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ORIGINAL RESEARCH PAPER

Transgenic potato overproducing L-ascorbic acid resisted an increase in methylglyoxal under salinity stress via maintaining higher reduced glutathione level and glyoxalase enzyme activity

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Abstract Salt-tolerance was studied in transgenic potato. It was conferred by overexpression of ascorbate pathway enzyme (D-galacturonic acid reductase, *GalUR*). As genetic engineering of the *GalUR* gene in potato enhances its ascorbic acid content (L-AsA), and subsequently plants suffered minimal oxidative stress-induced damage, we now report on the comprehensive aptness of this engineering approach for enhanced salt tolerance in transgenic potato (*Solanum tuberosum* L. cv. Taedong Valley). Potatoes overexpressing *GalUR* grew and tuberized in continuous presence of 200 mM of NaCl. The transgenic plants maintained a higher reduced to oxidized glutathione (GSH: GSSG) ratio together with enhanced activity of glutathione dependent antioxidative and glyoxalase enzymes under salinity stress. The transgenics resisted an increase in methylglyoxal that increased radically in untransformed control plants under salinity stress. This is the first report of genetic engineering of ascorbate pathway gene in maintaining higher level of GSH homeostasis along with higher glyoxalase activity inhibiting the accumulation in methylglyoxal (a potent cytotoxic compound) under salt stress. These results suggested the engineering of ascorbate pathway enzymes as a major step towards developing salinity tolerant crop plants.

Keywords Abiotic stress · Ascorbate · Transgenics · Antioxidant enzymes · Reduced glutathione · Methylglyoxal

Abbreviations

ASH	Ascorbate
APx	Ascorbate peroxidase
GalUR	D-Galacturonic acid reductase
GPx	Glutathione peroxidase
GR	Glutathione reductase
Gly I	Glyoxalase I
Gly II	Glyoxalase II
MG	Methylglyoxal
NADP	Nicotinamide adenine dinucleotide phosphate
GSSG	Oxidized glutathione
GSH	Reduced glutathione
ROS	Reactive oxygen species
UT	Untransformed control

Introduction

Adverse environmental factors, such as drought, salinity, heavy metal, heat, and cold, result in huge

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losses of crop yields. Abiotic stresses elicit complex cellular responses plant. The physiological and biochemical perspectives of salt tolerance mechanisms have been studied and several transgenic plants with enhanced salinity tolerance have been reported (Hasegawa et al. 2000; Singla-Pareek et al. 2001; Grover et al. 2003; Neill et al. 2003). These plants tolerated various stresses via a combination of different mechanisms. Adverse environmental factors cause generation of ROS and other cytotoxic compounds at cellular level, ultimately affecting the development of plants. Injuries associated with ROS, referred as oxidative stresses, are among the most profound destructive factors in plants (Xiong and Zhu 2002). Therefore, plants have developed a number of defense mechanisms including both enzymatic and non-enzymatic detoxification reactions and damage repair to protect themselves against these cytotoxins (Wang et al. 2003).

Vitamin C (L-ascorbic acid, AsA) is an important plant secondary metabolite as an anti-oxidant as well as a cell signaling modulator in a wide array of crucial physiological processes including cell division, growth regulation, and senescence (Smirnoff 1996; Conklin and Barth 2004; Pavet et al. 2005; Hemavathi et al. 2009, 2010a, b). Moreover, ascorbate acts as a co-factor for many enzymes thereby affecting the expression of genes involved in defense and hormone signaling pathways (Pastori et al. 2003; Tullio and Arrigoni 2004). It also participates in a variety of cellular processes, including cell wall growth and cell expansion, resistance to environmental stresses and senescence (Smirnoff and Wheeler 2000; Pavet et al. 2005). In plants, ascorbate interacts enzymatically as well as non-enzymatically in detoxification ROS as well as in the regeneration of vitamin E (Thomas et al. 1992). In plants, among the reducing substrate dependent peroxidases, ascorbate peroxidases (APx) are the major enzymes involved in intracellular H_2O_2 removal that utilizes ascorbate as an electron donor (Noctor et al. 1998). The APx catalyzes the first step of the ascorbate–glutathione cycle, a major antioxidant route operating in plant cells. In this cycle, ascorbate and reduced glutathione (GSH) are employed as reducing agents to detoxify H_2O_2. Besides APx, monodehydro-ascorbate reductase (MDHAR), dehydroascorbate reductase (DHAR) and glutathione reductase (GR) also catalyze important steps of this cycle (Alscher et al. 1997; Noctor and Foyer 1998). Reduced glutathione (a low molecular thiol compound) is found abundantly in plants and functions as a powerful cellular reductant which protects the plant against xenobiotics and several peroxides (May et al. 1998; Noctor et al. 1998). Nearly all cells display two forms of glutathione, oxidized (GSSG) and reduced (GSH), which interchange with each other to maintain the redox potential under different conditions. GSH is also involved in the expression of genes involved in improvement of redox regulation and stress tolerance (Wingsle and Karpinski 1996; Kocsy et al. 2001).

Methylglyoxal (MG) is a cytotoxic compound which reacts and/or modifies different molecules including DNA and proteins (Singla-Pareek et al. 2001; Yadav et al. 2005a, b, c). MG is a transition-state intermediate of both the triose-phosphates (dihydroxyacetone phosphate and glyceraldehydes 3-phosphate) of glycolysis pathway in eukaryotic cells (Kalapos 1999) which can also be synthesized via action of MG synthase present in the cell (Booth et al. 2003; Martins et al. 2001). Higher accumulation of MG is toxic to the cell as it inhibits cell proliferation 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 (Martins et al. 2001). However, MG concentration is regulated by glyoxalase system which depends on glutathione concentration under normal as well as stress conditions (Yadav et al. 2005a, b). Moreover, maintenance of higher GSH:GSSG ratio is prerequisite to maintain lower MG level (Yadav et al. 2005c) and protection from the ROS activity.

Ascorbic acid is synthesized by D-galacturonic acid pathway in plants (Smirnoff and Wheeler 2000). D-Galacturonic acid is reduced to L-galactonic acid by D-galacturonic acid reductase (GalUR) and is subsequently converted to L-galactono-1,4-lactone that is further oxidized to AsA by L-galactono1,4-lactone dehydrogenase (GalDH) (Smirnoff et al. 2001; Agius et al. 2003). We have shown earlier that transgenic potato plants overexpressing ascorbate pathway gene (*GalUR*) can tolerate different abiotic stresses (Hemavathi et al. 2009, 2010b) by maintaining higher activity of the ROS removing antioxidant enzymes as well as enhanced GSH:GSSG level. However, it is prerequisite to know the detailed mechanism of salt

tolerance, with the *GalUR* overexpressing transgenics. The results presented here showed that the transgenic potato overexpressing *GalUR* gene resulted in enhanced accumulation of ascorbic acid and resisted an overall increase in MG level under salinity stress, thus, reducing the MG toxicity. Concomitantly, a relatively higher GSH:GSSG ratio is also maintained in the transgenics which protects them from salinity induced oxidative stress. These parameters along with an enhanced antioxidative capacity of transgenic plants seem to confer enhanced salt stress tolerance.

Materials and methods

Plant material, growth conditions and salinity stress

The transgenic potato plants (*Solanum tuberosum* L., cv. Taedong valley) overexpressing *GalUR* with enhanced ascorbate (AsA) developed in our laboratory (Hemavathi et al. 2009, 2010b) were used for the experiments. The in vitro grown transgenic plants were maintained on MS medium with appropriate antibiotic. The in vitro cultured plants were further screened for the presence of the transgenes by PCR and Southern blot analysis. Single nodes cuttings from the in vitro propagated plants were sub-cultured in Pyrex glass culture tube (25 × 150 mm) containing MS (15 ml) medium (Murashige and Skoog 1962) supplemented with 3% (w/v) sucrose (non-tuber inducing medium) or 7% (w/v) sucrose (tuber-induction medium) and 200 mM NaCl as stress inducing agent. MS medium without NaCl served as negative control. The plants were maintained in a chamber under 16 h light conditions at 22°C for 4–5 weeks. Ten transgenic as well as untransformed plants were used for each treatment.

Protein extraction and enzyme assay

The transgenics and UT potato samples (leaves and tubers) collected from stressed (200 mM NaCl) and non-stressed medium were pulverized in pestle–mortar in presence of liquid N_2. Protein was extracted with an extraction buffer containing 0.1 M potassium phosphate buffer (pH 7.5), 50% (v/v) glycerol, 16 mM $MgSO_4$, 0.2 mM PMSF and 0.2% PVPP and centrifuged at 15,000×*g* for 30 min at 4°C and the supernatant was used for the determination of various enzyme activities. The protein content was determined by the Bradford method. For APx assay, 2 mM ASH was added to the extraction buffer while homogenizing the tissues and the activity was determined by monitoring the oxidation of ASH at 290 nm (Sgherri and Navari-Izzo 2000). GR and GST assay were done using the standard methods described (Foyer and Halliwell 1976; Habig and Jakoby 1981). The GPx activity was determined by the decrease in absorbance at 340 nm resulting from NADPH oxidation (Geiger et al. 1993). 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. DHAR activity was assayed as described by Nakano and Asada (1981). The reaction mixture contained 50 mM phosphate buffer (pH 7.0), 20 mM reduced glutathione, 2 mM dehydroascorbate and 100 μl crude protein. DHAR activity was determined by increase in absorbance at 265 nm (extinction coefficient 6.2 mM^{-1} cm^{-1}) due to the GSH-dependent production of AsA.

Glyoxalase I activity was assayed according to Ramaswamy et al. (1983) as a function of thioester formation (*S*-D-lactoyl glutathione) by measuring the rate of change of absorbance at 240 nm. The determination of glyoxalase II activity was done by monitoring the decrease in the absorbance at 240 nm due to the hydrolysis of the substrate, *S*-D-lactoylglutathione (Saxena et al. 2005). Specific activity of the enzymes was expressed in U/mg protein.

Analysis of glutathione, ascorbate and methylglyoxal content

Determination of glutathione reduced (GSH) and oxidized (GSSG) forms, was by the method of Griffith (1980). Total glutathione was determined at 412 nm using yeast-GR, 5,5′-dithio-bis-nitrobenzoic acid and NADPH. Determination of GSSG was done by incubating the mixture with 2-vinylpyridine at 25°C for 1 h to derivatize GSH before adding GR. The GSH content was calculated from the difference between total GSH and GSSG. A standard curve of GSH was used for quantification.

Ascorbate and DHA were assayed according to Kampfenkel et al. (1995) which is based upon the

reduction of Fe^{3+} to Fe^{2+} by AsA in acidic solution. Ascorbate was determined using 50 µl of the extracted solution by initially incubating for 20 min in 200 mM phosphate buffer solution (pH 7.4) and 1.5 mM dithiothreitol (DTT) to reduce DHA to AsA. After incubation, 200 µl 0.5% (w/v) *N*-ethylmaleimide (NEM) was added to remove excess DTT. AsA was assayed in a similar manner except that 400 µl deionized H_2O was substituted for DTT and NEM. Color developed in both the series of reaction mixtures with the addition of 10% trichloroacetic acid, 42% (v/v) phosphoric acid, 65 mM 2,2′-dipyridyl in 70% (v/v) ethanol, and 3% (w/v) $FeCl_3$. The reaction mixtures were then incubated at 42°C for 1 h and absorbance was recorded at 525 nm. A standard curve of ascorbate and DHA was established and used for quantification.

MG level were determined by extracting 0.3 g leaf in 3 ml 0.5 M perchloric acid following the protocol described (Yadav et al. 2005b). The assay mixture (1 ml) contained: 250 µl 7.2 mM 1,2-diaminobenzene, 100 µl 5 M perchloric acid and 650 µl sample extract. The absorbance was read at 336 nm. Final concentration of MG was calculated from standard curve and expressed in terms of µM/g FW.

Oxidative stress analysis

The nodal segments from transgenics and untransformed control (UT) plants were grown for 2–3 weeks on MS medium containing 200 mM NaCl alone or with different concentrations (10–15 mM) of reducing agents such as DTT and reduced glutathione (GSH). Effect of exogenous GSH on the survival and growth of UT or transgenic potato were measured. Similarly, the nodal cuttings of the transgenics and UT control potato were maintained on MS medium for 3–4 weeks containing 200 mM NaCl alone or with different concentrations (100–150 mM) of buthionine sulfoximine (BSO), an inhibitor of γ-glutamylcysteine synthetase (the first and rate limiting step of GSH biosynthesis) and data were recorded based on growth and tuberization pattern.

Statistical analysis

All the experiments were independently carried out at least three times with transgenic and UT control lines and each time with three replicates for all measurement. The means were analyzed using Statistical Analysis Software package program 9.1 (SAS, USA). Statistical differences were determined using one-way analysis of variance and standard error was calculated by using the *n* values of each experiments.

Results and discussion

Transgenic potatoes overexpressing *GalUR* (strawberry isolate) with higher ascorbic acid (L-AsA) were developed using pCAMBIA1302 vector under the control of a constitutive cauliflower mosaic virus 35S promoter via *Agrobacterium*-mediated transformation (Hemavathi et al. 2009, 2010b). Representative potatoes showed higher expression of the GalUR enzymes and higher L-AsA in plants including tubers. We have shown earlier that these transgenic plants grow and tuberize normally and their tuber formation remained unaltered under persisted stress (oxidative, salt & heavy metal) that was otherwise inhibitory for the growth of untransformed control plants (Hemavathi et al. 2009). However, these transgenics also maintained higher activities of the antioxidant enzymes together with higher gene expressions and higher proline content as well as less lipid peroxidation (Hemavathi et al. 2010b). The present study aimed at investigating the detailed mechanism behind the salt tolerance conferred by the overexpression of GalUR in the transgenic potato. The potato transgenics developed in the laboratory were maintained on the appropriate antibiotic containing medium and were screened by PCR and Southern blot analysis for confirmation of the transgenes insertion, before further stress analysis (Fig. 2a).

Transgenics show higher activity of various antioxidative enzymes

The activity of major ROS scavenging enzymes in the transgenics and UT samples (leaves & tubers) was determined which utilizes GSH for their action under normal and stressed (200 mM NaCl) conditions. Under the stress condition, the transgenics samples (leaves & tubers) showed 150–170% increase in GR and whereas in the WT plants it increased by 80% (Table 1). The activity of DHAR was increased by 170–190% in transgenics and 60% in WT plants. The increase in GPx and GST activity was almost double

Table 1 Effect of salt stress on activities of various antioxidative and glyoxalase pathway enzymes in leaves and tubers of UT control and transgenics (T) plants overexpressing *GalUR* gene

	UT	T (Leaves)	T (Tubers)
APx			
Control	18	32	34
NaCl	30	80	75
DHAR			
Control	10	18	21
NaCl	17	52	58
GPx			
Control	8	14	15
NaCl	11	30	32
GST			
Control	10.2	24	21
NaCl	15	28	34
GR			
Control	3	10	9
NaCl	5.5	25	23
Gly I			
Control	6	11	10
NaCl	10	26	24
Gly II			
Control	4	8	7
NaCl	6	19	17

The data are means of the specific activities from three separate assays of the leaves and tubers of three independent transgenic lines with three replicates

GR Glutathione reductase, *GPx* glutathione peroxidase, *APx* ascorbate peroxidase, *DHAR* dihydroreductase, *Gly I* glyoxalase I, *Gly II* glyoxalase II

For GR, GPx, PAX, SOD the units = nmol/min/mg protein and for Gly I and Gly II the units = units mg^{-1} protein

(110–115%) in transgenic samples and 45% in WT, while the APx activity increased by 120–150% in transgenics and by 45% in WT samples (Table 1) in response to salt. The basal level activity of these enzymes was higher in transgenic plants compared to the WT. The activity of these enzymes was further enhanced in response to salt indicating higher anti-oxidative capacity of the transgenics. Similar observation where anti-oxidant enzyme activity increased in tobacco plants overexpressing the glyoxalase pathway enzymes were reported by Yadav et al. (2005a, b). Higher activity of APx converts monodehydroascorbate (MDHA) and dehydroascorbate (DHA) to that of ASH. Further, a regeneration system comprising of MDHA reductase, DHA reductase and GR (Fig. 1) brings about the reduction of oxidized ASH using GSH (Foyer and Halliwell 1976). The increase in DHAR and GR activity in the plants ensures efficient regeneration of ascorbate, which can scavenge increased levels of H_2O_2 under stress (Fig. 1). GR catalyzes the reduction of GSSG to GSH in a NADPH-dependent reaction and its higher activity in GalUR overexpressing transgenic potato could result in higher GSH:GSSG ratio in transgenics. Improved tolerance to several oxidative stresses via increase in the activity of DHAR and GR (as another ascorbate–glutathione cycle enzyme) in stressed transgenics has been demonstrated earlier (Roxas et al. 1997; Foyer et al. 1995; Aono et al. 1995; Shigeoka et al. 2002). Further, the higher level of GPx may give tolerance to transgenics by degrading lipid peroxides which could damage cell membrane (Zhang and Kirkham 1996).

Transgenic plants maintain higher level of reduced glutathione under NaCl stress

The transgenics lines overexpressing the Vit C pathway enzymes were confirmed by PCR and Southern blot analysis (Fig. 2a) before applying salt stress. The glutathione ratio (GSH:GSSH) was further examined under unstressed and stressed (200 mM NaCl) conditions in different samples of the UT and transgenic potato. In case of untransformed plants, NaCl stress induced a 40% decline in the GSH:GSSG ratio (Fig. 2b) indicating an increase in GSSG levels in these plants. In contrast, salt stress induced a significant accumulation of GSH in transgenics, as high as 35% more (Fig. 2c) together with only 15% decrease in the GSH:GSSG ratio (Fig. 2b). Higher GSH:GSSG ratio could alter the redox state in plant cell and may activate special defense mechanisms like antioxidative enzymes (as mentioned in Fig. 1) via a redox signaling chain. Maintenance of GSH levels during unstressed and stress conditions can avert the generation of free radicals and, hence, tissue damage (Nagalakshmi and Prasad 2001). Further, in plant antioxidant systems, the ascorbate–GSH cycle and various cellular antioxidants like thiols and GSH (Foyer and Shigeoka 2001), play a major role in scavenging H_2O_2 in chloroplasts and cytosol (Foyer et al. 2001; Zhang and Kirkham 1996). However, like glutathione, ascorbate scavenges ROS both enzymatically and non-enzymatically and

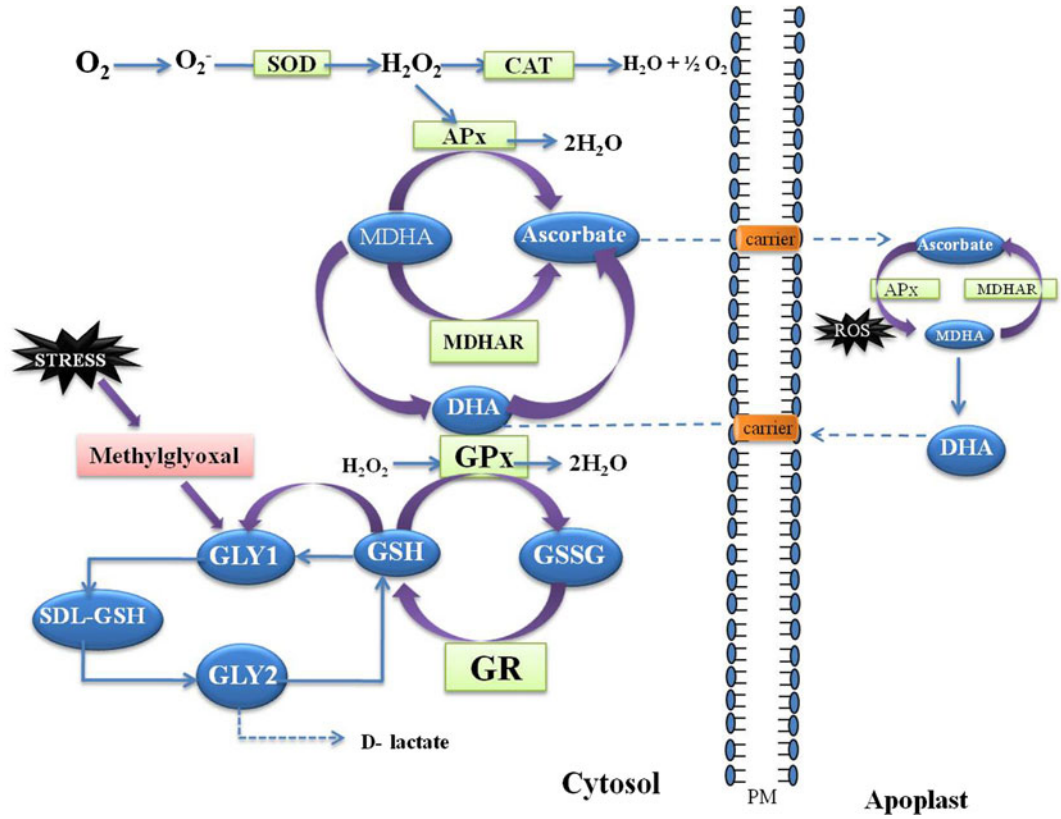

Fig. 1 Diagrammatic representation of detoxification of ROS and methylglyoxal via Glyoxalase–GSH–Ascorbate pathway. Methylglyoxal level is regulated by glyoxalase system depending on glutathione concentration under normal and stress conditions. During salinity stress, the scavenging of free radicals by APx increases MDHA and DHA via AsA regeneration system comprised of MDHAR, DHAR and GR which reduces DHA using GSH. GR catalyzes the reduction of GSSG to GSH to maintain the higher to GSH:GSSG in cytosol. The glyoxalase system utilizes the available GSH to detoxify the increased MG and GSH is recycled again via glyoxalase system

thus limits the lifetime of the ROS signal. In the potato transgenics the level of AsA and DHA also increased (Fig. 2d) which further strengthened the role of ascorbate as ROS scavenge.

The redox balance inside every cell is crucial for its normal functioning and GSH is well suited to act as redox sensor (Noctor et al. 1998). Reduced glutathione is a crucial reductant for the effective scavenging of ROS (Fig. 1) and for the maintenance of other antioxidants such as AsA and tocopherol (Alscher 1989). Tobacco overexpressing GR (Broadbent et al. 1995) and glyoxalase pathway genes (Yadav et al. 2005b) leads to elevated level of GSH with an enhanced stress tolerance. Similar results with increased AsA and GSH:GSSG ratios were reported in the transgenic tobacco plants expressing the DHAR gene (Kwon et al. 2003) and tobacco plants expressing CuZnSOD, APX and DHAR (Lee et al. 2007). Further, the higher antioxidant enzyme activity of GPx, DHAR, APx and DHAR in the *GalUR* transgenic potato (Table 1) may give higher stress tolerance to transgenics by degrading ROS which could lead to damaging cell membrane. GR catalyzes the reduction of GSSG to GSH in a NADPH-dependent reaction (Fig. 1) and its higher activity in transgenic potato could result in higher GSH:GSSG ratio in transgenics. These data suggest that the *GalUR* overexpressing transgenic plants respond in a positive manner to high NaCl stress, adjusting glutathione ratios to higher values, while UT control plants were unable to do so thereby indicating that this key antioxidant remain in the

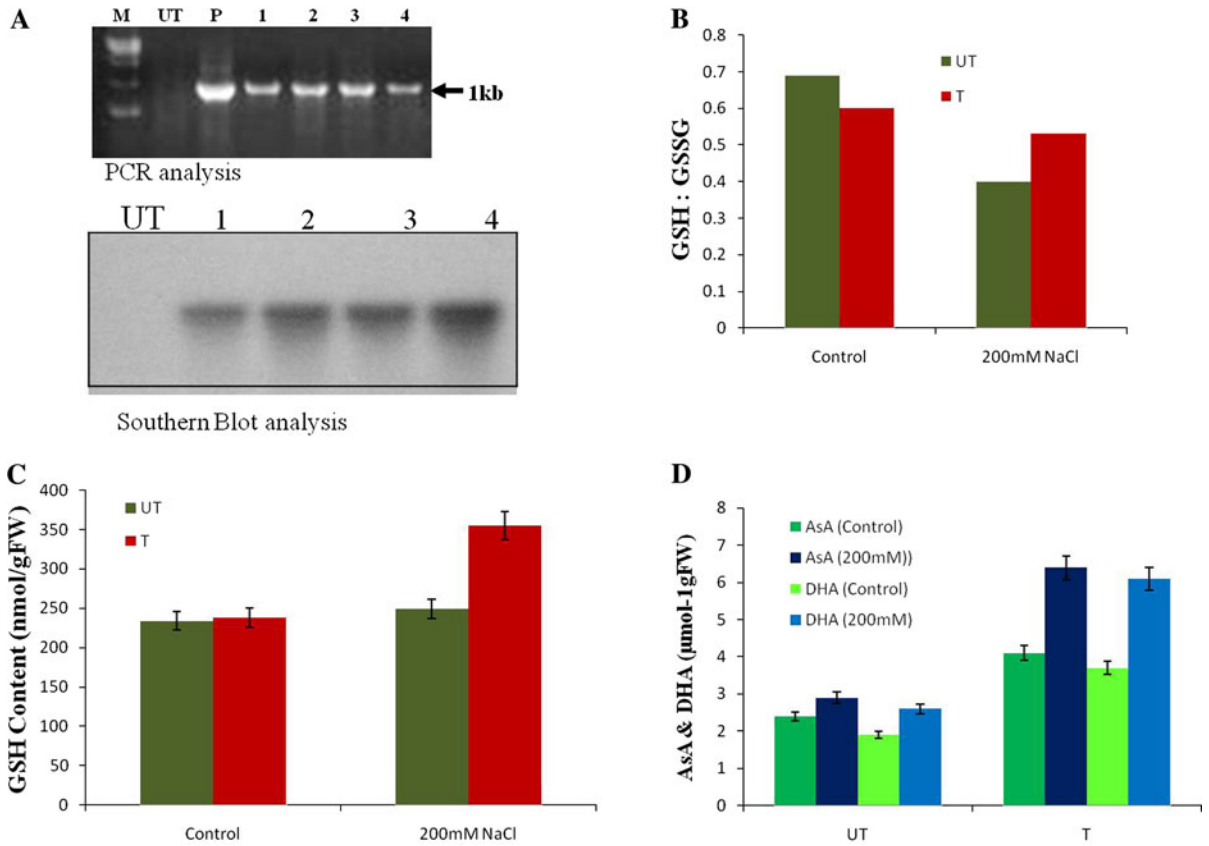

Fig. 2 **a** The transgenics were screened by PCR and Southern blot to confirm the integration of transgenics. **b** Effect of salt stress on GSH:GSSG ratio and **c** GSH level in UT and transgenic potato (including leaf and tuber) growing under stressed and non-stress conditions. A significant increase in the redox state of ascorbate. **d** Histogram showing the increase in redox state of the Asa and DHA in the UT and transgenic plants growing under stressed (200 mM NaCl) and non-stressed conditions. Data represent the mean of three replicates and of three independent experiments ±SD

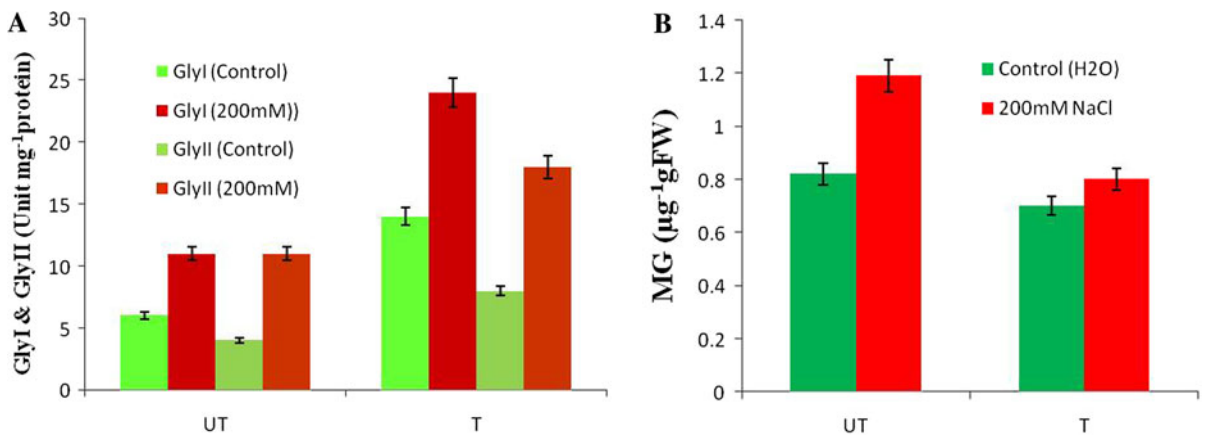

Fig. 3 Histograms showing **a** the activity of glyoxalase I and glyoxalase II enzymes and **b** relative MG levels in the transgenic and control potato exposed to 200 mM NaCl for 72 h. Data represent the mean of three replicates and of three independent experiments ±SD

highly reduced state compatible with continued cell function.

Transgenic potato exhibit higher glyoxalase activity and lower MG level under salinity stress

We determined the activity glyoxalase pathway enzymes, which also utilize GSH for their activity, under non-stress and stress (200 mM NaCl, 3 weeks) conditions in different samples of the UT and transgenic plants. Salt stress increased the activity of Gly I by 170–190% while Gly II by 160–175% in transgenics samples while these activity increases up to 55% in UT control plants (Fig. 3a). However, the basal level of activity of these enzymes was higher in transgenic plants (leaves and tubers) as compared to the UT control, ranging from 40 to 60% for Gly I, 35 to 40% for Gly II (Table 1). This increase in activity of the glyoxalase enzymes is due to increase in GSH:GSSG ratio in the transgenic potato as higher GSH is prerequisite for the higher activity of these enzymes (Yadav et al. 2005a, b).

MG is cytotoxic and its level increases during stress condition in animals, yeast (*Saccharomyces cerevisiae*), bacterial and plant systems (Singla-Pareekh et al. 2006). Since MG is the primary substrate of glyoxalase pathway and its levels increases in plants in response to various stresses, MG was quantified in the UT and transgenic potato growing unstressed and stressed (200 mM NaCl) conditions. In unstressed condition, MG level was almost similar in both UT and transgenics. However, under stressed condition, MG level increases twice in UT in comparison to that of the transgenics (Fig. 3b). However, a minimal level of MG is retained in the system under non stressed conditions seems to be important for normal development. These results indicate that higher activities of glyoxalase together with maintained higher ratio of GSH:GSSG in transgenics under NaCl stress, could detoxify MG beyond a definite level thus preventing its accumulation to higher toxic level in presence of salt.

The reduction of intracellular MG by overexpression of glyoxalase pathway enzymes (Yadav et al. 2005b) and aldose/aldehyde reductase (Oberschall et al. 2000) in transgenic tobacco leads to enhanced stress tolerance. The increase in concentration of MG reported in UT control plants could be due to the upregulation of enzymes of glycolysis and TCA cycle (Umeda et al. 1994). Under stress there may be increase in TCA and glycolysis cycle (Scaife 1969) and the increase in flux of triose phosphates could be converted to MG instead of giving only pyruvate. Importance of GSH in stress tolerance has been reported earlier, however the role of ascorbate pathway gene in maintaining GSH homeostasis along with higher glyoxalase activity has not been addressed in crop plants. Present work is the first study to illustrate the role of ascorbate pathway enzyme in maintaining the higher glyoxalase activity with higher GSH levels resulting in decrease in MG under salt stress.

Modulation of salinity tolerance by exogenous application of sulphydryl groups

Higher GSH:GSSG ratio also suggested that glutathione level maintenance is directly related to salt stress tolerance in the *GalUR* overexpressing transgenic potato. To further strengthen the role of glutathione, its level was modified via exogenous application of glutathione in MS basal medium with UT and transgenics. Here, growth and tuber induction of UT control and transgenics were compared under unstressed condition or 200 mM NaCl with GSH. When GSH was applied exogenously along with salt, not only transgenics but UT grew and develop tubers. Effect in terms of plants growth (Fig. 4a, c) and tuber formation (Fig. 4b) was even better at 10 mM as compared to 5 mM GSH (data not shown). Restoration in the growth of UT plants and tuber formation in the presence of supplemented GSH under salt stress indicated that a higher GSH:GSSG ratio might have been achieved by UT plants in the presence of exogenous GSH which usually maintain a lower GSH:GSSG ratio under stress. Similarly, the transgenic tobacco overexpressing the glyoxalase pathway enzymes (Yadav et al. 2005b, c; Singla-Pareek et al. 2006) resulted in higher levels of reduced glutathione. Gly I utilizes GSH to convert methylglyoxal (MG) into its thioester whereas the Gly II hydrolyzes this thioester to regenerate GSH (Singla-Pareekh et al. 2001). Since the glyoxalase I use GSH as a cofactor, the maintenance of higher level of GSH is important at cellular level. Further, the GSH is also used as cofactor in several antioxidant-removing

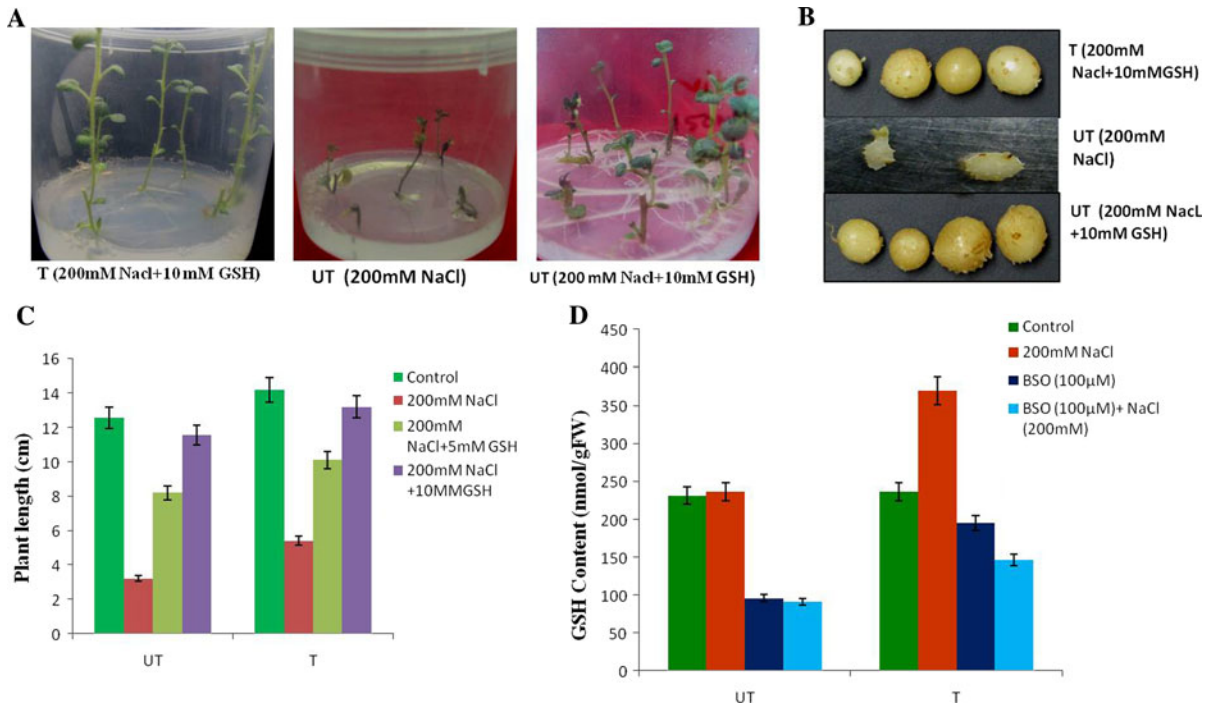

Fig. 4 Growth (**a**) and tuberization pattern (**b**) of UT and transgenic plants on the MS medium supplemented with GSH and (**c**) the histogram showing the growth pattern of the shoots in stressed medium with exogenously supplemented GSH. **d** Quantification of internal GSH after exogenous application of BSO in presence or absence of the salt in transgenic and UT. The values shown are the mean height of four nodal segments and of three independent experiments ±SE

enzymes which are produced under stress conditions; hence maintenance of higher level of GSH is prerequisite for the cell in order to survive under stress conditions.

Several antioxidant enzymes and also the glyoxalase system depend on GSH availability in the cytosol. Intracellular GSH maintenance is important in order to survive the stress condition. Thus, it was crucial to investigate the intracellular GSH biosynthesis in the transgenics. For this, the de novo synthesis of GSH was inhibited by transferring the in vitro maintained UT and transgenic plants to the medium containing buthionine sulfoximine (BSO) that inhibit γ-glutamylcysteine synthetase (Meister 1988) which is the first and rate-limiting enzyme of the GSH biosynthetic pathway. Growth of UT plants was severely inhibited while the *GalUR* transgenics showed almost normal growth (Fig. 4d) in the presence of BSO alone. This suggests that sufficient GSH is necessary to the system either through proficient recycling of GSH due to elevated glyoxalase activity or maintaining higher activity of ROS modulating enzyme via APX, GR, GPX and DHAR activity which ensure the maintenance of the GSH in the stress conditions. Similar results were also obtained with the overexpression of glyoxalase pathway enzyme which utilize and recycle the intracellular GSH (Yadav et al. 2005a, b). The de novo synthesis of glutathione has been correlated with high salt tolerance in transgenic tomato (Meister 1988).

Thus, an increase in antioxidative and glyoxalase pathway enzymes, together with maintenance of higher GSH:GSSG ratio, prevents MG accumulation which resulted in enhanced salinity tolerance in transgenic potato with higher ascorbic acid obtained from *GalUR* overexpression. In UT, salt stress increased the level of MG and decreased the GSH:GSSG ratio. Therefore, under salinity stress, antioxidative enzymes and the glyoxalase pathway enzymes might not be getting sufficient GSH for their action in UT plants. The transgenics maintained

higher GSH levels proving a major factor in conferring salinity tolerance. While, role of GSH has been shown in many other studies, its regulation via ascorbate pathway enzymes and its ameliorating role during salinity stress tolerance has not been shown previously.

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