Up-regulation of antioxidant and glyoxalase systems by exogenous glycinebetaine and proline in mung bean confer tolerance to cadmium stress

Mohammad Anwar Hossain · Mirza Hasanuzzaman · Masayuki Fujita

Published online: 24 November 2010
© Prof. H.S. Srivastava Foundation for Science and Society 2010

Abstract The present study investigates the possible mediatory role of exogenously applied glycinebetaine (betaine) and proline on reactive oxygen species (ROS) and methylglyoxal (MG) detoxification systems in mung bean seedlings subjected to cadmium (Cd) stress (1 mM CdCl₂, 48 h). Cadmium stress caused a significant increase in glutathione (GSH) and glutathione disulfide (GSSG) content, while the ascorbate (AsA) content decreased significantly with a sharp increase in hydrogen peroxide (H₂O₂) and lipid peroxidation level (MDA). Ascorbate peroxidase (APX), glutathione *S*-transferase (GST), glutathione peroxidase (GPX), and glyoxalase I (Gly I) activities were increased in response to Cd stress, while the activities of catalase (CAT), monodehydroascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR), glutathione reductase (GR) and glyoxalase II (Gly II) were sharply decreased. Exogenous application of 5 mM betaine or 5 mM proline resulted in an increase in GSH and AsA content, maintenance of a high GSH/GSSG ratio and increased the activities of APX, DHAR, MDHAR, GR, GST, GPX, CAT, Gly I and Gly II involved in ROS and

MG detoxification system as compared to the control and mostly also Cd-stressed plants, with a concomitant decrease in GSSG content, H₂O₂ and lipid peroxidation level. These findings together with our earlier findings suggest that both betaine and proline provide a protective action against Cd-induced oxidative stress by reducing H₂O₂ and lipid peroxidation levels and by increasing the antioxidant defense and MG detoxification systems.

Keywords Antioxidant defense · Glyoxalase system · Glycinebetaine · Proline · Cadmium stress · Reactive oxygen species · Mung bean

Abbreviations

AO	ascorbate oxidase
APX	ascorbate peroxidase
AsA	ascorbic acid
CAT	catalase
CDNB	1- chloro-2, 4-dinitrobenzene
DHA	dehydroascorbate
DHAR	dehydroascorbate reductase
DTNB	5,5'-dithio-bis (2-nitrobenzoic acid)
EDTA	ethylene diamine tetraacetic acid
Gly I	glyoxalase I
Gly II	glyoxalase II
GR	glutathione reductase
GSH	reduced glutathione
GSSG	oxidized glutathione
GPX	glutathione peroxidase
GST	glutathione <i>S</i> -transferase
MDA	malondialdehyde
MDHA	monodehydroascorbate
MDHAR	monodehydroascorbate reductase
MG	methylglyoxal
NTB	2-nitro-5-thiobenzoic acid

M. A. Hossain · M. Hasanuzzaman · M. Fujita (✉)
Department of Applied Biological Science,
Laboratory of Plant Stress Responses,
Faculty of Agriculture, Kagawa University,
Miki-cho, Kita-gun,
Kagawa 761-0795, Japan
e-mail: fujita@ag.kagawa-u.ac.jp

M. A. Hossain
Department of Genetics & Plant Breeding,
Bangladesh Agricultural University,
Mymensingh 2202, Bangladesh

M. Hasanuzzaman
Department of Agronomy, Sher-e-Bangla Agricultural University,
Dhaka 1207, Bangladesh

ROS	reactive oxygen species
SLG	S-D-lactoylglutathione
TBA	thiobarbituric acid
TCA	trichloroacetic acid

Introduction

Understanding the biochemical detoxification strategies that plants adopt against oxidative stress induced by accumulated metal ions is a key to manipulating metal tolerance in plants. Cadmium (Cd) is one of the most toxic metals for plants, due to its high solubility in water and phytotoxicity (Clemens 2006). The presence of an excessive amount of Cd in soil and water causes a range of morphological, biochemical and physiological responses leading to stunted growth and even plant death. Cd toxicity decreases stomatal density and conductance to CO₂ thus reducing leaf photosynthesis and interferes with uptake and distribution of nutrients and water (Popova et al. 2009). At the cellular level, Cd interacts with biomolecules such as protein and nucleic acids, is known to affect the enzyme activities and causes alterations in membrane permeability (Smeets et al. 2008). In addition, Cd stress is known to disturb redox homeostasis in plant cells and induce a burst of reactive oxygen species (ROS) (Hsu and Kao 2007a, b; Popova et al. 2009; Nouairi et al. 2009; Islam et al. 2010). ROS are necessary for inter- and intracellular signalling but at high concentrations they seriously disrupt normal metabolism leading to irreparable metabolic dysfunction and death (Gechev et al. 2006).

Plants have evolved enzymatic and non-enzymatic antioxidants to cope with ROS. These antioxidants include the enzymes superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPX), glutathione *S*-transferases (GST), ascorbate peroxidase (APX), dehydroascorbate reductase (DHAR), glutathione reductase (GR), and monodehydroascorbate reductase (MDHAR), water-soluble compounds such as ascorbate (AsA), glutathione (GSH) and flavonoids, and lipid-soluble compounds such as the carotenoids and tocopherols. The AsA–GSH cycle involves APX, DHAR, GR, MDHAR, reduced ascorbic acid (AsA), reduced glutathione (GSH) and NADPH in a series of cyclic reactions to detoxify H₂O₂ and also to regenerate AsA and GSH. Apart from its function directly to detoxify ROS and thereby to combat the potentially harmful effects of environmental stresses, the AsA–GSH cycle is also involved in redox sensing and signalling. Under stress conditions these redox signals could interfere with signalling networks complementary to the antioxidant system and regulate defence gene expression, thus coordinating the necessary readjustments in the redox-regulated plant

defence to overcome the oxidative stress (Mittler et al. 2004; Kuzniak and Sklodowska 2005). Additionally, CAT eliminates H₂O₂ by breaking it down to H₂O and O₂ and does not require any reducing equivalent (Mallick and Mohn 2000). GPX is considered to be an important ROS scavenger because of its broader substrate specifications and stronger affinity for H₂O₂ than those of catalase (Brigelius-Flohe and Flohe 2003). Importantly, GST catalyzes the detoxification of lipid peroxides and xenobiotics by conjugating them with GSH (Noctor et al. 2002) and helps them in sequestration into vacuole of a plant cell. These enzymes play a regulatory role in heavy metal-induced oxidative stress.

Recent studies in plants have demonstrated that methylglyoxal (MG) is produced in excessive amounts in response to various stresses including heavy metals (Yadav et al. 2005a, b; Singla-Pareek et al. 2006; Hossain et al. 2009). In addition to ROS, MG is also highly toxic to plant cells and by reacting with proteins, lipids, carbohydrates and DNA, they can lead to cell death in the absence of any protective mechanism. In plants, MG is detoxified mainly via the glyoxalase system that is comprised of two enzymes: glyoxalase I (Gly I) and glyoxalase II (Gly II). Gly I converts MG to S-D-lactoylglutathione (SLG) by utilizing GSH, while Gly II converts SLG to D-lactic acid, and during this reaction GSH is regenerated. The presence and characterization of both Gly I and II has been reported in many plants and the genes encoding these enzymes have been cloned and found to be regulated under various environmental conditions (Yadav et al. 2005a, b; Veena et al. 1999; Singla-Pareek et al. 2006, 2008; Hossain et al. 2009; Hossain and Fujita 2009, 2010). Overexpression of the glyoxalase pathway in transgenic tobacco and rice plants has been found to prevent an increase of ROS and MG under stress conditions by maintaining glutathione homeostasis and antioxidant enzyme levels (Yadav et al. 2005b; Singla-Pareek et al. 2006, 2008).

One of the most important physiologic strategies employed by higher plants under stress conditions is the accumulation of compatible solutes such as proline and betaine. Heavy metal stress up-regulates the enzymes in proline and betaine biosynthesis which confers an increase in stress tolerance (Bassi and Sharma, 1993a, b; Costa and Morel 1994; Mehta and Gaur 1999; Shao et al. 2008; Dinakar et al. 2009). The natural accumulation of proline/betaine, however, is not high enough to protect plants from stress-induced damage (Subbarao et al. 2001; Okuma et al. 2002; Tamura et al. 2003; Tamas et al. 2008). Under such condition their exogenous application may help to reduce the adverse effects of various environmental stresses including Cd stress (Yang and Lu 2005; Kumar and Yadav 2009; Huang et al. 2009; Islam et al. 2010; Hossain and Fujita 2010). In addition to their roles as osmoprotectants,

proline and betaine contribute to the protection of membranes, proteins and enzymes from the damaging effect of various stresses (Ashraf and Foolad 2007; Hossain and Fujita 2010). Furthermore, betaine and proline provide protection against oxidative stress by maintaining redox homeostasis (Chen et al. 2006; Hossain and Fujita 2010). Recently, it has been reported that betaine and proline alleviates Cd toxicity by detoxifying ROS and increasing antioxidant activity and GSH content in *Solanum* and tobacco BY-2 cells (Xu et al. 2009; Islam et al. 2010). To our knowledge, currently there is no information available on the possible beneficial effects of exogenous application of betaine and proline on MG detoxification system in plants grown under Cd stress. Although our previous study showed that both betaine and proline improve salinity tolerance in mung bean seedlings by enhancing antioxidant and MG detoxification systems (Hossain and Fujita 2010). In this study, we investigated the possible beneficial effects of exogenous proline and betaine on lipid peroxidation, intracellular ROS accumulation, and components of antioxidant defense and MG detoxification systems in mung bean seedlings subjected to Cd stress. In addition, we compared these results from Cd-stressed seedlings with our previous results from salinity stress (Hossain and Fujita 2010).

Materials and methods

Plant materials and stress treatments

Mung bean (*Vigna radiata* cv. Binamoog-1) seeds of uniform size were selected and surface-sterilized with 70% ethanol followed by washing several times with distilled water. The seeds were then soaked with distilled water for 20 min and sown in Petri plates (9 cm) lined with 4 layers of filter paper moistened with 15 ml of distilled water for germination under controlled conditions (light, 100 $\mu\text{mol photon m}^{-2} \text{ s}^{-1}$; temp, $25 \pm 2^\circ\text{C}$; RH, 65–70%) for 3 days. Germinated seedlings were then grown in Petri plates that contained 1000-fold diluted Hyponex solution (Type: 5-10-5, Hyponex, Japan). Six-day-old mung bean seedlings of approximately equal sizes were employed to experimentation. For Cd stress, seedlings were treated with Hyponex solution that contained 1 mM CdCl_2 in Petri plates lined with 4 layers of filter paper under the above conditions for 48 h. For Cd treatment in the presence of proline or betaine, seedlings were treated with 5 mM proline or 5 mM betaine with the above levels of CdCl_2 in Hyponex solution for 48 h. Control plants were grown in Hyponex solution only. In case of heavy metals, due to high binding capacity of filter paper to cations (Haluskova et al. 2009; Tamás et al. 2008; 2010; Kuriakose and Prasad

2008), application of 1 mM CdCl_2 concentration was required to obtain meaningful differences among the metabolites and the enzymatic activities within short period of time (48 h). Since the primary objectives of this investigation was to study the response of seedlings to Cd toxicity as well as biochemical mechanisms of proline- and betaine-induced Cd stress tolerance, these high concentrations were used throughout the experiment.

Extraction and analysis of ascorbate and glutathione

Mung bean leaves (0.5 g fresh weight) were homogenized in 1.5 ml ice-cold acidic extraction buffer (6% metaphosphoric acid containing 1 mM EDTA) using a mortar and pestle. Homogenates were centrifuged at $11,500 \times g$ for 15 min at 4°C and the supernatant was collected for analysis of ascorbate and glutathione.

Ascorbate content was determined following the method of Huang et al. (2005) with some modifications. The supernatant was neutralized with 0.5 M K-phosphate buffer (pH 7.0). The reduced ascorbate was assayed spectrophotometrically at 265 nm in 100 mM K-phosphate buffer (pH 5.6) with 0.5 unit of ascorbate oxidase (AO). A specific standard curve with AsA was used for quantification. The glutathione pool was assayed according to previously described methods (Yu et al. 2003) with modifications (Paradiso et al. 2008) utilizing 0.4 ml of aliquots of supernatant neutralized with 0.6 ml of 0.5 M K-phosphate buffer (pH 7.5). Based on enzymatic recycling, glutathione is oxidized by 5,5'-dithio-bis (2-nitrobenzoic acid) (DTNB) and reduced by NADPH in the presence of GR, and glutathione content is evaluated by the rate of absorption changes at 412 nm of 2-nitro-5-thiobenzoic acid (NTB) generated from the reduction of DTNB. GSSG was determined after removal of GSH by 2-vinylpyridine derivatization. Standard curves were generated with reduced and oxidized glutathione.

Enzyme extraction and assays

Using a pre-cooled mortar and pestle, 0.5 g of leaf tissue was homogenized in 1 ml of 50 mM ice-cold K-phosphate buffer (pH 7.0) containing 100 mM KCl, 1 mM ascorbate, 5 mM β -mercaptoethanol and 10% (w/v) glycerol. The homogenates were centrifuged at $11,500 \times g$ for 10 min and the supernatants were used for determination of enzyme activity. All procedures were performed at 0 – 4°C .

APX (EC: 1.11.1.11) activity was assayed following the method of Nakano and Asada (1981). The reaction buffer solution contained 50 mM K-phosphate buffer (pH 7.0), 0.5 mM AsA, 0.1 mM H_2O_2 , 0.1 mM EDTA, and enzyme extract in a final volume of 0.7 ml. The reaction was started by the addition of H_2O_2 and the activity was measured by

observing the decrease in absorbance at 290 nm for 1 min using an extinction co-efficient of $2.8 \text{ mM}^{-1} \text{ cm}^{-1}$.

MDHAR (EC: 1.6.5.4) activity was determined by the method of Hossain et al. (1984). The reaction mixture contained 50 mM Tris-HCl buffer (pH 7.5), 0.2 mM NADPH, 2.5 mM AsA, 0.5 unit of AO and enzyme solution in a final volume of 0.7 ml. The reaction was started by the addition of AO. The activity was calculated from the change in ascorbate at 340 nm for 1 min using an extinction co-efficient of $6.2 \text{ mM}^{-1} \text{ cm}^{-1}$.

DHAR (EC: 1.8.5.1) activity was determined by the procedure of Nakano and Asada (1981). The reaction buffer contained 50 mM K-phosphate buffer (pH 7.0), 2.5 mM GSH, and 0.1 mM DHA. The reaction was started by adding the sample solution to the reaction buffer solution. The activity was calculated from the change in absorbance at 265 nm for 1 min using extinction co-efficient of $14 \text{ mM}^{-1} \text{ cm}^{-1}$.

GR (EC: 1.6.4.2) activity was measured by the method of Cakmak et al. (1993). The reaction mixture contained 0.1 M K-phosphate buffer (pH 7.8), 1 mM EDTA, 1 mM GSSG, 0.2 mM NADPH, and enzyme solution in a final volume of 1 ml. The reaction was initiated with GSSG and the decrease in absorbance at 340 nm due to NADPH oxidation was recorded for 1 min. The activity was calculated using an extinction co-efficient of $6.2 \text{ mM}^{-1} \text{ cm}^{-1}$.

GPX (EC: 1.11.1.9) activity was measured as described by Elia et al. (2003) using H_2O_2 as a substrate. The reaction mixture consisted of 100 mM Na-phosphate buffer (pH 7.5), 1 mM EDTA, 1 mM NaN_3 , 0.12 mM NADPH, 2 mM GSH, 1 unit GR, 0.6 mM H_2O_2 and 20 μl of sample solution. The reaction was started by the addition of H_2O_2 . The oxidation of NADPH was recorded at 340 nm for 1 min and the activity was calculated using the extinction co-efficient of $6.62 \text{ mM}^{-1} \text{ cm}^{-1}$.

GST (EC: 2.5.1.18) activity was determined spectrophotometrically by the method Booth et al. (1961) with some modifications (Hossain et al. 2009). The reaction mixture contained 100 mM Tris-HCl buffer (pH 6.5), 1.5 mM GSH, 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 the increase in absorbance was measured at 340 nm for 1 min. The activity was calculated using the extinction co-efficient of $9.6 \text{ mM}^{-1} \text{ cm}^{-1}$.

CAT (EC: 1.11.1.6) activity was measured according to the method of Hossain et al. (2009) 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-phosphate 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 $39.4 \text{ M}^{-1} \text{ cm}^{-1}$.

Glyoxalase I (EC: 4.4.1.5) assay was carried out according to the method of Hossain et al. (2009). 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 0.7 ml. The reaction was started by the addition of MG and the increase in absorbance was recorded at 240 nm for 1 min. The activity was calculated using the extinction coefficient of $3.37 \text{ mM}^{-1} \text{ cm}^{-1}$.

Glyoxalase II (EC: 3.1.2.6) activity was determined according to the method of Principato et al. (1987) by monitoring the formation of GSH at 412 nm for 1 min. The reaction mixture contained 100 mM Tris-HCl buffer (pH 7.2), 0.2 mM DTNB and 1 mM S-D- lactoylglutathione (SLG) in a final volume of 1 ml. The reaction was started by the addition of SLG and the activity was calculated using the extinction co-efficient of $13.6 \text{ mM}^{-1} \text{ cm}^{-1}$.

Lipid peroxidation

The level of lipid peroxidation was measured in leaf tissue by estimating MDA, a decomposition product of the peroxidized polyunsaturated fatty acid component of the membrane lipid, using thiobarbituric acid (TBA) as the reactive material following the method of Heath and Packer (1968) with slight modifications (Hossain and Fujita 2010). The concentration of MDA was calculated by using the extinction co-efficient of $155 \text{ mM}^{-1} \text{ cm}^{-1}$ and expressed as $\text{nmol of MDA g}^{-1}$ fresh weight.

Measurement of H_2O_2

H_2O_2 was assayed according to the method described by Yu et al. (2003). H_2O_2 was extracted by homogenizing 0.5 g of leaf tissue with 3 ml of 50 mM K-phosphate buffer pH (6.5) at 4°C . The homogenate was centrifuged at $11,500\times g$ for 15 min. A 3-ml sample 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 $11,500\times g$ for 15 min at room temperature. The optical absorption of the supernatant was measured spectrophotometrically at 410 nm to determine the H_2O_2 content ($\epsilon=0.28 \mu\text{M}^{-1} \text{ cm}^{-1}$) and expressed as $\mu\text{mol g}^{-1}$ fresh weight.

Determination of protein

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

Statistical analysis

All data obtained were subjected to one-way analysis of variance (ANOVA) and the mean differences were com-

pared by a least significant difference (LSD) test using MSTAT-C. Differences at $P < 0.05$ were considered as significant.

Results

Cellular ascorbate and glutathione contents

A significant decrease (19%) in AsA content was observed due to Cd stress as compared to the control (Fig. 1). Proline- and betaine-supplemented Cd-stressed seedlings showed 5 and 2% decrease in AsA content as compared to control. However, proline- and betaine-supplemented Cd-stressed seedlings showed 17 and 21% higher in AsA content as compared to the seedlings treated with Cd alone.

Cd stress caused a significant increase in GSH content (113%) as compared to the control (Fig. 2a). Proline- and betaine-supplemented Cd-stressed seedlings showed 144 and 145% increase in GSH content as compared to control and its level was significantly higher than the seedlings treated with Cd alone.

A profound increase in GSSG content (96%) was observed in response to Cd stress (Fig. 2b). Proline- and betaine-supplemented Cd-stressed seedlings showed 38 and 5% increase in GSSG content as compared to control but the level was significantly lower than the seedlings treated with Cd only. Proline- and betaine-supplemented Cd-stressed seedlings showed 79 and 136% increase in GSSH/GSSG ratio as compared to the control (Fig. 2c). Seedlings treated with betaine showed significantly higher GSH/GSSG ratio than those treated with proline.

Fig. 1 Reduced ascorbate (AsA) contents in mung bean seedlings induced by proline and betaine under cadmium stress conditions. Cd, Cd+P and Cd+B indicates 1 mM CdCl₂, 1 mM CdCl₂+5 mM proline and 1 mM CdCl₂+5 mM betaine treatments, respectively. Each value is the mean±SE from four independent experiments. Bars with different letters are significantly different at $P < 0.05$

Fig. 2 Glutathione accumulation in mung bean seedlings induced by proline and betaine under cadmium stress conditions. a: reduced glutathione (GSH), b: oxidized glutathione (GSSG), c: GSH/GSSG ratio. Cd, Cd+P and Cd+B indicates 1 mM CdCl₂, 1 mM CdCl₂+5 mM proline and 1 mM CdCl₂+5 mM betaine treatments, respectively. Each value is the mean±SE from four independent experiments. Bars with different letters are significantly different at $P < 0.05$

Antioxidant enzymes

APX activity increased in response to Cd stress (Fig. 3a). Proline- and betaine-supplemented Cd-stressed seedlings

Fig. 3 Activities of APX (a), MDHAR (b), DHAR (c), GR (d), GPX (e), GST (f) and CAT (g) in mung bean seedlings induced by proline and betaine under cadmium stress conditions. Cd, Cd+P and Cd+B indicates 1 mM CdCl₂, 1 mM CdCl₂+5 mM proline and 1 mM CdCl₂+5 mM betaine treatments, respectively. Each value is the mean±SE from four independent experiments. Bars with different letters are significantly different at $P<0.05$.

also showed significant increased in APX activity (35 and 57% by proline and betaine, respectively) as compared to the control. Seedlings treated with betaine had significantly higher APX activity than those treated with proline.

MDHAR activity decreased significantly (24%) in response to Cd stress as compared to the control (Fig. 3b). Proline-supplemented Cd-stressed seedlings maintained similar level of activity as compared to the control but betaine-supplemented Cd-stressed seedlings showed significant increase in MDHAR activity (23%) as compared to the control. Proline- and betaine-supplemented Cd-stressed seedlings showed 27 and 54% higher in MDHAR activity as compared to the seedlings treated with Cd alone. Seedlings treated with betaine had higher activity than those treated with proline.

A significant decrease in DHAR activity was observed in response to Cd stress as compared to the control (Fig. 3c). Proline- and betaine-supplemented Cd-stressed seedlings showed significantly higher DHAR activity as compared to the seedlings treated with Cd alone.

GR activity decreased significantly (20%) under Cd stress in comparison with the control (Fig. 3d). Proline- and betaine-supplemented Cd-stressed seedlings showed (16 and 36%) increased in GR activity in comparison with the control. Both proline- and betaine-supplemented Cd-stressed seedlings showed significantly higher GR activity in comparison with the seedlings treated with Cd alone.

GPX activity was increased by 26% as compared to the control in response to Cd stress (Fig. 3e). Proline- and betaine-supplemented Cd-stressed seedlings showed 38 and 36% increase in GPX activity as compared to the control. However, GPX activity did not vary significantly irrespective of the presence or absence of proline or betaine.

GST activity was increased significantly (46%) in response to Cd stress (Fig. 3f) as compared to the control. Proline- and betaine-supplemented Cd-stressed seedlings showed 90 and 101% increase in GST activity as compared to the control. However, proline- and betaine-supplemented Cd-stressed seedlings showed significantly higher (30 and 39% by proline and betaine, respectively) GST activity as compared to the seedlings treated with Cd alone.

A significant decrease in CAT activity (55%) was observed in comparison with the control (Fig. 3g) in response to Cd stress. Proline- and betaine-supplemented Cd-stressed seedlings also showed significant decrease (47 and 38% by proline and betaine, respectively) in CAT

activity as compared to control. Betaine-supplemented Cd-treated seedlings showed significantly higher (37%) in CAT activity as compared to the seedlings treated with Cd alone.

Glyoxalase pathway enzymes

A slight increase in Gly I activity was observed in response to Cd stress (Fig. 4a). Proline- and betaine-supplemented Cd-stressed seedlings showed 24 and 29% increased in Gly I activity as compared to the control. However, both proline and betaine treated seedlings showed significantly higher Gly I activity as compared to seedlings treated with Cd alone.

Gly II activity decreased in response to Cd stress (Fig. 4b). Proline- and betaine-supplemented Cd treated seedlings showed 63 and 25% increase in Gly II activity as compared to the control. Proline- and betaine-treated seedlings showed significantly higher Gly II activity as compared to the seedlings treated with Cd alone; however, proline-treated seedlings had higher Gly I activity than those treated with betaine.

Fig. 4 Activities of Gly I (a) and Gly II (b) in mung bean seedlings induced by proline and betaine under cadmium stress conditions. Cd, Cd+P and Cd+B indicates 1 mM CdCl₂, 1 mM CdCl₂+5 mM proline and 1 mM CdCl₂+5 mM betaine treatments, respectively. Each value is the mean±SE from four independent experiments. Bars with different letters are significantly different at $P<0.05$

H₂O₂ content and lipid peroxidation (MDA)

Cd stress resulted in a significant increase (130%) in H₂O₂ content as compared to the control (Fig. 5a). Proline- and betaine-supplemented Cd-stressed seedlings also showed a significant increase in H₂O₂ content (92 and 98% by proline and betaine, respectively) as compared to control; however, the levels were significantly lower than the seedlings treated with Cd alone.

Lipid peroxidation levels in leaf tissues, measured as the content of MDA, is represented in Fig. 5b. A significant increase in lipid peroxidation (103%) was observed due to Cd stress. Proline- and betaine-supplemented Cd-stressed seedlings showed 48 and 43% increase in MDA content as compared to the control; however, the MDA levels were significantly lower (29 and 30% by proline and betaine, respectively) than the seedlings treated with Cd alone.

Fig. 5 Changes in H₂O₂ concentration (a) and lipid peroxidation (represented by MDA) (b) level in mung bean seedlings induced by proline and betaine under cadmium stress conditions. Cd, Cd+P and Cd+B indicates 1 mM CdCl₂, 1 mM CdCl₂+5 mM proline and 1 mM CdCl₂+5 mM betaine treatments, respectively. Each value is the mean $\pm$ SE from four independent experiments. Bars with different letters are significantly different at $P < 0.05$

Discussion

Plants, like all other organisms, have evolved a complex network of homeostatic mechanisms to minimize the damages from exposure to non-essential metal ions like Cd. Although the mechanism by which metals cause plant injury is not clearly understood, there is increasing evidence that, at least in part, metal toxicity is due to oxidative damage (Chao and Seo 2005; Hsu and Kao 2007a; Hu et al. 2009). One strategy for improving tolerance to Cd is altered cellular metabolism leading to accumulation of particular solutes that include nitrogen-containing compounds, such as proline and other amino acids, polyamines and quaternary ammonium compounds like betaine that stabilize proteins or stress proteins that protect plants to reduce the content of undesired heavy metals (Chen and Murata 2002). Proline and betaine accumulation in plants under metal stress has been widely reported (Sun et al. 2007; Costa and Morel 1994; Tamas et al. 2008). However, the mechanisms by which proline and betaine protect plant cells under heavy metal stress have remained unsolved. Recent studies in plants have demonstrated that exogenous application of betaine or proline at high concentration (1–20 mM) enhance tolerance to abiotic oxidative stress (Park et al. 2006; Hoque et al. 2007; Huang et al. 2009; Islam et al. 2010; Hossain and Fujita 2010). Extensive research findings support the idea that coordinated induction and regulation of the antioxidant and glyoxalase pathway enzymes are necessary to obtain substantial tolerance against oxidative stress. In this study, we investigated the possible regulatory role of exogenous proline and betaine in AsA and GSH content and the activities of AsA-GSH cycle and glyoxalase system enzymes including GST, GPX and CAT. It is hypothesized that the increased antioxidant protection through AsA-GSH cycle and glyoxalase pathway enzymes offered by proline and betaine might contribute to better protection against Cd-induced oxidative damages.

AsA is the most abundant antioxidant and serves as a major contributor to the cellular redox state and protects plants against oxidative damage resulting from a range of biotic and abiotic stresses (Smirnoff 2000). It is the substrate of cAPX and the corresponding organellar isoforms, which are critical components of the AsA-GSH cycle for H₂O₂ detoxification (Nakano and Asada 1981; Dalton et al. 1986). AsA can directly quench ¹O₂, O₂^{•-} and [•]OH. Therefore, elevated levels of endogenous AsA in plants are necessary to offset oxidative stress in addition to regulating other plant metabolic processes (Smirnoff 2000; Athar et al. 2008). Results obtained in this study reveal that Cd stress caused a sharp decrease in AsA content (Fig. 1). Our result is consistent with the recent reports of rice plants subjected to Cd toxicity (Hsu and Kao 2007b; Semane et al.

2007; Chao et al. 2010). But, pretreatment with AsA or L-galactono- γ -lactone (GL), a precursor of AsA, caused a reduction in Cd toxicity by enhancing the activities of antioxidant enzymes and by increasing gene expression (Zhao et al. 2005; Paradiso et al. 2008; Chao et al. 2010). The biosynthetic capacity of AsA is impaired under severe stress conditions because the AsA pool is generally determined by its rates of not only regeneration but also synthesis (Song et al. 2005). It has been reported that regeneration of AsA under Cd stress is insufficient or that AsA synthesis is lower than AsA catabolism (Schützendübel et al. 2001, 2002; Chao et al. 2010). Importantly, both proline and betaine-supplemented seedlings maintained significantly higher AsA content as compared to the seedling treated Cd alone. Therefore, both proline and betaine might play important roles in AsA regeneration through efficient functioning of MDHAR and DHAR (Yang et al. 2007).

APX is a key enzyme in the AsA-GSH cycle and plays a vital role in plant defense against oxidative stress by catalyzing the conversion of H_2O_2 to water. In our experiments, upon imposition of Cd stress, APX activity increased (Fig. 3a), which supports previous studies (Chao et al. 2010; Romero-Puertas et al. 2007; Hsu and Kao 2007b; Smeets et al. 2008). However, proline- and betaine-supplemented Cd-stressed seedlings showed a sharp increase in APX activity as compared to control as well as Cd-treated seedlings. Similar effects of proline and betaine on APX activity were also observed in plants in response to various stresses (Ma et al. 2006; Demiral and Türkan 2004). These results suggest that both exogenous proline and betaine could contribute in detoxification of H_2O_2 by enhancing APX activity under Cd stress.

The univalent oxidation of AsA leads to the formation of MDHA, which is converted to AsA by the action of NADPH-dependent MDHAR activity or disproportionates nonenzymatically to AsA and dehydroascorbate (DHA). DHA undergoes irreversible hydrolysis to 2, 3-diketogulonic acid or is recycled to AsA by DHAR, thereby capturing AsA before it is lost. Recent studies showed that both MDHAR and DHAR are equally important in regulating AsA levels and its redox state under oxidative stress condition (Eltayeb et al. 2006, 2007; Wang et al. 2010; Shalata et al. 2001). Results obtained in this study showed that both MDHAR and DHAR decreased along with a decreased level of AsA (Figs. 1 and 3b, c). These results indicate insufficient regeneration of AsA from MDHA and DHA under Cd stress conditions. However, both proline- and betaine-supplemented Cd-stressed seedlings mitigated the reduction of MDHAR and DHAR activity under Cd stress since proline and betaine can work as an enzyme protectant under stress (see Introduction). It is important to note that proline maintained higher AsA level by maintaining higher DHAR

activity whereas betaine maintained higher AsA level through enhancement of MDHAR activity indicating that both proline and betaine must have a role in AsA recycling under Cd stress. A high AsA level found in the present study also supports this.

GSH is the most abundant non-protein, sulfhydryl-containing molecule, and plays a vital role in the defense system of plants including redox regulation, conjugation of metabolites, detoxification of xenobiotics and homeostasis and cellular signalling that triggers adaptive responses (Foyer and Noctor 2005; Noctor et al. 2002; Hossain and Fujita 2010). Several authors have shown that an elevated GSH content is correlated with ability of plants to withstand Cd-induced oxidative stress (Chen and Kao 1995; Zhu et al. 1999; Reisinger et al. 2008). GSH takes part in the control of H_2O_2 levels and regeneration of AsA through the AsA-GSH cycle (Foyer and Noctor 2005). It can also function directly as a free radical scavenger by reacting with $^1\text{O}_2$, $\text{O}_2^{\cdot-}$, and $^{\cdot}\text{OH}$ (Larson 1988). GSH protects proteins against denaturation caused by the oxidation of protein thiol groups under stress. GSH is a substrate for GPX and GST and co-factor of Gly I, which are also involved in the removal of ROS, MG and endogenous toxic compounds. It is also involved in the transfer and storage of sulfur and in the detoxification of heavy metals where phytochelatin (PC) derived from GSH forms heavy metal complexes. Along with its oxidized form (GSSG), it acts as a redox couple important for maintaining the cellular homeostasis, playing a key role in diverse signaling systems in plants (Noctor 2006). Furthermore, GSH plays a protective role in salt tolerance by maintaining the redox state (Shalata et al. 2001). The increased level of the GSH pool is generally regarded as a protective response against oxidative stress (May and Leaver 1993; Xiang and Oliver 1998), although defense against stress situations sometimes occurs irrespective of the GSH concentration (Potters et al. 2004). In this study, Cd stress caused a significant increase in GSH content (Fig. 2a). Increase in GSH content in response to Cd stress were also reported in rice seedlings (Hu et al. 2009; Xu et al. 2009) and GSH metabolic genes coordinately respond to heavy metal stress (Xiang and Oliver 1998). The increase in GSSG content under Cd stress in the seedlings may partly be attributed to a decreased rate of GSH recycling or an increased rate of its degradation under the conditions of stress (Noctor and Foyer 1998; Hossain and Fujita 2010). Conversion of DHA to AsA requires extensive utilization of GSH (De Gara et al. 2000). The decreased levels of GSH in Cd-stressed seedlings may be attributed to an increase in DHAR activity which utilizes GSH as an electron donor in the reduction of DHA. However, in our experiment at severe Cd stress significant increases in GSSG content was observed although DHAR activity was decreased significantly (Figs. 2b and 3c). The formation of GSSG in Cd treated seedlings might be due to

the reaction of GSH with oxyradicals generated due to oxidative stress or due to enhancement of GPX and GST activity that decompose H_2O_2 and organic hydroperoxide or decrease GR activity (Shalata et al. 2001; Aravind and Prasad 2005; Hossain and Fujita 2010) (Fig. 3d, e, f). However, both proline- and betaine-supplemented Cd-stressed seedlings maintained a higher GSH and GSH/GSSG ratio as compared to the control and Cd-stressed seedlings (Fig. 2a, c). An increase in GSH content due to proline pretreatment and lower lipid peroxidation has recently been reported (Xu et al. 2009). Transgenic plants for higher proline and betaine biosynthesis also showed higher GSH, GSH/GSSG ratio and lower GSSG content as compared to wild-type under Cd medium and other stress conditions (Siripornadulsil et al. 2002; Yang et al. 2007). Therefore, both proline and betaine might play a significant role in maintaining higher GSH level either through efficient recycling or by modulating higher GSH synthesis (Kocsy et al. 2005; Hossain and Fujita 2010).

Biochemical and molecular studies have shown that GR plays an essential role in cell defense against reactive oxygen metabolites by sustaining the reduced status of glutathione and ascorbate pools which in turn maintain cellular redox state under stress (Ansel et al. 2006; Romero-Puertas et al. 2006). It has been observed that stress-tolerant plants tend to have high activities of GR (Sekmen et al. 2007; Aghaei et al. 2009). Additionally, overexpression of GR increases antioxidant activity and improves tolerance to oxidative stress (Noctor et al. 1998; Potters et al. 2004). In contrast, decreased GR activity results in increased stress sensitivity (Noctor and Foyer 1998). Results obtained in this investigation reveal that Cd stress significantly decreased GR activity (Fig. 3d). Decrease in GR activity due to Cd stress has been previously reported (Smeets et al. 2008; Gratao et al. 2008; Nouairi et al. 2009). Exogenous application of proline or betaine kept this activity significantly higher under Cd stress which is accompanied by lower GSSG content, indicates proline and betaine play significant roles in efficient recycling of GSSG. Similar protective role of exogenous proline and betaine were also observed in our previous findings (Hossain and Fujita 2010). Increased GR activity by proline and betaine under Cd stress conditions contributes to the maintenance of a higher GSH/GSSG ratio and increased GSH level which is used by DHAR and other GSH dependent enzymes involved in the antioxidant defense and glyoxalase systems (Hossain and Fujita 2010).

CAT is a key antioxidant enzyme, present exclusively in peroxisomes, which decomposes H_2O_2 . Our results indicated a sharp decline in CAT activity under Cd stress which suggests that this enzyme is unable to detoxify H_2O_2 generated by Cd stress (Fig. 3g). Decreases in CAT activity due to Cd stress were also reported in various plant species

(Romero-Puertas et al. 2006; Hu et al. 2009; Islam et al. 2010). But both proline- and betaine-supplemented Cd-stressed seedlings also had decreased CAT activity indicating that both are unable to increase CAT activity under severe Cd stress condition. However, both of them maintained slightly higher CAT activity in comparison with seedlings treated with Cd alone. Our results are also supported by the results of Xu et al. (2009) and Islam et al. (2010). Similar effects of proline and betaine on CAT activity were also observed in response to various stresses (Demiral and Türkan 2004; Khedr et al. 2003).

GSTs constitute a family of multifunctional enzymes present in both plants and animals. These dimeric enzymes catalyze the conjugation of GSH to a variety of electrophilic, hydrophobic, and often toxic substrates, thereby reducing their toxicity. It has recently been discovered that GPXs are ubiquitously occurring enzymes in plant cells that use GSH to reduce H_2O_2 and organic and lipid hydroperoxides. In this study, Cd stress lead to significant increases in GPX and GST activities compared to those of the control. The increase in GPX and GST activities even under severe Cd stress condition indicates that both GPX and GST enzymes may be more stable or more important for stress tolerance than other antioxidant enzymes (Fig. 3e & f). Increased GPX and GST activity due to Cd stress were also reported in tomato (Gratao et al. 2008), in rice seedlings (Hu et al. 2009), in *Arabidopsis* (Semane et al. 2007; Smeets et al. 2008), in barley root tips (Haluskova et al. 2009) and in *Brassica napus* (Nouairi et al. 2009). In the present investigation, the increases of GST and GPX were not high enough to protect cells from Cd-induced oxidative damage. However, the fact that exogenous proline and betaine increased GST and GPX activities and suppressed the production of H_2O_2 and MDA level indicates that both of them are able to reduce Cd-induced oxidative damage by increasing GST and GPX activities. Similar protective roles of exogenous proline and betaine were also observed in our previous experiments with mung bean seedlings under salinity stress (Hossain and Fujita 2010).

The accumulation of MG is indicative of abiotic stress conditions including heavy metals (Yadav et al. 2005a, b; Singla-Pareek et al. 2003; Hossain et al. 2009). MG causes a dose- and time-dependent depletion of GSH and increased GSH oxidation or formation of GSSG. At a high concentration of MG, GSH may be trapped as S-2-hydroxyacylglutathione, resulting in GSH depletion (Kalapos et al. 1992). Therefore, high amount of MG accumulation during stress could either act directly as a potent toxic agent affecting various plant processes or could deplete GSH. Higher Gly I and Gly II activities might protect plants against MG that is formed during abiotic stresses (Veena et al. 1999; Jain et al. 2002; Saxena et al. 2005; Hossain et al. 2009; Hossain and Fujita 2009). Overexpression of Gly I

and Gly II in transgenic plants inhibits an increase in MG level under Zn and salt stress condition and confers tolerance to heavy metal and high salt by increasing the GSH based detoxification system and decreasing lipid peroxidation (Veena et al. 1999; Singla-Pareek et al. 2003; Yadav et al. 2005a, b). A proteomic study of salt-tolerant barley also showed higher Gly I protein expression under salinity stress conditions (Witzel et al. 2009). In the present study, we observed a slight increase in Gly I activity while the Gly II activity decreased (Fig. 4a & b). An increase in Gly I activity in response to abiotic stresses including Cd and Zn, have also been reported in various plant species (Singla-Pareek et al. 2006; Hossain et al. 2009; Hossain and Fujita 2009, 2010). A decrease in Gly II activity was also observed in our previous study with onion callus subjected to Cd stress (Hossain and Fujita 2009). A decrease in Gly II activity might be due to inactivation or proteolytic degradation of enzymes. Therefore, a slight increase in Gly I activity and decrease of Gly II activity suggest that detoxification of MG via the glyoxalase system is not sufficient under severe Cd stress. However, both proline and betaine-treated seedlings could partially alleviate the stress-induced oxidative damage by maintaining higher Gly I and Gly II activities suggesting that both of them were able to enhance GSH regeneration and glutathione redox state via the glyoxalase system. The high GSH levels in turn facilitate phytochelatin synthesis and sequestration of heavy metal phytochelatin conjugates in the vacuole (Siripornadulsil et al. 2002).

Cd is a non-redox-active metal but it does elevate lipid peroxidation, contributing to a process of oxidative damage (Gratao et al. 2008). Cd stress has been shown to cause an abrupt increase in the amount of MDA in leaves of different crops (Hsu and Kao 2007a, b; Wang et al. 2008; Hu et al. 2009; Nouairi et al. 2009; Chao et al. 2010) and in most of the cases the increase in H_2O_2 content was correlated with a higher MDA level (Hsu and Kao 2007b; Hu et al. 2009; Islam et al. 2010; Wang et al. 2008). Control of the levels of H_2O_2 and MDA is thought to be a mechanism by which plants tolerate the stress (Hu et al. 2009; Hossain and Fujita 2010; Islam et al. 2010). Our results demonstrated a marked increase in H_2O_2 and MDA contents. The increase of H_2O_2 was correlated with the lipid peroxidation (Fig. 5a & b). A sharp increase in the level of H_2O_2 and lipid peroxidation in Cd-stressed seedlings resulted in increased oxidative damage probably due to impairment of the antioxidant defense and MG detoxification system. However, proline- and betaine-supplemented Cd-stressed seedlings maintained significantly lower H_2O_2 and MDA levels as compared to the seedlings treated with Cd alone (Fig. 5a & b), suggesting that both proline and betaine protect against Cd-dependent oxidative damage by enhancing antioxidant defense and MG detoxification systems (Demiral and Türkan 2004; Okuma et al. 2004; Park et al. 2006; Huang

et al. 2009; Hossain and Fujita 2010). Similar findings have been reported by other researchers demonstrating higher Cd tolerance by in vivo betaine or proline synthesis in plants (Siripornadulsil et al. 2002; Shao et al. 2008).

In conclusion, Cd stress induces a severe oxidative stress in mung bean seedlings where the antioxidant defense and glyoxalase systems seemingly fail to combat with the stress-induced oxidative damage. Exogenous applications of proline or betaine showed enhance tolerance to oxidative damage by enhancing ROS and MG detoxification systems. These findings together with our earlier findings (Hossain and Fujita 2010) suggest that both betaine and proline provide protective effects against Cd-induced oxidative stress by reducing H_2O_2 and lipid peroxidation levels and by increasing the antioxidant defense and glyoxalase systems. The information available concerning plants subjected to Cd and different concentrations of proline and betaine should provide a better understanding of the mechanisms of detoxification, which may help integrate biochemical genetics with plant breeding to produce stress-tolerant plants for detoxification or phytoremediation. However, further studies are required to elucidate the molecular mechanism and signalling pathways underlying the roles of proline and betaine in Cd tolerance of plants.

Acknowledgements Thanks are due to Dr. Md. Abdul Malek, Principal Scientific Officer, Plant Breeding Division, Bangladesh Institute of Nuclear Agriculture (BINA), Bangladesh Agricultural University (BAU) campus, Mymensingh-2202, Bangladesh for providing the seeds of *Vigna* cultivars.

References

- Aghaei K, Ehsanpour AK, Komatsu S (2009) Potato responds to salt stress by increased activity of antioxidant enzymes. *J Integr Plant Biol* 51:1095–1103
- Ansel DC, Franklin MLT, De Carvalho MHC, Lameta ADA, Fodil YZ (2006) Glutathione reductase in leaves of cowpea: cloning of two cDNAs, expression and enzymatic activity under progressive drought stress desiccation and abscisic acid treatment. *Ann Bot* 98:1279–1287
- Aravind PA, Prasad NV (2005) Modulation of cadmium induced oxidative stress in *Ceratophyllum demersum* by zinc involves ascorbate-glutathione cycle and glutathione metabolism. *Plant Physiol Biochem* 45:107–116
- Ashraf M, Foolad MR (2007) Roles of glycinebetaine and proline in improving plant abiotic resistance. *Environ Exp Bot* 59:206–16
- Athar HR, Khan A, Ashraf M (2008) Exogenously applied ascorbic acid alleviates salt-induced oxidative stress in wheat. *Environ Exp Bot* 63:224–231
- Bassi R, Sharma SS (1993a) Changes in proline content accompanying the uptake of zinc and copper by *Lemna minor*. *Ann Bot* 72:151–154
- Bassi R, Sharma SS (1993b) Proline accumulation in wheat seedlings exposed to zinc and copper. *Phytochem* 33:1339–1342
- Booth J, Boyland E, Sims P (1961) An enzyme from rat liver catalyzing conjugation. *Biochem J* 79:516–524

- Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:248–254
- Brigelius-Flohe R, Flohe L (2003) Is there a role of glutathione peroxidases in signaling and differentiation? *Biofactors* 17:93–102
- Cakmak I, Strbac D, Marschner H (1993) Activities of hydrogenperoxide scavenging enzymes in germinating wheat seeds. *J Exp Bot* 44:127–132
- Chao UH, Seo NH (2005) Oxidative stress in *Arabidopsis thaliana* exposed to cadmium is due to hydrogen peroxide accumulation. *Plant Sci* 168:113–120
- Chao YY, Hong CY, Kao CH (2010) The decline in ascorbic acid is associated with cadmium toxicity of rice seedlings. *Plant Physiol Biochem* 48:374–381
- Chen SL, Kao CH (1995) Glutathione reduces the inhibition of rice seedling root growth caused by cadmium. *Plant Growth Regul* 16:249–252
- Chen TH, Murata N (2002) Enhancement of tolerance of abiotic stress by metabolic engineering of betaines and other compatible solutes. *Curr Opin Plant Biol* 5:250–257
- Chen C, Wabduragala S, Becker DF, Dickmen MB (2006) Tomato QM-like protein protects *Saccharomyces cerevisiae* cells against oxidative stress by regulation intracellular proline levels. *Appl Environ Microbiol* 72:4001–4006
- Clemens S (2006) Evolution and function of phytochelatase synthases. *J Plant Physiol* 163:319–332
- Costa G, Morel JL (1994) Water relations, gas exchange and amino acid content in Cd-treated lettuce. *Plant Physiol Biochem* 32:561–570
- Dalton DA, Russell SA, Hanus FJ, Pascoe GA, Evans HJ (1986) Enzymatic reactions of ascorbate and glutathione that prevent peroxide damage in soybean root nodules. *Proc Natl Acad Sci USA* 83:3811–3815
- De Gara L, Paciolla C, De Tullio MC, Motto M, Arrigioni O (2000) Ascorbate-dependent hydrogen peroxide detoxification and ascorbate regeneration during germination of a highly productive maize hybrid: evidence of an improved detoxification mechanism against reactive oxygen species. *Physiol Plant* 109:7–13
- Demiral T, Türkan I (2004) Does exogenous glycinebetaine affect antioxidative system of rice seedlings under NaCl treatment? *J Plant Physiol* 161:1089–1100
- Dinakar N, Nagajyothi PC, Suresh S, Damodharam T, Suresh C (2009) Cadmium induced changes on proline, antioxidant enzymes, nitrate and nitrite reductases in *Arachis hypogaea* L. *J Environ Biol* 30:289–294
- Elia AC, Galarini R, Taticchi MI, Dorr AJM, Manilacci L (2003) Antioxidant responses and bioaccumulation in *Lathyrus melas* under mercury exposure. *Ecotoxicol Environ Saf* 55:162–167
- Eltayeb AE, Kawano N, Badawi G, Kaminaka H, Sanekata T, Morishima I (2006) Enhanced tolerance to ozone and drought stresses in transgenic tobacco overexpressing dehydroascorbate reductase in cytosol. *Physiol Plant* 127:57–65
- Eltayeb AL, Kawano N, Badawi GH, Kaminaka H, Sanekata T, Shibahar T, Inanaga S, Tanaka K (2007) Overexpression of monodehydroascorbate reductase in transgenic tobacco confers enhanced tolerance to ozone, salt and polyethylene glycol stresses. *Planta* 225:1255–1264
- Foyer CH, Noctor G (2005) Redox homeostasis and antioxidant signaling: a metabolic interface between stress perception and physiological responses. *Plant Cell* 17:1866–1875
- Gechev TS, Van Breusegem F, Stone JM, Denev L, Laloi C (2006) Reactive oxygen species as signals that modulate plant stress responses and programmed cell death. *BioEssays* 28:1091–1101
- Gratao PL, Monteiro CC, Antunes AM, Peres LEP, Azevedo RA (2008) Acquired tolerance to tomato (*Lycopersicon esculentum* cv Miro-Tom) plants to cadmium-induced stress. *Ann Appl Biol* 153:321–333
- Haluskova L, Valentovicova K, Huttova J, Mistrik I, Tamas L (2009) Effect of abiotic stresses on glutathione peroxidase and glutathione S-transferase activity in barley root tips. *Plant Physiol Biochem* 47:1069–1074
- Heath RL, Packer L (1968) Photoperoxidation in isolated chloroplast. I. Kinetics and stoichiometry of fatty acid peroxidation. *Arch Biochem Biophys* 125:189–198
- Hoque MA, Banu MNA, Okuma E, Amako K, Nakamura Y, Shimoishi Y, Murata Y (2007) Exogenous proline and glycinebetaine ingresses NaCl-induced ascorbate-glutathione cycle enzyme activities and proline improves salt tolerance more than glycinebetaine in tobacco Bright yellow-2 suspension- cultured cells. *J Plant Physiol* 164:553–561
- Hossain MA, Fujita M (2009) Purification of glyoxalase I from onion bulbs and molecular cloning of its cDNA. *Biosci Biotechnol Biochem* 73:2007–2013
- Hossain MA, Fujita M (2010). Evidence for a role of exogenous glycinebetaine and proline in antioxidant defense and methylglyoxal detoxification systems in mung bean seedlings under salt stress. *Physiol Mol Biol Plants* 16:19–29
- Hossain MA, Nakano Y, Asada K (1984) Monodehydroascorbate reductase in spinach chloroplasts and its participation in the regeneration of ascorbate for scavenging hydrogen peroxide. *Plant Cell Physiol* 25:385–395
- Hossain MA, Hossain MZ, Fujita M (2009) Stress-induced changes of methylglyoxal level and glyoxalase I activity in pumpkin seedlings and cDNA cloning of glyoxalase I gene. *Aust J Crop Sci* 3:53–64
- Hsu YT, Kao CH (2007a) Toxicity in leaves of rice exposed to cadmium is due to hydrogen peroxide accumulation. *Plant Soil* 298:232–241
- Hsu YT, Kao CH (2007b) Cadmium induced oxidative damage in rice leaves reduced by polyamines. *Plant Soil* 291:27–37
- Hu Y, Ge Y, Zhang C, Ju T, Cheng W (2009) Cadmium toxicity and translocation in rice seedlings are reduced by hydrogen peroxide pretreatment. *Plant Growth Regul* 59:51–61
- Huang C, He W, Guo J, Chang X, Su P, Zhang L (2005) Increased sensitivity to salt stress in ascorbate-deficient *Arabidopsis* mutant. *J Exp Bot* 56:3041–3049
- Huang Y, Bie Z, Liu Z, Zhen A, Wang W (2009) Protective role of proline against salt stress is partially related to the improvement of water status and peroxidase enzyme activity in cucumber. *Soil Sci Plant Nutr* 55:698–704
- Islam MM, Hoque MA, Okuma E, Banu MNA, Shimoishi Y, Nakamura Y, Murata Y (2010) Exogenous proline and glycinebetaine increase antioxidant enzyme activities and confer tolerance to cadmium stress in cultured tobacco cells. *J Plant Physiol* 166:1587–1597
- Jain M, Choudhary D, Kale RK, Sarin NB (2002) Salt and glyphosate-induced increase in glyoxalase I activity in cell lines of groundnut (*Arachis hypogaea*). *Physiol Plant* 114:499–505
- Kalapos MP, Garzo T, Antoni F, Mandl J (1992) Accumulation of S-D-lactoylglutathione and transient decrease of glutathione level caused by methylglyoxal load in isolated hepatocytes. *Biochim Biophys Acta* 1135:159–64
- Khedr AHA, Abbas MA, Wahid AAA, Quick WP, Abogadallah GM (2003) Proline induces the expression of salt-stress-responsive proteins and may improve the adaptation of *Pancreaticum maritimum* L. to salt-stress. *J Exp Bot* 54:2553–2562
- Kocsy G, Laurie R, Szalai G, Szilagyi V, Simon-Sarkadi L, Galiba G (2005) Genetic manipulation of proline levels affects antioxidants in soybean subjected to simultaneous drought and heat stresses. *Physiol Plant* 124:227–235
- Kumar V, Yadav SK (2009) Proline and betaine provide protection to antioxidant and methylglyoxal detoxification systems during cold

- stress and *Camellia sinensis* (L.) O.Kuntze. *Acta Physiol Plant* 31:261–269
- Kuriakose SV, Prasad MNV (2008) Cadmium stress affects seed germination and seedling growth in *Sorghum bicolor* (L.) Moench by changing the activities of hydrolyzing enzymes. *Plant Growth Regul* 54:143–156
- Kuzniak E, Skłodowska M (2005) Compartment-specific role of the ascorbate-glutathione cycle in the response to tomato leaf cells to *Botrytis cinerea* infection. *J Exp Bot* 413:921–933
- Larson RA (1988) The antioxidants of higher plants. *Phytochem* 27:969–978
- Ma QQ, Wang W, Li YH, Li DQ, Zou Q (2006) Alleviation of photoinhibition in drought-stressed wheat (*Triticum aestivum*) by foliar-applied glycinebetaine. *J Plant Physiol* 163:165–175
- Mallick N, Mohn FH (2000) Reactive oxygen species: response of algal cells. *J Plant Physiol* 157:183–193
- May MJ, Leaver CJ (1993) Oxidative stimulation of glutathione synthesis in *Arabidopsis thaliana* suspension cultures. *Plant Physiol* 103:621–627
- Mehta SK, Gaur JP (1999) Heavy-metal-induced proline accumulation and its role in ameliorating metal toxicity in *Chlorella vulgaris*. *New Phytol* 143:253–259
- Mittler R, Vanderauwera S, Gollery M, Breusegem FV (2004) Reactive oxygen gene network of plants. *Trends Plant Sci* 9:490–498
- Nakano Y, Asada K (1981) Hydrogen peroxide is scavenged by ascorbate-specific peroxidase in spinach chloroplasts. *Plant Cell Physiol* 22:867–880
- Noctor G (2006) Metabolic signalling in defence and stress: the central roles of soluble redox couples. *Plant Cell Environ* 29:409–425
- Noctor G, Foyer CH (1998) Ascorbate and glutathione: keeping active oxygen under control. *Annu Rev Plant Physiol Plant Mol Biol* 49:249–279
- Noctor G, Arisi A, Jouanin L, Kunert KJ, Rennenberg H, Foyer C (1998) Glutathione: biosynthesis, metabolism and relationship to stress tolerance explored in transformed plants. *J Exp Bot* 49:623–647
- Noctor G, Gomez L, Vanaker H, Foyer CH (2002) Interactions between biosynthesis, compartmentation and transport in the control of glutathione homeostasis and signaling. *J Exp Bot* 53:1283–1304
- Nouairi I, Ammar WB, Youssef NB, Miled DDB, Ghorbal MH, Zarrouk M (2009) Antioxidant defense system in leaves of Indian mustard (*Brassica juncea*) and rape (*Brassica napus*) under cadmium stress. *Acta Physiol Plant* 31:237–247
- Okuma E, Soeda K, Fukuda M, Tada M, Murata Y (2002) Negative correlation between the ratio of K^+ to Na^+ and proline accumulation in tobacco suspension cells. *Soil Sci Plant Nutr* 48:753–757
- Okuma E, Murakami Y, Shimoishi Y, Tada M, Murata Y (2004) Effects of exogenous application of proline and betaine on the growth of tobacco cultured cells under saline conditions. *Soil Sci Plant Nutr* 50:301–1305
- Paradiso A, Berardino R, de Pinto M, di Toppi LS, Storelli FT, de Gara L (2008) Increase in ascorbate-glutathione metabolism as local and precocious systemic responses induced by cadmium in durum wheat plants. *Plant Cell Physiol* 49:362–374
- Park EJ, Jeknic Z, Chen THH (2006) Exogenous application of glycinebetaine increases chilling tolerance in tomato plants. *Plant Cell Physiol* 47:706–714
- Popova LP, Maslenskova LT, Yordanova RY, Ivanova AP, Krantev AP, Szalai G, Janda T (2009) Exogenous treatment with salicylic acid attenuates cadmium toxicity in pea seedlings. *Plant Physiol Biochem* 47:224–231
- Potters G, Horemans N, Bellone S, Caubergs RJ, Trost P, Guisez Y (2004) Dehydroascorbate influences the plant cell cycle through a glutathione-independent reduction mechanism. *Plant Physiol* 134:1479–1487
- Principato GB, Rosi G, Talesa V, Giovannini E, Uolila L (1987) Purification and characterization of two forms of glyoxalase II from rat liver and brain of Wistar rats. *Biochem Biophys Acta* 911:349–355
- Reisinger S, Schiavon M, Norman T, Pilon-Smits EAH (2008) Heavy metal tolerance and accumulation in Indian mustard (*Brassica juncea* L.) expressing bacterial gamma-glutamylcysteine synthetase or glutathione synthetase. *Int J Phytoremed* 10:1–15
- Romero-Puertas MC, Corpas FJ, Sandalio LM, Leterrier M, Rodriguez-Serrano M, del Rio LA, Palma JM (2006) Glutathione reductase from pea leaves: response to abiotic stress and characterization of the peroxisomal isozyme. *New Phytol* 170:43–52
- Romero-Puertas M, Corpas FJ, Rodriguez-Serrano M, Gomez M, del Rio AL, Sandalio LM (2007) Differential expression and regulation of antioxidative enzymes by cadmium in pea plants. *J Plant Physiol* 164:1346–1357
- Saxena M, Bisht R, Roy DS, Sopory SK, Bhalla-Sarinn M (2005) Cloning and characterization of a mitochondrial glyoxalase II from *Brassica juncea* that is upregulated by NaCl, Zn and ABA. *Biochem Biophys Res Commun* 336:813–819
- Schützendübel A, Schwanz P, Teichmann T, Gross K, Langenfeld-Heyser R (2001) Cadmium-induced changes in antioxidative systems, hydrogen peroxide content, and differentiation in Scots pine roots. *Plant Physiol* 127:887–898
- Schützendübel A, Nikolova P, Rudolf C, Polle A (2002) Cadmium and H_2O_2 -induced oxidative stress in *Populus × canescens* roots. *Plant Physiol Biochem* 40:577–584
- Sekmen AH, Türkan I, Takio S (2007) Differential responses of antioxidative enzymes and lipid peroxidation to salt stress in salt-tolerant *Plantago maritima* and salt-sensitive *Plantago media*. *Physiol Plant* 131:399–411
- Semane B, Cuypers A, Smeets K, Van Belleghem F, Horemans F, Schat H, Vangronsveld J (2007) Cadmium responses in *Arabidopsis thaliana*: glutathione metabolism and antioxidative defence system. *Physiol Plant* 129:519–528
- Shalata A, Mittova V, Volokita M, Guy M, Tal M (2001) Response of the cultivated tomato and its wild salt-tolerant relative *Lycopersicon pennellii* to salt-dependent oxidative stress: the root antioxidative system. *Physiol Plant* 112:487–494
- Shao G, Chen M, Wang W, Zhang G (2008) The effect of salinity pretreatment on Cd accumulation and Cd-induced stress in *BADH*-transgenic and nontransgenic rice seedlings. *J Plant Growth Regul* 27:205–210
- Singla-Pareek SL, Ray M, Reddy MK, Sopory SK (2003) Genetic engineering of the glyoxalase pathway in tobacco leads to enhanced salinity tolerance. *Proc Natl Acad Sci USA* 100:14672–14677
- Singla-Pareek SL, Yadav SK, Pareek A, Reddy MK, Sopory SK (2006) Transgenic tobacco overexpressing glyoxalase pathway enzymes grow and set viable seeds in zinc-spiked soils. *Plant Physiol* 140:613–623
- Singla-Pareek SL, Yadav SK, Pareek A, Reddy MK, Sopory SK (2008) Enhancing salt tolerance in a crop plant by overexpression of glyoxalase II. *Transgenic Res* 17:171–180
- Siripornadulsil S, Traina S, Verma DPS, Sayre RT (2002) Molecular mechanisms of proline-mediated tolerance to toxic heavy metals in transgenic microalgae. *Plant Cell* 14:2837–2847
- Smeets K, Ruytinx J, Semane B, Belleghem FV, Remans T, Sanden SV, Vangronsveld J, Cupers A (2008) Cadmium-induced transcriptional and enzymatic alterations related to oxidative stress. *Environ Exp Bot* 63:1–8
- Smirnoff N (2000) Ascorbic acid: metabolism and functions of a multifaceted molecule. *Curr Opin Plant Biol* 3:229–235

- Song XS, Hu WH, Mao WH, Ogwen JO, Zhou YH, Yu JQ (2005) Response of ascorbate peroxidase isoenzymes and ascorbate regeneration system to abiotic stresses in *Cucumis sativus* L. *Plant Physiol Biochem* 43:1082–1088
- Subbarao GV, Wheeler RM, Levine LH, Stutte GW (2001) Glycinebetaine accumulation, ionic and water relations of red-beet at contrasting levels of sodium supply. *J Plant Physiol* 158:767–776
- Sun RL, Zhou QX, Sun FH, Jin CX (2007) Antioxidative defense and proline/phytochelatin accumulation in a newly discovered Cd-hyperaccumulator, *Solanum nigrum* L. *Environ Exp Bot* 60:468–476
- Tamas L, Dudikova J, Durcekova K, Haluskova L, Huttova J, Mistrik I, Olle M (2008) Alteration of the gene expression, lipid peroxidation, proline and thiol content along the barley root exposed to cadmium. *J Plant Physiol* 165:1193–1203
- Tamás L, Mistrik I, Huttová J, Halusková L, Valentovicová K, Zelinová V (2010) Role of reactive oxygen species-generating enzymes and hydrogen peroxide during cadmium, mercury and osmotic stresses in barley root tip. *Planta* 231:221–231
- Tamura T, Hara K, Yamaguchi Y, Koizumi N, Sano H (2003) Osmotic stress tolerance of transgenic tobacco expressing a gene encoding a membrane-located receptor-like protein from tobacco plants. *Plant Physiol* 131:454–462
- Veena, Reddy VS, Sopory SK (1999) Glyoxalase I from *Brassica juncea*: molecular cloning, regulation and its over-expression confer tolerance in transgenic tobacco under stress. *Plant J* 17:385–395
- Wang Z, Zhang Y, Huang Z, Huang L (2008) Antioxidant response of metal-accumulator and non-accumulator plants under cadmium stress. *Plant Soil* 310:137–149
- Wang Z, Zhang L, Xiao Y, Chen W, Tang K (2010) Increased vitamin C content accompanied by an enhanced recycling pathway confers oxidative stress tolerance in *Arabidopsis*. *J Integr Plant Biol* 52:400–409
- Witzel K, Weidner A, Surabhi GK, Börner A, Mock HP (2009) Salt stress-induced alterations in the root proteome of barley genotypes with contrasting response towards salinity. *J Exp Bot* 60:3546–3557
- Xiang C, Oliver DJ (1998) Glutathione metabolic genes coordinately respond to heavy metals and jasmonic acid in *Arabidopsis*. *Plant Cell* 10:1539–1550
- Xu J, Yin HX, Li X (2009) Protective effects of proline against cadmium toxicity in micropropagated hyperaccumulator, *Solanum nigrum* L. *Plant Cell Rep* 28:325–353
- Yadav SK, Singla-Pareek SL, Ray M, Reddy MK, Sopory SK (2005a) Methylglyoxal levels in plants under salinity stress are dependent on glyoxalase I and glutathione. *Biochem Biophys Res Commun* 337:61–67
- Yadav SK, Singla-Pareek SL, Ray M, Reddy MK, Sopory SK (2005b) Transgenic tobacco plants overexpressing glyoxalase enzymes resist an increase in methylglyoxal and maintain higher reduced glutathione levels under salinity stress. *FEBS Lett* 579:6265–6271
- Yang X, Lu C (2005) Photosynthesis is improved by exogenous glycinebetaine in salt-stressed maize plants. *Physiol Plant* 124:343–352
- Yang X, Wen X, Gong H, Lu Q, Yang Z, Tang Y, Liang Z, Lu C (2007) Genetic engineering of the biosynthesis of glycinebetaine enhances thermotolerance of photosystem II in tobacco plants. *Planta* 225:719–733
- Yu CW, Murphy TM, Lin CH (2003) Hydrogen peroxide-induces chilling tolerance in mung beans mediated through ABA-independent glutathione accumulation. *Funct Plant Biol* 30:955–963
- Zhao ZQ, Cai YL, Zhu YG, Kneer R (2005) Cadmium-induced oxidative stress and protection by L-Galactono-1, 4-lactone in winter wheat (*Triticum aestivum* L.). *Plant Nutr Soil Sci* 168:759–763
- Zhu YL, Pilon-Smits EAH, Tarun A, Weber SU, Jouanin L, Terry N (1999) Cadmium tolerance and accumulation in Indian mustard is enhanced by overexpressing γ -glutamylcysteine synthetase. *Plant Physiol* 121:1169–1177