Research Article

Evidence for a role of exogenous glycinebetaine and proline in antioxidant defense and methylglyoxal detoxification systems in mung bean seedlings under salt stress

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ABSTRACT

In mung bean seedlings, salt stress (300 mM NaCl) caused a significant increase in reduced glutathione (GSH) content within 24 h of treatment as compared to control whereas a slight increase was observed after 48 h of treatment. Highest oxidized glutathione (GSSG) content was observed after 48 h to treatment with a concomitant decrease in glutathione redox state. Glutathione peroxidase, glutathione *S*-transferase, and glyoxalase II enzyme activities were significantly elevated up to 48 h, whereas glutathione reductase and glyoxalase I activities were increased only up to 24 h and then gradually decreased. Application of 15 mM proline or 15 mM glycinebetaine resulted in an increase in GSH content, maintenance of a high glutathione redox state and higher activities of glutathione peroxidase, glutathione *S*-transferase, glutathione reductase, glyoxalase I and glyoxalase II enzymes involved in the ROS and methylglyoxal (MG) detoxification system for up to 48 h, compared to those of the control and mostly also salt stressed plants, with a simultaneous decrease in GSSG content, H_2O_2 and lipid peroxidation level. The present study suggests that both proline and glycinebetaine provide a protective action against salt-induced oxidative damage by reducing H_2O_2 and lipid peroxidation level and by enhancing antioxidant defense and MG detoxification systems. **[Physiol. Mol. Biol. Plants 2010; 16(1) : 19-29]** *E-mail : fujita@ag.kagawa-u.ac.jp*

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Abbreviations : *CDNB-1- Chloro-2,4-dinitrobenzene; 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 S-transferase; MDA-malondialdehyde; MG- methylglyoxal; NTB- 2-nitro-5-thiobenzoic acid; ROS- Reactive oxygen species; SLG; S*-D- lactoylglutathione*; TBA- thiobarbituric acid; TCA- trichloroactic acid*

INTRODUCTION

Salinity is one of the major abiotic stresses that adversely affect crop growth and productivity. High salt concentration causes an imbalance of cellular ions resulting in ion toxicity and osmotic stress while an increasing body of evidence suggests that high salinity induces the production of reactive oxygen species (ROS) and methylglyoxal (MG) in plants (Tanou *et al.,* 2009; Mittler, 2002; Munns, 2002; Yadav *et al.,* 2005a,b). ROS and MG are highly toxic to plant cell and by reacting with proteins, lipids, carbohydrates and DNA, they can lead to cell death in the absence of any protective mechanism (Foyer and Noctor, 1999; Hernandez *et al.,* 2001; Singla-Pareek *et al.,* 2006). Plant possess both enzymatic and non-enzymatic antioxidant defense systems to protect their cells against ROS and MG (Mittler, 2002; Apel and Hirt, 2004; Yadav *et al*., 2005a,b). Reduced glutathione (GSH), the most abundant low molecular weight thiol compound in plants functions as a stress indicator occurring in two distinct redox forms, promptly responding to oxidative stress. GSH is the key component of the antioxidant network that scavenges ROS either directly or indirectly by participating in the ascorbate-glutathione (AsA-GSH) cycle (Noctor and Foyer, 1998; Smirnoff, 2005).

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Detoxification of H_2O_2 through AsA-GSH cycle leads to transient increase of oxidized glutathione (GSSG) and disturb the glutathione homeostasis. This makes survival of the plant difficult under such adverse conditions. However, plants have glutathione reductase (GR) that catalyzes the conversion of GSSG to GSH and continuously provides GSH to AsA-GSH cycle. GPX is considered to be an important ROS scavenger because of its broader substrate specifications and stronger affinity for H_2O_2 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. GSH is also essential for MG metabolism in eukaryotes by the glyoxalase system, comprising two enzymes, glyoxalase I and glyoxalase II. Glyoxalase I converts MG to S-D-lactoylglutathione (SLG) by utilizing GSH, while glyoxalase II converts SLG to D-lactate and regenerate GSH (Thornalley, 1990). Furthermore, GSH plays a protective role in salt tolerance by the maintenance of the redox state (Shalata *et al.*, 2001). An increase in the level of the GSH pool is generally regarded as a protective response against oxidative stress (May and Leaver, 1993; Ruiz and Blumwald, 2002), although defense against stress situations sometimes occurs irrespective of the GSH concentration (Potters *et al.*, 2004).

A common response of plants to different abiotic stresses is the accumulation of osmolytes including proline and glycinebetaine (betaine) and elevated levels of proline and betaine accumulated in plants are correlated with enhanced stress tolerance (Chen *et al.,* 2000; Munns, 2005). In addition to their roles as osmoprotectants, proline and betaine also act as protectants of membranes, enzymes and other proteins (Bohnert and Jensen, 1996; Mäkelä *et al.*, 2000; Okuma *et al.*, 2004). Moreover, previous report suggest that exogenous proline and betaine protect enzymes, scavenge free radicals, and prevent oxidation under salt stress (Khedr *et al.*, 2003; Demiral and Turkan, 2004; Ma *et al.*, 2006; Ashraf and Foolad, 2007; Hoque *et al.*, 2007a,b) but it is not fully understood how exogenous proline and betaine alleviate salt induced oxidative damage. Besides this, little information is available about the protective roles of proline and betaine in glutathione metabolism and MG detoxification systems in plants exposed to oxidative stress although we earlier demonstrated that MG level, glyoxalase I and glyoxalase II activities had increased in response to various abiotic stresses in crop plants (Hossain *et al.*, 2009; Hossain and Fujita, 2009). It is expected that the up-regulation of the components of the antioxidant and glyoxalase systems offered by proline and betaine protects plant against NaCl-induced oxidative damage. In the present study, we investigated the effects of exogenous proline and betaine on the contents of glutathione and its redox states, activity of GSH-related enzymes involved in antioxidant defense and methylglyoxal detoxification systems in mung bean seedlings under salt stress.

MATERIALS AND METHODS

Plant materials and stress treatments

Mung bean (*Vigna radiata* L.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 - 100μmol photon $m^{-2}s^{-1}$; temp - 25 ± 2 °C; RH - 65-70 %) for three days. Germinated seedlings were then grown in hydroponic medium that contained 1000-fold diluted Hyponex solution (Type: 5-10-5, Hyponex, Japan). Five-day-old mung bean seedlings of approximately equal sizes were employed to experimentation. For salt stress, seedlings were treated with Hyponex solution that contained 300 mM NaCl and maintained under the above conditions. For salt treatment in the presence of proline or betaine, seedlings were treated with 15 mM proline or 15 mM betaine with the above level of NaCl in Hyponex solution. Control plants were grown in Hyponex solution only.

Assay of glutathione contents

Mung bean shoots (1 g fresh weight) were homogenized in 3 ml ice-cold acidic extraction buffer (6 % meta-phosphoric acid containing 1 mM EDTA) using a mortar and pestle. Homogenates were centrifuged at 11,500x g for 15 min at 4 °C. The supernatant was then neutralized with 0.5 M K-phosphate buffer (pH 7.5). Total glutathione content in shoot tissue was determined according to the procedure of Yu *et al.* (2002). Based on enzymatic recycling, glutathione is oxidized by 5,5[-dithio-bis (2-nitrobenzoic acid) (DTNB) and reduced by NADPH in the presence of glutathione reductase, 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. A specific standard curve with GSH was used. GSH was determined by subtraction of GSSG from the total glutathione concentration.

Enzyme extraction and assays

Using a pre-cooled mortar and pestle, 1 g of shoot tissue was homogenized in an equal volume of 50 mM ice-cold K-phosphate buffer (pH 7.0) containing 100 mM KCl, 1 % (w/v) ascorbate and 10 % (w/v) glycerol. The homogenates were centrifuged at 11,500x *g* for 10 min and the supernatants were used for determination of enzyme activity. All procedures were performed at 0-4 ºC.

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 coefficient of 6.62 $mM^{-1}cm^{-1}$.

GST (EC: 2.5.1.18) activity was determined spectrophotometrically by the method of Booth *et al.* (1961) with some modifications. The reaction mixture contained 100 mM Tris-HCl buffer (pH 6.5), 1.5 mM reduced glutathione (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 coefficient of 9.6 mM^{-1} 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 Na-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 the extinction coefficient of 6.2 $mM^{-1}cm^{-1}$.

Glyoxalase I (EC: 4.4.1.5) assay was carried out according to Hossain and Fujita (2009). Briefly, the assay mixture contained 100 mM K-phosphate buffer (pH 7.0), 15 mM magnesium sulfate, 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 $mM^{-1}cm^{-1}$.

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 $mM^{-1}cm^{-1}$.

Determination of protein

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

Lipid peroxidation

The level of lipid peroxidation was measured 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 Health and Packer (1968) with slight modifications. The shoot tissue (0.5 g) was homogenized in 3ml 5 % (w/v) trichloroacetic acid (TCA) and the homogenate was centrifuged at 11,500x g for 10 min. One ml supernatant was mixed 4 ml of TBA reagent (0.5 % of TBA in 20 % TCA). The reaction mixture was heated at 95 °C for 30 min in a water bath and then quickly cooled in an ice bath and centrifuged at 11,500x g for 15 min. The absorbance of the colored supernatant was measured at 532 nm and was corrected for non-specific absorbance at 600 nm. The concentration of MDA was calculated by using the extinction coefficient of 155 $mM^{-1}cm^{-1}$ and expressed as 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 shoot tissue with 3 ml of 50 mM K-phosphate buffer pH (6.5) at 4 °C. The homogenate was centrifuged at 11,500x 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 11,500x 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 (ϵ=0.28 $\mu mol^{-1}cm^{-1}$) and expressed as $\mu mol\ g^{-1}$ fresh weight.

Statistical analysis

All data obtained were subjected to one-way analysis of variance (ANOVA) and the mean differences were compared by a least significant difference (LSD) test using MSTAT-C. Differences at $P<0.05$ were considered significant.

RESULTS

Cellular glutathione contents

Salt stress induced a marked increase (114 %) in GSH content compared to that of the control within 24 h, whereas a drastic reduction (44 %) in GSH content was observed at 48 h over 24 h of treatment (Fig. 1A). Proline and betaine supplemented salt-treated seedlings showed a significant increase (116 and 102 %, respectively) in GSH level after 24 h of treatment compared to that of the control, but the GSH level at 48 h was significantly different from that of the control as well as that of salt-treated seedlings (Fig. 1A). The highest GSSG content (3.5-fold higher over the control) was found in seedlings treated with salt for 48 h. Proline and betaine supplemented salt-treated seedlings also showed significant increase in GSSG (3 and 1.9 fold, respectively) as compared to control (Fig. 1B) after 48 h of treatment, however, the level of GSSG was significantly lower than the seedlings treated with salt alone. After 24 h of incubation, salt stress caused a slight increase in the glutathione redox state compared to that of the control, which was significantly deceased at 48 h. However, both proline and betaine significantly contributed to the maintenance of the NaCl-induced redox state (Fig. 1C).

Activity of GSH-utilizing and regenerating enzymes

Importantly, both proline and betaine significantly maintained higher glutathione contents as well as glutathione redox state. Thus, to investigate the further protective mechanisms of proline and betaine against salt stress, GSH-associated enzymes involved in antioxidant defense and glyoxalase systems were assayed in seedlings after 24 h and 48 h of treatment.

In mung bean seedlings, GPX activity ranged from 0.111 to 0.203 nmol $min^{-1}mg^{-1}$ protein under both stressed and non-stressed conditions. Salt stress caused

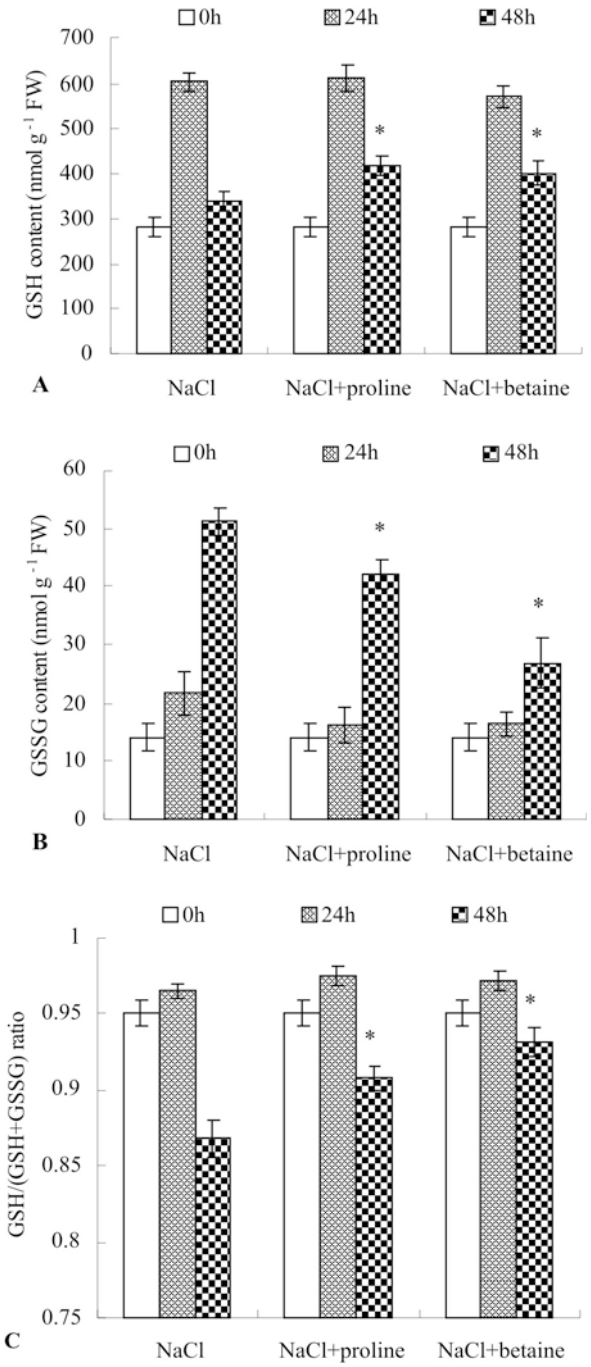

Fig. 1. Time course of glutathione accumulation in mung bean seedlings induced by proline and betaine under salt stress conditions. Five-day-old seedlings were exposed to 300 mM NaCl stress with or without 15 mM proline and 15 mM betaine for up to 48 h. A: reduced glutathione (GSH), B: oxidized glutathione (GSSG), C: glutathione redox state [GSH/ (GSH+GSSG)]. Each value is the mean ± SE from four independent experiments. * indicates significant difference between mean values at the same time of incubation over salt stress (P <0.05).

a significant increase in GPX activity within 24 h. After 48 h of salt treatment, the specific activity of GPX was 62 % higher than that of the control (Fig. 2A). Proline and betaine supplemented salt-stressed seedlings also increased GPX activity, but a significant increase over salt stress was observed in betaine-supplemented seedlings after 48 h of treatment.

GST activity was increased significantly in response to salt stress (Fig. 2B). Exogenously applied proline and betaine increased GST activity induced by salt stress by 8.6 % and 10 %, respectively, at 48 h. Time course results showed that, GST activity under the condition of salt stress did not vary significantly regardless the presence or absence of proline and betaine.

The pattern of induction of GR specific activities varied with the treatment period (Fig. 2C). Salt stress caused a significant increase (31 %) in GR activity compared to that of the control within 24 h, but a slight decrease in GR activity was observed after 48 h of salt treatment. Proline and betaine supplemented salt-stressed seedlings showed significant increased in GR activity up to 48 h (29 and 35 %, respectively). At the end of 48 h treatments, proline and betaine showed 10 % and 16 % higher in GR activity over salt stress. The effect of betaine on GR activity was more pronounced than that of proline but the difference was not significant.

A significant increase in glyoxalase I activity was observed in response to salt stress within 24 h (Fig. 2D), but a slight decrease was observed at 48 h. Exogenous betaine and proline mitigated the inhibition of NaCl-induced glyoxalase I activity. Both proline and betaine increased glyoxalase I activity induced by salt stress by 12 % and 17 %, respectively, at 24 h. Betaine caused a significant increase in activity over NaCl stress, but proline failed to do so.

Glyoxalase II activity was significantly increased in response to salt stress (Fig. 2E). Salt-stressed seedlings showed 1.2-fold and 1.5-fold higher glyoxalase II activity at 24 h and 48 h of treatment compared to that of the control. Both proline and betaine supplemented salt-treated seedlings showed an increase in glyoxalase II activity over salt stress, but a significant increase was observed after 48 h of treatment.

H_2O_2 contents and membrane lipid peroxidation (MDA)

Time course of H_2O_2 concentration and membrane lipid peroxidation in mung bean seedlings due to salt stress is presented in Fig. 3. Salt stress resulted in a significant increase (21 %) in H_2O_2 content within 24 h of treatment (Fig. 3A). H_2O_2 content was 48 % higher than that of the control after 48 h of salt treatment. Proline and betaine supplemented salt-stressed seedlings also showed continual increase in H_2O_2 content. However, the level of H_2O_2 content was significantly lower than the seedlings treated with salt alone after 48 h of treatment. After 48 h of treatment proline and betaine supplemented salt-stressed seedlings showed 12 % and 18 % decease in H_2O_2 content comparison to plants receiving NaCl alone.

Significant increase in lipid peroxidation (24 and 47 %, respectively) was observed after 24 h and 48 h of treatment as compared to the control (Fig. 3B). Proline- and betaine-supplemented salt-stressed seedlings also showed continual increase in MDA level. However the level of MDA was lower than the seedlings treated with salt alone. Proline and betaine supplemented salt- treated seedlings showed 8 % and 17 % deceases in MDA level after 48 h of treatment over salt stress. The effect of betaine on lipid peroxidation level was more prominent than that of proline, though there was no significant difference.

DISCUSSION

Incidences of NaCl stress in plants are mostly linked to osmoregulation problems and oxidative damage (Hernandez *et al.*, 1993). Plant accumulates compatible solute such as proline and betaine to mitigate the damaging effects of salt stress. The concentration of proline and betaine, however, are not high enough to adjust the osmotic potential in some plants under stress (Hamilton and Heckathorn, 2001; Okuma *et al.,* 2002). Exogenous application of proline and betaine thus provides a useful tool to study the mechanism governing tolerance/resistance reactions under saline condition. Plants employ an antioxidant defense system as well as a glyoxalase system against oxidative damage caused by ROS and MG. Up-regulation of the antioxidant defense and glyoxalase systems often correlates with the alleviation of oxidative damage and improved tolerance to salt stress (Veena *et al.*, 1999; Mittova *et al*., 2003a,b; Yadav *et al.*, 2005a,b; Hoque *et al.,* 2007a,b; Hossain and Fujita, 2009).

Thiol redox regulation is partially mediated through the redox state of the glutathione pool (GSH/GSSG) and ROS themselves are each thought to have important roles as environmental sensors and/or modulators of

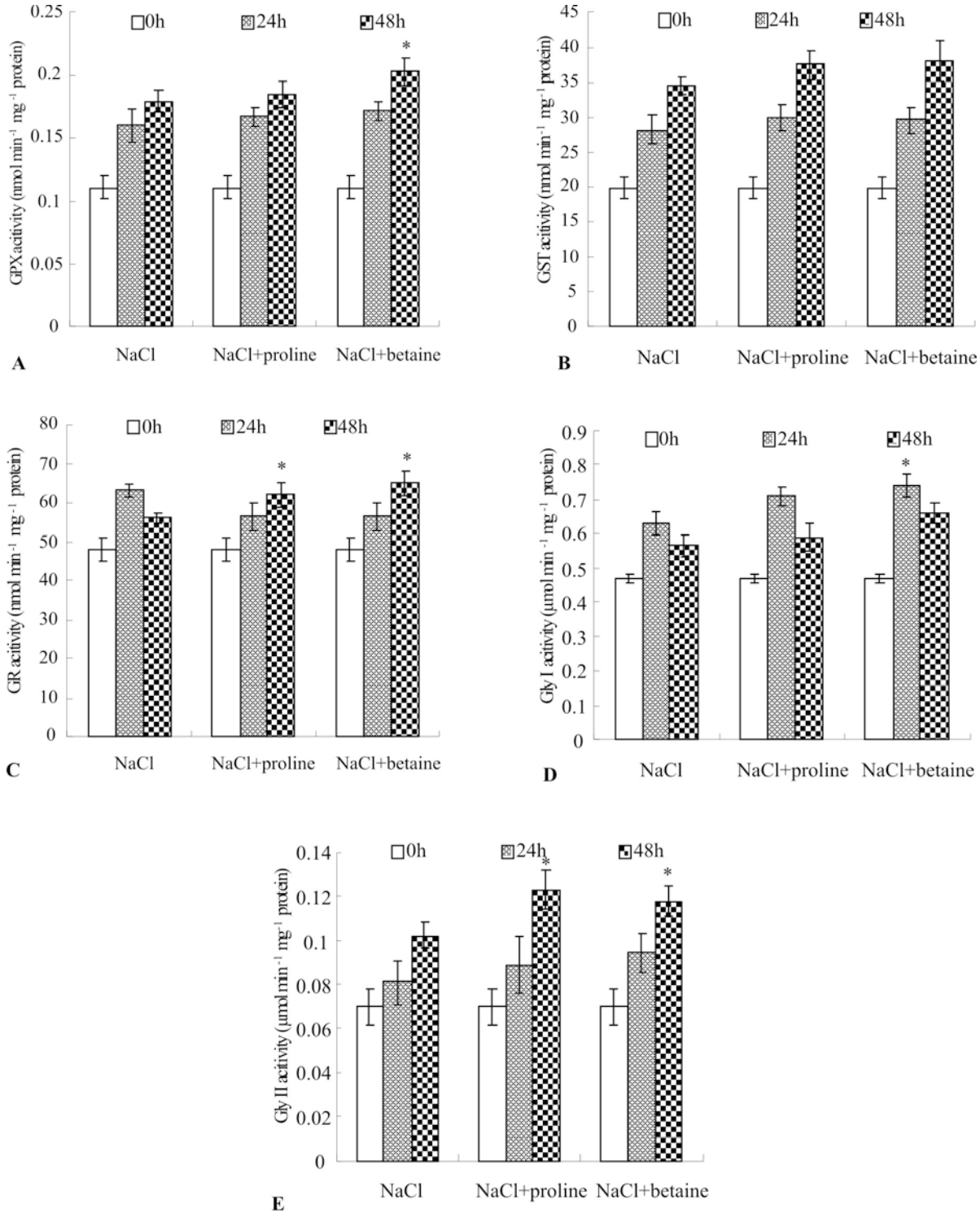

Fig. 2. Activities of GSH-utilizing and regenerating enzymes GPX (A), GST (B), GR(C), glyoxalase I (D) and glyoxalase II (E) in mung bean seedlings induced by proline and betaine under salt stress conditions. Five-day-old seedlings were exposed to 300 mM NaCl stress with or without 15 mM proline and 15 mM betaine for up to 48 h. Each value is the mean ± SE from four independent experiments. * indicates significant difference between mean values at the same time of incubation over salt stress (P <0.05).

global patterns of gene expression in development and defense (Alscher, 1989). Upon imposition of oxidative stress, the existing pool of GSH is converted to GSSG and GSH biosynthesis is stimulated (May and Leaver, 1993; Madamanchi *et al.*, 1994). Our results of glutathione (GSH-GSSG) estimation in salt-treated seedlings indicated that an increase GSH biosynthesis is an immediate response to salt stress and a drastic reduction of GSH concentration within 48 h due to high salt stress and simultaneous increase in oxidized glutathione (GSSG) (Fig. 1A-C), a true indication of oxidative stress. The formation of GSSG in salt-treated seedlings might be due to the reaction of GSH with oxyradicals generated due to oxidative stress or due to enhancement of GPX activity that decompose H_2O_2 (Aravind and Prasad, 2005; Shalata *et al*., 2001) as well as dehydroascorbate reductase (DHAR) activity (Hoque *et al.*, 2007b) which required GSH to regenerate ascorbate from dehydroascorbate. Proline and betaine supplemented salt-stressed seedlings maintained higher GSH level and glutathione redox state with simultaneous decrease in the oxidized GSSG (Fig. 1A-C). Proline and betaine probably modulate GSH levels by regulating its biosynthesis or by enabling efficient functioning of GR (Zhang and Kirkham, 1996). Results obtained in this study showed that salt stress caused a decrease in GR activity after 48 h, whereas both proline- and betaine-treated seedlings showed significant increases in GR activity (Fig. 2C) up to 48 h. These results agree with those of Demiral and Türkan (2004) showing that exogenous betaine increased GR activity in seedlings under a high salinity condition. Increased GR activity by proline and betaine under salt stress conditions contributes to maintenance of the glutathione pool in a reduced state, which is used by DHAR and other GSH-dependent enzymes involved in the antioxidant defense and glyoxalase systems.

Overexpression or higher activity of GPX and GST in plants increases antioxidant activity and improves tolerance to oxidative stress (Roxas *et al.*, 1997; Yoshimura *et al*., 2004; Yadav *et al*., 2005b). Furthermore, a high level of GPX-like protein in tobacco plants functions to remove unsaturated fatty acid hydroperoxides, leading to the maintenance of membrane integrity under various stresses including high NaCl stress (Yoshimura *et al.*, 2004). In this study, salt stress lead to significant increases in GPX and GST activities compared to those of the control, but a significant increase in GPX activity compared to that in salt-treated plants was observed after 48 h in betaine treated plants (Fig. 2A-B). The consistent increase in GPX and GST activities even under a severe salt stress condition indicates that both GPX and GST enzymes may be more stable or more important for stress tolerance than other antioxidant enzymes. Increased activities of GPX and GST in seedlings after 48 h under salt stress condition was accompanied by a decrease in the level of GSH (Figs. 1A, 2AB), indicating that an increased level of GSH is used by GPX and GST for detoxification of ROS and xenobiotics (endogenous cytotoxic compounds) and for membrane lipid peroxidation (Shalata *et al*., 2001; Mittova *et al.*, 2003b). However, the fact that exogenous proline and betaine increased GST and GPX activities and maintained higher GSH level indicates that both proline and betaine might play a significant role in GSH biosynthesis and metabolism. Transgenic tobacco plants overexpressing both GST and GPX also displayed improved tolerance to salinity and chilling stress (Roxas *et al.,* 1997).

Methylglyoxal is produced in the plant cell during normal physiological processes (Deswal *et al.*, 1993) and its level increased to a greater extent when plants get exposed to adverse environmental conditions including salinity, cold, drought and heavy metal stresses (Hossain *et al.,* 2009; Singla-Pareek *et al.,* 2006; Yadav *et al.*, 2005a,b). Higher glyoxalase I activity might protect plants against MG that is formed during oxidative stress (Hossain *et al.,* 2009; Jain *et al.,* 2002, Veena *et al.,* 1999). It has been shown that overexpression of glyoxalase I in transgenic plants resists an increase in MG level under a salt stress condition (Yadav *et al.,* 2005b) and confers tolerance to high salt and MG concentrations and heavy metals by increasing the antioxidant defense system and decreasing lipid peroxidation (Veena *et al.*, 1999; Singla-Pareek *et al.*, 2003; Yadav *et al.,* 2005a,b). In the present study, we observed a significant increase in glyoxalase I activity in salt-treated as well as proline and betaine-treated seedlings within 24 h (Fig. 2D), whereas glyoxalase I activity decreased gradually after 48 h of treatment, suggesting that detoxification of MG via the glyoxalase system is not sufficient under severe salt stress. The decreases in glyoxalase I activity in salt treated plant is probably due to the reduced glutathione content of the cell or inactivation of enzymes. However, both proline and betaine-treated seedlings could partially alleviate the stress-induced oxidative damage by maintaining higher glyoxalase I activity than that in salt stress only. Besides detoxification of MG, the glyoxalase system regenerates GSH that is trapped by MG to form SLG. Up-regulation of the glyoxalase II activity in

response to stress has been reported in plants (Hossain and Fujita, 2009; Saxena *et al.,* 2005) and overexpression of glyoxalase II under salinity maintains higher GSH level and GSH redox state (Yadav *et al.*, 2005b). A significant increase in glyoxalase II activity in salt-stressed cells (Fig. 2E) might lead to increased GSH generation and higher glutathione redox state. But in our study, we observed significant decrease GSH redox state although Gly II activity increased significantly. Decreased in GR activity (Fig. 2C) as well higher degradation of GSH are the reasons behind this. However, both proline and betaine increased glyoxalase II activity in accordance with GR activity, suggesting that both of them were able to enhance GSH regeneration and glutathione redox state via the glyoxalase system. The GSH levels found in the present study also support this.

H_2O_2 and MDA contents are routinely estimated parameters to assess the extent of oxidative stress. An increase in H_2O_2 and MDA contents upon salt stress has been reported in different plant species (Mittva *et al*., 2003a; Sheokand *et al*., 2008; Sumithra *et al*., 2006; Seckin *et al*., 2009) and control of the levels of H_2O_2 and MDA is thought to be a mechanism by which plants tolerate the stress (Chattopadhayay *et al.*, 2002; Bor *et al.,* 2003). An increase in the level of H_2O_2 and lipid peroxidation in salt-treated seedlings resulted in increased oxidative damage probably due to impairment of the antioxidant defense system. Conversely, a decreased level of H_2O_2 and MDA in both proline and betaine supplemented salt-treated seedlings (Fig. 3A,B) suggest that both proline and betaine protect against salt-dependent oxidative damage by enhancing antioxidant defense (Banu *et al*., 2009; Park *et al*., 2006; Hoque *et al.*, 2007a,b; Demiral and Turkan, 2004) and MG detoxification systems (Kumar and Yadav, 2009).

In conclusion, susceptibility of mung bean seedlings during severe salt stress was evident from the failure of H_2O_2 management and lipid peroxidation and reduction in GR and Gly I activity as well as GSH content including glutathione redox state. However, both proline and betaine supplemented salt treated seedlings maintained higher GSH level and glutathione redox state and higher activates of GPX, GST, GR, Gly I and Gly II. Therefore, apart from upregulation of AsA-GSH cycle (Hoque *et al.,* 2007b) co-ordinate induction of GSH, GR, GST, GPX, Gly I and Gly II by exogenous proline and betaine might play a protective role against salt stress induced oxidative damage. Taken as a whole, the

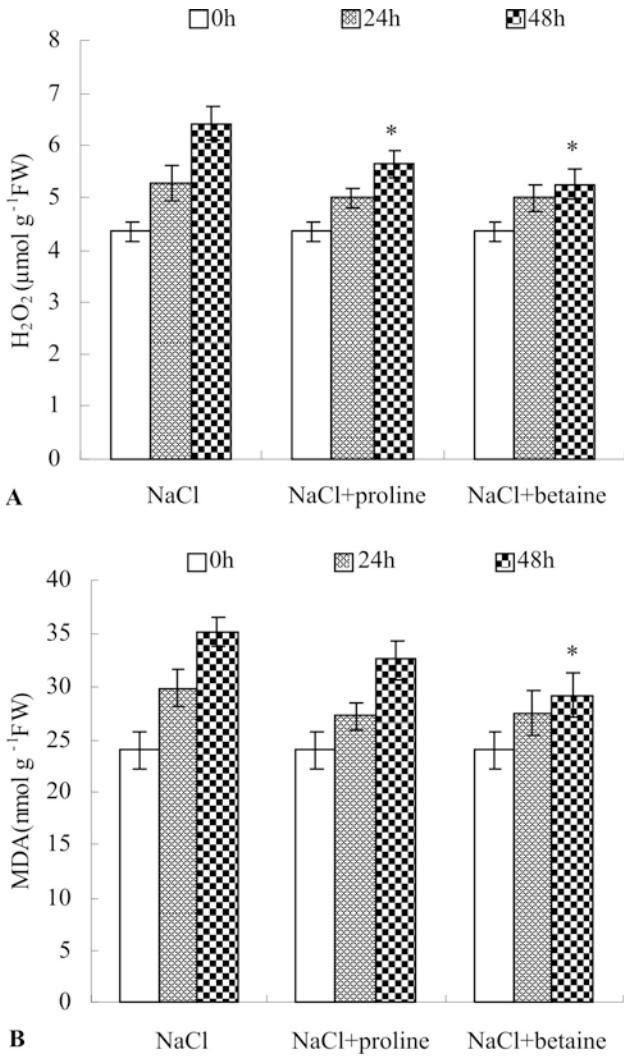

Fig. 3. Time course of changes in H_2O_2 concentration and lipid peroxidation (represented by MDA) in mung bean seedlings induced by proline and betaine under salt stress conditions. Five-day-old seedlings were exposed to 300 mM NaCl stress with or without 15 mM proline and 15 mM betaine for up to 48 h. A: H_2O_2 concentration, B: lipid peroxidation. Each value is the mean ± SE from four independent experiments. * indicates significant difference between mean values at the same time of incubation over salt stress (P <0.05).

results suggest that both exogenous proline and betaine can confer tolerance to salinity in mung bean seedlings by enhancement of the components of antioxidant defense and glyoxalase systems and exogenous application of these compounds also promise an alternative/additional way in genetic engineering studies to improve yield, production and quality under the condition of NaCl stress (Heuer, 2003).

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