Transgenic tobacco plants overexpressing glyoxalase enzymes resist an increase in methylglyoxal and maintain higher reduced glutathione levels under salinity stress

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Abstract **The mechanism behind enhanced salt tolerance conferred by the overexpression of glyoxalase pathway enzymes was studied in transgenic vis-à-vis wild-type (WT) plants. We have recently documented that salinity stress induces higher level accumulation of methylglyoxal (MG), a potent cytotoxin and primary substrate for glyoxalase pathway, in various plant species [Yadav, S.K., Singla-Pareek, S.L., Ray, M., Reddy, M.K. and Sopory, S.K. (2005) MG levels in plants under salinity stress are dependent on glyoxalase I and glutathione. Biochem. Biophys. Res. Commun. 337, 61–67]. The transgenic tobacco plants overexpressing glyoxalase pathway enzymes, resist an increase in the level of MG that increased to over 70% in WT plants under salinity stress. These plants showed enhanced basal activity of various glutathione related antioxidative enzymes that increased further upon salinity stress. These plants suffered minimal salinity stress induced oxidative damage measured in terms of the lipid peroxidation. The reduced glutathione (GSH) content was high in these transgenic plants and also maintained a higher reduced to oxidized glutathione (GSH:GSSG) ratio under salinity. Manipulation of glutathione ratio by exogenous application of GSSG retarded the growth of non-transgenic plants whereas transgenic plants sustained their growth. These results suggest that resisting an increase in MG together with maintaining higher reduced glutathione levels can be efficiently achieved by the overexpression of glyoxalase pathway enzymes towards developing salinity stress tolerant plants.**

Keywords: Glyoxalase pathway; Glutathione; Methylglyoxal; Antioxidative enzymes; Overexpression; Salt tolerance; Transgenic

1. Introduction

In plants salt tolerance mechanisms have been studied from physiological and biochemical perspectives and recently several transgenic plants with enhanced salinity tolerance have also been produced [2–5]. It has been proposed that these plants tolerate saline conditions by one or a combination of different mechanisms. Like other stresses, salinity stress also leads to generation of reactive oxygen species (ROS) which are detoxified either directly by non-enzymatic antioxidants (reduced glutathione (GSH), ascorbate (ASH), tocopherols and carotenoids, etc.) or by antioxidative enzymes (superoxide dismutase; ascorbate peroxidase (APx), glutathione peroxidase (GPx) and glutathione-*S*-transferase (GST), etc.) [6–8].

GSH is the most abundant low molecular thiol compound in plants. It is a powerful cellular reductant and protects against a range of peroxides, xenobiotics and heavy metals [9,10]. Recently, GSH has been shown to regulate the expression of genes whose products are involved in redox regulation and/or in enhancement of stress tolerance [11,12]. Almost all cells exhibit two forms of glutathione: oxidized (GSSG) and reduced (GSH), interchanging with each other to maintain the redox potential under different conditions. However, free radicals and oxidants oxidize the thiol group forming glutathione disulphide (GSSG). High cellular GSH:GSSG ratio, which is maintained by the action of glutathione reductase (GR) using reduced nicotinamide adenine dinucleotide phosphate (NADPH), is crucial for the redox state of the cell and determines the survival of cells under any adverse condition [13]. Redox balance inside every cell is crucial for its normal functioning and GSH is thought to be well suited to act as redox sensor [8,10,13].

Methylglyoxal (MG), a cytotoxic compound, can react with and modify different molecular targets including DNA and proteins. It is distributed throughout the diverse group of organisms including microorganisms, yeasts, animals [14] and recently found in higher plants [1]. It is produced as a non-enzymatic by-product of glycolysis [15] and also enzymatically from dihydroxyacetone phosphate in a reaction catalysed by MG synthase in bacteria [14]. The mechanism(s) of production of MG in plants is not yet understood.

The main MG catabolic pathway in eukaryotes is the glyoxalase system, comprising two enzymes, glyoxalase I (Gly I; *S*-D-lactoylglutathione:methylglyoxal lyase; EC 4.4.1.5) and glyoxalase II (Gly II; *S*-2-hydroxyacylglutathione hydrolase; EC 3.1.2.6). Gly I isomerizes the hemithioacetal formed non-enzymatically from MG and GSH, forming

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Abbreviations: APx, ascorbate peroxidase; ASH, ascorbate; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; GST, glutathione-*S*-transferase; Gly I, glyoxalase I; Gly II, glyoxalase II; MG, methylglyoxal; NADP, nicotinamide adenine dinucleotide phosphate; ROS, reactive oxygen species; WT, wild-type

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S-D-lactoylglutathione while Gly II catalyzes the hydrolysis of this thioester to D-lactate, regenerating GSH [16]. Although, it has been shown in animals that high concentration of MG decreases the level of total thiol and GSH [14], little is known about the relationship between MG formation and its degradation by glyoxalase system vis-à-vis concomitant change in the intracellular GSH level in plants. Recently in yeast it has been shown that MG is produced at a rate of approx. 0.1% of the glycolytic flux and that the glyoxalase pathway is the main detoxification pathway for MG [17]. However in addition to glyoxalase pathway, aldose reductases may also detoxify reactive aldehydes including MG [18].

We have shown earlier that transgenic tobacco plants overexpressing *Gly I* and *II* can tolerate high concentration of NaCl stress [19,20], but how glyoxalase overexpression enables transgenic plants to tolerate salt stress is an important aspect that remained to be explored. We recently reported that MG levels increase in plants following the exposure to NaCl stress [1]. The results presented here show that glyoxalase overexpression in transgenic plants resists an overall increase in the level of MG under salinity stress (due to its efficient detoxification), thus reducing the MG toxicity. Concomitantly, a relatively higher GSH:GSSG ratio is maintained in the transgenic plants which protects them from salinity induced oxidative stress. These parameters along with an enhanced antioxidative capacity of transgenic plants seem to confer enhanced stress tolerance.

2. Materials and methods

2.1. Plant material, growth conditions and salinity stress

T1 generation seeds of tobacco (*N. tabacum* var petit havana) transgenics overexpressing either *Gly I* (Nt*glyI*), or *Gly II* (Nt*glyII*) alone or both together (Nt*glyI*/*II*) in the same plant (double transgenic) were selected by germinating their seeds on either kanamycin or hygromycin or both antibiotics containing Murashige and Skoog medium (SIGMA) selection plates [20] . After one week, the germinated seedlings were further checked for the presence of the transgene and the positives for the transgene were transferred to pots containing vermiculite and finally to earthern pots kept in greenhouse with 16 h light photoperiod at 25 °C. Healthy green leaves of 1-month-old plants were detached and exposed to 200 mM NaCl or sterile distilled water (as experimental control) for 24 h and then used for various analyses.

2.2. Measurement of MG and lipid peroxidation

MG was extracted from leaf tissue (0.3 g) by homogenizing in 3 ml of 0.5 M perchloric acid. MG levels were measured in this extract following the protocol described in Yadav et al. [1]. The assay mixture of total 1 ml contained: 250 μl of 7.2 mM 1,2-diaminobenzene, 100 μl of 5 M perchloric acid and 650 μl of the sample extract (which was added the last) and the absorbance of the derivative was read at 336 nm. The final concentration of MG was calculated from standard curve and expressed in terms of μM/gFW. Lipid peroxidation was measured in terms of malondialdehyde (MDA) value by the reaction with thiobarbutaric acid according to Heath and Packer [21].

2.3. Measurement of glutathione and protein contents

Reduced (GSH) and oxidized glutathione (GSSG) were measured using the 5′-dithio-bis-(2-nitrobenzoic acid)/GSSG reductase recycling assay as described in previous studies [22]. GSH was estimated as the difference between total glutathione and that of GSSG. The protein content was assayed according to Bradford [23].

2.4. Enzyme extraction and activity determination

Tobacco leaves were homogenized with pestle and mortar in the presence of liquid nitrogen. The homogenous mixture was made with 1:2 (w/v) of extraction buffer containing 0.1 M potassium phosphate buffer (pH 7.5), 50% glycerol, 16 mM $MgSO_4$, 0.2 mM PMSF and 0.2% PVPP. The extract was centrifuged at $15000 \times g$ for 30 min at 4 °C and the supernatant was used for the determination of various enzyme activities. For APx assay, 2 mM ASH was added to the extraction buffer while homogenizing the tissue. GR and GST activities were determined by the standard methods already described [24,25]. GST was assayed by using 1-chloro-2,4-dinitrobenzene as a substrate. GPx (EC 1.11.1.9) activity was followed by the decrease in A_{340}, resulting from NADPH oxidation [26]. Total assay mixture of 1 ml contained 100 mM potassium phosphate buffer (pH 7.5), 2 mM GSH, 0.2 mM NADPH, 70 μM *tert*-butyl hydroperoxide, 1 U/ml GR and 10 μl of enzyme extract. APx (EC 1.11.1.11) activity was determined by monitoring the oxidation of ASH at 290 nm [27].

2.5. Oxidative stress analysis

T1 generation seeds of Nt*glyI*, Nt*glyII*, Nt*glyI*/*II* transgenics and wild-type (WT) plants were selected on MS media plates containing suitable antibiotics for 7 days. Seedlings were then transferred to the basal MS plates containing 200 mM NaCl alone or with different concentrations (5–15 mM) of reducing agents such as dithiothreitol (DTT), cysteine (Cys), reduced glutathione (GSH) and allowed to grow for next 12 days. For each experiment, plates were photographed and length of the seedlings was measured at appropriate time. Effect of exogenous GSSG on the survival and growth of WT and glyoxalase overexpressing transgenic tobacco seedlings was tested by germinating T1 seeds on MS selection media for 7 days and then transferred to MS media containing 10 mM GSSG and allowed to grow for next 12 days and photographed.

2.6. Statistical analysis

All experiments were independently carried out at least three times with three lines each of WT, Nt*glyI*, Nt*glyII*, Nt*glyI*/*II* and each time with three replicates (i.e., $n = 27$) for all the measurements, unless otherwise stated. For shoot length, mean of 6 seedlings was taken for four times (i.e., $n = 24$). The standard error was calculated by using the n values of each experiment.

3. Results and discussion

Transgenic tobacco plants overexpressing glyoxalase enzymes, under the control of a constitutive cauliflower mosaic virus 35 S promoter, were generated using pCAMBIA1304 vector through *Agrobacterium* mediated transformation. Three different types of transgenic plants were raised (i) those overexpressing *Gly I* (Nt*glyI*), (ii) those overexpressing *Gly II* (Nt*glyII*), and (iii) double transgenics overexpressing both *Gly I* and *Gly II* genes together (Nt*glyI*/*II*). We have shown earlier that these transgenic plants were able to grow and mature normally under non-stress conditions and their growth remained almost unaltered under persisted salinity that was otherwise inhibitory for the growth of WT plants [20]. It has been found that under stress, there is an increase in MG level on one hand [1] and also an increase in the ROS which could hamper the survival of plants under stress. In the transgenics, these processes may be suppressed, and antioxidants level may be maintained, thus leading to their survival under salt stress. The present experiments were designed to investigate the mechanism behind the salt tolerance conferred by the overexpression of glyoxalase pathway enzymes. Representative plants belonging to each category which were found to have constitutive high expression of Gly proteins (tested by western blotting) and higher enzyme activities were employed in the present study. It would be important to mention here that in all the tests performed in this study, the response of double transgenic plants (Nt*glyI*/*II*) was much better that either of the single gene transformants (Nt*glyI* or Nt*glyII*). In view of this we have mainly discussed the data for WT vis-à-vis Nt*glyI*/*II*.