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Methylglyoxal levels in plants under salinity stress are dependent on glyoxalase I and glutathione

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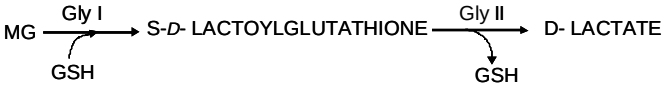
Abstract

Methylglyoxal (MG), a cytotoxic by-product produced mainly from triose phosphates, is used as a substrate by glyoxalase I. In this paper, we report on the estimation of MG level in plants which has not been reported earlier. We show that MG concentration varies in the range of 30–75 μM in various plant species and it increases 2- to 6-fold in response to salinity, drought, and cold stress conditions. Transgenic tobacco underexpressing glyoxalase I showed enhanced accumulation of MG which resulted in the inhibition of seed germination. In the glyoxalase I overexpressing transgenic tobacco, MG levels did not increase in response to stress compared to the untransformed plants, however, with the addition of exogenous GSH there was a decrease in MG levels in both untransformed and transgenic plants. The exogenous application of GSH reduced MG levels in WT to 50% whereas in the transgenic plants a 5-fold decrease was observed. These studies demonstrate an important role of glyoxalase I along with GSH concentration in maintaining MG levels in plants under normal and abiotic stress conditions.

Keywords: Methylglyoxal; Glyoxalase I; Salt stress; Glutathione; Plants; Rice; *Pennisetum*; *Brassica*; Tobacco; Abiotic stress

Methylglyoxal (MG) is a transition-state intermediate of both the triose-phosphates (dihydroxyacetone phosphate and glyceraldehyde-3-phosphate) of glycolysis pathway in eukaryotic cells [1]. It can also accumulate inside the cells by the leakage of 1,2-enediolate intermediate from the active site of triose phosphate isomerase [1]. Moreover, MG can also be synthesized through the action of MG synthase [2]. Higher level accumulation of MG is toxic to the cell as it inhibits cell proliferation [3] and 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 [2].

Glyoxalase system comprises of two enzymes, glyoxalase I (gly I) and glyoxalase II (gly II), which catalyze the detoxification of MG to D-lactate using reduced glutathione (GSH) as the cofactor and in the process GSH is recycled back [4,5]. The reaction catalyzed by gly I and gly II is as follows:



Glyoxalase enzymes have been extensively studied in microbial and animal systems [5,6]. The existence and widespread distribution of this shunt pathway indicate its universal role in primary metabolism [7,8]. Though very little work has been done in plants, differences in the pattern of enzyme distribution have been shown in Douglas-fir

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needles and their callus due to the presence and absence of MG, respectively [9], and the biological significance of this pathway under stress is just beginning to be explored [10–12]. Role of glyoxalases under stress in humans due to exercise, or in animals after irradiation or glycerol load in bacteria has been indicated [13]. However, recent studies in plants have demonstrated that different kind of stress conditions, such as salt and metal stress, results in enhanced expression of *gly I* [11]. Transgenic tobacco plants overexpressing *gly I* and *gly II* genes individually or together in the same plant indicated their role in salt tolerance [12]. However, whether or not MG accumulates in plants in response to stress conditions remains to be addressed.

Recently, it has been shown that MG can also act as a signal molecule [14] and hence its levels need to be regulated. MG levels have been measured in animal and yeast systems [15,16]. However, there are no reports on the estimation of MG in plants. The isolation, purification, and characterization of various enzymes, involved in the biosynthesis and breakdown of MG, has previously been reported in animals [17,18]. In the present study, we have standardized the protocol for the estimation of MG in plants and document that MG levels increase significantly in plants in response to salinity, drought, and cold stress conditions. Its levels were regulated by gly I and glutathione levels under normal and salt stress conditions.

Materials and methods

Standard curve for methylglyoxal. Different concentrations (10, 25, 50, and 100 μM) of pure MG (Sigma) were derivatized with 1,2-diaminobenzene and the absorbance of the resulting derivative was read by Cary 50 Bio UV-Visible spectrophotometer (Varian). One milliliter of total reaction mixture contained: 250 μL of 7.2 mM 1,2-diaminobenzene, 100 μL of 5 M perchloric acid, and double-distilled water. In the control, MG was not added to the reaction mixture.

Sample preparation for MG estimation in plants. About 0.3 g tissue was extracted in 3 mL of 0.5 M perchloric acid. After incubating for 15 min on ice, the mixture was centrifuged at 4 °C at 11,000*g* for 10 min. A colored supernatant was obtained in some plant extracts that was decolorized by adding charcoal (10 mg/mL), kept for 15 min at room temperature, and centrifuged at 11,000*g* for 10 min. Before using this supernatant for MG assay, it was neutralized by keeping for 15 min with saturated solution of potassium carbonate at room temperature and centrifuged again at 11,000*g* for 10 min. Neutralized supernatant was used for MG estimation.

Methylglyoxal assay. In a total volume of 1 mL, 250 μL of 7.2 mM 1,2-diaminobenzene, 100 μL of 5 M perchloric acid, and 650 μL of the neutralized supernatant were added in that order. The absorbance of the derivatized MG was read starting immediately at a broad spectrum range (200–500 nm) for 15 cycles of 1 min interval each.

HPLC-based methylglyoxal estimation. For standard curve, pure MG (1–10 μM) was used. In HPLC estimation, a total volume of 2 mL contained: 1 mL sample containing MG, 200 μL of 7.2 mM 1,2-diaminobenzene, 200 μL of 5 M perchloric acid, and 100 μL of 10 μM 2,3-dimethylquinoxaline (as internal standard). Samples were incubated at room temperature (25 °C) for 30 min and solid-phase extraction of the quinoxaline was performed as described by Cordeiro and Freire [16]. The mobile phase was 80% (v/v) 25 mM ammonium formate buffer (pH 3.4) and 20% (v/v) methanol. A volume of 100 μL was injected in a HPLC column (μ Bondapak 3.9 × 300 mm, RP-18C, Waters, Division of Millipore). Flux was set at 1 mL/min and quinoxalines were detected at 320 nm.

Plant growth conditions for MG measurements. Seeds of *gly I* transgenic tobacco overexpressing *gly I* gene in sense (NtS*gly I*) and antisense (NtAS*gly I*) orientations [11] and seeds of wild type (WT) tobacco plants were germinated. Either Murashige and Skoog (MS) basal or supplemented MS media containing either 5 mM GSH or 5 mM GSH and 200 mM NaCl were used for seed germination. The samples were placed under 16 h light photoperiod at 25 °C for 10 days before MG estimation. Seeds of rice (var. PB1 and IR64), tobacco, *Brassica*, and *Pennisetum* were germinated in pots containing vermiculite for 10 days and were subjected to either salinity (200 mM), cold (kept at 4 °C) or drought stress (withholding watering) for 24 h and fresh samples were used for MG estimation.

Results and discussion

Standardization of MG measurements in plants

Since there were no reports on the estimation of MG levels in plants, we followed the method of Cordeiro and Freire [16] with slight modifications. To measure MG, we standardized the chromatographic separation and spectrophotometric detection at 320 nm (HPLC) of MG which was derivatized with 1,2-diaminobenzene to form 2-methylquinoxaline (MQ). 2,3-Dimethylquinoxaline (DMQ) was used as the internal standard. We could separate the peaks of MQ and DMQ by using 80/20% (v/v) solution of 25 mM ammonium formate (pH 3.4)/methanol as mobile phase for HPLC. The retention times of MQ and DMQ were 9.9 and 13.2 min, respectively (Fig. 1A). The peaks obtained for plant samples were also exactly at the same time point. A proportional increase in the height of MQ was seen with the increasing concentration of pure MG (1–10 μM) whereas there was no change in the peak height of internal standard (5 μM was used in all the injection). On plotting the MG concentration and absorbance a linear curve was obtained (Fig. 1A, inset).

A simple spectrophotometer-based technique was also developed for estimating MG in plants. The plant sample was prepared as described in Materials and methods, and the absorbance of the derivatized MG was read at a broad spectrum range (200–500 nm) for 15 cycles of 1 min interval each. A clear peak (peak height 0.018) was observed at 332–336 nm with plant extract (Fig. 1Ba, peak marked with an arrow) and the height of the peak increased over a period of 15 min as more and more MG was getting derivatized during the measurement. To confirm that the peak observed at 332–336 nm with plant extract (Fig. 1Ba) was only due to the derivatization of MG with 1,2-diaminobenzene and not due to some other additional products, the neutralized and decolorized supernatant was incubated with purified gly I enzyme (10 U/mL) for 30 min that utilized MG. This resulted in the disappearance of the peak at 332–336 nm (Fig. 1Bb) and only a shift in base line was observed as reaction proceeded for 15 min. This clearly documented that the peak obtained was entirely due to the derivative of MG with 1,2-diaminobenzene and that there was no interference of other compounds.

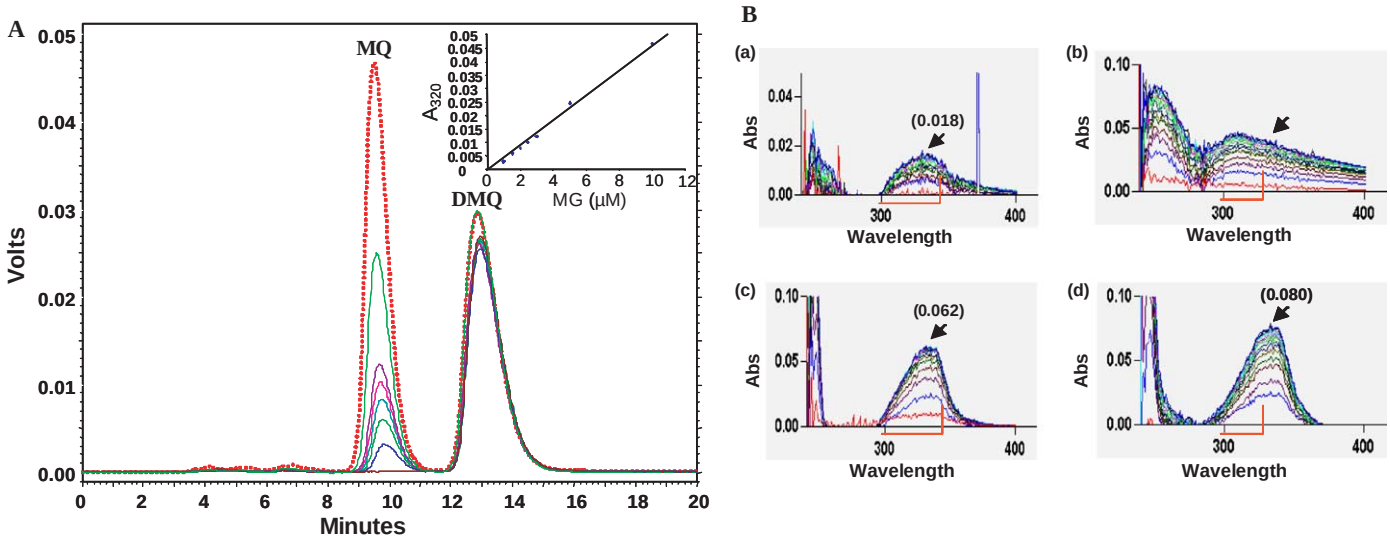

Fig. 1. Estimation of methylglyoxal (MG) levels in plants. (A) RP-HPLC-based analysis of quinoxalines for MG estimation. Chromatogram of the synthetic methylglyoxal (1–10 μM) showing the peaks of two distinct time points for 2-methylquinoxaline (MQ) and 2,3-dimethylquinoxaline (DMQ). Inset is a linear curve obtained by plotting the different concentrations of pure MG and the absorbance. (Ba) Absorption spectra for the derivatized MG in tobacco leaf extract supernatant by adding 1,2-diaminobenzene. The peak obtained at 332–336 nm is marked with an arrow, peak height 0.018. In all cases, the position of 332 nm has been marked with a red line on *X*-axis. (Bb) Absence of MG derivative peak at 332–336 nm (expected position of the peak has been marked with an arrow) when tobacco extract was incubated with gly I enzyme (10 U/mL) for 30 min at room temperature, only a shift in base line was seen as reaction proceeded for 15 min. (Bc) Absorption spectra for 10 μM pure derivatized MG (peak marked with an arrow, peak height 0.062). (Bd) Increase in peak height to 0.080 (equal to sum of absorbance of Ba and Bc, marked with an arrow) when absorption spectra were taken for the same sample extract (as used in Ba) in which an additional 10 μM pure MG was added.

To further confirm this, pure MG was directly derivatized that showed a peak (peak height 0.062) in the range of 332–336 nm (Fig. 1Bc). This derivatized pure MG was added with the sample supernatant (in which a derivative of MG was formed in the leaf extract with 1,2-diaminobenzene as shown in Fig. 1Ba), a single peak at the specified position was observed but the peak height in terms of absorbance increased from 0.018 (that of Fig. 1Ba) to 0.080 (Fig. 1Bd), which was equal to the sum of absorbance of derivatized MG in sample supernatant (Fig. 1Ba) and the derivatized pure MG (Fig. 1Bc). This result indicated that the nature of derivative formed with pure MG and MG from tobacco leaf extract was the same and therefore gave only a single peak with increased height (Fig. 1Bd).

Both the HPLC and spectrophotometer-based methods were found to be equally valid for MG estimations in plants. However, we find that the MG level measured in plant samples by spectrophotometer was about 15% less than that estimated by the HPLC method. The spectrophotometric method, however, is simpler and rapid, and can be used for comparative analysis of a large number of samples with certain degree of accuracy. However, for absolute measurements we recommend the HPLC method.

MG levels in plants under normal and stress conditions

The levels of MG were measured in leaves and roots of two monocot (*Oryza sativa* and *Pennisetum glaucum*) and two dicot (*Nicotiana tabacum* and *Brassica juncea*) plants under control and salt stress conditions. It was found that the basal level of MG was the same in leaves and roots, except in rice where its level was lower in roots (Fig. 2B). Under normal conditions, the MG level was lower in *Pennisetum* and tobacco than in rice and *Brassica* reflecting species-specific variations (Figs. 2A–D). During stress, *Pennisetum* showed a 3.5- and 2-fold increase in MG level in leaves and roots, respectively (Fig. 2A), whereas rice showed a 2.6- and 4-fold increase in leaves and roots, respectively (Fig. 2B). Tobacco showed a 2- and 1.8-fold increase in MG levels of leaves and root, respectively, whereas it was less than 2-fold in case of *Brassica* (Figs. 2C and D). Though no data are available for other plants, the range of MG reported is very wide in nature with as high as 300 μM MG in cultured Chinese hamster ovary cells and as low as 0.3 μM in yeast and in other animal systems [2,19]. In plants that we have studied, the MG levels range from 40 to 75 μM under normal conditions and from 75 to 200 μM under salinity stress. This shows that in general the plants have higher level of MG. These data also document that the accumulation of MG under salinity is a universal response in plants. The mechanism by which this occurs is not yet well understood. The increase in rate of glycolysis under stress could lead to higher MG level as it is produced mainly from triose phosphates [20].

To further check whether accumulation of MG in plants in response to stress is a general phenomenon, its levels were measured in two varieties of rice (PB1 and IR64) under drought, salinity, and cold stress conditions in shoot and root tissues. These data showed low level of MG in rice roots than shoots. The increase in MG was the largest

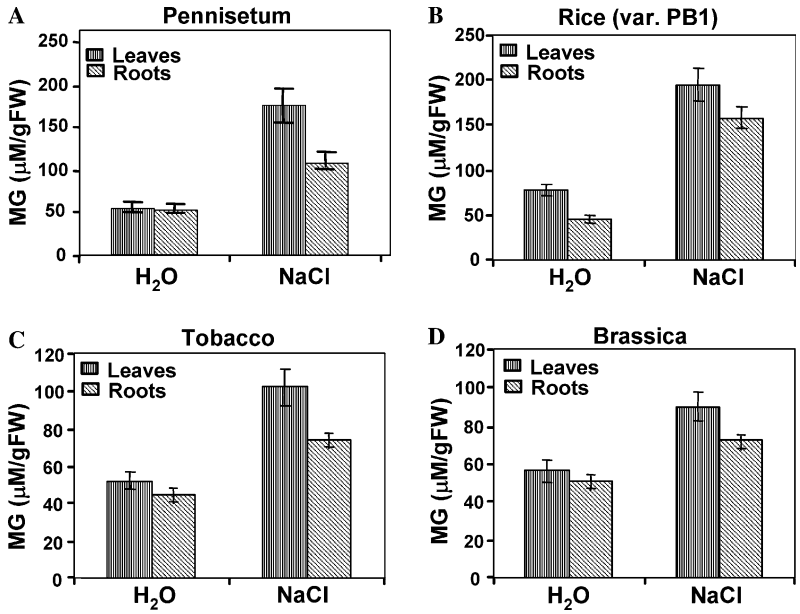

Fig. 2. Effect of salinity stress on MG levels in leaves and roots of different monocot and dicot plants. Ten-day-old seedlings were treated with 200 mM NaCl for 24 h, and their leaves and roots were used for MG level determination. (A) *P. glaucum*, (B) *O. sativa* (var. PB1), (C) *N. tabacum*, and (D) *B. juncea*.

(300 μM/gFW in shoot of both the varieties) during drought stress compared to salinity and cold stress (Table 1). The observation that under all three types of stress conditions, the MG levels increased, suggests that it is a general response to all abiotic stresses.

Glyoxalase I is important for detoxification of MG

Methylglyoxal is used as a substrate by gly I [5] as well as by aldose reductase [21,22]. To analyze the contribution of gly I in regulating the levels of endogenous MG, we used transgenic tobacco plants that either overproduced (NtS*gly I*) or underproduced (NtAS*gly I*) the gly I enzyme [11]. Both types of transgenic plants showed growth pattern similar to wild type during the T_0 generation. However, when T_1 seeds were germinated, the NtS*gly I* and WT tobacco seeds showed normal growth pattern whereas the NtAS*gly I* seeds failed to germinate (Fig. 3A). We measured the MG levels in T_1 seeds of the transgenic and non-transgenic lines. It was found that NtAS*gly I* seeds contain 132% higher MG as compared to WT and NtS*gly I* seeds (Fig. 3B). This suggests that NtAS*gly I* plants accumulated more MG due to low levels of the gly I enzyme availability that could not degrade the increasing level of MG leading to cytotoxicity and blocked seed germination. In fact, exogenous addition of MG has earlier been shown to inhibit plant growth and development [23]. These data suggest that gly I-dependent detoxification is the major pathway for MG catabolism, at least during the early stages of seed germination. Since the T_0 antisense plants, with lower level of gly I, showed both vegetative and reproductive growth [11], it is possible that some other detoxification processes may be involved during later stages of development or the level to which MG accumulates during normal and unstressed conditions may not be inhibitory to growth and development beyond seed germination. Recently, the role of aldose/aldehyde reductase (*ALR*) enzyme which uses NADPH, unlike gly I which uses GSH, for the reduction of reactive aldehydes such as 4-hydroxy-non-2-enal and methylglyoxal has been shown by over-expressing *ALR* in tobacco [22,24].

Exogenous GSH lowers MG level

The data in Fig. 3 showed that even if gly I levels are high as in NtS*gly I* plants, MG levels do not decrease below

Table 1
Effect of different stresses on MG (μM/gFW) levels in two varieties of rice

	IR64var.		PB1var.	
	Root	Shoot	Root	Shoot
Control	27.5 ± 1.6	62.3 ± 3.2	44.0 ± 2.4	74.8 ± 4.2
Drought	190.7 ± 9.8	304.4 ± 23.4	220.4 ± 18.5	301.0 ± 26.4
Salinity	124.4 ± 10.2	277.2 ± 18.6	153.2 ± 9.6	198.6 ± 14.2
Cold	73.4 ± 4.6	102.2 ± 6.4	104.5 ± 6.4	156.8 ± 10.2

Values are means of three replicates and two independent experiments ± SE.

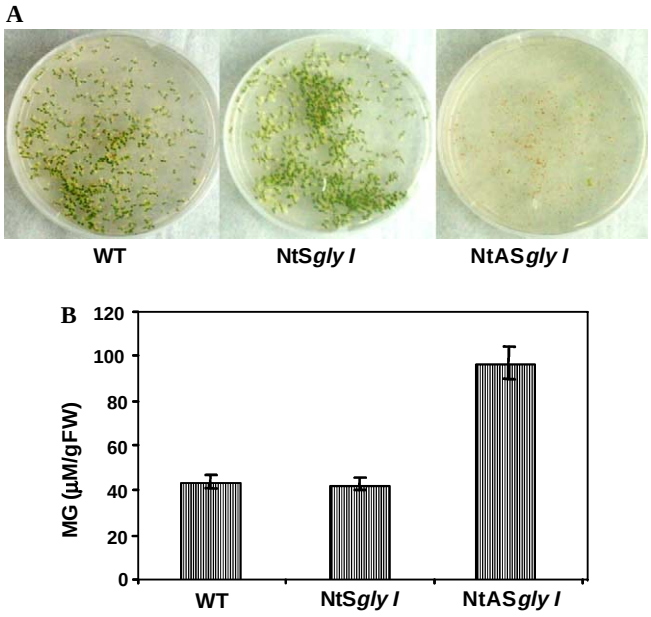

Fig. 3. Endogenous levels of MG in *gly I* sense and antisense transgenic tobacco plants during seed germination. (A) Seeds of NtS*gly I* (*gly I* gene overexpressing in sense orientation), NtAS*gly I* (*gly I* gene overexpressing in antisense orientation), and WT (wild type, non-transgenic) tobacco plants were germinated on MS medium. (B) MG level in seeds of NtS*gly I*, NtAS*gly I*, and WT tobacco plants.

a certain level. This suggests that there may be other factors limiting the degradation of MG. To check whether GSH, which is used as the cofactor by the gly I enzyme, could be the limiting factor, its effect on MG levels was analyzed in 10-day-old seedlings of WT, NtS*gly I*, and seeds of NtAS*gly I* (since seeds of NtAS*gly I* did not germinate) in the presence of different concentrations of GSH (0–10 mM) for 24 h. We found that WT seedlings showed a continuous decrease in MG level with increasing exogenous supply of GSH up to 10 mM (Fig. 4Ba). In case of NtS*gly I*, MG was decreased to 10 μM/gFW and this was achieved even at 5 mM GSH and thereafter, no further decrease in MG was observed (Fig. 4Bb). NtAS*gly I* seeds showed no decrease in MG levels (Fig. 4Bc). These results suggest that optimal levels of both GSH and gly I are needed for efficient detoxification of MG.

It was also seen that when the seeds were germinated on MS media containing 5 mM GSH, WT and NtS*gly I* germinated (Fig. 4A), whereas NtAS*gly I* seeds did not germinate (Fig. 4A). This shows that with very low level of gly I activity, seeds from NtAS*gly I* could not degrade the excess of MG even with the exogenous application of GSH.

Requirement of both GSH and high gly I for MG detoxification under stress

To check for the requirement of GSH and gly I for MG detoxification under stress conditions, where MG levels increase, seeds of WT and NtS*gly I* were germinated in the presence of 200 mM NaCl with 0–10 mM of GSH. Under these conditions, in absence of additional GSH, only NtS*gly I* seeds could germinate (data not shown) which indicated that the seeds overexpressing *gly I* in sense orientation were able to detoxify excess MG. As seen in Fig. 5B, the level of MG under stress in WT was around 94 μM whereas in NtS*gly I* it was only 50 μM. Methylglyoxal induces a dose- and time-dependent depletion of GSH and increases GSH oxidation or formation of GSSG. At high concentrations of MG, GSH may be trapped as

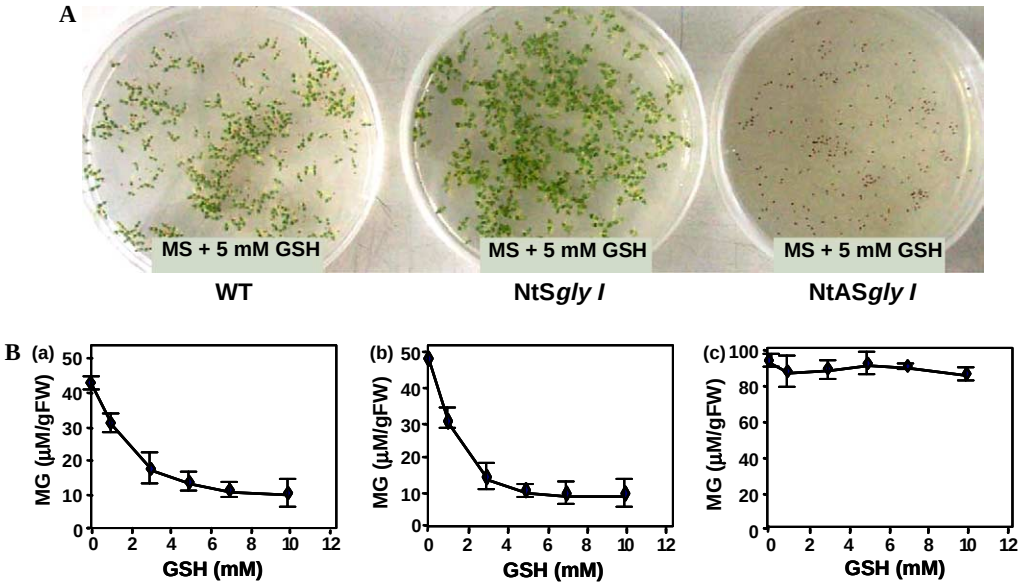

Fig. 4. Role of GSH in germination and regulation of MG level. (A) Phenotype of 10-day-old tobacco seedlings grown on MS media plates containing 5 mM GSH: WT, wild type; NtS*gly I*, *gly I* gene overexpressing in sense orientation; and NtAS*gly I*, *gly I* gene overexpressing in antisense orientation. (B) Seedlings (10-day-old) of WT (a), NtS*gly I* (b), and NtAS*gly I* (c) were incubated with different concentrations of GSH (0, 1, 3, 5, 7, and 10 mM) for 24 h and used for MG determination.

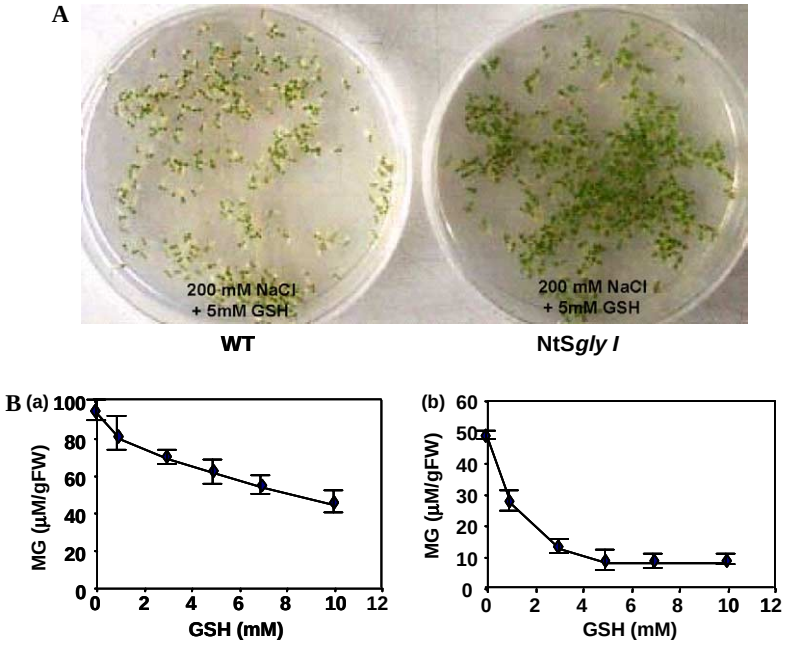

Fig. 5. Effect of salinity stress on seedling germination and MG level. (A) Phenotype of 10-day-old tobacco seedlings grown on MS media plates containing 200 mM NaCl in addition to 5 mM GSH: WT, wild type; NtS*gly I*, *gly I* gene overexpressing tobacco. (B) Seedlings (10-day-old) of WT (a), NtS*gly I* (b) were incubated with different concentrations of GSH (0, 1, 3, 5, 7, and 10 mM) for 24 h and used for MG determination.

S-2-hydroxyacylglutathione, resulting in GSH depletion [25,26]. GSH is an important metabolite as it is involved in the protection of plants from oxidative stress buildup due to various environmental stresses [27]. Therefore, higher amounts of MG accumulation during stress could either act directly as a potent toxic agent affecting various plant processes or it could deplete reduced glutathione (GSH) especially under reduced gly I situation. Under these situations, a smaller GSH pool would be available for other cellular activities [28,29].

To check whether exogenous GSH would be beneficial for reducing MG level under stress, seeds of WT and NtS*gly I* were germinated on MS media with different concentrations of GSH in addition to 200 mM NaCl. In WT seeds, the MG level decreased from 94 to 43 µM with an increase in the concentration of GSH in the growth medium (Fig. 5Ba) whereas in NtS*gly I*, MG level further decreased from 50 to 10 µM (Fig. 5Bb). With the addition of exogenous GSH (5 mM), germination of NtS*gly I* seeds was normal and importantly WT seeds also germinated under salt stress comparable to that of NtS*gly I* (Fig. 5A). This suggests that GSH can aid the plants to combat with increasing level of MG but GSH is useful only if plants have a competitive level of gly I enzyme which is lacking in NtAS*gly I* seeds. It was shown earlier that gly I, in addition to development, has a role during stress [10]. Our earlier studies also indicated that gly I overproducing tobacco plants tolerated a higher degree of salinity stress [11].

In conclusion, for the first time this study provides quantitative data on the levels of MG that are present in plants. We also show that under stress conditions the MG levels increase in all the plant systems tested and this seems to be a general stress response. There is a possibility that MG could therefore act as a signal for plants to respond to stress. We also show that the detoxification of MG occurs mainly through the glyoxalase pathway, at least during the early phase of seed germination. In addition to gly I, GSH may also be a limiting factor affecting the level of MG.

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