Inositol transporters AtINT2 and AtINT4 regulate arsenic accumulation in Arabidopsis seeds

Gui-Lan Duan1, Ying Hu1, Sabine Schneider2, Joseph McDermott3, Jian Chen4, Norbert Sauer2, Barry P. Rosen4, Birgit Daus5, Zijuan Liu6* and Yong-Guan Zhu6*

Arsenic contamination of groundwater and soils threatens the health of tens of millions of people worldwide. Understanding the way in which arsenic is taken up by crops such as rice, which serve as a significant source of arsenic in the human diet, is therefore important. Membrane transport proteins that catalyse arsenic uptake by roots, and translocation through the xylem to shoots, have been characterized in a number of plants, including rice. The transporters responsible for loading arsenic from the xylem into the phloem and on into the seeds, however, are yet to be identified.

Here, we show that transporters responsible for inositol uptake in the phloem in Arabidopsis also transport arsenic. Transformation of Saccharomyces cerevisiae with AtINT2 or AtINT4 led to increased arsenic accumulation and increased sensitivity to arsenite. Expression of AtINT2 in Xenopus laevis oocytes also induced arsenite import. Disruption of AtINT2 or AtINT4 in Arabidopsis thaliana led to a reduction in phloem, silique and seed arsenic concentrations in plants fed with arsenite through the roots, relative to wild-type plants. These plants also exhibited a large drop in silique and seed arsenic concentrations when fed with arsenite through the leaves. We conclude that in Arabidopsis, inositol transporters are responsible for arsenite loading into the phloem, the key source of arsenic in seeds.

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Arsenic is a Group 1 carcinogen1. This toxic metalloid is ubiquitous in soil and water due to weathering of minerals and to anthropogenic agricultural and industrial activities2. Arsenic in soil and water is taken up by plant roots and retained in edible tissues, representing the major sources of dietary arsenic3. It is estimated that rice contributes up to 50% of the total dietary arsenic for West Bengal and Bangladesh populations and up to 60% for the Chinese population4,5. Thus, reduction of arsenic in our food supply is essential for public health. A critical step in the accumulation of arsenic by plants is its transport across cellular membranes. Thus, the identification of responsible genes and gene products can lead to new strategies to reduce arsenic accumulation in rice straw than in grains. Phloem transport has been considered central for arsenic translocation to the grains, and approximately 90% of the As(III) in rice grains was transported via the phloem6,7. In addition, although the Lsi2 mutation significantly reduced arsenic accumulation in rice grains, it also led to reduced silicon transport, which results in poorer plant growth and yield8,9.

Therefore, it is of considerable importance to elucidate the pathways of arsenic loading into the phloem and from there into the seeds in terms of human exposure to arsenic. Depending on the growth conditions, S. cerevisiae takes up about 20% of total As(III) by the AQP Fps1p and about 80% by hexose transporters10. Mammalian GLUT1 also transports As(III) and MAs(V)11,25. Both yeast hexose transporters and GLUT1 belong to the monosaccharide transporter-like (MST-like) superfamily. MST-like transporters mediate the uptake of a wide range of substrates, including pentoses, hexoses and inositols26. A. thaliana inositol transporters (INTs) represent a subgroup within the MST-like superfamily27,28. We, therefore, considered the possibility that As(III) might be a substrate of INTs. The INT family in A. thaliana includes three genes that encode AtINT1, AtINT2, AtINT4 and a pseudogene, AtINT3, that does not encode a functional protein28. While AtINT1 is a tonoplastic protein29, AtINT2 and AtINT4 are

1State Key Laboratory of Urban and Regional Ecology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China. 2Molekulare Pflanzenphysiologie, Friedrich-Alexander-Universität Erlangen-Nürnberg, Erlangen 91058, Germany. 3Department of Biological Sciences, Oakland University, Rochester, Michigan 48309, USA. 4Department of Cellular Biology and Pharmacology, Florida International University, Herbert Wertheim College of Medicine, Miami, Florida 33199, USA. 5Department Analytical Chemistry, Helmholtz Centre for Environmental Research GmbH – UFZ, Permoserstrasse 15, Leipzig 04318, Germany. 6Key Laboratory of Urban Environment and Health, Institute of Urban Environment, Chinese Academy of Science, Xiamen 361021, China. *e-mail: liu2345@oakland.edu; ygzhu@rcees.ac.cn
plasma membrane H⁺-coupled transporters that are mainly expressed in the companion cells of phloem and mediate inositol uptake into the phloem and deliver mesophyll-derived inositol to the reproductive tissues28,30. We hypothesize that AtINT2 and AtINT4 are also functional arsenic transporters and are required for the long-distance transport of arsenite through the phloem and into A. thaliana seeds. Here we demonstrate that inositol transporters AtINT2 and AtINT4 express in yeast and oocytes and are key transporters regulating arsenic accumulation in plant seeds. In this study, the arsenic transport properties of AtINT2 and AtINT4 were examined by expression in yeast, X. laevis oocytes and A. thaliana. Here we demonstrate that inositol transporters AtINT2 and AtINT4 are also functional arsenic transporters and are required for the long-distance transport of arsenite through the phloem and into A. thaliana seeds. We propose that inositol transporters in crop plants such as rice may be the key to the introduction of arsenic into the food supply of the majority of the world’s population.

Results

AtINT2 and AtINT4 catalyse arsenic uptake in yeast and X. laevis oocytes. AtINT2 and AtINT4 were expressed in S. cerevisiae strain D458-1B28-31. This strain carries mutations in the ITR1 gene, which encodes an AtINT orthologue, and in the INO1 gene. Cells of yeast strain D458-1B expressing either AtINT2 or AtINT4 were more sensitive to As(III) than those with vector only (Fig. 1a). To further confirm the arsenic-sensitive phenotype, the AtINT2 and AtINT4 cDNAs were expressed in S. cerevisiae strain MG100, which has a disruption of the ACR3 gene that encodes an As(III) efflux transporter and is hypersensitive to As(III)32. MG100 expressing either AtINT2 or AtINT4 became even more sensitive to As(III) (Fig. 1b). These results indicated that either AtINT2 or AtINT4 expression elevated yeast sensitivity to As(III).

Yeast strains D458-1B expressing AtINT2, AtINT4 or containing the empty vector were treated with 50, 100, 250 and 500 μM As(III) for 24 h, and accumulation of arsenic was measured. D458-1B expressing AtINT2 or AtINT4 accumulated more arsenic than those with the empty vector under the same As(III) treatment (p < 0.001, Fig. 2a). In the 500 μM As(III) treatments, AtINT2 and AtINT4 expressing cells accumulated 2.2-fold and 2.5-fold, respectively, more arsenic than control. These results demonstrated that both AtINT2 and AtINT4 mediate the uptake of As(III). In this study, yeast strain D458-1B was used. This strain has a WT ACR3 gene, which encodes the primary arsenic efflux transporter. In this case, ACR3 would act in opposition to AtINTs; therefore, arsenic accumulation in D458-1B cells (Fig. 2a) was considerably lower than in an ACR3 deletion strain, such as the Δacr3 strain that was used to express Lsi (ref. 12).

The transport properties of the AtINT2 for As(III) were further analysed in X. laevis oocytes. Oocytes expressing AtINT2 exhibited significantly higher transport activity of As(III), which was approximately twofold higher than the control (p < 0.001, Fig. 2b). These results clearly showed that As(III) is transported by AtINT2.

Myo-inositol inhibits As(III) uptake by AtINT2 and AtINT4.

Yeasts D458-1B expressing AtINT2 or AtINT4 were treated with 250 μM As(III) and various concentrations of myo-inositol for 24 h. The concentrations of arsenic in yeast cells expressing AtINT2 or AtINT4 decreased correlating with the increase in myo-inositol in the growth medium (r² = 0.99, p < 0.001, Fig. 3). In contrast to that, arsenic concentrations in yeast cells containing the empty vector did not decrease significantly with increasing myo-inositol concentrations (Fig. 3). In the D458-1B strain, the AtINT orthologue (ITR1) gene is mutated28,30, so D458-1B transformed with vector could not accumulate arsenic through the INT pathway, thus the accumulation of arsenic was not affected by myo-inositol in the growth medium. However, in D458-1B expressing AtINT2 or AtINT4, arsenic accumulation in yeast and oocytes was observed with increasing concentrations of myo-inositol.
Kinetic parameters of AtINT2 and AtINT4. The Michaelis–Menten kinetics for As(III) uptake were investigated by treating yeast strains D458-1B expressing AtINT2 or AtINT4 with 2 µg ml⁻¹ myo-inositol and various concentrations of As(III) for 30 min (Fig. 4). Kinetic constants were calculated using a SigmaPlot transformation. For AtINT2, the $K_m$ for As(III) uptake was 219 µM As(III), and $V_{max}$ was 10 µg g⁻¹ yeast DW min⁻¹ ($r = 0.999$, $p < 0.0001$). For AtINT4, the $K_m$ for As(III) uptake was 174 µM As(III), and $V_{max}$ was 8.2 µg g⁻¹ yeast DW min⁻¹ ($r = 0.999$, $p = 0.0012$). The $K_m$ values for myo-inositol of AtINT2 and AtINT4 were 0.7–1.0 mM and 240 µM, respectively. Compared with the physiological substrate myo-inositol, the $K_m$ values for As(III) of both AtINT2 and AtINT4 were much lower, indicating that AtINT2 and AtINT4 have higher affinity for As(III) than to inositol.

AtINT2 and AtINT4 contribute to arsenic loading into phloem. To examine functions of AtINT proteins in the uptake and distribution of arsenic in A. thaliana, plants with T-DNA insertions in the genomic sequence of either AtINT2 or AtINT4 were obtained from the Arabidopsis Biological Resource Center (ABRC) at Ohio State University. Homozygous disruptions were confirmed by polymerase chain reaction-based genotyping. AtINT2-1 (Salk_1264_A07) and AtINT2-2 (Salk_065862 C) were found to be homozygous lines, each with a T-DNA insertion in the second intron of AtINT2 (Supplementary Fig. 1a,b). AtINT4-1 (Salk_082659. 41. 45.X) and AtINT4-2 (WiscDsLox293-296inv17) were also shown to be homozygous lines, each with a T-DNA insertion in the second exon of AtINT4 (Supplementary Fig. 2a,b). No AtINT2 or AtINT4 mRNA was detected in the respective mutants, indicating that these T-DNA insertion mutants are null alleles (Supplementary Figs 1c and 2c). AtINT2 or AtINT4 knockout mutants do not show alterations compared with WT plants during their life cycle. Usually, plants do not rely on inositol transport because they biosynthesize myo-inositol from glucose-6-phosphate.

To compare arsenic exuding from phloem, A. thaliana WT and mutant plants were grown in hydroponic MGRL solution at the flowering stage, As(III) was added to the nutrient solution to a final concentration of 50 µM. The plants were treated with As(III) for 1 week. Phloem exudates were collected and arsenic content in the exudates was analysed. Figure 5a shows that arsenic exuding from phloem of AtINT2 or AtINT4 mutants was significantly lower (by about 27–35%) than that from WT plants. These results are consistent with our hypothesis that AtINT2 and AtINT4 are involved in arsenic loading into phloem.

Arsenic accumulation and distribution was compared in different organs of WT and mutant plants. To this end, plants were grown in hydroponic MGRL solution containing 5 µM As(III). After plant maturation, arsenic concentrations in different organs were determined. Arsenic accumulated primarily in roots, with the concentration being approximately 12-fold higher than in shoots. The order of arsenic distribution was roots > shoots > empty siliques > seeds (Fig. 5b). The concentrations of arsenic in roots of mutants and WT were similar, while the concentrations in shoots, empty siliques and seeds were significantly lower in the mutants than in the corresponding organs of WT plants. Strikingly, AtINT2 or AtINT4 disruption resulted in a 45–64% reduction in arsenic accumulation in seeds (Fig. 5b). These results clearly demonstrate that AtINT2 and AtINT4 are necessary for arsenic accumulation in siliques and seeds of Arabidopsis. A similar situation has been described for the phloem-localized iron (Fe(II))— and manganese (Mn(II))—nicotianamine complex transporter OsYSL2 from rice. RNAi plants with a suppressed expression of OsYSL2 exhibit a reduced iron level within the shoots and seeds, similar to our results for INT mutants and arsenite translocation.
Figure 5 | Arsenic concentration in phloem exudates, xylem sap and plant tissues. **a**, Plants were grown in hydroponic solution, after 3 d treatment with 50–µM As(III), rosette leaves were harvested and phloem exudates were rapidly collected by an EDTA-facilitated method. **b**, Plants were grown in hydroponic solution, one week before harvesting, rosette leaves were brushed with a solution containing 50 µM As(III) and 0.1% Tween using a painting brush. Each plant was brushed with a 2 ml solution daily. After harvesting, the plants were separated into shoots (including upper stem and leaves that had not been brushed) and siliques (including seeds). **c**, Plants were grown in hydroponic solution, from flowering stage to harvest, As(II) was added to the nutrient solution to a final concentration of 5 µM. After harvesting, plants were separated into roots, shoots, empty siliques and seeds. **d**, Plants were grown in soil, after 3 d treatment with 5–µM As(III), xylem sap was collected. *P < 0.05 and **P < 0.01 statistically significant compared with WT. Averages and standard errors are shown; n = 4.

Discussion

It is becoming increasingly clear that plant aquaglyceroporins of the NIP subgroup such as the rice AQP Lsi1 catalyse the uptake of As(III) into roots12,13, and that the rice ArsB family member Lsi2 is responsible for the movement of As(III) from roots to shoots through the xylem12. The final piece of the puzzle is how arsenic is loaded from the shoots into the seeds of plants6. In this study, we show that the A. thaliana inositol transporters AtINT2 and AtINT4 catalyse As(III) loading into phloem and are necessary for arsenic accumulation in the seeds of this model plant. We speculate that knowledge of the pathway of arsenic accumulation in Arabidopsis seeds will shed light on the corresponding mechanism in rice, the main source of dietary arsenic for the majority of the world’s population. Understanding the loading mechanism of As(III) into rice grains, fruits or seeds of other crops is critical for enhancing food safety.

As(III) is the predominant arsenic species found in seeds, especially in rice grains3,36. A survey of arsenic speciation in Chinese rice showed that in market rice, 50–60% arsenic was present as As(III), and in rice collected from farmers’ fields in mining areas, 60–70% was As(III)35. Approximately 90% of As(III) in rice grains is delivered via the phloem19–23. However, prior to the present study, little was known about the mechanisms of arsenic loading and unloading during phloem transport6. Generally, solutes load into and unload from phloem through either the apoplastic or symplastic pathway. Aposplastic loading is driven thermodynamically via the proton motive force and conducted by plasma membrane transporters37. Symplastic loading is passive and conducted through plasmodesmata between adjacent cells38,39. In A. thaliana, AtINT2 and AtINT4 are located in the plasma membrane. Organ and tissue specificity of AtINT2 and AtINT4 expression showed that both AtINT2 and AtINT4 are strongly expressed in the vasculature, primarily in the companion cells of phloem, although there is also a little expression in root.
tissue. Functional analyses further demonstrated that AtINT2 and AtINT4 are H+-coupled symporters that are responsible for loading of inositol into the phloem to supply the developing seeds. In the present study we demonstrate that AtINT2 and AtINT4 also transport As(III) (Figs 1 and 2). Myo-inositol in the growth medium inhibited the uptake of As(III) by AtINT2 and AtINT4 (Fig. 3). The disruption of AtINT2 or AtINT4 significantly decreased arsenic concentration in phloem exudates (Fig. 5a), and subsequently significantly decreased arsenic concentration in shoots, silicles and seeds (Fig. 5b,c). Most importantly, arsenic accumulation in silicles and seeds decreased by half (Fig. 5c), but the ratios of each arsenic species in plants tissues were similar between the mutants and WT (Supplementary Fig. 4). Additionally, when plants were fed with arsenite through leaves, arsenic accumulation in shoots and silicles of mutants was significantly lower than those of WT (Fig. 5b). In contrast to that, arsenic concentrations in the xylem sap did not vary between WT and mutant plants (Fig. 5d). These results clearly demonstrate that inositol transporters AtINT2 and AtINT4 are responsible for arsenite loading into phloem, and essential for arsenite accumulation in A. thaliana seeds.

We conclude that AtINT2 and AtINT4 are responsible for the loading of arsenic from the apoplasm into the phloem (Supplementary Fig. 5). Our results are consistent with the tissue and cell specificity of expression. Nevertheless, a single mutation of AtINT2 or AtINT4 did not totally suppress translocation of arsenic into seeds (Fig. 5). This could be contributed to by AtINT4 in AtINT2 or AtINT4 in AtINT4, or other transporters may be also involved in arsenic loading to phloem. AtINT2 and AtINT4 are not expressed in young sink leaves, so it was anticipated that neither AtINT2 nor AtINT4 mutations would affect arsenic accumulation in seedlings treated with As(III) (Supplementary Fig. 3). Once entry into the companion cells of the phloem, arsenic passively diffuses through the plasmodesmata into the sieve elements and is finally released into the sink cells of seeds (Supplementary Fig. 5). As is the case for nutrients, unloading of arsenic from the phloem into the sink cells of plant seeds is likely to be mediated by specific transport proteins, and identification of these transporters should be a priority of future research.

In summary, we demonstrate here that inositol transporters AtINT2 and AtINT4 adventitiously catalyse loading of As(III) into the phloem, a possible pivotal step of arsenic translocation to the seeds of higher plants. To our knowledge, this is the first identification of transporters responsible for arsenic loading into phloem. If these findings prove to be applicable to rice, then inositol transporters may be candidates for future genetic modification to reduce the arsenic content in rice grain. If so, this discovery will enable development of new cultivars that accumulate lower amounts of arsenic in their grain without affecting yield production, a major advance toward mitigation of health risks posed by arsenic in rice.

Methods

Yeast constructs and arsenite sensitivity analysis. AtINT2 and AtINT4 were cloned into the yeast E. coli shuttle vectors NEV-N-Leu (ref. 30) (AtINT2) or NEV-E-Leu (ref. 41) (AtINT4); the constructs and the empty vectors were used to transform S. cerevisiae strain D458-1B (refs 28,30). In this study, AtINT2 and AtINT4 constructed plasmids were also transformed into S. cerevisiae strain MG100 (acr3Δ) (US patent US 2005026739 A1). Arsenite sensitivity phenotypic studies were performed as reported previously; cell growth was determined by light absorbance at 600 nm.

For the As(III) uptake assay, yeast strains D458-1B expressing AtINT2, AtINT4, or with empty vector were grown in 5 ml of liquid SD-Leu medium supplemented with 2 µg ml⁻¹ myo-inositol until mid-exponential phase. The cells in the cultures were harvested by centrifuge and resuspended in 50 ml of fresh SD-Leu medium containing 2 µg ml⁻¹ myo-inositol and different concentrations of As (50, 100, 250 and 500 µM). After 24 h of incubation (30 °C, 170 rpm), yeast cells were harvested for arsenic concentration determination. For substrate competition, mid-exponential phase yeast cells were treated with 250 µM As(III) and different concentrations of myo-inositol (0, 2, 4 and 8 µg ml⁻¹) for 24 h incubation (30 °C, 170 rpm). For kinetic assays, mid-exponential phase yeast cells were treated with 2 µg ml⁻¹ myo-inositol and various concentrations of As(III) (50, 100, 250 and 500 µM). After 30 min of incubation (30 °C, 170 rpm), yeast cells were harvested for arsenic concentration determination.

Expression of AtINTs in X. laevis oocytes and arsenite uptake. AtINT2 was cloned into plasmid pL in the BglII/KpnI. The primer sequences for constructions of different genes are as follows: forward primer 5′-GAGAGACTGATTGAGGAGGAAATAC-3′ (BglII site underlined); reverse primer 5′-GGCGGACCTCAGGACGTCT-3′ (KpnI site underlined). The plasmids were linearized by NotI digestion, and the capped RNA of NaPi-IIb1 was transcribed in vitro using a Message Machine T7 ultra kit (Ambion Co.). Stage V–VI X. laevis oocytes were isolated and treated with 0.2% collagenase A (Roche) for 2 h. Defoliated oocytes were injected with 25 ng (5 nL volume) of crRNA. Oocytes were then incubated in ND96 complete buffer (96 mM NaCl, 2 mM KCl, 1 mM MgCl₂, 1.8 mM CaCl₂, 5 mM Hepes, pH 5.5, supplemented with 1 mg ml⁻¹ gentamicin) for 3 d at 16 °C (ref. 43).

Accumulation of arsenicals in oocytes was assayed by incubation of the oocytes with 1 mM As(III) dissolved in ND96 buffer (pH 7.4) at room temperature for 30 min. After incubation, the oocytes were washed, dissolved in 70% nitric acid at 70 °C for 2 h, and then the arsenic concentration was analysed.

Plant treatments. To assay arsenic in phloem exudates, uniform homoygote and WT seedlings (10 d) were transferred from plates to hydroponic pots containing 5 l of MGRL nutrient solution. At the flowering stage, As(III) was added to the nutrient solution to a final concentration of 50 µM. On the third day of As(III) treatment, the nutrient solution was renewed with As(III), and, after 1 h, rosette leaves were harvested and weighed. Phloem exudates were rapidly collected by an EDTA-facilitated method. After 8 h of collection, phloem exudates solutions were passed through a 0.22 µm filter and stored at –4 °C until arsenic concentration determination.

To analyse total arsenic in mature A. thaliana tissues, from flowering stage to harvest, As(III) was added to the nutrient solution to a final concentration of 5 µM. After harvesting, plants were separated into roots, shoots, empty silicles and seeds. Samples were washed and dried for arsenic determination. To conduct leaf feeding experiments, 1 week before seed harvesting, both sides of the rosette leaves were brushed with a solution containing 50 µM As(III) and 0.1% Tween using a paint brush. Each plant was brushed with a 2 ml solution daily. After harvesting, the plants were separated into shoots (including upper stem and leaves that had not been brushed) and silicles (including seeds). Samples were washed and dried for arsenic determination.

For determination of the concentration of arsenic in the xylem sap of WT and mutants, soil-grown A. thaliana plants at the flowering stage were used. Treatment with 5 µM As(III) was performed for 3 days before xylem sap collection. Xylem sap collection was performed as described except that plants were not irrigated with NaCl. The xylem sap from two plants was pooled for each sample. Collected samples were stabilized by adding phosphoric acid to a final concentration of 10 mM, passed through a 0.22 µm filter and stored at 4 °C until determination of arsenic concentration.

Total arsenic analysis. For total arsenic analysis, yeast and plant subsamples were weighed and digested with 2.5 ml of concentrated nitric acid in a microwave oven (CEM Mars 5, CEM Corp.). Arsenic concentrations were determined by inductively coupled plasma mass spectrometry (Agilent Technologies 7500, USA).

Statistical analysis. Experiments using X. laevis oocytes, yeast and plant tissues adopted n = 4. Mean and standard errors were derived using SigmaPlot. Statistical differences were assessed by the Student pair-wise t-test. Data are presented as mean ± s.d. All P values < 0.05 were regarded as statistically significant.

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Author contributions

Y.G.Z., Z.J.L., B.P.R. and N.S. designed the research. G.L.D., Y. H., S.S., J.M., J.C. and B.D. performed research and analysed data. All authors were involved in extensive discussions and wrote the manuscript.

Additional information

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Competing interests

The authors declare no competing financial interests.