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EXOGENOUS PROLINE AND GLYCINEBETAINE ENHANCE GLUTATHIONE-DEPENDENT DEFENSE MECHANISMS AND MITIGATE CADMIUM TOXICITY IN CULTURED TOBACCO CELLS

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Abstract

Plants exposed to heavy metal stress, including cadmium (Cd), accumulate reactive oxygen species (ROS) and methylglyoxal(MG) that can cause cellular damage, leading to cell death. Efficient antioxidant defense and glyoxalase systems offered by proline and betaine might protect plants against Cd-induced damage. To elucidate the protection mechanisms of proline and betaine against Cd stress, we investigated the effects of exogenous proline and betaine on intracellular superoxide (O_2^-) level, contents of MG, carbonyl and phytochelatins (PCs), and activity of glutathione peroxidase (GPX), glyoxalase I and glyoxalsase II in cultured tobacco Bright Yellow-2 (BY-2) cells exposed to Cd stress. Intracellular O_2^- level, and MG, carbonyl, PC_2 and PC_3 contents were higher in Cd-stressed cells than non-stressed cells, whereas a decrease in PC_1 contents was observed in Cd-stressed cells. Cadmium stress caused significantly decrease in GPX, glyoxalase I and glyoxalase II activities in BY-2 cells. Exogenous application of proline or betaine to the Cd-stressed cells resulted in a decrease in O_2^- level, and MG and carbonyl contents but an increase in PC_1 and PC_3 contents. In Cd-stressed cells, exogenously applied proline or betaine led to an increase in GPX and glyoxalase I activities that are involved in the detoxification of H_2O_2 and MG, respectively. These results suggest that both proline and betaine provide protections against Cd toxicity by reducing carbonyl contents, and increment of the chelation with Cd and antioxidant defense and MG detoxification systems. The present study also demonstrates that betaine may contribute to the further protection against Cd toxicity due to a higher PC formation and GPX activity.

Keyword: Antioxidant defense, Betaine, Cadmium, Glyoxalase, Proline, Phytochelatins

Abbreviations: APX, ascorbate peroxidase; betaine, glycinebetaine; BY-2, Bright Yellow-2; CAT, catalase; GPX, glutathione peroxidase; GSH, Reduced glutathione; H_2O_2, hydrogen peroxide; MG, methylglyoxal; O_2^-, superoxide; PCs, phytochelatins; ROS, reactive oxygen species; SLG, S-D-lactylglutathione; SOD, superoxide dismutase

Introduction

Cadmium (Cd), a non essential heavy metal, is toxic to plants and disturbs various biochemical and physiological processes, leading to cell death and inhibition of growth (Rasheed *et al*., 2014; Islam *et al*., 2009a, b; Toppi and Gabbrielli, 1999; Sharma and Dietz, 2009). Similar to NaCl stress, Cd interferes with uptake and distribution of nutrients and water, and induces oxidative stress (Rasheed

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et al., 2014; Islam *et al*., 2009a, b; Toppi and Gabbrielli, 1999; Sharma and Dietz, 2009). Environmental stresses, including Cd, induce the production of ROS such as superoxide (O_2^-) and hydrogen peroxide (H_2O_2) in plant cells (Zouari *et al.*, 2016a,b; Toppi and Gabbrielli, 1999; Pitzschke *et al.*, 2006; Islam *et al*., 2009b; Xu *et al.*, 2009; Sharma and Dietz, 2009). Excess production of O_2^- and H_2O_2 is toxic to plants and causes oxidative damage to cellular constituents (Zouari *et al*., 2016a,b; Noctor and Foyer, 1998; Pitzschke *et al*., 2006; Banu *et al*., 2009; Islam *et al*., 2009b; Sharma and Dietz, 2009), although they act as signaling molecules that mediate many key physiological processes (Sharma and Dietz, 2009). Environmental stress leads to accumulation of methylglyoxal (MG), a cytotoxic compound, in higher plants (Yadav *et al*., 2005, 2008). Accumulation of MG results in a number of adverse effects such as increasing the degradation of proteins by modifying Arg, Lys and Cys residues, adducting with guanyl nucleotide in DNA, and inactivating antioxidant defense system (Martins *et al*., 2001).

One of the main adaptive mechanisms to Cd stress in plants is the accumulation of compatible solutes such as proline and glycinebetaine (betaine) (Zouari *et al*., 2016a,b; Rasheed *et al*., 2014; Islam *et al*, 2009a; Sharma and Dietz, 2006). Increasing levels of proline and betaine accumulated in plants are correlated with enhanced tolerance of salinity and heavy metal (Rasheed *et al*., 2014; Islam *et al*., 2009a; Sharma and Dietz, 2006). Besides functioning as osmoprotectant, proline and betaine maintain redox homeostasis (Hoque *et al*., 2008), suppress production of free radicals and reactive oxygen species (ROS) (Sharma and Dietz, 2006, Banu *et al*., 2009) and protect membranes, proteins and enzymes from the damaging effects of various stresses (Hoque *et al*. 2007a, Islam *et al*., 2009a). Moreover, exogenous proline and betaine have been shown to improve stress tolerance by up-regulating stress-protective proteins, preventing photoinhibition (Ma *et al*., 2006), and reducing lipid peroxidation (Demiral and Türkan, 2004; Banu *et al.*, 2009) and protein oxidation (Hoque *et al*., 2008).

Plants possess antioxidant defense and glyoxalase systems to protect their cells against the damaging effects of ROS and MG (Hoque *et al*., 2008; Noctor and Foyer, 1998; Sharma and Dietz, 2009; Yadav *et al*., 2005). Reduced glutathione (GSH) is the most abundant non-protein thiol in plants and plays an important role in the detoxification of ROS (Noctor and Foyer, 1998). Using GSH, glutathione peroxidase (GPX) catalyzes the reduction of H_2O_2, organic hydroperoxides and lipid peroxides. Glutathione is essential for MG metabolism in eukaryotes by the glyoxalase system, comprising two enzymes, glyoxalase I and glyoxalase II. Glyoxalase I catalyzes the formation of S-D-lactylglutathione (SLG) from the hemithioacetal formed non-enzymatically from MG and GSH, while glyoxalase II catalyzes the hydrolysis of SLG to regenerate GSH and liberate D-lactate (Thornalley, 1990). Glutathione is also used to synthesize phytochelatins (PCs), which play an important role in the detoxification of certain heavy metals (particularly Cd) in plants.

Efficient detoxification systems of ROS and MG are expected to play important roles in tolerance of plants to Cd stress. Exogenous proline and betaine increase antioxidant defense mechanisms in plant responses to various oxidative stresses (Demiral and Türkan, 2004; Ma *et al*., 2006). Our previous studies in NaCl-stressed cells, exogenous

proline and betaine improve tolerance and suppress cell death via enhancement of the antioxidant defense and MG detoxification systems (Hoque *et al*., 2007a, b, 2008; Banu *et al*., 2009). In Cd-stressed cells, proline acts as an antioxidant and thereby improves tolerance (Sharma and Dietz, 2006; Sharma and Dietz, 2009). Our recent reports demonstrate that exogenous proline and betaine increase intracellular proline and/or betaine contents, decrease intracellular H_2O_2 levels and lipid peroxidation, and increase the activities of superoxide dismutase (SOD), catalase (CAT) and ascorbate peroxidase (APX) to mitigate the detrimental effects of Cd stress (Islam *et al*., 2009a, b). The protective mechanisms of proline and betaine in plant responses to Cd stress remain to be elucidated. Therefore, we investigated the effects of exogenous proline and betaine on O_2^- level, contents of MG, carbonyl and PCs and activity of glutathione-associated enzymes glutathione peroxidase (GPX), glyoxalase I and glyoxalase II in cultured tobacco Bright Yellow-2 (BY-2) cells exposed to Cd stress. In this report, we also compared the results from Cd-stressed cells with our previous results from NaCl-stressed cells (Islam *et al*., 2009a, b; Hoque *et al*., 2007a, b, 2008; Banu *et al*., 2009).

Materials and methods

Culture of tobacco BY-2 cells

Suspension-cultured cells of tobacco (*Nicotiana tabacum* L. cv. Bright Yellow-2) were used as the source of Cd-unadapted cell lines (Islam *et al*., 2009). The standard medium was a modified LS medium (Hoque *et al*., 2007) in which KH_2PO_4 and thiamine-HCl were increased to 370 and 1 mg L^{-1}, respectively, supplemented with 3% sucrose and 1 μM 2,4-dichlorophenoxyacetic acid (Islam *et al*., 2009a). The Cd medium was the standard medium supplemented with 100 μM Cd (source of Cd was $CdCl_2 \cdot 2.5H_2O$). The 1 mM proline and 10 mM proline media were the Cd medium supplemented with 1 mM proline and 10 mM proline, respectively. The 1 mM betaine and 10 mM betaine media were the Cd medium supplemented with 1 mM betaine and 10 mM betaine, respectively. The cells were cultured and maintained as described by Islam *et al*. (2009a). Briefly, the cells were subcultured weekly by transferring suspension cells of a 7-d-old culture into 30 mL of different fresh media. The culture was incubated on a rotary shaker at 100 rpm and at 25°C in the dark. Cultured cells transferred to the different fresh media were used for different parameters at 4 d after inoculation.

Isolation of protoplasts and preparation of sample solution

Protoplasts were enzymatically isolated from BY-2 cells as described by Islam *et al*. (2009). The cells were subjected to occasional gentle swirling at 30°C for about 1 h in an enzyme solution, adjusted to pH 5.5, that contained 1% cellulase Onozuka RS (Yakult Honsha Co. Ltd., Tokyo, Japan), 0.1% pectolyase Y-23 (Seishin Pharmaceutical Co. Ltd., Tokyo, Japan), and 0.6 M sorbitol. Protoplasts were collected by centrifugation at 100*g* for 1 min. Protoplasts were broken up with a sonicator. The resulting suspension of broken protoplasts is referred to as the "sample solution" from now on.

Assay of intracellular O_2^- level

In order to measure intracellular O_2^- level by using a flow cytometer, protoplasts were isolated from tobacco BY-2 cultured cells. Isolated protoplasts were incubated with 10 μM dihydroethidium (DHE) at 37˚C for 30 min in the dark. Protoplasts were then

washed and re-suspended in sorbitol solution. Intracellular O_2^- levels were measured by flow cytometry.

Assay of MG contents

Methylglyoxal content was measured according to the method of Yadav et al. (2005) with some modifications. An aliquot of BY-2 cells (0.5 g fresh weight) was homogenized in 2 mL of PBS. The homogenate was centrifuged at 4˚C at 11,000*g* after incubating for 15 min on ice. The supernatant (650 µL) was incubated with 100 µL of 5 M perchloric acid for 10 min on ice. After centrifuging at 11,000*g* for 10 min, the supernatant was reacted with 250 µL of 7.2 mM 1,2-diaminobenzene. The absorbance of the derivative was read at 336 nm.

Assay of carbonyl contents

The carbonyl content was measured as described by Hoque *et al*. (2008). Proteins from BY-2 cells were extracted in 50 mM KH_2PO_4 buffer (pH 7.5) containing 10 mM Tris, 2 mM $MgCl_2$, 2 mM EGTA and 1 mM PMSF. Aliquots of extract were reacted with 10 mM 2,4-dinitrophenylhydrazine dissolved in 2.5 M HCl or 2.5 M HCl (control) for 1 h at room temperature. Proteins were precipitated with 20% trichloroacetic acid (w/v). After centrifuging, protein pellets were washed extensively with ethanol–ethylacetate (1:1) and dissolved in 6 M guanidine hydrochloride with 20 mM KH_2PO_4 (pH 2.3). After centrifuging at 9500*g* for 10 min, the absorbance was recorded at 380 nm and carbonyl content was calculated using the extinction coefficient of 22,000 M^{-1} cm^{-1}.

Assay of phytochelatins

Phytochelatins (PC) were measured according to the method of Sneller *et al.* (2000). Three main PCs occurring in cell (PC1, PC2 and PC3) were purified from cultured BY-2 cells. Five hundred micrograms of fresh weight of BY-2 cells were homogenized in 1998 µL of 6.3 mM diethylene triamine pentaacetate (DTPA) and 2 µL of 0.1% (v/v) trifluroacetate (TFA) with a mortar, pestle. The homogenate was centrifuged at 12,000*g* at 4^0C. Twenty microliters of supernatant were injected into the High performance liquid chromatography (HPLC) system for the measurement of PC_1, PC_2, PC_3. Before injection, the column was equilibrated in methanol and water, both containing 0.1% (v/v) TFA at flow of 0.5 mL/min. Absorbance at 412 nm were monitored in HPLC. Calibration curves of GSH were used in all measurement.

Assay of glutathione-associated enzymes
Glutathione peroxidase

Glutathione peroxidase (EC: 1.11.1.9) activity was assayed following the method of Hoque *et al*. (2008) using H_2O_2 as a substrate. The reaction mixture contained 100 mM Na-phosphate buffer (pH 7.5), 1 mM EDTA, 1 mM NaN_3, 0.12 mM NAPDH, 2 mM GSH, 1 unit GR, 0.6 mM H_2O_2 and 25 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 2 min when the extinction coefficient was 6.62 mM^{-1} cm^{-1}.

Glyoxalase I

Glyoxalase I (EC: 4.4.1.5) activity was measured according to Racker (1951). Hemimercaptal was produced by the preincubation at 25^0C for 10 min of a mixture containing 2 mM MG and 2 mM GSH in 100 mM Na-phosphate buffer (pH 6.8). The reaction was started by the formation of SDL when 50 µL of sample solution was added to

the hemimercaptal solution. The activity was calculated from the increase in absorbance at 240 nm for 1 min when the extinction coefficient was 3.31 mM^{-1} cm^{-1}.

Glyoxalase II

Glyoxalase II (EC: 3.1.2.6) activity was measured following the method of Antognelli *et al*. (2003). The reaction mixture consisted of 100 mM Tris-buffer (pH 7.4), 0.2 mM DTNB, 0.8 mM SLG and 50 µL of sample solution. The reaction was started by the addition of SLG. The activity was calculated from the increase in absorbance at 412 nm for 1 min when the extinction coefficient was 13.6 mM^{-1} cm^{-1}.

Determination of protein

Protein contents were measured as described by Islam *et al*. (2009) using BSA as a standard.

Statistical analysis

The significance of differences between mean values was compared by Student's *t*-test. Differences at $P<0.05$ were considered significant.

Results

Intracellular O_2^- levels

As shown in Fig. 1, intracellular O_2^- level in Cd-stressed BY-2 cells was 3-fold higher than that in non-stressed cells. To investigate whether proline or betaine could suppress Cd-induced O_2^- production, Cd-stressed cells were grown in the presence of proline or betaine. Exogenous application of proline at 1 mM and 10 mM resulted in a significant reduction of O_2^- production in Cd-stressed cells, whereas betaine only at 1 mM significantly reduced the production of O_2^-. However, the decreasing effect of proline on O_2^- production was more pronounced than that of betaine under Cd stress (Fig. 1).

Methylglyoxal contents

A significant increase in MG content in BY-2 cells was observed under Cd stress (Fig. 2). In comparison to non-stressed cells, Cd-stressed cells showed a 2.6-fold higher MG content. Exogenous proline at 1 mM (30%) and betaine at 1 mM (31%) and 10 mM (34%) caused significant reductions in MG content under Cd stress (Fig. 2).

Protein oxidation

To investigate the protective effects of proline and betaine against Cd-induced protein oxidation, carbonyl contents were measured in BY-2 cells cultured in the different media (Fig. 3). Cadmium stress caused a significant increment (3-fold) of carbonyl content in BY-2 cells. Exogenous proline (1 mM and 10 mM, 30-34%) and betaine (1 mM and 10 mM, 38%) resulted in significant reductions of carbonyl content under Cd stress. However, the carbonyl content in proline media was similar to that in betaine media (Fig. 3).

Phytochelatin contents

In tobacco BY-2 cells, phytochelatins such as PC_1, PC_2 and PC_3 were detected (Fig. 4). Cadmium-stressed cells showed a significant decrease (2-fold) in PC_1 contents, whereas exogenous application of both proline (44-106%) and betaine (115-125%) significantly increased PC_1 contents in Cd-stressed cells. On the other hand, levels of PC_2 and PC_3 were

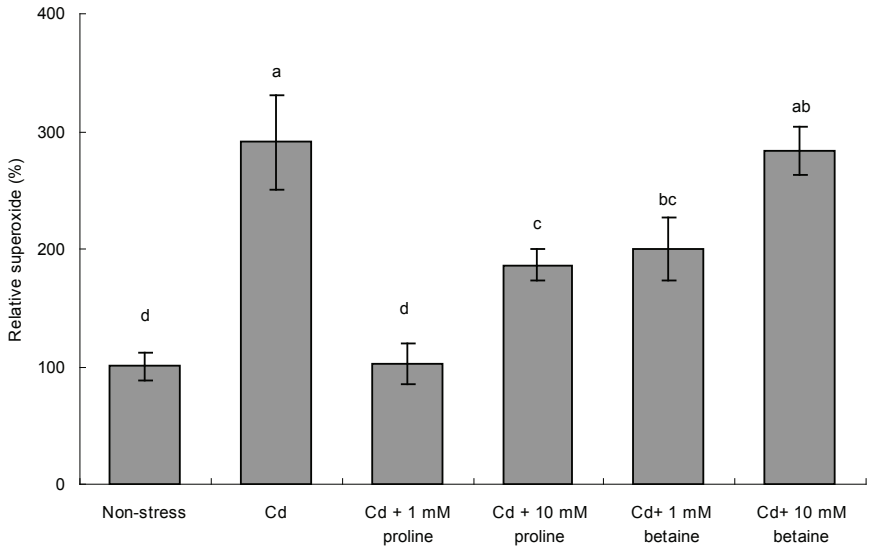

Fig. 1. Effect of proline and betaine on intracellular O_2^- levels in Cd-unadapted tobacco BY-2 suspension cells under Cd stress. Values represent the mean±SE from five independent experiments. Bars with the same letters are not significantly different at P<0.05. Non-stress, Cd, Cd+1 mM proline, Cd+10 mM proline, Cd+1 mM betaine and Cd+10 mM betaine indicate the standard medium, Cd medium, 1 mM proline medium, 10 mM proline medium, 1 mM betaine medium and 10 mM betaine medium, respectively as described in 'Materials and methods'.

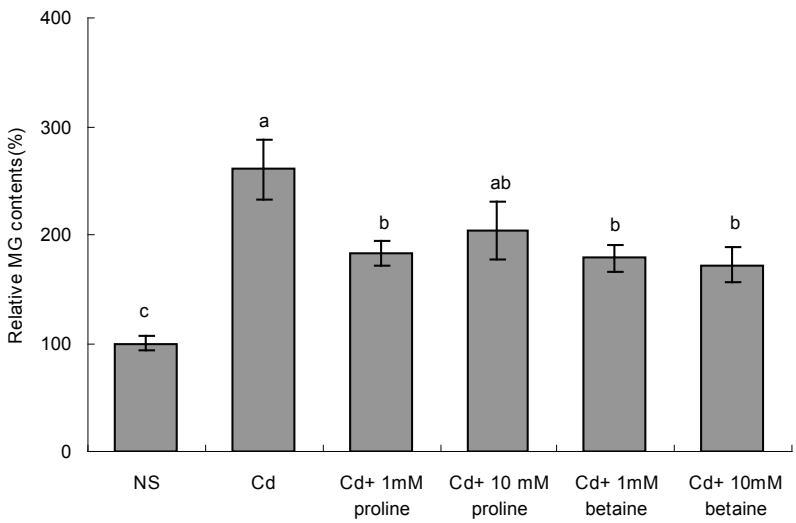

Fig. 2. Effect of proline and betaine on MG contents in Cd-unadapted tobacco BY-2 suspension cells under Cd stress. Values represent the mean±SE from five independent experiments. Bars with the same letters are not significantly different at P<0.05. Details of culture media are given in the legend of Figure 1.

significantly higher in Cd-stressed cells than non-stressed cells. Both exogenous proline (10 mM) and betaine (1 and 10 mM) resulted in a significant increase in PC_3 contents, whereas neither proline nor betaine increased PC_2 contents under Cd stress. It is also noted that the increasing effect of betaine on PC_3 contents was enormously higher than that of proline (Fig. 4).

Activity of glutathione-associated enzymes

A significant inhibition of GPX activity was observed in BY-2 cells in response to Cd stress (Fig. 5A). Exogenously applied proline (10 mM) and betaine (1 and 10 mM) significantly mitigated this inhibition under Cd stress. The mitigating effect of betaine on GPX activity was higher than that of proline (Fig. 5A).

As shown in Fig. 5B, a significant reduction in glyoxalase I activity was observed in response to Cd stress. Application of 1 mM proline as well as 1 and 10 mM betaine did not affect glyoxalase I activity in Cd-stressed cell, whereas 10 mM proline showed a significant increase in glyoxalase I activity in Cd-stressed cells (Fig. 5B).

Glyoxalase II activity was drastically decreased by Cd stress (Fig. 5C). Non-stressed cells showed 5-fold higher glyoxalase II activity than Cd-stressed cells. Under Cd stress, glyoxalase II activity did not vary significantly irrespective of the presence or absence of proline and betaine (Fig. 5C).

Discussion

The antioxidant defense mechanisms of proline and betaine have been reported in plant responses to various stresses including Cd stress (Zouari *et al*., 2016a,b; Ma *et al*., 2006; Sharma and Dietz, 2006; Xu *et al*.,

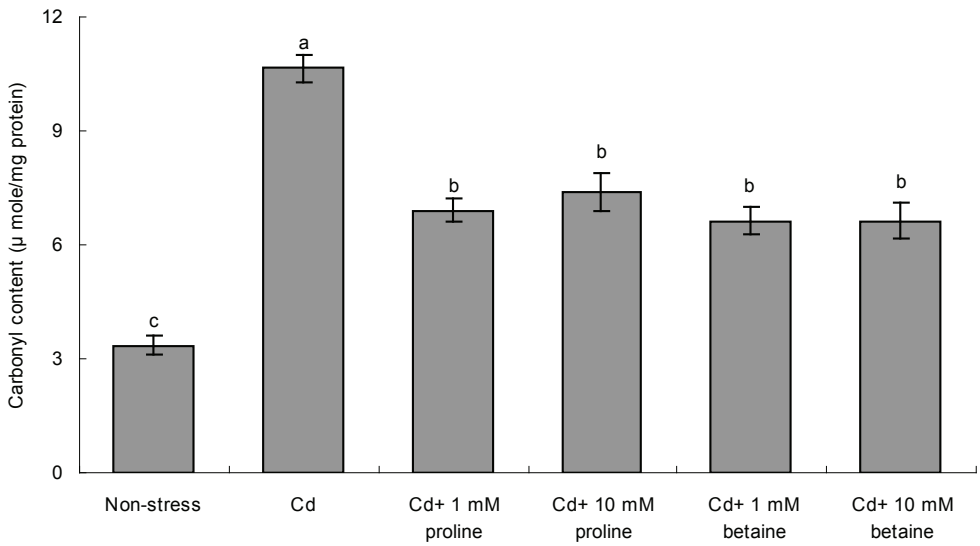

Fig. 3. Effects of proline and betaine on carbonyl contents (protein oxidation) in Cd-unadapted tobacco BY-2 suspension cells under Cd stress. Values represent the mean±SE from five independent experiments. Bars with the same letters are not significantly different at P<0.05. Details of culture media are given in the legend of Figure 1.

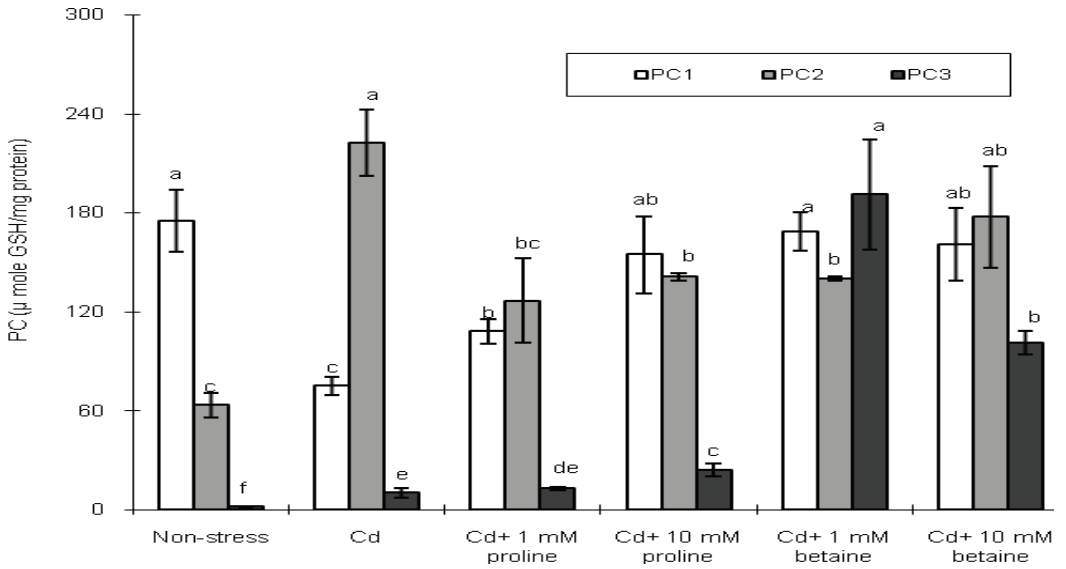

Fig. 4. Effect of proline and betaine on phytochelatins (PC_1, PC_2 and PC_3) in Cd-unadapted tobacco BY-2 suspension cells under Cd stress. Values represent the mean±SE from three independent experiments. Bars with the same letters are not significantly different at P<0.05. Details of culture media are given in the legend of Figure 1.

2009). In cultured tobacco BY-2 cells, we have shown that both exogenous proline and betaine improve salt tolerance (Hoque *et al*., 2007a, b, 2008; Banu *et al*., 2009) and Cd tolerance (Islam *et al*., 2009a, b). Proline improves tolerance to NaCl or Cd stress more than betaine because of superior ability of proline to enhance the antioxidant defense mechanisms (Hoque *et al*., 2007a, b, 2008; Banu *et al*., 2009; Islam *et al*., 2009a, b). In this study, we have attempted to clarify the role of proline and betaine in aspects of antioxidant defense as well as glyoxalase systems under Cd stress as the mechanism of proline-induced Cd tolerance is different from that of betaine-induced Cd tolerance (Islam *et al*., 2009a, b).

Accumulation of H_2O_2 and O_2^- induced by Cd has been reported in different plant species (Rasheed *et al*., 2014; Zouari *et al*., 2016a,b; Xu *et al*., 2009). We have earlier shown that Cd causes an increase in accumulation of H_2O_2 in BY-2 cells (Islam *et al.*, 2009b). Similar to the protective effects of proline and betaine against NaCl stress (Banu *et al*., 2009), both molecules suppress H_2O_2 accumulation and Evans blue-positive cells, and improve growth of BY-2 cells under Cd stress (Islam *et al*., 2009a, b). To investigate the further protective mechanisms of proline and betaine against Cd stress, intracellular O_2^- levels were measured in BY-2 cells. Exogenous application of proline or betaine to the Cd-stressed cells causes a reduction of O_2^- production, and that proline efficiently reduces this production (Fig. 1). Superoxide production is closely related to the activity of SOD. Our observations have indicated that the reduction of O_2^- levels in Cd-stressed cells in the presence of proline might correlate with the activity of SOD (Islam *et al*., 2009a; Fig.

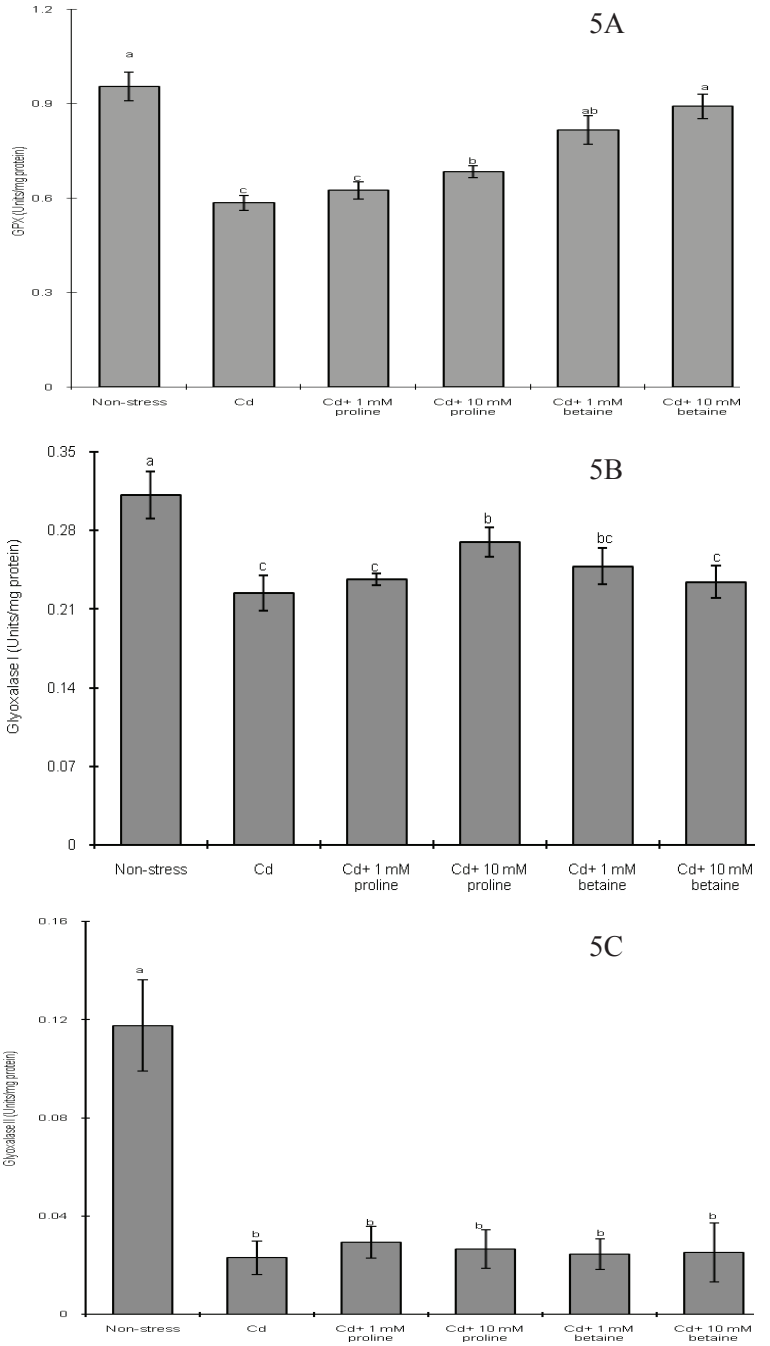

Fig. 5. Effect of proline and betaine on activity of GSH-associated enzymes glutathione peroxidase (A), glyoxalase I (B) and glyoxalase II (C) in Cd-unadapted tobacco BY-2 suspension cells. The activity of enzyme was expressed in units mg^{-1} protein. One unit of activity was defined as the µmol substrate metabolized per min. Values represent the mean±SE from five independent experiments. Bars with the same letters are not significantly different at P<0.05. Details of culture media are given in the legend of Figure 1.

1). These results suggest that both proline and betaine could scavenge H_2O_2 and O_2^- as well as provide protections against Cd toxicity via enhancement of the antioxidant defense mechanisms (Islam *et al*., 2009a, b; Fig. 1).

Salinity as well as Cd causes oxidative damage to cellular components. Similar to NaCl stress (Hoque *et al*., 2008; Banu *et al*., 2009), Cd stress results in significant increases in malondialdehyde (Islam *et al*., 2009a) and carbonyl contents (Fig 3) in BY-2 cells. An increase in carbonyl contents has also been observed in response to Cd stress in plant. A lower level of carbonyl contents in Cd-stressed cells in the presence of proline or betaine results in decreased oxidative damage to protein (Fig. 3). In NaCl-stressed cells, similar effects of proline and betaine on carbonyl contents have been observed (Hoque *et al*., 2008). These results suggest that both proline and betaine provides protection against Cd- as well as NaCl-induced oxidative damage.

In addition to its function to scavenge ROS either directly or indirectly by participating in the ascorbate-glutathione cycle, GSH serves as a precursor of peptides, known as PCs. Many heavy metals can induce PC production in plants, and Cd has been found to be most effective inducer of PCs. It has been shown that PC production is the main responsive mechanism to Cd stress in higher plants and plays a critical role in Cd detoxification by sequestering Cd into a less toxic form. In BY-2 cells, Cd stress induced the production of PC_2 and PC_3 but decreased PC_1 production. The production of PC_1 and PC_3 increased in Cd-stressed cells in the presence of proline or betaine. The effect of betaine on PC_3 production was much higher than that of proline (Fig. 4), demonstrating that betaine could effectively mitigate Cd toxicity by enhancing the chelation with Cd. These results are consistent with our previous results that exogenous betaine but not proline mitigates Cd toxicity by sequestering Cd (Islam *et al*., 2009a). In addition, proline could be involved in metal chelation in the cytosol and is a poor inducer of phytochelator synthesis (Hasan *et al*., 2009; Fig. 4) but betaine is a strong inducer of phytochelator synthesis (Fig. 4)

Glutathione peroxidase is an important ROS scavenger because of its broader substrate specifications and stronger affinity for H_2O_2 than CAT. *Arabidopsis thaliana GPX3* has been shown to play dual roles in H_2O_2 homeostasis, acting as a general scavenger and in H_2O_2 signal transduction (Miao *et al*., 2006). Overexpression or higher activity of GPX in plant increases antioxidant activity and improves tolerance to oxidative stress (Roxas *et al*., 2000). Cadmium stress has been shown to decrease in GPX activity (Hoque *et al*., 2006). Salt stress as well as Cd stress leads to a reduction in GPX activity, while both proline and betaine mitigate this reduction under both NaCl and Cd stress conditions (Hoque *et al*., 2008; Fig. 5A). Proline confers tolerance to NaCl or Cd more than betaine (Hoque *et al*., 2007a, b; Islam *et al*., 2009a, b), although betaine mitigates the reduction of GPX activity more than proline (Hoque *et al*., 2008; Fig. 5A). However, increased GPX activity by proline or betaine may contribute

to the reduction of H_2O_2 accumulation, and thereby improves tolerance to oxidative stresses including Cd.

Besides acting as a signaling molecule, increasing levels of MG in cells can cause oxidative damage to different molecules including DNA and proteins (Martins *et al*., 2001; Yadav *et al*., 2005). In this study, Cd induces MG accumulation in BY-2 cells. Conversely, both proline and betaine inhibit Cd-induced MG accumulation in Cd-stressed cells (Fig. 2), indicating that this inhibition may be a reason for higher activity of glyoxalase I, MG detoxifying enzyme, by exogenous application of proline or betaine. Overexpression of the glyoxalase pathway in transgenic plants has been found to resist an increase in MG level under abiotic stresses including heavy metal stresses (Yadav *et al*., 2005, 2008). Lower activity of glyoxalase I in Cd-stressed cells (Fig. 5B) indicates an insufficient detoxification of MG via glyoxalase system. Exogenous proline but not betaine showed a higher glyoxalase I activity in Cd-stressed cells (Fig. 5B) and similar results were also observed in NaCl-stressed cells (Hoque *et al*., 2008), suggesting that proline could contribute to the detoxification of MG by enhancing glyoxalase I activity in Cd- and NaCl-stressed cells. However, the reduction of MG contents does not correlate with the activity of glyoxalase I in Cd-stressed cells irrespective of the presence of proline or betaine (Fig. 2 and Fig. 5B). In addition to MG detoxification, glyoxalase system regenerates GSH by catalyzing glyoxalase II (Hossain *et al*., 2010). Up-regulation of the glyoxalase II activity in response to salinity and heavy metal stresses has been reported in plants (Saxena *et al*., 2005; Hossain *et al*., 2010). A drastic decrease in glyoxalase II activity was found in Cd-stressed cells even addition of proline or betaine (Fig. 5C). On the contrary, we have observed a higher glyoxalase I activity in NaCl-stressed cells irrespective of the presence or absence of proline and betaine (Hoque *et al*., 2008). No increment of the glyoxalase II activity by proline and betaine exhibits an inefficient regeneration of GSH via the glyoxalase system under NaCl or Cd stress (Fig. 5C; Hoque *et al*., 2008).

Conclusion

Both exogenous proline and betaine reduces oxidative damage to protein, enhance chelation with Cd, increase the activity of GSH-dependent enzymes involved in the detoxification of ROS and MG, and thereby mitigate Cd toxicity in tobacco BY-2 cells. Taken together, proline confers tolerance to Cd stress more than betaine, although betaine contributes to higher Cd sequestering, PC formation and GPX activity (Islam *et al*., 2009a, b). Further studies are required to clarify the protection mechanisms of proline and betaine in plant responses to Cd stress.

Figure 3. Effects of proline and betaine on carbonyl contents (protein oxidation) in Cd-unadapted tobacco BY-2 suspension cells under Cd stress. Values represent the mean±SE from five independent experiments. Bars with the same letters are not significantly different at $P<0.05$. Details of culture media are given in the legend of Figure 1.

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