Environmental and Experimental Biology (2014) 12: 73–81 *Original Paper*

A common bean (*Phaseolus vulgaris*) mutant with constitutively low cysteine desulfhydrase activity exhibits growth inhibition but uniquely shows tolerance to arsenate stress

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Abstract

A mutant designated as *pvcys* exhibiting huge deficiency in foliar L-cysteine desulfhydrase and D-cysteine desulfhydrase activity were isolated from an ethylmethane sulfonate-mutagenized M_2 population of a *Phaseolus vulgaris* L. genotype VL 63. The mutant showed growth inhibition and morpho-agronomic anomalies but exhibited high cysteine content and very low endogenous hydrogen sulfide concentration mainly due to crippling of cysteine degradation. Despite a normal glutathione and ascorbate redox pool, the mutant suffered oxidative stress due to over-accumulation of H_2O_2 and consequent membrane damage by lipid peroxidation. Uniquely, this oxidative load was relieved in the mutant upon imposition to 20 and 40 μM sodium arsenate through consumption of excess cysteine to meet the growing demand for glutathione and subsequently, to confer tolerance to arsenate-induced oxidative stress. Both L-cysteine desulfhydrase and D-cysteine desulfhydrase activity was constitutively low in the mutant, even in response to external stress. The *pvcys* mutation was monogenic recessive in inheritance.

Key words: arsenic, common bean, cysteine desulfhydrase, ethyl methane sulfonate, mutagenesis.
Abbreviations: As, arsenic; ASA, reduced ascorbate; Cys, cysteine; DCD, D-cysteine desulfhydrase; DHA, dehydroascorbate; EL, electrolyte leakage; EMS, ethyl methane sulfonate; LCD, L-cysteine desulfhydrase; GSSG, glutathione disulfide; GSH, reduced glutathione; MDA, malondealdehyde; N, nitrogen; NaOCl, sodium hypochlorite; ROS, reactive oxygen species; WT, wild type.

Introduction

Sulfur is a critical nutrient for metabolism, plant growth and development. It represents the ninth and least abundant essential macronutrient in plants (Höfgen, Hesse 2008). Cysteine (Cys) is the first stable and committed molecule in plant metabolism that contains both sulfur and nitrogen. It is the metabolic precursor for vital cellular components containing reduced S, reduced glutathione (GSH), homoglutathione, iron–sulfur clusters, vitamin cofactors like biotin and thiamin, and multiple secondary metabolites (Kopriva 2006; Davidian, Kopriva 2010; Takahashi et al. 2011; Birke et al. 2012; Kopriva et al. 2012). GSH is the most abundant low-molecular weight downstream thiol-metabolite with a plethora of functions in plant stress defense, hormone signaling, redox regulation, sexual plant reproduction and S homeostasis (Noctor et al. 2012; Traverso et al. 2013). The thiol of GSH is often involved in the redox cycle by thiol disulfide conversions. Versatility of this interchange for redox control has been demonstrated during salinity stress, detoxification of xenobiotics, exposure to heavy metals and metalloids and against biotic strains (Herschbach et al. 2010; Galant et al. 2011; Hossain, Komatsu 2012; Noctor et al. 2012; Fatma et al. 2013;Talukdar 2012a-d, 2013c; Talukdar, Talukdar 2014a).

Cellular control of Cys-homeostasis through its synthesis and degradation/consumption is of paramount importance for cells responding environmental stress (Park, Imlay 2003; Krueger et al. 2009; Álvarez et al. 2010). Studies have indicated that most of the endogenously synthesized hydrogen sulfide occurrs through the desulfuration of L-Cys and D-Cys by L-cysteine desulfhydrase (LCD) and D-cysteine desulfhydrase (DCD), respectively, rather than promoting Cys biosynthesis in high plants (Álvarez et al. 2010). An Arabidopsis mutant crippled in LCD activity exhibited premature leaf senescence along with increased Cys level and enhanced expression of senescence-associated genes and transcription factors (Álvarez et al. 2010). Rapeseed (*Brassica napus*) is able to react to fungal infection and releases H_2S as a result of increased LCD activity (Bloem et al. 2004). However, short-term exposure of *Brassica oleracea* to a high level of H_2S was observed to result in a decrease in sulfate reduction activity in the shoot, and also an increase in the thiol and Cys content in the shoot and root (Westerman et al. 2000). While depleting Cys level

escalates stress sensitivity (Lopez-Martin et al. 2008), free Cys, but not GSH, above a certain concentration threshold reportedly has the ability to exacerbate the prooxidant properties due to high reactivity of its thiol moiety (Park, Imlay 2003; Krueger et al. 2009). Thus, under non stress conditions, Cys levels should remain low (Álvarez et al. 2012).

Arsenic (As) is a ubiquitous toxic and carcinogenic metalloid (Gupta et al. 2008; Talukdar 2011, 2013a; Tripathi et al. 2012). Common bean (*Phaseolus vulgaris* L.) is a widely grown antioxidant-rich commercial food legume, but the crop is highly sensitive to As (Stoeva et al. 2005; Talukdar 2013b). As-induced oxidative damage and subsequent inhibition of growth has been reported in many other legume crops, like pea, chickpea, lentil, mung bean, and grass pea (Ahmed et al. 2006; Gupta et al. 2008; Talukdar 2013d, 2014). Being grown in aerobic fields, legumes are usually exposed to the arsenate (As V) form of As, which either directly or through conversion to highly toxic arsenite (As III) adversely affects plant growth by generating excess reactive oxygen species (ROS) and consequent oxidative damage to membrane structure and function (Finnegan, Chen 2012; Talukdar 2013e). The role of S-metabolisms and the Cys pool in modulation of GSH-mediated entire antioxidant defense subjected to arsenate stress has been demonstrated in rice, brassica, sunflower and lentil genotypes differing in tolerance (Chakraborty et al. 2009; Srivastava et al. 2009; Talukdar, Talukdar 2014b). Utilization of novel mutants in deciphering the intrinsic nature of GSH-mediated cellular defense during stress response has nicely been demonstrated in the model plant *Arabidopsis thaliana*, and in food legumes, like *Phaseolus vulgaris, Pisum sativum, Lens culinaris*, and *Lathyrus sativus* (Tsyganov et al. 2007; Talukdar 2012a, b; Talukdar and Talukdar 2013a,b). In the present study, two plants exhibiting severe deficiency in foliar LCD and DCD activity were isolated from an ethyl methane sulfonate (EMS)-induced M_2 generation and advanced to next generation to perform a detail study. The main goal of the present study was to (1) investigate the morpho-physiological changes, (2) measure the foliar LCD and DCD activity along with Cys and endogenous H_2S level, and (3) assess ascorbate (ASA) and GSH pools and their redox state under control and As (20 and 40 µM) treatment.

Materials and methods

Induced mutagenesis and plant materials

Fresh and healthy seeds of legume common bean (*Phaseolus vulgaris* L. cv. VL 63) presoaked with water (6 h) were treated with freshly prepared 0.15% aqueous solution of EMS (Sigma-Aldrich) for 8 h with intermediate shaking at 25 ± 2° C. M_1 seeds were sown treatment wise in completely randomized block design as reported earlier (Talukdar, Talukdar 2013b). During screening of antioxidant activity of M_2 plants in 2010, two variant plants, both showing very weak stems, leaf necrotic patches and abnormally low foliar activity of LCD and DCD, were detected. Seeds of these two variant plants (mean 100 seeds per plant) were harvested separately, and were sown in the next season (2013) to raise M_3 progeny. Leaf enzyme activity of respective progeny plants (a total of 185 plants) was again confirmed at M_3 generation, and based on this primary observation, the mutants were tentatively designated as *Phaseolus vulgaris L-/DCD-deficient mutant* (*pvcys*). Morphological, physiological and some biochemical characterizations of the mutant were performed on the M_3 progeny plants (Table 1). Pollen sterility was determined by staining freshly collected pollen grains with 1% acetocarmine solution and percentage of sterile pollens was calculated following Talukdar and Biswas (2007).

Culture conditions and arsenic-treatment protocols

Fresh and uniform-sized seeds of common bean cv. VL 63 and the mutant plants were surface sterilized with NaOCl (0.1%, w/v) and continuously washed under running tap water followed by distilled water. Seeds were germinated in the dark in two separate sets on moistened filter paper at 25 °C. Germinated seedlings were randomly placed in polythene pots (eight plants per pot) containing 250 mL of Hoagland's No 2 nutrient media and were allowed to grow for 10 d. The plants were then subjected to 20 µM and 40 µM sodium arsenate; technical grade, purity 98.5%, Sigma-Aldrich, Bangalore, India) using untreated plants as a control. The untreated (no added As) plant variety and mutant plants were designated as wild type (WT) and mutant control, respectively. Control and treated plants were allowed to grow for another 10 days. Nutrient solution was refreshed every alternate day to prevent depletion of nutrients as well as As in the course of the plant's exposure to the metalloid (Talukdar 2013b). The experiment was done in a completely randomized block design with four replicates in an environmentally controlled growth chamber under a 14 h photoperiod, 28/18 ± 2 °C, relative humidity of 70 ± 2%, and a photon flux density of 100 µmol m^{-2} s^{-1}.

Determination of foliar LCD and DCD activity

Foliar LCD (EC 4.4.1.1) activity was measured by the release of sulfide from Cys in a total volume of 1 mL consisting of 2.5 mM dithiothreitol, 0.8 mM L-Cys, 100 mM TRIS/HCl, pH 9.0, and enzyme extract (Bloem et al. 2004). The reaction was initiated by the addition of L-Cys. After incubation for 15 min at 37 °C the reaction was terminated by adding 100 µL of 30 mM $FeCl_3$ dissolved in 1.2 N HCl and 100 µL of 20 mM N,N-dimethyl-p-phenylenediamine dihydrochloride dissolved in 7.2 N HCl (Siegel 1965). The formation of methylene blue was determined at 670 nm. Solutions with different concentrations of sodium sulfide (Na_2S) were prepared, treated in the same way as

the assay samples, and were used for the quantification of enzymatically formed H_2S. DCD (EC 4.4.1.15) activity was determined in the same way, but D-Cys was used instead of L-Cys (Riemenschneider et al. 2005). The protein content of the supernatant was measured following Bradford (1976) using Bovine Serum Albumin as a standard. Four samples per treatment were collected with four replications in the assays.

Estimation of glutathione, ascorbate, and Cys in leaves

Reduced and oxidized forms of ascorbate and glutathione were measured following the methods of Law et al. (1983) and Griffith (1985), respectively. GSH and ASA redox was calculated as GSH / (GSH + GSSG) and ASA / (ASA + DHA). Cys content was measured spectrophotometrically (Perkin-Elmer, Lambda 35, Mumbai, India) at 560 nm following Gaitonde (1967).

Measurement of endogenous H_2S

Endogenous H_2S was determined by the formation of methylene blue from dimethyl-p-phenylenediamine in H_2SO_4 following Sekiya et al. (1982) and Chen et al. (2011), with some modifications. Leaves (0.5 g) were ground and extracted in 5 mL of phosphate buffer solution (pH 6.8, 50 mM) containing 0.1 M EDTA and 0.2 M ascorbate. The homogenate was mixed with 0.5 mL of 1 M HCl in a test tube to release H_2S, and H_2S was absorbed in a 1% (w/v) zinc acetate (0.5 mL) trap located in the bottom of the test tube. After 30 min of reaction, 0.3 mL of 5 mM dimethyl-p-phenylenediamine dissolved in 3.5 mM H_2SO_4 was added to the trap. Then 0.3 mL of 50 mM ferric ammonium sulfate in 100 mM H_2SO_4 was injected into the trap. The amount of H_2S in zinc acetate traps was determined spectrophometrically at 667 nm after leaving the mixture for 15 min at room temperature. Blanks were prepared by the same procedures without the zinc acetate solution.

Estimation of foliar H_2O_2 content, lipid peroxidation and electrolyte leakage

Fresh tissue of 0.1 g was powdered with liquid nitrogen and blended with 3mL acetone for 30 min at 4 °C. Then the sample was filtered through eight layers of gauze cloth. After addition of 0.15 g active carbon, the sample was centrifuged twice at 3 000 g for 20 min at 4 °C, then 0.2 mL 20 % $TiCl_4$ in HCl and 0.2 mL ammonia was added to 1 mL of the supernatant. After reaction, the compound was centrifuged at 3,000 g for 10 min, the supernatant was discarded, and the pellet was dissolved in 3 mL of 1 M H_2SO_4. H_2O_2 content was measured from the absorbance at 410 nm using a standard curve, following Wang et al. (2007). Membrane lipid peroxidation rate was determined by measuring the malondialdehyde (MDA) equivalents following Hodges et al. (1999). Electrolyte leakage (EL%) was measured following Dionisio-Sese and Tobita (1998).

Statistical analysis

The results presented are mean values ± standard errors obtained from at least four replicates. Significant differences from WT plants were calculated by the 'Student *t*-test' using Microsoft 'data analysis 2007'. Multiple comparisons among treatments were performed by ANOVA using software SPSS v. 10.0 (SPS Inc., USA), and means were separated by the Duncan's multiple range test. A probability of $P < 0.05$ was considered significant.

Results

Growth performance and L-/DCD activity in pvcys mutant under control and As-treated conditions

Compared to WT plants, the *pvcys* mutant exhibited reduction in growth parameters, as exhibited by significant decrease in stem height, leaf length, number (three leaves per 25 cm) and dry mass of shoot (Table 1). The intermodal distances (2.8 cm ± 0.9) were about 9-fold longer in the mutant in relation to WT plants (Fig. 1). Characteristic huge leaf necrotic patches with black-deep brown color appeared on the leaflet surface, stem, leaf petiole and pod walls (Fig. 1). Flowering was delayed, and seeds per pod, pods per plants and 100 seed weight were decreased (Table 1). Compared to WT, pollen sterility was about 2-fold higher in the mutant plants (Table 1). In comparison to WT plants, both LCD and DCD activity was significantly reduced in leaves of the mutant. LCD activity was nearly 8.50 % while DCD activity was about 10.10% of that of WT plants (Table 1).

During 10 days exposure to As under controlled hydroponic growth conditions, the *pvcys* mutant exhibited significant changes in various growth characteristics. Compared to mutant control, stem height was increased by about 2-fold, while shoot dry weight was higher by approximately 1.8-fold (Table 1), and number of necrotic spots on leaflet surface decreased at 20 µM As. Further improvement of growth traits coupled with increase in shoot dry weight over mutant control was noticed in the *pvcys* mutant subjected to 40 µM As. Lower pollen sterility was also noticed in the treated mutant (Table 1). Necrotic spots were conspicuously absent in the leaflet lamina, petiole and on pod wall and intermodal distances were reduced with increased leaf number (six/25 cm) in the 40 µM As-treated mutant (Fig. 2). Pollen sterility was very low at this concentration (Table 1). Growth traits of the *pvcys* mutant at this treatment protocol marginally varied in relation to those of untreated WT plants (Table 1). Compared to mutant control, no significant change was observed in measurable activities of both LCD and DCD in leaves of pvcys mutant under As- treatment regimes. Foliar mean LCD activity was about 8.75 % while DCD activity was about 10.00 % of WT plants (Table 1). Growth characteristics and enzyme activity marginally changed in WT plants under As-treatments (Table 1).

Table 1. Growth characteristics and leaf biochemical parameters in *Phaseolus vulgaris* WT genotype VL 63 and its mutant (M_3) line *pvcys* grown in field or hydroponically at untreated (control) and sodium arsenate (As; 20 and 40 µM) treated conditions. Data are means ± standard error from at least four replicates. * significantly ($P < 0.05$) different from WT plants in field conditions. Means followed by different lowercase letters indicate significant differences for a particular trait in hydroponically grown plants at $P < 0.05$ by ANOVA followed by Duncan's Multiple Range Tests. LCD, L-cysteine desulfhydrase; DCD, D-cysteine desulfhydrase; FM, fresh mass, GSH, reduced glutathione; GSSG, glutathione disulfide; ASA, reduced ascorbate; DHA, dehydroascorbate; MDA, malondialdehyde; EL, electrolyte leakage. No significant changes were observed in mother plants at 20 µM As, not shown in table

Traits	Field grown plants		Hydroponically grown plants				
	WT	Mutant	WT		Mutant		
	Control	Control	Control	As 40 µM	Control	As 20 µM	As 40 µM
Shoot height (cm)	155.5 ± 5.6	62.56± 3.9*	6.67 ± 1.3a	6.43 ± 1.4a	2.43 ± 0.6c	4.85 ± 0.9b	6.39 ± 0.6a
Leaflet length (cm)	4.19 ± 0.9	1.04 ± 0.3*	3.82 ± 0.8a	3.76 ± 0.7a	0.78 ± 0.2c	3.12 ± 0.8b	3.79 ± 0.6a
Shoot dry mass (g plant^{-1})	6.18 ± 0.03	1.21 ± 0.01*	0.17 ± 0.1a	0.16 ± 0.05a	0.04 ± 0.0c	0.074 ± 0.0b	0.15 ± 0.01a
Pollen sterility (%)	1.09 ± 0.1	2.17 ± 0.13*	1.10 ± 0.1c	1.12 ± 0.2c	2.20 ± 0.2a	1.76 ± 0.3b	1.15 ± 0.1c
LCD activity (nmol H_2S min^{-1} mg^{-1} protein)	19.38 ±0.64	1.64 ± 0.08*	21.38 ± 0.58a	20.38 ± 0.54a	1.64 ± 0.08b	1.64 ± 0.08b	1.87 ± 0.08b
DCD activity (nmol H_2S min^{-1} mg^{-1} protein)	16.15 ± 0.63	1.62 ± 0.06*	17.18 ± 0.53a	16.19 ± 0.47a	1.60 ± 0.04b	1.69 ± 0.09b	1.72 ± 0.10b
H_2S level (µmol g^{-1} FM)	0.067 ± 0.0	0.011 ± 0.0*	0.063 ± 0.0a	0.075 ± 0.0b	0.010 ± 0.0c	0.011 ± 0.0c	0.012 ± 0.0c
Cysteine content (nmol g^{-1} FW)	7.43 ± 0.54	59.89 ±0.61*	7.39 ± 0.51c	7.28 ± 0.49c	60.19 ± 0.63a	11.09 ± 0.45b	7.63 ± 0.59c
GSH (nmol g^{-1} FM)	181.5 ± 4.8	186.5 ± 5.3	189.4 ± 3.7b	291.4 ± 3.9a	177.8 ± 5.1b	288.7 ± 5.6a	290.3 ± 4.1a
GSSG (nmol g^{-1} FM)	23.8 ± 1.5	23.5 ± 1.3	22.9 ± 1.8a	29.9 ± 2.1a	23.3 ± 1.3a	30.3 ± 1.6a	29.8 ± 1.5a
GSH redox (GSH/GSH+GSSG)	0.884 ± 0.09	0.889 ± 0.10	0.892 ± 0.10a	0.910 ± 0.11a	0.881 ± 0.09a	0.905 ± 0.10a	0.906 ± 0.10a
ASA (nmol g^{-1} FM)	892.3 ± 6.5	809.3 ± 5.8	890.8 ± 7.1c	969.3 ± 7.5b	812.6 ± 6.3c	962.3 ± 6.9b	1012 ± 9.8a
DHA (nmol g^{-1} FM)	101.1 ± 3.5	101.4 ± 4.0	112.1 ± 3.8a	112.5 ± 3.1a	104.5 ± 3.7a	114.2 ± 2.9a	119.5 ± 2.9a
ASA redox (ASA/ASA+DHA)	0.900 ± 0.08	0.890 ± 0.10	0.888 ± 0.09a	0.895 ± 0.07a	0.886 ± 0.09a	0.894 ± 0.10a	0.894 ± 0.09a
H_2O_2 (µmol g^{-1} FM)	4.2 ± 0.8	17.6 ± 1.2*	4.4 ± 0.9b	4.5 ± 0.9b	17.4 ± 0.9a	4.7 ± 0.9b	4.6 ± 0.9b
MDA (nmol g^{-1} FM)	3.91 ± 0.7	23.9 ± 2.4*	4.0 ± 0.7b	4.1 ± 0.67b	19.9 ± 1.9a	3.87 ± 0.8b	3.89 ± 0.7b
EL (%)	4.82 ± 0.5	20.7 ± 2.7*	4.3 ± 0.6b	4.7 ± 0.67b	20.3 ± 2.5a	4.27 ± 0.6b	4.19 ± 0.7b

Changes in GSH, ASA, and Cys level in control and treated genotypes

Compared to WT plants, GSH, ASA and their respective redox state varied non-significantly in field grown *pvcys* mutant (Table 1). Foliar Cys level was, however, significantly higher in the mutant than that in WT plants. Upon exposure to 20 µM As, the total and redox pool of both GSH and ASA increased significantly in the mutant and mother genotypes but the Cys level reduced drastically (from the mutant control level) in the treated mutant and became close to WT level at 40 µM (Table 1). The GSH and ASA level was further increased in both WT and the mutant at 40 µM As (Table 1).

Endogenous foliar H_2S content

Measurable H_2S content was significantly higher in WT plants than that in mutant control (Table 1). Upon imposition of As-treatments, H_2S level showed moderate increase over WT in 40-µM treated mother genotypes. Compared to mutant control, no significant change in H_2S content was observed in leaves of As-treated mutant plants (Table 1).

Changes in foliar H_2O_2 content, lipid peroxidation and electrolyte leakage

Leaf H_2O_2 content, malondealdehyde (MDA) and EL% were significantly higher in the mutant plants under control conditions. Compared to WT, no significant increase in these three traits was, however, observed in As-treated mutant plants and WT genotypes (Table 1).

Genetic control of pvcys mutation and inheritance of As-tolerance

Crosses between WT and *pvcys* mutant yielded F_1 plants, which showed growth like WT (Table 2). In F_2 and corresponding test crosses, the characteristic mutant and WT phenotype segregated and showed good fit with 1:3 and 1:1 ratios, respectively (Table 2). The F_2 recessive plants

Fig. 1. A flowering twig of mutant plants (M) and its WT genotype VL 63, showing characteristic necrotic patches (arrows) on leaflet lamina, petiole, stem surface and on pod wall and longer intermodal distances in mutant twig in comparison to WT genotype.

Fig. 2. Mutant twig (M) after treatment with 40 µM sodium arsenate for 10 days in hydroponic conditions exhibited conspicuous absence of necrosis and reduced intermodal distances with leaf number comparable to WT genotype.

with mutant phenotype exhibited low LCD (8.65% of WT) and DCD (9.50% of WT) activities. Upon exposure to 20 and 40 µM As in same experimental protocol, all F_1 plants exhibited normal LCD and DCD activities and tolerance to As but the trait was segregated in F_2 and back cross, showing plants with low LCD and DCD activities and normal enzyme activity to fit well to 1:3 and 1:1, respectively (Table 2). The F_2 progeny plants with low LCD and DCD activity showed plant growth similar to WT plants under 20 and 40 µM As (data not shown).

Discussion

Retardation of growth traits coupled with appearance of severe necrosis in leaves and the pod wall of the common bean mutant *pvcys* isolated through EMS-mutagenized population of *P. vulgaris* cv. VL 63 was accompanied with huge deficiency in both LCD and DCD activities in its leaves. Significant decrease in shoot dry mass in the mutant was certainly been due to substantial reduction in stem height, leaflet size, number and pre-mature leaf senescence triggered by necrosis in leaf petiole and leaflet lamina. On the other hand, anomalies in reproductive parts were manifested by delayed flowering and high magnitude of pollen sterility, bringing about disturbances in grain yield components.

The inhibition in various morpho-agronomic traits in the mutant plants was remarkably associated with a high Cys level and normal (close to WT) GSH as well as ASA redox pool. A high Cys level in legume plants is a desirable trait, due to severe S-deficiency in grain legumes (Liao et al. 2013). In the present case, crippling of foliar both Cys-degrading enzyme activities presumably led to accumulation of free Cys in leaves. This was further substantiated by the extremely low level of endogenous H_2S content in the mutant plants. LCD and DCD play predominant roles in Cys-generated H_2S generation (Bloem et al. 2004; Álvarez et al. 2010, 2012; Chen et al. 2011), and although L-Cys desulfurase (another enzyme involved in Cys desulfuration reaction) activity was not studied in the present case, it seemed likely that mutagenesis induced a major blockage in the Cys degradation pathway of common bean. Although Cys constitutes the basis of plant thiol-metabolisms and building blocks of numerous plant primary and secondary metabolites, accumulated free Cys has high capacity to act as a prooxidant within cell (Park,

Table 2. Segregation of growth and arsenic tolerance as indicated by enzyme activity in *Phaseolus vulgaris* L. WT genotype VL 63 and its mutant line *pvcys*. Data in brackets indicate number of plants. Data are means ± standard error of at least four replicates. Segregation showed good fit to respective ratios at $P < 0.05$. [a] normal growth means growth traits similar to WT

Cross	F_1 phenotype[a] enzyme activity	F_2/test cross segregation		Ratio	X^2 value
		WT enzyme activity (plants)	Mutant enzyme activity (plants)		
Control					
VL 63 × *pvcys*	Normal growth	(151)	(52)	3:1	0.04
F1 × *pvcys*	–	(41)	(37)	1:1	0.20
LCD activity (nmol H_2S min^{-1} mg^{-1} protein)	20.38 ± 0.64	19.40 ± 0.64 (131)	1.68 ± 0.23 (43)	3:1	0.007
DCD activity (nmol H_2S min^{-1} mg^{-1} protein)	17.11 ± 0.39	16.32 ± 0.29 (128)	1.63 ± 0.21 (38)	3:1	0.39
Arsenate (20 µM) treated					
VL 63 × *pvcys*	Normal growth	(311)	–	–	–
LCD activity (nmol H_2S min^{-1} mg^{-1} protein)	22.38 ± 0.64	20.40 ± 0.64 (109)	1.68 ± 0.23 (39)	3:1	0.14
DCD activity (nmol H_2S min^{-1} mg^{-1} protein)	17.09 ± 0.39	17.12 ± 0.30 (128)	1.53 ± 0.21 (45)	3:1	0.09
Arsenate (40 µM) treated					
VL 63 × *pvcys*	Normal growth	(252)	–	–	–
LCD activity (nmol H_2S min^{-1} mg^{-1} protein)	18.38 ± 0.55	20.40 ± 0.64 (141)	1.70 ± 0.20 (49)	3:1	0.05
DCD activity (nmol H_2S min^{-1} mg^{-1} protein)	17.23 ± 0.33	17.12 ± 0.30 (140)	1.67 ± 0.17 (48)	3:1	0.03

Imlay 2003; Krueger et al. 2009; Álvarez et al. 2012). In the present study, no inhibition in morpho-agronomic characteristics was observed in WT plants, showing both LCD and DCD activities. Paradoxically enough, the high Cys level in the present mutant failed to trigger increase in GSH and ASA pool and to stimulate defense to mitigate growth inhibition. This created a blockage of Cys channeling to produce downstream thiol metabolites, required to maintain plant growth and mitigation of oxidative damage, and consequently led to its accumulation presumably to a prooxidant level. GSH exclusively requires Cys as one of its building blocks, but a normal level (close to WT) of GSH, GSSG and GSH redox state in the present mutant strongly suggested strict regulations of GSH biosynthesis within the cell in the backdrop of low LCD/DCD activities and in the absence of any exogenous stress signals. The lack of GSH-mediated defense stimulation presumably resulted in marked escalation in foliar H_2O_2 level in the mutant. H_2O_2 is a diffusible ROS and can induce oxidative damage over a certain limit by triggering membrane lipid peroxidation (Hodges et al. 1999; Foyer, Noctor 2003; Wang et al. 2007; Talukdar 2012d). MDA is a cytotoxic aldehyde generated through lipid peroxidation of membrane and marks the onset of oxidative stress in plants (Hodges et al. 1999; Foyer, Noctor 2003; Talukdar 2012c, d, 2013a). High H_2O_2 coupled with a rise in lipid peroxidation led to rise in EL% in leaves of the present mutant plants and presumably led to growth inhibition and leaf necrosis in the *pvcys* mutant. No such symptoms were observed in WT plants, suggesting intimate relationship between LCD/DCD activity, Cys level and GSH-mediated antioxidant defense in leaves of common bean.

Tolerance of *pvcys* mutant seedlings to two different regimes of As-treatment under a controlled hydroponic system was manifested by growth traits, pollen sterility and shoot dry mass close to WT plants. The complete disappearance of leaf and pod necrosis in As-treated mutant plants and short intermodal distances with higher leaf number presumably was associated with prevention of premature leaf senescence. This was a dramatic improvement of the growth characteristics of As-treated mutant plants in relation to mutant control plants. Reports are available regarding As-induced growth stimulation at low concentration of metalloid which was attributed to GSH-mediated up-regulation of antioxidant defense (Talukdar, Talukdar 2014b). In the present case both WT and the mutant plants showed no growth inhibition under As-treatment. Elevated level of GSH, ASA and their favorable redox state might be instrumental to prevent over-accumulation of H_2O_2 and subsequent ROS-induced oxidative damage to membrane integrity in both genotypes. However, it was noteworthy that the background of this tolerance to As-stress was completely different between WT and mutant plants; while LCD/DCD activity and the

Cys level remained unaltered (compared to WT) in treated WT, Cys level was drastically reduced to the WT level in the mutant plants. This decline in free Cys pool was certainly not due to increased LCD/DCD activity because there was no change in LCD and DCD activity, and endogenous H_2S level in the treated mutant compared to that of the mutant control remained low. The results clearly indicated constitutive deficiency in predominant Cys-degrading enzymes in the present bean mutant. Obviously, accumulated Cys pool in the mutant plants was consistently consumed to meet the growing cellular demand for GSH upon exposure to As and substantially relieved the mutant plant from the free Cys-load and its toxic level. With increasing GSH demand under elevated As concentration, Cys consumption was balanced with its synthesis, maintaining its pool close to that of WT plants. High GSH and ASA significantly helped mutant plants in scavenging of As-induced excess ROS. This was evidenced by non-significant changes in the H_2O_2 level compared to that in WT plants, thus lowering the lipid peroxidation rate and EL% from mutant control level. Absence of oxidative damage ultimately facilitated the mutant plants to maintain favorable growth performance even under high As exposure. However, determining to what extent mutant plants modulated its entire antioxidant defense through Cys and GSH in response to As exposure and whether low endogenous H_2S has any roles in defense cross-talk with GSH, require further study.

Inheritance studies revealed monogenic recessive control in the present bean genotype of the *pvcys* mutant. Significantly, As-tolerance in F_2 progeny plants was associated with growth traits and leaf non-enzymatic antioxidant metabolism similar as in WT plants. The unaltered activity level of LCD and DCD and low measurable H_2S level in these progeny plants confirmed constitutive under-expression of both the Cys-degrading enzymes and its true breeding inheritance in progeny plants. The present results clearly indicated that appearance of morpho-agronomic anomalies was orchestrated through a high Cys level which was consumed upon exposure to As. It seemed clear that severe deficiency in LCD and DCD activities in the present *pvcys* mutant was caused by recessive mutations in both loci (LCD and DCD) or in any one of the loci with pleiotropic effect on other loci controlling enzyme expression,s which were stably inherited and totally unresponsive to exogenous stress signal.

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Received 4 February 2014; received in revised form 4 April 2014; accepted 15 May 2014