ISSN: **0976-3031**

Available Online at http://www.recentscientific.com

International Journal of Recent Scientific Research
Vol. 3, Issue, 4, pp.234 - 243, April, 2012

International Journal of Recent Scientific Research

A GLUTATHIONE-OVERPRODUCING MUTANT IN GRASS PEA (*Lathyrus sativus* L.): ALTERATIONS IN GLUTATHIONE CONTENT, MODIOFICATIONS IN ANTIOXIDANT DEFENSE RESPONSE TO CADMIUM STRESS AND GENETIC ANALYSIS USING PRIMARY TRISOMICS

Dibyendu Talukdar[1]

Department of Botany, R.P.M. college campus, University of Calcutta, Uttarpara, Hooghly 712258, West Bengal, India

ARTICLE INFO

Article History:

Received 12th February, 2012
Received in revised form 20th March, 2012
Accepted 28th March, 2012
Published online 24th April, 2012

Key words:

Glutathione-overproducing mutant, Cadmium, Oxidative stress, *Lathyrus sativus* L, Cadmium tolerance locus, Leaf injury, Primary trisomics

ABSTRACT

A glutathione (GSH)-over producing mutant, *dwf1*, was isolated in grass pea (*Lathyrus sativus* L.). Possible cause of this overproduction and antioxidane defense response to cadmium (Cd) were investigated in nutrient solution, unsupplemented (control) or supplemented with 50 µM $CdCl_2$ and 1 mM L-buthionine-(*S,R*)-sulphoximine (BSO) in different combinations. Compared with mother variety, the mutant exhibited >2.5-fold increase in foliar GSH-content and normal or enhanced activities of antioxidant defense enzymes, enabling it to maintain quite normal growth even under Cd treatment. However, introduction of 1mM BSO in the medium alone or in combination with Cd reduced GSH level 3-3.5-fold in the mutant, confirming that high GSH-biosynthesis was required for its overproduction. The reduction of GSH redox state led to activities of defense enzymes below normal level with an obvious negative impact on growth of the mutant plants, and the effect was more severe in its mother plant. The result indicated central role of GSH played to prevent Cd-induced oxidative stress. Inheritance studies revealed involvement of two different loci, *cad L-1* and *liL-1*, in controlling cadmium tolerance and leaf injury, respectively. Primary trisomic analysis assigned *cadL-1* locus on extra chromosome of grass pea trisomic II in tight linkage with *liL-1* locus and indicated a possible pleiotropy of *cadL-1* locus on glutathione content of grass pea.

INTRODUCTION

Intracellular thiol redox status is a critical parameter in determining plant growth and development in response to continuous production of reactive oxygen species (ROS) (Bashandy *et al*., 2010, Foyer and Noctor, 2011). Among the ROS-scavenging machinery, ascorbate (ASC)-glutathione (GSH) cycle plays pivotal role in plant defense. This cycle, besides ascorbate, is continuously fueled by glutathione (reduced form: GSH; oxidized form: GSSG), the tripeptide γ-glutamylcysteinylglycine, as the principal antioxidant component. In plants, GSH is synthesized enzymatically from its constituent amino acids via two-step ATP-dependent pathway catalyzed by γ-glutamylcysteine synthetase (γ-ECS) and GSH synthetase (GSHS) (Hell and Bergmann, 1990). Owing to the presence of a strong nucleophilic thiol group on its cysteine residue, glutathione interacts with numerous cellular components as an efficient redox buffer, provides valuable information on cellular redox state and most importantly, regulates enzymatic processes in order to maximize defenses against stresses (Bashandy *et al*., 2010; Foyer and Noctor, 2011). It also 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 recycled to GSH by NADPH-dependent action of glutathione reductase (GR). The reduced ascorbate is used as a co-factor by ascorbate peroxidase or APX during scavenging of H_2O_2 (Asada, 2006). The H_2O_2 is one of the prominent oxidants within the cell, and is continuously generated in aerobic cells by photorespiration, activity of superoxide dismutase (SOD) and other reasons. The conjugation of GSH to heavy metals is accomplished by multifunctional enzymes glutathione *S*-tranferases (GSTs) (Dixon *et al*., 2009). Glutathione peroxidases (GPXs) are also involved in removal of organic peroxides and H_2O_2 using GSH or thioredoxins as electron donors (Navrot *et al*., 2006). The GSH/(GSH+GSSG) ratio, considered as GSH redox state, is likely to be far more influential in the antioxidant defense, control of gene expression and thionylation process than the absolute size of the glutathione pool (Noctor *et al*., 2011). The power of mutant analysis in elucidation of role of GSH in growth and development as well as heavy metal tolerance of plants has been studied

* Corresponding author: +91 9791636870
E-mail address: dibyendutalukdar9@gmail.com

in arrays of *Arabidopsis* mutants (Vernoux *et al*., 2000). Surprisingly, mutant identification regarding increased tolerance to heavy metals like cadmium in legume plants have rarely been performed and described to date, presumably because only few mutants showing altered heavy metal tolerance could be obtained (Tsyganov *et al*., 2007).

The value of a particular mutant is enhanced manifold once its genetic basis is elucidated and can be assigned on specific chromosome. The association of a gene to a specific chromosome can be determined from the modified segregation ratios of normal and recessive phenotypes in the diploid portion of F_2 progenies of primary trisomics (Singh, 2003). A complete set of primary trisomics (2n+1; 2n=15) has been identified and characterized in grass pea (2n=14), which along with other cytological tester stocks such as tetrasomics, reciprocal translocation lines and polyploids are now being used to associate a number of morphological mutants to their respective chromosomes (Talukdar, 2008, 2010a, 2009a, Talukdar and Biswas, 2007, 2008). This legume crop is known for its remarkable capacity to withstand various abiotic stresses (Vaz Patto *et al*., 2006; Talukdar, 2009b; Talukdar, 2011d) like salinity (Talukdar, 2011c), drought (Gengsheng *et al*., 2001) and heavy metals such as lead (Brunet *et al*., 2009) and arsenic (Talukdar, 2011b). Several NaCl-tolerant mutant lines have recently been isolated in grass pea through induced mutagenic techniques (Talukdar 2011a). One of the mutants designated as *dwf1* due to its dwarf stature was unique as it contained much higher amount of glutathione than its mother variety. Genetic basis of its dwarfism has been elucidated to some extent (Talukdar, 2009a, 2010b), but the reasons behind GSH over production and the genetic basis of metal tolerance of this mutant remained obscure. Present investigation has, therefore, been undertaken to explore the possible reasons behind the GSH over production by the mutant. Modulation of antioxidant defenses mediated by GSH and its response to cadmium treatment was investigated in the backdrop of GSH-overproduction and also, in the context of its depletion, treating with well known GSH inhibitor BSO, under control and cadmium-treatment. Genetic basis of cadmium tolerance has been ascertained by inheritance studies and primary trisomic analysis.

MATERIALS AND METHODS

Plant materials and growth conditions

Fresh and healthy seeds of *dwf1* plants and its mother control variety (*Lathyrus sativus* L. cv BioR-231) from the last growing season were surface sterilized with 70% ethanol for 2 min, rinsed twice in de-ionized water and then placed on water-moistened filter papers at dark to germinate at 25 °C. Germinated seedlings were immediately placed in polythene pots (10 plants $pots^{-1}$) containing 300 ml of Hoagland's No 2 nutrient media, and were permitted to grow for 15 d. The media were either unsupplemented (control plants) or was supplemented (three treatment sets) with $CdCl_2$, or BSO + $CdCl_2$ or BSO alone. For cadmium (Cd) treatment, 50 µM of $CdCl_2$ was added in the nutrient media. For BSO + $CdCl_2$ treatment, 1mM BSO (Sigma-Aldrich) was added, followed by 50 µM of $CdCl_2$. The concentrations were standardized by preliminary experiments. Seedlings were allowed to grow for another 15 d in six replicates each of control and treated sets. Respective nutrient solution in each set was refreshed in every alternate day to prevent depletion of nutrient as well as Cd and BSO in course of the plant's exposure to them. Seedlings were placed in a growing chamber which was conditioned following the manual of North Central Regional Committee, USA on Controlled Environment Technology (CBE 1994) with little modification for *Lathyrus* (day/night cycle of 14h/10h, at 22 °C, relative humidity of 70% and a photon flux density of 300µmol m^{-2} sec^{-1}). Mother variety and *dwf1* plants submitted to unstressed (0 µM $CdCl_2$) condition were used as mother control (MC) and mutant control (MuC), respectively, while those treated with Cd were designated as treated mother (TM) and treated mutant (MuT), respectively. Plants were harvested at 30 d growth period. Shoots and roots were rinsed thoroughly with sterile distilled water and oven dried at 65 °C for 72 h to weigh dry mass.

Estimation of glutathione contents and thiol synthetase enzyme assay

The concentrations of GSH and GSSG were determined with the enzyme recycling assay (Griffith 1980). Activity of γ-glutamylcysteine synthetase (γ-ECS; EC 6.3.2.2) and GSH synthetase (GSHS; EC 6.3.2.3) was assayed by HPLC quantification of the synthesized GSH as their MBB derivatives, following the methods of Matamoros *et al*., (1999).

Assay of antioxidant enzyme activities

Leaves collected from primary branches of MC, MuC and treated plants were used in biochemical studies. A pool of samples from six plants for each line was collected, and six 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, 2mM cysteine, 1% w/v PVP and 0.2% v/v Triton X-100. SOD (EC 1.15.1.1) activity was determined by the nitro-blue tetrazolium photochemical assay as described by Beyer and Fridovich (1987). The reaction mixture (3ml) contains 50 mM phosphate buffer (pH 7.8), 13 mM methionine, 75 µM NBT, 0.1 mM EDTA, 2µM riboflavin and 0.1 ml of enzyme extract. One unit of SOD was defined as the amount of protein causing a 50% NBT photoreduction. For the APX (EC1.11.1.11) activity, 20 mM ascorbate was added to the extraction buffer. 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). For DHAR (EC 1.8.51), tissue was extracted with 50mM K- phosphate buffer (pH 7.8), 1% PVP-10, 0.2mM EDTA and 10 mM β-mercaptoethanol. DHAR activity was determined by

following ascorbate formation at 265 nm (ε = 14.1 mM^{-1} cm^{-1}) for 3 min (Nakano and Asada, 1981). GR (EC 1.6.4.2) activity was determined with the same extraction medium as for DHAR but without β-mercaptoethanol and with 0.1% Triton X-100. Its activity was measured by monitoring glutathione-dependent oxidation of NADPH at 340 nm (ε = 6.22 mM^{-1} cm^{-1}) for 3 min (Dalton *et al*., 1986). CAT (EC 1.11.1.6) activity was assayed by measuring the disappearance of H_2O_2 at 240 nm (ε = 39.4 M^{-1} cm^{-1}) for 1 min (Aebi, 1984). GST (EC 2.5.1.18) 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 elute equivalent to 100 µg of protein. The reaction was initiated by the addition of 1 mM GSH (Sigma-Aldrich), and formation of S- (2,4-dinitrophenyl) glutathione 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 GR coupled assay to monitor the oxidation of GSH (Edwards, 1996). 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 was measured following the methodology described by Cheeseman (2006). Membrane electrolyte leakage (EL) was assayed by measuring the ions leaching from tissues into deionised water (Dionisio-Sese and Tobita, 1998). The EL was expressed as a percentage by the formula EL (%) = (EC_1) / (EC_2) ×100, where EC_1 is the initial electrical conductivity and EC_2 represents its final value.

Inheritance of cadmium tolerance and leaf injury

To trace the mode of inheritance of cadmium tolerance and leaf injury, the *dwf1* mutant line (M_4) was crossed with its mother control and three other varieties namely 'Hooghly Local', 'BioL-212', and 'B1' (showing Cd sensitivity in the form of reduced fresh and dry weight, high leaf injury, at 50 µM $CdCl_2$ pot culture method, data not presented) in all possible combinations, and F_1 and F_2 progenies were raised, successively. Simultaneously, the F_1 plants were also crossed with respective recessive parents to produce testcross progeny. Cd tolerance of F_1, F_2 and BC_1 progenies was carefully tested by pot nutrient medium supplemented with 50 µM of $CdCl_2$ for each plant following the above said protocol, and recessive phenotype for Cd tolerance was advanced to F_3 generation. A parallel earthen pot (30 cm) method containing mixture of soil: farm yard manure (3:1) and one plant pot^{-1} was also followed for genetic studies with application of 50 µM of $CdCl_2$ thrice a week (30 d) in a growing chamber (CBE, 1994) in triplicate. Leaf injury level in parent and segregating progenies were determined following Talukdar (2011a,d). Segregation data for Cd tolerance/sensitive and leaf injury (present/absent) from each cross were analyzed by $\chi 2$ test (single and joint segregation) to determine goodness-of-fit between observed and expected ratios (Table 2). Reciprocal crosses were made to determine the involvement of cytoplasmic factors of inheritance. Cross over value in percentage (cov%) was calculated from test cross data and incorporating this value in Kosambi's formula (Kosambi, 1944), map distance between linked genes was estimated.

Analysis of Cd tolerance and leaf injury traits in primary trisomics

The response (tolerance/sensitive and presence/absence of leaf injury) of *dwf1* mutant to Cd treatment was tested for association with seven different *Lathyrus* primary trisomics (LTr I-VII) to elucidate the possible chromosomal location of the gene/s controlling these two characters in grass pea. Crosses were made between each of the trisomics as the female parent and the mutant line as the male parent during winter 2009-10 in both pot protocols. Each of the F_1 plants was harvested individually. On the basis of unique leaflet and stipule characters, different trisomic phenotypes in segregating populations could be readily identified at seedling stages (Talukdar and Biswas, 2007), and their chromosome number was confirmed at meiotic metaphase-I. F_1 plants showing disomic phenotype (2n=14) were also identified by meiotic analysis. Trisomic F_2 plants were recovered from the selfed progeny of F_1 in the following season. BC_1 population was raised by crossing trisomic F_1 with corresponding recessive mutant parent. Meiotic chromosome association was studied in F_2 recessive homozygotes and BC_1 plants. In the segregating F_2 and BC_1 progenies, disomic and trisomic plants in different crosses were classified as dominant and recessive phenotype of plant response to Cd (tolerance/sensitive and presence/absence of leaf injury). Segregation of normal and mutant phenotypes in diploid portion of total population was examined by means of the $\chi 2$ test for normal disomic segregation ratio of 3:1 in F_2 and 1:1 for testcross. Significant deviations from this ratio were again tested for trisomic ratio of 8:1 in F_2 and 2:1 in testcross in the diploid portion of population. The modified segregation ratio along with recessive phenotype frequency in the diploid as well as in trisomic progeny was used to locate possible trisomic chromosome/s (critical chromosome) bearing gene/s (Table 3).

Statistical analysis

All values are means (± SE) of at least six independent experiments. Significance differences between control and each treatment was analyzed by Student's *t* test (at $p<0.05$), using software STATISTICA 6.0 (StatSoft, Inc. Tulsa, U.S.A).

RESULTS

Glutathione content, thiol-synthetase enzyme activities and ascorbate level

Compared with mother control, total glutathione content (GSH+GSSG) increased nearly 2.6-fold in leaves of *dwf1* plants (Table 1). GSH constituted 93% of total pool, while GSSG made up rest of the amount. The mother variety contained 96% GSH within the total pool. Upon Cd treatment, total and reduced GSH content had changed marginally in *dwf1* (Table 1). By contrast, GSH share reduced to 76% in TM plants, while GSSG level increased significantly (Table 1). Adding 1 mM BSO in the medium reduced GSH content by 3-(BSO alone) to 3.5-fold (Cd+BSO) in mutant plants and by 3-(BSO alone) to 3.7-fold (Cd+BSO) in mother plants. GSSG share in the mutant changed marginally under Cd+BSO treatment, but it continued to rise in mother plants. Redox state of GSH had changed, accordingly (Table 1).

Table 1 Foliar GSH, GSSG, GSH-redox state, reduced ascorbate (ASC) content and activities of γ-ECS and GSHS in *dwf1*mutant (A) and the mother plants (B) of *Lathyrus sativus* L. under different set of treatments

	Genotype	Control	50 µM $CdCl_2$,	Cd + 1mM BSO	1mM BSO
GSH (nmol g^{-1} FW)	A	704 ± 3.5	698 ± 3.3	199 ± 2.3*	235 ± 2.6*
	B	282 ± 4.2	224 ± 2.2	76 ± 1.6*	95 ± 1.9*
GSSG (nmol g^{-1} FW)	A	47 ± 1.2	50 ± 4.2	59 ± 1.8	30 ± 1.7
	B	10 ± 0.9	70 ± 2.7*	42 ± 1.8*	40 ± 1.2*
GSH redox	A	0.93 ± 2.5	0.93 ± 1.9	0.77 ± 1.3*	0.88 ± 2.7
(GSH/GSH+GSSG)	B	0.96 ± 2.2	0.76 ± 1.2*	0.64 ± 0.9*	0.70 ± 1.1*
ASC (nmol g^{-1} FW)	A	1761 ± 20	1739 ± 19	907 ± 18*	1017 ± 22*
	B	1842 ± 23	1136 ± 18	288 ± 10*	550 ± 18*
γ-ECS (nmol min^{-1} g^{-1}FW)	A	20.2 ± 2.0	19.8 ± 2.0	2.8 ± 1.0*	2.8 ± 1.4*
	B	6.6 ± 1.7	6.8 ± 1.1	1.9 ± 1.6*	1.9 ± 1.0*
GSHS (nmol min^{-1} g^{-1}FW)	A	27.3 ± 3.9	27.0 ± 3.0	3.2 ± 2.8*	2.9 ± 2.0*
	B	10.8 ± 1.8	9.9 ± 1.1	1.2 ± 1.8*	1.1 ± 1.0*

Data are mean ± SE (n = 6). Asterisks (*) denote the significant differences with respective control value at $p<0.05$. Control-nutrient media, seedlings were grown for 15 d under treatments.

Significant differences ($P<0.05$) were observed between MuC and MC plants in measurable activities of γ-ECS and GSHS (Table 1). Activity levels of both the enzymes showed an increase of 2.5-3-fold in leaves of MuC plants over MC under un-treated condition, and it was remained unperturbed in response to Cd treatment. Although marginal activity of GSHS was detected, BSO treatment (alone or Cd+BSO) reduced γ-ECS activity to a negligible level (Table 1). Compared with control, ASC content was nearly normal in Cd-treated mutant, but decreased significantly (about 1.9-fold) in mutant under Cd+BSO treatment. Reduction was more severe in mother plants exposed to Cd treatment and plummeted further (about 6.4-fold) after Cd+BSO treatment. Recovery of ASC content was observed in both plant types when BSO was added alone in the medium (Table 1).

Antioxidant enzyme activities, and H_2O_2, MDA and electrolyte leakage (EL%)

Activities of SOD, APX, DHAR, GR, GST, CAT and GPX in MuC plants were very close to MC plants (Figs. 1, 2). Upon imposition of Cd in the medium, APX, DHAR and GST levels increased by about 2-3-fold in MuT plants but decreased significantly in TM plants (Figs.1A, B, 2A) from its control values. Activities of GR and GPX were quite normal in MuT plants, but became low in TM plants (Figs. 1C, 2B). SOD and CAT activities in MuT plants changed non-significantly with MC value, but the CAT level increased by about 2.8-fold in TM plants (Figs. 1D, 2C). After Cd + BSO treatment, SOD level increased significantly in MuT (2-fold) and TM plants (2.8-fold). GR and GPX activities also increased by nearly 3-fold and 4.2-fold, respectively in MuT plants but decreased in TM plants (Figs. 1C, 2B). Activities of APX, DHAR, GST, and CAT declined in both mother and mutant plants but with higher magnitude in mother plants than the mutant. After BSO added alone, SOD and CAT reversed to its normal level, while others (except decrease in GPX level in mutant) followed similar trend like Cd + BSO treatment in mutant and mother plants (Figs. 1, 2).

Foliar H_2O_2 content and MDA level in mutant plants remained normal in Cd -treated mutant, but increased significantly after BSO was added alone or in combination with Cd in the medium (Table 2). Both the values in TM plants significantly exceeded its normal (MC) level in all sets of experiments. EL% had changed, accordingly (Table 2).

Dry Matter Accumulation and Leaf Injury Level

Not much change in shoot and root dry weight from control values was observed in mutant plants submitted to Cd treatment. Both values, however, declined significantly in mutant plants exposed to Cd+BSO and BSO treatments, showing more reduction in former treatment condition than the latter (Table 2). Compared with MC, the value in mother plants declined substantially under Cd treatment, and plummeted further once BSO was introduced in the medium (Table 2). Leaf injury level was 0 (absent as in control) in Cd-treated mutant, but elevated to 3 under Cd+BSO and again reduced to level 1 after BSO treatment alone. Injury level was 2 in Cd-treated mother plants and it increased to 4 after Cd+BSO treatments, but returned to level 1 under BSO treatment.

Table 2 Changes in H_2O_2 contents, lipid peroxidation (MDA), electrolyte leakage (EL%), dry weight of shoot and root of *dwf1*mutant (A) and the mother plants (B) of *Lathyrus sativus* L. under different set of treatments

Parameters	Genotype	Control	50 µm $CdCl_2$	Cd + 1mM BSO	1mM BSO
H_2O_2 (µmol g^{-1} FW)	A	2.44 ± 1.1	2.47 ± 1.4	4.19 ± 1.1*	3.08 ± 1.1*
	B	2.39 ± 1.2	3.12 ± 1.1*	4.38 ±1.1*	4.18 ± 1.2*
MDA (nmol g^{-1} FW)	A	2.53 ± 1.3	2.57 ± 1.1	3.53 ± 1.4*	3.10 ± 1.0*
	B	2.69 ± 2.3	3.03 ± 2.8*	3.89 ± 3.1*	3.80 ± 2.4*
EL%	A	8.0 ± 2.9	8.2 ± 2.3	12.9 ± 2.9*	10.8 ± 2.2*
	B	8.6 ± 2.7	19.8 ± 1.9*	22.9 ± 1.6*	21.9 ± 2.7*
Shoot dry weight (g $plant^{-1}$)	A	0.155 ± 0.01	0.153 ± 0.01	0.117± 0.02*	0.129 ± 0.01*
	B	0.160 ± 0.03	0.101 ± 0.05*	0.080 ± 0.05*	0.081± 0.01*
Root dry weight (g $plant^{-1}$)	A	0.179 ± 0.10	0.173 ± 0.10	0.110 ± 0.12*	0.109 ± 0.11*
	B	0.189 ± 0.18	0.110 ± 0.13*	0.077 ± 0.10*	0.081 ± 0.09*

Data are mean ± SE (n = 6). Asterisks (*) denote the significant differences with respective control value at $p<0.05$. Control-nutrient media, seedlings were grown for 15 d under treatments after 15 d growth in nutrient media.

Table 3 Segregation of cadmium tolerant and sensitive phenotypes in F_2 and BC_1 generations of crosses between *dwf1* and seven different primary trisomics (LTr types I-VII) of grass pea (*Lathyrus sativus* L.). For leaf injury, cross between *dwf1*× Tr II (bearing critical trisomic chromosome) has been presented only

Trisomic F_1	F_2 and BC_1 phenotype[b]									
	2n		Total	χ^2				2n+1		
	Sensitive	Tolerant		(1:1)	(2:1)	(3:1)	(8:1)	Sensitive	Tolerant	Total
(*dwf 1* × type I) selfed	101	30	131	-	-	0.11	20.88***	22	07	29
(*dwf 1*× type I) × *dwf1*	31	27	58	0.28	4.56*	-	-	10	02	12
(*dwf 1* × type II)[a] selfed	171	22	193	-	-	19.04***	0.02	49	00	49
(*dwf 1* × type II)[a] × *dwf1*	86	40	126	16.79***	0.14	-	-	11	00	11
(*dwf 1* × type III) selfed	69	21	90	-	-	0.13	13.61***	60	18	78
(*dwf 1* × type III) × *dwf1*	53	43	96	1.04	5.67*	-	-	17	04	21
(*dwf 1* × type IV) selfed	117	35	152	-	-	0.32	21.85***	50	15	65
(*dwf 1* × type IV) × *dwf1*	55	47	102	0.64	7.46**	-	-	18	08	26
(*dwf 1* × type V)selfed	145	45	190	-	-	0.18	30.42***	70	16	86
(*dwf 1* × type V) *dwf1*	41	36	77	0.32	6.24*	-	-	22	07	29
(*dwf 1* × type VI) selfed	71	22	93	-	-	0.09	14.83***	37	11	48
(*dwf 1* × type VI) × *dwf1*	46	40	86	0.42	6.72**	-	-	15	03	18
(*dwf 1* × type VII) selfed	98	30	128	-	-	0.32	17.55***	33	09	42
(*dwf 1* × type VII) × *dwf1*	26	22	48	0.33	9.38**	-	-	19	02	21
Leaf injury	Absent	Present	Total	F_2/BC_1				Absent	Present	total
(*dwf 1* × type II)[a] selfed	100	13	113	-	-	10.98***	0.01	22	00	22
(*dwf 1* × type II)[a] × *dwf1*	75	39	114	11.36***	0.03	-	-	13	00	13

* significant at 0.05 probability level,** significant at 0.01level and *** significant at 0.001 level; [a]-critical chromosome bearing trisomic; [b]-data pooled of two pot (plastic and earthen) methods (nutrient media+ Cd and soil mixtures+Cd, respectively)

Inheritance of Cd tolerance and leaf injury in *dwf1* mutant and glutathione content

Direct and reciprocal crosses between *dwf1* and four other varieties yielded F_1 plants, exhibiting reduced dry weight and high level (level 4) of leaf injury under Cd treatment in both plastic pot culture (nutrient media) and earthen pot (soil mixtures) methods. The characters, however, normal dry weight and absence of leaf injury (level 0) in 136 plants, reduced dry weight and leaf injury in 156 plants, normal dry weight but leaf injury in 30 and reduced dry weight with absence of leaf injury in 33 cases in a total of 355 plants, strongly ($\chi 2=864.61$, $P<0.05$) deviating from normal Mendelian dihybrid ratio of 9:3:3:1. Production of normal dry weight was taken as a criterion to ascertain tolerant phenotype, while reduced

dry weight was assessed as sensitive phenotype. Segregation of tolerant vs. sensitive phenotype (total of 100 vs. 313 plants in F_2 and 129 vs 138 in test cross) and leaf injury (absence vs. presence, 32 vs. 90 in F_2 and 34 vs.40 in test cross) in four crosses (*dwf1* × four varieties), however exhibited good fit ($\chi 2$=0.13, 0.09 for F_2, $\chi 2$=0.30, 0.48 for test cross at 1df, P<0.05) to the monohybrid 1:3 ratio in the F_2 generation and 1:1 in corresponding testcrosses (F_1× mutant) progenies, indicating complete dominance of Cd-sensitivity and presence of leaf injury over Cd-tolerance and absence of leaf injury. A designation of *CadL-1* (cadmium sensitive locus 1 in *Lathyrus*) for controlling Cd-sensitivity and *cadL-1* for Cd-tolerance and *LiL-1*/*liL-1* for presence/absence of leaf injury have been proposed for the preset study. In test cross of joint segregations derived from *dwf1*× four varieties, the four phenotypes (recovered in dihybrid F_2 progeny) appeared in 275, 300, 12 and 11 plants, respectively, showing significant deviation ($\chi 2$=511.61, P<0.05) from normal 1:1:1:1 ratio. Cov% (3.8%), calculated from test cross data, were incorporated into Kosambi's formula (Kosambi, 1944) and map distance between *cadL-1* and *liL-1* loci was estimated as 3.41cM. F_2 plants showing recessive phenotype were self-pollinated and the progeny plants (210) were true breeding for Cd-tolerant phenotypes in F_3 progeny of mutant lines (data not in table).

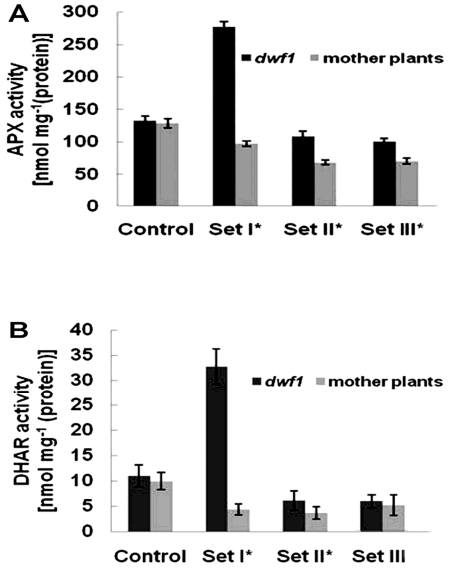

Fig. 1A, B

Among the four phenotypes recovered in F_2, plants of two parental types, namely normal dry weight and absence of leaf injury and reduced dry weight with leaf injury showed non-significant changes in total and reduced glutathione content in their leaves in comparison to *dwf1* mutant and control varieties, respectively. However, recombinant plant type with normal dry weight and leaf injury had GSH level (710 ± 4.2 nmol g^{-1} FW) close to *dwf1* mutant whereas plant with reduced dry weight and absence of leaf injury had GSH level (276 ± 1.9 nmol g^{-1} FW) nearly similar to control varieties.

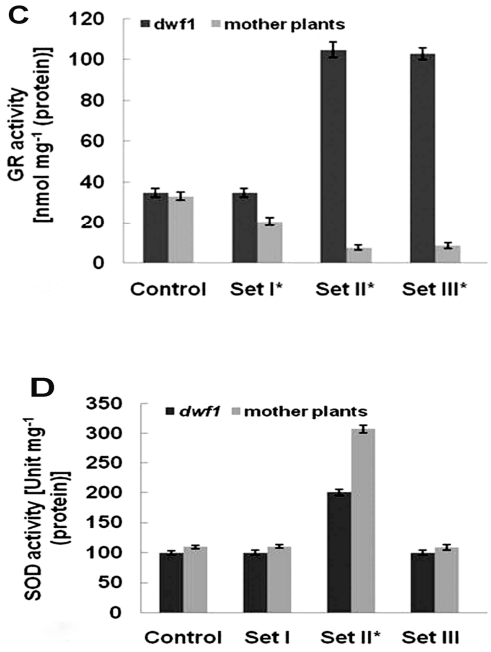

Fig. 1C, D

Segregation of *cadL-1* and *liL-I* loci in primary trisomics of grass pea

Linkage tests were conducted to study the association of *cadL-1* and *liL-1* with the extra chromosome of each of the seven different primary trisomics (LTr I-VII) isolated in grass pea. The results given in table 3 indicated that plants with recessive phenotype appeared in much less frequency in the diploid population of F_2 progenies obtained from the mutant × LTr-II than the other cross combinations between varieties and rest of the trisomic types. Segregation of Cd-sensitive (dominant) and tolerant (recessive) phenotypes and absence and presence of leaf injury in this cross combination showed significant deviations (P<0.001) from the expected normal disomic ratio of 3:1; however, there was a good fit to the expected trisomic ratio of 8:1 in F_2. Segregation in BC_1 populations also deviated from the expected disomic ratio of 1:1, but fitted well to the trisomic ratio of 2:1 (Table 3). None of the recessive homozygote plants was traced in the trisomic portion in this particular cross (Table 3), and all the recessive homozygotes recovered in the population were found to be diploids possessing 2n=14 chromosomes. On the other hand, segregation of dominant and recessive type in crosses between the mutants and rest of the trisomics showed significant deviations from the expected 8:1 ratio, but fitted well a normal disomic ratio of 3:1 in F_2 and 1:1 in corresponding testcrosses in the diploid population (Table 3, not shown for leaf injury). This was expected as frequency of recessive mutant plants was much higher in these crosses than in the former cross where trisomic II was involved. Also, an appreciable number of recessive homozygotes in 2n+1 portion of these crosses represented trisomic plants carrying one extra chromosome (2n+1=15) in their gametic complement.

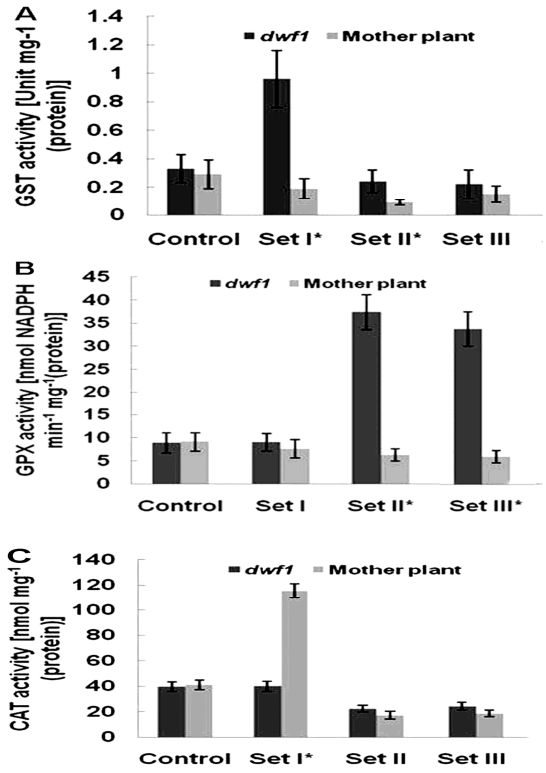

Fig. 2A, C

DISCUSSION

For the first time, a glutathione-overproducing mutant showing tolerance to Cd was isolated in grass pea. The over accumulation of glutathione, as confirmed by repeated measurements, was mainly due to significantly high activity of both γ-ECS and GSHS enzymes, involved in GSH biosynthesis. This was evidenced by inhibition of γ-ECS activity after addition of BSO in the medium and concomitant decrease in GSH level. It is worth mentioning that elevated GSH content led to high redox state (0.93) of glutathione in the mutant, and it was not perturbed even under high dose of Cd treatment. The γ-ECS is the principal but a rate-limiting enzyme in the committed first step of GSH-synthesis and its activity is known to be regulated by GSH through feedback inhibition (Hell and Bergmann, 1990, Jez *et al*., 2004) and also, by induction of stresses (Gómez *et al*., 2004). The consistency of foliar GSH levels in the present *dwf1* plants under control and Cd treatments suggested constitutive over-production of this thiol molecule, which was neither affected by feedback inhibition, nor was induced by elevated doses of Cd treatment. Although total glutathione content was close to MC value, GSH level and its redox state decreased significantly in Cd-treated mother plants. Transgenic tobacco plants showing enhanced glutathione biosynthetic capacity reportedly exhibited chlorotic/necrotic phenotypes as the consequences of oxidative stress (Creissen *et al*., 1999). Absence of such phenotypes and quite normal growth of *dwf1* plants even under high Cd concentration strongly contradicted this phenomenon in the present material. Within ASC-GSH cycle, high GSH levels in the mutant accelerated DHAR activity, which was then capable to supply enough ascorbate in its reduced form for efficient functioning of APX. Usual regeneration of GSH, however, was maintained by normal level of GR, which effectively used GSSG to adjust GSH redox pool within a certain range and thus, coped unexpectedly well with the massively increased GSH levels in the mutant. High GSH level also enhanced GST activity as this enzyme predominantly uses GSH as substrate in conjugation and subsequent removal of toxic components. This together with normal level of SOD and CAT led to efficient scavenging of ROS, preventing accumulation of excess H_2O_2 and membrane damage by lipid peroxidation in the Cd-treated mutant plant. Consequently, these ensured good growth as suggested by its quite normal root and shoot dry weight and conspicuous absence of leaf injury. The cascading effect of low GSH level in mother plants was felt in disruption of DHAR-GR-APX functions within ASC-GSH cycle. Low level of GPXs and GST simply aggravated the situation, and upregulation of CAT had no match to cope with rising level of H_2O_2. Presumably, this led to high percentage of membrane leakage due to increased lipid peroxidation (MDA content) with an obvious negative impact on growth (reduced dry weight and high leaf injury level) of Cd-treated mother plants.

After addition of BSO (alone or in combination with Cd), the fall of GSH level in the mutant plant was obvious. Remarkably, the GR activity was at control level in the mutant when Cd was imposed alone in the medium, but it accelerated to a significant level in Cd+BSO and BSO-treated mutant. This suggested induction of GR activity in response to depleting GSH biosynthesis in the mutant plants, and subsequently, led to higher GSH-regeneration capacity of the mutant than its mother plant, preventing huge fall of GSH redox state and loss of GSSG as a valuable substrate. Importance of GR in metabolism of intracellular H_2O_2 metabolism has been studied by *Arabidopsis* GR1 mutant (Mhamdi *et al*., 2010). The situation, however, was completely opposite in the present mother plants, where reducing GR level led to lowering of GSH redox state alarmingly with concomitant increase in GSSG level in leaves. The rising GSSG level is often taken as a marker of the degree of intracellular oxidative stress, also referred to as 'disulphide stress' (Queval *et al*., 2011). This type of stress was not severe in *dwf1* mutant, despite the fact that GSH deficiency (due to BSO treatment) under Cd treatment (Cd+BSO) not only crippled normal functioning of DHAR, APX and GSTs but also led to increase in SOD activity in the mutant. The rise in H_2O_2, lipid peroxidation product and percentage of electrolyte leakage in Cd+BSO-treated mutant had a negative impact on growth of mutant plants, but the situation was not as critical as the mother plants experienced. GSH-deficiency completely crippled antioxidant defense of the mother plants, and the situation got worse when SOD level (thus H_2O_2 generation) elevated but activity of CAT declined after addition of BSO. As CAT requires no reducing power to decompose H_2O_2, it plays a pivotal role in H_2O_2-scavenging

particularly when ASC-dependent APX is not fully functional due to paucity of co-factors as reported in an ASC-deficient *asfL-1* mutant of grass pea (Talukdar, 2011e).

The cascading effect of GSH-deficiency due to BSO treatment was also felt in regeneration capacity of ascorbate by DHAR. This was evidenced by quite normal level of ASC in Cd-treated mutants, showing increased DHAR activity, but by its sharp decline after imposition of Cd+BSO treatment. Apparently, low GSH availability after BSO treatment and increasing consumption of GSH molecule due to Cd treatment impeded normal functioning of DHAR, badly hampering ASC recycling process. Slight recovery of ASC pool in BSO (alone)-treated mutant, however, might be attributed to absence of Cd in the medium, facilitating limited available GSH to be used by DHAR. Further study, however, is needed to reveal the exact mechanism behind the recovery of ASC pool in BSO-treated mutant.

A simple monogenic recessive nature in inheritance of Cd-tolerance and absence of leaf injury phenotype has been revealed by genetic studies in the preset material of grass pea. Linkage studies and primary trisomic analysis assigned *cadl-1*, the locus responsible for Cd-tolerance, and *liL-1*, governing absence of leaf injury in Cd-treated mutant plant, on extra chromosome of grass pea trisomic II in closely linked state. Generally, presence or absence of leaf injury was studied as a symptom of oxidative stress in a plant. For the first time, two different genes have been detected in a legume crop for Cd-tolerance and leaf injury and assigned on specific chromosome. Remarkably enough, the high level of glutathione was always associated with tolerant phenotype (normal dry weight of shoot and root), but not with leaf injury, as found when total and reduced glutathione level was measured high in two types of F_2 and test cross progeny plants: tolerant with no injury (*cadL-1 cadL-1liL-1liL-1*, parental phenotype) and tolerant but with leaf injury (*cadL-1 cadL-1 LiL-1 Lil-1*, recombinant type). Additional evidence of this concept came from another type of recombinant plant, which exhibited Cd-sensitive phenotype (reduced dry weight) and complete absence of leaf injury (*CadL-1CadL-1 liL-1liL-1*) with no increase in foliar GSH level. The total and reduced glutathione content in leaves of this recombinant type was close to parental (mother variety) type-Cd-sensitive with high leaf injury (*CadL-1CadL-1LiL-1LiL-1*). Also, there was no recombinant type which showed high glutathione level but Cd-sensitive phenotype and vice versa. Therefore, it seems likely, that *cadL-1* locus has a pleiotropic effect on glutathione content in the present grass pea mutant line. The location of *cadL-1* locus on extra chromosome of LTr II also indicated that it was inherited independently with *df1* locus, responsible for typical *dwf1* dwarf phenotype in grass pea and was mapped on extra chromosome of trisomic I along with other morphological and isozyme markers (Talukdar, 2009a, 2010). Successful use of primary trisomics in locating *cadL-1* and *liL-1* on specific chromosome in the present study again confirmed role of grass pea trisomics as handy cytogenetic tool, the dosage effect of which has recently been exploited to reveal extra-chromosomal effect on antioxidant defense response (Talukdar, 2012).

Overproduction of GSH molecule in plant system has several commercial and academic implications (Noctor *et al*., 1998), and is potentially an interesting system to analyze the factors underlying higher glutathione contents and its consequences on plant metabolism. The present *dwf1* mutant is unique as it not only has the ability to overproduce GSH but is also capable to withstand Cd-induced oxidative stress without hampering GSH pools. Furthermore, it is clear that GSH-biosynthesis is required for its overproduction, and GSH is the key molecule in antioxidant defense of grass pea plant against Cd-induced oxidative stress. The monogenic simple inheritance of Cd-tolerance and leaf injury phenotype, their location on same chromosome in linked state and association of high GSH pool always with Cd-tolerant phenotype may be exploited for further analysis of genetic and intrinsic biochemical mechanisms of heavy metal tolerance in grass pea.

References

Aebi, H., 1984. Catalase *in vitro*. Methods in Enzymology, 105:121-126.

Asada, K., 2006. Production and scavenging of reactive oxygen species in chloroplasts and their functions. Plant Physiol., 141:391-396.

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. Plant Cell, 22:376-391.

Beyer, W.F. and Fridovich, I. 1987. Assaying for superoxide dismutase activity: some large consequences of minor changes in conditions. Ana. Biochem., 161:559-566.

Brunet, J., Varrault, G., Zuily-Fodil, Y. and Repellin, A. 2009. Accumulation of lead in the roots of grass pea (*Lathyrus sativus* L.) plant triggers systematic variation in gene expression in the shoots. Chemosphere, 77:1113-1120.

Cheeseman, J.M., 2006. Hydrogen peroxide concentrations in leaves under natural conditions. J. Exp. Bot., 10:2435-2444.

Council of Biology Editors, 1994. Scientific Style and Format: The CBE manual for authors, editors and publishers, Ed 6, CBE, Cambridge University Press, NY. Pp. 434-436.

Creissen, G., Firmin, J., Fryer, M., Kular, B., Leyland, N., Reynolds, H., Pastori, G., Wellburn, F., Baker, N., Wellburn, A. and Mullineaux, P. 1999 Elevated glutathione biosynthetic capacity in the chloroplasts of transgenic tobacco plants paradoxically causes increased oxidative stress. Plant Cell, 11:1277-1291.

Dalton, D.A., Russell, S.A., Hanus, F.J., Pascoe, G.A. and Evans, H.J. 1986. Enzymatic reactions of ascorbate and glutathione that prevent peroxide damage in soybean root nodules. Proc. Natl. Acad. Sci. USA, 83:3811-3815.

Dionisio-Sese, M.L. and Tobita, S. 1998. Antioxidant responses of rice seedlings to salinity stress. Plant Sci., 135:1-9.

Dixon, D.P., Hawkins, T., Hussey, P.J. and Edwards, R. 2009. Enzyme activities and subcellular localization of members of the *Arabidopsis* glutathione transferase superfamily. J. Exp. Bot., 60:1207-1218.

Edwards, R., 1996. Characterization of glutathione transferases and glutathione peroxidases in pea (*Pisum sativum*). Physiol. Plant., 98:594-604.

Foyer, C.H. and Noctor, G. 2011. Ascorbate and glutathione: the heart of the redox hub. Plant Physiol., 155:2-18.

Gengsheng, X., Kairong, C., Ji, L., Yafu, W. and Zhixio, Li. 2001. Water Stress and accumulation of β-N-Oxalyl-l-α,β-diaminopropionic acid in grass pea (*Lathyrus sativus*). J. Agric. Food Chem., 49:216–220.

Gómez, L.D., Vanacker, H., Buchner, P., Noctor, G. and Foyer, C.H. 2004. Intercellular distribution of glutathione synthesis in maize leaves and its response to short-term chilling. Plant Physiol. 34:1662-1671.

Griffith, O. W., 1980. Determination of glutathione disulphide using glutathione reductase and 2-vinylpyridine. Ana. Biochem, 106: 207-212.

Hell, R. and Bergmann, L. 1990. γ-Glutamylcysteine synthetase in higher plants: catalytic properties and subcellular localization. Planta, 180:603-612.

Jez, J.M., Cahoon, R.E. and Chen, S. 2004. *Arabidopsis thaliana* glutamate-cysteine ligase. Functional properties, kinetic mechanism, and regulation of activity. J. Biol. Chem., 279:33463-33470.

Kosambi, D. D., 1944. The estimation of map units from recombination values. Ann. Eugen. (London), 12: 172-175.

Li, Z-S., Zhen R-G. and Rea, P.A. 1995. 1-chloro-2, 4-dinitrobenzene-elicited increase in vacuolar glutathione-S-conjugate transport activity. Plant Physiol., 109:177-185.

Matamoros, M.A., Moran, J.F., Iturbe-Ormaetxe, I., Rubio, M.C. and Becana, M. 1999. Glutathione and homoglutathione synthesis in legume root nodules. Plant Physiol., 121:879-888.

Mhamdi, A., Hager, J., Chaouch, S., Queval, G., Han, Y., Taconna, L., Saindrenan, P., Gouia, H., Issakidis-Bourguet, E., Renou, J-P. and Noctor, G. 2010. Arabidopsis GLUTATHIONE REDUCTASE 1 is essential for the metabolism of intracellular H2O2 and to enable appropriate gene expression through both salicylic acid and jasmonic acid signaling pathways. Plant Physiol., 153: 1144-1160.

Nakano, Y. and Asada, K. 1981. Hydrogen peroxide is scavenged by ascorbate specific peroxidase in spinach chloroplast. Plant Cell Physiol., 22:867-880.

Navrot, N., Collin, V., Gualberto, J., Gelhaye, E., Hirasawa, M., Rey, P., Knaff, D.B., Issakidis, E., Jacquot, J.P. and Rouhier, N. 2006. Plant glutathione peroxidases are functional peroxiredoxins distributed in several subcellular compartments and regulated during biotic and abiotic stresses. Plant Physiol., 142:1364-1379.

Noctor, G., Arisi, M A-C., Jouanin, L. and Foyer, C.H. 1998. Manipulation of glutathione and amino acid biosynthesis in the chloroplast. Plant Physiol., 118:471-482.

Queval, G., Jaillard, D., Zechmann, B. and Noctor, G. 2011. Increased intracellular H_2O_2 availability preferentially drives glutathione accumulation in vacuoles and chloroplasts. Plant, Cell Environ., 34:21-32.

Saher, S., Piqueras, A., Hellin, E. and Olmos, E. 2004. Hyperhydricity in micropropagated carnation shoots: the role of oxidative stress. Physiol Plant., 120:152-161.

Singh, R. J., 2003 Plant Cytogenetics. Second edition, CRC Press, Boca Raton, Florida

Talukdar, D. and Biswas, A.K. 2007. Seven different primary trisomics in grass pea (*Lathyrus sativus* L.). I Cytogenetic characterization. Cytologia, 72: 385-396.

Talukdar, D. and Biswas, A.K. 2008. Seven different primary trisomics in grass pea (*Lathyrus sativus* L.).II. Pattern of transmission. Cytologia, 73:129-136.

Talukdar, D., 2008. Cytogenetic characterization of seven different primary tetrasomics in grass pea (*Lathyrus sativus* L.). Caryologia, 61:402-410.

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

Talukdar, D., 2009b. Recent progress on genetic analysis of novel mutants and aneuploid research in grass pea (*Lathyrus sativus* L.). Afric. J. Agric. Res., 4(13):1549-1559.

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. 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, 2:112-120.

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. Effect of arsenic-induced toxicity on morphological traits of *Trigonella foenum-graecum* L. and *Lathyrus sativus* L during germination and early seedling growth. Current Reserach Journal of Biological Science, 3:116-123.

Talukdar, D., 2011c. 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: 3619-3628.

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

Talukdar, D., 2011e. *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'.

Talukdar, D., 2012. The aneuploid switch: Extra-chromosomal effect on antioxidant defense through trisomic shift in *Lathyrus sativus* L. Indian Journal of Fundamental and Applied Life Sciences, 'in press'

Tsyganov, V.E., Belimov, A.A., Borisov, A.Y., Safronova, V.I., Georgi, M., Dietz, K-J. and Tikhonovich, I.A. 2007. A chemically induced new pea (*Pisum sativum*) mutant SGECD with increased tolerance to, and accumulation of cadmium. Annals of Botany, 99: 227-237.

Vaz Patto, M.C., Skiba, B., Pang, E., Ochatt, S., Lambein, F. and 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, R.C., Seeley, K.A., Reichheld, J-P., Muroy, S., Brown, S., Maughan, S.C., Cobbett, C.S., Montagu, M.V., Inzé, D., May, M.J. and Sung, Z.R. 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. Plant Cell, 12:97-109.

****