NIP1;1, an Aquaporin Homolog, Determines the Arsenite Sensitivity of Arabidopsis thaliana

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Arsenic [As(III)] is highly toxic to organisms, including plants. Very recently, transporters in rice responsible for As(III) transport have been described (Ma, J. F., Yamaji, N., Mitani, N., Xu, X. Y., Su, Y. H., McGrath, S. P., and Zhao, F. J. (2008) Proc. Natl. Acad. Sci. U. S. A. 105, 9931–9935), but little is known about As(III) tolerance. In this study, three independent As(III)-tolerant mutants were isolated from ethyl methanesulfonate-mutagenized M2 seeds of Arabidopsis thaliana. All three mutants carried independent mutations in Nodulin 26-like intrinsic protein 1;1 (NIP1;1), a homolog of an aquaporin. Two independent transgenic lines carrying promoter of transporting As(III). As content in the mutant plants was 30% lower than in wild-type plants. Promoter of transporting As(III) was highly expressed in roots, and GFP-NIP1;1 is localized to the plasma membrane. These data show that NIP1;1 is involved in As(III) uptake into roots and that disruption of NIP1;1 function confers As(III) tolerance to plants. NIP1;2 and NIP5;1, closely related homologs of NIP1;1, were also permeable to As(III). Although the disruption of these genes reduced the As content in plants, As(III) tolerance was not observed in nip1;2 and nip5;1 mutants. This indicates that As(III) tolerance cannot be simply explained by decreased As contents in plants.

Arsenic represents a major environmental contaminant in several regions of the world. Long-term exposure to As causes skin diseases and cancers in humans. In most As-contaminated areas, As ingested by humans is derived from groundwater that is naturally contaminated with As. Both drinking water and irrigation with As-contaminated groundwater contribute to human ingestion (2, 3). This is especially serious in West Bengal, India, and Bangladesh. Bangladesh has the most serious problem in terms of both the number of people affected and the severity of health problems (2, 3). The major ingestion pathway of As is drinking As-contaminated water, followed by eating foods grown with contaminated water. Crops cultivated with As-containing groundwater accumulate As in their edible parts (4, 5). Rice is the major crop in these areas and is known to accumulate high levels of As in its grains (4). In addition to health effects, As inhibits the growth of rice, which leads to a reduction in yield (6, 7). Both reducing As uptake and generation of As-tolerant plants, especially rice, are useful methods to circumvent problems associated with As contamination.

In the environment, both inorganic and organic As are present, and the inorganic form is more toxic. Two major inorganic As species in the environment are known: arsenate [As(V)] and arsenite [As(III)]. Transport processes of inorganic As have been studied in a number of organisms, and those involved in As uptake have been identified in bacteria, yeast, and animals (8). As(V) is taken up via phosphate transport systems, because As(V) (AsO$_4^{3-}$) is an analog of phosphate. The As(III) molecule is uncharged at neutral pH, and it enters the cell via aquaglyceroporins, which belong to the major intrinsic protein (MIP) family. Sanders et al. (9) first identified Glicp as an As(III) transporter by screening random-mutagenized Escherichia coli for antimonite [Sb(III)]-tolerant mutants. Sb(III), which is a congener of As(III), is also transported by aquaglyceroporins. Subsequently, Fps1p in yeast, AQP7 and AQP9 in humans, and LmAQP1 in Leishmania major have been identified as As(III) transporters (10, 11, 12). In addition to the MIP family, hexose permeases are also involved in As(III) uptake in yeast (13).

In plants, a number of studies have demonstrated the physiological properties and molecular mechanisms of inorganic As uptake. In rice, uptake kinetics of As(V) and As(III) follow the Michaelis-Menten equation (14), suggesting the presence of a transporter. In Arabidopsis thaliana, Pho1;1 and Pho1;4 are responsible for As(V) uptake (15). In the case of As(III), glycerol and Sb(III) inhibit As(III) uptake into rice roots (16). Because

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# The abbreviations used are: As, arsenic; GFP, green fluorescent protein; NIP1;1, Nodulin 26-like intrinsic protein 1;1; MES, 4-morpholineethanesulfonic acid; EMS, ethyl methanesulfonate; ICP-MS, inductively coupled plasma mass spectrometry.

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glycerol and Sb(III) are substrates for aquaglyceroporin, the finding suggests the involvement of aquaglyceroporins in As(III) uptake in plants. Very recently, involvement of aquaglyceroporins, including OsNIP2;1, have been reported in rice (17). In addition, in A. thaliana, NIP5;1, NIP6;1, and NIP7;1 are also permeable to As(III) in the yeast expression system (18, 19).

Considering that these transporters are involved in toxic As uptake into plants, disruption of these transporters is expected to confer As tolerance, as is the case in Escherichia coli and yeast. Disruption of phosphate transporters and NIP7;1 confer the As(V) tolerance and moderate As(III) tolerance to A. thaliana, respectively (15, 19).

After As enters the cell, As(V) is reduced to As(III) by a reductase. As(III) is excluded from the cell by ArsAB and Acr3p transporters in E. coli and yeast, respectively (8). In addition to exclusion, yeast has Ycf1p, an ABC transporter, which seques-
ters the As-glutathione complex into the vacuole (8). In plants, As-phytochelatin and As-glutathione complexes are found in As-treated plants (20), and the involvement of these complexes in As tolerance is being investigated.

Because the gene conferring moderate levels of As(III) tolerance to plants has been reported, we used a forward genetic approach to identify the gene(s) that confer strong tolerance to As(III) in A. thaliana. We isolated three As(III)-tolerant mutants by screening ethyl methanesulfonate (EMS)-mutagenized A. thaliana plants and identified a gene responsible for tolerance to As(III).

EXPERIMENTAL PROCEDURES

Plant Growth Conditions and Media—A. thaliana ecotype Col-0 was obtained from laboratory stock and was used for the physiological experiments. The seeds were surface-sterilized with bleach and sown onto half-strength Murashige-Skoog (MS) medium solidified with 1.2% Gellan Gum (Wako, Osaka, Japan) supplemented with 1% sucrose. The pH of the medium was adjusted with 0.05% MES and KOH to 5.7. For the arsenite trioxide treatment, arsenite trioxide (Wako) was added to the medium at the concentrations shown in individual experiments.

Physiological experiments. The seeds were surface-sterilized with bleach and sown onto half-strength MS medium containing 15 μM As(III). After incubation for 2 days at 4 °C, the plates were placed vertically, and the plants were grown at 22 °C for 7 days under a 16-h light/8-h dark photoperiod. The plants were washed with distilled water three times, dried at 65 °C for more than 2 days, and subjected to As determination with ICP-MS as described below.

As Accumulation in Plants—Seeds were sown on the half-strength MS medium containing 5 μM As(III). After incubation for 2 days at 4 °C, the plates were placed vertically, and the plants were grown at 22 °C for 7 days under a 16-h light/8-h dark photoperiod. The plants were washed with distilled water three times, dried at 65 °C for more than 2 days, and subjected to As determination with ICP-MS as described below.

Determination of As Concentration using ICP-MS—The samples, including oocytes and dried plants, were digested with concentrated HNO₃ at 110 °C. After complete digestion, the sample was dissolved in 0.08 N HNO₃ containing 10 ppb Ge.

72Ge was used as an internal standard. The mass 75 was monitored as the As signal. To remove the contribution from 40Ar35Cl, 77Se and 82Se were simultaneously monitored and the actual As signal was calculated with the equation given in the EPA 200.8 method (23).

Promoter-GUS Analysis—The DNA fragment corresponding to the promoter region of NIP1;1 was amplified with PCR using genomic DNA as template and the primers 5'-CGATT-TCCCGTGATCCGGTGCCAC-3' and 5'-GGTGCAC-TCCGGAGATCCGGCCATAGTGCAC-3' (Sall recognition site underlined). The amplified DNA fragment was digested with Sall, inserted into pENTR2B at the XmnI-Sall sites, and then transferred to the destination vector pMDC139 (24) using LR clonase (Invitrogen). Col-0 plants were transformed with the Agrobacterium-mediated floral dip method. GUS staining was performed as described previously (25).

Determination of Subcellular Localization using GFP—A GFP-NIP1;1 fusion gene was constructed as described below.
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NIP1;1 was amplified by PCR using the primers 5'-GGAATT-
CATGCGGATATCTCGGG-3' and 5'-GAGAGTCGACT-
CAATGTCTACCCATGTTCA-3' (underlines indicate EcoRI 
and Sall recognition sequences, respectively). The resulting 
DNA fragment was digested with EcoRI and Sall and inserted 
into pENTR2B (Invitrogen, Tokyo, Japan) at the EcoRI-XhoI sites 
and then transferred to the binary vector pMDC45 (24) 
using LR clonase (Invitrogen). nip1;1-1 plants were trans-
formed with the Agrobacterium tumefaciens-mediated floral 
dip method. After selection with hygromycin, GFP fluores-
cence of resistant plants was observed with fluorescence 
microscopy in the T1 generation to select the fluorescence-
emitting transgenic plants. The T2 generation was used for 
fluorescence observations using confocal laser-scanning 
microscopy.

RESULTS

Screening for As(III)-tolerant Mutants—To obtain insights 
into As(III) tolerance mechanisms, the EMS-mutagenized M2 
population of A. thaliana was screened for As(III)-tolerant 
mutants. We chose 15 μM As(III) as the selection condition. 
At this concentration, the root length of the wild type was 20% of 
that of plants growing on the As(III) minus medium after a 
10-day incubation. Approximately 30,000 M2 seeds were sown 
onto half-strength MS medium containing 15 μM As(III) and 
grown vertically for 10 days. Plants with roots more than two 
times longer than the wild type were selected. After confirma-
tion of the phenotype in the M3 generation, three independent 
mutants, 7-1, 9-1, and 10-1, were obtained (Fig. 1A). These 
were derived from independent batches of EMS-mutagenized M2 
populations. These mutant roots were more than three times 
longer than those of the wild type in the presence of 10, 15, and 
30 μM As(III), at which concentration the growth of wild-type 
roots was strongly inhibited (Fig. 1B).

The F2 population from crosses between Ler and 7-1 mutants 
was sown onto medium containing 15 μM As(III) and segre-
gated into tolerant, weakly tolerant, and sensitive phenotypes at 
a ratio of 1:2:1 (Fig. 1C). The same phenotype was observed in 
the populations of 9-1 and 10-1 (data not shown). These results 
indicate that the As(III)-tolerant phenotype is caused by a sin-
gle mutation, and the mutated alleles are all semidominant.

Identification of NIP1;1 as a Causal Gene for As(III) Tolerance—
We speculated that aquaglyceroporins might be involved in 
As(III) tolerance based on previous studies (9, 10, 11, 12, 17, 19). 
Among the NIP family in plants, some members of nodulin 
26-like intrinsic proteins (NIPs), which are classified as aquag-
lyceroporins in plants (26), were shown to transport small neu-
tral molecules including boric acid and silicic acid (21, 27). 
As(III) is also a small neutral molecule, and we speculated that 
As(III) tolerance may be due to mutation(s) in a NIP gene. In the 
A. thaliana genome, there are nine NIP genes (28), and we 
obtained seven T-DNA insertion mutants of NIPs (NIP1;1, 
nip1;1-1, NIP1;1-2, NIP3;1, NIP4;1, NIP5;1, NIP6;1, and NIP7;1) and exam-
ined their As(III) tolerance. Among NIP mutants examined, 
only NIP1;1 T-DNA insertion mutants (nip1;1-1, nip1;1-1) exhibited high tolerance to 15 μM As(III) (Fig. 2A, supplemental 
Fig. S1). In contrast, transgenic plants carrying T-DNA in 
NIP1;2, which is the most similar to NIP1;1 in terms of amino 
acid sequences among the NIP family members, did not show 
As(III) tolerance. Similarly, transgenic plants carrying T-DNA 
in NIP5;1, which mediates boric acid uptake from soil (21), also 
did not show As(III) tolerance (Fig. 2A). Furthermore, NIP7;1, 
which confers As(III) tolerance (19) did not confer strong tol-
erance in our experimental conditions (supplemental Fig. S1). 
As shown in Fig. 2B, nip1;1-1 and nip1;1-2 have T-DNA inser-
tions in the third intron and the fifth exon, respectively. The 
mRNA accumulation level was very low or not detected in nip1; 
1-1 and nip1;1-2, respectively, indicating that nip1;1-1 and 
nip1;1-2 are very strong and null alleles, respectively (Fig. 2C).

Because nip1;1 showed high tolerance to As(III), NIP1;1 genomic 
sequences of the As(III)-tolerant mutants 7-1, 9-1, and 
10-1 were determined. All of the mutants had a single nucleo-
tide substitution in NIP1;1 (Fig. 2B). Lines 7-1 and 9-1 mutants 
had mutations in the third and second exons, respectively. 
These mutations caused amino acid substitutions in the pro-
teins Gly63 to Glu63 and Thr188 to Ile188, respectively. The line 10-1 mutant had a mutation in the fourth exon/intron junction, which resulted in a larger transcript than the wild type (Fig. 2 D).

The sequence analysis of the RT-PCR product of 10-1 indicated that the third intron was not spliced out (data not shown). The third intron contains an in-frame stop codon, and the NIP1;1 transcript of the 10-1 line is likely to produce a short and abnormal protein.

To further confirm that NIP1;1 is the causal gene of As(III)-tolerance, mapping analysis was carried out using F2 population from crosses between Ler and As(III) tolerant mutants. Markers on chromosome 4 showed the strong linkage to As(III) tolerance phenotype, and the mutation was located between markers F24G24 and F9D16 (supplemental Fig. S2). NIP1;1 is located in this interval, further supporting that NIP1;1 is the causal gene of the phenotype.

NIP1;1 transcript accumulation was at a similar level among Col-0, 7-1, and 9-1 (Fig. 2 E). In 10-1, NIP1;1 transcript accumulation was reduced to 25% of that of Col-0 (Fig. 2 E). Taken together, these results establish that NIP1;1 is the causal gene for As(III)-tolerant mutants, and disruption of NIP1;1 function makes the plant tolerant to As(III).

NIP1;1 Transport As(III)—The fact that disruption of NIP1;1 confers strong As(III) tolerance suggests that NIP1;1 is an As(III) transporter that mediates toxic As(III) uptake into roots. To directly confirm the As(III) transport activity of the protein, we used a X. laevis oocyte expression system. A time course experiment showed that oocytes expressing NIP1;1 accumu-
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lated several times more As(III) than the control (Fig. 3A), demonstrating that NIP1;1 is capable of transporting As(III).

We also measured the transport activity of the mutant proteins corresponding to 7-1, 9-1, and 10-1 lines (Fig. 3B). The content of As(III) in oocytes expressing mutant proteins was similar to that of the water-injected oocytes, suggesting that all mutant proteins [7-1 (G63E), 9-1 (T188I), and 10-1] lost transport activity (Fig. 3B), and that Gly\(^{63}\) and Thr\(^{188}\) are important amino acid residues for the protein to function as an As(III) transporter. The lack of transport activity of the mutant alleles of NIP1;1 corresponded with the As(III) tolerance phenotype of the mutant plants (Fig. 1). We also measured the transport activity of NIP1;2 and NIP5;1 in oocytes (Fig. 3B). As(III) transport activity was detected in both proteins.

As Concentrations Were Reduced in nips Mutants—To examine the transport activity of NIPs in vivo, we measured As concentrations in the nip mutants. The mutant plants were grown vertically on plates containing 5 \(\mu\)M As(III) for 7 days, and As content in whole seedlings was determined. As shown in Fig. 3C, As concentrations in the nip1;1, nip1;2, and nip5;1 mutant plants were significantly lower than those in Col-0 plants. Taken together with the oocyte experiment, NIPs is active in As(III) transport in plants.

Tissue Specificity of NIP1;1 Expression—To identify tissue specificity of expression in detail, a NIP1;1 promoter region (−2317 bp to +18 from the first ATG) was fused in-frame with the GUS (β-glucuronidase) gene. Four independent transgenic lines were generated, and GUS staining patterns were observed in the T2 generation. GUS staining was observed both in shoots and roots. The staining in leaves was observed mostly in stomata (Fig. 4, A and B). The root-hypocotyl junction, especially in the root region, showed strong GUS activity. In roots, the patterns differed between primary and lateral roots. In lateral roots, both root tips and steles were stained, whereas in primary roots, steles but not root tips were stained (Fig. 4, D and E). The pattern was consistent among the four lines. We also determined mRNA accumulation in shoots and roots using real-time PCR. The NIP1;1 mRNA in roots was 20 times higher than that in shoots (Fig. 4F).

Subcellular Localization of NIP1;1—To identify the subcellular localization of NIP1;1, GFP-NIP1;1 fusion protein was expressed in the nip1;1-1 mutant under the cauliflower mosaic virus 35S-RNA promoter. About 50 independent lines were generated, and among these, GFP fluorescence was observed in 15 lines. These lines were subjected to the As(III) tolerance assay. Ten lines showed As(III)-sensitive phenotypes, and two of them (Nos. 7 and 8) were used (Fig. 5E). Sensitive phenotypes of the transgenic lines imply a complementation of the nip1;1-1 mutant, suggesting that GFP-NIP1;1 in these lines was functional (Fig. 5E). We also generated the transgenic plants carrying GFP-NIP1;1 construct as well as plants carrying NIP1;1-GFP construct under the control of the native NIP1;1 promoter. However, no fluorescence was observed after checking more than 20 lines for each construct (data not shown).

Subcellular localization of GFP fluorescence was observed with a confocal laser-scanning microscope, and two lines showed a similar fluorescence pattern (data not shown). In root tips, GFP fluorescence was observed in the cell periphery (Fig. 5, A and B).
A and B). This fluorescence pattern is different from that of tonoplast-localized proteins (29, 30). Vacuoles in cells near the root tips are observed as several vesicles. In the case of GFP-NIP1;1, we never observed such vesicle patterns, suggesting that GFP-NIP1;1 is localized to the plasma membrane. After plasmolysis treatment, the fluorescence mostly associated with plasma membrane, not with the cell walls (Fig. 5, C and D), further suggesting that GFP-NIP1;1 is localized to plasma membrane.

Interestingly, the fluorescence was observed at the distal side of the epidermal cells (Fig. 5, A and B, inset). Furthermore, GFP fluorescence was observed only in outer cell layers, even though it is driven by the 35S promoter, which is active in all cell layers in roots. It is likely that NIP1;1 transcript accumulation is differentially and post-transcriptionally regulated in a cell type-dependent manner.

**DISCUSSION**

In the present study, we demonstrated that NIP1;1 is the key determinant of As(III) tolerance in *A. thaliana*. We isolated three independent As(III) tolerant mutants in terms of root elongation by screening of 30,000 EMS-mutagenized M2 plants. All of them had mutations in NIP1;1 (Figs. 1 and 2). Moreover, nip1;1 mutant plants were highly tolerant to As(III), suggesting that NIP1;1 is the key determinant of As(III) tolerance. Although it has been reported that nip7;1 mutants are moderately tolerant to As(III) (19), the level of As(III) tolerance of nip1;1 mutant plants is much higher than that of nip1;1 under our experimental conditions (Fig. 2A and supplemental Fig. S1), suggesting that NIP1;1 is dominant over NIP7;1 in the determination of As(III) tolerance in *A. thaliana*.

NIP1;1 is able to transport As(III) (Fig. 3, A and B) and is localized to the plasma membrane in roots (Fig. 5). These data strongly suggest that NIP1;1 is involved in As(III) uptake from the medium into the roots. However, As(III) content in nip1;1 mutant plants was reduced to 70% of that of wild-type plants (Fig. 3C). It is possible that As(III) transport systems other than NIP1;1 account for 70% of As(III) uptake into roots. NIP1;2 and NIP5;1 are among the candidate genes for As(III) uptake based on the result of oocyte experiment (Fig. 3B). In addition, two molecular biological analyses have recently demonstrated that *A. thaliana* NIPs are involved in As(III) transport. One is by Bienert et al. (18), who showed that NIP5;1 and NIP6;1 are able to transport As(III) and Sb(III) using a yeast expression system. The other is by Isayenkov and Maathuis (19), who showed that T-DNA mutants of NIP7;1 are moderately tolerant to As(III) and have a reduced As(III) contents compared with wild type, and that NIP7;1 has As(III) transport activity in yeast. Taken together, NIP1;2, NIP5;1, NIP6;1, and NIP7;1, are major candidates for As(III) transport systems in *nip1;1* mutant plants. In the present study, we determined the As(III) concentrations in nip1;2 and nip5;1 mutant plants. As shown in Fig. 3C, disruption of NIP1;2 and NIP5;1 resulted in 10–15% reduction of As in plants, suggesting that these two genes are likely candidates for As(III) transport systems in *nip1;1* mutant plants.

Alternatively, the modest reduction in As content in *nip1;1* mutant plants can be explained if we assume a large part of As exists in the apoplastic space rather than the symplast. The cell wall may contribute to trap apoplastic As(III), which accounts for the lack of a drastic reduction in As(III) content in *nip1;1* mutant plants as well as *nip1;2* and *nip5;1* plants.

It is also intriguing that As(III) tolerance was observed in *nip1;1* mutant plants, while the reduction of As(III) contents in *nip1;1* mutant plants is 30%. As(III) tolerance was not observed in *nip1;2*, *nip5;1*, *nip6;1*, and *nip7;1* (Fig. 2A and supplemental Fig. S1) grown with 15 μM As(III), although all of them have the ability to transport As(III) (Refs. 18 and 19; Fig. 3B). Furthermore, a T-DNA insertion mutant of NIP5;1, which is expressed in roots and is localized to the plasma membrane (21), did not show As(III) tolerance (Fig. 2A). The expression level of NIP5;1 in roots was higher than that of NIP1;1 according to the MPSS database. These results suggest that the decrease in As(III) uptake is not the only reason for the high As(III) tolerance observed in *nip1;1* mutant plants.

One possible reason for the lack of As(III) tolerance in *nip1;2* and *nip5;1* is due to the cell type specificity of expression. NIP1;1 might be expressed in cells that are directly or indirectly important for root growth, where the expression levels of other NIP genes are not detected or very low. Actually, the expression pattern of NIP5;1 is in contrast to that of NIP1;1. NIP5;1 is expressed in epidermal, cortical, and endodermal cell, but weakly in stele (21).

Another possible explanation is that the disruption of NIP1;1 causes an altered distribution of physiological substrate in roots, which may lead to As(III) tolerance through some unidentified mechanisms. One candidate substrate is glycerol. It has been shown that NIP1;1 is able to transport glycerol in yeast (31). Physiological functions of glycerol in plants are less understood, but it has been suggested that glycerol is a component of cutin and suberin (32), which are hydrophobic cell wall barriers. Although the thickening of suberin layer in *nip1;1* mutant plants was not evident (data not shown), disruption of NIP1;1 may change a cell wall property and may lead to the prevention of As(III) influx into roots.

In conclusion, we have identified the key gene that determines As(III) tolerance in roots. Aquaglyceroporins probably exist in all plant species and have As(III) transport ability, because the As(III) transport activity have so far been observed in all aquaglyceroporins, regardless of species. In the course of writing this report, Ma et al. (17) showed that the silicic acid transporter Lsi1 (OsNIP2;1) mediates As(III) uptake from soils and that disruption of each gene reduces the As content in plants, although its contribution to As accumulation in rice grains is small. NIP genes could be used as a molecular marker for As(III) tolerance in crops, then they can be directly used to produce As-tolerant hybrid crops.

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