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Differential response of oxidative stress and thiol metabolism in contrasting rice genotypes for arsenic tolerance

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ABSTRACT

The mechanism of arsenic (As) tolerance was investigated on two contrasting rice (*Oryza sativa* L.) genotypes, selected for As tolerance and accumulation. One tolerant (Triguna) and one sensitive (IET-4786) variety were exposed to various arsenate (0–50 μM) levels for 7d for biochemical analyses. Arsenic induced oxidative stress was more pronounced in IET-4786 than Triguna especially in terms of reactive oxygen species, lipid peroxidation, EC and pro-oxidant enzymes (NADPH oxidase and ascorbate oxidase). However, Triguna tolerated As stress through the enhanced enzymes activities particularly pertaining to thiol metabolism such as serine acetyl transferase (SAT), cysteine synthase (CS), γ-glutamyl cysteine synthase (γ-ECS), γ-glutamyl transpeptidase (γ-GT), and glutathione-*S*-transferase (GST) as well as arsenate reductase (AR). Besides maintaining the ratio of redox couples GSH/GSSG and ASC/DHA, the level of phytochelatins (PCs) and phytochelatin synthase (PCS) activity were more pronounced in Triguna, in which harmonized responses of thiol metabolism was responsible for As tolerance in contrast to IET-4786 showing its susceptible nature towards As exposure.

1. Introduction

Arsenic is a food chain contaminant and class *I* carcinogen (Zhao et al., 2010). Currently, a considerable concern exists globally about arsenic (As) in potable water extracted from contaminated aquifers (Smedley and Kinniburgh, 2002). The sources of As include both natural (through dissolution of As compounds adsorbed onto pyrite ores into the water by geochemical factors) and anthropogenic (e.g., through use of insecticides, herbicides, and phosphate fertilizers, and from the semi-conductor industry) processes (Mondal et al., 2006). Arsenic contamination is prevailing in China, Russia and Vietnam and the situation is at its worst in the Bengal Delta (Bangladesh & West Bengal, India), where over 80 million people living in zones, having groundwater As level above 50 $\mu g\,L^{-1}$ (Smedley and Kinniburgh, 2002). Continuous irrigation of paddy fields with As contaminated groundwater has led to its build-up in paddy soil, with subsequent elevation of As in rice grain (Meharg and Rahman, 2003) and further its consumption constitutes a health risk for ~50% world population which depends on rice as a staple food (Norton et al., 2009).

Arsenic predominantly exists as inorganic arsenite (As^{III}) and arsenate (As^{V}) in nature. Arsenate is taken up by plants through high-affinity phosphate transporters (Meharg and Hartley-Whitaker, 2002; Tripathi et al., 2007) and disrupts energy flows in cells via competing with phosphate uptake. Being a redox active metalloid, As exposure also induces the generation of reactive oxygen species (ROS) through its intraconversion from one form to other and causing lipid peroxidation and associated toxicity (Srivastava et al., 2007; Mishra et al., 2008). However, an unrestrained increase in ROS level upon exposure to any abiotic stress including As may lead to oxidative stress (Srivastava et al., 2007). Therefore, the production and dismutation of ROS need to be harmonically balanced to allow plants to survive and grow both under normal and stressed conditions. To keep ROS production under control, plants are equipped with various enzymes and antioxidant compounds such as ascorbate (ASC) and glutathione (GSH) which are required for proper maintenance of redox state (Foyer and Noctor, 2009). NADPH plays important part in maintaining both ASC and GSH in a highly reduced state as an ultimate reductant. Such ROS-induced changes in ASC and/or GSH status are often taken as indicative of "oxidative stress". GSH is also required as a substrate for the synthesis of metalloid chelating ligands, the phytochelatins (PCs) (Grill et al., 2006). GSH is also used as reductant for enzymatic or nonenzymatic reduction of As^{V} to As^{III} (Bleeker et al., 2006), and further As^{III} is known to be complexed by both GSH and PCs (Raab et al., 2004).

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To combat As stress, plants modulate a number of pathways that operate not only to keep the cellular concentration of free metalloid ion to a minimum level (primary detoxification, e.g., thiol mediated complexation Bleeker et al., 2006); but also to prevent/repair any damage caused due to the presence of free ions at any point of time (secondary detoxification, e.g., antioxidant-mediated ROS quenching; Srivastava et al., 2007; Rai et al., 2011). A detailed investigation of PC-based As detoxification with respect to its effect on whole thiol metabolism might improve our understanding about the involvement of PCs in As detoxification. The increased rate of PC biosynthesis under As stress might induce sulphate uptake and reduction pathways (Rausch and Wachter, 2005) to fulfill the increased demand for cysteine and GSH. Cysteine is synthesized in the final step of the sulphate reduction pathway by the enzyme cysteine synthase (CS). The amount of GSH in a given organ is the result of the combined action of biosynthesis, consumption and degradation. GSH is synthesized in two ATP-dependent steps catalyzed by γ-glutamyl cysteine synthetase, a rate-limiting enzyme, and glutathione synthetase (GS) (Noctor and Foyer, 1998). It is consumed in a number of redox reactions to combat oxidative stress resulting into its oxidation to oxidized glutathione (GSSG), which is recycled into its reduced form by glutathione reductase (GR) (Mishra et al., 2008). In addition, GSH protects the plants against a range of toxicants by conjugating them or their metabolites through glutathione-*S*-transferases (GSTs) (Moons, 2003). Degradation of GSH, an important step in its metabolism, is supposed to be initiated by the enzyme called γ–glutamyl transpeptidases (γ-GTs), which is localized outside the cell membrane (Martin and Slovin, 2000) and in vacuoles (Nakano and Sekiya, 2005). This enzyme catalyzes the hydrolysis of uniquely linked N-terminal Glu from GSH, GSSG, GS-conjugates and probably PCs (Połeć-Pawlak et al., 2005).

Though, some authors demonstrated that As exposure interrupts morphological, physiological, and biochemical processes in genotypic manner in rice (Ahsan et al., 2008; Rai et al., 2011) and also affects growth, antioxidant responses and mineral nutrients (Dwivedi et al., 2010). However, no study has been performed till date to elucidate the response of thiol metabolic pathway including PC biosynthesis in rice in order to determine whether or not it has a role in the perception of stress induced during As exposure.

In order to obtain an insight into these mechanisms, two contrasting rice genotypes, Triguna (tolerant rice) and IET-4786 (sensitive rice) were selected on the basis of contrasting performance of growth, As accumulation, differential antioxidants and transcriptomic responses during As exposure (Dwivedi et al., 2010; Rai et al., 2011). It was hypothesized that tolerant variety must utilize the sulfur-containing metabolites for As detoxification via its overproduction to initiate the series of responses. To test this hypothesis, the present study was conducted to analyze the responses of contrasting rice genotypes in terms of oxidative stress, antioxidants, PCs and thiols biosynthesis related enzymes during As^V exposure.

2. Material and methods

2.1. Plant material and experimental conditions

Rice seeds of two contrasting cultivars (Triguna and IET-4786) obtained from Rice Research Station, Chinsurah, West Bengal, were grown in hydroponics. Seeds were disinfected in 0.1% $HgCl_2$ solution for 30 s, followed by thorough washing with deionized water and soaking in milli-Q for 24 h. These seeds were then transferred to Petri-dishes (3–4 day) kept in culture room at 26 °C in dark for proper germination. The plants were grown in hydroponic medium (Liu et al., 2004) for 10 day before treatment and then exposed to As^V (Na_2HAsO_4; 0, 10, 20, 50 μM) for 7 day. All the experiments conducted twice with three replicates for each treatment. After that plants were harvested, washed with milli-Q, blotted and used for the study of various parameters. Root and shoot length were measured by metric scale.

2.2. Estimation of superoxide radical, Hydrogen peroxide, lipid peroxidation, ion leakage and nitric oxide

The rate of superoxide radical (O_2^-) production was measured following the method of Chaitanya and Naithani (1994) by its capacity to reduce nitro blue tetrazolium (NBT) and is expressed as $\Delta A540\ min^{-1}\ mg^{-1}$ protein. For estimation of hydrogen peroxide (H_2O_2) levels, plants were homogenized in ice cold 50 mM potassium phosphate buffer (pH 7.0) containing 0.1% Triton X-100 and 1% PVP (w/v) (Milosevic and Slusarenko, 1996) and the level of H_2O_2 was measured according to Pick (1986). The assay was based on horseradish peroxidase-dependent oxidation of phenol red by H_2O_2 leading to the formation of a compound at alkaline pH, which exhibited significant absorbance at 600 nm. The amount of H_2O_2 was calculated using the ε of $19.8 \times 10^3\ M^{-1}\ cm^{-1}$ and is presented as mmol $H_2O_2\ g^{-1}$ fw. Lipid peroxidation was determined by estimation of the malondialdehyde (MDA) content following the method of Heath and Packer (1968) with slight modification as given earlier (Mishra et al., 2006). The amount of MDA was calculated by difference in absorbance at 532 and 600 nm using the ε of $155\ mM^{-1}\ cm^{-1}$. The ion leakage was measured in terms of electrical conductivity (EC) according to Devi and Prasad (1998). Metalloid exposed plants were washed with deionized water (Millipore, USA) and 500 mg of plant material was then transferred to 100 ml of deionised water for 24 h to facilitate maximum ion leakage and the EC of the water was recorded. The level of NO was measured by the determination of nitrite (NO_2^-) concentration using Griess reagent. Samples (0.2) were incubated with 1.8 ml of 100 mM potassium phosphate buffer (pH 7.0) and 0.2 ml of Griess reagent (1% sulfanilamide and 0.1% *N*-1-naphthylethylene-diamine dihydrochloride in 5% phosphoric acid) at room temperature for 10 min. Absorbance of the reaction mixture was recorded at 540 nm and the concentration of NO was determined using a standard curve prepared using the known concentrations of sodium nitrite (Green et al., 1982).

2.3. Assay of oxidases (NADPH oxidase and ascorbate oxidase)

For the assay of NADPH oxidase, control and metalloid–exposed plants were homogenized in 20 mM HEPES (pH 7.0) containing 1 mM EDTA, 1 mM EGTA, 1 mM phenylmethyl sulfonyl fluoride (PMSF), 2.5% polyvinylpyrrolidone (PVP) and 5% glycerol under chilled conditions. Homogenate was squeezed through four layers of cold cheese cloth and centrifuged at 12,000 × *g* for 15 min at 4 °C. Protein content of the supernatant was measured following Lowry et al. (1951). NADPH-dependent O_2^- generation by the enzyme NADPH oxidase (NOX; EC 1.6.3.1) was measured using NBT as an electron acceptor (Bielski et al., 1980), whose reduction was monitored at 530 nm. Monoformazan concentrations (and therefore O_2^- concentrations) were calculated using an extinction coefficient of $12.8\ mM^{-1}\ cm^{-1}$. The reaction mixture consisted of Tris buffer (50 mM Tris–HCl, pH 7.4), 5 mM NBT, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 5 mM NADPH and a suitable aliquot of enzyme extract. The selective reduction of NBT by O_2^- was calculated from the difference in the NBT reduction rate in the presence and absence of SOD (50–100 units ml^{-1}; Sigma). No NBT reduction with NADPH was observed in the absence of protein fractions.

For the assay of ascorbate oxidase plant tissue (500 mg) was homogenized in 1 ml of 50 mM phosphate buffer (pH 6.8) in a chilled mortar and pestle at 4 °C. The homogenate were centrifuged at 17,600 × *g* for 20 min, and the supernatants were used to assay AAO activity as described by Esaka et al. (1988). AAO activity was assayed by a following the decrease in absorbance at wavelength 265 nm of the reaction mixture containing 1 cm^3 of 100 μM ascorbic acid, 1.8 cm^3 of 50 mM sodium phosphate buffer (pH 6.8) and 0.2 cm^3 of enzyme extract. One unit of enzyme activity was defined as a decrease of 0.01 A_{265} per minute.

2.4. Estimation of thiol compounds

The cysteine content in plant material was estimated by using acid–ninhydrin reagent (Gaitonde, 1967). The level of GSH and GSSG was measured by following the protocol of Hissin and Hilf, 1976. Plant material (500 mg) was frozen in liquid nitrogen homogenized in 0.1 M sodium phosphate buffer (pH 8.0) containing 25% meta-phosphoric acid. The homogenate as centrifuged at 20,000 × *g* for 20 min at 4 °C and total glutathione (GSSG and GSH) content was determined fluorometrically in the supernatant after 15 min incubation with *o*-phthaldialdehyde (OPT). Fluorescence intensity was recorded at 420 nm after excitation at 350 nm on a Hitachi F 7000 fluorescence spectrophotometer.

2.5. Assays of enzymes of thiolic metabolism and antioxidant system

Control and As exposed plants were homogenized in buffers specific for each enzyme under chilled conditions. Homogenate was squeezed through four layers of cheese cloth and centrifuged at 12,000 × *g* for 15 min at 4 °C. Protein content of the supernatant was measured following the method of Lowry et al. (1951).