Chemical intervention for enhancing growth and reducing grain arsenic accumulation in rice

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ABSTRACT

Arsenic (As) is a ubiquitous environmental carcinogen that enters the human food chain mainly through rice grains. In the present study, we evaluated the potential of thiourea (TU; non-physiological reactive oxygen species scavenger) in mitigating the negative effects of arsenic (As) stress in indica rice variety IR64, with the overall aim to reduce grain As accumulation. At seedling stage, As+TU treatment induced the formation of more numerous and longer crown roots compared with As alone. The significant reduction in As accumulation was observed in As+TU treated seedling, which coincided with light-dependent suppression in the expression levels of aquaporins and photosynthesis-related genes in roots. The foliar-supplemented TU under As-stress maintained reducing redox conditions which decreased the rate of As accumulation to flag leaves and, eventually grain As by 0.53-fold compared with those of As treatment. The agronomic feasibility of TU was validated under naturally As contaminated sites of Nadia (West Bangal, India). The tiller numbers and crop productivity (kg seed/ha) of TU-sprayed plants were increased by 1.5- and 1.18-fold, respectively; while, grain As accumulation was reduced by 0.36-fold compared with those of water-sprayed control. Thus, this study established TU application as a sustainable solution for cultivating rice in As-contaminated field conditions.

KEY WORDS: Arsenic, aquaporins, crown root, root photosynthesis, thiourea.

INTRODUCTION

Arsenic (As) is a ubiquitous environmental toxin and is recognized as a group-1 carcinogen by the International Agency for Research on Cancer (IARC) (Humans, Organization & Cancer, 2004) and, as the most prioritized human toxin by the Agency for Toxic Substances and Disease Registry (ATSDR-2017; www.atsdr.cdc.gov). Globally, about 240 million people from over 70 countries spanning six inhabited continents are threatened by As poisoning. Most of the at-risk populations reside in Southeast Asian countries, including Bangladesh, Cambodia, China, India, Myanmar, Nepal, Pakistan and Vietnam, where the As concentrations in drinking water are much higher than the World Health Organization (WHO) standard (10 µg/L) (Naujokas, Anderson, Ahsan, Aposhian, Graziano, Thompson & Suk, 2013, Uppal, Zheng & Le, 2019). In addition to drinking water, another major route of As entry into humans is via As contaminated food, in particular, rice and rice-based products (Guillod-Magnin, Bruschweiler, Aubert & Haldimann, 2018, Hussain, Rengel, Qaswar, Amir & Zafar-ul-Hye, 2019, Upadhyay, Shukla, Yadav & Srivastava, 2018). In paddy soil-water, As builds up through the combination of natural (rock weathering and alluvial deposits) and anthropogenic (use of As-contaminated groundwater to irrigate) processes (Kumarathilaka, Seneweera, Meharg & Bundschuh, 2018). Although there are several inorganic and organic forms of As in water and soil, the major As species detected in rice grains include arsenite (As^{III}), arsenate (As^{V}), dimethylarsinic acid [$(CH_3)_2AsO_2H$, DMA(V)] and monomethylarsonic acid [$CH_3AsO_3^{-2}$, MMA]. In humans, chronic exposure to As not only causes various types of cancers, skin lesions, hyper/hypo-pigmentation and keratosis, but also increases the risk of lifestyle-related diseases, such as diabetes (Rahman, Sohel, Yunus, Alam, Nahar, Streatfield & Yunus, 2019, Xu, Fu, Wang, Hou & Pi, 2019).

Arsenic uptake and toxicity mechanisms in plants are well-explored. In brief, As^{V} uptake is mediated by phosphate transporters, while As^{III} mainly enters through nodulin 26 type-intrinsic proteins (NIPs, a subfamily of aquaglyceroporins). The entry of DMA and MMA inside the root is mediated by silicic acid transporter (Abbas, Murtaza, Bibi, Shahid, Niazi, Khan, Amjad, Hussain & Natasha, 2018, Garbinski, Rosen & Chen, 2019, Li, Wang & Song, 2016, Lindsay & Maathuis, 2017). Inside the plant, As^{V} interferes with phosphate dependent processes, such as oxidative phosphorylation and ATP synthesis, and As^{III} binds to sulfhydryl groups of a wide range of proteins, disturbing their functions. Both As^{V} and As^{III} induce the accumulation of reactive oxygen species (ROS), causing redox imbalance, and disturb nutrient homeostasis of the plants (Finnegan & Chen, 2012). Taken together, these changes reduce the overall plant growth and crop yield. In view of this, worldwide concerted research efforts are ongoing with dual objectives: first, to increase As-stress tolerance in plants for minimizing yield losses from As contaminated areas, and second, to reduce As accumulation in grains and make rice safe for human consumption. In light of this, multiple genes encoding transporters, thiol-metabolism related enzymes and regulatory proteins have been targeted through transgenic technology to enhance plant As tolerance and to reduce As accumulation (Lindsay & Maathuis, 2017), (Chen, Hua, Chen, Rathinasabapathi, Cao & Ma, 2019, Deng, Yamaji, Ma, Lee, Jeon, Martinoia, Lee & Song, 2018, Kumari, Rastogi, Shukla, Srivastava & Yadav, 2018, Shi, Wang, Chen, Tang, Wu, Salt, Chao & Zhao, 2016, Sun, Chen, Che, Konishi, Tang, Miller, Ma & Zhao, 2018). In parallel, application of low-cost and environmental friendly chemicals could be a sustainable option (Srivastava, Pasala, Minhas & Suprasanna, 2016). Being a non-genetic approach, the low-dose application of various chemicals faces relatively less regulatory restrictions (Srivasta*va et a*l., 2016). Thiourea (TU) is a non-physiological thiol-based reactive oxygen species

(ROS) scavenger (Gao, Wang, Sun & Epstein, 2008, Kelner, Bagnell & Welch, 1990) and anti-nitrification factor (Zacherl & Amberger, 1990) that has been shown to mitigate diverse environmental stresses, including salt (Kaya, Sonmez, Aydemir, Ashraf & Dikilitas, 2013, Srivastava, Sablok, Hackenberg, Deshpande & Suprasanna, 2017), drought (Vineeth, Kumar & Krishna, 2016), heat(Asthir, Thapar, Bains & Farooq, 2015), UV radiation (Pandey, Srivastava, Suprasanna & D'Souza, 2012), boron (Kaya, Sarioğlu, Akram & Ashraf, 2019), lead(Xalxo & Keshavkant, 2019) and arsenate (Srivastava, Srivastava, Mishra, D'Souza & Suprasanna, 2014). Since As-stress severity is expected to magnify under the coupled stress scenario of future climate (Muehe, Wang, Kerl, Planer-Friedrich & Fendorf, 2019), therefore, the present study was conducted to establish the mitigating potential of broad-range effective TU towards As^{III} stress conditions in rice, using the lab and field level studies. The main aim of the study was to unravel the mechanistic basis of TU-mediated enhanced growth and reduced grain As accumulation.

METHODS

PLANT MATERIALS, GROWTH CONDITIONS AND STRESS TREATMENTS

The entire study was performed on *Oryza sativa* var. IR-64. Seeds were surface sterilized with 30% ethanol for 3 min and then thoroughly washed with distilled water to remove traces of ethanol. Surface-sterilized seeds were soaked in distilled water under shaking condition (~100 rpm) at 25°C. After 14-16 h of incubation, seeds were allowed to germinate in tap water. After 48 h, germinated seeds were transferred to a customized PCR plate-based hydroponic setup containing modified Yoshida medium (Supplementary Table-1). After 15 d of growth under control conditions, seedlings were independently subjected to different treatments including control (Yoshida medium), As^{III} (prepared using $AsNaO_2$), As+TU and TU alone. Working concentrations of As^{III} and TU were 20 μM and 75 μM,

respectively. For As+TU and TU, pre-treatment with a similar concentration of TU was also administered for 24 h, which ensured that the presence of TU in seedlings before they actually face As-stress conditions. The pre-loading strategy has been demonstrated to maximize the impact of TU-mediated amelioration of As^{V} in rice (Srivasta*va et a*l., 2014). At every three-day cycle, the nutrient solutions were changed with fresh respective solution. After 10 d of treatment, differential phenotypes were qualitatively and quantitatively recorded in terms of numbers and lengths of crowns and main roots. After quantification, different organs including the main roots, crown roots, lower leaves (oldest) and upper leaves (youngest) were physically separated, washed thrice with chilled water and then dried at 65°C till constant weight. The dried tissue was weighed and then acid-digested for As quantification. In addition, at 20 d after stress, the differential phenotypes were further recorded in terms of main root lengths and shoot lengths. The microarray analysis was performed independently in roots and shoots 24 h after different treatments. The quantitative real-time PCR (qRT-PCR) analysis and hormone estimations were performed in roots at 24 h after different treatments. Additionally, for the selected genes, qRT-PCR was performed in roots at 24 h after treatment, independently under normal light-regime and complete dark conditions. The entire phenotyping work was performed in a plant growth chamber (Sanyo, Japan) having a daily cycle of a 14 h photoperiod with a light intensity of 150 μE m^{-2} s^{-1}, day/night temperatures of 25/22°C and relative humidity of 65-75%.

MICROARRAY ANALYSIS AND QUANTITATIVE REAL-TIME PCR

Total RNA was isolated using the miRVANA RNA Isolation kit (Thermo AM1560). The detailed protocols for RNA quality assessment and microarray analysis are provided as Supplementary Method-1. The quantitative real-time PCR was performed as per the method described previously (Srivasta*va et a*l., 2014).

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LC-MS BASED QUANTIFICATION OF PLANT HORMONES

SA and ABA levels were estimated using LC-MS. In brief, ~500 mg of liquid nitrogen ground samples was homogenized in twice volume of 90% methanol, sonicated for 2 min and then centrifuged at 12,000 rcf for 5 min at 4°C. Supernatant was transferred to fresh tube and spiked with internal standard Trytamine-D4. Spiked samples were dried under vacuum and reconstituted in 50 μl of 20% of methanol. Of this, 10 μl was injected in LC-MS and SA and ABA peaks were identified on retention time basis. The quantification was done by comparing peak area of individual sample with that of respective standards.

POT STUDIES WITH FOLIAR-APPLIED TU TREATMENT

Pot studies were performed at wire-house experimental farm of the Bhabha Atomic Research Centre, Mumbai (India). Plastic pots (without holes) filled with 14 kg of a mixture of paddy soil: decomposed cow dung manure (2:1) were prepared in four independent groups (five pots/group). Seeds were germinated and grown on the nursery bed till seedling stage. After 22 d, four healthy seedlings were transplanted into each pot. The recommended agronomic practices were followed for all four groups. This practice included the addition of a basal fertilizer dose of N:P:K (50:50:50 kg/ha), which was achieved by adding 4 g of Suphala (15:15:15) into the soil of each pot. In addition, a top dressing of nitrogenous fertilizer in the form of urea (0.72 g per pot) was added 40 and 60 d post-transplantation. All of the pots were watered weekly to maintain 3-4 cm of standing top water until the active grain filling stage. The four groups were named As (Group-1), As+TU (Group-2), TU (Group-3) and control (Group-4). Group-1 plants were treated with two doses of As (10 mg As^{III}/kg soil) at 20 and 67 d post-transplantation. Group-2 plants were treated with As treatment along with two foliar applications of TU (6.5 mM containing 0.01% Tween-20) at 50 and 65 d post-transplantation. Group-3 plants were treated with only two foliar applications of TU. Group-4

plants were not treated and served as controls. At 2, 10 and 15 d post-TU 2nd spray, flag leaves were harvested from different treatments and subjected to As estimation. The activity of glutathione reductase (GR) and levels of reduced (GSH) and oxidized (GSSG) glutathione were also quantified in flag leaves harvested at 10 d post-TU 2nd spray, as per the method described previously (Srivastava, Srivastava, Penna & D'Souza, 2011). Different agronomic traits, such as plant height, tiller number, panicle length, branches/panicle, flag leaf length and width, total dry biomass and seed yield/plant were measured at full plant maturity (approximately 100 d post-transplantation). Harvest index values under different treatments were also quantified using the following formula: [total seed weight (g) X 100]/total plant dry weight (g). After the harvest, the rhizospheric soil was collected at 15 cm from the top and dried for total As quantification.

FIELD EVALUATION IN NATURALLY ARSENIC CONTAMINATED SITES

Field evaluations were performed to validate pot study data. For this experiment, naturally arsenic contaminated site was selected in Nadia District (23°01'07.8"N-088°39'43.1"E, 23°01'14.3"N-88°38'24.7"E), West Bengal, India. Seeds of IR-64 variety were soaked in 6.5 mM TU for 6 h and then washed three times with distilled water. In parallel, seeds were also soaked independently in water, which served as water-spray control (WS). After soaking, seeds were planted in a nursery bed and seedlings were grown. After 21-25 d, both TU and WS treated seedlings were uprooted from the nursery and independently transplanted into adjoining plots of ~40 ft^2 size in both sites. In addition to seed treatment, TU treated plants were also treated with two foliar applications with TU (6.5 mM containing 0.01% Tween-20) at pre-flowering and grain filling stages, which were at 50 and 65 d post-transplantation, respectively. The trials were performed during July-November, 2018, and 5-7 and 4-6 cm of top water was maintained at site-1 and site-2, respectively, throughout the

life cycle. Site-1 and 2 were irrigated through surface pond and shallow tube wells wherein the As concentration in water ranged from 20-30 and 60-80 µg L^{-1}, respectively. Fertilizer amendment was performed as per locally adapted agronomic practices. In brief, N:P:K was provided in the form of Urea:Super phosphate:Muriate in the ratio of 120:60:40 kg ha^{-1}. N was applied in three tiers: ½ at the time of field preparation, and the other two (1/4 and 1/4) at early vegetative and tillering stages. A total of 25 plants were randomly selected, and various agronomic traits, such as dry biomass, tiller number, spikes/plant number, plant height, seed weight/plant, harvest index and seed yield, were quantified. The soil sampling was also done at three different stages and various parameters like pH and organic matter were quantified as per the method described previously(Upadhyay, Majumdar, Barla, Bose & Srivastava, 2019).

SYNCHROTRON BASED-ARSENIC MAPPING IN RICE GRAINS

Synchrotron based K-edge subtraction computed tomography technique was applied for 3D As mapping in rice grains. The data acquisition and analysis were carried out at BL-4 imaging beamline at Indus-2 Synchrotron source at available at RRCAT, Indore, India. The microCT scans were acquired below (11.8660 keV) and above (11.8675 keV) for the K-edge of arsenic (11.8667 keV) using monochromatic beam available at imaging beamline. The data was processed as per standard K-edge subtraction CT analysis and 2D and 3D images of rice samples were obtained. The 2D images show local spatial As distribution throughout the rice grains. Volume rendering of the obtained stack of microCT reconstructed slice images provides 3D distribution of As map in the grains. The mean gray values and standard deviation give a quantitative estimate of As accumulation in different treatments.

ELEMENTAL ANALYSIS

The analysis of As and other nutrient elements was performed using ICP-MS (Supplementary Method-2). The certified reference material (CRM) NIST 1568b rice flour (Supplementary Method-3) and blanks were included for quality assurance.

STATISTICAL ANALYSIS AND DATA DEPOSITION

All lab and field experiments were carried out in a completely randomized design and were repeated at least twice with triplicates to check reproducibility. One–way analysis of variance (ANOVA) was performed on all of the data to confirm the variability of the data and validity of the results. Duncan's multiple range test (DMRT) was performed to determine the significant difference between treatments using SPSS 17.0 statistical software. The microarray data corresponding to this study are available from NCBI Geo under accession number GSE71492.

RESULTS

DIFFERENTIAL ROOT ARCHITECTURE AND OVERALL PLANT PHENOTYPE

The post-germination phenotyping revealed differential phenotypes of rice seedlings under the As^{III} alone (As) and combined As^{III} and TU (As+TU) treatments (Fig. 1A-B). The major effect was observed in root-system architecture (RSA) until 10 d stress duration. Under As-stress, both the number as well as length of crown roots were increased by 1.32- (p=0.0365) and 2.2- (p=2.37E-07) fold, respectively compared with those of control. The response was further intensified under As+TU treatment, as both the number and length of crown roots were increased by 1.4- (p=0.0038) and 1.29- (p=0.0334) fold, respectively, compared with those of As alone treatment (Fig. 1C-D). Contrary to crown roots, both number and length of the main roots under As-stress were decreased by 0.48- (p=6.83E-07) and 0.81 (p=0.034) fold, respectively compared with those of control. As+TU treatment could not restore the negative impact of As on main root number. However, the main root length

was partially restored, as it was increased by 1.34-fold (p=0.0013) under As+TU compared with that of As treatment (Fig. 1E-F). Additionally, TU treated seedlings showed altered RSA, as the crown root length was increased by 1.69-fold (p=0.0003; Fig. 1D), while the main root number was decreased by 0.74-fold (p=0.0067; Fig. 1E) compared with that of control. No significant difference in the shoot phenotype between the As and As+TU treatments was observed until 10 d stress duration (Fig. 1A). The differential phenotypes were further recorded after 20 d stress duration. The main root length pattern remained unchanged; however, the differential shoot phenotype was also observed. The average shoot length under As+TU was increased by 1.13-fold (p=0.007) compared with that under As alone treatment. Additionally, shoot length under TU alone treatment was increased by 1.20-fold (p=0.001) compared with that of the control (Fig. 2).

In summary, the phenotyping results validated the potential of medium-supplemented TU to alter plant growth responses under both control as well as As-stress conditions. The differential phenotype in roots and shoots was evident at short- and long-term treatment duration, respectively.

ORGAN-WISE ARSENIC ACCUMULATION UNDER DIFFERENT TREATMENTS

In As-treated seedlings, As accumulation was observed maximally in main roots (94.8-fold) followed by crown roots (27.9-fold), lower leaves (15.6-fold) and upper leaves (7.9-fold) compared with those of control. In such organs, As accumulations were significantly decreased to 0.1-, 0.14-, 0.16-, 0.14-fold, respectively in As+TU treated seedlings compared with those of As alone. In both lower and upper leaves, As accumulation levels in As+TU treated seedlings were found comparable to that of control (Fig. 3).

ARSENIC TRANSCRIPTOME UPON TU SUPPLEMENTATION

To understand the mechanistic basis for TU-mediated amelioration of As-stress, microarray-based transcriptomic analysis was performed. The Venn analysis revealed that the majority of differentially expressed genes (DEGs) were shared between As and As+TU treatments in the roots (139) and shoots (216). However, we also identified As-specific DEGs (34 in root and 39 in shoot) and As+TU-specific DEGs (58 in root and 54 in shoot), suggesting that TU supplementation has a significant effect on the As induced transcriptome (Fig. 4A). The details of specific and shared DEGs across different treatments are provided in Supplementary Table-2 (root) and -3 (shoot). From Venn analysis, we attempted to identify TAR (TU modulated As Responsive) genes that maximally contributed to the As+TU phenotype. Here, we first pooled As-specific, As+TU-specific and As/As+TU-shared DEGs and computed the expression difference between As+TU and As treatments. Later, a 1-fold cut-off filter was applied over the As+TU and As expression differences, which identified a total of 54 TAR-R (Supplementary Table-4) and 26 TAR-S (Supplementary Table-5) genes in roots and shoots, respectively. The higher number of TAR-R genes than TAR-S genes is because TU was supplemented in hydroponic medium: hence, more response is expected in the roots. Further, TAR gene expression patterns were also studied using scatter plots (Fig. 4B). A strong positive correlation was observed between As and As+TU expression patterns for both TAR-R (r=0.76) and TAR-S (r=0.762) genes. However, the polynomial two-degree equation indicated comparatively lower negative slopes concomitant with higher positive slopes in As+TU compared with those in As-stress treatment. This result suggested that the As+TU transcriptome had two distinct components. The first component represented the resilient-DEGs (rDEGs) whose expression levels in As+TU treatment was shifted towards that of control. On the contrary, second component was amplified-DEGs (aDEGs) wherein the extent of up- or down-regulation in As+TU was further intensified compared with that of

As treatment (Fig. 4B). Further, TAR genes were analyzed using a heat map-based clustering approach that identified two independent clusters in both the TAR-R (Cluster-1 and 2; Supplementary Table-4) and TAR-S (Cluster-3 and 4; Supplementary Table-5) genes. Cluster-1 and 3 represent DEGs with lower expression levels in As+TU, while cluster-2 and 4 include DEGs with higher expression levels in As+TU, compared with those in As-stress (Fig. 4C). To understand the functional relevance of these clusters, we performed gene ontology (GO)-based enrichment analysis at the levels of biological process (BP), cellular component (CC) and molecular function (MF) (Fig. 4D). For TAR-R, cluster-1 (39 DEGs) was enriched with respect to photosynthesis (BP, GO:0015979) and thylakoid (CC, GO:0009579), while cluster-2 (15 DEGs) was mainly associated with abiotic stress stimulus (BP, GO:0009628), cytoplasmic (CC, GO:0044444), membrane-bounded organelle (CC, GO:0043227) and catalytic activity (MF, GO:0003824). TAR-S genes were enriched with respect to GO terms; however, due to fewer included genes, the significance level was found to be lower than that of TAR-R. Both cluster-3 (14 DEGs) and 4 (12 DEGs) were associated with metabolic process (BP, GO:0008152) and catalytic activity (MF, GO:0003824).

Thus, we identified TAR genes from both roots and shoots. The resilient- or amplified-type expressions of these genes together contributed to a distinct transcriptome response in As+TU compared with that in As stress.

EXPRESSION PROFILING OF AQUAPORIN ISOFORMS

The microarray results, although highlighted the significance of photosynthesis related (PR) and defence genes in mediating plant responses under As-stress, it could not explain the significantly reduced As accumulation in As+TU treated seedlings. Considering the major role of aquaporins in regulating As uptake (*Li et a*l., 2016, Ma, Yamaji, Mitani, Xu, Su, McGrath & Zhao, 2008), the expression levels of 33 aquaporin isoforms were quantified

in roots across different treatments. Of these, 23 and 19 aquaporin isoforms were significantly up-regulated in As and As+TU treated roots, respectively. In contrast, *OsNIP2;1 or OsLsi1* was downregulated by -3.02-fold in As+TU; while, *OsPIP1;1*, *2;1* and *2;2* were downregulated by ~2-fold in As-treated roots compared to those of control (Supplementary Table-6). Additionally, we computed the expression difference between As+TU and As treatment and the results clearly indicated that although aquaporin expression pattern was upregulated in both As+TU and As-treated roots; however, the magnitude of upregulation was significantly dampened under As+TU treatment (Supplementary Table-6). To highlight this, clustering analysis was performed on the aquaporin gene expression normalized data which yielded two independent clusters. The expression levels of 21 cluster-2 aquaporins was reduced upto 5-fold in As+TU, as compared with those in As treatment. These included *OsPIPs* (*1;2*, *2;4*, *2;5, 2;6* and *2;7*), *OsNIPs* (*1;1*, *1;2*, *1;3*, *2;1* and *4;1*), *OsTIPs* (*1;1*, *1;2*, *2;1*, *2;2*, *3;1*, *3;2*, *4;2*, *4;3* and *5;1*) and *OsSIPs* (*1;1* and *1;2*) (Fig. 5; Supplementary Table-6). Out of 12 cluster-1 aquaporins, *OsNIP1;4*, *OsNIP3;2*, *OsNIP3;3* and *OsPIP2;8* were also downregulated by more than 2-fold cut-off in As+TU than As-treated roots. The downregulated expression of 25 aquaporin isoforms (21 from cluster-2 and 4 from cluster-1) in As+TU compared to As-treated roots signifies TU potential for restoring the plant-water homeostasis, as demonstrated earlier in case of NaCl-treated mustard (Srivastava, Suprasanna, Srivastava & D'Souza, 2010). All such aquaporins can be included as another sub-group of rDEGs of TAR-R category. Few selected aquaporin isoforms such as *OsNIPs* (*1;1*, *1;2*, *1;4*, *3;3* and *4;1*), *OsPIPs (2;6, 2;8)* and *OsTIPs (1;1, 3;1, 3;2 and 5;1)* were also upregulated in TU alone treatment compared to those of control. Considering the regulatory roles of abscisic acid (ABA) and salicylic acid (SA) in regulating aquaporin expression, we quantified their levels in roots. While, ABA level was comparable under all the treatments

(Fig. 3B); SA was increased by 1.9- and 2.8-fold in As+TU and TU alone treatment, respectively compared with those of control. No significant change in SA was observed in As-treated roots (Fig. 3C).

LIGHT-DEPENDENT EXPRESSION OF TU MODULATED GENES IN ROOTS

The significant down-regulation of PR genes in As+TU treated roots indicated that TU mediated molecular effects might be light-dependent. To validate this, the expression levels of three PR genes were quantified in roots, independently under normal light-regime and complete dark conditions. Under normal light-cycle, the expression levels of PR genes like *Cyt-b6-FeS* (Cytochrome b6-f complex iron-sulfur subunit), CAB (Chlorophyll A-B binding protein) and *RBCS* (Ribulose bisphosphate carboxylase small chain) were found to be downregulated in As+TU than As alone treated roots (Fig. 6A-C), which validated the results derived from microarray analysis. Since, TIPs are known to increase during dark-adaptation (Uenishi, Nakabayashi, Tsuchihira, Takusagawa, Hashimoto, Maeshima & Sato-Nara, 2014), three top-ranked TIPs (*OsTIP1;2*, *2;1* and *4;3*) with maximum expression difference under As+TU and As treated roots (Supplementary Table-6) were also quantified under light-dark conditions. Like PR genes, under the normal light-cycle, the expression levels of these TIPs were found to be suppressed in As+TU treated roots than those of As alone (Fig. 6D-F). In contrast, neither PR genes nor TIPs showed any significant expression difference between As+TU and As treatments under the dark-adapted conditions (Fig. 6). In general, under both control and TU alone treatments, the expression levels of all the tested genes were significantly upregulated in roots of dark-adapted plants than those with normal light-regime; except for *Cyt-b6-FeS* and *OsTIP2;1* which were downregulated to 0.47- and 0.55-fold, respectively in TU alone treatment (Fig. 6).

GROWTH ENHANCEMENT AND GRAIN As REDUCTION UNDER FIELD CONDITIONS

The observation of TU-modulated physiological and molecular responses inspired us to determine whether As-induced negative effects can also be mitigated under field conditions. Initially, pot-studies were performed wherein TU treatment was given in the form of foliar spray. At both 2 and 10 d post-TU 2nd spray, As accumulation in As+TU treated flag leaves was decreased to 0.58- and 0.48-fold, compared with those of As treatment, respectively. However, at 15 d post-TU 2nd foliar spray, no significant difference in As accumulation was observed between As+TU and As-treated flag leaves (Fig. 7A). Besides, GR activity in As-treated flag leaves was also reduced to 0.54-fold compared with those under control. In contrast, no significant change in GR activity was observed under As+TU and TU treated flag leaves (Fig. 7C). Both GSH and GSSG levels in As-treated flag leaves were increased by 1.19- and 1.28-fold, respectively compared with those of control. Although, GSSG was found marginally decreased, GSH level in As+TU treated flag leaves was significantly increased by 1.13-fold compared with those under As treatment (Fig. 7D-E). No significant change either in GR activity or GSH and GSSG levels was observed in TU alone treated flag leaves (Fig. 7C-E). In addition, various growth and yield attributes were also quantified at the time of harvest (Table-1). The major effect of As-stress was observed in whole plant dry biomass, seed yield and harvest index, which were decreased to 0.50-, 0.41- and 0.69-fold, respectively, compared with those of control. In contrast, a significant improvement was observed under As+TU treatment, as these parameters were increased by 1.90-, 2.16- and 1.75-fold compared with those of As treatment. In case of TU treatment alone, the growth and yield attributes were found comparable, except that branches per panicle were 1.14-fold higher than the control value (Table-1). The elemental analysis was also performed in dehusked seeds harvested from plants given different treatments. The total As accumulation was reduced by 0.53-fold in As+TU compared with those of seeds

harvested from As-treated plants (Fig. 7F). As-stress reduced the levels of essential elements such as copper, manganese and zinc to 0.57-, 0.68- and 0.49-fold, respectively. Of these elements, copper was fully restored to the level of the control in As+TU treatment; however, manganese and zinc levels were comparable to those of As-treated seeds (Fig. 7G-I). The iron level remained unchanged under both As and As+TU treatment conditions, which is presumably the IR-64 variety specific response (Fig. 7J). No significant change in any of the tested elements was observed in the case of TU alone treated seeds (Fig. 7F-J).

The growth promotion effect of TU was further validated at naturally arsenic-contaminated site of Nadia district, West Bengal (India). Herein, seed treatment with similar concentration of TU was coupled with foliar application which significantly enhanced the plant growth and seed yield (Fig. 8). A major effect was observed in terms of tiller numbers, which was increased by 1.5-fold in TU sprayed plants compared with that observed for WS treatment (Fig. 8B). This result was concomitant with 1.34- and 1.18-fold increase in per plant seed and overall crop productivity (kg seeds per hectare), respectively. The synchrotron-based imaging indicated the uniform distribution of As in seeds harvested from WS as well as TU-sprayed plants, except for some localized lumps at the outside boundaries of the seed. The average grey-values indicated significant reduction in grain As accumulation in seeds of TU-sprayed plants (Fig. 8C). To validate this, the total As level was further quantified in dehusked seeds using ICP-MS. The data revealed significant reduction in grain As accumulation by 0.36-fold (p=0.0097) in seeds harvested from TU-sprayed plants compared with those of WS plants (Fig. 8D).

DISCUSSION

In the present study, we have investigated the molecular basis of TU for enhancing growth and tolerance under As-stress conditions in rice. Rice has a fibrous root system that

mainly consists of an embryonic primary/main root that develops from the radicle, embryonic/postembryonic crown roots and lateral roots. Crown roots are relatively thick and have relatively shorter and fewer lateral roots compared to the main root (Clark, MacCurdy, Jung, Shaff, McCouch, Aneshansley & Kochian, 2011). While the main root provides early vigor and establishes a framework to search for water and nutrients, crown roots provide lodging resistance and contribute to water and nutrients uptake (Rogers & Benfey, 2015). The exposure of As in the seedling stage imposed differential toxicity to main and crown root systems. The main root system was suppressed under As-stress; however, crown root length and density were increased (Fig. 1). This observation suggested the crucial role of crown roots in As-stress tolerance. The reduction in the main root under As-stress has been shown to be associated with altered expression of both auxin biosynthesis and transport genes (Ronzan, Piacentini, Fattorini, Della Rovere, Eiche, Riemann, Altamura & Falasca, 2018). Although, the role of crown roots has been demonstrated under drought stress in maize (Gao & Lynch, 2016), (Ahmed, Zarebanadkouki, Meunier, Javaux, Kaestner & Carminati, 2018); however, to the best of our knowledge, crown root function has not been explored under As-stress conditions. Under As+TU, the crown root response was further aggravated than As treatment (Fig. 1 C-D) that was associated with upregulated expression of a putative auxin efflux carrier (orthologous to Arabidopsis PIN-formed 5 protein) in As+TU treated roots (Supplementary Table-4). The auxin efflux carriers constitute a major component of polar auxin transport and are known to regulate the formation of crown roots in rice (Zhang, Li, Zhang, Yan, Wang, Zhao, Li, Qi, Sun & Zhu, 2012). The TU-triggered crown root response can be considered as an adaptive response of seedlings to survive better under As-stress conditions. Further, we analyzed As accumulation in different plant parts and found that overall As load, especially from the aerial parts, was significantly reduced in

As+TU treated seedlings than As alone treatment (Fig. 3). Owing to this, upon long-term treatment duration, improved shoot growth phenotype was also observed in As+TU compared with those under As-stress conditions (Fig. 2).

The majority of cluster-1 genes of TAR-R, which remained downregulated in As+TU treated roots compared with those under As treatment (Supplementary Table-4), encoded for PR components including ribulose bisphosphate carboxylase, photosystem I reaction center subunit, oxygen evolving enhancer protein and chlorophyll A-B binding protein. The exact function of PR genes in roots is not understood; however, their suppression is required for sustained root growth under phosphate (Pi) deficient conditions. An Arabidopsis *hps7* (hypersensitive to Pi starvation7) mutant, which has higher expression of PR genes in roots, showed increased ROS levels and hypersensitive root phenotype under the Pi deficient condition (Kang, Yu, Tian, Zhou, Li, Jiao & Liu, 2014). This observation inspired us to determine whether As^{III} exposure altered Pi levels in rice. The data revealed that Pi levels were decreased and increased in roots and shoots, respectively (Supplementary Fig. 1A-B). However, no significant changes in Pi status were noticed under As and As+TU treatments (Supplementary Fig. 1A). Thereafter, we quantified superoxide dismutase (SOD) activity and found that it was specifically increased in As-treated roots (Supplementary Fig. 1C). SOD constitutes the first line of defense towards oxidative damage and is responsible for the maintenance of redox homeostasis under various abiotic stresses such as salt and arsenic (Mittler, 2002). Owing to the ROS scavenging nature of TU, the redox status of As+TU treated roots would be maintained, as indicated by no significant change in SOD activity (Supplementary Fig. 1C). Thus, in spite of lower Pi level, balanced redox status facilitated the suppression of PR genes, leading to better root growth phenotype observed under As+TU treatment (Fig. 1C-F). Additionally, cluster-2 (TAR-R; Supplementary Table-4) and

cluster-4 (TAR-S; Supplementary Table-5), having abiotic stress related defense genes were upregulated, further contributing to improved phenotype under As+TU compared with that of As treatment.

Apart from PR genes, the expression levels of cluster-2 aquaporin isoforms, including *OsLsi1* which is the major transporter responsible for As^{III}-uptake (*Ma et a*l., 2008), were also suppressed (Fig. 5A), resulting in significantly reduced As accumulation in As+TU treated seedlings compared with those of As alone treatment (Fig. 3). For most of the cluster-2 aquaporins, the As-transport function has not yet been demonstrated, which opens up the new area for future investigations. In contrast to cluster-2, cluster-1 aquaporins like *OsPIP1;1* and *OsPIP2;1* with known functions to regulate water transport (Ding, Uehlein, Kaldenhoff, Guo, Zhu & Kai, 2019, Liu, Fukumoto, Matsumoto, Gena, Frascaria, Kaneko, Katsuhara, Zhong, Sun, Zhu, Iwasaki, Ding, Calamita & Kitagawa, 2013), were upregulated in As+TU than As treated roots (Fig. 5A), which represent compensatory mechanism of the plants to maintain their water status. Another aquaporin *OsTIP4;1*, which is proposed to transport As inside the vacuole (Dametto, Buffon, dos Reis Blasi & Sperotto, 2015), was also upregulated expression in As+TU treatment that might reduce As-toxicity by facilitating vacuolar As sequestration. Considering the critical role of ABA and SA in regulating the aquaporins (Kapilan, Vaziri & Zwiazek, 2018), the levels of these hormones were quantified. The results indicated higher accumulation of SA, which could drive the downregulated expression of aquaporin isoforms in As+TU treated roots (Fig. 5A and C). Earlier studies under salt stress conditions have confirmed that higher SA decreased the abundance of aquaporins in a ROS-dependent manner (Kapil*an et a*l., 2018). To sum-up, the enhanced accumulation of SA represents one of the mechanisms associated with TU-mediated reduced As accumulation through aquaporin downregulation and enhanced As-stress

tolerance. The exogenous SA has been demonstrated to strengthen redox homeostasis and ameliorate As-stress conditions in rice (Singh, Dixit, Kumar, Mishra, Kumar, Dixit, Singh, Dwivedi, Trivedi, Pandey, Dhankher, Norton, Chakrabarty & Tripathi, 2017) and maize (Kaya, Ashraf, Alyemeni, Corpas & Ahmad, 2020). Hence, TU being a redox molecule, can activate SA accumulation which will boost plant's ability to tolerate As-stress conditions. An additional evidence towards this hypothesis can also be derived from significantly increased accumulation of SA (Fig. 5C) and reduced root growth (Fig, 1 and 2) under the TU alone treatment. Similar to this, SA accumulation under stress-free scenario has been shown to attenuate the root growth (Tan, Abas, Verstraeten, Glanc, Molnar, Hajny, Lasak, Petrik, Russinova, Petrasek, Novak, Pospisil & Friml, 2020). Since, SA biosynthesis primarily takes place in chloroplast via isochorismate pathway (Lefevere, Bauters & Gheysen, 2020); therefore, the loss of TU-mediated suppression of PR genes and TIPs (Fig. 6), might be associated with reduced SA accumulation in As+TU treated roots of dark-adapted plants. Although, this need further investigations; however, it can be envisaged that TU-mediated molecular changes are light-dependent in nature, at least partially.

After exploring the molecular basis of TU action, we tested the potential of TU to promote rice growth and productivity under natural field conditions. Considering the size difference between young seedlings and mature plants, the TU dose was increased from 50 µM (laboratory level, medium-supplementation) to 6.5 mM (field-level, foliar spray). The dose selection was supported by the previous TU supplementation studies performed under field conditions to mitigate various abiotic stresses in different crops (Wakchaure, Minhas, Meena, Kumar & Rane, 2020, Waqas, Kaya, Riaz, Farooq, Nawaz, Wilkes & Li, 2019). The foliar application of TU significantly reduced the rate of As accumulation to flag leaves (Fig. 7A), which might be associated with TU ability to accelerate As^{V}-to-As^{III} reduction by

generating reducing redox environment inside the plants. The presence of As in the form of As^{III} promotes its complexation via GSH and phytochelatins (PCs) and subsequent sequestration into vacuole. This strategy operates mainly in nodes of rice plants and restricts As translocation from older leaves-to-flag leaves and to the grain (Chen, Moore, Miller, McGrath, Ma & Zhao, 2015a, S*hi et a*l., 2016). We quantified the level of GSH (a major redox buffer) and activity of GR (GSH regenerating enzyme) in flag leaves, at 10 d post TU 2^{nd} spray wherein the maximum difference between As and As+TU treatment in As accumulation was observed. The lack of reduction in GR activity (Fig. 7C) and higher GSH (Fig. 7D), along with relatively lower GSSG level (Fig. 7E), clearly indicated that the redox status in As+TU treated flag leaves was more reducing than those of As treatment. Incidentally, this also coincides with the chemical ability of TU to scavenge different types of ROS (G*ao et a*l., 2008, Keln*er et a*l., 1990). At the time of seed harvest, As level in rhizospheric soil of As+TU treated plants was found 2.78-fold higher than those of As-treated plants (Supplementary Fig. 2), suggesting either the reduced uptake and/or activated efflux of As in response to TU supplementation. Although, the down-regulated expression of well-characterized As-transporters (*Ma et a*l., 2008) like *OsNIP2;1/OsLsi1* (Supplementary Table-6) supported the reduced As-uptake; however, considering the reducing redox environment, As-efflux could also be activated in As+TU treated plants. Such a possibility is supported through transgenic studies wherein arsenate reductases (*OsHAC1;1* and *OsHAC1;2*) over-expressing rice lines have been demonstrated to have increased As-efflux than the wild-type plants(S*hi et a*l., 2016). The reduced rate of As translocation to flag leaves ultimately resulted into ~50% reduction in grain As accumulation, without jeopardizing the levels of other tested essential elements (Fig. 7G-J). The supplementation of BSO (L-buthionine-sulphoximine; a GSH biosynthesis inhibitor) is known to enhance grain As accumulation in

rice (Chen, Moore, Miller, McGrath, Ma & Zhao, 2015b), which is exactly opposite to TU effect. Thus, reduced redox status helps in restricting As translocation to grains. The genetic approaches like overexpression of single gene like *OsHAC1;1* or *OsHAC1;2* (S*hi et a*l., 2016) or pyramiding multiple genes(De*ng et a*l., 2018) like *ScYCF1* (*Saccharomyces cerevisiae* yeast cadmium factor1), *OsABCC1* (*Oryza sativa* C-type ATP-binding cassette transporter1) and γ-ECS (γ-glutamyl cysteine synthetase) have been demonstrated to reduce grain As level by 20 and 70%, respectively compared with those of wild-type. In view of this, TU-mediated chemical approach can serve as a viable non-genetic substitute for reducing grain As accumulation in rice. Apart from grain As reduction, another positive change in As+TU treated plants was observed in the form of improved growth and seed yield, compared with those of As treatment (Table-1). Since N:P:K:S requirement for rice cultivation is 120:50:50:13 (kg/ha), two foliar applications of 6.5 mM TU (500 litre/ha) will add only 0.3 and 1.6% of the total demand of N and S, respectively. Considering this, it is unlikely that TU spray can act as a nutritional supplement which is further substantiated through the comparative evaluation of TU and urea effects on lentil plants (Premaradhya, Shashidhar & Samuel Jeberson, 2018). Hence, the improved growth and seed yield observed under As+TU treatment is the manifestation of reduced As burden in the plants (Fig. 3). Apart from non-basmati IR-64, TU-mediated changes in plant growth, yield and grain As reduction were also validated in a basmati rice variety PB-1. Although, the extent of grain As reduction was found comparable (Supplementary Fig. 3); the TU-mediated growth and yield enhancement effects in PB-1 were less pronounced than IR-64. These results could be because PB-1 showed an As tolerant phenotype, and hence TU effect was less evident (Supplementary Table-7). LC-MS analysis revealed that residual TU in seeds of both IR-64 and PB-1 was non-detectable (LOQ or lower quantification limit: 0.2 mg/kg). Since,

NOAEL (No Observed Adverse Effect Limit) of TU is 6.88 mg TU/kg body weight/day (Srivastava *et al*., 2016), seeds harvested from TU sprayed plants can be considered as safe for human consumption.

Under field conditions, TU-mediated growth and yield enhancement has been demonstrated even in absence of stress (Pandey, Srivastava, D'Souza & Penna, 2013, Premaradhya *et al*., 2018); however, in our pot experiment, such responses under TU alone treatment were found comparable to those of control (Table-1). This discrepancy clearly suggested that TU-dependent beneficial effects are dependent upon multi-level interactions between plant, soil and overall environmental conditions. Hence, we attempted to validate the efficacy of TU in IR-64 rice variety under naturally As contaminated sites of Nadia district (West Bengal, India). Herein, we also performed 6 h seed treatment with TU so that early seedling vigor could be maintained during transplantation in As contaminated soils (Supplementary Table-8). The significant increase in growth and yield related attributes along with reduction in grain-As levels have also been observed under the realistic field conditions (Fig. 8A-B). The synchrotron-based imaging indicated homogenous As distribution in seeds including the outermost bran layer as well as endosperm. This finding clearly suggested that post-production processes of rice like milling or polishing will only be partially effective in reducing As toxicity, from the viewpoint of human consumption. In addition, the As level was uniformly reduced in seeds from TU-sprayed plants (Fig. 8C), further supporting that TU supplementation reduced the net rate of As translocation to grains. Recently, using the multi-year field study, TU applicability has also been successfully demonstrated in locally grown rice varieties of Nadia(Upadhyay, Majumdar, Barla, Bose & Srivastava, 2020). Hence, these results together supported that TU supplementation could be included as a routine agronomic practice under the As-contaminated fields.

CONCLUSIONS

In conclusion, the microarray analysis at seedling stage revealed that medium-supplemented As+TU treatment down- and up-regulated the expression levels of PR genes and auxin efflux carrier, respectively in roots and upregulated the expression of defense related genes in both root and shoot, compared with those of As-stress conditions. In addition, the expression levels of majority of aquaporin isoforms were also downregulated in As+TU, compared with those of As stress, which coincided with increased SA accumulation in roots. Such changes at molecular level along with the significant reduction in As accumulation ultimately resulted into improved growth phenotype under As+TU treatment. At mature plant stage, the foliar applications of TU generated the reducing redox conditions that decreased the rate of As accumulation towards flag-leaves, and also the overall As accumulation in grains. In addition, the crop yield was also increased with no significant residual TU accumulation in seeds. Thus, the study highlighted the mechanistic basis of TU-mediated As-tolerance, which supported the agronomic feasibility of using TU supplementation for enhancing growth and reducing grain As accumulation under the realistic field conditions.

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REFERENCES

Abbas G., Murtaza B., Bibi I., Shahid M., Niazi N.K., Khan M.I., Amjad M., Hussain M. & Natasha (2018) Arsenic Uptake, Toxicity, Detoxification, and Speciation in Plants: Physiological, Biochemical, and Molecular Aspects. *Int J Environ Res Public Health*, **15**.

Ahmed M.A., Zarebanadkouki M., Meunier F., Javaux M., Kaestner A. & Carminati A. (2018) Root type matters: measurement of water uptake by seminal, crown, and lateral roots in maize. *Journal of experimental botany*, **69**, 1199-1206.

Asthir B., Thapar R., Bains N.S. & Farooq M. (2015) Biochemical responses of thiourea in ameliorating high temperature stress by enhancing antioxidant defense system in wheat. *Russian Journal of Plant Physiology*, **62**, 875-882.

Chen Y., Hua C., Chen J., Rathinasabapathi B., Cao Y. & Ma L.Q. (2019) Expressing arsenite antiporter PvACR3; 1 in rice (Oryza sativa L.) decreases inorganic arsenic content in rice grains. *Environmental science & technology*.

Chen Y., Moore K.L., Miller A.J., McGrath S.P., Ma J.F. & Zhao F.-J. (2015a) The role of nodes in arsenic storage and distribution in rice. *Journal of experimental botany*, **66**, 3717-3724.

Chen Y., Moore K.L., Miller A.J., McGrath S.P., Ma J.F. & Zhao F.J. (2015b) The role of nodes in arsenic storage and distribution in rice. *J Exp Bot*, **66**, 3717-3724.

Clark R.T., MacCurdy R.B., Jung J.K., Shaff J.E., McCouch S.R., Aneshansley D.J. & Kochian L.V. (2011) Three-dimensional root phenotyping with a novel imaging and software platform. *Plant physiology*, **156**, 455-465.

Dametto A., Buffon G., dos Reis Blasi É.A. & Sperotto R.A. (2015) Ubiquitination pathway as a target to develop abiotic stress tolerance in rice. *Plant signaling & behavior*, **10**, e1057369.

610Deng F., Yamaji N., Ma J.F., Lee S.K., Jeon J.S., Martinoia E., Lee Y. & Song W.Y. (2018)
611 Engineering rice with lower grain arsenic. *Plant Biotechnol J*, **16**, 1691-1699.
612Ding L., Uehlein N., Kaldenhoff R., Guo S., Zhu Y. & Kai L. (2019) Aquaporin PIP2;1 affects
613 water transport and root growth in rice (Oryza sativa L.). *Plant Physiol Biochem*, **139**,
614 152-160.
615Finnegan P.M. & Chen W. (2012) Arsenic toxicity: the effects on plant metabolism. *Front*
616 *Physiol*, **3**, 182.
617Gao Q., Wang G., Sun Y. & Epstein I.R. (2008) Simultaneous tracking of sulfur species in
618 the oxidation of thiourea by hydrogen peroxide. *J Phys Chem A*, **112**, 5771-5773.
619Gao Y. & Lynch J.P. (2016) Reduced crown root number improves water acquisition under
620 water deficit stress in maize (Zea mays L.). *Journal of experimental botany*, **67**, 4545-
621 4557.
622Garbinski L.D., Rosen B.P. & Chen J. (2019) Pathways of arsenic uptake and efflux. *Environ*
623 *Int*, **126**, 585-597.
624Guillod-Magnin R., Bruschweiler B.J., Aubert R. & Haldimann M. (2018) Arsenic species in
625 rice and rice-based products consumed by toddlers in Switzerland. *Food Addit*
626 *Contam Part A Chem Anal Control Expo Risk Assess*, **35**, 1164-1178.
627Humans I.W.G.o.t.E.o.C.R.t., Organization W.H. & Cancer I.A.f.R.o. (2004) *Some drinking-*
628 *water disinfectants and contaminants, including arsenic* (vol. 84). IARC.
629Hussain S., Rengel Z., Qaswar M., Amir M. & Zafar-ul-Hye M. (2019) Arsenic and Heavy
630 Metal (Cadmium, Lead, Mercury and Nickel) Contamination in Plant-Based Foods.
631 In: *Plant and Human Health, Volume 2*, pp. 447-490. Springer.

Kang J., Yu H., Tian C., Zhou W., Li C., Jiao Y. & Liu D. (2014) Suppression of photosynthetic gene expression in roots is required for sustained root growth under phosphate deficiency. *Plant physiology*, **165**, 1156-1170.

Kapilan R., Vaziri M. & Zwiazek J.J. (2018) Regulation of aquaporins in plants under stress. *Biol Res*, **51**, 4.

Kaya C., Ashraf M., Alyemeni M.N., Corpas F.J. & Ahmad P. (2020) Salicylic acid-induced nitric oxide enhances arsenic toxicity tolerance in maize plants by upregulating the ascorbate-glutathione cycle and glyoxalase system. *J Hazard Mater*, **399**, 123020.

Kaya C., Sarioğlu A., Akram N.A. & Ashraf M. (2019) Thiourea-mediated Nitric Oxide Production Enhances Tolerance to Boron Toxicity by Reducing Oxidative Stress in Bread Wheat (Triticum aestivum L.) and Durum Wheat (Triticum durum Desf.) Plants. *Journal of Plant Growth Regulation*, 1-16.

Kaya C., Sonmez O., Aydemir S., Ashraf M. & Dikilitas M. (2013) Exogenous application of mannitol and thiourea regulates plant growth and oxidative stress responses in salt-stressed maize (Zea mays L.). *Journal of Plant Interactions*, **8**, 234-241.

Kelner M.J., Bagnell R. & Welch K.J. (1990) Thioureas react with superoxide radicals to yield a sulfhydryl compound. Explanation for protective effect against paraquat. *J Biol Chem*, **265**, 1306-1311.

Kumarathilaka P., Seneweera S., Meharg A. & Bundschuh J. (2018) Arsenic speciation dynamics in paddy rice soil-water environment: sources, physico-chemical, and biological factors-a review. *Water research*, **140**, 403-414.

Kumari P., Rastogi A., Shukla A., Srivastava S. & Yadav S. (2018) Prospects of genetic engineering utilizing potential genes for regulating arsenic accumulation in plants. *Chemosphere*, **211**, 397-406.

Lefevere H., Bauters L. & Gheysen G. (2020) Salicylic Acid Biosynthesis in Plants. *Frontiers in Plant Science*, **11**.

Li N., Wang J. & Song W.Y. (2016) Arsenic Uptake and Translocation in Plants. *Plant Cell Physiol*, **57**, 4-13.

Lindsay E.R. & Maathuis F.J.M. (2017) New Molecular Mechanisms to Reduce Arsenic in Crops. *Trends Plant Sci*, **22**, 1016-1026.

Liu C., Fukumoto T., Matsumoto T., Gena P., Frascaria D., Kaneko T., Katsuhara M., Zhong S., Sun X., Zhu Y., Iwasaki I., Ding X., Calamita G. & Kitagawa Y. (2013) Aquaporin OsPIP1;1 promotes rice salt resistance and seed germination. *Plant Physiol Biochem*, **63**, 151-158.

Ma J.F., Yamaji N., Mitani N., Xu X.-Y., Su Y.-H., McGrath S.P. & Zhao F.-J. (2008) Transporters of arsenite in rice and their role in arsenic accumulation in rice grain. *Proceedings of the National Academy of Sciences*, **105**, 9931-9935.

Mittler R. (2002) Oxidative stress, antioxidants and stress tolerance. *Trends in plant science*, **7**, 405-410.

Muehe E.M., Wang T., Kerl C.F., Planer-Friedrich B. & Fendorf S. (2019) Rice production threatened by coupled stresses of climate and soil arsenic. *Nat Commun*, **10**, 4985.

Naujokas M.F., Anderson B., Ahsan H., Aposhian H.V., Graziano J.H., Thompson C. & Suk W.A. (2013) The broad scope of health effects from chronic arsenic exposure: update on a worldwide public health problem. *Environmental health perspectives*, **121**, 295-302.

Pandey M., Srivastava A.K., D'Souza S.F. & Penna S. (2013) Thiourea, a ROS scavenger, regulates source-to-sink relationship to enhance crop yield and oil content in Brassica juncea (L.). *PLoS One*, **8**, e73921.

680Pandey M., Srivastava A.K., Suprasanna P. & D'Souza S.F. (2012) Thiourea mediates
681 alleviation of UV-B stress-induced damage in the Indian mustard (Brassica juncea
682 L.). *Journal of Plant Interactions*, **7**, 143-150.
683Premaradhya N., Shashidhar K. & Samuel Jeberson R. (2018) Effect and profitability of foliar
684 application of thiourea on growth and yield attributes of lentil (Lens culinaris L.) under
685 Manipur Conditions of North-East, India. *Int. J. Curr. Microbiol. App. Sci*, **7**, 1040-
686 1050.
687Rahman M., Sohel N., Yunus F.M., Alam N., Nahar Q., Streatfield P.K. & Yunus M. (2019)
688 Arsenic exposure and young adult's mortality risk: A 13-year follow-up study in
689 Matlab, Bangladesh. *Environ Int*, **123**, 358-367.
690Rogers E.D. & Benfey P.N. (2015) Regulation of plant root system architecture: implications
691 for crop advancement. *Current Opinion in Biotechnology*, **32**, 93-98.
692Ronzan M., Piacentini D., Fattorini L., Della Rovere F., Eiche E., Riemann M., Altamura M. &
693 Falasca G. (2018) Cadmium and arsenic affect root development in Oryza sativa L.
694 negatively interacting with auxin. *Environmental and Experimental Botany*, **151**, 64-
695 75.
696Shi S., Wang T., Chen Z., Tang Z., Wu Z., Salt D.E., Chao D.Y. & Zhao F.J. (2016)
697 OsHAC1;1 and OsHAC1;2 Function as Arsenate Reductases and Regulate Arsenic
698 Accumulation. *Plant Physiol*, **172**, 1708-1719.
699Singh A.P., Dixit G., Kumar A., Mishra S., Kumar N., Dixit S., Singh P.K., Dwivedi S., Trivedi
700 P.K., Pandey V., Dhankher O.P., Norton G.J., Chakrabarty D. & Tripathi R.D. (2017)
701 A protective role for nitric oxide and salicylic acid for arsenite phytotoxicity in rice
702 (Oryza sativa L.). *Plant Physiol Biochem*, **115**, 163-173.

Srivastava A., Suprasanna P., Srivastava S. & D'Souza S. (2010) Thiourea mediated regulation in the expression profile of aquaporins and its impact on water homeostasis under salinity stress in Brassica juncea roots. *Plant science*, **178**, 517-522.

Srivastava A.K., Pasala R., Minhas P.S. & Suprasanna P. (2016) Plant bioregulators for sustainable agriculture: integrating redox signaling as a possible unifying mechanism. In: *Advances in Agronomy*, pp. 237-278. Elsevier.

Srivastava A.K., Sablok G., Hackenberg M., Deshpande U. & Suprasanna P. (2017) Thiourea priming enhances salt tolerance through co-ordinated regulation of microRNAs and hormones in Brassica juncea. *Sci Rep*, **7**, 45490.

Srivastava A.K., Srivastava S., Mishra S., D'Souza S.F. & Suprasanna P. (2014) Identification of redox-regulated components of arsenate (As(V)) tolerance through thiourea supplementation in rice. *Metallomics*, **6**, 1718-1730.

Srivastava A.K., Srivastava S., Penna S. & D'Souza S.F. (2011) Thiourea orchestrates regulation of redox state and antioxidant responses to reduce the NaCl-induced oxidative damage in Indian mustard (Brassica juncea (L.) Czern.). *Plant Physiol Biochem*, **49**, 676-686.

Sun S.K., Chen Y., Che J., Konishi N., Tang Z., Miller A.J., Ma J.F. & Zhao F.J. (2018) Decreasing arsenic accumulation in rice by overexpressing OsNIP1;1 and OsNIP3;3 through disrupting arsenite radial transport in roots. *New Phytol*, **219**, 641-653.

Tan S., Abas M., Verstraeten I., Glanc M., Molnar G., Hajny J., Lasak P., Petrik I., Russinova E., Petrasek J., Novak O., Pospisil J. & Friml J. (2020) Salicylic Acid Targets Protein Phosphatase 2A to Attenuate Growth in Plants. *Curr Biol*, **30**, 381-395 e388.

Uenishi Y., Nakabayashi Y., Tsuchihira A., Takusagawa M., Hashimoto K., Maeshima M. & Sato-Nara K. (2014) Accumulation of TIP2; 2 aquaporin during dark adaptation is partially PhyA dependent in roots of Arabidopsis seedlings. *Plants*, **3**, 177-195.

Upadhyay M.K., Majumdar A., Barla A., Bose S. & Srivastava S. (2019) An assessment of arsenic hazard in groundwater–soil–rice system in two villages of Nadia district, West Bengal, India. *Environmental geochemistry and health*, **41**, 2381-2395.

Upadhyay M.K., Majumdar A., Barla A., Bose S. & Srivastava S. (2020) Thiourea supplementation mediated reduction of grain arsenic in rice (Oryza sativa L.) cultivars: A two year field study. *Journal of Hazardous Materials*, 124368.

Upadhyay M.K., Shukla A., Yadav P. & Srivastava S. (2018) A review of arsenic in crops, vegetables, animals and food products. *Food chemistry*.

Uppal J.S., Zheng Q. & Le X.C. (2019) Arsenic in drinking water–Recent examples and updates from Southeast Asia. *Current Opinion in Environmental Science & Health*.

Vineeth T., Kumar P. & Krishna G. (2016) Bioregulators protected photosynthetic machinery by inducing expression of photorespiratory genes under water stress in chickpea. *Photosynthetica*, **54**, 234-242.

Wakchaure G., Minhas P., Meena K.K., Kumar S. & Rane J. (2020) Effect of plant growth regulators and deficit irrigation on canopy traits, yield, water productivity and fruit quality of eggplant (Solanum melongena L.) grown in the water scarce environment. *Journal of Environmental Management*, **262**, 110320.

Waqas M.A., Kaya C., Riaz A., Farooq M., Nawaz I., Wilkes A. & Li Y. (2019) Potential Mechanisms of Abiotic Stress Tolerance in Crop Plants Induced by Thiourea. *Front Plant Sci*, **10**, 1336.

749Xalxo R. & Keshavkant S. (2019) Melatonin, glutathione and thiourea attenuates lead and
750 acid rain-induced deleterious responses by regulating gene expression of
751 antioxidants in Trigonella foenum graecum L. *Chemosphere*, **221**, 1-10.
752Xu Y., Fu J., Wang H., Hou Y. & Pi J. (2019) Arsenic Exposure and Lifestyle-Related
753 Diseases. In: *Arsenic Contamination in Asia*, pp. 83-118. Springer.
754Zacherl B. & Amberger A. (1990) Effect of the nitrification inhibitors dicyandiamide, nitrapyrin
755 and thiourea onNitrosomonas europaea. *Fertilizer Research*, **22**, 37-44.
756Zhang Q., Li J., Zhang W., Yan S., Wang R., Zhao J., Li Y., Qi Z., Sun Z. & Zhu Z. (2012)
757 The putative auxin efflux carrier OsPIN3t is involved in the drought stress response
758 and drought tolerance. *The Plant Journal*, **72**, 805-816.

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FIGURE LEGENDS

Fig. 1: Post-germination phenotyping of rice seedlings under short-term stress treatments. The 15-day-old hydroponically grown rice seedlings (Variety: IR64) were subjected to different treatments including control (Yoshida medium), arsenite (As), arsenite+thiourea (As+TU) and thiourea alone (TU). Working concentrations of As^{III} and TU were set to 20 and 75 μM, respectively. For As+TU and TU, pre-treatment with a similar concentration of TU was also administered for 24 h. After 10 d of treatment, differential phenotypes were recorded qualitatively (A-B) and quantitatively in terms of numbers and lengths of crown (C-D) and main (E-F) roots. The box plots represented data from three biological replicates (n=25; ~8-9 plants from each replicate). The mean values±SE were compared on the basis of *p*-values calculated using Student's t-test. The experiment was repeated twice to validate reproducibility.

Fig. 2: Post-germination phenotyping of rice seedlings under long-term stress treatments. The 15 d old hydroponically grown rice seedlings were subjected to different treatments including control (Yoshida medium), arsenite (As); arsenite+thiourea (As+TU) and thiourea alone (TU). Working concentrations of As^{III} and TU were set as 20 and 75 mM, respectively. For As+TU and TU, pre-treatment with similar concentration of TU was also given for 24 h. After 20 d of treatment, differential phenotype was recorded qualitatively (A) as well as quantitatively in terms of root (B) and shoot (C) length. The box plots represented data from three biological replicates (n=25; ~8-9 plants from each replicate). The mean values were compared on the basis of *p*-value calculated using student t-test. The experiment was repeated twice to validate reproducibility.

Fig. 3: Organ-level arsenic quantification in rice seedlings under different treatments. The 15-day-old hydroponically grown rice seedlings (Variety: IR64) were subjected to

different treatments including control (Yoshida medium), arsenite (As), arsenite+thiourea (As+TU) and thiourea alone (TU). Working concentrations of As^{III} and TU were set to 20 and 75 μM, respectively. For As+TU and TU, pre-treatment with a similar concentration of TU was also administered for 24 h. After 10 d of treatment, different organs such as main root, crown root, lower leaf and upper leaf were harvested separately and then dried till they achieve constant weight. Total As levels were quantified using ICP-MS. Different letters on the bar graphs have been placed on the basis of the LSD value derived from SPSS software (DMRT, $p \leq 0.05$).

Fig. 4: Microarray analysis for the identification of differentially expressed genes (DEGs) in rice seedlings under different treatments. The 15-day-old hydroponically grown rice seedlings (Variety: IR64) were subjected to different treatments including control (Yoshida medium), arsenite (As), arsenite+thiourea (As+TU) and thiourea alone (TU). Working concentrations of As^{III} and TU were set to 20 and 75 μM, respectively. For As+TU and TU, pre-treatment with a similar concentration of TU was also administered for 24 h. At 24 h after treatment, root and shoot samples were separately harvested from two independent biological replicates and total RNA was isolated for microarray analysis. The results were represented in the form of (A) Venn diagrams showing overlapping and treatment-specific DEGs; (B) Scatter plots displaying expression patterns of Thiourea modulated Arsenic Responsive (TAR) DEGs, separately for root (TAR-R) and shoot (TAR-S); (C) Heat map-based clustering of TAR-R and TAR-S DEGs and (C) Gene ontology-based functional enrichment analysis of gene cluster-1 (C-1), 2 (C-2), 3 (C-3) and 4 (C-4). Refer Supplementary table-4 and 5 for information about gene annotation and fold-change of gene clusters associated TAR-R and TAR-S DEGs, respectively.

Fig. 5: Real-time PCR based expression profiling of aquaporin isoforms and hormone accumulation in rice seedlings under different treatments. The 15 d old hydroponically grown rice seedlings were subjected to different treatments including control (Yoshida medium), arsenite (As); arsenite+thiourea (As+TU) and thiourea alone (TU). Working concentrations of As^{III} and TU were set as 20 and 75 μM, respectively. For As+TU and TU, pre-treatment with similar concentration of TU was also given for 24 h. The expression profiling of aquaporin isoforms (A) and quantification of hormones like ABA (B) and SA (C) were performed in roots at 24 h after stress. The heat-map represent normalized expression across different treatments compared with those of control. Expression values were normalized using tubulin gene as a reference. Different letters on the bar graphs have been placed on the basis of the LSD value derived from SPSS software (DMRT, $p \leq 0.05$). Refer supplementary table-9 for the details of the gene-specific primers.

Fig. 6: Expression profiling of selected TIPs and photosynthesis related genes in roots under light and dark conditions. The 15 d old hydroponically grown rice seedlings were subjected to different treatments including control (Yoshida medium), arsenite (As); arsenite+thiourea (As+TU) and thiourea alone (TU). Working concentrations of As^{III} and TU were set as 20 and 75 μM, respectively. For As+TU and TU, pre-treatment with similar concentration of TU was also given for 24 h. The expression analysis of TIPs (*OsTIP1;2*, *2;1* and *4;3*) and photosynthesis related genes like *Cyt-b6-FeS* (Cytochrome b6-f complex iron-sulfur subunit), CAB (Chlorophyll A-B binding protein) and *RBCS* (Ribulose bisphosphate carboxylase small chain) was performed in roots at 24 h after stress, independently under normal light-cycle (16 h light and 8 h dark) and complete dark conditions. Expression values were normalized using tubulin gene as a reference. Different letters on the bar graphs have

been placed on the basis of the LSD value derived from SPSS software (DMRT, $p \leq 0.05$). Refer supplementary table-9 for the details of the gene-specific primers.

Fig. 7: Pot trial with IR-64 rice variety under different treatments. Pot studies were performed with plants given different treatments such as control, As, As+TU and TU. A total of two doses of As (10 mg As^{III}/kg soil) were given at 20 and 67 d post-transplantation. For As+TU, As treatment was supplemented with two foliar applications of TU (6.5 mM containing 0.01% Tween-20) at 50 and 65 d post-transplantation. For TU alone treatment, two foliar applications of TU were performed. Plants given no treatment served as control. At 2, 10 and 15 d post-TU 2nd spray, the As accumulation was quantified in flag leaves of plants given different treatments (A). Besides, at 10 d post-TU 2nd spray, activity of GR (C) and levels of GSH (D) and GSSG (E) were also quantified in flag leaves. The representative panicles were shown (B) and various agronomic traits were quantified at full maturity of the plants (refer Table-1). The levels of As (F) and other mineral nutrients including copper (G), manganese (H), zinc (I) and iron (J) were quantified from dehusked seeds harvested from plants given different treatments. Different letters on the bar graphs have been placed on the basis of the LSD values derived from SPSS software (DMRT, $p \leq 0.05$).

Fig. 8: Field studies with IR-64 rice variety under naturally arsenic-contaminated sites of Nadia district, West Bengal (India). Naturally arsenic-contaminated site was selected in Nadia District (23°01'07.8"N-88°39'43.1"E, 23°01'14.3"N-88°38'24.7"E), West Bengal, India. Seeds were soaked either in 6.5 mM TU or water (denoted as WS for water-soaked) for 6 h, and then plants were raised using normal agronomic practices (refer to the methods section for details). In addition to seed treatments, TU treated plants were also given two foliar applications of TU (6.5 mM containing 0.01% Tween-20) at pre-flowering and grain filling stages, which occurred at 50 and 65 d post-transplantation, respectively. Representative

plant under WS and TU treatments was shown qualitatively. The corresponding transverse-section (at ~5 cm above root-shoot junction) was shown beneath to highlight the change in tiller numbers (A). Various agronomic parameters were quantified from 25 randomly selected plants (B). As accumulation levels in seed was quantified using the synchrotron-based imaging (C) and ICP-MS (D). The mean values were compared on the basis of *p*-values calculated using Student's t-tests.