

The *Arabidopsis thaliana* aquaglyceroporin AtNIP7;1 is a pathway for arsenite uptake

Stanislav V. Isayenkov, Frans J.M. Maathuis*

Biology Department Area 9, University of York, York YO10 5DD, United Kingdom

Received 5 March 2008; revised 11 April 2008; accepted 11 April 2008

Available online 22 April 2008

Edited by Julian Schroeder

Abstract We studied the effect of loss of function in the NIP subfamily II in *Arabidopsis thaliana* to assess their potential role(s) in arsenite (AsIII) uptake. Loss of function in AtNIP7;1 led to increased plant tolerance to AsIII and reduced total As in planta. AtNIP7;1 expression in various yeast backgrounds increased AsIII sensitivity. In the *acr3Δ* yeast genotype, AtNIP7;1 caused a moderate increase in AsV tolerance. Short-term As uptake in *fsp1Δ* expressing AtNIP7;1 was significantly larger than that in the empty vector control.

The data suggest that AtNIP7;1 can mediate AsIII transport and contributes to AsIII uptake in plants.

© 2008 Federation of European Biochemical Societies. Published by Elsevier B.V. All rights reserved.

Keywords: Arsenic; Arsenite; Aquaporin; Uptake; *Arabidopsis thaliana*

1. Introduction

Arsenic is a mineral which predominantly occurs in the environment in two inorganic forms, fully oxidised as arsenate (AsV) and in reduced form as arsenite (AsIII). It is ubiquitous in many natural environments and toxic to all life forms [1]. Many plants readily take up both AsV and AsIII which negatively impacts on growth. Arsenic therefore has adverse effects on agriculture in many areas, particularly in South East Asia where natural occurrence of arsenic is high and its accumulation in crops provides the risk of arsenic entering the food chain. Although in general AsV is more prevalent in the environment and its uptake in plants is therefore more widespread, AsIII toxicity is particularly problematic in wetland grown crops where reducing conditions pertain such as paddy fields for rice production.

Uptake of AsV in plants has been shown to occur through inorganic phosphate transporters [2], most likely due to the chemical similarities between arsenate and phosphate. As a phosphate analogue, AsV interferes with crucial metabolic processes such as oxidative phosphorylation and ATP synthesis. In planta, AsV is detoxified through its reduction to AsIII and subsequent processes that include chelation with phytochelatin [3] and vacuolar compartmentation.

At equimolar concentrations, AsIII is more toxic than AsV. AsIII has a propensity to bind to sulfhydryl groups and thus

has significant detrimental effects on general protein functioning. In analogy with bacteria [4], yeast [5] and mammals [6], it has been postulated that AsIII may enter plants through aquaporin-like transporters, specifically the so-called aquaglyceroporins [4,7]. The latter form a subfamily within the aquaporin family and have pore structures that slightly deviate from other aquaporins. The altered selectivity filter in the pore region allows aquaglyceroporins to conduct small non-polar moieties such as glycerol [4,7], silicon [8], and boron [9]. AsIII would move through aquaglyceroporins as the non-polar As(OH)₃.

Improving As tolerance in crops may require a multifaceted approach [1] but must be based on a firm understanding of the underlying mechanisms by which As is taken up, distributed through plants and compartmentalised. No specific plant AsIII transporters have been identified to date. To identify potential gene products involved in AsIII uptake, we analysed loss of function mutants in aquaglyceroporins of *Arabidopsis* and identified a mutant which lacks NIP7;1 that showed increased tolerance to AsIII. Yeast complementation and AsIII uptake assays show that NIP7;1 is capable of mediating AsIII transport.

2. Materials and methods

2.1. Plant material

T-DNA insertion mutants were received from NASC for all members of the metalloid channeling aquaglyceroporins belonging to subgroup II of the nod26-like intrinsic proteins (NIPs) [4]. For NIP5;1 SALK_122287 and SALK_012572 were acquired, for NIP6;1 SALK_146323 was acquired and for NIP7;1 SALK_057023, SALK_042590 and SAIL_1165_G05 were acquired. Homozygous lines were identified for each of these using LB primers (<http://signal.salk.edu/cgi-bin/tdnaexpress>) and gene-specific primers as designed by the SALK site isect tool (<http://signal.salk.edu/tdnaprimers.html>). Seedling RNA was collected from homozygous lines for RT-PCR analysis using 5' and 3' primers.

Wild-type, *Arabidopsis thaliana* (L.) ecotype Columbia (0) and mutant plant seeds were surface sterilised and placed on agar plates (~10 seeds for each genotype per plate). Growth medium composition was as described previously [10] and contained 1.25 mM KNO₃, 0.5 mM Ca(NO₃)₂, 0.5 mM MgSO₄, 0.625 mM KH₂PO₄ as macronutrients. AsV and AsIII were added to the growth medium as K₃AsO₄ and As₂O₃, respectively, in varying concentrations as indicated in the text. After stratification at 4 °C for two days, plates were transferred to a growth room with the following conditions (12 h, 200 μmol m⁻² s⁻¹ light intensity, 23/20 °C day/night temperature, RH 70–80%). seedling growth determination, plants were grown for 14 days on plates. Three to five plates were grown for each treatment.

2.2. Cloning of NIP7;1

cDNA from RNA isolated from *Arabidopsis* seedlings was used to obtain a full length clone of NIP7;1. NIP7;1 was amplified with primers AtNIP7;1_FOR GCAAGCTTAAAATGAATGGTGAGG-

*Corresponding author. Fax: +44 (0) 1904 328666.
E-mail address: fjm3@york.ac.uk (F.J.M. Maathuis).

CACGGTCAAGAGTAGT and AtNIP7;_REV GCGAATTCT-TAACGTAAAAGTGAAGAAACGGAAGGGGA. As PCR template, 20 ng of cDNA were used. PCR was performed in 50 μ l volume with 1 \times Phusion HF PCR buffer, 200 μ M of each dNTP, 3% DMSO and 1 \times Phusion polymerase (New England BioLabs, Herts, UK). The cycling protocol was: 95 $^{\circ}$ C for 30 s; 36 cycles at 95 $^{\circ}$ C for 10 s, 72 $^{\circ}$ C for 30 s; final extension at 72 $^{\circ}$ C for 10 min. The full length NIP7;1 was cloned into the HindIII and EcoRI sites of pYES2 (Invitrogen, Paisley, UK) and used to transform the yeast strains.

2.3. Expression of NIP7;1 in yeast

Three yeast genotypes were transformed with pYES-NIP7;1 and pYES-EV. The W303-1A parental strain, the *acr3* Δ mutant [11] which lacks the endogenous yeast arsenite efflux transporter ACR3 and the *fps1* Δ strain that lacks the arsenite uptake aquaglyceroporin FSP1 [5]. Growth of the six genotypes was assessed by pre-growth of cultures in SD-glucose medium to an OD₆₀₀ of around 1.0. After equalisation of ODs for the different genotypes, 2 μ l, 10-fold dilution series were deposited on plates using SD medium with galactose as carbon source and with the AsIII or AsV concentrations as indicated in the text.

2.4. Plant AsIII uptake assays

AsIII uptake was determined in seedlings after growth for seven days on plates containing the standard growth medium supplemented with 5, 10 or 15 μ M AsIII (As₂O₃). AsIII uptake in mature plants was determined by exposing hydroponically grown mature plants (4-week-old) to 50 μ M AsIII for 6, 18 and 40 h. For both seedling and mature plants, material was harvested by freezing in liquid nitrogen. Frozen material was ground and approximately 100 mg was digested in 3 ml concentrated nitric acid and 2 ml hydrogen peroxide in a CEM MARS5 microwave (CEM, Buckingham, UK). Digests were analysed on a Pye Unicam 701 ICP-OES spectrometer (Cambridge, UK).

2.5. Yeast AsIII uptake assays

AsIII uptake in yeast was essentially carried out as described in Ref. [5]: cells were grown in 50 ml liquid SC medium to an OD₆₀₀ of ~1, harvested and resuspended in fresh medium and exposed to 20 μ M AsIII for 30 or 60 min. Cells were then harvested, washed in ice cold distilled water, and boiled in 10 ml of water for 5 min. Total As was determined in the water sample by ICP-OES. Uptake assays were performed on three independently grown yeast cultures.

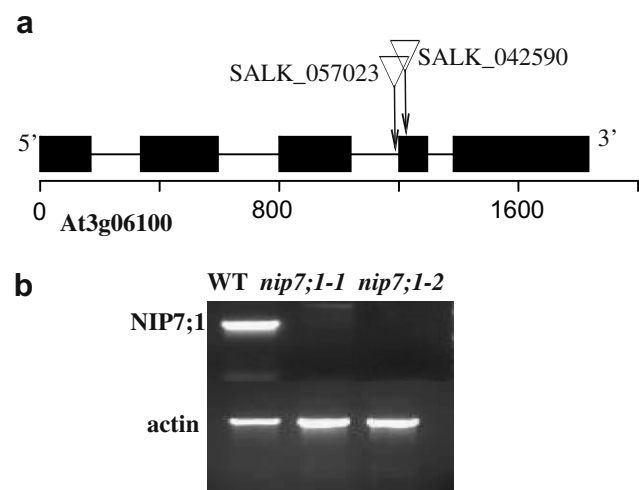


Fig. 1. Identification of T-DNA insertion mutants for AtNIP7;1. (a) Schematic view of the two T-DNA insertions in AtNIP7;1 (At3g06100). Line SALK_057023 locates to the 3rd intron whereas line SALK_042590 inserts at the beginning of the 4th exon. (b) RT-PCR data obtained from leaf mRNA, using *AtNIP7;1* full length primers and actin primers as control showing the absence of transcript in lines *nip7;1-1* and *nip7;1-2* (SALK_057023 and SALK_042590, respectively).

3. Results and discussion

3.1. Loss of function mutants in NIP7;1 show improved AsIII tolerance

The *Arabidopsis* genome contains around 35 genes belonging to the major intrinsic protein (MIP) superfamily [12]. Within the MIP family, the subfamily of nod26-like intrinsic proteins (NIPs) has nine members and in turn divides into subgroups I and II based on amino acid composition of the Ar/R (aromatic/arginine residue) part of the selectivity filter in the channel pore [4,7,12]. Subgroup II contains NIP5;1, NIP6;1 and NIP7;1 which contain the Ala/Val/Ala/Arg sequence in their Ar/R region [7]. NIP5;1 has been shown to function as a boron transporter in planta [9] whereas the function of NIP6;1 and 7:1 remain to be elucidated, although NIP7;1 may be involved in silicon transport [8].

Similar aqua(glycero)porin type transporters were previously suggested to function as AsIII uptake pathways in bac-

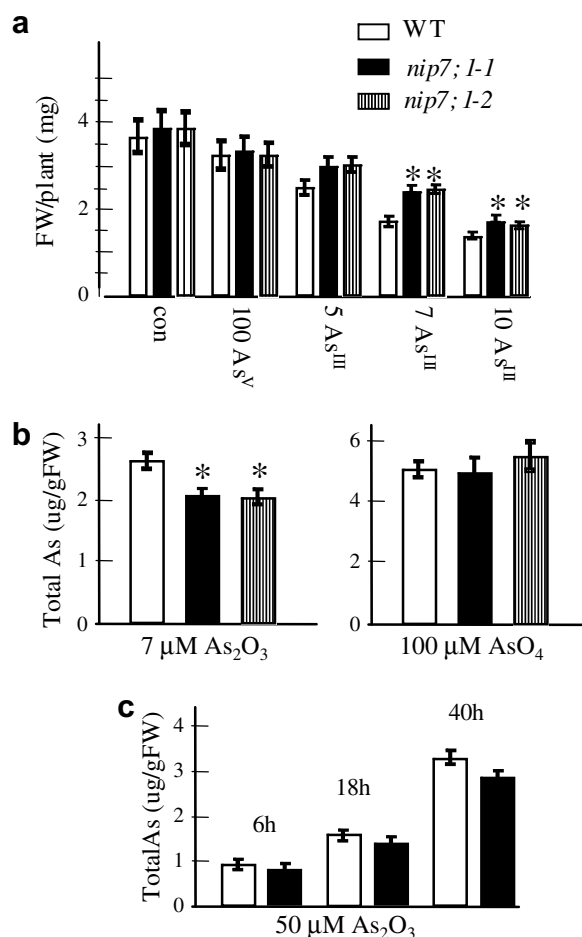


Fig. 2. AtNIP7;1 expression affects plant growth and As accumulation. (a) Seedling growth on agar plates in control conditions and in the presence of 100 μ M AsV or 5, 7 and 10 μ M AsIII. Growth is significantly better for both *nip7;1-1* and *nip7;1-2* loss of function lines when exposed to AsIII but not AsV. Asterisks indicate significant differences at the 5% levels. (b) Total As in seedlings after growth on plates containing 7 μ M AsIII or 100 μ M AsV. Asterisks indicate significant differences at the 5% level. (c) Total As in 4-week-old mature plants growing in hydroponics after exposure to 50 μ M AsIII for 6, 18 or 40 h.

teria, yeast and mammals [1]. For plants too there are data suggesting that aquaporin type transporters are responsible for AsIII uptake [13] but no specific proteins were identified and we therefore proceeded to acquire loss of function mutants in all three members of the NIP subgroup II.

PCR and RT-PCR analysis identified one genuine loss of function mutant for NIP5;1 and two for NIP6;1 (SALK_012572 and SALK_046323 respectively, data not shown). Two independent loss of function mutants were also identified for NIP7;1, (*nip7;1-1* and *nip7;1-2* in SALK_057023 and SALK_042590, respectively) which carry T-DNA inserts at the end of the 3rd intron and beginning of the 4th exon, respectively (Fig. 1a). Both lines showed a complete absence of NIP7;1 transcript (Fig. 1b). The five mutant lines were grown together with wild-type plants in control conditions and on plates with various concentrations of AsIII (As₂O₃) and AsV (K₂HAsO₄). No significant growth difference was observed between wild-type plants and *nip5;1* or wild-type and *nip6;1* loss of function lines, either in the presence of elevated AsIII or AsV (data not shown). However, both *nip7;1-1* and *nip7;1-2* lines showed significantly improved growth in the presence of AsIII but not AsV (Fig. 2a) with the largest growth difference occurring at 7 μM. This suggested that NIP7;1 plays a role in AsIII uptake and we decided to further analyse this hypothesis.

3.2. Loss of function mutants in NIP7;1 show reduced AsIII uptake

The level of total As in seedlings grown on AsIII containing plates was significantly lower in both *nip7;1* genotypes compared to wild-type plants (Fig. 2b) when plants were exposed to 7 μM AsIII in the growth medium. No significant difference in total As was present after growth on 100 μM AsV. Although not significant, the observed difference between wild-type and *nip7;1* seedlings was mirrored in mature plants that were exposed over much shorter times to AsIII: After 40 h exposure to 50 μM AsIII there was a reduction in tissue As in the *nip7;1* mutant (Fig. 2c).

3.3. AtNIP7;1 affects As tolerance and As uptake in yeast

Expression of AtNIP7;1 in various yeast genotypes modified their arsenic tolerance. We expressed AtNIP7;1 in wild-type, *acr3Δ* and *fps1Δ* yeast to test its effect on As tolerance and As uptake. Acr3p is predicted to be a plasma membrane localised protein with 10 transmembrane domains [11]. Disruption of *ACR3* led to a 5-fold increase in yeast sensitivity to AsIII, presumably due to a lack of extrusion of inorganic AsIII or of chelated AsIII into the external medium. The *acr3Δ* genotype is also more sensitive to AsV. In most organisms, including yeast, AsV is believed to be quickly reduced to AsIII by arsenate reductases [14]. Thus, the loss of Acr3p causes increased accumulation of AsIII in the cell and therefore a larger degree of growth inhibition [11]. When expressed in *acr3Δ* yeast, NIP7;1 increased AsIII sensitivity (Fig. 3a) indicating that NIP7;1 contributes to cellular AsIII load.

Fps1p encodes an aquaporin of the yeast MIP family, capable of bidirectional glycerol transport [5]. Disruption of *FPS1* affects cellular osmotic homeostasis but also improves yeast arsenite tolerance, primarily through reduced uptake of this compound. The significant increase in AsIII tolerance in the *fps1Δ* background suggests that Fps1p is a major constituent of yeast AsIII uptake. AtNIP7;1 expression in the *fps1Δ* yeast to a large extent removed the relative AsIII tolerance of this genotype and made *fps1Δ:NIP7;1* cells grow similarly to wild-type cells in the presence of AsIII.

An increase in AsIII sensitivity was also recorded for wild-type yeast cells expressing AtNIP7;1. In the wild-type, the endogenous routes such as Fps1p are still available but NIP7;1 can provide an extra pathway for AsIII uptake.

Since AsV is rapidly reduced to AsIII in the cytosol and *ACR3* is the main AsIII extrusion system in yeast, the presence of *ACR3* activity may also impact on AsV toxicity. We therefore grew *acr3Δ:EV* and *acr3Δ:NIP7;1* in the presence of varying AsV concentrations (Fig. 3). A small but consistent improvement in *acr3Δ:NIP7;1* growth was observed relative to *acr3Δ:EV* cells. These data indicate that NIP7;1 may mediate AsIII efflux in the absence of *ACR3* and thus reduce AsV toxicity.

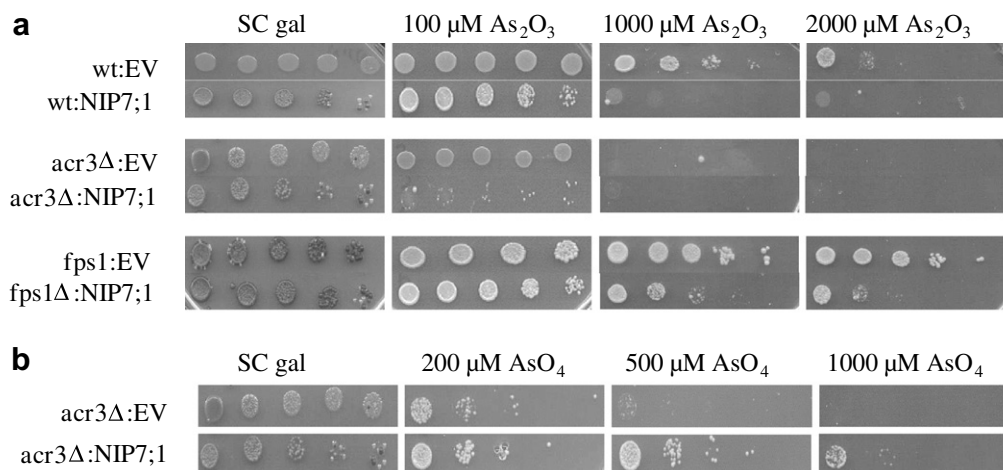


Fig. 3. Expression of AtNIP7;1 in yeast affects As tolerance. (a) Expression of AtNIP7;1 in wild-type, *acr3Δ* or *fps1Δ* yeast increases yeast sensitivity to AsIII present in the growth substrate. Note that endogenous AsIII tolerance varies for the different yeast genotypes and is the largest in *fps1Δ*, intermediate in wild-type and the least in *acr3Δ* cells. The increased AsIII sensitivity suggests AtNIP7;1 contributes to AsIII uptake in all three backgrounds. (b) The *acr3Δ* genotype lacks the predominant AsIII extrusion mechanism and expression of AtNIP7;1 moderately improves AsV tolerance. AsV is reduced to AsIII intracellularly and AtNIP7;1 may improve AsV tolerance by increasing AsIII efflux capacity.

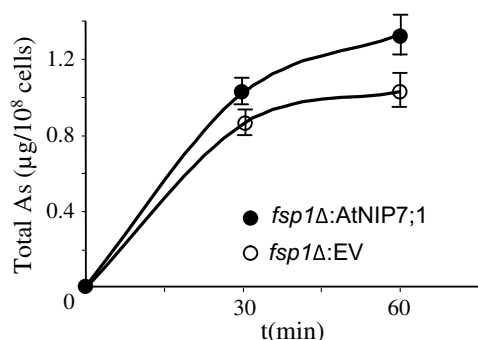


Fig. 4. Expression of AtNIP7;1 in *fsp1Δ* yeast increases yeast AsIII uptake. Short-term AsIII uptake from a 20 μM As_2O_3 solution is significantly greater in yeast which express AtNIP7;1 compared to the empty vector (EV) control leading to higher total As contents in the *fsp1Δ:AtNIP7;1* cells. Data are the average \pm S.D. of three independent uptake assays.

Summarised, the above data strongly suggest that AtNIP7;1 can mediate AsIII uptake when expressed in various yeast genotypes, irrespective of the presence of endogenous AsIII uptake pathways. The data from *acr3Δ* furthermore suggest that AsIII movement through NIP7;1 can also occur in the opposite direction and therefore improve AsV tolerance by reducing AsIII accumulation.

We further tested the capacity of NIP7;1 to mediate AsIII uptake by measuring short-term AsIII uptake in *fsp1Δ* expressing AtNIP7;1 in comparison to cells expressing the empty vector. Fig. 4 shows that short-term uptake of AsIII in *fsp1Δ:EV* is smaller than in *fsp1Δ:NIP7;1* confirming the notion that NIP7;1 is a major conduit for AsIII entry into yeast.

4. Conclusions

As with all aquaporins, aquaglyceroporins are extremely efficient water transporters with rates of up to 10^9 s^{-1} at 1 MPa osmotic gradients [7,12]. Maximum rates for solute fluxes through this type of aquaporin are generally much lower but in many physiological conditions, osmotic gradients may be small or non-existent. In such cases, net fluxes through aquaglyceroporins may largely exist of solute rather than water fluxes but quantitative data are yet to emerge.

The aquaglyceroporin AtNIP7;1 is expressed at considerable levels in both root and shoot tissue (<https://www.genevestigator.ethz.ch>) where it may participate in the movement of small, non-polar compounds. Our results show that loss of function in AtNIP7;1 improves *Arabidopsis* AsIII tolerance and reduces AsIII uptake into the plant root. Furthermore, heterologous expression of AtNIP7;1 in yeast shows it affects yeast growth on AsIII and increases yeast AsIII uptake. These findings greatly suggest that AtNIP7;1 forms part of an AsIII uptake pathway in *A. thaliana*.

In the yeast *acr3Δ* genotype, NIP7;1 also improved AsV tolerance, indicating AsIII movement through NIP7;1 can be bidirectional in yeast. However, we did not find similar effects in plants. Recently, evidence was provided for AsIII efflux from rice and tomato roots [15] but this was shown to be sen-

sitive to the uncoupler CCCP (carbonyl cyanide *m*-chlorophenylhydrazone) and therefore likely to involve an active transport system rather than a passive, aquaporin mediated mechanism.

The identification of specific gene-products that mediate AsIII uptake may allow us to genetically engineer plants where this function is altered, either to increase uptake for phytoremediation purposes [1,16] or to reduce AsIII in the food chain [1].

Acknowledgements: We are grateful to Dr. Michael Thorsen and Dr. Markus Tamas (Goeteborg University) for donating the *fsp1Δ* yeast strain. We thank Maria Budarina for technical assistance.

References

- [1] Tripathi, R.D., Srivastava, S., Mishra, S., Singh, N., Tuli, R., Gupta, D.K. and Maathuis, F.J.M. (2007) Arsenic hazards: strategies for tolerance and remediation by plants. *Trends Biotech.* 25, 158–165.
- [2] Asher, C.J. and Reay, P.F. (1979) Arsenic uptake by barley seedlings. *Aust. J. Plant Physiol.* 6, 459–466.
- [3] Pickering, I.J., Prince, R.C., George, M.J., Smith, R.D., George, G.N. and Salt, D.E. (2000) Reduction and coordination of arsenic in Indian mustard. *Plant Physiol.* 122, 1171–1177.
- [4] Bienert, G.P., Schuessler, M.D. and Jahn, T.P. (2008) Metalloids: essential, beneficial or toxic? Major intrinsic proteins sort it out. *Trends Biochem. Sci.* 33, 20–26.
- [5] Wysocki, R., Chery, C.C., Wawrzycka, D., Van Hulle, M., Cornelis, R., Thevelein, J.M. and Tamas, M.J. (2001) The glycerol channel Fps1p mediates the uptake of arsenite and antimonite in *Saccharomyces cerevisiae*. *Mol. Microbiol.* 40, 1391–1401.
- [6] Liu, Z.J., Shen, J., Carbrey, J.M., Mukhopadhyay, R., Agre, P. and Rosen, B.P. (2002) Arsenite transport by mammalian aquaglyceroporins AQP7 and AQP9. *Proc. Natl. Acad. Sci. USA* 99, 6053–6058.
- [7] Wallace, I.S., Choi, W. and Roberts, D.M. (2006) The structure, function and regulation of the nodulin 26-like intrinsic protein family of plant aquaglyceroporins. *BBA* 1758, 1165–1175.
- [8] Ma, J.F., Tamai, K., Yamaji, N., Mitani, N., Konishi, S., Katsuhara, M., Ishiguro, M., Murata, Y. and Yano, M. (2006) A silicon transporter in rice. *Nature* 440, 688–691.
- [9] Takano, J., Wada, M., Ludewig, U., Schaaf, G., von Wiren, N. and Fujiwara, T. (2006) The *Arabidopsis* major intrinsic protein NIP5;1 is essential for efficient boron uptake and plant development under boron limitation. *Plant Cell* 18, 1498–1509.
- [10] Gobert, A., Isayenkov, S., Voelker, C., Czempinski, K. and Maathuis, F.J.M. (2007) The two-pore channel TPK1 gene encodes the vacuolar K^+ conductance and plays a role in K^+ homeostasis. *Proc. Natl. Acad. Sci. USA* 104, 10726–10731.
- [11] Wysocki, R., Bobrowicz, P. and Ulaszewski, S. (1997) The *Saccharomyces cerevisiae* ACR3 gene encodes a putative membrane protein involved in arsenite transport. *J. Biol. Chem.* 272, 30061–30066.
- [12] Maurel, C. (2007) Plant aquaporins: novel functions and regulation properties. *FEBS Lett.* 581, 2227–2236.
- [13] Meharg, A.A. and Jardine, L. (2003) Arsenite transport into paddy rice (*Oryza sativa*) roots. *New Phytol.* 157, 39–44.
- [14] Raab, A., Feldmann, J. and Meharg, A.A. (2004) The nature of arsenic-phytochelatin complexes in *Holcus lanatus* and *Pteris cretica*. *Plant Physiol.* 134, 1113–1122.
- [15] Xu, X.Y., McGrath, S.P. and Zhao, F.J. (2007) Rapid reduction of arsenate in the medium mediated by plant roots. *New Phytol.* 176, 590–599.
- [16] Huang, J.W., Poynton, C.Y. and Elles, M.P. (2004) Mechanisms of arsenic hyperaccumulation in *Pteris* species: root As influx and translocation. *Environ. Sci. Technol.* 38, 3412–3417.