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A metabolic diversion in the upstream thiol cascade of cysteine-deficient lentil (*Lens culinaris* Medik.) mutants induces arsenate tolerance by modulating downstream antioxidant defense

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Abstract: The functional interplay between upstream and downstream thiol cascades was investigated in a cysteine-deficient mutant (*cysLc1*), a catalase-deficient (*catLc2*) mutant, a double mutant (showing deficiency of both cysteine (Cys) (upstream) and catalase (downstream antioxidant defense) (*cysLc1*/*catLc2*)), and the VL125 genotype of lentil under the following treatments: a) 50 µM sodium arsenate (As); and b) As + 1 mM L-buthionine-sulfoximine (BSO). Both *cysLc1* and the double mutant experienced As-induced oxidative stress due to the lack of responsiveness of the entire thiol cascade and antioxidant defense. Contrastingly, the *catLc2* mutant and VL125 exhibited As tolerance. Under the As + BSO treatment, glutathione (GSH) synthesis was inhibited, but sulfate transport and Cys synthesis were differentially regulated in the four genotypes. The *cysLc1* and *catLc2* mutants induced Cys desulfuration pathways and generated huge endogenous hydrogen sulfide, stimulating ascorbate-mediated antioxidant defense and catalases as an alternative mechanism of As tolerance under low GSH redox. The nonresponsiveness of this alternate route, coupled with a crippled ascorbate-mediated antioxidant defense, led to huge Cys build-up and ROS overaccumulation in VL125 and the double mutant, which consequently experienced As-induced growth inhibition. The study indicated that a metabolic diversion in an upstream thiol cascade through Cys desulfuration is imminent for Cys homeostasis and modulation of the downstream antioxidant defense against As toxicity when the Cys consumption route towards GSH is blocked.

Key words: Ascorbate, glutathione, hydrogen sulfide, cysteine homeostasis, mutants, gene expression

1. Introduction

Sulfur (S) is an essential nutrient for plant metabolism, growth, and development. Plants generally take up S from the soil in the form of sulfate, which is reduced to sulfide. Sulfide is incorporated by O-acetylserine (thiol) lyase (OAS-TL) into the amino acid skeleton of O-acetylserine (OAS) to form cysteine (Cys) (Davidian and Kopriva, 2010; Hell and Wirtz, 2011; Takahashi et al., 2011; Talukdar and Talukdar, 2014). Cys is not only an important amino acid in proteins, but also a precursor for numerous essential biomolecules. It can mitigate oxidative damage using its own antioxidant properties (Takahashi et al., 2011; Genisel et al., 2015). Cys can be directly incorporated into glutathione (GSH), the low molecular weight tripeptide and the most important downstream regulator of cellular S status in plants. GSH is synthesized by γ-glutamylcysteine synthetase (γ-ECS) in a rate-limiting way. This action can be specifically inhibited by L-buthionine-sulfoximine (BSO) (Noctor et al., 2012). The use of BSO has therefore become a reliable strategy to assess the response of GSH and GSH-dependent antioxidant defense to induced stress. Within the ascorbate (AsA)-GSH cycle, dehydroascorbate reductase (DHAR) regenerates AsA using GSH as a reductant and produces glutathione disulfide (GSSG). AsA is used by ascorbate peroxidase (APX) as an exclusive cofactor during scavenging of H_2O_2, generated by superoxide dismutase (SOD) during dismutation of superoxide radicals, photorespiration, and from other sources. Within the GSH cycle, glutathione reductase (GR) catalyzes the reduction of GSSG to GSH, and with the help of DHAR it maintains high AsA-redox and a favorable GSH/GSSG ratio. This is necessary for the tight control of metal-induced ROS scavenging (Noctor et al., 2012; Anjum et al., 2014; Turan and Ekmekçi, 2014). Outside the AsA-GSH cycle, catalases (CAT) metabolize H_2O_2, which can oxidize GSH through the action of GSH-S-transferase (GST) (Finnegan and Chen, 2012). While sulfate transport and Cys synthesis play key roles in the upstream thiol cascade, GSH and the GSH-dependent defense constitute the downstream thiol cascade during plants' response to stress.

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Knowledge regarding the metabolic diversion of Cys into different routes to regulate plant stress response has recently been widened with the discovery of hydrogen sulfide (H_2S) as a prominent signaling molecule in plants (Calderwood and Kopriva, 2014). Apart from assimilatory sulfate reduction, endogenous H_2S in higher plants is generated through the desulfuration of Cys by L-cysteine desulfhydrase (LCD) and D-cysteine desulfhydrase (DCD) (Álvarez et al., 2010). H_2S participates in diverse physiological activities to promote plant growth processes and stress tolerance (Chen et al., 2011; Calderwood and Kopriva, 2014; Talukdar, 2015). Although these reports seem to be encouraging in terms of the positive effects of H_2S in plant systems, it is still not clear whether these effects are from H_2S alone or are mediated by downstream thiol antioxidant metabolites like GSH. A balance between Cys synthesis and its degradation/consumption to H_2S/GSH may thus have immense significance not only in maintaining Cys homeostasis, but also in regulating sulfide status and its effects on antioxidant defense in plants (Álvarez et al., 2010).

Arsenic (As) is a ubiquitous toxic metalloid. Food crops such as rice, pulses, and vegetables grown on As-contaminated soil can accumulate high levels of As in roots, shoots, and seeds (Gupta et al., 2008; Bhattacharya et al., 2010; Talukdar, 2013). Lentil (*Lens culinaris* Medik.) is a widely grown antioxidant-rich commercial food legume, but like other edible legumes, the crop is As-sensitive (Gunes et al., 2009; Talukdar, 2013). Primary investigation revealed significant As accumulation in lentil organs and seeds (Talukdar, 2013). Being grown in aerobic fields, legumes are usually exposed to the arsenate form of As, which either directly or through conversion to the highly toxic arsenite adversely affects plant growth by generating excess reactive oxygen species (ROS) and consequent oxidative damage to membrane structures and functions (Gunes et al., 2009; Talukdar, 2013). S metabolism components, and particularly GSH and phytochelatins (PCs), play central roles during As detoxification (Rai et al., 2011; Tripathi et al., 2012). However, it is not known whether metabolic channeling in the routes of Cys metabolism and endogenous H_2S has any roles in the As response of crop plants. The thiol cascade and downstream antioxidant defense mechanism of As tolerance is also poorly understood in lentils. Two Cys-deficient mutants and two CAT-deficient mutants have recently been characterized in ethyl methanesulfonate (EMS)-mutagenized population of lentils (Talukdar and Talukdar, 2013; Talukdar, 2014a). The mutants were self-fertile and provided different genetic backgrounds suitable to dissect and track the intrinsic metabolic events of lentil crops responding to arsenate stress. The major objectives of the current study were to explore: 1) the responses of Cys metabolizing (synthesis and desulfuration) enzymes; 2) the roles of GSH and the response of antioxidant defense enzymes; and 3) the involvement of H_2S in conferring As tolerance to leaves of lentil genotypes subjected to arsenate treatment. BSO was used along with As to elucidate the response of the thiol cascade and antioxidant defense to As stress in the absence of GSH synthesis and low GSH-redox.

2. Materials and methods

2.1. Plant material, growth conditions, and treatment protocol

Fresh seeds of four lentil (*Lens culinaris* Medik.) genotypes, viz. cultivar VL125, one cysteine-deficient *Lens culinaris* mutant 1 (*cysLc1*), one catalase-deficient *Lens culinaris* mutant 2 (*catLc2*), and one double mutant (*cysLc1*/*catLc2*), were surface sterilized with NaOCl (0.1%, w/v) and continuously washed under running tap water, followed by distilled water. Both single mutants were genetically characterized as recessive mutations (Talukdar and Talukdar, 2013; Talukdar, 2014a). The *cysLc1* mutant, isolated from EMS-mutagenized (0.10% EMS, 6 h) M_2 population of the lentil genotype L 414, exhibited low foliar OAS-TL activity (21% of the wild type) and a low seed Cys level (25% of the wild type) (Talukdar, 2014a). The *catLc2* mutant was isolated from EMS-treated (0.5% EMS, 6 h) M_2 generation of the lentil cultivar VL125 and showed CAT activity of 11.22% of the wild type in M_2 (Talukdar and Talukdar, 2013). The double mutant was isolated with a double recessive mutation, having deficiency of both foliar OAS-TL and CAT activity in F_2 generation of *cysLc1* × *catLc2* through intercrossing (Talukdar, unpublished). Seeds of VL 125 were collected from the Pulses and Oilseed Research Station in Berhampore, West Bengal, India, and grown for two seasons (2008 and 2009) at a private farm in Kalyani (22°59' N/88° 29' E), West Bengal, India (Talukdar and Talukdar, 2013). Seeds were allowed to germinate in the dark in two separate sets on moistened filter paper at 25 °C. Germinated seedlings were randomly placed in polythene pots (10 cm in diameter and 12 cm high, 10 plants $pots^{-1}$) containing 250 mL of Hoagland's No. 2 nutrient media, and were allowed to grow for 10 days. Seedlings of all the four genotypes were then subjected to: a) 50 μM of sodium arsenate (As, MW 312.01 g mol^{-1}; technical grade, purity 98.5%, Sigma-Aldrich) treatment; and b) As + 1 mM BSO (Sigma-Aldrich). Then they were allowed to grow for another 10 days. Untreated plants were used as controls; a cultivar was used as a mother control (MC) and three mutant controls, MuCys, MuCat, and MuCysCat, were used for *cysLc1*, *catLc2*, and the double mutant, respectively. The experiment was carried out in a completely randomized block design in an environmentally controlled growing chamber under a 14 h photoperiod,

28/18 (± 2 °C), relative humidity of 70 ± 2%, and a photon flux density of 150 μmol m^{-2} s^{-1}. The nutrient solution was refreshed three times per week, and all experiments were conducted in triplicate with four replicates. After 10 days, As-exposed seedlings were harvested along with the controls, carefully washed with distilled water, blotted gently, and oven-dried at 60 °C until constant weight. The root and shoot dry weights were measured and the leaves were used for metabolic and molecular analysis.

2.2. Determination of As content

The As concentrations in dried root and shoot samples were measured by digestion methods (HNO_3–$HClO_4$ mixture at 3:1, v/v) using flow injection hydride generation atomic absorption spectrophotometer (Perkin-Elmer, FIA-HAAS Analyst 400) and keeping a Standard Reference Materials of tomato leaves (item number 1573a from the US National Institute of Standards and Technology) for part of the quality assurance/quality control protocol as detailed earlier (Talukdar, 2013). The translocation factor (TF) is the ratio between the level of As in shoots and that in roots.

2.3. Estimation 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).

2.4. Assay of GSH, AsA, Cys, and thiol metabolizing enzymes

The reduced and oxidized forms of AsA and GSH were measured following Law et al. (1983) and Griffith (1980), respectively. The AsA and GSH redox states were calculated as AsA/ (AsA + DHA) and GSH/ (GSH + GSSG), respectively. For enzyme assay, plant tissue was homogenized in buffers specific for each enzyme under chilled conditions. The homogenate was squeezed through four layers of cheese cloth and centrifuged at 12,000 × *g* for 15 min at 4 °C. The protein content of the supernatant was measured following Bradford (1976) using bovine serum albumin (BSA) as a standard. Serine acetyltransferase (SAT; EC 2.3.1.30) activity was assayed following Blaszczyk et al. (2002). An enzyme unit was considered as the amount of enzyme catalyzing the acetylation of 1 pmol of L-serine per minute. The OAS-TL (EC 2.5.1.47) activity was assayed by measuring the production of L –Cys (Saito et al., 1994). Cys content was measured spectrophotometrically (Perkin-Elmer, Lambda 35) at 560 nm following Gaitonde (1967). A blank control with all compounds except OAS was maintained, and the linearity of the assay was checked with 2.5 and 10 μL of added crude leaf extract. Assays of γ-ECS (EC 6.3.2.2), PC synthase (PCS; EC 2.3.2.15), LCD (EC 4.4.1.1), and DCD (EC 4.4.1.15) were conducted following Seelig and Meister (1984), Howden et al. (1995), Bloem et al. (2004), and Riemenschneider et al. (2005), respectively.

2.5. Assay of antioxidant enzymes

Leaf tissue (250 mg) was homogenized in 1 mL of 50 mM K-phosphate buffer (pH 7.8) containing 1 mM ethylenediaminetetraacetic acid, 1 mM dithiothreitol, and 2% (w/v) polyvinyl pyrrolidone using a chilled mortar and pestle kept in an ice bath. The homogenate was centrifuged at 15,000 × *g* and 4 °C for 20 min. Clear supernatant was used for enzyme assays. For measuring APX activity, the tissue was separately ground in homogenizing medium containing 2.0 mM AsA in addition to the other ingredients. All assays were done at 25 °C. Soluble protein content was determined using BSA as a standard (Bradford, 1976). SOD (EC 1.15.1.1) activity was determined by nitro blue tetrazolium (NBT) photochemical assay (Beyer and Fridovich, 1987) and was expressed as unit min^{-1} mg^{-1} protein. One unit of SOD was equal to that amount causing a 50% decrease of SOD-inhibited NBT reduction. APX (EC 1.11.1.11) activity (nmol AsA oxidized min^{-1} mg^{-1} protein) was assayed according to Nakano and Asada (1981) with H_2O_2-dependent oxidation of AsA, followed by a decrease in the absorbance at 290 nm (ε = 2.8 mM^{-1} cm^{-1}). DHAR (EC 1.8.5.1) and GR (EC 1.6.4.2) enzyme activities were measured following Nakano and Asada (1981) and Carlberg and Mannervik (1985), respectively, as detailed earlier (Talukdar, 2013b, 2014c). CAT (nmol min^{-1} mg^{-1} protein) was assayed at 240 nm (ε = 39.4 M^{-1} cm^{-1}) (Zhang et al., 2009). GST (EC 2.5.1.18) activity was assayed following Li et al. (1995).

2.6. Estimation of foliar H_2O_2 content, lipid peroxidation, and electrolyte leakage (EL%)

Leaf H_2O_2 content was measured following Wang et al. (2007). Lipid peroxidation was determined by measuring the malondialdehyde (MDA) content at 532 nm (ε = 155 mM^{-1} cm^{-1}; Saher et al., 2004). EL% was assayed by measuring the ions leaching from tissues into deionized water (Dionisio-Sese and Tobita, 1998).

2.7. Relative gene expression analysis through quantitative RT-PCR

First strand cDNA was synthesized from DNA-free intact RNA with oligo-dT primers and with MmuLV reverse transcriptase enzyme kit (Chromous Biotech), following manufacturer's instructions. Quantitative RT-PCR of first strand cDNA was run on an ABI Step-One (Applied Biosystems) real-time PCR machine. Amplification was done in a total reaction volume of 50 μL, containing 2.0 μL of template (first-strand cDNA), 2.0 μL of forward and reverse primer each with 50 nM μL^{-1} concentration, 2× PCR SYBR green ready mixture (Fast Q-PCR Master Mix, Chromous Biotech) (25.0 μL), and 19.0 μL of DEPC water. Primers for selected genes were constructed by Primer Express V. 3.0 software (Applied Biosystems) with the search for available reports on *Lens culinaris* (Talukdar and Talukdar, 2014). The sequences (5′ → 3′) of the forward

(F) and reverse (R) primers in each of the candidate genes are presented in Table 1. The qRT-PCR cycling stages consisted of initial denaturation step at 94 °C (3 min), followed by 35 cycles at 94 °C (5 s), 62 °C (10 s), 72 °C (10 s), and a final extension stage at 72 °C (2 min). Samples for qRT-PCR were run in four biological replicates with three technical replicates/biological replicates. RT-PCR reaction mixtures were loaded onto 2% agarose gels in TAE buffer. A 100-bp DNA ladder was run on every gel. The mRNA levels were normalized against *EF1-α* as the housekeeping gene, and the relative (to control) expression of target genes was calculated. Details of information on candidate genes are presented in the supplementary table.

2.8. Statistical analysis

Data are presented as means ± standard error (SE) of at least four replicates. Variance analysis was performed on all experimental data, and the significance ($P < 0.05$) of means was determined by Duncan's multiple range test using SPSS, v. 10.0.

3. Results

3.1. Plant dry weight and pattern of As accumulation

After 10 days of As exposure, *cysLc1* and the double mutant *cysLc1/catLc2* exhibited significant ($P < 0.05$) reduction in dry weights of shoots and roots in comparison with the controls (Figure 1A). After 10 days of As + BSO exposure, the shoot dry weight reduced significantly in VL125, whereas *cysLc1/catLc2* showed further reduction in dry weight as compared with its performance under exposure to 50 µM As alone (Figure 1A). Changes were not significant in the rest of the cases (Figure 1A).

Under the 50 µM As treatment alone, As accumulation was markedly higher in roots than in shoots of VL125 and *catLc2*, while an opposite scenario was observed in the *cysLc1* mutant (Figure 1B). In the *cysLc1/catLc2* double mutant, As accumulated in nearly equal amounts in shoots and roots. As concentration in shoots increased significantly over that in roots in all the genotypes under the As + BSO condition. The shoot/root TF ratio was less

Table 1. Oligonucleotide primer sequence (5′ → 3′) used in qRT-PCR reactions.

Candidate genes	Forward primers	Reverse primers
LcSultr2;1	GATCATAGTTAAACTTCCACACAACG	CGTAAATTCATCACATGCAATAACCG
LcSultr2;2	GCTTGGATCCAGGGCATAGAGCTTC	AATTGGATCCATCAACACAACTCGG
OAS-TLA	CTCACAAGATTCAAGGGATAGGA	GTCATGGCTTCCGCTTCTTTC
OAS-TLB	GGATCCGCAGTGTCTGTACCAACGAAA	GACGTCTCACAATTCTGGCTTCAT
LcSAT1;1	AGCCATACTTCCTTTATCTCTGAGTG	AACAGTATATGTACTCTCAGCAGTAAC
LcSAT1;2	GGACCATACTTCCTTTATCTCTGAGTG	CTACTAGCAATAATCAACCTTTTCATC
γ-ECS	TTCTTGGGCTTGGCTTTCA	TTCCCTTTGGCATTATTGGTATGT
PCS	CCTAATGGAATCTGATGTGCCTT	CTTCTTTGACAGCGACGAGCCTT
LcLCD	GATGCAATGTATTTGTCTCTTTTTC	TTTCTTTTTATAATCTTTTGCTCCC
LcDCD	AGAACAGTTCTCTCTTTTTGTCGA	GGACAGTCCACCTTAGAGGCTAGA
Cu/ZnSODI	GCTTCCATATCCATGCCTTG	AGCTACTCTGCCACCAGCAT
Cu/ZnSODII	TTGCATTTCAACTGGACCAC	GACCACCTTTCCCAAGATCA
MnSOD	GGCGGAGGTCATATTAACCA	AAGCCACACCCATCCAGAC
FeSOD	GCACCACAGAGCTTATGTAG	GGAGTGGATGATGATGGTTC
APXI	GACTTGGCCCTGGCGTTGTTGCT	TCCAGAACCGTCCTTGTAAGTGC
APXII	GAGGAGAGTGAAGGCAAAGCAT	GTCAGTCAAGCTCGCATACGATA
APXIII	CTTCTCCAGCCGATCAAAGA	AGGACATTGGTCAGGTCCAG
DHAR	TTGAGGTTGCTGCCAAGGCT	TGGGATCCTTGCTCTTGAGG
GRI	GAAATTGCTAGTCTATGCGTCA	AGCAAACTCCAAGGCAATGT
GRII	GGTCTGCTCTTCACGTAGACCGCT	AATGGCTGTGGGTGATGTCCGAA
GSTI	TTGTCAGCAAAACATGACGA	GAAACAAATACGTGACAGAATC
GSTII	TCCATTCGACACTTGGTTCA	'GTGGCTATTAAGATCCAACATG
CAT	CCTGTCATTGTGCGTTTCTC	CTCCCACCTTAATGGCCTCT
EF-1α	TGTCGACTCTGGGAAGTCAA	CTCTTTCCCTTTCAGCCTTG

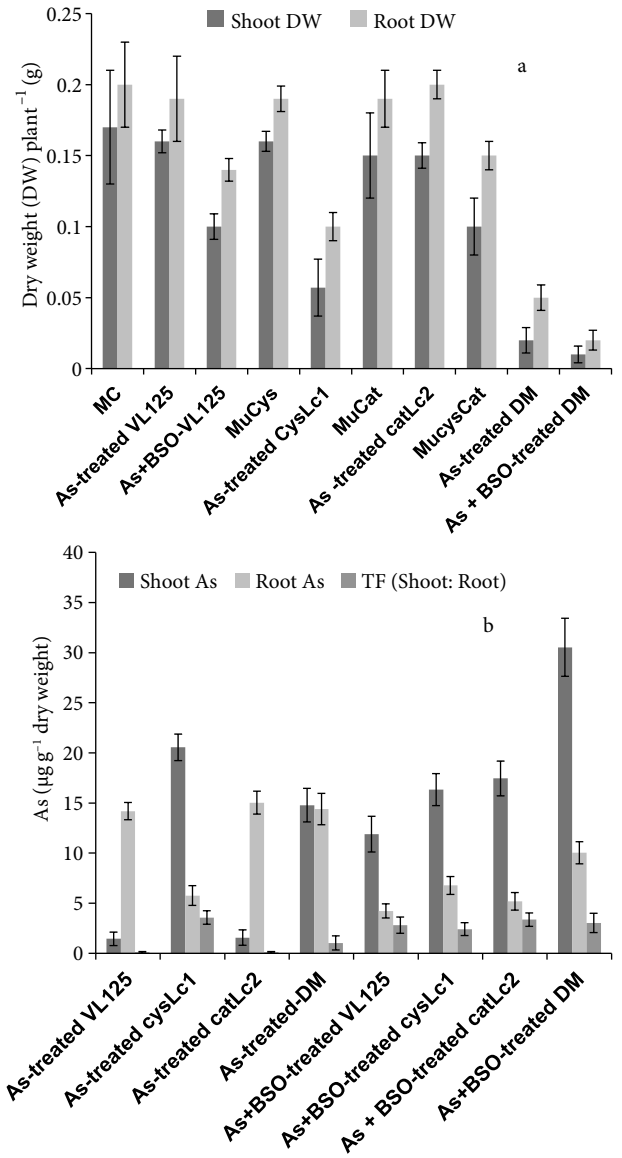

Figure 1. Changes in: a) per plant shoot and root dry weight (g); and b) the As-accumulation and transfer (TF Shoot/root) ratio in the *Lens culinaris* Medik., genotype VL 125, the two mutants (*cysLc1* and *catLc2*), and the double mutant (*cysLc1*/*catLc2*) in the untreated controls (0 µM of As), the As (Sodium arsenate, 50 µM)-treated plants, and the As + 1 mM BSO (L-buthionine-sulfoximine)-treated plants. Bars indicate the replicates of the three independent experiments. Means followed by different uppercase letters for shoots, lowercase letters for roots, and lowercase letters with primes were significantly different at $P < 0.05$ using ANOVA, followed by Duncan's multiple range test.

than 0.1 in the As-treated VL125 and *catLc2*, 1.0 in the double mutant, and more than 1.0 in the As-treated *cysLc1* mutant. Under As + BSO treatment, the TF ratio of As (shoot/root) increased significantly in all four genotypes, but the highest (10-fold) increase was recorded in the double mutant (Figure 1B).

3.2. Effects on GSH and AsA levels

The total GSH and its redox state increased significantly over the controls in the *catLc2* mutant and VL125 at 50 µM As (Table 2). GSH and AsA levels significantly decreased, while their oxidized forms increased substantially in the *cysLc1* and the double mutants under As treatment (Table 2). Under the As + BSO condition, GSH redox declined nearly fivefold in both mutants, but AsA redox increased significantly over that of the MuCat in the *catLc2* mutant and approached the control value in the *cysLc1* mutant (Table 2). Reduced GSH and AsA redox was observed in VL125 and the double mutant under As + BSO treatment (Table 2). The change was not significant in the rest of the cases.

3.3. Foliar Cys level and thiol-metabolizing enzymes

Foliar Cys content in the *cysLc1* mutant was constitutively low, showing only 20% of the wild type under the untreated (MuCys) condition. Leaf Cys levels decreased significantly in the *cysLc1* and double mutant at 50 µM As, but approached the control levels under the As + BSO condition (Table 2). Cys levels in VL125 and the double mutant were tripled only under As + BSO treatment; under the same treatment they partially changed for the *catLc2* mutant (Table 2). The H_2S levels significantly increased over their respective values in MuC in the As + BSO-treated *cysLc1* and *catLc2* mutants, but varied marginally in the rest of the cases (Table 2).

The activities of OAS-TL, γ-ECS, and PCS increased significantly in the *catLc2* mutant and VL125, but decreased markedly in the *cysLc1* and double mutant under 50 µM As treatment (Table 3). Under the As + BSO condition, γ-ECS activity was not detectable, while PCS levels decreased substantially in all four genotypes. OAS-TL activities were reduced in the *catLc2* mutant only (Table 3). LCD and DCD activities were increased by 2.5- and 2.7-fold over those values in MuC in both mutants under BSO treatment. Enzyme activity did not change significantly in the rest of the cases (Table 3).

3.4. Response of antioxidant enzymes and oxidative stress parameters

Foliar SOD activity was significantly higher in all the genotypes across treatments. Under As treatment alone, APX, DHAR, CAT, GR, and GST activities declined significantly in the *cysLc1* and double mutants (Table 3). CAT activity was constitutively low in the *catLc2* mutant and partially low in the double mutant throughout the treatment regimens (Table 3). GR and GST activities in As-treated VL125 and *catLc2* increased significantly over their respective controls. Under As + BSO treatment, GST activity was reduced markedly in all four genotypes. Only

Table 2. Changes in foliar GSH and GSSG (nmol g^{-1} FW), GSH redox [GSH/(GSH+GSSG)], AsA and DHA (nmolg^{-1} FW), AsA redox [AsA/(AsA+DHA)], Cysteine (Cys, nmol g^{-1} FW), and H_2S (nmol g^{-1} FW) in the genotype (*Lens culinaris* Medik.) VL125, *cysLc1*, *catLc2*, and double mutant *cysLc1*/*catLc2* under 50 μM As (sodium arsenate) treatment and As + 1 mM BSO treatment for 10 days.

Traits	MC (VL 125)	MuCys	MuCat	MucysCat	As-treated				As + BSO-treated			
					VL 125	*cysLc1*	*catLc2*	*cysLc1*/ *catLc2*	VL125	*cysLc1*	*catLc2*	*cysLc1*/ *catLc2*
GSH	181.3 ± 2.2c	36.27 ± 1.8e	209.9 ± 4.3c	125.8 ± 2.2d	378.8 ± 4.9b	11.78 ± 0.9f	619.1 ± 6.3a	32.9 ± 1.3e	39.8 ± 1.1e	36.8 ± 1.1e	45.9 ± 0.9e	30.5 ± 1.1e
GSSG	21.2 ± 1.1g	9.23 ± 1.1f	30.8 ± 1.5e	23.7 ± 1.1e	40.8 ± 1.9d	39.2 ± 1.8d	86.9 ± 1.7c	134.1 ± 1.8b	150.3 ± 2.5b	191.2 ± 2.9a	211.7 ± 2.4a	170.0 ± 2.9a
GSH redox	0.89 ± 0.9a	0.79 ± 0.09b	0.87 ±0.07a	0.84 ±0.06a	0.90 ±0.07a	0.23 ±0.07c	0.87 ± 0.07a	0.19 ± 0.01c	0.21 ± 0.03c	0.16 ± 0.02d	0.17 ± 0.04d	0.15 ± 0.03d
AsA	852.0 ± 15a	690.2 ± 9.8b	656.8 ± 10b	675.4 ± 8.9b	839.3 ± 12a	100.9 ± 1.8d	661.8 ± 10b	133.7 ± 1.2c	120.5 ± 2.0c	539.2 ± 7.8b	615.6 ±0.08b	90.8 ± 2.0d
DHA	102.1 ± 7.6d	96.8 ± 5.7d	198.6 ± 4.9c	111.6 ± 3.9d	99.7 ± 3.4d	650.1 ± 9.5a	200.6 ± 4.9c	498.3 ± 2.7b	457.5 ± 1.9b	96.8 ± 5.7d	156.9 ±0.05c	489.2 ± 4.8b
AsA redox	0.89 ±0.07a	0.87 ± 0.06a	0.76 ±0.06b	0.86 ±0.09a	0.89 ±0.06a	0.13 ± 0.05d	0.77 ± 0.08b	0.21 ± 0.05c	0.21 ± 0.01c	0.85 ± 0.07a	0.80 ±0.07a	0.16 ± 0.01d
Cys	8.98 ±0.49c	4.82 ± 0.08e	9.04 ±0.51c	6.15 ±0.67d	8.11 ±0.37c	0.98 ±0.51f	6.28 ± 0.46d	3.19 ± 0.61	27.39 ± 1.6a	7.20 ± 0.61c	9.27 ±0.78c	18.51 ± 1.12b
H_2S	0.04 ±0.01c	0.03 ± 0.01c	0.05 ±0.01c	0.03 ±0.03c	0.04 ±0.01c	0.03 ±0.01c	0.06 ± 0.01b	0.03 ± 0.01c	0.04 ± 0.01c	0.07 ± 0.01b	0.19 ±0.07a	0.03 ± 0.01c

Data are means ± standard error of four replicates. Means followed by different lowercase letters indicate significant differences for a particular trait at $P < 0.05$ by ANOVA, followed by Duncan's multiple range tests.

CAT activities increased significantly in the *cysLc1* mutant. In the *catLc2* mutant, APX, DHAR, and GR activities were markedly enhanced under As + BSO treatment (Table 3). Except SOD, activities of all the enzymes were significantly reduced in the As + BSO-treated VL125 and double mutant, with more severe effect in the double mutant (Table 3).

Foliar H_2O_2, MDA, and EL% increased significantly in the As-exposed *cysLc1* and double mutants. Under the As + BSO condition, these three traits were markedly elevated in VL125 and further enhanced in the double mutant, but did not change significantly in the *cysLc1* and *catLc2* mutants (Table 4).

3.5. Changes in gene expression

With As treatment alone, two putative sulfate transporter genes *LcSultr2;1* and *LcSultr 2;2* and transcripts of OAS-TLA, OAS-TLB, γ-ECS, and PCS were significantly upregulated in VL125 and the *catLc2* mutant, but the latter four were reduced markedly in the *cysLc1* and double mutants (Figures 2A and 2B). Under the As + BSO condition, the *LcSultr 2;1*, *LcSultr 2;2*, and OAS-TLA isoforms were upregulated, while OAS-TL B was downregulated in the *catLc2* mutant (Figures 2A and 2B). In *cysLc1*, OAS-TLA was constitutively repressed, but OAS-TLB changed insignificantly with As + BSO treatment. No γ-ECS transcript was detected, while PCS expression was downregulated in the four genotypes. Transcripts of LCD and DCD increased to the tune of nearly threefold in *cysLc1* and *catLc2*. The change was not significant in the rest of the cases (Figures 2A and 2B).

Under As treatment alone, four isoforms of SOD were detected, of which Cu/Zn SOD I and Cu/Zn SOD II were significantly upregulated in all four genotypes (Figures 3A and 3B). Moreover, one Mn SOD and one Fe SOD transcript was also induced in the As-treated *cysLc1* and *catLc2* mutants. Expressions of APX I, APX II, DHAR, and CAT isoforms declined significantly in the *cysLc1* and double mutants in comparison with their respective controls (Figure 3A). CAT expression was constitutively low in the *catLc2* mutant throughout the treatment regimes. Expressions of GR I, GR II, GST I, and GST II were significantly induced over their respective controls in As-treated VL125 and *catLc2*, while they were substantially downregulated in the *cysLc1* and double mutants exposed

Table 3. Changes in the activities of foliar OAS-TL (nmol Cys min^{-1} mg^{-1} protein), SAT (U mg^{-1} protein), γ-ECS (nmol γ-EC min^{-1} mg^{-1} protein), PCS (nmol GSH eq min^{-1} mg^{-1} protein), LCD (nmol H_2S min^{-1} mg^{-1} protein), DCD (nmol H_2S min^{-1} mg^{-1} protein), SOD (U mg^{-1} protein), APX (μmol AsA oxi min^{-1} mg^{-1} protein), DHAR (μmol AsA formed min^{-1} mg^{-1} protein), GR (nmol NADPH oxi min^{-1} mg^{-1} proten), GST (Units mg^{-1} protein), and CAT (nmol H_2O_2 min^{-1} mg^{-1} protein) in the lentil (*Lens culinaris* Medik.) genotype VL125, *cysLc1*, *catLc2*, and double mutant *cysLc1/catLc2* exposed to 50 μM As (sodium arsenate) and As + 1 mM BSO treatments for 10 days.

Traits	MC (VL 125)	MuCys	MuCat	MucysCat	As-treated				As + BSO-treated			
					VL 125	*cysLc1*	*catLc2*	*cysLc1/ catLc2*	VL125	*cysLc1*	*catLc2*	*cysLc1/ catLc2*
OAS-TL	22.9 ± 1.9c	5.04 ±1.78d	18.5 ± 0.9c	13.26 ± 0.6c	43.51 ±2.1b	3.67 ±0.45e	54.21 ± 2.6a	3.44 ± 0.74e	19.58 ±1.54c	4.96 ±0.78d	23.97 ±2.01c	12.98 ±1.1c
SAT	0.69 ±0.04a	0.71 ±0.04a	0.65 ±0.03a	0.78 ±0.10a	0.68 ±0.04a	0.66 ±0.03a	0.67 ±0.07a	0.80 ±0.07a	0.68 ±0.03a	0.70 ±0.04a	0.68 ±0.06a	0.79 ±0.04a
γ-ECS	0.56 ±0.03c	0.49 ± 0.02d	0.61 ±0.04c	0.50 ±0.03d	0.79 ±0.05b	0.23 ±0.01e	0.88 ± 0.08a	0.19 ± 0.001e	ND	ND	ND	ND
PCS	0.78 ±0.10c	0.69 ± 0.09d	0.87 ±0.11c	0.75 ±0.08c	1.29 ±0.09b	0.29 ± 0.03e	1.69 ± 0.12a	0.37 ± 0.04e	0.10 ± 0.001f	0.09 ± 0.001f	0.12 ± 0.05f	0.05 ± 0.002g
LCD	16.41 ±1.39b	4.41 ± 0.60d	11.76 ±1.44b	7.67 ±0.98c	16.03 ±1.42b	4.21 ±0.68d	12.04 ± 1.51b	8.03 ± 1.01c	15.33 ± 1.31b	10.53 ± 1.88b	31.75 ± 2.21a	7.59 ± 0.95c
DCD	10.44 ±0.89b	3.67 ± 1.18d	9.86 ±0.91b	6.71 ±0.67c	11.00 ±0.91b	3.33 ±1.15d	9.06 ± 1.04b	6.03 ± 0.67c	9.77 ± 0.08b	8.78 ± 0.34b	27.06 ±0.97a	6.11 ± 0.78c
SOD	130.8 ± 3.4a	141.6 ± 3.9a	130.6 ± 3.3a	153.8 ± 4.1a	194.8 ± 5.0b	191.5 ± 4.2b	177.7 ± 3.5b	196.3 ± 3.2b	229.9 ± 4.1a	231.3 ± 4.6a	217.5 ± 4.5a	276.8 ± 4.8a
APX	121.5 ± 3.2c	133.5 ± 3.5b	119.9 ± 3.0c	139.9 ± 3.9b	126.5 ± 3.5c	75.5 ± 1.5d	123.9 ± 3.0c	59.3 ± 1.9e	56.7 ± 1.8e	129.5 ± 3.0c	178.9 ± 3.3a	39.9 ± 2.5f
DHAR	0.49 ± 0.08c	0.59 ± 0.08b	0.67 ± 0.09b	0.55 ± 0.07c	0.51 ± 0.07c	0.28 ± 0.03d	0.63 ± 0.05b	0.25 ± 0.04d	0.23 ± 0.08d	0.60 ± 0.06b	0.89 ± 0.09a	0.15 ± 0.05e
GR	41.6 ± 1.3c	41.9 ± 1.4c	39.6 ± 1.8c	36.6 ± 1.2c	71.9 ± 1.9b	19.9 ± 1.0d	83.5 ± 2.2a	16.3 ± 1.22d	20.5 ± 1.5d	37.7 ± 1.1c	85.3 ± 2.2a	6.6 ± 0.8e
GST	0.16 ±0.05c	0.19 ± 0.07c	0.22 ±0.08c	0.16 ± 0.02c	0.39 ±0.08b	0.10 ±0.01d	0.49 ± 0.10a	0.08 ±0.001d	0.04 ±0.001e	0.03 ±0.002e	0.07 ±0.01d	0.02 ±0.001e
CAT	80.4 ± 5.8b	57.3 ± 4.1c	8.91 ± 2.8e	33.7 ± 4.2d	78.4 ± 5.3b	23.8 ±0.87d	8.53 ± 2.34e	17.8 ± 1.75e	28.3 ± 1.92d	228.6 ± 10.9a	8.50 ± 2.3e	11.76 ± 0.98e

Data are means ± standard error of four replicates. Means followed by different lowercase letters indicate significant differences for a particular trait at $P < 0.05$ by ANOVA, followed by Duncan's multiple range tests. ND-not detected.

to As-treatment alone (Figures 3A and 3B). In the As + BSO-treated *cysLc1* mutant, CAT expression was upregulated by about 5.5-fold, while expressions of APX I and II, DHAR, and GR I and II were close to the control values. Expressions of APX I and II, DHAR, and GR I and II were significantly upregulated and a new isoform, APX III, was also induced in the As + BSO-treated *catLc2* mutant (Figures 3A and 3B). GSTs were reduced, but SOD isoforms changed marginally in all genotypes (Figures 3A and 3B).

4. Discussion

4.1. Growth response and As accumulation pattern

Growth inhibition is one the most significant and tractable physiological phenomena during As toxicity of crop plants (Malik et al., 2012; Talukdar, 2013). The lentil genotypes in the present study exhibited utterly contrasting responses to the 50 μM As treatment. While plant growth was not affected in VL125 and the *catLc2* mutant, growth was severely inhibited in the *cysLc1* and double mutants, and the magnitude of inhibition was greater in shoots than in roots. This might be due to higher As accumulation in the

Table 4. Changes in foliar H_2O_2 (μmol g^{-1} FW), malondialdehyde (MDA, nmol g^{-1} FW), and electrolyte leakage (EL%) in the lentil (*Lens culinaris* Medik.) genotype VL125, *cysLc1*, *catLc2*, and double mutant *cysLc1/catLc2* exposed to 50 μM As (sodium arsenate) and As + 1 mM BSO treatments for 10 days.

Traits	MC (VL 125)	MuCys	MuCat	MucysCat	As-treated				As + BSO-treated			
					VL 125	*cysLc1*	*catLc2*	*cysLc1/ catLc2*	VL125	*cysLc1*	*catLc2*	*cysLc1/ catLc2*
H_2O_2	4.4 ± 0.7d	4.8 ± 0.6d	3.4 ± 0.4e	5.3 ± 0.7c	4.5 ± 0.8d	12.9 ± 0.9b	3.7 ± 0.6e	18.9 ± 0.8a	16.3 ± 0.5a	4.9 ± 0.7d	4.0 ± 0.9d	22.7 ± 1.3a
MDA	4.1 ± 0.5 d	4.7 ± 0.7d	4.1 ± 0.8d	4.9 ± 0.6d	4.3 ± 0.6d	14.7 ± 0.7b	4.5 ± 0.8d	19.8 ± 1.3a	14.8 ± 1.3b	5.1 ± 0.6c	3.9 ± 0.7d	20.7 ± 1.7a
EL%	3.3 ± 0.5e	3.9 ± 0.6e	3.5 ± 0.5e	5.0 ± 0.9c	3.5 ± 0.6e	17.1 ± 0.9b	3.5 ± 0.7e	20.5 ± 1.0a	13.2 ± 0.8b	4.1 ± 0.7d	4.3 ± 0.5d	22.5 ± 1.2a

Data are means ± standard error of four replicates. Means followed by different lowercase letters indicate significant differences for a particular trait at $P < 0.05$ by ANOVA, followed by Duncan's multiple range test.

roots of VL125 and *catLc2* than in their shoots, and the higher As in shoots of *cysLc1* than in *cysLc1* roots. This fact was supported by the lower shoot/root transfer ratio (<1.0) in VL125 and *catLc2*, and the higher (>1.0) ratio in the *cysLc1* mutant. Obviously, a considerable amount of As was prevented to go upward by the VL125 and *catLc2* roots. In contrast, shoots were exposed to high As in the *cysLc1* mutant. In this scenario, the greater inhibition of shoot dry weight than that of root weight in the double mutant, despite nearly equal amounts of As levels (TF = 1.0) in shoots and roots, indicated the photosynthetic organs had higher sensitivity to As exposure than the underground parts. Unlike As hyperaccumulators, most crop plants possess mechanisms to retain/detoxify much of their As burden in the root (Stoeva et al., 2005; Gunes et al., 2009; Khan et al., 2009; Talukdar, 2013). The GSH pool facilitated GSH-As binding, thus preventing the translocation of As from the root to the shoot in the VL125 and *catLc2* mutant in the present study. Contrastingly, low GSH levels (due to Cys deficiency) in *cysLc1* and in the double mutant resulted in some perturbations in GSH-As binding, leading to upward translocation of As. Thus, in the presence of BSO in the medium, the low GSH pool impeded sequestration and detoxification of As in the roots. This resulted in maximum translocation of As to the shoots of all four genotypes with highest accumulation in the double mutant. However, the higher ratio of shoot/root As accumulation became more detrimental in VL125 and the double mutant in the presence of BSO, as evidenced by their reduced dry weight. In this backdrop, high As accumulation and shoot dry weight as per MuC levels in the *cysLc1* and *catLc2* mutants strongly indicated greater As tolerance and localized detoxification capability of the genotypes compared with those found in VL125 and their double mutant.

4.2. Response of the thiol cascade to As treatment

Accumulating evidence indicates the critical importance of S in As tolerance and detoxification (Tripathi et al., 2012). GSH plays central role in the process (Finnegan and Chen, 2012). Increased GSH levels pushed its corresponding redox states above 0.8, favorable to maintain high thiolic capability and antioxidant defense in the *catLc2* mutant and VL125 exposed to As in the present study. Gene and protein expression studies have confirmed that transcriptional regulation of sulfate uptake/transport and the antioxidant system are the keys to As tolerance (Chakrabarty et al., 2009; Rai et al., 2011; Talukdar and Talukdar, 2014). Significant upregulation of *LcSultr2;1* and *LcSultr2;2* in leaves of the As-treated *catLc2* mutant and VL125 strongly indicated induction of sulfate transporters involved in xylem loading of sulfate and its subsequent transport from root to shoot. This is necessary to meet the growing S demand to detoxify As in photosynthetic organs. During its synthesis, GSH exclusively requires Cys as one of its building blocks. The significant increases in OAS-TL, γ-ECS, and PCS activities in VL125 and the *catLc2* mutant subjected to As-treatment alone might be due to elevated expressions of OAS-TL A and B, γ-ECS, and PCS transcripts and were instrumental to maintain a steady supply of Cys and its downstream thiol metabolites like GSH and PCs. Stimulation of this entire thiol cascade indicated the fact that high As exposure necessitated greater thiol demand. This is being met through the coordinated induction of the thiol cascade in both genotypes. Obviously, the normal levels (close to those of the controls) of Cys in the As-treated mutant and VL125 strongly indicate the availability of enough thiol

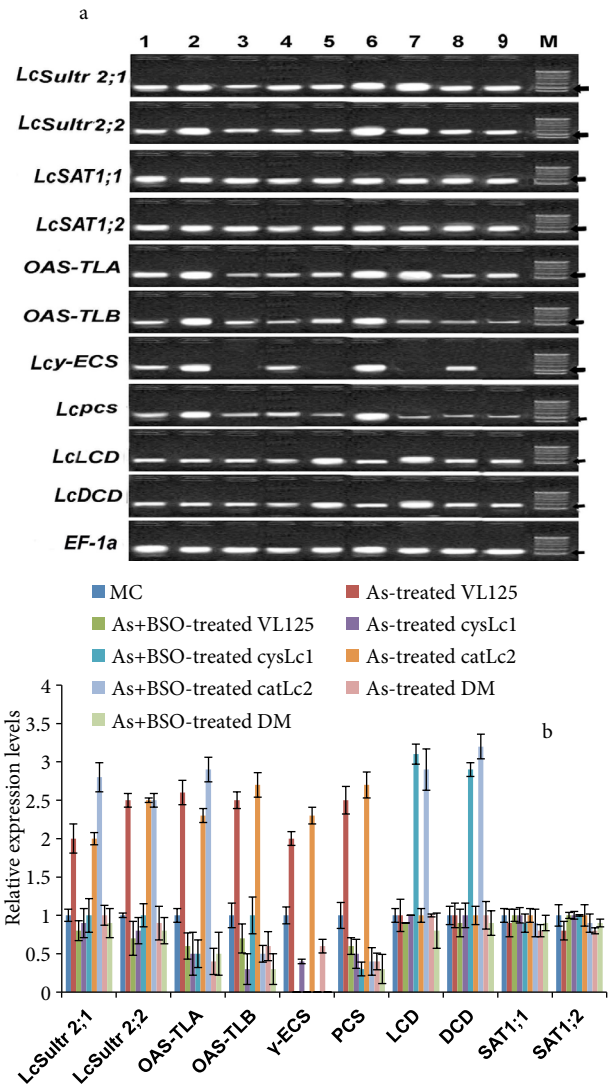

Figure 2. Transcript analysis of two sulfate transporters, SAT, OAS-TL, γ-ECS, PCS, DCD, and LCD isoforms in leaves of VL 125, the two mutants (*cysLc1* and *catLc2*), and the double mutant (*cysLc1/catLc2*) of *Lens culinaris* Medik. in the untreated controls (0 µM of As), the As (Sodium arsenate, 50 µM)-treated plants, and the As + 1 mM BSO (L-buthionine-sulfoximine)-treated plants by: a) qRT-PCR, followed by 2% agarose gel electrophoresis with *EF-1α* used for cDNA normalization; and b) their relative expression levels. Bars indicate replicates with average of three technical replicates/biological replicates. Asterisk denotes the significant changes (up- or downregulation) in relation to the control (set as 1) at $P < 0.05$. Lane 1: control (value set in 1 for controls); lane 2: As-treated VL125; lane 3: As + BSO-treated VL125; lane 4: As-treated *cysLc1*; lane 5: As+BSO-treated *cysLc1*; lane 6: As-treated *catLc2*; lane 7: As + BSO-treated *catLc2*; lane 8: As-treated DM (double mutant); lane 9: As + BSO-treated DM (double mutant); M-100-bp DNA marker (M) (arrow 200 bp).

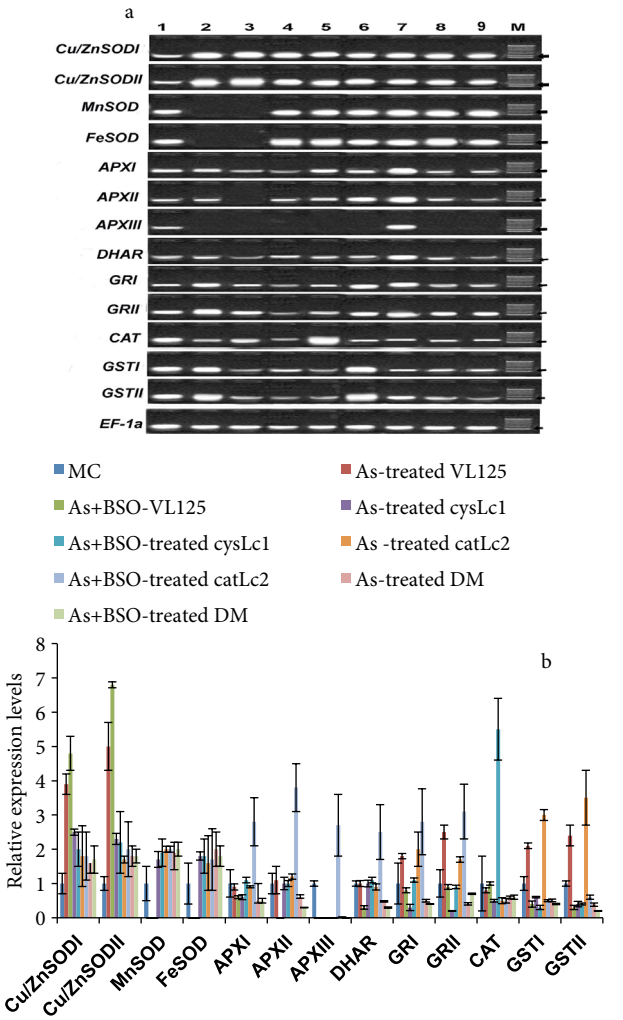

Figure 3. Transcript analysis of SODs, APXs, DHAR, GRI, GRII, CAT, and GST I and GST II isoforms in leaves of VL 125, the two mutants (*cysLc1* and *catLc2*), and the double mutant (*cysLc1/catLc2*) of *Lens culinaris* Medik. in untreated controls (0 µM of As), the As (Sodium arsenate, 50 µM)-treated plants, and the As + 1 mM BSO (L-buthionine-sulfoximine)-treated plants by: a) qRT-PCR, followed by 2 % agarose gel electrophoresis with *EF-1α* used for cDNA normalization; and (b) their relative expression levels. Bars indicate replicates with average of three technical replicates/biological replicates. Asterisk denotes the significant changes (up- or downregulation) in relation to the control (set as 1) at $P < 0.05$. Lane 1: control (value set in 1 for controls); lane 2: As-treated VL125; lane 3: As + BSO-treated VL125; lane 4: As-treated *cysLc1*; lane 5: As+BSO-treated *cysLc1*; lane 6: As-treated *catLc2*; lane 7: As + BSO-treated *catLc2*; lane 8: As-treated DM (double mutant); lane 9: As + BSO-treated DM (double mutant); M-100-bp DNA marker (M) (arrow 200 bp).

pools to meet the escalating consumption of downstream thiol moieties. Cys has its own antioxidant and reducing power, which mitigates salt-induced oxidative damage in barley seeds without affecting antioxidant defense components (Genisel et al., 2015). In the present study, the nonresponsiveness of sulfate transporters accompanied by significant downregulations of OAS-TL, γ-ECS, and PCS expressions might have reduced Cys levels and lowered GSH/PC in both the *cysLc1* and double mutants under As treatment alone. Both genotypes already have genetic backgrounds of a low Cys pool, which further declined due to As exposure. Crop plants like *Brassica*, rice, and lentils exhibited As-induced upregulation of different S transporters and GSH/PC transcripts, but there were genotypic differences in their mechanisms of As tolerance (Chakrabarty et al., 2009; Srivastava et al., 2009; Talukdar and Talukdar, 2014).

4.3. The thiol cascade and downstream antioxidant defense under As treatment

Proper augmentation of upstream thiolic capability with downstream antioxidant defense components is essential in the As tolerance mechanism. Enhanced GR activity in the As-treated VL125 and *catLc2* mutant ensured effective recycling of GSH and favorable GSH-redox by preventing excess build-up of GSSG. This capability was effectively aided by enhanced GST activity and favorable AsA balance in response to As exposure. GR and GST activity in both genotypes increased mainly due to over twofold upregulation of both *GRI* and *GRII* and nearly three- to fourfold induction of *GST I* and *II* transcripts in the presence of As. In contrast, downregulation of both GR and GST expressions resulted in decreased activity of GR and GST, respectively, and concomitant reduction of GSH-redox in the As-treated *cysLc1* and double mutants. The GR transcript was not responsive in As-treated *B. juncea* (Srivastava et al., 2009), but was elevated in Indian mustard (Khan et al., 2009). In agreement with the present results, GST activity and its transcripts were upregulated in *Arabidopsis*, rice, lentils, and *Brassica* subjected to As treatment (Abercrombie et al., 2008; Chakrabarty et al., 2009; Srivastava et al., 2009; Talukdar and Talukdar, 2014). Barring GR and GSTs, no other downstream antioxidant component like APX, DHAR, and CAT was found elevated (over the values of the controls) in the *catLc2* mutant and VL125 genotype under As treatment alone. However, significant increase in SOD activity in all four genotypes under As exposure occurred through elevated expressions of Cu/Zn SOD I and Cu/Zn SOD II transcripts along with Mn SOD and Fe SOD transcripts in the *cysLc1* and *catLc2* mutants. This indicates that As induced excess generation of superoxide radicals and participation of different cellular compartments during dismutation of superoxide radicals. This is in agreement with earlier reports in As-treated lentils (Talukdar and Talukdar, 2014) and *Arabidopsis* (Abercrombie et al., 2008). Although stimulated SOD activity has the capacity to generate excess H_2O_2 as a byproduct, the H_2O_2 level was contained within the control level in As-treated *catLc2* and VL125. In contrast, the absence of any induction in upstream thiol metabolism as well as in downstream antioxidant defense led to the generation of excess H_2O_2 and elevated lipid peroxidation in the photosynthetic organs of the As-treated *cysLc1* and double mutants. This marked the onset of As-induced oxidative stress in both genotypes.

4.4. Thiol cascade components and antioxidant defense under the As + BSO combination

Perhaps one of the most intriguing questions in the present investigation was the tolerance of the *catLc2* and *cysLc1* mutants to the combined treatment of As and BSO. Both mutants effectively counterbalanced As-induced oxidative stress by maintaining normal (close to MuC) dry weight in presence of BSO, despite complete inhibition of γ-ECS activity and significant downregulation of PCS transcripts. Furthermore, the absence of GST I and repression of GST II isoform led to a sharp decline in GST activity in the As + BSO-treated mutants. This indicated insufficient availability of GSH pool and concomitant collapse of GSH-dependent antioxidant defense. Barring these similarities, the two mutants differed sharply with each other in their mechanisms of As tolerance in the presence of BSO. Significant upregulation of both group 2 transporters and the OAS-TLA isoform in the *catLc2* mutant indicated stimulation of the upstream thiol cascade. Despite nearly a fivefold decrease in the GSH redox state, constitutively low CAT levels and concomitant reduction in GST and PCS activity, foliar H_2O_2 and lipid peroxidation levels were managed within the control levels by the mutant. Apart from APX I, all isoforms of APX, DHAR, and GR overexpressed and a new isoform, APX III, originated in the BSO-treated mutant. Unlike *catLc2*, induced CAT expressions were accompanied with normal (close to MuCys) levels of the upstream thiol cascade and APX, DHAR, and GR activity effectively prevented As-induced oxidative damage in the *cysLc1* mutant. In contrast, BSO exposure aggravated As stress in VL125 and the double mutant plant, exhibiting huge accumulation of H_2O_2 and concomitant onset of oxidative stress.

4.5. Cys homeostasis and modulation of antioxidant defense via H_2S under As + BSO treatment

Remarkably enough, Cys levels in both mutants were similar to those in MuC, despite blockage of Cys channeling to GSH by BSO; in order to ascertain that, the Cys degradation pathway was studied. LCD and DCD play predominant roles in Cys desulfuration and consequent Cys homeostasis via H_2S generation (Chen et al., 2011; Talukdar, 2014b, 2015). H_2S has the ability to regulate Cys synthesis

through OAS-TL in the Cys synthase complex (Hell and Wirtz, 2011). In the present study, stimulated LCD/DCD activity in both mutants led to fair increases in measurable H_2S levels. This might have triggered the association of the enzyme complex and consequent reduction in OAS-TL activity. OAS-TL expression was constitutively low in the *cysLc1* mutant. However, it was regulated in the *catLc2* mutant under As + BSO treatment by counterbalancing the upregulation of OAS-TLA with downregulation of the OAS-TLB isoform. Obviously, along with induced desulfuration, this upstream regulation of Cys synthesis was necessitated to prevent excess build-up of free Cys in view of a blockage of its downstream channeling to GSH. Thus, it seems clear that H_2S maintained Cys homeostasis in the *catLc2* mutant by: a) counterbalancing the pace of its synthesis (via OAS-TL); and b) inducing its degradation via increased LCD/DCD activity. In the As + BSO-treated *cysLc1* mutant, the Cys level was maintained through its degradation. This homeostasis in the Cys level is utterly important as Cys can mitigate oxidative stress by using its own reducing and antioxidant properties, as recently observed in salt-stressed barley seedlings (Genisel et al., 2015). However, bean and *Arabidopsis* mutants, deficient in Cys desulfuration pathway, exhibited overaccumulation of free Cys, which led to excess ROS-mediated oxidative imbalance (Álvarez et al., 2010; Talukdar, 2014b). In the present study, the absence of any induction in LCD/DCD activity in the BSO-treated VL125 and double mutant resulted in overaccumulation of Cys. Similarly, the induction of Cys desulfuration in the Arabidopsis *des1* mutant and grass pea *rlfL-1* mutant strongly confirmed that Cys desulfuration is an essential way to avoid the toxic accumulation of Cys within cells (Álvarez et al., 2010; Talukdar and Talukdar, 2014).

The induction of H_2S generation in the As + BSO-treated *catLc2 and cysLc1* mutants was accompanied with the stimulation of AsA-dependent antioxidant defense. In the present study, H_2S seems to play dual roles: it maintained and stabilized the Cys level and induced AsA-dependent antioxidant defense in the *catLc2 and cysLc1* mutants exposed to As and BSO. The huge rise in the H_2S level could be considered a 'H_2S burst', presumably mediated through cellular signals generated due to low GSH-redox under As exposure. Significant enhancement of AsA-redox along with elevated APX, DHAR, and GR activity in the *catLc2* and CAT activity in the *cysLc1* mutants resulted in a marked decrease in H_2O_2 content, lipid peroxidation, and membrane leakage. Presumably, H_2S mitigated As toxicity by inducing AsA-mediated antioxidant defense in the presence of BSO. Certainly, GSH was not the sole receiver of the H_2S effects, which were percolated through a GSH-independent way against As-toxicity in the mutants. GSH synthesis was also blocked in the As + BSO-treated VL125 and double mutant, but the absence of any induction in H_2S production possibly led to lowering of the AsA-dependent antioxidant defense of the genotypes. Together, the results pointed out that BSO treatment impeded GSH-dependent enzymatic defense. However, the mutants effectively induced AsA-mediated defense components in different ways to manage As-induced ROS levels in the photosynthetic organs.

The present study on four lentil genotypes revealed the functional interplay between Cys-generated H_2S upstream and GSH-dependent antioxidant defense downstream thiol cascades in the presence of As. While As-induced oxidative stress was observed in the *cysLc1* and double mutants, there was As tolerance in the *catLc2* mutant and VL125. However, upon the combined addition of BSO and As, both mutants induced the Cys degradation pathway to generate endogenous H_2S and stimulated AsA-mediated antioxidant defense and CAT as an alternative route of As tolerance under low GSH redox (due to BSO). Failure to induce these mechanisms to alternate to GSH led to a huge Cys build-up, which, coupled with the crippled AsA-mediated defense, resulted in ROS overaccumulation and consequent oxidative damage in VL125 and the double mutant. The results suggested that metabolic diversion in the upstream thiol cascade through Cys desulfuration is imminent when the Cys consumption route towards GSH is blocked. Moreover, the endogenous H_2S surge holds the key to cellular Cys homeostasis and modulation of the downstream antioxidant defense against As toxicity.

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Supplementary table

Gene name	Accession no.	Primer sequence(5′ → 3′)	Amplicon bp	Ct value (log of mean)	Tm (°C)
Sultr2;1	JG293684	F-GATCATAGTTAAACTTCCACACAACG R- CGTAAATTCATCACATGCAATAACCG	470	1.89	57/56
Sultr2;2	AK244423)	F-GCTTGGATCCAGGGCATAGAGCTTC R- AATTGGATCCATCAACACAACTCGG	475	1.79	58/57
OAS-TLA	AJ006024	F-CTCACAAGATTCAAGGGATAGGA R- GTCATGGCTTCCGCTTCTTTC	409	1.81	59/59
OAS-TLB	EF193211	F-GGATCCGCAGTGTCTGTACCAACGAAA R- GACGTCTCACAATTCTGGCTTCAT	399	1.76	59.5/ 59.4
SAT1;1	Phvul.001G170600	F-AGCCATACTTCCTTTATCTCTGAGTG R- AACAGTATATGTACTCTCAGCAGTAAC	318	1.59	60/60
SAT1;2	Phvul.010G110600	F-GGACCATACTTCCTTTATCTCTGAGTG R- CTACTAGCAATAATCAACCTTTTCATC	289	1.61	59.6/ 59.5
γ-ECS	JG293631	F-TTCTTGGGCTTGGCTTTCA R- TTCCCTTTGGCATTATTGGTATGT	62	1.59	60/60
PCS	JG293973	F-CCTAATGGAATCTGATGTGCCTT R- CTTCTTTGACAGCGACGAGCCTT	198	1.67	57.8/57.7
LcLCD	Glyma01g40510	F-GATGCAATGTATTTGTCTCTTTTTC R- TTTCTTTTTATAATCTTTTGCTCCC	200	1.65	58/58
LcDCD	Glyma09g02450	F-AGAACAGTTCTCTCTTTTTGTCGA R- GGACAGTCCACCTTAGAGGCTAGA	157	1.68	60/59.9
Cu/ZnSODI	JG294022	F-GCTTCCATATCCATGCCTTG R- AGCTACTCTGCCACCAGCAT	305	1.42	60/59
Cu/ZnSODII	X56435	F-TTGCATTTCAACTGGACCAC R- GACCACCTTTCCCAAGATCA	224	1.39	59.7/ 59.6
MnSOD	X60170	F-GGCGGAGGTCATATTAACCA R- AAGCCACACCCATCCAGAC	315	1.43	60.1/ 60.0
FeSOD	AY426764	F-GCACCACAGAGCTTATGTAG R- GGAGTGGATGATGATGGTTC	436	1.41	60/ 60
APXI	JG294047	F-GACTTGGCCCTGGCGTTGTTGCT R- TCCAGAACCGTCCTTGTAAGTGC	150	1.46	60/59
APXII	JG293953	F-GAGGAGAGTGAAGGCAAAGCAT R- GTCAGTCAAGCTCGCATACGATA	159	1.49	60.2/ 60.0
APXIII	JG293952	F-CTTCTCCAGCCGATCAAAGA R- AGGACATTGGTCAGGTCCAG	118	1.45	60/ 60
DHAR	JG294084	F-TTGAGGTTGCTGCCAAGGCT R- TGGGATCCTTGCTCTTGAGG	127	1.41	59.9/59.8
GRI	X60373	F-GAAATTGCTAGTCTATGCGTCA R- AGCAAACTCCAAGGCAATGT	400	1.39	52.8 / 52.6
GRII	X98274	F-GGTCTGCTCTTCACGTAGACCGCT R- AATGGCTGTGGGTGATGTCCGAA	324	1.38	54.7 / 54.3
GSTI	J03197, M20363	F-TTGTCAGCAAAACATGACGA R- GAAACAAATACGTGACAGAATC	217	1.48	60/59
GSTII	AF243365	F-TCCATTCGACACTTGGTTCA R- GTGGCTATTAAGATCCAACATG	229	1.47	61/ 60
CAT	JG294070	F-CCTGTCATTGTGCGTTTCTC R- CTCCCACCTTAATGGCCTCT	440	1.37	54.1 / 54.0
EF-1α	X96555	F-TGTCGACTCTGGGAAGTCAA R- CTCTTTCCCTTTCAGCCTTG	198	1.49	60 /60