# *Arabidopsis thaliana* NIP7;1 is Involved in Tissue Arsenic Distribution and Tolerance in Response to Arsenate

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# Highlights

* *Arabidopsis thaliana* NIP7:1 knockout plants are more tolerant to arsenate.
* *AtNIP7;1* affects long-distance transport of arsenic through the xylem and phloem.
* Loss of function in *AtNIP7;1* leads to reduced seed arsenic levels.

# Abstract

The *Arabidopsis* aquaglyceroporin *NIP7;1* is involved in uptake and tolerance to the trivalent arsenic species arsenite. Here we show that *NIP7;1* is also involved in the response to pentavalent arsenate. Loss of function of *NIP7;1* improved tolerance to arsenate and reduced arsenic levels in both the phloem and xylem, resulting in altered arsenic distribution between tissues. There was no clear correlation between growth and shoot arsenic concentration. This is the first report detailing the involvement of a *NIP* transporter in response to arsenate. The data suggest that these proteins are relevant targets for breeding and engineering arsenic tolerance in crops.

# Keywords

Arsenic; Arsenate; NIP7;1; Tolerance*; Arabidopsis thaliana*

# Introduction

Arsenic (As) is a toxic metalloid found naturally in the environment. High levels of inorganic As contamination have been reported from more than 70 countries worldwide, and its entry into the food chain through drinking water and accumulation in crops poses a serious health risk to millions of people [1]. Arsenic is readily taken up by plants including important crop species such as rice, which is a major contributor to dietary As intake. Arsenic is also phytotoxic, affecting plant growth and ultimately reducing yield.

Arsenic is present in soils primarily in the inorganic forms arsenate (AsV) and arsenite (AsIII). AsV is the most abundant form in aerobic soils, whereas in anaerobic conditions such as flooded paddy fields, AsIII predominates. Once taken up by plants, AsV is rapidly reduced to AsIII which has a high affinity for sulphhydryl groups and so can be detoxified through complexation with thiol rich peptides and subsequent compartmentalisation in the vacuole [2, 3]. The speciation of As impacts on its bioavailability and so on its uptake and accumulation in plants. AsV is a phosphate analogue and competes for uptake through phosphate transporters (such as PHT1;1 and PHT1;4 in *Arabidopsis*) [4, 5] whereas AsIII is taken up through aquaglyceroporins [6].

Aquaglyceroporins, a sub-family of the aquaporins, mediate the bidirectional transport of glycerol and other small uncharged solutes, including metalloids, across the membrane. Members of the NIP (nodulin-26-like intrinsic proteins) and PIP (plasma membrane intrinsic proteins) subfamilies of aquaporins have been shown to facilitate AsIII transport in plants in addition to transporting essential nutrients such as silicon and boron [7-13].

There are nine members of the NIP subfamily in *Arabidopsis*, of which six have been shown to transport AsIII so far. Loss-of-function mutations in five *Arabidopsis* NIPs (*nip1;1, nip1;2, nip3;1, nip5;1* and *nip7;1*) resulted in reduced plant As levels, while three displayed increased tolerance to growth in AsIII (*nip1;1, nip3;1* and *nip7;1*) [12-14]. The reduced As content of these mutants suggests that they may play a role in AsIII uptake but does not rule out alternative functions such as translocation and distribution of As. To date, only NIP3;1has been implicated in the root-to-shoot translocation of AsIII, with *nip3;1* plants accumulating significantly less As in the shoots after exposure to AsIII [13]. *Arabidopsis* NIPs have distinct tissue expression patterns. *NIP5;1, NIP3;1* and *NIP1;1* are expressed predominantly in the root. In the root, NIP5;1 localises to the distal side of epidermal cells, NIP1;1 is localised to the stele while NIP3;1 is expressed throughout the root [13-15]. *NIP7;1* is most highly expressed in developing anthers where it is proposed to deliver boron to developing pollen [16] but is also present in many other tissues in both roots and shoots (Figure 1) [17].

Several NIPs confer resistance to AsV when expressed in yeast strains lacking an AsIII efflux system, suggesting that NIPs are able to increase tolerance by mediating transport of AsIII to the external medium [11, 14]. The rice aquaporin NIP2;1/Lsi1 can efflux AsIII from rice roots, [18] and enhanced efflux of AsIII was observed in transgenic *Arabidopsis* roots expressing the rice PIP OsPIP2;6 when treated with AsV [9]. Bi-directional transport of AsIII through NIPs could contribute differentially to AsIII and AsV tolerance and detoxification in plants, and so may have implications for As tolerance in crops grown in aerobic soils where AsV predominates.

In order to improve tolerance to As and reduce accumulation in crops, a more detailed understanding of the mechanisms governing As uptake, efflux, translocation and accumulation in separate parts of the plant is required. The potential role of NIPs in these processes has yet to be unravelled and here we describe the role of NIP7;1 in As uptake, translocation and efflux in response to AsV. Our data suggest that NIP7;1 affects seed As deposition and tolerance to AsV by altering the distribution of As between root and shoot tissue.

# Materials and Methods

## Plant Materials and Growth Conditions

The T-DNA insertion mutant in NIP7;1 was obtained from NASC (SALK\_057023). It was previously shown to be a loss of function mutant as described [12].

WT *Arabidopsis thaliana* (L.) ecotype Columbia(0) and mutant seeds were sown on F2+S (Levington UK) soil and stratified at 4°C for 48hrs before transfer to 16hr light/8 hr dark conditions with night-day temperatures of 20-23°C for 21 days. Plants were transferred to 1-litre hydroponics boxes containing half-strength MS medium and allowed to acclimatise for 24hrs before treatment. AsV was added to the growth medium as different concentrations of KH2AsO4 as indicated in the text.

## Arsenic Content Analyses

Arsenic content was analysed in shoot and root tissue of plants exposed to 150µM AsV in hydroponics for 1, 7 and 14 days. Roots were washed in ice-cold desorption buffer (1mM K2HPO4, 0.5mM Ca(NO3)2 and 5mM MES-pH 5.6) for 15 minutes to remove apoplastic As and rinsed in dH2O. Seeds were collected from plants exposed to 150µM AsV in hydroponics for two weeks. Plant tissue was dried at 80°C for 48hrs and weighed. Samples were digested in HNO3 at 70°C for 24hrs and the As content determined using an ICP-OES (Thermo iCAP 7000 series).

## Arsenic Efflux Experiment

Three week old plants were exposed to 350µM AsV in hydroponics for 24hrs. Roots were rinsed in dH2O and placed in 1.5ml of As free medium for 6 and 24hrs. The As content of the medium, shoot and root tissue was analysed as described above.

## Arsenic Content of Xylem and Phloem Sap

Three week old WT and *nip7;1* plants were exposed to 150µM AsV in hydroponics for three weeks under 8hr light/16hr dark growth conditions. Phloem exudates were collected as described by Tetyuk, O. *et al.* 2013 [19].

For collection of xylem sap, WT and *nip7;1* plants were grown on soil for 5 weeks before transfer to hydroponics. After a few days, 150µM AsV was added to the medium for 24hrs. The weight of the plants and medium was taken at the start and end of treatment to enable calculation of transpiration rate. The bolt was cut using a sharp blade around 5cm above the level of the rosette. Decapitated plants were transferred to a pressure chamber (Digital Plant Water Potential Apparatus EL540-300) and around 20 kiloPascal pressure was applied. Xylem sap was collected for 20 mins and the volume was made up to 2.2mls using 5% HNO3­. As content analysis was as described above.

# Results

## *NIP7;1* Knockout Plants Are More Tolerant to AsV

We previously characterised the SALK\_057023 insertion line showing it is a genuine *NIP7;1* loss of function mutant [12]. Homozygous *nip7;1* mutant seedlings were grown on agar plates containing different concentrations of AsIII and AsV [12]. The fresh weight of *nip7;1* seedlings treated with AsIII was significantly larger than wild type (WT) while no difference was observed on AsV. To investigate under more physiological conditions, we grew mature *nip7;1* and WT plants in hydroponics. Plants were exposed for two weeks to a range of AsV concentrations and relative growth rates (RGR) were determined. At the highest concentration tested, plant weights were reduced by 40% compared to control conditions. Figure 2 shows that *nip7;1* plants were more tolerant than WT to AsV. Relative to growth in control conditions, *nip7;1* plants grew significantly faster than WT when exposed to 50, 100 and 150µM AsV for 1 week (Figure 2A). After two weeks in treatment, (when plants were beginning to flower) increased tolerance of *nip7;1* plants was still apparent (Figure 2B).

## *NIP7;1* has an Impact on As Distribution in Plants Exposed to Arsenate

In theory, *NIP7;1* may affect the concentration of As in different tissues by facilitating the influx, efflux or long distance transport of As throughout the plant. Each of these processes could impact on overall tolerance. Since it is unlikely that *NIP7;1* has a role in AsV uptake, because NIPs are not permeable to AsV, we focused on the potential roles of *NIP7;1* in the distribution of As between plant organs and tissues.

WT and *nip7;1* plants were exposed to 150µM AsV in hydroponics and the As content of root and shoot tissues was analysed after 1, 7 and 14 days. After 1 day, the As concentration was significantly lower in the shoots of *nip7;1* plants compared to WT, while there was no difference in the root As concentration (Figure 3A and 3B). The shoot/root ratio of As concentration (S:R As) in the knockout plants was also significantly lower than in WT, indicating a possible role of *NIP7;1* in root to shoot transfer of As (Figure 3C). After 7 days, the concentration of As in both root and shoot tissue was higher than after 1 day in both genotypes (Figure 3A and 3B). However the knockout plants no longer had a lower As concentration in the shoot compared to WT. After 14 days, both genotypes had accumulated even higher concentrations of As but at this time point the shoot concentration of *nip7;1* plants was significantly higher than that of WT plants (Figure 3A and 3B). The As S:R of *nip7;1* plants was also significantly higher than that of WT after 14 days exposure (Figure 3C). At this stage, the total plant concentration of As was actually higher in *nip7;1* plants (Figure 3D) in spite of a greater RGR (Figure 2B).

Since NIP7;1 expression is generally high in reproductive tissues, the As content of seeds was analysed fromplants exposed to 150µM AsV in hydroponics for 14 days. Figure 4 shows that in spite of higher shoot As levels, seeds from the *nip7;1* plants contained significantly less As than WT.

## NIP7;1 Affects As Efflux and Long Distance Transport in Plants Exposed to AsV

To investigate the role of *NIP7;1* in As efflux from the roots, WT and *nip7;1* plants were exposed to AsV for 24hrs before transfer to As free medium. The amount of As in the shoot, root and medium was measured after 6 and 24 hours. Initially (6hrs after transfer) there was no difference between WT and *nip7;1* plants with respect to the proportion of As effluxed but after 24hrs, efflux from *nip7;1* roots was significantly higher than WT (Figure 5A). If *NIP7 ;1* is involved in efflux from the roots, knockout plants would be expected to efflux less As to the external medium than WT plants. The observed higher efflux of As from the mutants suggests that NIP7;1 alters As sequestration in root tissues in a manner that facilitates efflux. For example, loss of function of *NIP7;1* may affect the long distance transport of As resulting in an increased local concentration of As in the root, which would promote efflux from the root down a comparatively larger concentration gradient. To explore this hypothesis, the As content in the xylem sap of WT and *nip7;1* plants was measured.

Mature WT and *nip7;1* plants were exposed to 150µM AsV in hydroponics for 24hrs prior to collection of xylem sap from the stems. The concentration of As in the xylem sap was measured in parallel with water loss rates to enable calculation of root to shoot As flux. The concentration of As in the xylem sap of *nip7;1* plants was significantly lower than in WT (Figure 5B). The flux of As through the xylem was also lower in the knockout, but the difference was not significant (p=0.053, Figure 5C).

The lower As concentration in the xylem could account for the reduced As concentration observed in the shoots of *nip7;1* plants after 1 day exposure to AsV (Figure 3A). However, shoot As concentration is a function of both As import from the xylem and export via the phloem. The As content of the phloem was therefore measured by sampling petiole exudate of mature leaves of plants exposed to 150µM AsV. Compared to WT, *nip7;1* plants exuded a smaller proportion of the leaf As content into the phloem (Figure 5D).

# Discussion

Previous studies have focused on the role of NIPs in response to AsIII. NIP1;1, NIP3;1 and NIP7;1 have all been shown to affect tolerance to AsIII with concomitant alterations in AsIII influx and As tissue levels [12-14]. These studies suggest that members of the NIP subfamily in *Arabidopsis* have diverse roles in AsIII tolerance and affect AsIII uptake and translocation, observations that are likely related to their unique expression patterns. However, so far, no studies have investigated the role of NIPs in response to AsV.

*NIP7;1* conferred tolerance to AsV on yeast strains lacking the AsIII efflux transporter ScACR3p, indicating that NIP7;1 is capable of bi-directional transport of AsIII [12]. This suggested that NIP7;1 may have a role in AsIII efflux from plants, and thus could contribute to AsV tolerance. In this case, we would expect *nip7;1* plants to be more sensitive to AsV treatment. However, our data show that *nip7;1* plants are actually more tolerant, growing faster than WT when exposed to different concentrations of AsV (Figure 2). To explore how loss of function of *NIP7;1* results in increased AsV tolerance, we studied tissue As levels and As concentrations in the long distance transport systems, the xylem and phloem. Loss of function in NIP7;1 led to dynamic changes in shoot:root ratios and reduced levels of As in both the xylem and phloem (Figure 3 and 5). *NIP7;1* therefore affects multiple aspects of As homeostasis in the plant, particularly net translocation from the root to shoot.

When exposed to AsV, *nip7;1* plants were shown to efflux more As from the roots than WT plants (Figure 5A). Increased efflux from *nip7;1* plants could occur as an indirect effect of reduced translocation of As to the shoot through the xylem. Although overall root As levels remained steady (Figure 3B), an increased local As concentration in specific parts of the root could drive greater efflux, possibly through other NIP proteins located at the epidermis (such as *NIP3;1* or *NIP5;1*) [13, 20]. An alternative explanation for the increased net efflux of As is that knockout of *NIP7;1* reduces uptake of AsIII from the medium. The majority of AsV taken up by the plant will be rapidly reduced to AsIII prior to efflux from the root [3, 21]. In the absence of *nip7;1*, less of the effluxed AsIII may be recirculated back into the root.

In the short term (24hrs exposure to AsV), the As concentration in the shoot of *nip7;1* plants (and S:R As) is lower than that of WT (Figure 3A and 3D), suggesting that the reduction in xylem flux in the knockout is more important than the reduced phloem flux. This is not surprising since, in general, phloem flux is around a factor three to four fold lower than xylem flux [22]. The observed short term reduction in As shoot:root ratio could contribute to the AsV tolerance growth phenotype of *nip7;1* plants. However, after a longer period of exposure to AsV (7 to 14 days), increased tolerance is still apparent in *nip7;1* plants despite similar or even higher concentrations of As in the shoot compared to WT (Figure 2). Since the shoot As concentrations increase with length of exposure, reduced levels of recirculation through the phloem in *nip7;1* plants may become relatively more important, resulting in an increased S:R As compared to WT. However, other factors such as more efficient As sequestration in the knockout or differences in As speciation, cannot be ruled out.

Increased As tolerance in the presence of increased As accumulation has been reported before. For example, Catarecha et al*.*, (2007) [5] describe a *PHT1;1* (high-affinity phosphate transporter) allele which has a reduced rate of AsV uptake but leads to increased As accumulation and tolerance. It was postulated that the slower rate of uptake may enable more efficient detoxification of AsV in the form of enhanced AsV reduction, complexation and sequestration in the vacuole, ultimately leading to increased accumulation of As without increased phytotoxicity. It is possible that the reduced flux of As through the xylem and phloem of *nip7;1* plants has a similar effect on detoxification of As in the shoot, resulting in improved tolerance and increased accumulation over time.

Interestingly, knockout of *NIP7;1* resulted in significantly reduced As concentrations in the seeds of AsV exposed plants, with *nip7;1* seeds accumulating 2.7 fold less As than WT. This suggests *NIP7;1* has an important role in As loading into the seeds. Since mineral deposition in seeds is largely mediated by the phloem, this is likely to be an effect of the reduced As flux through the phloem in *nip7;1* plants. A similar mechanism was recently reported for loss of function mutants in inositol transporters [23]: null mutants in AtINT2 and AtINT4 showed reduced seed As content inplants treated with AsIII, which was attributed to a role of these proteins in AsIII loading of the phloem. However *NIP7;1* is highly expressed in the reproductive tissues (Figure 1), particularly in developing anthers [16], and so a direct effect of *NIP7;1* on seed As accumulation cannot be ruled out.

In conclusion, *NIP7;1* plays an important role in the response to AsV: Loss of function *nip7;1* plantsare more tolerant to AsV and NIP7;1 affects the spatio-temporal distribution of plant As probably via its effect on loading of As into both the xylem and phloem. Interestingly, initial responses to As exposure may not be indicative of the long term response and furthermore, tolerance is not necessarily correlated to (shoot) As concentration. These phenomena could have implications for future research: increased accumulation of As in shoot tissue would be beneficial in the context of phytoremediation purposes while increased tolerance could reduce the negative effect of As on crop yield and reduced accumulation of As in the seed could help reduce As exposure through the food chain. Thus, the reported insights could help inform strategies for engineering As tolerance in crops grown in aerobic soils where AsV is the predominant As species.

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# Figure Legends

Figure 1. *NIP7;1* is expressed in several different tissues throughout the inflorescence, shoot and root. Data were obtained from the GENEVESTIGATOR database [17]. Values are means ± SEs.

Figure 2. Plants with the *nip7;1* genotype showed increased tolerance to AsV. (A) RGR of plants grown for 1 week in hydroponics in control, 50, 100, 150 and 200µM AsV conditions. (B) RGR of plants grown for 2 weeks in the same conditions as (A). At least three individuals of each genotype were grown in each condition. Plants were weighed at the start, middle and end of treatment. Data are from at least 6 independent assays, values are means ± SEs expressed relative to control conditions. Asterisks indicate significant differences between genotypes at p<0.05 for each treatment using an unpaired t-test.

Figure 3. *NIP7;1* alters the tissue distribution of As in plants exposed to AsV. (A-B,D) Total As concentrations in shoots (A), roots (B) and whole plants (D) grown in 150µM AsV in hydroponics for 1, 7 and 14 days. (C) Shoot to root As ratio of the same plants. Three individuals were pooled per sample. Data are from 6 independent experiments, values are means ± SEs and asterisks indicate significant differences between genotypes at p<0.05 for each time point using an unpaired *t*-test.

Figure 4. *NIP7;1* knockout plants accumulate less As in the seed. Values are mean concentrations of seed from four individuals of each genotype after growth in 150µM AsV. Asterisks indicate significant differences between genotypes at p<0.05 using an unpaired *t*-test.

Figure 5. *NIP7;1* affects efflux of As from roots and is involed in xylem and phloem loading of As. (A) As efflux from WT and *nip7;1* plants following exposure to 350µM AsV. Efflux is expressed as percentage of total As in the medium. Data are from at least four independent assays. (B) Total As concentration in the xylem sap of mature plants exposed to 150µM AsV. (C) Flux of As through the xylem of mature plants exposed to 150µM AsV. Flux is calculated as the rate of transpiration (water lost per gram of plant fresh weight) multiplied by xylem sap As concentration. The experiment was repeated three times. (D) Phloem As concentrations of plants exposed to 150µM AsV. The As in the phloem exudate is expressed relative to the As concentration of the leaves. Exudates were collected from 15 leaves of each genotype (5 from each of 3 individuals) per assay. Data are from four independent assays. Values are means ± SEs. Asterisks indicate significant differences between genotypes at p<0.05 using unpaired *t*-tests.









