Investigating the origin and transport of methylated arsenic species in plants

A thesis presented for the degree of Doctor of Philosophy in Biological Science at the University of Aberdeen

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Declaration

I hereby declare that the work presented in this thesis has been performed by myself, unless otherwise stated, and has not been accepted in any previous application for a degree. All quotations have been distinguished by quotation marks, and all sources of information are acknowledged by reference to the authors.

Charlotte Lomax,

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Summary

Inorganic arsenic is a highly toxic element known to cause various cancers and other diseases in humans. Arsenic contamination is widespread worldwide, particularly in South-East Asia where arsenic-contaminated groundwater is used for drinking and rice cultivation. Unlike other cereals, paddy rice can efficiently accumulate arsenic in the grain. Rice is a staple food for around 50% of the world's population, and so arsenic accumulation in rice is of great concern. Arsenite, As(III), is the predominant form of arsenic within plants, but rice grains often contain significant proportions of organic arsenic species. The most common of these are dimethylarsinic acid (DMA) and monomethylarsonic acid (MMA).

Arsenic methylation is a well-characterised detoxification mechanism in micro-organisms and animals, but it was unknown whether plants were also able to methylate inorganic arsenic. A variety of plant species were exposed to inorganic arsenic under axenic conditions, and additional treatments including nutrient deficiency and symbiosis with root-nodulating bacteria were also investigated. In all plant species tested, and under all treatments, exposure to inorganic arsenic resulted in detection solely of inorganic arsenic in plant tissues. When present in the growth medium, rice was able to take up MMA and DMA, and MMA and DMA were also detected in shoots of rice grown in soils from the UK and Bangladesh. Sub-optimal preservation of arsenic species in soil water samples, and the high translocation efficiency of organic arsenic in plants, explains why MMA and DMA are detected in plants more frequently than soil.

The uptake of undissociated MMA by rice roots is predominantly facilitated by OsNIP2;1 (OsLsi1), a member of the NIP subfamily of aquaporins, which also accounts for 50% of root DMA uptake. Therefore the transport of MMA and DMA by other NIP aquaporins was investigated using heterologous expression in *Xenopus laevis* oocytes. OsNIP1;1 and OsNIP3;3 were permeable to MMA, as well as arsenite, silicon and water. Additionally, OsNIP3;2 was found to be permeable to arsenite and boron. However, uptake of DMA was not observed for oocytes expressing any NIP gene, including *OsNIP2;1*. The role of

different regions of NIP aquaporins in substrate selectivity is discussed, as well as limitations of expressing plant proteins in *Xenopus* oocytes.

MMA and DMA have acid dissociation constants (pK_{a1}) of 4.19 and 6.14 respectively, and so increasing the pH of the medium increases the proportion of dissociated, charged complexes. Inorganic arsenate has a low pK_{a1} of 2.21, and as a chemical analogue of phosphate is taken up by phosphate transporters in plants. Rice plants overexpressing the high-affinity phosphate transporter *OsPT8*, and the phosphate-starvation transcription factor *OsPHR2*, were used to determine if the phosphate transport pathway also contributes to transport of MMA and DMA.

In hydroponic culture, shoots of the *OsPT8*-overexpression line contained significantly more MMA and DMA than wild-type. Additionally, the presence of phosphate in the medium significantly decreased root uptake of MMA and DMA, by both *OsPT8*-overexpressor and wild-type rice plants. Expression of *OsPT8* in *Xenopus* oocytes conferred significant uptake of arsenate and MMA compared to the control. Roots and shoots of the *OsPHR2*-overexpression line contained significantly more MMA than wild-type when the exposure medium was at pH 7.0, but not at pH 5.5. However, pH does not only affect the dissociation of MMA and DMA. Phosphate transporters are more active under lower pH conditions due to the increase in protons for co-transport. Therefore, the effect of lower pH on MMA and DMA transport is the net result of enhanced phosphate transporter activity, and decreased availability of dissociated MMA and DMA for transport.

Overall the work contained in this thesis shows that methylated arsenic species are not formed within plants, and are transported by two different classes of transporters depending on the pH of the medium.

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List of abbreviations and acronyms

ABC	ATP-binding cassette
ADP	Adenosine diphosphate
AM	Arbuscular myccorhizae
ANOVA	Analysis of variance
AR	Arsenate reductase
As(III)	Arsenite
As(V)	Arsenate
As3MT	Arsenite methyltransferase
ATP	Adenosine triphosphate
ATSDR	Agency for Toxic Substances and Disease Registry
b.w.	Body weight
BMDL	Benchmark dose lower confidence limit
cDNA	Complementary DNA
cRNA	Complementary RNA
DMA	Dimethylarsinic acid (pentavalent)
DW	Dry weight
EDTA	Ethylenediaminetetraacetic acid
EFSA	European Food Safety Authority
EU	European Union
FAO	Food and Agriculture Organization of the United Nations
FW	Fresh weight
GSH	Glutathione
HPLC	High performance liquid chromatography
IARC	International Agency for Research on Cancer

ICP-MS	Inductively coupled plasma mass spectrometry
ICP-OES	Inductively coupled plasma optical emission spectroscopy
JECFA	Joint FAO/WHO Expert Committee on Food Additives
LOD	Limit of detection
LOQ	Limit of quantitation
LSD	Least significant difference
MBS	Modified Barth's saline
MCL	Maximum contaminant level
MIP	Major intrinsic protein
MMA	Monomethylarsonic acid (pentavalent)
MRL	Minimal risk level
NAD	Nicotinamide adenine dinucleotide (oxidised form)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
NIP	Nodulin26-like intrinsic protein
NPOC	Non-purgeable organic carbon
PC	Phytochelatin
PIP	Plasma membrane intrinsic protein
pK_a	Acid dissociation constant (logarithmic scale)
PT	Phosphate transporter
PTWI	Provisional tolerable weekly intake
ROS	Reactive oxygen species
SAM	*S*-adenosylmethionine
SE	Standard error
Tetra	Tetramethylarsonium ion
TF	Translocation factor (ratio of shoot to root As concentration)
TIP	Tonoplast intrinsic protein

TMA	Trimethylarsine
TMAO	Trimethylarsine oxide
TOC	Total organic carbon
WHO	World Health Organization

Note: ‘MMA’ and ‘DMA’ are used throughout the thesis to represent the pentavalent forms of these As species. When referred to, trivalent MMA and DMA are designated as MMA(III) and DMA(III).

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1. General introduction

1.1 Chemical properties of arsenic

Arsenic (As) is the twentieth most abundant element in the Earth's crust and is widely distributed in the environment (Mandal & Suzuki, 2002). Arsenic compounds are potent poisons and have been implicated in the deaths of several historical figures including Napoleon Bonaparte, American president Zachary Taylor and Chinese Emperor Guangxu.

A member of group V of the periodic table, As is classified as a metalloid; having properties of both metals and non-metals. Inorganic arsenic can occur in several oxidation states (-3, 0, +3 and +5), but exists mainly in the environment as one of two oxidation states: +5 termed arsenate, and +3 termed arsenite. In solution pH and redox potential (Eh) are the dominant factors that control As speciation. Under oxidising conditions arsenate ($H_2AsO_4^-$, $HAsO_4^{2-}$) is dominant, whereas arsenite ($H_3AsO_3^0$) is predominant under reducing conditions below pH 9 (Supplementary Figure 1.1).

In addition to these inorganic As species, organoarsenicals including monomethylarsonic acid (MMA), dimethylarsinic acid (DMA), trimethylarsine oxide (TMAO), tetramethylarsonium ion (Tetra), arsenobetaine, arsenocholine, and numerous arsenosugars have been detected in various organisms and environmental samples (Rezanka & Sigler, 2008; Figure 1.1). The mechanism of As methylation is discussed in **Section 1.5.2** and **Section 1.5.3**. The properties of As species is affected not only by their structure, but also by the pH of the medium, as the acid dissociation constant (pK_a) of As compounds varies greatly (Supplementary Figure 1.2).

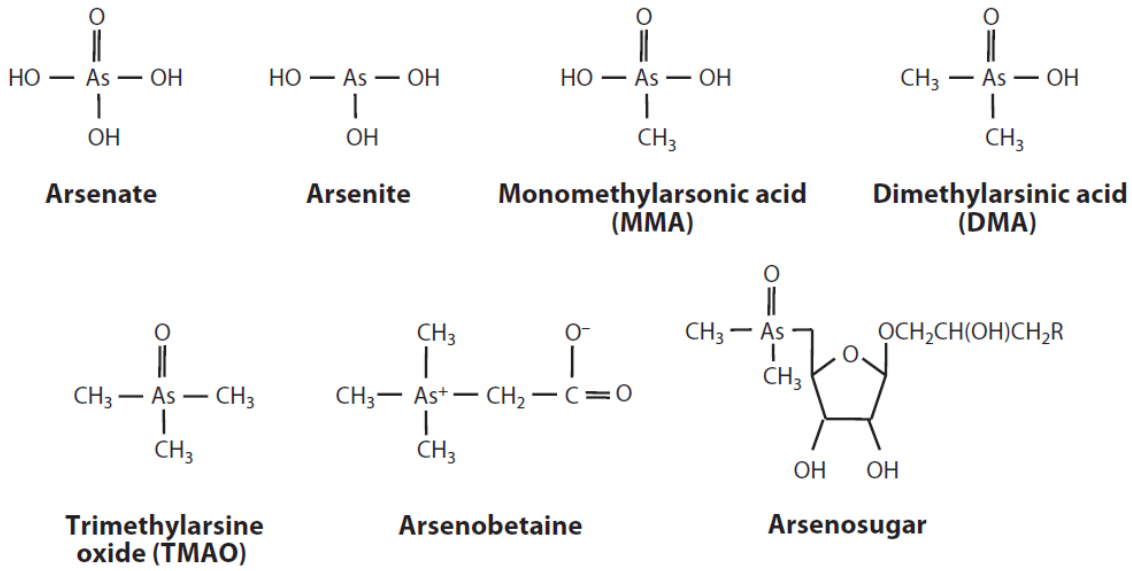


Figure 1.1 Structure of common As compounds, taken from Zhao *et al.*, (2010b).

1.2 Environmental occurrence of arsenic

The global average concentration of As in soils is around 6 mg kg^{-1}, however the value varies greatly geographically (Mandal & Suzuki, 2002). Arsenic levels in certain areas are elevated due to natural processes including volcanic eruptions and the weathering of rocks containing minerals of As. Anthropogenic activities including the mining and smelting of precious metals, burning of fossil fuels and use of As-containing herbicides, pesticides, fungicides and wood preservatives accounts for around 75% of environmental As (Mandal & Suzuki, 2002). In the United States (USA), As is also found in broiler chicken feed in the form of roxarsone (3-nitro-4-hydroxyphenylarsonic acid) which is excreted in the manure, and is often added to soil as fertiliser (Christen, 2001).

Arsenic-contamination of groundwater is a growing global problem, affecting countries including Bangladesh, Nepal, Mongolia, Taiwan, Pakistan, China, Chile and the USA (Chakraborti *et al.*, 2002; Brammer & Ravenscroft, 2009; Thakur *et al.*, 2011; Rodríguez-Lado *et al.*, 2013). Arsenic can be released into groundwater through four mechanisms: reductive dissolution, alkaline desorption, sulphide oxidation, and geothermal activity. In South-East Asia reductive dissolution; whereby the reduction of ferric iron by microbial activity liberates adsorbed As from sediments, is the main process of As release into groundwater (Ravenscroft *et al.*, 2009). Arsenic is also the fourteenth most abundant

element in seawater, with concentrations generally between 1 to 8 μg L^{-1} (Mandal & Suzuki, 2002).

1.2.1 Environmental arsenic speciation

Arsenate [As(V)] is predominant in aerobic soils, but generally has low mobility due to a strong binding affinity for minerals including ferrihydrite and goethite (Fitz & Wenzel, 2002). Arsenite [As(III)] is the main species of As in anaerobic soils such as flooded rice paddies, and due to a high pK_a of 9.22 (Figure S1.2) is present predominantly as undissociated arsenous acid [$As(OH)_3$] making it much more mobile (Zhao *et al.*, 2009). Additionally, organic As species including MMA, DMA, TMAO and arsenobetaine have been detected in soils (Takamatsu *et al.*, 1982; Bednar *et al.*, 2002a; Geiszinger *et al.*, 2002; Huang & Matzner, 2006).

In seawater, inorganic As is predominant, with a ratio of arsenate to arsenite of 10:1, arising from microbial reduction of the more thermodynamically-stable arsenate (Johnson, 1972). Additionally, groundwater largely contains only inorganic As, whereas lakes and ponds can contain small amounts of methylated As species such as MMA and DMA (Mandal & Suzuki, 2002).

1.3 Arsenic toxicity

Arsenic is highly toxic to almost all organisms, and was one of the first chemicals identified to cause cancer in humans (Smith *et al.*, 2002). Chronic exposure to concentrations of inorganic As in drinking water above 50 μg L^{-1} has been associated with skin lesions including melanosis and keratosis; cancers of the bladder, liver, lung, kidney and skin; and other conditions including diabetes mellitus, hypertension, decreased liver and kidney function, respiratory problems, and neurological disorders (for review see NRC, 1999). Long-term exposure to As-contaminated drinking water may also negatively affect pregnancy and foetal development, as both inorganic and methylated arsenicals are able to cross the placental membrane. Arsenic exposure during pregnancy in humans increases the incidence of spontaneous abortion, stillbirth, preterm birth, low birth weight and birth defects (NRC, 1999). Additionally, smoking and poor nutritional status

and have been found to exacerbate As toxicity in humans (Ferreccio *et al.*, 2000; Maharjan *et al.*, 2007).

The toxicity of As is exploited in medicine, and historically As compounds were used to treat various ailments including psoriasis, asthma, eczema and haemorrhoids (for review see Dilda & Hogg, 2007). More practical treatments include Salvarsan, which was used to treat syphilis, caused by the bacterium *Treponema pallidum*, and trypanosomiasis (sleeping sickness), caused by the parasitic protozoan parasite *Trypanosoma brucei* sp. Arsenic trioxide (As_2O_3) under the name Trisenox, is currently used to treat acute promyelocytic leukaemia (Sanz *et al.*, 2005).

Arsenic is also toxic to plants and was used historically as a herbicide and defoliant. In rice, As causes 'straighthead' in which the panicles are erect rather than deflexed and in extreme cases do not form at all (Marin *et al.*, 1992). Arsenic-contaminated groundwater used to irrigate paddy fields decreased rice grain yield from nine to seven, and three to two tonnes per hectare in successive field trials in Bangladesh (Panaullah *et al.*, 2009).

On a cellular level, arsenite has a very strong binding affinity for sulphydryl groups of proteins and so can disrupt various cellular processes; whereas arsenate is a phosphate-analogue and can displace phosphate from biological molecules including ATP to form non-functional ADP-As (Hughes, 2002; Rezanka & Sigler, 2008). Despite being a well-known carcinogen, As does not interact directly with DNA, and instead causes genotoxic effects through generation of reactive oxygen species (ROS), direct inhibition of DNA repair enzymes, and alteration of DNA methylation patterns (Schuhmacher-Wolz *et al.*, 2009).

Until recently it was thought that As methylation; resulting mainly in MMA, DMA and to a lesser extent TMAO, was the main mammalian detoxification pathway for inorganic As (see **Section 1.5** for the biochemical pathway of As methylation). Pentavalent MMA and DMA were found to be ten times less cytotoxic than arsenate, and 1000-fold less cytotoxic than arsenite to mouse macrophage cell cultures (Sakurai *et al.*, 1998). Additionally, methylated As species present in food and water are less well absorbed during digestion (compared to 80-90% absorption of ingested inorganic As), and are more readily excreted in the urine (NRC, 1999). However, intermediates in the methylation

pathway MMA(III) and DMA(III), that is MMA and DMA containing trivalent As, are more cytotoxic, genotoxic and more potent inhibitors of certain enzymes than arsenite or arsenate (Thomas *et al.*, 2001). Although these intermediates are assumed to be short-lived, *in vitro* MMA(III) and DMA(III) were found to be associated with cytosolic proteins and so may be able to produce toxic effects (Styblo & Thomas, 1997). Additionally, due to advances in As speciation techniques, MMA(III) and DMA(III) have been detected in human urine after exposure to moderately high concentrations of As in drinking water (Mandal *et al.*, 2004). Based on data from *in vitro* exposure of mammalian cell cultures, the rank order of arsenicals based on their genotoxicity is proposed to be: DMA(III)≈ MMA(III) > arsenite > arsenate >> MMA ≈ DMA (Schuhmacher-Wolz *et al.*, 2009).

1.3.1 Legislation

In 1967 the Joint FAO (Food and Agriculture Organization of the United Nations)/WHO (World Health Organization) Expert Committee on Food Additives (JECFA) concluded that in the absence of sufficient data, the maximum daily load of As should be set at 50 µg kg body weight (b.w.)$^{-1}$ day^{-1} (JECFA, 1967). In 1983, due to emerging evidence of the link between inorganic As and lung and skin cancer, the Committee decreased the provisional maximum tolerable daily intake for inorganic As to 2.0 µg kg b.w.$^{-1}$ day^{-1} (JECFA, 1983). Based on this value, in 1988 the JECFA set a provisional tolerable weekly intake (PTWI) for inorganic As of 15 µg kg b.w.$^{-1}$ (JECFA, 1989).

In 2011 the JECFA concluded from epidemiological studies that the benchmark dose lower confidence limit for a 0.5% increase on the incidence of lung cancer ($BMDL_{0.5}$) was 3.0 µg kg^{-1} b.w. day^{-1}. Therefore the Committee withdrew the previous PTWI for inorganic As (equivalent to 0.21 µg kg b.w.$^{-1}$ day^{-1}) as it was too close to the $BMDL_{0.5}$ value (JECFA, 2011). Currently the JECFA has not issued a new PTWI for inorganic As. In 2007 the U.S. Agency for Toxic Substances and Disease Registry (ATSDR) set a minimal risk level (MRL) for chronic oral inorganic As exposure of 0.3 µg kg b.w.$^{-1}$ day^{-1} (ATSDR, 2007). In 2009 the European Food Safety Authority (EFSA) concluded from epidemiological data that the benchmark dose lower confidence limit for a 1% increase on the incidence of lung cancer ($BMDL_{01}$) for inorganic As is also 0.3 µg kg b.w.$^{-1}$ day^{-1} (EFSA, 2009).

In 2009, the WHO International Agency for Research on Cancer (IARC) classified MMA and DMA to Group 2B: possibly carcinogenic to humans. Arsenobetaine and 'other organic arsenic compounds that are not metabolised in humans' were assigned to Group 3: not classifiable as to its carcinogenicity to humans (IARC, 2009). The ATSDR has set a MRL for chronic oral exposure to DMA at 20 μg kg b.w.$^{-1}$ day^{-1}, and for MMA at 10 μg kg b.w.$^{-1}$ day^{-1} (ATSDR, 2007).

The guideline concentration of As in drinking water set by the World Health Organization (WHO) and European Union (EU) is 10 μg L^{-1}, however in Bangladesh and other developing countries the limit is set at 50 μg L^{-1} (WHO, 1993; Council of the European Union, 1998; Alam *et al.*, 2002). Until recently the maximum contaminant level (MCL) for As in drinking water in the USA was also 50 μg L^{-1}, but was lowered to 10 μg L^{-1} in 2006, because the cancer risk from drinking water containing 50 μg L^{-1} As is estimated to be as high as 13 per 1000 people (Smith *et al.*, 1992; Smith *et al.*, 2002).

The Food Standards Agency (FSA) estimated the daily intake for an average UK adult to be 1.33 μg kg b.w.$^{-1}$ day^{-1} total As, and 0.018-0.082 μg kg b.w.$^{-1}$ day^{-1} inorganic As (Food Standards Agency, 2004).

1.4 Exposure routes

Due to advances in analytical techniques, the number of studies into the As content of various foodstuffs has risen dramatically in recent years. In areas with As-contaminated water, drinking water is the primary source of As in the human diet (Kile *et al.*, 2007). However, as strategies to remediate, or find alternative sources of drinking water are installed, the contribution of non-rice food sources to As intake will increase. Additionally, inorganic As is classified as a non-threshold carcinogen, and so all sources should be studied in order to minimise human exposure.

1.4.1 Drinking water

In the 1970s, international aid agencies began a tubewell-installation programme in Bangladesh to combat the high infant mortality due to consumption of surface water contaminated with pathogens responsible for cholera and dysentery. However, the groundwater reached by the tubewells is contaminated with inorganic As, and now

endangers the lives of millions of people in Bangladesh and West Bengal, India (Chakraborti *et al.*, 2002). It is thought that up to half of Bangladesh's 10 million tubewells are contaminated, pumping water above the 10 μg L^{-1} WHO guideline, and that around 85 million people in Bangladesh alone are at risk of As poisoning (BGS, 1999; Hossain, 2006). However, the problem is not confined to the Bengal Delta region; contamination of groundwater with As is a growing problem and has been reported for over 70 countries including China and the USA (Brammer & Ravenscroft, 2009).

1.4.2 Rice

Rice (*Oryza sativa*) is the primary source of inorganic As for populations not exposed to As-contaminated drinking water in developed countries such as the USA (Meacher *et al.*, 2002; Meliker *et al.*, 2006). Recently, consumption of As in rice was found to increase the prevalence of micronuclei in urothelial cells, an indicator of DNA damage, in a human population from West Bengal, India not exposed to elevated As concentrations in drinking water (Banerjee *et al.*, 2013). There is no international limit for As in food, however the Chinese government set a regulatory limit of 150 μg kg^{-1} inorganic As in rice (USDA Foreign Agricultural Service, 2006).

Consumption of rice also represents a major route of As exposure to people living in areas with As-contaminated drinking water, such as Bangladesh (Kile *et al.*, 2007; Ohno *et al.*, 2007). This is partly due to the reliance of these populations on rice, which provides 73% of the calorific intake of people in Bangladesh (Ninno & Dorosh, 2001). The relative contribution of dietary sources of As increases, as drinking water As concentrations decrease (Kile *et al.*, 2007). Even if alternative uncontaminated sources of water could be found, irrigation of crops has deposited thousands of tonnes of As in the soils of this region (Meharg & Rahman, 2003; Meharg, 2004).

Unlike other cereals, rice is able to accumulate a significant amount of As in the grain (Williams *et al.*, 2007b). This is due to two main factors: the first being that under flooded paddy conditions in which rice is typically cultivated, arsenite, the more bioavailable species of As predominates (Xu *et al.*, 2008). However this does not fully account for the increased grain As levels in rice compared to other cereal crop species. When supplied with arsenite in hydroponic culture, the translocation of As from root to shoot was double

that in rice than for wheat (*Triticum aestivum*) or barley (*Hordeum vulgare*; Su *et al*., 2010). The second factor is that rice accumulates silicon (Si) up to 10% shoot dry weight (Ma *et al.*, 2006), and that arsenous acid [$As(OH)_3$] is an analogue of silicic acid [$Si(OH)_4$]. Arsenous acid and silicic acid have similar molecular diameters, 4.11 Å and 4.38 Å respectively, and both have high pK_as, 9.22 and 9.84 respectively, meaning they exist mainly as undissociated complexes below pH 8 (Ma *et al.*, 2008). Silicon, although not considered an essential plant nutrient, has many beneficial effects in plants such as increased resistance to pathogens and insect pests, increased drought and salt tolerance, and reduced lodging (Ma, 2004; Ma & Yamaji, 2006). Low Si-accumulation by rice results in a significant reduction in both the yield and quality of grain produced (Ma & Yamaji, 2006). Arsenite transporters in plants are discussed in detail in **Section 1.8.2**.

A global 'normal' range of As concentrations in rice grains has been proposed of 80 to 200 μg kg^{-1}, based on analysis of 204 commercial samples originating from various countries including China and the USA (Zavala & Duxbury, 2008). Analysis of 901 commercially available white rice samples originating from uncontaminated sources revealed a mean total As concentration of 150 μg kg^{-1}, which is within the proposed normal range (Meharg *et al.*, 2009). However, samples from Egypt contained extremely low As concentrations, averaging 50 μg kg^{-1}, and samples from France and the USA contained the highest concentrations with means of 280 and 250 μg kg^{-1} respectively. Additionally, modelling global As concentrations in rice must take into account the contribution of the individual countries to global rice supply (Meharg *et al.*, 2009). The highest reported As concentration for field-grown rice grain is 1835 μg kg^{-1}, found in a highly contaminated region of Bangladesh (Meharg & Rahman, 2003).

The highest As concentration of the 204 rice samples analysed by Zavala and Duxbury (2008) was 714 μg kg^{-1} in a brown long-grain sample purchased in the USA, and on average brown rice contained 40% more As than white rice. Brown rice contains higher As concentrations than white rice as a large proportion of As is located in the bran, pericarp and subaleurone and layers which are removed by polishing (Ren *et al.*, 2006; Rahman *et al.*, 2007; Meharg *et al.*, 2008b; Moore *et al.*, 2010).

Arsenic speciation in rice grains shows considerable geographical and genotypic variation. Generally inorganic As and DMA are predominant but their proportions are highly variable. The reasons for this are discussed in detail in **Chapter 3**. Synchrotron x-ray fluorescence microtomography revealed that treatment with inorganic As or MMA resulted in As located primarily in the ovular vascular trace; the source of nutrient influx into grain located opposite the embryo, whereas DMA treatment resulted in As spread throughout the grain including the endosperm (Carey *et al.*, 2010; Carey *et al.*, 2011). NanoSIMS analysis revealed that in rice grains with high DMA concentrations, As is primarily located in the subaleurone region. In contrast, in grains from plants treated with inorganic As, the majority of As was in the aleurone cells near the ovular vascular trace (Moore *et al.*, 2013).

1.4.3 Rice products

Due to the high levels of As in rice grains, certain rice products have also been found to contain significant quantities of As. Rice crackers and crisped rice breakfast cereals were found to contain high percentages of inorganic As (75-90%), with a total As concentration of 390 μg kg^{-1} found in one brand of rice crackers (Sun *et al.*, 2009). Rice bran is marketed as a health food because it is a good source of fibre and high in minerals, vitamins and antioxidants. However, commercially available rice bran products were found to contain As concentrations up to 1980 μg kg^{-1}, with iAs accounting for 70% of the total on average (Sun *et al.*, 2008). Brown rice syrup, made from whole brown rice grains, is commonly used as a sweetener in processed foods. Samples of brown rice syrup purchased in the UK contained between 80 and 330 μg kg^{-1} total As, and over 70% of As in the highest sample was found to be inorganic (Signes-Pastor *et al.*, 2009). Similar As concentrations were found in samples of brown rice syrup purchased in the USA, where total As ranged between 80 and 410 μg kg^{-1} As. Inorganic As was predominant in the brands containing lower total concentrations of As, however almost equal proportions of inorganic As and DMA were found in the sample with the highest As concentration (Jackson *et al.*, 2012a).

Baby rice, recommended for weaning children due to its low allergen potential, purchased in the UK was found to contain As concentrations up to 310 μg kg^{-1}, and 35% of the products sampled would be banned in China (Meharg *et al.*, 2008c). Infant foods purchased in Spain, including baby rice, were also found to contain significant quantities

of As, up to a maximum of 2310 μg kg^{-1} in a fish-based food. Inorganic As only made up a small portion of the total As in this sample (see **Section 1.4.5** for As speciation in seafood), however inorganic As was predominant in rice and meat-based foods (Carbonell-Barrachina *et al.*, 2012). Another study into infant formulas, purees and infant foods on sale in the USA found and that inorganic As was predominant in all samples. Estimated servings of formula, pear purees and infant foods would give median As exposures of 0.05, 0.20 and 0.39 μg kg $b.w.^{-1}$ day^{-1} respectively (Jackson *et al.*, 2012b). Although the infant formulas and purees tested do not exceed the 0.3 μg kg $b.w.^{-1}$ day^{-1} $BMDL_{01}$/MRL (ATSDR, 2007; EFSA, 2009), other sources of inorganic As may be present in the child's diet such as drinking water or rice milk. Furthermore, evidence is emerging that As exposure in childhood can negatively affect health into adult life (Vahter, 2008).

Finally, rice milk samples purchased from supermarkets in the UK were also found to contain alarming quantities of As. All had As concentrations above the WHO drinking water guideline of 10 μg L^{-1}, with a maximum concentration of 30 μg L^{-1}, and inorganic As accounted for 82% of the total As on average (Meharg *et al.*, 2008a). In the UK, babies and toddlers (under 4.5 years) are advised against consuming rice milk to limit their intake of inorganic As (Food Standards Agency, 2009). Arsenic concentrations in toddler formulas containing brown rice syrup were more than 20-fold higher than those lacking brown rice syrup. Additionally inorganic As concentrations in the reconstituted soy-based formula were 1.5 to 2.5-fold higher than the WHO drinking water guideline (Jackson *et al.*, 2012a).

1.4.4 Fruit and vegetables

Reported As concentrations in vegetables vary greatly, with a maximum of 3.99 μg kg^{-1} reported in Kachu sak (*Colocasia antiquorum*) from Bangladesh (Das *et al.*, 2004). However, the tubewell water used for irrigation in the sample area was highly contaminated, with a mean concentration of 520 μg L^{-1} As; over 50 times the WHO standard. The average As content of vegetable samples grown on contaminated soils in West Bengal, Chile and Bangladesh respectively were 0.075 μg g^{-1} DW (Signes-Pastor *et al.*, 2008), 0.114 μg g^{-1} FW (Munoz *et al.*, 2002) and 0.210 μg g^{-1} DW (Alam *et al.*, 2003). It is again noteworthy that the mean As concentration in tubewell water in the study area

of Bangladesh was well above the WHO limit at 240 μg L^{-1}, and that soil As concentrations averaged 13.3 mg kg^{-1} (Alam *et al.*, 2003).

A study of 88 apple and grape juices purchased in the USA found that 10% of samples had As concentrations higher than the WHO drinking water standard (10 μg L^{-1}), and that inorganic As was predominant (Consumer Reports, 2012). Another study into the As content of apple juice from the USA reported that one-third of the 38 samples tested contained As 'nearly at or above' the drinking water limit (Wilson *et al.*, 2012). Arsenic levels in fruit juices are of particular concern because they are consumed by children, which due to their small body size, are at higher risk of As poisoning. Apple juice, particularly, can contain high concentrations of As due to the historical use of As-containing pesticides, such as lead arsenate ($PbHAsO_4$) in orchards (MacLean & Langille, 1981).

1.4.5 Meat and seafood

Arsenic may also enter the food chain through consumption of meat products or seafood. Arsenic levels in fish and seafood have consistently been reported amongst the highest of all food products investigated (Dabeka *et al.*, 1993; Tsuda *et al.*, 1995; Schoof *et al.*, 1999; Llobett *et al.*, 2003; Al Rmalli *et al.*, 2005). Additionally, seafood accounted for around 90% of total dietary As in a study of U.S. citizens (Adams *et al.*, 1994). However, in all samples tested inorganic As represents a very low proportion of the total As in seafood. The majority instead consists mainly of arsenobetaine, with minor amounts of MMA, DMA, TMAO, arsenocholine and arsenosugars reported (Edmonds & Francesconi, 1993; Borak & Hosgood, 2007). Arsenobetaine and 'other organic arsenic compounds that are not metabolised in humans' are assigned to Group 3: "not classifiable as to its carcinogenicity to humans" by the International Agency for Research on Cancer (IARC, 2009). MMA and DMA are in Group 2B: "possibly carcinogenic to humans", and so diets high in seafood might still be of concern.

Rice straw is often fed to cattle in many countries, and was found to contain around 10 mg kg^{-1} As when rice plants were irrigated with water containing 50 μg L^{-1} As; the Bangladeshi drinking water limit (Abedin *et al.*, 2002a). Milk produced by cattle from five areas of Bangladesh was analysed and found to contain an average of 26.2 μg L^{-1} As. Cows

were exposed to As through rice straw and drinking water, containing an average of 1114.4 μg kg^{-1} and 89.6 μg L^{-1} As respectively (Ghosh *et al.*, 2013). Cow dung cakes, found to contain As concentrations of up to 8997 μg kg^{-1}, can be burnt as a kitchen fuel providing an additional route of As exposure through inhalation of ash and particulate matter (Pal *et al.*, 2007). Arsenic in the meat of animals fed rice straw has not yet been investigated, but will represent a further exposure route for people living in areas with As-contaminated water (Abedin *et al.*, 2002a; Das *et al.*, 2004).

Recently, a market basket survey of chicken was conducted in the USA. Conventionally-reared chicken (treated with roxarsone) was found to contain the same amount of total As as organically-reared chicken, however the concentration of inorganic As was three times higher in both raw and cooked samples of conventional chicken (Nachman *et al.*, 2013). Mean concentrations of around 20 to 50 mg kg^{-1} total As have been reported in roxarsone-fed poultry litter, which is often used by farmers to fertilise their crops (Arai *et al.*, 2003; Garbarino *et al.*, 2003). In fresh poultry litter roxarsone is the predominant As species, but is rapidly degraded by anaerobic bacteria liberating inorganic As (Garbarino *et al.*, 2003; Stolz *et al.*, 2007). It is estimated that chicken production in the Delmarva peninsula, USA produces 20 to 50 metric tonnes of As annually (Hancock *et al.*, 2001). In contrast to roxarsone-fed poultry, eggs and excreta from hens exposed to As-contaminated drinking water in Bangladesh, were found to contain just 19.2 μg kg^{-1} and 1440 μg kg^{-1} As respectively (Ghosh *et al.*, 2012).

Llama meat is part of the staple diet of people living in the Andean highlands of Argentina, and was found to contain up to 280 μg kg^{-1} total As (originally published in Spanish, reviewed in Bundschuh *et al.*, 2012). Average As concentrations in goat's milk from Illapata, Chile were found to be 440 μg L^{-1}; far exceeding the Chilean limit of 120 μg L^{-1} total As in foods (Bundschuh *et al.*, 2012). Arsenic was transferred to livestock from their primary foodstuff, alfalfa, which is irrigated with local groundwater containing incredibly high As concentration of 1300 μg L^{-1}. Arsenic concentrations in cow's milk from neighbouring Camarones, also exceeded the national limit with an average of 260 μg L^{-1}. Cheeses made from the caprine or bovine milk were also analysed, and contained 420 and 300 μg kg^{-1} total As respectively (originally published in Spanish, reviewed in Bundschuh *et al.*, 2012).

1.4.6 Herbal medicines and supplements

There is no international guideline for As in medicines or supplements, but the Singapore Ministry of Health set a maximum permissible level of As at 5000 μg kg^{-1} (Koh & Woo, 2000), and the Chinese government has set a maximum contaminant concentration of As in Chinese herbal medicines at 2000 μg kg^{-1} (Ministry of Health of the People's Republic of China, 2005). A study of 247 traditional Chinese medicines on sale in Queensland, Australia found that based on the recommended daily dose, five would exceed the total tolerable daily intake of As for females. Alarmingly, one of the samples gave 276-fold more As than the tolerable daily intake (based on the WHO guideline, and mean daily Australian dietary exposure; Cooper *et al.*, 2007). Additionally, analysis of As concentration and speciation in Chinese herbal medicines sampled directly from fields or purchased at markets, found that inorganic As was generally predominant (Liu *et al.*, 2013).

A study of 16 dietary supplements on sale in Denmark conducted by Hedegaard *et al.* (2013), found As concentrations ranging from 580 to 5000 μg kg^{-1}. Arsenic speciation varied with the type of sample; plant-based supplements were dominated by inorganic As, whereas for algal-based samples inorganic As accounted for just 15% of the total on average. Based on the recommended dose of the supplements, daily inorganic As exposure was calculated to be up to 13.0 μg day^{-1} (4.14 g day^{-1} of Chuan Xin Lian containing 5000 μg kg^{-1} total As), which for a 60 kg adult would provide 72% of the 0.3 μg kg $b.w.^{-1}$ day^{-1} $BMDL_{01}$/MRL (ATSDR, 2007; EFSA, 2009).

1.5 Arsenic tolerance mechanisms

Arsenic is a highly toxic element, but is also ubiquitous in the environment, and so As tolerance mechanisms are present in virtually all organisms. However, there are some exceptions and As-hyperaccumulating plants including the Chinese brake fern *Pteris vittata* (Ma *et al.*, 2001) have been identified, as well as dissimilatory arsenate-reducing microbes which use arsenate as an electron donor in respiration (for review see Oremland & Stolz, 2003). Recent reports that a bacterium can survive using As instead of phosphorus (Wolfe-Simon *et al.*, 2011) have since been refuted, as DNA extracted from strain GFAJ-1 was found to contain phosphorus (Erb *et al.*, 2012; Reaves *et al.*, 2012).

1.5.1 Reduction

It may seem paradoxical for arsenate to be reduced to arsenite which is more toxic (see **Section 1.3**) however, the detoxification mechanisms of methylation and complexation both require As to be present in reduced form. Arsenate can be reduced to arsenite non-enzymatically by glutathione (GSH), but it is thought that this occurs too slowly to be physiologically relevant (Zhao *et al.*, 2009).

Arsenate reductases (ARs) have been extensively studied in micro-organisms. Disruption of *arsC* from *Escherichia coli* and *ACR2* in yeast (*Saccharomyces cerevisiae*) resulted in arsenate sensitivity (Mukhopadhyay & Rosen, 1998; Mukhopadhyay & Rosen, 2002). Although structurally unrelated, both ArsC and ACR2 require GSH for arsenate reduction. ACR2 homologues bear structural similarity to the catalytic domain of the human Cdc25 phosphatase. Expression of the catalytic domains of *hCDC25B* and *hCDC25C* in *E. coli* mediated arsenate reduction in the presence of GSH (Bhattacharjee *et al.*, 2010).

In mammals, there is an emerging model of adventitious arsenate reduction by phosphorolytic enzymes. Arsenylated compounds produced by these enzymes are reduced by various thiols liberating arsenite (Gregus *et al.*, 2009; Németi & Gregus, 2009). This mechanism could explain arsenate reductase activity observed for a diverse range of enzymes including glyceraldehyde-3-phosphate dehydrogenase, mitochondrial ATP synthase, glycogen phosphorylase-a and purine nucleoside phosphorylase (Németi & Gregus, 2002; Gregus & Németi, 2005; Németi & Gregus, 2007; Németi *et al.*, 2010).

Arsenate reductases with homology to yeast ACR2 have been identified in plant species including *Arabidopsis thaliana* (Dhankher *et al.*, 2006), rice (Duan *et al.*, 2007), As-tolerant *Holcus lanatus* (Bleeker *et al.*, 2006) and the As hyperaccumulator *P. vittata* (Duan *et al.*, 2005). Knockdown of *AtACR2* expression using RNA interference (RNAi) was reported to increase sensitivity to arsenate, and cause over 10-fold higher As accumulation in shoots compared to wild-type (Dhankher *et al.*, 2006). Additionally, an Arabidopsis knockdown mutant (caused by a T-DNA insertion in the *AtACR2* promoter), was hypersensitive to arsenate and accumulated around 6-fold more As in the shoots (Nahar *et al.*, 2012). However, T-DNA insertion lines of *AtACR2* characterised by Liu *et al.* (2012) accumulated similar concentrations of As in the shoots to the wild-type, and was only more tolerant to

arsenate under extremely low phosphate concentrations (1 µM). Furthermore, As speciation in Arabidopsis was unaffected by either knockout or overexpression of *AtACR2*. The authors propose that multiple pathways of arsenate reduction may exist in plants, as has been demonstrated for mammals.

An arsenate-activated glutaredoxin from *P. vittata*, *PvGRX5*, conferred tolerance to arsenite and arsenate when expressed in *E. coli* strain AW3110 which lacks the *ars* operon (Sundaram *et al.*, 2008). Expression of *PvGRX5* in Arabidopsis increased tolerance to arsenite and arsenate, and significantly decreased As accumulation in leaves (Sundaram et al., 2009). Additionally, a cytosolic triosephosphate isomerase from *P. vittata* was found to increase arsenate tolerance when expressed in *E. coli* lacking ArsC (Rathinasabapathi *et al.*, 2006).

1.5.2 Arsenic methylation by mammals

Soon after exposure to inorganic As, the methylated species MMA and predominantly DMA, were detected in human urine (Braman & Foreback, 1973). Early *in vitro* studies, performed mainly using hepatic tissue from mouse or rat, found that *S*-adenosylmethionine (SAM) and GSH are required for methylation of inorganic As (Buchet & Lauwerys, 1985; Buchet & Lauwerys, 1988). SAM is considered to be the universal donor of methyl groups in mammalian systems. As3MT, As(III) methyltransferase, is responsible for catalysing the transfer of a methyl group from SAM to reduced As, and was first purified from rat liver cytosol (Lin *et al.*, 2002). It is thought that the reductant power *in vivo* is provided by thioredoxin (Trx) which is regenerated by Trx-reductase and NADPH, as is the case in *Staphylococcus aureus* (Messens *et al.*, 1999; Thomas *et al.*, 2004). Interestingly certain mammals including guinea pigs (*Cavia porcellus*) appear to be unable to methylate As, but do not appear to be any more As-sensitive (Aposhian, 1997; Healy *et al.*, 1997).

1.5.3 Arsenic methylation by microbes

Since As3MT was identified as the enzyme responsible for converting arsenite to DMA in mammals (Thomas *et al.*, 2004), over 200 homologues have been identified in archaea, fungi, metazoa and bacteria (Bhattacharjee & Rosen, 2007). Some have been termed ArsM as they are downstream of an *arsR* gene, encoding an As-responsive transcriptional

repressor which controls transcription of *ars* operons (Xu *et al.*, 1998). The model *E. coli arsRDABC* operon also includes *arsC*; encoding an arsenate reductase, *arsB*; encoding a plasma membrane arsenite/proton antiporter, *arsA*; encoding an ATPase which fuses with ArsB to form an active arsenite pump, and *arsD*; encoding a chaperone that delivers arsenite to ArsAB (Carlin *et al.*, 1995; Lin *et al.*, 2006). Expression of *arsM*, from the soil bacterium *Rhodopseudomonas palustris*, alone conferred arsenite tolerance to a strain of *E. coli* lacking enodogenous As resistance genes (Qin *et al.*, 2006). ArsM proteins are able to sequentially methylate arsenite, with trimethylarsine gas as the end-product (Qin *et al.*, 2009).

1.5.4 Arsenic methylation by plants

A gene containing the *arsM* family UbiE/Coq5 motif was found to be up-regulated under arsenate exposure (Norton *et al.*, 2008). However, this gene lacks three conserved cysteine residues found in all proven As methyltransferases (Ye *et al.*, 2012) and has not yet been characterised. Arsenic methylation has not been demonstrated in higher plants and is the focus of **Chapter 3**.

1.5.5 Complexation

In humans it is estimated that 99% of cellular arsenite is thiol-bound (Kitchin & Wallace, 2005). Glutathione is the most abundant low molecular weight thiol in human cells, with concentrations ranging between 0.5 and 10 mM. At physiological pH, trivalent As species will form the As-glutathione conjugates As(III)-GS_3, MMA(III)-GS_2 and DMA(III)-GS. Detection of As-conjugates in biological samples is uncommon due to the extraction procedures used, and the instability and short half-life of these complexes (for review see Leslie, 2012). However, As(III)-GS_3 and MMA(III)-GS_2 were identified in the bile of rats treated with arsenite (Kala *et al.*, 2000). See **Section 1.7.3** for transporters of As complexes in micro-organisms and animals**.**

Phytochelatins (PCs) are heavy metal-binding peptides synthesised by plants from glutathione, and have the general structure of $(\gamma\text{-Glu-Cys})_n$-Gly, where *n* can be as high as 11, but generally lies between two and five (Cobbett, 2000). Exposure to either arsenate or arsenite induces PC-synthesis (Schmoger *et al.*, 2000). Numerous As-PC and As-GS complexes have been identified in various plants, with As(III)-PC_3 predominant in both

sunflower (*Helianthus annuus*) and *H. lanatus* (Raab *et al.*, 2004; Raab *et al.*, 2005). Additionally, significant proportions of As(III)-PC_4 were detected in duckweed (*Wolffia globosa*) and Arabidopsis (Liu *et al.*, 2010b; Zhang *et al.*, 2012). Exposure of sunflower to MMA produced only trivalent arsenicals such as MMA(III)-$(PC)_2$ (Raab *et al.*, 2005). Transporters of As-complexes in plants are discussed in **Section 1.8.3**.

1.6 Transport in plants

In As-sensitive plants, reduction and conjugation prevent As translocation to the aerial tissues, presumably to protect the vital photosynthetic and reproductive tissues. However, the most common organic species, MMA and DMA, are readily translocated from roots to shoots. This has been observed in plants including tomato (Burlo *et al.*, 1999), turnip (Carbonell-Barrachina *et al.*, 1999; Yao *et al.*, 2009), pepper (Száková *et al.*, 2007), rice (Marin *et al.*, 1992), radish (Tlustoš *et al.*, 2002), cotton (Marcuswyner & Rains, 1982) and *Spartina patens* and *Spartina alterniflora* (Carbonell-Barrachina *et al.*, 1998). Perhaps most significantly, in a study of 46 plant species from 13 different families, Raab *et al.* (2007b) found that all but one species (*Melica aristata*) had shoot to root translocation factors (TFs) higher for DMA than that for arsenate; with the average of all species studied almost ten times higher.

One explanation for the higher translocation efficiency of pentavalent DMA and MMA is that, unlike arsenite, they are not conjugated to thiols such as phytochelatins (Raab *et al.*, 2007b). A pentavalent DMA-GS complex was identified in cabbage (*Brassica oleracea*), but the high levels of sulphur in members of the Brassicaceae may mean they contain unusual As complexes (Raab *et al.*, 2007c). Arsenic-thiol complexes are only stable at acidic pH, and so are proposed to be located in the vacuole (Sneller *et al.*, 2000), which limits As translocation to the shoots. Arsenic-thiol complexes have not been identified in xylem or phloem sap (Pickering *et al.*, 2000; Raab *et al.*, 2005; Ye *et al.*, 2010) and decreased complexation in PC-deficient *cad1-3*, and GSH-deficient *cad2-1* Arabidopsis mutants, resulted in increased accumulation of As in shoots (Liu *et al.*, 2010b). Complexation with PCs was also shown to decrease As translocation from shoots to rice grain. Foliar sprays with BSO (L-buthionine-sulphoxime), a potent inhibitor of γ-glutamylcysteine synthetase, the first enzyme in the glutathione biosynthesis pathway,

decreased shoot As concentrations but increased As concentrations in husk and brown rice (Duan *et al.*, 2011). Furthermore, the low levels of As-complexation in the As hyperaccumulator *P. vittata* is proposed as one mechanism resulting in the exceptionally high foliar concentrations of As in this species (Zhao *et al.*, 2003; Su *et al.*, 2008).

Recently, the mechanisms of As transport to rice grain were investigated by feeding flag leaves and excised panicles (Carey *et al.*, 2010; Carey *et al.*, 2011; Zhao *et al.*, 2012). Pulsing excised panicles with DMA resulted in ten-fold higher grain As concentrations than those treated with arsenite, arsenate or As(III)-GS_3. Additionally, stem-girdling treatment revealed that 90% of arsenite is transported to developing grains via the phloem, compared to 55% for DMA (Carey *et al.*, 2010). Feeding flag leaves with arsenite or arsenate resulted in very little remobilisation of As to developing grains, whereas treatment with MMA or DMA gave 100-fold higher grain As concentrations than the inorganic As treatments (Carey *et al.*, 2011). Using radioactive ^{73}As, Zhao *et al.* (2012) showed that inorganic As fed to flag leaves was exported to tissues in the order of stem > rachis > grain > other leaves. Furthermore, when roots were fed with ^{73}As, only around 10% was translocated to the shoots, and only 3.3% of total ^{73}As in aboveground tissues within the grain. Steam-girdling at the base of the panicle decreased grain ^{73}As by 97% (Zhao *et al.*, 2012).

1.7 Arsenic transporters in micro-organisms and animals

Due to their different chemical properties, different As species are transported by diverse transporter families and will be discussed separately.

1.7.1 Arsenate

Arsenate ($H_2AsO_4^-$/ $HAsO_4^{2-}$; see Figure S1.2 for dissociation) is a chemical analogue of phosphate ($H_2PO_4^-$/ HPO_4^{2-}) and due to similar size; 2.66 Å and 2.52 Å respectively, shape and acid dissociation constants, is thought to be taken up by phosphate transporters. Both of the inorganic phosphate transporters in *E. coli*, Pst and Pit, are able to transport arsenate (Willsky & Malamy, 1980). Two high-affinity; Pho84 and Pho89, and three low-affinity phosphate transporters; Pho87, Pho90 and Pho91, have been identified in yeast (Persson *et al.*, 2003). A screen of yeast mutants identified *pho84*, and the *pho86 pho87*

double mutant as arsenate tolerant (Bun-ya *et al.*, 1996). Pho86 is located in the endoplasmic reticulum (ER) and is necessary for targeting of Pho84 to the plasma membrane under phosphate-limiting conditions (Lau *et al.*, 2000).

Mammals possess five inorganic phosphate transporters that mediate transport of phosphate across the plasma membrane, which are classified into two families; type II and type III sodium/phosphate co-transporters (Virkki *et al.*, 2007). Expression of the rat isoforms of all five transporters; *rNaPiIIa*, *rNaPiIIb*, *rNaPiIIc*, *rPit1* and *rPit2*, in *Xenopus laevis* oocytes resulted in significant arsenate uptake in the absence of phosphate. Furthermore, when expressed in *Xenopus* oocytes, *NaPi-IIb1* from zebrafish (*Danio rerio*) facilitated arsenate uptake, which was coupled with sodium ions and inhibited by phosphate (Beene *et al.*, 2011).

The presence of 1 mM inorganic phosphate in the medium decreased arsenate uptake of oocytes expressing the rat phosphate transporters by an average of 96.3% (Villa-Bellosta & Sorribas, 2010). The average concentration of phosphate in human plasma was found to be 1.15 ± 0.19 mM (Walser, 1961), therefore the physiological significance of arsenate transport by phosphate transporters in mammals is probably minimal. This is supported by the finding that arsenate uptake by mammalian cell lines is generally negligible compared to arsenite (Hirano *et al.*, 2003), apart from when phosphate-free culture medium is used (Huang & Lee, 1996). However, one of the transporters, rNaPiIIb, displayed a much higher affinity for arsenate and is highly expressed in enterocytes, so may be involved in uptake of ingested arsenate (Villa-Bellosta & Sorribas, 2010).

1.7.2 Arsenite

The first arsenite transporter to be identified was the glycerol facilitator from *E. coli*, GlpF. Arsenite uptake in the *glpF* mutant is reduced by 80% (Meng *et al.*, 2004). In solution arsenite is present as arsenous acid [$As(OH)_3$] which is an analogue of glycerol. GlpF belongs to the major intrinsic protein (MIP) superfamily of aquaporins. Aquaporins facilitate diffusion of small neutral solutes across membranes, and as they are not coupled to an energy source facilitate bi-directional transport of substrates depending on the concentration gradient. In yeast, Fps1, a homologue of GlpF, was also shown to be permeable to arsenite (Wysocki *et al.*, 2001).

Aquaporins AQP7 and AQP9, from mouse (*Mus musculus*), rat (*Rattus norvegicus*) and man (*Homo sapiens*), have been shown to transport arsenite when heterologously expressed in yeast or *Xenopus* oocytes (Liu *et al.*, 2002; Liu *et al.*, 2004b; McDermott *et al.*, 2010). However, arsenite permeability is not a common feature of all mammalian aquaporins, and arsenite transport was not observed for hAQP3 or hAQP10 (Liu *et al.*, 2004b). Additionally, expression of zebrafish *AQP3*, *AQP3l*, *AQP9a*, *AQP9b* and *AQP10* in *Xenopus* oocytes facilitated uptake of arsenite (Hamdi *et al.*, 2009).

Interestingly, uptake of arsenite by yeast lacking *Fps1* (*fps1*Δ) in the absence of glucose was only reduced by 25%, and arsenite uptake was inhibited by glucose, galactose, fructose and mannose. Yeast has 18 hexose permeases, and expression of *HXT1*, *HXT3*, *HXT4*, *HXT5*, *HXT7* or *HXT9* restored arsenite transport in strain HD300; which lacks all endogenous hexose transporters (Liu *et al.*, 2004a). The glucose transporter GLUT1, isolated from either rat or human, was also able to transport arsenite when expressed in yeast and *Xenopus* oocytes (Liu *et al.*, 2006a).

Transport of arsenite by hexose transporters may seem strange, but polymerisation of three arsenous acid [$As(OH)_3$] molecules is predicted to form a six-membered ring with structural similarity to glucose (Meng *et al.*, 2004). Alternatively, the binding site of hexose transporters may be occupied by two or three arsenite monomers (Liu *et al.*, 2006a). Lastly, mammalian glucose transporters are proposed to facilitate water transport (Fischbarg *et al.*, 1990), and so arsenite may be transported via the water-translocation pathway (Liu *et al.*, 2006a).

Members of the organic anion transporting polypeptide (OATP) family have also been implicated in arsenite transport in humans. Expression of *OATPB* in the human colon adenocarcinoma Caco-2 cell line was upregulated under arsenite treatment, and silencing *OATPB* caused a significant decrease cellular arsenite accumulation (Calatayud *et al.*, 2012). Expression of *OATPC* in the human embryonic kidney HEK-293 cell line significantly increased uptake of arsenite and arsenate, although the difference was greater for arsenite treatment (Lu *et al.*, 2006).

Micro-organisms also possess two families of arsenite efflux transporters. Members of the ArsB family are widespread in prokaryotes, whereas Acr3 members have been

identified in bacteria, archaea, fungi and the As-hyperaccumulating fern *P. vittata* (Wysocki *et al.*, 1997; Rosen, 1999; Fu *et al.*, 2009; Indriolo *et al.*, 2010). ArsB from *E. coli* is best characterised and is an arsenite/proton antiporter, but can fuse with ArsA to form an ATP-driven arsenite pump (Dey & Rosen, 1995). Acr3 transporters belong to the bile/arsenite/riboflavin transporter (BART) superfamily (Mansour *et al.*, 2007). Acr3 from yeast is best characterised, and is an arsenite/proton antiporter (Maciaszczyk-Dziubinska *et al.*, 2011).

1.7.3 Methylated arsenic

Aquaporins have also been shown to transport methylated As species. Interestingly, yeast expressing *hAQP9* accumulated significantly more trivalent MMA [MMA(III)] than arsenite (Liu *et al.*, 2006b). Furthermore, expression of *hAQP9* in *Xenopus* oocytes resulted in uptake of pentavalent MMA and DMA. Uptake of MMA and DMA decreased with increasing medium pH, demonstrating that the undissociated, neutral forms of MMA and DMA are the substrates of hAQP9 (McDermott *et al.*, 2010). Expression of zebrafish *AQP3l* and *AQP10* facilitated significant uptake of MMA(III) in *Xenopus* oocytes (Hamdi *et al.*, 2009). However, yeast Fps1 was not permeable to trivalent MMA, suggesting that arsenite transport is not a predictor of permeability to trivalent MMA (Liu *et al.*, 2006b).

The mammalian glucose transporter, GLUT1 also facilitated uptake of trivalent MMA when expressed in *Xenopus* oocytes, but unlike arsenite, MMA(III) transport was not inhibited by glucose (Liu *et al.*, 2006a). Site-directed mutagenesis in the rat isoform to increase water permeability also increased MMA(III) uptake (Jiang *et al.*, 2010). This suggests that water and MMA(III) share a common transport mechanism that differs from that of sugars, and possibly arsenite (Liu *et al.*, 2006a; Jiang *et al.*, 2010).

1.7.4 Conjugated arsenic

Once chelated to glutathione, As-GS complexes are transported into the vacuole or out of the cell by members of the ATP-binding cassette (ABC) transporter superfamily. In yeast arsenite-glutathione complexes are transported into the vacuole by Ycf1 (Ghosh *et al.*, 1999). Ycf1 belongs to the multidrug resistance-associated protein (MRP) subfamily of ABC transporters. Ycf1 has been found to mediate the transport of complexes such as cadmium Cd(II)-GS_2; mercury Hg(II)-GS_2; lead Pb(II)-GS_2; selenium Se(IV)-GS_2 and

antimony Sb(III)-GS_3 in addition to arsenite As(III)-GS_3 (Ghosh *et al.*, 1999; Gueldry *et al.*, 2003; Song *et al.*, 2003; Prévéral *et al.*, 2006; Lazard *et al.*, 2011).

Humans possess nine MRP members, also known as ABCC transporters, of which MRP1/ABCC1 and MRP2/ABCC2 are best characterised (Leslie, 2012). ABCC1 and ABCC2 both transport As(III)-GS_3 and MMA(III)-GS_2 (Kala *et al.*, 2000; Leslie *et al.*, 2004; Carew *et al.*, 2011). *ABCC2* is highly expressed in hepatocytes and is proposed to have a critical role in transporting As-conjugates, including As(III)-GS_3 and MMA(III)-GS_2 into the bile for excretion (Kala *et al.*, 2000).

1.8 Arsenic transporters in plants

1.8.1 Arsenate

Arsenate, as a chemical analogue of phosphate, is thought to be taken up by phosphate transporters at the root surface. Indirect evidence for this theory comes from studies reporting the inhibition of arsenate uptake by phosphate (including: Ullrich-Eberius *et al.*, 1989; Abedin *et al.*, 2002b), and increased arsenate tolerance of Arabidopsis phosphate transporter mutants (Shin *et al.*, 2004; Gonzalez *et al.*, 2005; Catarecha *et al.*, 2007). Additionally, arsenate represses phosphate starvation responses in Arabidopsis, possibly through a shared arsenate/phosphate signalling pathway (Catarecha *et al.*, 2007). Furthermore, arsenate tolerance in *H. lanatus* appears to involve post-translational modification of the high-affinity phosphate transporter system (Meharg & Macnair, 1990; Macnair *et al.*, 1992; Meharg & MacNair, 1992; Meharg *et al.*, 2013), and phosphate transporters in *P. vittata* appear to have a higher affinity for arsenate than those from non-tolerant species (Wang *et al.*, 2002; Poynton *et al.*, 2004).

Evidence for the uptake of arsenate by phosphate transporters in rice came from Wu *et al.* (2011) who found that in hydroponic culture, overexpression of the high-affinity phosphate transporter *OsPT8* significantly increased concentrations of both arsenate and arsenite in roots, shoots and xylem sap compared to wild-type when exposed to arsenate. Additionally, mutation in OsPHF1 (*phosphate transporter traffic facilitator1*) significantly decreased root uptake of ^{33}P and arsenate compared to the background, an OsPHR2-overexpression line (Wu *et al.*, 2011).

Uptake kinetics revealed that OsPT8 has a high affinity for both phosphate and arsenate (Wu *et al.*, 2011). Furthermore, arsenate induced significant inward currents, similar to those for phosphate, measured by two-electrode voltage clamp of *Xenopus* oocytes expressing the high-affinity phosphate transporter from barley *HvPht1;1*, (Preuss *et al.*, 2011). However, As concentrations in grains and straw of *OsPT8*-overexpression lines grown in flooded soils were no different to wild-type, suggesting that the phosphate transport pathway makes little contribution to As accumulation in rice grain grown under flooded paddy conditions (Wu *et al.*, 2011).

1.8.2 Arsenite

The transporters responsible for the uptake, and transport to the stele for xylem loading, of silicon in rice roots were identified as OsLsi1 and OsLsi2 respectively (Ma *et al.*, 2006; Ma *et al.*, 2007a). Casparian strips present in the endodermis and exodermis of rice roots prevent the apoplastic flow of solutes to the vascular tissue. Therefore the localisation of OsLsi1 to the distal plasma membrane, and OsLsi2 to the proximal plasma membrane, of exodermal and endodermal cells permits effective transcellular transport of Si (Ma *et al.*, 2007a). Based on sequence similarity, OsLsi1 was identified as a member of the MIP superfamily, belonging to the Nodulin26-like intrinsic protein (NIP)-subfamily and is also known as OsNIP2;1. OsLsi2 is an active efflux transporter with no similarity to OsLsi1, and instead shares low sequence similarity with the arsenite efflux transporter, ArsB from *E. coli* (Meng *et al.*, 2004; Ma *et al.*, 2007a).

Due to the similar size, shape and charge of arsenous acid [$As(OH)_3$] and silicic acid [$Si(OH)_4$], it was hypothesised that Si transporters were also able to transport arsenite. Transport activity of OsLsi1 for arsenite was confirmed through heterologous expression in *Xenopus* oocytes and yeast, and *lsi1* and *lsi2* mutant shoots contained 71% and 25% As compared to wild-type respectively (Ma *et al.*, 2008). Expression of *OsLsi1* sensitised yeast to arsenite, but intriguingly increased tolerance to arsenate. This is due to the fact that OsLsi1 facilitates bi-directional arsenite transport, and so facilitated the diffusion of arsenite resulting from arsenate reduction. OsLsi1 was found to be mediate arsenite efflux from rice roots after arsenate exposure in hydroponic culture (Zhao *et al.*, 2010b).

Other members of the NIP subfamily of aquaporins in rice; OsNIP1;1, OsNIP2;2 (Lsi6), OsNIP3;1 and OsNIP3;2, have also shown arsenite transport in *Xenopus* oocytes or yeast (Bienert *et al.*, 2008; Ma *et al.*, 2008; Mitani *et al.*, 2008). In Arabidopsis, AtNIP1;1, AtNIP1;2, AtNIP5;1, AtNIP6;1 and AtNIP7;1 have demonstrated permeability to arsenite in *Xenopus* oocytes or yeast, and also by increased arsenite tolerance of *atnip1;1* and *atnip7;1* mutants (Bienert *et al.*, 2008; Isayenkov & Maathuis, 2008; Mitani *et al.*, 2008; Kamiya *et al.*, 2009; Mitani-Ueno *et al.*, 2011).

Recently, Mosa *et al.* (2012) demonstrated that members of the plasma membrane intrinsic proteins (PIP)-subfamily of aquaporins are also involved in arsenite transport in rice. Expression of *OsPIP2;4*, *OsPIP2;6* and *OsPIP2;7* resulted in arsenite accumulation in *Xenopus* oocytes. Additionally, Arabidopsis expressing *OsPIP2;4*, *OsPIP2;6* and *OsPIP2;7* were more tolerant to arsenite, although root and shoot total As content was not significantly different from wild-type. Although OsPIP1;2 and OsPIP1;3 showed no arsenite transport in *Xenopus* oocytes, root expression of all five PIP genes studied was significantly down-regulated during short-term exposure (12 hours) to 100 µM arsenite.

Although no homologues of the yeast arsenite effluxer, ACR3 are found in higher plants such as Arabidopsis and rice, the As-hyperaccumulating fern *P. vittata* expresses two similar genes; *PvACR3* and *PvACR3;1* (Indriolo *et al.*, 2010). Gametophytes of *P. vittata* with reduced expression of *PvACR3* were more sensitive to arsenite than both wild-type, and those with decreased expression of *PvACR3;1*. Additionally, arsenite and arsenate treatment upregulated expression of *PvACR3* in gametophytes, whereas expression of *PvACR3;1* was unaffected by As treatment. PvACR3 is localised to the tonoplast, and so is proposed to mediate transport of arsenite into the vacuole (Indriolo *et al.*, 2010).

Recently the gene encoding yeast ACR3 (*ScACR3*) was introduced into Arabidopsis and rice (Ali *et al.*, 2012; Duan *et al.*, 2012). In Arabidopsis, expression of *ScACR3* resulted in increased tolerance to arsenite and arsenate of transgenic protoplasts, seedlings grown on agar plates and hydroponically-cultured mature plants (Ali *et al.*, 2012). ScACR3 localised to the plasma membrane, and increased As efflux of protoplasts and roots treated with arsenate. Expression of *ScACR3* did not significantly affect the concentration of As in tissues of transgenic Arabidopsis, but did affect As partitioning, with an increase

translocation factor (TF) which is the ratio of As in the shoot to root (Ali *et al.*, 2012). Very similar results were observed for Arabidopsis transformed with *PvACR3* (Chen *et al.*, 2013b).

Expression of *ScACR3* in rice resulted in some phenotypic differences compared to Arabidopsis (Duan *et al.*, 2012). Root efflux of arsenite was significantly increased by expression of *ScACR3*, however, As concentrations in roots and shoots were significantly lower than in the wild-type, with no difference in As translocation. Most importantly, As concentrations in *ScACR3*-expressing straw, flag leaves, husks and brown rice grains were significantly decreased compared to wild-type (Duan *et al.*, 2012). Therefore introduction of *ACR3* genes could be utilised to decrease As concentrations in edible crops, or to increase As-accumulation for phytoremediation (Chen *et al.*, 2013b).

1.8.3 Methylated arsenic

DMA and MMA are the major organic As species present in the terrestrial environment. Li *et al.* (2009) recently discovered that OsLsi1 is also responsible for the uptake of undissociated pentavalent methylated arsenic species in rice roots. The *lsi1* mutant lost around 80% uptake capacity for MMA and 50% for DMA. In contrast, mutations in *lsi2* had little effect on uptake. Additionally, *Xenopus* oocytes expressing *OsLsi1* accumulated significantly more MMA than control, water-injected oocytes.

However, MMA and DMA have relatively low acid dissociation constants (pK_{a1}) of 4.19 and 6.14 respectively (Figure S1.2), and increasing the pH of the medium greatly decreased uptake of MMA and DMA by wild-type rice (Li *et al.*, 2009a). The transporters responsible for the uptake of the dissociated forms of MMA and DMA have yet to be identified in plants, and are the subject of **Chapter 5**.

1.8.4 Conjugated arsenic

Arsenic-PC complexes are only stable under acidic conditions such as within the vacuole (Sneller *et al.*, 2000). It was presumed that an ABC protein was responsible for the transport of these complexes into the vacuole (Bleeker *et al.*, 2006), however the ABC superfamily is very large; with 131 members in Arabidopsis (Jasinski *et al.*, 2003). Recently the major vacuolar arsenite-PC transporters were identified in Arabidopsis (Song *et al.*, 2010). AtABCC1 and AtABCC2 were identified from the sensitivity of the single mutants to

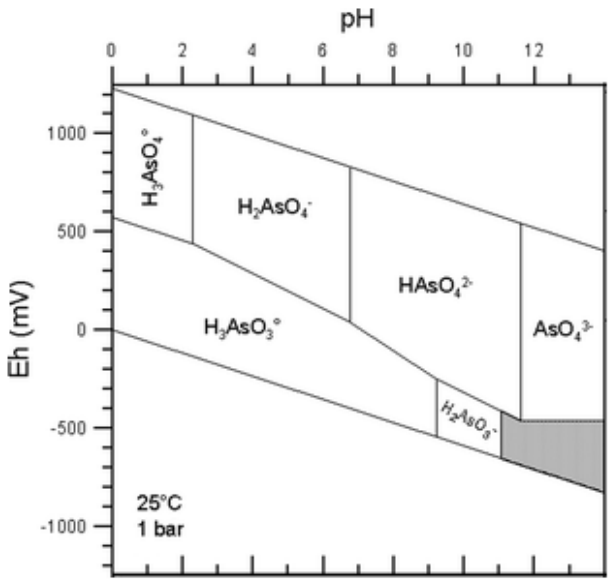
a sodium salt of MMA, however increased sensitivity to arsenate was only observed in the *atabcc1 atabbc2* double mutant, indicating some functional redundancy (Song *et al.*, 2010). Intact vacuoles isolated from the Arabidopsis *atabcc1 atabbc2* double mutant took up just 15% of the As(III)-PC_2 complexes compared to wild-type vacuoles, demonstrating that these transporters are the major pathway for As(III)-PC accumulation in vacuoles. The low level residual As(III)-PC_2 uptake was proposed to be mediated by AtABCC11 and AtABCC12; which show high homology to AtABCC1 and AtABCC2 (Song *et al.*, 2010). ABC transporters generally show broad substrate specificity, and AtABCC1 and AtABCC2 were also found to be permeable to complexes of mercury, including Hg(II)-PC and Hg(II)-GS_2 (Park et al., 2012). The rice genome encodes 12 putative full-size ABC transporters belonging the ABCC/MRP subfamily (Jasinski et al., 2003) however, none of these have yet been characterised.

1.9 Aims

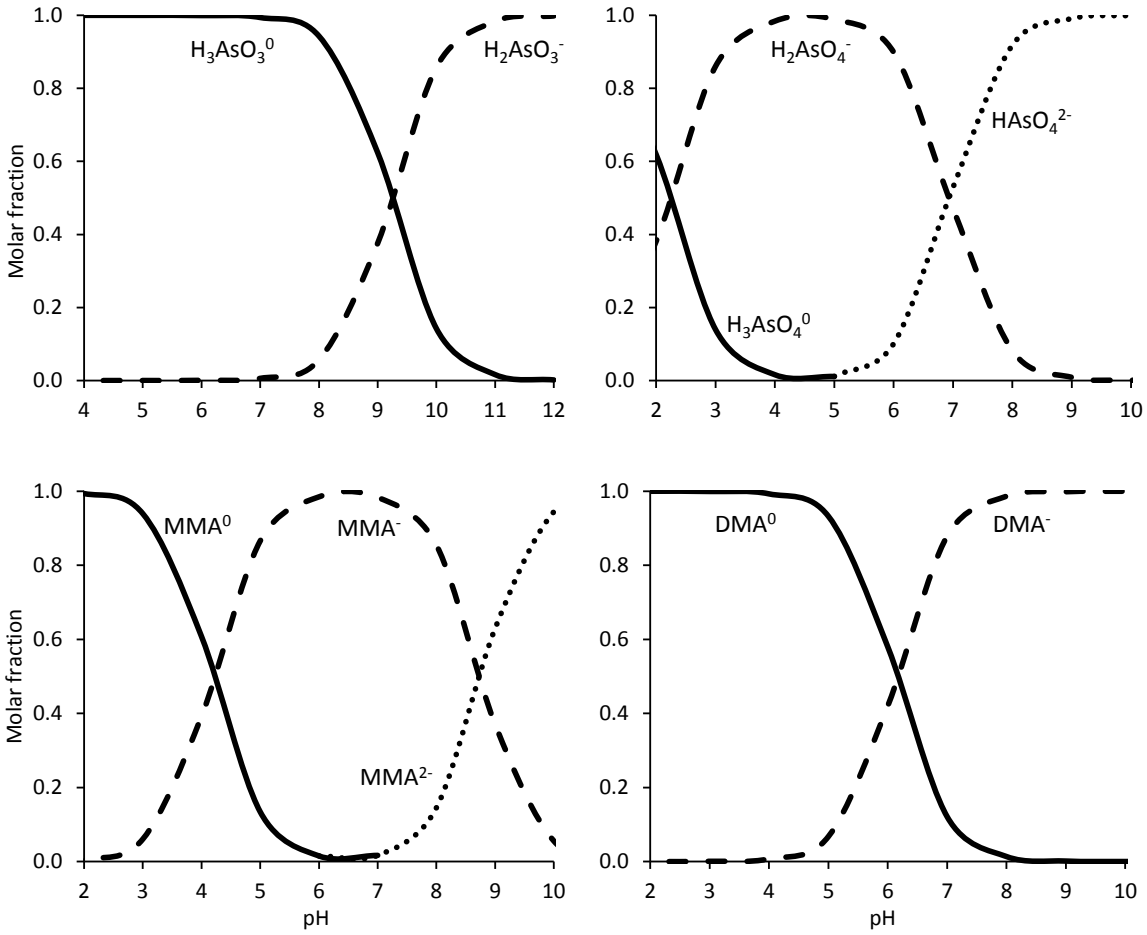
The two major objectives of this thesis were to determine the origin of methylated As species in plants, and to identify the transport pathway of these organic As species. The chemical properties of MMA and DMA mean that they are strongly affected by changes in pH, and so two distinct classes of plant transporters were investigated. Therefore the main aims of the thesis were:

1. To determine whether plants are capable of methylating As under a range of conditions including nutrient stress and symbiosis with root-nodulating bacteria.
2. To characterise the substrate specificity of rice aquaporins OsNIP1;1, OsNIP3;2 and OsNIP3;3 using heterologous expression in *Xenopus laevis* oocytes.
3. To investigate the contribution of the phosphate transport pathway to the uptake and translocation of MMA and DMA using rice mutants grown in hydroponic culture.

1.10 Supplementary information



Supplementary Figure 1.1 Eh-pH diagram for aqueous inorganic As species, taken from Marini & Accornero (2007). Shaded areas represent uncertainty in relationships between As species under high pH and strongly reducing conditions.



Supplementary Figure 1.2 Dissociation curves of arsenite (pK_a = 9.22), arsenate (pK_{a1} = 2.21, pK_{a2} = 6.95), MMA (pK_{a1} = 4.19, pK_{a2} = 8.77) and DMA (pK_a = 6.14).

2. General materials and methods

All chemicals used were of analytical grade or better, and water used to make reagents was ultrapure (18.2 MΩ). All seeds were stored in the dark at 4°C before use.

2.1 Analysis of total arsenic concentration

2.1.1 ICP-MS: Agilent 7500ce

From October 2009 to August 2012, total As concentration in digested materials was determined by ICP-MS (Agilent 7500ce; Agilent Technologies, Santa Clara, CA, USA). An autosampler (Cetac ASX-520; Teledyne Cetac Technologies, Omaha, NE, USA) was connected to the water-jacketed nebuliser of the ICP-MS. Samples were diluted 5-fold by the Agilent Integrated Sample Introduction System (ISIS). A solution of 250 ppb of mixed internal standard elements (Li^6, Sc, Ge, Y, In, Tb, Bi) was mixed continuously with the diluted sample through a peristaltic pump. Signals at *m/z* 75 (As), 72 (Ge) and 35 (Cl) were collected with a dwell time of 0.5 seconds for As and Ge, and 0.2 seconds for chlorine. The possible polyatomic interference of $^{40}Ar^{35}Cl$ on *m/z* 75 was removed by the Agilent Octopole Reaction System operating in helium (He) gas mode. The As signal (*m/z* 75) was normalised by the germanium (Ge) signal (*m/z* 72) to correct any drift during analysis.

Standards were made using a 1000 ppm As stock solution (*Certipur*®; Merck Millipore, Darmstadt, Germany) traceable to SRM (standard reference material) from NIST (National Institute of Standards and Technology) in 5% HNO_3, to match the matrix of the samples. Blanks and check standards were repeated throughout the run of samples to ensure accuracy and precision of measurements, and assess any possible contamination or carry-over. Limits of detection (LOD) and quantitation (LOQ) for As in digested plant material were 0.60 and 2.00 µg L^{-1} respectively (calculated from blank values: LOD = 3-fold, and LOQ = 10-fold blank).

2.1.2 ICP-MS: PerkinElmer NexION

From September 2012 onwards, total concentration of elements in digested materials was determined by ICP-MS (PerkinElmer NexION 300X; PerkinElmer, Waltham, MA, USA).

An autosampler (Cetac ASX-520; Teledyne Cetac Technologies, Omaha, NE, USA) was connected to the water-jacketed nebuliser of the ICP-MS via a one*FAST* high-throughput sample introduction system (one*FAST*; Elemental Scientific, Omaha, NE, USA). A solution of 25 ppb of mixed internal standard elements (Li^6, Sc, Ge, Y, In, Tb, Bi) was mixed continuously with sample through a peristaltic pump.

For analysis of total As concentration, the possible polyatomic interference of $^{40}Ar^{35}Cl$ on was removed by operating in Kinetic Energy Discrimination (KED) mode using He gas. The As signal (*m/z* 75) was normalised by the Ge signal (*m/z* 72) to correct any drift during analysis. For analysis of total boron (B) concentration, the B signals (*m/z* 10 and 11) were normalised by the lithium (Li) signal (*m/z* 6) to correct any drift during analysis. To limit contamination of B from borosilicate glass, all standards and reagents were made in plasticware. For analysis of total Ge concentration the Ge signal (*m/z* 72) was normalised by the yttrium (Y) signal (*m/z* 89).

Standards were made using 5% HNO_3, to match the matrix of the samples, and 1000 ppm stock solutions of the elements of interest (*Certipur*®; Merck Millipore, Darmstadt, Germany) traceable to SRM from NIST. Blanks and check standards were repeated throughout the run of samples to ensure accuracy and precision of measurements, and assess any possible contamination or carry-over. Limits of detection (LOD) and quantitation (LOQ) for As in digested plant material were 0.09 and 0.30 µg L^{-1} respectively (calculated from blank values: LOD = 3-fold, and LOQ = 10-fold blank).

2.2 Statistical analysis

Means, standard errors and *t*-tests were performed using Microsoft Excel (Redmond, WA, USA). ANOVAs were performed using GenStat (VSN International, Hemel Hempstead, UK). For ANOVA, when necessary data were transformed (log_{10} or square root) to homogenise sample variances.

3. Origin of methylated arsenic in plants

3.1 Literature review

3.1.1 Occurrence of methylated As in soils

Arsenic speciation of soils is generally dominated by inorganic As, but MMA and DMA have been detected in orchard soils (Takamatsu et al., 1982), paddy fields (Takamatsu et al., 1982), acidic fens (Huang & Matzner, 2006) and cotton fields (Bednar et al., 2002a). Acids or chelating agents must be added to soil water extracts to prevent precipitation of oxidised iron compounds which can sorb soluble As. Infrequent detection of methylated As in soil waters may be due to sample preparation, as it was found that acidification with hydrochloric acid (HCl) preserved DMA peaks better than the previously recommended chelator, EDTA (Bednar *et al.*, 2002b; Zhao *et al.*, 2013b).

3.1.2 Occurrence of methylated As in plants

A number of studies have reported finding methylated As species in plant samples. A comprehensive index of studies, including the plant species analysed and the As species determined, originally compiled by Meharg and Hartley-Whitaker (2002) and Panda *et al.* (2010), updated to contain all studies up until September 2013 can be found in Supplementary Table 3.1. However the occurrence of organic As species in plants alone does not provide proof for the existence of an As-methylation pathway *in planta*.

After indications that phosphate (and nitrate) deficiency increase As-methylation in marine algae, Nissen and Benson (1982) reported that methylated As species were detected in phosphate and nitrate deficient tomato plants (*Solanum lycopersicum*); and to a lesser extent in those deficient in phosphate only. The majority of methylated species were detected in the leaves of plants from both treatments. Leaves contained mostly DMA, roots primarily contained MMA, and shoots were intermediate; containing roughly equal proportions of the two species. The authors discounted the possibility that the methylated As originated from microbes associated with the roots, as there were considerable amounts of methylated As in the leaves. However, As speciation was determined by 2D-chromatography which is difficult to interpret, and as plants were grown under non-sterile conditions methylation by microbes cannot be ruled out. Also

the high translocation efficiency of DMA is now well-established (see **Section 1.6**), and so methylated As taken up from the growth medium would be found predominantly in leaves.

Significant concentrations of methylated As was detected in moso bamboo (*Phyllostachys pubescens* Mazel) shoots in a market basket survey of seven Chinese provinces (Zhao *et al.*, 2006). Methylated species were not detected in any of the 21 other edible terrestrial plants sampled, including tomato. Inorganic As was generally predominant, but in one sample DMA accounted for 71% of total As. DMA was detected in all samples analysed whereas MMA was only present in seven of 15 samples. The authors propose this is because MMA is an intermediate during the methylation process. Interestingly winter bamboo shoots, which grow underground, all contained a high proportion of DMA, whereas DMA was only present in one of three samples of spring bamboo shoots which grow aboveground. Organic species were not detected in soil samples taken from one of the bamboo farms (Zhao *et al.*, 2006). However, this may be because the concentration was below detection limits, or due to low extraction efficiency. High proportions of methylated As species were also reported in the aboveground tissues of red clover (*Trifolium pratense*) growing on an arsenopyrite vein in Austria. Unlike the other plant species investigated *T. pratense* was found to contain a high proportion of methylated species (62%), of which MMA was predominant. The authors proposed that the close association with symbiotic bacteria in Fabaceae may influence As methylation (Geiszinger *et al.*, 2002).

3.1.3 Occurrence of methylated As in rice

Arsenic speciation in rice grain is dominated by inorganic As and DMA, but their proportions are highly variable, with inorganic As accounting for 11 to 91% of total grain As (Heitkemper *et al.*, 2001). Low proportions of other As species including MMA and Tetra are also occasionally detected, but usually only in samples with abnormally high As concentrations (Batista *et al.*, 2011; Hansen *et al.*, 2011; Kuramata *et al.*, 2011). Arsenic speciation in rice grains shows considerable genotypic and geographical variation (Norton *et al.*, 2009b; Pillai *et al.*, 2010; Kuramata *et al.*, 2013; Lei *et al.*, 2013). Based on differences in grain As speciation, Zavala *et al.* (2008) proposed that rice be split in to two groups; inorganic or DMA type. Rice produced in the USA, China and Australia was

categorised as DMA type, whereas rice from the rest of Asia and Europe was classified as inorganic type. The authors proposed that these differences were due to different capacities for As methylation between the regional cultivars.

3.1.4 Arsenic methylation *in vitro*

Cullen *et al.* (1989) reported detecting 4% DMA in cell suspension cultures of Madagascar periwinkle (*Catharanthus roseus*) treated with MMA for 12 days. The majority of As remained as MMA, however 1% inorganic As was also detected. Exposure to DMA resulted in 12% of the total As present as MMA, with 'traces' of inorganic As and trimethylarsenicals and the majority remaining as DMA. However the authors do not provide any detailed information on the As speciation data (replicates, total As, extraction efficiency etc.) or the analytical method used.

Arsenic methylation by cell extracts of bentgrass, *Agrostis capillaris* (formerly known as *Agrostis tenuis*), has been also been reported (Wu *et al.*, 2002). Plants were grown hydroponically with or without arsenate, and cell extracts were obtained from plants after three days exposure. Arsenic methyltransferase activity was then measured in the cell extracts with an assay using ^{3}H-radiolabelled SAM. Arsenic methylation activity was detected in leaf extracts from plants exposed to arsenate, with lower activity in control plants not exposed to As. Root extracts showed low activity in both treatments. In leaf extracts MMA was the predominant early product, but during longer assay times DMA accumulation exceeded that of MMA. The authors suggest that arsenate is taken up by roots and reduced to arsenite before methylation in leaves.

However the authors did not describe how often the nutrient solution was changed and significant microbial, and possibly algal, growth would occur during the four week growth period. Furthermore the cell extraction and protein fractionation procedures undertaken appear to make no attempt to exclude proteins not originating from bentgrass. The authors concede that methyltransferases may incorporate ^{3}H-SAM into compounds other than methylated As, and 'background methylation' was substantial in assays conducted without arsenite using extracts of plants not exposed to As. Finally, despite the method being based on the determination of ^{3}H-radioactivity, there was no evidence that the methylated As compounds actually contained ^{3}H.

3.1.5 Arsenic methylation by transgenic rice

Recently an *arsM* gene from the soil bacterium *Rhodopseudomonas palustris* was introduced into rice (Meng *et al.*, 2011). When exposed to arsenite, roots of the transgenic plants did contain small quantities of MMA and DMA; however inorganic As was still predominant accounting for over 99% of total As. Interestingly methylated As was not detected in shoots of the transgenic plants, however significantly more volatile As (presumed to be TMA(III) but not identified) was collected in chemotraps from plants expressing *arsM*. In all hydroponic experiments MMA and DMA were not detected in the wild-type plants, however significant proportions of DMA were present in wild-type field-grown grain and husks; accounting for 14.1 and 7.6% of total As respectively.

Overall rice expressing *arsM* contained significantly lower total As concentrations in roots, evolved 10-fold more volatile As, and had significantly lower concentrations of arsenite and arsenate in grain and husk. The amount of volatile As collected amounted to just 0.06% of plant total As, however some volatile As may have been lost from the chemotraps when the nutrient solution was renewed. Therefore although only low levels of As methylation were observed, these results provide a promising method of reducing As accumulation in rice (Meng *et al.*, 2011).

3.2 Aims

Therefore, the aim of the experiments within this chapter was to determine whether As methylation occurs within plants, or if methylated As species are taken up by plants from the surrounding medium.

3.3 Materials and methods

Generally, plants were grown under axenic conditions and exposed to different As species in the growth medium until harvest. Any replicates contaminated with fungi or bacteria were discarded and excluded from analysis. Arsenic speciation of the root and shoot tissues was determined by HPLC-ICP-MS (see **Section 3.3.6**). Growth conditions used for all rice experiments were 27°C/20°C day/night temperatures, 14-h photoperiod with a light intensity of 300 µmol m^{-2} s^{-1}. Tomato and red clover plants were grown under a constant temperature of 22°C, 16-h photoperiod with a light intensity of 250 µmol m^{-2} s^{-1}.

3.3.1 Axenically-grown rice

Obtaining sterile rice seedlings was problematic and so Plant Preservative Mixture™ solution (PPM™; Plant Cell Technology, Washington, DC, USA) was included in the solution to germinate seedlings after seed sterilisation, but was not added to the agar medium.

Rice seed (*Oryza sativa* cv. Nipponbare) were de-husked and sterilised by washing twice with 70% (v/v) ethanol, followed by 30 minutes in 1% (v/v) active sodium hypochlorite (NaClO). Seed were washed thoroughly with sterile water and left to germinate in 1% (v/v) PPM™ solution for 5-7 days in the growth conditions for rice given above. After germination, 4 seedlings were placed on full-strength Yoshida nutrient solution [1.43 mM NH_4NO_3, 0.32 mM $NaH_2PO_4{\cdot}2H_2O$, 0.51 mM K_2SO_4, 0.10 mM $CaCl_2{\cdot}2H_2O$, 1.64 mM $MgSO_4{\cdot}7H_2O$, 9.47 µM $MnCl_2{\cdot}4H_2O$, 0.075 µM $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$, 0.02 mM H_3BO_3, 0.15 µM $ZnSO_4{\cdot}7H_2O$, 0.16 µM $CuSO_4{\cdot}5H_2O$, 0.04 mM $FeCl_3{\cdot}6H_2O$, 0.07 mM citric acid, pH 5.5; Yoshida *et al.* (1976)] set with 1% (w/v) agar in coupled Magenta™ vessels (Sigma, St. Louis, MO, USA) and were grown under the same conditions until harvest. To investigate the effect of nutrient deficiency, concentrations of nitrogen (N) and phosphorus (P) in the growth medium were decreased from 2.86 and 0.32 mM in the control Yoshida medium, to 200 and 10 µM respectively.

3.3.2 Axenically-grown tomato

Tomato (cv. Alicante) seed were sterilised in 1% (v/v) active NaClO for 10 minutes, washed thoroughly with sterile water and 4 seed were placed on half-strength Murashige

and Skoog (MS) growth medium [9.4 mM KNO_3, 10.3 mM NH_4NO_3, 0.62 mM KH_2PO_4, 1.5 mM $CaCl_2 \cdot 2H_2O$, 0.75 mM $MgSO_4 \cdot 7H_2O$, 0.05 mM H_3BO_3, 0.05 mM $Na_2EDTA \cdot 2H_2O$, 0.05 mM $FeSO_4 \cdot 7H_2O$, 0.05 mM $MnSO_4 \cdot H_2O$, 0.05 µM $CoCl_2 \cdot 6H_2O$, 0.05 µM $CuSO_4 \cdot 5H_2O$, 0.52 µM $Na_2MoO_4 \cdot 2H_2O$, 2.5 µM KI, 15.0 µM $ZnSO_4 \cdot 7H_2O$, pH 5.8; Murashige and Skoog (1962)] set with 1% (w/v) agar in each Phytatray™ II box (Sigma). Nutrient deficiency was also investigated in tomato, and the concentrations of N and P were also reduced to 200 and 10 µM, from 30.0 and 0.62 mM in the control half-strength MS medium respectively.

3.3.3 Red clover with nodulation

The experiments with red clover were conducted as a modified most probable number plant-infection assay (MPN; Vincent, 1970). Red clover seed were sterilised in 95% (v/v) H_2SO_4 for 5 minutes, rinsed thoroughly with sterile water and germinated on 1% (w/v) agar for 3 days. One germinated seedling was placed on each slope of sterile quarter-strength Hewitt's nutrient solution [0.37 mM $MgSO_4 \cdot 7H_2O$, 0.33 mM Na_2HPO_4, 1.0 mM $CaCl_2 \cdot 6H_2O$, 0.50 mM K_2SO_4, 6.25 µM NaFeEDTA, 2.5 µM NaCl, 1.25 µM H_3BO_3, 0.25 µM $MnSO_4 \cdot 4H_2O$, 0.04 µM $ZnSO_4 \cdot 7H_2O$, 0.03 µM $CuSO_4 \cdot 5H_2O$, 0.005 µM $CoSO_4 \cdot 6H_2O$, 0.002 µM $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, pH 6.5; Hewitt (1966)], lacking major nitrogen sources, set with 1.5% (w/v) agar in boiling tubes. After one week, 100 µL of *Rhizobium leguminosarum* (bv. *trifolii*) cultured in yeast extract-mannitol broth (YM; 54.89 mM mannitol, 2.87 mM K_2HPO_4, 0.81 mM $MgSO_4 \cdot 7H_2O$, 1.71 mM NaCl, 39.64 mM $CaCO_3$, 0.04% (w/v) yeast extract; pH 6.8) was placed on each plant's root system. Rhizobium were verified by culturing on YM agar with Congo red (54.89 mM mannitol, 2.87 mM K_2HPO_4, 0.81 mM $MgSO_4 \cdot 7H_2O$, 1.71 mM NaCl, 0.04% (w/v) yeast extract, 1.5% (w/v) agar, 0.025% (w/v) Congo red; pH 6.8). Most rhizobia absorb the red dye weakly, whereas most other bacteria take it up strongly (Hirsch & Skinner, 1992). Control plants were grown on medium amended with 0.26 mM NH_4NO_3 and were inoculated with 100 µL sterile YM. Before harvest, nodule number was recorded and 4 or 5 plants with similar numbers of nodules were grouped together to form a single replicate for As extraction and speciation.

3.3.4 Soil grown rice

Soil was collected from a paddy field in Faridpur, Bangladesh and an arable field at Rothamsted, UK, sieved to 4 mm and air-dried. The Bangladeshi soil contained 34 mg kg^{-1}

total As, and the UK soil (containing 11 mg kg^{-1} total As) was amended with 10 mg As kg^{-1}, in the form of sodium arsenate ($Na_2HAsO_4 \cdot 7H_2O$). 150 g of each soil was weighed into coupled Magenta™ vessels, 25 mg NH_4NO_3 was added to all soils and 300 mg glucose was added to those in the glucose treatment. The soils were flooded with de-ionised water and incubated at 20°C for one month to induce reducing conditions. Autoclaving was carried out three times in total with week-long intervals at 20°C between autoclaving. After the second and third rounds of autoclaving, soil sterility was tested by spreading 10 µL of standing water onto 1/10^{th} tryptic soy agar (TSA; 0.3% (w/v) tryptic soy broth, 1.5% (w/v) Bacto Agar) plates incubated at 25°C. After 5 days plates were inspected visually for the presence of microbial colonies. Rice (cv. Nipponbare) seed, dehusked and sterilised as above, were germinated in 1% (v/v) PPM™ solution for 14 days before planting in the flooded soils. After 42 days growing under the same conditions for rice described above, shoots were harvested and As extracted. Standing water above the soil was collected using a syringe. Pore water was collected by centrifuging 70 g soil for 10 minutes at 3600 rpm. Both soil water samples were immediately acidified with 6 M HCl to keep the pH between 1-2, filtered through a sterile 0.2 µm filter, and immediately analysed for As speciation using HPLC-ICP-MS.

3.3.5 Arsenic extraction from plant tissues for As speciation

All the plants within one vessel were grouped together to form a single replicate (apart from red clover). After sufficient growth (30-38 days) plants were removed from agar and shoots, roots and remnants of the seed coat were separated using a scalpel. Shoots were rinsed with de-ionised water, blotted dry and weighed before grinding in liquid nitrogen using a mortar and pestle. Roots were rinsed with de-ionised water and submerged in 250 mL ice-cold desorption solution (1 mM K_2HPO_4, 0.5 mM $Ca(NO_3)_2$, 5 mM MES, pH 6.0; Xu *et al.*, 2007). After 15 minutes, roots were removed, blotted dry, weighed and ground in liquid nitrogen. The seed remnant was discarded. Arsenic speciation in the growth medium was measured by removing approximately 1 g of agar from the area beneath the root mat using a spatula. Finely ground shoots and roots, and agar samples were transferred to 30 mL vials with 20 mL of phosphate buffer solution (2 mM NaH_2PO_4, 0.2 mM Na_2-EDTA, pH 6.0), and sonicated for one hour at 100% ultrasound power (Fisherbrand FB11002; Thermo Fisher Scientific Inc., Waltham, MA, USA); keeping the

samples as cold as possible. Samples were then double-filtered through Whatman 40s and sterile 0.45 µm filters into 2 mL vials for analysis by HPLC-ICP-MS.

3.3.6 Analysis of arsenic speciation

Arsenic speciation in plant extracts and soil waters was determined using HPLC-ICP-MS (Agilent LC1100 series and Agilent ICP-MS 7500ce), as described by Li *et al.* (2009a) with modification: arsenite, arsenate, DMA and MMA were separated using method 1, and arsenite, MMA(III), MMA(V) and DMA were separated using method 2. Both methods use an anion-exchange column fitted with a guard column (Hamilton PRP X-100; Hamilton Company, Reno, NV, USA). For method 1 the mobile phase consisted of 6.6 mM $NH_4H_2PO_4$, 6.6 mM NH_4NO_3, 0.2 mM EDTA and 3% methanol (pH adjusted to 6.2). For method 2 the same solution was used but diluted by 75% with deionised water. In both methods the mobile phase was run isocratically at 0.7 mL min^{-1}. The outlet of the separation column was connected to the nebuliser of the ICP-MS, see **Section 2.1.1**. Germanium (Ge) was continuously mixed with the post-column solution to act as an internal standard. Arsenic species in the samples were quantified by external calibration curves with peak areas. Figure 3.1A gives an example chromatogram produced from mixed standards of arsenite, arsenate, MMA and DMA.

3.3.7 NPOC analysis

Total organic carbon (TOC) of pore water samples was measured as non-purgeable organic carbon (NPOC) by wet chemical UV oxidation (TOC-V WP; Shimadzu Corporation, Kyoto, Japan) by the Rothamsted Research Analytical Unit.

3.4 Results

3.4.1 Investigating As methylation in rice

Preliminary experiments found no difference in As speciation or concentration of rice exposed to arsenite or arsenate (Supplementary Figure 3.1) so all further experiments use arsenate only. Rice exposed to 10 µM arsenate under axenic conditions only contained inorganic As, predominantly arsenite, in roots and shoots (Figures 3.1B; 3.1C).

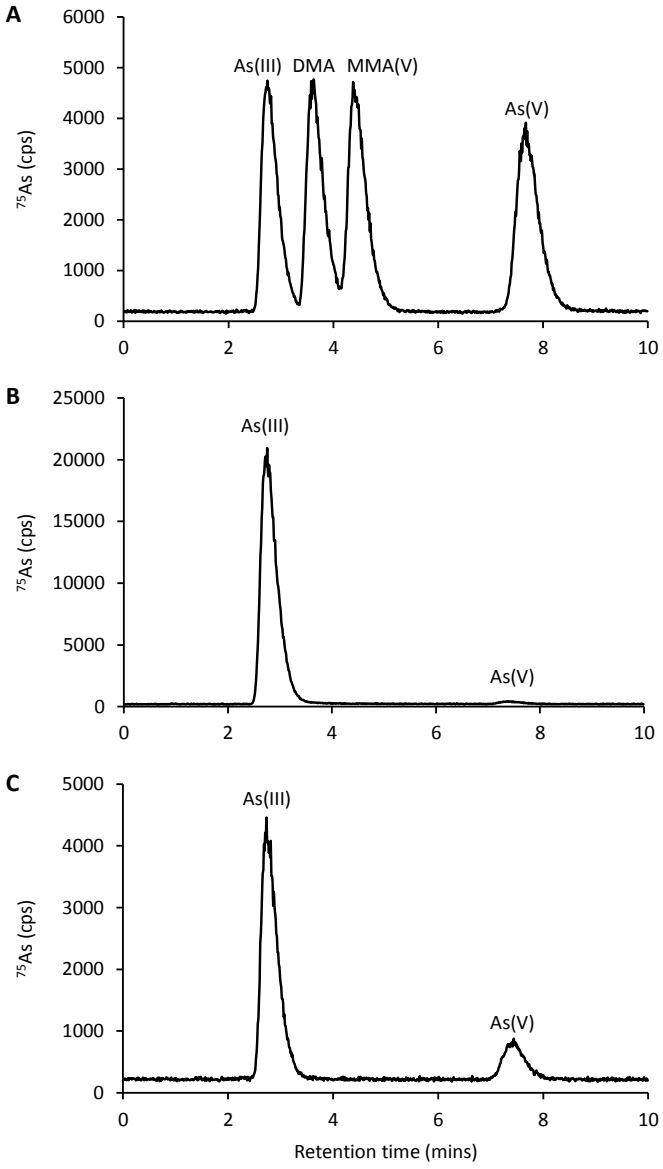


Figure 3.1 Chromatograms obtained through HPLC-ICP-MS of **A**, standards containing 50 µg As L^{-1} arsenite, DMA(V), MMA(V) and arsenate; and representative chromatograms of As speciation in extracts of rice (cv. Nipponbare) **B**, roots and **C**, shoots exposed to 10 µM arsenate under axenic conditions. cps = counts per second.

Therefore, due to the reports of Nissen and Benson (1982) that As methylation in tomato was increased by nutrient deficiency; rice plants were grown axenically and exposed to arsenate in growth medium with reduced concentrations of nitrogen (low N), phosphorus (low P) or both (low NP).

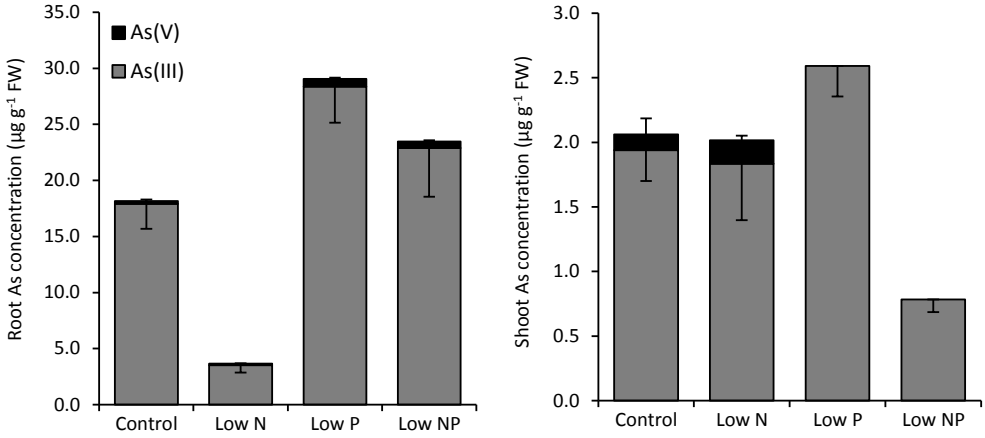


Figure 3.2 Mean arsenic speciation of rice plants under nutrient deficiency exposed to 10 µM arsenate in axenic culture. Plants were grown in Magenta boxes containing Yoshida's agar with full nutrients (Control), 200 µM N (Low N), 10 µM P (Low P), or 200 µM N and 10 µM P (Low NP); for 30 days. Error bars represent SE (*n* = 2-4).

However, deficiency of nitrogen, phosphorus or both nitrogen and phosphorus did not affect As speciation, and after exposure to arsenate only inorganic As was detected in rice tissues (Figure 3.2). Although nutrient deficiency did not induce As methylation, some differences in As concentrations of roots and shoots were observed. Shoots under the low NP treatment, and roots under the low N treatment, contained significantly lower As concentrations than control ($P < 0.05$). Additionally, shoot fresh weights were significantly decreased ($P < 0.001$) in all treatments with reduced nutrient concentrations compared to the control (Figure S3.2).

Interestingly, the low N treatment significantly increased the root to shoot translocation factor (TF) of As compared to control ($P < 0.001$). The reason for this has not been investigated; however nitrogen deficiency may decrease the pool of GSH or PCs able to complex arsenite for sequestration in vacuoles. Non-chelated arsenite would then be translocated to the shoot more efficiently than in the control treatment, as has been demonstrated in Arabidopsis (Liu *et al.*, 2010b).

To ensure that all of these findings were not due to lack of detection of methylated As species, rice was grown axenically in the presence of 5 µM pentavalent MMA or DMA.

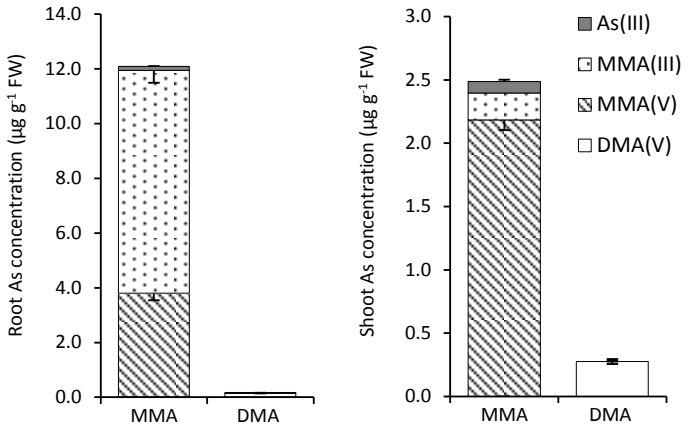


Figure 3.3 Mean arsenic speciation in rice plants exposed to 5 µM MMA or DMA in axenic culture. Plants were grown in Magenta boxes filled with Yoshida's agar for 30 days. Error bars represent SE (*n* = 5).

Analysis of root and shoot samples showed that under DMA treatment, shoots contained DMA only, whereas low concentrations of MMA, accounting for 17.8% of the total on average, were detected in roots (Figure 3.3; Table S3.2). MMA exposure resulted in As present in shoots predominantly as MMA, but also some MMA with trivalent As [MMA(III)] (8.6%) and arsenite (3.6%). The same three As species were present in roots but MMA(III) was predominant, accounting for 67.5% of the total As on average. Interestingly, MMA exposure significantly increased root fresh weight compared to control plants not exposed to As and those under DMA treatment (Figure S3.3; $P < 0.001$).

The MMA stock solution used contained 2.5% arsenite as an impurity; however the DMA stock solution was pure and so MMA detected in roots of plants exposed to DMA may result from de-methylation. Li *et al.* (2009a) also detected trivalent MMA in rice roots, accounting for 13.6% of total As, after exposure to pentavalent MMA in hydroponic culture for 24 hours. Additionally, MMA was not detected in an extraction of the agar medium surrounding roots under DMA treatment (data not shown).

All experiments with rice were conducted using Nipponbare, a japonica cultivar used to sequence the rice genome (Goff *et al.*, 2002). Therefore an experiment was conducted using two other rice cultivars; Italica carolina another japonica, and Kasalath an indica, to

ensure that lack of As methylation is not a trait specific to Nipponbare.

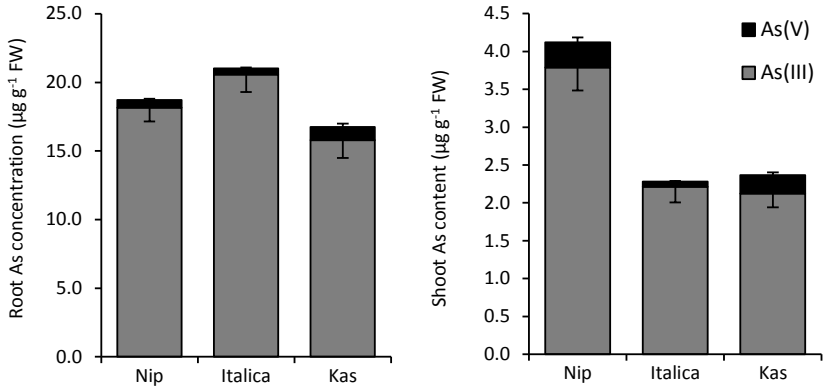


Figure 3.4 Mean arsenic speciation of different rice cultivars exposed to 10 µM arsenate in axenic culture. Nip = Nipponbare (japonica), Italica = Italica carolina (japonica) and Kas = Kasalath (indica). Plants were grown in Magenta boxes containing Yoshida's agar for 30 days. Error bars represent SE (n = 5).

Methylated As species were not detected in roots or shoots of any of the rice cultivars exposed to arsenate under axenic conditions (Figure 3.4). There were however, differences in As uptake and translocation between the cultivars. The greatest of these was that the TF (ratio of shoot:root As concentration) of Nipponbare was significantly higher than that of Italica carolina and Kasalath ($P < 0.001$). Additionally, Italica carolina appears to be most sensitive to As, because unlike the other two cultivars, both root and shoot fresh weight were significantly decreased by arsenate compared to the As-free control ($P < 0.001$; Figure S3.4).

3.4.2 Investigating As methylation in tomato and red clover

Nissen and Benson (1982) reported that As methylation in tomato was increased by nutrient deficiency. However, reduced concentrations of N and P in the nutrient medium did not induce As methylation in axenically-grown rice (Figure 3.2). Therefore, As metabolism in tomato was investigated by exposing plants to arsenate under axenic conditions with reduced concentrations of N and P in the medium.

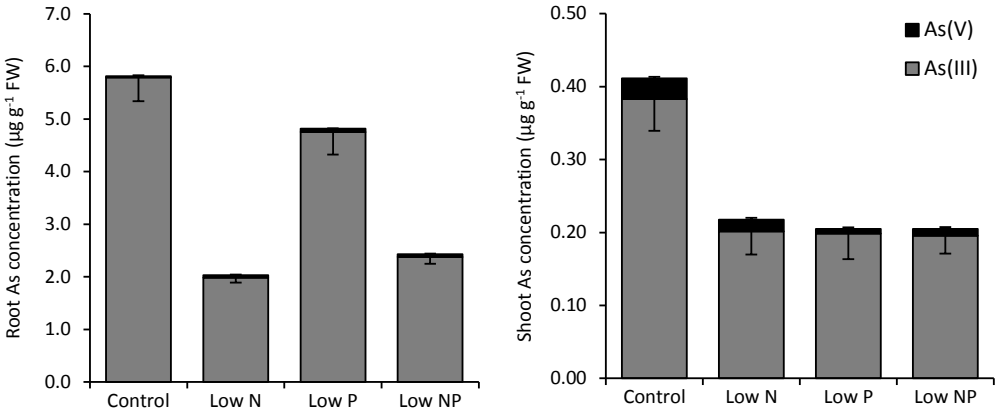


Figure 3.5 Mean arsenic speciation of tomato plants exposed to 10 µM arsenate. Plants were grown in Phytatray II™s filled with half-strength MS medium set with 1% agar, with full nutrients (Control), 200 µM N (Low N), 10 µM P (Low P), or 200 µM N and 10 µM P (Low NP); for 37 days. Error bars represent SE (*n* = 6).

However, once again only inorganic As was detected in plants exposed to arsenate (Figure 3.5). For tomato, induced N and P deficiency generally resulted in significantly decreased total As concentrations in roots and shoots compared to control ($P < 0.01$), with the exception of shoots under low P treatment. An As-free control was included in the experiment to measure the effect of As on growth of tomato plants (Figure S3.5). The presence of arsenate in the nutrient medium had no effect on root weight, but did significantly decrease shoot fresh weight compared to the As-free control ($P < 0.001$).

Interestingly, nutrient deficiency increased several fresh weight measurements. In particular, the low NP treatment resulted in significantly increased root fresh weight (mean 0.069 g in the control and 0.254 g in low NP treatment; Figure S3.5). Increased root growth to aid nutrient acquisition in response to deficiency is often observed, however shoots under low N and low NP treatments were also significantly larger than the control treatment ($P < 0.001$; Figure S3.6). Similarly to rice, the TF of As in tomato under low N treatment was also increased compared to control, however this difference was not significant ($P = 0.09$).

Since methylated As species were not detected in axenically-grown rice or tomato, even under nutrient deficiency, As speciation of red clover plants was investigated. Geiszinger *et al.* (2002) reported very high proportions of MMA in red clover, and suggested that association with symbiotic root-nodulating bacteria may affect As speciation in the plants.

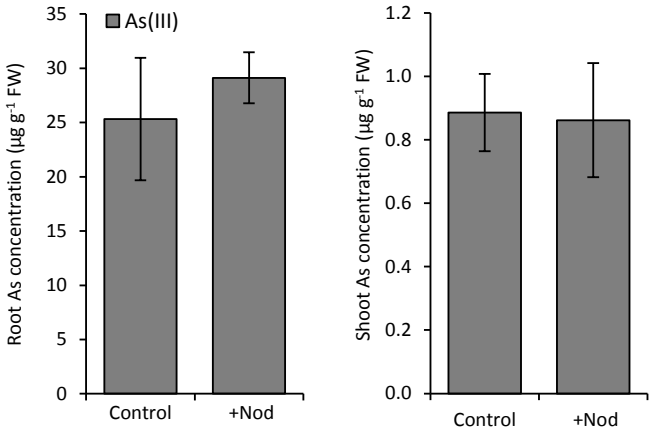


Figure 3.6 Mean arsenic speciation of red clover plants exposed to 10 µM arsenate with (+Nod) or without (Control) inoculation with *Rhizobium leguminosarum* (bv. *trifolii*). Plants were grown in boiling tubes on slopes of quarter-strength Hewitt's agar; the control treatment received ammonium nitrate, for 57 days. Error bars represent SE (n = 6).

Inoculation of red clover plants with *R. leguminosarum* (bv. *trifolii*) resulted in an average of 5.3 ± 1.1 root nodules per plant. However the presence of root nodules had no significant effect on the total As concentration or As speciation in red clover, and only arsenite was detected in roots and shoots under both treatments (Figure 3.6). Inoculation with root-nodulating *Rhizobium* significantly increased root fresh weight (data not shown; $P < 0.05$) of red clover, most likely due to the presence of root nodules.

A further experiment was conducted with red clover, as the previous study found only arsenite in root and shoots when plants were exposed to arsenate, showing a great capacity for As reduction. Additionally, root As concentrations were comparable to rice, and over four-fold higher than those for tomato (Figures 3.2, 3.5). Therefore, red clover was again grown with or without nodulation from *R. leguminosarum*, with 5 or 10 µM arsenate, or 5 µM DMA in the growth medium. An As-free control was also included to determine whether As influences nodule formation.

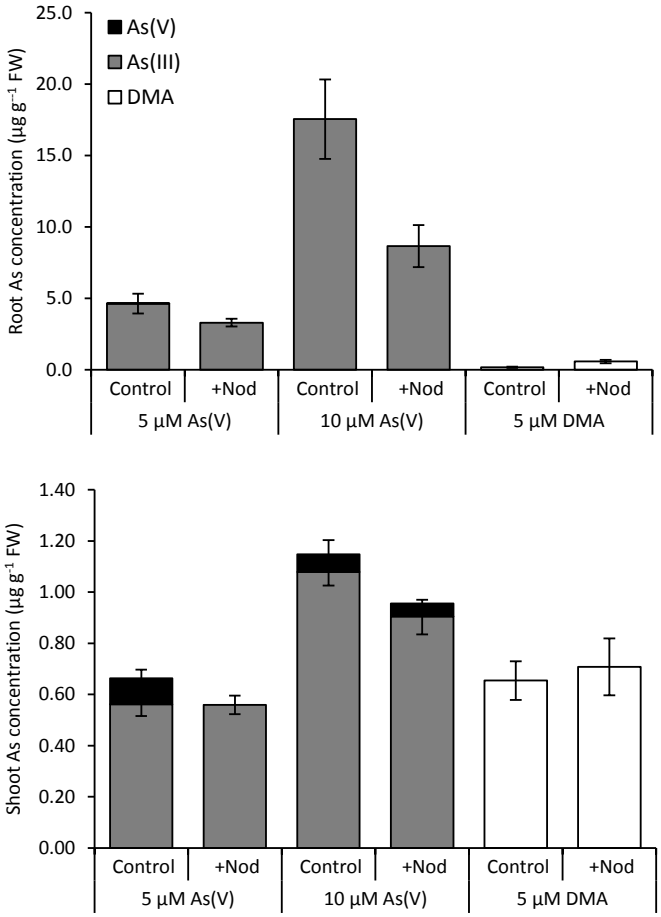


Figure 3.7 Mean arsenic speciation of red clover plants exposed to arsenic with (+Nod) or without (Control) inoculation with *Rhizobium leguminosarum* (bv. *trifolii*); the control treatment received ammonium nitrate. Plants were grown in boiling tubes on slopes of quarter-strength Hewitt's agar for 57 days. Error bars represent SE (*n* = 5-7).

Again, no methylated As species were detected in plants exposed to arsenate (Figure 3.7). However, unlike the previous experiment, arsenate was present in shoots of plants in the arsenate treatments, accounting for 0-27% of the total As. Additionally, root As concentrations in the 10 µM arsenate treatment were not as high as found previously (Figure 3.6). Inoculation of plants with *Rhizobium* significantly decreased root As concentration in the 10 µM arsenate treatment ($P = 0.015$); which again is in contrast to the previous experiment. The reason for this discrepancy remains unclear.

Only DMA was detected in plants in the DMA treatment, unlike rice roots which contained MMA after DMA exposure (Figure 3.3). However, concentrations of DMA in clover roots was very low (2 of 7 replicates actually had no detectable As species in root extracts) and so the products of de-methylation may be present in roots, but at extremely low concentrations. DMA was the only As species present in clover shoot extracts also,

although at higher, quantifiable, concentrations. In their study, Geiszinger *et al.* (2002) only sampled plant shoots. It is possible that red clover shows the same high level of translocation efficiency for MMA as DMA, so any MMA present in the soil solution would be quickly transported to the aerial tissues.

The presence of 5 or 10 µM As in the medium had no effect on the number of nodules present on individual plant roots in the +Nod treatments (P = 0.231). Additionally, As treatment had no effect on shoot fresh weight (Figure S3.7; P = 0.307), and the only significant differences in root weights were between the control and +Nod treatments within the different As treatments, again most likely due to the presence of root nodules.

3.4.3 Rice grown in flooded soil

Having confirmed that plants are able to take up methylated As species if they are present in the growth medium, the formation of MMA and DMA in soil was investigated. Two soils were used; a Bangladeshi paddy soil (Faridpur; BGN) and a UK arable soil (Rothamsted: UK), and rice plants were grown under flooded conditions.

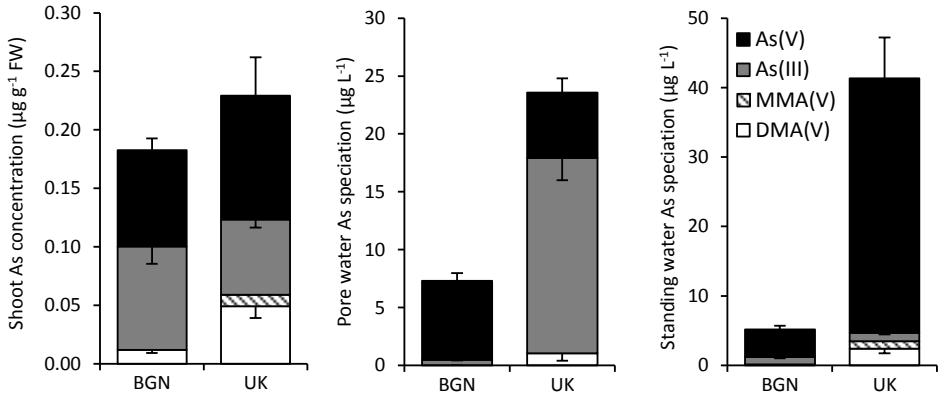


Figure 3.8 Mean arsenic speciation in rice (cv. Nipponbare) and waters from 2 soils: BGN = Bangladeshi paddy soil and UK = UK soil. Plants were grown in Magenta boxes containing flooded soil for 57 days. Error bars represent SE (n = 5).

Due to difficulty in removing roots from soil only rice shoots were analysed. Rice shoots grown in the Bangladeshi soil contained DMA (6.3% of the total As), and in the UK soil contained both MMA and DMA, accounting for 3.4% and 21.7% of the total As respectively (Figure 3.8; Table S3.3). Additionally soil pore water and standing water

collected at the end of the experiment also showed the presence of MMA and DMA; at higher concentrations in the UK soil than in the Bangladeshi soil.

An attempt was made to sterilise the soils to act as a control treatment, however although soils appeared sterile after autoclaving and incubating three times, when this was checked again after plant growth, all replicates were found to be non-sterile. Also a further treatment included the addition of glucose to the soils, as it was hypothesised that this would promote microbial activity, and so increase As methylation (Figures S3.8; S3.9). Interestingly, the addition of glucose significantly increased shoot total As for both soils compared to the autoclaved and control treatments ($P < 0.001$). Standing and soil pore water total As concentrations were significantly increased by autoclaving for both soils ($P < 0.001$), and DMA and MMA concentrations in pore water were also significantly higher compared to the control treatment ($P = 0.004$ and $P < 0.001$ respectively). Interactions between the As speciation of rice shoots with soil pore and standing waters were investigated. The only significant relationship found was between DMA concentrations in standing water and rice shoots from the UK soil amended with glucose (Figure S3.10).

Total organic carbon (TOC) was determined for the pore water samples (Figure S3.9), and was around 50 and 15-fold higher in the autoclaved samples compared to the control and glucose treatments for the Bangladeshi and UK soils respectively. Autoclaving is known to destroy soil structure and cause the release of organic carbon as well as manganese, N, P and sulphur (Berns *et al.*, 2008). The increase in soluble essential nutrients may actually promote microbial growth and explain why As methylation was increased in the autoclaved treatment compared to the control. Additionally the breakdown of soil structure may modify the redox potential and increase the soluble, bioavailable fraction of As. Surprisingly the addition of glucose to the soils did not increase TOC in the pore water, however the long duration of soil incubation and plant growth (70 days in total) may mean that all the glucose added was respired by micro-organisms.

Therefore although sterile soil was not achieved, it is clear that methylated As is formed in soils, and is taken up by plants.

3.5 Discussion

Methylated As species were not detected in rice, tomato or red clover tissues exposed to arsenate under axenic conditions, even with nutrient deficiency or symbiosis with root-nodulating bacteria (Figures 3.1; 3.2; 3.5; 3.6). Methylated As was detected however in rice plants exposed to MMA and DMA, demonstrating that plants are able to take these As species up when they are present in the growth medium (Figure 3.3; Table S3.2). MMA and DMA were also detected in rice shoots grown in flooded soil from Bangladesh or the UK, and were also present in the soil pore and standing waters (Figure 3.8).

Furthermore, Arao *et al*., (2011) reported that addition of the antibiotic chloramphenicol to nutrient solution significantly decreased DMA formation under MMA treatment in hydroponic culture of rice. It is highly likely that microbial methylation explains the presence of methylated As species in plants cultured in non-sterile media reported previously (Nissen & Benson, 1982; Quaghebeur & Rengel, 2003; Raab *et al.*, 2007a). Using GeoChip analysis, Lomax *et al.* (2012) found that *arsM* genes were highly abundant in a Bangladeshi paddy soil.

Recently, several studies have reported an increase in MMA and DMA in soils after the addition of fertilisers with high organic matter contents such as dried distillers' grain (DDG; Jia *et al.*, 2012), biogas slurry (Jia *et al.*, 2013b) and rice straw (Jia *et al.*, 2013a). The addition of organic matter is thought to enhance As mobilisation through competition for sorption sites and by lowering the redox potential (Jia *et al.*, 2013b). Additionally, increasing the organic matter content of 14 different paddy soils by addition of rice straw was found to increase the abundance of microbial *arsM* genes by an average of almost 140% (Jia *et al.*, 2013a).

The results also suggest that the methyltransferase containing a UbiE/Coq5 motif found to be upregulated by arsenate exposure (Norton *et al.*, 2008), does not function as an As methyltransferase *in planta* (Ye *et al.*, 2012). When data from several market basket surveys were combined, the relationship between total grain As and the proportion of inorganic As varied greatly by region (Zhao *et al.*, 2013a). Rice from Asia (including Bangladesh, India, China and Thailand) shows a strong linear relationship, whilst rice from

the U.S. displayed a hyperbolic pattern; reaching a maximum around 0.15 mg kg^{-1}. Rice produced in Europe (including Italy, Spain and France) was more variable and displayed an intermediate relationship. Recent studies have found that differences in grain As speciation are due to environmental conditions, rather than genetic differences between cultivars. Norton *et al.* (2009a) grew 13 rice cultivars at sites in Bangladesh, India and China under normal field conditions and found that site had the biggest influence on grain DMA percentage. There were also significant effects of genotype and site by genotype interaction; however these contributed only slightly to grain As speciation in comparison to site. Therefore there is little evidence to support the classification of rice cultivars to DMA or inorganic type by Zavala *et al.* (2008).

Although unable to further methylate MMA, rice roots clearly have a capacity to reduce MMA to trivalent MMA (Figure 3.3; Table S3.2). Interestingly, in humans MMA reduction is the rate-limiting step in As methylation (Zakharyan *et al.*, 2001). Recently it was reported that rice plants are also able to reduce TMAO to volatile TMA gas (Jia *et al.*, 2012). Trivalent DMA is very unstable (Gong *et al.*, 2001) which may explain the lack of detection of this As species in plants, although it has been found in human urine (Le *et al.*, 2000). Rice plants may even be able to demethylate As, as MMA was detected in roots after exposure to DMA (Figure 3.3; Table S3.2). MMA has also been detected in the As hyperaccumulators, *P. vittata* and *Pteris cretica* and As-tolerant *Boehmeria nivea* exposed to DMA in sand culture (Huang *et al.*, 2008), and in radish (*Raphanus sativus*) grown in soil amended with DMA. De-methylation of DMA (Huang *et al.*, 2007) and MMA (Yoshinaga *et al.*, 2011) mediated by micro-organisms has also been observed in soils, however the significance of this process to the global As biogeocycle is not yet fully understood.

The lack of methylated As species in red clover with nodulation from *R. leguminosarum* (Figures 3.6; 3.7) is unsurprising as a BLAST search (tBlastn) failed to find any genes with homology to *arsM* from *Rhodopseudomonas palustris* (accession number: NP_948900.1; Qin *et al.*, 2006) in the genomes of *Rhizobium* species. However, the possibility cannot be excluded that As methylation by other symbiotic micro-organisms may result in accumulation of methylated As in the host plant. For example, Ultra *et al*., (2007) found elevated DMA concentrations in soil surrounding sunflower roots colonised with the arbuscular mycorrhizal (AM) fungus *Glomus aggregatum*. Furthermore, AM fungal

hyphae were found to facilitate transport of arsenate in a split-compartment pot experiment (Meding & Zasoski, 2008), and shoot DMA concentrations of maize (*Zea mays*) were higher in plants inoculated with the AM fungus *G. mosseae* compared to the uninoculated control (Yu *et al.*, 2009). However rice plants inoculated with *G. intraradices* contained only inorganic As after exposure to arsenate for 10 days in sand culture (Chen *et al.*, 2013a). Phosphate is transferred from AM fungi to the host in the form of polyphosphate, however polyarsenate is too unstable to be translocated in the same way (Smith *et al.*, 2010). A further BLAST search (tBlastn) using the protein sequence of *arsM* from *R. palustris* (Qin *et al.*, 2006) did not find any homologous genes in the genomes of members of the phylum Glomeromycota. However, difficulties in culturing AM fungi mean that these species are underrepresented in genetic databases. Further investigation into the contribution of AM fungi to the uptake of As from soils and As methylation is needed.

3.6 Conclusions

Taken together, these results show that diverse plant species are unable to methylate inorganic As, and that these As species instead originate from soil micro-organisms. The high translocation efficiency MMA and DMA, coupled with suboptimal preservation of organic As species in soil water extracts, explains why methylated As species are more frequently detected in plants than soil.

Geographical variation in As speciation in rice is likely to be caused by environmental factors, and possibly different microbial communities within the soil. Genotypic differences in As speciation in rice (Norton *et al.*, 2009b; Pillai *et al.*, 2010) are most likely due to variation in the uptake and/or translocation of methylated As. Undissociated pentavalent MMA and DMA are taken up by rice roots via Lsi1 (OsNIP2;1; Li *et al.*, 2009a), and variation in the expression of *OsNIP2;1* has been reported between rice cultivars (Ma *et al.*, 2007b). Possible transport systems of the methylated As species MMA and DMA, are discussed in the following two chapters.

The majority of the experiments within this chapter; along with hydroponic data from W.-J. Liu, and soil micro-array data from L. Wu, was accepted for publication in 2011: Lomax *et al*., 2012. Methylated arsenic species in plants originate from soil microorganisms. *New Phytologist* **193**: 665-672. A copy of the manuscript can be found in the Appendix.

3.7 Supplementary information

Supplementary Table 3.1 Detection of organic As species in terrestrial plant samples. Originally compiled by Meharg and Hartley-Whitaker (2002) and Panda *et al.* (2010), updated to contain references until September 2013.

Organic As species detected in different plant species	Reference
Monomethylarsonic acid (MMA)	
Crowberry (*Empetrum nigrum*)	Bergqvist and Greger (2012)
Scots pine (*Pinus sylvestris*)	Bergqvist and Greger (2012)
Cattail (*Typha latifolia*)	Larios *et al.* (2012)
Bearded creeper (*Crupina vulgaris*)	Larios *et al.* (2012)
Teasel (*Dipsacus fullonum*)	Ruiz-Chancho *et al.* (2011)
European alder (*Alnus glutinosa*)	Ruiz-Chancho *et al.* (2011)
Heather (*Calluna vulgaris*)	Šlejkovec *et al.* (2010)
Gorse (*Ulex europaeus*)	Šlejkovec *et al.* (2010)
Blackberry (*Rubus ulmifulmus*)	Šlejkovec *et al.* (2010)
Moss (*Brachythecium cf. Reflexum*)	Ruiz-Chancho *et al.* (2008)
Hard rush (*Juncus inflexus*)	Ruiz-Chancho *et al.* (2008)
Downy oak (*Quercus pubescens*)	Ruiz-Chancho *et al.* (2008)
Boxtree (*Buxus sempervirens*)	Ruiz-Chancho *et al.* (2008)
Broom (*Sarothamnus scoparius*)	Ruiz-Chancho *et al.* (2008)
False brome (*Brachypodium phoenicoides*)	Ruiz-Chancho *et al.* (2008)
Carrot (*Daucus carota*)	Yathavakilla *et al.* (2008)
Slender wheat grass (*Agropyron trachycaulum*)	Mir *et al.* (2007)
Bluejoint (*Calamagrostis canadensis*)	Mir *et al.* (2007)
Field horsetail (*Equisetum arvense*)	Mir *et al.* (2007)
Moso bamboo (*Phyllostachys pubescens*)	Zhao *et al.* (2006)
Spike rush (*Eleochairs* spp.)	Zheng *et al.* (2003)
Red clover (*Trifolium pratense*)	Geiszinger *et al.* (2002)
Rice (*Oryza sativa*)	Heitkemper *et al.* (2001)
Apple (*Malus domestica*)	Caruso *et al.* (2001)
Foxtail barley (*Hordeum jubatum*)	Koch *et al.* (2000)
Agrostis scabra	Koch *et al.* (2000)
Moss (*Drepanocladus* sp.)	Koch *et al.* (2000)
Green spleenwort (*Asplenium viride*)	Kuehnelt *et al.* (2000)
Tufted hair grass (*Deschampsia cespitosa*)	Kuehnelt *et al.* (2000)
Norway spruce (*Picea abies*)	Kuehnelt *et al.* (2000)
White Alder (*Alnus incana*)	Kuehnelt *et al.* (2000)
Wild strawberry (*Fragaria vesca*)	Kuehnelt *et al.* (2000)
Bilberry (*Vaccinium myrtilis*)	Kuehnelt *et al.* (2000)
Cowberry (*Vaccinium vitis-idaea*)	Kuehnelt *et al.* (2000)
Yarrow (*Achillea millefolium*)	Kuehnelt *et al.* (2000)
Shady horsetail (*Equisetum pretense*)	Kuehnelt *et al.* (2000)
Sedge (*Scirpus* sp.)	Kuehnelt *et al.* (2000)

Monomethylarsonic acid (MMA) continued..	
Cedar (*Thuja plicata*)	Kuehnelt *et al.* (2000)
N- and P-deficient tomato (*Solanum lycopersicum*)	Nissen and Benson (1982)
Dimethylarsinic acid (DMA)	
Blueberry (*Cyanococcus* sp.)	Koch *et al.* (2013)
Cattail (*Typha latifolia*)	Larios *et al.* (2012)
Bearded creeper (*Crupina vulgaris*)	Larios *et al.* (2012)
Heather (*Erica andevalensis*)	Márquez-García *et al.* (2012)
Teasel (*Dipsacus fullonum*)	Ruiz-Chancho *et al.* (2011)
Heather (*Calluna vulgaris*)	Šlejkovec *et al.* (2010)
Gorse (*Ulex europaeus*)	Šlejkovec *et al.* (2010)
Blackberry (*Rubus ulmifulmus*)	Šlejkovec *et al.* (2010)
Beetroot (*Beta vulgaris*)	Száková *et al.* (2010)
Moss (*Brachythecium* cf. Reflexum)	Ruiz-Chancho *et al.* (2008)
European alder (*Alnus glutinosa*)	Ruiz-Chancho *et al.* (2008)
Hard rush (*Juncus inflexus*)	Ruiz-Chancho *et al.* (2008)
Downy oak (*Quercus pubescens*)	Ruiz-Chancho *et al.* (2008)
Boxtree (*Buxus sempervirens*)	Ruiz-Chancho *et al.* (2008)
Broom (Sarotha*mnus scoparius*)	Ruiz-Chancho *et al.* (2008)
Moss (*Hydnum cupressiforme*)	Ruiz-Chancho *et al.* (2008)
Fern (*Dryopteris filix-max*)	Ruiz-Chancho *et al.* (2008)
False brome (*Brachypodium phoenicoides*)	Ruiz-Chancho *et al.* (2008)
Moss (*Hydnum* sp.)	Ruiz-Chancho *et al.* (2008)
Carrot (*Daucus carota*)	Yathavakilla *et al.* (2008)
Purple loosestrife (*Lythrum salicaria*)	Mir *et al.* (2007)
Panicle aster (*Aster lanceolatus*)	Mir *et al.* (2007)
Canada goldenrod (*Solidago canadensis*)	Mir *et al.* (2007)
Blue vervain (*Verbena hastata*)	Mir *et al.* (2007)
Moso bamboo (*Phyllostachys pubescens*)	Zhao *et al.* (2006)
Spike rush (*Eleochairs* spp.)	Zheng *et al.* (2003)
Red clover (*Trifolium pratense*)	Geiszinger *et al.* (2002)
Ribwort plantain (*Plantago lanceolata*)	Geiszinger *et al.* (2002)
Cock's-foot grass (*Dactylis glomerata*)	Geiszinger *et al.* (2002)
Rice (*Oryza sativa*)	Heitkemper *et al.* (2001)
Apple (*Malus domestica*)	Caruso *et al.* (2001)
Foxtail barley (*Hordeum jubatum*)	Koch *et al.* (2000)
Agrostis scabra	Koch *et al.* (2000)
Moss (*Drepanocladus* sp.)	Koch *et al.* (2000)
Broad buckler fern (*Dryopteris dilate*)	Kuehnelt *et al.* (2000)
Green spleenwort (*Asplenium viride*)	Kuehnelt *et al.* (2000)
Tufted hair grass (*Deschampsia cespitosa*)	Kuehnelt *et al.* (2000)
Norway spruce (*Picea abies*)	Kuehnelt *et al.* (2000)
White Alder (*Alnus incana*)	Kuehnelt *et al.* (2000)
Wild strawberry (*Fragaria vesca*)	Kuehnelt *et al.* (2000)
Bilberry (*Vaccinium myrtilis*)	Kuehnelt *et al.* (2000)
Cowberry (*Vaccinium vitis-idaea*)	Kuehnelt *et al.* (2000)

Dimethylarsinic acid (DMA) continued..	
Yarrow (*Achillea millefolium*)	Kuehnelt *et al.* (2000)
Sedge (*Scirpus* sp.)	Kuehnelt *et al.* (2000)
Cedar (*Thuja plicata*)	Kuehnelt *et al.* (2000)
Fleabane (*Erigeron* sp.)	Koch *et al.* (1999)
N- and P-deficient tomato (*Solanum lycopersicum*)	Nissen and Benson (1982)
Trimethylarsine oxide (TMAO)	
Labrador tea (*Rhododendron groenlandicum*)	Koch *et al.* (2013)
Teasel (*Dipsacus fullonum*)	Ruiz-Chancho *et al.* (2011)
Heather (*Calluna vulgaris*)	Šlejkovec *et al.* (2010)
Moss (*Brachythecium* cf. Reflexum)	Ruiz-Chancho *et al.* (2008)
Boxtree (*Buxus sempervirens*)	Ruiz-Chancho *et al.* (2008)
European alder (*Alnus glutinosa*)	Ruiz-Chancho *et al.* (2008)
Hard rush (*Juncus inflexus*)	Ruiz-Chancho *et al.* (2008)
Downy oak (*Quercus pubescens*)	Ruiz-Chancho *et al.* (2008)
Broom (*Sarothamnus scoparius*)	Ruiz-Chancho *et al.* (2008)
Moso bamboo (*Phyllostachys pubescens*)	Zhao *et al.* (2006)
Spike rush (*Eleochairs* spp.)	Zheng *et al.* (2003)
Red clover (*Trifolium pratense*)	Geiszinger *et al.* (2002)
Ribwort plantain (*Plantago lanceolata*)	Geiszinger *et al.* (2002)
Cock's-foot grass (*Dactylis glomerata*)	Geiszinger *et al.* (2002)
Bilberry (*Vaccinium myrtilis*)	Kuehnelt *et al.* (2000)
Broad buckler fern (*Dryopteris dilate*)	Kuehnelt *et al.* (2000)
Green spleenwort (*Asplenium viride*)	Kuehnelt *et al.* (2000)
Tufted hair grass (*Deschampsia cespitosa*)	Kuehnelt *et al.* (2000)
Norway spruce (*Picea abies*)	Kuehnelt *et al.* (2000)
European Larch (*Larix deciduas*)	Kuehnelt *et al.* (2000)
White Alder (*Alnus incana*)	Kuehnelt *et al.* (2000)
Wild strawberry (*Fragaria vesca*)	Kuehnelt *et al.* (2000)
Cowberry (*Vaccinium vitis-idaea*)	Kuehnelt *et al.* (2000)
Raspberry (*Rubus idaeus*)	Kuehnelt *et al.* (2000)
Yarrow (*Achillea millefolium*)	Kuehnelt *et al.* (2000)
Shady horsetail (*Equisetum pretense*)	Kuehnelt *et al.* (2000)
Tetramethylarsonium ion (Tetra)	
Teasel (*Dipsacus fullonum*)	Ruiz-Chancho *et al.* (2011)
Boxtree (*Buxus sempervirens*)	Ruiz-Chancho *et al.* (2011)
Rice (*Oryza sativa*)	Hansen *et al.* (2011)
Moss (*Brachythecium* cf. Reflexum)	Ruiz-Chancho *et al.* (2008)
Spike rush (*Eleochairs* spp.)	Zheng *et al.* (2003)
Red clover (*Trifolium pratense*)	Geiszinger *et al.* (2002)
Ribwort plantain (*Plantago lanceolata*)	Geiszinger *et al.* (2002)
Broad buckler fern (*Dryopteris dilatata*)	Kuehnelt *et al.* (2000)
Green spleenwort (*Asplenium viride*)	Kuehnelt *et al.* (2000)
Wild strawberry (*Fragaria vesca*)	Kuehnelt *et al.* (2000)
Cowberry (*Vaccinium vitis-idaea*)	Kuehnelt *et al.* (2000)

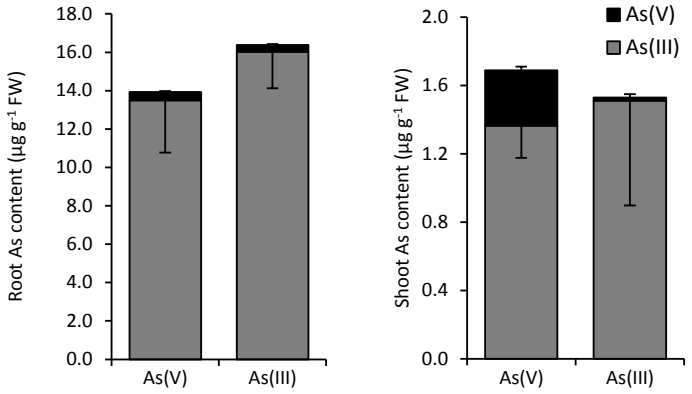
Tetramethylarsonium ion (Tetra) continued..	
Moss (*Drepanocladus* sp.)	Koch *et al.* (2000)
Monkey flower (*Mimulus* sp.)	Koch *et al.* (1999)
Arsenobetaine (AsB)	
Labrador tea (*Rhododendron groenlandicum*)	Koch *et al.* (2013)
Tufted hair grass (*Deschampsia cespitosa*)	Kuehnelt *et al.* (2000)

Supplementary Table 3.2 Mean ± SE arsenic speciation (μg g^{-1} fresh weight) of rice plants exposed to 5 μM MMA or DMA in axenic culture (*n* = 5).

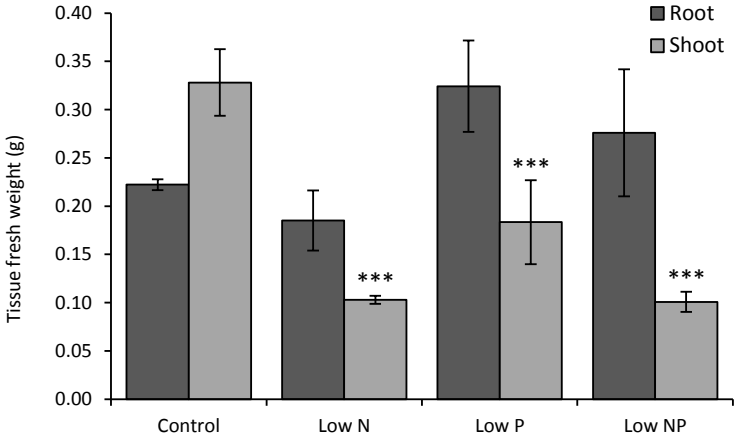
Arsenic treatment	Tissue	Arsenic species detected			
		As(III)	DMA	MMA(V)	MMA(III)
MMA	Root	0.141 ± 0.017		3.795 ± 0.256	8.151 ± 0.457
	Shoot	0.089 ± 0.005		2.183 ± 0.080	0.214 ± 0.003
DMA	Root		0.137 ± 0.006	0.030 ± 0.003	
	Shoot		0.276 ± 0.020		

Supplementary Table 3.3 Mean ± SE arsenic speciation of rice shoots (μg g^{-1} fresh weight) and pore and standing water samples (μg L^{-1}) from rice plants grown in flooded soils (*n* = 5).

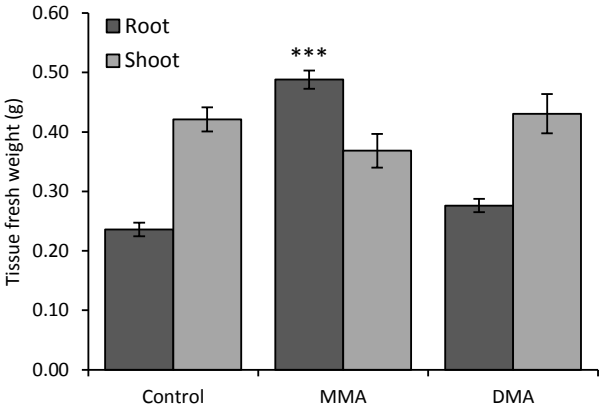
Sample	Soil	As(V)	As(III)	MMA(V)	DMA(V)
Rice shoot	BGN	0.082 ± 0.010	0.088 ± 0.015		0.012 ± 0.003
	UK	0.106 ± 0.033	0.064 ± 0.007	0.01 ± 0.004	0.049 ± 0.010
Pore water	BGN	6.807 ± 0.669	0.483 ± 0.047		
	UK	5.646 ± 1.231	16.88 ± 1.922		1.051 ± 0.645
Standing water	BGN	3.947 ± 0.533	1.065 ± 0.193		0.162 ± 0.124
	UK	36.67 ± 5.88	1.182 ± 0.247	1.075 ± 0.285	2.432 ± 0.689



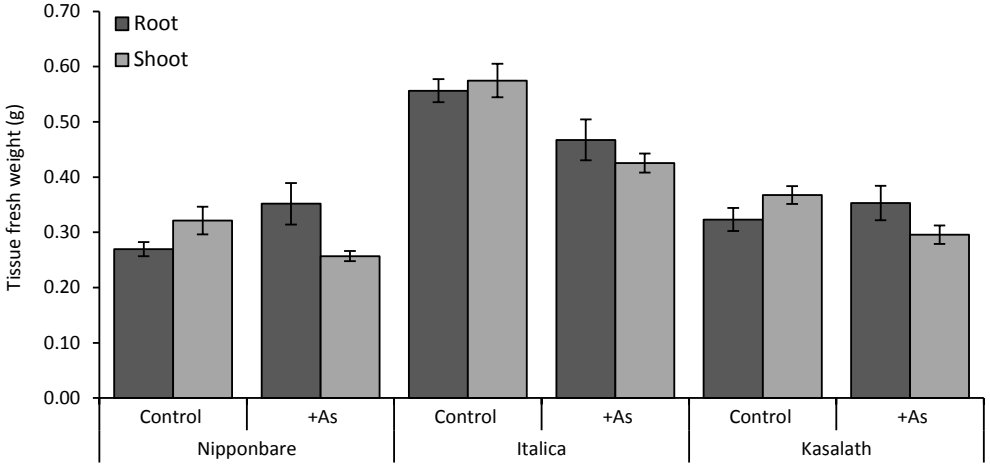
Supplementary Figure 3.1 Mean arsenic speciation of rice plants (cv. Nipponare) exposed to 10 µM arsenate or arsenite in axenic culture. Plants were grown on Petri dishes containing Yoshida's agar for 10 days before transfer to dishes containing agar amended with As for a further 16 days. Error bars represent SE (*n* = 5-7).



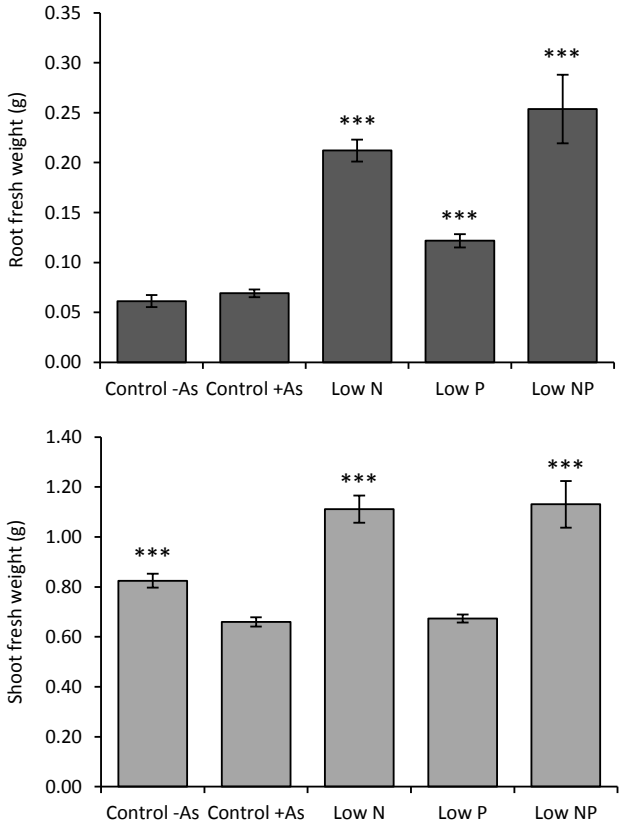
Supplementary Figure 3.2 Mean fresh weight of rice plants under nutrient deficiency exposed to 10 µM arsenate in axenic culture. Plants were grown in Magenta boxes containing Yoshida's agar with full nutrients (Control), 200 µM N (Low N), 10 µM P (Low P), or 200 µM N and 10 µM P (Low NP); for 30 days. Error bars represent SE (*n* = 2-4). *** = *P* < 0.001, significant difference from control (ANOVA).



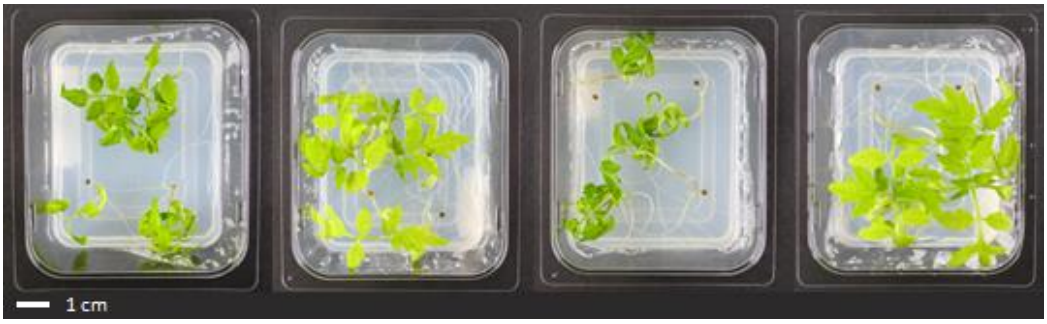
Supplementary Figure 3.3 Mean fresh weight of rice plants exposed to 5 µM MMA or DMA in axenic culture. Plants were grown in Magenta boxes filled with Yoshida's agar for 30 days. Error bars represent SE (*n* = 5). *** = *P* < 0.001 significant difference from control (ANOVA).



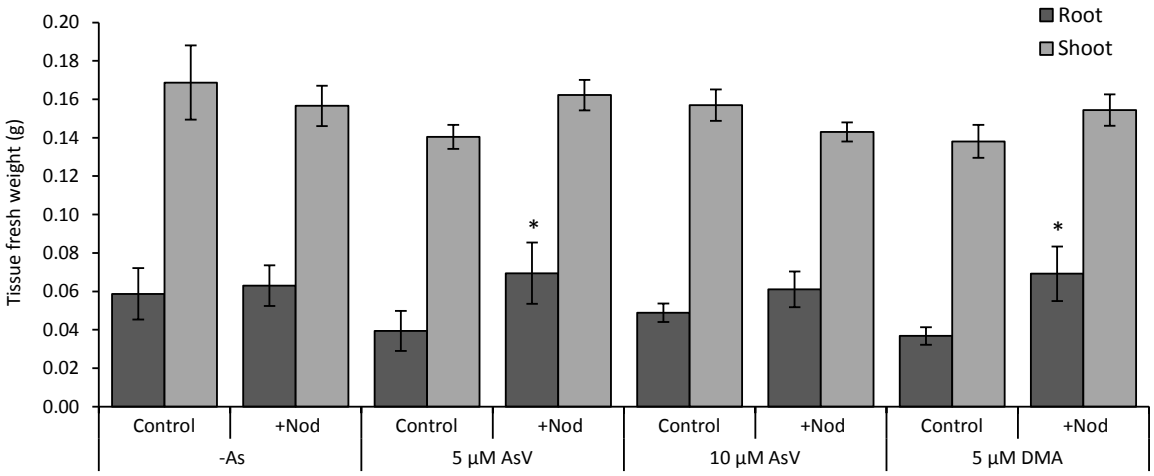
Supplementary Figure 3.4 Mean fresh weights of different rice cultivars grown in the absence (Control) or presence of 10 µM arsenate (+As) in axenic culture. Plants were grown in Magenta boxes containing Yoshida's agar for 30 days. Error bars represent SE (*n* = 5).



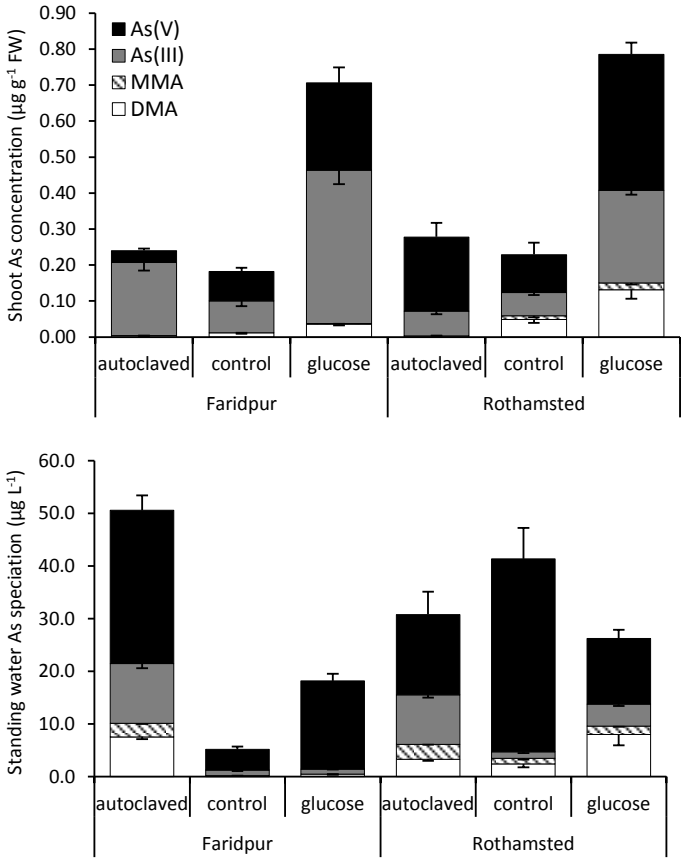
Supplementary Figure 3.5 Mean fresh weight of tomato plants exposed to 10 µM arsenate. Plants were grown in Phytatray II™s filled with half-strength MS medium set with 1% agar with full nutrients (Control +As), 200 µM N (Low N), 10 µM P (Low P), or 200 µM N and 10 µM P (Low NP); for 37 days. A treatment with full nutrients without arsenate (Control -As) was also included. Error bars represent SE (n = 6). *** = P < 0.001 significant difference from control +As (ANOVA).



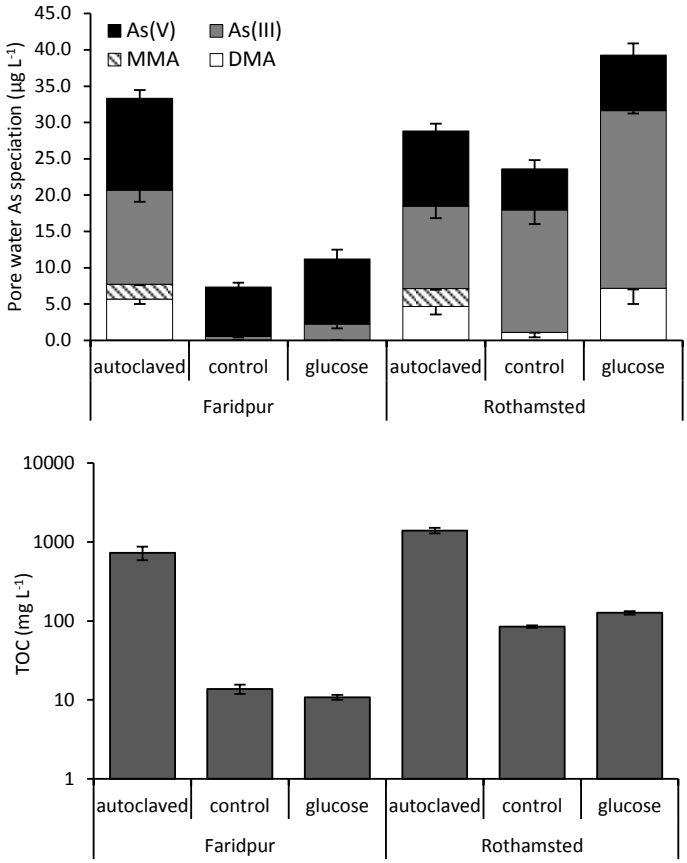
Supplementary Figure 3.6 Photos of tomato plants exposed to 10 µM arsenate. Plants were grown in Phytatray II™s filled with half-strength MS medium set with 1% agar with full nutrients (Control), 200 µM N (Low N), 10 µM P (Low P), or 200 µM N and 10 µM P (Low NP); for 37 days.



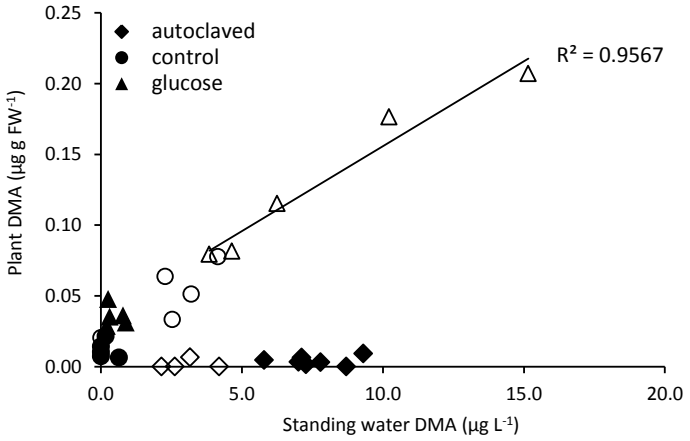
Supplementary Figure 3.7 Mean fresh weights of red clover plants exposed to arsenic with (+Nod) or without (Control) inoculation with *Rhizobium leguminosarum* (bv. *trifolii*); the control treatment received ammonium nitrate. Plants were grown in boiling tubes on slopes of quarter-strength Hewitt's agar for 57 days. Error bars represent SE (n = 4-7). * = $P < 0.05$ significant difference from control (ANOVA).



Supplementary Figure 3.8 Mean arsenic speciation in rice (cv. Nipponbare) shoots and standing water samples. Plants were grown in Magenta boxes containing flooded soil for 57 days. Error bars represent SE (n = 5).



Supplementary Figure 3.9 Mean arsenic speciation and non-purgeable organic carbon (TOC) of pore water samples. Rice plants were grown in Magenta boxes containing flooded soil for 57 days. Error bars represent SE (*n* = 5).



Supplementary Figure 3.10 Interaction between mean rice shoot and standing water DMA concentrations. Rice plants were grown in Magenta boxes containing flooded soil for 57 days. Filled symbols represent samples from the Bangladeshi soil, and open symbols represent samples from the UK soil.

4. Transport of methylated arsenic: NIP aquaporins

Both MMA and DMA have been detected in paddy soils (Takamatsu *et al.*, 1982), and are consistently found in rice grain (for review see Zhao *et al.*, 2010c). The finding that plants are unable to methylate As (**Chapter 3**) poses questions about the transport pathway of these As species. MMA and DMA have lower pK_{a1} values than arsenite, but higher than arsenate, and so will exist as both charged and uncharged forms in the physiological pH range of around 4.0 to 8.5 (Smith & Raven, 1979; Figure S1.2).

4.1 Literature review

Recently aquaporins in rice and humans have been reported to transport undissociated MMA and DMA. The rice aquaporin OsLsi1 (OsNIP2;1) was shown to transport undissociated pentavalent MMA and DMA in hydroponic culture. Increasing the pH of the exposure medium, decreases the proportion of undissociated complexes, and decreased As uptake by rice roots. Direct uptake of MMA was also shown for *Xenopus* oocytes expressing *OsLsi1* (Li *et al.*, 2009a). Additionally, oocytes expressing the human aquaporin *hAQP9* took up significantly more pentavalent MMA and DMA when the pH of the exposure solutions was decreased (McDermott *et al.*, 2010).

4.1.1 Aquaporins

Aquaporins belong to the major intrinsic protein (MIP) superfamily, and are integral membrane proteins which mediate the passive flow of small polar molecules, such as water and glycerol, across membranes. Transport is not coupled to an energy source, and so aquaporins have been shown facilitate bi-directional transport of substrates depending on the concentration gradient (Meinild *et al.*, 1998; Bienert *et al.*, 2008; Maciaszczyk-Dziubinska *et al.*, 2010; Zhao *et al.*, 2010a). The phenomenon of channel-mediated water transport was first observed by Benga *et al.* (1986) in human red blood cells. Subsequently, a 28 kDa protein was purified from erythrocytes (Denker *et al.*, 1988), and expressed in *Xenopus* oocytes where water transport was directly demonstrated (Preston & Agre, 1991; Preston *et al.*, 1992). The protein, then known as CHIP28 for ‘channel-like

integral protein of 28 kDa', was renamed aquaporin-1 (AQP1). In 1999, the crystal structure of AQP1 at 4.5 Å resolution was published by Agre's group (Mitsuoka *et al.*, 1999), and further studies revealed the structural features that determine aquaporin selectivity (Murata *et al.*, 2000). In 2003, Peter Agre was jointly-awarded the Nobel Prize for Chemistry for his role in 'the discovery of water channels'.

Aquaporins have been identified in almost all classes of life from bacteria to humans, and are highly divergent in higher plants. The human genome encodes just 13 aquaporins (Ishibashi *et al.*, 2009), whilst Arabidopsis has 35 (Johanson *et al.*, 2001), and rice has 33 or 39 (Sakurai *et al.*, 2005; Bansal & Sankararamakrishnan, 2007). Historically the term 'aquaporin' was used exclusively for water-transporting MIPs, but now applies to all MIP superfamily members (Hachez & Chaumont, 2010). Those permeable to water only are known as strict-aquaporins or orthodox-aquaporins, whereas aquaporins permeable to water and small uncharged molecules such as glycerol are often termed aquaglyceroporins.

Aquaporins are relatively small with an average molecular weight between 28 and 30 kDa. Typically they contain six transmembrane helices (1-6) connected by five loops (A-E) with both the N- and C- terminal domains protruding into the cytoplasm. Two half-helices (loops B and D) form a characteristic 'hourglass'-like seventh transmembrane domain, and bring together two highly conserved NPA (asparagine-proline-alanine) motifs (Jung *et al.*, 1994). The quaternary structure is a tetramer with a fifth putative pore at the centre.

Plant aquaporins are classified into four subfamilies based on their sequences and subcellular-localisations in Arabidopsis: tonoplast intrinsic proteins (TIPs), plasma membrane intrinsic proteins (PIPs), Nodulin 26-like intrinsic proteins (NIPs) and small basic intrinsic proteins (SIPs; Johanson *et al.*, 2001). Additionally a further three subfamilies were proposed based on sequences from the moss *Physcomitrella patens*; GlpF-like intrinsic proteins (GIPs), hybrid intrinsic proteins (HIPs), and uncategorised X-intrinsic proteins (XIPs; Gustavsson *et al.*, 2005; Danielson & Johanson, 2008). XIP members have since been identified in higher plants including poplar (*Populus trichocarpa*), tobacco (*Nicotiana tabacum*) and tomato (Bienert *et al.*, 2011; Lopez *et al.*, 2012).

4.1.2 Selectivity of aquaporins

Aquaporins are channels that allow passive diffusion of substrates across membranes, but are selective on the basis of size and charge. Two constrictions within the pore have been identified as selectivity filters. The first is the NPA region, which has been shown to orient water molecules to force them to pass through the pore in single-file (Tajkhorshid *et al.*, 2002). Aquaporins with single substitutions of all three NPA motif residues have been identified; however those for asparagine are least common. Mutation of asparagine to serine (N76S) in the first NPA motif of rat AQP1 caused the aquaporin to leak sodium ions. The authors propose that the critical role of asparagine in cation exclusion is the reason for the overwhelming prevalence of this residue in NPA motifs (Wree *et al.*, 2011). Interestingly, mutations of the NPA motifs of rat AQP4 affected membrane targeting. Both A99T and A215T mutant proteins were retained in the ER, whereas native AQP4 is localised to the plasma membrane (Guan *et al.*, 2010).

The second region involved in substrate selectivity, located approximately 8 Å from the NPA region towards the cytoplasmic face of the pore, is the aromatic/arginine (ar/R) filter. This consists of four residues; one each from helices 2 and 5, and two from loop E (H2, H5, LE_1, LE_2). Typically the region contains aromatic residues, such as phenylalanine (F), tyrosine (Y) or tryptophan (W), with a conserved arginine (R) residue in the fourth position (LE_2). The size of the ar/R filter of the mammalian strict-aquaporin AQP1 (FHCR) was determined by high-resolution X-ray crystallography to exactly fit a single water molecule (Sui *et al.*, 2001). Searching the MIPMod database (Gupta *et al.*, 2012), shows that plant PIPs generally have an ar/R filter similar to that of AQP1 (FHTR). However, as can be seen in Supplementary Table 4.1, members of the NIP subfamily have particularly diverse ar/R regions.

Although aquaporins can be permeable to a range of solutes, protons must be excluded to avoid losing the proton motive force which drives numerous cellular processes. This is particularly difficult as protons can move along columns of water molecules by hydrogen bond exchange, forming transient hydronium ions, known as a ‘proton-wire’ (Pomes & Roux, 1996). Both the NPA region and ar/R filter have been separately implicated in proton exclusion by aquaporins (de Groot & Grubmuller, 2005). However, recently a model of synergistic interaction between the two selectivity filters has emerged (Li *et al.*,

2011a). The amide groups of asparagine residues present in the NPA motifs interact with the oxygen atoms of water molecules. This prevents them from forming hydrogen bonds with adjacent water molecules and so inhibits proton permeation (Murata *et al.*, 2000). The ar/R filter increases the overall free energy barrier to proton transport through electrostatic repulsion and can also orient water molecules to prevent proton-wire formation (Li *et al.*, 2011a).

Finally, the extracellular and cytosolic vestibules of aquaporins may contribute to solute pre-selection. For example, the strict mammalian aquaporin AQP1 has hydrophilic vestibules, whereas these regions of the glycerol facilitator from *E. coli*, GlpF, are more hydrophobic (Sui *et al.*, 2001).

4.1.3 Substrates of aquaporins

Since the purification and characterisation of hAQP1 from red blood cells (Smith & Agre, 1991; Preston *et al.*, 1992), aquaporins have been identified in a diverse range of organisms. The genomes of terrestrial plants, in particular, encode a large number of aquaporins, which have been proposed to play a role in the adaptation of green plants to life on land (Danielson & Johanson, 2010; Anderberg *et al.*, 2011). The archetypal aquaporin from spinach (*Spinacia oleracea*) SoPIP2;1 is water-specific (Johansson *et al.*, 1998), whilst GmNod26 from soybean (*Glycine max*) is permeable to both water and glycerol (Rivers *et al.*, 1997; Dean *et al.*, 1998). Early research on aquaporins focussed on transport of water and glycerol, whereas recently a number of novel substrates have been identified.

Carbon dioxide is a neutral molecule, with a diameter similar to that of water. Transport of carbon dioxide by aquaporins was first demonstrated by heterologous expression of hAQP1 in *Xenopus* oocytes (Nakhoul *et al.*, 1998). hAQP1 is highly expressed in red blood cells, and tissues involved in gas-exchange including the pulmonary capillaries and vascular smooth muscle (Preston & Agre, 1991; Effros *et al.*, 1997; Shanahan *et al.*, 1999). The physiological relevance of transport of carbon dioxide by aquaporins has been debated. This was mainly due to data from artificial and simulated membranes, which indicated that carbon dioxide could pass through the lipid bilayer with lower energy cost than transport by aquaporins (Verkman, 2002; Hub & de Groot, 2006). However, studies

of biological membranes generally found carbon dioxide permeability to be 10 to 1000-fold lower than values obtained for pure lipid bilayers (Uehlein *et al.*, 2012).

Overexpression of NtAQP1 increased photosynthetic rate of tobacco plants proportionally to the level of carbon dioxide in the atmosphere, suggesting that plant aquaporins may also be permeable to carbon dioxide (Uehlein *et al.*, 2003). Additionally, overexpression of native *NtAQP1* in tobacco, and expression of *HvPIP2;1* from barley in rice, were found to significantly increase the conductance of carbon dioxide by mesophyll cells (g_m; Hanba *et al*., 2004; Flexas *et al*., 2006). However, expression of *HvPIP2;1* also affected several important physical characteristics of rice leaves including mesophyll cell size, cell wall thickness, stomatal density, stomatal size and Rubisco levels (Hanba *et al.*, 2004). Although overexpression of native NtAQP1 did not affect any physical leaf traits (Flexas *et al.*, 2006). Reduced photosynthetic rate of knock-out mutants, and heterologous expression in yeast, demonstrated that AtPIP1;2 transports carbon dioxide in Arabidopsis (Heckwolf *et al.*, 2011; Uehlein *et al.*, 2012). Interestingly, analysis of NtAQP1 in yeast suggested that carbon dioxide was transported through the pore at the centre of the aquaporin tetramer (Otto *et al.*, 2010).

Another 'unconventional permeant' of aquaporins is hydrogen peroxide. By expressing a range of aquaporins in strains of yeast with increased sensitivity to oxidative stress, Bienert *et al.* (2007) demonstrated that hAQP8, AtTIP1;1 and AtTIP1;2 are permeable to hydrogen peroxide. Using the mammalian cell line, HEK 293, Miller *et al.* (2010) confirmed transport of hydrogen peroxide by hAQP8, and also reported permeability for hAQP3. Decreased growth of yeast expressing *AtPIP2;1*, *AtPIP2;4*, *AtTIP2;3* or *AtNIP2;1* on medium containing hydrogen peroxide suggests that these aquaporins are also able to transport hydrogen peroxide (Dynowski *et al.*, 2008). In Arabidopsis roots, expression of *AtPIP2;1*, *AtPIP2;2*, *AtPIP2;3*, *AtPIP2;4*, *AtPIP2;5*, *AtPIP2;6*, *AtPIP2;7* and *AtPIP2;8* was down-regulated following hydrogen peroxide treatment. Additionally, expression of *AtPIP2;2*, *AtPIP2;4*, *AtPIP2;5* and *AtPIP2;7* increased sensitivity of yeast to hydrogen peroxide (Hooijmaijers *et al.*, 2012). Interestingly, simulations of SoPIP2;1 found that in contrast to carbon dioxide, hydrogen peroxide and urea permeate through the individual aquaporin pores (Dynowski *et al.*, 2008).

Finally, identification of metalloids, including arsenite (see **Sections 1.7.2**; **1.8.2**), as substrates of aquaporins has become increasingly common in recent years. With a pK_a of 11.8, antimonite, like silicic acid and arsenite, is almost entirely present as undissociated $Sb(OH)_3$ at physiological pH. A mutagenesis screen revealed that GlpF from *E. coli* is permeable to antimonite [Sb(III)] (Sanders *et al.*, 1997). Additionally, rat AQP7 and mouse AQP9 were shown to transport antimonite when expressed in yeast or *Xenopus* oocytes (Liu *et al.*, 2002). A screen of plant NIP aquaporins in yeast and oocytes demonstrated that AtNIP5;1, AtNIP6;1 and AtNIP7;1 from Arabidopsis; OsNIP2;1, OsNIP2;2 and OsNIP3;2 from rice; LjNIP5;1 and LjNIP6;1 from lotus (*Lotus japonicus*), as well as rat AQP9, Fps1 from yeast and GlpF from *E. coli*, are permeable to antimonite (Bienert *et al.*, 2008). Additionally, the *atnip1;1* mutant was found accumulate less Sb, and so was more tolerant to antimonite than wild-type, and *atnip1;2* and *atnip5;1* mutants (Kamiya *et al.*, 2009). Wu and Beitz (2007) speculate that the size and shape of arsenite and antimonite make discrimination from glycerol impossible, meaning that all 'aquaglyceroporins' may be permeable to these toxic metalloids.

Unlike antimony and arsenic, boron is essential for higher plants as a component of cell walls (for review see O'Neill *et al.*, 2004). Boric acid has a pK_a of 9.24 and so at physiological pH is present predominantly as undissociated $B(OH)_3$. *AtNIP5;1* was found to be upregulated in boron-deficient roots of Arabidopsis, and when expressed in *Xenopus* oocytes was found to transport boric acid and water (Takano *et al.*, 2006). AtNIP6;1 is 83.1% similar to AtNIP5;1 at the amino acid level, and when expressed in *Xenopus* oocytes was permeable to boric acid but not water (Tanaka *et al.*, 2008). The final member of the NIPII-subgroup of aquaporins in Arabidopsis, AtNIP7;1, was found to transport boric acid, urea and glycerol in *Xenopus* oocytes but is impermeable to water. However, an unusual tyrosine residue in transmembrane helix 2 (Tyr^{81}) occludes the pore, and so this residue was modified (Y81C) for characterisation (Li *et al.*, 2011b). AtNIP7;1 is expressed in developing anthers, and so the authors propose that Tyr^{81} may be involved in regulating AtNIP7;1 to prevent B toxicity in the pollen. Assays in yeast demonstrated that barley HvPIP1;3 and HvPIP1;4 are both permeable to boric acid, however their expression in plants was unaffected by boron deficiency or toxicity (Fitzpatrick & Reid, 2009). Finally, mutation or overexpression of *Fps1* altered boric acid tolerance and B

uptake of yeast, suggesting that Fps1 may also be permeable to boric acid (Nozawa *et al.*, 2006).

4.1.4 Regulation of aquaporins

The major role of aquaporins in water transport means their function must be tightly regulated to maintain water homeostasis. Regulation of aquaporins first occurs through altered gene expression depending on factors including cell-type, developmental stage and environmental conditions. However aquaporins can also undergo a variety of post-translational modifications such as phosphorylation, glycosylation and methylation, and respond to environmental factors such as pH, calcium ions and pressure (for review see Maurel *et al.*, 2008). All together these mechanisms allow organisms to fine tune aquaporin activity.

Phosphorylation of soybean GmNod26 was the first reported post-translational modification of a plant aquaporin (Weaver & Roberts, 1991). Direct evidence has shown that spinach SoPIP2;1, formally known as PM28A, and kidney bean (*Phaseolus vulgaris*) PvTIP3;1, formally called α-TIP, are also phosphorylated *in planta* (Johnson & Chrispeels, 1992; Johansson *et al.*, 1998). Additionally, mutagenesis studies in *Xenopus* oocytes suggest that a serine residue near to the first NPA motif, present in both SoPIP2;1 and PvTIP3;1, is also a target of several protein kinases (Maurel *et al.*, 1995; Johansson *et al.*, 2000). Phosphorylation of aquaporins in response to water and temperature stress has been reported for several plant species. It has been proposed that phosphorylation of key serine residues the N- and C- cystosolic termini causes rapid, reversible channel gating (Johansson *et al.*, 2000), however the exact mechanism is yet to be elucidated. In the case of mammalian AQP2, phosphorylation of Ser^{256} in the N-terminus causes relocalisation from intracellular vesicles to the apical plasma membrane (Brown, 2003).

Approximately one-quarter to one-third of newly synthesised human AQP2 monomers are glycosylated. Glycosylation was found not to alter tetramerisation or selectivity, but was essential for AQP2 to exit the Golgi and be correctly targeted to the plasma membrane (Hendriks *et al.*, 2004). In plants, glycosylation is thought to be involved in the regulation of McTIP2;1 from ice plant (*Mesembryanthemum crystallinum*). Under osmotic stress induced by mannitol treatment, a glycosylated isoform of McTIP2;1 was detected,

and McTIP2;1 was re-localised from the tonoplast to higher density membrane fractions. Treatment with various inhibitors revealed that McTIP2;1 is also phosphorylated during re-distribution (Vera-Estrella *et al.*, 2004).

AtPIPs isolated from Arabidopsis roots were the first reported methylated membrane proteins in plants. Residues in the N-terminus of members of both the PIP1 and PIP2 subclasses were found to be methylated, dimethylated or acetylated (Santoni *et al.*, 2006). Additionally, methylation of glutamate and lysine residues in the C-terminus of PIP2;2 from broccoli (*Brassica oleracea*) has been reported (Casado-Vela *et al.*, 2010). However, analysis of *atpip2;1* mutants in membrane vesicles showed that methylation of Lys^{3} or Glu^{6} is not required for water transport (Santoni *et al.*, 2006), and the role of methylation in regulating aquaporin activity is currently unknown.

Activity of aquaporins can also be regulated by pH and calcium ions (Ca^{2+}). In the case of bovine AQP0, pH and Ca^{2+} were found to have separable effects on water permeability. pH sensitivity is modulated by a histidine residue in loop A; which is postulated to re-orient water molecules so they cannot enter the pore, while regulation by Ca^{2+} involves calmodulin binding to the C-terminus (Németh-Cahalan & Hall, 2000); which may also re-organise water molecules to prevent their passage through AQP0 (Chaumont *et al.*, 2005). Heterologous expression in *Xenopus* oocytes revealed that water transport of AtPIP1;2, AtPIP2;1 and AtPIP2;2 was abolished when the cytosolic pH was lowered from 7 to 6 (Tournaire-Roux *et al.*, 2003). Structural modelling of AtPIP2;2 suggests that under low pH a histidine residue in loop D (His^{197}) is protonated, which stabilises interactions between basic residues in the N-terminus and acidic residues in loop D. This blocks the pore and abolishes water transport (Chaumont *et al.*, 2005). This model is supported experimentally as mutating His^{197} to an acidic aspartate residue (H197D) results in a pH-insensitive, constitutively open channel. Additionally, substituting His^{197} with a basic lysine residue (H197K) results in a channel with very little water permeability (Tournaire-Roux *et al.*, 2003).

High resolution crystal structures of human AQP1 and *E. coli* GlpF have shown that these aquaporins exist as homotetramers (Fu *et al.*, 2000; Murata *et al.*, 2000). However, several plant TIPs and PIPs have been shown to exist as heterotetramers. When

expressed in *Xenopus* oocytes, *ZmPIP1;2* from maize (*Zea mays*) demonstrated little to no water permeability. However, co-expression with different *ZmPIP2* genes showed co-operative water transport. The authors suggest that an interaction between PIP1s and PIP2s is required for correct trafficking of PIP1s to the plasma membrane (Fetter *et al.*, 2004). The requirement of PIP2 isoforms for the correct targeting of PIP1 proteins to the plasma membrane has been confirmed in other plant species including tobacco, grape (*Vitis vinifera*) and durum wheat (*Triticum turgidum*; Mahdieh *et al*., 2008; Vandeleur *et al*., 2009; Ayadi *et al*., 2011) . Modelling mutations of residues in loop E altered putative interactions between ZmPIP1 and ZmPIP2 isoforms (Chaumont *et al.*, 2005). However, loop A was implicated in interactions between *Beta vulgaris* BvPIP1 and BvPIP2 isoforms (Jozefkowicz *et al.*, 2013). Recently, using a bimolecular fluorescence complementation (BiFC) assay in yeast, heterotetramers of AtTIP1;2, AtTIP2;1 and AtTIP3;1 were observed. Interestingly, AtTIP1;2 and AtTIP2;1 were also found to interact with AtPIP2;1 (Murozuka *et al.*, 2013).

4.1.5 NIPs

Members of the NIP subfamily are classified by their structural similarity to the archetypal GmNod26, however they are functionally diverse. Soybean nodulin-26 was one of the first aquaporins identified in higher plants, and is abundant in the symbiosome membrane which forms during nodulation of leguminous plants by members of the soil bacteria family Rhizobiaceae (Fortin *et al.*, 1987). GmNod26 has since been characterised by expression in *Xenopus* oocytes and purified membrane vesicles, and has been shown to transport water, glycerol and formamide, but not urea (Rivers *et al.*, 1997; Dean *et al.*, 1998; Table S4.1).

Generally NIPs show relatively low water permeability and are able to mediate the flux of various uncharged solutes, thought to depend on the composition of the ar/R region. The Arabidopsis genome encodes nine NIP aquaporins (Johanson *et al.*, 2001), and rice has 10 or 13 (Sakurai *et al.*, 2005; Bansal & Sankararamakrishnan, 2007). The NIP subfamily is further divided into three subgroups depending on their pore size, which is largely determined by the composition of the ar/R region (Rouge & Barre, 2008; Figure 4.1). OsLsi1 (OsNIP2;1) belongs to subgroup III due to a large pore size of ≥ 6 Å, compared with 3.5 Å and ≤ 5 Å for subgroups I and II respectively (Liu *et al.*, 2009).

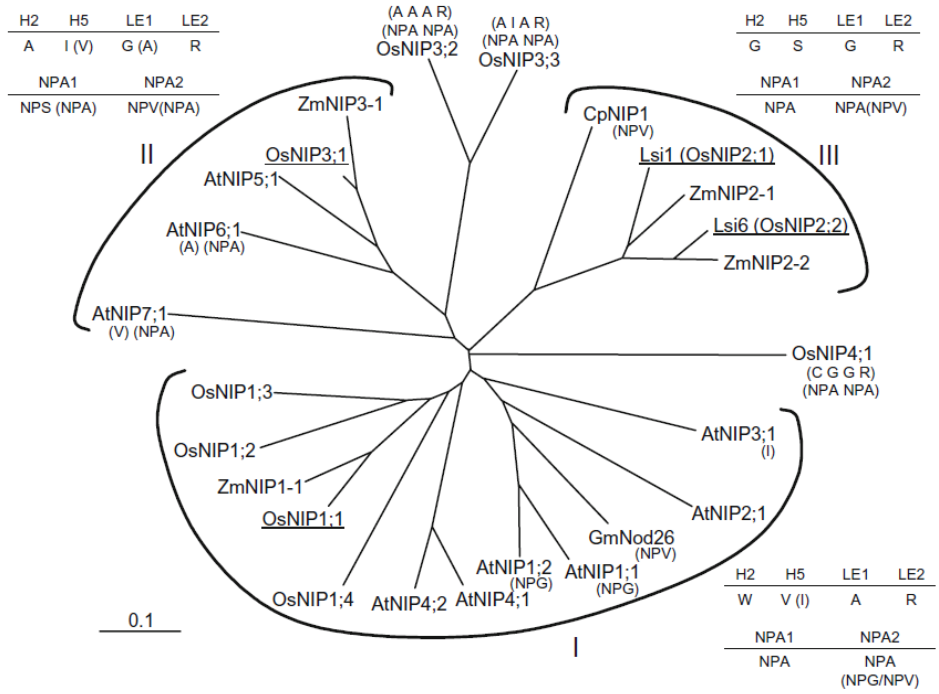


Figure 4.1 Phylogenetic tree of plant aquaporins constructed by *Mitani et al*., (2008). NIPs from rice (Os-), Arabidopsis (At-) and maize (Zm-), as well as soybean nodulin-26 (GmNod26) and courgette (*Cucurbita pepo*) CpNIP1 were classified into three subgroups based on the two NPA motifs and ar/R selectivity filter. The NPA motifs and the ar/R residues of each subgroup are given, and minor substitutions of those residues are in parentheses.

Unlike other plant aquaporins, NIP members show permeability to a range of solutes, and have been implicated in the transport of essential nutrients. A comprehensive index of reported substrate permeability of NIP subfamily members can be found in Supplementary Table 4.1. For example, AtNIP6;1 was identified to mediate boron uptake in Arabidopsis roots (Takano *et al.*, 2006), and NIP members from several plant species have been shown to transport silicon (Ma *et al.*, 2006; Yamaji *et al.*, 2008; Chiba *et al.*, 2009; Mitani *et al.*, 2009; Grégoire *et al.*, 2012; Montpetit *et al.*, 2012). Boron is transported by NIPs in the form of boric acid [$B(OH)_3$], which at 2.57 Å, is a similar size to urea (2.62 Å; Takano *et al.*, 2006). Silicon and arsenite, however, are transported in the form of silicic acid [$Si(OH)_4$] and arsenous acid [$As(OH)_3$], which at 4.38 Å and 4.11 Å respectively, are significantly larger molecules (Wu & Beitz, 2007; Ma *et al.*, 2008).

The ar/R filter of aquaporins has been shown to play a crucial role in proton exclusion. Additionally, site-directed mutagenesis experiments have found that substrate specificity of aquaporins can be altered by modifying the tetrad of residues which make up this filter. Wallace and Roberts (2005) found that modifying the first residue of the ar/R filter

of AtNIP6;1 from alanine to tryptophan (A119W) conferred transport properties similar to GmNod26 when expressed in *Xenopus* oocytes. Native AtNIP6;1 is impermeable to water, but transports urea and glycerol, whereas GmNod26, and the mutated AtNIP6;1, transport water, glycerol and formamide but exclude urea. AtNIP6;1 differs from GmNod26 in the NPA region as well as the ar/R filter. However substitution of valine to alanine (V252A) in the second NPA motif had no effect on transport. The authors concluded that the H2 position of the ar/R filter is critical in determining substrate specificity of NIPs (Wallace & Roberts, 2005). However, Mitani-Ueno *et al.* (2011) found that substitution of the H2 residue of OsLsi1 (G88A) had no effect on transport of silicon, arsenite, boron or water when expressed in *Xenopus* oocytes. Substitution of the H5 residue (S207I), on the other hand, abolished silicon, boron and water uptake, and significantly decreased arsenite transport. Interestingly, mutation of both the H2 and H5 residues of the ar/R filter (A117G-S236S), and NPA motifs (S139A-V250A) of AtNIP5;1 to mimic the pore of OsLsi1 did not confer Si transport ability (Mitani-Ueno *et al.*, 2011). Therefore residues other than those in the NPA and ar/R filters may also be involved in substrate specificity of NIP aquaporins.

Unlike other solutes, arsenite permeability is common to members across the three different subgroups (Zhao *et al.*, 2009; Table S4.1). For rice, in addition to OsLsi1 (OsNIP2;1) from group III; OsNIP1;1 (group I), OsNIP2;2 (group III), OsNIP3;1 (group II) and OsNIP3;2 have all shown arsenite transport ability when expressed in *Xenopus* oocytes or yeast (Bienert *et al.*, 2008; Ma *et al.*, 2008; Mitani *et al.*, 2008). Furthermore arsenite transport has also been demonstrated for AtNIP1;1 and AtNIP2;1 from group I; and AtNIP5;1, AtNIP6;1 and AtNIP7;1 from group II (Bienert *et al.*, 2008; Isayenkov & Maathuis, 2008; Mitani *et al.*, 2008; Kamiya *et al.*, 2009; Mitani-Ueno *et al.*, 2011). In fact the only NIP aquaporin found not to transport arsenite is OsNIP3;1 (Ma *et al.*, 2008). This is especially surprising given that the size of the arsenous acid molecule is larger than the predicted pore of group I NIPs. Furthermore, arsenite permeability was affected less by mutations in the ar/R residues of OsLsi1 and AtNIP5;1 than silicon or boron (Mitani-Ueno *et al.*, 2011).

4.2 Aims

Having concluded that methylated As species in plants originate from soil micro-organisms, the aim of the experiments within this chapter is therefore to identify and characterise the transporters responsible for the transport of undissociated MMA and DMA in rice. The major silicon and arsenite transporter in rice roots, OsLsi1 (OsNIP2;1), has been shown to transport MMA and DMA, therefore other members of the NIP aquaporin subfamily may also be permeable to undissociated MMA and DMA.

The transporters of dissociated MMA and DMA complexes, which increase in abundance with increasing pH, is the subject of **Chapter 5**.

4.3 Materials and methods

For comprehensive protocols on heterologous expression of plant proteins in *Xenopus* oocytes see Theodoulou and Miller (1995).

4.3.1 Heterologous expression in *Xenopus* oocytes

The construct for expressing *OsLsi1* and the human aquaporin *hAQP9* in oocytes were gifts from Prof J.F. Ma (Okayama University, Japan) and Dr Z.J-. Liu (Oakland University, USA) respectively. The oocyte expression constructs of *OsNIP1;1*, *OsNIP3;2* and *OsNIP3;3* were made by Dr Y. Chen (Rothamsted Research, UK) from cDNA of rice (cv. Nipponbare).

Competent *E. coli* cells were transformed using heat shock with plasmid pX*β*G-ev1 containing the cDNA of *OsLsi1* or *hAQP9*, or with plasmid pT7TS containing the cDNA of *OsNIP1;1*, *OsNIP3;2, OsNIP3;3*, or *HvPIP2;*2. Plasmids were purified using a Qiagen QIAprep Spin miniprep kit according to manufacturer's instructions. Once purified, the plasmids were linearised using an appropriate restriction enzyme (SpeI pX*β*G-ev1, and BamHI for pT7TS) and cRNA synthesised *in vitro* using the Ambion mMessage mMachine kit according to manufacturer's instructions (T3 for pX*β*G-ev1, and T7 for pT7TS). The cRNA produced has a 7-methyl guanosine cap structure at the 5' end, and a poly-adenosine tail on the 3' end, which increases RNA stability in the oocytes. The synthesised cRNAs were purified by phenol/chloroform extraction and precipitation with isopropanol, and were stored at -80°C.

Ovaries of adult female *Xenopus laevis* frogs were collected from the University of Cambridge and stored in modified Barth's solution (MBS; 88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 15 mM Hepes, 0.3 mM $CaNO_3$, 0.41 mM $CaCl_2$, 0.82 mM $MgSO_4$, 10 µg mL^{-1} sodium penicillin and 10 µg mL^{-1} streptomycin sulphate; pH 7.4; osmolality 195±5 mmol kg^{-1}). Oocytes were isolated by separating lobes into clusters of around 30 oocytes using forceps, and gentle swirling in 1 mg mL^{-1} type II collagenase dissolved in calcium-free MBS (88 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 15 mM Hepes, 0.82 mM $MgSO_4$, 10 µg mL^{-1} sodium penicillin and 10 µg mL^{-1} streptomycin sulphate; pH 7.4; osmolality 195±5 mmol kg^{-1}) for around 45 minutes. Oocytes were then rinsed thoroughly with MBS containing calcium to prevent degradation by the collagenase. Healthy stage V or VI oocytes were selected by visual inspection and kept in small Petri dishes coated with a

Nunclon™Δ surface (Nunc, Naperville, IL, USA). All instruments for handling oocytes were stored in 70% ethanol and MBS (both normal and Ca-free) were autoclaved before the addition of antibiotics.

Healthy oocytes were injected with approximately 50 nL of 1 ng nL^{-1} cRNA using a non-filamented borosilicate glass capillary pulled into a fine tip of 5-10 µm diameter connected to a compressed air picolitre injector (Medical Systems PLi-100). Injection tips were baked at 180°C for 12 hours before use to degrade ribonucleases (RNases) and were calibrated to give around 20 injections of 1 µL of water. Non-injected oocytes, as well as those injected with 50 nL of nuclease-free water, serve as controls. After injection, oocytes were stored at 20°C with twice daily washing in MBS. After two days incubation transport assays were conducted.

4.3.2 Oocyte transport assays

To measure water permeability, single oocytes were osmotically-challenged by placing them in MBS diluted with de-ionised water. Oocytes were imaged using a Leica MZ8 microscope linked to a DCF300FX camera taking a photo every 20 seconds for a total of three minutes. The diameter of the oocytes was then measured using ImageJ (NIH; Bethesda, MD, USA) and converted to volumes by using the formula $V = 4/3\ \pi\ r^3$.

Germanium hydroxide [$Ge(OH_4)$] was prepared by adding NaOH to a solution of germanium chloride ($GeCl_4$). All solutions for B analysis were made in plasticware to avoid contamination from borosilicate glass. After exposure to MBS amended with As, Ge or B (see figure legends for exact uptake conditions), oocytes were rinsed at least 6 times with clean MBS and several oocytes forming one replicate were placed in centrifuge tubes. Oocytes were then digested with 0.4 mL HNO_3 heated to 90°C for 45 minutes in a water batch. Once cooled, samples were diluted to 7 or 8 mL with ultrapure water and analysed for total As, Ge or B concentration by ICP-MS (see **Section 2.1**).

Table 4.1 LOD and LOQ for elements analysed in digested *Xenopus* oocytes calculated from blank values (LOD = 3-fold, and LOQ = 10-fold blank) in ng $oocyte^{-1}$.

Analyte	LOD	LOQ
As	0.021	0.070
Ge	0.027	0.090
B	0.047	0.150

4.4 Results

Due to variability between batches of *Xenopus* oocytes, data are presented with all tested genes and treatments in a single figure. Specific conditions for uptake, including the concentration of the substrate and the incubation time, can be found in the figure legends. In all cases water-injected oocytes were included as a negative control, and OsLsi1 as a positive control for arsenite uptake and water permeability (discussed below).

4.4.1 Transport of arsenite and MMA

To validate the methods of cRNA synthesis, oocyte injection and arsenite uptake, *OsLsi1* (*OsNIP2;1*) was used as a positive control. A plasma membrane aquaporin from barley (*Hordeum vulgare*) previously found to be highly water permeable, *HvPIP2;2*, was also included as a positive control for oocyte swelling upon osmotic challenge (Besse *et al.*, 2011).

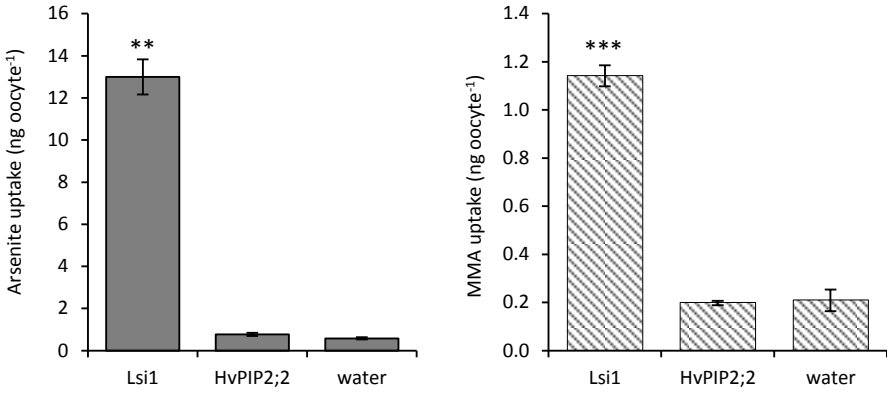


Figure 4.2 Mean arsenic content of *Xenopus* oocytes expressing *OsLsi1* and *HvPIP2;2*. Oocytes were exposed to MBS containing 0.1 mM arsenite at pH 7.4 for 30 minutes, or MMA at pH 4.5 for 30 minutes. After exposure oocytes were rinsed thoroughly and split into replicates of 5-6 oocytes for analysis. Error bars represent SE (n = 3-4). ** = $P < 0.01$, *** = $P < 0.001$ significant difference from control (water) of Student's t-test.

Expression of *OsLsi1* resulted in significantly higher uptake of arsenite and MMA compared to the water-injected control oocytes (Figure 4.2). HvPIP2;2, included as a positive control for water permeability, shows no arsenite or MMA uptake. Recently, Mosa *et al.* (2012) reported that OsPIP2;4, OsPIP2;6 and OsPIP2;7 are able to transport arsenite, however when expressed in oocytes *OsPIP1;2* and *OsPIP1;3* showed no arsenite uptake.

Arsenite and MMA permeability of other NIP members was tested. OsNIP1;1 belongs to subgroup I (Figure 4.1), and has previously been shown to be permeable to arsenite when expressed in *Xenopus* oocytes (Ma *et al.*, 2008; Mitani *et al.*, 2008). OsNIP3;2 and OsNIP3;3 are not assigned to a subgroup in the NIP phylogeny constructed by Mitani *et al.*, (2008), but have been classified to group II by other studies (Wallace *et al.*, 2006; Bienert *et al.*, 2008; Grégoire *et al.*, 2012). *OsNIP3;2* was found to confer arsenite sensitivity when expressed in yeast (Bienert *et al.*, 2008) but OsNIP3;3 has not previously been studied.

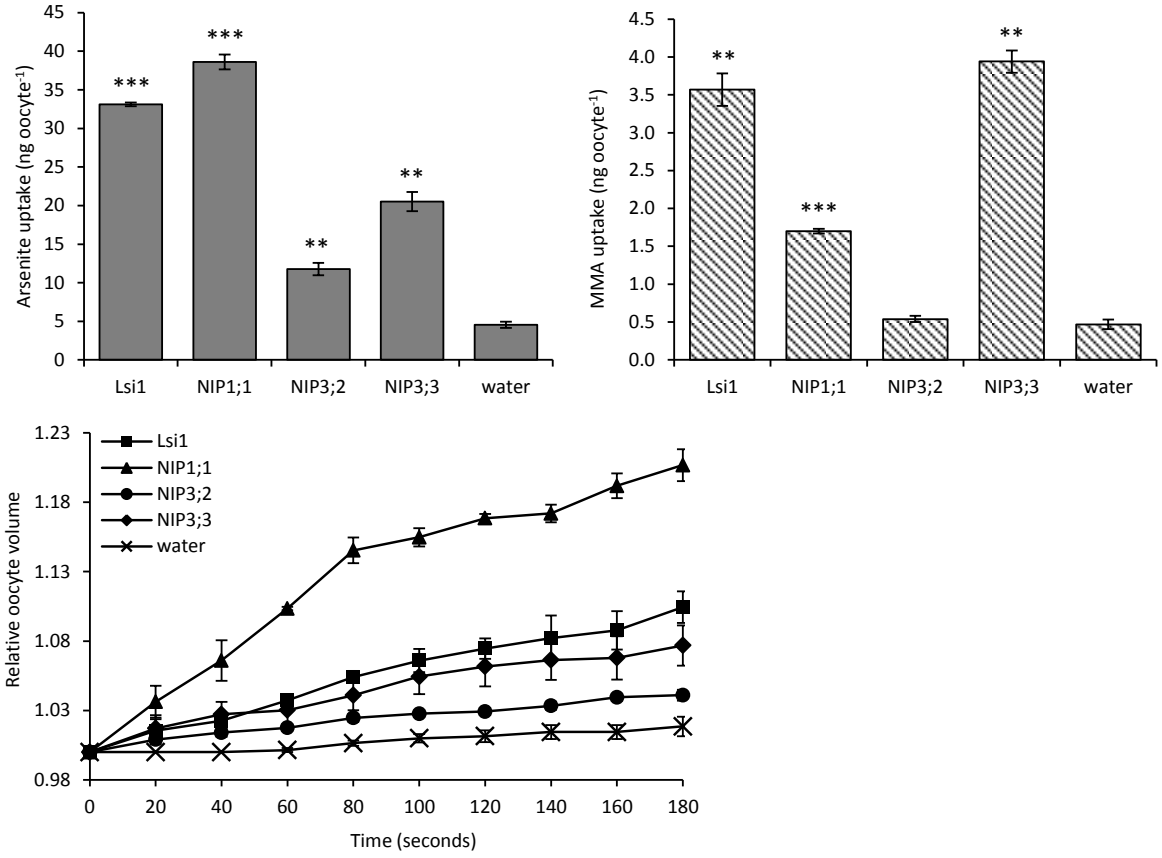


Figure 4.3 Mean arsenite, MMA and water uptake of *Xenopus* oocytes expressing *OsLsi1*, *OsNIP1;1*, *OsNIP3;2*, and *OsNIP3;3*. Oocytes were exposed to MBS containing 1 mM arsenite at pH 6.0 for 60 minutes; 1 mM MMA at pH 6.0 for 60 minutes; or MBS diluted to 20% for 3 minutes. After As exposure oocytes were rinsed thoroughly and split into replicates of 9-15 oocytes for analysis. Error bars represent SE (n = 2-4). ** = $P < 0.01$, and *** = $P < 0.001$ significant difference from control (water) of Student's t-test.

Although expression of *OsNIP1;1*, *OsNIP3;2*, and *OsNIP3;3* all increased the capacity for oocytes to accumulate arsenite, there were differences between the genes (Figure 4.3). Expression of *OsLsi1* and *OsNIP1;1* increased As content of the oocytes by 7.3- and 8.5-

fold on average respectively compared to the water-injected control. However oocytes expressing *OsNIP3;2* accumulated only 2.6-fold more As on average than the control oocytes. OsNIP3;3 was intermediate, with 4.5-fold more As accumulation on average compared to the control.

As found previously (Figure 4.2), OsLsi1 demonstrated permeability to MMA. Expression of *OsNIP3;3* also greatly increased MMA uptake, whereas expression of *OsNIP1;1* gave only a moderate increase, and oocytes expressing *OsNIP3;2* were no different from the water-injected control. Interestingly, the pattern of arsenite uptake for the oocytes expressing the different genes is not reflected in MMA uptake. Therefore NIP aquaporins which are highly permeable to arsenite may not necessarily show significant transport of other As species.

Additionally, all genes showed higher water permeability than the water-injected control. The increase was greatest for OsNIP1;1, whereas oocytes expressing *OsNIP3;2* showed only a small increase compared to the control oocytes.

4.4.2 Transport of DMA

Although transport of MMA has previously been reported for oocytes expressing *OsLsi1*, uptake of DMA by a plant transporter is unreported to date. The only transporter showing direct DMA uptake is the human aquaporin, hAQP9, which was highly permeable to both MMA and DMA when expressed in *Xenopus* oocytes (McDermott *et al.*, 2010).

DMA uptake was not observed in five independent batches of oocytes (data not shown). In all cases, expression of *OsLsi1* and other NIP genes was confirmed by positive results for arsenite uptake. A variety of modifications to the assay conditions were tested, such as increasing the concentration of DMA in the exposure medium, increasing the incubation time in the treatment solution, and decreasing the pH of the exposure medium (to increase the proportion of undissociated DMA complexes). Additionally the exact method reported by McDermott *et al.* (2010) was tested, whereby oocytes were exposed to 1 mM As at pH 6.0 for 60 minutes.

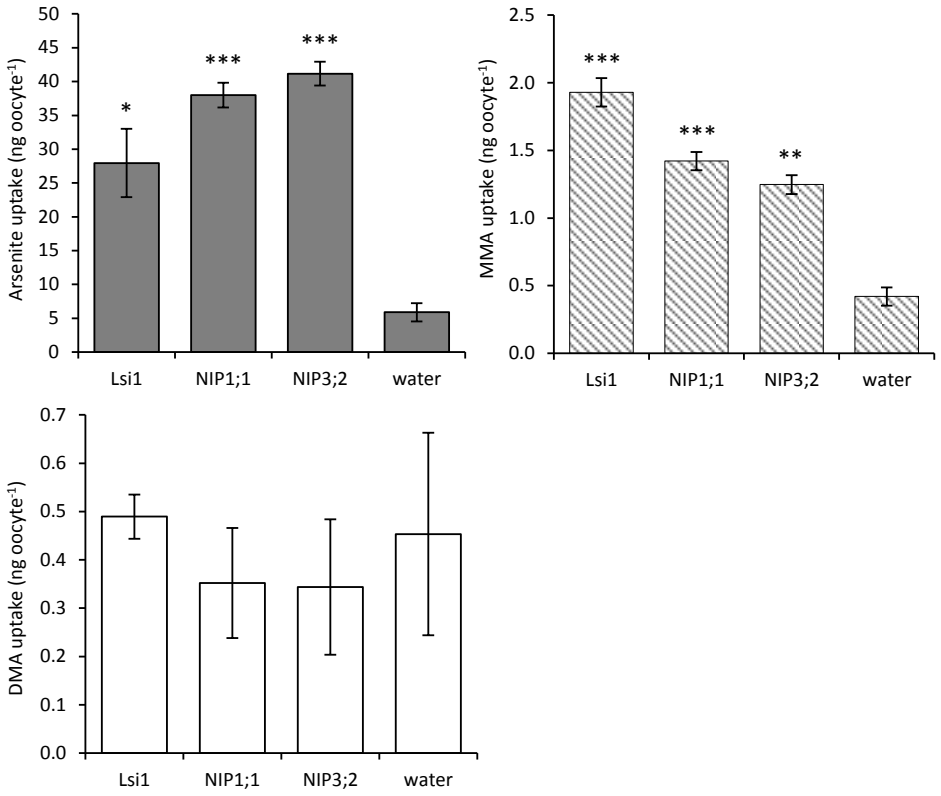


Figure 4.4 Mean arsenic content of *Xenopus* oocytes expressing *OsLsi1*, *OsNIP1;1* and *OsNIP3;2*. Oocytes were exposed to MBS containing 1 mM arsenite, MMA or DMA at pH 6.0 for 60 minutes. After exposure oocytes were rinsed thoroughly and split into replicates of 5-11 oocytes for analysis. Error bars represent SE (n = 3). * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$ significant difference from control (water) of Student's t-test.

However, DMA transport was not observed for any NIP (Figure 4.4). Additionally, OsNIP1;1 and OsNIP3;2 demonstrated permeability to arsenite and MMA. This is in contrast to the previous experiment which found little difference in arsenite uptake, and no difference in MMA transport, of OsNIP3;2 compared to the control (Figure 4.3).

To investigate DMA transport by OsNIP3;3, oocytes were incubated in MBS containing 0.1 mM DMA at pH 7.0 for 90 minutes. The optimum pH for storing *Xenopus* oocytes is 7.4-7.6 (Theodoulou & Miller, 1995), so a compromise must be achieved between a pH which preserves the integrity of the cells; especially for longer exposure times, and a pH at which a significant proportion of MMA and DMA complexes are undissociated.

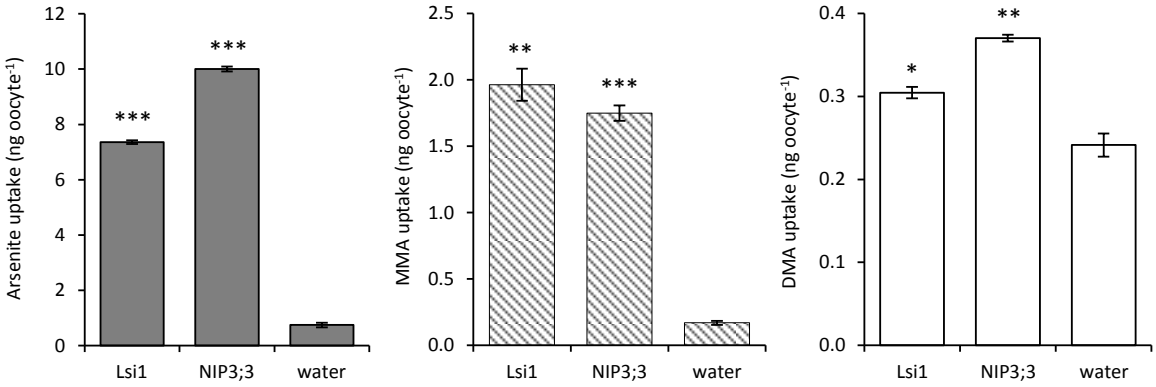


Figure 4.5 Mean arsenic content of *Xenopus* oocytes expressing *OsLsi1* and *NIP3;3*. Oocytes were exposed to MBS containing 0.1 mM arsenite at pH 7.4 for 30 minutes, 0.1 mM MMA at pH 4.5 for 45 minutes, or 0.1 mM DMA at pH 7.0 for 90 minutes. After exposure oocytes were rinsed thoroughly and split into replicates of 6-8 oocytes for As analysis. Error bars represent SE (n = 3). * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$ significant difference from control (water) of Student's *t*-test.

Oocytes expressing *OsNIP3;3* and *OsLsi1* did accumulate more As than the control after exposure to DMA (Figure 4.5). Although this increase was statistically significant, the absolute difference is very small. Interestingly oocytes expressing *OsNIP3;3* showed significantly higher arsenite uptake than those expressing *OsLsi1* ($P < 0.01$), although there was no difference in MMA uptake ($P = 0.21$).

High uptake of DMA by oocytes expressing *hAQP9* has been reported (McDermott *et al.*, 2010), and so an oocyte-expression construct was obtained to act as a positive control.

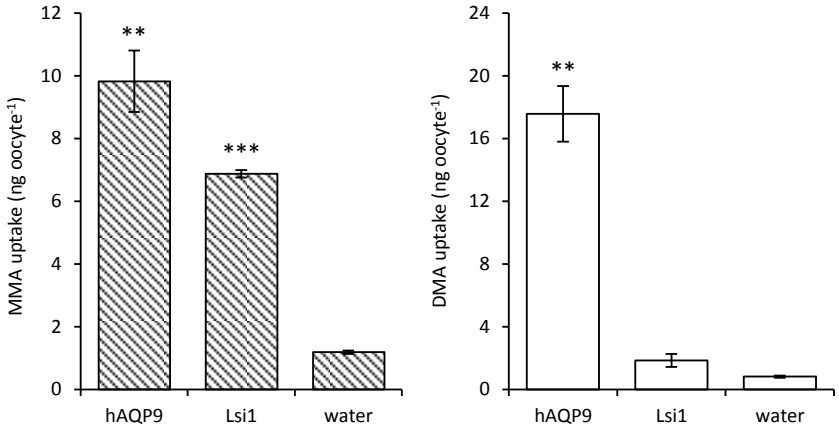


Figure 4.6 MMA and DMA uptake of *Xenopus* oocytes expressing *Lsi1* and *hAQP9*. Oocytes were exposed to MBS containing 1 mM MMA or DMA at pH 6.0 for 60 minutes. After exposure oocytes were rinsed thoroughly and split into replicates of 7-10 oocytes for analysis. Error bars represent SE (n = 3). ** = $P < 0.01$, *** = $P < 0.001$, significant difference from control (water) of Student's *t*-test.

Although MMA uptake by oocytes expressing *OsLsi1* is similar to that of *hAQP9*, there is a vast difference in DMA uptake (Figure 4.6). This result validates the methods for DMA exposure and As analysis of oocytes. On average DMA uptake was 2.6-fold higher in *OsLsi1*-expressing oocytes compared to the control, however this was not significant ($P = 0.06$).

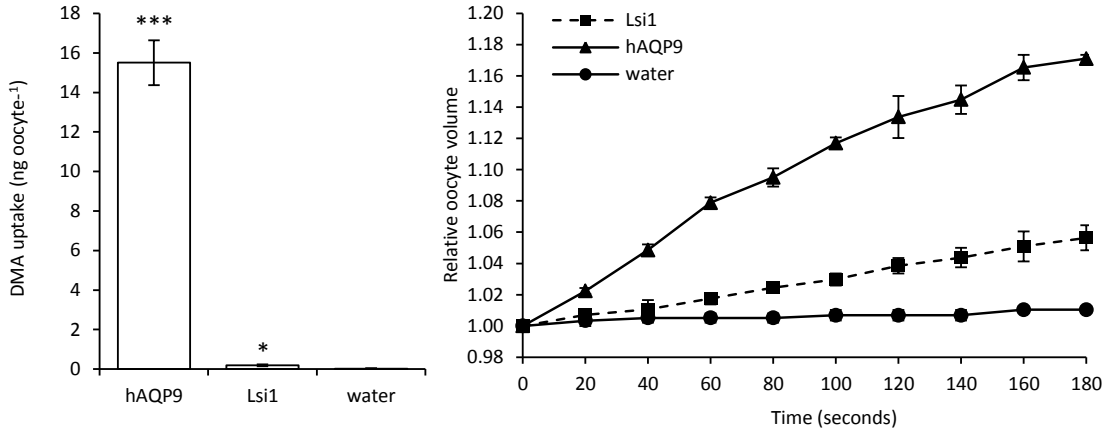


Figure 4.7 DMA uptake and water permeability of *Xenopus* oocytes expressing *OsLsi1* and *hAQP9*. For DMA uptake, oocytes were exposed to MBS containing 1 mM DMA at pH 6.0 for 60 minutes. After exposure oocytes were rinsed thoroughly and split into replicates of 5-10 oocytes for analysis. For water permeability, oocytes were exposed to MBS diluted by 50% with oocyte diameter measured every 20 seconds. Error bars represent SE (n = 3-4). * = $P < 0.05$, *** = $P < 0.001$, significant difference from control (water) of Student's t-test.

When repeated, oocytes expressing *hAQP9* again showed considerably higher DMA uptake than those expressing *OsLsi1* (Figure 4.7). However, in this batch of oocytes DMA uptake was statistically significantly higher in *OsLsi1*-expressing oocytes compared to the water-injected control. Additionally, the increase in volume of oocytes under osmotic challenge, was much greater for those expressing *hAQP9* than *OsLsi1*.

4.4.3 Transport of other substrates

To further characterise OsNIP1;1, OsNIP3;2 and OsNIP3;3, permeability to some common substrates of aquaporins; water, germanium and boron, was investigated. OsLsi1 has been reported to be permeable to all substrates tested except glycerol (see Table S4.1).

Silicon permeability was measured by exposing oocytes to a solution containing germanic acid [$Ge(OH)_4$], as an analogue of silicic acid [$Si(OH)_4$]. It is not possible to use Si directly as it spontaneously and irreversibly polymerises at higher concentrations. Plant roots take

up Si and Ge through the same transport pathway, but Ge causes toxicity in the leaves and so can be used to screen for Si transport mutants (Rains *et al.*, 2006; Nikolic *et al.*, 2007).

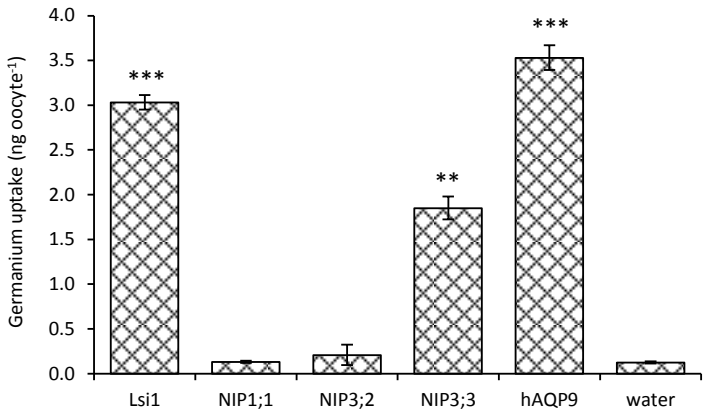


Figure 4.8 Mean germanium content of *Xenopus* oocytes expressing *OsLsi1, OsNIP1;1*, *OsNIP3;2*, *OsNIP3;3* and *hAQP9*. Oocytes were exposed to MBS containing 0.1 mM germanium hydroxide at pH 7.4 for 30 minutes. After exposure oocytes were rinsed thoroughly with MBS and split into replicates of 7-9 oocytes for analysis. Error bars represent SE (n = 3). ** = $P < 0.01$, *** = $P < 0.001$ significant difference from control (water) of Student's t-test.

Both OsLsi1 and OsNIP3;3 show good permeability to Ge (Figure 4.8), whereas oocytes expressing *OsNIP1;1* or *OsNIP3;2* are similar to the water-injected control. hAQP9 was also included in the assay and shows high transport of Ge, which has not previously been reported.

Water permeability of was investigated by measuring oocyte swelling under osmotic challenge. Previously, OsLsi1 was found to be permeable to water (Figures 4.3; 4.7) and so HvPIP2;2 was not required to act as a positive control. Boron transport was measured by exposing oocytes to a solution containing boric acid [$B(OH)_3$].

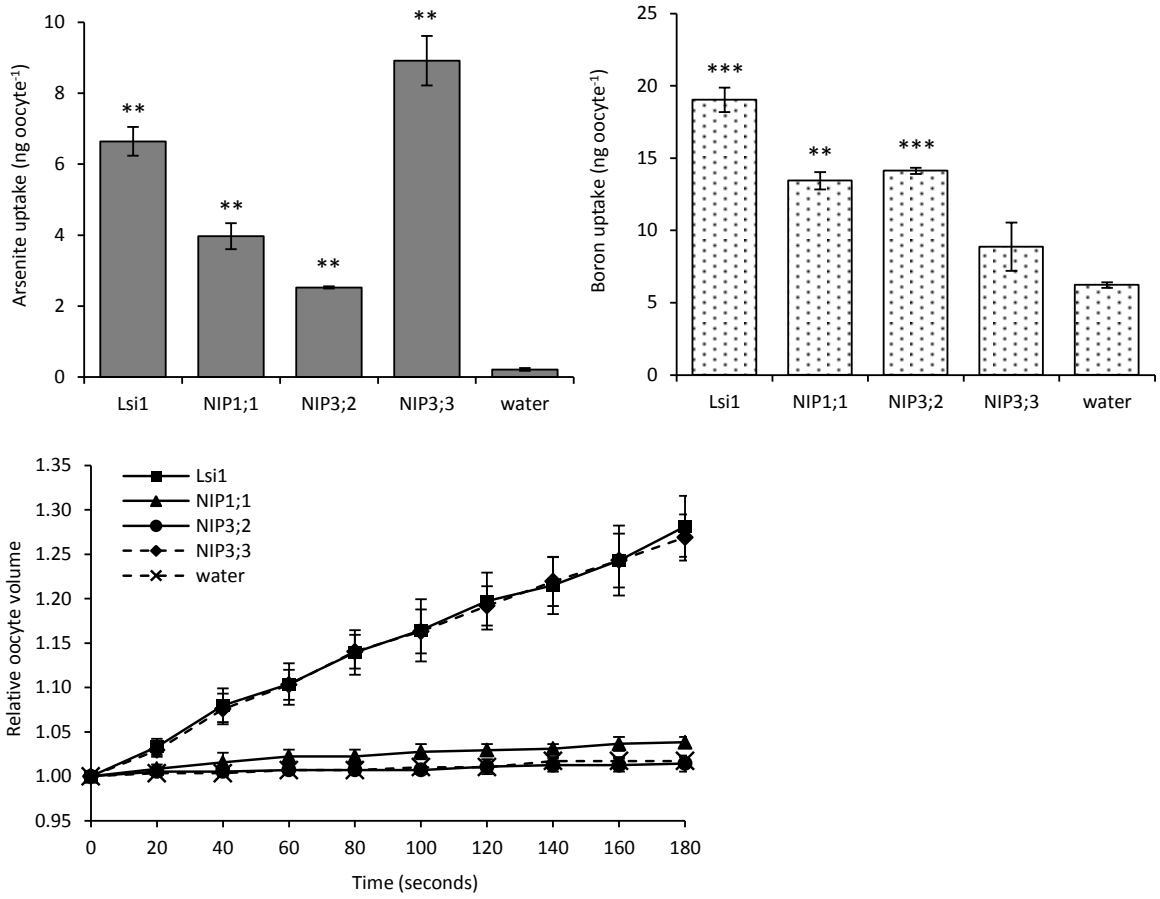


Figure 4.9 Mean arsenic, boron and water uptake of *Xenopus* oocytes expressing *OsLsi1*, *OsNIP1;1*, *OsNIP3;2*, and *OsNIP3;3*. Oocytes were exposed to MBS containing 0.1 mM arsenite at pH 7.4 for 30 minutes; 5 mM boric acid at pH 7.4 for 60 minutes or MBS diluted to 20%. After exposure to arsenite or boron oocytes were rinsed thoroughly and split into replicates of 5-7 oocytes for analysis. Error bars represent SE (n = 2-4). ** = $P < 0.01$, *** = $P < 0.001$, significant difference from control (water) of Student's t-test.

Expression of *OsLsi1*, *OsNIP1;1* and *OsNIP3;2* all significantly increased boron uptake compared to the control oocytes (Figure 4.9). However oocytes expressing *OsNIP3;3* were not significantly different from the water-injected control.

Additionally, oocytes expressing *OsNIP3;3* showed high water permeability, but those expressing *OsNIP3;*2 and *OsNIP1;1* were similar to the water-injected controls (Figure 4.9). Previously *OsNIP1;1* expression significantly increased water permeability of oocytes (Figure 4.3). Oocytes expressing *OsNIP1;1* in the same batch did take up arsenite, so the difference is unlikely to be due to lack of expression and remains unclear.

4.5 Discussion

The ability of *OsLsi1* (*OsNIP2;1*) to transport arsenite and MMA when expressed in *Xenopus* oocytes was confirmed (Figures 4.2; 4.3; 4.4; 4.5; S4.1). Additionally, OsNIP1;1 and OsNIP3;3 were also shown to transport MMA (Figures 4.3; 4.4; 4.5; S4.1). Expression of *OsNIP3;2* in oocytes resulted in significant MMA uptake in one batch of oocytes (Figure 4.4), but no measurable MMA uptake in two others (Figures 4.3; S4.1). In two of the experiments MMA uptake was proportional to arsenite uptake, i.e. transport of both arsenite and MMA was either high or low (Figures 4.3; 4.4), but in the third experiment arsenite uptake was high whereas MMA uptake was absent (Figure S4.1). Therefore, further investigation is required into the permeability of OsNIP3;2 to MMA (see below for discussion on limitations of using *Xenopus* oocytes).

Ma *et al.* (2008) found that *OsNIP1;1* was only weakly expressed in rice roots, and Sakurai *et al.* (2005) reported relatively high expression in both young and old leaves. Expression of *OsNIP3;2* and *OsNIP3;3* was found to be highest in older leaves (Sakurai *et al.*, 2005). Arsenite uptake by roots was reduced by 53% in the *lsi1* mutant compared to wild-type, and MMA uptake was reduced by 80% (Ma *et al.*, 2008; Li *et al.*, 2009a). Therefore although OsNIP1;1 and OsNIP3;3 probably play a minor role in uptake of MMA by rice roots, they may be involved in root to shoot translocation or transport to grain. Furthermore, higher expression in old leaves may suggest a role in retranslocation of methylated As from flag leaves during grain filling (Carey *et al.*, 2011). Due to the high toxicity of As, it is imperative that all transporters permeable to As are identified, especially those involved in transporting As to rice grain.

In all experiments; with different batches of oocytes and varying exposure conditions, OsLsi1, OsNIP1;1 and OsNIP3;3 show higher uptake of arsenite than MMA (Figures 4.2; 4.3; 4.4; 4.5; S4.1). The difference in total As content of oocytes after exposure to MMA suggests that these NIP aquaporins are less permeable to MMA than arsenite. However, MMA uptake was either conducted at pH 4.5 (Figures 4.2; 4.5; S4.1) or 6.0 (Figures 4.3; 4.4) whereby just 32.9% and 1.53% of MMA complexes are undissociated respectively, compared to arsenite which is 99.9% undissociated at both pH 4.5 and 6.0 (Figure S1.2).

Therefore it is difficult to draw conclusions on the relative permeability of NIPs to MMA without more evidence, such as competition studies or rice mutants.

Unlike MMA, consistent uptake of DMA by *Xenopus* oocytes expressing plant NIPs was not observed (Figures 4.5; 4.6; data not shown). Expression of *OsLsi1* and *OsNIP3;3* did significantly increase DMA uptake statistically (Figure 4.5; 4.7); but the absolute differences compared to the water-injected controls were very small. Expression of *hAQP9* resulted in considerable uptake of DMA (Figures 4.6; 4.7) and validated the DMA uptake assay. In humans, DMA, and to a lesser extent MMA, are produced in the liver by activity of the As3MT enzyme (Thomas *et al.*, 2004). *hAQP9* is predominantly expressed in hepatocytes, where it is proposed to facilitate transport of both arsenite into cells, and DMA and MMA into the bloodstream for excretion (McDermott *et al.*, 2010). Therefore, hAQP9 may have evolved to transport methylated As to increase detoxification. This is in contrast to plants, which cannot methylate As (Lomax *et al.*, 2012). Uptake of DMA by rice roots is significantly slower than that of inorganic As and MMA (Raab *et al.*, 2007b), and so longer incubation may be necessary to observe DMA transport by *Xenopus* oocytes expressing rice NIPs.

Interestingly, NIPs from rice show less variation in the NPA motifs than those from Arabidopsis. Of the ten NIP aquaporins expressed by rice, nine of them possess two unmodified NPA motifs (Figure 4.1). The lack of B transport by OsNIP3;3 (Figure 4.9) is particularly interestingly as OsNIP3;3 has the same ar/R filter as the Arabidopsis boron transporter, AtNIP6;1 (Tanaka *et al.*, 2008; Table S4.1). However, AtNIP6;1 has a substitution in the second NPA motif (NPA-NPV) which may be required for boron transport (Figure 4.1). Furthermore, OsNIP3;1 has mutations in both motifs, NPS-NPV, and is proposed to mediate boron transport in rice (Hanaoka & Fujiwara, 2007).

The highly conserved NPA motifs may suggest that substrate specificity of rice NIP aquaporins is predominantly determined by the ar/R filter. OsNIP3;2 and OsNIP3;3 have similar ar/R filters, differing by just one residue; AAAR for OsNIP3;2, and AIAR for OsNIP3;3, but show very different transport profiles. Whereas OsNIP3;3 shows permeability to arsenite, MMA and silicon (Figures 4.3, 4.5; 4.8; 4.9; S4.1), OsNIP3;2 shows limited arsenite and MMA transport, and no silicon uptake (Figures 4.3; 4.8; 4.9;

S4.1). Additionally, OsNIP3;3 is more permeable to water than OsNIP3;2 (Figures 4.3; 4.9). However, substrate permeability of aquaporins may rely on residues other than those in the NPA and ar/R regions. Analysis of mutated forms of *E. coli* AqpZ by X-ray crystallography revealed that residues in loops C and E which do not line the pore or interact directly with substrates also contributed to selectivity (Savage *et al.*, 2010). Additionally, the extracellular and cytoplasmic regions of aquaporins may act as pre-selectivity filters (Sui *et al.*, 2001).

Oocytes expressing *OsLsi1*, *OsNIP3;3* and *hAQP9* all showed significantly higher Ge uptake than the water-injected control (Figure 4.7). Transport of Ge by OsNIP3;3 is surprising as that it was believed that Si transport was limited to members of subgroup III with the classic GSGR pore (Mitani *et al.*, 2008). Additionally, site-directed mutagenesis studies found that Si transport was lost when the ar/R filter of OsLsi1 was changed to GIGR (Mitani-Ueno *et al.*, 2011). The ar/R filter of OsNIP3;3 has an isoleucine residue in the H5 (second) position, which makes silicon transport seemingly more unlikely.

The lack of arsenite uptake by *HvPIP2;2*-injected oocytes (Figure 4.2), is interesting given the recent finding that *OsPIP2;4*, *OsPIP2;6* and *OsPIP2;7* are highly permeable to arsenite when expressed in oocytes (Mosa *et al.*, 2012). The authors speculated that lack of arsenite uptake by *OsPIP1;2* and *OsPIP1;3* was due to incompatibility between certain transporters and the *Xenopus* expression system. The requirement of PIP2-aquaporins for correct targeting of PIP1 isoforms to the plasma membrane has been demonstrated in various plant species (Fetter *et al.*, 2004; Mahdieh *et al.*, 2008; Vandeleur *et al.*, 2009; Ayadi *et al.*, 2011). *HvPIP2;2* shows high water-permeability when expressed in *Xenopus* oocytes (Besse *et al.*, 2011), and so correct targeting is implied. Therefore it may be interesting to compare PIPs from rice with those from other plant species, to see what factors determine arsenite permeability in PIP-aquaporins.

Expression of *hAQP9* in *Xenopus* oocytes resulted in considerably higher accumulation of DMA compared to those expressing *OsLsi1* (Figures 4.6; 4.7). Interestingly, expression of *hAQP9* gave similar levels of arsenite and MMA uptake compared to *OsLsi1* (Figures 4.6; S4.1). However, when the pH of the exposure medium was decreased from 6.0 to 4.5, MMA uptake by *hAQP9*-expressing oocytes was far greater than that for *OsLsi1* (Figures

4.6; S4.1). Additionally, hAQP9 appears to be more permeable to water than OsLsi1 (Figure 4.7). The reason for the differences may be the ar/R filters of the transporters; FACR for hAQP9, and GSGR for OsLsi1. The main difference is the bulky, aromatic phenylalanine residue in the pore of hAQP9, compared to the smaller glycine residues of OsLsi1. Members of NIP subgroup I have an aromatic tryptophan residue in the first position of the ar/R filter (H2), and have been shown to transport arsenite (see Table S4.1). OsNIP1;1 is permeable to MMA (Figures 4.3; 4.4) but to a lesser extent than hAQP9 (Figure S4.1).

An explanation other than substrate affinity could also account for higher MMA, DMA and water transport of hAQP9 compared to OsLsi1. hAQP9 is a human aquaporin and may be more highly expressed in *Xenopus* oocytes. One of the main differences between plant and animal genes is codon usage. For example, the codon GCG shows the biggest difference in frequency between *Xenopus laevis* (4.7‰) and *Oryza sativa* (26.6‰) as given in the Codon Usage Database (Nakamura *et al.*, 2000). Optimising the primary structure plant genes has been shown to increase expression of plant nitrate transporters and aquaporins in *Xenopus* oocytes (T. Miller, personal communication). Overall, the average difference in codon frequency between rice and *Xenopus* is 4.90‰, compared to 3.48‰ for human and *Xenopus* genes.

Another difference which may affect transporter activity in *Xenopus* oocytes is post-translational modification. Although the complete mechanism of aquaporin regulation is not currently understood, it has been demonstrated that plant aquaporins can be phosphorylated, methylated, acetylated, and glycosylated. Therefore differences between animal and plant protein modifications may mean that activities of transporters in *Xenopus* oocytes are different to those *in planta*. Furthermore, the intracellular and extracellular conditions such as pH and concentration of salts; particularly sodium chloride, are very different between *Xenopus* oocytes and plant cells (Miller & Zhou, 2000). The key histidine residue responsible for the pH-dependant gating of SoPIP2;1 (Törnroth-Horsefield *et al.*, 2006) is not conserved in NIPs, suggesting that either a different residue is protonated, or a different mode of regulation (Hove & Bhave, 2011). Finally, expression of plant genes has been found to modify the activity of endogenous

transporters in *Xenopus* oocytes, which can be misinterpreted as properties of the foreign protein (Miller & Zhou, 2000).

4.6 Conclusions

Permeability to arsenite and MMA was demonstrated for *Xenopus* oocytes expressing *OsNIP1;1*, and *OsNIP3;3*, as well as *OsLsi1*. Additionally, OsNIP1;1 and OsNIP3;3 were shown to transport water, and OsNIP3;3 is also permeable to germanium, an analogue of silicon. Finally, OsNIP1;1 and OsNIP3;2, as well as OsLsi1, demonstrated permeability to boric acid. Expression of *OsNIP3;2* resulted in permeability to arsenite, but varying levels of MMA transport.

Consistent, significant DMA uptake by *Xenopus* oocytes was not observed for any rice NIP, including *OsLsi1*. Transport of DMA by OsLsi1 is inferred by the finding that *lsi1* mutant rice roots accumulated 49% less DMA compared to wild-type (Li *et al.*, 2009a). The experimental methods were validated by the use of hAQP9 as a positive control for DMA and MMA uptake. Expression of *hAQP9* was also shown to increase uptake of germanium. The difficulties of using *Xenopus* oocytes to characterise plant transporters; such as differential codon usage, post-translational modifications and membrane-targeting, as well as variation between individual cells and batches of cells, may be the cause of inconsistent MMA uptake by OsNIP3;2, and possibly the lack of observed DMA transport.

Both OsNIP1;1 and OsNIP3;3 are expressed at low levels in rice roots, but are more highly expressed in older leaves. Root uptake of MMA is dominated by OsLsi1, demonstrated by the *lsi1* mutant, which accumulated 80% less MMA in the roots compared to wild-type (Li *et al.*, 2009a). Therefore, although OsNIP1;1 and OsNIP3;3 probably only play a minor role in MMA uptake by rice roots, they may be more important in translocation from roots to shoots, or to the grain.

Expression of the human aquaporin, *hAQP9*, conferred considerable transport of MMA and DMA compared to both control oocytes, and those expressing rice NIPs including OsLsi1. Interestingly, transport of arsenite was similar for hAQP9 and the rice aquaporins. Additionally, OsNIP3;2 and OsNIP3;3 displayed significant differences in transport ability for MMA, germanium and water, despite only a single residue difference in the selectivity region of the two transporters. Therefore, it may be possible to modify plant NIPs to

decrease transport of arsenite, whilst maintaining transport ability for essential substrates.

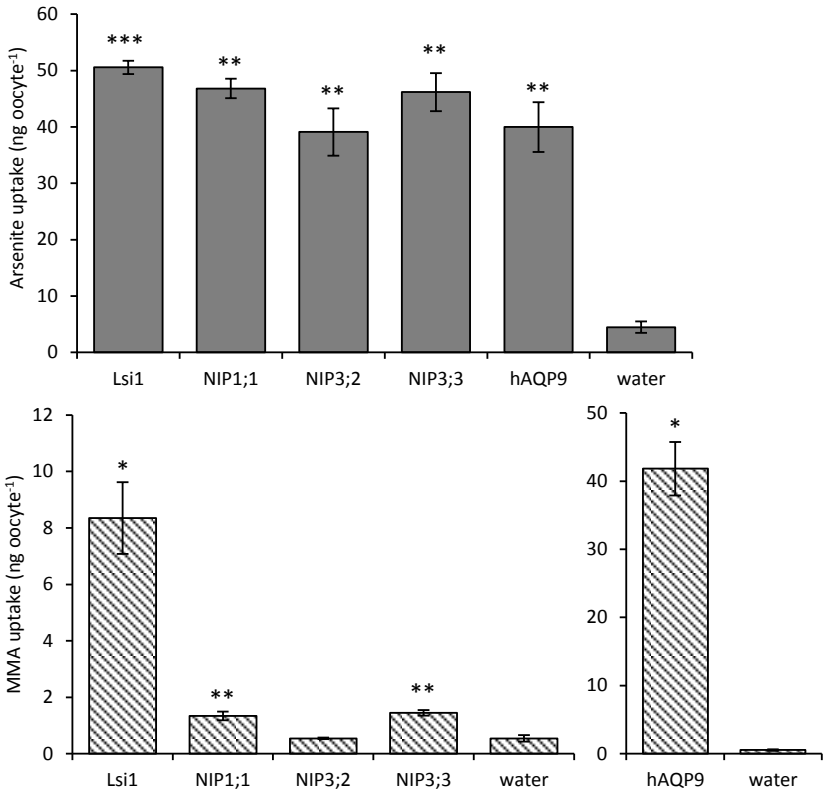
4.7 Supplementary information

Supplementary Table 4.1 Summary of characterisation of NIPs from Arabidopsis (At-), courgette (Cp-), *Equisetum arvense* (Ea-), soybean (Gm-), hydrangea (Hm-), barley (Hv-), *Lotus japonicas* (Lj-), rice (Os-), pea (Ps-), *Pinus taeda* (Pt-), wheat (Ta-) and maize (Zm-). The experimental methods are given after the specific reference and are: heterologous expression in *Xenopus laevis* oocytes ([1]), yeast ([2]) or Arabidopsis ([3]); knock-out or RNAi mutants ([4]), and ([5]) sub-cellular localisation.

Group	Name	ar/R	H_2O	As	Si	B	Sb	urea	glycerol	other	References
I	AtNIP1;1	WVAR		✓			✓		✓		Weig and Jakob (2000)[2]; Kamiya *et al.* (2009)[1,4,5]; Kamiya and Fujiwara (2009)[2,4]
I	AtNIP1;2	WVAR		✓			×		✓	H_2O_2	Weig and Jakob (2000)[2]; Dynowski *et al.* (2008)[2]; Kamiya *et al.* (2009)[1,4]; Kamiya and Fujiwara (2009)[4]
I	AtNIP2;1	WVAR	×			×	×	×	×	Lactic acid	Mizutani *et al.* (2006)[2,5]; Choi and Roberts (2007)[1,5]; Bienert *et al.* (2008)[2]
II	AtNIP5;1	AIGR	✓	✓	×	✓	✓				Takano *et al.* (2006)[1,4,5]; Bienert *et al.* (2008)[2]; Kamiya *et al.* (2009)[1,4]; Kamiya and Fujiwara (2009)[4]; Mitani-Ueno *et al.* (2011)[1]
II	AtNIP6;1	AIAR	×	✓		✓	✓	✓	✓	Formamide	Wallace and Roberts (2005)[2]; Bienert *et al.* (2008)[2]; Tanaka *et al.* (2008)[1,4,5]
II	AtNIP7;1	AVGR	×	✓		✓	✓	✓	✓		Bienert *et al.* (2008)[2]; Isayenkov and Maathuis (2008)[2,4]; Li *et al.* (2011b)[1]
III	CpNIP1	GSGR	✓					✓	×		Klebl *et al.* (2003)[2]
II	EaNIP3;1	STAR			✓						Grégoire *et al.* (2012)[1,3]
II	EaNIP3;3	STAR			✓						Grégoire *et al.* (2012)[1,3]
II	EaNIP3;4	STAR			✓						Grégoire *et al.* (2012)[1,3]
I	GmNod26	WVAR	✓					×	✓	Ammonia, Formamide	Rivers *et al.* (1997)[1]; Dean *et al.* (1998)[1]; Wallace *et al.* (2002)[1]; Hwang *et al.* (2010)*
II	HmPALT1	TIAR								Al	Negishi *et al.* (2012)[2,3,5]

Group	Name	ar/R	H_2O	As	Si	B	Sb	urea	glycerol	other	References
III	HvNIP2;1	GSGR	✓	✓	✓	✓					Chiba *et al.* (2009)[1,5]; Schnurbusch *et al.* (2010)[1,2,4];
II	LjNIP5;1	AIGR		✓			✓				Bienert *et al.* (2008)[2]
II	LjNIP6;1	TIAR		✓			✓				Bienert *et al.* (2008)[2]
I	OsNIP1;1	WVAR		✓							Bienert *et al.* (2008)[2]; Ma *et al.* (2008)[1]
III	OsNIP2;1	GSGR	✓	✓	✓	✓	✓	✓	×	Se, MMA, DMA	Ma *et al.* (2006)[1,4,5]; Bienert *et al.* (2008)[2]; Ma *et al.* (2008)[1]; Mitani *et al.* (2008)[1]; Li *et al.* (2009a)[1,4]; Schnurbusch *et al.* (2010)[2,4]; Zhao *et al.* (2010d)[2,4]; Mitani-Ueno *et al.* (2011)[1]; Montpetit *et al.* (2012)[1,3]
III	OsNIP2;2	GSGR		✓	✓		✓				Bienert *et al.* (2008)[2]; Ma *et al.* (2008)[1]; Mitani *et al.* (2008)[1]; Yamaji *et al.* (2008)[4,5]; Yamaji and Ma (2009)[4]
II	OsNIP3;1	AIGR		✓							Ma *et al.* (2008)[1]; Hanaoka and Fujiwara (2007)[5]
II	OsNIP3;2	AAAR		✓			✓				Bienert *et al.* (2008)[2]
I	PsNIP-1	WVAR	✓						✓		Schuurmans *et al.* (2003)[1]
I	PtNIP1;1	WVAR	✓						✓		Ciavatta *et al.* (2001)[1,2]
III	TaLsi1	GSGR			✓						Montpetit *et al.* (2012)[1,3,5]
III	ZmNIP2;1	GSGR			✓			✓			Mitani *et al.* (2009)[1,5]; Gu *et al.* (2012)[2]
III	ZmNIP2;2	GSGR			✓			✓			Mitani *et al.* (2009)[1,5]
III	ZmNIP2;4	GSGR			✓			✓			Gu *et al.* (2012)[2]

*Recombinant GmNOD26 was characterised in isolated proteoliposomes of *Pichia pastoris* by Hwang *et al.* (2010).



Supplementary Figure 4.1 Arsenite and MMA uptake of *Xenopus* oocytes expressing *OsLsi1, OsNIP1;1*, *OsNIP3;2*, *OsNIP3;3* and *hAQP9*. Oocytes were exposed to MBS containing 1 mM arsenite at pH 7.4, or 1 mM MMA at pH 4.5, for 60 minutes. After exposure oocytes were rinsed thoroughly and split into replicates of 3-7 oocytes for analysis. Error bars represent SE (n = 2-4). * = $P < 0.05$, ** = $P < 0.01$, *** = $P < 0.001$ significant difference from control (water) of Student's *t*-test.

5. Transport of methylated arsenic: Phosphate transporters

NIP aquaporins are permeable to the undissociated (uncharged) forms of MMA and DMA. However due to their pK_a values, dissociated (charged) forms of MMA and DMA will exist in the physiological pH range of around 4.0 to 8.5 (Smith & Raven, 1979; Figure S1.2). When studying undissociated MMA and DMA uptake via OsNIP2;1 (Lsi1), Li *et al.* (2009a) observed that increasing the pH of the medium decreased As uptake (by decreasing the proportion of undissociated complexes available for uptake). However, the amount of MMA taken up by rice roots at pH 5.5 and 6.5 was higher than predicted by the proportion of undissociated complexes. Additionally the rice *lsi1* mutant retained 50% of root DMA uptake (Li *et al.*, 2009a). Therefore, a transport pathway for dissociated methylated As species may exist.

5.1 Literature review

Phosphate transporters (PTs) are well known to transport inorganic arsenate in plants (see **Section 1.8.1**) which, like inorganic phosphate (Pi), exists almost entirely as charged forms at physiological pH ($H_2AsO_4^-$ or $HAsO_4^{2-}$). Due to the similarity of arsenate and the dissociated forms of MMA and DMA, phosphate transporters may also be able to facilitate the transport of MMA and DMA at higher pH levels. Supportive evidence for this hypothesis comes from the finding that phosphate starvation increased DMA uptake by maize seedlings by ten-fold, compared to only a two-fold increase in arsenate uptake (Abbas & Meharg, 2008).

5.1.1 Phosphate transport

Phosphorus is an essential plant nutrient as a component of ATP (adenosine triphosphate), nucleic acids and phospholipids, and is also involved in regulating enzyme function and signal transduction. In soils, phosphate can exist in several different forms, but is predominantly taken up by plants in the form of orthophosphate, Pi (Ullrich-Eberius *et al.*, 1981; Tu *et al.*, 1990). Although the total concentration of P in soils can be relatively high, the average concentration of orthophosphate in the soil solution is around 1 µM,

and seldom exceeds 10 µM (Bieleski, 1973). The concentration of P within plant cells is estimated to be in the millimolar range, and so phosphate uptake by roots is strongly against the concentration gradient.

The Arabidopsis genome contains at least five distinct classes of membrane proteins displaying phosphate transport activity. Four of the families are named Pht1, Pht2, Pht3 and Pht4 whereas the fifth is called the plastidic phosphate translocator group (pPT; Muchhal *et al.*, 1996; Daram *et al.*, 1999; Takabatake *et al.*, 1999; Knappe *et al.*, 2003; Guo *et al.*, 2008). It is thought that only members of the Pht1 family facilitate uptake of phosphate from the external medium. Members of the other four families are implicated in phosphate transport across internal membranes.

Pht1 members are thought to be high-affinity phosphate transporters, and belong to the phosphate-proton symporter (PHS) family; part of the major facilitator superfamily (MFS). Pht1 transporters (PTs) are predicted to have 12 transmembrane (TM) domains, with both the N- and C-terminal domains in the cytoplasm. A large hydrophilic, cytoplasmic loop connects TM domains 5 and 6, giving the characteristic '6+6' structure of MFS members (Marger & Saier, 1993). Electrophysiological studies have shown that PTs are secondary active transporters, coupling the transport of orthophosphate to the proton gradient across the plasma membrane (Ullrich-Eberius *et al.*, 1981). It has been estimated that four protons are co-transported with each phosphate anion (Sakano, 1990).

Interestingly, when expressed in oocytes, the low-affinity barley transporter, *HvPht1;6* was able to transport sulphate, nitrate and chloride in addition to phosphate (Preuss *et al.*, 2010). However oocytes expressing *HvPht1;1* showed no response to external nitrate or sulphate using two-electrode voltage clamp, whereas external phosphate and arsenate induced significant inward currents (Preuss *et al.*, 2011). Additionally, nitrate and sulphate did not compete with ^{32}Pi for uptake by *LePT1*-expressing yeast (Daram *et al.*, 1998), and the presence of chloride, sulphate or nitrate also had no effect on Pi uptake of yeast expressing *AtPht2;1* (Daram *et al.*, 1999; Versaw & Harrison, 2002). Therefore the affinity of PTs to anions other than phosphate may show variation between individual transporters.

5.1.2 Rice phosphate transporters

Based on homology with the yeast phosphate transporter Pho84, the rice genome contains 13 putative transporters belonging to the Pht1 high-affinity phosphate transporter family named OsPT1-13 (Paszkowski *et al.*, 2002). So far six of the 13 genes have been characterised.

OsPT11 and OsPT13 are involved in acquiring phosphate from arbuscular myccorhizal (AM) fungi (Paszkowski et al., 2002; Glassop et al., 2007). Expression of *OsPT11* is induced exclusively upon colonisation by AM fungi. OsPT11 was able to complement the yeast *pho84* mutant under low phosphate supply and so is putatively characterised as a high-affinity transporter (Paszkowski *et al.*, 2002). Unlike *OsPT11*, *OsPT13* is expressed in non-myccorhizal roots but is significantly upregulated by AM fungal colonisation. This suggests a dual role of OsPT13 in phosphate transport in non-myccorhizal roots and acquisition of phosphate from AM fungi (Glassop *et al.*, 2007).

Both *OsPT2* and *OsPT6* are expressed mainly in leaves under normal conditions, but expression in roots is strongly induced under P-deficient conditions (Ai et al., 2009). Expression of both *OsPT2* and *OsPT6* in roots was found to be strongly down-regulated under colonisation with the AM fungus *G. intraradices* (Paszkowski *et al.*, 2002). Expression of *OsPT6* was able to restore growth of yeast strain MB192, which lacks endogenous high-affinity PTs, under low phosphate conditions. Analysis of ^{33}Pi uptake in the transformed yeast gave an apparent K_m of 97 µM Pi for OsPT6. However, expression of *OsPT2* did not functionally complement the mutant yeast, and was instead identified as a low-affinity transporter, with a putative role in internal phosphate transport and translocation (Ai et al., 2009).

OsPT8 was found to be expressed independently of phosphate supply in various tissues including root tips and lateral roots, leaves, stamen and grain. Analysis of ^{32}Pi uptake in *Xenopus* oocytes and yeast strain MB192 expressing *OsPT8* had apparent K_m values of 27 and 23 µM Pi respectively (Jia *et al.*, 2011). RNAi and T-DNA insertion lines of *OsPT8* had significantly decreased seed setting rate and increased phosphate concentrations in the panicle axis, demonstrating a critical role of OsPT8 in transporting phosphate to the developing grains (Jia et al., 2011). Overexpression of *OsPT8* causes excessive

accumulation of P, especially in the shoots, with P toxicity symptoms present in leaves when grown under high phosphate supply (Jia *et al.*, 2011; Wu *et al.*, 2011).

Finally, *OsPT1* expression was detected in various cells of both rice roots and shoots, and was unaffected by phosphate supply (Sun *et al.*, 2012). Expression of *OsPT1* in the yeast *pho84* mutant complemented growth at low phosphate levels, and uptake of ^{33}Pi of the transformants gave an apparent K_m of 177 µM Pi. Under sufficient phosphate supply, shoots of overexpression and RNAi lines contained significantly more and less P than wild-type respectively (Sun *et al.*, 2012).

5.1.3 Regulators of PTs: OsPHR2

In Arabidopsis the MYB transcription factor, AtPHR1 (phosphate starvation response1), plays a key role in phosphate-starvation signalling. AtPHR1 binds to the imperfect palindromic sequence of GNATATNC, also known as the PHR1 binding sequence (P1BS), which is found in the upstream region of all phosphate starvation-induced genes, apart from *AtPHR1* itself (Rubio *et al.*, 2001).

Two homologous genes were found in rice, designated *OsPHR1* and *OsPHR2*, however only OsPHR2 appears to be a functional homologue of AtPHR1 (Zhou *et al.*, 2008). Analysis of the promoter region of phosphate starvation induced (PSI) genes and yeast one-hybrid assays suggested that another P1BS-like motif is required for OsPHR2 binding. Of the 13 rice PTs only *OsPT2*, *OsPT3*, *OsPT7*, *OsPT10* and *OsPT11* have both P1BS and P1BS-like motifs in their promoter (Wu *et al.*, 2013). The remaining PSI PTs may be under the regulation of other members of the phosphate signalling pathway, including OsPHO2 which is negatively regulated by OsPHR2 via the mature microRNA, miR399 (Bari *et al.*, 2006). OsSPX1 is also downstream of OsPHR2, and was found to regulate the expression of PSIs including *OsPT2*, *OsPT3*, *OsPT6*, and *OsPT8* (Wang *et al.*, 2009).

Overexpression of *OsPHR2* under phosphate-sufficient conditions leads to excessive accumulation of phosphate in root and shoots; enhanced root elongation and root hair growth; and up-regulation of phosphate transporters including *OsPT9* in root, and *OsPT1*, *OsPT5*, *OsPT7*, *OsPT9*, and *OsPT12* in shoot (Zhou *et al.*, 2008). The accumulation of excess phosphate in *OsPHR2*-overexpression (*PHR2-ov*) lines is mainly attributed to the up-regulation of *OsPT2*, as shoot phosphate concentrations were reduced by 70% in

PHR2-ov/*pt2* double mutants compared to *PHR2-ov* (Liu *et al.*, 2010a). Expression of *OsPT8* in roots and shoots is also enhanced in PHR2-ov (Wu *et al.*, 2011).

5.1.4 Regulators of PTs: OsPHF1

OsPHR2 regulates the transcription of PTs, but their activity can also be regulated post-translationally. In Arabidopsis, mutations in *AtPHF1* (*phosphate transporter traffic facilitator1*) resulted in reduced phosphate uptake and increased arsenate tolerance (Gonzalez *et al.*, 2005). AtPHF1 is localised to the endoplasmic reticulum (ER) and is required for correct trafficking of AtPht1;1 (and possibly other members of the Pht1 family) to the plasma membrane (Bayle *et al.*, 2011).

Two homologous genes were found in rice, designated *OsPHF1* and *OsPHF1L*, however only OsPHF1 seems to be a functional homologue of AtPHF1 (Chen *et al.*, 2011). Screening of an EMS (ethylmethanesulphonate)-generated mutant library for arsenate tolerance and low P accumulation identified three independent *osphf1* mutants. Additionally, overexpression of *OsPHF1* resulted in excessive accumulation of P in both roots and shoots, causing growth inhibition and phosphate toxicity symptoms in leaves. OsPHF1 was also localised to the ER, and regulated proper trafficking of OsPT2 and OsPT8 to the plasma membrane. Interestingly, despite significant upregulation under phosphate limiting conditions, *OsPHF1* expression was found not to be under the regulation of OsPHR2 (Chen *et al.*, 2011).

5.2 Aims

Undissociated MMA was shown to be substrate of NIP aquaporins in **Chapter 4**, and previous studies (Li *et al.*, 2009a). However DMA transport by NIPs was very limited. Additionally the low pK_{a1} of MMA and DMA means dissociated MMA and DMA complexes will be present in the physiological pH range. Arsenate is transported by phosphate transporters, and so may also be able to transport charged methylated As species. Therefore the aim of the experiments within this chapter is to determine whether phosphate transporters can transport dissociated MMA and DMA in rice.

5.3 Materials and methods

5.3.1 Hydroponic culture of rice phosphate transport mutants

Three transgenic lines were grown in hydroponic culture: PT8-ov; an overexpression line of *OsPT8* driven by the cauliflower mosaic virus (CaMV) 35S promoter, PHR2-ov; an overexpression line of *OsPHR2* also driven by the CaMV 35S promoter, and *phf1*; a mutation in *OsPHF1* isolated from an EMS-mutagenised population of PHR2-ov seeds. Wild-type for PT8-ov and PHR2-ov is Nipponbare, and for *phf1* is PHR2-ov. These lines were a generously provided by Prof P. Wu (Zhejiang University, China) and have been characterised previously for phosphate and arsenate transport (Zhou *et al.*, 2008; Wu *et al.*, 2011).

Rice seed were surface sterilised with 0.5% (v/v) active NaClO for 15 mins, rinsed and soaked in de-ionised water overnight, and placed on a nylon net floating on a 0.5 mM $CaCl_2$ solution. After one week the solution was replaced with modified half-strength Kimura nutrient solution with decreased phosphate concentration (0.091 mM KNO_3, 0.183 mM $Ca(NO_3)_2$, 0.274 mM $MgSO_4$, 0.183 mM $(NH_4)_2SO_4$, 10 µM KH_2PO_4, 1.0 µM $MnCl_2$, 3.0 µM H_3BO_3, 0.1 µM $(NH_4)_6Mo_7O_{24}$, 0.4 µM $ZnSO_4$, 0.2 µM $CuSO_4$, 80 µM NaFe(III)-EDTA; pH 5.5). After a further week individual seedlings were transferred to 300 mL Magenta™ boxes (Sigma) filled with half-strength Kimura solution (as above but 0.091 mM KH_2PO_4). The nutrient solution was renewed twice weekly. The growth conditions were 16 hour photoperiod with a light intensity of more than 350 µmol m^{-2} s^{-1}, day/night temperatures of 28°C/25°C, and relative humidity of 70%. To minimise changes in pH during the exposure period, As-amended nutrient solution lacked ammonium sulphate and was buffered with 5 mM MES or Hepes for pH 5.5 or 7.0 respectively.

After As exposure (exact conditions are given in figure legends) aliquots of nutrient solution were collected to monitor changes in pH during the uptake period. Roots and shoots were separated and rinsed with de-ionised water. Apoplastic As was removed from roots by submerging in desorption solution (1 mM K_2HPO_4, 0.5 mM $Ca(NO_3)_2$, 5 mM MES, pH 6.0; Xu *et al.*, 2007) for 15 minutes.

Shoots and roots were oven-dried at 60°C overnight before weighing into 25 mL Pyrex digestion tubes. For digestion, 5 mL HNO_3/$HClO_4$ (85/15 v/v) acid was added to each

sample. Pre-digestion at room temperature lasted for between 12-48 hours depending on sample weight. The heating program: 3 hours at 60°C, 1 hour at 100°C, 1 hour at 120°C and finally 2 hours at 175°C, was run overnight in an acid-scrubbed fume cupboard. The next day 5 mL of 20% (v/v) HNO_3 was added to each sample and heated at 80°C for 30 minutes. Approximately 10 mL of water was then added, and reheated at 80°C for a further 30 minutes. Samples were allowed to cool completely before making up to 20 mL with water, giving a final concentration of 5% (v/v) HNO_3. Blanks and the certified reference materials BCR-414 (plankton; As concentration 6.82 ± 0.28 mg kg^{-1}) or NIST 1568a (rice flour; As concentration 0.29 ± 0.03 mg kg^{-1}) were included in all batches for quality assurance.

Total As concentration in samples was determined by ICP-MS (see **Section 2.1**), and other elements including P were analysed by ICP-OES.

5.3.2 Analysis of total concentration of elements by ICP-OES

Analysis of the total concentration of P and As in digested samples was carried out by ICP-OES (PerkinElmer Optima 7300 DV; Waltham, MA, USA) by the Rothamsted Research Analytical Unit.

5.3.3 Oocyte transport assays

The construct for expressing *OsPT8* in oocytes (pT7TS backbone) was a gift from Dr X. Fan (Nanjing Agricultural University, China). cRNA was produced and injected into oocytes as described in **Section 4.3.1**. BamHI was used for plasmid linearization, and the T7 mMessage mMachine kit (Ambion) was used to synthesise the cRNA.

To measure As uptake, oocytes were incubated in MBS (see **Section 4.3.1**) amended with 1 mM arsenate, MMA or DMA at pH 7.4 for 60 minutes. After exposure oocytes were rinsed at least 6 times with clean MBS and between 5 and 11 oocytes, forming one replicate, were placed in centrifuge tubes. Pooled oocytes were then digested with 0.4 mL HNO_3 heated in a water bath at 90°C for 45 minutes. Cooled samples were diluted to a final volume of 7 mL with ultrapure water and analysed for total As concentration by ICP-MS (see **Section 2.1.2**).

Phosphate uptake was measured by the method given in Ai *et al.* (2009), modified from Vanveldhoven and Mannaerts (1987). Briefly, oocytes were incubated in MBS amended with 0.5 mM phosphate (NaH_2PO_4) for 14 hours. After exposure oocytes were rinsed thoroughly with ice-cold MBS, and single oocytes were placed in centrifuge tubes. 250 µL of ultrapure water was added to each sample and oocytes were lysed in a sonicating bath at 100% ultrasound power for around 30 seconds (Fisherbrand FB11002; Thermo Fisher Scientific Inc., Waltham, MA, USA). 25 µL aliquots of the lysed oocyte samples were placed in the wells of a 96-well plate and diluted to a final volume of 250 µL. 50 µL of 1.75% $(NH_4)_6Mo_7O_{24}{\cdot}4H_2O$ in 6.3 N H_2SO_4 was added to each well. After incubating for 10 minutes, 50 µL of 0.035% malachite green in 0.35% polyvinyl alcohol was added to each well. Samples were incubated at room temperature for a further 30 minutes before absorbance was measured at 610 nm (Thermo Varioskan; Thermo Fisher Scientific Inc., Waltham, MA, USA). Standards containing a range of phosphate concentrations were included in duplicate in each plate, and were used to construct calibration curves to measure the phosphate concentration of oocyte samples.

5.4 Results

5.4.1 Arsenic uptake by a rice *PT8*-overexpression line

OsPT8 is a high-affinity phosphate transporter, and previous studies have found that when overexpressed, rice plants accumulate increased phosphorus especially in the shoots (Jia *et al.*, 2011; Wu *et al.*, 2011). To investigate the role of OsPT8 in the uptake of dissociated methylated As, hydroponically-grown wild-type (Nipponbare) and *OsPT8* overexpressing (PT8-ov) rice plants were exposed to MMA or DMA in the nutrient solution, in the absence (-P) or presence (+P; 0.091 mM) of phosphate, and at pH 5.5 or 7.0 for 48 hours. At pH 5.5, 95.3% of MMA and 18.6% of DMA complexes are dissociated. At pH 7.0 the proportion increases to 99.8% for MMA and 87.9% for DMA.

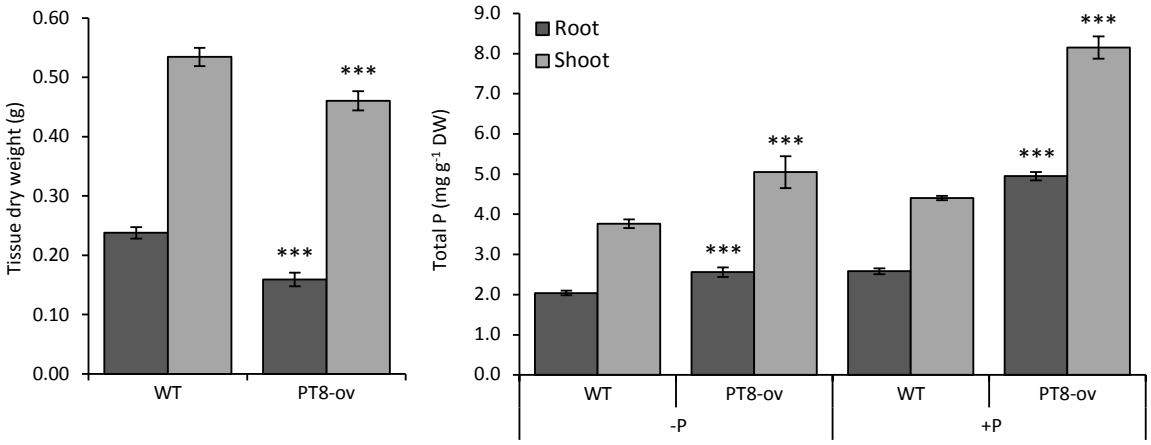


Figure 5.1 Mean dry weights and total P concentration of hydroponically-grown rice plants. WT = Nipponbare, PT8-ov = *OsPht1;8* over-expression line. Plants were grown in half-strength Kimura nutrient solution for 34 days before treatment in the absence (-P) or presence (+P) of phosphate. Error bars represent SE (n = 32 for DW; n = 16 for P). *** = $P < 0.001$ significant difference from wild-type (ANOVA).

Both root and shoot dry weights of the PT8-ov line were significantly decreased compared to wild-type ($P < 0.001$; Figure 5.1). The other treatments (pH, As species and ± P) had no effect on dry weight and so the mean of all treatments is presented. Additionally, roots and shoots of PT8-ov contained significantly higher concentrations of P than wild-type, especially in the +P treatment ($P < 0.001$; Figure 5.1). Both pH and As treatments had no effect on the P concentration of plants. Jia *et al.* (2011) found that when grown under both high (300 µM) and low phosphate (15 µM) conditions, PT8-ov plants accumulated significantly more P in their shoots than wild-type. Furthermore, under normal phosphate supply, root and shoot biomass of PT8-ov was significantly decreased compared to wild-

type, presumably due to phosphate toxicity. Tips of older leaves of the PT8-ov line, in particular, presented areas of chlorosis and necrosis (see Figure S5.1).

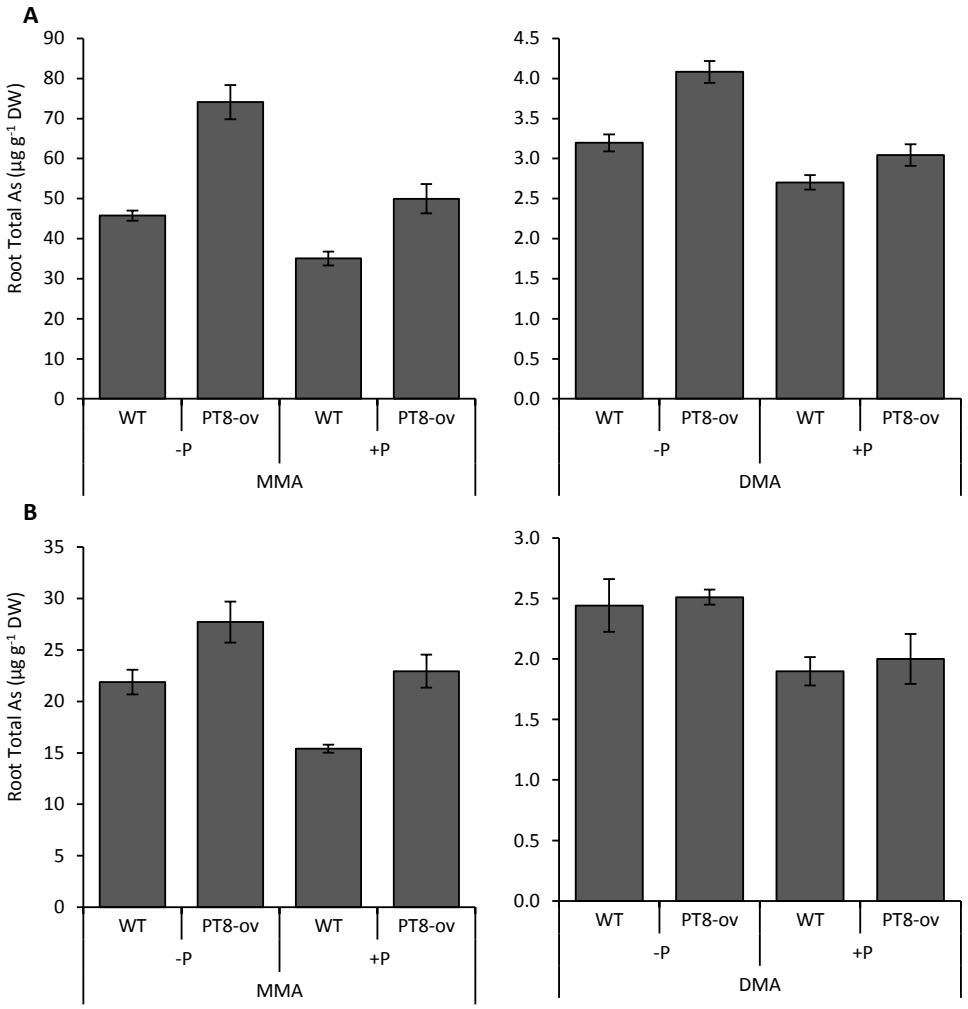


Figure 5.2 Mean root As concentration of hydroponically-grown rice plants exposed to 5 µM MMA or DMA, with (+P) or without (-P) phosphate, at **A**, pH 5.5 or **B**, 7.0 for 48 hours. WT = Nipponbare, PT8-ov = *OsPht1;8* over-expression line. Plants were grown in half-strength Kimura nutrient solution for 34 days before As treatment. Error bars represent SE (*n* = 4).

Total As concentrations in roots (Figure 5.2) were analysed by 4-way ANOVA (see Tables S5.1; S5.2 for full output). The treatment with the greatest overall effect was phosphate ($P < 0.001$), which did not interact significantly with any of the other factors. Three two-way interactions were also significant; As x pH ($P < 0.001$), As x genotype ($P < 0.001$) and genotype x pH ($P = 0.04$).

Root As concentrations were significantly lower in the +P treatment compared to the -P treatment, regardless of the pH or As species in the exposure medium, or the genotype.

Root MMA uptake was significantly higher than DMA uptake under both pH treatments, but the difference was significantly greater at pH 5.5 than pH 7.0. This effect was independent of P treatment and genotype.

The PT8-ov line had significantly higher root As concentrations compared to wild-type after exposure to either As species, although the difference between the genotypes was greater under MMA treatment than DMA treatment.

Finally, root As concentrations of the PT8-ov line were significantly higher than wild-type, irrespective of P treatment or As species, but the difference between the genotypes was significantly greater at pH 5.5 than at pH 7.0.

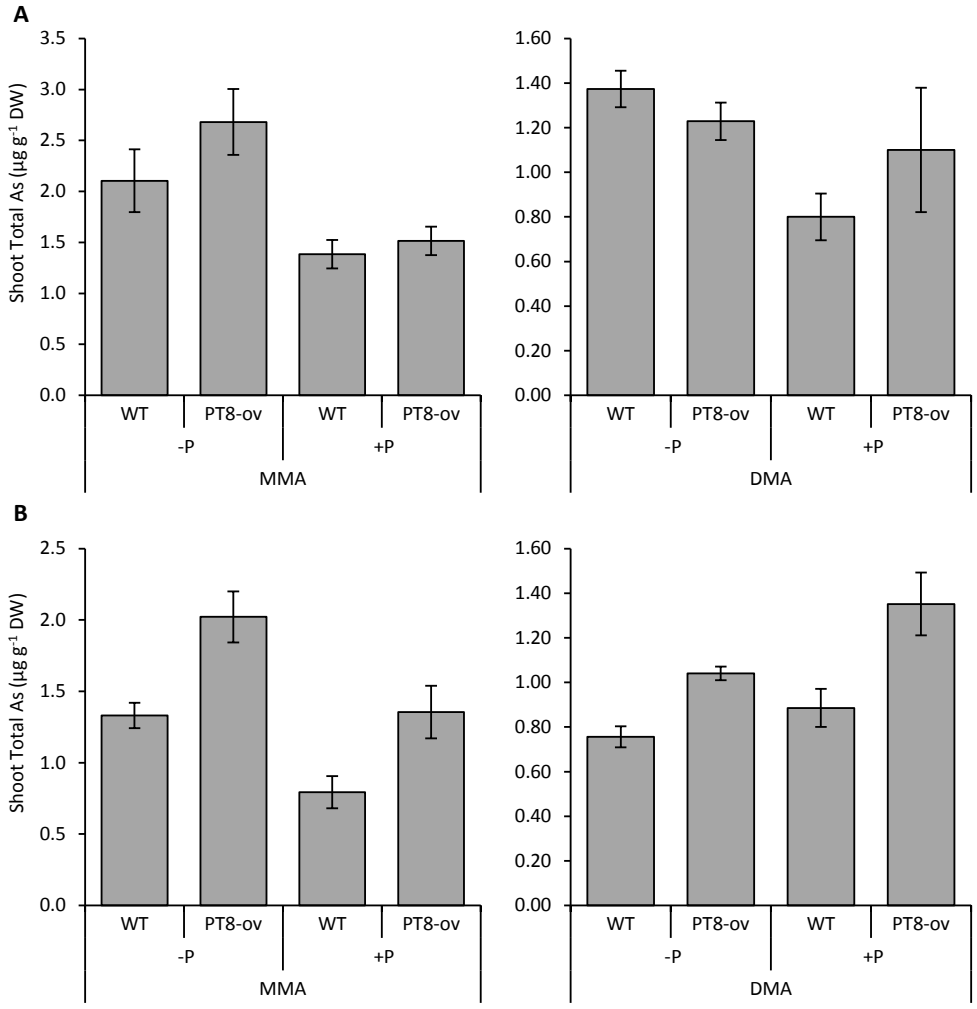


Figure 5.3 Mean shoot As concentration of hydroponically-grown rice plants exposed to 5 µM MMA or DMA, with (+P) or without (-P) phosphate, at **A**, pH 5.5 or **B**, 7.0 for 48 hours. WT = Nipponbare, PT8-ov = *OsPht1;8* over-expression line. Plants were grown in half-strength Kimura nutrient solution for 34 days before As treatment. Error bars represent SE (*n* = 4).

Total As concentrations in shoots (Figure 5.3) were also analysed by 4-way ANOVA (see Tables S5.3; S5.4 for full output) and show some interesting differences from the root data. The treatment with the greatest overall effect on shoot As, was genotype ($P < 0.001$), which did not interact significantly with any of the other factors. Two two-way interactions were also significant; As x P ($P < 0.001$), and P x pH ($P = 0.029$).

Shoots of the PT8-ov line had significantly higher As concentrations than wild-type, regardless of the pH, presence of phosphate, or As species in the exposure medium.

Exposure to MMA in combination with -P treatment, resulted in significantly higher shoot As concentrations than exposure to DMA with both P treatments, or MMA under +P treatment.

Finally, shoot As concentration was significantly higher in plants exposed to medium lacking phosphate at pH 5.5, compared to those under +P treatment, or at pH 7.0 with either P treatment.

5.4.2 Heterologous expression of *OsPT8* in *Xenopus* oocytes

Expression of *OsPT8* in *Xenopus* oocytes allows uptake of dissociated MMA and DMA to be measured directly. Arsenate was included as a positive control.

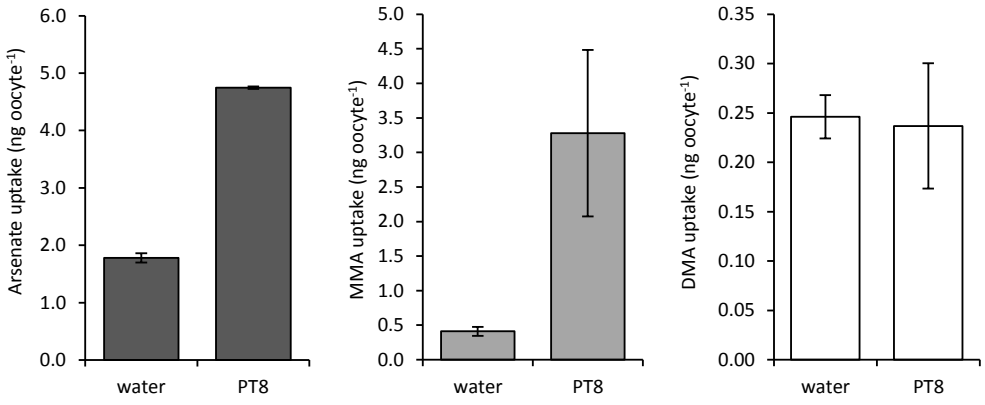


Figure 5.4 Mean As content of oocytes expressing *OsPT8*. Oocytes were exposed to MBS containing 1 mM arsenate, MMA or DMA at pH 7.4 for 60 minutes. After exposure oocytes were rinsed thoroughly and split into replicates of 5-11 oocytes for analysis. Error bars represent SE (n = 2-3).

Oocytes expressing *OsPT8* showed considerably enhanced uptake of arsenate and MMA compared to the water-injected controls (Figure 5.4). However, after exposure to DMA

there was no difference in As content of oocytes injected with *OsPT8* or water. An attempt was made to repeat this experiment, and measure phosphate uptake, in another batch of oocytes. However for an unknown reason, the cRNA was not expressed and no difference was seen between the OsPT8 and water-injected oocytes under any treatment (data not shown).

5.4.3 Arsenic uptake by rice *PHR2*-overexpression and *phf1* mutants

To further investigate the contribution of the phosphate transport pathway to the uptake and translocation of dissociated pentavalent MMA and DMA, two transgenic rice lines; PHR2-ov, *phf1*, and wild-type (Nipponbare) were grown in hydroponic culture and exposed to arsenate, MMA or DMA in medium lacking phosphate, at pH 5.5 (where 95.3% of MMA complexes and 18.6% DMA complexes are dissociated) for 24 hours.

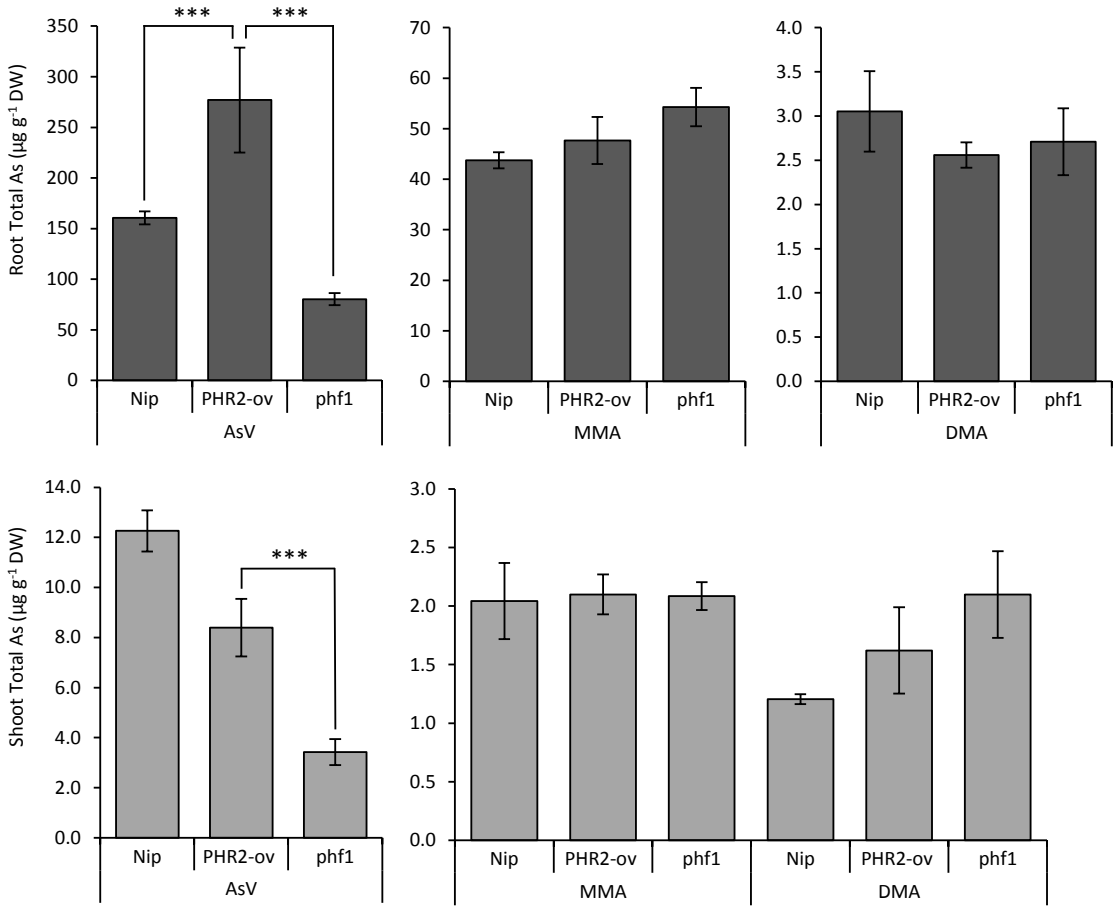


Figure 5.5 Mean As concentration of hydroponically-grown rice plants exposed to 5 µM arsenate (AsV) MMA or DMA, without phosphate, at pH 5.5 for 24 hours. Nip = Nipponbare; PHR2-ov = *OsPHR2* over-expression line in a Nipponbare background; and phf1 = o*sphf1* mutant in the PHR2-ov background. Plants were grown in half-strength Kimura nutrient solution for 43 days before As treatment. Error bars represent SE (n = 3-4). *** = $P < 0.001$ significant difference in flagged comparisons with background (ANOVA).

When previously characterised, PHR2-ov was found to accumulate excessive P in the shoots (Zhou *et al.*, 2008), whereas *phf1* showed decreased root and shoot ^{33}P uptake compared to PHR2-ov (Wu *et al.*, 2011). Consistent with these reports, at the end of the growth period, PHR2-ov shoot P concentrations were significantly higher than Nipponbare, and *phf1* shoot P concentrations were significantly lower than PHR2-ov (Figure S5.2). Additionally, root dry weight was significantly decreased in both PHR2-ov compared to wild-type, and *phf1* compared to PHR2-ov. Shoot dry weight of *phf1* was also significantly decreased compared to PHR2-ov.

Exposure to arsenate gave the highest root As concentrations, followed by MMA, with DMA treatment resulting in the lowest root As (Figure 5.5). Shoots followed a similar pattern, but the difference between MMA and DMA is much smaller. Irrespective of genotype, arsenate and MMA have similar average TFs (translocation factors) of 0.05 and 0.04 respectively, whereas that of DMA is considerably higher at 0.61.

No significant differences were found in As concentration between Nipponbare and PHR2-ov, or PHR2-ov and *phf1* in roots or shoots exposed to MMA or DMA. Root As was significantly higher in the PHR2-ov line compared to background (Nipponbare), and was significantly lower in the *phf1* mutant compared to background (PHR2-ov), under arsenate treatment. However in shoots under arsenate treatment, only the difference between *phf1* and PHR2-ov was significant.

The experiment was also conducted with the pH of the exposure medium at 7.0, whereby 99.8% of MMA complexes and 87.9% of DMA complexes are dissociated.

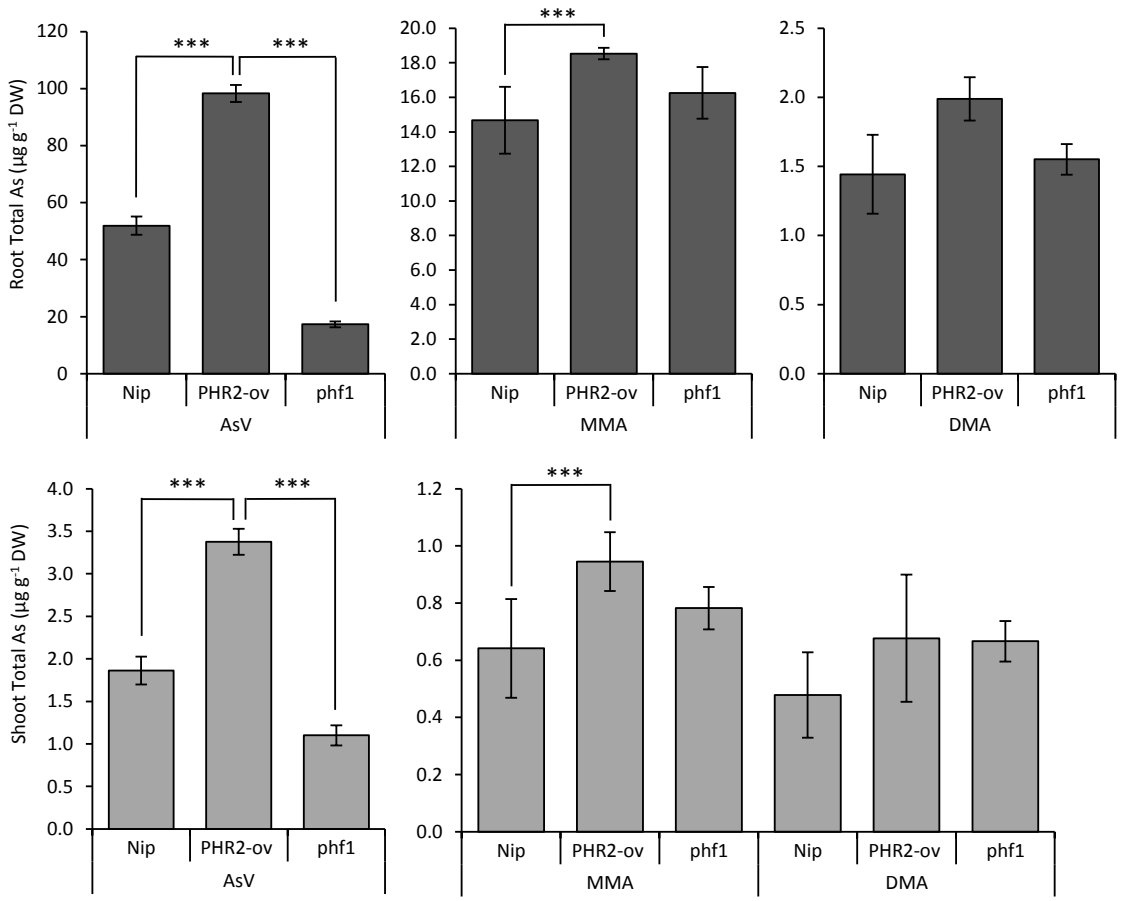


Figure 5.6 Mean As concentration of hydroponically-grown rice plants exposed to 5 µM arsenate (AsV) MMA or DMA, without phosphate, at pH 7.0 for 24 hours. Nip = Nipponbare; PHR2-ov = *OsPHR2* over-expression line in a Nipponbare background; and phf1 = o*sphf1* mutant in the PHR2-ov background. Plants were grown in half-strength Kimura nutrient solution for 40 days before As treatment. Error bars represent SE (*n* = 3-4). *** = *P* < 0.001 significant difference in flagged comparisons with background (ANOVA).

Differences between the experiments conducted on the PHR2-ov and *phf1* lines at pH 5.5 and 7.0 cannot be analysed directly as the plants varied greatly, especially in biomass (Figures S5.2; S5.3). However, overall trends in the data can be compared.

The results for As exposure at pH 7.0 are generally similar to those at pH 5.5, but there are some interesting differences. Firstly, under arsenate treatment both root and shoot As concentrations of the PHR2-ov line were significantly higher than background (Nipponbare), whereas root and shoot As concentrations of the *phf1* mutant were significantly decreased compared to PHR2-ov (Figure 5.6). Additionally PHR2-ov roots and shoots had significantly higher As concentration after exposure to MMA compared to wild-type. Again no differences in As concentration of tissues after exposure to DMA was observed between the genotypes.

Exposure to arsenate at pH 7.0 also resulted in the highest root and shoot As concentrations. Again MMA treatment gave an intermediate root As concentration, whereas little difference was seen in shoots after exposure to MMA or DMA. In this experiment arsenate and MMA both have average TFs of 0.05, whilst the TF for DMA is again much greater at 0.37; but is lower than for the experiment conducted at pH 5.5.

5.5 Discussion

The ability of OsPT8 to transport MMA and arsenate was clearly demonstrated by *Xenopus* oocytes expressing *OsPT8* (Figure 5.4). Although the experiment has not been repeated in different batches of oocytes, and phosphate transport by OsPT8 has not been confirmed, the preliminary data are very strong. Rice plants overexpressing *OsPT8* also had significantly higher As concentrations in roots and shoots after exposure to arsenate compared to wild-type when grown hydroponically (Figure S5.4). Uptake of arsenate and MMA was comparable for *OsPT8*-injected oocytes, which may indicate that the affinity of OsPT8 is similar for both substrates. This is in contrast to the NIP aquaporins, which show higher arsenite uptake than MMA (see **Chapter 4**). By measuring root uptake of the PT8-ov line in hydroponic experiments, Wu *et al.* (2011) determined the K_m of OsPT8 to be 11.4 and 14.2 µM for phosphate and arsenate respectively. Further investigation into the affinity of PTs to MMA and DMA is required.

The independent, highly significant, effect of P treatment on root As uptake of OsPT8-ov and wild-type plants (Figure 5.2) indicates that there is competition between phosphate, and MMA and DMA for root uptake. Alternatively, the lack of phosphate in the uptake medium may induce expression of phosphate starvation induced genes, including phosphate transporters. The treatments were applied for 48 hours before harvest, in which time the phosphate starvation response is induced and genes including *OsPT2*, *OsPT4* and *OsPT8* are upregulated (Kamiya *et al.*, 2013; Wu *et al.*, 2013).

Shoot As concentration was significantly increased in the OsPT8-ov line compared to wild-type, irrespective of the other treatments (Figure 5.3). Two independently generated OsPT8-ov lines show excessive P accumulation in the shoots, whilst root P concentration is only moderately increased (Jia *et al.*, 2011; Wu *et al.*, 2011; Figures 5.1; S5.1). The mean translocation factor (TF), measured as the ratio of As in paired root and shoot samples, was also significantly higher for OsPT8-ov plants compared to wild-type; regardless of the P, pH and As treatments ($P = 0.012$; Figure S5.5). Therefore, the significant increase in shoot uptake and translocation of MMA and DMA is consistent with the phenotype of phosphate over accumulation in the shoots of of *OsPT8*-overexpressor lines.

Overexpression of *OsPHR2* causes increased expression of several PTs, including *OsPT8* (Zhou *et al.*, 2008), and shows significantly increased As concentrations in roots and shoots when exposed to arsenate at both pH levels (Figures 5.5; 5.6). However unlike the PT8-ov line, there was no difference in MMA uptake between PHR2-ov and wild-type at pH 5.5 (Figure 5.5). Yet there was a significant difference in both root and shoot As concentration between the genotypes under MMA treatment, when the exposure medium was at pH 7.0 (Figure 5.6). The effect of pH on As uptake by all genotypes is discussed below.

Although arsenate uptake was significantly decreased in roots and shoots of the *phf1* line compared to background (PHR2-ov) at both pH levels, no differences were seen between the *phf1* and PHR2-ov under MMA or DMA treatment (Figures 5.5; 5.6). The mutation in *osphf1* causes retention of several PTs in the ER, causing significantly decreased biomass and tissue P concentrations (Chen *et al.*, 2011; Figures S5.2; S5.3). However, plants do retain the ability to take up some phosphate and arsenate, presumably via PTs not regulated by OsPHF1 (Wu *et al.*, 2011). Additionally, for the relatively small differences in MMA and DMA transport to be observed, variation between individual replicates must be low. However, as the large standard error bars present in Figures 5.5 and 5.6 demonstrate, plant-to-plant variation in the *phf1* line is relatively high, and so may mask any differences arising from As treatment.

For all the tested genotypes (Nipponbare, PT8-ov, PHR2-ov, *phf1*) As uptake was higher at pH 5.5 (Figures 5.2; 5.3; 5.5) than at pH 7.0 (Figures 5.2; 5.3; 5.6). This is particularly apparent for arsenate uptake, which was 3.1 and 6.6-fold greater at pH 5.5 compared to pH 7.0, for wild-type roots and shoots respectively. At lower pHs there is a greater concentration of protons for co-transport by PTs. For example the yeast high-affinity phosphate transporter, Pho84 has a pH optimum of 4.5 (Berhe *et al.*, 1995). Therefore, higher arsenate uptake under low pH is most likely due to higher activity of PTs. However changes in pH also affect the dissociation of MMA and DMA, and decreasing the pH decreases the proportion of dissociated complexes (Figure S1.2). Therefore, the effect of low pH on MMA and DMA transport is the net result of enhanced PT activity, and decreased availability of dissociated MMA and DMA.

A decrease in dissociation causes an increase in the proportion of undissociated MMA and DMA complexes. Transport of the neutral forms of MMA and DMA by NIP aquaporins (see **Chapter 4**) is thought to be the major pathway of methylated As uptake in rice. This is demonstrated by the *oslsi1* mutant, which causes around 80% decrease in MMA, and 50% decrease in DMA root uptake compared to wild-type (Li *et al.*, 2009a). The change in dissociation of MMA between pH 5.5 and 7.0 is only 4.51% (4.67% undissociated at pH 5.5 and 0.15% at pH 7.0), but uptake of MMA was 2.1-fold higher in absence of phosphate, and 2.3-fold higher in the presence of phosphate, at pH 5.5 compared to pH 7.0 for wild-type roots (Figure 5.2).

The difference in root As between PT8-ov and wild-type was significantly greater under MMA treatment than DMA treatment (Figure 5.2). Additionally, for DMA the change in dissociation between pH 5.5 and pH 7.0 is much greater (69.23%; 81.36% undissociated at pH 5.5 and 12.13% at pH 7.0), however pH had the smallest effect on DMA transport; DMA uptake was 1.3 and 1.8-fold greater at pH 5.5 compared to pH 7.0, for wild-type roots and shoots respectively. Furthermore, the uptake of MMA was significantly increased in roots and shoots of the PHR2-ov line compared to wild-type, but DMA concentrations were unaffected (Figure 5.6). Finally, DMA uptake was also not apparent for *Xenopus* oocytes expressing *OsPT8* (Figure 5.4). However DMA uptake by rice roots is slower than for inorganic As or MMA (Raab *et al.*, 2007b), and so longer incubation time may be necessary to observe differences in DMA uptake by oocytes. Overall, these results suggest that the phosphate transport pathway contributes less to the transport of DMA than MMA. The additional methyl group does not significantly alter the size of the DMA molecule, but does increase hydrophobicity. This property may allow DMA to be transported by another class of membrane proteins, and explain the differences in transport of DMA and MMA.

5.6 Conclusions

Significantly increased concentrations of MMA and DMA in rice shoots were found in plants overexpressing the high-affinity phosphate transporter, *OsPT8*. Additionally, the presence of phosphate in the uptake medium was found to significantly decrease uptake of MMA and DMA, for both PT8-ov and wild-type. Furthermore, uptake of MMA by *Xenopus* oocytes demonstrated directly that OsPT8 is able to transport MMA. Finally, shoot As concentrations were significantly increased after MMA exposure at pH 7.0 for rice plants overexpressing the phosphate starvation transcription factor, *OsPHR2*. Taken together, these results show that the phosphate transport pathway does contribute to the uptake and translocation MMA and DMA in rice.

Uptake of arsenate, MMA and DMA by rice roots in hydroponics was lower at pH 7.0 compared to pH 5.5 for all genotypes. At lower pHs there is an increase in protons for co-transport by PTs, but a decrease in dissociated MMA and DMA complexes. Additionally, the proportion of undissociated MMA and DMA complexes increases, which are substrates of NIP aquaporins. Therefore the contribution of PTs to methylated As transport is directly dependent on the pH, which determines the both the dissociation of MMA and DMA, and the proton gradient for co-transport.

Although pH, P treatment and overexpression of *OsPT8* did significantly affect DMA transport, the effects on MMA uptake were much greater. Additionally, overexpression of *OsPHR2* caused significantly increased transport of arsenate and MMA compared to wild-type, whereas DMA uptake was unaffected. Taken together these results indicate that the phosphate transport pathway contributes less to the transport of DMA than MMA. The more hydrophobic nature of DMA may mean that another transport pathway for this As species exists.

5.7 Supplementary information

Supplementary Table 5.1 Four-way ANOVA of log_{10} root As (μg g^{-1} DW) data. See Figure 5.2 for raw means. Significant treatments/interactions are in bold.

Treatment	d.f.	s.s.	m.s.	v.r.	F pr.
As	1	18.95745	18.95745	7533.91	<.001
Genotype	1	0.16957	0.16957	67.39	<.001
P	**1**	**0.21884**	**0.21884**	**86.97**	**<.001**
pH	1	1.12232	1.12232	446.02	<.001
As.Genotype	**1**	**0.04829**	**0.04829**	**19.19**	**<.001**
As.P	1	0.00302	0.00302	1.20	0.279
Genotype.P	1	0.00041	0.00041	0.16	0.689
As.pH	**1**	**0.14795**	**0.14795**	**58.79**	**<.001**
Genotype.pH	**1**	**0.01123**	**0.01123**	**4.46**	**0.04**
P.pH	1	0.00050	0.00050	0.20	0.658
As.Genotype.P	1	0.00112	0.00112	0.44	0.508
As.Genotype.pH	1	0.00029	0.00029	0.12	0.735
As.P.pH	1	0.00111	0.00111	0.44	0.510
Genotype.P.pH	1	0.00812	0.00812	3.23	0.079
As.Genotype.P.pH	1	0.00116	0.00116	0.46	0.501
Residual	48	0.12078	0.00252		
Total	63	20.81215			

d.f. = degrees of freedom; s.s. = sum of squares; m.s. = mean squares; v.r. = variance ratio; F pr. = *F* probability.

Supplementary Table 5.2 Means tables of significant treatments and interactions from 4-way ANOVA of root As (μg g^{-1} DW) data. Log-transformed values are given in parentheses. Different superscript letters indicate significant difference at the 5% LSD level (0.0252 for single, and 0.0357 for 2-way interactions of log_{10} means).

P	+P	-P
	8.095 (0.908)[a]	10.597 (1.025)[b]

As.Genotype	WT	PT8-ov
MMA	27.040 (1.432)[a]	38.896 (1.590)[b]
DMA	2.503 (0.398)[c]	2.795 (0.446)[d]

As.pH	5.5	7.0
MMA	49.136 (1.691)[a]	21.399 (1.330)[b]
DMA	3.212 (0.507)[c]	2.178 (0.338)[d]

Genotype.pH	5.5	7.0
WT	10.824 (1.034)[a]	6.252 (0.796)[b]
PT8-ov	14.581 (1.164)[c]	7.456 (0.873)[d]

Supplementary Table 5.3 Four-way ANOVA of log_{10} shoot As (μg g^{-1} DW) data. See Figure 5.3 for raw means. Significant treatments/interactions are in bold.

Treatment	d.f.	s.s.	m.s.	v.r.	F pr.
As	1	0.3379	0.3379	38.97	<.001
Genotype	**1**	**0.4708**	**0.4708**	**54.29**	**<.001**
P	1	0.2051	0.2051	23.66	<.001
pH	1	0.2054	0.2054	23.68	<.001
As.Genotype	1	0.0303	0.0303	3.49	0.068
As.P	**1**	**0.2824**	**0.2824**	**32.56**	**<.001**
Genotype.P	1	0.0093	0.0093	1.08	0.304
As.pH	1	0.0126	0.0126	1.45	0.234
Genotype.pH	1	0.0027	0.0027	0.31	0.579
P.pH	**1**	**0.0439**	**0.0439**	**5.06**	**0.029**
As.Genotype.P	1	0.0001	0.0001	0.01	0.925
As.Genotype.pH	1	0.0066	0.0066	0.76	0.386
As.P.pH	1	0.0034	0.0034	0.39	0.536
Genotype.P.pH	1	0.0000	0.0000	0.00	0.984
As.Genotype.P.pH	1	0.0001	0.0001	0.01	0.939
Residual	48	0.4162	0.0087		
Total	63	2.0267			

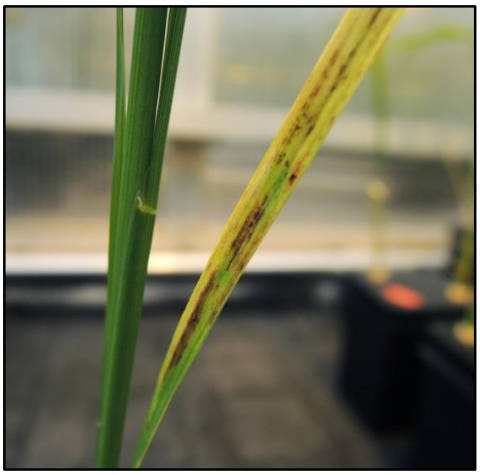
d.f. = degrees of freedom; s.s. = sum of squares; m.s. = mean squares; v.r. = variance ratio; F pr. = *F* probability.

Supplementary Table 5.4 Means tables of significant treatments and interactions from 4-way ANOVA of shoot As (μg g^{-1} DW) data. Log-transformed values are given in parentheses. Different superscript letters indicate significant difference at the 5% LSD level (0.0468 for single, and 0.0662 for 2-way interactions of log_{10} means).

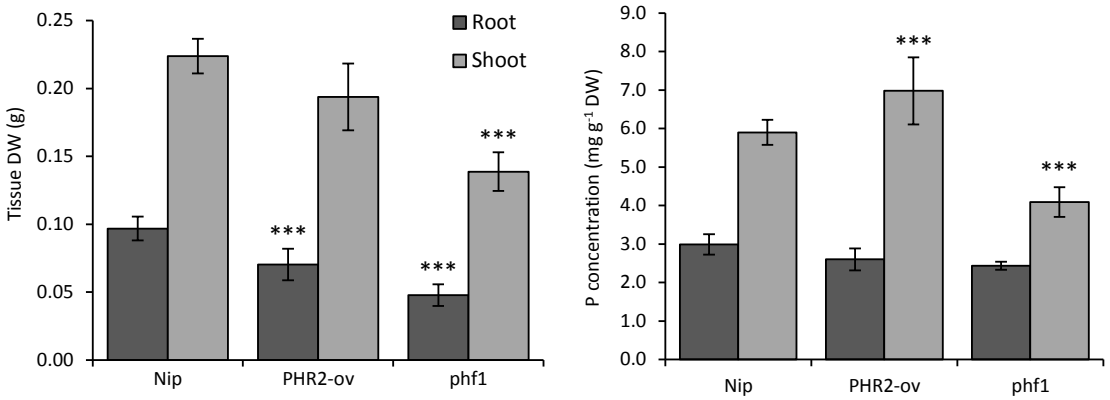
Genotype	WT	PT8-ov
	1.045 (0.019)[a]	1.552 (0.191)[b]

As.P	+P	-P
MMA	1.135 (0.055)[a]	2.000 (0.301)[b]
DMA	1.102 (0.042)[a]	1.052 (0.022)[a]

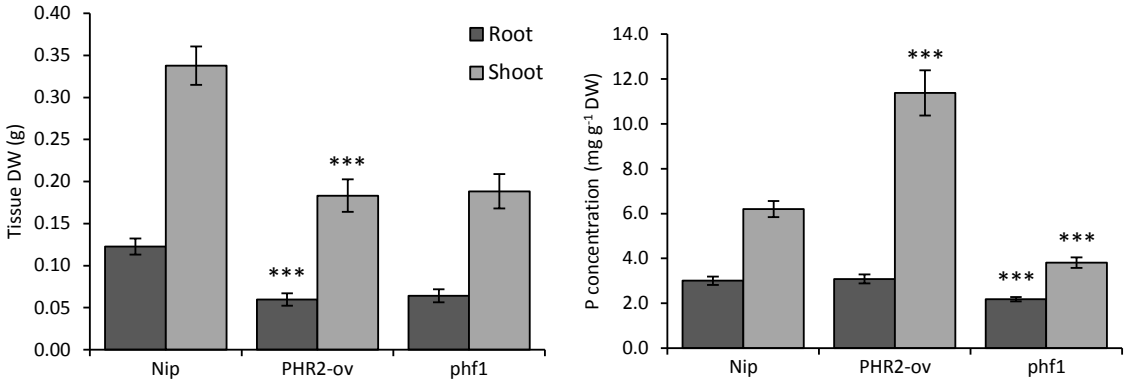
P.pH	5.5	7.0
+P	1.199 (0.079)[a]	1.042 (0.018)[a]
-P	1.754 (0.244)[b]	1.199 (0.079)[a]



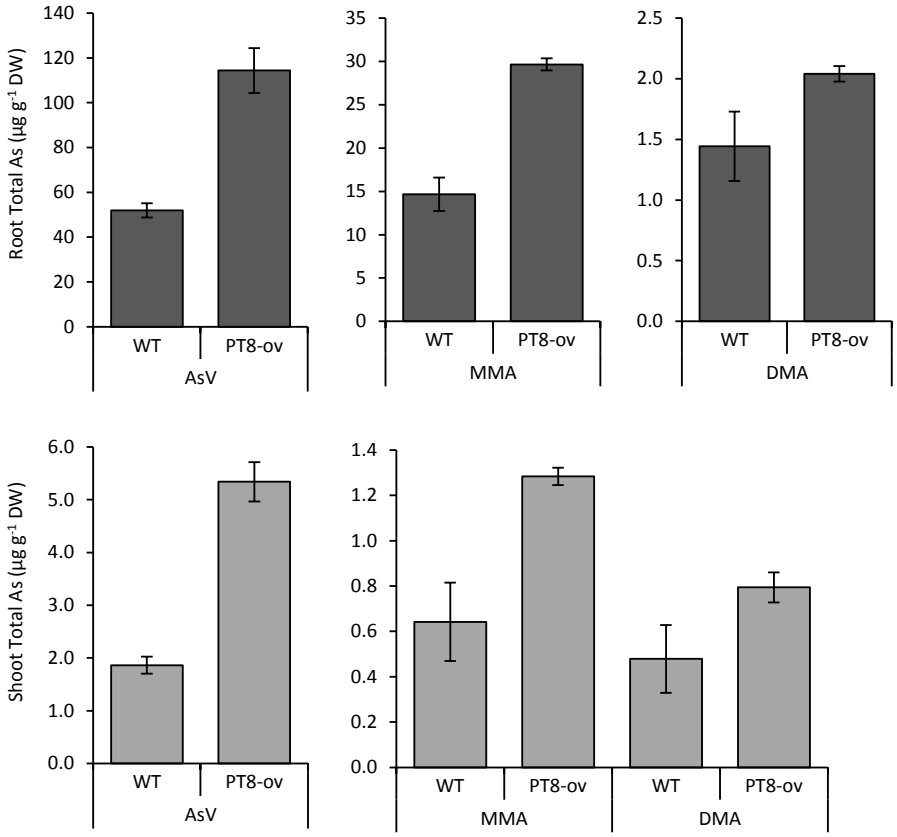
Supplementary Figure 5.1 Typical phosphate toxicity symptoms present in older leaves of the *OsPT8*-overexpression line. Rice seed (PT8-ov and WT) were germinated in low phosphate (10 µM) nutrient solution for 7 days, then grown for a further 29 days in modified half-strength Kimura B nutrient solution (91 µM phosphate).



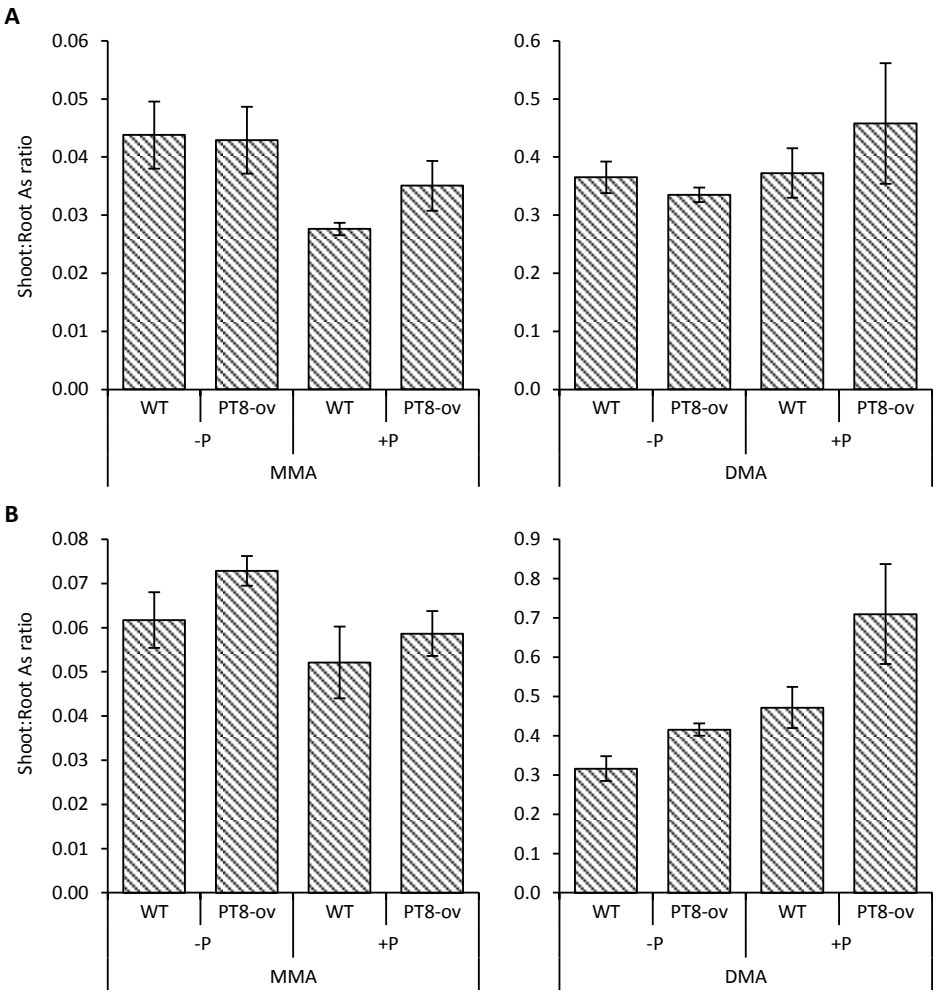
Supplementary Figure 5.2 Mean dry weight and total P concentration of hydroponically-grown rice. Nip = Nipponbare, PHR2-ov = *OsPHR2* over-expression line, and phf1 = o*sphf1* mutant. Plants were grown in half-strength Kimura nutrient solution for 43 days before As treatment. Error bars represent SE (*n* = 9-12). *** = *P* < 0.001 significant difference from background (ANOVA).



Supplementary Figure 5.3 Mean dry weight and total P concentration of hydroponically-grown rice. Nip = Nipponbare, PHR2-ov = *OsPHR2* over-expression line, and phf1 = o*sphf1* mutant. Plants were grown in half-strength Kimura nutrient solution for 40 days before As treatment. Error bars represent SE (*n* = 9-12). *** = *P* < 0.001 significant difference from background (ANOVA).



Supplementary Figure 5.4 Mean root As concentration of hydroponically-grown rice plants exposed to 5 µM arsenate (AsV), MMA or DMA, without phosphate, at 7.0 for 24 hours. WT = Nipponbare, PT8-ov = *OsPht1;8* over-expression line. Plants were grown in half-strength Kimura nutrient solution for 40 days before As treatment. Error bars represent SE (*n* = 3-4).



Supplementary Figure 5.5 Mean shoot:root As ratio (TF) of hydroponically-grown rice plants exposed to 5 µM MMA or DMA, with (+P) or without (-P) phosphate, at **A**, pH 5.5 or **B**, 7.0 for 48 hours. WT = Nipponbare, PT8-ov = *OsPht1;8* over-expression line. Plants were grown in half-strength Kimura nutrient solution for 34 days before As treatment. Error bars represent SE (*n* = 4).

6. General discussion

The work presented in this thesis focussed on two specific aims: firstly to determine whether plants are able to methylate inorganic As, and secondly to identify and characterise transporters responsible for the transport of MMA and DMA into and within plants.

6.1 Arsenic methylation in plants

The data presented in **Chapter 3** demonstrate that rice, tomato and red clover are unable to methylate inorganic As, and instead take up MMA and DMA from the growth medium. The species tested are from diverse plant families (Poaceae, Solanaceae and Fabaceae), and so it is unlikely that plants are unable to methylate As. The additional treatments of nutrient deficiency or symbiosis with root-nodulating bacteria, did not affect the As species present in roots or shoots. Exposure to inorganic As in axenic culture lasted for between 16 and 57 days, allowing sufficient time or any As-tolerance mechanisms to be induced. Additionally, the presence of arsenate in the nutrient agar had minimal effects on plant growth, and so the possible lack of As methylation due to overwhelming As-toxicity can also be ruled out.

Generally, inorganic As dominates soil extracts, however MMA and DMA were present in pore and standing water samples of flooded soils from Bangladesh and the UK, and have been reported in some studies (Takamatsu *et al.*, 1982; Bednar *et al.*, 2002a; Geiszinger *et al.*, 2002; Huang & Matzner, 2006; Huang & Matzner, 2007). Chemical preservation of soil extracts before analysis is necessary to prevent As adsorbing to insoluble ferric compounds. However, it was recently discovered that hydrochloric acid preserves DMA peaks for speciation better than EDTA (Zhao *et al.*, 2013b). Organic arsenicals may be present in soil from their past use as herbicides or pesticides; as was common practise in cotton fields and orchards (MacLean & Langille, 1981; Bednar *et al.*, 2002a), or from organic fertilisers. Additionally, considerable As-contamination of phosphate fertilisers applied to the Rothamsted Park Grass Experiment between 1888 and 1947 was recently

discovered (Hartley *et al.*, 2013). Alternatively methylated As may result from atmospheric deposition or microbe-mediated As methylation (Huang *et al.*, 2011).

In the USA, a large proportion of rice is produced on former cotton fields, which may explain the higher proportion of organic As in grain produced there (Williams *et al.*, 2007a; Zavala *et al.*, 2008; Zhao *et al.*, 2013a). The half-life of MMA and DMA applied to soils was estimated to be around 20 days in a lab-based study, but these As species were still detectable 1.5 years after application to an experimental field (Woolson *et al.*, 1982). More recently, little change in DMA speciation was observed under anaerobic conditions over a 3 month period, and although a large proportion of MMA was degraded to inorganic As, some was also methylated to DMA (Shimizu *et al.*, 2011b). MMA and DMA can bind to iron oxyhydroxides in soil (Shimizu *et al.*, 2011a; Shimizu *et al.*, 2011b), and sorption is affected by factors including redox potential, pH and organic matter (Bowell, 1994). Furthermore, the persistence of MMA and DMA in certain soils may be due to the scale of As-containing compounds applied; it is estimated that over 3,000 tonnes per year of MMA was applied to cotton fields in the USA in the 1990's (Bednar *et al.*, 2002a). Alternatively, the past use of As-containing compounds may have increased the methylation capacity of the microbial communities in these soils.

No change in As speciation was detected in arsenate-amended soil microcosms fumigated with formaldehyde, in contrast to control microcosms which contained MMA and DMA after 10 days incubation (Turpeinen *et al.*, 2002). Additionally, methylation of MMA to DMA was significantly decreased by the addition of the antibiotic chloramphenicol to nutrient solution used to grow rice, compared to the antibiotic-free control (Arao *et al.*, 2011). Arsenic methylation by micro-organisms is well characterised, and several recent studies have reported links between fertiliser application and organic As in soils (Jia *et al.*, 2012; Jia *et al.*, 2013a; Jia *et al.*, 2013b; Norton *et al.*, 2013; Zhao *et al.*, 2013a), microbial *arsM* abundance (Jia *et al.*, 2013a; Zhao *et al.*, 2013b) and rice grain As speciation (Jia *et al.*, 2013b; Zhao *et al.*, 2013b).

Total As concentrations in soil pore water were increased by addition of farm yard manure (Norton *et al.*, 2013), and fertilisers including dried distillers' grain, biogas slurry and rice straw were found to increase in MMA and DMA in soils (Jia *et al.*, 2012; Jia *et al.*,

2013a; Jia *et al.*, 2013b). The addition of organic matter is thought to enhance As mobilisation through competition for sorption sites and by lowering the redox potential (Jia *et al.*, 2013b; Norton *et al.*, 2013). Increasing the organic matter content of 14 different paddy soils by addition of rice straw was found to increase the abundance of microbial *arsM* genes by an average of almost 140% (Jia *et al.*, 2013a). Recently, Zhao *et al.* (2013b) found that six soils from diverse geographical locations exhibited very different profiles of As mobilisation and methylation. Interestingly, formation of MMA and DMA was influenced more by pH and dissolved organic carbon than *arsM* abundance. GeoChip analysis measures the copy number and diversity of *arsM* genes, not the expression level, which may explain the lack of correlation (Lomax *et al.*, 2012; Zhao *et al.*, 2013b).

6.2 Transport of MMA and DMA

Having concluded that As methylation does not occur in plants, the question of how these organic As species are taken up by, and transported within plants is raised. Different families of transporters are implicated in transporting arsenite and arsenate due to the inherent differences in their chemical properties. Arsenite has a high pK_a and so mainly exists as an undissociated, neutral species with structural similarity to silicic acid and glycerol. Whereas the much lower pK_a of arsenate means it is almost entirely dissociated, and an analogue of inorganic phosphate. Therefore the intermediate pK_as of MMA and DMA were hypothesised to cause these organic As species to be transported by both aquaporins and phosphate transporters depending on the pH.

6.2.1 Transport of undissociated MMA and DMA

The NIP aquaporin OsNIP2;1 (OsLsi1), was identified as the major route of silicon and arsenite uptake in rice roots (Ma *et al.*, 2006; Ma *et al.*, 2008). Unlike silicic acid, arsenite is a common substrate of NIP aquaporins. Using the rice *lsi1* mutant and expression in *Xenopus* oocytes, Li *et al.* (2009a) demonstrated that OsLsi1 also facilitates root uptake of undissociated MMA and DMA. The *lsi1* mutant lost 80% and 50% of the uptake capacity for MMA and DMA respectively (Li *et al.*, 2009a). Therefore other NIP aquaporins may facilitate the remaining MMA and DMA uptake by roots, or be involved in transport

within plants. *OsNIP4;1* is highly expressed in anthers and so may be involved in transport of As to grain (Nguyen *et al.*, 2013). However, aquaporins facilitate diffusion of substrates, and so over time considerable transport could be achieved even from low levels of expression.

In **Chapter 4**, three NIP aquaporins from rice were characterised by heterologous expression in *Xenopus* oocytes. OsNIP1;1 was found to be permeable to arsenite, MMA, boric acid and water, but not germanic acid. Germanic acid is an analogue of silicic acid, widely-used as a tracer for silicon transport in plants (Nikolic *et al.*, 2007). OsNIP3;3 facilitated transport of arsenite, MMA, water and germanic acid, but not boric acid. OsNIP3;2 was permeable to arsenite and boric acid, but not germanic acid, and showed limited water transport. Unfortunately expression of *OsNIP3;2* gave inconsistent levels of MMA uptake in *Xenopus* oocytes.

Significant uptake of DMA by oocytes expressing *OsLsi1* was observed once, however in further batches of oocytes there was no difference between those injected with *OsLsi1* and water. This was due to slightly decreased DMA uptake of *OsLsi1*-injected oocytes, and increased DMA content of the water-injected control oocytes. However, even in the one positive result, As content of oocytes expressing *OsLsi1* exposed to DMA for 60 minutes was almost 25-fold lower than those exposed to arsenite for 30 minutes. To act as a positive control, a construct of the human aquaporin, AQP9 was obtained. When characterised previously, hAQP9 was found to be highly permeable to pentavalent MMA and DMA, as well as trivalent MMA (McDermott *et al.*, 2010). Uptake of DMA by oocytes expressing *hAQP9* was considerably greater than those expressing *OsLsi1*. Uptake of germanic acid, arsenite and MMA mediated by hAQP9 and OsLsi1 was broadly similar. However, when the pH of the medium was decreased to from 6.0 to 4.5, MMA transport by hAQP9 was 5-fold higher than that of OsLsi1. The proportion of undissociated MMA complexes increases from 1.53% at pH 6.0, to 32.9% at pH 4.5, however, as both OsLsi1and hAQP9 are permeable to neutral MMA the reason for this difference is unknown.

In humans, AQP9 is highly expressed in the liver, which is the main site of As methylation. Therefore hAQP9 may have evolved to efficiently transport DMA, as a product of As

methylation, into the blood for excretion (McDermott *et al.*, 2010). Plants cannot methylate As (Lomax *et al.*, 2012), instead detoxifying As through complexation and sequestration in the vacuole, and so any ability to transport MMA and DMA is assumed to be adventitious. The aromatic/arginine (ar/R) filter of the two proteins is very different; FACR for hAQP9 and GSGR for OsLsi1, and may be the cause of differences in selectivity.

However, different levels of protein expression or activity in the *Xenopus* oocytes could also explain the differences in DMA transport of hAQP9 and OsLsi1. Codon usage preference is greater between *Xenopus laevis* and *Oryza sativa*, than between *Xenopus laevis* and *Homo sapiens* (determined using the Codon Usage Database; Nakamura *et al.*, 2000). Furthermore, a variety of post-translation modifications have been identified for plant aquaporins (for review see Chaumont *et al.*, 2005). Although the function of many of these modifications is not clear, it is reasonable to assume that processes such as methylation, phosphorylation and glycosylation will either be different in the *Xenopus* oocyte to those *in planta*, or be absent. Furthermore, Bienert *et al.* (2008) found that when expressed in yeast, OsNIP3;2 conferred the same level of arsenite sensitivity as OsLsi1 (OsNIP2;1), indicating high permeability to arsenite. This is in contrast with the results presented, and in addition to inconsistent MMA uptake, may indicate incompatibility between *OsNIP3;2* and in the *Xenopus* expression system.

In theory, transported substrates of NIP aquaporins would be related to their selectivity filters. For instance, the lack of boric acid transport by OsNIP3;3 is interesting, as OsNIP3;3 has the same ar/R filter as the Arabidopsis boron transporter, AtNIP6;1 (AIAR; Tanaka *et al.*, 2008). However, AtNIP6;1 has a substitution in the second NPA motif (NPA-NPV), and OsNIP3;1, which is proposed to mediate boron transport in rice (Hanaoka & Fujiwara, 2007), has mutations in both motifs (NPS-NPV). Therefore is seems that an NPV motif is critical for boron transport. Interestingly, the other nine expressed NIP aquaporins in rice have two conserved NPA motifs. This may suggest that substrate specificity is predominantly determined by the ar/R filter.

The ar/R filters of OsNIP3;2 (AAAR) and OsNIP3;3 (AIAR) differ by a single reside, but the two proteins display different properties when expressed in *Xenopus* oocytes. Whereas OsNIP3;3 shows permeability to arsenite, MMA and silicon, OsNIP3;2 shows limited

arsenite and MMA transport, and no silicon uptake. Additionally, OsNIP3;3 is more permeable to water than OsNIP3;2. However, substrate permeability of aquaporins may rely on residues other than those in the NPA motifs and ar/R region. The most likely of these are the remaining pore-lining residues, and the extracellular and cytoplasmic regions; which may act as pre-selectivity filters (Sui *et al.*, 2001; Wu & Beitz, 2007; Mitani-Ueno *et al.*, 2011).

Finally, members of the PIP-subfamily of aquaporins were also shown to transport arsenite (Mosa *et al.*, 2012), and so could also be permeable to MMA and DMA. OsPIP2;4, OsPIP2;6 and OsPIP2;7 transported arsenite in *Xenopus* oocytes and increased tolerance to arsenite when expressed in Arabidopsis (Mosa *et al.*, 2012). *OsPIP2;4* and *OsPIP2;6* are most highly expressed in the reproductive tissues such as anther and ovary (Nguyen *et al.*, 2013) and so may be involved in As transport to grain.

6.2.2 Transport of dissociated MMA and DMA

The contribution of the phosphate transport pathway to MMA and DMA transport was investigated using two rice overexpressors and one mutant line. Overexpression of *OsPT8* (PT8-ov) causes excessive accumulation of phosphate, especially in the shoots, with P toxicity symptoms present in leaves when grown under high phosphate supply (Jia *et al.*, 2011; Wu *et al.*, 2011; Figure S5.1). Overexpression of *OsPHR2* under phosphate-sufficient conditions leads to excessive accumulation of phosphate in root and shoots and up-regulation of phosphate transporters including *OsPT8* and *OsPT9* in roots, and *OsPT1*, *OsPT5*, *OsPT7*, *OsPT8*, *OsPT9*, and *OsPT12* in shoots (Zhou *et al.*, 2008; Wu *et al.*, 2011). The *phf1* mutant was isolated from an EMS-mutagenised population of PHR2-ov seeds based on arsenate tolerance, and showed decreased root and shoot ^{33}P uptake compared to PHR2-ov (Wu *et al.*, 2011).

Roots of both wild-type and PT8-ov contained significantly less As when phosphate was present in the uptake medium; at either pH 5.5 or 7.0, and for both MMA and DMA treatment. The effect was highly significant and did not interact with the other treatments, suggesting that there is competition between phosphate, and MMA and DMA for root uptake. Alternatively, upregulation of phosphate transporter expression during the 48 hour treatment may account for the increase in uptake of MMA and DMA. Shoot

As concentrations were significantly higher in the PT8-ov line compared to wild-type, irrespective of the other treatments. Two independently generated OsPT8-overexpression lines excessively accumulate P in the shoots, whilst root P concentration is only moderately increased (Jia *et al.*, 2011; Wu *et al.*, 2011). The mean ratio of As in paired root and shoot samples was also significantly higher for PT8-ov plants compared to wild-type; regardless of the other treatments. Therefore, the significant increase of MMA and DMA in the shoot is consistent with the phosphate-accumulating phenotype of *OsPT8*-overexpression.

Both root and shoot As concentrations of the PHR2-ov line were significantly higher than wild-type under MMA treatment at pH 7.0. However, there were no significant differences between PHR2-ov and wild-type at pH 5.5. There was a considerable amount of plant-to-plant variation of the PHR2-ov line which may mask other significant differences, but pH also has conflicting effects on MMA and DMA transport.

For all the tested genotypes (Nipponbare, PT8-ov, PHR2-ov and *phf1*) As uptake (arsenate, MMA and DMA) was higher at pH 5.5 than at pH 7.0. At lower pHs there is an increase in protons for symport through phosphate transporters, but a decrease in dissociated MMA and DMA complexes. Furthermore, as the proportion of dissociated MMA and DMA complexes decreases, the proportion of undissociated complexes increases. Increased transport of MMA and DMA under lower pH conditions could be due to either; increased activity of phosphate transporters compensating for the decrease in undissociated complexes, or increased transport of undissociated MMA and DMA by NIP aquaporins. The observation that the *lsi1* mutation causes a decrease of 80% of MMA, and 50% of DMA root uptake compared to wild-type at pH 5.5 (Li *et al.*, 2009a), suggests that low pH increases MMA and DMA uptake by increasing the proportion of undissociated complexes for transport by NIP aquaporins.

6.3 Overall conclusions

Transport of undissociated MMA via aquaporins, predominantly OsLsi1 (OsNIP2;1), is the major route for MMA uptake by rice roots (Li *et al.*, 2009a). However, once inside plant cells MMA and DMA, due to their pK_a values, are more sensitive to changes in pH than arsenite and arsenate. Furthermore, Carey *et al.* (2011) found that MMA and DMA, unlike inorganic As, were readily retranslocated from flag leaves to developing rice grains. Phosphate is remobilised from senescing leaves to sink tissues, which is facilitated by increased expression of phosphate transporters (Himelblau & Amasino, 2001; Zhu *et al.*, 2003). Therefore phosphate transporters may make a greater contribution to transport of MMA and DMA within plants. Moreover, transport by aquaporins relies on existing chemical concentration gradients, and so phosphate transporters, which are driven by proton co-transport, may be responsible for the accumulation of MMA and DMA in certain tissues.

OsLsi1 is implicated in the uptake of undissociated pentavalent DMA in rice roots (Li *et al.*, 2009a), and significant differences in DMA transport were observed for the *OsPT8*-overexpression line. However, neither of these differences was as great as for MMA. Furthermore, the rice *lsi1* mutant retained 50% of DMA uptake, compared to just 20% for MMA (Li *et al.*, 2009a). Taken together, these results show that both the phosphate transport pathway and NIP aquaporins contribute less to the transport of DMA than MMA. The chemical nature of DMA may mean that it is transported by another, yet unidentified, family of transporters.

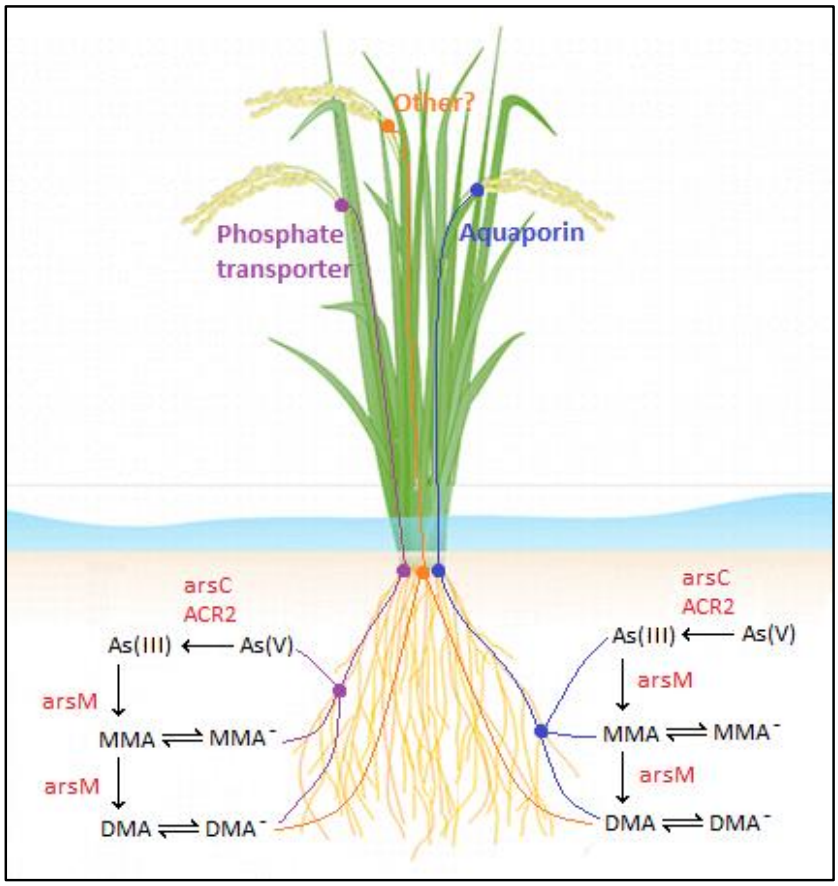


Figure 6.1 Diagram showing formation of MMA and DMA and transport pathways in rice.

Overall the model of As methylation and transport in plants is as follows (see Figure 6.1): microbe-mediated As methylation causes an increase in MMA and DMA in the soil solution. Phosphate transporters mediate uptake of dissociated MMA and DMA, and NIP aquaporins transport undissociated MMA and DMA, into rice roots. The contribution of the different pathways depends on the pH; which modifies phosphate transporter activity and determines the dissociation of MMA and DMA, and the availability of phosphate; as phosphate competes with dissociated MMA and DMA for transport via phosphate transporters. Once inside plants, MMA and DMA are translocated with high efficiency to the aerial tissues, especially rice grains.

6.4 Additional studies

Determining the protein level of the NIP aquaporins and hAQP9 in *Xenopus* oocytes would be useful to link the level of expression to As uptake. For instance, the seemingly higher permeability of hAQP9 to MMA and DMA, may be due to increased expression rather than substrate selectivity. If necessary, the plant expression vectors could be modified to better match the *Xenopus laevis* codon preferences and enhance expression. Additionally, the assay for DMA uptake of *Xenopus* oocytes should be developed, possibly by increasing the incubation time, or by using ^{73}As radiolabeled DMA for better quantitation at low levels of uptake.

Further investigation into *Xenopus* oocytes expressing *OsPT8* is also required. Although significant uptake of arsenate and MMA was observed, there was no uptake of DMA and phosphate transport was not determined. Experiments could also look into the effect of the pH of the exposure medium, and phosphate competition assays.

Identification of the transporters that load MMA and especially DMA, into the vascular tissues would also be valuable. Other candidate DMA transporters, possibly members of the solute carrier superfamily, should also be studied.

Finally, plant studies are required to fully understand the transport of MMA and DMA in plants. Heterologous expression can characterise individual transporters, but mutants would ideally be used to determine the contribution of these transporters to the overall uptake and translocation of MMA and DMA, especially to rice grain. A critical role in phosphate transport to developing grains means that knocking-down, rather than knocking-out expression of should be used to study the role of *OsPT8* in grain As accumulation. Another potential candidate would be *OsPT2*, which may be involved in re-translocation of phosphate, and MMA and DMA, to rice grains. OsPIP1;1 and OsPIP2;1 are the most highly expressed aquaporins in anthers and so may also be of interest.

6.5 Future prospects

Recently the *arsM* gene from the soil bacterium *Rhodopseudomonas palustris* was expressed in rice. Transgenic plants gave off more volatile As, and had significantly lower concentrations of As in grains (Meng *et al.*, 2011). This is a promising method of reducing As accumulation in rice, however caution is also required. Although pentavalent MMA and DMA are less toxic in cell cultures (Sakurai *et al.*, 1998), they could be reduced to trivalent MMA and DMA, which were found to be more toxic than inorganic As (Thomas *et al.*, 2001).

Possible agronomic solutions to reduce As accumulation include growing rice aerobically (Xu *et al.*, 2008; Talukder *et al.*, 2012) and addition of silicon fertilisers (Li *et al.*, 2009b). However, compromised yield and the cost of fertilisers may make these options less practical, especially in rural areas. Phytoremediation with *Pteris vittata* has significantly reduced As in rice grain in glasshouse experiments (Ye *et al.*, 2011; Mandal *et al.*, 2012). However, the land required for phytoremediation may also make this strategy unsuitable for most rice growers.

A potential strategy would be to exploit the As-accumulating phenotype of rice to combine phytoremediation with an edible food crop. However, much more knowledge about the transport pathways of As to grain is needed, as well as maintaining yields and preventing reintroduction into the food chain through the use of rice tissues to feed livestock or as a fertiliser.

7. References

Abbas M, Meharg A. 2008. Arsenate, arsenite and dimethyl arsinic acid (DMA) uptake and tolerance in maize (*Zea mays* L.). *Plant and Soil* **304**: 277-289.

Abedin MJ, Cresser MS, Meharg AA, Feldmann J, Cotter-Howells J. 2002a. Arsenic accumulation and metabolism in rice (*Oryza sativa* L.). *Environmental Science & Technology* **36**: 962-968.

Abedin MJ, Feldmann J, Meharg AA. 2002b. Uptake kinetics of arsenic species in rice plants. *Plant Physiology* **128**: 1120-1128.

Adams M, Bolger P, Gunderson E. 1994. Dietary intake and hazards of arsenic. *Arsenic Exposure and Health. Special Issue of Environmental Geochemistry and Health. Science and Technology Letters* **16**: 41-50.

Ai PH, Sun SB, Zhao JN, Fan XR, Xin WJ, Guo Q, Yu L, Shen QR, Wu P, Miller AJ, Xu GH. 2009. Two rice phosphate transporters, OsPht1;2 and OsPht1;6, have different functions and kinetic properties in uptake and translocation. *Plant Journal* **57**: 798-809.

Al Rmalli SW, Haris PI, Harrington CF, Ayub M. 2005. A survey of arsenic in foodstuffs on sale in the United Kingdom and imported from Bangladesh. *Science of the Total Environment* **337**: 23-30.

Alam MGM, Allinson G, Stagnitti F, Tanaka A, Westbrooke M. 2002. Arsenic contamination in Bangladesh groundwater: a major environmental and social disaster. *International Journal of Environmental Health Research* **12**: 236-253.

Alam MGM, Snow ET, Tanaka A. 2003. Arsenic and heavy metal contamination of vegetables grown in Samta village, Bangladesh. *Science of the Total Environment* **308**: 83-96.

Ali W, Isner J-C, Isayenkov SV, Liu W, Zhao F-J, Maathuis FJM. 2012. Heterologous expression of the yeast arsenite efflux system ACR3 improves *Arabidopsis thaliana* tolerance to arsenic stress. *New Phytologist* **194**: 716-723.

Anderberg H, Danielson J, Johanson U. 2011. Algal MIPs, high diversity and conserved motifs. *BMC Evolutionary Biology* **11**: 110.

Aposhian HV. 1997. Enzymatic methylation of arsenic species and other new approaches to arsenic toxicity. *Annual Review of Pharmacology and Toxicology* **37**: 397-419.

Arai Y, Lanzirotti A, Sutton S, Davis JA, Sparks DL. 2003. Arsenic speciation and reactivity in poultry litter. *Environmental Science & Technology* **37**: 4083-4090.

Arao T, Kawasaki A, Baba K, Matsumoto S. 2011. Effects of arsenic compound amendment on arsenic speciation in rice grain. *Environmental Science & Technology* **45**: 1291–1297.

ATSDR. 2007. *Toxicological profile for arsenic*. Atlanta, GA: U.S. Department of Health and Human Services, Agency for Toxic Substances and Disease Registry.

Ayadi M, Cavez D, Miled N, Chaumont F, Masmoudi K. 2011. Identification and characterization of two plasma membrane aquaporins in durum wheat (*Triticum turgidum* L. subsp. durum) and their role in abiotic stress tolerance. *Plant Physiology and Biochemistry* **49**: 1029-1039.

Banerjee M, Banerjee N, Bhattacharjee P, Mondal D, Lythgoe PR, Martínez M, Pan J, Polya DA, Giri AK. 2013. High arsenic in rice is associated with elevated genotoxic effects in humans. *Scientific Reports* **3**.

Bansal A, Sankararamakrishnan R. 2007. Homology modeling of major intrinsic proteins in rice, maize and Arabidopsis: comparative analysis of transmembrane helix association and aromatic/arginine selectivity filters. *Bmc Structural Biology* **7**.

Bari R, Pant BD, Stitt M, Scheible WR. 2006. PHO2, microRNA399, and PHR1 define a phosphate-signaling pathway in plants. *Plant Physiology* **141**: 988-999.

Batista BL, Souza JMO, De Souza SS, Barbosa Jr F. 2011. Speciation of arsenic in rice and estimation of daily intake of different arsenic species by Brazilians through rice consumption. *Journal of Hazardous Materials* **191**: 342-348.

Bayle V, Arrighi J-F, Creff A, Nespoulous C, Vialaret J, Rossignol M, Gonzalez E, Paz-Ares J, Nussaume L. 2011. *Arabidopsis thaliana* high-affinity phosphate transporters exhibit multiple levels of posttranslational regulation. *Plant Cell* **23**: 1523-1535.

Bednar AJ, Garbarino JR, Ranville JF, Wildeman TR. 2002a. Presence of organoarsenicals used in cotton production in agricultural water and soil of the southern United States. *Journal of Agricultural and Food Chemistry* **50**: 7340-7344.

Bednar AJ, Garbarino JR, Ranville JF, Wildeman TR. 2002b. Preserving the distribution of inorganic arsenic species in groundwater and acid mine drainage samples. *Environmental Science & Technology* **36**: 2213-2218.

Beene LC, Halluer J, Yoshinaga M, Hamdi M, Liu Z. 2011. Pentavalent Arsenate Transport by Zebrafish Phosphate Transporter NaPi-IIb1. *Zebrafish* **8**: 125-131.

Benga G, Popescu O, Pop VI, Holmes RP. 1986. *p*-(Chloromercuri)benzenesulfonate binding by membrane proteins and the inhibition of water transport in human erythrocytes. *Biochemistry* **25**: 1535-1538.

Bergqvist C, Greger M. 2012. Arsenic accumulation and speciation in plants from different habitats. *Applied Geochemistry* **27**: 615-622.

Berhe A, Fristedt U, Persson BL. 1995. Expression and purification of the high-affinity phosphate transporter of *Saccharomyces cerevisiae*. *European Journal of Biochemistry* **227**: 566-572.

Berns AE, Philipp H, Narres HD, Burauel P, Vereecken H, Tappe W. 2008. Effect of gamma-sterilization and autoclaving on soil organic matter structure as studied by solid state NMR, UV and fluorescence spectroscopy. *European Journal of Soil Science* **59**: 540-550.

Besse M, Knipfer T, Miller AJ, Verdeil JL, Jahn TP, Fricke W. 2011. Developmental pattern of aquaporin expression in barley (*Hordeum vulgare* L.) leaves. *Journal of Experimental Botany* **62**: 4127-4142.

BGS. 1999. *Groundwater Studies for Arsenic Contamination in Bangladesh.* London, UK.

Bhattacharjee H, Rosen B 2007. Arsenic Metabolism in Prokaryotic and Eukaryotic Microbes. 371-406.

Bhattacharjee H, Sheng J, Ajees AA, Mukhopadhyay R, Rosen BP. 2010. Adventitious Arsenate Reductase Activity of the Catalytic Domain of the Human Cdc25B and Cdc25C Phosphatases. *Biochemistry* **49**: 802-809.

Bieleski RL. 1973. Phosphate pools, phosphate transport, and phosphate availability. *Annual Review of Plant Physiology and Plant Molecular Biology* **24**: 225-252.

Bienert GP, Bienert MD, Jahn TP, Boutry M, Chaumont F. 2011. *Solanaceae* XIPs are plasma membrane aquaporins that facilitate the transport of many uncharged substrates. *Plant Journal* **66**: 306-317.

Bienert GP, Moller ALB, Kristiansen KA, Schulz A, Moller IM, Schjoerring JK, Jahn TP. 2007. Specific aquaporins facilitate the diffusion of hydrogen peroxide across membranes. *Journal of Biological Chemistry* **282**: 1183-1192.

Bienert GP, Thorsen M, Schussler MD, Nilsson HR, Wagner A, Tamas MJ, Jahn TP. 2008. A subgroup of plant aquaporins facilitate the bi-directional diffusion of $As(OH)_3$ and $Sb(OH)_3$ across membranes. *Bmc Biology* **6**: 15.

Bleeker PM, Hakvoort HWJ, Bliek M, Souer E, Schat H. 2006. Enhanced arsenate reduction by a CDC25-like tyrosine phosphatase explains increased phytochelatin accumulation in arsenate-tolerant *Holcus lanatus*. *Plant Journal* **45**: 917-929.

Borak J, Hosgood HD. 2007. Seafood arsenic: Implications for human risk assessment. *Regulatory Toxicology and Pharmacology* **47**: 204-212.

Bowell RJ. 1994. Sorption of arsenic by iron oxides and oxyhydroxides in soils. *Applied Geochemistry* **9**: 279-286.

Braman RS, Foreback CC. 1973. Methylated forms of arsenic in environment *Science* **182**: 1247-1249.

Brammer H, Ravenscroft P. 2009. Arsenic in groundwater: A threat to sustainable agriculture in South and South-east Asia. *Environment International* **35**: 647-654.

Brown D. 2003. The ins and outs of aquaporin-2 trafficking. *American Journal of Physiology-Renal Physiology* **284**: F893-F901.

Buchet JP, Lauwerys R. 1985. Study of inorganic arsenic methylation by rat-liver *in vitro* - relevance for the interpretation of observations in man *Archives of Toxicology* **57**: 125-129.

Buchet JP, Lauwerys R. 1988. Role of thiols in the *in vitro* methylation of inorganic arsenic by rat-liver cytosol. *Biochemical Pharmacology* **37**: 3149-3153.

Bun-ya M, Shikata K, Nakade S, Yompakdee C, Harashima S, Oshima Y. 1996. Two new genes, *PHO86* and*PHO87*, involved in inorganic phosphate uptake in *Saccharomyces cerevisiae*. *Current Genetics* **29**: 344-351.

Bundschuh J, Nath B, Bhattacharya P, Liu C-W, Armienta MA, Moreno López MV, Lopez DL, Jean J-S, Cornejo L, Lauer Macedo LF, Filho AT. 2012. Arsenic in the human food chain: the Latin American perspective. *Science of the Total Environment* **429**: 92-106.

Burlo F, Guijarro I, Carbonell-Barrachina AA, Valero D, Martinez-Sanchez F. 1999. Arsenic species: Effects on and accumulation by tomato plants. *Journal of Agricultural and Food Chemistry* **47**: 1247-1253.

Calatayud M, Barrios JA, Velez D, Devesa V. 2012. In Vitro Study of Transporters Involved in Intestinal Absorption of Inorganic Arsenic. *Chemical Research in Toxicology* **25**: 446-453.

Carbonell-Barrachina AA, Aarabi MA, DeLaune RD, Gambrell RP, Patrick WH. 1998. The influence of arsenic chemical form and concentration on *Spartina patens* and *Spartina alterniflora* growth and tissue arsenic concentration. *Plant and Soil* **198**: 33-43.

Carbonell-Barrachina AA, Burlo F, Valero D, Lopez E, Martinez-Romero D, Martinez-Sanchez F. 1999. Arsenic toxicity and accumulation in turnip as affected by arsenic chemical speciation. *Journal of Agricultural and Food Chemistry* **47**: 2288-2294.

Carbonell-Barrachina AA, Wu X, Ramirez-Gandolfo A, Norton GJ, Burlo F, Deacon C, Meharg AA. 2012. Inorganic arsenic contents in rice-based infant foods from Spain, UK, China and USA. *Environmental Pollution* **163**: 77-83.

Carew MW, Naranmandura H, Shukalek CB, Le XC, Leslie EM. 2011. Monomethylarsenic Diglutathione Transport by the Human Multidrug Resistance Protein 1 (MRP1/ABCC1). *Drug Metabolism and Disposition* **39**: 2298-2304.

Carey AM, Norton GJ, Deacon C, Scheckel KG, Lombi E, Punshon T, Guerinot ML, Lanzirotti A, Newville M, Choi YS, Price AH, Meharg AA. 2011. Phloem transport of arsenic species from flag leaf to grain during grain filling. *New Phytologist* **192**: 87-98.

Carey AM, Scheckel KG, Lombi E, Newville M, Choi Y, Norton GJ, Charnock JM, Feldmann J, Price AH, Meharg AA. 2010. Grain Unloading of Arsenic Species in Rice. *Plant Physiology* **152**: 309-319.

Carlin A, Shi WP, Dey S, Rosen BP. 1995. The *ars* Operon of *Escherichia coli* Confers Arsenical and Antimonial Resistance. *Journal of Bacteriology* **177**: 981-986.

Caruso JA, Heitkemper DT, B'Hymer C. 2001. An evaluation of extraction techniques for arsenic species from freeze-dried apple samples. *Analyst* **126**: 136-140.

Casado-Vela J, Muries B, Carvajal M, Iloro I, Elortza F, Martínez-Ballesta MC. 2010. Analysis of Root Plasma Membrane Aquaporins from *Brassica oleracea*: Post-Translational Modifications, de novo Sequencing and Detection of Isoforms by High Resolution Mass Spectrometry. *Journal of Proteome Research* **9**: 3479-3494.

Catarecha P, Segura MD, Franco-Zorrilla JM, Garcia-Ponce B, Lanza M, Solano R, Paz-Ares J, Leyva A. 2007. A mutant of the *Arabidopsis* phosphate transporter PHT1;1 displays enhanced arsenic accumulation. *Plant Cell* **19**: 1123-1133.

Chakraborti D, Rahman MM, Paul K, Chowdhury UK, Sengupta MK, Lodh D, Chanda CR, Saha KC, Mukherjee SC. 2002. Arsenic calamity in the Indian subcontinent - What lessons have been learned? *Talanta* **58**: 3-22.

Chaumont F, Moshelion M, Daniels MJ. 2005. Regulation of plant aquaporin activity. *Biology of the Cell* **97**: 749-764.

Chen J, Liu Y, Ni J, Wang Y, Bai Y, Shi J, Gan j, Wu Z, Wu P. 2011. OsPHF1 regulates the plasma membrane localization of low- and high-affinity Pi transporters and determines Pi uptake and translocation in rice. *Plant Physiology*.

Chen XW, Wu FY, Li H, Chan WF, Wu C, Wu SC, Wong MH. 2013a. Phosphate transporters expression in rice (*Oryza sativa* L.) associated with arbuscular mycorrhizal fungi (AMF) colonization under different levels of arsenate stress. *Environmental and Experimental Botany* **87**: 92-99.

Chen YS, Xu WZ, Shen HL, Yan HL, Xu WX, He ZY, Ma M. 2013b. Engineering Arsenic Tolerance and Hyperaccumulation in Plants for Phytoremediation by a *PvACR3* Transgenic Approach. *Environmental Science & Technology* **47**: 9355-9362.

Chiba Y, Mitani N, Yamaji N, Ma JF. 2009. HvLsi1 is a silicon influx transporter in barley. *Plant Journal* **57**: 810-818.

Choi WG, Roberts DM. 2007. Arabidopsis NIP2;1, a major intrinsic protein transporter of lactic acid induced by anoxic stress. *Journal of Biological Chemistry* **282**: 24209-24218.

Christen K. 2001. Chickens, manure, and arsenic. *Environmental Science & Technology* **35**: 184A-185A.

Ciavatta VT, Morillon R, Pullman GS, Chrispeels MJ, Cairney J. 2001. An Aquaglyceroporin Is Abundantly Expressed Early in the Development of the Suspensor and the Embryo Proper of Loblolly Pine. *Plant Physiology* **127**: 1556-1567.

Cobbett CS. 2000. Phytochelatins and their roles in heavy metal detoxification. *Plant Physiology* **123**: 825-832.

Consumer Reports 2012. Arsenic in your juice.In. *Consumer Reports Magazine*. 22-27.

Cooper K, Noller B, Connell D, Yu J, Sadler R, Olszowy H, Golding G, Tinggi U, Moore MR, Myers S. 2007. Public health risks from heavy metals and metalloids present in traditional Chinese medicines. *Journal of Toxicology and Environmental Health-Part a-Current Issues* **70**: 1694-1699.

Council of the European Union. 1998. *Council Directive 98/83/EC of 3 November 1998 on the quality of water intended for human consumption.* Official Journal of the European Communities **L 330**.

Cullen WR, Hettipathirana D, Reglinski J. 1989. The effect of arsenicals on cell suspension cultures of the Madagascar periwinkle (*Catharanthus roseus*). *Applied Organometallic Chemistry* **3**: 515-521.

Dabeka RW, McKenzie AD, Lacroix GMA, Cleroux C, Bowe S, Graham RA, Conacher HBS, Verdier P. 1993. Survey of arsenic in total diet food composites and estimation of dietary-intake of arsenic by Canadian adults and children. *Journal of Aoac International* **76**: 14-25.

Danielson JA, Johanson U. 2010. Phylogeny of major intrinsic proteins. *Adv Exp Med Biol* **679**: 19-31.

Danielson JAH, Johanson U. 2008. Unexpected complexity of the Aquaporin gene family in the moss *Physcomitrella patens*. *Bmc Plant Biology* **8**.

Daram P, Brunner S, Persson BL, Amrhein N, Bucher M. 1998. Functional analysis and cell-specific expression of a phosphate transporter from tomato. *Planta* **206**: 225-233.

Daram P, Brunner S, Rausch C, Steiner C, Amrhein N, Bucher M. 1999. Pht2;1 Encodes a Low-Affinity Phosphate Transporter from Arabidopsis. *The Plant Cell* **11**: 2153-2166.

Das HK, Mitra AK, Sengupta PK, Hossain A, Islam F, Rabbani GH. 2004. Arsenic concentrations in rice, vegetables, a fish in Bangladesh: a preliminary study. *Environment International* **30**: 383-387.

de Groot BL, Grubmuller H. 2005. The dynamics and energetics of water permeation and proton exclusion in aquaporins. *Current Opinion in Structural Biology* **15**: 176-183.

Dean RM, Rivers RL, Zeidel ML, Roberts DM. 1998. Purification and Functional Reconstitution of Soybean Nodulin 26. An Aquaporin with Water and Glycerol Transport Properties†. *Biochemistry* **38**: 347-353.

Denker BM, Smith BL, Kuhajda FP, Agre P. 1988. Identification, purification, and partial characterization of a novel M_r 28,000 integral membrane protein from erythrocytes and renal tubules. *Journal of Biological Chemistry* **263**: 15634-15642.

Dey S, Rosen BP. 1995. Dual mode of energy coupling by the oxyanion-translocating ArsB protein. *Journal of Bacteriology* **177**: 385-389.

Dhankher OP, Rosen BP, McKinney EC, Meagher RB. 2006. Hyperaccumulation of arsenic in the shoots of *Arabidopsis* silenced for arsenate reductase (ACR2). *Proceedings of the National Academy of Sciences of the United States of America* **103**: 5413-5418.

Dilda PJ, Hogg PJ. 2007. Arsenical-based cancer drugs. *Cancer Treatment Reviews* **33**: 542-564.

Duan G-L, Hu Y, Liu W-J, Kneer R, Zhao F-J, Zhu Y-G. 2011. Evidence for a role of phytochelatins in regulating arsenic accumulation in rice grain. *Environmental and Experimental Botany* **71**: 416-421.

Duan G, Kamiya T, Ishikawa S, Arao T, Fujiwara T. 2012. Expressing *ScACR3* in Rice Enhanced Arsenite Efflux and Reduced Arsenic Accumulation in Rice Grains. *Plant and Cell Physiology* **53**: 154-163.

Duan GL, Zhou Y, Tong YP, Mukhopadhyay R, Rosen BP, Zhu YG. 2007. A CDC25 homologue from rice functions as an arsenate reductase. *New Phytologist* **174**: 311-321.

Duan GL, Zhu YG, Tong YP, Cai C, Kneer R. 2005. Characterization of arsenate reductase in the extract of roots and fronds of Chinese brake fern, an arsenic hyperaccumulator. *Plant Physiology* **138**: 461-469.

Dynowski M, Schaaf G, Loque D, Moran O, Ludewig U. 2008. Plant plasma membrane water channels conduct the signalling molecule H_2O_2. *Biochemical Journal* **414**: 53-61.

Edmonds JS, Francesconi KA. 1993. Arsenic in seafoods - human health-aspects and regulations. *Marine Pollution Bulletin* **26**: 665-674.

Effros RM, Darin C, Jacobs ER, Rogers RA, Krenz G, Schneeberger EE. 1997. Water transport and the distribution of aquaporin-1 in pulmonary air spaces. *Journal of Applied Physiology* **83**: 1002-1016.

EFSA. 2009. Scientific opinion on arsenic in food. *EFSA Journal* **7**: Article 1351.

Erb TJ, Kiefer P, Hattendorf B, Günther D, Vorholt JA. 2012. GFAJ-1 Is an Arsenate-Resistant, Phosphate-Dependent Organism. *Science* **337**: 467-470.

Ferreccio C, Gonzalez C, Milosavjlevic V, Marshall G, Sancha AM, Smith AH. 2000. Lung cancer and arsenic concentrations in drinking water in Chile. *Epidemiology* **11**: 673-679.

Fetter K, Van Wilder V, Moshelion M, Chaumont F. 2004. Interactions between plasma membrane aquaporins modulate their water channel activity. *Plant Cell* **16**: 215-228.

Fischbarg J, Kuang KY, Vera JC, Arant S, Silverstein SC, Loike J, Rosen OM. 1990. Glucose transporters serve as water channels. *Proceedings of the National Academy of Sciences* **87**: 3244-3247.

Fitz WJ, Wenzel WW. 2002. Arsenic transformations in the soil-rhizosphere-plant system: fundamentals and potential application to phytoremediation. *Journal of Biotechnology* **99**: 259-278.

Fitzpatrick KL, Reid RJ. 2009. The involvement of aquaglyceroporins in transport of boron in barley roots. *Plant Cell and Environment* **32**: 1357-1365.

Flexas J, Ribas-Carbo M, Hanson DT, Bota J, Otto B, Cifre J, McDowell N, Medrano H, Kaldenhoff R. 2006. Tobacco aquaporin NtAQP1 is involved in mesophyll conductance to CO_2 *in vivo*. *Plant Journal* **48**: 427-439.

Food Standards Agency. 2004. *Total and inorganic arsenic in the 1999 total diet study.*

Food Standards Agency. 2009. *Survey of total and inorganic arsenic in rice drinks.* **Food Survey Information Sheet 02/09**.

Fortin MG, Morrison NA, Verma DPS. 1987. Nodulin-26, a peribacteroid nodulin is expressed independently of the development of the peribacteroid compartment. *Nucleic Acids Research* **15**: 813-824.

Fu DX, Libson A, Miercke LJW, Weitzman C, Nollert P, Krucinski J, Stroud RM. 2000. Structure of a glycerol-conducting channel and the basis for its selectivity. *Science* **290**: 481-486.

Fu HL, Meng Y, Ordonez E, Villadangos AF, Bhattacharjee H, Gil JA, Mateos LM, Rosen BP. 2009. Properties of arsenite efflux permeases (Acr3) from *Alkaliphilus metalliredigens* and *Corynebacterium glutamicum*. *Journal of Biological Chemistry* **284**: 19887-19895.

Garbarino JR, Bednar AJ, Rutherford DW, Beyer RS, Wershaw RL. 2003. Environmental fate of roxarsone in poultry litter. I. Degradation of roxarsone during composting. *Environmental Science & Technology* **37**: 1509-1514.

Geiszinger A, Goessler W, Kosmus W. 2002. Organoarsenic compounds in plants and soil on top of an ore vein. *Applied Organometallic Chemistry* **16**: 245-249.

Ghosh A, Awal MA, Majumder S, Mostofa M, Khair A, Islam MZ, Rao DR. 2012. Arsenic in Eggs and Excreta of Laying Hens in Bangladesh: A Preliminary Study. *Journal of Health Population and Nutrition* **30**: 383-393.

Ghosh A, Majumder S, Awal MA, Rao DR. 2013. Arsenic Exposure to Dairy Cows in Bangladesh. *Archives of Environmental Contamination and Toxicology* **64**: 151-159.

Ghosh M, Shen J, Rosen BP. 1999. Pathways of As(III) detoxification in *Saccharomyces cerevisiae*. *Proceedings of the National Academy of Sciences of the United States of America* **96**: 5001-5006.

Glassop D, Godwin RM, Smith SE, Smith FW. 2007. Rice phosphate transporters associated with phosphate uptake in rice roots colonised with arbuscular mycorrhizal fungi. *Canadian Journal of Botany* **85**: 644-651.

Goff SA, Ricke D, Lan TH, Presting G, Wang RL, Dunn M, Glazebrook J, Sessions A, Oeller P, Varma H, Hadley D, Hutchinson D, Martin C, Katagiri F, Lange BM, Moughamer T, Xia Y, Budworth P, Zhong JP, Miguel T, Paszkowski U, Zhang SP, Colbert M, Sun WL, Chen LL, Cooper B, Park S, Wood TC, Mao L, Quail P, Wing R, Dean R, Yu YS, Zharkikh A, Shen R, Sahasrabudhe S, Thomas A, Cannings R, Gutin A, Pruss D, Reid J, Tavtigian S, Mitchell J, Eldredge G, Scholl T, Miller RM, Bhatnagar S, Adey N, Rubano T, Tusneem N, Robinson R, Feldhaus J, Macalma T, Oliphant A, Briggs S. 2002. A draft sequence of the rice genome (*Oryza sativa* L. ssp *japonica*). *Science* **296**: 92-100.

Gong ZL, Lu XF, Cullen WR, Le XC. 2001. Unstable trivalent arsenic metabolites, monomethylarsonous acid and dimethylarsinous acid. *Journal of Analytical Atomic Spectrometry* **16**: 1409-1413.

Gonzalez E, Solano R, Rubio V, Leyva A, Paz-Ares J. 2005. PHOSPHATE TRANSPORTER TRAFFIC FACILITATOR1 is a plant-specific SEC12-related protein that enables the endoplasmic reticulum exit of a high-affinity phosphate transporter in *Arabidopsis*. *Plant Cell* **17**: 3500-3512.

Grégoire C, Remus-Borel W, Vivancos J, Labbe C, Belzile F, Belanger RR. 2012. Discovery of a multigene family of aquaporin silicon transporters in the primitive plant *Equisetum arvense*. *Plant Journal* **72**: 320-330.

Gregus Z, Németi B. 2005. The glycolytic enzyme glyceraldehyde-3-phosphate dehydrogenase works as an arsenate reductase in human red blood cells and rat liver cytosol. *Toxicological Sciences* **85**: 859-869.

Gregus Z, Roos G, Geerlings P, Nemeti B. 2009. Mechanism of Thiol-Supported Arsenate Reduction Mediated by Phosphorolytic-Arsenolytic Enzymes. *Toxicological Sciences* **110**: 282-292.

Gu RL, Chen XL, Zhou YL, Yuan LX. 2012. Isolation and characterization of three maize aquaporin genes, *ZmNIP2;1*, *ZmNIP2;4* and *ZmTIP4;4* involved in urea transport. *Bmb Reports* **45**: 96-101.

Guan X-G, Su W-H, Yi F, Zhang D, Hao F, Zhang H-G, Liu Y-J, Feng X-C, Ma T-H. 2010. NPA motifs play a key role in plasma membrane targeting of aquaporin-4. *IUBMB Life* **62**: 222-226.

Gueldry O, Lazard M, Delort F, Dauplais M, Grigoras I, Blanquet S, Plateau P. 2003. Ycf1p-dependent Hg(II) detoxification in *Saccharomyces cerevisiae*. *European Journal of Biochemistry* **270**: 2486-2496.

Guo B, Jin Y, Wussler C, Blancaflor EB, Motes CM, Versaw WK. 2008. Functional analysis of the Arabidopsis PHT4 family of intracellular phosphate transporters. *New Phytologist* **177**: 889-898.

Gupta AB, Verma RK, Agarwal V, Vajpai M, Bansal V, Sankararamakrishnan R. 2012. MIPModDB: a central resource for the superfamily of major intrinsic proteins. *Nucleic Acids Research* **40**: D362-D369.

Gustavsson S, Lebrun AS, Norden K, Chaumont F, Johanson U. 2005. A novel plant major intrinsic protein in *Physcomitrella patens* most similar to bacterial glycerol channels. *Plant Physiology* **139**: 287-295.

Hachez C, Chaumont F 2010. Aquaporins: A Family of Highly Regulated Multifunctional Channels. In: Jahn T, Bienert G eds. *MIPs and Their Role in the Exchange of Metalloids*: Springer New York, 1-17.

Hamdi M, Sanchez MA, Beene LC, Liu QY, Landfear SM, Rosen BP, Liu ZJ. 2009. Arsenic transport by zebrafish aquaglyceroporins. *Bmc Molecular Biology* **10**.

Hanaoka H, Fujiwara T. 2007. Channel-mediated boron transport in rice. *Plant and Cell Physiology* **48**: S227-S227.

Hanba YT, Shibasaka M, Hayashi Y, Hayakawa T, Kasamo K, Terashima I, Katsuhara M. 2004. Overexpression of the barley aquaporin HvPIP2;1 increases internal CO_2 conductance and CO_2 assimillation in the leaves of transgenic rice plants. *Plant and Cell Physiology* **45**: 521-529.

Hancock T, Denver J, Riedel G, Miller C 2001. Source, transport, and fate of arsenic in the Pocomoke River Basin, a poultry dominated Chesapeake Bay Watershed.In. *Proceedings of Arsenic in the Environment Workshop*: Citeseer.

Hansen HR, Raab A, Price AH, Duan GL, Zhu YG, Norton GJ, Feldmann J, Meharg AA. 2011. Identification of tetramethylarsonium in rice grains with elevated arsenic content. *Journal of Environmental Monitoring* **13**: 32-34.

Hartley TN, Macdonald AJ, McGrath SP, Zhao FJ. 2013. Historical arsenic contamination of soil due to long-term phosphate fertiliser applications. *Environmental Pollution* **180**: 259-264.

Healy SM, Zakharyan RA, Aposhian HV. 1997. Enzymatic methylation of arsenic compounds: IV. *In vitro* and *in vivo* deficiency of the methylation of arsenite and monomethylarsonic acid in the guinea pig. *Mutat Res* **386**: 229-239.

Heckwolf M, Pater D, Hanson DT, Kaldenhoff R. 2011. The *Arabidopsis thaliana* aquaporin AtPIP1;2 is a physiologically relevant CO_2 transport facilitator. *Plant Journal* **67**: 795-804.

Hedegaard RV, Rokkjaer I, Sloth JJ. 2013. Total and inorganic arsenic in dietary supplements based on herbs, other botanicals and algae-a possible contributor to inorganic arsenic exposure. *Analytical and Bioanalytical Chemistry* **405**: 4429-4435.

Heitkemper DT, Vela NP, Stewart KR, Westphal CS. 2001. Determination of total and speciated arsenic in rice by ion chromatography and inductively coupled plasma mass spectrometry. *Journal of Analytical Atomic Spectrometry* **16**: 299-306.

Hendriks G, Koudijs M, van Balkom BWM, Oorschot V, Klumperman J, Deen PMT, van der Sluijs P. 2004. Glycosylation Is Important for Cell Surface Expression of the Water Channel Aquaporin-2 but Is Not Essential for Tetramerization in the Endoplasmic Reticulum. *Journal of Biological Chemistry* **279**: 2975-2983.

Hewitt EJ. 1966. *Sand and water culture methods used in the study of plant nutrition*: Commonwealth Agricultural Bureaux.

Himelblau E, Amasino RM. 2001. Nutrients mobilized from leaves of *Arabidopsis thaliana* during leaf senescence. *Journal of Plant Physiology* **158**: 1317-1323.

Hirano S, Cui X, Li S, Kanno S, Kobayashi Y, Hayakawa T, Shraim A. 2003. Difference in uptake and toxicity of trivalent and pentavalent inorganic arsenic in rat heart microvessel endothelial cells. *Archives of Toxicology* **77**: 305-312.

Hirsch PR, Skinner FA 1992. The identification and classification of *Rhizobium* and *Bradyrhizobium*. In: Board RGJ, D.

Skinner, F. A. ed. *Identification Methods in Applied and Environmental Microbiology*. Oxford: Blackwell Scientific Publications, 45-65.

Hooijmaijers C, Rhee JY, Kwak KJ, Chung GC, Horie T, Katsuhara M, Kang H. 2012. Hydrogen peroxide permeability of plasma membrane aquaporins of *Arabidopsis thaliana*. *Journal of Plant Research* **125**: 147-153.

Hossain MF. 2006. Arsenic contamination in Bangladesh - An overview. *Agriculture Ecosystems & Environment* **113**: 1-16.

Hove RM, Bhave M. 2011. Plant aquaporins with non-aqua functions: deciphering the signature sequences. *Plant Molecular Biology* **75**: 413-430.

Huang J-H, Hu K-N, Decker B. 2011. Organic Arsenic in the Soil Environment: Speciation, Occurrence, Transformation, and Adsorption Behavior. *Water Air and Soil Pollution* **219**: 401-415.

Huang J-H, Matzner E. 2007. Mobile arsenic species in unpolluted and polluted soils. *Science of the Total Environment* **377**: 308-318.

Huang JH, Matzner E. 2006. Dynamics of organic and inorganic arsenic in the solution phase of an acidic fen in Germany. *Geochimica Et Cosmochimica Acta* **70**: 2023-2033.

Huang JH, Scherr F, Matzner E. 2007. Demethylation of dimethylarsinic acid and arsenobetaine in different organic soils. *Water Air and Soil Pollution* **182**: 31-41.

Huang R-N, Lee T-C. 1996. Cellular Uptake of Trivalent Arsenite and Pentavalent Arsenate in KB Cells Cultured in Phosphate-Free Medium. *Toxicology and Applied Pharmacology* **136**: 243-249.

Huang ZC, Chen TB, Lei M, Liu YR, Hu TD. 2008. Difference of toxicity and accumulation of methylated and inorganic arsenic in arsenic-hyperaccumulating and -hypertolerant plants. *Environmental Science & Technology* **42**: 5106-5111.

Hub JS, de Groot BL. 2006. Does CO2 permeate through aquaporin-1? *Biophysical Journal* **91**: 842-848.

Hughes MF. 2002. Arsenic toxicity and potential mechanisms of action. *Toxicology Letters* **133**: 1-16.

Hwang JH, Ellingson SR, Roberts DM. 2010. Ammonia permeability of the soybean nodulin 26 channel. *Febs Letters* **584**: 4339-4343.

IARC. 2009. *A review of human carcinogens. Part C: Arsenic, metals, fibres, and dusts*. Lyon, France: International Agency for Research on Cancer.

Indriolo E, Na G, Ellis D, Salt DE, Banks JA. 2010. A vacuolar arsenite transporter necessary for arsenic tolerance in the arsenic hyperaccumulating fern *Pteris vittata* is missing in flowering plants. *Plant Cell* **22**: 2045-2057.

Isayenkov SV, Maathuis FJM. 2008. The *Arabidopsis thaliana* aquaglyceroporin AtNIP7;1 is a pathway for arsenite uptake. *Febs Letters* **582**: 1625-1628.

Ishibashi K, Hara S, Kondo S. 2009. Aquaporin water channels in mammals. *Clinical and Experimental Nephrology* **13**: 107-117.

Jackson BP, Taylor VF, Karagas MR, Punshon T, Cottingham KL. 2012a. Arsenic, Organic Foods, and Brown Rice Syrup. *Environmental Health Perspectives* **120**: 623-626.

Jackson BP, Taylor VF, Punshon T, Cottingham KL. 2012b. Arsenic concentration and speciation in infant formulas and first foods. *Pure and Applied Chemistry* **84**: 215-223.

Jasinski M, Ducos E, Martinoia E, Boutry M. 2003. The ATP-Binding Cassette Transporters: Structure, Function, and Gene Family Comparison between Rice and Arabidopsis. *Plant Physiology* **131**: 1169-1177.

JECFA. 1967. *Specifications for the identity and purity of food additives and their toxicological evaluation: some emulsifiers and stabilizers and certain other substances* WHO Technical Report Series **373**. Geneva, Switzerland.

JECFA. 1983. *Toxicological evaluation of certain food additives and contaminants.* WHO Food Additives Series **18**. Geneva, Switzerland.

JECFA. 1989. *Evaluation of certain food additives and contaminants.* WHO Technical Report Series **24**. Geneva, Switzerland.

JECFA. 2011. *Safety evaluation of certain contaminants in food.* WHO Food Additives Series **63**. Geneva, Switzerland.

Jia H, Ren H, Gu M, Zhao J, Sun S, Zhang X, Chen J, Wu P, Xu G. 2011. The Phosphate Transporter Gene OsPht1;8 Is Involved in Phosphate Homeostasis in Rice. *Plant Physiology* **156**: 1164-1175.

Jia Y, Huang H, Sun GX, Zhao FJ, Zhu YG. 2012. Pathways and Relative Contributions to Arsenic Volatilization from Rice Plants and Paddy Soil. *Environmental Science & Technology* **46**: 8090-8096.

Jia Y, Huang H, Zhong M, Wang FH, Zhang LM, Zhu YG. 2013a. Microbial arsenic methylation in soil and rice rhizosphere. *Environ Sci Technol* **47**: 3141-3148.

Jia Y, Sun GX, Huang H, Zhu YG. 2013b. Biogas slurry application elevated arsenic accumulation in rice plant through increased arsenic release and methylation in paddy soil. *Plant and Soil* **365**: 387-396.

Jiang X, McDermott JR, Ajees AA, Rosenac BP, Liu ZJ. 2010. Trivalent arsenicals and glucose use different translocation pathways in mammalian GLUT1. *Metallomics* **2**: 211-219.

Johanson U, Karlsson M, Johansson I, Gustavsson S, Sjovall S, Fraysse L, Weig AR, Kjellbom P. 2001. The complete set of genes encoding major intrinsic proteins in Arabidopsis provides a framework for a new nomenclature for major intrinsic proteins in plants. *Plant Physiology* **126**: 1358-1369.

Johansson I, Karlsson M, Johanson U, Larsson C, Kjellbom P. 2000. The role of aquaporins in cellular and whole plant water balance. *Biochimica Et Biophysica Acta-Biomembranes* **1465**: 324-342.

Johansson I, Karlsson M, Shukla VK, Chrispeels MJ, Larsson C, Kjellbom P. 1998. Water transport activity of the plasma membrane aquaporin PM28A is regulated by phosphorylation. *Plant Cell* **10**: 451-459.

Johnson DL. 1972. Bacterial reduction of arsenate in sea water. *Nature* **240**: 44-45.

Johnson KD, Chrispeels MJ. 1992. Tonoplast-Bound Protein Kinase Phosphorylates Tonoplast Intrinsic Protein. *Plant Physiology* **100**: 1787-1795.

Jozefkowicz C, Rosi P, Sigaut L, Soto G, Pietrasanta LI, Amodeo G, Alleva K. 2013. Loop A Is Critical for the Functional Interaction of Two *Beta vulgaris* PIP Aquaporins. *PloS one* **8**.

Jung JS, Preston GM, Smith BL, Guggino WB, Agre P. 1994. Molecular structure of the water channel through aquaporin CHIP. The hourglass model. *Journal of Biological Chemistry* **269**: 14648-14654.

Kala SV, Neely MW, Kala G, Prater CI, Atwood DW, Rice JS, Lieberman MW. 2000. The MRP2/cMOAT transporter and arsenic-glutathione complex formation are required for biliary excretion of arsenic. *Journal of Biological Chemistry* **275**: 33404-33408.

Kamiya T, Fujiwara T. 2009. Arabidopsis NIP1;1 Transports Antimonite and Determines Antimonite Sensitivity. *Plant and Cell Physiology* **50**: 1977-1981.

Kamiya T, Islam R, Duan G, Uraguchi S, Fujiwara T. 2013. Phosphate deficiency signaling pathway is a target of arsenate and phosphate transporter OsPT1 is involved in As accumulation in shoots of rice. *Soil Science and Plant Nutrition* **59**: 580-590.

Kamiya T, Tanaka M, Mitani N, Ma JF, Maeshima M, Fujiwara T. 2009. NIP1;1, an Aquaporin Homolog, Determines the Arsenite Sensitivity of *Arabidopsis thaliana*. *Journal of Biological Chemistry* **284**: 2114-2120.

Kile ML, Houseman EA, Breton CV, Smith T, Quamruzzaman O, Rahman M, Mahiuddin G, Christiani DC. 2007. Dietary arsenic exposure in Bangladesh. *Environmental Health Perspectives* **115**: 889-893.

Kitchin KT, Wallace K. 2005. Arsenite binding to synthetic peptides based on the Zn finger region and the estrogen binding region of the human estrogen receptor-α. *Toxicology and Applied Pharmacology* **206**: 66-72.

Klebl F, Wolf M, Sauer N. 2003. A defect in the yeast plasma membrane urea transporter Dur3p is complemented by *CpNIP1*, a Nod26-like protein from zucchini (*Cucurbita pepo* L.), and by *Arabidopsis thaliana* delta-TIP or gamma-TIP. *Febs Letters* **547**: 69-74.

Knappe S, Flugge UI, Fischer K. 2003. Analysis of the plastidic *phosphate translocator* gene family in Arabidopsis and identification of new *phosphate translocator*-homologous transporters, classified by their putative substrate-binding site. *Plant Physiology* **131**: 1178-1190.

Koch I, Dee J, House K, Sui J, Zhang J, McKnight-Whitford A, Reimer KJ. 2013. Bioaccessibility and speciation of arsenic in country foods from contaminated sites in Canada. *Science of the Total Environment* **449**: 1-8.

Koch I, Feldmann J, Wang LX, Andrewes P, Reimer KJ, Cullen WR. 1999. Arsenic in the Meager Creek hot springs environment, British Columbia, Canada. *Science of the Total Environment* **236**: 101-117.

Koch I, Wang LX, Ollson CA, Cullen WR, Reimer KJ. 2000. The predominance of inorganic arsenic species in plants from Yellowknife, Northwest Territories, Canada. *Environmental Science & Technology* **34**: 22-26.

Koh HL, Woo SO. 2000. Chinese proprietary medicine in Singapore - Regulatory control of toxic heavy metals and undeclared drugs. *Drug Safety* **23**: 351-362.

Kuehnelt D, Lintschinger J, Goessler W. 2000. Arsenic compounds in terrestrial organisms. IV. Green plants and lichens from an old arsenic smelter site in Austria. *Applied Organometallic Chemistry* **14**: 411-420.

Kuramata M, Abe T, Kawasaki A, Ebana K, Shibaya T, Yano M, Ishikawa S. 2013. Genetic diversity of arsenic accumulation in rice and QTL analysis of methylated arsenic in rice grains. *Rice* **6**.

Kuramata M, Abe T, Matsumoto S, Ishikawa S. 2011. Arsenic accumulation and speciation in Japanese paddy rice cultivars. *Soil Science and Plant Nutrition* **57**: 248-258.

Larios R, Fernandez-Martinez R, LeHecho I, Rucandio I. 2012. A methodological approach to evaluate arsenic speciation and bioaccumulation in different plant species from two highly polluted mining areas. *Science of the Total Environment* **414**: 600-607.

Lau WTW, Howson RW, Malkus P, Schekman R, O'Shea EK. 2000. Pho86p, an endoplasmic reticulum (ER) resident protein in *Saccharomyces cerevisiae*, is required for ER exit of the high-affinity phosphate transporter Pho84p. *Proceedings of the National Academy of Sciences of the United States of America* **97**: 1107-1112.

Lazard M, Ha-Duong NT, Mounie S, Perrin R, Plateau P, Blanquet S. 2011. Selenodiglutathione uptake by the *Saccharomyces cerevisiae* vacuolar ATP-binding cassette transporter Ycf1p. *Febs Journal* **278**: 4112-4121.

Le XC, Lu X, Ma M, Cullen WR, Aposhian HV, Zheng B. 2000. Speciation of Key Arsenic Metabolic Intermediates in Human Urine. *Analytical Chemistry* **72**: 5172-5177.

Lei M, Tie BQ, Zeng M, Qing PF, Song ZG, Williams PN, Huang YZ. 2013. An arsenic-contaminated field trial to assess the uptake and translocation of arsenic by genotypes of rice. *Environmental Geochemistry and Health* **35**: 379-390.

Leslie EM. 2012. Arsenic-glutathione conjugate transport by the human multidrug resistance proteins (MRPs/ABCCs). *Journal of Inorganic Biochemistry* **108**: 141-149.

Leslie EM, Haimeur A, Waalkes MP. 2004. Arsenic transport by the human multidrug resistance protein 1 (MRP1/ABCC1) - Evidence that a tri-glutathione conjugate is required. *Journal of Biological Chemistry* **279**: 32700-32708.

Li H, Chen HN, Steinbronn C, Wu BH, Beitz E, Zeuthen T, Voth GA. 2011a. Enhancement of Proton Conductance by Mutations of the Selectivity Filter of Aquaporin-1. *Journal of Molecular Biology* **407**: 607-620.

Li RY, Ago Y, Liu WJ, Mitani N, Feldmann J, McGrath SP, Ma JF, Zhao FJ. 2009a. The Rice Aquaporin Lsi1 Mediates Uptake of Methylated Arsenic Species. *Plant Physiology* **150**: 2071-2080.

Li RY, Stroud JL, Ma JF, McGrath SP, Zhao FJ. 2009b. Mitigation of Arsenic Accumulation in Rice with Water Management and Silicon Fertilization. *Environmental Science & Technology* **43**: 3778-3783.

Li T, Choi WG, Wallace IS, Baudry J, Roberts DM. 2011b. *Arabidopsis thaliana* NIP7;1: An Anther-Specific Boric Acid Transporter of the Aquaporin Superfamily Regulated by an Unusual Tyrosine in Helix 2 of the Transport Pore. *Biochemistry* **50**: 6633-6641.

Lin S, Shi Q, Nix FB, Styblo M, Beck MA, Herbin-Davist KM, Hall LL, Simeonsson JB, Thomas DJ. 2002. A novel *S*-adenosyl-L-methionine : arsenic(III) methyltransferase from rat liver cytosol. *Journal of Biological Chemistry* **277**: 10795-10803.

Lin YF, Walmsley AR, Rosen BP. 2006. An arsenic metallochaperone for an arsenic detoxification pump. *Proceedings of the National Academy of Sciences of the United States of America* **103**: 15617-15622.

Liu F, Wang ZY, Ren HY, Shen CJ, Li Y, Ling HQ, Wu CY, Lian XM, Wu P. 2010a. OsSPX1 suppresses the function of OsPHR2 in the regulation of expression of *OsPT2* and phosphate homeostasis in shoots of rice. *Plant Journal* **62**: 508-517.

Liu QP, Wang HS, Zhang ZH, Wu JS, Feng Y, Zhu ZJ. 2009. Divergence in function and expression of the NOD26-like intrinsic proteins in plants. *Bmc Genomics* **10**: 13.

Liu W, Schat H, Bliek M, Chen Y, McGrath SP, George G, Salt DE, Zhao F-J. 2012. Knocking Out *ACR2* Does Not Affect Arsenic Redox Status in *Arabidopsis thaliana*: Implications for As Detoxification and Accumulation in Plants. *PloS one* **7**: e42408.

Liu WJ, Wood BA, Raab A, McGrath SP, Zhao FJ, Feldmann J. 2010b. Complexation of Arsenite with Phytochelatins Reduces Arsenite Efflux and Translocation from Roots to Shoots in Arabidopsis. *Plant Physiology* **152**: 2211-2221.

Liu XJ, Zhao QL, Sun GX, Williams P, Lu XJ, Cai JZ, Liu WJ. 2013. Arsenic speciation in Chinese Herbal Medicines and human health implication for inorganic arsenic. *Environmental Pollution* **172**: 149-154.

Liu ZJ, Boles E, Rosen BP. 2004a. Arsenic trioxide uptake by hexose permeases in *Saccharomyces cerevisiae*. *Journal of Biological Chemistry* **279**: 17312-17318.

Liu ZJ, Carbrey JM, Agre P, Rosen BP. 2004b. Arsenic trioxide uptake by human and rat aquaglyceroporins. *Biochemical and Biophysical Research Communications* **316**: 1178-1185.

Liu ZJ, Sanchez MA, Jiang X, Boles E, Landfear SM, Rosen BP. 2006a. Mammalian glucose permease GLUT1 facilitates transport of arsenic trioxide and methylarsonous acid. *Biochemical and Biophysical Research Communications* **351**: 424-430.

Liu ZJ, Shen J, Carbrey JM, Mukhopadhyay R, Agre P, Rosen BP. 2002. Arsenite transport by mammalian aquaglyceroporins AQP7 and AQP9. *Proceedings of the National Academy of Sciences of the United States of America* **99**: 6053-6058.

Liu ZJ, Styblo M, Rosen BP. 2006b. Methylarsonous acid transport by aquaglyceroporins. *Environmental Health Perspectives* **114**: 527-531.

Llobett JM, Falco G, Casas C, Teixido A, Domingo JL. 2003. Concentrations of arsenic, cadmium, mercury, and lead in common foods and estimated daily intake by children, adolescents, adults, and seniors of Catalonia, Spain. *Journal of Agricultural and Food Chemistry* **51**: 838-842.

Lomax C, Liu W-J, Wu L, Xue K, Xiong J, Zhou J, McGrath SP, Meharg AA, Miller AJ, Zhao F-J. 2012. Methylated arsenic species in plants originate from soil microorganisms. *New Phytologist* **193**: 665-672.

Lopez D, Bronner G, Brunel N, Auguin D, Bourgerie S, Brignolas F, Carpin S, Tournaire-Roux C, Maurel C, Fumanal B, Martin F, Sakr S, Label P, Julien JL, Gousset-Dupont A, Venisse JS. 2012. Insights into *Populus* XIP aquaporins: evolutionary expansion, protein functionality, and environmental regulation. *Journal of Experimental Botany* **63**: 2217-2230.

Lu W-J, Tamai I, Nezu J-i, Lai M-L, Huang J-d. 2006. Organic anion transporting polypeptide-C mediates arsenic uptake in HEK-293 cells. *Journal of Biomedical Science* **13**: 525-533.

Ma JF. 2004. Role of silicon in enhancing the resistance of plants to biotic and abiotic stresses. *Soil Science and Plant Nutrition* **50**: 11-18.

Ma JF, Tamai K, Yamaji N, Mitani N, Konishi S, Katsuhara M, Ishiguro M, Murata Y, Yano M. 2006. A silicon transporter in rice. *Nature* **440**: 688-691.

Ma JF, Yamaji N. 2006. Silicon uptake and accumulation in higher plants. *Trends in Plant Science* **11**: 392-397.

Ma JF, Yamaji N, Mitani N, Tamai K, Konishi S, Fujiwara T, Katsuhara M, Yano M. 2007a. An efflux transporter of silicon in rice. *Nature* **448**: 209-U212.

Ma JF, Yamaji N, Mitani N, Xu XY, Su YH, McGrath SP, Zhao FJ. 2008. Transporters of arsenite in rice and their role in arsenic accumulation in rice grain. *Proceedings of the National Academy of Sciences of the United States of America* **105**: 9931-9935.

Ma JF, Yamaji N, Tamai K, Mitani N. 2007b. Genotypic difference in silicon uptake and expression of silicon transporter genes in rice. *Plant Physiology* **145**: 919-924.

Ma LQ, Komar KM, Tu C, Zhang W, Cai Y, Kennelley ED. 2001. A fern that hyperaccumulates arsenic. *Nature* **409**: 579-579.

Maciaszczyk-Dziubinska E, Migdal I, Migocka M, Bocer T, Wysocki R. 2010. The yeast aquaglyceroporin Fps1p is a bidirectional arsenite channel. *Febs Letters* **584**: 726-732.

Maciaszczyk-Dziubinska E, Migocka M, Wysocki R. 2011. Acr3p is a plasma membrane antiporter that catalyzes As(III)/H^+ and Sb(III)/H^+ exchange in *Saccharomyces cerevisiae*. *Biochimica Et Biophysica Acta-Biomembranes* **1808**: 1855-1859.

MacLean KS, Langille WM. 1981. Arsenic in orchard and potato soils and plant tissue. *Plant and Soil* **61**: 413-418.

Macnair MR, Cumbes QJ, Meharg AA. 1992. The genetics of arsenate tolerance in yorkshire fog, *Holcus lanatus* L. *Heredity* **69**: 325-335.

Maharjan M, Watanabe C, Ahmad SA, Umezaki M, Ohtsuka R. 2007. Mutual interaction between nutritional status and chronic arsenic toxicity due to groundwater contamination in an area of Terai, lowland Nepal. *Journal of Epidemiology and Community Health* **61**: 389-394.

Mahdieh M, Mostajeran A, Horie T, Katsuhara M. 2008. Drought stress alters water relations and expression of PIP-type aquaporin genes in *Nicotiana tabacum* plants. *Plant Cell Physiol* **49**: 801-813.

Mandal A, Purakayastha TJ, Patra AK, Sanyal SK. 2012. Phytoremediation of arsenic contaminated soil by *Pteris vittata* L. II. Effect on arsenic uptake and rice yield. *International Journal of Phytoremediation* **14**: 621-628.

Mandal BK, Ogra Y, Anzai K, Suzuki KT. 2004. Speciation of arsenic in biological samples. *Toxicology and Applied Pharmacology* **198**: 307-318.

Mandal BK, Suzuki KT. 2002. Arsenic round the world: a review. *Talanta* **58**: 201-235.

Mansour NM, Sawhney M, Tamang DG, Vogl C, Saier MH. 2007. The bile/arsenite/riboflavin transporter (BART) superfamily. *Febs Journal* **274**: 612-629.
Marcuswyner L, Rains DW. 1982. Uptake, accumulation, and translocation of arsenical compounds by cotton. *Journal of Environmental Quality* **11**: 715-719.
Marger MD, Saier MH, Jr. 1993. A major superfamily of transmembrane facilitators that catalyse uniport, symport and antiport. *Trends in Biochemical Sciences* **18**: 13-20.
Marin AR, Masscheleyn PH, Patrick WH. 1992. The influence of chemical form and concentration of arsenic on rice growth and tissue arsenic concentration. *Plant and Soil* **139**: 175-183.
Marini L, Accornero M. 2007. Prediction of the thermodynamic properties of metal-arsenate and metal-arsenite aqueous complexes to high temperatures and pressures and some geological consequences. *Environmental Geology* **52**: 1343-1363.
Márquez-García B, Perez-Lopez R, Ruiz-Chancho MJ, Lopez-Sanchez JF, Rubio R, Abreu MM, Nieto JM, Cordoba F. 2012. Arsenic speciation in soils and *Erica andevalensis* Cabezudo & Rivera and *Erica australis* L. from São Domingos Mine area, Portugal. *Journal of Geochemical Exploration* **119**: 51-59.
Maurel C, Kado RT, Guern J, Chrispeels MJ. 1995. Phosphorylation regulates the water channel activity of the seed-specific aquaporin α-TIP. *Embo Journal* **14**: 3028-3035.
Maurel C, Verdoucq L, Luu DT, Santoni V. 2008. Plant aquaporins: Membrane channels with multiple integrated functions. *Annual Review of Plant Biology* **59**: 595-624.
McDermott JR, Jiang X, Beene LC, Rosen BP, Liu ZJ. 2010. Pentavalent methylated arsenicals are substrates of human AQP9. *Biometals* **23**: 119-127.
Meacher DM, Menzel DB, Dillencourt MD, Bic LF, Schoof RA, Yost LJ, Eickhoff JC, Farr CH. 2002. Estimation of multimedia inorganic arsenic intake in the US population. *Human and Ecological Risk Assessment* **8**: 1697-1721.
Meding SM, Zasoski RJ. 2008. Hyphal-mediated transfer of nitrate, arsenic, cesium, rubidium, and strontium between arbuscular mycorrhizal forbs and grasses from a California oak woodland. *Soil Biology & Biochemistry* **40**: 126-134.
Meharg A, MacNair M. 1992. Suppression of the high affinity phosphate uptake system: a mechanism of arsenate tolerance in *Holcus lanatus* L. *Journal of Experimental Botany* **43**: 519-524.
Meharg AA. 2004. Arsenic in rice - understanding a new disaster for South-East Asia. *Trends in Plant Science* **9**: 415-417.
Meharg AA, Deacon C, Campbell RCJ, Carey AM, Williams PN, Feldmann J, Raab A. 2008a. Inorganic arsenic levels in rice milk exceed EU and US drinking water standards. *Journal of Environmental Monitoring* **10**: 428-431.
Meharg AA, Hartley-Whitaker J. 2002. Arsenic uptake and metabolism in arsenic resistant and nonresistant plant species. *New Phytologist* **154**: 29-43.
Meharg AA, Lombi E, Williams PN, Scheckel KG, Feldmann J, Raab A, Zhu YG, Islam R. 2008b. Speciation and localization of arsenic in white and brown rice grains. *Environmental Science & Technology* **42**: 1051-1057.
Meharg AA, Macnair MR. 1990. An altered phosphate-uptake system in arsenate-tolerant *Holcus lanatus* L. . *New Phytologist* **116**: 29-35.
Meharg AA, Rahman M. 2003. Arsenic contamination of Bangladesh paddy field soils: Implications for rice contribution to arsenic consumption. *Environmental Science & Technology* **37**: 229-234.
Meharg AA, Sun GX, Williams PN, Adomako E, Deacon C, Zhu YG, Feldmann J, Raab A. 2008c. Inorganic arsenic levels in baby rice are of concern. *Environmental Pollution* **152**: 746-749.
Meharg AA, Williams PN, Adomako E, Lawgali YY, Deacon C, Villada A, Cambell RCJ, Sun G, Zhu YG, Feldmann J, Raab A, Zhao FJ, Islam R, Hossain S, Yanai J. 2009. Geographical Variation in Total and Inorganic Arsenic Content of Polished (White) Rice. *Environmental Science & Technology* **43**: 1612-1617.

Meharg C, Khan B, Norton G, Deacon C, Johnson D, Reinhardt R, Huettel B, Meharg AA. 2013. Trait-directed *de novo* population transcriptome dissects genetic regulation of a balanced polymorphism in phosphorus nutrition/arsenate tolerance in a wild grass, *Holcus lanatus*. *New Phytologist* DOI: 10.1111/nph.12491

Meinild AK, Klaerke DA, Zeuthen T. 1998. Bidirectional water fluxes and specificity for small hydrophilic molecules in aquaporins 0-5. *Journal of Biological Chemistry* **273**: 32446-32451.

Meliker JR, Franzblau A, Slotnick MJ, Nriagu JO. 2006. Major contributors to inorganic arsenic intake in southeastern Michigan. *International Journal of Hygiene and Environmental Health* **209**: 399-411.

Meng X-Y, Qin J, Wang L-H, Duan G-L, Sun G-X, Wu H-L, Chu C-C, Ling H-Q, Rosen BP, Zhu Y-G. 2011. Arsenic biotransformation and volatilization in transgenic rice. *New Phytologist* **191**: 49-56.

Meng YL, Liu ZJ, Rosen BP. 2004. As(III) and Sb(III) uptake by G1pF and efflux by ArsB in *Escherichia coli*. *Journal of Biological Chemistry* **279**: 18334-18341.

Messens J, Hayburn G, Desmyter A, Laus G, Wyns L. 1999. The essential catalytic redox couple in arsenate reductase from *Staphylococcus aureus*. *Biochemistry* **38**: 16857-16865.

Miller AJ, Zhou JJ. 2000. *Xenopus* oocytes as an expression system for plant transporters. *Biochimica Et Biophysica Acta-Biomembranes* **1465**: 343-358.

Miller EW, Dickinson BC, Chang CJ. 2010. Aquaporin-3 mediates hydrogen peroxide uptake to regulate downstream intracellular signaling. *Proceedings of the National Academy of Sciences of the United States of America* **107**: 15681-15686.

Ministry of Health of the People's Republic of China. 2005. *Maximum Levels of Contaminants in Foods.* **GB2762**. Beijing, China.

Mir KA, Rutter A, Koch I, Smith P, Reimer KJ, Poland JS. 2007. Extraction and speciation of arsenic in plants grown on arsenic contaminated soils. *Talanta* **72**: 1507-1518.

Mitani-Ueno N, Yamaji N, Zhao FJ, Ma JF. 2011. The aromatic/arginine selectivity filter of NIP aquaporins plays a critical role in substrate selectivity for silicon, boron, and arsenic. *Journal of Experimental Botany* **62**: 4391-4398.

Mitani N, Yamaji N, Ma JF. 2008. Characterization of substrate specificity of a rice silicon transporter, Lsi1. *Pflugers Archiv-European Journal of Physiology* **456**: 679-686.

Mitani N, Yamaji N, Ma JF. 2009. Identification of Maize Silicon Influx Transporters. *Plant and Cell Physiology* **50**: 5-12.

Mitsuoka K, Murata K, Walz T, Hirai T, Agre P, Heymann JB, Engel A, Fujiyoshi Y. 1999. The Structure of Aquaporin-1 at 4.5-Å Resolution Reveals Short α-Helices in the Center of the Monomer. *Journal of Structural Biology* **128**: 34-43.

Mizutani M, Watanabe S, Nakagawa T, Maeshima M. 2006. Aquaporin NIP2;1 is mainly localized to the ER membrane and shows root-specific accumulation in *Arabidopsis thaliana*. *Plant and Cell Physiology* **47**: 1420-1426.

Montpetit J, Vivancos J, Mitani-Ueno N, Yamaji N, Remus-Borel W, Belzile F, Ma JF, Belanger RR. 2012. Cloning, functional characterization and heterologous expression of *TaLsi1*, a wheat silicon transporter gene. *Plant Molecular Biology* **79**: 35-46.

Moore KL, Hawes CR, McGrath SP, Zhao FJ, Grovenor CRM. 2013. High resolution SIMS analysis of arsenic in rice. *Surface and Interface Analysis* **45**: 309-311.

Moore KL, Schroder M, Lombi E, Zhao FJ, McGrath SP, Hawkesford MJ, Shewry PR, Grovenor CRM. 2010. NanoSIMS analysis of arsenic and selenium in cereal grain. *New Phytologist* **185**: 434-445.

Mosa KA, Kumar K, Chhikara S, McDermott J, Liu ZJ, Musante C, White JC, Dhankher OP. 2012. Members of rice plasma membrane intrinsic proteins subfamily are involved in arsenite permeability and tolerance in plants. *Transgenic Research* **21**: 1265-1277.

Muchhal US, Pardo JM, Raghothama KG. 1996. Phosphate transporters from the higher plant *Arabidopsis thaliana*. *Proceedings of the National Academy of Sciences of the United States of America* **93**: 10519-10523.

Mukhopadhyay R, Rosen BP. 1998. *Saccharomyces cerevisiae ACR2* gene encodes an arsenate reductase. *Fems Microbiology Letters* **168**: 127-136.

Mukhopadhyay R, Rosen BP. 2002. Arsenate reductases in prokaryotes and eukaryotes. *Environmental Health Perspectives* **110**: 745-748.

Munoz O, Diaz OP, Leyton I, Nunez N, Devesa V, Suner MA, Velez D, Montoro R. 2002. Vegetables collected in the cultivated Andean area of northern Chile: Total and inorganic arsenic contents in raw vegetables. *Journal of Agricultural and Food Chemistry* **50**: 642-647.

Murashige T, Skoog F. 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia Plantarum* **15**: 473-&.

Murata K, Mitsuoka K, Hirai T, Walz T, Agre P, Heymann JB, Engel A, Fujiyoshi Y. 2000. Structural determinants of water permeation through aquaporin-1. *Nature* **407**: 599-605.

Murozuka E, Hanisch S, Pomorski TG, Jahn TP, Schjoerring JK. 2013. Bimolecular fluorescence complementation and interaction of various *Arabidopsis* major intrinsic proteins expressed in yeast. *Physiologia Plantarum* **148**: 422-431.

Nachman KE, Baron PA, Raber G, Francesconi KA, Navas-Acien A, Love DC. 2013. Roxarsone, Inorganic Arsenic, and Other Arsenic Species in Chicken: A U.S.-Based Market Basket Sample. *Environmental Health Perspectives* **121**: 818-824.

Nahar N, Rahman A, Mos M, Warzecha T, Algerin M, Ghosh S, Johnson-Brousseau S, Mandal A. 2012. *In silico* and *in vivo* studies of an *Arabidopsis thaliana* gene, *ACR2,* putatively involved in arsenic accumulation in plants. *Journal of Molecular Modeling* **18**: 4249-4262.

Nakamura Y, Gojobori T, Ikemura T. 2000. Codon usage tabulated from international DNA sequence databases: status for the year 2000. *Nucleic Acids Research* **28**: 292-292.

Nakhoul NL, Davis BA, Romero MF, Boron WF. 1998. Effect of expressing the water channel aquaporin-1 on the CO_2 permeability of *Xenopus* oocytes. *American Journal of Physiology-Cell Physiology* **274**: C543-C548.

Negishi T, Oshima K, Hattori M, Kanai M, Mano S, Nishimura M, Yoshida K. 2012. Tonoplast- and Plasma Membrane-Localized Aquaporin-Family Transporters in Blue Hydrangea Sepals of Aluminum Hyperaccumulating Plant. *PloS one* **7**.

Németh-Cahalan KL, Hall JE. 2000. pH and Calcium Regulate the Water Permeability of Aquaporin 0. *Journal of Biological Chemistry* **275**: 6777-6782.

Németi B, Gregus Z. 2002. Mitochondria Work as Reactors in Reducing Arsenate to Arsenite. *Toxicology and Applied Pharmacology* **182**: 208-218.

Németi B, Gregus Z. 2007. Glutathione-dependent reduction of arsenate by glycogen phosphorylase - A reaction coupled to glycogenolysis. *Toxicological Sciences* **100**: 36-43.

Németi B, Gregus Z. 2009. Mechanism of Thiol-Supported Arsenate Reduction Mediated by Phosphorolytic-Arsenolytic Enzymes. *Toxicological Sciences* **110**: 270-281.

Németi B, Regonesi ME, Tortora P, Gregus Z. 2010. Polynucleotide Phosphorylase and Mitochondrial ATP Synthase Mediate Reduction of Arsenate to the More Toxic Arsenite by Forming Arsenylated Analogues of ADP and ATP. *Toxicological Sciences* **117**: 270-281.

Nguyen MX, Moon S, Jung KH. 2013. Genome-wide expression analysis of rice aquaporin genes and development of a functional gene network mediated by aquaporin expression in roots. *Planta* **238**: 669-681.

Nikolic M, Nikolic N, Liang YC, Kirkby EA, Romheld V. 2007. Germanium-68 as an Adequate Tracer for Silicon Transport in Plants. Characterization of Silicon Uptake in Different Crop Species. *Plant Physiology* **143**: 495-503.

Ninno Cd, Dorosh PA. 2001. Averting a food crisis: private imports and public targeted distribution in Bangladesh after the 1998 flood. *Agricultural Economics* **25**: 337-346.

Nissen P, Benson AA. 1982. Arsenic Metabolism In Fresh-Water and Terrestrial Plants. *Physiologia Plantarum* **54**: 446-450.

Norton GJ, Adomako EE, Deacon CM, Carey AM, Price AH, Meharg AA. 2013. Effect of organic matter amendment, arsenic amendment and water management regime on rice grain arsenic species. *Environmental Pollution* **177**: 38-47.

Norton GJ, Duan GL, Dasgupta T, Islam MR, Lei M, Zhu YG, Deacon CM, Moran AC, Islam S, Zhao FJ, Stroud JL, McGrath SP, Feldmann J, Price AH, Meharg AA. 2009a. Environmental and Genetic Control of Arsenic Accumulation and Speciation in Rice Grain: Comparing a Range of Common Cultivars Grown in Contaminated Sites Across Bangladesh, China, and India. *Environmental Science & Technology* **43**: 8381-8386.

Norton GJ, Islam MR, Deacon CM, Zhao FJ, Stroud JL, McGrath SP, Islam S, Jahiruddin M, Feldmann J, Price AH, Meharg AA. 2009b. Identification of low inorganic and total grain arsenic rice cultivars from Bangladesh. *Environmental Science & Technology* **43**: 6070–6075.

Norton GJ, Lou-Hing DE, Meharg AA, Price AH. 2008. Rice-arsenate interactions in hydroponics: whole genome transcriptional analysis. *Journal of Experimental Botany* **59**: 2267-2276.

Nozawa A, Takano J, Kobayashi M, Von Wirén N, Fujiwara T. 2006. Roles of BOR1, DUR3, and FPS1 in boron transport and tolerance in *Saccharomyces cerevisiae*. *Fems Microbiology Letters* **262**: 216-222.

NRC. 1999. *Arsenic in Drinking Water*. Washington, USA: National Academy of Sciences.

O'Neill MA, Ishii T, Albersheim P, Darvill AG. 2004. RHAMNOGALACTURONAN II: Structure and Function of a Borate Cross-Linked Cell Wall Pectic Polysaccharide. *Annual Review of Plant Biology* **55**: 109-139.

Ohno K, Yanase T, Matsuo Y, Kimura T, Rahman MH, Magara Y, Matsui Y. 2007. Arsenic intake via water and food by a population living in an arsenic-affected area of Bangladesh. *Science of the Total Environment* **381**: 68-76.

Oremland RS, Stolz JF. 2003. The ecology of arsenic. *Science* **300**: 939-944.

Otto B, Uehlein N, Sdorra S, Fischer M, Ayaz M, Belastegui-Macadam X, Heckwolf M, Lachnit M, Pede N, Priem N, Reinhard A, Siegfart S, Urban M, Kaldenhoff R. 2010. Aquaporin Tetramer Composition Modifies the Function of Tobacco Aquaporins. *Journal of Biological Chemistry* **285**: 31253-31260.

Pal A, Nayak B, Das B, Hossain MA, Ahamed S, Chakraborti D. 2007. Additional danger of arsenic exposure through inhalation from burning of cow dung cakes laced with arsenic as a fuel in arsenic affected villages in Ganga-Meghna-Brahmaputra plain. *Journal of Environmental Monitoring* **9**: 1067-1070.

Panaullah GM, Alam T, Hossain MB, Loeppert RH, Lauren JG, Meisner CA, Ahmed ZU, Duxbury JM. 2009. Arsenic toxicity to rice (*Oryza sativa* L.) in Bangladesh. *Plant and Soil* **317**: 31-39.

Panda SK, Upadhyay RK, Nath S. 2010. Arsenic Stress in Plants. *Journal of Agronomy and Crop Science* **196**: 161-174.

Park J, Song WY, Ko D, Eom Y, Hansen TH, Schiller M, Lee TG, Martinoia E, Lee Y. 2012. The phytochelatin transporters AtABCC1 and AtABCC2 mediate tolerance to cadmium and mercury. *Plant Journal* **69**: 278-288.

Paszkowski U, Kroken S, Roux C, Briggs SP. 2002. Rice phosphate transporters include an evolutionarily divergent gene specifically activated in arbuscular mycorrhizal symbiosis. *Proceedings of the National Academy of Sciences* **99**: 13324-13329.

Persson B, Lagerstedt J, Pratt J, Pattison-Granberg J, Lundh K, Shokrollahzadeh S, Lundh F. 2003. Regulation of phosphate acquisition in *Saccharomyces cerevisiae*. *Current Genetics* **43**: 225-244.

Pickering IJ, Prince RC, George MJ, Smith RD, George GN, Salt DE. 2000. Reduction and coordination of arsenic in Indian mustard. *Plant Physiology* **122**: 1171-1177.

Pillai TR, Yan WG, Agrama HA, James WD, Ibrahim AMH, McClung AM, Gentry TJ, Loeppert RH. 2010. Total Grain-Arsenic and Arsenic-Species Concentrations in Diverse Rice Cultivars under Flooded Conditions. *Crop Science* **50**: 2065-2075.

Pomes R, Roux B. 1996. Structure and dynamics of a proton wire: A theoretical study of H^+ translocation along the single-file water chain in the gramicidin a channel. *Biophysical Journal* **71**: 19-39.

Poynton CY, Huang JWW, Blaylock MJ, Kochian LV, Elless MP. 2004. Mechanisms of arsenic hyperaccumulation in *Pteris* species: root As influx and translocation. *Planta* **219**: 1080-1088.

Preston GM, Agre P. 1991. Isolation of the cDNA for erythrocyte integral membrane protein of 28 kilodaltons: member of an ancient channel family. *Proceedings of the National Academy of Sciences* **88**: 11110-11114.

Preston GM, Carroll TP, Guggino WB, Agre P. 1992. Appearance of water channels in *Xenopus* oocytes expressing red cell CHIP28 protein. *Science* **256**: 385-387.

Preuss CP, Huang CY, Gilliham M, Tyerman SD. 2010. Channel-Like Characteristics of the Low-Affinity Barley Phosphate Transporter PHT1;6 When Expressed in *Xenopus* Oocytes. *Plant Physiology* **152**: 1431-1441.

Preuss CP, Huang CY, Tyerman SD. 2011. Proton-coupled high-affinity phosphate transport revealed from heterologous characterization in *Xenopus* of barley-root plasma membrane transporter, HvPHT1;1. *Plant Cell and Environment* **34**: 681-689.

Prévéral S, Ansoborlo E, Mari S, Vavasseur A, Forestier C. 2006. Metal(loid)s and radionuclides cytotoxicity in *Saccharomyces cerevisiae*. Role of YCF1, glutathione and effect of buthionine sulfoximine. *Biochimie* **88**: 1651-1663.

Qin J, Lehr CR, Yuan CG, Le XC, McDermott TR, Rosen BP. 2009. Biotransformation of arsenic by a Yellowstone thermoacidophilic eukaryotic alga. *Proceedings of the National Academy of Sciences of the United States of America* **106**: 5213-5217.

Qin J, Rosen BP, Zhang Y, Wang GJ, Franke S, Rensing C. 2006. Arsenic detoxification and evolution of trimethylarsine gas by a microbial arsenite *S*-adenosylmethionine methyltransferase. *Proceedings of the National Academy of Sciences of the United States of America* **103**: 2075-2080.

Quaghebeur M, Rengel Z. 2003. The distribution of arsenate and arsenite in shoots and roots of *Holcus lanatus* is influenced by arsenic tolerance and arsenate and phosphate supply. *Plant Physiology* **132**: 1600-1609.

Raab A, Feldmann J, Meharg AA. 2004. The nature of arsenic-phytochelatin complexes in *Holcus lanatus* and *Pteris cretica*. *Plant Physiology* **134**: 1113-1122.

Raab A, Ferreira K, Meharg AA, Feldmann J. 2007a. Can arsenic-phytochelatin complex formation be used as an indicator for toxicity in *Helianthus annuus*? *Journal of Experimental Botany* **58**: 1333-1338.

Raab A, Schat H, Meharg AA, Feldmann J. 2005. Uptake, translocation and transformation of arsenate and arsenite in sunflower (*Helianthus annuus*): formation of arsenic-phytochelatin complexes during exposure to high arsenic concentrations. *New Phytologist* **168**: 551-558.

Raab A, Williams PN, Meharg A, Feldmann J. 2007b. Uptake and translocation of inorganic and methylated arsenic species by plants. *Environmental Chemistry* **4**: 197-203.

Raab A, Wright SH, Jaspars M, Meharg AA, Feldmann J. 2007c. Pentavalent arsenic can bind to biomolecules. *Angewandte Chemie-International Edition* **46**: 2594-2597.

Rahman MA, Hasegawa H, Rahman MM, Miah MAM. 2007. Accumulation of arsenic in tissues of rice plant (*Oryza sativa* L.) and its distribution in fractions of rice grain. *Chemosphere* **69**: 942-948.

Rains DW, Epstein E, Zasoski RJ, Aslam M. 2006. Active silicon uptake by wheat. *Plant and Soil* **280**: 223-228.

Rathinasabapathi B, Wu S, Sundaram S, Rivoal J, Srivastava M, Ma LQ. 2006. Arsenic resistance in *Pteris vittata* L.: identification of a cytosolic triosephosphate isomerase based on cDNA expression cloning in *Escherichia coli*. *Plant Molecular Biology* **62**: 845-857.

Ravenscroft P, Brammer H, Richards K. 2009. *Arsenic Pollution: A Global Synthesis*: John Wiley & Sons.

Reaves ML, Sinha S, Rabinowitz JD, Kruglyak L, Redfield RJ. 2012. Absence of Detectable Arsenate in DNA from Arsenate-Grown GFAJ-1 Cells. *Science* **337**: 470-473.

Ren X, Liu Q, Wu D, Shu Q. 2006. Variations in concentration and distribution of health-related elements affected by environmental and genotypic differences in rice grains. *Rice Science* **13**: 170-178.

Rezanka T, Sigler K. 2008. Biologically active compounds of semi-metals. *Phytochemistry* **69**: 585-606.

Rivers RL, Dean RM, Chandy G, Hall JE, Roberts DM, Zeidel ML. 1997. Functional analysis of nodulin 26, an aquaporin in soybean root nodule symbiosomes. *Journal of Biological Chemistry* **272**: 16256-16261.

Rodríguez-Lado L, Sun GF, Berg M, Zhang Q, Xue HB, Zheng QM, Johnson CA. 2013. Groundwater Arsenic Contamination Throughout China. *Science* **341**: 866-868.

Rosen BP. 1999. Families of arsenic transporters. *Trends in Microbiology* **7**: 207-212.

Rouge P, Barre A. 2008. A molecular modeling approach defines a new group of Nodulin 26-like aquaporins in plants. *Biochemical and Biophysical Research Communications* **367**: 60-66.

Rubio V, Linhares F, Solano R, Martin AC, Iglesias J, Leyva A, Paz-Ares J. 2001. A conserved MYB transcription factor involved in phosphate starvation signaling both in vascular plants and in unicellular algae. *Genes & Development* **15**: 2122-2133.

Ruiz-Chancho MJ, Lopez-Sanchez JF, Rubio R. 2011. Occurrence of methylated arsenic species in parts of plants growing in polluted soils. *International Journal of Environmental Analytical Chemistry* **91**: 844-855.

Ruiz-Chancho MJ, Lopez-Sanchez JF, Schmeisser E, Goessler W, Francesconi KA, Rubio R. 2008. Arsenic speciation in plants growing in arsenic-contaminated sites. *Chemosphere* **71**: 1522-1530.

Sakano K. 1990. Proton/Phosphate Stoichiometry in Uptake of Inorganic Phosphate by Cultured Cells of *Catharanthus roseus* (L.) G. Don. *Plant Physiology* **93**: 479-483.

Sakurai J, Ishikawa F, Yamaguchi T, Uemura M, Maeshima M. 2005. Identification of 33 rice aquaporin genes and analysis of their expression and function. *Plant and Cell Physiology* **46**: 1568-1577.

Sakurai T, Kaise T, Matsubara C. 1998. Inorganic and methylated arsenic compounds induce cell death in murine macrophages via different mechanisms. *Chemical Research in Toxicology* **11**: 273-283.

Sanders OI, Rensing C, Kuroda M, Mitra B, Rosen BP. 1997. Antimonite is accumulated by the glycerol facilitator GlpF in *Escherichia coli*. *Journal of Bacteriology* **179**: 3365-3367.

Santoni V, Verdoucq L, Sommerer N, Vinh J, Pflieger D, Maurel C. 2006. Methylation of aquaporins in plant plasma membrane. *The Biochemical Journal* **400**: 189-197.

Sanz MA, Fenaux P, Lo Coco F, European APLGE. 2005. Arsenic trioxide in the treatment of acute promyelocytic leukemia. A review of current evidence. *Haematologica-the Hematology Journal* **90**: 1231-1235.

Savage DF, O'Connell JD, Miercke LJW, Finer-Moore J, Stroud RM. 2010. Structural context shapes the aquaporin selectivity filter. *Proceedings of the National Academy of Sciences of the United States of America* **107**: 17164-17169.

Schmoger MEV, Oven M, Grill E. 2000. Detoxification of arsenic by phytochelatins in plants. *Plant Physiology* **122**: 793-801.

Schnurbusch T, Hayes J, Hrmova M, Baumann U, Ramesh SA, Tyerman SD, Langridge P, Sutton T. 2010. Boron Toxicity Tolerance in Barley through Reduced Expression of the Multifunctional Aquaporin HvNIP2;1. *Plant Physiology* **153**: 1706-1715.

Schoof RA, Yost LJ, Eickhoff J, Crecelius EA, Cragin DW, Meacher DM, Menzel DB. 1999. A market basket survey of inorganic arsenic in food. *Food and Chemical Toxicology* **37**: 839-846.

Schuhmacher-Wolz U, Dieter HH, Klein D, Schneider K. 2009. Oral exposure to inorganic arsenic: evaluation of its carcinogenic and non-carcinogenic effects. *Critical Reviews in Toxicology* **39**: 271-298.

Schuurmans J, van Dongen JT, Rutjens BPW, Boonman A, Pieterse CMJ, Borstlap AC. 2003. Members of the aquaporin family in the developing pea seed coat include representatives of the PIP, TIP, and NIP subfamilies. *Plant Molecular Biology* **53**: 655-667.

Shanahan CM, Connolly DL, Tyson KL, Cary NRB, Osbourn JK, Agre P, Weissberg PL. 1999. Aquaporin-1 is expressed by vascular smooth muscle cells and mediates rapid water transport across vascular cell membranes. *Journal of Vascular Research* **36**: 353-362.

Shimizu M, Arai Y, Sparks DL. 2011a. Multiscale Assessment of Methylarsenic Reactivity in Soil. 1. Sorption and Desorption on Soils. *Environmental Science & Technology* **45**: 4293-4299.

Shimizu M, Arai Y, Sparks DL. 2011b. Multiscale Assessment of Methylarsenic Reactivity in Soil. 2. Distribution and Speciation in Soil. *Environmental Science & Technology* **45**: 4300-4306.

Shin H, Shin HS, Dewbre GR, Harrison MJ. 2004. Phosphate transport in Arabidopsis: Pht1;1 and Pht1;4 play a major role in phosphate acquisition from both low- and high-phosphate environments. *Plant Journal* **39**: 629-642.

Signes-Pastor AJ, Deacon C, Jenkins RO, Haris PI, Carbonell-Barrachina AA, Meharg AA. 2009. Arsenic speciation in Japanese rice drinks and condiments. *Journal of Environmental Monitoring* **11**: 1930-1934.

Signes-Pastor AJ, Mitra K, Sarkhel S, Hobbes M, Burlo F, Groot WTd, Carbonell-Barrachina AA. 2008. Arsenic speciation in food and estimation of the dietary intake of inorganic arsenic in a rural village of West Bengal, India. *Journal of Agricultural and Food Chemistry* **56**: 9469-9474.

Šlejkovec Z, van Elteren JT, Glass HJ, Jeran Z, Jacimovic R. 2010. Speciation analysis to unravel the soil-to-plant transfer in highly arsenic-contaminated areas in Cornwall (UK). *International Journal of Environmental Analytical Chemistry* **90**: 784-796.

Smith AH, Hopenhaynrich C, Bates MN, Goeden HM, Hertzpicciotto I, Duggan HM, Wood R, Kosnett MJ, Smith MT. 1992. Cancer risks from arsenic in drinking-water. *Environmental Health Perspectives* **97**: 259-267.

Smith AH, Lopipero PA, Bates MN, Steinmaus CM. 2002. Public health - Arsenic epidemiology and drinking water standards. *Science* **296**: 2145-2146.

Smith BL, Agre P. 1991. Erythrocyte M_r 28,000 transmembrane protein exists as a multisubunit oligomer similar to channel proteins. *Journal of Biological Chemistry* **266**: 6407-6415.

Smith FA, Raven JA. 1979. Intracellular pH and its regulation. *Annual Review of Plant Physiology and Plant Molecular Biology* **30**: 289-311.

Smith SE, Christophersen HM, Pope S, Smith FA. 2010. Arsenic uptake and toxicity in plants: integrating mycorrhizal influences. *Plant and Soil* **327**: 1-21.

Sneller FEC, van Heerwaarden LM, Koevoets PLM, Vooijs R, Schat H, Verkleij JAC. 2000. Derivatization of phytochelatins from *Silene vulgaris*, induced upon exposure to arsenate and cadmium: Comparison of derivatization with Ellman's reagent and monobromobimane. *Journal of Agricultural and Food Chemistry* **48**: 4014-4019.

Song WY, Park J, Mendoza-Cozatl DG, Suter-Grotemeyer M, Shim D, Hortensteiner S, Geisler M, Weder B, Rea PA, Rentsch D, Schroeder JI, Lee Y, Martinoia E. 2010. Arsenic tolerance in Arabidopsis is mediated by two ABCC-type phytochelatin transporters. *Proceedings of the National Academy of Sciences of the United States of America* **107**: 21187-21192.

Song WY, Sohn EJ, Martinoia E, Lee YJ, Yang YY, Jasinski M, Forestier C, Hwang I, Lee Y. 2003. Engineering tolerance and accumulation of lead and cadmium in transgenic plants. *Nature Biotechnology* **21**: 914-919.

Stolz JF, Perera E, Kilonzo B, Kail B, Crable B, Fisher E, Ranganathan M, Wormer L, Basu P. 2007. Biotransformation of 3-nitro-4-hydroxybenzene arsonic acid (roxarsone) and release of inorganic arsenic by *Clostridium* species. *Environmental Science & Technology* **41**: 818-823.

Styblo M, Thomas DJ. 1997. Binding of arsenicals to proteins in an *in vitro* methylation system. *Toxicology and Applied Pharmacology* **147**: 1-8.

Su YH, McGrath SP, Zhao FJ. 2010. Rice is more efficient in arsenite uptake and translocation than wheat and barley. *Plant and Soil* **328**: 27-34.

Su YH, McGrath SP, Zhu YG, Zhao FJ. 2008. Highly efficient xylem transport of arsenite in the arsenic hyperaccumulator *Pteris vittata*. *New Phytologist* **180**: 434-441.

Sui HX, Han BG, Lee JK, Walian P, Jap BK. 2001. Structural basis of water-specific transport through the AQP1 water channel. *Nature* **414**: 872-878.

Sun G, Williams PN, Zhu Y, Deacon C, Carey AM, Raab A, Feldmann J, Meharg AA. 2009. Survey of arsenic and its speciation in rice products such as breakfast cereals, rice crackers and Japanese rice condiments. *Environment International* **35**: 473-475.

Sun GX, Williams PN, Carey AM, Zhu YG, Deacon C, Raab A, Feldmann J, Islam RM, Meharg AA. 2008. Inorganic arsenic in rice bran and its products are an order of magnitude higher than in bulk grain. *Environmental Science & Technology* **42**: 7542-7546.

Sun SB, Gu MA, Cao Y, Huang XP, Zhang X, Ai PH, Zhao JN, Fan XR, Xu GH. 2012. A Constitutive Expressed Phosphate Transporter, OsPht1;1, Modulates Phosphate Uptake and Translocation in Phosphate-Replete Rice. *Plant Physiology* **159**: 1571-1581.

Sundaram S, Rathinasabapathi B, Ma LQ, Rosen BP. 2008. An arsenate-activated glutaredoxin from the arsenic hyperaccumulator fern *Pteris vittata* L. regulates intracellular arsenite. *Journal of Biological Chemistry* **283**: 6095-6101.

Sundaram S, Wu S, Ma LQ, Rathinasabapathi B. 2009. Expression of a *Pteris vittata* glutaredoxin *PvGRX5* in transgenic *Arabidopsis thaliana* increases plant arsenic tolerance and decreases arsenic accumulation in the leaves. *Plant Cell and Environment* **32**: 851-858.

Száková J, Havlik J, Valterova B, Tlustos P, Goessler W. 2010. The contents of risk elements, arsenic speciation, and possible interactions of elements and betalains in beetroot (*Beta vulgaris*, L.) growing in contaminated soil. *Central European Journal of Biology* **5**: 692-701.

Száková J, Tlustos P, Goessler W, Pavlikova D, Schmeisser E. 2007. Response of pepper plants (*Capsicum annum* L.) on soil amendment by inorganic and organic compounds of arsenic. *Archives of Environmental Contamination and Toxicology* **52**: 38-46.

Tajkhorshid E, Nollert P, Jensen MØ, Miercke LJW, O'Connell J, Stroud RM, Schulten K. 2002. Control of the Selectivity of the Aquaporin Water Channel Family by Global Orientational Tuning. *Science* **296**: 525-530.

Takabatake R, Hata S, Taniguchi M, Kouchi H, Sugiyama T, Izui K. 1999. Isolation and characterization of cDNAs encoding mitochondrial phosphate transporters in soybean, maize, rice, and *Arabidopsis*. *Plant Molecular Biology* **40**: 479-486.

Takamatsu T, Aoki H, Yoshida T. 1982. Determination of arsenate, arsenite, monomethylarsonate, and dimethylarsinate in soil polluted with arsenic. *Soil Science* **133**: 239-246.

Takano J, Wada M, Ludewig U, Schaaf G, von Wiren N, 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.

Talukder ASMHM, Meisner CA, Sarkar MAR, Islam MS, Sayre KD, Duxbury JM, Lauren JG. 2012. Effect of water management, arsenic and phosphorus levels on rice in a high-arsenic soil–water system: II. Arsenic uptake. *Ecotoxicology and Environmental Safety* **80**: 145-151.

Tanaka M, Wallace IS, Takano J, Roberts DM, Fujiwara T. 2008. NIP6;1 is a boric acid channel for preferential transport of boron to growing shoot tissues in *Arabidopsis*. *Plant Cell* **20**: 2860-2875.

Thakur JK, Thakur RK, Ramanathan AL, Kumar M, Singh SK. 2011. Arsenic Contamination of Groundwater in Nepal-An Overview. *Water* **3**: 1-20.

Theodoulou FL, Miller AJ. 1995. *Xenopus* oocytes as a heterologous expression system for plant-proteins. *Molecular Biotechnology* **3**: 101-115.

Thomas DJ, Styblo M, Lin S. 2001. The cellular metabolism and systemic toxicity of arsenic. *Toxicology and Applied Pharmacology* **176**: 127-144.

Thomas DJ, Waters SB, Styblo M. 2004. Elucidating the pathway for arsenic methylation. *Toxicology and Applied Pharmacology* **198**: 319-326.

Tlustoš P, Goessler W, Szakova J, Balik J. 2002. Arsenic compounds in leaves and roots of radish grown in soil treated by arsenite, arsenate and dimethylarsinic acid. *Applied Organometallic Chemistry* **16**: 216-220.

Törnroth-Horsefield S, Wang Y, Hedfalk K, Johanson U, Karlsson M, Tajkhorshid E, Neutze R, Kjellbom P. 2006. Structural mechanism of plant aquaporin gating. *Nature* **439**: 688-694.

Tournaire-Roux C, Sutka M, Javot H, Gout E, Gerbeau P, Luu D-T, Bligny R, Maurel C. 2003. Cytosolic pH regulates root water transport during anoxic stress through gating of aquaporins. *Nature* **425**: 393-397.

Tsuda T, Inoue T, Kojima M, Aoki S. 1995. Market basket and duplicate portion estimation of dietary intakes of cadmium, mercury, arsenic, copper, manganese, and zinc by Japanese adults. *Journal of Aoac International* **78**: 1363-1368.

Tu SI, Cavanaugh JR, Boswell RT. 1990. Phosphate-uptake by excised maize root-tips studied by *in vivo* ^{31}P nuclear-magnetic-resonance spectroscopy. *Plant Physiology* **93**: 778-784.

Turpeinen R, Pantsar-Kallio M, Kairesalo T. 2002. Role of microbes in controlling the speciation of arsenic and production of arsines in contaminated soils. *Science of the Total Environment* **285**: 133-145.

Uehlein N, Lovisolo C, Siefritz F, Kaldenhoff R. 2003. The tobacco aquaporin NtAQP1 is a membrane CO_2 pore with physiological functions. *Nature* **425**: 734-737.

Uehlein N, Sperling H, Heckwolf M, Kaldenhoff R. 2012. The *Arabidopsis* aquaporin PIP1;2 rules cellular CO_2 uptake. *Plant Cell and Environment* **35**: 1077-1083.

Ullrich-Eberius CI, Novacky A, Fischer E, Lüttge U. 1981. Relationship between energy-dependent phosphate-uptake and the electrical membrane-potential in *Lemna gibba G1*. *Plant Physiology* **67**: 797-801.

Ullrich-Eberius CI, Sanz A, Novacky AJ. 1989. Evaluation of Arsenate- and Vanadate-Associated Changes of Electrical Membrane Potential and Phosphate Transport in *Lemna gibba* G1. *Journal of Experimental Botany* **40**: 119-128.

Ultra VUY, Tanaka S, Sakurai K, Iwasaki K. 2007. Arbuscular mycorrhizal fungus (*Glomus aggregatum*) influences biotransformation of arsenic in the rhizosphere of sunflower (*Helianthus annuus* L.). *Soil Science and Plant Nutrition* **53**: 499-508.

USDA Foreign Agricultural Service. 2006. *People's Republic of China FAIRS Product Specific Maximum Levels of Contaminants in Foods.* Global Agriculture Information Network Report **CH6064**.

Vahter M. 2008. Health effects of early life exposure to arsenic. *Basic & Clinical Pharmacology & Toxicology* **102**: 204-211.

Vandeleur RK, Mayo G, Shelden MC, Gilliham M, Kaiser BN, Tyerman SD. 2009. The role of plasma membrane intrinsic protein aquaporins in water transport through roots: diurnal and drought stress responses reveal different strategies between isohydric and anisohydric cultivars of grapevine. *Plant Physiology* **149**: 445-460.

Vanveldhoven PP, Mannaerts GP. 1987. Inorganic and organic phosphate measurements in the nanomolar range. *Analytical Biochemistry* **161**: 45-48.

Vera-Estrella R, Barkla BJ, Bohnert HJ, Pantoja O. 2004. Novel Regulation of Aquaporins during Osmotic Stress. *Plant Physiology* **135**: 2318-2329.

Verkman AS. 2002. Does aquaporin-1 pass gas? - An opposing view. *Journal of Physiology-London* **542**: 31-31.

Versaw WK, Harrison MJ. 2002. A chloroplast phosphate transporter, PHT2;1, influences allocation of phosphate within the plant and phosphate-starvation responses. *The Plant Cell* **14**: 1751-1766.

Villa-Bellosta R, Sorribas V. 2010. Arsenate transport by sodium/phosphate cotransporter type IIb. *Toxicology and Applied Pharmacology* **247**: 36-40.

Vincent JM. 1970. *A Manual for the Practical Study of Root-Nodule Bacteria*. Oxford: Blackwell Scientific.

Virkki LV, Biber J, Murer H, Forster IC. 2007. Phosphate transporters: a tale of two solute carrier families. *American Journal of Physiology-Renal Physiology* **293**: F643-F654.

Wallace IS, Choi WG, Roberts DM. 2006. The structure, function and regulation of the nodulin 26-like intrinsic protein family of plant aquaglyceroporins. *Biochimica Et Biophysica Acta-Biomembranes* **1758**: 1165-1175.

Wallace IS, Roberts DM. 2005. Distinct transport selectivity of two structural subclasses of the nodulin-like intrinsic protein family of plant aquaglyceroporin channels. *Biochemistry* **44**: 16826-16834.

Wallace IS, Wills DM, Guenther JF, Roberts DM. 2002. Functional selectivity for glycerol of the nodulin 26 subfamily of plant membrane intrinsic proteins. *Febs Letters* **523**: 109-112.

Walser M. 1961. Ion association. IV. Interactions between calcium, magnesium, inorganic phosphate, citrate and protein in normal human plasma. *Journal of Clinical Investigation* **40**: 723-&.

Wang C, Ying S, Huang HJ, Li K, Wu P, Shou HX. 2009. Involvement of OsSPX1 in phosphate homeostasis in rice. *Plant Journal* **57**: 895-904.

Wang JR, Zhao FJ, Meharg AA, Raab A, Feldmann J, McGrath SP. 2002. Mechanisms of arsenic hyperaccumulation in *Pteris vittata*. Uptake kinetics, interactions with phosphate, and arsenic speciation. *Plant Physiology* **130**: 1552-1561.

Weaver CD, Roberts DM. 1991. Phosphorylation of nodulin-26 by a calcium-dependent protein-kinase. *Faseb Journal* **5**: A426-A426.

Weig AR, Jakob C. 2000. Functional identification of the glycerol permease activity of *Arabidopsis thaliana* NLM1 and NLM2 proteins by heterologous expression in *Saccharomyces cerevisiae*. *Febs Letters* **481**: 293-298.

WHO. 1993. *Guidelines for drinking-water quality, Second edition, Vol. 1. Recommendations.* Geneva, Switzerland.

Williams PN, Raab A, Feldmann J, Meharg AA. 2007a. Market basket survey shows elevated levels of As in South Central US processed rice compared to California: Consequences for human dietary exposure. *Environmental Science & Technology* **41**: 2178-2183.

Williams PN, Villada A, Deacon C, Raab A, Figuerola J, Green AJ, Feldmann J, Meharg AA. 2007b. Greatly enhanced arsenic shoot assimilation in rice leads to elevated grain levels compared to wheat and barley. *Environmental Science & Technology* **41**: 6854-6859.

Willsky GR, Malamy MH. 1980. Effect of arsenate on inorganic phosphate transport in *Escherichia coli*. *Journal of Bacteriology* **144**: 366-374.

Wilson D, Hooper C, Shi XY. 2012. Arsenic and Lead in Juice: Apple, Citrus, and Apple-Base. *Journal of Environmental Health* **75**: 14-20.

Wolfe-Simon F, Blum JS, Kulp TR, Gordon GW, Hoeft SE, Pett-Ridge J, Stolz JF, Webb SM, Weber PK, Davies PCW, Anbar AD, Oremland RS. 2011. A Bacterium That Can Grow by Using Arsenic Instead of Phosphorus. *Science* **332**: 1163-1166.

Woolson EA, Aharonson N, Iadevaia R. 1982. Application of the high-performance liquid chromatography-flameless atomic absorption method to the study of alkyl arsenical herbicide metabolism in soil. *Journal of Agricultural and Food Chemistry* **30**: 580-584.

Wree D, Wu BH, Zeuthen T, Beitz E. 2011. Requirement for asparagine in the aquaporin NPA sequence signature motifs for cation exclusion. *Febs Journal* **278**: 740-748.

Wu B, Beitz E. 2007. Aquaporins with selectivity for unconventional permeants. *Cellular and Molecular Life Sciences* **64**: 2413-2421.

Wu JH, Zhang R, Lilley RM. 2002. Methylation of arsenic *in vitro* by cell extracts from bentgrass (*Agrostis tenuis*): effect of acute exposure of plants to arsenate. *Functional Plant Biology* **29**: 73-80.

Wu P, Shou HX, Xu GH, Lian XM. 2013. Improvement of phosphorus efficiency in rice on the basis of understanding phosphate signaling and homeostasis. *Current Opinion in Plant Biology* **16**: 205-212.

Wu ZC, Ren HY, McGrath SP, Wu P, Zhao FJ. 2011. Investigating the Contribution of the Phosphate Transport Pathway to Arsenic Accumulation in Rice. *Plant Physiology* **157**: 498-508.

Wysocki R, Bobrowicz P, Ulaszewski S. 1997. The *Saccharomyces cerevisiae ACR3* gene encodes a putative membrane protein involved in arsenite transport. *Journal of Biological Chemistry* **272**: 30061-30066.

Wysocki R, Chery CC, Wawrzycka D, Van Hulle M, Cornelis R, Thevelein JM, Tamas MJ. 2001. The glycerol channel Fps1p mediates the uptake of arsenite and antimonite in *Saccharomyces cerevisiae*. *Molecular Microbiology* **40**: 1391-1401.

Xu C, Zhou TQ, Kuroda M, Rosen BP. 1998. Metalloid resistance mechanisms in prokaryotes. *Journal of Biochemistry* **123**: 16-23.

Xu XY, McGrath SP, Meharg AA, Zhao FJ. 2008. Growing rice aerobically markedly decreases arsenic accumulation. *Environmental Science & Technology* **42**: 5574-5579.

Xu XY, McGrath SP, Zhao FJ. 2007. Rapid reduction of arsenate in the medium mediated by plant roots. *New Phytologist* **176**: 590-599.

Yamaji N, Ma JF. 2009. A transporter at the node responsible for intervascular transfer of silicon in rice. *Plant Cell* **21**: 2878-2883.

Yamaji N, Mitatni N, Ma JF. 2008. A transporter regulating silicon distribution in rice shoots. *Plant Cell* **20**: 1381-1389.

Yao LX, Li GL, Dang Z, He ZH, Zhou CM, Yang BM. 2009. Arsenic speciation in turnip as affected by application of chicken manure bearing roxarsone and its metabolites. *Plant and Soil* **316**: 117-124.

Yathavakilla SKV, Fricke M, Creed PA, Heitkemper DT, Shockey NV, Schwegel C, Caruso JA, Creed JT. 2008. Arsenic speciation and identification of monomethylarsonous acid and monomethylthioarsonic acid in a complex matrix. *Analytical Chemistry* **80**: 775-782.

Ye J, Rensing C, Rosen BP, Zhu Y-G. 2012. Arsenic biomethylation by photosynthetic organisms. *Trends in Plant Science* **17**: 155-162.

Ye W-L, Wood BA, Stroud JL, Andralojc PJ, Raab A, McGrath SP, Feldmann J, Zhao F-J. 2010. Arsenic speciation in phloem and xylem exudates of castor bean. *Plant Physiology* **154**: 1505-1513.

Ye WL, Khan MA, McGrath SP, Zhao FJ. 2011. Phytoremediation of arsenic contaminated paddy soils with *Pteris vittata* markedly reduces arsenic uptake by rice. *Environmental Pollution* **159**: 3739-3743.

Yoshida S, Forno D, Cock JH, Gomez KA. 1976. *Laboratory Manual for Physiological Studies of Rice*: The International Rice Research Institute: Los Banos, Laguna, Philippines.

Yoshinaga M, Cai Y, Rosen BP. 2011. Demethylation of methylarsonic acid by a microbial community. *Environmental Microbiology* **13**: 1205-1215.

Yu Y, Zhang SZ, Huang HL, Luo L, Wen B. 2009. Arsenic Accumulation and Speciation in Maize as Affected by Inoculation with Arbuscular Mycorrhizal Fungus *Glomus mosseae*. *Journal of Agricultural and Food Chemistry* **57**: 3695-3701.

Zakharyan RA, Sampayo-Reyes A, Healy SM, Tsaprailis G, Board PG, Liebler DC, Aposhian HV. 2001. Human monomethylarsonic acid (MMA(V)) reductase is a member of the glutathione-S-transferase superfamily. *Chemical Research in Toxicology* **14**: 1051-1057.

Zavala YJ, Duxbury JM. 2008. Arsenic in rice: I. Estimating normal levels of total arsenic in rice grain. *Environmental Science & Technology* **42**: 3856-3860.

Zavala YJ, Gerads R, Gurleyuk H, Duxbury JM. 2008. Arsenic in rice: II. Arsenic speciation in USA grain and implications for human health. *Environmental Science & Technology* **42**: 3861-3866.

Zhang X, Uroic MK, Xie W-Y, Zhu Y-G, Chen B-D, McGrath SP, Feldmann J, Zhao F-J. 2012. Phytochelatins play a key role in arsenic accumulation and tolerance in the aquatic macrophyte *Wolffia globosa*. *Environmental Pollution* **165**: 18-24.

Zhao F-J, Ago Y, Mitani N, Li R-Y, Su Y-H, Yamaji N, McGrath SP, Ma JF. 2010a. The role of the rice aquaporin Lsi1 in arsenite efflux from roots. *New Phytologist* **186**: 392-399.

Zhao F-J, Ago Y, Mitani N, Li R-Y, Su Y-H, Yamaji N, McGrath SP, Ma JF. 2010b. The role of the rice aquaporin Lsi1 in arsenite efflux from roots. *New Phytologist* **182**: 392-399.

Zhao F-J, McGrath SP, Meharg AA. 2010c. Arsenic as a Food Chain Contaminant: Mechanisms of Plant Uptake and Metabolism and Mitigation Strategies. *Annual Review of Plant Biology* **61**: 535-559.

Zhao F-J, Zhu Y-G, Meharg AA. 2013a. Methylated Arsenic Species in Rice: Geographical Variation, Origin, and Uptake Mechanisms. *Environmental Science & Technology* **47**: 3957-3966.

Zhao FJ, Harris E, Yan J, Ma JC, Wu LY, Liu WJ, McGrath SP, Zhou JZ, Zhu YG. 2013b. Arsenic Methylation in Soils and Its Relationship with Microbial *arsM* Abundance and Diversity, and As Speciation in Rice. *Environmental Science & Technology* **47**: 7147-7154.

Zhao FJ, Ma JF, Meharg AA, McGrath SP. 2009. Arsenic uptake and metabolism in plants. *New Phytologist* **181**: 777-794.

Zhao FJ, Stroud JL, Khan MA, McGrath SP. 2012. Arsenic translocation in rice investigated using radioactive ^{73}As tracer. *Plant and Soil* **350**: 413-420.

Zhao FJ, Wang JR, Barker JHA, Schat H, Bleeker PM, McGrath SP. 2003. The role of phytochelatins in arsenic tolerance in the hyperaccumulator *Pteris vittata*. *New Phytologist* **159**: 403-410.

Zhao R, Zhao MX, Wang H, Taneike Y, Zhang XR. 2006. Arsenic speciation in moso bamboo shoot - A terrestrial plant that contains organoarsenic species. *Science of the Total Environment* **371**: 293-303.

Zhao XQ, Mitani N, Yamaji N, Shen RF, Ma JF. 2010d. Involvement of Silicon Influx Transporter OsNIP2;1 in Selenite Uptake in Rice. *Plant Physiology* **153**: 1871-1877.

Zheng J, Hintelmann H, Dimock B, Dzurko MS. 2003. Speciation of arsenic in water, sediment, and plants of the Moira watershed, Canada, using HPLC coupled to high resolution ICP-MS. *Analytical and Bioanalytical Chemistry* **377**: 14-24.

Zhou J, Jiao F, Wu Z, Li Y, Wang X, He X, Zhong W, Wu P. 2008. *OsPHR2* is involved in phosphate-starvation signaling and excessive phosphate accumulation in shoots of plants. *Plant Physiology* **146**: 1673-1686.

Zhu T, Budworth P, Chen W, Provart N, Chang H-S, Guimil S, Su W, Estes B, Zou G, Wang X. 2003. Transcriptional control of nutrient partitioning during rice grain filling. *Plant Biotechnology Journal* **1**: 59-70.

8. Appendix