Functional studies of *AtACR2* gene putatively involved in accumulation, reduction and/or sequestration of arsenic species in plants

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Abstract: Food-based exposure to arsenic is a human carcinogen and can severely impact human health resulting in many cancerous diseases and various neurological and vascular disorders. This project is a part of our attempts to develop new varieties of crops for avoiding arsenic contaminated foods. For this purpose, we have previously identified four key genes, and molecular functions of two of these, *AtACR2* and *AtPCS1*, have been studied based on both *in silico* and *in vivo* experiments. In the present study, a T-DNA tagged mutant, (SALK_143282C with mutation in *AtACR2* gene) of *Arabidopsis thaliana* was studied for further verification of the function of *AtACR2* gene. Semi-quantitative RT-PCR analyses revealed that this mutant exhibits a significantly reduced expression of the *AtACR2* gene. When exposed to 100 μ M of arsenate (AsV) for three weeks, the mutant plants accumulated arsenic approximately three times higher (778 μ g/g d. wt.) than that observed in the control plants (235 μ g/g d. wt.). In contrast, when the plants were exposed to 100 μ M of arsenite (AsIII), no significant difference in arsenic accumulation was observed between the control and the mutant plants (535 μ g/g d. wt. and 498 μ g/g d. wt., respectively). Also, when arsenate and arsenite was measured separately either in shoots or roots, significant differences in accumulation of these substances were observed between the mutant and the control plants. These results suggest that *AtACR2* gene is involved not only in accumulation of arsenic in plants, but also in conversion of arsenate to arsenite inside the plant cells.

Key words: *Arabidopsis thaliana*; arsenate reductase 2 gene; arsenic accumulation; arsenic speciation; IC-ICP-DRC-MS; RT-PCR.

Abbreviations: ACR2, arsenate reductase 2; As, arsenic; AsIII, arsenite; AsV, arsenate; DMA, dimethylarsinic acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Gpase, glycogen phosphorylase; Grx, glutaredoxin; GSH, glutathione; ICP-DRC-MS, inductively coupled plasma dynamic reaction cell mass spectrometry; ICV, initial calibration verification; MMA, monomethylarsonic acid; MS, Murashige-Skoog; MT, mutant type; PNPase, purine nucleoside phosphorylase; PT-Pase, protein tyrosine phosphatase; WT, wild type.

Introduction

Arsenic (As) contaminated ground water together with anthropogenic activities, such as mining, chemical industries, use of arsenic-based pesticides or irrigation of cultivated crops with As-contaminated water lead to severe contamination of human foods (Bundschuh et al. 2012; Halder et al. 2012; Neidhardt et al. 2012; Rahman et al. 2014, 2015). In the As-contaminated environment different organic and inorganic As compounds have been identified, but the inorganic arsenite, (AsIII) and arsenate (AsV) are the most common. Generally, AsV is the dominant species in the soil solution under oxidizing conditions, whereas AsIII is the predominant species under moderately reducing conditions (Wood et

al. 2002). AsV is a chemical analogue of phosphate and is readily taken up into plant roots by the high-affinity phosphate channel (Meharg et al. 2002; Ali et al. 2009). After being taken up by the plant, AsV can produce toxicity itself by substituting phosphate in phosphorylation reactions, or after being reduced to AsIII, which is more toxic than AsV due to its high affinity for protein thiols (Lutsenko & Arguello 2012).

Plant populations originating from strongly AsV-enriched soils have been shown to exhibit substantially enhanced levels of AsV tolerance (Tripathi et al. 2007). Plant arsenate reductases belong to the so-called ACR family. The model plant *Arabidopsis thaliana* has a homologue of the catalytic domain of CDC25 that has been shown to reduce arsenate (Bleeker et al. 2006).

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This enzyme, called alternatively AtACR2, AtASR and AtCDC25, has been identified and characterised as an arsenate reductase both in microorganisms and in plants (Bleeker et al. 2006; Dhankher et al. 2006; Nahar et al. 2012). Disruption of arsenate reductase gene leads to arsenate hypersensitivity in all *Escherichia coli*, *Saccharomyces cerevisiae* and *A. thaliana* (Duan et al. 2007).

The mechanisms of arsenate reduction in microorganisms have been studied and described extensively (Messens & Silver 2006; Bhattacharjee & Rosen 2007). Three types of cytosolic arsenate reductases have been identified: (i) the first type is the ArsC of *E. coli*, which uses glutaredoxin (Grx) and glutathione (GSH) as reductants; (ii) the second type of ArsC is from *Staphylococcus aureus* and *Bacillus subtilis*, which uses thioredoxin as a reductant; and (iii) the third type is the Acr2p from eukaryotic organisms, such as yeast and the parasitic protozoa *Leishmania major*, which also uses Grx and GSH as reductants (Zhou et al. 2004; Bhattacharjee & Rosen 2007). In addition, Acr2p is a member of the protein tyrosine phosphatase (PTPase) superfamily, which includes the human cell cycle dual-specificity phosphatases CDC25s (cell division cycle).

Based on the sequence homology to the yeast Acr2p, plant homologues of ACR2 have been cloned and characterised from *A. thaliana* (Dhankher et al. 2006), *Holcus lanatus* (Bleeker et al. 2006), rice (Duan et al. 2007) and the As hyperaccumulator *Pteris vittata* (Ellis et al. 2006). In *A. thaliana*, the ACR2 gene identified earlier as Arath; CDC25 has a dual specificity of tyrosine phosphatase that may have a role in cell cycle regulation (Landrieu et al. 2004). But under normal growth conditions, the ACR2 mutants of *A. thaliana* (T-DNA insertion lines and RNA interference knockdown lines) does not exhibit any abnormal phenotypes (Bleeker et al. 2006). Similarly, ACR2 genes from rice (*Oryza sativa* L.), *H. lanatus* and *P. vittata* are also CDC25-like genes (named *OsACR2*, *HLACR2*, *PvACR2*, respectively). Interestingly, Arath; CDC25 (*AtACR2*) and two isoforms of *OsACR2* exhibit phosphatase activity (Landrieu et al. 2004; Duan et al. 2007), whereas yeast Acr2p and *PvACR2* do not (Ellis et al. 2006). Expression of plant ACR2 genes (*AtACR2*, *OsACR2* and *PvACR2*) in the *E. coli* mutant lacking *ArsC* or the yeast mutant lacking *Acr2p* restored their resistance to arsenate (Dhankher et al. 2006; Ellis et al. 2006; Duan et al. 2007). Furthermore, ACR2 proteins purified from *E. coli* overexpressing *OsACR2;1*, *OSACR2;2*, *PvACR2* or *HLACR2* (also named *HLAsr*) could catalyse GSH/Grx-dependent arsenate reduction (Bleeker et al. 2006; Ellis et al. 2006; Duan et al. 2007). These studies demonstrate the ability of the plant ACR2 to reduce arsenate in heterologous systems.

Mammalian enzymes have been shown to be capable of reducing arsenate *in vitro* in the presence of an appropriate thiol, including purine nucleoside phosphorylase (PNPase), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and glycogen phosphorylase (GPase) (Gregus & Nemeti 2002, 2005, 2007). It is not known

if plant PNPase, GAPDH or equivalents of GPase can reduce arsenate in the presence of thiols. Nevertheless, it appears that arsenate reduction may be catalysed partly by non-specific enzymes.

In this report we have studied accumulation of arsenic species in an *A. thaliana* SALK mutant, in which the ACR2 gene has been partially inactivated by insertion of a T-DNA tag in the genome. Our results revealed that the ACR2 gene has significant effect on the arsenic redox status in *A. thaliana*. Our findings also suggest that accumulation of arsenic in plants is a complex process, which might be regulated by many genes (Lund et al. 2010; Bundschuh et al. 2012). Thus, inactivation of a single gene, such as ACR2, is not enough to significantly effect on accumulation of total arsenics in *A. thaliana*.

Material and methods

Preparation of plant material

In this work three Columbia ecotype variants of *A. thaliana* plants were used. These were: (i) wild-type (WT) plants; (ii) vector transformed transgenic plants (VC); and (iii) a T-DNA tagged SALK mutant (MT; SALK_143282C) harbouring a T-DNA insertion tag in the upstream region of the *AtACR2* gene. In all experiments, both WT and VC plants were used as controls.

Seeds of WT and VC plants were produced previously at the University of Skövde, Sweden, whereas the seeds of SALK mutant were kindly supplied by The Arabidopsis Stock Center in Nottingham (NASC; <http://arabidopsis.info/>). All seeds were vernalised by storing them at +4°C in dark for at least seven days. Prior to germination, all seeds were surface sterilised by immersing them in 70% ethanol for 5 min. After five washes with sterile distilled water, the seeds were sown under axenic conditions onto Petri dishes containing 20 mL of solidified Murashige-Skoog (MS) medium (Murashige & Skoog 1962). MS basal salt medium (Sigma-Aldrich; Cat. No. M5524) was supplemented with 20 g/L sucrose and 8 g/L agarose (Sigma; Cat. No. A9539-500G). The pH of the medium was adjusted to 5.8. Plants were grown in the growth chambers maintaining 16 h photoperiod with a light intensity of 350 $\mu\text{mol m}^{-2} \text{s}^{-1}$, day:night temperatures 22:19°C and 70% relative humidity. For isolation of RNA, three-week old plants were collected from the Petri dishes, excess agar was washed off with sterile water, submerged into liquid nitrogen and stored at -80°C until use.

Arsenic exposure of plants and sample collection

Seven-day old seedlings of MT, WT and VC lines germinated on MS medium were transferred under axenic conditions onto MS medium containing either arsenate (AsV; sodium arsenate dibasic heptahydrate; $\text{Na}_2\text{HAsO}_4 \cdot 7\text{H}_2\text{O}$ from Sigma-Aldrich; Cat. No. A6756) or arsenite (AsIII; sodium meta arsenite; NaAsO_2 from Sigma-Aldrich; Cat. No. 71287). Arsenic exposure was conducted for three weeks and each treatment contained 15 seedlings with eight replicates. Plants exposed to 100 μM AsV or 100 μM AsIII were used for determination of arsenic accumulation in shoots and roots. Furthermore, we have also investigated if there is any correlation between the time of exposure and the amount of accumulated arsenic in plants. To achieve this, the plants were exposed to 100 μM AsV or 100 μM AsIII

Fig. 1. Schematic diagram of the *AtACR2* gene (to scale) with exons (encoding the C-terminal catalytic domain), introns and 3'- and 5'-UTRs, as indicated. The relative positions of T-DNA insertion are shown with LB/RB (LB – left border and RB – right border). WT-Col represents the *ACR2* gene in wild-type, whereas MT-N59 represents the inactivated *ACR2* gene in the SALK mutant.

and shoot samples were collected after one, two and three weeks.

On the other hand, plants exposed to 100 μ M AsV for three weeks were also used for determination of arsenic species in roots and shoots. At the end of exposure time, the plants were pulled out of the medium, washed 4–5 times with deionized water to remove any surface contamination and separated into two groups – (a) shoots and (b) roots. Plant samples were then oven dried at 55°C for 4 days. Dried tissues were ground by using mortar and pestle and stored at +4°C until further use (for measurement of arsenic concentration).

Analysis of gene expression by RT-PCR

To verify whether the *AtACR2* (*At5g03455*) gene in the SALK mutant (SALK_143282C) was indeed knocked out or knocked down, we performed RT-PCR. Total RNA from three-week old plants was isolated by using RNeasy Plant Mini Kit (Qiagen, Valencia, CA; Cat. No. 74904) and treated with RNase free DNase (Qiagen; Cat. No. 79254). RT-PCR was performed using the Robust-TI RT-PCR Kit (Finnzymes, Espoo, Finland; Cat. No. F-580L). For each sample the total volume of reaction mixture was adjusted to 50 μ L containing 2,000 ng RNA, 1.5 mM $MgCl_2$, 1X PCR buffer, 200 mM dNTP, 10 pmol of forward and reverse primers, 5 U AMV-RT and 2 U DyNAzyme EXT DNA polymerase. PCR tubes containing the reaction mixture were then placed in the thermocycler (Flexcycler, model: Block assembly 96G). The RT-PCR cycle setups for the first strand cDNA synthesis and the second strand DNA synthesis were carried out as described by Nahar et al. (2012). The following primers were used for RT-PCR: Art-Forward 5'-AAATGGCGATGGCGAGAA-3' and Art-Reverse 5'-AATCGCCCTTGCAAGGAAC-3'. The primers were specific to the first and the third exons of *ACR2* gene. The transcription level of *A. thaliana* actin gene was analysed similarly as described by Nahar et al. (2012) and used as a control. For visualization and separation of the target cDNA fragment, 10 μ L of the amplified product were then loaded onto agarose gel for electrophoresis.

Analysis of total arsenic by using ICP-DRC-MS

From each treatment aliquots of each ground materials (approximately 0.025–0.05 g) were digested in a Hot Block Digestion Apparatus with HNO_3 and H_2O_2 as specified in EPA, Method 3050B (Elke et al. 1996). The resulting sample digests were analysed for accumulation of total arsenic

by using inductively coupled plasma dynamic reaction cell mass spectrometry (ICP-DRC-MS).

Analysis of arsenic species by IC-ICP-DRC-MS

From each treatment aliquots of ground materials (approximately 0.025–0.05 g) were transferred to polypropylene centrifuge tubes followed by addition of 50% MeOH (v/v). Extraction mixtures were then placed in an inverting shaker, shaken at 80 rpm, for at least three hours. Each sample extract was then briefly centrifuged (7,500 rpm for 1 min) and the supernatant was filtered (0.45 μ m) directly into a sealed autosampler vial. Extracts were then analysed by ion chromatography ICP-DRC-MS. Prior to sample analysis, all calibration curves were adjusted by using the second source of standards, also known as initial calibration verification (ICV) standards. The analysis of arsenic species was carried out immediately following sample collection or extraction. For each batch of samples, the analysis was completed within 12 h. Analyses were repeated to confirm whether there was any change in As species during analysis. To test the stability of As species in shoots and roots, two sets of control samples as well as matrix spikes were included in the described experiments. This was done for monitoring any potential species conversion attributable to either the applied extraction procedure or sample matrices. One set contained only arsenate, whereas the other set contained arsenite, monomethylarsonic acid (MMA), and dimethylarsinic acid (DMA).

Data analysis

Significance of treatment effects was tested by one way ANOVA employing PRISM 6.0 version. Treatment means were compared based on the least significant difference. All experiments were carried out with three repetitions and eight replications.

Results

Expression of *ACR2* gene in the SALK mutant

RT-PCR was performed to verify whether the T-DNA tag in the SALK mutant (Fig. 1) indeed resulted in inactivation or partially reduced expression of *ACR2* gene. These results are presented in Figure 2. The intensity of the amplified bands obtained in the SALK mutant was approximately three-times less than that

obtained in the WT or VC control plants (data not shown). Reduced expression of *ACR2* mRNA in the

mutant plants suggests that this gene in the SALK mutant is partially inactivated (knocked down) by T-DNA insertion. Expression of *ACR2* gene in the VC control plants was identical to that observed in WT control plants (Fig. 2). For this reason all the results obtained in the mutant plants were compared with those obtained in the WT control plants.

Accumulation of total arsenic in plants
To verify if the target gene *ACR2* is indeed involved in arsenic accumulation in *A. thaliana*, the SALK mutant and the control plants were exposed to 100 μ M of arsenate (AsV) or 100 μ M arsenite (AsIII) independently. After three weeks of exposure, accumulation of arsenic in these plants was determined by using ICP-DRC-MS as described in the Materials and methods. These results are shown in Figure 3a. When exposed

Fig. 3. Effect of *ACR2* gene on growth and accumulation of arsenic in *A. thaliana*. (a) Total arsenic accumulation was measured in shoots by ICP-DRC-MS. Blue bars represent wild-type control plants (WT), the red bars represent SALK mutant plants (MT-N59) and green bars represent vector control plants. Error bars (I) are SE ($n = 7-8$). (b) Analysis of plant growth. Seven-day old seedlings of wild-type (WT) and SALK mutant (MT) were exposed to arsenate (100 μ M) or arsenite (100 μ M) for three weeks.

Fig. 4. Arsenic speciation in shoots and roots of *A. thaliana*. Seven-day old seedlings were exposed to arsenate (100 μ M) for three weeks. Shoots and roots were collected, dried and the arsenic content was determined by ion chromatography ICP-DRC-MS (μ g/g d. wt.). (a) As species in roots and (b) in shoots of *A. thaliana*. Error bars (I) are SE ($n = 7-8$).

to 100 μ M AsV for three weeks, the amount of arsenic accumulated in the shoots of the SALK mutant was approximately three-fold higher (778 μ g/g d. wt.) than that (235 μ g/g d. wt.) accumulated in the shoots of control plants (Fig. 3a). In contrast, when the SALK mutant was exposed to 100 μ M AsIII for three weeks, the amount of arsenic accumulated in the shoots (498 μ g/g d. wt.) did not differ significantly from that (535 μ g/g d. wt.) accumulated in the shoots of WT control plants (Fig. 3a).

Furthermore, several distinct phenotypic differences between the control and the mutant plants were observed when exposed to 100 μ M AsV or AsIII. The mutant plants were affected negatively showing relatively retarded growth and a higher level of stress (the leaves of these plants were smaller in size, started to turn yellow exhibiting symptoms of early decay-ing/senescence) as compared to those observed in WT control plants (Fig. 3a).

Accumulation of arsenic species in roots and shoots

For determination of arsenic species in shoots and roots, both mutant and control plants were exposed to 100 μ M AsV for three weeks as described above. Different species of arsenic were extracted quantitatively from the air-dried plant material with an average recovery of $94.1\% \pm 5\%$. The recovery was determined as the sum of the extracted As species (measured by ion chromatography ICP-DRC-MS) relative to the total As concentrations in plant material (measured in plant digests by ICP-MS). The recovery was slightly better ($98\% \pm 5\%$) when it was expressed as the total As concentration in the plant material measured in plant digests by ICP-DRC-MS (data not shown). Most of the As species detected in roots and shoots were the inorganic arsenic species, AsV and AsIII. These results are presented in Figure 4. Figure 4a indicates that amount of AsV accumulated in roots of the mutant plants was 4.5 fold higher (1,767 μ g/g d. wt.) than that observed in roots of the control plants (389 μ g/g d. wt.). However, the amount of AsIII accumulated in roots of the

mutant plants was about 1.6 fold lower (2,188 μ g/g d. wt.) than that of observed in roots of the control plants (3,408 μ g/g d. wt.). On the other hand, in shoots of the mutant plants accumulation of both AsV (474 μ g/g d. wt.) and AsIII (398 μ g/g d. wt.) was respectively 5 fold and 3 fold higher than those observed in shoots of the control plants (AsV 99 μ g/g d. wt. and AsIII 126 μ g/g d. wt.), respectively (Fig. 4b). The amount of organic arsenic species like MMA and DMA was detectable neither in shoots nor in roots and for this reason data on these arsenic species are not presented.

Discussion

Accumulation of arsenic in plants and microorganisms has been studied previously by many researchers (Mukhopadhyay et al. 2000; Bleeker et al. 2006; Dhankher et al. 2006; Bhattacharjee et al. 2007). Unfortunately, until today there are a very few reports on accumulation of arsenic species in different parts of the plants (roots, shoots, etc.), which has important implications for how As is redistributed within the plants (Zhao et al. 2009). Previously, by employing the *in silico* and *in vivo* studies on *A. thaliana*, we have postulated that the *AtACR2* gene is involved in reduction of arsenate (AsV) to arsenite (AsIII) in the plant cells (Nahar et al. 2012). One advantage of this explanation is that arsenite (but not arsenate) can be sequestered in the vacuoles of the plant cells although it is more toxic than AsV (Cobbett 2000; Mukhopadhyay et al. 2000; Dhankher et al. 2006; Blum et al. 2007). To verify the postulated function of the *AtACR2* gene (*At5g03455*), we have included a SALK mutant of *A. thaliana* (Landrieu et al. 2004) in our investigations. As indicated in the database, this mutant harbours a T-DNA insertion tag in the upstream exon of the *ACR2* gene (Fig. 1). Because of this insertion, the *ACR2* gene should be either knocked-down (reduced activity, partial inactivation) or knocked-out (inactive). Results obtained in the RT-PCR analyses confirmed that expression of *ACR2* gene (mRNA) in the SALK

mutant was reduced partially (Fig. 2). For our studies, a mutant harbouring a completely knocked-out *ACR2* gene without exhibiting any transcriptional or translational activity could be the best option. Unfortunately, we could not find any such mutant in the Arabidopsis Stock Centre (<http://arabidopsis.info/>). Even with partially reduced activity of the *ACR2* gene, the mutant plants when exposed to 100 μ M arsenate for three weeks accumulated total arsenic approximately three-fold higher than the WT control plants (Fig. 3a). Previously, we and several other researchers have shown similar results while working with other *ACR2* mutants (Dhankar et al. 2006; Nahar et al. 2012). However, when the plants were exposed to arsenate for one week, no significant difference in arsenic accumulation could be seen between WT and MT plants. Similar results were also shown by Liu et al. (2012). One of the explanations for this observation could be the short exposure time and/or other physiological conditions for plants growth.

To understand the mechanisms of arsenate transport through phosphate channel and its metabolism in higher plants, we have analysed arsenic species in WT and MT plants of *A. thaliana*. During extraction or post extraction storage period, the arsenic species, particularly the phytochelatin complexes, PC-AsIII, may dissociate into PC and free AsIII, with the latter getting oxidized to AsV if the pH of the extraction media is 7.2 (Meharg & Hartley-Whitaker 2002). AsIII can also be gradually oxidized to AsV during aging and drying of plant material (Webb et al. 2003). Therefore, care has been taken when extracting As species from the plant matrix to ensure that the extraction and sample preparation do not modify the *in situ* As speciation in materials derived from plants. However, different amount of AsIII and AsV were obtained from the plant material by using the same extraction method even though plants had been exposed to different amounts of AsV (Van den Broeck et al. 1998) or the same plants had been harvested in different seasons (Koch et al. 2000). The cited reports indicate that both AsV and AsIII occur in plants that have been exposed only to AsV. Moreover, both AsV and AsIII were found in shoots of *Pteris vittata* with the help of X-ray absorption near-edge structure spectroscopy (Lombi et al. 2002). Because this technique allows for As speciation in plant material without the need for its extraction, it is unlikely that the AsV (25% of total As) reported in *P. vittata* would be an artifact of sample preparation. In the present study, different proportions/amount of AsIII and AsV were measured in WT and MT plants of *A. thaliana*, depending on the exposure time to AsV (Fig. 3a,b). By comparing the level of accumulation of arsenic species in the mutant and control plants it can be concluded that the *ACR2* gene of *A. thaliana* is indeed involved in reduction of AsV to AsIII (Fig. 4a,b). Identification of As species in roots and shoots will facilitate future research to understand and explain the mechanisms of As transport from root to shoot of the plant. Future works on better understanding the function of the *ACR2* gene should also include investigation

on the presence of As species in xylem and phloem of the plant, through which arsenic might be transported from roots to the shoots.

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