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Application of glycine reduces arsenic accumulation and toxicity in *Oryza sativa* L. by reducing the expression of silicon transporter genes

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ABSTRACT

The present study was intended to investigate the role of amino acid glycine in detoxification of As in *Oryza sativa* L. The growth parameters such as, shoot length and fresh weight were decreased during As(III) and As(V) toxicity. However, the application of glycine recovered the growth parameters against As stress. The application of glycine reduced the As accumulation in all the treatments, and it was more effective against As(III) treatment and reduced the accumulation by 68% in root and 71% in shoot. Similarly, the translocation of As from root to shoot, was higher against As(III) and As(V) treatments, whereas, reduced upon glycine application. The translocation of Fe and Na was also affected by As, which was lower under As(III) and As(V) treatments. However, the application of glycine significantly enhanced the translocation of Fe and Na in the shoot. Besides, the expression of lower silicon transporters i.e. Lsi-1 and Lsi-2 was observed to be significantly suppressed in the root with the application of glycine against As treatment. Similarly, the expression of three GRX and two GST gene isoforms were found to be significantly increased with glycine application. Simultaneously, the activities of antioxidant enzymes i.e. L-arginine dependent NOS, SOD, NTR and GRX were found to be significantly enhanced in the presence of glycine. Increased activities of antioxidant enzymes coincided with the decreased level of TBARS and H_2O_2 in rice seedlings. Overall, the results suggested that the application of glycine reduces As accumulation through suppressing the gene expression of lower silicon transporters and ameliorates As toxicity by enhancing antioxidants defense mechanism in rice seedlings.

1. Introduction

Arsenic (As) is a metal(loid), poses a significant threat to plants, particularly in rice, causing health hazards to rice patrons (Williams et al., 2009; Zhao et al., 2010). Arsenic naturally exists in two abundant inorganic forms i.e., arsenite [As(III)] and arsenate [As(V)]. Arsenite is predominant under anoxic paddy fields, easily uptaken by plants, whereas, As(V) in aerobic conditions. As(III) is 2–10 times more toxic than As(V), causing morphological and physiological disorders in plants (Tripathi et al., 2007; Ahsan et al., 2008; Tchounwou et al., 2012). The As related problem is very acute in South East Asian countries and these areas produces 90% of world rice. Rice is a major staple food to half of the world population. It accumulates As more efficiently than other cereals crops (Williams et al., 2007) as it grows under flooded conditions that lead to As(III) uptake through nodulin-26 like intrinsic protein (NIP) aquaporin channel (Lsi-1 and Lsi-2) (Ma et al., 2008). However, As(V) enters into the cell through specific phosphate transporters (Zhao et al., 2010). In most of the As-resistant organisms, As(V) is reduced to As(III) and expelled outside the cell or sequestered into the vacuoles facilitated by glutathione–S-transferase (GST) (Dubey et al., 2016). To detoxify the toxicity induced by the As, plants increases its antioxidant enzymes and non-enzymatic products like ascorbate-glutathione pools (Alscher et al., 2002; Azevedo et al., 2002). Plants under As stress also synthesise amino acids, thiol-containing proteins and other metabolites to counter against the stress (Cobbett, 2001; Hassinen et al., 2011).

Amino acids (AAs) such as glycine, proline, cysteine and histidine plays a crucial role in providing tolerance to the plants under stress conditions. The level of these amino acids increases under heavy metal exposure in plants (Davies et al., 1987; Rai, 2002). Some stress responsive amino acids act as an osmoregulator and phytochelator against various stresses (Tripathi et al., 2013). Dave et al. (2013) have demonstrated that glutamic acid (Glu), Gly and cysteine (Cys) are involved in As detoxification by the formation of glutathione and

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phytochelatins (PC). Dwivedi et al. (2010b) demonstrated that higher exposure of As to the rice plants decreases the levels of some AAs. However, the few AAs play a crucial role in amelioration of heavy metal toxicity. Glycine is a hydrophobic amino acid, which has no alkyl group. It is involved in the biosynthesis of glutathione whose, concentration increases during metal stress. To detoxify the toxicity generated by As, plants increases its antioxidant enzymes like superoxide dismutase (SOD), catalase (CAT), glutaredoxin (GRX) and non-enzymatic products like hydrogen peroxide (H_2O_2), thiobarbituric acid reactive substance (TBARS) and glutathione (GSH) pools (Alscher et al., 2002; Azevedo et al., 2002). Plants under As stress synthesise AA, thiol-containing proteins and other metabolites for defending the stress (Cobbett, 2001; Hassinen et al., 2011). As an abundant source of nitrogen, glycine acts as a direct precursor of ammonium after urea, which regulates the nitrogen cycle of plants (Schiller et al., 1998). The accumulation of glycine also influences the level of other AAs e.g. serine and glutamate. Waditee et al. (2005) reported that the application of glycine in *Arabidopsis* also enhances the levels of betaine, which reduces the toxic effects of different stresses.

In lights of these findings, the present study was aimed to investigate the effects of glycine on the As accumulation, modulation of As(III) specific transporters and responses of antioxidant defense mechanism against As stress.

2. Materials and methods

2.1. Growth conditions and experimental design

The experimental design consists of different treatments of As(III) and As(V) along with glycine lower (L) and glycine higher (H) and their respective controls. The experimental control is wholly untreated plants and designated as “C” while the treatments of glycine are designated as Gly(L) and Gly(H), reported as glycine controls. Rice cultivar, Usar-3, was obtained from Narendra Dev Agriculture University, Faizabad, Uttar Pradesh. The seeds were germinated and grown as explained by Dubey et al. (2016). Twenty five uniform (10 cm) seedlings were placed in 150 ml beaker, containing 100 ml of 100% Hewitt nutrient solution (HNS), prepared with Milli-Q water (pH 6.8–7.0) (Hewitt, 1966). The composition ($\mu g\ ml^{-1}$) of Hewitt nutrient solution included N (168), P (41), K (156), Mg (36), Ca (160), S (48), Fe (2.8), Mn (0.55), B (0.54), Cu (0.064), Zn (0.065) and Mo (0.048). All the treatments contained four biological replicates. After 7d of growth in the nutrient solution, different treatments were provided as As(III) ($4\ \mu g\ ml^{-1}$) and As(V) ($4\ \mu g\ ml^{-1}$), using salts of $NaAsO_2$, $Na_2HAsO_4{\cdot}7H_2O$ (Sigma-Aldrich, USA) respectively.

For convenience, the treatments were abbreviated as “C” for wholly untreated plants, As(III) (4) for arsenite $4\ \mu g\ ml^{-1}$ (~53.3 μM), As(III) (4) + Gly(L)” for arsenite $4\ \mu g\ ml^{-1}$ and 3 mM Gly, As(III)(4) + Gly(H) for arsenite $4\ \mu g\ ml^{-1}$ and 4 mM Gly, As(V)(4) for arsenate $4\ \mu g\ ml^{-1}$ (~53.3 μM), As(V)(4) + Gly(L) for arsenate $4\ \mu g\ ml^{-1}$ and Gly 3 mM, As (V)(4) + Gly(H) for arsenate $4\ \mu g\ ml^{-1}$ and Gly 4 mM.

2.2. Determination of antioxidants and stress markers

TBARS content ($mmol\ g^{-1}$ fw) was measured as mentioned in Heath and Packer (1968) using $\varepsilon = 0.155\ M\ g^{-1}$ fw given for MDA-TBA adduct. The H_2O_2 content ($nmol\ g^{-1}$ fw) was estimated according to the method described by Velikova et al. (2000). For estimation of antioxidant enzyme activities, fresh samples of leaves (~300 mg each) were used, following the procedure described by Dubey et al. (2016). Superoxide dismutase (SOD) (EC 1.15.1.1) activity was measured spectrophotometrically at 560 nm following Beauchamp and Fridovich (1971) and presented as $U\ mg^{-1}$ protein, where 1U of SOD activity is the amount of protein required to inhibit 50% of initial reduction of nitro-blue tetrazolium (NBT). NOS activity was performed according to the method of Corpas et al. (2006). Glutathione reductase (GR) (EC 1.6.4.2) activity was assayed following Smith et al. (1988) and represented as $U\ mg^{-1}$ protein, where 1U is the conversion of 1 mM of oxidized glutathione (GSSG) min^{-1} to reduced glutathione (GSH).

2.3. Elemental analysis

The metal(loid) contents (As) were determined, following the method of Kumar et al. (2013). Plant tissues (leaf ~500 mg and root ~300 mg) were digested on a hot plate using HNO_3:$HClO_4$ (3:1). Elements [Cu, Fe, Zn and Mn ($\mu g\ ml^{-1}$)] were analyzed using AAS (GBC Avanta Σ), whereas, for As ($\mu g\ l^{-1}$), AAS was fitted with a hydrate generator (MDS 2000) using NaH_2BO_4 + NaOH (3 M) and HCl (3 M). The values are presented in $\mu g\ g^{-1}$ dw (dry weight) and the translocation factor (TF) is the ratio of the elements in shoot divided by root.

2.4. Gene expression analysis using qRT-PCR

Total RNA (ribonucleic acid) from the shoot after 7 days of treatment, was extracted using the Spectrum Plant Total RNA Kit (Sigma-Aldrich, USA), followed by treatment with RNAase-free DNase I (deoxiribonuclease I) (Sigma-Aldrich, USA). Quantitative real-time PCR (qRT-PCR) was carried out using 2 μl of the cDNA (complementary DNA) corresponding to the set of selected genes in a reaction containing 2 × PCR Master Mix (Thermo Scientific, USA). Lower silicon transporters, namely Lsi-1 and Lsi-2, two CC (cystein-cystein) type GRX (*Os01g27140* and *Os01g13950*), one CPYC (cystein, proline, tyrosine and cystein) type (*Os02g40500*) and one GRL (glutaredoxin like) type GRX (*Os01g61350*) genes were taken for the study. The expression of two glutathione-S-transferase (GST) genes, namely (*Os09g20220* and *Os02g38160*) was also studied using specific set of primers. Three technical replicates of each biological replicate were taken for the qRT-PCR analysis. The primers for rice ubiquitin gene were used as an internal control to ensure that equal amounts of cDNA were used in all the reactions. The PCR reaction was carried out using the following cycle conditions: an initial denaturation at 94 °C for 2 min, 40 PCR cycles were performed at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s, followed by a final 5-min extension at 72 °C. After obtaining the “ct” value for each reaction, the relative expression was calculated by 2^-delta Ct method. The list of selected genes and oligonucleotide primers (Eurofins, India) used for each gene are listed in the additional file (S. Table 1).

2.5. Statistical analysis

All the values are average of four replicates. The data were subjected to Duncan's Multiple Range Test (DMRT) for the analysis of significant difference between the means ($p < 0.05$). All the values are represented as percentage increase or percentage decrease with respect to the respective values in wholly untreated control seedlings, or otherwise mentioned.

3. Results

3.1. Glycine affected the morphology and accumulation of elements

Both the treatments of As i.e. As(III) and As(V) reduced the growth parameters of rice seedlings, where As(III) was found to be more toxic. However, with the application of glycine, shoot length and fresh weight were recovered (Table 1). The maximum recovery in shoot length (11%) and fresh weight (23%) was observed with the application of Gly (H) to the As(V) treated rice seedlings, as compared to their respective control. However, Gly(L) applied to As(III) recovered the root length (21%), shoot length (5%) and fresh weight (17%), as compared to the As(III) treatment alone.

Accumulation of the As in root was higher than shoot against both the As species (Table 2). The application of glycine reduced the As