

# Identification of redox-regulated components of arsenate (As<sup>V</sup>) tolerance through thiourea supplementation in rice†

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Arsenic (As) is a ubiquitously present environmental carcinogen that enters into the human food chain through rice grains. In our previous research, the application of thiourea (TU; a non-physiological thiol based ROS scavenger) has been demonstrated to enhance salt and UV stress tolerance as well as the crop yield under field conditions. These effects were associated with the ability of TU to maintain plant redox homeostasis. Since As stress also induces redox imbalance, the present research was initiated to evaluate the efficiency of TU in regulating As tolerance/accumulation in rice. The supplementation of TU (75 μM) to As<sup>V</sup> (25 μM) improved the root growth and also reduced the As concentration by 56% in the aerial parts, which could be attributed to significant downregulation of the Lsi2 transporter responsible for the translocation of As from root to shoot. The fact that these effects were not due to direct interaction between As and TU was confirmed from complexation studies using HPLC-(ICP-MS)-(ESI-MS). Short-term kinetic studies of GSH levels and the GSH/GSSG ratio confirmed the establishment of differential redox states in As and As + TU treated seedlings. The real-time RT-PCR based comparative expression profiling under As with/without TU treatment identified Sultr1;1 and Sultr1;2 as major redox-regulated sulfate transporters. Their specific induction in shoots coupled with enhanced root-to-shoot sulfate translocation (analyzed using <sup>35</sup>S-sulfate as a radiotracer) was observed under TU supplementation. Furthermore, the level of thiolic metabolites (PC2 in roots and GSH and PC3 in shoots) and activities of sulfur metabolism enzymes (ATP sulfurylase and cysteine synthase in roots and 5'-adenylylsulfate reductase in shoot) were also increased with As + TU as compared to As treatment. Thus, this study utilizes the interaction between As and TU to identify the critical redox regulated components of As tolerance in rice.

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## Introduction

Arsenic (As) is a ubiquitously present environmental toxin and is recognized as a group-1 carcinogen by the International Agency for Research on Cancer (IARC). The health of nearly 150 million people worldwide from over 70 countries spanning six inhabited continents is threatened from As hazards. The major route of As contamination for humans is either through

drinking water or crops and fodders,<sup>1</sup> mainly rice.<sup>2</sup> Thus, different strategies are being developed to obtain low grain arsenic rice, either through conventional breeding/variety selection or by modern transgenics; however, these approaches will still take some time to come into use under field conditions. Under this milieu, the strategy with the most potential is supposed to be the management of agronomic practices to provide an immediate and sustainable solution to reduce As load in rice grains. Various approaches have been demonstrated to hold potential, e.g. growing rice with less irrigation,<sup>3</sup> supply of silicate minerals<sup>4</sup> and phosphorus<sup>5</sup> and inoculation with arsenic-tolerant soil fungi<sup>6</sup> and mycorrhiza.<sup>7</sup>

Inorganic As is a prevalent form present in the environment, existing as arsenate (AsO<sub>4</sub><sup>3-</sup>, As<sup>V</sup>) or arsenite (AsO<sub>3</sub><sup>3-</sup>, As<sup>III</sup>), depending upon the pH and redox potential of the environment.<sup>8</sup> Although the mode of toxicity of the two forms of As is different, As toxicity, in general, is associated with the induction of sulfur deficiency, oxidative stress and alteration of redox states.<sup>9-11</sup> Sulfur is an essential element for plant growth. There is a family of sulfate transporters (classified as groups-1 to 4)

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which takes up sulfur in the form of inorganic sulfate.<sup>12</sup> Inside the plant, sulfate is first activated to adenosine-5'-phosphosulfate (APS) by ATP sulfurylase, and then reduced to sulfite by APS reductase (APR). Sulfite is reduced to sulfide, which is incorporated by cysteine synthase into *O*-acetylserine to form cysteine.<sup>13</sup> The key enzyme of the sulfur assimilation pathway is APR, which is regulated by transcription factor Long Hypocotyl 5 (HY5) in a demand-driven and light-dependent manner.<sup>14</sup> The major proportion of sulfur reduction takes place in shoot chloroplasts, which is supported by the light regulated nature of HY5.<sup>15</sup> Glutathione (GSH;  $\gamma$ -Glu-Cys-Gly) and phytochelatins (PCs; GSH oligomers) are the important sulfur-containing compounds responsible for As complexation, vacuolar sequestration and maintenance of redox states.<sup>16–18</sup> The importance of sulfur is also due to the fact that its supply affects As uptake, translocation and accumulation in rice plants.<sup>19,20</sup> The relevance of redox state in the regulation of As toxicity<sup>9</sup> and for the activation of downstream signaling events is known.<sup>21</sup> Thus, it was hypothesized that a plant's As stress tolerance may be enhanced by avoiding redox imbalance. In our earlier research, we used thiourea (TU), as an external agent, to maintain the plant's redox balance under salt and UV stress.<sup>22,23</sup> TU is a non-physiological thiol and its broad range ROS scavenging activity in biological systems is well documented.<sup>24</sup> The positive effect of TU was also demonstrated in the enhancement of source-to-sink sucrose translocation,<sup>25</sup> in identification of the signaling and effector components of salt tolerance<sup>26</sup> and to improve the crop yield and oil content of Brassica.<sup>27</sup> In the present work, the effect of the interaction between As and TU was utilized for the identification of redox regulatory mechanisms of As tolerance in rice. The efficacy of TU in reducing the As load was also assessed.

## Materials and methods

### Plant material, growth conditions and treatment

The study was performed on *Oryza sativa* var. IR64. Seeds were surface sterilized with 30% ethanol for 3 min and then washed thoroughly with distilled water to remove traces of ethanol. The seeds were then soaked in distilled water under shaking conditions ( $\sim 100$  rpm) at 25 °C. The volume of water was adjusted so as to provide sufficient air to seeds while shaking. After 14–16 h of incubation, seeds were uniformly spread on a Petri plate and then allowed to germinate under dark conditions. A customized circular thermocol disc was made, which had the capacity to hold 18 seedlings. The 4 d old seedlings were fixed on these discs and then placed in a 1 L beaker with 800 ml of 1/2 Kimura solution supplemented with different treatments such as As<sup>V</sup> (prepared using the salt Na<sub>2</sub>HAsO<sub>4</sub>), As + TU and TU. One separate set was maintained as a control. All the sets were transferred into a plant growth chamber (Sanyo, Japan) with a daily cycle of a 14 h photoperiod with a light intensity of 150  $\mu\text{E m}^{-2} \text{s}^{-1}$ , day/night temperatures of 25/22 °C and a relative humidity of 65–75%. After 12 d of growth, differential phenotype was recorded in terms of dry weight/seedlings and average root and shoot lengths. Dry weights were measured

after drying the samples to a constant weight in an oven. A similar set-up was employed for the measurement of arsenic content, level of various thiols and activities of sulfur metabolism related enzymes. The roots and shoots were harvested and stored at  $-80$  °C until analysis. The harvesting time was fixed at 1 PM for each batch of experiments. For the measurement of short-term <sup>35</sup>S-sulfate uptake kinetics, the redox couple (GSH and GSSG) and real-time RT-PCR based expression profiling, seedlings were grown for 15 d under control conditions and then subjected to different treatments. For As + TU and TU, pre-treatment with TU was performed for 24 h. In order to study the light-dependent regulation, the treatments were given at 9 AM and then 1, 4 and 8 h harvesting of roots and shoots was performed, and samples were stored at  $-80$  °C until analysis. The concentrations of As<sup>V</sup> and TU were 25  $\mu\text{M}$  and 75  $\mu\text{M}$ , respectively.

### Arsenic measurement

For each treatment, seedlings were washed thoroughly in ice-cold milli-Q water to remove adsorbed As. The roots and shoots were then separated and oven-dried at 80–85 °C until a constant dry weight was obtained. The dried tissue ( $\sim 100$  mg) was kept in 1 ml of concentrated HNO<sub>3</sub> overnight at room temperature and then digested at 120 °C. The residue was then diluted in 10 ml of milli-Q water and subjected to As estimation using ICP-MS. The certified reference material (CRM) NIST 1568a rice flour from and blanks were included for quality assurance.

### *In vitro* complexation studies of arsenic with glutathione and thiourea

To check the complexation of As with thiourea, various combinations of As (4 to 40 mM, either As<sup>III</sup> or As<sup>V</sup>) and thiourea (33 to 330 mM), with and without GSH (3.3 to 33 mM) were tested. The substances were dissolved in degassed water or 0.1% formic acid and allowed to react for 12–15 h under nitrogen. The complexes were analyzed by HPLC-(ICP-MS)-(ESI-MS).

An HP1100 HPLC system (Agilent Technologies Böblingen, Germany) with an auto-sampler cooled to 4 °C was used. The separation was performed on a reverse-phase C18, Waters Atlantis column (150 mm  $\times$  4.6 mm  $\times$  5  $\mu\text{m}$ , 100 Å) using a gradient of 0.1% (v/v) formic acid (A) and 0.1% formic acid in 20% (v/v) methanol (B) at a flow rate of 1 ml min<sup>-1</sup>. Post-column, the flow was split in a ratio of 1 : 1 into the ICP-MS and ESI-MS. The 6130 quadrupole LC/MS system (Agilent Technologies Böblingen, Germany) was used as a molecule-specific detector for postcolumn detection of the As complexes by their molecular ion peaks. The MSD was used in the positive ionization mode from *m/z* 50 to *m/z* 1000 with an API electrospray head. The settings chosen were: capillary voltage of 4000 V, nebulizer pressure of 40 psi, drying gas flow of 12 L min<sup>-1</sup> at 350 °C, quadrupole temperature of 100 °C, and fragmenter voltage of 80 V. The ICP-MS 7500ce (Agilent Technologies Böblingen, Germany) was used for the element-specific detection of As. The instrument was equipped with a microconcentric nebulizer (flow rate < 100  $\mu\text{L min}^{-1}$ ), a Peltier cooled spray chamber, and oxygen as an additional plasma gas. The instrument was used in the soft extraction mode. The instrument settings were checked daily for As sensitivity and optimized when necessary.



### Fluorescence HPLC based estimation of various thiols

For the measurement of various thiols, liquid nitrogen ground plant samples (~400 mg) were extracted in buffer [diethylene-triamine pentaacetic acid (DTPA; 6.3 mM) and trifluoroacetic acid (TFA; 0.1% v/v)]. The extraction was performed on an equal volume basis and the supernatant was collected after centrifuging at 13 000g for 10 min at 4 °C. The supernatant (250 µL) was added to 615 µL of HEPES buffer [HEPES (200 mM), DTPA (6.3 mM; pH 8.2)]. To this mixture, 25 µL of tris(2-carboxyethyl)-phosphine hydrochloride (TCEP; 20 mM; as a disulfur reductant) and 10 µL of *N*-acetyl-L-cysteine (0.5 mM; as an internal standard) were added and the final mix was pre-incubated at 45 °C for 10 min in a water bath. This step is required to ensure that all thiols are in a reduced state so that maximum derivatization can occur. For monobromobimane (mBBR) based derivatization, 10 µL of mBBR (50 mM) was added and the mix was incubated in the dark in a water bath for 30 min at 45 °C. The reaction was terminated by the addition of 100 µL of acetic acid (10 mM). The derivatized samples were filtered with 0.22 micron nylon syringe filters and then stored at -20 °C for HPLC analyses. The separation and analysis of various thiols (GSH, cysteine and PCs) was carried out on reverse-phase HPLC (Waters, USA) with a Purospher RP-18e column (Merck) using a gradient of solvent A (99.9% acetonitrile + 0.1% TFA) and B (89.9% water + 10% acetonitrile + 0.1% TFA) at a flow rate of 1 ml min<sup>-1</sup> as described in Minocha *et al.*<sup>28</sup> Fluorescence intensity with an excitation wavelength of 380 nm and an emission wavelength of 470 nm was recorded using a fluorescence detector (Waters 474). The chromatograms were recorded and analyzed using Empower software.

### Measurement of activities of sulfur metabolism related enzymes

The liquid nitrogen ground plant samples (~500 mg) were homogenized in extraction buffer (1 ml), squeezed through four layers of cheesecloth and then centrifuged at 12 000g for 15 min at 4 °C. The specific extraction buffer was used for each enzyme as described previously by Hartmann *et al.*<sup>29</sup> The detailed methodology for the measurement of enzyme activity is given in the ESI,† S-1. The protein content in the sample was measured as per the protocol of Lowry *et al.*<sup>30</sup>

### Measurement of redox state in terms of GSH/GSSG ratio

The level of reduced (GSH) and oxidized (GSSG) glutathione was determined fluorometrically using *o*-phthaldialdehyde (OPT) as a fluorophore by following the protocol of Hissin and Hilf.<sup>31</sup>

### Short-term uptake kinetics using <sup>35</sup>S-sulfate as a radiotracer

For <sup>35</sup>S-sulfate radiotracer uptake kinetics, the hydroponic solutions of the seedlings given different treatments were supplemented independently with <sup>35</sup>S-sulfate (2 MBq L<sup>-1</sup>). After 1, 4 and 8 h, the root and shoot parts were separately harvested and <sup>35</sup>S-sulfate levels were measured by scintillation counting. For scintillation counting, seedlings were removed from the radioactive solution and then rinsed with the ice-cold non labeled nutrient solution (3 times for 20 s each). Root and

shoot samples were weighed separately and then digested in 10 ml of HCl (1 N) at room temperature. After 7 d, 100 µL of digested extract was mixed with 5 ml of scintillation cocktail [naphthalene (30 g), PPO (2 g), ethylene glycol (100 ml), methanol (50 ml) were mixed and the volume made up to 500 ml with dioxane] and then counted on protocol 2 of a TRI-CARB 2100 TR liquid scintillation analyzer (Packard, Canberra), as described previously.<sup>32</sup> The efficiency of the counter used was 95%.

### Primer design and real-time PCR based expression profiling of sulfate and arsenite transporter (low silicon 2; Lsi2)

All the primers used for real-time PCR were from the exon-intron boundary and designed using a web-based Quant-prime tool.<sup>33</sup> The details of the primers are given in ESI,† S-2. The specificity of all primers was confirmed by sequence analysis of RT-PCR amplicons. The DNA-free total RNA was extracted using a mirVANA kit (AM1560, Ambion). The 260/280 and 260/230 ratios greater than 2 and the intactness of rRNA bands (28/18 s) in denaturing gel electrophoresis were considered as quality control of RNA to be used for further analysis. RNA (2 µg) was subjected to cDNA synthesis using Superscript III RT (18080-093; Invitrogen) following the manufacturer's protocol. Real-time PCR was carried out using Rotor-Gene 6600 (Corbett Life Science; www.corbettlifescience.com). Reactions were set up by combining 10 µL of SyBr green PCR reaction mix (Sigma; S 4320) with 2.5 µL of 1 : 5 diluted cDNA templates, 1.5 µL each of forward and reverse primer (10 mM each), and 4.5 µL of PCR grade water (Sigma W 1754). For gene expression analyses, the reference gene (tubulin) and one target gene were analyzed per run, and reactions were carried out in triplicates for each sample. The following PCR protocols were followed: 95 °C for 15 min; 40 cycles at 94 °C for 20 s, 55 °C for 30 s, and 72 °C for 30 s followed by 72 °C for 10 min and melting curve analysis. The data of the Ct value (cycle threshold) were calculated for target/reference genes for each treatment and respective control, and then log<sub>2</sub> expression fold difference was calculated using REST-384 version 2 software. For both up- and down-regulation, a 1.5-fold change was set as the cutoff for detecting significant changes in expression.

### Statistical analysis

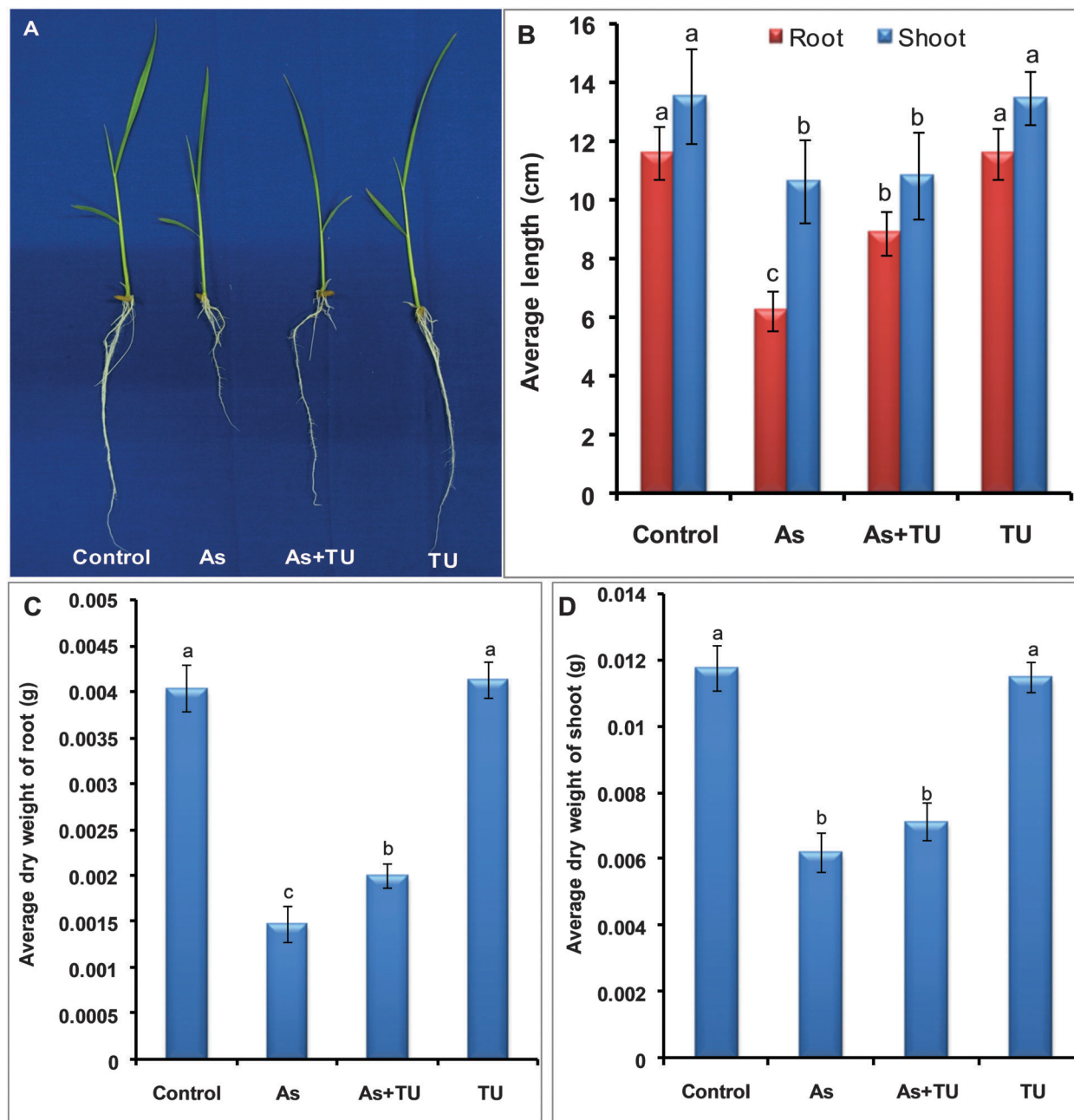
The experiments were carried out in a completely randomized design. All the experiments were repeated at least twice to check reproducibility. One-way analysis of variance (ANOVA) was performed on all the data to confirm the variability of data and the validity of results. Duncan's multiple range test (DMRT) was performed to determine the significant difference between treatments using the statistical software SPSS 17.0.

## Results

### Thiourea supplementation partially alleviated arsenic stress

The post-germination phenotyping was performed under different treatments to evaluate the effectiveness of TU supplementation. The analysis revealed differential phenotype of





**Fig. 1** Differential phenotype of *Oryza sativa* seedlings. The rice seedlings were grown for 4 d under control conditions and then subjected to different treatments such as control, arsenic ( $\text{As}^{\text{V}}$ ; 25  $\mu\text{M}$ ); arsenic ( $\text{As}^{\text{V}}$ ; 25  $\mu\text{M}$ ) + thiourea (TU; 75  $\mu\text{M}$ ), and thiourea alone (TU; 75  $\mu\text{M}$ ) for 12 d. Differential growth phenotype (A), average root and shoot length (B), average dry weight of root (C) and shoot (D) were analyzed. The data represents the mean  $\pm$  SE of three biological replicates. The experiment was repeated twice to check its reproducibility. The different letters on the bar graphs have been placed on the basis of the LSD value derived from SPSS software (DMRT,  $P < 0.05$ ).

seedlings subjected to As with/without TU treatments (Fig. 1A). There was a significant reduction in root and shoot lengths of 46 and 21%, respectively, under As stress as compared to the control. The supplementation of TU increased the root length (Fig. 1B) and the dry weight (Fig. 1C) by 42 and 13%, respectively, as compared to those of seedlings treated with As alone. No significant differences were observed in the length and dry weight of shoots between As and As + TU treated seedlings (Fig. 1B). The phenotype of the seedlings subjected

to TU treatment alone was comparable to that of the control (Fig. 1A–D).

#### Level of arsenic in different plant parts

In roots, the concentration of As was not significantly different in As (2710  $\mu\text{g g}^{-1}$  DW) and As + TU (2825  $\mu\text{g g}^{-1}$  DW) treatments (Fig. 2A). However, TU supplementation significantly reduced the As concentration in the aerial parts of rice seedlings. The As + TU treated seedlings showed a 56% reduction in As concentration



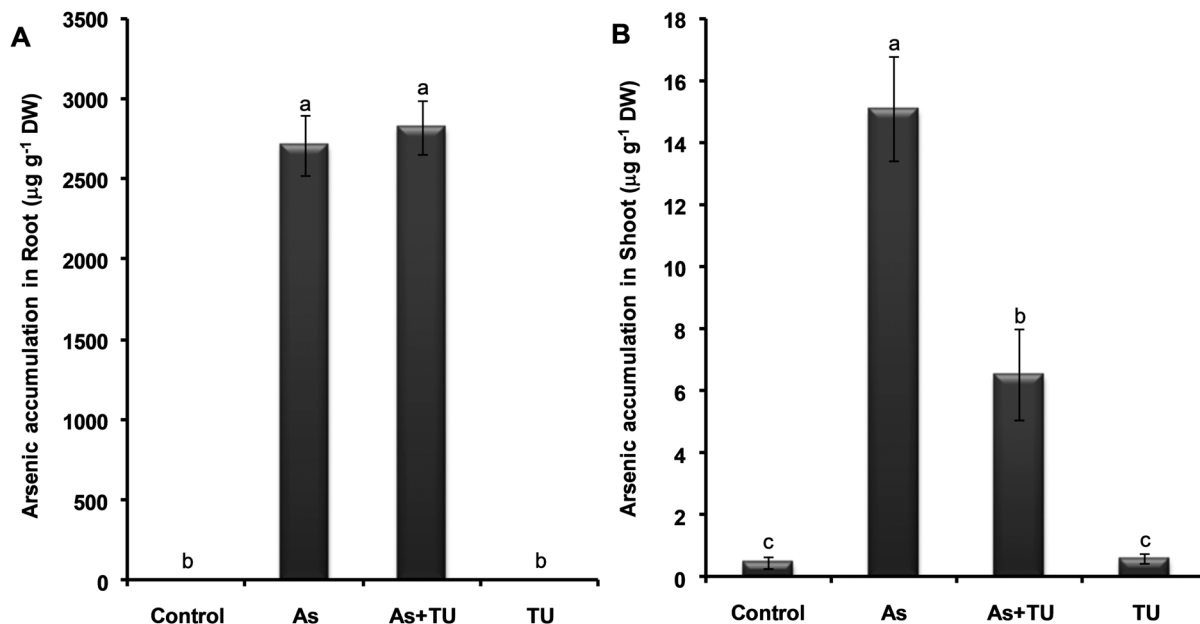


Fig. 2 ICP-MS based estimation of arsenic level. The rice seedlings were grown for 4 d under control conditions and then subjected to different treatments such as control, arsenic (As<sup>V</sup>; 25 µM); arsenic (As<sup>V</sup>; 25 µM) + thiourea (TU; 75 µM), and thiourea alone (TU; 75 µM) for 12 d. The roots (A) and shoots (B) were harvested and used for the As estimation. The data represents the mean ± SE of five biological replicates. The experiment was repeated twice to check its reproducibility. The different letters on the bar graphs have been placed on the basis of the LSD value derived from SPSS software (DMRT,  $P < 0.05$ ).

in the shoots as compared to that of treatment with As alone (Fig. 2B). By taking into account root and shoot dry weight data and As concentration, the total As content in the root and shoot (µg) was calculated. It was found that total root As content per plant increased from 4.07 µg with As alone to 5.65 µg for As + TU, while the total shoot As content per plant decreased significantly from 0.092 µg to 0.046 µg.

#### Lack of complexation between arsenic and thiourea

To check the possibility of As complexation with TU, an *in vitro* experiment was performed and analyzed by HPLC coupled in parallel to ICP-MS as an element specific detector and ESI-MS as a molecule specific detector (Fig. 3). The complexes of As with TU and/or GSH which could form are: As-TU<sub>3</sub>, GS-As-TU<sub>2</sub>, GS<sub>2</sub>-As-TU, As-GS<sub>3</sub>. The reaction mixtures containing As<sup>V</sup> in all

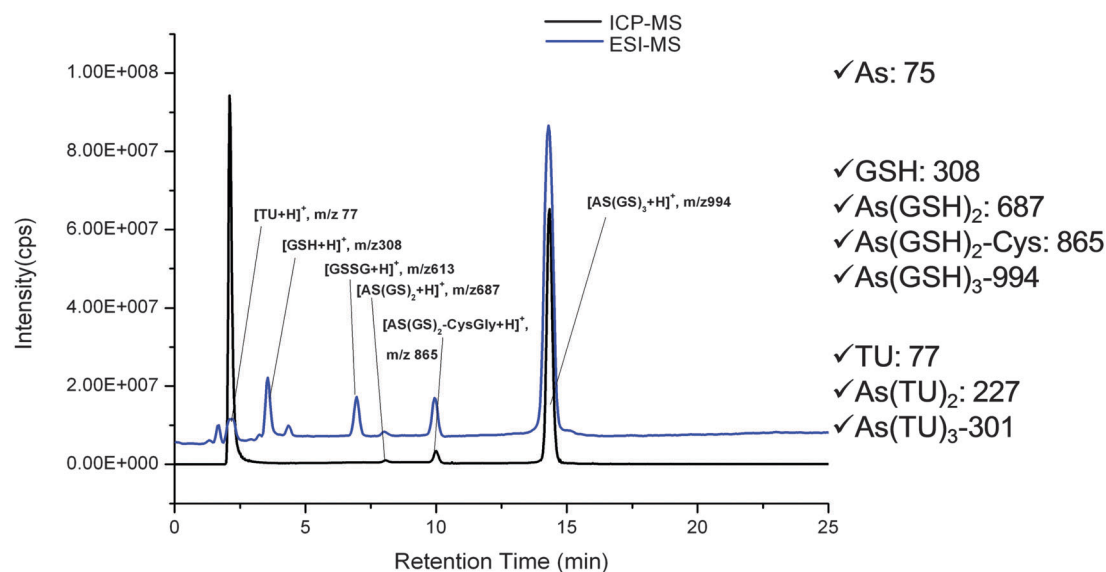


Fig. 3 *In vitro* complexation study of arsenic with glutathione (GSH) and thiourea (TU). HPLC-ICP-MS/ESI-MS chromatograms of the reaction mixture containing As(III), thiourea and glutathione. ESI-MS (blue line) data in scan mode and ICP-MS  $m/z$  75 (As) (black line) data were measured in parallel. ICP-MS traces showed four species of As which correspond to inorganic As and various complexes of GSH according to their  $m/z$  signals in ESI-MS, as indicated in the figure. None of the complexes contained thiourea. The experiment was repeated twice to check its reproducibility.



combinations and  $\text{As}^{\text{III}}$  without GSH showed only one peak in ICP-MS, corresponding to inorganic As. However, the reaction mixture containing  $\text{As}^{\text{III}}$ , TU and GSH showed four As species in ICP-MS. ESI-MS showed strong a signal at  $m/z$  values of 75, 687, 865 and 994 corresponding to inorganic As,  $\text{As}^{\text{+}}\text{-GS}_2$ ,  $\text{GS}_2\text{-As-CysGly} + \text{H}^+$  and  $\text{As-GS}_3 + \text{H}^+$  for the ICP-MS peaks. None of the peaks corresponding to As-TU complexes were detected in ESI-MS. Thiourea, reduced GSH and oxidized GSH were also detected by ESI-MS, showing signals at  $m/z$  77, 308, and 613 respectively for  $[\text{M} + \text{H}]^+$ .

### Thiourea treatment modulates the level of various thiols

Fluorescence HPLC based detection was performed for thiols such as cysteine and GSH (Fig. 4A) and phytochelatins (Fig. 4B). The levels of most of the thiols were significantly increased in both the roots and shoots under As and As + TU treatment. In roots, the cysteine, GSH and PC4 contents increased about 10-, 2.4- and 22-fold in both As and As + TU treatments, respectively, as compared to that of the control. This was in contrast to PC2, which specifically increased 56-fold under As + TU as compared to As treatment. No significant induction in the level of PC3 was observed under any treatment (Fig. 4B). In the shoot, the cysteine content was increased 1.15-fold for both As and As + TU treatments as compared to that of the control. In contrast, the GSH level increased 1.8- and 2.8-fold under As and As + TU treatment, respectively, as compared to that of the control. The level of PC3 was increased 2.63-fold in As + TU as compared to that of any other treatment. The level of PC2 was found to be same under As and As + TU treatments, while

that of PC4 was increased for As (1.7-fold) but decreased for As + TU (0.5-fold) treatments, as compared to that of the control (Fig. 4B). For treatment with TU alone, no significant change in the level of any thiol was observed in roots (Fig. 4A), however in shoots, the cysteine, GSH and PC2 contents were significantly increased as compared to those of the control (Fig. 4A and B). To measure the extent of As chelation by thiols (GSH + PCs), molar ratios of  $-\text{SH}$  to As (analyzed in fresh samples) were calculated.<sup>34</sup> The molar ratios of  $-\text{SH}$  to As were 0.109 and 0.122 for As and As + TU in roots. Hence, a maximum of about 3.6% and 4.1% As would be chelated by thiols in roots, assuming a stoichiometry of three SH to one As. In contrast,  $-\text{SH}$  to As molar ratios were very high in shoots for both As (27) and As + TU (76) treatment, suggesting an excess of thiols and that all As may be chelated.

### Activities of sulfur metabolism related enzymes

The activities of sulfur metabolism related enzymes such as ATP sulfurylase (APS), 5'-adenylsulfate reductase (APR) and cysteine synthase (CS) were measured in the roots and shoots of seedlings subjected to different treatments. The APS activity was increased 4- and 1.19-fold in As + TU treated roots and shoots, respectively, as compared to that of the control. For treatments with As and TU alone, no significant differences in APS activity were observed in roots as well as in shoots (Fig. 5A and B). The APR activity in shoots was decreased and increased by 45% and 77% under As and As + TU treatments, respectively, compared with that of the control (Fig. 5C). No APR activity could be detected in roots. The light mediated regulation of

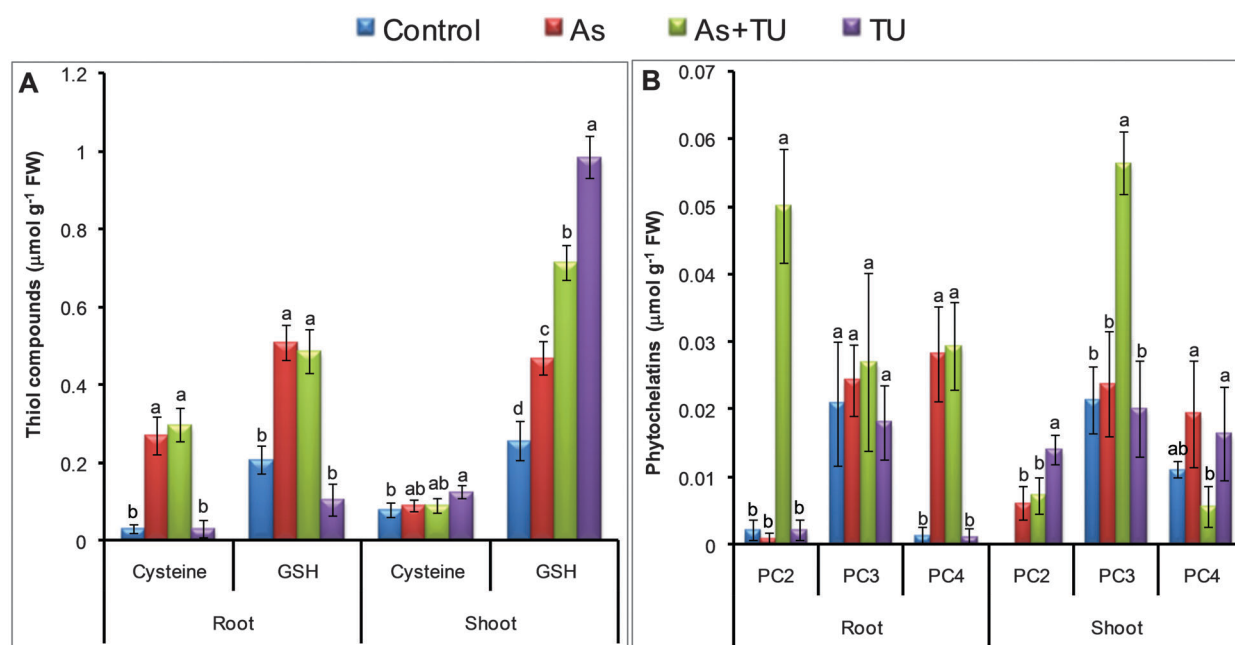


Fig. 4 Fluorescence HPLC based estimation of various thiolic metabolites. The rice seedlings were grown for 4 d under controlled conditions and then subjected to different treatments such as control, arsenic ( $\text{As}^{\text{V}}$ ; 25  $\mu\text{M}$ ); arsenic ( $\text{As}^{\text{V}}$ ; 25  $\mu\text{M}$ ) + thiourea (TU; 75  $\mu\text{M}$ ) and thiourea alone (TU; 75  $\mu\text{M}$ ) for 12 d, and HPLC based estimation of reduced glutathione (GSH) and cysteine (A) and phytochelatins (B) was performed. The data represent the mean  $\pm$  SE of three biological replicates. The experiment was repeated twice to check its reproducibility. The different letters on the bar graphs have been placed on the basis of the LSD value derived from SPSS software (DMRT,  $P < 0.05$ ).



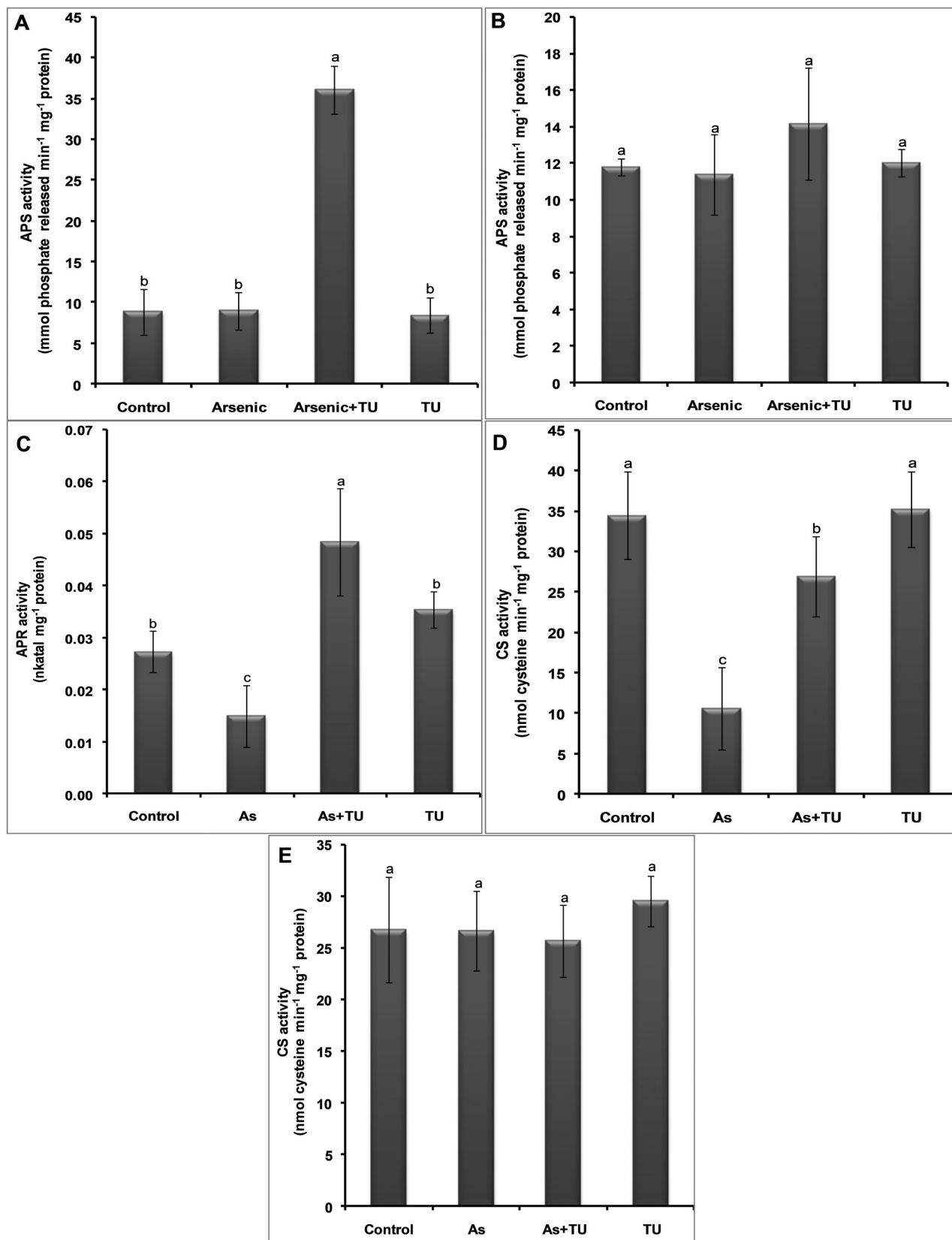


Fig. 5 Measurement of activities of sulfur metabolism related enzymes. The rice seedlings were grown for 4 d under control conditions and then subjected to different treatments such as control, arsenic ( $\text{As}^{\text{V}}$ ; 25  $\mu\text{M}$ ); arsenic ( $\text{As}^{\text{V}}$ ; 25  $\mu\text{M}$ ) + thiourea (TU; 75  $\mu\text{M}$ ) and thiourea alone (TU; 75  $\mu\text{M}$ ) for 12 d. APS (ATP sulfurylase; A: root and B: shoot), APR (5'-adenylylsulfate reductase; C: shoot; no activity detected in roots) and CS (Cysteine synthase; D: root and E: shoot) activities were assayed. The data represent the mean  $\pm$  SE of three biological replicates. The experiment was repeated twice to check its reproducibility. The different letters on the bar graphs have been placed on the basis of the LSD value derived from SPSS software (DMRT,  $P < 0.05$ ).



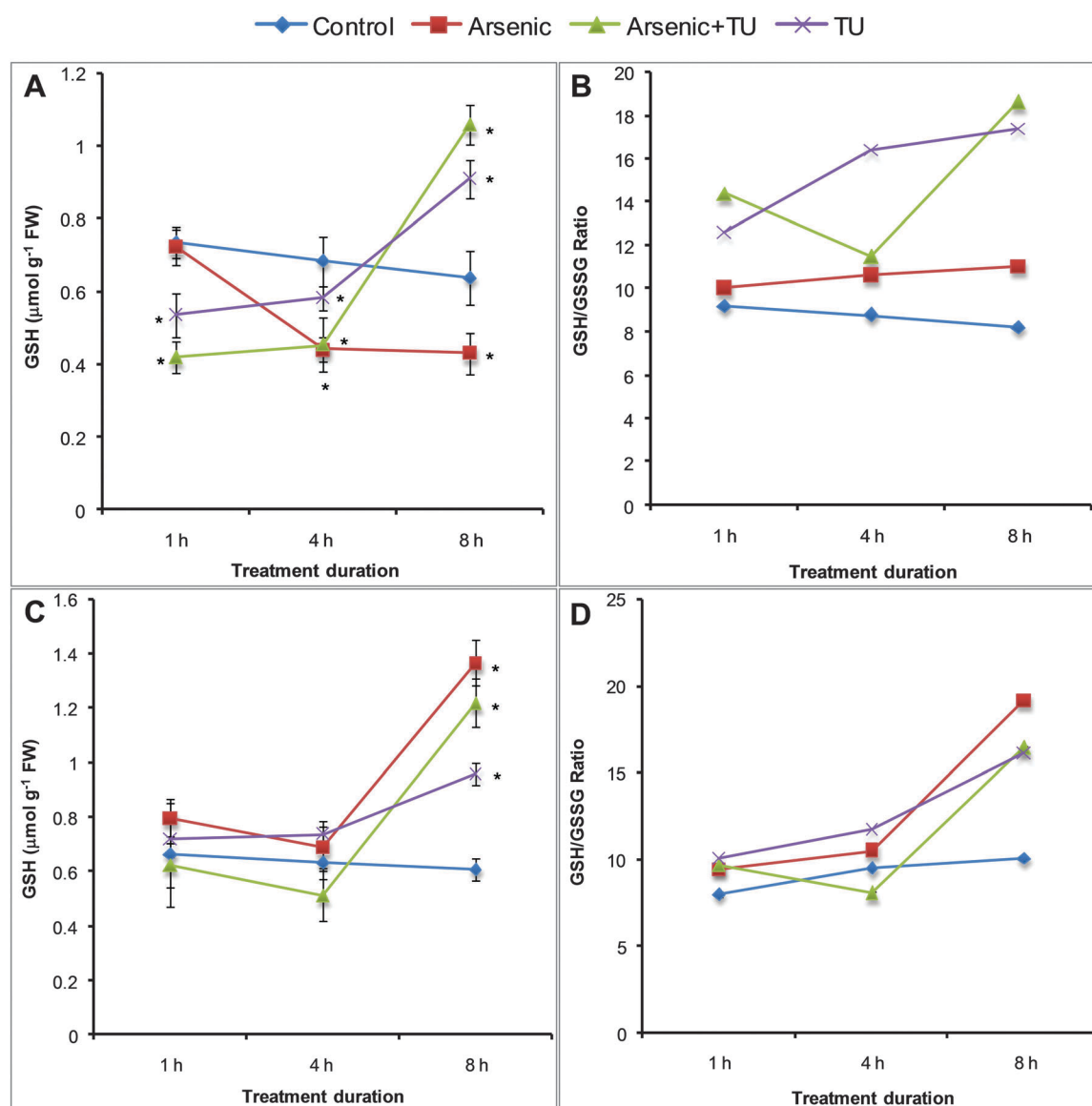
APR activity might be responsible for its significantly low activity in roots, which could not be detected. The CS activity in roots was decreased by 70 and 20% under As and As + TU treatments, respectively, as compared to that of control and TU treatments (Fig. 5D). In shoots, no significant difference in CS activity was observed under any treatment (Fig. 5E).

#### Thiourea mediates modulation of cellular redox state

In roots, under As stress, the GSH content decreased in a time-dependent manner and the maximum decrease of 32% was observed at 8 h. Under As + TU treatment and treatment with TU alone, the GSH level remained lower than that of the control until 4 h and a sharp increase was observed at 8 h (66% and

42% increase for As + TU and TU treatments, respectively, as compared to that of the control; Fig. 6A). In contrast with the GSH level, the GSH/GSSG ratio was found to be higher for all treatments compared to that of the control, with the maximum being at 8 h when the ratios were 1.35-, 2.26- and 2.1-fold higher for treatments with As, As + TU and TU alone, respectively (Fig. 6B).

In shoots, no significant difference in the GSH level was seen until 4 h under any treatment. At 8 h, the GSH levels were increased 2.25-, 2- and 1.58-fold in treatment with As, As + TU and TU alone, respectively, as compared to that of the control (Fig. 6C). The response of the GSH/GSSG ratio was similar to that of the GSH level in all treatments (Fig. 6D).



**Fig. 6** Measurement of the redox state in terms of the GSH level and the GSH/GSSG ratio. The rice seedlings were grown hydroponically for 15 d under control conditions and then subjected to different treatments such as control, arsenic ( $\text{As}^{\text{V}}$ ; 25  $\mu\text{M}$ ); arsenic ( $\text{As}^{\text{V}}$ ; 25  $\mu\text{M}$ ) + thiourea (TU; 75  $\mu\text{M}$ ) and thiourea alone (TU; 75  $\mu\text{M}$ ). After 1, 4 and 8 h of treatment, the GSH level (A: root; C: shoot) and the GSH/GSSG ratio (B: root; D: shoot) were measured. For As + TU and TU alone, 24 h pretreatment of TU was also given. The data represent the mean  $\pm$  SE of three biological replicates. The experiment was repeated twice to check its reproducibility. Asterisks (\*) have been placed on the basis of the LSD value derived from SPSS software (DMRT,  $P < 0.05$ ).



### Differential translocation of sulfate from root to shoot: $^{35}\text{S}$ -sulfate based radiotracer study

In roots, the  $^{35}\text{S}$ -sulfate progressively increased in a time dependent manner under all treatments. The control roots showed maximum uptake at 1 h with minimum uptake at 8 h. In TU-treated roots, the uptake was initially slow until 4 h, and then there was an increase in  $^{35}\text{S}$ -sulfate uptake at 8 h. In As and As + TU treatments, the level of  $^{35}\text{S}$ -sulfate was almost the same until 4 h. However at 8 h, the  $^{35}\text{S}$ -sulfate levels were increased by 1.91- and 1.12-fold, respectively under As and As + TU treatments as compared to the control (Fig. 7A).

In shoots, initially at 1 h, the level of  $^{35}\text{S}$ -sulfate was almost same in control, As and TU alone treatments but was slightly higher in As + TU treatment. With increasing time,  $^{35}\text{S}$ -sulfate uptake increased in all treatments. However, the uptake of  $^{35}\text{S}$ -sulfate was lower in the control and As treatments as compared to TU alone and As + TU treatments, with the lowest uptake being in control treatments. At 8 h, the total  $^{35}\text{S}$ -sulfate levels in As + TU and treatment with TU alone were increased 3.42- and 2.96-fold, respectively, as compared to the control (Fig. 7B).

### Expression profiling of different classes of sulfate (Sultrs) and $\text{As}^{\text{III}}$ (Lsi2) transporters in root and shoot

In roots, Sultr1;1, 1;2, 2;1 and 3;3 were up-regulated in both As and As + TU treatments, however, the level of regulation was comparatively higher for As than for As + TU treatment. Additionally, the higher level of expression was maintained until 8 h in As for Sultr1;1, 1;2 and 2;1 but not in As + TU. Additionally, few isoforms were regulated in a treatment-

specific manner, *viz.*, the up-regulation of Sultr1;3 in As + TU and Sultr3;4 in As, at 4 h and Sultr4;1 in As at 4 h and 8 h. In treatment with TU alone, the levels of most of the sulfate transporters were either significantly down-regulated or not significantly affected in roots for all time points, except for Sultr1;2 (at 1 h) and Sultr3;3 (at 8 h) which were 2.19- and 2.48-fold up-regulated, respectively. The expression of Lsi2 was not changed under any treatment until 4 h of treatment. At 8 h, Lsi2 was down-regulated 3- and 2.5-fold, respectively under As + TU and TU treatments, as compared to that of the control (Table 1A).

In shoots, under As stress, Sultr1;1 and 1;2 were either down-regulated or remained level with the control except for Sultr1;1 at 1 h. This was in contrast to As + TU, where the down-regulation of Sultr1;1 and 1;2 was limited to 1 h; beyond which a time-dependent increase was observed in their expression. The levels at 8 h were 6.89- and 3.91-fold up-regulated for Sultr1;1 and 1;2, respectively. The profiles of the remaining Sultrs' responses 1 h after treatment were also different between As with/without TU treatment. As treatment was associated with the induction of Sultr2;1 and down-regulation of Sultr3;2 and 3;3, while As + TU treatment caused up-regulation of Sultrs 1;3, 2;2, 3;1 and 4;1. In shoots treated with TU alone, the profiles of most of the Sultrs were comparable to that of As + TU; however, the extent of change was significantly greater. At 4 h, Sultrs 1;1, 1;2 and 2;1 were 1.21-, 1.3-, and 1.41-fold higher in As + TU but 11.59-, 10.36-, and 7-fold higher under treatment with TU alone. One isoform showing a major difference in expression pattern between TU and As + TU was Sultr3;4, which was up-regulated in TU (ranging from 0.75- to 1.51-fold at different time points)

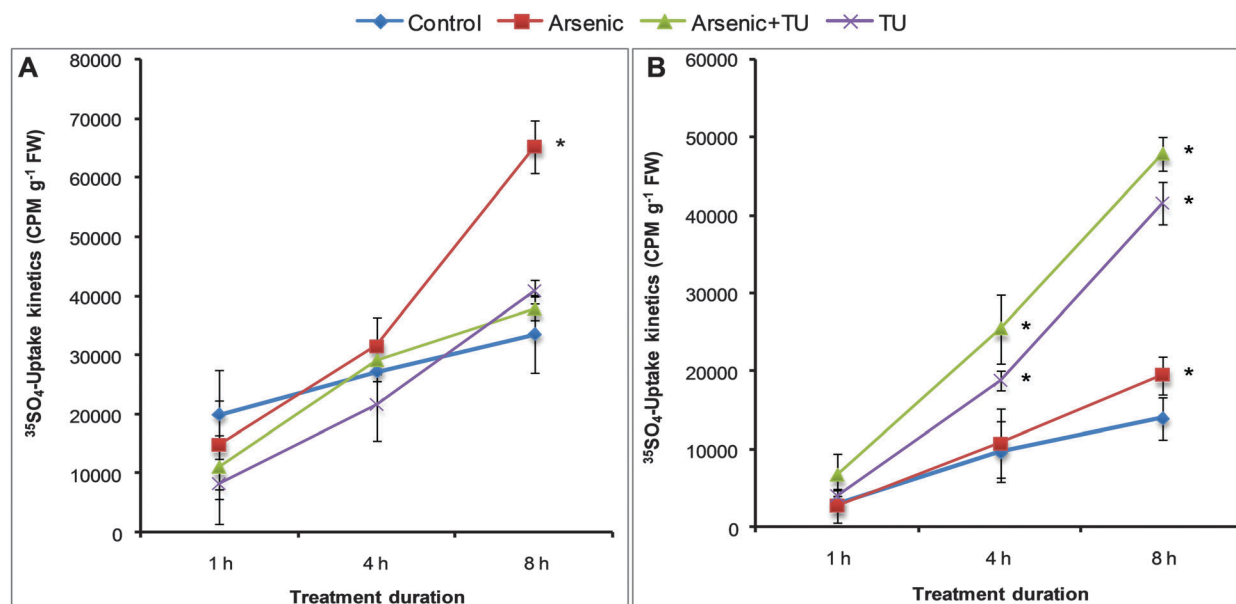


Fig. 7 Short-term  $^{35}\text{S}$ -sulfate uptake kinetics. The rice seedlings were grown hydroponically for 15 d under control conditions and then subjected to different treatments such as control, arsenic ( $\text{As}^{\text{V}}$ ; 25  $\mu\text{M}$ ); arsenic ( $\text{As}^{\text{V}}$ ; 25  $\mu\text{M}$ ) + thiourea (TU; 75  $\mu\text{M}$ ) and thiourea alone (TU; 75  $\mu\text{M}$ ). All treatment solutions were supplemented with  $^{35}\text{S}$ -sulfate (2 MBq  $\text{L}^{-1}$ ). After 1, 4 and 8 h of treatment, the roots (A) and shoots (B) were harvested and the  $^{35}\text{S}$ -sulfate levels were measured by scintillation counting. For As + TU and TU alone, 24 h pretreatment of TU was also given. The data represents the mean  $\pm$  SE of three biological replicates. The experiment was repeated twice to check its reproducibility. Asterisks (\*) have been placed on the basis of the LSD values derived from SPSS software (DMRT,  $P < 0.05$ ).



**Table 1** Expression fold difference ( $\log_2$ ) of different sulfate transporters (Sultrs) and As<sup>III</sup> specific transporters (Lsi2) measured using real time RT-PCR. Rice seedlings were grown hydroponically for 15 d under control conditions and then subjected to different treatments such as control, arsenic (As<sup>V</sup>; 25  $\mu$ M); arsenic (As<sup>V</sup>; 25  $\mu$ M) + thiourea (TU; 75  $\mu$ M) and thiourea alone (TU; 75  $\mu$ M). After 1, 4 and 8 h of treatment, RNA was extracted from roots (A) and shoots (B) and used for real-time RT-PCR. For As + TU and TU alone, 24 h pretreatment of TU was also given. The data represents the mean  $\pm$  SE of three biological replicates. For both up (indicated in bold) and down (indicated in italic) regulation, a 1.5-fold change was set as the cutoff and was considered as a significant change. The details of gene-specific primers are mentioned in ESI, S-2†

	Arsenic			Arsenic + TU			TU		
	1 h	4 h	8 h	1 h	4 h	8 h	1 h	4 h	8 h
<b>A</b>									
Sultr1;1	-1.43	<b>4.467</b>	<b>3.859</b>	1.135	<b>3.733</b>	1.306	0.95	-3.427	-3.058
Sultr1;2	-1.36	<b>3.292</b>	<b>2.298</b>	<b>2.165</b>	<b>2.91</b>	0.009	<b>2.19</b>	0.294	-0.867
Sultr1;3	0.34	1.332	-0.032	-0.325	<b>1.985</b>	0.419	-0.22	-0.931	0.158
Sultr2;1	0	<b>5.641</b>	<b>3.636</b>	0.26	<b>3.553</b>	0.612	-0.15	-1.616	-2.976
Sultr2;2	-1.16	0.972	-0.207	-1.82	0.03	-0.411	-1.145	-1.901	0.113
Sultr3;1	1.3	-0.548	-1.112	0.73	-0.975	-0.671	0.665	-2.351	-1.152
Sultr3;2	0.21	0.842	-0.277	-1.16	0.525	-0.381	-2.41	-1.516	1.283
Sultr3;3	1.05	<b>2.157</b>	-0.407	-0.555	<b>1.74</b>	0.754	-1.725	-1.756	<b>2.488</b>
Sultr3;4	-0.405	<b>2.087</b>	0.243	-0.9	0.99	-2.361	-1.375	-0.926	-2.127
Sultr3;6	0.05	0.75	0.143	-0.315	-0.47	-0.831	-0.945	-2.466	-1.597
Sultr4;1	0.94	<b>2.587</b>	<b>2.273</b>	0.545	1.435	-0.356	0.885	-2.001	-1.112
Lsi-2	-0.11	0.552	-1.481	-0.725	-0.94	-3.051	-0.385	-1.271	-2.472
<b>B</b>									
Sultr1;1	<b>3.88</b>	-2.379	-0.451	-2.585	1.214	<b>6.893</b>	-6.21	<b>11.597</b>	<b>8.17</b>
Sultr1;2	0.275	-0.107	-0.114	-5.39	1.309	<b>3.919</b>	-8.37	<b>10.36</b>	<b>5.037</b>
Sultr1;3	-0.45	-1.142	0.346	<b>1.835</b>	-0.506	-1.541	<b>1.775</b>	-1.32	-1.643
Sultr2;1	<b>3.445</b>	<b>1.563</b>	0.248	0.95	1.413	1.015	0.485	7	1.379
Sultr2;2	-0.465	0.553	1.086	<b>2.385</b>	0.759	-0.271	<b>1.715</b>	-0.135	1.067
Sultr3;1	-0.43	-1.697	-0.324	<b>1.995</b>	-1.116	0.154	1.325	-1.295	-0.708
Sultr3;2	-2.01	-0.507	0.661	-0.335	-0.831	0.044	0.72	-1.46	-0.163
Sultr3;3	-2.46	-1.187	0.286	0.715	-0.516	-1.731	<b>1.515</b>	-1.995	-2.678
Sultr3;4	-0.505	0.568	-0.324	0.115	0.239	-0.576	0.755	<b>1.695</b>	<b>1.512</b>
Sultr3;6	0.25	-1.617	0.276	1.17	-0.886	0.869	0.18	-0.02	1.217
Sultr4;1	0.335	-1.787	-0.194	<b>1.97</b>	-0.951	0.354	1.06	0.87	-0.283

but not in As + TU (ranging from -0.58- to 0.24-fold at different time points) (Table 1B).

## Discussion

In an earlier study, TU supplementation has been demonstrated to impart salt tolerance through the maintenance of cellular energetics<sup>35</sup> and redox homeostasis.<sup>22</sup> Since these are also the major determinants of As stress tolerance in plants,<sup>9,11</sup> the present study was performed to evaluate the efficiency of TU for ameliorating As-induced damage and to implicate the significance of redox homeostasis in As stress tolerance. Initially, post-germination phenotyping of rice was performed using a range of As concentrations (5–50  $\mu$ M) on the basis of average root length and the IC<sub>50</sub> value (25  $\mu$ M) was calculated (data not shown). Then, a range of TU concentrations (10–200  $\mu$ M) were tested along with 25  $\mu$ M As (data not shown) and 75  $\mu$ M TU was found to be optimum, which could partially revert the seedling phenotype (in terms of root length) (Fig. 1). The lack of complete phenotype reversal indicates that there are redox independent factors in As induced damage, and hence, redox homeostasis alone may not alleviate overall toxicity. The physiological thiol GSH has been evaluated in earlier studies for stress amelioration against As<sup>36</sup> and cadmium.<sup>37</sup> However, being a physiological thiol, it may also modulate a range of metabolic pathways in

addition to the redox state. Such a possibility is comparatively low for TU, which is a non-physiological thiol, and hence the observed effects can be correlated to the redox state with a greater certainty. It has been confirmed in our earlier studies, using HyPer-transformed Arabidopsis lines (Srivastava *et al.*, unpublished research) as well as through biochemical methods,<sup>22</sup> that TU supplementation generates a reduced redox state. A TU-mediated shift in redox state towards the reducing direction might be responsible for partial stress amelioration against As stress. The As level was analyzed to test whether improved root growth in As + TU was associated with a decline in As. Surprisingly, As concentration in roots was not significantly affected. In fact, owing to the increase in root dry weight, the total root As content per plant in As + TU was even higher than for treatment with As alone. However, both As concentration and total shoot As content per plant were significantly reduced in shoots under As + TU treatment as compared to treatment with As alone (Fig. 2). This suggested that the loading of As into the xylem for root-to-shoot transport is affected under TU treatment. To test this hypothesis, the expression level of Lsi2 (a silicon or As<sup>III</sup> exporter) was analyzed in roots under different treatments. Owing to the localization of OsLsi2 on the proximal side of epidermal and endodermal cells, it is involved in the translocation of As from root to shoot.<sup>11</sup> Although the present study deals with As<sup>V</sup>, As<sup>III</sup> specific transporters were analyzed because, inside the plants, As<sup>V</sup> has been shown to be



rapidly converted into As<sup>III</sup>.<sup>38</sup> Under As + TU treatment, Lsi2 expression was down-regulated in roots which might be responsible for decreasing the As levels in the shoots. These are interesting data which signify the redox state as an important regulator of As uptake and translocation in rice. This is further supported by the findings of Liu *et al.*<sup>39</sup> and Duan *et al.*,<sup>40</sup> where BSO (*L*-buthionine sulfoximine, a GSH biosynthesis inhibitor known for creating an oxidized redox environment) treatment has been demonstrated to enhance root-to-shoot or shoot-to-grain As translocation in Arabidopsis and rice. Since the application of TU under field conditions is already established, the present result of TU mediated reduction of root-to-shoot As translocation can have implications for reducing As load from rice grains.

In spite of the decrease in As level, no significant differences in shoot growth were observed between As and As + TU treatment (Fig. 1). This might be either due to the short duration of the experiment or due to the differences in As concentration not being sufficient to produce visible differences in shoot growth. In contrast, root growth was improved under As + TU treatment compared with As treatment, despite the fact that As concentration was not significantly different between two treatments (Fig. 1A and B). There may be two possible reasons for this observation. Firstly, there might be improved tolerance against As toxicity through enhanced antioxidant potential. Such a mechanism has been suggested for TU supplemented *Brassica juncea* seedlings subjected to salt stress.<sup>22</sup> Secondly, the level of free As might be variable between the two treatments, which may be achieved through efficient vacuolar sequestration of As mediated through some unknown redox-dependent transporter, or by As complexation either by TU itself due to presence of a thiol group (–SH) or by GSH and PCs. The possibility of As complexation with TU was evaluated *in vitro* using HPLC coupled with parallel ICP-MS and ESI-MS. The data obtained indicated that the formation of As-TU complexes was not feasible (Fig. 3) and was ruled out as one of the possible mechanisms for reducing free As levels in roots. The induction of *in built* tolerance mechanisms of As complexation *via* thiolic metabolites was then studied. Thiol metabolism is regarded as a major determinant of As tolerance<sup>41</sup> as well as As accumulation in plants.<sup>39,40</sup> The fluorescence HPLC based profiling of various thiols was performed in both roots and shoots (Fig. 4) and significant differences were observed for PC2 in roots and GSH, PC3 and PC4 in shoots between As and As + TU treatments. However, the molar ratio of total thiols (GSH + PC2 + PC3 + PC4)-to-As confirmed that the major portion of As would be present as a non-chelated form in the roots for both As and As + TU treatments. This indicated that a positive effect of TU on root growth was not dependent upon GSH/PCs mediated improved As complexation. This might be due to a preference for long-term As storage, as uncomplexed As, similar to what has been demonstrated for seaweeds.<sup>42</sup> Thus, the possibility of a vacuolar transporter mediating the transport of uncomplexed As does exist, as discovered in the lower plant *Pteris vittata*.<sup>43</sup> In contrast, thiols were present in excess in the shoot and all As might be present as complexed

species in both treatments. The higher levels of GSH and PCs may play a role as redox buffers. This was also evident from the significant accumulation of cysteine, GSH and PC2 in treatment with TU alone. Although GSH is an established redox buffer,<sup>17</sup> the role of PCs in redox balancing is only just emerging.<sup>18</sup> Furthermore, the sulfur assimilation was also studied to explain the differential synthesis of GSH and PCs under different treatments. A significant increase was observed in the activities of APS and CS in roots (Fig. 5A and D) and APR in shoots (Fig. 5C) under As + TU treatment as compared to As treatment. This suggests that the regulation of these enzymes is also under redox control. The redox-dependent regulation of APR has previously been shown.<sup>14</sup>

Although the chemical action of TU in the scavenging of a broad range of biological ROS is well established,<sup>24</sup> to obtain a measure of the redox state kinetics of plants in the initial stages of As stress, GSH levels and GSH/GSSG ratios in rice seedlings were measured. The GSH/GSSG ratio was selected as it is considered the major determinant of the cellular redox state.<sup>17</sup> In As + TU and TU alone treated roots, the GSH/GSSG ratio was significantly higher than that for As treatment at all time points. In contrast, a differential redox state in shoots was seen only at 8 h after treatment, wherein both the GSH level and the GSH/GSSG ratio were higher in all treatments as compared to the control (Fig. 6C and D). In order to correlate these changes of redox status with sulfur metabolism, measurements of sulfate uptake kinetics were performed under similar treatment conditions using <sup>35</sup>S-sulfate as a radiotracer. The comparative analysis of the <sup>35</sup>S-sulfate level in As and As + TU treatment confirmed that root-to-shoot translocation of sulfate, rather than its uptake, is the rate limiting step behind the As mediated induction of sulfur deficiency.<sup>44,45</sup> Furthermore, the differential translocation observed under As with/without TU also confirmed that the process is redox regulated. In order to identify the associated candidate genes, the quantitative real-time PCR based comparative expression profiling of sulfate transporters was performed. In roots, the overall down-regulation of Sultrs in TU pretreated seedlings suggested their regulation in a demand driven manner.<sup>12</sup> However, the expression of Sultr1;2, which is the major high-affinity sulfate transporter in plants, was increased at 1 h and not significantly down-regulated at 8 h under treatment with TU alone, which would have maintained the basal sulfate uptake. The plant's improved sulfur status under TU supplementation was also evident, as the comparatively higher and extended expression level of selected group-1 (Sultr1;1 and 1;2), -2 (Sultr2;1), -3 (Sultr3;3 and 3;4) and -4 (4;1) transporters was observed only in As treated roots and not under As + TU treatment. The expression profiling was correlated with radiotracer data, where the sulfate content in roots at 8 h under As was much higher than for any other treatment (Fig. 7A). The enhanced root-to-shoot sulfate translocation observed under As + TU and TU treatments was attributable to significant up-regulation of Sultrs 1;1 and 1;2 in shoots. These results suggested a tissue-specific function for Sultr1;1/1;2. In roots, they played a vital role in sulfate uptake, while in shoots they were responsible for sulfate unloading to facilitate the root-to-shoot translocation.



Apart from redox regulation, these Sultrs were also found to be light-regulated, as their enhanced expression was observed only after 9 AM. Light-dependent regulation of Sultr1;2 has already been demonstrated.<sup>46</sup> This is justified as maximum sulfate assimilation occurs only during the daytime. The early induction (1 h) of Sultr2;2 (low-affinity transporter), Sultr3;1<sup>47</sup> and Sultr4;1 (for vacuolar sulfate remobilization) transporters in the shoots upon As + TU treatment might have contributed towards the higher sulfate content observed even at the 1 h time point in comparison to other treatments. This was probably to compensate for the down-regulation of Sultr1;1 and Sultr1;2 at 1 h, and suggest transporters other than those of group 1 are not light-regulated, however this needs to be assessed further. The significantly different signatures of Sultrs observed in roots and shoots under As, As + TU and TU treatments suggest that their expression is co-ordinately regulated by the plant's sulfur demand, redox status and light. Recently, the regulatory role of a plant's sulfur status<sup>48</sup> and redox state<sup>49</sup> has been established for the model plant *Arabidopsis thaliana*. To the best of our knowledge, this is the first study where spatial-, temporal-, and redox-regulation of Sultrs has been studied in rice.

In conclusion, this study implicates the importance of redox homeostasis for ameliorating As stress in rice through the use of TU, a non-physiological thiol based ROS scavenger. Under As stress, TU supplementation mediated the redox balance that led to the down-regulation of transporters for As translocation (Lsi2) leading to a reduction in As level from aerial parts. This was simultaneous with the up-regulation of sulfate transporters (Sultr1;1 and 1;2), enhanced root-to-shoot sulfate translocation and increased activities of sulfur assimilation related enzymes, which ultimately result in partial amelioration of the effects observed under As stress. Thus, the findings not only signify the importance of redox-regulatory mechanisms for enhancing a plant's tolerance against As stress and for reducing As load in rice grains, but also widen the range of TU application for ameliorating abiotic stress in crop plants.

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