Arsenic stress affects the expression profile of genes of 14-3-3 proteins in the shoot of mycorrhiza colonized rice

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Abstract The intimate association between the arbuscular mycorrhizal fungi and host plants helps the latter in phosphate acquisition in exchange of carbohydrates and in enhanced stress tolerance. Similarly, the ubiquitous 14-3-3 protein family is known to be a major regulator of plant metabolism and stress responses. However, the involvement of mycorrhiza and plant 14-3-3 proteins interaction in plant response to environmental stimuli, such as arsenic (As) stress, is yet unknown. In this study, we analysed the impact of the As stress on the expression profile of 14-3-3 genes in the shoot of mycorrhiza colonized rice (*Oryza sativa*) plants. Ten day old rice seedlings were kept for 45 days for mycorrhizal colonisation (10 g inoculum per 120 g soilrite) and were then subjected to 12.5 μ M arsenate [As(V)] exposure for 1 and 3 days, in hydroponics. Arsenate stress resulted in significant change in expression of 14-3-3 protein genes in non-colonized and mycorrhiza colonized rice plants which indicated As mediated effects on 14-3-3 proteins as well as interactive impact of mycorrhiza colonization. Indeed, mycorrhiza colonization itself induced up-regulation of all 14-3-3 genes in the absence of

As stress. The results thus indicate that 14-3-3 proteins might be involved in As stress signalling and the mycorrhiza induced As stress response of the rice plants.

Keywords 14-3-3 proteins · Arsenic · Mycorrhiza · Rice · Stress

Introduction

The 14-3-3 proteins were first identified in the 1960's. These are phosphoserine-binding proteins that regulate activities of a wide array of targets through direct protein–protein interactions (Xu et al. 2012a). Several lines of evidence suggest that in higher plants 14-3-3 proteins bind to a wide range of transcription factors and signalling molecules and play vital roles in plant growth regulation, and signalling and responses to different biotic and abiotic stresses (Chen et al. 2006; Xu and Shi 2006; Xu et al. 2012a; Liu et al. 2016). The over-expression of the *Arabidopsis* 14-3-3 gene, *AtGF14 λ* , in cotton resulted in improved water-stress tolerance (Yan et al. 2004). In contrast, over-expression of *Glycine soja* 14-3-3 gene, *GsGF14o*, in *Arabidopsis thaliana* resulted in decreased drought tolerance during seed germination and seedling growth (Sun et al. 2014). Fungal infection and salt treatment were found to result in up-regulation of *ZmGF14-6* in maize plants, whereas drought stress caused down-regulation of this gene. When rice plants with constitutive expression of *ZmGF14-6* were generated, they showed increased drought tolerance (Campo et al. 2012).

Arsenic is a ubiquitously present toxic metalloid and a potential human carcinogen (Sharma 2012; Pathare et al. 2013; Srivastava et al. 2014). Plants exposed to As stress show altered morphological, physiological, and

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biochemical processes, such as decreased germination, inhibition of shoot and root growth, generation of reactive oxygen species and lipid peroxidation (Abedin and Meharg 2002; Somenahally et al. 2011; Huang et al. 2012). In the Indian subcontinent, rice is an important food crop and an accumulator of As in its grains (Nath et al. 2014) making it a major source of As exposure to humans (Sohn 2015). In rice, 14-3-3 proteins are encoded by a multigene family. With the help of Real-Time RT-PCR, the expression profile of rice 14-3-3 protein family was examined in response to different defense signalling compounds and biotic and abiotic stress stimuli, and results suggested that at least four 14-3-3 genes (*OsGF14b*, *OsGF14c*, *OsGF14e* and *OsGF14f*) were differentially induced by various stress stimuli (Chen et al. 2006). Furthermore, *OsGF14e* has been implicated to have a role in disease resistance through negative effects on the induction of plant defense response genes and cell death (Manosalva et al. 2011). Although the gene expression of 14-3-3 proteins under various environmental stress has been investigated (Chen et al. 2006; Xu and Shi 2006; Manosalva et al. 2011; Campo et al. 2012; Xu et al. 2012a), expression profiling of the rice 14-3-3 gene family in response to As stress has not yet been studied.

Majority of the terrestrial plants form symbiotic association with the arbuscular mycorrhizal fungi (AMF) that provide the nutrients especially phosphate to plants in exchange of carbohydrates. Several studies have reported that mycorrhiza colonized plants show improved growth under various stresses like salinity (Evelin et al. 2009; Kaya et al. 2009; Ardakani et al. 2011; Evelin and Kapoor 2014), drought (Tian et al. 2013; Li et al. 2014) and heavy metal toxicity (Schutzendubel and Polle 2002; Luo et al. 2014). In case of As, AMF inoculated *Plantago lanceolata* plants were found to have higher biomass and lower As in roots as compared to non-inoculated plants (Orlowska et al. 2012). Inoculation of AMF enhanced grain yield of upland rice without increasing grain As concentrations in As-spiked soils (Wu et al. 2014). AMF colonization has also been found to mitigate As toxicity and reduce As concentration in soybean plants (Spagnoletti et al. 2016). Further, the interaction effect of As stress and AMF colonisation on the expression of host genes has also been studied (Chen et al. 2012, 2013a). It was found that expression of As transporters, *Lsi1* and *Lsi2*, was significantly decreased while that of important phosphate transporter, *OsPT11*, increased, which lead to improved phosphate status and reduced As uptake in AMF inoculated plants as compared to non-inoculated plants. However, the effect of As and AMF interaction on the expression of gens of 14-3-3 proteins is unknown. This study aims to investigate the combined effect of AMF colonisation and As stress on the expression profile of 14-3-3 protein genes.

Our goal was to identify differentially expressed 14-3-3 proteins in AMF colonized rice under As stress.

Materials and methods

Rice seed germination, growth and AMF inoculation

Germination and inoculation of rice seeds with AMF was carried out as indicated by Chen et al. (2013a) with some minor modifications. Seeds of rice variety IR64 (*Oryza sativa* L.) were surface sterilized with 70 % ethanol for 1 min, 0.1 % mercuric chloride for 5 min, followed by three washes in sterile distilled water. Sterilized seeds were germinated on petriplates containing moist cotton. Germinated seeds were transferred to Kimura B nutrient solution, which was renewed every 3rd day, and grown hydroponically for 10 day in Plant Growth Chamber (Sanyo, Japan) having a daily cycle of a 14-h photoperiod with a light intensity of $150 \mu\text{E m}^{-2} \text{s}^{-1}$, day/night temperature of $25 \pm 2^\circ\text{C}$ and relative humidity of 65–75 %. The 10 day old rice seedlings of uniform height (10 cm) were then transferred to plastic pots (14 cm height and 7 cm diameter) containing 10 g *Glomus intraradices* (new name: *Rhizophagus irregularis*) inoculum per 120 g soilrite. Control plants were inoculated with autoclaved inoculums. During the colonisation period, plants were supplemented with Kimura B nutrient solution (containing 0.009 mM of KH_2PO_4) twice a week and irrigated with distilled water as and when needed. Plants were allowed to grow for 45 days for the colonisation of AMF.

Arsenic treatments to the non-colonized (NCo) and colonized (Co) rice plants

After seedling growth for 45 days, the non-colonized (NCo) and colonized (Co) plants were carefully removed from soilrite, washed slowly without damaging the roots and then transferred to hydroponics solution containing different arsenate [As(V)] concentrations. Ten individual plants from each, NCo and Co set were harvested for analysis of shoot length, root length, fresh weight per seedling and mycorrhizal colonisation percentage. Our hydroponic setup included glass beakers of 250 ml capacity, containing two types of solutions viz. 200 ml of As (V) solution ($12.5 \mu\text{M}$) prepared in Kimura B medium and 200 ml of Kimura B medium without As. The treatment conditions were as follows: NCo plants, Co plants, NCo plants + As(V) ($12.5 \mu\text{M}$) and Co plants + As(V) ($12.5 \mu\text{M}$). Each beaker contained 2 individual rice plants and each treatment had 6 beakers as replicates. During treatment the concentration of KH_2PO_4 was 0.09 mM, as against the 0.009 mM concentration during colonisation.

Harvesting of plant material

After As(V) treatment, plants were harvested at two time durations viz. 1 day and 3 day. At each time point, plants ($n = 6$) from 3 replicate beakers were harvested. Before harvesting, desorption of adsorbed As(V) was carried out as indicated by Pathare et al. (2013). The roots were immersed in ice-cold distilled water thrice for 20 min each, in order to remove the apoplastic As(V) before As estimation. After desorption, plants were separated into root and shoot, ground in liquid nitrogen and stored at -80°C for further analysis. One subset of the samples was oven dried for analysis of total As.

RNA isolation and real-time PCR based expression profiling of 14-3-3 genes

All the primers used for real-time PCR were from exon–intron boundary and designed using web-based Quantprime tool (Arvidsson et al. 2008). The details of the primers are given in Table 1. Specificity of all primers was confirmed by sequence analysis of RT-PCR amplicons. The protocols for RNA extraction, cDNA synthesis, real-time PCR analyses and $\log_2$ fold-calculation were same as described in our earlier work (Srivastava et al. 2014).

Arsenic quantification

Total As content was measured as described in Pathare et al. (2013). Oven dried plant material was digested with 1 ml of concentrated HNO_3 on a heating block at 108°C for 1 h followed by heating at 200°C till the samples were dry. The residue was dissolved in 10 ml of milli-Q water. Arsenic concentrations were determined on an atomic absorption spectrophotometer (GBC 906AA, Australia) coupled to a hydride generation system (HG3000). In each analytical batch, reagent blanks and spiked samples were included in the acid digestion to assess the accuracy of the chemical analysis. Analytical data quality was ensured through repeated analysis of standard reference materials.

AMF colonisation

Root colonisation was determined by using the root staining technique described by Phillips and Hayman (1970). The percent root colonisation was measured using the grid-line intersection method (Giovannetti and Mosse 1980). After staining, fine root segments of Co plants were washed with distilled water and subsequently treated with 10 % KOH for 1 h followed by repeated washing with sterile distilled water. Roots segments were then treated with 1 % HCl for 3 min and stained with 0.05 % Trypan blue. Stained root segments were then dispersed in a plate with gridlines and assessed for colonisation under a dissecting microscope.

Statistical analysis

Student's t test was performed to measure the significance level between the treatment conditions at 95 % confidence interval and is represented by * and ** for $P \leq 0.05$ and $P \leq 0.01$ respectively.

Results

Effect of mycorrhizal colonisation on the plant growth

The mycorrhizal colonization was established after 45 days of growth, which was evident from microscopic analysis of stained roots (Fig. 1a, b). The phenotypic analysis revealed a marked difference between the NCo and Co rice plants in terms of shoot length, root length and fresh weight per seedling (Fig. 1c). Effects of mycorrhizal colonisation on the shoot length, root length and fresh weight per seedling are indicated in Fig. 2. Co plants had significantly greater ($P < 0.01$) shoot and root lengths (Fig. 2a). The average shoot and root length of Co plants was about 28 and 44 % greater than the NCo plants. In addition, the Co plants

Table 1 Details of the primers used for the quantitative real-time PCR of 14-3-3 protein genes in rice

Gene name	Forward primer (5′–3′)	Backward primer (5′–3′)
<i>OsGF14a</i>	GGACTTCTGATATGCAGGACGATG	TGCTCATCCTCAGGCTTGTTG
<i>OsGF14b</i>	AGGACATTGCTTTGGCTGAG	TTACTGCCCTCGCTGGAG
<i>OsGF14c</i>	CGTTTGACGAAGCCATCTCC	CTAGTAGAACAGGAGAAGAATC
<i>OsGF14d</i>	AATGCATACAAGGCTGCTCAGGAC	TTGAGTGCAAGCCCAAGCCTTATG
<i>OsGF14e</i>	GATATTGCCCTGGCAGAGTTG	GAGATATCGGAAGTCCACAGC
<i>OsGF14g</i>	TGATATGGCTGATGATGCAGACG	CACCACACCCTTGTTGTTGTTG
<i>OsGF14h</i>	TGGCTGAATTTAGCACTGGAAGTG	TTGCCACTTGGCTAGCTCTCTC
Tubulin	TTTGTGTTGCGGCAATCTGGTG	AGTGGCATACTTGAATCCTTGC

Fig. 1 Microscopic analysis of AMF colonization after staining with Trypan Blue: mycorrhizal hyphae (a) and vesicles (b). The growth of non-colonized (NCo) and colonized (Co) rice plants (c)

Fig. 2 Effect of mycorrhizal colonization on **a** shoot and root length and **b** fresh weight per seedling. Non-colonized (NCo) and colonized (Co) rice plants are indicated by *open bars* and *black bars*

showed significantly higher ($P < 0.01$) fresh weight per seedling (about 28 %) than the NCo rice plants (Fig. 2b).

Arsenic concentration in NCo and Co plants

Total As content of the NCo and Co rice plants was measured in order to ascertain the effect of mycorrhizal colonisation on the As concentration by the host plant. The levels of As in the shoot and root tissues of NCo and Co plants are shown in Fig. 3. Arsenic concentration increased with time in both NCo and Co seedlings. Arsenic concentration was substantially higher in the roots as compared to the shoots of the NCo as well as Co rice seedlings. Levels of As in the roots of NCo plants were 40 and 89 $\mu\text{g g}^{-1}\text{dw}$ at 1 and 3 days, respectively whereas in Co plants the As levels were significantly lower ($P < 0.05$) compared to NCo i.e. 24.5 and 47 $\mu\text{g g}^{-1}\text{dw}$ at 1 and 3 days, respectively (Fig. 3a). No significant difference ($P > 0.05$) was observed between the shoot As levels in the NCo and Co plants at 1 days. As levels in the shoot were

respectively. All values are mean $\pm$ SD ($n = 6$). Student t test was performed to test the significance level. ** on the bars represents $P < 0.01$. Note that the scales of Y axis are different

1.3 and 2.4 $\mu\text{g g}^{-1}\text{dw}$ at 1 and 3 days, respectively in NCo plants, while shoots of Co plants accumulated 2.4 and 3.2 $\mu\text{g g}^{-1}\text{dw}$ of As. Interestingly, at both the time durations, the amount of As accumulated in the shoots of Co plants was higher ($P = 0.03$) compared to NCo plants.

Effect of As stress on the expression profile of 14-3-3 genes in the NCo and Co rice

By using real-time RT-PCR, we analysed the expression patterns of seven genes of rice 14-3-3 proteins. In general, As stress resulted in significant down-regulation of most of the 14-3-3 protein genes at either single or both durations in NCo-plants (Fig. 4). After 1 days of As stress, the expression of *OsGF14c*, *OsGF14e* and *OsGF14g* genes declined (1.29-, 1.22- and 1.25-fold, respectively), while *OsGF14a*, *OsGF14b*, *OsGF14d* and *OsGF14h* genes showed non-significant changes. At 3 days, *OsGF14a* showed maximum down-regulation (2.8-fold) followed by

Fig. 3 Total As concentrations in root (a) and shoot (b) at two time durations viz. 1 and 3 days in rice plants exposed to arsenate stress. As concentrations in the non-colonized (NCo) and colonized (Co) rice

plants are indicated by *open bars* and *black bars* respectively. All values are mean $\pm$ SD ($n = 3$). Student *t* test was performed to test the significance level. *Bars represents $P < 0.05$

Fig. 4 Effect of As stress on the expression profile of seven 14-3-3 protein genes in the shoot of non-colonized and colonized rice plants. Treatment conditions are represented by different types of bars. *Open bars* and *black bars* indicate Non-colonized (NCo) and Colonized

(Co) plants respectively treated with As for 1 days, whereas, *dotted bars* and *bars with checks* indicate Non-colonized (NCo) and Colonized (Co) plants respectively treated with As for 3 days

OsGF14b, *OsGF14h*, *OsGF14c* and *OsGF14d* (1.8-, 1.5-, 1.2-, and 1.1-fold, respectively).

Under As stress, the expression of the 14-3-3 proteins in the shoot of Co rice plants varied considerably compared to the NCo plants (Fig. 4). At 1 days, *OsGF14a*, *OsGF14b*, *OsGF14d*, and *OsGF14h* genes were up-regulated by 1.0-, 0.7-, 0.6-, and 0.9-fold, respectively, whereas *OsGF14c* showed a down-regulation of 0.6-fold. At 3d, As stress decreased the expression of *OsGF14a*, *OsGF14b* and *OsGF14h* by 1.5-, 0.6- and 0.7-fold, respectively. In contrast, *OsGF14g* was up-regulated by 0.8-fold. Thus, time-dependent change in expression pattern of 14-3-3 genes was observed, e.g. *OsGF14a*, *OsGF14b*, *OsGF14d* and

OsGF14h which were up-regulated at 1 days showed down-regulation after 3 days of As stress in Co plants. Further, As stress caused differential response of 14-3-3 genes in NCo and Co plants either in terms of fold-level or opposite response (Fig. 4).

Effect of mycorrhizal colonisation on the expression profile of 14-3-3 genes in rice plants

Analysis of 14-3-3 gene expression levels in the colonized plants not subjected to As stress, reveals that mycorrhizal colonisation had resulted in the up-regulation of all the seven rice 14-3-3 genes at both the time durations (Fig. 5).

Fig. 5 Effect of mycorrhizal colonization on the expression profile of seven 14-3-3 protein genes in the shoot of colonized rice plants. These were the colonized plants that were not subjected to As stress. 1 and 3 days indicate the time periods after their transfer to hydroponic solution

OsGF14a gene showed maximum up-regulation (2.3-fold) at 1 days followed by *OsGF14d*, *OsGF14g*, *OsGF14h*, *OsGF14c* and *OsGF14e* (1.8-, 1.4-, 1.2-, 1.1- and 1.0-fold, respectively). At 3 days, *OsGF14e* gene showed maximum up-regulation of 2.9-fold while *OsGF14c* showed the least up-regulation of 0.7-fold. The expression levels of *OsGF14a*, *OsGF14b*, *OsGF14c* and *OsGF14d* decreased with time from 1 to 3 days in contrast to *OsGF14e*, *OsGF14g* and *OsGF14h* whose up-regulation increased at 3 days compared to 1 days.

Discussion

14-3-3 proteins, a family of ubiquitous regulators, are known to play important roles in several biological processes in plants such as metabolic regulation, plant-pathogen interactions, abiotic stress tolerance, adaptation to nutrient deficiency, signal transduction etc. (Cooper et al. 2003; Roberts 2003; Chen et al. 2006; Xu et al. 2012b). In this study, we used a real-time RT-PCR in order to investigate the effect of As stress on the expression profile of 14-3-3 protein genes in the shoot of NCo and Co rice plants. Our results indicate that 14-3-3 genes respond differentially in the NCo and Co rice plants in response to As stress.

Previous studies indicated that 14-3-3 proteins are involved in plant responses to environmental stress like salt, oxidative stress, pathogen attack, nutrient deficiency etc. Xu and Shi (2006) observed that salt stress resulted in significant up-regulation of four tomato 14-3-3 genes viz. *TFT1*, *TFT4*, *TFT7* and *TFT10*. Similarly, a differential expression of four rice 14-3-3 proteins viz. *OsGF14b*, *OsGF14c*, *OsGF14e* and *OsGF14f* was observed (Chen et al. 2006) in response to plant-pathogen interaction,

defence signalling compounds and diverse abiotic stimuli. Furthermore, the transcripts of three rice 14-3-3 protein genes (*OsGF14b*, *OsGF14c* and *OsGF14e*) were induced in response to several environmental stresses including drought (Cooper et al. 2003). These evidences suggest that 14-3-3 proteins are induced in a variety of stresses thus supporting the notion that they are important for stress response regulation.

Heavy metals, including metalloid As, severely affect plant growth through interaction with plant sulfhydryl groups, membrane disruption, disturbed ionic homeostasis and oxidative stress (Shri et al. 2009). Plant plasma membrane H^+ ATPase plays important role in maintenance of ion homeostasis and are regulated by 14-3-3 protein binding (Janicka-Russak et al. 2012). A few observations have indicated that the activity of plasma membrane ATPase is altered under heavy metal stress (Ahn et al. 2001; Janicka-Russak et al. 2008; Kabala et al. 2008; Janicka-Russak et al. 2012). Similarly, plasma membrane H^+ ATPase activity and 14-3-3 protein accumulation increased when cucumber plants were treated with Cd and Cu stress for 6 days (Janicka-Russak et al. 2012). 14-3-3 proteins play indispensable role in the activation of plasma membrane H^+ ATPases (Camoni et al. 2012; Chen et al. 2013b, 2015). In this study, with the aid of real time RT-PCR we observed that As stress resulted in differential expression patterns of 14-3-3 genes in the shoot of NCo and Co plants. As stress led to significant up-regulation of five genes viz. *OsGF14b*, *OsGF14d*, *OsGF14h* (at 1 days time point) and *OsGF14e* and *OsGF14g* (at 3 days time point) in the NCo plants (Fig. 3). Similar up-regulation of *OsGF14b*, *OsGF14d* and *OsGF14g* was observed under heavy metal stress viz. Cu and Cd (Yao et al. 2007). *OsGF14a*, *OsGF14c* and *OsGF14g* were up-regulated in the Co plants in response to As, in contrast to their down-regulation in the NCo plants subjected to As stress (Fig. 3). *OsGF14c* is shown to be associated with the ATPase-synthase complexes in the thylakoid, plasma, and vacuolar membranes and to be involved in plant stress responses (Cooper et al. 2003). An up-regulation of *OsGF14c* in Co plants under As stress, in our study, indicates that it might be an important component of mycorrhiza induced signalling pathway and stress tolerance mechanism under As stress. Although the exact role of 14-3-3 proteins in heavy metal response, especially As, is largely unknown. One possibility is that at least some of these genes might be involved in As stress signalling pathways via interactions with proteins like H^+ ATPases, whose role in heavy metal stress tolerance is known. H^+ ATPases create a pH and electrical gradient across plasma membrane which acts as a driving force for secondary transport of ions and nutrients into and out of the cells (Bucher 2007; Wang et al. 2014). Besides their regulation by 14-3-3 proteins (Fuglsang et al.

2011; Xu et al. 2012c), activity of plant H^+ ATPases is also regulated by mycorrhizal colonisation (Gianinazzi-Pearson et al. 2000). Hence, further research aimed at mycorrhiza mediated As stress tolerance in rice needs to concentrate on the elucidating the role of H^+ ATPase and 14-3-3 proteins. Our current study expands upon the previous findings of the rice 14-3-3 family (Zhang et al. 2001; Cooper et al. 2003; Liu et al. 2004; Chen et al. 2006) and would serve as a starting point to explore their functions in mycorrhiza mediated As stress response mechanisms.

Conclusions

Our study provides a preliminary information about the effect of As(V) stress on the expression of shoot 14-3-3 protein genes in mycorrhiza colonized rice plants. The work identified As(V) responsive 14-3-3 genes (*OsGF14a*, *OsGF14b*, *OsGF14c*, *OsGF14g* and *OsGF14h*) showing the significant change in their expression in both time-dependent and mycorrhiza-colonization dependent manner. This identification of As(V) responsive 14-3-3 genes would provide a platform for future research in elucidating the roles of 14-3-3 proteins in mycorrhiza mediated As stress tolerance.

Authors' Contribution VP conducted experiments and wrote MS. SS designed the experiment and corrected MS. BS assisted in statistical analysis. PS designed the experiment and finalized the MS.

Compliance with ethical standards

Conflict of interest Authors declare that they have no conflict of interest.

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