., 1999; Kalapos et al., 1992) and recently in plant systems (Yadav et al., 2005a; Singla-Pareek et al., 2006). A high level of MG accumulation is toxic to cells as it inhibits cell proliferation (Ray et al.,1994) and results in a number of adverse effects such as increasing the degradation of proteins and inactivating the antioxidant defense system (Martin et al., 2001).

Most organisms protect themselves from the deleterious effects of MG by detoxifying it with the help of the glyoxalase pathway, which is comprised of two enzymes: glyoxalase I, which uses GSH as a cofactor for the conversion of MG to *S*-D lactoylglutathione, and glyoxalase II (hydroxyacyl glutathione hydrolase; EC 3.1.2.6), which gives GSH back to the system, leading to the production of D-lactate. The reaction catalyzed by glyoxalase I and glyoxalase II is as follows:



This pathway has been reported from a diverse group of organisms, including humans, mice, protozoa, fungi, bacteria and plants. Recently, it has been reported that MG levels were increased significantly in plants in response to salinity, drought, and cold stresses (Yadav et al., 2005a, b; Singla-Pareek et al., 2006). Likewise, an increased glyoxalase I activity was reported in rapidly dividing and non-differentiated cells/tissues compared with the activity in differentiated tissues (Deswal et al.,1993; Paulus et al.,1993; Ramaswamy et al.,1983, 1984 ; Seraj et al.,1992). Treatments that stimulate cell growth, including hormones (auxins, cytokinins, etc.) and blue light also increased glyoxalase I activity (Chakravarty and Sopory, 1998). Conversely, inhibition of cell growth resulted in lower levels of glyoxalase I activity (Deswal et al., 1993; Paulus et al., 1993; Sethi et al., 1988). Glyoxalase I from tomato and *Brassica* were shown to be upregulated under salt, water and heavy metal stresses (Espartero et al., 1995; Veena et al., 1999). However, whether the accumulation of MG and upregulation of glyoxalase I activity in plants in response to various stresses is a common phenomenon or not remains to be addressed. Although the importance of the glyoxalase pathway in stress tolerance in plants has recently been demonstrated (Veena et al., 1999), the component enzymes have not been characterized in detail. The nature of glyoxalase I in plants is of particular interest as several variants of this enzyme have evolved through the process of gene duplication and 3D domain swapping (Cameron et al., 1997). Cloning of the glyoxalase I gene would be useful not only for obtaining a better understanding of its physiological role but also for obtaining information on the genetic structure of this enzyme. Therefore, the present study was undertaken to investigate the regulation of methylglyoxal level and glyoxalase I activity due to different abiotic stresses as well as molecular characterization of glyoxalase I gene from pumpkin.

Materials and Methods

Plant materials and stress treatments

To raise seedlings, mature pumpkin (*Cucurbita maxima* Duch.) seeds were sown in vermiculite saturated with deionized water and incubated in the dark at 25ºC. Five-day-old seedlings were used for various stress treatments. Before use, seedlings were removed from vermiculite and all traces of vermiculite were washed off carefully with deionized water. For temperature stress, seedlings were placed into two separate cups each containing 20 ml of distilled water and incubated at 4ºC and 42ºC. For drought stress treatment, seedlings were placed in a cup without water and kept at 25ºC.

Seedlings were placed in 20 ml of 300 mM NaCl solution for salt stress. One mM $CdCl_2$ solution was also used as heavy metal stress. To study the effect of white light, seedlings were placed in a cup containing 20 ml of distilled water and exposed to white light (60 µmol photon $m^{-2}s^{-1}$) and illuminated for 12, 24 and 48 h at 25ºC. To observe the hormonal effect on pumpkin glyoxalase I, 50 µM 2,4-D solution and 50 µM ABA solution were used. As carbonyl compounds and aldehydes, 25 mM MG solution was used for chemical stress. Four seedlings were used in each treatment and were incubated for 24 h in the dark. Seedlings incubated with 20 ml of distilled water in the dark at 25ºC were used as controls.

Sample preparation for MG estimation

Methylglyoxal was estimated basically according to the method of Yadav et al. (2005a) with some modification. About 0.5 g hypocotyl tissue was homogenized in 3 mL of 0.5 M perchloric acid. After incubating for 15 min on ice, the mixture was centrifuged at 4ºC for 10 min at 11,000 g. The supernatant was decolorized by adding charcoal (10 $mgmL^{-1}$), kept for 15 min at room temperature, and centrifuged at 11,000 g for 10 min. Before using this supernatant for MG assay, it was neutralized by keeping for 15 min with saturated solution of potassium carbonate at room temperature and centrifuged again at 11,000 g for 10 min. The neutralized supernatant was used for MG estimation.

Methylglyoxal assay

In a total volume of 1ml, 250 µL of 7.2 mM 1, 2-diaminobenzene, 100 µL of 5 M perchloric acid, and 650 µL of the neutralized supernatant were added in that order. The absorbance at 335 nm of the derivatized MG was read after 25 min in a Hitachi U-2000 spectrophotometer (Hitachi, Japan). The final concentration of MG was calculated from the standard curve and expressed in terms of $\mu molg^{-1}FW$.

Preparation of crude enzyme solution

After incubation for different stress treatments, cotyledon and roots were removed from the seedlings, and hypocotyls were homogenized in an equal volume of 50 mM potassium phosphate buffer (pH 7.0) containing 100 mM KCl, 1% (w/v) ascorbate and 10% (w/v) glycerol with a pre-cooled mortar and pestle. The homogenates were centrifuged at 11,500 g for 10 min and the supernatant was used as a crude enzyme solution for GST and CAT assays. For glyoxalase I assay, proteins were precipitated by ammonium sulphate at 65% saturation from the crude enzyme solution and centrifuged at 11,500 g for 10 min. The precipitate was dissolved in a minimum volume of buffer and transferred to a dialyzed membrane, dialyzed against 10 mM potassium phosphate buffer (pH 7.0) overnight, and then used for glyoxalase I assay. All procedures were performed at 0-4ºC.

Pumpkin cDNA library construction

Total RNA (1.29 mg) was obtained from 23 g of callus treated with 180 µM 2,4-D for 2 days according to the method of Vries et al. (1988). A 2,4-D treated-pumpkin callus cDNA-lambda ZAP II library was constructed using 3 µg of purified poly(A)$^+$mRNA with a titer of 2.5 x 10^5 pfu (plaque-forming units) for the library as recommended by the manufacturer (Stratagene).

Immunoscreening of cDNA library

Twelve thousand pfu of the pumpkin cDNA library were plated for primary screening. Plaques were formed on NZY top agarose at 42ºC for 4 h. To lift plaques, a nitrocellulose filter (Hybond ECL, Amersham) treated with 10 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was put on the surface of the agarose where plaques had formed and incubated at 39ºC for 3.5 h. After incubation, the filter was removed from the agarose and subjected to immunodetection based on Amer- sham's ECL using anti glyoxalase I antiserum. The immuno-positives plaques were purified through two more rounds of screening under the above conditions.

In vivo excision and sequencing of screened phagemids

The purified cDNA clones were rescued from the phage following Stratagene's *in vivo* excision protocol. The cDNAs rescued in pBluescript SK(-) were sequenced using a DNA sequencer (Pharmacia) and the sequences were translated into protein sequences. Nucleotide sequences and deduced amino acid sequences were analyzed using the GENETYX software system (Software Development Co., Tokyo).

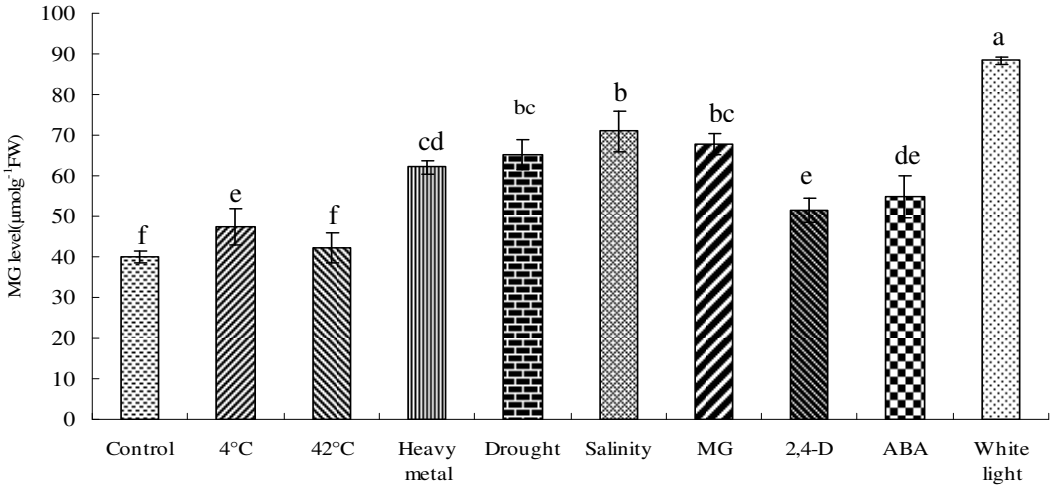


Fig 1. Effects of various stresses on MG levels in pumpkin seedlings. Five-day-old seedlings were treated with low temperature (4ºC), high temperature (42ºC), heavy metal (1 mM $CdCl_2$), drought, salinity (300 mM NaCl), MG (25 mM), 2,4-D (50 µM), ABA (50 µM) and white light (60 µmol photon m^{-2} s^{-1}) stresses for 24 h. Results were obtained from four independent experiments and bars indicate standard error. Bars with different letters are significantly different at P≤ 0.05.

Expression in E. coli

The open reading frame of each clone encoding glyoxalase I was found to be in-frame with the β-galactosidase gene α-complementation particle in pBluescript SK(-). In order to express glyoxalase I as a fusion protein, XL1-blue cells were transformed with the phagemids and cultivated at 37ºC in LB medium supplemented with 50 µg ml^{-1} ampicillin and 1 mM IPTG for 19 h. The cells were collected and lysed in an equal volume of 50 mM potassium phosphate buffer (pH 7.0) containing 100 mM KCl, 1% (w/v) ascorbate and 10% (w/v) glycerol with a pre-cooled mortar and pestle. Cellular debris was pelleted after centrifugation (11,500 *g* at 4 ºC for 10 min), and the supernatant was used for glyoxalase I assays. pBluescript with inserted DNA was used as controls.

Assay of enzymatic activities and protein quantification

Glyoxalase I (EC: 4.4.1.5) assay was carried out according to Chakravarty and Sopory (1998) with slight modification. Briefly, the assay mixture contained 100 mM K-phosphate buffer (pH 7.0), 15 mM magnesium sulphate, 1.7 mM reduced glutathione and 3.5 mM methylglyoxal in a final volume of 0.8 ml. The reaction was started by the addition of MG and the formation of thioester was measured by observing the increase of absorbance at 240 nm for 1 min in a Shimadzu spectrophotometer (Shimadzu, Kyoto, Japan) and the activity was calculated using the extinction coefficient of 3.37 $mM^{-1}cm^{-1}$. GST (EC: 2.5.1.18) activity was determined spectrophotometrically by the method of Booth et al. (1961) with some modifications. The reaction mixture contained 100 mM potassium phosphate buffer (pH 6.5), 1.5 mM reduced glutathione, 1 mM 1-chloro- 2,4-dinitrobenzene (CDNB), and enzyme solution in a final volume of 0.7 ml. The enzyme reaction was initiated by the addition of CDNB and A_{340} was measured at 25˚C for 1 minute. The activity was calculated using the extinction coefficient of 9.6 $mM^{-1}cm^{-1}$. CAT (EC: 1.11.1.6) activity was measured according to the method of Chance and Maehly (1955) by monitoring the decrease of absorbance at 240 nm for 1 min caused by the decomposition of H_2O_2. The reaction mixture contained 50 mM K-P buffer (pH 7.0), 15 mM H_2O_2 and enzyme solution in a final volume of 0.7 ml. The reaction was initiated with enzyme extract and the activity was calculated using the extinction co-efficient of 39.4 $M^{-1}cm^{-1}$.

The protein concentration of each sample was determined by the method of Bradford (1976) using BSA as protein standard.

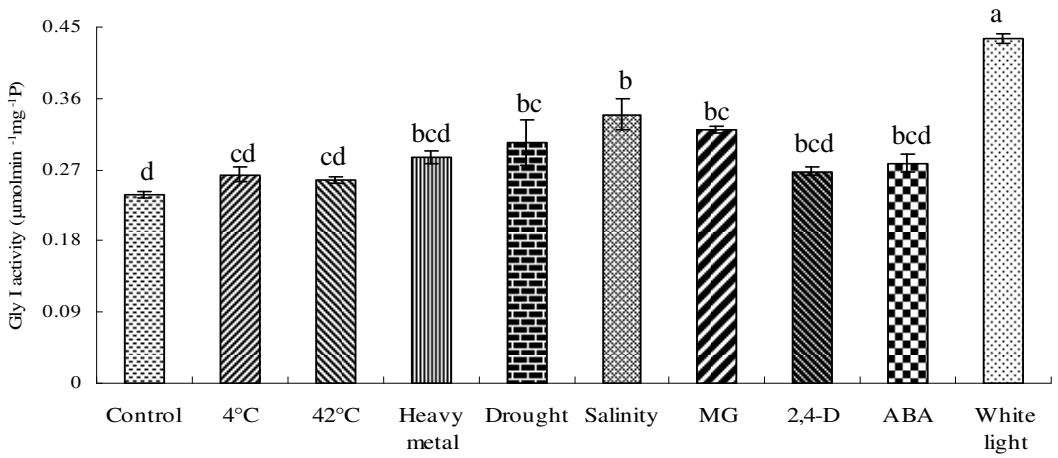


Fig 2. Effects of various stresses on glyoxalase I activities in pumpkin seedlings. Five-day-old seedlings were treated with low temperature (4ºC), high temperature (42ºC), heavy metal (1 mM $CdCl_2$), drought, salinity (300 mM NaCl), MG (25 mM), 2,4-D (50 μM), ABA (50 μM) and white light (60 μmol photon $m^{-2}s^{-1}$) stresses for 24 h. Results were obtained from four independent experiments and bars indicate standard error. Bars with different letters are significantly different at P≤ 0.05.

Northern blot hybridization analysis

After treatment, only hypocotyls were frozen immediately in liquid nitrogen. RNA was isolated using an RNeasy Plant Mini Kit (QIAGEN) following the manufacturer's instructions. Total RNA (5 μg in each lane) was subjected to electrophoresis in a 1% (w/v) agarose gel that contained 5% (v/v) formaldehyde and blotted onto Hybond-N (Amersham). All hybridization and washing conditions were *as per* DIG Nucleic Acid Detection Kit (Boehringer Mannheim, Mannheim, Germany). For hybridization, we synthesized labeled RNA probes covering the 3′ flanking region of specific cDNA (Pumpkin glyoxalase I, Accession no. AB303333) cloned into pBluescript SK(-) with T7 RNA polymerase using a Boehringer DIG RNA Labeling Kit (SP6/T7). The labeled probes bound specifically to respective RNA molecules, and hybridized RNA molecules were visualized using a DIG Luminescent Detection Kit (Boehringer Mannheim, Mannheim, Germany).

Statistical analysis

All data obtained was subjected to one-way analysis of variance (ANOVA) and the significance of difference between the mean values was compared by Duncan's multiple range test using MSTAT-C. Differences at P ≤ 0.05 were considered significant.

Results

Methylglyoxal levels in plants under normal and stress conditions

To check whether the upregulation of MG in plants in response to various stresses is a common phenomenon, its levels were measured in five-day-old pumpkin seedlings under control as well as various stressful conditions, including drought, salinity, cold, high temperature, white light, heavy metal, MG, 2,4-D and ABA stresses. It was found that methylglyoxal levels increased significantly due to different stress treatments within 24 hours, and the levels ranged from 39.96 to 88.40 μmolg $^{-1}$ FW under control and various stress conditions (Fig. 1). White light caused the highest induction (2.21-fold) of methylglyoxal level followed by salinity (1.77-fold), methylglyoxal (1.69-fold), drought (1.63-fold), heavy metal (1.55-fold) and ABA (1.37-fold) stresses. The rapid increase of MG level in plants due to different stresses clearly suggested that it is a general response to all abiotic stresses.

Glyoxalase I activity under normal and stress conditions

To verify whether the increased MG level was responsible for the elevation of enzymatic activity,

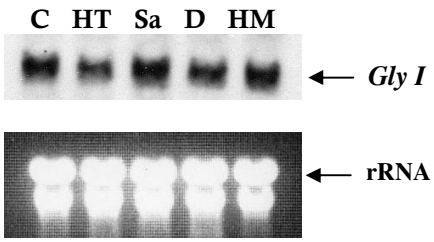


Fig 3. Northern blot analysis of induction of pumpkin glyoxalase I mRNA by high temperature (HT), salinity (Sa), drought (D) and heavy metal (HM) stresses. For Northern blot analysis pumpkin seedlings were treated with the above stresses for 24 h. The control is expressed as C. Each lane received 5 µg of total RNA.

we then measured the glyoxalase I activity in pumpkin seedlings under the same experimental conditions and observed a significant increase in glyoxalase I activity in response to various stresses (Fig. 2). A sharp increase of glyoxalase I activity (1.82-fold) was observed due to white light stress followed by salinity (1.42-fold), methylglyoxal (1.34-fold), drought (1.27-fold) and heavy metal (1.19- fold) stresses within 24 hours. These findings were in accordance with the MG levels upregulated by different stress treatments.

Upregulation of glyoxalase I transcript level under normal and stress conditions

To obtain further insights, we also examined the effects of stresses at transcriptional level by Northern blotting (Fig. 3,4). A noticeable increase in the *Gly I* transcript was observed due to different stress treatments. The rapid accumulation of the *Gly I* transcript by various inducers clearly suggests towards the role of this gene in early stress responses. Maximum induction of *Gly I* transcript was observed by white light stress followed by salinity, MG and heavy metal stresses (Fig. 3, 4). These findings were also in accordance with MG level and glyoxalase I activity. However, increases in MG level, glyoxalase I activity and *Gly I* transcript level due to white light is a new finding.

Differential responses of glyoxalase I, glutathione-S-transferase (GST) and catalase (CAT) activities due to white light treatment

To determine whether the enhanced expression of glyoxalase I activity is correlated with exposure time, we further conducted a time course experiment with pumpkin seedlings under white light condition. The experiment showed that when seedlings that had been grown in the dark were subjected to a white light condition, the glyoxalase I activity increased up to 24 hours and then gradually decreased (Fig. 5). However, the specific activities of two ROS-scavenging enzymes (GST and CAT) were down regulated upon exposure to light (Fig. 6, 7).

Isolation of glyoxalase I cDNA clone and over-expression in E. coli

An expression cDNA library was constructed using mRNA prepared from pumpkin callus and it was immounoscreened with the anti-glyoxalase I antiserum. Five positive clones were obtained from three rounds of screening. Three of the clones were chosen randomly and sequenced. The pumpkin glyoxalase I cDNA (AB 303333) consists of 975-bp nucleotides encoding a polypeptide of 185 amino acids (Fig. 8) having a predicted molecular weight of 20,772.14 Da and a predicted isoelectric point of 5.15. In order to determine whether this cDNA encodes glyoxalase I protein, we expressed the protein that the cDNA encoded as a fusion protein of β-galactosidase in XL1-blue cells in the presence of 1 mM IPTG. Following induction with IPTG, the overexpressed *Gly1* showed 1800-fold higher glyoxalase I activity compared with cells transformed with vector alone. In Western blotting, the fusion protein expressed in the *E. coli* cells was bound with anti-glyoxalase I antiserum (data not shown), indicating again that the cDNA was that of glyoxalase I.

The sequence analysis of the cloned gene encoding for glyoxalase I enzyme of glyoxalase pathway from pumpkin (Accession no. AB 303333) showed significant homology with other known glyoxalase I sequences of plants present in the database (Fig. 9). The deduced amino acid sequence of *Cucurbita maxima* showed maximum identity with *Cicer arietinum, Glycine max* and *Brassica juncea* (88%, 86% and 85% respectively) and it showed 83% identity with *Arachis hypogaea* and 79% identity with *Avicennia marina*.

Discussion

In an attempt to determine whether the upregulation of MG levels in plants in response to various stresses is a common phenomenon, MG levels were measured under various stressful conditions in this study. Significant increases of MG levels were

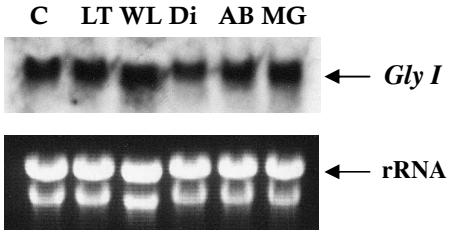


Fig 4. Northern blot analysis of induction of pumpkin glyoxalase I mRNA by low temperature (LT), white light (WL), 2, 4-D (Di), abscisic acid (AB) and methylglyoxal (MG) stresses for 24 hours. For Northern blot analysis pumpkin seedlings were treated with the above stresses for 24 h. The control is expressed as C. Each lane received 5 µg of total RNA.

observed due to different stress treatments, whereas sharp increase were observed by white light, salinity, chemical, drought and heavy metal stresses (Fig. 1). Elevated levels of MG due to stress treatments have also been reported recently in plant systems (Yadav et al., 2005a; Singla-Pareek et al., 2006). However, the mechanism(s) of the production of MG in plants has been not elucidated. It is thought that MG could be generated by removal of the phosphoryl group of triose phosphates produced during glycolysis or following the degradation of lipid peroxides (as in animals), which generates products like 4-hydroxynon-2-enal and MG (Vander et al.,1995), but it is not known whether this pathway is present in plants. Under stress conditions, cells become metabolically active, which is mirrored by upregulation of enzymes involved in glycolysis and TCA cycles (Umeda et al., 1994; Espartero et al., 1995; Sommer et al., 2001; Scaife, 1969) and as a result flux of triose phosphates increases which, instead of giving only pyruvate could be converted to MG. We showed that MG levels increased under stress conditions, and this seems to be a general stress response. There is a possibility that MG could therefore act as a signal for plants to respond to stress.

Several research groups have reported that the activity of glyoxalase I was affected by various exogenous factors and abiotic stress treatments including salt, water and heavy metal stresses (Chakravarty & Sopory, 1998; Espartero et al., 1995; Veena et al., 1999). In the present study, we also observed a significant increase of glyoxalase I activity as well as glyoxalase I transcript level due

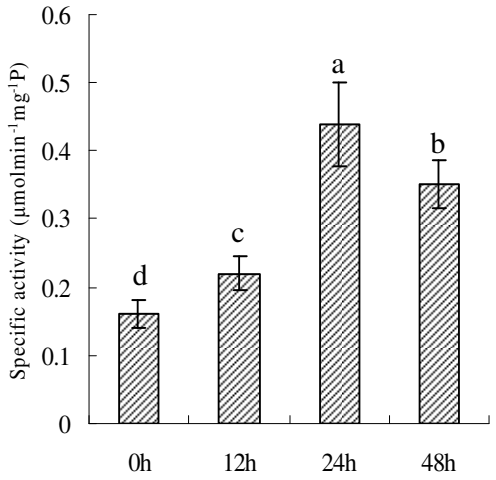


Fig 5. Changes in relative specific activities of glyoxalase I in pumpkin seedlings exposed to white light. Results were obtained from four independent experiments and bars indicate standard error. Bars with different letters are significantly different at $P \leq 0.05$.

to different stress treatments, and the results were in accordance with the MG levels (Fig. 1, 2, 3, 4). Significant positive correlation ($r = 0.93^{***}$) between MG level and glyoxalase I activity, indicating that the glyoxalase system might be required for the detoxification of MG formed, both spontaneously and enzymatically, from troise phosphates. In this regard, glyoxalase I could be expected to be a house-keeping protein present in all cells. Although up-regulation of glyoxalase I activity in response to salt, water deficit, ABA and heavy metal stress treatments has been reported earlier in plant (Espartero et al., 1995; Yadav et al., 2005b; Veena et al., 1999), this is the first report on stress-induced increases of glyoxalase I activity, *Gly I* transcript level and methylglyoxal levels in plants. It is conceivable that an elevated level of glyoxalase I activity is required to remove methylglyoxal, a toxic and unavoidable by-product of triosephosphate metabolism produced in ample amounts under normal and various stressful conditions. Increase in glyoxalase I activity during stress tolerance may also indicate active metabolic status of the cell, in which cell division and growth are compromised in order to conserve energy for mobilization of resources towards stress tolerance and defense strategies. The gene expression profile of glyoxalase I also showed true reflection of possible changes in activity levels due to different abiotic stresses.

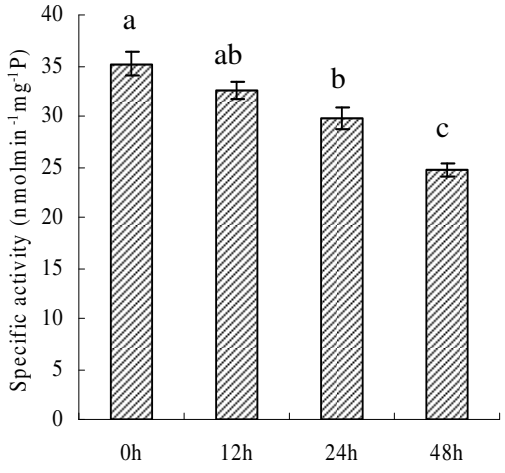


Fig 6. Changes in relative specific activities of GST in pumpkin seedlings exposed to white light. Results were obtained from four independent experiments and bars indicate standard error. Bars with different letters are significantly different at $P \leq 0.05$.

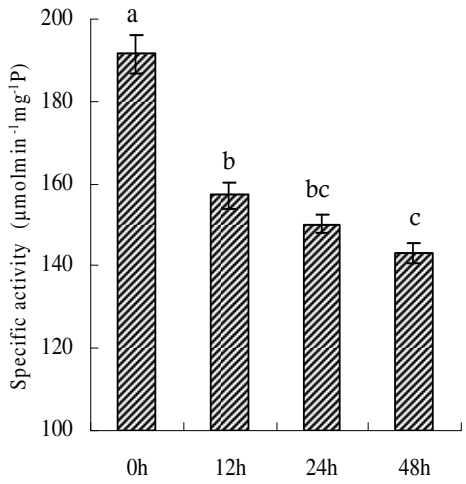


Fig 7. Changes in relative specific activities of CAT in pumpkin seedlings exposed to white light. Results were obtained from four independent experiments and bars indicate standard error. Bars with different letters are significantly different at $P \leq 0.05$.

Besides detoxification of methylglyoxal, the glyoxalase system might also play a role in providing tolerance to stress by recycling glutathione that would be 'trapped' spontaneously by methylglyoxal to form hemithioacetal (Creightion et al.,1988; Thornalley,1990), thereby maintaining glutathione homeostasis. Methylglyoxal has been shown to decrease the level of protein thiol (Basjarab & Balasubramanian, 1990) and the level of reduced glutathione (Leocini et al., 1989; Kalapos et al., 1992). It is known that reduced glutathione is essential for effective scavenging of toxic compounds (such as H_2O_2 and organic H_2O_2) and for maintenance of other antioxidants such as ascorbates and tocopherols (Alscher, 1989). In addition, GSH is known to stimulate a variety of defence responses in plants (May et al., 1998; Wingate et al., 1988). Moreover, overexpression of glyoxalase genes involved in the regulation of glutathione homeostasis (namely, glutathione reductase, glutathione S-transferase/ glutathione peroxidase) in transgenic plants has been shown to result in an increased tolerance against oxidative stress (Broadbent et al., 1995; Noctor et al., 1998; Roxas et al., 1997; Yadav et al., 2005b). Light is a very important environmental factor, and many species have evolved sophisticated photosensory systems enabling them to respond appropriately. species have evolved sophisticated photosensory systems enabling them to respond appropriately. The effect of light on plant growth and development is evident during the transition from a dark-grown (etiolated) to a light-grown (de-etiolated) morphology. This transition can be regarded as light stress because upon exposure to light, seedlings undergo a number of dramatic changes, including a significant reduction in the rate of elongation, opening in the apical hock, expansion of true leaves, development of mature chloroplasts, pigment synthesis, and the assembly of the photosynthesis in the thylakoids (Yang et al., 2007). All of these processes are accomplished by and depend on the differential expression of a large number of genes. In this study, we observed significant increases in methylglyoxal level and glyoxalase I activity as well as *Gly I* transcript level for up to 24 hours of white light treatment, whereas glyoxalase I activity decreased gradually after 24 hours . The results of this study suggested the regulative mechanisms of glyoxalase I enzymes related to chloroplast morphogenesis of the pumpkin seedlings by light. In contrast to glyoxalase I, the activity of two antioxidant and ROS-scavenging enzymes decreased gradually, whereas a sharp reduction of CAT activity was observed within 12 hours of light treatment. Several research groups have also been reported the decrease of antioxidant enzyme in etiolated seedlings upon exposure to light (Yang et al., 2007; Cano et al., 2006). Here, we need to

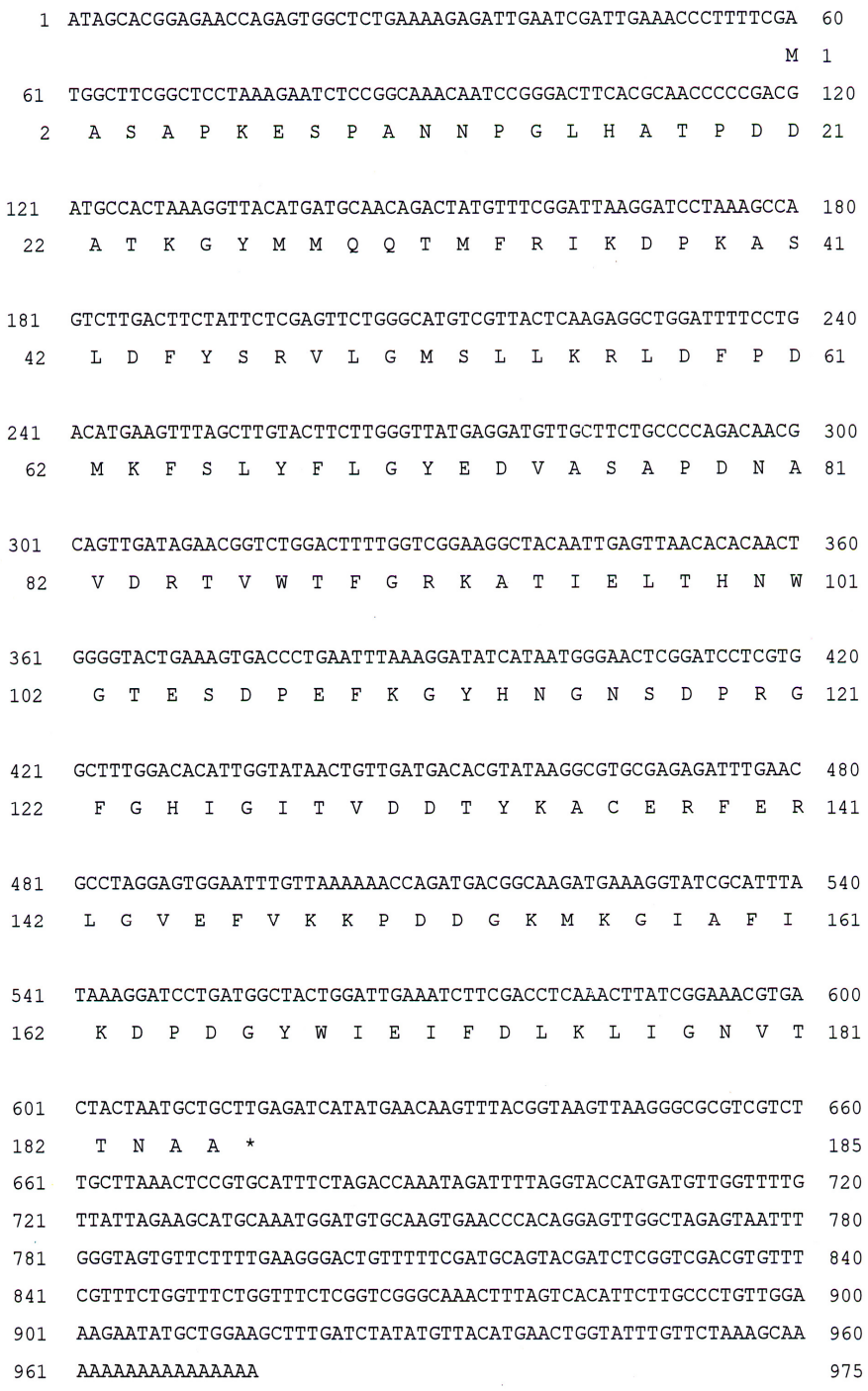


Fig 8. Nucleotide sequence of pumpkin glyoxalase I (short-type) cDNA and deduced amino acid sequence.

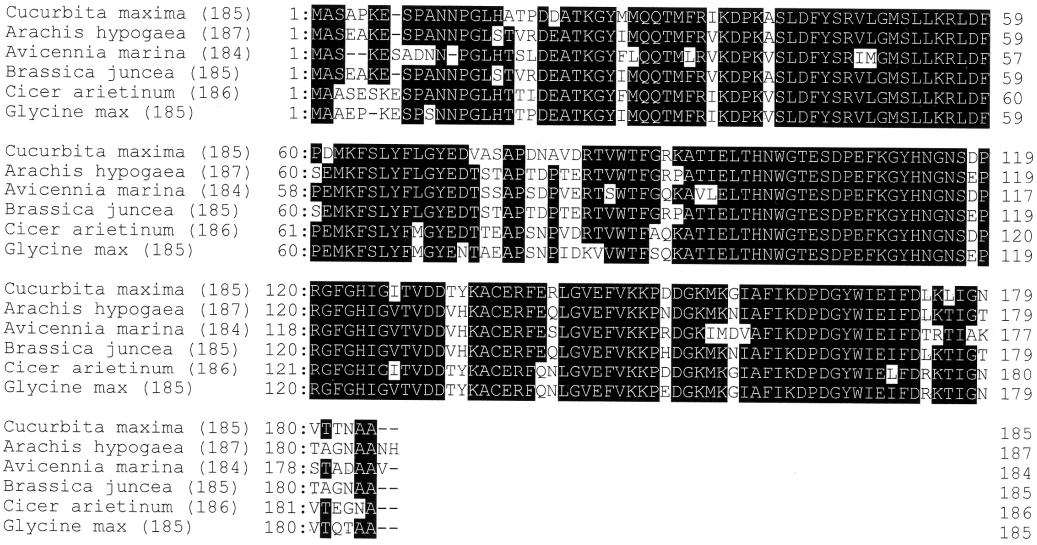


Fig 9. Multi-sequence alignment of the deduced amino acid sequence of the *Gly I* cDNA clone from *Curcurbita maxima* and previously reported glyoxalase I sequences found in the database. Identical residues are in a dark background.

clarify that we germinated the pumpkin seedlings for five days under a dark condition and we also found GST and CAT accumulated much more abundantly in etiolated pumpkin seedlings than in green ones (Fig. 6,7). Our results implied that etiolated pumpkin seedlings may possibly be under an oxidative stress, which can be released in an early stage of de-etiolation. Therefore, we propose two possible causes of the increase in methylglyoxal level as well as glyoxalase I activity in seedlings under white light conditions. One is that under white light conditions cells become more metabolically active (switching from heterotrophic to autotrophic metabolism), which is responsible for the increases of MG levels, glyoxalase I activity and *Gly I* transcript level. The other possible reason is increase of reduced glutathione (GSH), a co-factor of glyoxalase I, by the consequent removal of oxidative stress from the seedlings upon exposure to light. Further studies with measurement of GSH and activities of other enzymes involved in the antioxidant system will be helpful in drawing any such conclusion. As a part of our study to understand the structure of glyoxalase I gene, we have cloned and characterized glyoxalase I cDNA from pumpkin for the first time. The ORF of *Gly I* cDNA codes for 185 amino acids having a predicted molecular weight of 20,772.14 Da and a predicted isoelectric point of 5.15. Based on amino acid number, pumpkin glyoxalase I was classified as short-type glyoxalase I and pumpkin glyoxalase I showed significant homology with other known glyoxalase I sequences of plants.

Finally, it can be concluded that increase of methylglyoxal levels due to different stress treatments seems to be a basic cellular response. Accordingly, the concentration of MG that elicits glyoxalase I activity and glyoxalase I transcript demonstrated in this study seems to be physiologically relevant. Glyoxalase I enzyme plays an important role in the detoxification of MG produced in plants under normal and stressful conditions. Overexpression of *Gly I* gene under white light condition suggests a role in photoautotrophic transition (de-etiolation process) of plants.

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