Plant Aquaporins and Metalloids

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Abstract The metalloids represent a group of physiologically important elements, some of which are essential or at least beneficial (boron and silicon) for plant growth and some of which are toxic (arsenic, antimony and germanium). Exposure to and availability of metalloids can have major effects on plant fitness and yield and can seriously downgrade the end-use quality of certain crop products. Plants have evolved various membrane transport systems to regulate metalloid transport both at the cellular and whole plant level. To date, the channel proteins referred to as aquaporins (AQPs) represent the most favored candidates ensuring metalloid homeostasis. AQPs are found in all living organisms. From bacteria to mammals and also in plants, several distinct AQP subfamilies facilitate the transmembrane diffusion of the set of physiologically and environmentally important metalloids. A subgroup of the Nodulin26-like intrinsic protein AQP subfamily (NIPs) has been designated as functional metalloidoporins. NIPs are the only known transport protein family in the plant kingdom which are essential for the uptake, translocation, or extrusion of various uncharged metalloid species. This chapter describes the various features, and particularly the metalloid transport properties of plant AQPs, and illustrates their physiologically important contributions to metalloid homeostasis. Their intimate involvement in metalloid transport underlines their relevance to plant nutrition, detoxification of toxic mineral elements phytoremediation, phytomining, and biofortification.

1 The Metalloids

The metalloids represent a group of elements whose physical and chemical properties define them as being neither metals nor nonmetals. The six elements falling into this class are boron (B), silicon (Si), arsenic (As), antimony (Sb), germanium (Ge), and tellurium (Te). Selenium (Se), polonium (Po), and astatine (At) also belong to

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pk_{a1}	protonated [metalloid acid]-H		>90% protonated acid	deprotonated $[\text{metalloid base}]^-$	
9.25	boric acid	H_3BO_3	pH < 8.30	$[H_4BO_4]^-$	borate
9.51	silicic acid	H_4SiO_4	pH < 8.56	$[H_3SiO_4]^-$	silicate
9.23	arsenous acid	H_3AsO_3	pH < 8.28	$[H_2AsO_3]^-$	arsenite
2.26	arsenic acid	H_3AsO_4	pH < 1.31	$[H_2AsO_4]^-$	arsenate
11.8	antimonous acid	H_3SbO_3	pH < 10.85	$[H_2SbO_3]^-$	antimonite
2.85	antimonic acid	H_3SbO_4	pH < 1.9	$[H_2SbO_4]^-$	antimonate
9.0	germanic acid	H_2GeO_3	pH < 8.05	$[HGeO_3]^-$	germanate
2.57	selenous acid	H_2SeO_3	pH < 1.62	$[HSeO_3]^-$	selenite
1.74	selenic acid	H_2SeO_4	pH < 0.79	$[HSeO_4]^-$	selenate

Fig. 1 pH-dependent acid-base equilibrium of hydroxylated metalloid acids. The *green color* indicates the chemical form and structural formula of the metalloid which predominates at the physiological pH range. Only neutral forms of metalloid acids are channeled by metalloidoporins. pKa values of the metalloid acids and the structural formula are given. The pH range in which more than 90 % of the metalloid acid occurs in its fully protonated acid species is displayed

the group but are less commonly designated as such. The lack of an unambiguous set of defining criteria reflects the dependence of many of their physical and chemical properties on ambient temperature and pressure, as well as on their crystal lattice/crystal structure. The metalloids have a metallic appearance but are brittle. They are electrical semiconductors, can alloy with metals, and typically form amphoteric to weakly acidic oxides (Fig. 1). Their abundance in the Earth's crust varies from Si – the second most abundant element after oxygen, constituting ~25 % by mass of the Earth's crust (Lombi and Holm 2010) – to At, of which not more than 25 g is present in the total Earth' s crust at any given time (Lombi and Holm 2010).

The biological significance of the metalloids ranges from essential through beneficial to toxic. B is required for plant growth (Marschner 2012); Si is not generally recognized as essential, except for a few algal species and members of the *Equisetaceae* (Epstein 1994), although it is recognized as being beneficial for growth in many species. Se is essential in the human diet and for the growth of some algae, but is not so for plants (Pilon-Smits and Quinn 2010). As, Sb, Ge, and Te are all considered to be (phyto)toxic. The molecular form and the concentration of metalloids are both important in assessing the reaction of a plant to exposure. The impact of beneficial and essential metalloids on a given plant's metabolism can be summarized, *pace* Paracelsus: "the only difference between a nutrient and a poison is the dose."

2 The "Major Intrinsic Proteins" or Aquaporins

The large family of "major intrinsic proteins" comprises transmembrane-spanning channel proteins, found in almost all life forms (the exceptions being certain thermophilic *Archaea* and intracellular bacteria) (Abascal et al. 2014). The term "aquaporin" (AQP) is widely used as a synonym. Despite their sequence variation at the amino acid level, crystal structures acquired to date imply a high degree of conservation. The AQPs form tetramers: each monomer constitutes a functional channel on its own and is composed of six transmembrane-spanning helices (TMHs) with five connecting loops (loop A to loop E) and two cytoplasmic termini (see chapter "Structural Basis of the Permeation Function of Plant Aquaporins"). They define a narrow path across various cellular membranes, including the plasma membrane, the endoplasmic reticulum, the mitochondria, the vacuole, the vesicles involved in the trafficking pathway, the tonoplast, and the chloroplast (Maurel et al. 2015). They facilitate the diffusion of water and small uncharged solutes and have been shown by various means to control water homeostasis. In plants, they function to import water into the root from the soil, to transport it from the root to the shoot, to drive osmotic force-driven growth, and to ensure cytoplasmic osmolarity (Maurel et al. 2015; Chaumont and Tyerman 2014; see chapters "Aquaporins and Root Water Uptake" and "Aquaporins and Leaf Water Relations"). AQPs also have an impact on the uptake, translocation, sequestration, and extrusion of uncharged and physiologically important compounds such as glycerol (Richey and Lin 1972; Luyten et al. 1995), nitric oxide (NO) (Herrera et al. 2006), hydrogen peroxide (H_2O_2) (Bienert et al. 2006, 2007; Dynowski et al. 2008), urea (CH_4N_2O) (Liu et al. 2003), ammonia (NH_3) (Jahn et al. 2004; Loqué et al. 2005), lactic acid (Tsukaguchi et al. 1998; Choi and Roberts 2007; Bienert et al. 2013), and acetic acid (Mollapour and Piper 2007). Of note in the context of this chapter, they also transport arsenous acid (H_3AsO_3) (Bienert et al. 2008a, b; Ma et al. 2008; Kamiya et al. 2009), boric acid (H_3BO_3) (Takano et al. 2006; Tanaka et al. 2008; Hanaoka et al. 2014), silicic acid (H_4SiO_4) (Ma et al. 2006), antimonous acid (H_3SbO_3) (Bienert et al. 2008a; Kamiya et al. 2009), germanic acid (H_4GeO_4) (Ma et al. 2006; Hayes et al. 2013), and selenous acid (H_2SeO_3) (Zhao et al. 2010a, b) (Fig. 2).

AQPs allow the passage of a single continuous file of molecules. While a few ion-mediating AQPs have been identified (reviewed by Yool and Campbell 2012), the consensus, based on chemical species selectivity, is that only non-charged molecules are able to pass through the majority of AQP channels. However, compared to animal AQPs, not many plant AQPs have been assessed for being permeable to ions. The selectivity and transport capacity of each isoform are determined by the identity of the amino acids aligned along the channel pathway (see also chapter "Structural Basis of the Permeation Function of Plant Aquaporins"). The so-called "aromatic/arginine" (ar/R) selective filter, situated on the luminal side of the membrane, comprises four residues (R1–R4), located in TMH2 (R1), TMH5 (R2), and loop E (R3 and R4); this structure

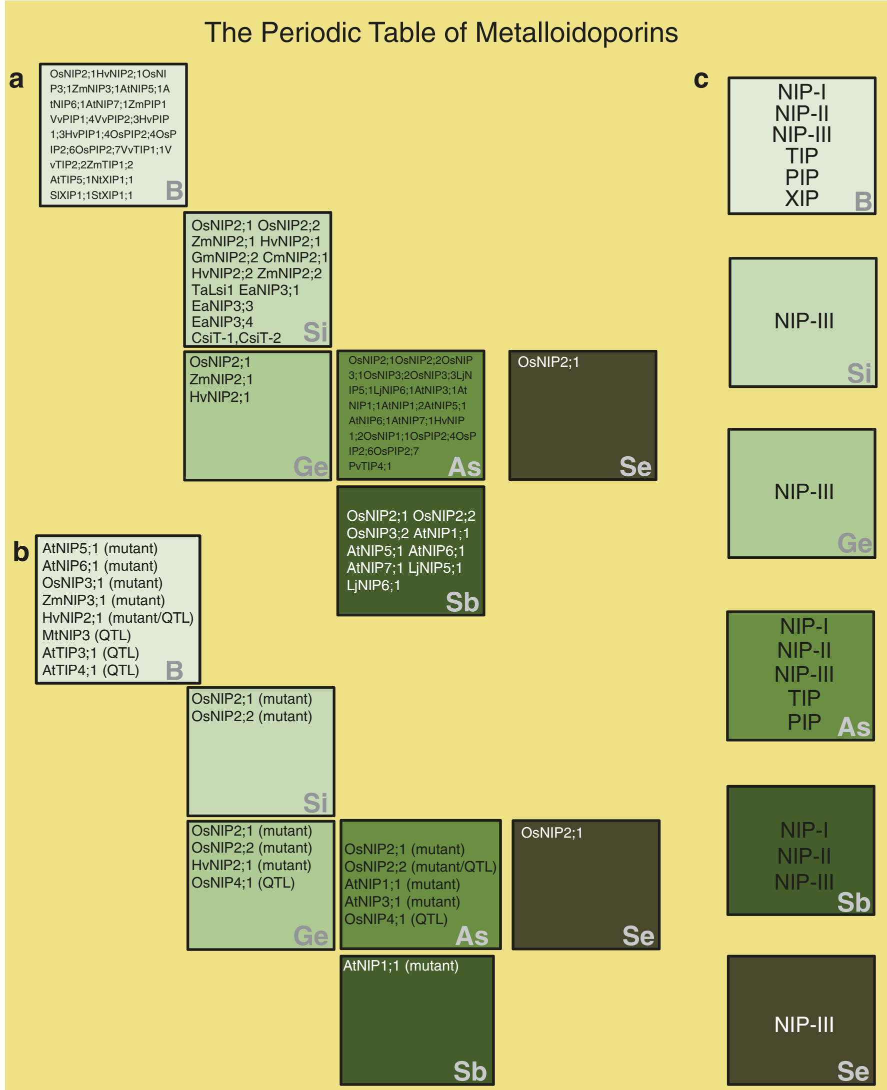

Fig. 2 The periodic table of metalloidoporins. (**a**) Aquaporin channel proteins, which were shown to be permeable to the corresponding metalloid acid in transport assays performed in plants, or heterologous expression systems (i.e., plants, frog oocytes, or yeasts) are listed. (**b**) Listed aquaporins have either been identified to occur in quantitative trait loci genomic regions linked to the tolerance toward toxicity or deficiency of the corresponding metalloid species (indicated by "QTL") or which, when being silenced or knocked out in planta (indicated by "mutant"), caused obvious metalloid deficiency or tolerance phenotypes. (**c**) Phylogenetic or functional plant aquaporin groups which were shown to be permeable to the corresponding metalloid acid in transport assays performed in plants or heterologous expression systems (plants, frog oocytes, or yeasts) are listed

forms a size exclusion barrier and the hydrogen bond environment necessary for the efficient transport of a particular substrate (Murata et al. 2000). A second selectivity filter, the so-called "NPA" motif (asparagine-proline-alanine or variants thereof), is formed by the two membrane-embedded half-helices of loop A and loop E, each containing the conserved AQP signature. The "NPA"motifs meet in the center of the membrane, forming a narrow hydrophilic cavity (Murata et al. 2000) and are responsible for the exclusion of water-mediated proton and ion transport.

A major difference between plants and other organisms is the large number of AQP isoforms encoded by plant genomes (Abascal et al. 2014). While the norm in bacteria, fungi, and mammals is 2–13 genes per genome (Agre and Kozono 2003), the moss *Physcomitrella patens* and the lycophyte *Selaginella moellendorffii* encode, respectively, 23 and 19 *AQP*s (Danielson and Johanson 2008; Anderberg et al. 2012). Higher plant genomes harbor from 30 to 70 isoforms: the number in *Arabidopsis thaliana* is 35 (Johanson et al. 2001), in cabbage (*Brassica oleracea*) 67 (Diehn et al. 2015), in Chinese cabbage (*Brassica rapa*) 57 (Diehn et al. 2015), in poplar (*Populus trichocarpa)* 55 (Gupta and Sankararamakrishnan 2009), in banana (*Musa* sp.) 47 (Hu et al. 2015), in castor bean (*Ricinus communis*) 47 (Zou et al. 2015), in soybean (*Glycine max*) 66 (Zhang et al. 2013), in potato (*Solanum tuberosum*) 41 (Venkatesh et al. 2013), in tomato (*Solanum lycopersicum*) 47 (Sade et al. 2009; Reuscher et al. 2013), in cotton (*Gossypium hirsutum*) 71 (Park et al. 2010), in rice (*Oryza sativa*) 33 (Sakurai et al. 2005), and in maize (*Zea mays*) at least 36 (Chaumont et al. 2001).

Based on their sequence, the AQPs have been classified into two major subgroups, which in both bacteria and mammalians reflect their contrasting functionality: the orthodox AQPs (AQPs) act as channels for water and small solutes such as ammonia or hydrogen peroxide, while the aquaglyceroporins (GLPs) are responsible for the transport of solutes, such as glycerol or urea. In plants, the congruence between phylogeny and functionality is less clear. The sequences present in higher plants cluster phylogenetically with the AQPs and have been arranged into five distinct subfamilies, namely, the nodulin26-like intrinsic proteins (NIPs), the plasma membrane intrinsic proteins (PIPs), the tonoplast intrinsic proteins (TIPs), the small basic intrinsic proteins (SIPs), and the as yet poorly characterized X intrinsic proteins (XIPs) (Chaumont et al. 2001; Johanson et al. 2001; Danielson and Johanson 2008). XIPs are found in many, but not all, species within the section *Magnoliopsida* (they are not present in species belonging to the Brassicaceae), but have not been identified in any section *Liliopsida* species to date (Danielson and Johanson 2008). Analyses of the genomes of lower plants and algae have revealed several mostly not yet functionally characterized but clearly distinct AQP subfamilies (Anderberg et al. 2011; Khabudaev et al. 2014). For some plant AQPs (notably the NIPs and XIPs), certain specific sequence features, along with their functionality, have been taken to suggest a functional equivalence with the GLPs.

3 Non-plant AQP and GLP-Mediated Metalloid Transport

The transport of glycerol mediated by GLPs is an important component of carbon metabolism and osmoregulation in bacteria, *Archaea*, protozoans, and mammals (Hara-Chikuma and Verkman 2006; Laforenza et al. 2015; Ahmadpour et al. 2014). Some GLPs are better described as "metalloidoporins" (Pommerrenig et al. 2015), since they fulfill physiologically important metalloid channel functions, thereby ensuring cellular metalloid homeostasis. Representative examples for such functional metalloidoporin GLPs are isoforms, which are part of As resistance (ars) operons. For example, the As resistance operons in bacteria such as *Escherichia coli* comprise the five genes *arsR*, *arsD*, *arsA*, *arsB*, and *arsC* (Rosen and Tamas 2010). The presence of arsenate ($H_2AsO_4^-$) in the growing medium activates *arsR*, which encodes a regulatory protein; the products of *arsC* and *arsD* are, respectively, an arsenate reductase and an arsenate binding metallochaperone, which together deliver arsenite ($H_2AsO_3^-$) to the ATP-driven extrusion pump encoded by *arsA* and *arsB* (Rosen and Tamas 2010). In the bacterial species *Sinorhizobium meliloti*, *Mesorhizobium loti*, *Caulobacter crescentus*, and *Ralstonia solanacearum,* a gene encoding a GLP aquaporin, which functions as an As-permeable channel, replaces the *arsB*-encoded efflux pump (Yang et al. 2005). These cases demonstrate that certain bacteria have adapted AQPs to handle As efflux and that an inheritable link between AQPs and metalloid transport exist (Yang et al. 2005). A further exciting link between metalloid transport and AQP function is represented in the actinomycete *Salinispora tropica*, where a GLP sequence has been fused to the sequence encoding an arsenate reductase domain, resulting in the translation of a dual function protein (Wu et al. 2010). The N-terminal GLP channel protein shows a greater selectivity for H_3AsO_3 than for either water or glycerol (Mukhopadhyay et al. 2014) and facilitates the efflux of H_3AsO_3 out of the cells directly at its site of production catalyzed by the C terminal arsenate reductase region of the protein (Wu et al. 2010). This spatially identical site of production and transport has the advantage that toxic As species do not pass through the cytoplasm before reaching their efflux site.

In *Saccharomyces cerevisiae*, the GLP FpsI acts normally as an osmoregulator. When the yeast cells are exposed to H_3AsO_3 stress, *FpsI* transcription is downregulated, and the preexisting FpsI in the cell will be inactivated in a phosphorylation-dependent manner (reviewed by Maciaszczyk-Dziubinska et al. 2012). Once inactivated, short-term H_3AsO_3 uptake is prevented; after a longer exposure to the stress, the abundance of *FpsI* transcript rises, which increases the efficiency of H_3AsO_3 efflux. The required concentration gradient is established in the yeast cell via the exudation of glutathione, which enables the exported H_3AsO_3 to be extracellularly chelated (Thorsen et al. 2012). Mammalian GLPs have also been identified as participating in As detoxification. This was demonstrated by the impaired ability of AQP9-null mice and mouse hepatocytes to dispose of As and which therefore suffer an increased severity of toxicity symptoms (Carbrey et al. 2009; Shinkai et al. 2009). Reviews by Mukhopadhyay et al. (Mukhopadhyay et al. 2014) and by

Maciaszczyk-Dziubinska et al. (Maciaszczyk-Dziubinska et al. 2012) have detailed how non-plant AQP channels support the bidirectional cross-membrane movement of metalloids in a range of organisms.

The above-depicted examples of non-plant AQP and GLP-mediated metalloid transport processes are listed to demonstrate that the link between AQPs and metalloid transport is non-incidental in nature and is given across kingdoms. Diverse organisms independently evolved different AQP-employing strategies to regulate the transport and homeostasis of various metalloids. The adaption of AQPs to act as metalloidoporins is, on the one hand, based on the chemical characteristics of the channel and, on the other, on the physicochemical properties of uncharged hydroxylated metalloid species resembling those of glycerol, the suggested original substrate of AQPs. The size of undissociated hydroxy-metalloid acids (Fig. 1) and their volume, dipole moment, surface charge distribution, and ability to form hydrogen bonds are all reminiscent of glycerol. All these attributes are decisive for the efficient passage though the AQP pore and metalloids behave as effective molecular transport mimics of glycerol (Porquet and Filella 2007). The experimental data derived from bacteria to mammals did significantly change the view on how membrane permeability to metalloids might be regulated in planta. The long-held assumption that uncharged metalloids are solely transported across plant membranes via a process of passive nonprotein-facilitated diffusion has had to be reconsidered in the light of the discovery of metalloid-permeable plant AQPs.

The following observations support the view that plant membranes can obstruct the diffusion of metalloids and that plant AQPs, like their GLP counterparts, offer the means to adjust membrane permeability appropriately: (1) concentration gradients across membranes of uncharged metalloid species have been detected (Meharg and Jardine 2003; Dordas et al. 2000; Dordas and Brown 2000), (2) the permeability coefficients for B measured in certain plant vesicles are significantly higher than those measured in synthetic liposomes (Dordas et al. 2000; Dordas and Brown 2000), (3) the transmembrane transport of B and As can be inhibited by potent AQP blockers (Meharg and Jardine 2003; Dordas et al. 2000), while (4) glycerol acts as a competitor for As flux (Meharg and Jardine 2003). As described subsequently, a number of both target-oriented and nontargeted approaches have revealed that certain plant AQPs (and especially members of the NIP subfamily) are physiologically important metalloidoporins.

4 NIP-Mediated Metalloid Transport in Plants

The evolutionary origin of the NIPs is unclear. Phylogenetically, they cluster with bacterial and archaeal NIP-like proteins, forming a basal lineage within the AQPs distinct from the aquaporin Z-like or glycerol uptake facilitator-like proteins (Abascal et al. 2014). Their phylogeny provides support for the notion that plant NIPs were originally acquired via horizontal gene transfer from the prokaryotic chloroplast progenitor (Abascal et al. 2014), but the alternative route of convergent

functional evolution cannot be totally excluded. The plant NIPs can be phylogenetically divided into subgroups NIP1 through NIP5, which are remarkably well conserved across species (Danielson and Johanson 2010; see also chapter "The Nodulin 26 Intrinsic Protein Subfamily"). Note that the numerals "1" to "5" designating the five phylogenetically NIP subgroups do not match with the designated numerals designating *NIP* genes within one species. The low level of node support and the various polytomies that arise in *NIP* phylogenies emphasize the uncertain evolutionary relationships obtained between the *NIP* subgroups and isoforms (Danielson and Johanson 2010; Abascal et al. 2014). Based on the amino acid composition of the ar/R constriction region, three functional groups (NIP-I through -III) have been recognized (Wallace and Roberts 2004; Mitani et al. 2008; see chapter "The Nodulin 26 Intrinsic Protein Subfamily"). The three functional NIP subgroups are represented in all higher plants, although NIP-III is largely confined to section *Liliopsida* species (Danielson and Johanson 2010).

The soybean NIP GmNOD26 was the first plant AQP to be described (Fortin et al. 1987; see chapter "The Nodulin 26 Intrinsic Protein Subfamily") and became the eponym of the NIP subfamily. It is the major proteinaceous constituent of the root nodule membranes (Fortin et al. 1987; Dean et al. 1999). Transport assays designed to assess the permeability of diverse functional NIP subgroups have shown that glycerol, NH_3, CH_4N_2O, water, H_2O_2, and metalloids can all be transported via these proteins (Bienert and Chaumont 2011). To date, however, in planta evidence for physiologically relevant non-metalloid transport is lacking. NIPs are not only channel metalloids but are also essentially required for their transport into and within the plant. Evidence gathered from genetic, physiological, and molecular biology experiments argues for them having a major impact on metalloid homeostasis. Indeed, they are the only protein family in plants known to be essential for the uptake, translocation, as well as extrusion of a number of uncharged metalloids (Fig. 2).

4.1 NIP-Mediated Transport of Boron

B has long been recognized as essential for plant growth (Warrington 1923); nevertheless, the only known function of B surrounds the formation of borate ester bridges within the primary cell wall, which serve to crosslink rhamnogalacturonan-II (RG-II) monomers. Dimerized RG-II contributes to the overall network of pectic polysaccharides (Funakawa and Miwa 2015). In a standard plant cell wall, >90 % of RG-II monomers are dimerized, and although the overall proportion of cell wall pectin represented by RG-II is only around 10 %, it is clear that the quantity of free and cross-linked RG-II is critical for cell differentiation and elongation, as well as for plant growth and development (Funakawa and Miwa 2015). Insufficient crosslinking induced by B-deficient growing conditions has a deleterious effect on plant growth and results in dwarfed plants (O'Neill et al. 2001). *Magnoliopsida* species tend to have a higher B demand than those in the class of *Liliopsida*, which

correlates with the quantity of RG-II found within the cell wall (Pérez et al. 2003). B deficiency manifests itself in the form of meristematic defects, abnormal cell differentiation, and a compromised expansion of the stem, leaf, and vascular system. Flowering – especially pollen development – and pollen tube growth are also highly sensitive to B deficiency (Marschner 2012). While the molecular roles of B are enigmatic, the last years have provided detailed understanding on B transport mechanisms in plants.

The transcription of NIP-II genes in roots such as *AtNIP5;1* and its orthologs in various plant species responds rapidly to B starvation (Takano et al. 2006; Hanaoka et al. 2014; Zhou et al. 2015). *NIP5;1* transcripts of Arabidopsis, citrus, and rice are strongly upregulated within 24 h after the onset of B-deficient conditions. Reverse genetic approaches in Arabidopsis and rice using NIP-II knockout and silenced plants have shown that B uptake into the roots requires a functional *AtNIP5;1* and *OsNIP3;1*, respectively (Takano et al. 2006; Hanaoka et al. 2014). The heterologous expression of *AtNIP5;1*, *AtNIP6;1*, and *OsNIP3;1* promotes the transport of H_3BO_3 in yeast, frog oocytes, and plants, demonstrating that they are all functional B transporters (Takano et al. 2006; Tanaka et al. 2008; Hanaoka et al. 2014). *Atnip5;1* and *Atnip6;1* knockouts display characteristic symptoms of B deficiency, i.e., reduced stability of the epidermis abolished apical dominance and perturbed cell differentiation (Takano et al. 2006; Tanaka et al. 2008). While *AtNIP5;1* is expressed in the root epidermis and operates to move H_3BO_3 into the root, the *AtNIP6;1* product is deposited in young leaf phloem companion and parenchyma cells, where it presumably is involved in unloading H_3BO_3 from the xylem into the phloem (Takano et al. 2006; Tanaka et al. 2008) (see also chapter "Plant Aquaporin Trafficking").

Under B-deficient conditions, the shoot growth of *Atnip6;1* knockouts is restricted, suggesting that AtNIP6;1 is important for the allocation of B to developing and meristematic tissue (Tanaka et al. 2008). Under such conditions, both *Atnip5;1* and *Atnip6;1* knockouts form largely sterile flowers. In rice, OsNIP3;1 has been shown as responsible for the uptake of B into the root, its translocation into the shoot, and its unloading from the xylem into the phloem in the mature leaf (Hanaoka et al. 2014). Its encoding gene is strongly transcribed in the root exodermis and in the cells surrounding the vascular bundles in both the root and shoot. When the *OsNIP3;1* gene is silenced, neither the total B concentration nor its distribution between the shoot and root is disturbed, provided that the conditions are not B-deficient; however, when the supply of B is limiting, the shoot's B content is significantly decreased. This indicates different regulations of AtNIP5;1 and its ortholog OsNIP3;1. Consistent with this result, a map-based cloning approach targeting the *Dwarf and tiller-enhancing* 1 (*dte-1*) rice mutant identified *OsNIP3;1* as the candidate gene underlying the mutated locus (Liu et al. 2015); the mutant displays B deficiency symptoms when the supply of B is suboptimal (Liu et al. 2015). These results clearly indicate the crucial function of NIPs in plant B homeostasis. *ZmNIP3;1*, the maize ortholog of *OsNIP3;1*, has been similarly identified thanks to its positional cloning to underlie the phenotype of the *tassel-less1* (*tsl-1*) mutant (Durbak et al. 2014). This mutant produces not only an aberrant flower, but its vegetative growth resembles that of a B-deficient maize plant. When expressed heterologously, *ZmNIP3;1* facilitates

the uptake of B into both frog oocytes and yeast cells (Durbak et al. 2014). Tissue B content is suboptimal in the *tassel-less1* mutant, and the mutant phenotype can be rescued by providing a source of B. *ZmNIP3;1* transcript is highly abundant in the wild-type silk and (to a lesser extent) in the tassel and root, a distribution which is dissimilar to that shown by its *A. thaliana* and rice orthologs. The *tassel-less 1* mutant carries a gene encoding for a mutated ZmNIP3;1 protein resulting in a nonfunctional channel protein (Durbak et al. 2014, Leonard et al. 2014).

Even though they differ with respect to their spatial transcription profile, each of the *Atnip5;1*, *Atnip6;1*, *Osnip3;1* (*dte-1*), and *Zmnip3;1* (*ts-l1*) loss-of-function mutants expresses a normal phenotype, provided that the supply of B is non-limiting; however, when this is not the case, the plants remain stunted, their apical dominance is compromised, and they suffer from inflorescence defects and reproductive sterility (Takano et al. 2006; Tanaka et al. 2008; Durbak et al. 2014; Hanaoka et al. 2014; Liu et al. 2015). The NIP-II group AQP isoforms are therefore crucial for the uptake and distribution of B within the plant not just in section *Magnoliopsida* species, which have a relatively high B requirement, but also in section *Liliopsida* ones, which do not need as much B for growth (Marschner 2012).

Excessive soil B is phytotoxic. B is taken up in the transpiration stream, so tends to accumulate initially in more mature leaves (Nable et al. 1997). As a result, B toxicity manifests itself as leaf chlorosis/necrosis, spreading from the leaf margin into the center of the leaf (Nable et al. 1997; Shatil-Cohen and Moshelion 2012). Barley (which, like all of the cereals, has a relatively low B requirement) is particularly sensitive to B toxicity (Schnurbusch et al. 2010). The genomic region of barley associated with B tolerance harbors *HvNIP2;1*. The mapping population progeny carrying the *HvNIP2;1* allele that is present in the B tolerant mapping parent (the Algerian landrace Sahara 3771) exhibits a higher level of tolerance and accumulates less B in their leaves than those which carry the alternative allele from cv. Clipper. The level of *HvNIP2;1* transcript increases from the root tip to the basal root region in both parental lines, but its abundance is up to 15-fold higher in the roots of the sensitive parent (Schnurbusch et al. 2010). A sequence comparison of the alternative *HvNIP2;1* coding sequences identified only one base variation, while the predicted encoded proteins are identical. The *HvNIP2;1* upstream sequence (up to −1377 nt) is wholly monomorphic, so the differential transcription of the gene has been concluded to reflect sequence variation even further upstream (Schnurbusch et al. 2010). Based on its H_4SiO_4 permeability both in frog oocytes and *in planta*, and its tissue distribution, the barley protein HvNIP2;1 is also thought to have an impact on the supply of the metalloid Si, even though no correlation could be established between *HvNIP2;1* transcription and the plant's Si uptake capacity (Chiba et al. 2009).

In *Medicago truncatula*, Bogacki et al. (2013) show that 95 % of the phenotypic variation for B tolerance displayed by the progeny of a cross between two contrasting parents could be linked to two microsatellite loci, which flank a cluster of five predicted AQP genes. Among them, only one (*MtNIP3*) is transcribed in the leaf and root. While the transcript levels are low and indistinguishable in the roots of tolerant and sensitive types, a fourfold difference in the leaf is observed, and the

leaf B concentration is correlated with the phenotype, suggesting that *MtNIP3* is likely the gene underlying B tolerance (Bogacki et al. 2013). Based on the observed B distribution, it can be excluded that an enhanced B translocation from the roots is responsible for the differential B tolerance between these genotypes. It has been suggested that the redistribution of B from the symplast to the apoplast of leaves and subsequent leaching through rain and/or the removal of B via guttation represents the basis for the observed MtNIP3-dependent tolerance (Bogacki et al. 2013).

These examples demonstrate that the regulation of NIP-metalloidoporin activity and expression are important mechanisms for plants to adapt to either B-deficient or toxic environmental conditions.

NIPs are not the only proteins known to be involved in B transport in plants. The first B transporter to be described was identified from the analysis of an *A. thaliana* mutant in which shoot, but not root growth, was severely inhibited by B deficiency (Takano et al. 2002). The product of the mutated gene *AtBOR1* was shown to mediate the xylem loading of B. BOR proteins share homology with the Slc4 bicarbonate transporters (Parker and Boron 2013) and are predicted to form 14 plasma membrane-spanning helices. Potentially, a secondary active transport process is responsible for the BOR-mediated efflux of B (Parker and Boron 2013). The substrate used by HvBOT1, a sodium-dependent BOR transport protein from barley, was demonstrated to be the borate anion $H_4BO_4^-$ (Nagarajan et al. 2015). $H_4BO_4^-$ represents highly likely also the substrate of other BOR proteins. BOR-type transporters and NIP-II group AQPs cooperatively regulate B influx and efflux in a species-dependent manner. In rice, *OsNIP3;1* – but not *OsBOR1* – is expressed in the stele, while in the exodermis and endodermis, the genes are co-expressed (Nakagawa et al. 2007). In contrast, in *A. thaliana* AtBOR1 and AtNIP5;1 together control the radial transport of B to the vascular system in various cell types together, and are co-expressed in the endodermis (Takano et al. 2008, 2010).

A responsive metalloid transport system is of biological importance because plants can face sudden changes in the availability of these elements. Several *AtNIP5;1* gene homologs, the products of which are both able to channel H_3BO_3 and are known to be important for B uptake, are transcriptionally upregulated when the availability of B is limiting but downregulated when B is in oversupply (Takano et al. 2006; Tanaka et al. 2008; Hanaoka et al. 2014; Zhou et al. 2015; Martínez-Cuenca et al. 2015). The *AtNIP5;1* 5′-UTR is particularly important both for the induction of *AtNIP5;1* transcription and for its mRNA degradation under B-sufficient conditions (Tanaka et al. 2011). A similar regulatory role has been suggested for the almost identical 5′-UTR of *OsNIP3;1* in B-deficient conditions and after B resupply. While the molecular basis for this upregulation is unknown, an 18 bp sequence within the *AtNIP5;1* 5′ UTR has been shown to be responsible for the rapid destabilization of *AtNIP5;1* mRNA when the plants are oversupplied with B, shortening the mRNA's half-life to about 30 % compared to plants grown under B-limiting conditions (Tanaka et al. 2011). This specific 18 bp sequence also influences the abundance of other tested downstream mRNA sequences in a B concentration-dependent manner (Tanaka et al. 2011), leading to the suggestion that a number of genes are regulated via a B-dependent mRNA (de-)stabilization or translational

efficiency mechanism. It remains to be shown if the mRNA destabilization is caused by a direct interaction between H_3BO_3 and the ribose sugar component of the RNA, as ribose moieties can chemically interact with H_3BO_3 or via other yet unknown mechanisms.

4.2 NIP-Mediated Transport of Silicon

Si is a non-essential element for most plants, but it does exert some highly beneficial effects on growth and productivity (Ma et al. 2002; Ma and Yamaji 2015). The presence of silica in plant tissue has been associated with an enhancement to certain plants' tolerance to drought, salinity, extreme temperature stress, and nutrient imbalance, as well as providing physical strength to the stem and leaves, thereby increasing lodging resistance in the field (Ma and Yamaji 2015). In addition, small herbivores typically avoid feeding on grasses that deposit significant quantities of silica in their leaves and digest them rather inefficiently. High silica contents also protect plants from fungal pathogens. The element has been designated as quasi-essential for rice (Epstein 1994), and Si fertilizers (the bioavailable form is silicic acid [H_4SiO_4]) are widely used in rice production in various continents (Ma and Yamaji 2015). The tissue concentration of Si in the aerial part of the plant varies across species from 0.1 % to 10 % of dry weight and by 5–10 % from rice cultivar to rice cultivar (Ma and Takahashi 2002).

The first higher plant Si transporter to be identified was OsNIP2;1 (syn. OsLsi1) (Ma et al. 2006). The *low-silicon* (*lsi*) mutant displays severe Si deficiency symptoms; the mutated gene product differs from that of the wild type by a single residue. The substitution of ala132 by thr132 significantly alters the protein conformation, resulting in a loss of its channel functionality. RNAi-induced suppression of *OsNIP2;1* expression in cv. Nipponbare reduces Si uptake considerably, producing a phenotype resembling that of the *lsi1* mutant (Ma et al. 2006). The wild-type gene product localizes to the exodermis and endodermis and to root zones, which are decisive for and intimately associated with Si uptake. The expression of *OsNIP2;1* in frog oocytes results in a de novo capacity to transport H_4SiO_4, but not glycerol or water (Ma et al. 2006). OsNIP2;1 is a NIP-III AQP, a class of protein typically characterized by an ar/R selectivity filter comprising gly, ser, gly, and arg. The small size of these four residues leads to the formation of a pore diameter that is somewhat larger than those produced by NIP-I and -II proteins. Once Si is taken up by rice roots, more than 95 % of it is translocated from the roots to the shoots (Ma and Takahashi 2002). In the shoot, OsNIP2;2 is responsible for the unloading of H_4SiO_4 from the xylem sap into the cytoplasmic leaf space (Yamaji and Ma 2009). This protein is polar-localized to the adaxial side of xylem parenchyma cells in the leaf sheath and blade (Yamaji and Ma 2009). Transpirational water loss drives the gradual polymerization of H_4SiO_4 into amorphous silica, which is deposited as a double layer beneath the cuticle (Ma and Takahashi 2002). In *OsNIP2;2* knockout plants, H_4SiO_4 accumulates in the leaf guttation sap, and an altered pattern of silica deposition in the

leaf is observed (Yamaji and Ma 2009). NIP-III isoforms permeable to H_4SiO_4 and important for the uptake and distribution of Si have been identified in barley (HvLsi1 [HvNIP2;1] and HvLsi6 [HvNIP2;2]: Chiba et al. 2009; Yamaji et al. 2012), wheat (TaLsi1: Montpetit et al. 2012), maize (ZmLsi1 [ZmNIP2;1] and ZmLsi6 [ZmNIP2;2]: Mitani et al. 2009a), cucumber (CsiT-1 and CsiT-2: Wang et al. 2015), pumpkin (CmNIP2;1: Mitani et al. 2011), and soybean (GmNIP2;2: Deshmukh et al. 2013).

OsNIP2;1, HvNIP2;1, ZmNIP2;1, and TaLsi1 channels are present mainly in the root and are known to be required both for the uptake of H_4SiO_4 into the plant and for its transport toward the vasculature (Ma et al. 2006; Montpetit et al. 2012; Mitani et al. 2009a; Chiba et al. 2009). *OsNIP2;2*, *HvNIP2;2*, *ZmNIP2;2*, *GmNIP2;1*, *GmNIP2;2*, *CmNIP2;1*, *CSiT1*, and *CSiT2* transcripts are all detectable in both the root and the shoot; the function of their products in rice, barley, and maize is considered to lie in xylem unloading in the leaf sheath and blade (Yamaji and Ma 2009; Yamaji et al. 2012; Mitani et al. 2009a); an additional function in rice is the intervascular transfer of nutrients at the nodes (Yamaji and Ma 2009; Yamaji et al. 2015).

While the abovementioned NIP-IIIs all share a capacity to transport H_4SiO_4, the various orthologs differ from one another with respect to both their spatial expression and their transcriptional response to specific stimuli. For example, *OsNIP2;1*, *OsNIP2;2*, *GmNIP2;1*, and *GmNIP2;2* are all downregulated by the presence of H_4SiO_4 (Ma et al. 2006; Yamaji and Ma 2009; Deshmukh et al. 2013), whereas *ZmNIP2;1*, *TaLsi*1, and *HvNIP2;1* are nonresponsive (Chiba et al. 2009; Mitani et al. 2009a; Montpetit et al. 2012). OsNIP2;1 is abundant in the exodermis and endodermis in primary and lateral roots where casparian strips exist (Ma et al. 2006); both *HvNIP2;1* and *ZmNIP2;1* are active in the epidermis, hypodermis, and cortex (Chiba et al. 2009; Mitani et al. 2009a); CmNIP2;1 is ubiquitous throughout the root (Mitani et al. 2011); OsNIP2;2/Lsi6 homologs in rice, barley, and maize are deposited throughout the root tip and in xylem parenchyma in the leaf (Yamaji et al. 2008; Yamaji et al. 2012; Yamaji and Ma 2009; Mitani et al. 2009a). The herbaceous perennial horsetail (*Equisetum arvense*) is one of the highest accumulators of Si in the plant kingdom (Chen and Lewin 1969). It encodes nine NIPs (EaNIP3;1 through 9), of which EaNIP3;1, EaNIP3;3, and EaNIP3;4 have each been shown to be permeable to H_4SiO_4 and to feature a distinct amino acid residue composition in their selectivity filter, namely, composed of ser, thr, ala, and arg (Grégoire et al. 2012).

The composition of cereal and horsetail Si channel ar/R selectivity filters is too variable for it to be usable as a diagnostic for Si transporters. Nonetheless, an in silico analysis has identified a phenylalanine in TMH6 and a polar serine/threonine residue in TMH5 that are shared by all Si-permeable NIP-III group proteins while being absent from all other NIPs (Pommerrenig et al. 2015). However, whether these residues are indeed critical for H_4SiO_4 selectivity has yet to be experimentally verified.

NIP-III group channels are encoded by the genomes of both *Liliopsida* and *Magnoliopsida* species, including the Gramineae, Arecaceae, Musaceae, Solanaceae, Rosaceae, Cucurbitaceae, Leguminosae, Vitaceae, Rubiaceae, and Rutaceae, as well as in the species *Amborella trichopoda*, which has been placed at, or near the base of,

the angiosperm lineage (Ma and Yamaji 2015). *A. thaliana* lacks any *NIP-III* genes. Note that the presence of a *NIP-III* gene(s) does not correlate with an enhanced capacity to accumulate Si. For example, NIP-III group isoforms are produced by tomato, which is a non-accumulator (Mitani and Ma 2005). Thus, NIP-IIIs likely fulfill also other physiological functions – an example is the previously mentioned barley HvNIP2;1 protein, associated with B tolerance (Schnurbusch et al. 2010).

NIP channels are not the only plant proteins able to transport Si. The Lsi2-type transporters have been designated as putative anion transporters (Ma et al. 2007; Mitani et al. 2009b; Mitani-Ueno et al. 2011; Yamaji et al. 2015); they form 11 predicted plasma membrane-spanning helices and remove Si from the cell via a secondary active process driven by the establishment of a proton gradient across the plasma membrane (Ma et al. 2007). In rice, an Lsi2 homolog governs the uptake and transport of $H_3AsO_3/H_2AsO_3^-$ and its translocation into the grain (Ma et al. 2008). Lsi2-type transporters are found in many *Magnoliopsida* (including *A. thaliana*) and *Liliopsida* species (Ma and Yamaji 2015). The function of the *A. thaliana* homolog (encoded by *At*1*g*02260) is still unknown. The cooperation of Lsi2-type transporters and NIP-III channels is required for cell-to-cell Si transport (reviewed by Ma and Yamaji 2015). In some cases, NIP channels and Lsi2-type efflux transporters are located within the same cell type but with opposite polarity; in other cases, they appear in adjacent cell layers. A mathematical modeling approach has calculated that the polar localization of the two transporter types (NIPs and Lsi2-type transporters) at the exodermis and endodermis is optimal with respect to an energy efficient and high capacity Si uptake into the rice root (Sakurai et al. 2015).

4.3 NIP-Mediated Transport of Arsenic

As is an acutely toxic and carcinogenic though relatively abundant and highly bioavailable metalloid, which can enter the human food chain via contaminated water or plant biomass (mainly via staple crops) (Meharg and Zhao 2012). The most common forms present in soil are $H_2AsO_4^-$ and H_3AsO_3. In well-aerated (oxidative) soils, the former type predominates, while the latter type is associated with hypoxic (reducing) conditions. Both forms are readily taken up by plants (Meharg and Zhao 2012). Arsenate ($H_2AsO_4^-$) and phosphate ($H_2PO_4^-$), the salts of arsenic acid (H_3AsO_4) and phosphoric acid (H_3PO_4), share a similar tetrahedral structure, pK_a, molecular volume, and electrostatic behavior. Thus, being chemical analogs, $H_2AsO_4^-$ can readily replace $H_2PO_4^-$, entering the plant via phosphate transporters (Zangi and Filella 2012). High affinity phosphate transporters are unable to distinguish between the two compounds (Zangi and Filella 2012; Li et al. 2015). Once taken up, $H_2AsO_4^-$ forms As-adducts which are typically short-lived and nonfunctional compared to the physiologically functional P-adducts; an example is the formation and rapid autohydrolysis of $H_2AsO_4^-$-ADP, initiating a futile cycle which uncouples oxidative phosphorylation and interferes with enzymes regulated by phosphorylation (Finnegan and Chen 2012). As most arable soils are not hypoxic,

most of the As taken up by plants is in the form $H_2AsO_4^-$. Shortly after entering the root, it is enzymatically or nonenzymatically reduced to $H_2AsO_3^-$ and then protonated to form H_3AsO_3 (Finnegan and Chen 2012). The reduction of $H_2AsO_4^-$ to $H_2AsO_3^-$ is a common detoxification strategy used by most organisms, including plants (Bienert and Jahn 2010b). In the form $H_2AsO_3^-$/H_3AsO_3, As is more easily transported than $H_2AsO_4^-$, but its toxicity is enhanced by its ready reactivity with sulfur groups, thereby inactivating enzymes for which their functionality depends on cysteine residues or dithiol cofactors (Finnegan and Chen 2012). In non-hyperaccumulators, most of the $H_2AsO_3^-$/H_3AsO_3 taken up is chelated by glutathione or a metallothionein and sequestered into root cell vacuoles by the action of ABC transporters; alternatively it can be effluxed out of the cells (Li et al. 2015). The $H_2AsO_3^-$/H_3AsO_3 which is neither compartmentalized nor effluxed is distributed throughout the plant either actively by members of the secondary active Si-transporting Lsi2-type transporter family or passively along a concentration gradient by NIPs which transport Si and B (see elsewhere in this chapter; Pommerrenig et al. 2015; Li et al. 2015).

In bacteria, fungi, fish, and mammals (including humans), H_3AsO_3 is transported by specific GLPs (reviewed in Bienert and Jahn 2010a; Maciaszczyk-Dziubinska et al. 2012; Mukhopadhyay et al. 2014). Evidence supporting the involvement of AQPs in As transport has been obtained from kinetic uptake studies of the rice root (Meharg and Jardine 2003). In particular, when $H_2AsO_3^-$ was supplied to rice roots, As uptake can be partially inhibited by alternative AQP substrates (such as glycerol and antimonite) or by the AQP inhibitor $HgCl_2$ (Meharg and Jardine 2003). Consequently, Meharg and Jardine postulated already in 2003 that H_3AsO_3 is transported across plant plasma membranes via MIPs/AQPs. In 2008, three studies independently and congruently demonstrated in direct uptake experiments that certain plant NIPs are permeable to H_3AsO_3 (Isayenkov and Maathuis 2008; Bienert et al. 2008a; Ma et al. 2008). The effect of exposing plants to $NaAsO_2$ and As trioxide (As_2O_3) on uptake and growth implies strongly that the uncharged H_3AsO_3 molecule permeates plant NIPs ($NaAsO_2$ and As_2O_3 form H_3AsO_3 in aqueous solution). A detailed study has shown that H_3AsO_3 shares several physicochemical and structural characteristics with the canonical NIP substrate glycerol, further supporting the idea that it is transported in planta through AQP channels (Porquet and Filella 2007).

A number of rice (OsNIP2;1, OsNIP2;2, and OsNIP 3;2), *A. thaliana* (AtNIP5;1, AtNIP6;1, and AtNIP7;1), and *Lotus japonicus* (LjNIP5;1 and LjNIP6;1) proteins have been tested for their ability to abolish the As tolerance displayed by certain *S. cerevisiae* yeast strains (Fig. 2); all of them significantly increase the level of sensitivity to $NaAsO_2$ (Bienert et al. 2008a). When the yeast is cultured on a medium containing $H_2AsO_4^-$, the NIP proteins also facilitate the efflux of the H_3AsO_3 generated in vivo through enzymatic reduction of $H_2AsO_4^-$ (Bienert et al. 2008a), clearly demonstrating the bidirectional flux of H_3AsO_3 carried out by plant NIPs.

The physiological consequences of NIP-mediated H_3AsO_3 transport have been revealed by exposing an *Osnip2;1* knockout rice line (defective in Si uptake) to $H_2AsO_3^-$. The accumulation of As in the mutant's shoot and root is reduced by, respectively, 71 % and 53 % compared to that recorded for a wild-type plant grown

in a medium lacking H_4SiO_4 (Ma et al. 2008). The presence of H_4SiO_4 reduces As uptake in the wild type but not in the *Osnip2;1* mutant plants, indicating a competitively inhibited flux of the two substrates through the native OsNIP2;1 channel. A short-term uptake assay has demonstrated that As uptake by the mutant is 57 % less than that of the wild type (Ma et al. 2008). The conclusion is that OsNIP2;1 is responsible for H_3AsO_3 uptake in planta (Ma et al. 2008). The addition of $H_2AsO_4^-$ to the growth medium promotes OsNIP2;1-mediated H_3AsO_3 efflux (Zhao et al. 2010b). The suggestion here was that NIPs are able to reinforce As detoxification by effluxing H_3AsO_3 out of the roots after its intracellular formation through the reduction of $H_2AsO_4^-$, provided that the rhizosphere environment is permissive. When challenged with organic (methylated) molecules involving As, the *Osnip2;1* mutant takes up only half the amount of either monomethylarsonic acid (CH_5AsO_3) or dimethylarsinic acid ($C_2H_7AsO_2$) taken up by wild-type plants (Li et al. 2009). The heterologous expression of *OsNIP2;1* in frog oocytes has shown that this NIP facilitates both the influx and efflux of H_3AsO_3, as well as that of CH_5AsO_3 and $C_2H_7AsO_2$ (Ma et al. 2008; Li et al. 2009). The indications are therefore that OsNIP2;1 represents an important bidirectional channel for a range of uncharged As species and represents the major uptake pathway for these species into rice.

A screen of an EMS mutagenized population of *A. thaliana* was used by Kamiya et al. (Kamiya et al. 2009) to identify individuals compromised for root growth in the presence of $H_3AsO_3/H_2AsO_3^-$. The three selected mutants all carry a mutation in the *AtNIP1;1* coding sequence. The heterologous expression of each of the mutant alleles in frog oocytes has shown that they specify a nonfunctional As-impermeable AtNIP1;1 channel. The abundance of wild-type *AtNIP1;1* transcript was 20 times higher in the root than the shoot, and a promoter-GUS fusion analysis showed that the *AtNIP1;1* promoter is active in the stomata, the root-hypocotyl junction, the lateral root tip and stele, and the primary root stele (Kamiya et al. 2009). These data suggest that AtNIP1;1 contributes to As uptake into Arabidopsis roots. Similarly, AtNIP3;1 has been shown to participate in both As uptake and root-to-shoot translocation in plants subjected to H_3AsO_3 stress (Xu et al. 2015). Several independent *Atnip3;1* loss-of-function mutants display a clear improvement in their level of H_3AsO_3 tolerance, as expressed by their aerial growth and their reduced ability to accumulate As in the shoot (Xu et al. 2015). The *Atnip3;1/Atnip1;1* double mutant exhibits a strong degree of H_3AsO_3 tolerance; its root and shoot continue to grow even in the presence of normally toxic levels of H_3AsO_3. *AtNIP3;1* promoter activity is confined largely to the root, although not in the root tip (Xu et al. 2015). The overall conclusion is that AtNIP3;1 participates in H_3AsO_3 uptake and root-to-shoot translocation (Xu et al. 2015).

Studies based on a range of heterologous expression systems have demonstrated that members of all three functional NIP subclasses have the ability to channel uncharged As species. The outcome of expressing the rice (*OsNIP1;1*, *OsNIP2;1*, *OsNIP2;2*, and *OsNIP3;1*) and *A. thaliana* (*AtNIP1;1*, *AtNIP1;2*, *AtNIP5;1*, and *AtNIP7;1*) genes in frog oocytes is an increased influx of H_3AsO_3, moreover the expression of *AtNIP3;1*, *HvNIP1;2*, *HvNIP2;1*, *HvNIP2;2,* and *OsNIP3;3* in yeast enhances the cells' sensitivity to H_3AsO_3 providing additional evidence for the H_3AsO_3 permeabilities of NIPs (Fig. 2; Ma et al. 2008; Kamiya et al. 2009; Katsuhara et al. 2014; Xu et al. 2015).

A QTL mapping study in rice, based on a cross between the $H_2AsO_4^-$ tolerant cv. Bala and the sensitive cv. Azucena, was able to identify three genomic regions harboring genes determining the tolerance of the former cultivar (Norton et al. 2008). Analysis of the progeny suggested that an individual needs only to inherit any two of the three tolerance loci from cv. Bala for it to be tolerant. One of the three QTL regions harbored two genes which were differentially regulated when the plants were exposed to As stress: one encodes an aminoacylase and the other is *OsNIP4;1*; both are more actively transcribed in the tolerant parent (Norton et al. 2008). The latter gene is particularly significant as NIPs are implicated in the transport of H_3AsO_3 into the root. However, the heterologous expression of *OsNIP4;1* – unlike that of other NIPs – in an As-sensitive yeast cell line does not increase their H_3AsO_3 sensitivity (Katsuhara et al. 2014). The mechanistic basis of OsNIP4;1 on H_3AsO_3 tolerance remains to be determined.

OsNIP3;1, required for the uptake and translocation of H_3BO_3 (Hanaoka et al. 2014), also transports H_3AsO_3 when expressed in frog oocytes (Ma et al. 2008). *OsNIP3;1* is downregulated in response to an elevated supply of $H_3AsO_3/H_2AsO_3^-$ but not of $H_2AsO_4^-$ (Chakrabarty et al. 2009). As-responsive downregulation may help to lower the level of OsNIP3;1-mediated As root uptake under B-deficient conditions. All acquired information on As transport mechanisms controlling As fluxes into and within plants, particularly to edible plant parts such as rice grains, is highly valuable for the development of breeding strategies or the engineering of minimal-As-accumulating plants.

Two independent analyses have failed to identify any QTL linked to either *OsNIP2;1* or *OsNIP3;1* associated with the grain content of either $H_2AsO_3^-$/ $H_2AsO_4^-$ or $C_2H_7AsO_2$ (Kuramata et al. 2013; Norton et al. 2014). However, one QTL region (harboring *OsNIP2;2*) has been identified as contributing to the methylated As content of the grain (Kuramata et al. 2013). When tested at the seedling stage, both the shoot and root As contents in an *OsNIP2;2* knockout line are indistinguishable from those recorded in the wild type (Ma et al. 2008). Since *OsNIP2;2* is expressed in the node below the panicle after the onset of grain filling (Yamaji and Ma 2009), it has been suggested that differences in the grain $C_2H_7AsO_2$ content are due to a genotype-dependent transport efficiency and/or expression of *OsNIP2;2* (Kuramata et al. 2013). Carey et al. (Carey et al. 2010; Carey et al. 2011) have shown that $C_2H_7AsO_2$ is highly mobile in the panicle vascular system and is readily translocated into the grain. Whether OsNIP2;2 is permeable to either CH_5AsO_3 or $C_2H_7AsO_2$ remains to be shown.

So far, the indication is that the toxic metalloid As (both in its reduced and uncharged forms) transport in plants is handled largely by NIPs. Whether NIP-mediated H_3AsO_3 transport is simply an adventitious nonphysiological side activity, as a consequence of the compound's structural similarity to that of certain other essential metalloid nutrient substrates, or whether it has evolved as a genetically or physiologically implemented detoxification strategy along the lines of the GLPs in microbes, still remains to be resolved. Given that plants are sessile, it may well be that, in addition to their efflux activity from the root, the involvement of NIPs in As cell-to-cell translocation adds to the final compartmentalization of As-phytochelatin

complexes in vacuoles of specific As-tolerant cell types (Moore et al. 2011) and/or an ability to protect As-sensitive cells. The latter two hypothesized roles of NIPs may also be supported by the observation that when AtNIP1;2- and AtNIP5;1-mediated As transport are disrupted *in planta*, the level of H_3AsO_3 tolerance is not increased, even though the tissue As content is markedly lowered (Kamiya et al. 2009). These findings indicate that H_3AsO_3 tolerance cannot be solely explained by a decreased As content in plants. The importance of gaining a better understanding of the regulation and mode of As transport has practical importance, as it will guide breeding strategies to selectively route As fluxes to targeted locations within or outside of crop plants depending on the objectives (i.e., accumulation, enrichment in, or exclusion from certain tissues) and to generate crop varieties that take up little or no As or at least do not translocate it to the edible parts of the plant.

4.4 NIP-Mediated Transport of Antimony

Trivalent and pentavalent Sb species have no known physiological role for plants, rather they are toxic (Kamiya and Fujiwara 2009). Homologous and heterologous expression systems have been used to show that various NIPs (Bienert et al. 2008a, b; Kamiya and Fujiwara 2009) and mammalian and microbial GLPs (reviewed by Maciaszczyk-Dziubinska et al. 2012) facilitate the movement of trivalent uncharged Sb species. The expression of *AtNIP5;1*, *AtNIP6;1* and *AtNIP7;1*, *LjNIP5;1* and *LjNIP6;1*, and *OsNIP3;2* and *OsNIP2;1* in a metalloid-tolerant yeast mutant abolishes the tolerance when the transformants were exposed to $C_8H_4K_2O_{12}Sb_2$ (potassium antimonyl tartrate) (Bienert et al. 2008a, b). The two independent *AtNIP1;1* T-DNA insertion mutants mentioned above in the context of tolerance to H_3AsO_3 are also able to both maintain root growth in the presence of toxic levels of $C_8H_4K_2O_{12}Sb_2$ and limit the accumulation of Sb (Kamiya and Fujiwara 2009). As the knockout of other NIPs (such as AtNIP1;2 and AtNIP5;1) expressed in the root do not reduce Sb sensitivity, it is likely that AtNIP1;1 is responsible, at least in part, for regulating and mediating the entry of Sb (Kamiya and Fujiwara 2009). Thus, NIPs belonging to each of the three functional subgroups NIP-I (AtNIP1;1), NIP-II (AtNIP5;1, AtNIP6;1, AtNIP7;1, LjNIP5;1, LjNIP6;1, and OsNIP3;2), and NIP-III (OsNIP2;1) facilitate the transport of Sb across plant membranes (Fig. 2). The Sb concentrations used in yeast and *A. thaliana* toxicity assays (up to 100 μM) do not occur in natural soils (Bienert et al. 2008a; Kamiya and Fujiwara 2009). Nevertheless, localized pollution associated with certain industrial activity has led to heavy loading with Sb_2O_3, so knowledge of Sb transport mechanisms is of relevance in the context of phytoremediation measures based on either Sb hyperaccumulators or on crop plants able to restrict the quantity of Sb translocated to edible parts. The likelihood is that the involvement of NIPs in the transport of trivalent Sb is an adventitious feature of these channels, which are presumed to have evolved as a means of transporting metalloids of physiological significance such as boric acid

or silicic acid. Various microbial GLPs have also proven to be Sb permeable (reviewed by Maciaszczyk-Dziubinska et al. 2012; Zangi and Filella 2012; Mukhopadhyay et al. 2014; Mandal et al. 2014), even though there is no known biological requirement for this element. The nonspecificity of AQP/GLP channels is exploited in some cases in order to infiltrate curative drugs into parasitic or abnormal cells (notably cancerous cells). For example, Sb-containing drugs used to kill certain protozoan parasites are effectively taken up by the target organism via their AQP transport systems (Mandal et al. 2014). Two of the major drugs used to combat leishmaniasis are the pentavalent antimonials sodium stibogluconate (Pentostam) and meglumine antimoniate (Glucantime) (Mukhopadhyay et al. 2014). One of the five AQPs of *Leishmania major* (LmAQP1) is known to be involved in the as yet mechanistically non-understood uptake process of these drugs. Both experimentally induced and naturally occurring mutations in *LmAQP1* have been shown to reduce the uptake of Sb and hence increase the parasite's tolerance of the drugs (Mandal et al. 2014).

A similar scenario applies with respect to the arsenical drug melarsoprol, which enters the target cell via an AQP; drug resistance arises when the AQP is mutated to a form that hinders the free passage of the drug (Baker et al. 2012; Alsford et al. 2012). As pointed out in a recent review (Pommerrenig et al. 2015), it has been suggested that antimonous acid (H_3SbO_3) is the form of Sb generally permeating through NIPs and other GLPs when $C_8H_4K_2O_{12}Sb_2$ (antimony potassium tartrate) is provided as the source of Sb source in toxicity assays. This conclusion is largely based on the physicochemical similarity of H_3SbO_3 with H_3AsO_3 (Porquet and Filella 2007). Salts of H_3SbO_3 formally exist. In water, they form a gelatinous precipitate, which is formed by antimony trioxide ($Sb_2O_3 * H_2O$) which is itself potentially formed by $C_8H_4K_2O_{12}Sb_2$. However, the uncharged H_3SbO_3 is suggested to be metastable and, thus, does not occur in nature in significant quantities (Vink 1996). Some doubt remains therefore as to the form of Sb that permeates AQPs. Therefore, scientific efforts should be initiated to assess which Sb species permeates AQPs.

4.5 NIP-Mediated Transport of Germanium

Due to the absence of any known biological function and the rarity of Ge in most soils, the permeability of certain NIPs to this element is again likely a serendipitous effect of the structural similarity of Ge compounds to those formed by other physiologically significant metalloids. The bioavailable forms of Ge are the polar tetrahedral ortho-acid (H_4GeO_4) and the nonpolar, planar meta-acid form (H_2GeO_3), the chemical properties of which resemble, respectively, H_4SiO_4 and H_3BO_3 (Fig. 1). Neither of these forms has been exhaustively quantified in natural soils, the rhizosphere, or within plant tissue. The element is present in many silicate minerals in quantities of up to a few ppm; an estimate of the mean soil Ge concentration is 1.6 mg kg^{-1} (Rosenberg 2009). The dissociation behavior of germanic acid (pK_{a1}=9)

resembles that of H_3BO_3 and H_4SiO_4, suggesting that at physiological pH, the prevalent form is non-charged and therefore capable of being transported by NIPs (Fig. 1). It has been long assumed that the uptake and translocation properties of Ge are similar to those shown by Si (Nikolic et al. 2007, Takahashi et al. 1976a, b). Plants containing high amounts of Si (particularly grasses) tend to be more sensitive to excess Ge than those containing little Si (Nikolic et al. 2007). The Ge concentration in soil-grown plants ranges from 0.01 mg kg^{-1} (*Magnoliopsida* species) to 1 mg kg^{-1} (*Poaceae* species), reflecting the more effective H_4SiO_4 transporter machinery present in grasses (Ma and Yamaji 2015), which comprises the NIP-IIIs and the Lsi2-type efflux transporters. The former facilitate the passive transport of Si across the plasma membrane between the apoplast/soil solution and plant cells down concentration gradients (Ma et al. 2006), while the latter are responsible for the efflux of Si from the cell (Ma et al. 2007).

Long before the discovery of Si and Ge transporters (Ma et al. 2006, 2007) and the molecular basis for the dual transport functions of NIPs and Lsi2-type transporters was described (reviewed by Ma and Yamaji 2015), existing knowledge of the chemical similarity between Si and Ge hydroxylated compounds was exploited in the use of Ge as an Si analog in toxicity screens (Ma et al. 2002; Nikolic et al. 2007). This form of screen was used to identify the rice *lsi* mutants (Ma et al. 2002). Subsequent mapping approaches identified the underlying responsible NIP aquaporin (*OsNIP2;1* and *OsNIP2;2*) and Lsi2-type transporter (*OsLsi2* and *OsLsi3*) genes (Ma et al. 2006; Ma et al. 2007; Yamaji and Ma 2009; Yamaji et al. 2015). The radioactive ^{68}Ge isotope and the non-radioactive isotopes in the form of germanic oxide (GeO_2) are frequently used as chemical tracer analogs for studying Si transport features of certain NIPs *in planta* as well as in the *S. cerevisiae* and *Xenopus laevis* frog oocyte heterologous expression systems (Ma et al. 2006; Nikolic et al. 2007; Schnurbusch et al. 2010; Mitani-Ueno et al. 2011; Gu et al. 2012; Hayes et al. 2013; Bárzana et al. 2014). A genome-wide association mapping study in rice has shown that some Ge sensitive loci coincide with known QTL underlying Si or As accumulation, but none map in the vicinity of either *OsNIP2;1* or *OsNIP2;2* (Talukdar et al. 2015). A QTL associated with Ge sensitivity lies within 200 Kbp of *OsLsi2*. *OsNIP4;1* (Os01g02190) is located within the genomic region of the detected loci. *OsNIP4;1* is strongly expressed in the inflorescence and particularly in the anthers (Liu et al. 2009). However, substrate selectivity data are not available, making it difficult to interpret its function with respect to Ge tolerance. The chemical similarities between the nonpolar, planar H_3BO_3 and H_2GeO_3 have prompted Hayes et al. (Hayes et al. 2013) to use Ge treatment as a surrogate for the effect of B toxicity on barley and wheat. A barley cultivar showing a mild reaction to the presence of GeO_2 is also tolerant to high levels of B; the underlying basis for B tolerance is a very low transcript abundance of *HvNIP2;1*, the gene implicated as encoding a B and Si transporter (Schnurbusch et al. 2010).

In summary, the nonspecific selectivity of NIP-IIIs being permeable to Si, B, and Ge represents a valuable feature, allowing to use Ge as a suitable tracer in science to mimic and characterize Si and B transport processes or to screen graminaceous crop populations for altered functions of NIP-III channels and related proteins

(Hayes et al. 2013). Ge is an important element for the semiconductor industry. However, unlike most metalloids and metals, it is not generally found in concentrated form in nature, so it has been suggested that plant accumulators could be exploited to extract it from contaminated but also agricultural soils. In this context, NIPs could potentially be engineered to increase the efficiency of the extraction process, allowing Ge to be recovered from biomass grown for the purpose of phytomining. Ge could then be extracted from, e.g., plant digestates of bioenergy crops or from straw or other not used plant residuals as a second add-on “yield” value.

4.6 NIP-Mediated Transport of Selenium

Se is essential in the human and animal diet, but is not essential for plant growth. The biologically active form of Se is the derived amino acid selenocysteine, which is inserted into bacterial, archeal, and eukaryotic mRNA by a specific tRNA. Because of the lower reduction potential of selenocysteine compared to cysteine itself, this compound has an important role in the catalytic sites of glutathione peroxidases and thioredoxin reductases, which act as protectants against oxidative stress (Lobanov et al. 2009). Vegetables and fruits represent the major source of dietary Se. The content of Se within plant tissue is rather low, presumably because it has no benefit for the plant; nevertheless, the element is readily taken up from the soil (Pilon-Smits and Quinn 2010). Thus, a suggested strategy to counteract Se deficiency in the diet is Se biofortification of staple crops, which would require the selection of Se accumulators or effective translocators of Se into the edible part of the plant.

The most prominent forms of soil Se are selenite ($HSeO_3^-$) and selenate ($HSeO_4^-$), with the latter predominating in well-aerated soils. The similar structure and pK_a values of selenate and sulfate result in the former being recognized and transported by sulfate transporters (Sors et al. 2005). The cross talk between selenate and sulfur metabolism makes this transport system unfavorable in the context of biofortification, as modifications to sulfur transport may have detrimental effects on a range of important traits, thereby outweighing any advantages of enhanced Se accumulation (Bienert and Chaumont 2013). H_2SeO_3 is a diprotic weak acid with pk_{a1} and pk_{a2} vales of 2.57 and 6.6, respectively, so that at physiological pHs it exists predominantly in the form of both $HSeO_3^-$ and SeO_3^{2-} (Fig. 1). Phosphate transporters (such as rice OsPT2) have been implicated in the active uptake of $HSeO_3^-$ into the root (Zhang et al. 2014). Under acidic conditions, selenous acid (H_2SeO_3) predominates (Fig. 1). The standard AQP inhibitors $HgCl_2$ and $AgNO_3$ both inhibit the uptake of H_2SeO_3 into the rice and maize root (Zhang et al. 2012; Zhang et al. 2010). Supplying $HSeO_3^-$ in a kinetic study of Se uptake into the maize root has shown that, when grown in an acidic (pH 3) medium, uptake is mostly in the form H_2SeO_3 (Zhang et al. 2010). Se uptake kinetics follow a linear trend which may suggest that the limiting step is a channel-mediated transport mechanism.

The first plant H_2SeO_3 transporter to be identified was OsNIP2;1 (Zhao et al. 2010); when grown in the presence of $HSeO_3^-$, the loss-of-function mutant *Osnip2;1*

accumulates significantly less Se in its shoot and xylem sap than does the wild type. In contrast, the mutant and the wild type accumulate an equal amount of Se when grown on a medium supplemented with $HSeO_4^-$. Further experiments have revealed that H_2SeO_3 is most likely the Se form transported by OsNIP2;1 (Zhao et al. 2010a). The ability of OsNIP2;1 to transport Se has been further demonstrated by heterologously expressing it in yeast (Zhao et al. 2010a). NIPs may be involved in the intercellular transport of Se as well as in its uptake. Once $HSeO_4^-$ is taken up, it is reduced to $HSeO_3^-$ in both the chloroplast and the cytoplasm, before being further reduced to the Se^{2-} ion and hence incorporated into selenocysteine or selenomethionine; these amino acids can be nonspecifically incorporated into proteins instead of cysteine, leading to toxicity (Pilon-Smits and Quinn 2010). Still unresolved is whether (1) NIP isoforms of plant species other than rice are permeable to Se, (2) the permeability of NIPs to H_2SeO_3 is a feature of only the H_4SiO_4-permeable NIP-III isoforms present in both *Liliopsida* and *Magnoliopsida* species, and (3) the engineering of *NIP*s could represent viable means of directing Se flux in staple crops.

5 PIP-Mediated Metalloid Transport in Plants

On the basis of their sequence, the PIPs are the most homogeneous of the plant AQPs and also the most numerous (Anderberg et al. 2012). Two PIP subgroups (PIP1 and PIP2) are recognized and share a sequence identity above 50 %. The PIP1s have a longer N terminal and a shorter C terminal domain than the PIP2s, as well as having a shorter extracellular loop A (Chaumont et al. 2001). *PIP1* and *PIP2* genes behave differently when heterologously expressed in frog oocytes: in general, only PIP2s are able to induce a significant level of transmembrane water movement (Fetter et al. 2004; see chapter "Heteromerization of Plant Aquaporins"). When a *PIP1*/*PIP2* pair cloned from several section *Liliopsida* and *Magnoliopsida* species is co-expressed in frog oocytes, their products interact to modify their trafficking into and/or stability within the host membrane, thereby cooperating to synergistically increase water permeability (see chapter "Heteromerization of Plant Aquaporins"). A combination of physiological and molecular genetic evidence indicates that PIP water channels are highly important for the plant's water homeostasis (Maurel et al. 2015; Chaumont and Tyerman 2014). A small number of PIPs have been shown to be permeable to molecules other than water, including H_2O_2 and urea (reviewed by Maurel et al. 2015), and of note in the context of this chapter, they also transport uncharged metalloid species.

5.1 PIP-Mediated Transport of Boron

Direct evidence for the involvement of PIPs in B transport is fragmentary. Maize ZmPIP1;1 was the first plant AQP shown to have the capacity to transport H_3BO_3: the heterologous expression of *ZmPIP1;1* in frog oocytes results in a 30 % increase

in B permeability over that achieved in control oocytes or those expressing ZmPIP3 (renamed ZmPIP2;5), AtNLM1, or EcGlpF (Dordas et al. 2000). The H_3BO_3 permeability of plasma membranes isolated from squash (*Cucurbita pepo*) vesicles is partially inhibited by the AQP inhibitors $HgCl_2$ and phloretin and is reversibly rescued by treatment with 2-mercaptoethanol. As mentioned earlier, this sort of compound-dependent on-off transport behavior is indicative of AQP-mediated transport. Dordas et al. (2000) have suggested that some H_3BO_3 enters the plant cell via passive diffusion through the plasma membrane lipid bilayer, while the rest is transported through PIP1 channels. Therewith this study provided the first experimental indication that plant AQPs are involved in metalloid transport and particularly in B transport. Subsequently, it has been shown that transferring either maize plants or transgenic tobacco plants overexpressing GFP:ZmPIP1 to a B-deficient medium for about 1 h results in the rapid disappearance of ZmPIP1 channels from the root apex cell plasma membrane (Goldbach et al. 2002). The implication is that the *ZmPIP1* product cannot be directly involved in B uptake under B-deficient conditions, since otherwise its upregulation would have been expected, as is the case for *AtNIP5;1* (Takano et al. 2006). Instead, the removal of B-permeable proteins from the plasma membrane may serve to prevent an undesirable loss of B from the root. A possible hypothesis is that the B permeability shown by certain PIPs only functions when the supply of B is non-limiting; alternatively, it may be that the removal of PIPs from the plasma membrane is independent of any potential H_3BO_3 channeling activity associated with these membrane pores. Based on yeast toxicity growth assays, H_3BO_3 permeability has also been inferred for the grapevine PIP isoforms VvTnPIP1;4 and VvTnPIP2;3 (Sabir et al. 2014).

The barley HvPIP1;3 and HvPIP1;4 resemble ZmPIP1;1 at the sequence level, and localize to the plasma membrane in both heterologous and native expression systems, in contrast to many PIP1s derived from other species (see chapter "Heteromerization of Plant Aquaporins"). The B permeability of these PIP1s has been investigated using a yeast toxicity growth assay (Fitzpatrick and Reid 2009). Both proteins increase the sensitivity of the yeast cells to exogenously supplied B, and an analysis of the cellular B content has confirmed that both are capable of mediating the uptake of B (Fitzpatrick and Reid 2009). The quantitative response of these HvPIP1s to a variation in the external concentration of B is unclear, since the transcription of their genes is unresponsive to the B nutritional status of the plant. In contrast, the transcription of both *OsPIP2;4* and *OsPIP2;7* does respond to the rice plant B nutritional status: they are downregulated in the shoot and strongly upregulated in the root when the external concentration of B is raised (Kumar et al. 2014). The heterologous expression of *OsPIP2;4* and *OsPIP2;7* in a yeast mutant frequently used to assess As permeability results in an increased sensitivity to B and in a significantly higher accumulation of B. When these proteins are constitutively expressed in *A. thaliana*, the plants produce more biomass and longer roots when being exposed to high levels of B but do not accumulate either more or less B. However, a short-term kinetic uptake assay has suggested that the stems and roots of the *OsPIP2*-expressing plants contain more B than do those of the wild type (Kumar et al. 2014). While the outcomes of heterologous expression clearly imply that certain PIPs are permeable to B, it remains to be demonstrated that the observed

differences in B content of plants derive from a capacity of the PIPs to transport B, rather than reflecting a secondary effect of an AQP function unrelated to B transport. For example, a PIP-mediated change in the flux of water will alter the plant water status and hence its transpiration rate. As the transport of B within the plant depends strongly on the volume of the transpiration stream, an altered tissue B status can occur independently of active B uptake. The failure to measure tissue Ca in the above study is unfortunate, since B and Ca share a similar mobility through the xylem and distribution within the plant. A critical experiment would be to demonstrate whether or not plants experiencing a dissimilar B transport and PIP protein amount are also differentiated with respect to transpiration rate. Why ZmPIP1;3/PIP1;4 and ZmPIP2;2 are impermeable to B despite sharing a high level of sequence similarity with ZmPIP1;1 remains a puzzle (Bárzana et al. 2014). In brief, the assumption is that certain PIP1 and PIP2 isoforms possess residual permeability to H_3BO_3 sufficient to facilitate its transmembrane transport when expressed in a heterologous expression context; however, irrefutable evidence for their participation in B transport in plants is still lacking.

5.2 PIP-Mediated Transport of Arsenic

To date, the only claim that PIPs can be permeable to H_3AsO_3 was made by Mosa et al. (Mosa et al. 2012), who were able to demonstrate the downregulation of *OsPIP1;2*, *OsPIP1;3*, *OsPIP2;4*, *OsPIP2;6*, and *OsPIP2;7* in the root and shoot in response to $H_2AsO_3^-$ treatment. The heterologous expression of OsPIP2;4, *OsPIP*2;6, and *OsPIP*2;7 in frog oocytes caused increased As uptake, and the constitutive expression of *OsPIP2;4*, *OsPIP2;6*, and *OsPIP2;7* in *A. thaliana* results in an enhancement to the plant's tolerance toward $H_2AsO_3^-$, in contrast to the expectation that the transgene products should have increased the uptake of As (Mosa et al. 2012). The transgenic plants, however, show no evidence of an increased accumulation of As in either their shoot or their root.

The responsiveness of *PIP*s to As stress is a feature displayed by a number of plant species. As in rice, the abundance of five *Brassica juncea PIP1* and eight *PIP2* transcripts is reduced by exposing the plants to $H_3AsO_3/H_2AsO_3^-$ stress (Srivastava et al. 2013). Whether the observed variation was influenced, even in part, by diurnal cycling (which is known to affect *PIP* expression, see review by Heinen et al. 2009) cannot be ascertained. A subsequent whole genome transcriptome profiling of *B. juncea* subjected to $H_3AsO_3/H_2AsO_4^-$ stress has identified *PIP1;1* and *PIP2;2* as both being significantly downregulated by the stress (Srivastava et al. 2015). The stress also decreases the tissue water content of the plants, which inhibits seedling growth; at the same time increases are induced with respect to the production of reactive oxygen species, the extent of lipid peroxidation and in the level of root oxidation (Srivastava et al. 2013). Given that reactive oxygen species act to downregulate *PIP2* in the root (Hooijmaijers et al. 2012) and to drive the internalization of plasma membrane-localized PIPs (Wudick et al. 2015), it has yet to be resolved

whether the altered state of *PIP* transcription is a direct effect of the As stress or whether it is rather a secondary effect, generated, for example, by a raised level of reactive oxygen species.

While direct uptake assays in heterologous expression systems provide a line of evidence suggesting the permeability of specific PIPs to metalloids, it remains puzzling why orthologous isoforms, despite their sharing a high degree of overall sequence homology and being 100 % identical in the regions of the protein known to determine selectivity (the NPA motifs and the ar/R selectivity filters) and reach the plasma membrane in the heterologous expression systems, are nevertheless impermeable to As and other metalloids.

6 TIP-Mediated Metalloid Transport in Plants

The TIPs are localized in the tonoplast (the vacuolar membrane). Vacuolar subtypes are characterized by a specific set of TIP isoforms dependent on the developmental stage of the plant and the cell differentiation status (Jauh et al. 1999). The TIPs make an important contribution to cellular osmoregulation, turgor, osmo-sensing, cell growth, and vacuolar differentiation, thanks to their capacity to transport water across the tonoplast (reviewed in Maurel et al. 2015). The various TIP subgroups are highly variable with respect to sequence, especially within their ar/R selectivity filter, resulting in a broad substrate spectrum, including urea (Liu et al. 2003, Soto et al. 2008), NH_3 (Jahn et al. 2004; Loqué et al. 2005), glycerol (Gerbeau et al. 1999; Li et al. 2008), H_2O_2 (Bienert et al. 2007) and various metalloids (as discussed below). It has been suggested that these transport functions are additive to the water transport function.

6.1 TIP-Mediated Transport of Boron

The heterologous expression of maize *ZmTIP1;2* in yeast increases the host cells' sensitivity to the presence of H_3BO_3 in the growth medium and increases H_3BO_3 flux in an iso-osmotic swelling assay when being expressed in frog oocytes (Bárzana et al. 2014). No attempt has been made so far to test whether this increased B permeability can be explained by a rise in the passive transmembrane diffusion of H_3BO_3 through the lipid bilayer induced by an increased rate of water transport. The substrate selectivity of the grapevine TIPs VvTnTIP1;1 and VvTnTIP2;2 has been assessed by expressing them in yeast, and both proteins strongly induce the cells' sensitivity to externally supplied B (Sabir et al. 2014). The potential physiological significance of these vacuolar-localized proteins to plant B homeostasis has not been investigated, either in conditions of B under- or oversupply. The *A. thaliana* pollen-specific gene *AtTIP5;1* appears to be induced by B stress, and its ectopic expression in the rest of the plant significantly increases the level of the plant

tolerance to normally toxic levels of B (Pang et al. 2010). The interpretation of these outcomes might be that the plant is able to sequester B into the vacuoles when B is oversupplied. While the pollen specificity of *AtTIP5;1* has been ascribed to the high demand for B during pollen germination and pollen tube growth, the way in which AtTIP5;1 affects the transport of B within the pollen remains to be demonstrated. There is no convincing molecular or physiological evidence as yet for the involvement of TIPs in B homeostasis.

A QTL mapping approach targeting B efficiency in *A. thaliana* has been described by Zeng et al. (Zeng et al. 2008). The focus was on a trait referred to as a "B efficiency coefficient" (BEC), defined as the ratio between the seed yield of a given genotype grown under limiting B conditions and its seed yield when grown under non-limiting conditions. Five QTL have been identified, of which three – including the largest effect one named *AtBE1–2* – map within the same genomic region as a QTL for seed yield under limiting B conditions. The *AtBE1–2* harboring region also contains the *BOR1* homolog *BOR5* (*At*1*g*74810) and *AtTIP3;1* (*At*1*g*73190), while the *AtBE2* region contains *AtTIP4;1* (*At*2*g*25810). The implication is that at least two TIPs may well contribute to B efficiency, although as yet neither *TIP* gene product has been associated with B homeostasis. No *NIP* gene maps within any of QTL regions associated with either BEC or seed yield under limiting B conditions. Transcription profiling of contrasting B deficiency-tolerant citrus rootstocks has revealed that again a *TIP4;1* gene variant is substantially upregulated within the first 24 h of exposure to B deficiency but only in the tolerant genotype (Zhou et al. 2015). The significance of B to vacuolar function (if any) and the B storage capacity of different vacuole types remain obscure.

6.2 TIP-Mediated Transport of Arsenic

The As hyperaccumulator fern species *Pteris vittata* tolerates high concentrations of As in the growth substrate. The species reduces $H_2AsO_4^-$ to $H_2AsO_3^-$, which is then moved into the lamina of its fronds, where it is stored as free $H_3AsO_3/H_2AsO_3^-$. Few of the proteins contributing to these transport processes have yet been described. Indriolo et al. (Indriolo et al. 2010) have isolated the genes *PvACR3* and *PvACR3;1*, which encode proteins similar to the active ACR3 $H_2AsO_3^-$ efflux permease present in yeast. Like its yeast ortholog, PvACR3 actively transports As and localizes it to the vacuolar membrane in the gametophyte, where it is presumably detoxified. He et al. (He et al. 2015) have transformed a *P. vittata* cDNA library into yeast in an attempt to identify further As transporting proteins via a functional complementation assay. The screen has revealed *PvTIP4;1* gene, which encodes a protein permeable to $H_3AsO_3/H_2AsO_3^-$. Within its native species, *PvTIP4;1* transcription is largely confined to the roots. Unlike other TIP family members, PvTIP4;1 localizes to the plasma membrane rather than to the tonoplast. The capacity of PvTIP4;1 to transport As has been explored in both yeast and *A. thaliana*. Its heterologous expression in yeast results in an increased sensitivity to externally supplied $H_2AsO_3^-$ and in an

increased uptake of As; furthermore, the mutation of the cysteine residue in the R3 position of its ar/R selectivity filter abolishes its ability to transport As (He et al. 2015). The constitutive expression of *PvTIP4;1* in *A. thaliana* boosts the accumulation of As and causes $H_2AsO_3^-$ sensitivity.

The conclusion is that certain TIPs are As permeable and that As sequestration is probably adopted for physiological As detoxification. Evidence, albeit indirect, showing that some TIPs can influence membrane permeability to metalloids has arisen from a study of the hydrangea (*Hydrangea macrophylla*) TIP1 HmPALT1, which, when heterologously expressed in yeast, facilitates the transmembrane diffusion of a not determined form of the Al^{3+} ion (Negishi et al. 2012). The form of Al transported across the tonoplast may be aluminum hydroxide (H_3AlO_3), an uncharged compound which shares some physicochemical similarities to certain AQP-channeled metalloid species.

7 XIP-Mediated Metalloid Transport in Plants

7.1 XIP-Mediated Transport of Boron

The plant and fungal AQP subfamily denoted as XIPs was first discovered by Danielson and Johanson (2008). While XIPs occur in many sections, *Magnoliopsida* species, the *Brassicaceae* spp. (including *A. thaliana*), and *Poaceae* lack any *XIP*s (Abascal et al. 2014). It is possible that other AQP isoforms have adopted the function of XIPs in these taxa. Based on the nature of their selectivity filter, the XIPs resemble the NIPs more closely than they do either the TIPs or the PIPs (Bienert et al. 2011). Their absence from both *A. thaliana* and rice, the two leading model plant species, reasons that little is known of their physiological role in plants. Initial studies support the notion that XIPs are not highly permeable to water, but favor larger uncharged solutes (Bienert et al. 2011; Lopez et al. 2012). The expression of six *Solanaceae XIP*s (*NtXIP1;1α* and *NtXIP1;1β*, *StXIP1;1α* and *StXIP1;1β*, *SlXIP1;1α* and *SlXIP1;1β*) in yeast results in an increased sensitivity to externally supplied H_3BO_3 (Bienert et al. 2011), suggesting the permeability of XIPs to H_3BO_3. The evidence supports the idea that the XIPs contribute to metalloid transport in plants, but this suggestion needs experimental confirmation. Whether XIPs facilitate the transport of other metalloids such as H_3AsO_3 or H_4SiO_4 remains to be seen.

8 Outlook

Given the rarity of At, Po, and Te and the lack of any biological significance for any of these metalloids in most organisms, any potential AQP-mediated transport associated with them is unlikely to be of any biological importance (Pommerrenig et al. 2015). At present, whether uncharged forms of these trace elements are transported

in planta by AQPs is unknown. A number of challenges and open questions associated with plant AQP-mediated metalloid transport need to be addressed to complement the present knowledge. These are: (1) Which plant AQPs are permeable to which metalloid(s)? (2) Which metalloid-permeable AQPs are physiologically and actively involved in metalloid metabolism or response reactions? (3) How are plant AQPs regulated at the transcriptional and posttranslational level in response to metalloid exposure? (4) How do plant AQPs cooperatively orchestrate the transport of a given metalloid in one plant species? (5) What sequence motifs determine the metalloid selectivity of an AQP? (6) How can the ability of AQPs to transport and modify metalloid level and distribution be exploited to generate plants showing tolerance to either a high or a low level of metalloid? The answers to these questions will bear on the potential of plants to be exploited for certain agricultural conditions, for phytoremediation, for phytomining, or for biofortification. Finally, it will be interesting to analyze in an evolutionary and ecophysiological context when and where the ability of plant AQPs to channel metalloids was transformed into a main channel function.

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