Tansley review

Arsenic uptake and metabolism in plants

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Summary

Arsenic (As) is an element that is nonessential for and toxic to plants. Arsenic contamination in the environment occurs in many regions, and, depending on environmental factors, its accumulation in food crops may pose a health risk to humans. Recent progress in understanding the mechanisms of As uptake and metabolism in plants is reviewed here. Arsenate is taken up by phosphate transporters. A number of the aquaporin nodulin26-like intrinsic proteins (NIPs) are able to transport arsenite, the predominant form of As in reducing environments. In rice (*Oryza sativa*), arsenite uptake shares the highly efficient silicon (Si) pathway of entry to root cells and efflux towards the xylem. In root cells arsenate is rapidly reduced to arsenite, which is effluxed to the external medium, complexed by thiol peptides or translocated to shoots. One type of arsenate reductase has been identified, but its *in planta* functions remain to be investigated. Some fern species in the Pteridaceae family are able to hyperaccumulate As in above-ground tissues. Hyperaccumulation appears to involve enhanced arsenate uptake, decreased arsenite-thiol complexation and arsenite efflux to the external medium, greatly enhanced xylem translocation of arsenite, and vacuolar sequestration of arsenite in fronds. Current knowledge gaps and future research directions are also identified.

I. Introduction

Arsenic (As) is a well-known human toxin which has arguably influenced human history more than any other toxic element or compound (e.g. Nriagu, 2002). In recent decades, millions of people have suffered from As poisoning as a result of drinking As-contaminated water extracted from shallow tube wells in South and Southeast Asia (Nordstrom, 2002). Soil As

contamination has also occurred in some areas as a result of mining activities, use of arsenical herbicides, insecticides and wood preservatives, and irrigation with As-contaminated groundwaters. Excessive uptake of As by crop plants may present a food safety problem. This is exemplified by recent findings that rice (*Oryza sativa*) is particularly efficient in As uptake from paddy soil, leading to accumulation in rice grain at concentrations that may pose a health risk to people consuming large amounts of rice in their diet (Williams *et al.*, 2007; Zhu *et al.*, 2008). Understanding how plants take up and metabolize As is important for developing mitigation measures to counter the problem of food-chain contamination by As. Meanwhile, the discovery of As-hyperaccumulating fern species (Ma *et al.*, 2001) has attracted much attention and further research focusing on understanding the mechanisms behind this extraordinary phenomenon and evaluation of the phytoremediation potential of various As hyperaccumulators.

Much progress has been made on plant As uptake and metabolism since the last Tansley Review on the topic by Meharg & Hartley-Whitaker (2002), which focused on As speciation, toxicity and resistance/tolerance mechanisms. This review will present recent progress in the understanding of physiological, biochemical and molecular mechanisms of As uptake, metabolism and hyperaccumulation by plants, and highlight the knowledge gaps that require further research.

II. Mechanisms of arsenic uptake and efflux

1. Arsenate uptake

Arsenate (As(V)) is the main As species in aerobic soils. It has a strong affinity for iron oxides/hydroxides in soil; thus the concentrations of arsenate in soil solutions are usually low. Wenzel *et al.* (2002) reported ≤ 53 nM arsenate in the soil solutions from a range of uncontaminated and moderately contaminated soils and up to 2.3 μM in a highly contaminated soil. Many hydroponic studies have used much higher concentrations of arsenate than those found in soil solution, and their environmental relevance has been questioned (Fitz & Wenzel, 2002).

Physiological and electrophysiological studies have shown that arsenate and phosphate share the same transport pathway in higher plants, with the transporters having a higher affinity for phosphate than for arsenate (e.g. Asher & Reay, 1979; Ullrich-Eberius *et al.*, 1989; Meharg *et al.*, 1994). The uptake mechanism involves cotransport of phosphate or arsenate and protons, with stoichiometry of at least $2H^+$ for each $H_2PO_4^-$ or $H_2AsO_4^-$ (Ullrich-Eberius *et al.*, 1989). A number of phosphate transporters have been characterized in plants (Rausch & Bucher, 2002; Bucher, 2007). There are over 100 phosphate transporters in the Phosphate transporter 1 (Pht1) family, most of which are strongly expressed in roots and are likely to be involved in phosphate uptake from the external medium (Bucher, 2007). In *Arabidopsis thaliana*, two phosphate transporters, Pht1;1 and Pht1;4, play a significant role in phosphate acquisition from both low- and high-phosphorus (P) environments (Shin *et al.*, 2004). The *A. thaliana* double mutant *pht1;1Δ4Δ* was much more resistant to arsenate than the wild type, indicating that Pht1;1 and Pht1;4 mediate arsenate uptake (Shin *et al.*, 2004). In the *A. thaliana* mutant defective in phosphate transporter traffic facilitator 1 (PHF1), the trafficking of the Pht1;1 protein from the endoplasmic reticulum to the plasma membrane is impaired (González *et al.*, 2005). This mutant was much more resistant to arsenate than the wild type, further supporting a role of Pht1;1 in arsenate uptake. Recently, Catarecha *et al.* (2007) identified an arsenate-tolerant mutant of *A. thaliana*, *pht1;1-3*, which harbours a semidominant allele coding for the high-affinity phosphate transporter PHT1;1. Rather intriguingly, the *pht1;1-3* mutant displays the dual phenotypes of decreased arsenate uptake in the short-term and increased As accumulation over a longer period of growth. As the wild-type plants suffered from severe As toxicity, it is perhaps not surprising that their As accumulation capacity was curtailed compared with the mutant. Acquisition of knowledge about phosphate transporters and their regulation in plants will undoubtedly lead to a better understanding of the arsenate uptake mechanisms in plants. Specifically, it would be interesting to determine the relative selectivity of different transporters for phosphate and arsenate, and to examine allelic variation in this selectivity.

Reduced uptake of arsenate is a well-known mechanism of arsenate resistance employed by many plant species, which is achieved through a suppression of the high-affinity phosphate/arsenate uptake system in the resistant plants (reviewed by Meharg & Hartley-Whitaker, 2002).

2. Arsenite uptake

Arsenite (As(III)) is the dominant As species in reducing environments such as flooded paddy soils (Marin *et al.*, 1993; Takahashi *et al.*, 2004; Xu *et al.*, 2008). Thermodynamically, reduction of arsenate to arsenite can occur quite readily at intermediate redox potentials (Inskeep *et al.*, 2002). Flooding of paddy soils leads to mobilization of arsenite into the soil solution and enhanced As bioavailability to rice plants (Xu *et al.*, 2008). The arsenite concentration in soil solutions from flooded paddy soils typically varies from 0.01 to 3 μM; these concentrations are generally higher than those of arsenate found in uncontaminated aerobic soils. Arsenous acid ($As(OH)_3$) has a pK_a of 9.22. Therefore, arsenite, despite its name referring to an oxyanion, is actually present in solution predominantly as an undissociated neutral molecule at pH < 8.

Little was known about the mechanisms of arsenite uptake in plants until recently. Research on arsenite uptake mechanisms in plants has benefited greatly from the knowledge gained from microbial studies. In *Escherichia coli*, yeast and humans, some aquaglyceroporins, a subfamily of the aquaporin superfamily with larger pores to allow passage of neutral

molecules such as glycerol, can transport arsenite (reviewed by Bhattacharjee & Rosen, 2007). These include the *E. coli* glycerol facilitator GlpF, the yeast glycerol channel protein Fps1p, and the mammalian aquaglyceroporins AQP7 and AQP9. An alternative mechanism, other than that involving aquaglyceroporins, has also been identified in yeast. The addition of glucose inhibited arsenite uptake by 80%, and the deletion of hexose permease genes led to a much reduced uptake of arsenite in yeast, suggesting that hexose permeases catalyse the majority of arsenite uptake (Liu *et al.*, 2004b).

Plant roots are capable of rapidly taking up arsenite from the external medium. Short-term (20 min) uptake experiments with excised rice roots showed that the maximum (V_{max}) of arsenite influx was comparable to that of arsenate in the absence of phosphate, but the concentration at which the influx is half V_{max} (K_m) was higher (Abedin *et al.*, 2002b). At higher concentrations (> 100 μM), that is, in the low-affinity range, arsenite influx was substantially faster than arsenate (Abedin *et al.*, 2002b; Meharg & Jardine, 2003). Furthermore, unlike arsenate uptake, arsenite uptake was inhibited by glycerol and antimonite, but not by phosphate. Based on competition experiments, Meharg & Jardine (2003) suggested that arsenite may be taken up by aquaporin channels in plant roots.

Recently, evidence that some plant aquaporin channels can mediate arsenite influx has been obtained from three independent studies (Bienert *et al.*, 2008b; Isayenkov & Maathuis, 2008; Ma *et al.*, 2008). Bienert *et al.* (2008b) expressed a number of plant genes encoding the nodulin26-like intrinsic proteins (NIPs), a subfamily of the plant aquaporin family, in yeast. They found that the expression of *AtNIP5;1* and *AtNIP6;1* from *A. thaliana*, *OsNIP2;1* and *OsNIP3;2* from rice, and *LjNIP5;1* and *LjNIP6;1* from *Lotus japonicus* increased the sensitivity of yeast to arsenite and antimonite, as well as As accumulation in the yeast cells. Interestingly, AtNIP5;1 has been identified as a boric acid transporter essential for boron (B) uptake by *A. thaliana* roots (Takano *et al.*, 2006). Despite the arsenite transport activity of AtNIP5;1 and AtNIP6;1 when expressed heterologously in yeast (Bienert *et al.*, 2008b), the *A. thaliana* T-DNA insertion lines of these two genes showed no significant difference from the wild type in growth in the presence of elevated concentrations of either arsenite or arsenate, suggesting that these two proteins do not contribute significantly to arsenite transport *in planta* (Isayenkov & Maathuis, 2008). These authors identified AtNIP7;1 as a possible candidate for arsenite transport in *A. thaliana*. The T-DNA insertion lines of *AtNIP7;1* were more resistant to arsenite than the wild type, and accumulated approx. 25% less As when grown in agar plates containing 7 μM arsenite. Expression of *AtNIP7;1* in yeast increased arsenite sensitivity.

Arsenite uptake is of particular importance for rice and other aquatic plants with their roots growing in anaerobic or semi-anaerobic environments. Recently, Ma *et al.* (2008) have identified OsNIP2;1, also named Lsi1 because of its primary function as a silicon (Si) transporter (Ma *et al.*, 2006), as a major pathway for the entry of arsenite into rice roots. Expression of *Lsi1* in *Xenopus laevis* oocytes and in yeast markedly increased the uptake of arsenite, but not of arsenate. Mutation of *Lsi1* in rice (*lsi1* mutant) resulted in a *c.* 60% loss in the short-term (30-min) arsenite influx to roots compared with wild-type rice. These data indicate that arsenite shares the Si transport pathway for entry into rice root cells. This is not surprising because arsenite and silicic acid have two important similarities: both have a high pK_a (9.2 and 9.3 for arsenous acid and silicic acid, respectively); and both molecules are tetrahedral with similar sizes. *Lsi1* is strongly expressed in rice roots and its expression is further enhanced in plants not supplied with Si (Ma *et al.*, 2006). The Lsi1 protein is localized on the plasma membrane of the distal side of both exodermis and endodermis cells, where Casparian strips occur.

Ma *et al.* (2008) showed that, in addition to Lsi1, three other NIP channel proteins in rice, OsNIP1;1, OsNIP2;2 (also named Lsi6) and OsNIP3;1, are also able to mediate arsenite influx into *X. laevis* oocytes expressing these genes. While OsNIP2;2 is permeable to silicic acid, OsNIP1;1 and OsNIP3;1 are not (Mitani *et al.*, 2008); the latter (OsNIP3;1) has been shown to mediate B uptake in rice roots (Takano *et al.*, 2008). However, unlike Lsi1, OsNIP1;1, OsNIP2;2 and OsNIP3;1 are expressed at very low levels in rice roots, and thus are unlikely to play a significant role in arsenite influx. Indeed, the loss of function lines of OsNIP2;2 did not show a significant decrease in arsenite uptake (Ma *et al.*, 2008).

NIPs represent one of the four subfamilies of the plant major intrinsic proteins (MIPs), commonly called aquaporins, the other three being plasma membrane intrinsic proteins (PIPs), tonoplast intrinsic proteins (TIPs), and small basic intrinsic proteins (SIPs) (Chaumont *et al.*, 2005; Maurel *et al.*, 2008). NIPs have low to no water permeability and the ability to transport multiple uncharged solutes of varying sizes including glycerol, urea, ammonia, boric acid and silicic acid (Wallace *et al.*, 2006), as well as arsenite (Bienert *et al.*, 2008b; Isayenkov & Maathuis, 2008; Ma *et al.*, 2008). They are sometimes called aquaglyceroporins (Wallace *et al.*, 2006), although some of the NIPs (e.g. OsNIP2;1) have little permeability to glycerol (Ma *et al.*, 2006; Mitani *et al.*, 2008), and there is no direct evidence for a physiological role in plants of glycerol transport through NIPs (Bienert *et al.*, 2008b). Phylogenetic studies suggest that NIPs were acquired early at the beginning of plant evolution by horizontal gene transfer of a bacterial homologue of aquaporins, whose founding member is the bacterial GlpF that can also transport arsenite (Zardoya *et al.*, 2002; Wallace *et al.*, 2006). There are nine and 10–13 members of the NIP subfamily in the *A. thaliana* and rice genomes, respectively (Forrest & Bhave, 2007; Maurel *et al.*, 2008). The substrate selectivity of aquaporins is mainly controlled by two pore constrictions, one formed by the highly conserved asparagine–proline–alanine (NPA) boxes and the other the aromatic/arginine (ar/R) selectivity filter (Wallace *et al.*, 2006; Maurel *et al.*, 2008). Based on homology modelling

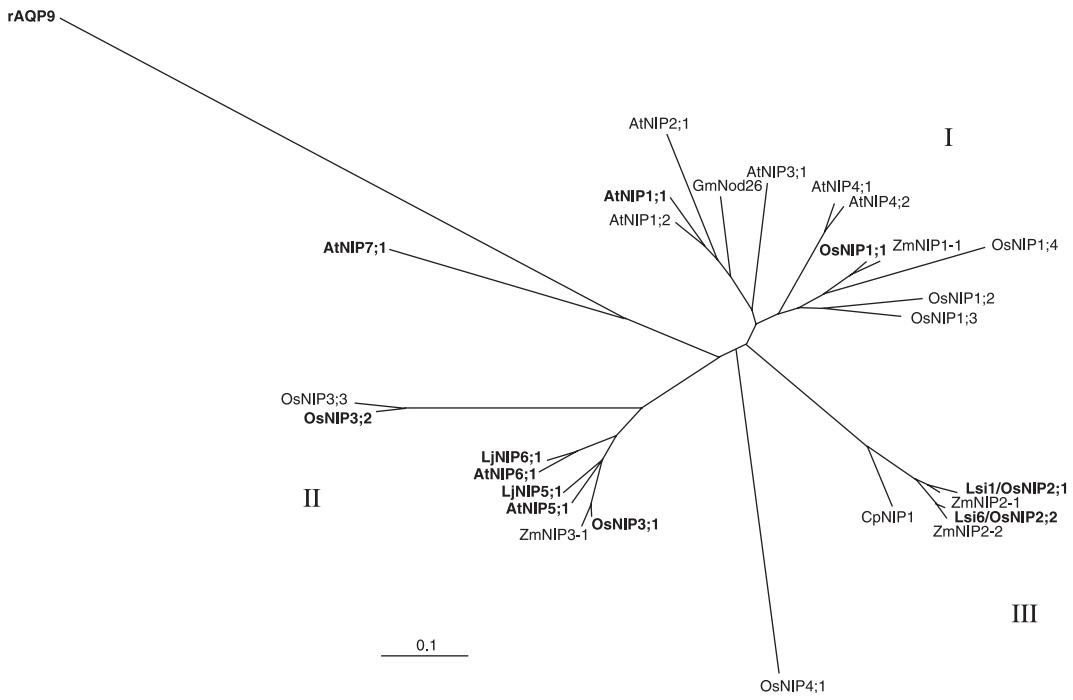

Fig. 1 Phylogenetic tree of plant nodulin26-like intrinsic protein (NIP) channel proteins. The NIPs that have been shown to be permeable to arsenite are shown in bold. At, *Arabidopsis thaliana*; Os, *Oryza sativa*; Zm, *Zea mays*; Lj, *Lotus japonica*; Gm, *Glycine max*; Cp, *Cucurbita pepo*; rAQP9, mammalian aquaglyceroporin 9. I, II and III represent three subgroups of the NIP proteins. The amino acid sequences of NIPs were aligned by Clustal W (http://www.ebi.ac.uk/Tools/clustalw2).

of pore structures at the ar/R selectivity filter, NIPs have been subdivided into two (Wallace *et al.*, 2006) or three subgroups (Mitani *et al.*, 2008) (Fig. 1). The NIP I subgroup includes the archetype nodulin 26 and is permeable to water, glycerol and lactic acid. The members of NIP II subgroup have a predicted larger pore size than those of the NIP I subgroup, and are permeable to larger solutes such as urea, formamide and boric acid, but with a much reduced water permeability (Wallace *et al.*, 2006). The NIP III proteins transport silicic acid; the ar/R region of these proteins contains residues of smaller size, thus forming a larger constriction site compared with other NIP subgroups. However, it appears that arsenite permeability is a property widespread in all NIP subgroups (Fig. 1), suggesting that transport of arsenite is not controlled by the ar/R selectivity filter. It is likely that more members of the NIP protein family will be found to be permeable to arsenite.

To date, there is no report of arsenite permeability in the PIP, TIP and SIP channel proteins in plants. Whether these proteins can transport arsenite remains to be investigated. PIPs have a narrow pore structure typical of orthodox, water-selective aquaporins (Maurel *et al.*, 2008) and are, therefore, not likely to be permeable to arsenite. By contrast, it is possible that some TIP channels may be permeable to arsenite and contribute to arsenite transport into the vacuoles.

While Lsi1 transports arsenite into rice root cells, a different transporter, Lsi2, has been found to mediate arsenite efflux in the direction of xylem (Ma *et al.*, 2008) (Fig. 2). Lsi2 was initially identified as an Si efflux transporter (Ma *et al.*, 2007). Lsi2 is also localized at the exodermis and endodermis of rice roots, but, in contrast to Lsi1, at the proximal side. Therefore, the pathway of Si transport from the external medium to the stele involves the influx of silicic acid mediated by the aquaporin channel Lsi1 (Ma *et al.*, 2006), and the efflux of Si towards the stele mediated by Lsi2 (Ma *et al.*, 2007). Mutation in *Lsi2* in two independent rice mutants led to a marked decrease (66–75%) in As accumulation in shoots compared with wild types (Ma *et al.*, 2008). Arsenite concentrations in xylem sap from the mutants were much lower than those in xylem sap from wild-types. Moreover, addition of Si to the nutrient solution inhibited arsenite transport to the xylem and accumulation in the shoots in the wild-type rice, but not in the two *lsi2* mutants. Rice is a strong accumulator of Si, with Si concentration in the shoots typically varying from 5 to 10%. The efficient Si uptake pathway in rice also allows inadvertent passage of arsenite, thus explaining why rice is efficient in accumulation of As.

When both the *lsi1* and *lsi2* mutants and their wild-types were grown to maturity in a field experiment, the *lsi2* mutants were found to contain significantly lower concentrations of As in straw and grain than the wild-type rice, whereas the differences between the *lsi1* mutant and its wild-type were not statistically significant (Ma *et al.*, 2008). Therefore, although loss of function of Lsi1 affects short-term arsenite influx and

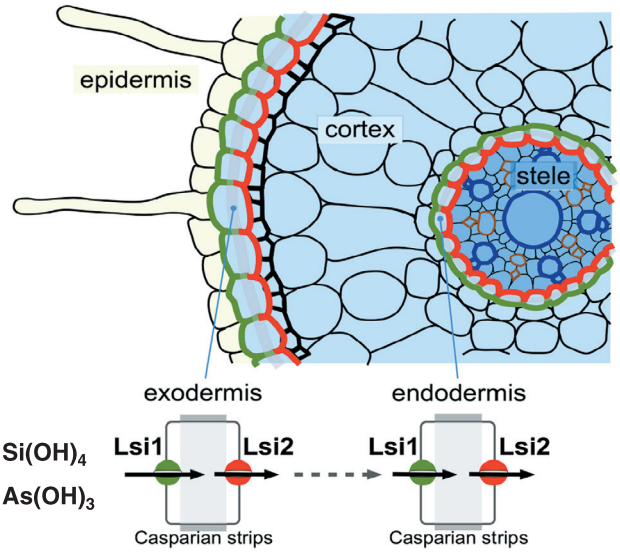

Fig. 2 Arsenite uptake pathway in rice (*Oryza sativa*) roots. Modified from Ma *et al*. (2007). Lsi1 and Lsi2 are Si influx and efflux transporters, respectively.

As accumulation in rice, the efflux of arsenite toward xylem mediated by Lsi2 is the crucial step in controlling As accumulation in rice shoots and grain over a longer growth period. It has been reported that additions of silicate inhibited As accumulation by rice when arsenate was the form of As added to the nutrient solution; yet this effect was not attributable to a direct competition between Si and arsenate because they do not share the same transporters (Guo *et al*., 2005, 2007). These observations can now be explained by the involvement of Lsi2. Arsenate taken up by rice roots is reduced in the root cells to arsenite, which is transported towards the xylem via the Si/arsenite effluxer Lsi2 and is subject to competitive inhibition from Si. Applying Si fertilizers to rice crops may prove to be an effective way of mitigating the problem of excessive transfer of As from paddy soil to rice grain. In addition, Si application can increase rice yield by alleviating biotic and abiotic stresses (Ma & Yamaji, 2006).

3. Uptake of methylated arsenic species

Methylated As species, such as monomethylarsonic acid (MMA) and dimethylarsinic acid (DMA), are found in some soils, but are usually minor As species (Francesconi & Kuehnelt, 2002). They may originate from past use of arsenical pesticides/herbicides, or may be synthesized by soil micro-organisms and algae. MMA and DMA can be taken up by plants, but generally less efficiently than inorganic arsenate or arsenite (Marin *et al*., 1992; Carbonell-Barrachina *et al*., 1998; Burló *et al*., 1999; Carbonell-Barrachina *et al*., 1999). Raab *et al*. (2007b) compared As uptake by 46 plant species exposed to 13.3 μM arsenate, MMA or DMA for 24 h. They found that plants on average took up about half the MMA and a fifth of the amount of DMA compared with arsenate absorption.

The mechanisms of MMA and DMA uptake by plant roots are at present unknown. Abedin *et al*. (2002b) found that the concentration-dependent uptake of MMA into rice roots can be described by Michaelis–Menten kinetics, whereas the DMA uptake did not conform to either a Michaelis–Menten or a linear function. A later study by Abbas & Meharg (2008) showed that the DMA uptake into maize (*Zea mays*) roots can be described by a Michaelis–Menten plus linear function. In these studies, the short-term (20-min) uptake of DMA and MMA was much smaller that that of arsenate or arsenite. Surprisingly, the pretreatment of P starvation of maize increased DMA uptake by 90%, compared with a 50% enhancement of the arsenate influx (Abbas & Meharg, 2008). Caution is warranted when interpreting the data from these short-term uptake experiments, because MMA and DMA trapped in the root apoplast, unlike inorganic As, are difficult to remove by washing with a phosphate solution (Raab *et al*., 2007b). Because of this uncertainty, the uptake data do not simply equate to the influx into the root symplast.

4. Efflux of As species

Following uptake of arsenate by roots, some of the arsenate is lost from the cells via efflux to the external medium (Xu *et al*., 2007); this is similar to the situation for phosphate, which can also be lost via efflux especially under high-P conditions (Mimura, 1999). The mechanism of arsenate efflux is not known, but may be similar to that of phosphate efflux which is thought to be via anion channels (Mimura, 1999).

Xu *et al*. (2007) showed that arsenate added to the aerated nutrient solution was rapidly converted to arsenite by the roots of tomato (*Lycopersicon esculentum*) and rice. Microbes living in the nutrient solution or root exudates contributed little to arsenate reduction to arsenite. This is surprising because arsenate is expected to be stable in the aerobic environment. Phosphate inhibits arsenate uptake and the subsequent production of arsenite in the external medium, suggesting that the arsenite is extruded by root cells following arsenate reduction inside the cells. Indeed, efflux of both arsenate and arsenite was observed when tomato roots preloaded with arsenate were transferred to an As-free medium. Furthermore, the protonophore carbonylcyanide m-chlorophenylhydrazone (CCCP) inhibited the efflux of arsenite, suggesting that the efflux is linked to the proton gradient across the plasma membranes, or is metabolically dependent. Arsenite efflux by roots has been observed in other plant species, including *A. thaliana*, *Holcus lanatus*, wheat (*Triticum aestivum*), barley (*Hordeum vulgare*) and maize (*Zea mays*), but not in the As hyperaccumulator *Pteris vittata* (discussed in more detail Section VII). Within 24 h of exposure to arsenate, arsenite efflux was approximately 3 times the amount of As accumulated in the plants, suggesting rapid cycling of As between plant roots and the medium

(F. J. Zhao *et al.*, unpublished). It appears that arsenite efflux by roots is very rapid immediately following arsenate uptake, and diminishes once the arsenate supply is withheld, possibly because cellular arsenite is complexed with thiols and sequestered in the vacuoles (see Section IV). The rapid conversion of arsenate to arsenite in the external medium raises a question regarding previous hydroponic studies where As speciation was not monitored.

In aerobic soils, arsenite is oxidized rapidly to arsenate either chemically by reactions with manganese oxide (e.g. Oscarson *et al.*, 1981) or by arsenite-oxidizing microbes (Macur *et al.*, 2004). Thus, soil, plant roots and microbes are likely to be engaged constantly in the reduction–oxidation cycle of arsenate–arsenite. Studies using rhizoboxes to enable measurement of As speciation in the rhizosphere showed accumulation of arsenite close to the vicinity of sunflower (*Helianthus annuus*) and maize roots (Ultra *et al.*, 2007a,b; Vetterlein *et al.*, 2007), suggesting that efflux of arsenite occurs in soil-grown plants.

The mechanisms of arsenite efflux from plant roots remain to be elucidated. In microbes, arsenate reduction followed by arsenite efflux is a common and important mechanism of As detoxification (Bhattacharjee & Rosen, 2007). In *E. coli*, arsenite efflux is mediated by either ArsB or the ArsAB complexes. ArsB is a secondary efflux protein coupled to the proton-motive force. ArsB can also associate with ArsA, an ATPase, to form a pump that is much more efficient than ArsB alone at extruding arsenite from the cells (Dey *et al.*, 1994). In yeast and fungi, a different type of efflux carrier protein, Acr3p, is responsible for arsenite efflux, although the mechanism of Acr3p is possibly similar to that of ArsB, relying on the proton-motive force for energy (Wysocki *et al.*, 1997). The CCCP sensitivity of arsenite efflux by tomato roots (Xu *et al.*, 2007) suggests a possibility of ArsB- or Acr3p-like carriers for the efflux. However, direct evidence has yet to be obtained.

Another possible mechanism responsible for arsenite efflux from plant roots involves aquaporin channels, some of which allow bidirectional passage of solutes (Mitani *et al.*, 2008). Unlike the efflux carriers or efflux pumps described above, aquaporin-mediated arsenite efflux occurs through diffusion when the internal arsenite concentration exceeds that in the external medium. In the legume symbiont *Sinorhizobium meliloti*, the arsenic resistance (ars) operon includes an aquaglyceroporin (aqpS) in place of arsB, which confers arsenate resistance possibly through arsenite efflux (Yang *et al.*, 2005). Bienert *et al.* (2008b) showed that expression of *AtNIP5;1*, *AtNIP6;1*, *OsNIP2;1*, *OsNIP3;1* and *LjNIP6;1* in yeast significantly enhanced its tolerance to arsenate. This is interpreted as the NIP channels mediating efflux of arsenite, which was produced by the reduction of arsenate inside the yeast cells. Similarly, Isayenkov & Maathuis (2008) reported that expression of AtNIP7;1 in the yeast *acr3Δ* mutant resulted in a small but consistent increase in arsenate tolerance, suggesting that AtNIP7;1 may mediate arsenite efflux in the absence of ACR3. Bienert *et al.* (2008a) argued that aquaporins/aquaglyceroporin channel-mediated extrusion of arsenite is an ancient mechanism for As detoxification. However, it is not clear whether a similar mechanism is involved in plants.

Is arsenite efflux by plant roots a detoxification mechanism, as has been shown for microbes? This question can only be answered unequivocally when the transporters responsible for the efflux are identified. Knockout or knockdown lines of these transporters would then allow a detailed examination of their roles in As accumulation, efflux and tolerance. In a study comparing arsenite efflux in arsenate-resistant and nonresistant ecotypes of *H. lanatus*, Logoteta *et al.* (2008) found that arsenite efflux was proportional to arsenate uptake in both ecotypes, with the resistant ecotype having a much lower arsenate uptake, as has been demonstrated before (Meharg & Macnair, 1992). This finding suggests that arsenite efflux is not enhanced in the resistant ecotype, which has evolved as a result of the selection pressure of high As availability in soil. However, this observation does not rule out the possibility that arsenite efflux is a constitutive, rather than adaptive, mechanism of As detoxification. Although arsenite can be re-absorbed by roots, the presence of rapid efflux machinery would logically lead to a decreased As burden in the root cells.

III. Rhizosphere interactions

1. Arsenic speciation in the rhizosphere

Chemical and biological processes taking place in the rhizosphere may influence the speciation of As and its bioavailability to plants. Although arsenate is the predominant species in aerobic soils, anaerobic microsites may exist, leading to transient formation of arsenite, especially in the rhizosphere where microbial activity and oxygen consumption are highest. Furthermore, as discussed in the previous section, both plant roots and microbes extrude arsenite. Therefore, arsenate and arsenite may coexist in the vicinity of plant roots under generally aerobic conditions (Ultra *et al.*, 2007a,b; Vetterlein *et al.*, 2007). Thus, it is possible that plants growing in aerobic soils will encounter and absorb some arsenite, as well as arsenate.

Aquatic plants growing in anaerobic soils can release oxygen to their rhizosphere through aerenchyma, resulting in the oxidation of ferrous iron (Fe^{2+}) and the formation of iron plaques, consisting mainly of ferrihydrite, on the root surfaces. The Fe oxides formed have a strong adsorptive capacity for arsenate. Concentrations of As in the rice iron plaques were about 5-fold higher than those in root tissues (Liu *et al.*, 2006). Studies using X-ray absorption spectrometry (XAS) showed that most (70–80%) of the As sorbed by the iron plaque on the roots of *Phalaris arundinacea*, *Typha latifolia* and rice was arsenate, with the remaining (20–30%) As being arsenite (Hansel *et al.*, 2002; Blute *et al.*, 2004; Liu *et al.*, 2006).

Because of this strong sink, As concentrations in the rhizosphere soils were found to be 5–9 times higher than those in the bulk soils in a riparian floodplain (Voegelin *et al.*, 2007). How does the iron plaque affect As uptake by plants? On the one hand, the formation of the plaque enriches As in the rhizosphere, which may lead to increased As uptake by plants (Otte *et al.*, 1991). On the other hand, the iron plaque may sequester As and form a barrier that decreases the entry of As into plants (Liu *et al.*, 2004a). Short-term uptake experiments with excised rice roots showed that the presence of iron plaques decreased arsenate uptake, probably as a result of the adsorption effect, but enhanced arsenite uptake (Chen *et al.*, 2005). The reasons for the latter are not clear.

2. The role of mycorrhizal fungi

The symbiosis between mycorrhizal fungi and plants benefits the host plants by increasing the acquisition of nutrients, especially P. It is, therefore, pertinent to examine whether this symbiosis may influence As uptake and resistance in the host plants, and, if so, how. In the majority of studies, though not all (e.g. Knudson *et al.*, 2003), symbiosis with mycorrhizal fungi was found to confer As resistance in the host plants. This effect may be produced by several possible mechanisms. First, mycorrhizal colonization may suppress the high-affinity phosphate transport system in plant roots, leading to less uptake of arsenate (Gonzalez-Chavez *et al.*, 2002). Secondly, mycorrhizal fungi may enhance As resistance in the host plants by effluxing As to the external medium. This mode of action was clearly demonstrated by Sharples *et al.* (2000a,b), who reported that the ericoid mycorrhizal fungus *Hymenoscyphus ericae* from an As-contaminated site developed arsenate resistance by extruding arsenite into the medium following arsenate uptake, a mechanism that is common in bacteria and fungi (see Section II). Thirdly, by enhancing P acquisition and improving the P nutrition of the host plant, mycorrhizal fungi improve plant growth, causing a dilution of As concentrations in plant tissues (Liu *et al.*, 2005; Ahmed *et al.*, 2006; Chen *et al.*, 2007), or increasing cytoplasmic inorganic P. Mycorrhizal plants often have a higher P/As concentration ratio, which is likely to benefit the plant through enhanced tolerance to As. Finally, there is circumstantial evidence that mycorrhizal fungi may restrict As translocation from roots to shoots (Gonzalez-Chavez *et al.*, 2002; Chen *et al.*, 2007; Ultra *et al.*, 2007b), although how this control is exerted is unclear and requires further investigation.

Ultra *et al.* (2007a,b) reported the presence of methylated As species in the rhizosphere of sunflower (*H. annuus*) plants when inoculated with the arbuscular mycorrhizal (AM) fungus *Glomus aggregatum*. DMA was found at low but detectable concentrations in the rhizosphere soil but not in the bulk soil, nor in the rhizosphere soil of uninoculated plants. Mycorrhizal fungi may be able to mediate As biomethylation and release some of the methylated As in the rhizosphere.

IV. Arsenic metabolism *in planta*

1. Arsenate reduction

Analysis of As speciation in plant tissues generally shows that As is predominantly present in the As(III) oxidation state, even though plants had been exposed to arsenate. For example, As(III) accounted for 96–100% of the As in the roots and shoots of *Brassica juncea* (Pickering *et al.*, 2000), 97–100% in the leaves of *A. thaliana* (Dhankher *et al.*, 2002) and 92–99% in the roots of tomato and rice (Xu *et al.*, 2007). This means that, following uptake, arsenate is reduced efficiently to arsenite in plant cells, and that most plants have a high capacity for arsenate reduction. A nuclear magnetic resonance study showed that arsenate can be reduced to arsenite *in vitro* by glutathione (Delnomdedieu *et al.*, 1994). However, this chemical reaction is thought to be too slow to account for the efficient reduction taking place in plant cells. Bleeker *et al.* (2006) found that at least 90% of the reduction capacity in the root protein extracts of *H. lanatus* was enzymatic.

The mechanisms of arsenate reduction in microbes have been studied and described extensively (Messens & Silver, 2006; Bhattacharjee & Rosen, 2007). Three types of cytosolic arsenate reductases have been identified: *E. coli* ArsC which uses glutaredoxin (Grx) and glutathione (GSH) as reductants, a second type of ArsC from *Staphylococcus aureus* and *Bacillus subtilis* which uses thioredoxin as a reductant, and Acr2p from eukaryotic organisms such as yeast and the parasitic protozoa *Leishmania major*, which also uses Grx and GSH as reductants (Bhattacharjee & Rosen, 2007). Acr2p is a member of the protein tyrosine phosphatase (PTPase) superfamily, which includes the human cell cycle dual-specificity phosphatases CDC25s (cell division cycle).

Based on the sequence homology to the yeast Acr2p, plant homologues of ACR2 have been cloned and characterized from *A. thaliana* (Dhankher *et al.*, 2006), *H. lanatus* (Bleeker *et al.*, 2006), rice (Duan *et al.*, 2007) and the As hyperaccumulator *P. vittata* (Ellis *et al.*, 2006). In *A. thaliana*, the *ACR2* gene was earlier identified as Arath;CDC25, which is a dual-specificity tyrosine phosphatase that may have a role in cell cycle regulation (Landrieu *et al.*, 2004a,b), although *A. thaliana* T-DNA insertion lines and RNA interference (RNAi) knockdown lines of this gene did not exhibit any phenotypes of abnormal growth under normal growth conditions (Bleeker *et al.*, 2006; Dhankher *et al.*, 2006). Similarly, *ACR2* genes from rice, *H. lanatus* and *P. vittata* are also *CDC25*-like genes. Interestingly, Arath;CDC25 (AtACR2) and two isoforms of OsACR2 exhibit phosphatase activity (Landrieu *et al.*, 2004a; Duan *et al.*, 2007), whereas yeast Acr2p and PvACR2 do not (Ellis *et al.*, 2006). Expression of plant *ACR2* genes (*AtACR2*, *OsACR2* and *PvACR2*) in the *E. coli* mutant lacking *ArsC* or the yeast mutant lacking *Acr2p* restored their resistance to arsenate (Dhankher *et al.*, 2006; Ellis *et al.*, 2006; Duan *et al.*, 2007). Furthermore, purified ACR2 proteins from *E. coli*

overexpressing *OsACR2;1*, *OSACR2;2*, *PvACR2* or *HlACR2* (also named *HlAsr*) can catalyse GSH/Grx-dependent arsenate reduction (Bleeker *et al.*, 2006; Ellis *et al.*, 2006; Duan *et al.*, 2007). These studies demonstrate the ability of the plant ACR2 to reduce arsenate in heterologous systems.

In *A. thaliana*, the *AtACR2* transcript appears to be expressed constitutively at low levels in all organs, with roots having a higher level of protein expression than shoots (Dhankher *et al.*, 2006). In rice, *OsACR2;1* is expressed in both root and shoots, whereas *OsACR2;2* is expressed mainly in roots (Duan *et al.*, 2007). The expression of both *OsACR2* and *HlACR2* (*HlAsr*) is induced by arsenate exposure (Bleeker *et al.*, 2006; Duan *et al.*, 2007). By contrast, the expression of *PvACR2* in *P. vittata* gametophytes is constitutive and not inducible by arsenate exposure (Ellis *et al.*, 2006).

Although the *in vitro* enzyme assays and heterologous expression studies described above have established plant ACR2 proteins as capable of reducing arsenate, their role in arsenate reduction *in planta* has not been resolved unequivocally. *Arabidopsis thaliana* knockdown lines of *AtACR2* using an RNAi construct showed increased arsenate sensitivity, but only at a high level of arsenate exposure (Dhankher *et al.*, 2006). Assays of crude protein extracts from roots of wild-type *A. thaliana* showed two components of arsenate reductase activity: a constitutive component present in plants unexposed to arsenate and an arsenate-inducible component; the activity of the latter was approximately half of the former (Bleeker *et al.*, 2006). Interestingly, a T-DNA insertion mutant of the *AtACR2* gene contained the same level of the constitutive reductase activity as the wild type, but lost the activity of the inducible component. It has been hypothesized that the inducible component is attributable to AtACR2, whereas the constitutive component represents an as yet unidentified pathway(s) of arsenate reduction *in vivo*. This would explain why As speciation in *A. thaliana* roots and shoots was dominated by As(III) even in the T-DNA knockout or RNAi knockdown lines of *AtACR2* (F. J. Zhao *et al.*, unpublished; Om P. Dhankher, personal communication). Similarly, although deletion of *ScAcr2p* in yeast resulted in increased sensitivity to arsenate, analysis of As speciation revealed that the extent of arsenate reduction to arsenite was not affected (David Salt, personal communication).

It is possible that other enzymes or pathways of arsenate reduction exist in plants. Recently, Rathinasabapathi *et al.* (2006) reported that a cytosolic triosephosphate isomerase (TPI) isolated from *P. vittata* may be involved in arsenate reduction directly or indirectly. Expression of the *PvTPI* gene in the *E. coli* strain lacking *ArsC* increased its arsenate resistance, as well as increasing the percentage of cellular arsenic present as arsenite. TPI is an enzyme involved in glycolysis, catalysing the reversible interconversion of the triose phosphate isomers dihydroxyacetone phosphate and D-glyceraldehyde 3-phosphate. The mechanism by which PvTPI enhances arsenate resistance in *E. coli*, and whether this enzyme plays a role in As metabolism *in planta*, remain unclear. Several mammalian enzymes have been shown to be capable of reducing arsenate *in vitro* in the presence of an appropriate thiol, including purine nucleoside phosphorylase (PNPase), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and glycogen phosphorylase (GPase) (Gregus & Nemeti, 2002, 2005; Nemeti & Gregus, 2007). It is not known if plant PNPase, GAPDH or equivalents of GPase can reduce arsenate in the presence of thiols. Nevertheless, it appears that arsenate reduction may be catalysed partly by nonspecific enzymes.

2. Complexation and sequestration of arsenic

Arsenite has a high affinity to the sulphhydryl (–SH) groups of peptides such as GSH and phytochelatins (PCs). *In vitro* studies showed that GSH and arsenite form a $(GS)_3$-arsenite complex with cysteinyl sulphhydryl as the arsenite binding site (Delnomdedieu *et al.*, 1994). The complex is stable in the pH range from 1.5 to 7.0–7.5, but dissociates at higher pH. The toxicity of arsenite is thought to be caused by the binding of arsenite to the –SH groups of proteins, thus altering protein structure or interfering with the catalytic sites of enzymes (Meharg & Hartley-Whitaker, 2002). Inorganic arsenate does not form complexes with thiol compounds, but pentavalent As in DMA can bind to GSH when it is activated by sulphide (Raab *et al.*, 2007c). Raab *et al.* (2007c) identified the dimethylarsinothioyl glutathione (DMAS-GS) complex from the sulphur-rich plant species *Brassica oleracea* (cabbage).

There is strong evidence that complexation of arsenite by PCs is an important mechanism of As detoxification, and hence tolerance, in As-nonhyperaccumulating plants. Exposure to arsenate or arsenite induces a large response in the synthesis and accumulation of PCs in plants (Grill *et al.*, 1987; Maitani *et al.*, 1996; Sneller *et al.*, 1999; Schmöger *et al.*, 2000; Srivastava *et al.*, 2007; Schulz *et al.*, 2008). Inhibition of PC synthesis by treatment with L-buthionine-sulphoxime (BSO), a potent inhibitor of γ-glutamylcysteine synthetase, leads to hypersensitivity to As (Schmöger *et al.*, 2000; Hartley-Whitaker *et al.*, 2002; Schat *et al.*, 2002). The most convincing evidence for an essential role of PCs in As detoxification was obtained with the *A. thaliana* mutant *cad1-3* (cadmium sensitive), which lacks the functional PC synthase; this mutant produces little PCs and is 10–20-fold more sensitive to arsenate than the wild type (Ha *et al.*, 1999).

Intact PCs-As complexes have been isolated from plant tissues (Sneller *et al.*, 1999; Schmöger *et al.*, 2000). In a recent study using high-performance liquid chromatography with inductively coupled plasma mass spectrometry (HPLC-ICP-MS) and molecule-specific electrospray-ionization mass spectrometry (ES-MS), Raab *et al.* (2005) identified up to 14 different species of As complexes, including As(III)-$(PC_2)_2$, As(III)-PC_3, GS-As(III)-PC_2 (GS being GSH bound to arsenite via the –SH of cysteine), MMA-PC_2 and some other unidentified species, in sunflower exposed to arsenate or arsenite. The GS-As(III)-PC_2 complex was the dominant complex initially,

followed by As(III)-PC_3 as the predominant As-PC complex. Similarly, in the arsenate-tolerant *H. lanatus*, As(III)-PC_3 was the dominant complex (Raab *et al.*, 2004). These studies show that arsenite is mainly coordinated with three –SH groups. In *Rauvolfia serpentia* cell culture (Schmöger *et al.*, 2000), in *Silene vulgaris* (Sneller *et al.*, 1999; Schat *et al.*, 2002) and in tolerant populations of *H. lanatus* (Hartley-Whitaker *et al.*, 2002), the molar ratio of PCs-SH to As is ≥ 3:1. Pickering *et al.* (2000) and Dhankher *et al.* (2002) used XAS to determine the speciation of As in *B. juncea* and *A. thaliana*. They found that nearly all (96–100%) As in roots and shoots was bound to thiol groups. By contrast, Raab *et al.* (2005) found that < 60% of the As extracted from sunflower roots and leaves was complexed with thiols. Both speciation techniques have their strengths and weaknesses. In the case of HPLC-ICP-MS/ES-MS, As species are extracted from plant tissues with aqueous solutions and separated on a suitable HPLC column before identification and quantification of As species. Decomposition or *de novo* synthesis of As species during the extraction step is a possibility. In the case of XAS, speciation and quantification are based on fitting the spectra of the sample to those of the model compounds; the results are often limited by the availability and the small number of model compounds used. Also, minor species may not be quantified reliably. Recently, Bluemlein *et al.* (2008) made a direct comparison of the two techniques and obtained comparable results for As speciation in *Thunbergia alata*. The HPLC-ICP-MS/ES-MS method showed that 55–64% of the As was As(III) bound to thiol compounds, while XAS gave 53% as As(III) bound to thiols, 38% as uncomplexed arsenite and 9% as arsenate.

Although PCs play a constitutive role in arsenite detoxification, it is less clear whether elevated PC synthesis contributes to the adaptively enhanced tolerance of plants colonizing As-contaminated sites. When PC synthesis was suppressed by the inhibitor BSO, both tolerant and nontolerant *H. lanatus* plants became hypersensitive to arsenate and arsenite (Schat *et al.*, 2002; Bleeker *et al.*, 2006). This means that both plants rely on a PC-dependent detoxification mechanism. Under equivalent arsenate stress, measured as the effective external concentration resulting in a 50% inhibition of root elongation, PC production was 15- to 20-fold higher in the tolerant clones than in the nontolerant clone of *H. lanatus*, suggesting a role of PC synthesis in the adaptive tolerance (Hartley-Whitaker *et al.*, 2001). However, recent studies by Bleeker *et al.* (2006) suggest that it is not the capacity of PC synthesis *per se*, but rather the expression and activity of the arsenate reductase HlAsr (HlACR2), that differentiates the tolerant from the nontolerant plants of *H. lanatus*. They argued that a higher arsenate reduction driven by HlAsr leads to more production of PCs in the tolerant plants. By contrast, Arnetoli *et al.* (2008) found that a mine population of *Silene paradoxa* was more tolerant to arsenate than a nonmine population, but the mine plants accumulated much less PCs than the nonmine plants even though they accumulated similar concentrations of As in roots. This suggests that the mine population of *S. paradoxa* does not rely on enhanced PC synthesis for its adaptively increased tolerance. The discussion in Section VI will demonstrate that hypertolerance of As hyperaccumulators is not attributable to enhanced PC production.

Because of the role of PCs in As tolerance, transgenic plants overexpressing PC synthase genes or the genes involved in the synthesis of the PC precursor GSH may be expected to be more tolerant to arsenate or arsenite. This has indeed been demonstrated in a number of studies. Dhankher *et al.* (2002) showed that overexpression of two *E. coli* genes, the *ArsC* gene for increased arsenate reduction and the gene encoding γ-glutamylcysteine synthetase (γ-ECS) for increased GSH synthesis, in *A. thaliana* leaves substantially increased arsenate tolerance. Moreover, As accumulation in the shoots, measured as shoot As concentration, was increased by 2–3-fold; but note that the *E. coli ArsC* gene was overexpressed only in leaves, whereas *E. coli γ-ECS* was constitutively overexpressed in both shoot and root tissues. Li *et al.* (2004) overexpressed *AtPCS1* in *A. thaliana* using a strong constitutive *A. thaliana* actin-2 expression cassette. The transgenic lines produced more thiol peptides, including several unidentified compounds, than the wild type in response to arsenate exposure, particularly in the root tissues. The *AtPCS1*-overexpressing lines were much more tolerant to arsenate but, paradoxically, became hypersensitive to cadmium (Cd). In this study, As accumulation in shoots was not enhanced by *AtPCS1* overexpression, presumably because complexation of arsenite with thiols in roots does not favour transport of As from roots to shoots (see Section V). Similarly, transgenic plants of *B. juncea* expressing the *A. thaliana AtPCS1* gene under the control of its native promoter showed a moderate increase in tolerance to arsenate, but not As accumulation in shoots (Gasic & Korban, 2007). Interestingly, while *A. thaliana* plants overexpressing *AtPCS1* in the cytoplasm were more tolerant to arsenate, targeting *AtPCS1* to *A. thaliana* chloroplasts induced a marked sensitivity to arsenate (Picault *et al.*, 2006).

There is little knowledge regarding the cellular and subcellular distribution of As in nonhyperaccumulator plants, as elemental mapping techniques currently available may not be sensitive enough for the relatively low concentrations of As normally found in plant tissues, or may not provide sufficiently high resolution for the investigation of subcellular localization. Preserving the *in situ* As speciation during sample preparation presents a further significant challenge if the aim is to quantify the subcellular distribution of different As species. It is assumed that arsenite–thiol complexes formed in the cytoplasm are subsequently transported into and sequestered in the vacuoles, where the acidic pH (~5.5) is favourable to the stability of the complexes. By analogy, virtually all of the Cd and its binding peptides in the protoplasts isolated from tobacco (*Nicotiana tabacum*) leaves were found to be localized in the vacuoles (Vögeli-Lange & Wagner, 1990). In yeast, the

vacuolar membrane transporter yeast cadmium factor 1p (Ycf1p) confers resistance to a variety of toxic metals and metalloids including Cd and As. Ycf1p is a member of the yeast multidrug resistance-associated protein (MRP) subfamily of ATP-binding cassette (ABC) proteins, and mediates transport of glutathione-S-conjugated metals and metalloids, such as Cd-$(GS)_2$ (Li *et al.*, 1997) and As(III)-$(GS)_3$ (Ghosh *et al.*, 1999). In *Schizosaccharomyces pombe*, Cd-PC complexes are transported into the vacuole by Heavy Metal Tolerance 1 (HMT1), which is also a member of the ABC family (Ortiz *et al.*, 1995). Presumably HMT1 can also transport As(III)-PC complexes, although no direct evidence has been reported. Vacuolar transporters similar to HMT1 or Ycf1p have not been reported in plants. Nevertheless, transport assays showed that As(III)-$(GS)_3$ was efficiently transported into the tonoplast membrane vesicles prepared from *H. lanatus* roots in a MgATP-dependent and charge-neutral fashion, suggesting that an ABC transporter is involved (Bleeker *et al.*, 2006). No difference in the transport rate was found between the arsenate-tolerant and nontolerant plants of *H. lanatus*, suggesting that there is no adaptive enhancement of this process in the tolerant plants. Free arsenite was also taken up into the vesicles, but at only approximately one-fifth of the rate of that of As(III)-$(GS)_3$.

3. Methylation

Methylated As species, such as MMA, DMA and trimethylarsine oxide (TMAO), have been found in plant samples (Francesconi & Kuehnelt, 2002; Meharg & Hartley-Whitaker, 2002). In field-collected samples, these methylated As species may originate from the soil. However, in hydroponic cultures where no methylated As species were present in the medium, DMA and/or MMA was found in plant tissues or xylem sap at low concentrations, usually < 1% of the total As concentration (Quaghebeur & Rengel, 2003; Raab *et al.*, 2007a; Xu *et al.*, 2007), suggesting that *de novo* methylation of As occurs in plants. In rice grain, methylated As, mainly DMA, can account for very little or up to ~90% of the total As (Williams *et al.*, 2005; Meharg *et al.*, 2008; Xu *et al.*, 2008; Zavala *et al.*, 2008). The proportion of methylated As in total As differed between rice genotypes (Liu *et al.*, 2006), and was found to increase with total As concentration in grain in an experiment involving different As additions to soil and water management regimes (Xu *et al.*, 2008). The location of As methylation in rice is not clear.

The pathway and enzymology of As methylation in plants have not been elucidated. Wu *et al.* (2002) showed that cell extracts from *Agrostis capillaris* exhibited As methylation activity in an *in vitro* assay with ^{3}H-labelled *S*-adenosyl-L-methionine (SAM) as the methyl donor. The activity was found in leaf but not root extracts, and was induced by pre-exposure of the plants to arsenate. MMA was the initial product of methylation, but over a longer assay time DMA accumulated. It is likely that As methylation in plants follows the Challenger pathway, which has been well established in fungi and bacteria (reviewed by Bentley & Chasteen, 2002). In microbes, arsenite is methylated by *S*-adenosylmethyltransferase using the methyl donor SAM. The product of this reaction is pentavalent MMA(V), which is reduced by a reductase to trivalent MMA(III) with thiols (e.g. glutathione). Methylation and reduction steps continue to produce di- and tri-methyl compounds including DMA(V), DMA(III), TMAO and trimethylarsine (TMA), with the end product TMA being a volatile gas. Genes encoding *S*-adenosylmethyltransferase are widespread in microbial genomes. The *arsM* gene encoding such an enzyme has been isolated from the soil bacterium *Rhodopseudomonas palustris* (Qin *et al.*, 2006). When expressed heterologously in an As-sensitive strain of *E. coli*, arsM conferred As tolerance by catalysing the formation of a number of methylated intermediates from arsenite and volatilization loss of TMA from both the cells and the medium (Qin *et al.*, 2006; Yuan *et al.*, 2008).

In a rice microarray study (Norton *et al.*, 2008), a gene annotated as a methyltransferase (Os02g51030) was up-regulated by exposure to arsenate in the growth medium. This gene contains a ubiquinone/coenzyme Q5 (UbiE/Coq5) family protein motif, which is also present in the *arsM* genes from bacteria and archaea (Qin *et al.*, 2006). Whether this gene is responsible for As methylation in rice remains to be investigated. It is also not known if plants can produce and volatilize TMA as micro-organisms do. Any potential volatilization of As is likely to be small though, as the total As content in sunflower after 1 d of exposure to arsenate remained unchanged over the following 32 d without As exposure to roots (Raab *et al.*, 2007a).

V. Long-distance translocation of arsenic

1. Translocation from roots to shoots

Unlike P, As has, generally, a low mobility with respect to translocation from roots to shoots in plants except hyperaccumulators. In wild-type *A. thaliana*, 73 and 2.6% of P and As, respectively, taken up by roots was distributed to the shoots (Quaghebeur & Rengel, 2004). The inefficient root to shoot translocation is also reflected in the generally low ratios of shoot As to root As concentrations in plants supplied with inorganic As: 0.01–0.03 in tomato (Burló *et al.*, 1999), 0.03–0.1 in two salt marsh wetland plant species, *Spartina patens* and *Spartina alterniflora* (Carbonell-Barrachina *et al.*, 1998), ~0.12 in *B. juncea* (Pickering *et al.*, 2000), and 0.11–0.31 in rice (Marin *et al.*, 1992). In a more comprehensive study including 46 plant species, Raab *et al.* (2007b) reported a range from 0.01 to 0.9, with a median of 0.09, in arsenate-treated plants. Although DMA is taken up by roots inefficiently compared with other As species, it is translocated more efficiently from roots to shoots; the shoot to root concentration ratio varied from 0.02 to 9.8, with a median of 0.8 (Raab *et al.*, 2007b).

Table 1 Arsenic (As) speciation and mobility in xylem sap

Species	As species supplied to plants	As species (%)			Ratio of xylem sap As to external As	References
		Arsenate	Arsenite	DMA		
Rice (*Oryza sativa*)	Arsenate	0–19	81–100	0	0.32–0.49	F. J. Zhao *et al.* (unpublished);
	Arsenite	0.2–5.5	94.5–99.8	0	0.30–0.56	Ma *et al*. (2008)
Barley (*Hordeum vulgare*)	Arsenate	39.8	60.2	0	0.09	F. J. Zhao *et al.* (unpublished)
	Arsenite	16.8	83.2	0	0.07	
Holcus lanatus	Arsenate	34.5	65.5	0	0.05	Logoteta *et al*. (2008)
Cucumber (*Cucumis sativus*)	Arsenate	9.6	86.6	3.7	0.18	Mihucz *et al*. (2005)
	Arsenite	10.3	86.2	3.4	0.30	
Brassica juncea	Arsenate	41	59	0	0.04	Pickering *et al*. (2000)
Tomato (*Lycopersicon esculentum*)	Arsenate	5.1	93.0	2.8	0.05	Xu *et al*. (2007)
	Arsenite	10.5	86.7	1.9	0.02	
Pteris vittata	Arsenate	4.6	95.4	0	34–73	Su *et al*. (2008)
	Arsenite	2.1	97.9	0	37–49	

DMA, dimethylarsinic acid.

Why is As translocation from roots to shoots limited? The explanation is probably that arsenate is reduced to arsenite rapidly in roots, followed by complexation with thiols and possibly sequestration in the root vacuoles. When the *A. thaliana* arsenate reductase AtACR2 was silenced using RNAi, As accumulation in the shoots increased markedly; the ratio of shoot:root As concentrations increased from 0.01 in the wild type to 0.25 in the RNAi lines (Dhankher *et al.*, 2006). The authors suggested that blocking AtACR2 would lead to more arsenate in the roots available for xylem transport to the shoots, presumably via the phosphate transport pathway. Intriguingly, Bleeker *et al.* (2006) obtained the opposite results, with the T-DNA insertion lines of AtACR2 showing less As translocation to the shoots.

Studies with the *A. thaliana* phosphate mutant *pho1*, which is defective in xylem loading of phosphate, showed no effect on As distribution to the shoots (Quaghebeur & Rengel, 2004). Furthermore, the *pho2* mutant over-accumulates P in the shoots as a result of a mutation in an E2 conjugase gene involved in P-starvation signalling, but did not over-accumulate As in the shoots (Quaghebeur & Rengel, 2004). These results suggest that As is not loaded into the xylem mainly as the phosphate analogue arsenate. The forms of As transported in the xylem exudates have been investigated in a number of studies. Care must be taken to preserve the As speciation in the sap samples because arsenite may be easily oxidized to arsenate. In all of the plant species studied, arsenite is the predominant form of As in the xylem sap, accounting for 60–100% of the total As (Table 1). Arsenate is a minor species even when plants are fed arsenate. Methylated As is rarely detected in xylem sap. Mihucz *et al.* (2005) and Xu *et al.* (2007) found the presence of DMA in the xylem sap from cucumber (*Cucumis sativus*) and tomato plants, but only at < 4% of the total As. Inorganic arsenite appears to be the main As species that is transported from the root cortical cells to the xylem vessels, because there is no evidence of arsenite-PC or arsenite-GS complexes in the xylem sap of *B. juncea* or sunflower (Pickering *et al.*, 2000; Raab *et al.*, 2005). The fact that arsenite is the dominant form of As in xylem sap is not surprising, considering that arsenate is reduced rapidly in roots.

Plant species vary widely in the xylem mobility of As, as reflected by the ratio of As concentration in the xylem sap to that in the external nutrient solution (Table 1). This ratio is well below 1 in nonhyperaccumulating plants, among which rice stands out as the most efficient in transporting As to the xylem, probably a consequence of the high expression of the Si/arsenite effluxer Lsi2. The As hyperaccumulator *P. vittata*, which has extraordinary xylem mobility for As (discussed further in Section VI), contrasts markedly with nonhyperaccumulating plants.

2. Phloem transport

There is little knowledge of the extent and mechanisms of As transport in the phloem. In rice, As concentrations decrease markedly in the order of roots > stems and leaves > husks > grain (Abedin *et al.*, 2002a; Liu *et al.*, 2006; Xu *et al.*, 2008), suggesting that remobilization of As from stems and leaves to grain, if any, may be limited. However, the contributions of xylem-versus phloem-derived As to the accumulation in grain need to be evaluated experimentally. There is evidence that PCs and other thiol peptides can be transported through phloem from leaves to roots in *A. thaliana* (Chen *et al.*, 2006; Li *et al.*, 2006). Cadmium is likely to be transported as Cd-GS or Cd-PC complexes in the phloem of *Brassica napus* (Mendoza-Cózatl *et al.*, 2008). Whether As(III)-PC or As(III)-GS complexes can be transported in phloem has not been investigated. As these complexes are not very stable at pH > 7.5, their transport in phloem may not be favoured because of the slightly alkaline pH of phloem sap.

VI. Arsenic hyperaccumulation

1. Occurrence

Since the first discovery of As hyperaccumulation in *P. vittata* (Ma *et al.*, 2001), a total of 12 As hyperaccumulators have been identified, all of them belonging to the Pteridaceae family of ferns and most being found within the *Pteris* genus (Supporting Information Table S1). Ferns are early land plants, but the Pteridaceae family arrived relatively late in terms of fern evolution (Meharg, 2003). Not all of the *Pteris* species are As hyperaccumulators, so it would be interesting to investigate the phylogenetic relationship between the hyperaccumulating and nonhyperaccumulating fern species. It is likely that more As-hyperaccumulating fern species will be found, because only a small proportion of the *c.* 400 species within Pteridaceae have been tested for As accumulation to date.

Arsenic hyperaccumulation by *Pteris* species is a constitutive trait, with plants originating from As-contaminated and As-noncontaminated environments showing broadly similar hyperaccumulating abilities (Zhao *et al.*, 2002; Wang *et al.*, 2007). The ability is present in both sporophytes and gametophytes (Gumaelius *et al.*, 2004). Accumulation of > 2% As in the frond dry weight has been reported (Wang *et al.*, 2002; Gumaelius *et al.*, 2004), although the limit of tolerance is between 5000 and 10 000 mg kg^{-1} (Lombi *et al.*, 2002; Tu & Ma, 2002).

2. Mechanisms of As hyperaccumulation

The As hyperaccumulation phenotype is a result of a combination of several physiological processes, although little is known about the underpinning molecular mechanisms. Hypertolerance is found in all naturally evolved hyperaccumulators; As hyperaccumulators have a much higher degree of tolerance than the nonhyperaccumulator species within the *Pteris* genus (Caille *et al.*, 2005; Singh & Ma, 2006). Compared with the nonhyperaccumulator *Pteris ensiformis*, *P. vittata* possesses a higher antioxidant capacity and also maintains a lower concentration of reactive oxygen species (Srivastava *et al.*, 2005; Singh *et al.*, 2006). By contrast to nonhyperaccumulators, which rely on PC complexation for As detoxification and tolerance, very little of the As accumulated in the roots and fronds of *P. vittata* and *Pteris cretica* (~1–3% of the total As) is complexed with PCs (Zhao *et al.*, 2003; Raab *et al.*, 2004; Zhang *et al.*, 2004). The majority of As (60–90% of the total As) in the fronds of *Pteris* species is inorganic arsenite (Francesconi *et al.*, 2002; Lombi *et al.*, 2002; Wang *et al.*, 2002; Zhang *et al.*, 2002; Webb *et al.*, 2003; Pickering *et al.*, 2006; Su *et al.*, 2008), which appears to be stored in the vacuoles (Lombi *et al.*, 2002; Pickering *et al.*, 2006). It may be prohibitively costly for plants to evolve PC-dependent As hyperaccumulation, because at the 1 : 3 stoichiometric ratio of As:S, the amount of S required to complex 2500 mg As kg^{-1} in the fronds of *P. vittata* would have exceeded the total amount of S typically accumulated by the plants (Zhao *et al.*, 2002). Vacuolar sequestration of arsenite is therefore the key mechanism of As detoxification in the hyperaccumulator ferns. How this is achieved, and especially the identity of the vacuolar transporters responsible for arsenite transport across the tonoplast, deserve further investigation.

Arsenate is likely to be the main form of As taken up by the hyperaccumulator ferns as they grow on aerobic soils, and the uptake is via the phosphate transport system as in nonhyperaccumulator plants (Wang *et al.*, 2002; Poynton *et al.*, 2004). Compared with nonhyperaccumulator ferns, *P. vittata* and *P. cretica* have a higher arsenate influx (Poynton *et al.*, 2004; Caille *et al.*, 2005) and a lower K_m, indicating higher affinity of the transporter for arsenate (Poynton *et al.*, 2004). The most striking difference lies in the efficiency of root to shoot translocation, exemplified by the large ratios of shoot to root As concentrations in the As hyperaccumulators (typically 5–25; e.g. Tu & Ma, 2002; Tu *et al.*, 2002; Zhao *et al.*, 2002). The ratio of the As concentration in the xylem sap of *P. vittata* to that in the nutrient solution was about 2 orders of magnitude higher than that in the nonhyperaccumulators (Table 1). Su *et al.* (2008) showed that the majority (93–98%) of the As in the xylem sap of *P. vittata* was in the form of arsenite, regardless of whether the plant was treated with arsenate or arsenite. Roots or rhizoids of *P. vittata* are likely to be the main location of arsenate reduction, with arsenite being preferentially loaded into the xylem. This is consistent with the findings of Duan *et al.* (2005), who found the activity of the glutathione-dependent arsenate reduction only in the roots of this hyperaccumulator. Although the gene encoding an arsenate reductase has been cloned from *P. vittata* gametophytes (Ellis *et al.*, 2006), its *in planta* role and its contribution to overall arsenate reduction have not been ascertained. Other enzymes (e.g. TPI and Grx5) may be directly or indirectly involved (Rathinasabapathi *et al.*, 2006; Sundaram *et al.*, 2008).

Also, hyperaccumulators differ from nonhyperaccumulators in that there is minimal efflux of arsenite from the roots of *P. vittata* to the external medium (Su *et al.*, 2008). This, together with little PC complexation of arsenite in *P. vittata* roots (Zhao *et al.*, 2003), may explain the highly efficient xylem transfer in hyperaccumulators. Consistent with this model is the observation that the ease of root to shoot translocation of As is inversely related to the degree of arsenite–thiol co-ordination in roots (Huang *et al.*, 2008). It is also possible that transporters mediating arsenite efflux from cortical cells towards xylem are highly expressed in As hyperaccumulators, although their identities are not yet known.

VII. Conclusions

There has been substantial progress in understanding the pathways of As uptake and metabolism in plants, especially regarding arsenite transport, arsenate reduction and As

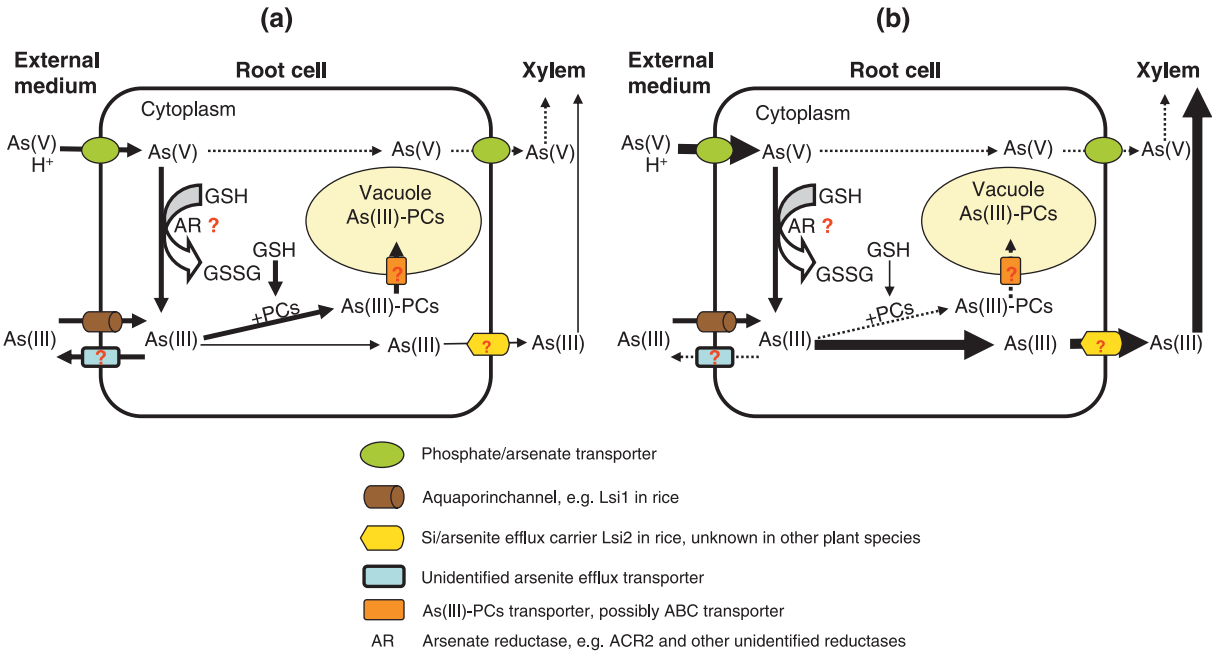

Fig. 3 Schematic diagram of arsenic (As) uptake and metabolism in roots of nonhyperaccumulators (a) and hyperaccumulators (b). Line thickness relates to flux rate, with the dotted line indicating the slowest rate. Question marks indicate major knowledge gaps. ABC, ATP-binding cassette; ACR, arsenic compound resistance; GSSG, oxidized glutathione; GSH, glutathione; Lsi, Si influx transporter; PC, phytochelatin.

complexation and speciation. Current understanding of the As uptake and metabolism pathways in As hyperaccumulators and nonhyperaccumulators is summarized schematically in Fig. 3. Arsenic is a nonessential element and its uptake by plants is an inadvertent event driven by transporters for essential/beneficial elements. While the shared phosphate/arsenate transport system has been well established, arsenite transport via aquaporins and the Si effluxer represents new knowledge. Arsenite transport is much more prevalent than previously thought, because arsenite is the main species of As that is translocated from roots to shoots, which is also the bottleneck of As accumulation in plant shoots. Because these transporters play essential roles in plants, it is not conceivable to block As entry into plants by knocking out the responsible transporters without affecting plant functions. However, it is likely that there is considerable allelic variation in the affinities of the transporters for As species, thus allowing exploitation of those variants of transporters that can better discriminate against As.

Gaps in our knowledge are also illustrated in Fig. 3. *In planta* arsenate reduction probably involves multiple pathways catalysed by different enzymes; only one enzyme has been identified to date. The transporters responsible for arsenite efflux to the external medium, the transport of arsenite or arsenite–thiol complexes into the vacuole and the transport of arsenite towards the xylem (other than in rice) have yet to be identified. The genes and enzymes responsible for As methylation in plants are still unknown. Internal redistribution of As, especially from vegetative tissues to grain, is poorly understood. Molecular understanding of As hyperaccumulation remains rudimentary. Future research should address these knowledge gaps, taking advantages of modern analytical tools for As speciation and a combination of physiological and molecular approaches.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Table S1 List of arsenic hyperaccumulator species

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