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Research Article

Rapid response of rice plants to arsenite toxicity

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Abstract

Absorption of arsenic (As) by plant root is an essential activity that bridges the soil As concentration and physiological responses which can be monitored soon after exposure to As. In the present study, physiological responses to short exposure to arsenite (As^{III}) was compared in two indigenous rice cultivars one adapted to temperate and humid (TH) and the second one to warm and humid (WH) climates. Twenty day-old plants were exposed to 75 µM As^{III}, 6h. Expression of certain transporters, antioxidant system, and As detoxifying proteins were noticeable. The levels of aquaporin transcripts OsNIP 2;1 (Lsi1), OsPIP1; 3, and OsPIP2;6 significantly decreased in both cultivars. A more prominent reduction of aquaporin transcripts was observed in the WH cultivar and was accompanied by a lower As^{III} uptake as compared to the TH. The activity of superoxide dismutase, catalase, ascorbate peroxidase, and glutathione reductase enhanced in both cultivars, indicating the attempts to protect plants against As^{III}-induced oxidative damage. These antioxidant responses were more pronounced in the WH cultivar and were accompanied by remarkably enhanced levels of reduced glutathione (a non-protein thiol) and phytochelatin, particularly in shoots. Although both cultivars responsed to As^{III} very rapidly, based on the data presented here, it is likely that long-term adaptation of WH cultivar to warm and humid climate has driven genomic changes resulting in As^{III} sequestration and other protective strategies against As^{III} toxicity.

Keywords: Aquaporin, Antioxidant system, Arsenite, Rice, Thiol compounds

Introduction

Arsenic (As) is one of the most harmful toxic metalloids that is naturally present in many soils and ground waters and also can be deposited in the environment due to human industrial activities. In flooded paddy soils and rice fields, As is reduced from arsenate (As^{V}) to arsenite (As^{III}) which is the most toxic inorganic form of As. Therefore, crops such as rice (*Oryza sativa*) are the main As^{III} entrance to the human food chain (Moore *et al*., 2010). As a general observation, As uptake systems are adventitious (Garbinski *et al*., 2019). Most plants would tend not to accumulate As, but it enters cells via uptake systems through other nutrients and minerals such as aquaporins (AQPs).

In rice plants, the efficient Si uptake pathway allows inadvertent passage of As^{III} due to their chemical similarity; they both exist as neutral forms in paddy soils, i.e., arsenous acid-$As(OH)_3$ and silicic acid-$Si(OH)_4$ (Ma *et al*., 2008). Recent studies have shown that As^{III} is taken up through silicon transporters e.g., Lsi1 (OsNIP2;1) which is the major entry route of As^{III} into rice roots (Zhao *et al*., 2010; Mosa *et al*., 2012). Also, other NIPs including OsNIP1;1, OsNIP2;2, OsNIP3;1, and OsNIP3;2 show permeability to As^{III} (Bienert *et al*., 2008; Ma *et al*., 2008). Moreover, plasma membrane intrinsic proteins (PIPs, another subfamily of the AQPs), including OsPIP2;4, OsPIP2;6 and OsPIP2;7, are also involved in As^{III} transport (Mosa *et al*., 2012).

Arsenic is not only nonessential element for plants, but also adversely affects their growth and development. Damage of cell membranes, reduction of transpiration intensity, and increased production of reactive oxygen species (ROS) are major consequences of As toxicity to plants. Oxidative stress interrupts the balance between oxidizing reactions and antioxidants that can lead to oxidation and modification of cellular amino acids/proteins, and ultimately cell death (Srivastava *et al*., 2011). Oxidative stress is combated by plants through a well-developed enzymatic and non-enzymatic antioxidant system. Superoxide dismutase (SOD), catalase (CAT), peroxidase (POD), ascorbate peroxidase (APX), as well as glutathione reductase (GR) are the most important enzymatic antioxidants with different affinities to their substrates. Ascorbate, α-tocopherol, and reduced glutathione (GSH) are well known representatives of non-enzymatic antioxidants. Meanwhile, GSH and another sulfur-containing ligand, phytochelatin (PC) are able to detoxify As via conjugation and subsequent transportation of As into vacuoles by ABC transporters (Srivastava *et al*., 2011; Song *et al*., 2014).

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To the best of our knowledge, the differential uptake and detoxification strategies of rice cultivars from different habitats after a short exposure to a high concentration of As^{III} have not been studied yet. In the present study the response to As^{III} was evaluated in two rice cultivars; *Oryza sativa* Indica cv. Hashemi which was adapted to temperate and humid (TH) and *O. sativa* Indica cv. Amber which has been adapted to warm and humid (WH) climates (Tayefeh *et al*., 2018). Harmonized strategies from reducing As^{III} uptake by increasing its conjugation accompanied by improvement of antioxidant system in restoring redox stability of rice plants are discussed.

Material and methods

Plant materials, As^{III} treatment and determination of its content: Seeds of two rice cultivars were surface sterilized by immersing in 1% sodium hypochlorite (NaClO) solution for 5 min followed by rinsing with deionized water three times. Then, the seeds were allowed to germinate between two layers of moistened culture paper for seven days. Uniform seedlings were selected and transferred to aerated hydroponic cultures containing Kamachi nutrient solution (Kamachi *et al*., 1991), pH 5.5. The plants were grown in growth chamber with a 16 h light/8 h dark photoperiod, 24±2 °C, relative humidity 60% and 107 µmol $m^{-2}s^{-1}$ PPFD (photosynthetic photon flux density). Nutrient solutions were renewed every 3 days.

Arsenite was supplied in the form of sodium arsenite (Na_2HAsO_2). A preliminary study was conducted applying different concentrations from 0 to 250 µM of As^{III}, and the plant growth was monitored in a time course manner. Based on the results of this study, 75 µM was selected as a concentration at which the plant growth was inhibited. Higher concentrations were severely detrimental leading to death of the plants after 12 h treatment. Therefore, 20 day-old plants were treated with 75 µM As^{III} for 6 h. The plants were harvested at different intervals, thoroughly washed with deionized water and gently blotted. The plant roots and shoots were separated, frozen with liquid N_2, and stored at -80$^{\circ C}$ until they were used for biochemical analysis.

Total arsenic concentrations in plant samples were determined by ICP-MS and Atomic absorption. In brief, the samples were weighed into digestion tubes and digested in high purity concentrated nitric acid (HNO_3, 65%) and hydrogen peroxide (H_2O_2, 30%) at a temperature up to 200 °C for ICP-MS and 140 °C for atomic absorption analysis. Then, the samples were diluted and analyzed using an ICAP Q (Thermo Fischer Scientific, Waltham, USA) with a FAST sample introduction system (Elemental Scientific, Omaha, USA). Calibration was performed using single element standard solutions and a 1 ppb in solution was used as internal standard. Analysis was performed in both standard and collision (i.e. Kinetic Energy Discrimination, KED) modes, using helium as a collision gas for the latter. Comparison of standard and collision modes demonstrated that potential interference from ArCl on ^{75}As is not an issue for these samples. In case of atomic absorption analysis after heating the samples at 140 °C the clear solutions then again heated to 180 °C to boil off the nitric acid. After cooling, the residue was taken up in 10 ml of a solution containing 10 % HCl, 5 % ascorbic acid and 10 % KI. Arsenic concentrations were measured in duplicate using a Shimadzu spectra AA-6200 Atomic Absorption Spectrophotometer with a hydride generator (WHG 103A).

ROS and antioxidant assay: The root and shoot samples of 20 day-old seedlings were extracted with 50 mM Na-Pi buffer. Measurement of hydroxyl-radical ($OH^{\bullet}$) was quantified via degradation of 2-deoxyribose and the reaction of the resulting compounds with thiobarbituric acid (TBA) (Nemati *et al*., 2018).

Determination of hydrogen peroxide (H_2O_2) was conducted based on the reaction of the sample extract with trichloracetic acid (TCA) in the presence of KI. Absorbance of the mixture solution was read at 390 nm by a double beam spectrophotometer (Cintra 6, GBC, Dandenong, Vic., Australia) (Rezaei *et al*., 2011).

Ferric ion reducing antioxidant power (FRAP) of sample extracts was determined based on oxidation-reduction of potassium ferricyanide and ferric chloride in the presence of TCA. Ascorbic acid was used as a positive control (Oyaizu, 1986).

Free radical scavenging capacity of samples extracts was determined using the stable 2.2'diphenyl picrylhydrazyl radical (DPPH). Ascorbic acid (10 mg mL^{-1}) was used as a positive control (Bemani *et al*., 2012).

For determination of antioxidant enzymatic activities, the samples were extracted with HEPES-KOH buffer (50 mM, pH 7.8) containing 0.1 mM EDTA. Protein content of the extract was measured by Bradford method *et al*. (1976). The activity of superoxide dismutase (SOD) was determined based on the inhibition of reduction of nitroblue tetrazolium chloride (NBT) and production of formazan. One unit of SOD activity was defined as the amount of enzyme that resulted in 50% inhibition of the rate of NBT reduction at 560 nm. The activity of catalase was monitored by measuring H_2O_2 consumption and decreasing of absorbance at 240 nm. Peroxidase activity of the sample extract was determined by measuring oxidation rate of guaiacol in the presence of H_2O_2 at 470 nm. The activity of APX was monitored by oxidation of ascorbate as a specific substrate and the decrease in absorbance at 290 nm. The rate constant was calculated using the extinction coefficient of 2.8 $mM^{-1}cm^{-1}$. Glutathione reductase activity was determined following oxidation of NADPH at 340 nm in 1 mL of reaction mixture containing 100 mM Na-phosphate buffer (pH 7.8), 2 mM EDTA, 0.2 mM NADPH and 0.5 mM oxidized glutathione (GSSG) (Rao *et al*., 1996).

In order to measure GSSG and GSH contents of the samples, ophthaldialdehyde (OPA) was added to the

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extract and the absorbance was read by a fluorescence spectrophotometer (Perkin Elmer LS55, USA) with excitation at 350 nm and emission at 420 nm (Hissin and Hilf, 1976).

Thiols were extracted by sulfosalicylic acid containing diethylene triamine pentaacetic acid (DTPA). Non-Protein thiols (NPTs) were analyzed after reaction of thiols with 5,5′-dithio-2-nitrobenzoic acid (DTNB) and using the molecular extinction coefficient of 13,600 M^{-1} cm^{-1} at 412 nm. The content of phytochelatin was calculated as PCs = NPT-(GSH+GSSG) (Duan *et al*., 2011).

Peroxidation of membrane lipids was measured by measuring complex of TBA with malondialdehyde (MDA) at 532 nm. Corrections were used for the interference of complexation of TBA with non-specific substrates at 440 and 600 nm. An extinction coefficient of 157 $mM^{-1}cm^{-1}$ was used (Du and Bramlage, 1992).

Determination of amino acids: The samples were extracted with EtOH 80% followed by centrifugation, evaporation of the supernatant, and adding full name (OPA). Amino acids were eluted on a Prevail C18 column (250 mm × 4.6 mm I.D, 5 µm, Alltech) using HPLC system (Agilent Technologies 1260, CA, USA), equipped with a FLD HP 1100 detector. Mobile phase was composed of 25 mM Na-pi containing 35% MeOH and 15% acetonitrile. Fluorescence detection and quantification were carried out by excitation at 230 nm and emission at 460 nm (Biermann *et al*., 2013). To measure of proline content, the samples were extracted with 3% sulfosalicylic acid. Acetic acid and ninhydrin were added to the supernatant and boiled for 1 h and the absorbance was read at 520 nm (Bates *et al*., 1973).

Quantitative expression analysis of As transporters: In order to determine the role of AQPs in As^{III} uptake by roots, quantitative RT–PCR (qRT–PCR) was performed and the changes in transcripts of OsNIP 2;1 (LSI1) and OsPIP1; 3, and OsPIP2; (as representative members of PIP1 and PIP2) were analyzed in rice roots. Total RNA was isolated using Hybrid-R™kit (Cinna Gen, Iran) following the manufacturer's instructions. Reverse transcription was performed to synthesize cDNA by using the RT Kit. Specific primers were designed on the basis of sequence information obtained from NCBI for *Oryza sativa* as follows: OsPIP1; 3 forward primer: CTGGTGATCGATGAAGCTAG; reverse primer: ACACAAGTACCATTTCTCACAC; OsPIP2; forward primer: GCCAGGTGCATGATTTGTT; reverseprimer: GCCGAAGCAGTTTGTATCTC; OsNIP;1 forward primer: GCCAGCAACAACTCGAGAACAA; reverse primer: CATGGTAGGCATGGTGCCGT. Quantitative real time qRT-PCR was carried out in ABI step one real-time PCR detection system using SYBR-Green. Relative expression level was calculated using 2−ΔΔCT method (Livak and Schmittgen, 2001). Rice beta actin was used as an internal control to normalize genes expression.

Statistical analysis: All the experiments were repeated twice with similar results. The data were expressed as the mean values ± the standard deviation (SD). Statistical analysis was performed using LSD (least significant difference), and the differences between the treatments were expressed as significant at a level of $P \leq 0.05$.

Results and discussion

Arsenite uptake: In order to monitor rapid absorption of As^{III} by rice roots, the plants were exposed to 75 µM As^{III} for 6 h and harvested at different intervals. Negligible amount of As^{III} was found in the rice seeds and were transferred to the seedlings before any treatment (Fig. 1). During the treatment, As^{III} was rapidly absorbed reaching maximal levels of 96 and 70 µg/g DW in TH and WH, respectively (Fig. 1). After 6 h exposure to As^{III}, more than half of total As^{III} content was found in the roots than shoots of both cultivars (Fig. 1).

Arsenite was easily and rapidly absorbed by both the examined rice cultivars and accumulated more in roots than in shoots. Involvement of Lsi1 (OsNIP2;1), OsPIP1; 3 and OsPIP2; 4 in transport of As^{III} into the rice plant have previously been shown (Ma *et al*., 2008; Mosa *et al*., 2012). Diminished expression of these genes, although to some extent may be resulted from downregulation of whole metabolism of the plant (Finnegan and Chen, 2012), indicates that the plants attempt to restrict As^{III} uptake. Regulation of As uptake systems to restrict influx of this toxic metalloids has been reported previously in yeast and mammalian cells (Jochem *et al*., 2019; Garbrinski *et al*., 2019; Wysocki *et al*., 2001).

Redox assessment and As^{III} detoxifying agents: The DPPH radical scavenging capacity (DPPH RSC) of shoots was overall higher than of roots in both the examined cultivars (ca. 2.5-3 folds) (Fig. 2a-b). Exposure to As^{III} significantly reduced the DPPH RSC of roots to 53% and 75% of their corresponding controls in TH and WH, respectively (Fig. 2a). Also, arsenic treatment reduced the DPPH RSC of shoots, but again the reduction was more pronounced in TH than in WH (Fig. 2b). A remarkable reduction was observed in FRAP of rice roots after exposure to As^{III} (54% and 23% of the controls in TH and WH, respectively, at 6 h) (Fig. 2c). Meanwhile, The FRAP was reduced in shoots of As^{III}-treated plants albeit with a much moderate tendency. Although the FRAP of shoots of both cultivars after 6 h As^{III} treatment were almost identical, again shoot FRAP reduction was more prominent in TH than WH (20% and 11% of the controls in TH and WH, respectively) (Fig. 2d).

The initial activity of SOD was lower in TH roots than in WH (Table 1). Treatment with As^{III} significantly increased SOD activity of roots in both cultivars, and the rate of increase was found to be more pronounced in WH. The initial activity of CAT was identical in both cultivars, but the arsenite treatment increased it up to2.8 and 2 folds of their control levels in roots and shoots

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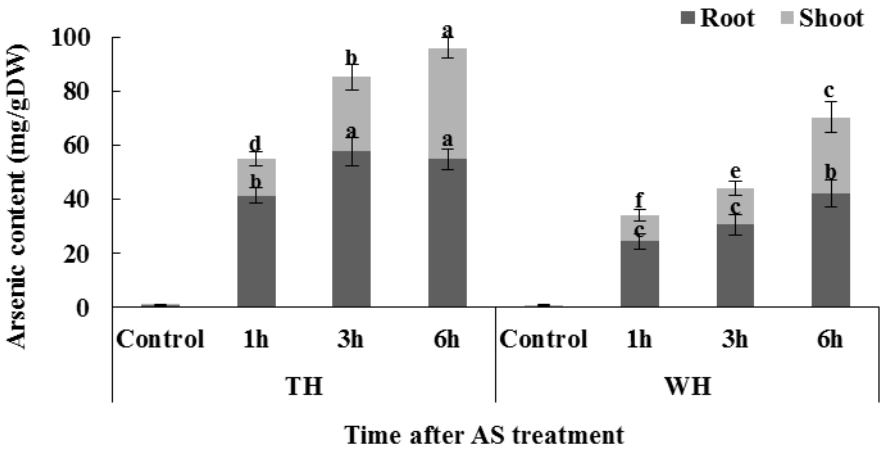

Fig. 1. Arsenite content of rice cultivars after exposure to 75 µM As^{III} for 0, 1, 3, and 6h. Data are presented as mean of three independent experiments with 20 plants each. The standard deviations are not shown, however their ratios to corresponding mean values were less than 4%. TH and WH, the cultivar adapted to temperate – humid and warm-humid climates, respectively.

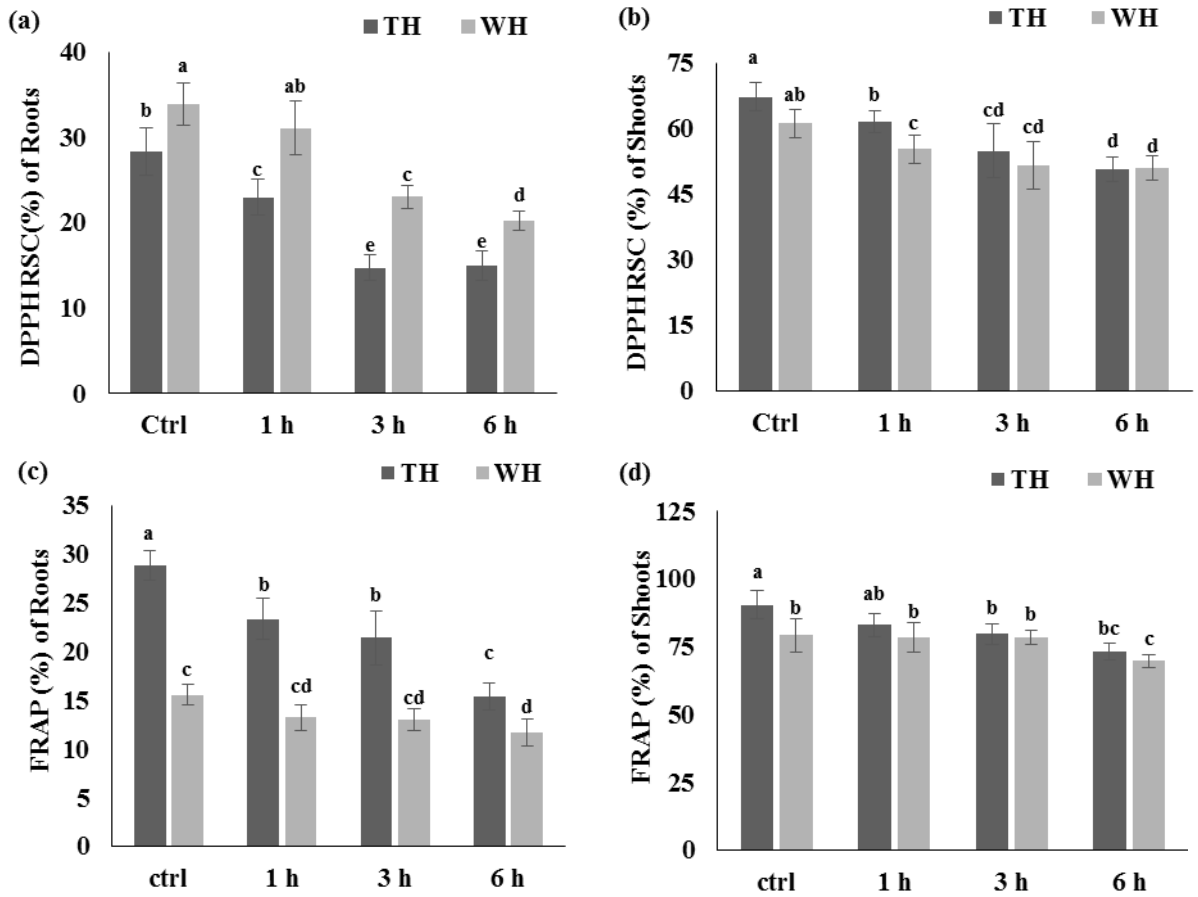

Fig. 2. Radical scavenging and ferric ion reducing potential of two rice cultivars, before and after exposure to 75 µM As^{III}. Data are presented as mean ±SD, n=3. Bars with different letters in each cultivar show significant different at P≤0.05 according to LSD test. TH and WH, the cultivar adapted to temperate – humid and warm-humid climates, respectively.

of TH, respectively. In WH, CAT activity increased up to 3.3 and 2.3 fold of their corresponding controls in roots and shoots, respectively.

The activity of GR in both cultivars was similar before treatment with As^{III}. Exposure of plants to As^{III} significantly increased GR activity of roots and shoots in both cultivars, although the rate of increase was more pronounced in WH. The initial activity of POD was higher in roots of TH than of WH. Treatment with As^{III} significantly enhanced POD activity in both cultivars, but the rate of increase was more prominent in WH. An increase of about 2-3 folds of the controls was observed

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Table 1. Alteration of antioxidant enzymes activity of rice plants before and after exposure to 75 µM As^{III}

			SOD (unit.mg protein^{-1})	CAT (ΔA240.mg protein^{-1})	GR (µmol NADPH oxidised min^{-1} mg protein^{-1})	POD (µmol H_2O_2 reduced min^{-1} mg^{-1} protein)	APX (ΔAbs290 mprotein^{-1})
TH	Root	Control	17.45±2.1^{c}	15.14±1.3^{d}	3.47±0.8^{c}	0.67±0.04^{c}	2.66±0.9^{c}
		1 h	21.24±3.2^{c}	18.32±2.2^{c}	5.14±1.3^{c}	0.73±0.06^{c}	4.42±1.2^{b}
		3 h	30.25±2.0^{b}	36.12±1.8^{b}	6.67±0.7^{b}	0.78±0.03^{b}	5.69±1.5^{a}
		6 h	33.25±0.9^{a}	43.43±0.9^{a}	8.38±0.9^{a}	0.91±0.05^{a}	5.08±1.3^{a}
	Shoot	Control	12.03±1.7^{d}	25.38±2.7^{c}	14.66±1.2^{c}	1.26±0.08^{b}	4.25±0.28^{c}
		1 h	14.25±1.1^{c}	30.34±1.8^{b}	15.45±0.9^{c}	1.29±0.05^{b}	7.24±0.75^{b}
		3 h	19.24±2.8^{b}	46.34±2.2^{a}	18.43±1.9^{b}	1.41±0.03^{a}	11.76±2.3^{a}
		6 h	25.31±2.1^{a}	48.27±1.6^{a}	22.99±1.3^{a}	1.46±0.04^{a}	12.43±1.4^{a}
WH	Root	Control	25.25±2.1^{d}	16.52±2.1^{d}	3.47±0.7^{d}	0.31±0.02^{c}	3.32±0.5^{d}
		1 h	33.05±1.3^{c}	25.19±2.8^{c}	6.55±1.4 ^{c}	0.31±0.04^{c}	4.27±0.4^{c}
		3 h	48.23±3.2^{b}	44.32±3.4^{b}	8.88±1.6^{b}	0.38±0.06^{b}	6.23±0. 6^{b}
		6 h	62.25±4.3^{a}	55.15±3.6^{a}	12.42±2.3^{a}	0.49±0.07^{a}	8.23±0. 7^{a}
	Shoot	Control	14.78±3.2^{d}	29.41±3.2^{d}	15.37±0.9^{c}	1.94±0.03^{c}	4.56±0.23^{d}
		1 h	18.83±5.1^{c}	38.28±4.8^{c}	17.06±1.3^{c}	2.25±0.05^{b}	9.63±0.75^{c}
		3 h	27.45±7.4^{b}	60.21±3.1^{b}	21.72±2.7^{b}	2.68±0.07^{a}	13.49±1.17^{b}
		6 h	35.53±4.8^{a}	67.32±2.5^{a}	28.59±3.1^{a}	2.89±0.08^{a}	15.63±1.08^{a}

Data are presented as mean ±SD, n=3. Bars with different letters in each column are significantly different at P≤0.05 according to LSD test.

in the activity of APX of both cultivars after exposure to As^{III}, but the rate was found to be higher in WH.

The content of GSH and the GSH/GSSG ratio was significantly increased in both roots and shoots of TH upon exposure of the plants to As^{III} up to 3 h of the treatment (Table 2). The same tendency was observed in the content of GSH and the GSH/GSSG ratio of WH but with a more prominent rate which persisted toward the end of treatment. Exposure to As^{III} significantly and continuously increased the contents of PC and NPT during the treatment in both rice cultivars. However, it was noticeable, that the rates of enhancement of PC and NPT were more pronounced in shoots than roots and in particular of WH than TH.

In comparison with the control condition, exposure to As^{III} increased H_2O_2 content of roots of TH and WH to 149% and 135% of their control, respectively (Fig. 3a). The rate of increase of H_2O_2 content of shoots was more outstanding in TH than in WH (Fig. 3b). The fact that the initial content of $OH^{\bullet}$ in roots and shoots of TH was lower than in WH, the rate of its increase upon As^{III} treatment was more prominent (Fig. 3 c, d). The rate of membrane lipid peroxidation of roots and shoots of WH, particularly after 6 h exposure to As^{III} was remarkably lower than in TH (Fig. 3 e,f).

Among all detected essential amino acids of rice plants, the alterations of Glu, Cys, Gly, and Pro were more prominent in As^{III}-treated plants relative to the amino acid levels in the control plants. The Pro content of both cultivars increased during the period of exposure to As^{III} (Table 3). In TH cultivar the highest contents of Glu, Cys, Gly were observed 3 h after As^{III} treatment whereas in WH cultivar the increase of aforementioned amino acids was more pronounced and lasted for 6 h (Table 3).

Plant root is the first organ which encounters As^{III}, accumulates it and expresses its toxicity (Das *et al.*, 2013). A significant correlation has been reported between the reduced growth attributes raised from As toxicity and the As accumulation rate. The total As^{III} accumulated in WH cultivar was about 70% of TH.

One inevitable toxic effect of As for plants is overproduction of ROS that adversely affects plant metabolism. A clear relationship between As stress, redox homeostasis and antioxidant capacity has been reported (Sharma and Dietz, 2006). Exposure to As^{III} significantly reduced DPPH and FRAP of both cultivars, indicating As^{III}-induced oxidative stress and the rate of their reduction was more outstanding in roots than shoots. Superoxide anions ($O_2^{\bullet-}$) were the first destructive ROS which were produced. Catalytic dismutation of $O_2^{\bullet-}$ to H_2O_2 and water is carried out by SOD and serves as a source for production of other species i.e., $OH^{\bullet}$ and H_2O_2. The latter is relatively long-lived (1^{ms}) and therefore more dangerous than other ROS.

Increase of SOD activity in plants must be followed by increased activity of enzymes that are responsible for H_2O_2 elimination, such as CAT, APX, and POD (Sharma *et al.*, 2012). Although all H_2O_2 scavenger enzymes act in a cooperative or synergistic way, it is more likely that CAT effectively eliminates H_2O_2, thereby regulates the activity of APX (Ghanati *et al.*, 2005). The latter can scavenge H_2O_2 that has not been removed by catalase and requires a specific reductant with a higher affinity for H_2O_2 (ascorbate), allowing for the scavenging of small amounts of H_2O_2, in more specific locations (shoots). This explains why the most elevated rate of CAT activity was observed in roots, while of APX was detected in shoots of As^{III}-treated plants. Glutathione reductase is another H_2O_2 removing enzyme which functions in ascorbate-glutathione cycle using GSH as a specific substrate.

Besides, the enzyme catalyzes the reduction of

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Table 2. The content of thiol containing compound of rice plants before and after exposure to 75 µM As^{III}

			GSH	NPT	GSH/GSSG	PC
			(µmol/g FW)			
TH	Root	Control	4.09±0.1^{c}	22.45±1.2^{d}	13.53±1.3^{b}	18.36±1.2^{c}
		1 h	4.89±0.2^{b}	27.68±1.8^{c}	14.82±0.8^{b}	22.79±1.6^{b}
		3 h	6.91±0.2^{a}	40.23±1.3^{a}	18.67±0.3^{a}	29.32±1.2^{a}
		6 h	4.52±0.3^{b}	36.25±1.4^{b}	10.71±0.4^{c}	32.41±1.4^{d}
	Shoot	Control	6.22±0.1^{c}	62.24±2.3^{d}	16.19±0.7^{b}	56.02±1.2^{c}
		1 h	6.91±0.2^{b}	66.25±1.6^{c}	17.08±1.3^{b}	59.34±1.8^{b}
		3 h	9.14±0.3^{a}	74.59±2.0^{b}	19.95±0.9^{a}	65.45±2.0^{a}
		6 h	6.33±0.2^{c}	92.18±2.8^{a}	11.20±0.2^{c}	81.48±3.4^{d}
WH	Root	Control	4.48±0.3^{d}	23.01±0.6^{d}	12.21±0.1^{d}	18.52±1.8^{d}
		1 h	5.17±0.5^{c}	26.26±2.1^{c}	12.48±0.3^{c}	21.09±1.2^{c}
		3 h	7.85±0.8^{b}	38.51±0.4^{b}	14.06±0.2^{b}	30.66±1.7^{b}
		6 h	9.89±0.5^{a}	43.42±0.9^{a}	16.57±0.4^{a}	35.53±2.1^{a}
	Shoot	Control	5.02±0.5^{d}	63.71±2^{d}	20.14±0.3^{d}	58.68±4.1^{d}
		1 h	6.22±04^{c}	74.63±3.2^{c}	23.69±0.5^{c}	68.4±3.3^{c}
		3 h	8.77±0.7^{b}	104.43±4.1^{b}	29.87±0.8^{b}	95.66±5.2^{b}
		6 h	9.75±0.3^{a}	130.98±4.8^{a}	32.59±1.2^{a}	121.22±5.8^{a}

Data are presented as mean ± SD, n=3. Bars with different letters in each column are significantly different at P≤0.05 according to LSD test.

Table 3. The content of prolin and amino acids precursor of glutathione in rice plants before and after exposure to 75 µM As^{III}

			Glu	Gly	Cys	Pro
			µg/g FW			
TH	Root	Control	45.40±2.1^{b}	25.73±1.3^{b}	28.04±1.8^{b}	17.02±0.4^{d}
		1 h	42.19±3.2^{b}	24.23±2.2^{b}	24.33±1.3^{c}	19.23±1.6^{c}
		3 h	66.14±2.0^{a}	40.32±1.8^{a}	41.23±0.7^{a}	25.23±2.4^{b}
		6 h	35.03±0.9^{c}	20.32±0.9^{c}	16.01±0.9^{d}	31.23±2.3^{a}
	Shoot	Control	57.60±1.7^{c}	55.44±2.7^{c}	25.46±1.2^{c}	23.27±1.8^{c}
		1 h	59.66±1.1^{c}	62.10±1.8^{b}	30.33±0.9^{b}	25.23±1.5^{c}
		3 h	70.21±0.8^{a}	66.84±2.2^{a}	36.71±1.9^{a}	30.25±1.3^{b}
		6 h	65.81±2.1^{b}	63.42±1.6^{b}	31.57±1.3^{b}	32.62±0.9^{a}
WH	Root	Control	49.57±2.1^{c}	24.64±2.1^{d}	19.99±0.7^{c}	11±1.2^{c}
		1 h	55.33±1.3^{b}	34.31±2.8^{c}	28.08±1.4^{b}	12.18±0.9^{c}
		3 h	81.09±3.2^{a}	52.22±3.4^{b}	74.08±3.6^{a}	14.63±0.7^{b}
		6 h	43.33±4.3^{d}	61.84±3.6^{a}	74.65±4.3^{a}	16.54±1.1^{a}
	Shoot	Control	60.86±3.2^{c}	30.57±1.2^{c}	20.41±0.9^{c}	31.09±1.3^{c}
		1 h	71.19±5.1^{b}	36.16±1.8^{b}	24.86±1.3^{b}	32.15±1.5^{c}
		3 h	94.93±7.4^{a}	42.74±2.1^{a}	36.96±2.7^{a}	39.51±2.4^{b}
		6 h	73.83±4.8^{b}	43.42±2.5^{a}	39.01±3.1^{a}	55.23±3.1^{a}

Data are presented as mean ± SD, n=3. Bars with different letters in each graph are significantly different at P≤0.05 according to LSD test.

GSSG to GSH. Glutathione has important functions as a scavenger of O_2^-, H_2O_2 and $OH^•$ to maintain the reduced state of plant cell (Noctor and Foyer, 1998). Also, glutathione contributes to As detoxification since it is a building block of PC and since GSH can directly bind to As^{III} to form the $As(GS)_3$ complex. It is well-established that not only the pool of GSH but also the GSH/GSSG ratio (i.e., high levels of GSH but lower level of GSSG) are both important to maintain the redox status of the cell, meanwhile may modulate some pathways including synthesis of glutathione itself. A comparison between the two examined rice cultivars clearly showed higher GSH and GSH/GSSG in WH. Altogether, the results reveal the high potential of the WH cultivar to reduce As^{III} uptake, minimizing oxidative damage, and maintain redox homeostasis.

However, it is noteworthy that not only transporters and redox enzymes, but also biotransformation as well as biosynthetic pathways for conjugation of arsenic are critical determinants of As resistance (Li *et al*., 2016; Chandrakar *et al*., 2017). Among arsenic species, As^{III} has a high affinity to the thiol (-SH) group of peptides and proteins. Plants form As^{III} complex with S-containing ligands to diminish the free As^{III} concentration and transport the As^{III}-thiol complexes into vacuoles through ABC transporters (Chen *et al*., 2017).

The most prominent conjugated As forms include $As(GS)_3$, $(GS)As^{III}$-PC_2, As^{III} -PC_3, and As^{III}-$(PC2)_2$ (Garbinski *et al*., 2019). A positive correlation was observed between the duration of exposure of rice cultivars to As^{III} and the amount of NPT, GSH and PCs. It is accepted that despite the sequestration of As in the vacuoles within root cell, still an appreciable amount of

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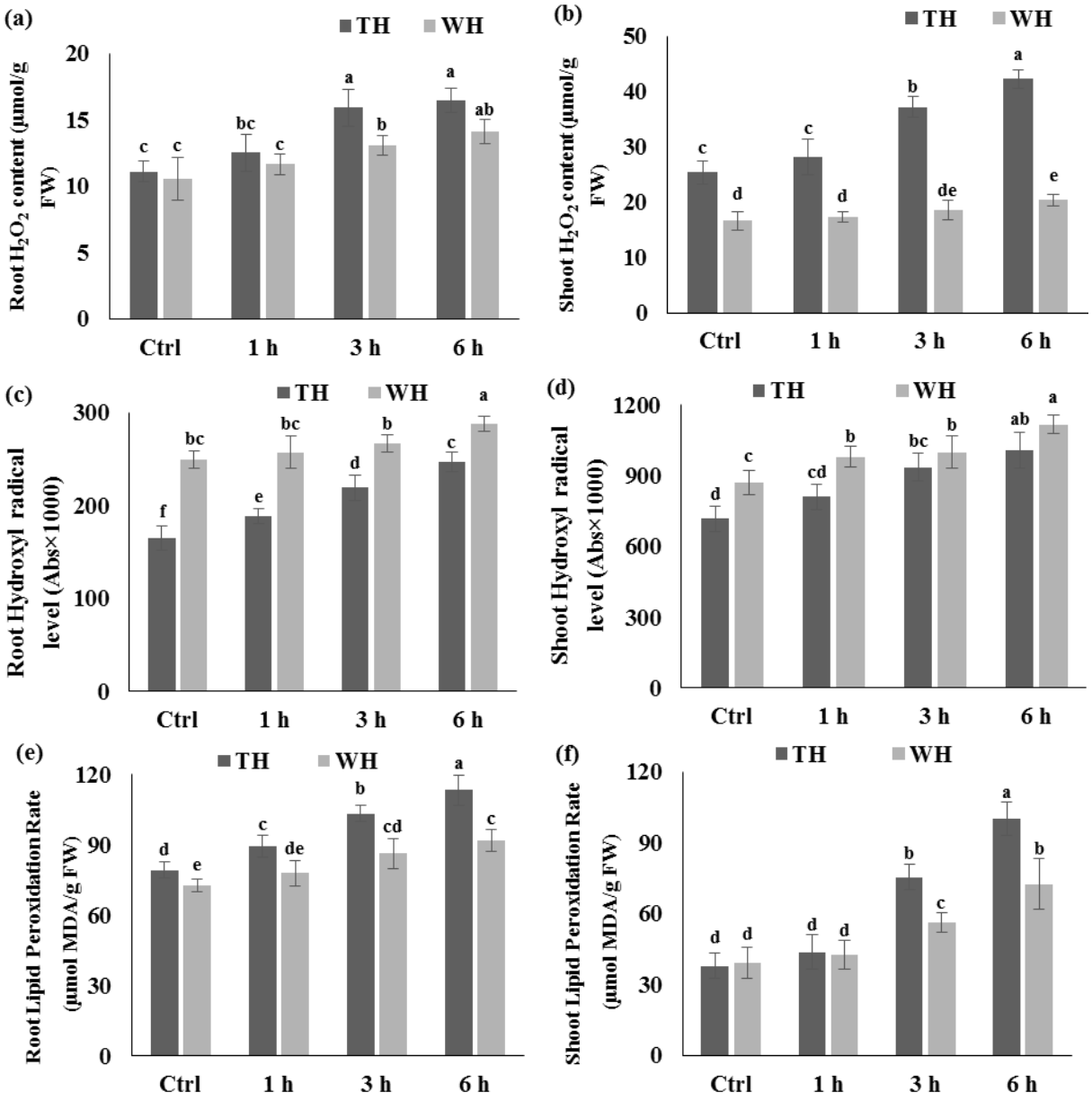

Fig. 3. Effect of As^{III} on H_2O_2 and hydroxyl radical contents and membrane lipid peroxidation rate of rice cultivars before and after treatment with 75 µM As^{III}. Data are presented as mean ± SD, n=3. Bars with different letters in each cultivar show significant different at P≤0.05 according to LSD test. TH and WH, the cultivar adapted to temperate – humid and warm-humid climates, respectively.

arsenic can be transported to rice shoots (Mitra *et al*., 2017). Remarkably high amounts of PC were found in shoots of WH during As treatment, emphasizing again that besides lower As uptake, this cultivar has the potential to efficiently detoxify As in shoots and therefore rescue photosynthetic apparatus.

Another positive correlation was observed between GSH, PC, and their amino acid precursors i.e., glutamic acid (Glu), glycine (Gly), and cysteine (Cys). Meanwhile, glutamic acid is used to produce proline (Pro) via a phosphorylation reaction catalyzed by glutamate kinase. In agreement with previous reports (Mishra and Dubey, 2006), the content of Pro significantly increased after exposure of rice plants to As^{III}. Different functions have been attributed to Pro accumulated under various abiotic stresses i.e., regulation of osmotic potential and redox state, and scavenging of ROS (Szabados and Savoure, 2010). The results presented here suggest that under As^{III} stress glutamate kinase activity is regulated through a feedback mechanism by the content of Pro. An increased level of free Pro causes allosteric regulation of glutamate kinase activity to inhibit excessive production of Pro and the preferred utilization of Glu for GSH and PC synthesis (Fujita *et al*., 2003).

Expression level of As transporters: Quantitative RT-PCR analysis of AQPs as putative As transporters showed that under control conditions, the expression levels of OsNIP2; 1 and OsPIP2; 6 were almost identical in the roots of both cultivars, whereas the

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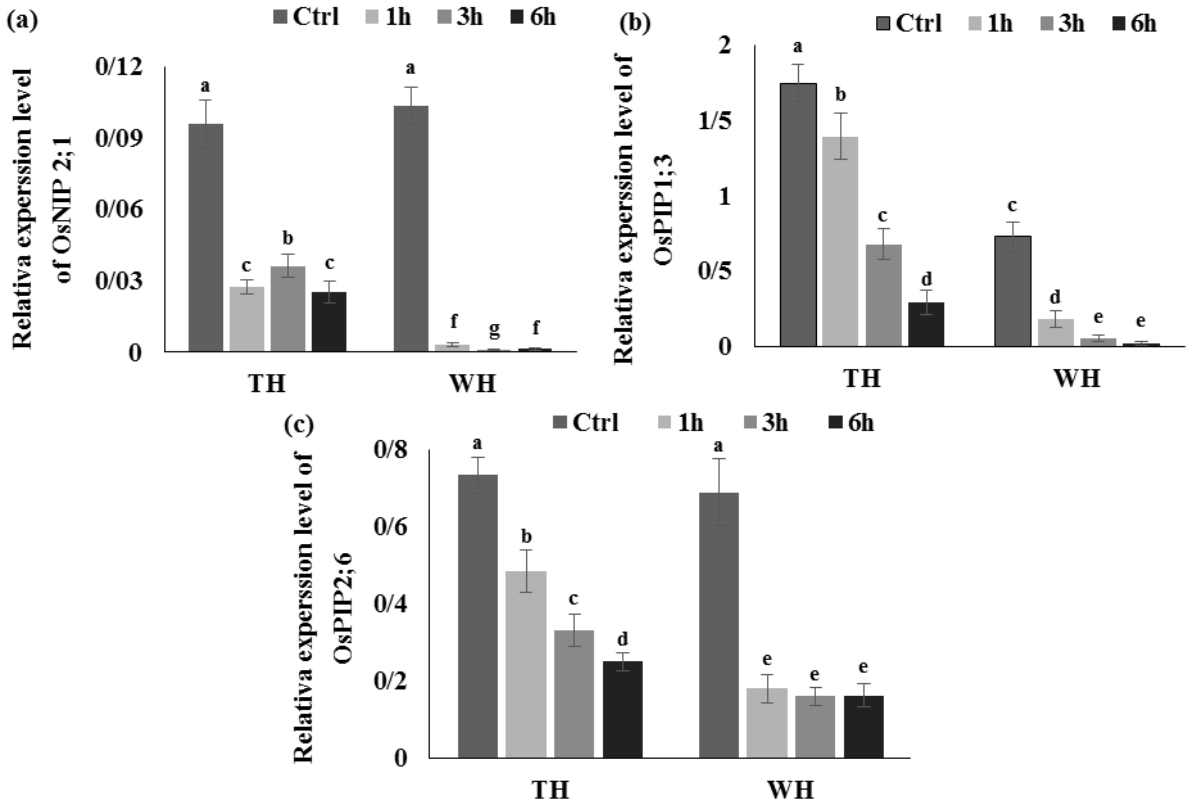

Fig. 4. Expression levels of OsNIP2;1 (a), OsPIP1;3 (b), and OsPIP 2.6 genes in roots of rice cultivars. Data are presented as mean ± SD, n=3. Bars with different letters in each cultivar show significant different at P≤0.05 according to LSD test. TH and WH, the cultivar adapted to temperate – humid and warm-humid climates, respectively.

expression level of OsPIP1; 3 was about 2-fold higher in TH compared to the WH (Fig. 4). One hour of arsenite treatment resulted in reduced expression level of OsNIP2;1 to 26% and 1.4% of the corresponding control levels in TH and WH, respectively (Fig. 4a). At the 6 h of the treatment the expression of OsPIP1;3 was only 4% and 2.5% of their corresponding control levels in TH and WH, respectively (Fig. 4b). During the As^{III} treatment, transcripts of OsPIP2;6 gradually decreased in TH roots to 34% of the control, whereas in WH they decreased to 24% of the control at 1 h and did not significantly change for the rest of the treatment period (Fig. 4c).

Similar to many other membrane proteins, aquaporin genes are temporally and spatially expressed in a specific pattern not only during ontogeny but also under environmental challenges (Yaneff *et al*., 2015). Among the examined aquaporines, OsNIP2;1 showed the highest rate of down regulation. Although we did not examine the expression pattern of other NIPs, downregulation of OsNIP2;1 which was more prominent in WH, perhaps at least in part, explains the lower As^{III} accumulation in this cultivar, compared to TH.

Plant aquaporins function bi-directionally and therefore are involved in not only water uptake but also water lose (Mosa *et al*., 2012). The results showed that during exposure to As^{III}, the TH cultivar lost more water than WH and was accompanied by lower rate of reduction in water (51% vs 34%). It has been shown that PIP1–PIP2 interaction and the formation of heterotetramers represent interesting post-translational regulatory mechanism, mainly increase of water conductance (Yaneef *et al*., 2015; Vajpai *et al*., 2018). In comparison with TH, higher reduction of both OsPIP2;6 and OsPIP1;3 transcripts in WH may have function in less water loose under As stress conditions.

Conclusion

On the whole, the results presented here show that orchestrated functions of minimizing As^{III} uptake via downregulated expression of transporter genes in addition to the enhanced redox enzymatic activities, and improved conjugating metabolites are employed by the WH cultivar to mitigate As^{III} toxicity for the plant. The long-term adaptation of WH cultivar to hot climate has driven genomic changes resulting in As^{III} sequestration and other protective strategies against As^{III} toxicity. The high ability of WH to sequester As^{III} provides the plant with reduced As^{III} translocation to grains, thereby decreases the risk associated with rice consumption as a staple food. Such cultivars therefore, can be suggested not only as a good candidate for further molecular investigations on As^{III} transport system, but also for sustainable development of agricultural programs in As-contaminated soils.

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