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An Induced Glutathione-deficient Mutant in Grass Pea (*Lathyrus sativus* L.): Modifications in Plant Morphology, Alterations in Antioxidant Activities and Increased Sensitivity to Cadmium

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ABSTRACT

A glutathione-deficient mutant, designated as *gshL-1*, was isolated in 350 Gy gamma ray treated M_2 progeny of grass pea (*Lathyrus sativus* L.) var. 'BioL-212'. Its fitness was tested in control (un-stressed) and in four different concentrations (5, 10, 25 and 50 µM) of $CdCl_2$. The mutant, containing only 15% of total glutathione in leaves and 20% of that in roots of mother control (MC) variety, exhibited slow growth, poor root development and significant reduction in biomass production, in comparison to MC plants. The impact was more severe in Cd-treated plants. The huge deficiency in total and reduced glutathione (GSH) content was mainly due to significantly low level of glutathione reductase activity, triggering a massive disruption in antioxidant defense systems in both leaves and roots of the mutant. Interestingly, glutathione biosynthesis was not affected in the mutant. Within ascorbate-GSH cycle, low GSH availability presumably impeded the normal functioning of dehydroascorbate reductase (DHAR), badly hampering the regeneration of reduced ascorbate in the mutant. The cascading effect of low GSH pool was also felt in scavenging of hydrogen peroxide due to below normal activity of ascorbate peroxidase and glutathione peroxidase, although catalase level was significantly high in both untreated and Cd-treated mutant plants. Furthermore, nearly 7-fold decrease in activity of GSH-S-transferases (GSTs) and accumulation of total phytochelatins only 10% of MC plants might led to increased sensitivity of the mutant plant to the imposed Cd stress. The results indicated potentiality of the induced mutagenesis in creation of novel mutants to explore the regulation of antioxidant redox homeostasis, mechanism of reactive oxygen species scavenging and tolerance to metal toxicity in grass pea.

Keywords: ascorbate-glutathione cycle, catalase, H_2O_2, phytochelatins, redox state

Abbreviations: ABA, abscisic acid; **Cd**, cadmium; **EMS**, ethyl methane sulfonate; **FAD**, flavin adenine dinucleotide; **GSH**, reduced glutathione; ***gshL-1***, glutathione-deficient *Lathyrus* type-1 mutant; **GSSG**, glutathione disulfide or oxidized glutathione; **H_2O_2**, hydrogen peroxide; **PC**, phytochelatin; **ROS**, reactive oxygen species

INTRODUCTION

Mutation is defined as a change in the heritable constitution of an organism. It is an important source of genetic variation and one of the major driving forces in evolution of living organisms. Mutation occurs naturally at very low frequencies. However, it can be induced at much higher frequencies using physical, chemical and biological mutagens (Kharkwal and Shu 2009). The use of induced mutation in crop improvement has proven to be an effective approach to improve yield, quality and resistance to biotic and abiotic stresses (Maluszynski *et al*. 1995; Nichterlain *et al*. 2000; Tiryaki and Staswick 2002). There are examples of successful mutagenic induction of tolerance to abiotic stresses, such as salt tolerance in *Hordeum vulgare* cv. 'Golden Promise' (Forster 2001), *Oryza sativa* cv. 'Nipponbare' (Thang *et al*. 2010), *Chrysanthemum morifolium* cv. 'Maghi Yellow' (Hossain *et al*. 2006), *Musa acuminata* cv. 'Dwarf Cavendish' (Miri *et al*. 2009) or drought and cold tolerance in rice (Maluszynski *et al*. 1995; Thang *et al*. 2010), and aluminium tolerance in *Hordeum vulgare* (Nawrot *et al*. 2001) and in *Pisum sativum* 'bronze' mutants (Guinel and LaRue 1993). As the crop plants including legumes experience increasing stress in different perspectives, a broader range of variability can give wider scope of selection for their tolerance to stress. Induced mutagenesis has provided a clean, flexible, workable, non-hazardous and low-cost alternative to genetically modified organisms (GMOs) for crop improvement (Jain 2010).

Grass pea (*Lathyrus sativus* L.), the only cultivated member under the genus *Lathyrus*, is an annual leguminous crop used for human consumption and animal feedstock, extensively in Indian subcontinent, Australia, the Mediterranean regions and to a lesser extent in South America, China and parts of Europe (Biswas 2007). This crop is known for its remarkable hardiness in low input and marginal environments and tolerance to biotic and abiotic stresses (Vaz Patto *et al*. 2006; Biswas 2007). Due to low genetic variability and inter-specific incompatibility in natural population, technique of mutation breeding was adopted as a valuable supplement to conventional plant breeding for creation of additional genetic variations in grass pea (Rybinski 2003; Talukdar 2009a). In recent times, a good number of desirable morphological mutations has been isolated and characterized in induced mutant population of this crop (Talukdar 2009b, 2011e). The power of mutagenic treatment was also manifested in induction of various structural and numerical aberrations and polyploidy in grass pea. A robust cytological stock comprising of trisomics, tetrasomics, double trisomics, various reciprocal translocations and autotetraploid lines has recently been developed in this crop (Talukdar 2009c, 2010a, 2010b). This mutant stock has been successfully used to characterize and map the desirable morphological and isozyme markers on specific chromosomes of grass pea (Talukdar 2009a, 2010c, 2011b, 2011d). Very recently, an ascorbate-deficient and two flavonoid-

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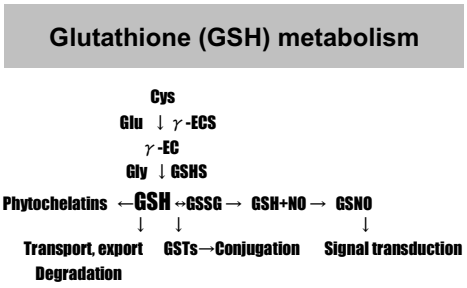

Fig. 1 Glutathione (GSH) biosynthetic pathway and possible routes of its utilization in higher plants. γ-ECS - γ–glutamylcysteine synthetase, GSHS - Glutathione synthetase.

deficient mutants have been detected in gamma ray and EMS-mutagenized population of grass pea, respectively, and the latter has already been mapped on grass pea chromosomes along with isozyme loci related to antioxidant metabolites, using primary trisomics (Talukdar 2012a, 2012b).

The heavy metal cadmium (Cd) is readily taken up by plant species and interferes with cellular metabolic processes, resulting in growth inhibition and low biomass accumulation (Dixit *et al*. 2001; Sandalio *et al*. 2001). Cd induces oxidative stress by generating free radicals and reactive oxygen species (ROS). These species unbalances the cellular redox system in favour of oxidised form, resulting in oxidative damage to lipids, proteins, pigments, and nucleic acids. Plants possess well-developed antioxidant defence systems, comprising of several enzymes and low molecular weight molecules. Mutational approach offers a powerful tool to study the genetic, physiological and molecular mechanisms protecting plants against metal toxicity (Tsyganov *et al*. 2007). Mutant isolation is one of the effective ways to isolate and clone the target genes and analyze their functions (Thang *et al*. 2010). Surprisingly, mutant selections regarding increased tolerance and accumulation have rarely been performed and described to date, presumably because only few mutants showing altered heavy metal tolerance could be obtained. In the model plant, *Arabidopsis*, cadmium-sensitive mutants *cad1* and *cad2* (Howden and Cobbett 1992; Howden *et al*. 1995; Cobbett *et al*. 1998), copper-sensitive mutant *cup1-1* (van Vliet *et al*. 1995) and Mn, Cu and Zn over-accumulating *man1* mutant (Delhaize 1996) were developed through induced mutagenesis. In pea, increased tolerance and accumulation of Cd were induced in an ethyl methane sulfonate (EMS)-treated mutant (Tsyganov *et al*. 2007). These mutants have been used extensively to reveal underlying genetic and physiological mechanisms of heavy metal homeostasis and tolerance in plants.

Glutathione (reduced form: GSH; oxidized form: GSSG), the tripeptide γ-glutamylcysteinylglycine, is the major source of non-protein thiols in most plant cells and plays pivotal roles in plant defense against ROS-induced oxidative stress, presence of xenobiotics and heavy metal toxicity (Bergmann and Rennenberg 1993). Its role in germination of pollen grains and gametophyte development in higher plants has recently been elucidated (Zechmann 2011). In plants, GSH is synthesized enzymatically from its constituent amino acids via a two-step ATP-dependent pathway (Alscher 1989; Hell and Bergmann 1990). The first step, catalyzed by γ-glutamylcysteine synthetase (γ-ECS), forms γ-glutamylcysteine and the second, catalyzed by GSH synthetase (GSHS), produces GSH through the addition of glycine residue (**Fig. 1**). Among the ROS-scavenging machinery, ascorbate (ASC)-glutathione (GSH) cycle plays a pivotal role in plant defense, where GSH plays a central role in regeneration of reduced ascorbate from its oxidized form dehydroascorbate (DHA) through the reactions catalyzed by dehydroascorbate reductase (DHAR). The resultant glutathione disulfide or GSSG is then reduced back to GSH by NADPH-dependent action of glutathione reductase (GR). This homodimeric flavoprotein is extremely important in maintaining GSH:GSSG ratios within the cell. The balance in favor of reduced thiol group makes this redox couple ideally suited to information transduction (Noctor *et al*. 2002). The GSH/GSH+GSSG ratio is likely to be far more influential in the antioxidant defense, control of gene expression and thionylation process of numerous components than the absolute size of the glutathione pool (Noctor *et al*. 2002). Using the reduced ascorbate as a co-factor, ascorbate peroxidase (APX) reduces H_2O_2 to H_2O. Besides the APX in ASC-GSH cycle, catalases (CAT) which are exclusively located in peroxisomes also degrades photorespiratory H_2O_2 to H_2O, but without consuming any type of reducing power. The H_2O_2 is a predominant oxidant within the cell and is continuously generated in aerobic cells by photorespiration, activity of superoxide dismutase (SOD) and other reasons. GSH in reduced form protects many thiol containing enzymes from being oxidized by maintaining high redox state (Foyer and Noctor 2011). It is also the precursor of phytochelatins (PCs) which binds heavy metals like Cd through chelation and the PC-metal complex is sequestered in the vacuoles (Rauser 1995). The PC synthase (γ-Glu-Cys dipeptidyl transpeptidase) catalyzes the synthesis of PCs by transferring a γ-Glu-Cys moiety of GSH to GSH or to other PCs (Zenk 1996). The conjugation of GSH to heavy metals is accomplished by multifunctional enzymes glutathione *S*-tranferases (GSTs) (Marrs 1996). Glutathione peroxidases (GPXs) are also involved in removal of organic peroxides and H_2O_2 using GSH or thioredoxins as electron donors (Noctor *et al*. 2006). Transgenic tobacco overexpressing GST/GPX reportedly exhibited enhanced antioxidant capacity under stress (Roxas *et al*. 1997).

The power of mutant analysis in elucidation of role of GSH as a redox component in growth and development of plants has been demonstrated in *Arabidopsis rml1* mutant showing inhibition of root growth due to almost absence (nearly 3% of wild type) of GSH (Vernoux *et al*. 2000). Its importance in heavy metal tolerance was well characterized in *cad1* and *cad2* mutants of *Arabidopsis*, and the latter was used to reveal the interplay between thioredoxin and GSH systems in *Arabidopsis* auxin signaling (Bashandy *et al*. 2010). The cadmium sensitivity of these two mutants was mainly due to deficiency in PC synthase (Howden and Cobbett 1992; Howden *et al*. 1995) and γ-glutamylcysteine synthetase (Cobbett *et al*. 1998) activity, respectively. The Cd tolerant pea mutants exhibited elevated concentration of GSH in the roots. In grass pea, transcripts of several antioxidant enzymes were analysed in lead-induced oxidative stress (Brunet *et al*. 2009), while morpho-physiological variations were studied in the plants submitted to arsenic (Talukdar 2011c). The compensatory role of GSH in antioxidant defense of this crop has recently been elucidated in an induced mutant line, exhibiting huge deficiency (58-81%) in cellular ascorbate level (Talukdar 2012b). As part of a broader strategy to develop a mutant stock useful in understanding the stress tolerance mechanism, mutation was induced through different doses of gamma rays in the present author's laboratory during 2008-09. On the basis of preliminary observations, two plants showing weak growth and rolled leaflets were isolated in 350 Gy gamma ray treated M_2 progeny of improved grass pea (*Lathyrus sativus* L.) var. 'BioL-212'. Although initially it seemed to be an aneuploid type, later it was found to be diploid but deficient in glutathione content in roots and leaves. Detail investigation was carried out at M_3 generation using both unstressed control and cadmium-induced stress conditions. The objectives of the present work were to study 1) the morphology of the mutant, 2) the cause/s of glutathione deficiency, 3) its effect on antioxidant defense systems within ascorbate-glutathione cycle and outside it, and 4) alterations in thiol containing enzymatic defense, such as GSTs, GPXs and PC synthase in control and in response to cadmium treatments. The comparative response of GSH-deficient mutant in the two different environments could help in the global understanding of GSH-mediated cellular events and its effects on growth and development of grass pea plants.

MATERIALS AND METHODS

Induced mutagenesis and detection of glutathione-deficient mutant in field

Fresh and healthy seeds of an improved grass pea cv. 'BioL-212' were irradiated with different doses (50, 100, 150, 200, 250, 300, 350 and 400 Gy) of gamma rays to induce mutation. This is an improved cultivar with low seed neurotoxin (<0.1%) content, and exhibited very high tolerance to salinity stress (Talukdar 2011g). Therefore, it was selected for the present study. A total of 800 treated seeds were sown to raise the M_1 generation in separate plots (treatment-wise) with 30 and 50 cm uniform distances between plants and rows, respectively. Seeds of individual M_1 plants in all the treatments along with control were separately harvested and sown in different rows in a randomised block design to raise M_2 progenies. After careful examination, two plants were detected in 350 Gy-irradiated populations by their characteristic weak growth and rolled leaflets (**Fig. 2**). These two 'variant' plants were subjected to rigorous screening in subsequent selfed-generation, and found to be self-fertile with normal diploid (2n=2x=14) chromosome complement, but highly deficient in total extractable glutathione content in leaves and roots. After necessary confirmation of its GSH levels at M_3 generation, the progeny of these two 'variant' plants were designated as *gshL-1* (glutathione-deficient *Lathyrus* type-1) mutant.

Fig. 2 The *gshL-1* mutant with its mother plant at early seedling stage. (**A**) Reduced height. (**B**) Characteristic rolled leaflets (→) of the mutant.

Culture conditions and Cd treatment

Grass pea seeds obtained from M_3 plants of *gshL-1* mutant and its mother variety 'BioL-212' were allowed to germinate at dark in two separate sets on moistened filter paper at 25°C. Germinated seedlings were randomly placed in polythene pots (10 plants pot^{-1}) containing 300 ml of Hoagland's No 2 nutrient media (Hoagland and Arnon 1938) and were allowed to grow for 14 d. The media were, then, supplemented with five different concentrations (0, 5, 10, 25 and 50 µM) of $CdCl_2$ and were allowed to grow for another 14 d. Six replicates for each treatment were prepared to give a total of 30 pots. Nutrient solution was refreshed in every alternate day to prevent depletion of nutrient as well as Cd in the course of the plant's exposure to the metal. Seedlings and plants were placed in a growing chamber with a 14-h photoperiod, at 25°C, humidity of 70% and a photon flux density of 200 µmol m^{-2} s^{-1}. Mutant plants submitted to unstressed (0 µM $CdCl_2$) and Cd-treated conditions were used as mutant control (MuC) and negative control (NC), respectively, whereas mother variety was regarded as mother control (MC) for 0 µM $CdCl_2$ and treated mother (TM) for rest of the treatment regimes (5, 10, 25 and 50 µM $CdCl_2$).

Determination of dry mass and Cd content

Plants were harvested at 30 d growth period. Shoots and roots were separated carefully and rinsed thoroughly with sterile distilled water. Lengths of seedling and root were measured. Data given were the average of at least six independent experiments ± standard error (SE). Plant samples were, then, oven dried at 65 °C for 72 h for determination of dry mass or immediately frozen in liquid nitrogen, and stored at -80 °C for future analysis. The roots and leaves were digested in a HNO_3-$HClO_4$ (3: 1, v/v) mixture and Cd concentration was determined using an atomic absorption spectrophotometer (Perkin-Elmer, Analyst 300).

Estimation of thiols and thiol synthetase assays

Non-protein thiols (NPT) were extracted by homogenizing frozen plant material in 10% sulfosalicylic acid solution. The homogenate was centrifuged for 5 min at 13,000 × *g* at 4°C, and the supernatants were immediately assayed for sulphydryl groups and GSH. The NPT content was measured spectrophotometrically with Ellman's reagent: 0.25 cm^3 of acid soluble supernatant was neutralized with 0.5 cm^3 of Na-phosphate buffer (143 mM, pH = 7.5), 0.02 cm^3 of 5,5'dithiobis[2-nitrobenzoic acid] (0.6 mM) (Sigma-Aldrich, St. Louis, MI, USA) was then added to the solution, followed by the measurement at A_{412}. Total glutathione and oxidized glutathione (GSSG) were estimated following the method of Anderson (1985). The assay was based on sequential oxidation of glutathione by 5,5'dithiobis[2-nitrobenzoic acid] and reduced by NADPH-dependent glutathione reductase (GR). To quantify GSSG content, 2-vinylpyridine (Sigma-Aldrich, St. Louis, MI, USA) was added to the extract in order to eliminate the GSH. After 1 h of incubation in 25°C, diethylether was added to remove the 2-vinylpyridine from the extract. Total phytochelatins (PCs) content was calculated by subtracting the amount of GSH from the total amount of NPT following the principles of De Vos *et al*. (1992).

The activity of γ-glutamylcysteine synthetase (γ-ECS; EC 6.3.2.2) was measured according to the method of Creissen *et al*. (1999). Briefly, samples (50 mg) were ground in a mortar in 0.1M Tris-HCl, pH = 7.5, 5 mM EDTA (10 ml g^{-1}) with 1 g g^{-1} PVP-30 and acid-washed sand. The homogenate was centrifuged twice at 15,000 × *g* for 15 min. The γ-ECS assay was performed in a 500 µL reaction mixture containing 0.1 M Tris-HCl, pH 7.5, 0.1 M $MgCl_2$, 10 mM ATP, 50 mM Na-L-glutamate, 8 mM phosphocreatine, 2 U of creatine phosphokinase, and 200 µL of cleared supernatant. The mixture was incubated under anaerobic conditions at 37°C for 10 min. before the reaction was commenced with the addition of 50 µL of 20 mM L-cysteine. Samples were collected at 10-min intervals, the reaction was stopped, and the products were derivatized with monobromobimane (MBB), separated and quantified by standard HPLC (flow rate 1 ml min^{-1}, excitation = 390 nm, emission = 482 nm, three times per sample) method. γ-ECS activity was expressed as nmol of γ-EC formed min^{-1} g^{-1} fresh weight.

Activity of GSH synthetase (GSHS) was assayed by HPLC quantification of the synthesized GSH as their MBB derivatives, following the methods of Matamoros *et al*. (1999). The reaction mixture contained 125 mM Tris-HCl (pH = 8.5), 50 mM KCl, 25 mM $MgCl_2$, 5 mM ATP, 5 mM PEP, 5 U of pyruvate kinase, 5 mM DTE, 0.5 mM γ-EC, and 5 mM glycine in a total volume of 100 µL. After preincubation at 30°C for 3 min, the reaction was initiated by adding 100 µL of samples and stopped by derivatization of aliquots. GSHS activity was expressed as nmol min^{-1} g^{-1} fresh weight.

For PC synthase (EC 2.3.2.15) assay, approximately 2 g of seedling tissue was frozen in liquid N, and then ground in a chilled mortar in extraction buffer (0.05 M Tris-HC1, pH 8.01, 0.01 M β-mercaptoethanol, 10% [v/v] glycerol; 1 mL of buffer per g of tissue). The homogenate was centrifuged at 10,000 × *g* for 13 min and the supernatant was used to assay for PC synthase, according to the protocol of Howden *et al*. (1995).

Assay of antioxidant enzyme activities

The mature fully expanded leaves (grown on primary branches) and roots were collected from MuC, MC, NC and TM plants for biochemical studies. A pool of samples from six plants for each line was collected, and three independent experiments were performed. All operations were performed at 0-4 °C, except mentioned otherwise. Samples (1g) were ground with a mortar and pestle and homogenized in an extraction medium containing 50

mM K-phosphate buffer pH 7.8, 0.1 mM EDTA, 2 mM cysteine, 1% w/v PVP-10 and 0.2% v/v Triton X-100. All the chemicals and reagents were purchased from Sigma-Aldrich, St. Louis, MI, USA.

For the APX (EC1.11.1.11) activity, 20 mM ascorbate was added to the extraction buffer. The extracts were filtered through two layers of cheesecloth, and the homogenate was centrifuged at 14,000 × *g* for 20 min, at 4°C. The supernatant fraction was filtered through a column containing 1 mL of Sephadex G-50 equilibrated with the same buffer used in homogenization. The hydrogen peroxide-dependent oxidation of ascorbate was followed by a decrease in the absorbance at 290 nm with extinction constant 2.8 mM^{-1} cm^{-1} following the method of Nakano and Asada (1981).

SOD (EC 1.15.1.1) activity was determined by the nitro-blue tetrazolium photochemical assay method as described by Beyer and Fridovich (1987). The reaction mixture (3 ml) contains 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 75 µM NBT, 0.1 mM EDTA, 2 µM riboflavin and 0.1 ml of enzyme extract. One unit of SOD was defined as the amount of protein causing a 50% NBT photoreduction.

DHAR (EC 1.8.51) was extracted with 50 mM K- phosphate buffer (pH 7.8), 1% PVP-10, 0.2 mM EDTA and 10 mM β-mercaptoethanol. DHAR activity was determined by following ascorbate formation at 265 nm ($\varepsilon = 14.1$ mM^{-1} cm^{-1}) for 3 min (Nakano and Asada 1981).

GR (EC 1.6.4.2) was extracted with the same medium as for DHAR but without β-mercaptoethanol and with 0.1% Triton X-100, and its activity was measured by monitoring glutathione-dependent oxidation of NADPH at 340 nm ($\varepsilon = 6.22$ mM^{-1} cm^{-1}) for 3 min (Dalton *et al*. 1986).

CAT (EC 1.11.1.6) was extracted in 50 mM K-phosphate buffer (pH 7.0) and 0.5% PVP-10, and its activity was assayed by measuring the reduction of H_2O_2 at 240 nm ($\varepsilon = 39.4$ M^{-1} cm^{-1}) for 1 min (Aebi 1984).

For the estimation of GST (EC 2.5.1.18) activity, 1 g plant samples were extracted in 5 ml medium containing 50 mM phosphate buffer, pH 7.5, 1 mM EDTA and 1 mM DTT. The enzyme activity was assayed in a reaction mixture containing 50 mM phosphate buffer, pH 7.5, 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) and the eluate equivalent to 100 µg of protein. The reaction was initiated by the addition of 1 mM GSH (Sigma-Aldrich, St. Louis, MI, USA) and formation of S- (2,4-dinitrophenyl) glutathione (DNP-GS) was monitored as an increase in absorbance at 334 nm to calculate the GST specific activity (Li *et al*. 1995).

Total GPX (EC 1.15.1.1) activity was determined from 1 g plant tissues extracted in 3 ml of 0.1 M Tris-HCl, pH 7.5, containing 2 mM DTT and 1 mM EDTA. The enzyme activity was ascertained by using cumene hydroperoxide (both selenium and non-selenium enzyme types) as a substrate and GSH-reductase (GR) coupled assay to monitor the oxidation of GSH (Edwards 1996). The reaction mixture consisted of 0.1 M phosphate buffer, pH 7.0, containing 0.2% (w/v) Triton X-100, 0.24 unit of GR, 1 mM GSH, 0.15 mM NADPH, and 1 mM cumene hydroperoxide. After addition of enzyme eluate, cuvettes were incubated at 30°C for 10 min, and NADPH was added to measure the basal rate of GSH oxidation by monitoring the absorbance at 340 nm for 3 min. The reaction was initiated by addition of cumene hydroperoxide, and GPX activity was expressed as change in absorbance at 340 nm mg^{-1} protein min^{-1}.

Estimation of lipid peroxidation, H_2O_2 levels and electrolyte leakage

Lipid peroxidation was determined by measuring the malondialdehyde (MDA) content at 532 nm with extinction coefficient of 155 mM^{-1} cm^{-1} (Saher *et al*. 2004). Hydrogen peroxide (H_2O_2) content of leaves and roots was measured following the methodology described by Cheeseman (2006). Tissue samples were homogenized in the extraction medium 0.1 M K-phosphate (pH 6.4) supplemented with 5 mM KCN. The assay mixture contained 250 µM ferrous ammonium sulphate, 100 µM sorbitol, 100 µM xylenol orange, and 1% ethanol in 25 mM H_2SO_4. Changes in absorbance were determined by the difference in absorbance between 550 nm and 800 nm, and H_2O_2 contents were calculated from a standard curve. Membrane electrolyte leakage (EL) was assayed by measuring the ions leaching from tissues into deionised water. The EL was expressed as a percentage by the formula EL (%) = $(EC_1)/(EC_2) \times 100$, where EC_1 is the initial electrical conductivity and EC_2 represents its final value (Dionisio-Sese and Tobita 1998).

Statistical analysis

All values presented here are means (± SE) of at least six independent experiments. Significance ($P < 0.05$) of differences between control and each treatment was determined by student *t*-test (two-tailed) using Microsoft Excel software 'data analysis' 2007.

RESULTS

Plant growth and cadmium (Cd) accumulation by mother variety and *gshL-1* plants

The *gshL-1* mutant, isolated in 350 Gy gamma ray irradiated progeny in the improved grass pea variety 'BioL-212', exhibited slow growth, inward rolling of leaflets, reduced seedling length, absence of stipule, very short tendril, and reduced number of leaflets $leaf^{-1}$ (**Fig. 2**) and produced a significant growth inhibition measured as dry weight. Shoot dry weight of 30 d old mutant plants reduced by about 1.5-times in comparison to its mother variety in control (MC) condition (**Table 1**). Root growth was also significantly inhibited in *gshL-1* plants in control (MuC) condition (**Table 1**). Root length in the mutant decreased to 2.4-fold of MC

Table 1 Growth parameters and Cd concentration in 30d-old mother control (MC) variety 'BioL-212' and *gshL-1* mutant plants of grass pea (*Lathyrus sativus* L.) in control (0 µM $CdCl_2$) and four $CdCl_2$-treatment (5, 10, 25 and 50 µM). Data are means ± SE of six independent experiments. Asterisk (*) denotes significant difference from respective control value at $P < 0.05$.

Parameters[a]	$CdCl_2$ concentration (µM)				
	0 (control)	5	10	25	50
Total seedling length (cm)					
Variety	30.3 ± 0.09	31.0 ± 0.11	28.8 ± 0.12	27.6 ± 0.03	19.7 ± 0.17*
Mutant	19.5 ± 0.04	19.1 ± 0.09	10.0 ± 0.11*	7.6 ± 0.05*	2.5 ± 0.09*
Shoot dry weight (g $plant^{-1}$)					
Variety	0.095 ± 0.21	0.095 ± 0.27	0.090 ± 0.19	0.091 ± 0.20	0.046 ± 0.22*
Mutant	0.063 ± 0.19	0.063 ± 0.19	0.029 ± 0.21*	0.019 ± 0.19*	0.013 ± 0.29*
Root length (cm)					
Variety	12.4 ± 0.9	13.0 ± 0.7	12.3 ± 0.3	11.9 ± 0.8	7.4 ± 0.9*
Mutant	5.2 ± 0.8	4.9 ± 0.7	3.5 ± 1.0*	2.2 ± 0.6*	1.7 ± 0.7*
Root dry weight (g $plant^{-1}$)					
Variety	0.176 ± 0.11	0.181 ± 0.30	0.191 ± 0.20*	0.195 ± 0.19*	0.161 ± 0.22*
Mutant	0.058 ± 0.17	0.052 ± 0.11	0.029 ± 0.18*	0.013 ± 0.16*	0.006 ± 0.21*
Cd concentration (µg g^{-1} dry weight)					
Variety (leaf)	0.07 ± 0.3	18.10 ± 4.7*	42.09 ± 1.7*	74.10 ± 1.9*	80.11 ± 2.7*
Variety (root)	1.75 ± 0.9	2.19 ± 2.7	4 ± 23*	3.3 ± 37	5.1 ± 58*
Mutant (leaf)	0.03 ± 0.1	0.03 ± 0.2	0.08 ± 0.5*	0.07 ± 0.11*	0.05 ± 0.3
Mutant (root)	1.18 ± 0.10	2.89 ± 1.2	64.4 ± 10*	66 ± 9.7*	59.6 ± 6.3*

plants. This feature was accompanied with reduction in number of lateral roots in MuC plants. In comparison to MC plants, root dry weight of 30 d-old MuC plants reduced by approximately 3.03-fold.

Growth of both shoot and root in mother variety 'BioL-212' and *gshL-1* mutant was also studied in four different (5, 10, 25 and 50 μM) concentrations of $CdCl_2$. Cd caused continuous growth inhibition in *gshL-1* shoot (NC plants), and lowest estimate (0.013 g plant^{-1}) was taken at 50 μM (**Table 1**). Shoot dry weight changed non-significantly throughout the treatment regimes in variety 'BioL-212' except in 50 μM, where it was reduced abruptly 2-fold from control value (**Table 1**). Root length in NC plants decreased significantly ($P < 0.05$) by 1.5-fold of MuC (5.2 cm) in 10 μM $CdCl_2$, and the lowest value (1.7 cm) was recorded in 50 μM. Significant reduction of root length in treated variety 'BioL-212' (TM) was measured only at 50 μM $CdCl_2$ (**Table 1**). Barring 50 μM treatment, lateral root development was not affected in TM plants. Its number started to reduce from 10 μM in NC plants, and no lateral root development was noticed at 50 μM. However, thickening of lateral roots was observed in Cd-treated NC plants. Root dry weight in TM plants increased considerably from MC value at elevated Cd concentrations, but decreased below this level at 50 μM $CdCl_2$. Compared to MuC, root dry weight in the mutant had decreased by about 2-fold in 10 μM, 4.5-fold in 25 μM, and 10-fold in 50 μM of $CdCl_2$ (**Table 1**). No visible symptoms of toxicity, except growth retardation, however, were observed in mother variety and mutant plants under Cd stress.

Cd accumulation in roots and leaves followed different trends in mother variety and *gshL-1* plants under elevated Cd levels. Accumulation was much higher in the roots than in the leaves of the mutant, while completely reverse trend was noticed in its mother plants (**Table 1**). In leaves of mother plants and roots of mutant, Cd level began to increase from 5 μM, showed steep rise at 10 μM and then became stabilized (**Table 1**). By contrast, no concentration-dependent accumulation was observed in *gshL-1* leaves, and the accumulation was also very negligible (**Table 1**).

Thiol contents in *gshL-1* mutant

The thiol pools in *gshL-1* mutant were determined by estimating glutathione and extractable phytochelatin (PCs) contents in leaves and roots of 30-d-old mutant, and it was compared with mother plants (**Tables 2, 3**). In control condition, *gshL-1* mutant (MuC) contained only 15% of total glutathione in leaves and approximately 20% of that in roots of MC. Among the total glutathione pool, reduced glutathione (GSH) constituted about 94% in MC plants, but the share sharply decreased to 37% in both organs of the MuC plants (**Tables 2, 3**). Rest of the amounts was made up of oxidized glutathione (GSSG). The redox state of gluta-

Table 2 Leaf glutathione (GSH and GSSG) contents [nmol g^{-1}(FW)], their redox states, total phytochelatin contents [nmol g^{-1} (FW)] and activities of γ-ECS and GSHS [nmol min^{-1} g^{-1} (FW)] and PC synthase (nmol GSH eq min^{-1} mg^{-1} of protein) enzymes in 30 d-old mother variety 'BioL-212' and *gshL-1* mutant of grass pea (*Lathyrus sativus* L.) in control (0 μM $CdCl_2$) and four $CdCl_2$-treatment (5, 10, 25 and 50 μM). Data are means ± SE of six independent experiments.

Parameters[a]	$CdCl_2$ concentration (μM)				
	0 (control)	5	10	25	50
GSH (A)	297 ± 11.2	293 ± 11.5	282 ± 9.0	287 ± 13.7	157 ± 9.8*
GSH (B)	18.2 ± 1.6	15 ± 1.1	8 ± 1.3*	6 ± 2.0*	4 ± 1.2*
GSSG (A)	18 ± 4.2	20.1 ± 5.0	15 ± 1.2	28 ± 1.9	3 ± 0.9*
GSSG (B)	30.1 ± 3.5	33 ± 2.7	42 ± 3.2*	40 ± 4.0*	26.2 ± 1.6*
GSH redox (A)	0.942 ± 5.2	0.930 ± 5.0	0.949 ± 4.7	0.911 ± 6.5	0.980 ± 6.6
GSH redox (B)	0.370 ± 6.2	0.310 ± 5.0	0.160 ± 3.3*	0.130 ± 2.8*	0.133 ± 1.9*
Total PCs (A)	228.8 ± 26.3	230 ± 15.0	233.2 ± 19.0	239 ± 27.0	492.3 ± 45.5*
Total PCs (B)	22.9 ± 5.9	20.2 ± 5.1	15.9 ± 3.7	ND	ND
γ-ECS (A)	6.8 ± 1.1	6.6 ± 1.1	6.9 ± 1.3	7.7 ± 2.0	6.9 ± 1.2
γ-ECS (B)	6.6 ± 1.3	9.1 ± 1.8	12.9 ± 2.0*	13.5 ± 2.1*	12.2 ± 3.1*
GSHS (A)	10.8 ± 1.2	11 ± 1.1	12 ± 1.7	11.9 ± 1.6	10.8 ± 0.9
GSHS (B)	11.8 ± 1.7	13.6 ± 1.0	16.9 ± 2.0*	16.7 ± 3.1*	16.2 ± 1.1*
PC synthase (A)	3.19 ± 0.9	3.20 ± 1.0	3.22 ± 1.1	3.30 ± 2.0	10.2 ± 3.1
PC synthase (B)	0.45 ± 0.06	0.39 ± 0.1	0.21 ± 0.3*	ND	ND

[a] A-mother plants, B-*gshL-1* mutant, GSH redox-GSH/(GSH+GSSG), PCs-phytochelatin contents, γ-ECS-γ-glutamylcysteine synthetase, GSHS-glutathione synthetase, ND-not detected

* significantly different from respective control value at $P < 0.05$

Table 3 Root glutathione (GSH and GSSG) contents [nmol g^{-1}(FW)], their redox states, total phytochelatin contents [nmol g^{-1} (FW)] and activities of γ-ECS and GSHS [nmol min^{-1}g^{-1} (FW)] and PC synthase (nmol GSH eq min^{-1} mg^{-1} of protein) enzymes in 30d-old mother variety 'BioL-212' and *gshL-1* mutant of grass pea (*Lathyrus sativus* L.) in control (0 μM $CdCl_2$) and four $CdCl_2$-treatment (5, 10, 25 and 50 μM). Data are means ± SE of six independent experiments.

Parameters[a]	$CdCl_2$ concentration (μM)				
	0 (control)	5	10	25	50
GSH (A)	367 ± 15.0	366 ± 10.8	329 ± 8.5	340 ± 23	169 ± 11.3*
GSH (B)	30 ± 3.0	29 ± 3.0	11 ± 2.1*	8 ± 2.3*	6 ± 2.2*
GSSG (A)	33 ± 4.2	30 ± 3.8	31 ± 2.5	30 ± 2.2	7 ± 1.2*
GSSG (B)	50 ± 5.5	51 ± 4.8	64 ± 7.2*	52 ± 3.5*	24 ± 2.3*
GSH redox (A)	0.918 ± 6	0.920 ± 5	0.911 ± 2	0.918 ± 6	0.960 ± 8.2
GSH redox (B)	0.370 ± 6	0.361 ± 5.3	0.140 ± 1.3*	0.133 ± 2.3*	0.200 ± 1.9*
Total PCs (A)	292.8 ± 20	290 ± 25	293.5 ± 33	289 ± 30	519.8 ± 50*
Total PCs (B)	29.7 ± 8	30 ± 5	20.1 ± 2.7*	12.6 ± 1.7*	5.0 ± 1.2*
γ-ECS (A)	7.3 ± 1.1	7.5 ± 1.5	8.1 ± 2.1	9.8 ± 2.2	9.1 ± 3.1
γ-ECS (B)	8.0 ± 1.7	9.8 ± 2.1	16.7 ± 2.9*	17 ± 2.6*	12.8 ± 3.5*
GSHS (A)	11.2 ± 1.4	11.3 ± 1.6	13.4 ± 2.7	12.8 ± 2.0	12.1 ± 1.9
GSHS (B)	12.2 ± 2.0	17.5 ± 2.2	20 ± 2.7*	18.1 ± 3.2*	20 ± 2.1*
PC synthase (A)	2.11 ± 0.6	2.10 ± 0.5	2.13 ± 0.9	2.39 ± 2.7	6.80 ± 4.1
PC synthase (B)	0.31 ± 0.02	0.29 ± 0.1	0.18 ± 0.3*	0.07 ± 0.3*	0.03 ± 0.7*

[a] A-mother plants, B-*gshL-1* mutant, GSH redox-GSH/(GSH+GSSG), PCs-phytochelatin contents, γ-ECS-γ-glutamylcysteine synthetase, GSHS-glutathione synthetase

* significantly different from respective control value at $P < 0.05$

thione, as calculated by GSH/(GSH+GSSG), was estimated at around 0.370 in the mutant, a 2.5-fold decline from MC value under unstressed condition. In both plant types, however, roots accumulated higher amount of GSH and GSSG than in the leaves (**Tables 2, 3**).

Upon imposition of $CdCl_2$ treatment, MC plants showed no significant change in the GSH, GSSG and total glutathione pool up to 25 µM in both tissues. Thereafter, it had reduced sharply, and a decrease by 1.9-2.2-fold for GSH and 5-6-fold in GSSG contents was estimated at 50 µM (**Tables 2, 3**). The redox state, however, remained unchanged. By contrast, a dose-dependent decrease in GSH content was exhibited in *gshL-1* mutant (NC) from 10 µM Cd, and lowest concentration was detected at 50 µM $CdCl_2$ in both organs. Compared with MuC, the reduction was 56-78% in leaf, and was 64-80% in roots of the mutant plants across 5-50 µM Cd concentrations. Like MuC plants, GSSG content remained higher than GSH levels, but as the treatment progressed the total glutathione pool (GSH+GSSG) in NC plants had reduced substantially in relation to MuC plants. The redox state had reduced accordingly (**Tables 2, 3**).

Phytochelatin (PCs) accumulation between mother plants and *gshL-1* mutant varied significantly. MuC plants contained total PCs only 10% of MC plants (**Tables 2, 3**). In both plant types, PCs accumulation was considerably higher in the roots than in the leaves. In the mutant, this level remained unchanged up to 5 µM Cd, but thereafter, it declined abruptly. No PCs were detected in leaves from 25 µM Cd, while it became negligible in roots. PCs content changed marginally when MC plants were submitted to Cd treatment. However, at 50 µM Cd, this content increased by about 2-fold in relation to MC (**Tables 2, 3**).

Ascorbate pool in *gshL-1* and its mother plants

Reduced form of ascorbate content [nmol g^{-1} (fresh weight)] in leaves (1998 ± 42.7) and roots (155 ± 10) of MuC plants was very close to MC value (2012 ± 45 in leaves, 161.8 ± 23 in roots), but decreased abruptly by about 2-fold in the mutant at 10 µM $CdCl_2$ and lowest value (330 ± 15 in leaves, 80.8 ± 10 in roots) was recorded at 50 µM Cd. Considering both leaves and roots, dehydroascorbate or DHA content [nmol g^{-1} (fr. wt.)] ranged between 123.5-173.3 in MC and between 148-151.5 in MuC plants. DHA content increased over MuC by about 1.2-3.5-fold in the Cd-treated mutant, and highest level (227-239) was recorded at 50 µM Cd (not in table).

Thiol synthesis enzyme activities

The mother plants exhibited non-significant changes in activities of both γ-glutamylcysteine synthetase (γ-ECS) and GSH synthetase (GSHS) even under different levels of Cd treatments (**Tables 2, 3**). Activity levels of both enzymes in MuC plants were very close to MC plants. However, upon imposition of Cd treatment, their activities began to increase in both organs of the mutant plants, and it became significant ($P < 0.05$) from 10 µM $CdCl_2$ treatment for both enzymes. Considering both leaf and root, the increase was nearly 2-fold for γ-ECS and 1.4-1.6-fold for GSHS (**Tables 2, 3**) PC synthase activity was approximately 7-fold lower in the MuC than in the MC plants, and no activity was detected in mutant leaves at 25 µM and at 50 µM Cd treatments. Enzyme activity remained at control level in mother plants up to 25 µM. After that, it had sharply increased over MC value by about 3.2-fold at 50 µM $CdCl_2$ (**Tables 2, 3**).

Antioxidant enzyme activities

Significant ($P < 0.05$) alterations in enzymatic activities of antioxidants were observed in *gshL-1* mutant in relation to its mother plants. Activities of foliar SOD in the mutant remained close to MC level across the treatments but significantly increased at 10 µM and 25 µM Cd treatments in roots. Thereafter, it declined at 50 µM Cd, but remained higher than the control (both MC and MuC) (**Figs. 3A, 3B**). Among the enzymes participating in ascorbate-glutathione cycle, activities of APX, DHAR and GR reduced to a considerable extent in *gshL-1* mutant in relation to its mother plants (**Figs. 3C-H**). Comparing both leaves and roots, APX activity reduced 2-2.4-fold in MuC plants and further declined by about 4-10-fold in the mutant under Cd treatment (NC). DHAR level in the mutant was as per with mother plants up to 5 µM Cd, but it decreased significantly from 10 µM Cd and > 2-fold reduction in its activity took place at Cd concentrations higher than this. GR activity was nearly 8-10-fold lower in the mutant than in the mother variety, and its activity remained unchanged throughout the treatment period in relation to MuC. GR level fell sharply in the mother plants only at 50 µM Cd, but remained 3 times higher than in the mutant at this concentration (**Figs. 3G-H**).

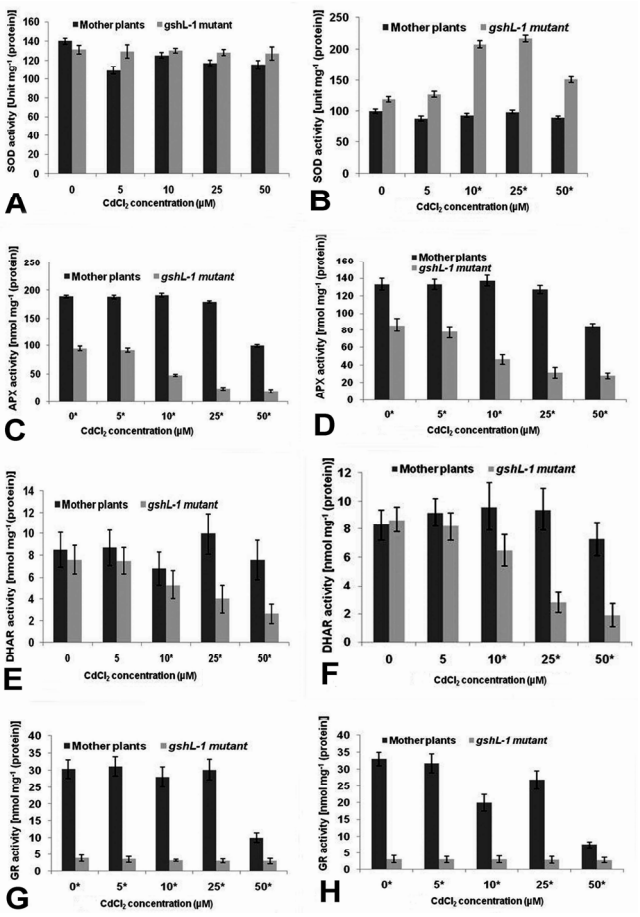

Fig. 3 Activities of SOD and three ascorbate-glutathione cycle enzymes in leaves and roots of mother plants 'BioL-212' and *gshL-1* mutant in grass pea (*Lathyrus sativus* L.). (**A**) SOD-leaf. (**B**) SOD-root. (**C**) APX-leaf. (**D**) APX-root. (**E**) DHAR-leaf. (**F**) DHAR-root. (**G**) GR-leaf. (**H**) GR-root. Control treatment (0 µm Cd) denotes mother control (MC) for mother variety and mutant control (MuC) for *gshL-1* mutant. Asterisk (*) denotes the significant differences ($P < 0.05$, *t*-test, n=6) between mother plants and the mutant for the trait at that particular Cd concentration.

Among the three other antioxidant enzymes outside the ascorbate-glutathione cycle, activities of CAT increased 2.4 (leaf)-3.4 (root)-fold (**Figs. 4A, 4B**), while GPX level decreased by nearly 2(leaf)-2.4 (root)-fold in MuC plants and no activity could be detected in leaves of mutant plants after 10 µM (**Figs. 4C, 4D**). Similarly, measurable GST activity reduced around 7-fold in both tissues of the MuC plants in comparison to MC and became undetectable from 25 µM (**Figs. 4E, 4F**). Barring a decline at 50 µM Cd, there was no change in GPX activity in the mother plants, while GST level followed Cd concentration-dependent increase up to 25 µM and then slipped considerably.

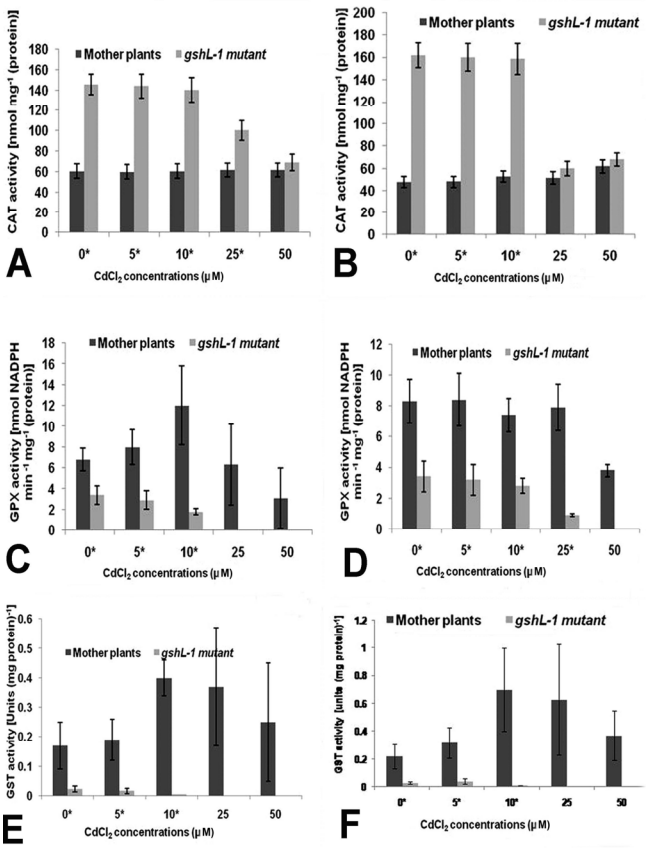

Fig. 4 Antioxidant enzyme activities outside ascorbate-glutathione cycle in leaves and roots of mother plants 'BioL-212' and *gshL-1* mutant in grass pea (*Lathyrus sativus* L.). (**A**) CAT-leaf. (**B**) CAT-root. (**C**) GPX-leaf. (**D**) GPX-root. (**E**) GST-leaf. (**F**) GST-root. Control treatment (0 µm Cd) denotes mother control (MC) for mother variety and mutant control (MuC) for *gshL-1* mutant. Asterisk (*) denotes the significant differences ($P < 0.05$, *t*-test, n=6) between mother plants and the mutant for the trait at that particular Cd concentration.

Hydrogen peroxide accumulation, lipid peroxidation and electrolyte leakage

Accumulation of H_2O_2 showed a significant increase that was more pronounced in the roots than in the leaves of the mutant plants (**Table 4**). Compared with MC, the level of H_2O_2 in roots of Cd-treated mutant plants gradually increased from 2-fold (at 10 µM) to 2.9-fold (at 50 µM). Enhanced rate of lipid peroxidation was also recorded as indicated by gradually increasing malondialdehyde (MDA) contents in the mutant plants exposed to Cd treatment (**Table 4**). The MDA content increased significantly over MC in both tissues of the mutant from 10 µM Cd, while non-significant variation was observed in mother plants. Likewise, electrolyte leakage (EL%) enhanced by 1.5-fold in leaves and about 2-fold in roots of the MuC plants. It increased further by 2.2-2.5-fold in leaves and > 3-fold in roots of the Cd-treated mutant plants. MC level of EL% increased marginally only at 50 µM. Otherwise, it was quite normal (**Table 4**).

DISCUSSION

gshL-1: the biochemical mutant in grass pea isolated through induced mutagenesis

Induced mutagenic techniques have been successfully used to elucidate the role of different antioxidant metabolites and intrinsic biochemical mechanisms in response to different abiotic stresses. In grass pea, this technique has, so far, been used to isolate desirable mutations in relation to different yield components and obviously, to low seed neurotoxin (β-N-Oxalyl-L α, β-diamino propionic acid or ODAP) content (Talukdar 2009b). Although known for its remarkable hardiness and moderate tolerance to various abiotic stresses, virtually nothing is known about underlying defense mechanism of this crop against heavy metal-induced oxidative stress. Very recently, several NaCl-tolerant mutant lines showing alterations in antioxidant defense systems have been isolated (Talukdar 2011f) and levels of tolerance to salinity have also been determined in grass pea (Talukdar 2011g). As ODAP is an amino acid, it may have some roles in stress response mechanism of this crop (Lambein *et al*. 1990; Zhang *et al*. 2003). It is suspected that biosynthesis of ODAP in grass pea plants is related to thiol metabolism of this plant through the activity of enzyme cysteine synthase (Ikegami *et al*. 1993). A correlation of seed ODAP content with water and salinity stress has been reported in different genotypes and induced mutant lines of grass pea (Cocks *et al*. 2000; Xing *et al*. 2001; Yang *et al*. 2004; Talukdar 2011a). Furthermore, a remarkable phytoremediatory effect of grass pea roots against lead-induced oxidative stress is directly related to root glutathione pool and GSH-mediated antioxidant defense mechanism of this crop (Brunet *et al*. 2008, 2009). In this backdrop, identification and isolation of *gshL-1* mutant through induced mutagenesis assume significance, and to the best of my knowledge, this is the first of this kind of mutant among leguminous crops.

Table 4 Leaf and root hydrogen peroxide (H_2O_2-µmol g^{-1} fresh weight), MDA content (nmol g^{-1} fresh weight) and electrolyte leakage (EL%) in 30 d-old mother control (MC) variety 'BioL-212' and *gshL-1* mutant plants of grass pea (*Lathyrus sativus* L.) in control (0 µM $CdCl_2$) and four $CdCl_2$-treatment (5, 10, 25 and 50 µM). Data are means ± SE of six independent experiments.

Parameters[a]	$CdCl_2$ concentration (µM)				
	0 (control)	5	10	25	50
Leaf					
H_2O_2 (A)	3.69 ± 0.3	4.82 ± 1.9	3.73 ± 0.5	4.18 ± 1.3	5.23 ± 1.0*
H_2O_2 (B)	3.78 ± 1.0	4.23 ± 3.1	4.37 ± 1.3	6.17 ± 2.6*	6.59 ± 1.4*
MDA (A)	2.87 ± 0.9	2.90 ± 1.0	3.11 ± 1.8	2.90 ± 1.5	4.00 ± 2.2
MDA (B)	3.33 ± 1.4	3.28 ± 1.3	7.44 ± 1.2*	8.78 ± 2.3*	10.81 ± 1.2*
EL% (A)	8.99 ± 1.3	9.01 ± 2.1	7.52 ± 1.9	7.75 ± 1.4	9.53 ± 2.5
EL% (B)	12.80 ± 2.3	12.72 ± 2.5	18.73 ± 1.3*	20.84 ± 3.1*	21.35 ± 3.3*
Root					
H_2O_2 (A)	2.44 ± 0.3	3.09 ± 1.3	3.12 ± 1.1	3.80 ± 2.0	5.31 ± 1.7*
H_2O_2 (B)	3.75 ± 1.9	5.19 ± 2.7*	6.25 ± 2.1*	6.48 ± 3.0*	9.75 ± 4.9*
MDA (A)	2.69 ± 1.1	2.70 ± 1.0	2.90 ± 2.1	3.08 ± 1.3	4.02 ± 1.6
MDA (B)	3.59 ± 1.7	3.61 ± 1.4	5.18 ± 1.9*	5.29 ± 2.1*	8.12 ± 3.5*
EL% (A)	11.53 ± 2.9	12 ± 3.1	10.84 ± 2.6	10.36 ± 2.2	12.31 ± 3.1
EL% (B)	23.57 ± 3.6	26.03 ± 4.2	33.48 ± 6.6*	46.06 ± 7.0*	48.77 ± 12.9*

[a] A-mother plants, B-*gshL-1* mutant

* significantly different from respective control value at $P < 0.05$

gshL-1 is deficient in glutathione pool and exhibited growth inhibition with increased cadmium sensitivity

Compared to its mother plants, *gshL-1* mutant exhibited huge decrease of total glutathione content in its leaves and roots. Repeated measurements revealed that the mutant contained only 15% of total glutathione in leaves and 20% of that in roots of MC variety. The share decreased further after imposition of Cd treatment, as evidenced by 56%-80% reduction of total glutathione in NC plants in relation to MuC value. In both treated and un-treated conditions, the reduction in total glutathione content was mainly due to the sharp decline in reduced glutathione (GSH) pool, while its oxidized form, the glutathione disulfide (GSSG), increased under high cadmium treatments. The accumulation of GSSG resulted in significant reduction of GSH redox state in the *gshL-1* mutant, and this condition is often taken as a marker of the degree of intracellular oxidative stress, also referred to as 'disulphide stress' (Queval *et al*. 2011).

Plant growth expressed as dry weight of shoot and root was severely affected in the *gshL-1* mutant. The effect was, however, more pronounced on the roots than on the shoots of the mutant plants. Reduction in shoot dry weight was mainly due to stunted growth of aerial portions manifested by reduced seedling height, rolled-short leaflets, absence of stipules, shorter tendril and decreased number of leaflets $leaf^{-1}$. On the other hand, decreased length in roots and reduced number of lateral roots led to nearly 3.03-fold decline in root dry weight of the mutant in relation to its mother plants in unstressed condition. Although no visible symptom of toxicity, except retardation of growth, was prominent on any parts of the mutant plants, the inhibition of growth accelerated when the *gshL-1* plants were submitted to $CdCl_2$ treatment. A concentration-dependent decrease for both shoot and root growth was noticed in the mutant with a significant effect from 10 μM $CdCl_2$. Its mother plants performed better, maintaining normal growth up to 25 μM $CdCl_2$ and showing reduction in dry weight, thereafter. This suggested higher sensitivity of *gshL-1* mutant to cadmium treatment than its mother plants. Interestingly, thickening of lateral roots took place in the mutant plants only after imposition of Cd treatment. In agreement with this finding, Rodríguez-Serrano *et al*. (2006) attributed this phenomenon to the Cd-dependent interferences with cambium differentiation and cell division in the Cd-treated pea roots. Thus, it is reasonable to say that depletion of glutathione redox pool in the present grass pea mutant severely impeded the normal root growth. Significant decrease in biomass accumulation indicated increased sensitivity of the mutant to the Cd-induced stress. This inhibition of root growth has been attributed to a block in cell cycle progression and associated halt in root meristem development in glutathione-deficient *Arabidopsis rml1* mutant (Vernoux *et al*. 2000). The increased Cd-sensitivity due to glutathione deficiency was also studied extensively in series of *Arabidopsis* mutants, like *cad1* and *cad2* (Howden *et al*. 1995; Cobbett *et al*. 1998). Inhibition of growth was reported in Cd-sensitive genotypes of different plants including legumes like *Pisum sativum* (Lozano-Rodriguez *et al*. 1997; Dixit *et al*. 2001) and *Medicago sativa* (Ortega-Villasante *et al*. 2005). Higher accumulation of cadmium along with complete normal growth of roots, however, was observed in Cd-tolerant pea mutant, induced by ethylmethane sulfonate (Tsyganov *et al*. 2007). Peculiarly, a higher accumulation of Cd, but lower sensitivity of roots to its accumulation than leaves was observed in pea and tomato roots (Sandalio *et al*. 2001; Ben Ammar *et al*. 2008). Significant accumulation of heavy metal and concomitant decrease in root biomass accumulation was also found in arsenic-sensitive genotypes of *Lathyrus sativus* L. and *Trigonella foenum-graecum* L. (Talukdar 2011c). Higher accumulation of Cd and normal biomass production in the present mother plants indicated tolerance of this grass pea variety to cadmium to a certain limit.

GSH-deficiency crippled the formation of phytochelatins and possibly, altered Cd accumulation in the mutant

As an obvious consequence, depletion in GSH pool in *gshL-1* mutant badly hampered the production of PCs, another thiol-peptide involved in detoxification or homeostasis of heavy metals (Rauser 1995), like Cd (Howarth *et al*. 2003; Nocito *et al*. 2006). This was evidenced by simultaneous reduction of both GSH and PCs content in MuC and NC and also by normal level of both GSH and PCs in mother plants up to 25 μM Cd. Significantly, the decrease of GSH level in mother plants at 50 μM was associated with dramatic increase in PCs content. Decline in GSH content due to enhanced PCs synthesis was also observed in a number of plant species (De Vos *et al*. 1992; Xiang and Oliver 1998). The enzyme PC synthase is known to be activated by metal ions and uses GSH as a substrate to catalyze the synthesis of PCs (Zenk 1996). Significant decrease of its activity in the present grass pea mutant might be due to paucity of enough GSH within cell and indicated its failure to up-regulate its activity even under high Cd treatment. By contrast, normal level of PC synthase activity in Cd-treated mother plants maintained PC content as per MC level up to 25 μM Cd and enhancement of its activity at 50 μM resulted in increase of PCs content. Interestingly, GSH content in mother plants declined only at this treatment level, indicating a higher trigger point of the mother plants for the onset of phytochelatin synthesis at the expenses of GSH. As limited available GSH is extensively consumed in order to meet the growing challenge of oxidative stress, PCs synthesis and its transportation was becoming increasingly difficult for the mutant. Perhaps, because of this reason, PCs were undetected in the leaves of the mutant from 25 μM $CdCl_2$ treatment, when GSH content was extremely low. Although much lower than its mother plants, PCs was detectable in the roots of the mutant plants even when it was submitted to 50 μM Cd. Cadmium is a thiol-reactive toxicant, and the long-distance transport of γ-glutamylcysteine containing thiol-peptides like GSH and PCs has potential implications for the cotransport of bound thiol-reactive substances like cadmium (Li *et al*. 2006). Translocation of Cd from root to shoot has been proposed to occur via the xylem of Indian mustard in a PC-independent manner (Salt *et al*. 1995). Li *et al*. (2006) in *Arabidopsis* mutants explained the inability of thiol-peptide-metal chelate complexes to move upwards, leading to its sequestration in the root. In contrast with this view, Gong *et al*. (2003), however, found no difficulties in PC-mediated long distance xylem transport of Cd up to leaves, leading to reduced accumulation of cadmium in roots of *Arabidopsis cad1-3* mutant. Song *et al*. (2010) identified two ATP binding cassette transporters (ABCC) for shuttling arsenite (ASIII)-PC_2 into plant vacuoles. The higher sensitivity of the present *gshL-1* roots than the leaves to Cd could be explained by its inability to transport Cd to the aerial parts of the plant. As root is the first organ to encounter the metal treatment, it seems likely that glutathione deficiency and concomitant decrease in PCs synthesis disrupted thiol-dependent upward movement of cadmium in the present mutant, resulting in over-accumulation of Cd in the mutant roots and its negligible amount in the leaves. Although detail mechanism is not known, existence of this thiol-mediated transport of Cd in grass pea was evidenced by over-accumulation of this metal higher in the leaves than in the roots of the mother control plants which possessed quite normal level of both GSH and PCs and showed no symptom of Cd-induced toxicity. Therefore, it seems likely that GSH deficiency and decrease in phytochelatin contents resulted in increased Cd-sensitivity of *gshL-1* mutant, as also reported earlier in *cad1* mutant of *Arabidopsis* (Howden *et al*. 1995).

Glutathione deficiency in *gshL-1* mutant was accompanied with impairment in its recycling, but with normal synthesis

Besides the synthesis by two-step enzymatic reactions, glutathione is constantly regenerated by NADPH-dependent reduction of the disulphide bond of oxidized glutathione (GSSG) through enzymatic actions of glutathione reductase (GR) in the ascorbate-glutathione cycle. GR plays a central role in maintaining the glutathione redox pool heavily in favor of reducing form, thus, facilitating this principal antioxidant component to function as an efficient redox buffer in numerous cellular metabolic events during the process of plant growth and development and also during stress (Edwards *et al*. 1994; Noctor *et al*. 2002; Contour-Ansel *et al*. 2006). The GSH content in the present *gshL-1* mutant had reduced by about 92-95%, while GSSG level had increased by >2-fold in relation to its mother plants. Remarkably, GSH biosynthesis was quite normal in the mutant, as observed by quite normal functioning of both γ-ECS and GSHS enzymes in both leaf and roots. It is noteworthy that activities of these two enzymes even increased when *gshL-1* plants were subjected to Cd-stress. The demand-driven acceleration in GSH synthesis was mainly attributed to its increasing consumption in response to Cd-induced oxidative stress, due to GSH conversion to PCs and formation of GSH-Cd conjugates (Xiang and Oliver 1998; Noctor *et al*. 2002; Kolb *et al*. 2010; Queval *et al*. 2011). Despite normal biosynthesis in the present *gshL-1* mutant, GSH level remained very low in control and gradually reduced at elevated Cd-concentrations. This apparent conflicting result strongly indicated that maintenance of GSH redox pool in the *gshL-1* mutant required sources other than its biosynthesis. Several lines of evidences suggested that regeneration of GSH by GR has more implications than its synthesis in glutathione homeostasis and stress tolerance in plants (Foyer *et al*. 1995; Dixit *et al*. 2001). Poplar plants overexpressing GR showed increased GSH contents and tolerance to photoinhibition (Foyer *et al*. 1995). Similar findings were reported in tobacco (Mullineaux *et al*. 1994) and in pea (Broadbent *et al*. 1995), strongly implicating that GR activity is the principal factor that influences glutathione levels through controlling the capacity of regeneration of GSH from GSSG (Noctor *et al*. 2002). It is, therefore, reasonable to say that huge deficiency in GSH level in the present grass pea mutant was mainly due to defect in its regeneration capability, orchestrated by abnormally low level of GR activity in the mutant which was not recovered even through induction of high Cd-treatment. This suggested constitutive down-regulation of GR enzyme in the present material. As GR plays pivotal role in plant antioxidant defense, up-regulation of its activity was found in different plants including legumes under heavy metal stress (Dixit *et al*. 2001; Reddy *et al*. 2005; Contour-Ansel *et al*. 2006; Mishra *et al*. 2006). In grass pea, increased GR activity was found associated with their tolerance to lead-induced phytotoxicity (Brunet *et al*. 2009), NaCl-induced stress (Talukdar 2011f, 2011h) and in the backdrop of severe ascorbate-deficiency (Talukdar 2012b). This is, certainly, not the case for the present mutant. All the isoforms of GR are known to be highly specific for GSSG, and if the reduced enzyme is not reoxidized by GSSG reversible inactivation follows (Arscott *et al*. 1989). This can be ruled out as a possible reason for GR inactivation in *gshL-1* mutant as GSSG content increased in both tissues. Like GSH, GSSG can regulate GR expression as the pea GR gene contains a putative GSSG binding site (Creissen *et al*. 1992). Despite nearly 2-fold increase in GSSG level over its mother plants, *gshL-1* mutant showed no up-regulation of GR by GSSG. Unlike other GSH-deficient plant mutant like *rml1*, *gsh1/2*, *pad 2-1*, *cad1* and *cad2*, *gshL-1* is unique as this mutant was originated through gamma ray induced mutagenesis, and is deficient in glutathione only due to impairment in GR activity, not due to inactivation of γ-ECS, PC-synthase or GSHS in thiol-biosynthesis process. However, a *gr1* knock out mutant, showing inactivation of GR cytosolic isoform was isolated in Arabidopsis. This mutant showed decreased GSH-GSSG ratio, but in contrast to the present *gshL-1* in grass pea, it was not deficient in total glutathione concentration and produced no evidence of generalized oxidative stress (Marty *et al*. 2009; Mhamdii *et al*. 2010). Extensive accumulation of GSSG and thioredoxin dependent poor growth was observed in a glutathione reductase null mutant of yeast (Muller 1996).

Low GSH availability impeded the GSH-dependent recycling of reduced ascorbate in the ascorbate-glutathione cycle

GSH is a major water-soluble antioxidant in plant cells (Alscher 1989). A major function of GSH in protection against oxidative stress is the recycling of ascorbate from its oxidized form, the dehydroascorbate (DHA) through GSH-mediated activity of dehydrascorbate reductase (DHAR) in the ascorbate-glutathione cycle (Foyer and Halliwell 1976; Nakano and Asada 1981). In this pathway, glutathione acts as a recycled intermediate in the reduction of H_2O_2 using electrons derived, ultimately, from H_2O. Due to low level of GR and concomitant decrease in GSH availability in the *gshL-1* mutant, the normal interplay of GR-DHAR was severely disrupted, and the effect was more pronounced at elevated Cd-treatment. Although DHAR activity was initially as per control level, it reduced drastically at higher Cd concentrations. This resulted in significant decline in ascorbate regeneration capacity of both leaves and roots of the mutant. This GSH-dependent and DHAR-mediated recycling of reduced ascorbate is the major source of cellular ascorbate pool (Chen *et al*. 2003; Talukdar 2012b), and has immense significance in compensating the antioxidant defense to balance the reduced pool of glutathione. This was not fully possible in case of *gshL-1* mutant as this regeneration machinery collapsed partially due to huge deficiency of GSH in both tissues of the mutant.

Accumulation of H_2O_2 and MDA indicated failure of GSH-mediated H_2O_2-lipid peroxide scavenging machinery in the *gshL-1* mutant under cadmium stress

The cascading effect of GSH-deficiency was felt in disruption and alteration of different antioxidant defense enzymes involved in H_2O_2-metabolism in the mutant. The ASC-GSH cycle is the key H_2O_2-scavenging machinery in plants, and with at least seven isozymes, APX is the most prolific enzyme in this cycle (Asada 2006). However, this antioxidant enzyme exclusively requires reduced ascorbate as a co-factor to function efficiently (Noctor and Foyer 1998; Asada 2006). In the present case, SOD activity in *gshL-1* mutant (PC) was as per with MC plants, but APX activity was 2-2.4-fold lower in the mutant than MC. Presumably, disruption of steady supply of reduced ascorbate due to below normal activity of DHAR in the GSH-deficient mutant plants prevented the APX to function efficiently in removal of H_2O_2. Hossain and Asada (1984) and Cruz de Carvalho (2008) reported that APX activity was greatly reduced in low ascorbate concentration due to rapid inactivation of chloroplast isoforms, particularly in the presence of H_2O_2. Thus, the situation got worse when *gshL-1* plants were submitted to Cd treatment, as increased SOD activity generated excess H_2O_2, which might be instrumental for further lowering of APX activity at higher Cd-treatment.

The crippling of APX capacity to scavenge H_2O_2 was accompanied with significant reduction of both GPX and GST activities but increased activity of CAT in the mutant. Both GPX and GST can use GSH as a potential substrate, while CAT requires no reducing substrate to metabolize H_2O_2. Since H_2O_2 is a predominant cellular oxidant, its accumulation to toxic level can not be allowed particularly in places where thiol-regulated enzymes are functioning (Mittler 2002). Conversely, as a stable and diffusible ROS signal,

H_2O_2 has the ability to regulate the induction of GPX/GST (Mhamdi *et al*. 2010; Queval *et al*. 2011) and CAT (Miao *et al*. 2006). Besides Cd-PCs complex, intracellular sequestration of Cd ions can also be achieved through conjugation of Cd-GSH complex, a reac-tion catalyzed by GST enzymes (Dixon *et al*. 2009). A 2-2.4 decrease in GPX activity and nearly 7-fold reduction in GST level in the *gshL-1* mutant severely jeopardized the prospect of efficient scavenging of H_2O_2 and lipid peroxides and intracellular sequestration of heavy metal in the present mutant. This was evidenced by the facts that accumulation of both H_2O_2 and MDA (a product of lipid peroxidation) took upward trend with concomitant decrease in activities of both GPX and GST in both tissues of the mutant at elevated Cd treatment. This confirmed the failure of H_2O_2 to induce the activities of these two enzymes in MuC and in Cd-stressed mutant plants. Increased activities of these two prominent thiol-based enzymes were observed in *Pisum sativum* L. plants under Cd-stress (Dixit *et al*. 2001) and in roots and shoots of *Lathyrus sativus* L., *Cicer arietinum* L. and *Macrotyloma uniflorum* (Lam.) plants submitted to lead treatment (Reddy *et al*. 2005; Brunet *et al*. 2009). Certainly, low GSH availability in *gshL-1* mutant led to inadequate response of both GPX and GST even under high Cd treatment. H_2O_2 level is well correlated with MDA content, and therefore, both these compounds are routinely used as the biochemical markers of oxidative stress (Bandeoỷlu *et al*. 2004; Cheeseman 2006; Cruz de Carvalho 2008). A good correlation between the intracellular H_2O_2 availability and the status of glutathione pool has been suggested and oxidative perturbation of GSH redox state is somehow linked to CAT activities through transmission of H_2O_2 signal (Mhamdi *et al*. 2010; Quevel *et al*. 2011). This idea has been strengthened by the present case, where among H_2O_2-metabolizing enzymes, only CAT activity increased, while APX and GPX decreased abnormally. Yet, CAT activity alone could not compensate this huge deficiency, despite a significant increase, resulting in H_2O_2 accumulation in the mutant tissues. Consequently, this led to oxidative damage of membrane through increased lipid peroxidation as manifested by significant enhancement in percentage of electrolyte leakage in the mutant. Its mother plants, however, showed efficient scavenging of ROS through normal functioning of H_2O_2-metabolizing enzymes and proper sequestration of Cd through GST/PC synthase activity. This pre-vented H_2O_2 over-accumulation and minimized Cd-induced oxidative damage, although, decrease in antioxidant capacity to a certain extent became evidenced only at 50 µM Cd, indicating cadmium sensitivity of this grass pea variety at this level.

CONCLUSION

In conclusion, the results showed that *gshL-1* mutant is under severe oxidative stress and became highly sensitive to cadmium treatment. GR inactivation is the primary cause of GSH-deficiency in this mutant, triggering a massive disruption in activities of different antioxidant enzymes and the disturbances in phytochelatin synthesis. All these ultimately led to decrease in growth and development of the mutant plants, the rate of which accelerated under cadmium treatment as a symptom of its higher sensitivity to cadmium-induced oxidative stress than its mother plants. The 10 µM Cd level was toxic to *gshL-1* mutant, while its mother variety BioL-212 showed cadmium sensitivity only at 50 µM Cd treatment. Present study clearly revealed origin of a new biochemical mutant in grass pea through induction of mutagenic techniques. The potentials of this unique mutant may be exploited in grass pea to explore the underlying thiol-based regulation of antioxidant defense system against heavy metal-induced stress and more specifically, to reveal the link between thiol metabolism and role of neurotoxin in the process of remarkable stress tolerance in this hardy legume crop.

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REFERENCES

Aebi H (1984) Catalase *in vitro*. *Methods in Enzymology* **105**, 121-126
Alscher RG (1989) Biosynthesis and antioxidant function of glutathione in plants. *Physiologia Plantarum* **77**, 457-464
Anderson ME (1985) Tissue glutathione. In: Greenwald RA (Ed) *Handbook of Methods for Oxygen Radical Research*, CRC Press, Boca Raton, Florida, pp 317-323
Arscott LD, Drake DM, Williams Jr. CH (1989) Inactivation-reactivation of two-electron reduced *Escherichia coli* glutathione reductase involving a dimer-monomer equilibrium. *Biochemistry* **28**, 3591-3598
Asada K (2006) Production and scavenging of reactive oxygen species in chloroplasts and their functions. *Plant Physiology* **141**, 391-396
Bandeoğlu E, Eyidoğan F, Yücel M, Öktem A (2004) Antioxidant responses of shoots and roots of lentil to NaCl-salinity stress. *Plant Growth Regulation* **42**, 69-77
Bashandy T, Guilleminot J, Vernoux T, Caparros-Ruiz D, Ljung K, Meyer Y, Reichheld J-P (2010) Interplay between the NADP-linked thioredoxin and glutathione systems in *Arabidopsis* auxin signaling. *The Plant Cell* **22**, 376-391
Ben Ammar W, Mediouni C, Tray B, Ghorbel MH, Jemal F (2008) Glutathione and phytochelatin contents in tomato plants exposed to cadmium. *Biologia Plantarum* **52 (2)**, 314-320
Bergmann L, Rennenberg H (1993) Glutathione metabolism in plants. In: de Kok LJ, Stulen I, Rennenberg H, Brunold C, Rauser WE (Eds) *Sulfur Nutrition and Sulfur Assimilation in Higher Plants*, SPB Academic Publishing, The Hague, pp 109-123
Beyer WF, Fridovich I (1987) Assaying for superoxide dismutase activity: Some large consequences of minor changes in conditions. *Analytical Biochemistry* **161**, 559-566
Biswas AK (2007) Induced mutation in grass pea (*Lathyrus sativus* L.). In: Ochatt SJ, Jain SM (Ed) *Underutilised and Neglected Crops, Herbs and Spices*, Science Press, Enfield, USA, pp 19-39
Broadbent P, Creissen GP, Kular B, Wellburn AR, Mullineaux P (1995) Oxidative stress responses in transgenic tobacco containing altered levels of glutathione reductase activity. *The Plant Journal* **8**, 247-255
Brunet J, Repellin A, Varrault G, Terryn N, Zuily-Fodil Y (2008) Lead accumulation in the roots of grass pea (*Lathyrus sativus* L.): A novel plant for phytoremediation systems? *Comptes Rendus Biologies* **331**, 859-864
Brunet J, Varrault G, Zuily-Fodil Y, Repellin A (2009) Accumulation of lead in the roots of grass pea (*Lathyrus sativus* L.) plants triggers systematic variation in gene expression in the shoots. *Chemosphere* **77**, 1113-1120
Cheeseman JM (2006) Hydrogen peroxide concentrations in leaves under natural conditions. *Journal of Experimental Botany* **10**, 2435-2444
Chen Z, Young TE, Ling J, Chang S-C, Gallie DR (2003) Increasing vitamin C content of plants through enhanced ascorbate recycling. *Proceedings of the National Academy of Sciences USA* **100**, 3525-3530
Cobbett CS, May MJ, Howden R, Rolls B (1998) The glutathione-deficient, cadmium-sensitive mutant, *cad2-1*, of *Arabidopsis thaliana* is deficient in γ-glutamylcysteine synthetase. *The Plant Journal* **16 (1)**, 73-78
Cocks P, Siddique K, Hanbury C (2000) Impact of stress on neurotoxins. In: Cocks P, Siddique K, Hanbury C (Ed) *Lathyrus: A New Grain Legume*, RIRDC, Australia, pp 4-15
Contour-Ansel D, Torres-Franklin ML, Cruz de Carvalho MH, D'Arcy-Lameta A, Zuily-Fodil Y (2006) Glutathione reductase in leaves of cowpea: Cloning of two cDNAs, expression and enzymatic activity under progressive drought stress, desiccation and abscisic acid treatment. *Annals of Botany* **98**, 1279-1287
Creissen G, Edwards EA, Enard C, Wellburn A, Mullineaux P (1992) Molecular characterization of glutathione reductase cDNAs from pea (*Pisum sativum* L.). *The Plant Journal* **2**, 129-131
Creissen G, Firmin J, Fryer M, Kular B, Leyland N, Reynolds H, Pastori G, Wellburn F, Baker N, Wellburn A, Mullineaux P (1999) Elevated glutathione biosynthetic capacity in the chloroplasts of transgenic tobacco plants paradoxically causes increased oxidative stress. *The Plant Cell* **11**, 1277-1291
Cruz de Carvalho MH (2008) Drought stress and reactive oxygen species: Production, scavenging and signaling. *Plant Signaling and Behavior* **3**, 156-165
Dalton DA, Russell SA, Hanus FJ, Pascoe GA, Evans HJ (1986) Enzymatic reactions of ascorbate and glutathione that prevent peroxide damage in soybean root nodules. *Proceedings of the National Academy of Sciences USA* **83**, 3811-3815
Delhaize E (1996) A metal-accumulator mutant of *Arabidopsis thaliana*. *Plant Physiology* **111**, 849-855
De Vos CHR, Vonk MJ, Vooijs R, Schat H (1992) Glutathione depletion due to copper-induced phytochelatin synthesis causes oxidative stress in *Silene*

cucubalus. *Plant Physiology* **98**, 853-858
Dionisio-Sese ML, Tobita S (1998) Antioxidant responses of rice seedlings to salinity stress. *Plant Science* **135**, 1-9
Dixit V, Pandey V, Shyam R (2001) Differential antioxidative responses to cadmium in roots and leaves of pea (*Pisum sativum* L. cv. Azad). *Journal of Experimental Botany* **52 (358)**, 1101-1109
Dixon DP, Hawkins T, Hussey PJ, Edwards R (2009) Enzyme activities and subcellular localization of members of the *Arabidopsis* glutathione transferase superfamily. *Journal of Experimental Botany* **60**, 1207-1218
Edwards Anne E, Enard C, Creissen GP, Mullineaux M (1994) Synthesis and properties of glutathione reductase in stressed peas. *Planta* **192**, 137-143
Edwards R (1996) Characterization of glutathione transferases and glutathione peroxidases in pea (*Pisum sativum*). *Physiologia Plantarum* **98**, 594-604
Forster BP (2001) Mutation genetics of salt tolerance in barley: An assessment of Golden Promise and other semi-dwarf mutants. *Euphytica* **120**, 317-328
Foyer CH, Halliwell B (1976) Presence of glutathione and glutathione reductase in chloroplasts: A proposed role in ascorbic acid metabolism. *Planta* **133**, 21-25
Foyer CH, Noctor G (2011) Ascorbate and Glutathione: The heart of the redox hub. *Plant Physiology* **155**, 2-18
Foyer CH, Souriau N, Perret S, Lelandais M, Kunert KJ, Pruvost C, Jouanin L (1995) Overexpression of glutathione reductase but not glutathione synthetase leads to increases in antioxidant capacity and resistance to photoinhibition in poplar trees. *Plant Physiology* **109**, 1047-1057
Gong J-M, Lee DA, Schroeder JI (2003) Long distance root-to-shoot transport of phytochelatins and cadmium in *Arabidopsis*. *Proceedings of the National Academy of Sciences USA* **100**, 10118-10123
Guinel FC, LaRue TA (1993) Excessive aluminium accumulation in pea mutant E107 (*brz*). *Plant and Soil* **157**, 75-82
Hell R, Bergmann L (1990) γ-Glutamylcysteine synthetase in higher plants: Catalytic properties and subcellular localization. *Planta* **180**, 603-612
Hoagland DR, Arnon DI (1938) The water-culture method for growing plants without soil. *University of California, College of Agriculture, Experimental Station Circular Berkeley, CA*, pp 347-353
Hossain MA, Asada K (1984) Purification of dehydroascorbate reductase from spinach and its characterization as a thiol enzyme. *Plant Cell Physiology* **25**, 85-92
Hossain Z, Mandal AKA, Datta SK, Biswas AK (2006) Isolation of a NaCl-tolerant mutant of *Chrysanthemum morifolium* by gamma radiation: *In vitro* mutagenesis and selection by salt stress. *Functional Plant Biology* **33**, 91-101
Howden R, Cobbett CS (1992) Cadmium-sensitive mutants of *Arabidopsis thaliana*. *Plant Physiology* **100**, 100-107
Howden R, Goldsbrough PB, Andersen CR, Cobbett CS (1995) Cadmium-sensitive, *cad1* mutants of *Arabidopsis thaliana* are phytochelatin deficient. *Plant Physiology* **107**, 1059-1066
Howarth JR, Domínguez-Solís JR, Gutiérrez-Alcalá G, Wray JL, Romero LC, Gotor C (2003) The serine acetyltransferase gene family in *Arabidopsis thaliana* and the regulation of its expression by cadmium. *Plant Molecular Biology* **51**, 589-598
Ikegami F, Ongena G, Sakai R, Itagaki S, Kobori M, Ishikawa T, Kuo YH, Lambein F, Murakoshi F (1993) Biosynthesis of β-(isoxazolin-5-on-2-yl)-alanine by cysteine synthase in *Lathyrus sativus*. *Phytochemistry* **33**, 93-98
Jain SM (2010) Mutagenesis in crop improvement under the climate change. *Romanian Biotechnological Letters* **15 (2)**, 88-106
Kharkwal MC, Shu QY (2009) The role of induced mutations in world food security. In: Shu QY (Ed) *Induced Plant Mutations in the Genomics Era*, Proceedings of the Joint FAO/IAEA International Symposium, Vienna, Austria, pp 33-38
Kolb D, Müller M, Zellnig G, Zechmann B (2010) Cadmium induced changes in subcellular glutathione contents within glandular trichomes of *Cucurbita pepo* L. *Protoplasma* **243**, 87-94
Lambein F, Ongena G, Kuo YH (1990) β-isoxazolinone-alanine is involved in the biosynthesis of the neurotoxin, β-N-oxalyl-L-α,β-diaminopropionic acid. *Phytochemistry* **29**, 3793-3796
Li Y, Dankher OP, Carreira L, Smith AP, Meagher RB (2006) The shoot-specific expression of γ-Glutamylcysteine synthetase diorects the long-distance transport of thiol-peptides to roots conferring tolerance to mercury and arsenic. *Plant Physiology* **141**, 288-298
Li Z-S, Zhen R-G, Rea PA (1995) 1-chloro-2,4-dinitrobenzene-elicited increase in vacuolar glutathione-*S*-conjugate transport activity. *Plant Physiology* **109**, 177-185
Lozano-Rodríguez E, Hernández LE, Bonay P, Carpena-Ruiz RO (1997) Distribution of cadmium in shoot and root tissues of maize and pea plants: physiological disturbances. *Journal of Experimental Botany* **48 (306)**, 123-128
Maluszynski M, Ahloowalia BS, Sigurbjörnsson B (1995) Application of *in vivo* and *in vitro* mutation techniques for crop improvement. *Euphytica* **85 (1-3)**, 303-315
Marrs KA (1996) The functions and regulation of glutathione-*S*-transferase in plants. *Annual Review of Plant Physiology and Plant Molecular Biology* **47**, 127-158
Matamoros MA, Moran JF, Iturbe-Ormaetxe I, Rubio MC, Becana M (1999) Glutathione and homoglutathione synthesis in legume root nodules. *Plant Physiology* **121**, 879-888
Marty L, Siala W, Schwarzländer M, Fricker MD, Wirtz M, Sweetlove LJ, Meyer Y, Meyer AJ, REichheld JP, Hell R (2009) The NADPH-dependent thioredoxin system constitutes a functional backup for cytosolic glutathione reductase in *Arabidopsis*. *Proceedings of the National Academy of Sciences USA* **106**, 9109-9114
Mhamdi A, Hager J, Chaouch S, Queval G, Han Yi, Taconnat L, Saindrenan P, Gouia H, Issakidis-Bourguet E, Renou J-P, Noctor G (2010) *Arabidopsis* GLUTATHIONE REDUCTASE 1 plays a crucial role in leaf responses to intracellular hydrogen peroxide and in ensuring appropriate gene expression through both salicyclic acid and jasmonic acid signaling pathways. *Plant Physiology* **153**, 1144-1160
Miao Y, Lv D, Wang P, Wang X-C, Chen J, Miao C, Song C-P (2006) An *Arabidopsis* glutathione peroxidase functions as both a redox transducer and a scavenger in abscisic acid and drought stress responses. *The Plant Cell* **18**, 2749-2766
Miri SM, Mousavi A, Naghavi MR, Mirzail M, Talaei AR, Khiabani MN (2009) Analysis of induced mutants of salinity resistant banana (*Musa acuminata* cv. Dwarf Cavendish) using morphological and molecular markers. *Iranian Journal of Biotechnology* **7 (2)**, 86-92
Mishra S, Srivastava S, Tripathi RD, Kumar R, Seth CS, Gupta DK (2006) Lead detoxification by coontail (*Ceratophyllum demersum* L.) involves induction of phytochelatins and antioxidant system in response to its accumulation. *Chemosphere* **65**, 1027-1039
Mittler R (2002) Oxidative stress, antioxidants and stress tolerance. *Trends in Plant Science* **7**, 405-410
Muller EGD (1996) A glutathione reductase mutant of yeast accumulates high levels of oxidized glutathione and requires thioredoxin for growth. *Molecular Biology of the Cell* **7**, 1805-1813
Mullineaux P, Creissen G, Broadbent P, Reynolds H, Kular B, Wellburn A (1994) Elucidation of the role of glutathione reductase using transgenic plants. *Biochemical Society Transactions* **22**, 931-936
Nakano Y, Asada K (1981) Hydrogen peroxide is scavenged by ascorbate specific peroxidase in spinach chloroplast. *Plant Cell Physiology* **22**, 867-880
Nawrot M, Szarejko I, Maluszynski M (2001) Barley mutants with increased tolerance to aluminium toxicity. *Euphytica* **120**, 345-356
Nichterlain K, Bohlman H, Niclen S, Maluszynski M (2000) Achievements and trends of using induced mutations in crop improvement. In: *DAEBRNS Symposium on the use of Nuclear and Molecular Techniques in Crop Improvement*, 2000, Mumbai, pp 27-35
Nocito FF, Lancilli C, Crema B, Fourcroy P, Davidian JC, Sacchi GA (2006) Heavy metal stress and sulphate uptake in maize roots. *Plant Physiology* **141**, 1138-1141
Noctor G, Foyer CH (1998) Ascorbate and glutathione: Keeping active oxygen under control. *Annual Review Plant Physiology Plant Molecular Biology* **49**, 249-279
Noctor G, Gomez L, Vanacker H, Foyer CH (2002) Interactions between biosynthesis, compartmentation and transport in the control of glutathione homeostasis and signaling. *Journal of Experimental Botany* **53**, 1283-1304
Ortega-Villasante C, Rellán-Álvarez R, del Campo FF, Carpena-Ruiz RO, Hernández LE (2005) Cellular damage induced by cadmium and mercury in *Medicago sativa*. *Journal of Experimental Botany* **56 (418)**, 2239-2251
Queval G, Jaillard D, Zechmann B, Noctor G (2011) Increased intracellular H_2O_2 availability preferentially drives glutathione accumulation in vacuoles and chloroplasts. *Plant, Cell and Environment* **34**, 21-32
Rauser WE (1995) Phytochelatins and related peptides. *Plant Physiology* **109**, 1141-1149
Reddy A, Kumar S, Jyothsna Kumari G, Thimmanaik S, Sudhakar C (2005) Lead induced changes in antioxidant metabolism of horsegram (*Macrotyloma uniflorum* (Lam.) Verdc.) and bengalgram (*Cicer arietinum* L.). *Chemosphere* **60**, 97-104
Rodríguez-Serrano M, Romero-Puertas MC, Zabalza A, Corpas FJ, Gómez M, del Río LA, Sandalio LM (2006) Cadmium effect on oxidative metabolism of pea (*Pisum sativum* L.) roots: Imaging of reactive oxygen species and nitric oxide accumulation *in vivo*. *Plant Cell Environment* **29**, 1532-1544
Roxas VP, Smith RK, Allen ER, Allen RD (1997) Over-expression of glutathione-*S*-transferase/glutathione peroxidase enhances the growth of transgenic tobacco seedlings during stress. *Nature Biotechnology* **15**, 968-991
Rybinski W (2003) Mutagenesis as a tool for improvement of traits in grass pea (*Lathyrus sativus* L.). *Lathyrus Lathyrism Newsletter* **3**, 27-31
Saher S, Piqueras A, Hellin E, Olmos E (2004) Hyperhydricity in micropropagated carnation shoots: The role of oxidative stress. *Physiologia Plantarum* **120**, 152-161
Sandalio LM, Dalurzo HC, Gómez M, Romero-Puertas MC, del Río LA (2001) Cadmium-induced changes in the growth and oxidative metabolism of pea plants. *Journal of Experimental Botany* **52 (364)**, 2115-2126
Salt DE, Prince RC, Pickering IJ, Raskin I (1995) Mechanisms of cadmium mobility and accumulation in Indian mustard. *Plant Physiology* **109**, 1427-1433
Song W-Y, Park J, Mendoza-Cózatl DG, Suter-Grotemeyer M, Shim D, Hörtensteiner S, Geisler M, Weder B, Rea PA, Rentsch D, Schroeder JI, Lee Y, Martinoia E (2010) Arsenic tolerance in *Arabidopsis* is mediated by two ABCC-type phytochelatin transporters. *Proceedings of the National*

Academy of Sciences USA **107**, 21187-21192

Talukdar D (2009a) Dwarf mutations in grass pea (*Lathyrus sativus* L.): Origin, morphology, inheritance and linkage studies. *Journal of Genetics* **88**, 165-175

Talukdar D (2009b) Recent progress on genetic analysis of novel mutants and aneuploid research in grass pea (*Lathyrus sativus* L.). *African Journal of Agricultural Research* **4**, 1549-1559

Talukdar D (2009c) Development of cytogenetic stocks through induced mutagenesis in grass pea (*Lathyrus sativus*): Current status and future prospects in crop improvement. *Grain Legume (CSIC, Spain)* **54**, 30-31

Talukdar D (2010a) Reciprocal translocations in grass pea (*Lathyrus sativus* L.). Pattern of transmission, detection of multiple interchanges and their independence. *Journal of Heredity* **101**, 169-176

Talukdar D (2010b) Cytogenetic characterization of induced autotetraploids in grass pea (*Lathyrus sativus* L.). *Caryologia* **63 (1)**, 62-72

Talukdar D (2010c) Allozyme variations in leaf esterase and root peroxidase isozymes and linkage with dwarfing genes in induced dwarf mutants of grass pea (*Lathyrus sativus* L.). *International Journal of Genetics and Molecular Biology* **2 (6)**, 112-120

Talukdar D (2011a) Flower and pod production, abortion, leaf injury, yield and seed neurotoxin levels in stable dwarf mutant lines of grass pea (*Lathyrus sativus* L.) differing in salt stress responses. *International Journal of Current Research* **2**, 46-54

Talukdar D (2011b) Genetics of pod indehiscence in *Lathyrus sativus* L. *Journal of Crop Improvement* **25**, 1-15

Talukdar D (2011c) Effect of arsenic-induced toxicity on morphological traits of *Trigonella foenum-graecum* L. and *Lathyrus sativus* L during germination and early seedling growth. *Current Research Journal of Biological Sciences* **3**, 116-123

Talukdar D (2011d) Cytogenetic analysis of a novel yellow flower mutant carrying a reciprocal translocation in grass pea (*Lathyrus sativus* L.). *Journal of Biological Research - Thessaloniki* **15**, 123-134

Talukdar D (2011e) Bold-seeded and seed coat colour mutations in grass pea (*Lathyrus sativus* L.): Origin, morphology, genetic control and linkage analysis. *International Journal of Current Research* **3**, 104-112

Talukdar D (2011f) Isolation and characterization of NaCl-tolerant mutations in two important legumes, *Clitoria ternatea* L. and *Lathyrus sativus* L.: Induced mutagenesis and selection by salt stress. *Journal of Medicinal Plants Research* **5 (16)**, 3619-3628

Talukdar D (2011g) Morpho-physiological responses of grass pea (*Lathyrus sativus* L.) genotypes to salt stress at germination and seedling stages. *Legume Research* **34 (4)**, 232-241

Talukdar D (2011h) The aneuploid switch: Extra-chromosomal effect on antioxidant defense through trisomic shift in *Lathyrus sativus* L. *Indian Journal of Fundamental and Applied Life Sciences* **1 (4)**, 263-273

Talukdar D (2012a) Flavonoid-deficient mutants in grass pea (*Lathyrus sativus* L.): Genetic control, linkage relationships and mapping with aconitase and S-nitrosoglutathione reductase isozyme loci. *The Scientific World Journal* in press

Talukdar D (2012b) *asfL1*: An ascorbate deficient, semi-dwarf mutant exhibits alterations in ascorbate-glutathione redox status and antioxidant defense in grass pea (*Lathyrus sativus* L.). *Biologia Plantarum* in press

Thang NB, Wu J, Zhou W, Shi C (2010) The screening of mutants and construction of mutant library for *Oryza sativa* cv. Nipponbare via ethyl methane sulphonate inducing. *Biologia* **65 (4)**, 660-669

Tiryaki I, Sttaswick PE (2002) An *Arabidopsis* mutant defective in jasmonate response is allelic to the auxin-signalling mutant *axr1*. *Plant Physiology* **130**, 887-894

Tsyganov VE, Belimov AA, Borisov AY, Safronova VI, Georgi M, Dietz K-J, Tikhonovich IA (2007) A chemically induced new pea (*Pisum sativum*) mutant SGECD with increased tolerance to, and accumulation of cadmium. *Annals of Botany* **99**, 227-237

Van Vilet C, Anderson CR, Cobbett CS (1995) Copper-sensitive mutant of *Arabidopsis thaliana*. *Plant Physiology* **109**, 871-878

Vaz Patto M, Skiba B, Pang E, Ochatt S, Lambein F, Rubiales F (2006) *Lathyrus* improvement for resistance against biotic and abiotic stresses: From classical breeding to marker assisted selection. *Euphytica* **147**, 133-147

Vernoux T, Wilson RC, Seeley KA, Reichheld J-P, Muroy S, Brown S, Maughan SC, Cobbett CS, Montagu MV, Inzé D, May MJ, Sung ZR (2000) The *ROOT MERISTEMLESS/CADMIUM SENSITIVE 2* gene defines a glutathione-dependent pathway involved in initiation and maintenance of cell division during postembryonic root development. *The Plant Cell* **12**, 97-109

Xiang C, Oliver DJ (1998) Glutathione metabolic genes coordinately respond to heavy metals and jasmonic acid in *Arabidopsis*. *The Plant Cell* **10**, 1539-1550

Xing G-S, Cui K-R, Li J, Li J, Wang Y-F, Li Z-X (2001) Water stress and accumulation of β-*N*-Oxalyl-l-α,β-diaminopropionic acid in grass pea (*Lathyrus sativus*). *Journal of Agricultural Food Chemistry* **49 (1)**, 216-220

Yang HM, Zhang XY, Wang GX (2004) Relationships between stomatal character, photosynthetic character and seed chemical composition in grass pea at different water availabilities. *The Journal of Agricultural Science* **142**, 675-681

Zechmann B, Koffler BE, Russell SD (2011) Glutathione synthesis is essential for pollen germination *in vitro*. *BMC Plant Biology* **11**, 54

Zenk MH (1996) Heavy metal detoxification in higher plants – a review. *Gene* **179**, 21-30

Zhang J, Xing GM, Yan ZY, Li ZX (2003) Beta-*N*-oxalyl-L-alpha, beta-diaminopropionic acid protects the activity of glycolate oxidase in *Lathyrus sativus* seedlings under high light. *Russian Journal of Plant Physiology* **50 (5)**, 618-622