**The aquaporin OsNIP3;2 is involved in arsenite uptake by rice roots**

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**Summary**

* Previous studies have shown that the Nodulin 26-like (NIP) aquaporin protein Lsi1 (OsNIP2;1) is involved in arsenite [As(III)] uptake in rice. However, the function of other rice NIPs remains unknown.
* We analysed the permeability of OsNIP3;2 to As(III) in *Xenopus laevis* oocytes. The expression pattern and subcellular localization of OsNIP3;2 were determined using quantitative RT-PCR, promoter-GUS (β-glucuronidase) assay and transient expression of OsNIP3;2:GFP in rice and tobacco protoplasts. As(III) uptake and As distribution in the roots of the *OsNIP3;2* knockout mutants and wild type were investigated.
* OsNIP3;2 was permeable to As(III) when expressed in oocytes. The expression of *OsNIP3;2* was suppressed by 5 µM As(III), but enhanced markedly by 20 and 100 µM of As(III). *OsNIP3;2* was predominantly expressed in the lateral roots and the stele region of the primary roots. The protein was located on the plasma membrane. Knockout of *OsNIP3;2* significantly decreased As concentration in the roots but had little effect on shoot As concentration. Synchrotron µ-XRF showed decreased As accumulation in the stele of the lateral roots in the mutants compared with wild type.
* OsNIP3;2 is involved in As(III) uptake by rice lateral roots, but its contribution to As accumulation in the shoots is limited.

**Key words:** aquaporin, arsenic, arsenite, lateral root, NIP, rice,

**Introduction**

Arsenic (As) is a metalloid well known for its toxicity to human and animals. Exposure to excessive As causes numerous diseases, such as cancers, diabetes, cardio vascular disease and developmental disorders (Abdul *et al.*, 2015). In south and southeast Asia, millions of people have suffered from As poisoning due to elevated levels of As in the drinking water (Brammer & Ravenscroft, 2009). In these regions, As-laden groundwater has been extracted to irrigate rice crops, resulting in the elevation of As in the soil and rice grain (Meharg & Rahman, 2003; Dittmar *et al.*, 2010). Rice is the staple food for approximately half of the world population, for which rice consumption constitutes a major dietary source of inorganic As (Kile *et al.*, 2007; Li *et al.*, 2011).

Compared with other cereal crops, paddy rice is about 10 fold more efficient at accumulating As (Williams *et al.*, 2007). This is caused by the mobilization of arsenite [As(III)] in flooded paddy soil (Xu *et al.*, 2008) and the highly efficient uptake of As(III) by the silicon (Si) pathway in rice (Ma *et al.*, 2008). Due to the similarity in the physiochemical properties between arsenous acid and silicic acid, the rice Si transporters Lsi1 and Lsi2 are able to transport As(III) as well (Ma *et al.*, 2008). Lsi1 and Lsi2 act as the influx and efflux transporters, respectively, which are polarly localized on the distal and proximal sides of both exodermis and endodermis in rice roots and form a one-way transport system (Ma *et al.*, 2006, 2007). This localization pattern facilitates the transport of the substrates such as Si(OH)4 and As(III) through the Casparian bands into the stele for xylem loading.

Lsi1 (OsNIP2;1) belongs to the Nodulin 26-like membrane intrinsic proteins (NIPs) subfamily of the major intrinsic protein (MIP or aquaporin) super family (Perez Di Giorgio *et al.*, 2014). Aquaporins are passive transporters which allow small neutral molecules such as water, glycerol and urea to pass the channel following the osmotic or concentration gradient. All aquaporin proteins share a highly conserved structure with six alpha-helical hydrophobic helices, five inter-helical loops and two additional membrane-embedded domains, namely two NPA (Asn-Pro-Ala) boxes in loop B and E, and an ar/R (aromatic/arginine) filter (Forrest & Bhave, 2007; Ludewig & Dynowski, 2009). The two NPAs are important for water permeability (Benga, 2012; Kosinska Eriksson *et al.*, 2013) whereas the ar/R constriction region is the narrowest point of the entire channel which determines the selectivity by size exclusion. While almost all NIP aquaporins in rice have the identical two NPA motifs (except for OsNIP3;1 which carries a NPV and a NPS), the ar/R compositions are different.

There are 10 and 9 genes encoding NIP aquaporins in the rice and *Arabidopsis thaliana* genomes, respectively (Maurel *et al.*, 2015). NIP aquaporins can be divided into three subfamilies (Mitani *et al.*, 2008) based on the structure of the ar/R filter formed by two residues from helix 2 (H2) and helix 5 (H5), as well as two residues from loop E (LE1 and LE2). However, NIPs have also been grouped into four (Abascal *et al.*, 2014), five (Danielson & Johanson, 2010), or six (Soto *et al.*, 2012) subgroups in different phylogeny studies. In this paper we follow the three subgroup assignment of Mitani *et al.* (2008). The ar/R filters of the subgroups I, II and III are composed of WV(I)AR, AI(V)G(A)R and GSGR, respectively. The permeability to Si appears to be restricted to the subgroup III members Lsi1 and Lsi6 (OsNIP2;2) (Ma *et al.*, 2006; Mitani *et al.*, 2008; Yamaji *et al.*, 2008), whereas the subgroup II members AtNIP5;1, AtNIP6;1 and OsNIP3;1 have been identified as boric acid transporters (Takano *et al.*, 2006; Tanaka *et al.*, 2008; Hanaoka *et al.*, 2014). In contrast, many members of all three subgroups appear to be permeable to As(III) (Isayenkov & Maathuis, 2008; Kamiya *et al.*, 2009; Zhao *et al.*, 2009; Mitani-Ueno *et al.*, 2011; Mukhopadhyay *et al.*, 2014), suggesting a less stringent structural requirement for As(III) permeability than for Si and boron (B) permeability. Despite the widespread As(III) permeability, to date only Lsi1 among the rice NIP proteins has been shown to be involved in As(III) uptake in rice (Ma *et al.*, 2008). The role of other rice NIP aquaporins in As(III) uptake remains untested.

In this study, we investigated the role of the subgroup II NIP member OsNIP3;2 (LOC\_Os08g05590) in As(III) uptake by rice roots. Its permeability to As(III) and Si(OH)4 was tested in *Xenopus laevis* oocytes. The gene expression in roots under different concentrations of As(III) was studied by quantitative real time RT-PCR (Q-PCR). Promoter-GUS (β-glucuronidase) and GFP-tagged protein were used to study the expression pattern of the gene and the subcellular localization of the protein. Rice mutants of *OsNIP3;2* were used to study As(III) root uptake combining of inductively coupled plasma mass spectrometry (ICP-MS) and synchrotron µX-ray Fluorescence (µ-XRF) mapping.

**Materials and Methods**

**Plant Materials and Culture**

Rice (*Oryza sativa* cv. Nipponbare) plants were grown in hydroponics either in a glasshouse (photoperiod 12 hr, light intensity 350 µmol m-2 s-1, day/night temperatures 25°C/20°C, 70% relative humidity) or in a growth cabinet (photoperiod 12 hr, light intensity 300 µmol m-2 s-1, day/night temperatures 27°C/20°C, 80% relative humidity). The ½ strength Kimura nutrient solution was used, with the following composition: 0.091 mM KNO3, 0.183 mM Ca(NO3)2, 0.274 mM MgSO4, 0.1 mM KH2PO4, 0.183 mM (NH4)2SO4, 0.5 µM MnCl2, 3 µM H3BO3, 0.1 µM (NH4)6Mo7O24, 0.4 µM ZnSO4, 0.2 µM CuSO4, 20 µM NaFe(III)-EDTA, and 2 mM MES (pH adjusted to 5.5 with NaOH).

**cRNA synthesis and uptake assays in *Xenopus laevis* oocytes**

The open reading frame (ORF) of *OsNIP3;2* was cloned by PCR from rice (*Oryza sativa*, cv. Nipponbare) root cDNA with following primers: forward 5’-GAAGATCTATGGAAGGGGGCAAGATGAGC-3’ and reverse 5’-GGACTAGTCTACAGCTTGATTGCAAAAT-3’. The PCR product was digested with *Bgl*II/*Spe*I and ligated into the oocyte expression vector pT7TS/FLAG generated from pT7TS (Tong *et al.*, 2005) by adding a FLAG tag before and in frame with the cloning site of *Bgl*II. The construct was sequenced (Eurofins) and confirmed. *Lsi1* cDNA in vectors pXßG-ev1 (Ma, J. F. *et al.*, 2008) was provided by Professor Jian Feng Ma from Okayama University, Japan. Capped mRNA (cRNA) was synthesized using T7 (*OsNIP3;2*) and T3 (*Lsi1*) mMESSAGE mMACHINE *in vitro* transcription kit (Ambion) respectively following the manufacturer’s instructions.Oocytes of *Xenopus laevis* were removed and treated as described previously (Zhou *et al.*, 1998). Stage V or VI oocytes were chosen for injection with 50 nl of cRNA (1 µg µl-1) or equal volume of RNase-free water as a negative control. The injected oocytes were incubated in Modified Barth Solution (MBS) for two days before assays. cRNA and water injected oocytes were exposed to 100 µM of As(III) for 60 min or 100 µM of Ge(OH)4 (as the analogue of Si(OH)4 (Nikolic *et al.*, 2007)) for 30 min respectively in MBS (pH7.4). The oocytes were washed six times in MBS and digested with HNO3 at 90°C. As and Ge concentrations in the digests were determined by inductively coupled plasma mass spectrometry (ICP-MS, NexION 300, PerkinElmer). The ar/R pore regions were modelled using 'DeepView PdbViewer' v 3.7 (<http://www.expasy.org/spdbv/>) with the A subunit of the Spinach SoPIP2;1 (ID 110537, PDB code 4IA4) served as a template.

**Quantitative real time RT-PCR**

Three week rice seedlings (cv. Nipponbare) grown in hydroponics were exposed to different concentrations (0 - 100 µM) of As(III) for different durations. Each treatment included three biological replicates with two plants in each replicate. Total root RNA was isolated with RNeasy Plant Mini Kit (Giagen) following the manufacturer’s instructions. cDNA was synthesized with SuperScript® III Reverse Transcriptase (Invitrogen) and Q-PCR was performed on a C1000TM Thermal Cycler system (BIO\_RAD) with SYBR® Green JumpStart™ Taq ReadyMix™ (Sigma). Rice *HistonH3* was used as a reference gene. The primers used are as follows: *OsNIP3;2* forward 5’-GTGGCAGTCGGAGCAACAATCAT-3’ and reverse 5’-CTTCTCCAGCTACAGCACCAAGTG-3’; *Lsi1* forward 5’-ATCTACTTCCTGGGCCCAGT-3’ and reverse 5’-AGGAGAGCTTCTGGGAGGAG-3’; *Lsi2* forward 5’-ATCACCTTCCCCAAGTTCC-3’ and reverse 5’-CAGCTCCCTCCAGTACATGC-3’; *HistonH3* forward 5’-GGTCAACTTGTTGATTCCCCTCT-3’ and reverse 5’-AACCGCAAAATCCAAAGAACG-3’. Relative expression was calculated as previously described (Rieu & Powers, 2009).

**Generation of transgenic rice and detection of GUS expression**

A 2451 bp fragment of *OsNIP3;2* upstream sequence before the ATG was amplified from the Nipponbare genomic DNA using primers: forward 5’-TTAATTAATGGACAACGCAAATATTTCCT-3’ and reverse 5’-GGCGCGCCGTTCAAAGGCAAGCTACTAATCA-3’. The PCR product was ligated into *EcoR*V digested pBlueScript II SK(+) vector and the sequence was confirmed (Eurofins). The *OsNIP3;2* promoter was subcloned as a *Pac*I/*Asc*I fragment into a pS1aG-3 (Tang *et al.*, 2012) shuttle vector to replace the original *HvPht1;1* promoter before a GUS report gene. The construct was introduced in Nipponbare rice via Agrobacterium-mediated transformation as described previously (Tang *et al.*, 2012). Two independent transgenic lines were produced and analysed for GUS signal.

**Subcellular localization of OsNIP3;2**

To investigate the subcellular localization of OsNIP3;2, the *OsNIP3;2* full coding cDNA was cloned into pSAT6-EGFP-C1 (GenBank accession No. AY818377) vector between E*coR*I and H*ind*III. Then the 35S:eGFP:OsNIP3;2 fragment was sub-cloned into the vector pRCS2-ocs-nptII (GenBank accession No. DQ005456) with PI-PspI. The construct was transformed to *Agrobacterium* strain GV3101. Empty vector pCAMBIA-1302 (GenBank accession No. AF234298) with a 35S:GFP construct was used as a control. The two constructs were transformed into rice protoplasts using PEG-mediated transformation as previously described (Tang *et al.*, 2012). The red-fluorescent FM®4-64 dyes were used as a plasma membrane marker. 35S:eGFP:OsNIP3;2 and pCAMBIA-1302 were also transiently expressed in tobacco (*Nicotiana benthamiana*) leaf epidermal cells as described previously (Sparkes *et al.*, 2006). The protoplasts were observed using Confocal Laser Scanning Microscope (Zeiss).

**Pore modelling**

Ar/R selectivity regions of Lsi1 and NIP3;2 were modelled using 'DeepView PdbViewer' v 3.7 (http://www.expasy.org/spdbv/). The A subunit of the Spinach SoPIP2;1 (ID 110537, PDB code 4IA4) served as a template. Pore regions are shown perpendicular to the membrane with the apoplastic and cytoplasmic compartments located at the top and bottom of the diagram.

**Identification of *OsNIP3;2* mutants and As(III) uptake assays**

To analyse the function of OsNIP3;2 in plants, a T-DNA line *nip3;2-1 (*AAY E05, Flanking Sequence Tag GenBank accession No. CL517700) from Oryza Tag Line (http://oryzatagline.cirad.fr/index.htm), France and a *Tos17* line *nip3;2-2* (NE6003, Flanking Sequence Tag GenBank accession No. AG209363) from Genome Resource Center (https://tos.nias.affrc.go.jp/), Japan were obtained. Both lines were generated in the background of cv. Nipponbare. Genotyping of *nip3;2-1* and *nip3;2-2* was performed by PCR with two primers from genomic sequence (on two sides of the insertion point) and one primer from inserted sequences (used with one of the genomic primers). The primers used were as follows: *nip3;2-1* forward 5’-TTCCGCTTTTCCGAAATCCA-3’ and reverse 5’-TCCCTTTGACGGCGAACGAT-3’ on genomic sequence, a reverse primer 5’-GTCTGGACCGATGGCTGTGT-3’ on inserted sequence; *nip3;2-2* forward 5’-ATATACTTAACTAATCACATTGC-3’ and reverse 5’-AATTTACTTACTGCATTGGG-3’ on genomic sequence, a forward primer 5’-ATATACTTAACTAATCACATTGC-3’ on inserted sequence. RT-PCR was used to confirm the knock-out of *OsNIP3;2* using primers from *OsNIP3;2* coding sequence and span the insertion sites of both *nip3;2-1* and *nip3;2-2*. The primers used were forward 5’- TGTTACTGTCCCTCCCATGC-3’ and reverse 5’-CGAGGATTTGTGCAGCAATG-3’. RT-PCR was conducted on the cDNAs synthesized from root total RNAs of wild type (WT) and mutant plants.

*OsNIP3;2* mutants and the WT were grown in hydroponics with five replicates (two plants each pot with 900 ml of nutrient solution). The plants were exposed to different concentration of As(III) and Ge(OH)4 as shown in the Results sections. Shoots and roots were separated and dried at 60°C for three days.

**Soil pot experiment**

A soil pot experiment was conducted to investigate the effect of *OsNIP3;2* mutation on As accumulation in the above-ground tissues of rice. Due to insufficient seeds of *nip3;2-1*, only *nip3;2-2* and WT were used in the experiment. A paddy soil containing 12.5 mg kg-1 total As was collected from the experimental farm of Nanjing Agricultural University, China. Each pot was filled with 15 kg soil amended with sufficient amounts of basal nutrients. Soil was flooded with water to maintain a 2 - 3 cm layer of standing water above the soil surface. One plant each of the mutant and WT was planted into each pot. There were four replicates. Plants were grown in a net enclosure with natural sunlight and ambient conditions in Nanjing, China during June - October 2015. At maturity, rice plants were harvested, separated into different tissues and dried at 60°C for three days.

**Determination of As and Ge concentrations**

Plant samples were finely ground and digested with HClO4/HNO3 (15:85 v/v) (Zhao *et al.*, 1994). The As and Ge concentrations were determined by ICP-MS (NexION 300X, PerkinElmer). Blanks and certified reference materials (CRMs, rice flour NIST1568a and spinach leaves GBW10015) were included in the analysis. The total As concentrations obtained for the CRMs were 0.31 ± 0.007 and 0.23 ± 0.02 mg kg-1 (mean ± SD, n = 3), respectively, which were in good agreement with the certified values (0.29 ± 0.03 and 0.23 ± 0.03 mg kg-1 for rice flour and spinach leaves, respectively).

**Synchrotron µ-XRF**

Three-week old seedlings of Nipponbare, *nip3;2-1* and *nip3;2-2* were exposed to 10 µM As(III) for two weeks in a hydroponic culture. Segments of mature roots at approximately 4 cm from the root tip were cut and placed into a planchette coated with hexadecane. The samples were frozen at -196 °C with a pressure of 210 MPa for 30s using a Leica HPM100 high pressure freezer. The frozen samples were freeze substituted, embedded in resin and sectioned into 7 µm thickness as described previously (Moore *et al.*, 2014). The adjacent sections of 1 µm were stained with Toluidine blue for optical images. Synchrotron µ-XRF was undertaken at the Diamond Light Source (Unite Kingdom) on the I18 microfocus beamline. The incident X-ray energy was set to 12.4 keV using a Si(111) monochromator. The X-ray fluorescence spectra were collected using a Si drift detector. The beam size and step size were both 2 µm. Quantification of the concentrations of As and other elements of interest in the samples were carried out using an external calibration with XRF reference materials.

**Statistical analysis**

One-way ANOVAs were performed using GenStat 18th edition (VSN International, Hemel Hempstead, United Kingdom). Least significant difference (LSD) was used to compare the means of treatments or rice lines.

**Results**

**Permeability of OsNIP3;2 to As(III) and silicic acid**

Because the ar/R site in aquaporins is the narrowest constriction of the pore, it serves as the primary selectivity filter for rejection of large molecules (Forrest & Bhave, 2007). The four amino acid residues of the OsNIP3;2 ar/R filter (AAAR) are very different from those of Lsi1 (GSGR). The diameters of the ar/R sites of OsNIP3;2 and Lsi1 were estimated by simulations based on the X-ray structure of the spinach aquaporin SoPIP2;1 (Tornroth-Horsefield *et al.*, 2006). As shown in Fig. 1a, the narrowest parts in the channels of OsNIP3;2 and Lsi1 are both located between the residues in loop E (LE1 and LE2). The two pores were predicted to have the same diameter of 0.53 nm.

To compare the permeability of OsNIP3;2 and Lsi1 for As(III) and Si(OH)4 (using Ge(OH)4 as an analogue (Nikolic *et al.*, 2007)), *OsNIP3;2* and *Lsi1* were heterologously expressed in *Xneopus* *laevis* oocytes. Lsi1 was highly permeable to both As(III) and Ge(OH)4 (Fig. 1b and c), which was consistent with previous data (Ma *et al.*, 2006, 2008; Mitani *et al.*, 2008). OsNIP3;2 was also permeable to As(III), but the As(III) uptake was 20% lower than the oocytes expressing *Lsi1* (Fig. 1b). In contrast, OsNIP3;2 exhibited no permeability to Ge(OH)4 (Fig. 1c).

**Expression of *OsNIP3;2*** **is regulated by As(III)**

Q-PCR was used to monitor the responses of *OsNIP3;2* expression in rice roots (cv. Nipponbare) to different levels of As(III) exposure. *Lsi1* and *Lsi2* were also included for comparison. In the first experiment, plants were exposed to a relatively low level of 5 µM As(III) for between 1 h and 6 d. Compared with the control treatment, 5 µM As(III) decreased the expression of *Lsi1*, *Lsi2* and *OsNIP3;2* in the roots, although the effect was not significant at all time points measured (Fig. 2a - c). In both the control and 5 µM As(III) treatments, the expression level of *OsNIP3;2* was substantially lower than those of *Lsi1* and *Lsi2*.

In the second experiment, plants were exposed to toxic levels of 20 and 100 µM As(III) for 1 - 24 h. Compared with the control (no As(III)), the expression of both *Lsi1* and *Lsi2* in roots was severely repressed by both levels of As(III) at 6 h and 24 h (Fig. 2d and e). In contrast, the expression of *OsNIP3;2* was significantly enhanced by both 20 and 100 µM As(III) at 6 and 24 h (Fig. 2f). At 20 µM As(III), the *OsNIP3;2* transcript level was 3 and 2 times higher than that in the control at 6 and 24 h, respectively. At 100 µM As(III), the *OsNIP3;2* transcript level was 55 and 38 times higher than that in the control at 6 and 24 h, respectively.

**Expression pattern of *OsNIP3;2* in rice root**

The tissue expression pattern of *OsNIP3;2* was visualised in the roots of transgenic rice expressing the GUS reporter gene under the control of the *OsNIP3;2* promoter. The GUS activity was detected in the elongation and mature zone of primary roots, but not in the root tips (Fig. 3a). Further transverse and longitudinal sectioning studies showed that the gene is predominantly expressed within the stele region of the primary roots (Fig. 3b and c). Strong expression was also observed in the lateral roots (Fig. 3d), where the GUS signal was detected in most cell layers (Fig. 3e).

**OsNIP3;2 is localized at the plasma membrane**

To study the sub-cellular localization of OsNIP3;2, the GFP and OsNIP3;2 fusion protein under the control of a CaMV 35S promoter was transiently expressed in rice protoplasts. A GFP reporter driven by a CaMV 35S promoter was used as a control. The OsNIP3;2:GFP fluorescence signal was visualized at the plasma membrane, showing a co-localization with the plasma membrane marker FM®4-64, whereas the control GFP signal was observed in the cytoplasm (Fig. 4). In a further transient assay in tobacco (*N. benthamiana*) leaves, OsNIP3;2:GFP signal was also found to be localized on the plasma membrane (Fig. S1).

**The role of OsNIP3;2 in As(III) uptake by rice roots**

Two loss-of-function mutants*,* named *nip3;2-1* and *nip3;2-2*, respectively, were obtained. Alignment of flanking sequence of the two mutant lines with the genomic DNA sequence of *OsNIP3;2* confirmed that *nip3;2-1* and *nip3;2-2* carry a insertion at the first and the second exon respectively (Fig. 5a). Knock out of *OsNIP3;2* expression in the homozygote plants was confirmed by RT-PCR (Fig. 5a). The growth of the WT and the mutant plants were comparable in hydroponic experiments (Fig. 5b).

To investigate the function of *OsNIP3;2* with regard to As (III) uptake in plants, two hydroponic experiments were conducted. To minimize the effect of As(III) exposure on the *OsNIP3;2* transcript abundance in roots, the first experiment used 1 h exposure to 5 or 20 µM of As(III). At both levels of As(III) exposure, As concentrations in the roots of *nip3;2-1* and *nip3;2-2* were significantly lower than those of the WT plants, by 16 - 22% and 17 - 22% at 5 and 20 µM As(III), respectively (Fig. 5c). However, no significant difference was found in the shoot As concentrations. In the second experiment, plants were exposed to 5 µM As(III) and 10 µM Ge(OH)4 for 8 days. Similar to the first experiment, As concentrations in the mutant roots were significantly lower (by 23 - 25%) than the WT, whereas there was no significant difference in the shoot As concentration (Fig. 5d). There was also no significant difference between the mutants and WT plants in either root or shoot Ge concentrations (Fig. 5e).

**OsNIP3;2 mutants accumulate less As in the lateral roots than WT plants**

Synchrotron µ-XRF mapping was employed to image As distribution in the roots of *OsNIP3;2* mutants and WT after plants were exposed to 10 µM As(III) for 2 weeks. In WT plants, As was found to accumulate strongly in the stele of the primary roots, especially in the pericycle and the endodermis cells (Fig. S2). Fig. 6a shows a lateral root branching out from the primary root in a WT plant, exhibiting strong As accumulation in the stele of the primary root and in the lateral root. In the lateral roots of WT, As was also mainly accumulated in the stele. In contrast, As accumulation was greatly reduced in the lateral roots of both *nip3;2-1* and *nip3;2-2* (Fig. 6 b and c). The As concentrations in the scanned lateral root sections of WT, *nip3;2-1* and *nip3;2-2* reached a maximum of 791, 39 and 259 mg kg-1, respectively. In the stele region of the primary roots, no consistent difference between the WT and the mutants was found, although As in *nip3;2-1* appeared tobe distributed in a smaller region compared to WT (Fig. S2).

**Knock-out of *OsNIP3;2* has no significant effect on grain As accumulation**

To investigate if OsNIP3;2 contributes to As accumulation in rice grain, *nip3;2-2* and WT plants were grown in a soil pot experiment up to maturity. Arsenic concentration follows the order of leaf > stem > panicle > husk > grain. However, there was no significant difference between the mutant and WT in As concentrations in any of the above-ground tissues (Fig. 7). There was also no significant difference in the total biomass, tiller number and the number of fertile grain between the mutant and the WT (data not shown).

**Discussion**

Among the 10 members of the NIP family in the rice genome, until now only Lsi1 (OsNIP2;1) has been shown to play an important role in As(III) uptake in rice (Ma  *et al.*, 2008). Several other rice NIP aquaporins, including OsNIP3;2, are also permeable to As(III) when expressed heterologously in *Xenopus laevis* oocytes or yeast (Bienert *et al.*, 2008; Ma *et al.*, 2008). However, their role in As(III) uptake or translocation in rice has not been investigated.

In the present study, OsNIP3;2 was found to have a high permeability to As(III) when expressed in *Xenopus laevis* oocytes, corroborating the yeast data presented by Bienert *et al*. (2008). The As(III) permeability of OsNIP3;2 was only slightly lower than that of Lsi1 (Fig. 1). However, unlike Lsi1, OsNIP3;2 is not permeable to Ge(OH)4/Si(OH)4, even though the two aquaporins have similar pore size at the ar/R constriction site according to model simulations (Fig. 1), suggesting structural features other than the pore size may also be involved in determining the selectivity for substrates. Consistent with this observation, Mitani-Ueno et al. (Mitani-Ueno *et al.*, 2011) showed that changing the ar/R selectivity filter of AtNIP5;1, a B transporter, to that of Lsi1 did not result in a gain of the Si transport activity. Most plant NIP aquaporins tested to date show As(III) permeability, whereas Si permeability is restricted to a few NIP proteins (Bienert *et al.*, 2008; Ma *et al.*, 2008; Mitani *et al.*, 2008; Mitani-Ueno *et al.*, 2011), suggesting a less stringent structural requirement for the permeability of As(III) than for Si.

Different from *Lsi1* which is predominantly expressed in the exodermis and endodermis cells of rice roots, *OsNIP3;2* was found to be mainly expressed in the stele of the primary roots and in most cell layers in the lateral roots (Fig. 3). The tissue specificity of *OsNIP3;2* expression is more similar to that of the B transporter *OsNIP3;1*, although the latter is also expressed in the exodermis cells of the primary roots (Hanaoka *et al.*, 2014). At the subcellular level, OsNIP3;2 was found to be localized at the plasma membrane (Fig. 4), which is consistent with other rice NIP proteins (Ma & Yamaji, 2006; Yamaji & Ma, 2009; Hanaoka *et al.*, 2014). However, the transcript level of *OsNIP3;2* in the bulk root tissue was much lower than that of *Lsi1* or *Lsi2* (Fig. 2). There were also differences in their response to As(III) exposure. The expression of all three genes was down-regulated by exposure to a non-toxic level (5 µM) of As(III). At higher concentrations of As(III), the expression of *OsNIP3;2* was greatly increased, whereas both the *Lsi1* and *Lsi2* transcripts were greatly decreased. Down-regulation in the expression of these genes by As(III) exposure may be a feed-back response mechanism to limit As(III) uptake by roots. However, the reason for the greatly increased expression of *OsNIP3;2* at high As(III) concentrations remains unclear.

In short-term hydroponic experiments, As(III) accumulation in the roots of two *OsNIP3;2* knockout mutants was found to be significantly lower than WT (Fig. 5), suggesting that OsNIP3;2 plays a role in As(III) uptake into rice roots. However the contribution of OsNIP3;2 was modest, with *c.* 20% decrease in the mutants compared with WT. This effect is smaller than that previously reported for Lsi1 with a decrease of *c.* 50% in As accumulation in the roots of the mutant (Ma *et al.*, 2008). Furthermore, *OsNIP3;2* mutation did not affect the accumulation of Ge, an analogue of Si, which is consistent with the transporter not being permeable to Ge(OH)4/Si(OH)4 when expressed in *Xenopus laevis* oocytes.

Synchrotron µ-XRF mapping showed strong accumulation of As in the stele region of the primary roots (Fig. S2), which is consistent with previous studies on rice, wheat and cowpea (Moore *et al.*, 2011; Kopittke *et al.*, 2012, 2014). Using high-resolution secondary ion mass spectrometry (NanoSIMS), Moore *et al.* (2011) further showed that As was preferentially sequestered in the vacuoles of the pericycle and endodermal cells of rice roots. In the present study, strong As accumulation in the stele region of the lateral roots and in the junction where lateral roots branching out from the primary roots was also observed (Fig. 6a). Furthermore, *nip3;2* mutants were found to accumulate lower levels of As in the steles of the lateral roots (Fig. 6c). This result, together with the observation that *OsNIP3;2* is strongly expressed in the lateral roots (Fig. 3), suggests that OsNIP3;2 plays a role in As(III) uptake in the lateral roots. However, mutation in *OsNIP3;2* did not significantly affect As accumulation in the shoots in either hydroponic or soil pot experiments (Fig. 5 and 7). This may imply that As(III) taken up by lateral roots is not efficiently translocated to the shoots. The study by Ma *et al.* (2008) showed that the As(III) efflux towards the stele mediated by Lsi2 plays a more important role in controlling As(III) accumulation in rice shoots than the As(III) influx mediated by Lsi1. The observed down-regulation of Lsi2 by As(III) exposure (Fig. 2) may further restrict the translocation of As(III) from the roots to the shoots. Therefore, it is perhaps not surprising that mutation in *OsNIP3;2* did not significantly affect As(III) concentrations in the shoots.

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**Figure Legends**

**Fig. 1** OsNIP3;2 is able to transport As(III) . (a) Comparison of the ar/R pore size and structure of Lsi1 and OsNIP3;2. The narrowest part of the pore is indicated by yellow bars. AA residue colour coding: white: carbon backbone; red: oxygen; dark blue: nitrogen; light blue: hydrogen. (b) and (c) As and Ge concentrations in cRNA injected oocytes exposed to 100 µM of As(III) and Ge(OH)4 respectively. Values = mean ± SE (n=3). \* P<=0.05, \*\* P<=0.01 (ANOVA test with the H2O injected control)

**Fig. 2** Regulation of *Lsi1*, *Lsi2* and *OsNIP3;2* expression by As(III). Nipponbare seedling roots grown in hydroponic were exposed to different concentrations of As(III) respectively. Relative expression of *Lsi1* (a and d)*,* *Lsi2* (b and e)*,* and *OsNIP3;2* (c and f) was shown in values = mean ± SE (n=3). \* P<=0.05, \*\* P<=0.01 (ANOVA test between without (0 µM) and with As(III) (5, 20 and 100 µM) respectively)

**Fig. 3** Expression pattern of *OsNIP3;2* in rice root. A 2451 bp promoter region was cloned before a GUS reporter gene and the construct was introduced into rice (Nipponbare). (a) Primary root. (b) Transverse and (c) Longitudinal section of the primary root in (a). (d) Primary root with lateral roots developed. (e) Transverse section of a lateral root. Bar (a) = 1 mm, Bar (b) and (c) = 100 µm, Bar (d) = 2 mm, Bar (e) = 200 µm. C: cortex, EN: endodermis, EP: epidermis, EX: exodermis, S: stele, SC: sclerenchyma

**Fig. 4** OsNIP3;2 was localized on plasma membrane. GFP reporter gene was fused in frame with OsNIP3;2 driven by a CaMV 35S promoter. The construct was expressed transiently in rice (Nipponbare) stem protoplast. FM®4-64 dye was used as a marker for plasma membrane. Bar = 10 µm

**Fig. 5** Knock-out of OsNIP3;2 reduced the As accumulation in root in rice. (a) Schematic diagram of *OsNIP3;2* mutants and knock-out of *OsNIP3;2* in *nip3;2-1* and *nip3;2-2* by RT-PCR. (b) Comparison of the dry weight of six week old Nipponbare and *nip3;2* plants grown in hydroponics. (c) As concentrations of two week rice seedlings exposed to 5 and 20 µM of As(III) for 1 h in nutrient solution respectively. As (d) and Ge (e) concentrations in shoot and root of two week seedlings exposed to 5 µM of As(III) and 10 µM of Ge(OH)4 in nutrient solution for eight days. Values = mean ± SE (n=5). \* P<=0.05, \*\* P<=0.01 (ANOVA test compared to Nipponbare)

**Fig. 6** Synchrotron µ-XRF mapping of As in lateral root cross sections. Plants were exposed to 10 µM As(III) for 2 weeks in hydroponic solution. Root sections at approximately 4 cm from the tip were cut and prepared with high pressure freezing, freeze substitution and embedded in resin. Samples for synchrotron were sectioned at 7 µm thickness and adjacent 1 μM sections were stained with Toluidine blue for optical images. Synchrotron µ-XRF was performed with a step size = 2 µm and X-ray fluorescence was detected using a silicon drift detector. (a) Stained Nipponbare root section and quantification of arsenic accumulation in the squire region. (b) Stained optical images of lateral root sections. (c) Quantification of arsenic accumulation across the lateral sections close to (b). NB, Nipponbare. Scale bars in (a) = 100 μm, (b) and (c) = 50 μm. EN: endodermis, EP: epidermis, EX: exodermis, SC: sclerenchyma

**Fig. 7** As concentrations of different tissues in rice grown in soil. Values = mean ± SE (n=4)

**Supporting information**

**Fig. S1** Subcellular localization of OsNIP3;2 in tobacco leaf protoplast. OsNIP3;2:GFP fusion protein was expressed transiently in tobacco (*N. benthamiana*) leaf protoplast driven by a CaMV 35S promoter. Bar = 10 µm

**Fig. S2** Stained optical images and synchrotron µ-XRF quantification of arsenic in rice primary root cross sections. Scale bars = 100 μm. C: cortex, EN: endodermis, EP: epidermis, EX: exodermis, MXI: early metaxylem vessel, MX II: late metaxylem vessel, P: pericycle, SC: sclerenchyma