Arsenic transport in plants

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Abstract

Arsenic (As), a metalloid occurring ubiquitously in nature in organic and inorganic forms, is classified as a potent carcinogen. Among the inorganic forms which are more toxic, As^{III} has a high affinity to bind with sulfhydryl groups of the amino acid cysteine, affecting many key metabolic processes such as fatty acid metabolism and glutathione production. As^{V} is a phosphate analogue and can substitute the inorganic phosphate affecting nucleotide synthesis and energy homeostasis of the cell. Much of the research on As in plants focuses on rice as it is the major source of dietary As intake and is often grown in areas with aquifers containing high amounts of As which are prevalent in south east Asian countries. Currently there is a great need to understand how plants deal with As, both from the perspective as potential sources of dietary As but also as potential mechanisms for phytoremediation. We are therefore using various approaches to identify and characterise plant membrane proteins involved in transport of As.

NIPs (nodulin like intrinsic proteins) are a subgroup of plant aquaporins reported to be involved in bidirectional transport of As^{III}. Loss of function mutants in *Arabidopsis* NIPs (*nip5;1, nip6;1* and *nip7;1*) were identified and analysed for their role in As uptake, efflux and translocation. The data showed that *nip5;1* and *nip6;1* may be involved in the efflux of As. The data for total As concentration in root and shoot tissues showed that among these mutants only *nip6;1* has a higher fraction of total As in the shoots compared to wild type. Lower efflux and more translocation suggest that this isoform (*nip6;1*) may be involved in vacuolar sequestration of As. Interestingly, *nip7;1* showed a higher efflux of As^{III} compared to other mutants and wild type. This suggests that NIP7;1 might have a role in the vacuolar sequestration or translocation because the loss of function resulted in more cytosolic As^{III} due to lower sequestration or translocation, which made more As^{III} available for the efflux.

The *Saccharomyces cerevisiae* gene ACR3 is involved in As^{III} efflux from the cytosol. We have transformed it into *Arabidopsis* and rice to assess if this can improve plant As tolerance. The results showed that expression of ACR3 affects plant growth. It appears that both in *Arabidopsis* and rice ACR3 may be involved in the efflux and translocation of As, as was shown by the results at the cellular, seedling and mature plant levels. ACR3 expression could be a potential means for phytoremediation of As in *Arabidopsis* because it increases As translocation to shoot tissue.

In addition, yeast was used as a heterologous expression system to screen cDNA libraries from rice (*Oryza sativa*) and *Arabidopsis thaliana* using the yeast strains (*ycf1*Δ and *acr3*Δ) to identify (new) transporters that are involved in As transport in plants. No growth tolerant/sensitive phenotype was observed in any yeast strain with both expression libraries suggesting the absence of putative new transporters/proteins involved in As transport.

The information obtained from this study can be used in future research. ACR3-like genes involved in As tolerance from other organisms can be potentially useful in plants. Based on the results from NIPs, the isoforms involved in As efflux (*nip5;1* and *nip6;1*) can be used to generate double knock out mutants to see if these have an additive/synergistic effect on As transport that will add to the knowledge of As transport *in planta*.

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Author's declaration

Unless otherwise acknowledged, I declare that the work presented here is original.

Some of the work presented here has been published in the following articles.

Chapter 1

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Chapter 4

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

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Chapter 1

1 Introduction

1.1 The occurrence and usage of arsenicals

Arsenic (As) is a metalloid, which occurs ubiquitously in the earth's crust. In addition, As is often present in the atmosphere, in soils and ground waters. Natural processes like weathering of rocks and volcanic emissions, and human activities such as combustion of fossil fuels, mining, smelting of ores or the application of arsenical pesticides, herbicides and wood preservatives, are the main sources which contribute to As contamination in the environment (Smedley and Kinniburgh, 2002).

Arsenic exists in either organic or inorganic form but is normally not encountered in its elemental state. Typically, the inorganic fraction contains oxygenated As anions or more complex As salts with for example sulphur and iron of which arsenopyrite (FeAsS) is the most abundant (Brewster, 1992, Chatterjee, 1994). However, the most prevalent inorganic As species are the pentavalent As^{V} arsenate (occurring as $H_2AsO_4^-$ and $HAsSO_4^{2-}$ in most environments) and the trivalent As^{III}, arsenite (As_2O_3) which dissolves as $As(OH)_3$.

Although large numbers of different organic As compounds can be found in organisms, including arsenolipids and arsenosugars, those that persist in the environment tend to be mostly methylated arsenicals such as monomethylarsonic acid (MMA^{V}) and dimethylarsinic acid (DMA^{V}) (Meharg and Hartley-Whitaker, 2002). In addition, environmental organic arsenicals may derive from pesticides, herbicides and preservatives.

Medicinal properties of arsenic have been exploited by humans for a considerable time: For example, the German pharmacologist Paul Ehrlich introduced the arsenic compound arsphenamine for the treatment of syphilis in 1909 (Yarnell, 1983, Van Den Enden, 1999). Arsenic compounds have also been used to treat diseases like trypanosomiasis, amoebic dysentery, sleeping fever and promyelocytic leukaemia (National Academy of Sciences, 1977). Besides their medicinal use, arsenicals have been employed since the Bronze age when they were often added during smelting to create a harder metal alloy. More recent applications include widespread usage in the production of herbicides, pesticides, insecticides and defoliants (Orme and Kegley, 2006). Furthermore, arsenic helps remove impurities during glass making, it is a colorant in fireworks and a doping material in semiconductor manufacturing (Ishiguro, 1992).

1.2 Arsenic toxicity

Arsenic is toxic to all living organisms. It has been defined as a group 1 carcinogen and is placed in the highest health hazard category by the international agency for research on cancer (IARC, 1987, Naidu et al., 2006, National Research Council, 2001). Elevated risks of cancer of the lung, skin and prostate result from prolonged exposure to medium and high levels of arsenicals (arsencosis). In addition, low level chronic arsencosis can cause non cancerous afflictions such as hypo- and hyperpigmentation, keratosis, heart problems and diabetes (IPCS, 2001).

At the cellular level, arsenic toxicity depends to a large extent on the nature of the arsenical: As^{III} has a high affinity to bind with sulfhydryl groups found in the amino acid cysteine. Binding of arsenic to these residues disrupts protein structure and protein-protein interactions thus affecting many key metabolic processes in the cell such as fatty acid metabolism, glucose uptake and glutathione production (Gochfeld, 1997, Young, 2000). In addition, the binding ability of As^{III} to the reducing agent glutathione can lead to glutathione depletion and therefore increased levels of damaging reactive oxygen species (ROS) (Bhattacharjee et al., 2008).

Being a phosphate analogue, As^{V} can substitute inorganic phosphate in a plethora of biochemical processes. For example, synthesis of triphosphate nucleotides like ATP can be affected which impacts on energy homeostasis, carbon metabolism and nucleic acid synthesis. This can also negatively affect DNA repair and DNA methylation and thus impact on gene expression (Mead, 2005b).

Organic arsenic can take many forms such as methylated species, arsenic betaine, arsenosugars and arsenolipids. Generally, the toxicity of these compounds is lower than that of inorganic arsenic species (Tamaki and Frankenberger, 1992). However, there may be exceptions to this rule: in the plants *Spartina patens* and radish, DMA^{V} was the most toxic arsenicals found (Carbonell-Barrachina et al., 1998). The increased mobility of methylated arsenicals such as MMA^{III} can also lead to toxicities that are in effect greater than that of inorganic arsenic. Petrick et al., (2000) found that in human hepatocytes cytotoxicity decreased as: $MMA^{III} > As^{III} > As^{V} > MMA^{V} = DMA^{V}$.

The damaging effects of arsenicals on humans manifest themselves predominantly through contact with arsenic in contaminated drinking water and through the food chain.

In combination these factors account for around 99% of the total human arsenic ingestion (Ontario Ministry of the Environment, 2001). It is estimated that more than 100 million people are exposed to water which contains arsenic above the WHO safety limit of 0.01 ppm (Nordstrom, 2002) and arsenic contamination of ground water is a particularly big problem in countries with naturally elevated arsenic soil contents such as Bangladesh, India , China, Vietnam, United States of America and Mexico (O'Neill, 1995, Smedley and Kinniburgh, 2002, Chowdhury et al., 2000, Fazal et al., 2001).

Arsenic contamination through food consumption results mainly from crop irrigation with arsenic contaminated water. The problem is particularly acute where rice is concerned for several reasons (Williams et al., 2005). The flooded conditions in which paddy rice is cultivated leads to mobilisation of As^{III} which would otherwise be sequestered in the soil (Xu et al., 2008). Among the cereals, rice accumulates relatively high proportions of arsenic in its edible parts with amounts of grain arsenic that range from 0.08-0.20 mg/kg (Zavala and Duxbury, 2008). Thus, with rice being the major calorific food stuff for billions and its main production in south east Asian countries that have high levels of arsenic in their aquifers, rice forms a major focus of research to mitigate human arsenic contamination. Exposure to arsenic can also result from diets that are rich in (shell)fish: In these organisms, arsenic is mainly present as arsenobetaine and arsenocholine which are relatively non-toxic.

1.3 Arsenic resistance and transport in non plant organisms

1.3.1 General mechanisms of arsenic detoxification

In most organisms detoxification of arsenicals relies on multiple strategies but some general mechanisms can be identified. Pentavalent arsenic compounds (e.g. arsenate) are often rapidly reduced to As^{III} which has a high affinity for -SH groups and thus can be bound to sulfhydryl rich chelators such as glutathione and phytochelatins. Conjugation removes the reactivity and allows relatively safe intra- and intercellular transport of the complexed arsenic, for example into tissues and cellular compartments with low sensitivity. In both prokaryotes and mammals, generic methylation of mainly trivalent arsenic occurs which can increase mobility and volatility of arsenicals. This allows secretion of methylated arsenic such as through the skin and urine in mammals and direct release into the atmosphere by bacteria.

1.3.2 Arsenic transport and resistance in prokaryotes

In prokaryotes, arsenate and arsenite enter through phosphate transporters and aquaglyceporins respectively. *Escherichia coli* contains two phosphate transporters Pit and Pst that participate in As^{V} transport (Rosenberg et al., 1977). The Pit system provides the main arsenate uptake pathway (Willsky and Malamy, 1980a, Willsky and Malamy, 1980b). Two main pathways for As^{III} uptake were also identified in prokaryotes: the *E. coli* glycerol facilitator, GlpF, was the first bacterial member of the aquaporin family to be identified and this protein is ubiquitously distributed across prokaryotes. Although the physiological function of GlpF is in glycerol uptake, it was shown to also be permeable to arsenite: A glpF deletion strain of *E. coli* still showed an 80% decrease in arsenite uptake compared to wild type (Bhattacharjee and Rosen, 2007).

Prokaryotic arsenic resistance is controlled by the *ars*RBC operon. *ars*R is a small metallo-regulatory protein that functions as an As-sensing repressor protein (figure 1.1). The presence of As removes arsR from its binding site to initiate expression of the operon. *arsC* encodes the glutathione dependent reductase which reduces As^{V} to As^{III} and *arsB* extrudes the As^{III} from the cell using H^{+} antiport as a driving force (Tripathi et al., 2007a). A second family of arsenate reductases, which is also widely distributed in bacteria, is typified by the *arsC* gene product of *Staphylococcus aureus* but relies on thioredoxin as the source of reducing potential rather than glutathione.

In some bacteria the *ars*RBC operon acquired extra genes and evolved into *ars*RDABC (Lin et al., 2006). arsA is an ATPase which can bind to arsB converting the As^{III} efflux carrier to an ATP driven extraction pump which presumably has a much larger capacity than arsB alone. arsD is a weak As^{III} responsive repressor of the operon with a function similar to arsR (Rosen, 2002).

In some bacteria additional detoxification mechanisms have been identified: the *Sinorhizobium meliloti ars* operon includes an aquaglyceroporin (*aqpS*) instead of *arsB* (Yang et al., 2005). aqpS may provide another pathway for As^{III} extrusion from the cell. In *Rhodopseudomonas palustris* the *arsM* is part of the *ars* operon and encodes an As^{III}-S-adenosyl-methionine-methyltransferase, regulated by the *arsR* repressor (figure 1.1). This enzyme mediates the sequential methylation of As^{III} to volatile trimethylarsine oxide which is released into the atmosphere (Qin et al., 2006).

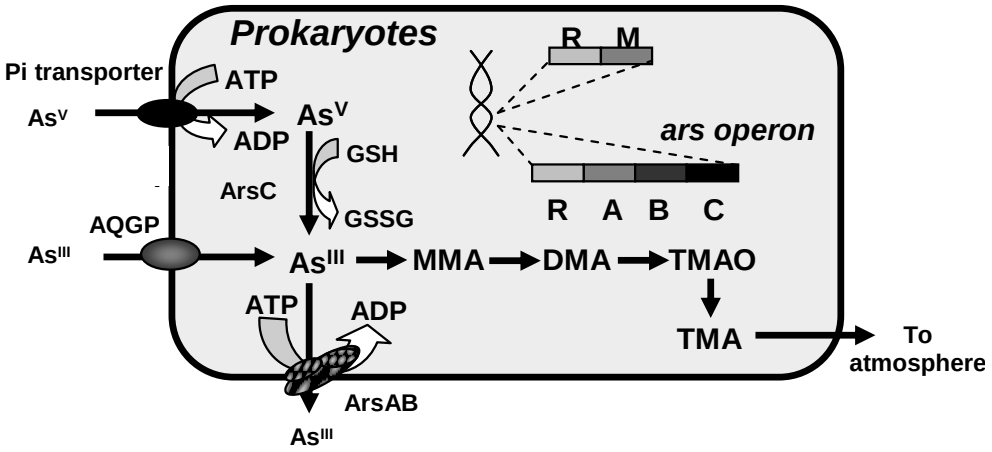


Figure 1.1 Arsenic transport in prokaryotes

The main uptake of arsenate (As^V) into bacterial cells occurs via PhoS, PstC, and PstB phosphate (Pi) transporters. Arsenite (As^{III}) enters the bacterial cells via the GlpF aquaglyceroporin (AQGP). As^V is reduced to As^{III} by the bacterial ArsC arsenate reductase using glutathione (GSH) as reductant. ArsB, an As^{III}:H^{+}-antiporter, or ArsAB, an As^{III} ATPase, extrude As^{III} into the external environment. In addition, As^{III} can be released into the environment in volatile form after subsequent methylation steps carried out by As^{III}-S-adenosylmethionine methyltransferase. MMA: monomethylarsonic acid, DMA: dimethylarsinic acid, TMAO: trimethylarsine oxide, TMA trimethylarsine.

1.3.3 Arsenic transport and resistance in yeast

The eukaryotic model system *Saccharomyces cerevisiae* has been extensively studied in relation to arsenic tolerance and detoxification. In yeast As^V enters cells through high affinity phosphate transporters such as Pho84 (figure 1.2), whereas As^{III} influx occurs through the aquaglyceroporin Fps1 (Wysocki et al., 2001, Tripathi et al., 2007a). In addition, it has been proposed that glucose permeases are involved in yeast arsenic uptake (Liu et al., 2004a). Expression of yeast hexose carriers restored As sensitivity in the yeast mutant Δ*fps1* which is As^{III} tolerant (Liu et al., 2006).

Arsenic tolerance in yeast is provided by the gene cluster *ACR1, ACR2* and *ACR3. ACR1* encodes a putative transcription factor that regulates *ACR2* and *ACR3* transcription, possibly by directly sensing cellular As levels. *ACR2* encodes an arsenate reductase and *ACR3* has been shown to encode a plasma membrane expressed As^{III}-

efflux transporter (figure 1.2). The gene cluster therefore provides a mechanism for sensing, reduction and efflux of As. Nevertheless, a second pathway for detoxification is present in yeast in the form of vacuoles: cytosolic As^{III} complexed with glutathione can be sequestered into this compartment through an ABC-type transporter, YCF1 that also transports conjugates of other harmful compounds (Ghosh et al., 1999).

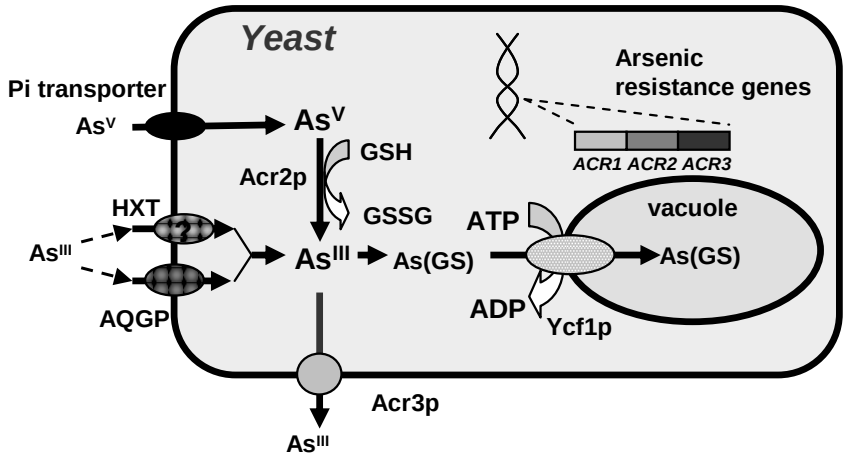


Figure 1.2 Arsenic transport in yeast

In yeast, uptake of arsenate is facilitated by Pho87 type phosphate transporters, and arsenite is taken up mainly via the AQGP Fps1p but may also enter cells through hexose permeases (HXTs). The reduction of arsenate to arsenite in yeast cells is provided by the Acr2p arsenate reductase via glutathione reduction (GSH to GS). Removal of cytoplasmic As^{III} can occur through conjugation to glutathione ($As(GS)_3$) which is sequestered into vacuoles by the ABC transporter Ycf1p, or through extrusion of As^{III} via the plasma membrane carrier Acr3.

1.3.4 Arsenic transport and resistance in mammals

Ingested As^{V} is taken up in the gut via carriers that normally process phosphate such as the Na^{+}:Pi cotransporter present in the intestinal membranes. The main mechanisms for As^{III} uptake in mammals are aquaglyceroporins (AQPs) and the hexose permeases (HXTs). Expression of the rat AQP9 in yeast increased uptake of both As^{III} and MMA^{III} (Liu et al., 2002b). Expression of either AQP7 and AQP9 from rat or the human AQP7 and AQP9 in *Xenopus* oocytes also increased As^{III} uptake (Liu et al., 2004b).

Recent work has shown that Glut1, a mammalian glucose permease, can facilitate uptake of As^{III} and MMA^{III} when expressed in yeast and *Xenopus* oocytes. Interestingly,

competition between glucose and MMA^{III} did not take place, suggesting that translocation of each substrate occurs independently in this protein (Liu et al., 2006).

Membrane proteins are also key players in arsenic removal from mammalian cells. Members of two subfamilies (MRP and MRD) of ABC transporters were shown to be involved in arsenic efflux from cells. Exposure of rat derived cell lines to various arsenicals led to elevated expression of MRP1, MRP2 and MDR1 (Liu et al., 2001, Kojima et al., 2006). In contrast, mice carrying loss of function mutations in these transporters are considerably more sensitive to arsenic toxicity and accumulated more arsenicals in their tissues (Liu et al., 2002a). Close association of GSTP1, a glutathione transferase, and MRPs and MDRs in membrane vesicles suggests that complexation of As^{III} compounds by GSH provides the substrate for the ABC transporters which mediate extrusion of arsenicals from the cell (Leslie et al., 2004).

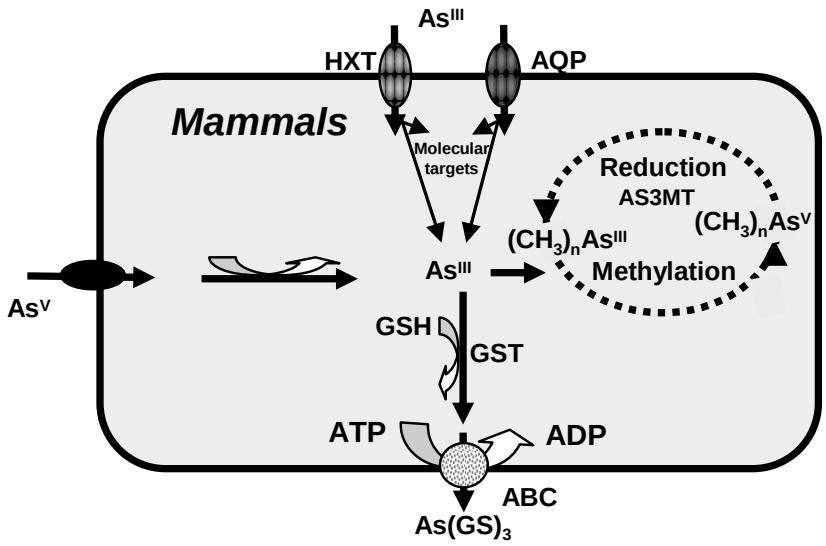


Figure 1.3 Arsenic transport in mammals

Like in yeast, uptake of As^{III} in mammalian cells can occur via aquaporins such as AQP7 and AQP9 and via HXTs. Specific proteins responsible for arsenate uptake and arsenate reduction in mammals have yet to be identified. The main efflux mechanisms for As^{III} in mammals appear to be ABC transporters from the MRP and MDR subfamilies. Methylation of As^{III} by arsenic methyltransferases such as AS3MT increases mobility of arsenicals in the body and facilitates removal through skin and urine.

1.4 Arsenic detoxification and transport in plants

1.4.1 Arsenate uptake

Arsenic is readily taken up by plant roots, in most cases as arsenate (As^V) the dominant form of arsenic in aerobic environments (Meharg and Hartley-Whitaker, 2002). Being a phosphate analogue, As^V and phosphate share the same uptake pathways. Specific transporters have been identified that are believed to mediate a large part of the observed As^V influx (figure 1.4A) and these include the *A. thaliana* Pht1;1 and 1;4 high and medium affinity phosphate uptake systems (Shin et al., 2004). However, the affinity of different phosphate transporters for As^V is higher in the hyperaccumulator *Pteris vittata* compared to the non accumulator plant species (Poynton et al., 2004, Wang et al., 2002).

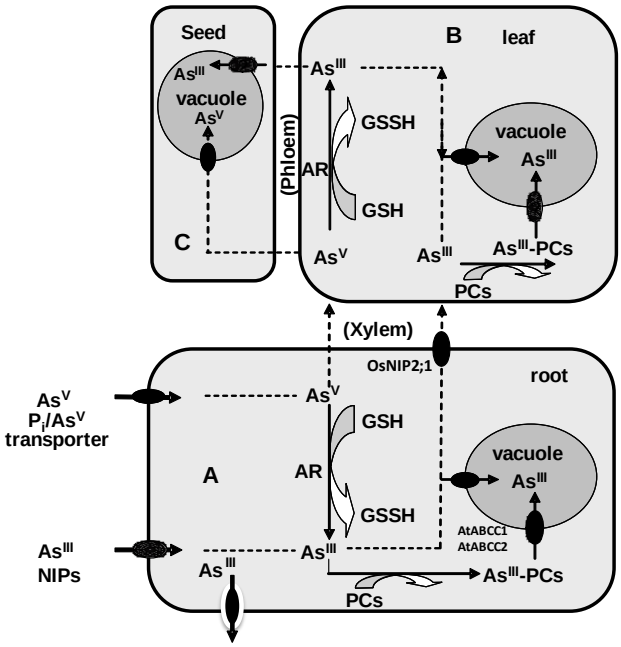


Figure 1.4 Mechanisms of As transport in plants

(A) Plants take up As^V through phosphate transporters (Pi) such as those belonging to the PHT1 family. As^{III} influx occurs through aquaglyceroporins of the NIP (nodulin like intrinsic protein) subfamily. Similarly, there may be As^{III} efflux towards the external medium. As^V is reduced to As^{III} by arsenate reductase (AR) using glutathione (GSH) as a reductant. As^{III} can form complexes with thiol groups from glutathione and phytochelatins (PCs) to lower its cytotoxicity. The complexed As^{III} is sequestered into the vacuole via AtABCC1 and AtABCC2 in *Arabidopsis* and inorganic As^{III} through unknown transporters. Inorganic As^V and As^{III} are the

major arsenicals found in the xylem sap of plants. In rice, As^{III} is translocated to the xylem via aquaporin (OsNIP2;1) (B) Most plant species act as 'excluders' i.e. a very small proportion of arsenic is translocated to shoot tissue where similar reduction and sequestration mechanisms are present. (C) Via the phloem, some of the total arsenic content ends up in the vacuoles and other tissues of edible parts such as seeds. Although there is no hard evidence of As transport to the vacuole, however the recent studies on the localisation of As in rice grain using the NanoSIMS technology showed that As mainly occurs as DMA in the subaleurone layer of the grain with high degree of mobility(Carey et al., 2012, Moore et al., 2010).

1.4.2 Arsenite uptake

It is clear from multiple studies that plant arsenic toxicity mainly derives from exposure to inorganic arsenate (As^{V}) and arsenite (As^{III}). Since As^{V} is rapidly reduced, intracellular arsenic is predominantly in the form of inorganic or complexed As^{III}. The molecular identity of the proteins that participate in uptake, efflux, compartmentation and long distance transport of As^{III} is largely unknown but great progress has been achieved recently in identifying a number of them, particularly those associated with As^{III} uptake (figure 1.4A).

In anaerobic soils arsenic mostly exists as inorganic As^{III} due to abundant reducing activity of microbial organisms. Thus in most aquatic plants, uptake of As^{III} is at the root of arsenic toxicity. Although there were reports that NIPs (Nodulin26-like intrinsic proteins), a subgroup of plant aquaporins, were involved in bidirectional transport of As^{III} until recently no direct evidence existed. However, recently it was reported that a loss of function mutation in AtNIP7;1 led to increased As^{III} tolerance and that this aquaglyceroporin contributes to As^{III} uptake in *Arabidopsis* roots (Isayenkov and Maathuis, 2008). AtNIP5;1 and AtNIP6;1 affect As^{III} sensitivity when heterologously expressed in yeast, Bienert et al., (2008b) indicating that these isoforms can also transport As^{III}. Furthermore AtNIP1;1 is involved in As^{III} uptake and its disruption results in As^{III} tolerance in *Arabidopsis thaliana* (Kamiya et al., 2009).

Arsenite uptake is of prime importance concerning rice and other aquatic plants, as the roots of these plants grow in anaerobic reducing conditions. Physiological studies suggest that As^{III} is transported through the aquaporin OsNIP2;1(Lsi1) which was previously shown to function as a Si uptake pathway. Expression of Lsi1 in yeast and

Xenopus oocytes showed increased capacity for As^{III} uptake, while the loss of function in rice *lsi1* showed a 60% decrease in As^{III} uptake capacity for the short term uptake analyses (Ma et al., 2008a). In addition, other rice aquaporins such as OsNIP1;1 and OsNIP2;2 (Lsi6), when expressed in *Xenopus laevis* oocytes were able to increase As^{III} uptake, but due to low expression levels in rice these aquaporins were unable to show any significant role in As^{III} uptake (Ma et al., 2008a). Similarly, the expression of OsNIP2;1 and OsNIP3;2 increased the sensitivity to As^{III} when heterologously expressed in yeast (Bienert et al., 2008b).

1.4.3 Uptake of methylated As

Organic forms of arsenic occur in some soils but usually in a small proportion, and consist mostly of methylated arsenic, such as MMA^V and DMA^V. Uptake of these compounds by plants is lower than that for inorganic As species (Raab et al., 2007, Carbonell-Barrachina et al., 1998).

It has recently been reported that in rice, the aquaporin Lsi1 is involved in the uptake of pentavalent methylated As species and the loss of function in rice *lsi1* led to an 80% reduction in the uptake capacity for MMA and 50% for DMA compared to wildtype plants (Li et al., 2009).

1.4.4 As metabolism

Plants vary greatly in arsenic tolerance from sensitive species that include all major crops, to tolerant plants such as Indian mustard and several populations of the grass *Holcus lanatus*, to extremophiles such as the hyperaccumulator *Pteris vittata* (Chinese break fern) which can accumulate 2% of its dry weight as arsenic (Wang et al., 2002). In spite of this ecophysiological diversity many common responses to arsenic exposure have been observed. For example, most intracellular As^V is reduced to As^{III} by the action of specific arsenate reductases. Such proteins have been identified in many plant species including *Arabidopsis* (Bleeker et al., 2006), *H. lanatus* (Dhankher et al., 2006), *P. vittata* (Ellis et al., 2006) and rice (Duan et al., 2007). Many plant arsenate reductases are bifunctional and also show tyrosine phosphatase activity. They are homologous to the human cell-cycle dual-specificity phosphatases CDC25s, and often contain the conserved motif HCX_5R. Expression of plant arsenate reductases in *E. coli* and yeast mutants that lack endogenous reductases restored As^V resistance and several have been found to be induced by plant exposure to As^V (Dhankher et al., 2006, Duan et al., 2007,

Ellis et al., 2006). However, the physiological relevance of these enzymes is not always clear since in some species disruption of their activity had little impact on arsenic tolerance and alternative mechanisms of As^{V} reduction may be present in plants (Zhao et al., 2009).

1.4.5 As complexation and sequestration

Arsenic toxicity is largely manifested in the cytoplasm and a common mechanism of detoxifying cytoplasmic metals and metalloids is the complexation via sulphur bonds (Rosen, 2002, Tripathi et al., 2007a). Although variation between species is likely, it is believed that a substantial proportion of As^{III} may be chelated in this way to minimise cytoplasmic exposure. In plants, this is typically carried out by glutathione and glutathione-based phytochelatins (PCs) which have a general structure $(\lambda\text{-Glu-Cys})_n$-Gly. The –SH functional group of the cysteine in glutathione and PCs has a high affinity for As^{III}. Many studies point to the essential role of PCs in both constitutive and adaptive plant tolerance to arsenic (Raab et al., 2004). For example, an *Arabidopsis* mutant that lacked the capacity to synthesise PCs was found to become hypersensitive to arsenate (Ha et al., 1999). Interestingly however, the proportion of complexed arsenic in hyperaccumulators such as *P. vittata* (Pickering et al., 2006, Zhao et al., 2003) and *P. cretica* (Raab et al., 2004) is very small, suggesting that such species rely on tolerance mechanisms that do not involve complexation of arsenic. Any exposure to inorganic As (As^{III} and As^{V}) resulted in the production and accumulation of PCs in plants (Srivastava et al., 2007, Schulz et al., 2008, Grill et al., 1987) . The tolerant and non tolerant populations of *H. lanatus* showed hypersensitivity, when PC synthesis was inhibited by using buthionine sulphoximine (BSO), which inhibits the activity of γ-glutamylcysteine synthetase (Schat et al., 2002, Bleeker et al., 2006). However, arsenate stress induces a 15-20 fold increase in PCs in the tolerant populations suggesting that the phenomenon of PC production may be an adaptive mechanism of tolerance (Hartley-Whitaker et al., 2001) . Recently it has been reported that the loss of function in the vacuolar ABC transporters, AtABCC1 and AtABCC2 increased the sensitivity to As. The expression of these genes in yeast increased As tolerance and accumulation, suggesting a role in the sequestration of complexed As to the vacuole (Song et al., 2010).

1.4.6 As translocation

In most plants only a fraction of the arsenic is translocated to shoot tissue, a widespread tolerance mechanism that is also observed for other toxic ions. However, in hyperaccumulators the opposite is the case: the majority of potentially harmful metals/metalloids is deposited in the shoot. This is exemplified in *P. vittata* where around 80% of the arsenic is in the shoot. In contrast, only around 5-10% of total arsenic ends up in leaves of non-accumulating species such as the fern *P. tremula* (Caille et al., 2005), *Arabidopsis* (Isayenkov and Maathuis, 2008) and rice (Ma et al., 2008a). The form in which arsenic is translocated from root to shoot appears to be mainly as inorganic As^{III}, accounting for 60–100% of the total arsenic (Ma et al., 2008a, Pickering et al., 2006, Zhao et al., 2009). The As^{V} translocation is limited as most of it is reduced in the roots to As^{III}, which forms complexes with PCs and is sequestered to the vacuole (Zhao et al., 2008). An *Arabidopsis* RNAi silenced line for AtACR2 showed increased As concentration in the shoots, suggesting higher availability of As^{V} for translocation using the phosphate transport system (Dhankher et al., 2006). However, the loss of function mutants in AtACR2 showed the opposite result, having less translocation (Bleeker et al., 2006). In *Arabidopsis*, the loss of function mutants deficient in the synthesis of glutathione (*cad2-1*) and PCs (*cad3-1*) were exposed to As^{V}. Both the mutants showed 20 times higher sensitivity to As^{V} compared to wild type with a higher As concentration in the shoots and enhanced efflux in the roots (Liu et al., 2010).

1.4.7 As^{III} efflux through aquaporins and other systems

Arsenic tolerance in prokaryotes and unicellular eukaryotes relies heavily on As^{III} efflux and the relevant membrane transporters have been well characterised (Bhattacharjee and Rosen, 2007). The question whether As^{III} efflux is an important component of arsenic tolerance in plants has yet to be answered definitively but several studies have shown that plants release As^{V} and As^{III} into the external medium (Zhao et al., 2009). As^{III} efflux occurs soon after plants are exposed to arsenate but the mechanistic details of this process are unclear (Xu et al., 2007).

The recent work on the role of plant aquaglyceroporins in As^{III} uptake revealed some evidence that the same class of protein may also contribute to the loss of As^{III} from cells. Bienert et al., (2008) showed that expression of group II NIPs from *A. thaliana*,

rice and *L. japonicus* in the yeast strain *acr3*Δ that lacks its As^{III} efflux carrier, significantly enhanced yeast tolerance to arsenate. Similarly, Isayenkov and Maathuis (2008) reported that expression of AtNIP7;1 in the same yeast background resulted in a small but consistent increase in As^{V} tolerance. In both cases, these data were interpreted as evidence for NIP channels forming a shunt pathway for the efflux of arsenite which was produced by the reduction of arsenate inside the yeast cells. There are precedents for aquaglyceroporins acting in cellular As^{III} extrusion notably in the earlier mentioned *S. meliloti* which expresses *aqpS* in its bacteroid membrane to release As^{III}.

Theoretically, similar mechanisms could operate and be beneficial in plants, especially for species exposed to As^{V}. In the case of As^{III} exposed plants like rice, such a mechanism could easily create futile cycling of As^{III} and appears of less advantage. A definitive answer regarding the physiological relevance of aquaglyceroporin mediated As^{III} release has yet to be reached.

As^{III} extrusion from the cytosol through aquaporin-type transporters is by definition a passive mechanism dependent on the concentration gradient and may not be very efficient in a lot of conditions. Many organisms show the presence of energy coupled systems to remove As^{III} from the cytoplasm such as the H^{+} coupled bacterial ArsB, the ATP dependent arsAB or the yeast ACR3 which is also believed to be H^{+} coupled (Wysocki et al., 1997, Rosen, 2002). Whether plants carry out active As^{III} extrusion is not clear; In tomato roots preloaded with As^{V}, As^{III} efflux was found to be sensitive to the protonophore CCCP (carbonylcyanide chlorophenyl hydrazone) suggesting an active mechanism may be involved (Xu et al., 2007). However, CCCP-induced depolarisation would also affect passive fluxes.

An *acr3*Δ yeast complementation approach using a cDNA library from *P. vittata* revealed the presence of an ACR3 like transporter in this species, which is localised in the tonoplast and involved in the vacuolar sequestration of complexed As^{III} (Indriolo et al., 2011). A recent study where the yeast ACR3 was expressed in rice showed that it increased As^{III} efflux from the roots and resulted in lower As concentration in the roots, shoots and grain (Duan et al., 2011). However, the expression of ACR3 in *Arabidopsis* resulted in higher efflux of As^{III} but a higher translocation to the shoots (Ali et al., 2012).

The similarity in size and pKa of As^{III} and nutritional minerals such as boric acid and silicic acid means that endogenous mechanisms for the latter substrates may also form potential As^{III} efflux systems. Both boric acid and silicic acid are moved through the xylem and can be sequestered in vacuoles, two processes that involve efflux steps. Loss of function in the silicon efflux system Lsi2 impacts greatly on plant arsenic distribution, with a large reduction in both xylem and grain As^{III} concentration (Ma et al., 2008a). In addition, the xylem As^{III} levels are reduced by silicon indicating that Lsi2 is responsible for a significant proportion of As^{III} loading into the vascular tissue. In combination with the data obtained from the Lsi1 loss of function mutant, the results suggest that in rice, arsenic uptake and distribution closely parallels that of silicon, a nutrient required in large amounts by rice. This may severely limit the scope to engineer rice with improved arsenic tolerance.

Efflux from the cytosol into the large lytic vacuole is a further generic detoxification pathway that has been shown to function in plants with regards to toxic minerals such as Na^{+} and heavy metals like Pb^{2+} and Cd^{2+}. The latter are likely to be chelated to compounds such as glutathione and PCs before vacuolar deposition. The form in which vacuolar arsenic is stored appears to be species dependent: For the hyperaccumulator *P. vittata* it was concluded that vacuolar arsenic consisted almost exclusively of inorganic As^{III} (Pickering et al., 2006), whereas in non accumulators such as *H. lanatus*, a large proportion was shown to be complexed (Raab et al., 2004). In *Arabidopsis*, it has been reported recently that the two phytochelatin transporters AtABCC1 and AtABCC2 are involved in the vacuolar sequestration of PC-As^{III} complexes (Song et al., 2010). However, the proteins that mediate vacuolar sequestration in tonoplast enriched microsomes from *H. lanatus* roots, glutathione complexed As^{III} was efficiently transported into the vesicles after addition of MgATP and the authors suggested that this phenomenon could be mediated by ABC type transporters (Bleeker et al., 2006).

1.5 Background and rationale for thesis

Arsenic (As) contamination of drinking water and the food chain has arisen as a global health problem. It is important to know how plants deal with As both from the perspective as potential sources of dietary As but also as potential mechanisms for phytoremediation. Among crops, rice is efficient in the accumulation of As in the edible

part, as it is mostly grown in paddies that are often irrigated with As contaminated water.

Ideally this problem should be solved at the source, i.e. through soil remediation and usage of non-contaminated irrigation water. However, this is unlikely to be achieved in the near future and different strategies may be required to limit arsenic entry into the food chain. Approaches to reduce arsenic uptake in crops, especially into edible parts, would provide a viable alternative, and rely on different techniques:

Arsenic tolerance was shown to be a quantitatively inherited character. In rice there are huge variations in As content among genotypes from different parts of the world (Norton et al., 2008). Crossing varieties with different As backgrounds might be interesting in order to study the inheritance pattern of the genes involved in As tolerance.

In rice, silicon and As^{III} share the same uptake pathway. Thus, it may be possible to use soils with high Si content resulting from volcanic ash which might lead to less uptake of As^{III}. In addition, adding Si fertilizers may be useful in preventing As accumulation in paddy conditions (Zhao et al., 2010c).

Recently, significant progress has been made in the identification and characterization of proteins responsible for As transport in pflants. Different strategies could be used to limit the entry of As into the seed particularly in rice. It is not possible to completely stop the entry of As because of shared transporters with essential nutrients. However, there is a possibility to identify the variants in different transporters which are selective against As.

To limit the uptake, it is highly desirable to retain As in the roots. This may be achieved by the overexpression of genes involved in As^{V} reduction and PC synthesis in combination with vacuolar sequestration. Similarly, the overexpression of those NIP isoforms involved in the efflux of As^{III} and silencing of isoforms that contribute to As uptake using RNAi could be useful in reducing As uptake.

Phytoremediation of As contaminated soils would be another alternative to reduce mobilization of As into the food chain. The Chinese brake fern (*Pteris vittata*) has the natural ability to hyperaccumulate As in its fronds. However, this fern grows only in warm and humid conditions and cannot be used in many environments. So there is a

need to identify other species with the same ability or to modify plants for the said purpose. Genetic engineering tools can be used to increase the efficiency of plants to hyperaccumulate As. Strategies like increasing the uptake of As, reducing the efflux, chelation and sequestration of As at the root level and enhancing the capacity of root to shoot translocation would help in efficient accumulation of As.

Having the basic knowledge of arsenic resistance and metabolic mechanisms in microbes, plant scientists are now in a position to exploit this and test if similar pathways are functional in plants. The unexplored mechanisms of As uptake and distribution in plants need further research. From As uptake to its accumulation in shoots and grain many transport mechanism are involved which are still unknown. In plants, NIPs may be involved in the bidirectional movement of As, so there is a need for different NIP mutants to be evaluated for their As tolerance. Similarly, the mechanisms already characterised in other organisms like the efflux carrier Acr3p and vacuolar sequestration via YCF1 in yeast could be used to screen for similar mechanisms in plants.

1.6 Aims of the project

The transport of As in plants needs further research by exploring the mechanisms of As efflux and translocation to the shoots and grain. Until now no specific transporters have been identified for As^{III} efflux. In addition to that, so far non of the efflux transporter identified in microbes has been functionally analysed in plants. The results from the present study will demonstrate if the As tolerance transporters from microbes can be useful in plants. This information will also be useful for plant scientists to utelise the microbial tolerance mechanisms in plants for biotic and abiotic stresses. Beside the microbial system, the transporters reported to be in As transport in plants will be further analysed. This will help in using the systems already available in plants by selection for different agronomical characters.

The main focus of research is to ensure the food safety by limiting the entry of As into the edible plant parts using multiple strategies. The aim of my project is to test whether the expression of *ScACR3* (a plasma membrane located transporter involved in the efflux of As in yeast) affects As tolerance in *Arabidopsis* and rice. In addition NIPs are

reported to be involved in the bidirectional transport of As^{III}. Therefore, I also analysed the roles of *Arabidopsis* NIPs in As transport.
The objectives of this work are as follows:

1. To study the roles of *Arabidopsis* NIPs in As uptake, efflux and translocation.
2. To study how expression of the yeast As^{III} transporter *ScACR3* affects As tolerance in plants (rice and *Arabidopsis*).
3. Screening of cDNA libraries from rice (*Oryza sativa*) and *Arabidopsis thaliana* to identify (new) transporters that are involved in As transport.

Chapter 2

2 The roles of *Arabidopsis* NIPs in As uptake, efflux and translocation

2.1 Introduction

Plant aquaporins are membrane proteins which are involved in the transport of water and neutral molecules (Maurel et al., 2008). There are 35 and 33 isoforms of aquaporins identified in *Arabidopsis* and rice respectively (Wallace and Roberts, 2005, Bansal and Sankararamakrishnan, 2007, Sakurai et al., 2005).

Based on homology and localisation studies, plant aquaporins cluster in four subfamilies called PIPs (plasma membrane intrinsic proteins), TIPs (tonoplast intrinsic proteins), SIPs (small basic intrinsic proteins) and NIPs (nodulin26-like intrinsic proteins). Among these, the NIPs are specific to plants with 10 and 9 members in rice and *Arabidopsis* respectively (Wallace and Roberts, 2004, Bansal and Sankararamakrishnan, 2007).

On the basis of the Ar/R (aromatic/arginine) domain composition, the plant NIP subfamily can be further divided into groups I, II and III (Fig. 2.1D) with group I containing the archetypal Nodulin26 and NIPs with permeability to water, glycerol, and lactic acid. NIPs in groups II and III have a predicted larger pore size than those of the NIP I group with permeability for larger solutes such as antimonite, urea, formamide, silicic acid and boric acid, but very low water permeability. In contrast to group I members, group II and III isoforms are sometimes referred to as metalloid transporters and also include the outlier AtNIP7;1. In the previous studies, NIPs were classified into two groups and AtNIP7;1 was classified as an outlier. However, in the recent classification of NIPs into three groups, AtNIP7;1 has been placed in group II. (Isayenkov and Maathuis, 2008, Wallace and Roberts, 2005, Takano et al., 2006, Tanaka et al., 2008).

The tertiary structure of aquaporin subunits is composed of a two times three transmembrane domain (TMD) structure (Fig. 2.1A) which both contain the canonical NPA motif. The two domains fold onto each other with the NPA motifs forming part of the central restrictive pore, and is often referred to as the ‘hourglass model’ (Wallace et al., 2006). Four subunits form a functional aquaporin, each with a central pore (Fig. 2.1C).

Aquaporin selectivity is mainly derived from the Ar/R (aromatic-arginine) pore region which is located a few angstroms from the canonical NPA-NPA motif (Fig. 2.1B). It

consists of two residues from TMD2 and TMD5 and two residues from loop E. The composition of the Ar/R domain defines the pore size, pore hydrophobicity and hydrogen bonding between pore and substrate and hence greatly impacts on selectivity and function of aquaporins.

Archetypal aquaporins contain a narrow pore with a width of around 0.3 nm, just wider than a water molecule (~0.28 nm). This tight fit and an energy barrier near the channel NPA motifs that shapes the pore water-wire, ensure high water selectivity, a very low proton conductance and no or very little permeability for any other substrates. However, in several of the plant aquaporin subfamilies such as the NIPs, the Ar/R composition creates a larger pore size thus allowing additional substrates to permeate such as neutral metalloids, undissociated acids and small solutes like glycerol. This class of aquaporin is therefore often referred to as aquaglyceroporins.

Several isoforms in this group have been shown to fulfil physiological functions in planta: AtNIP5;1 was shown to catalyse B uptake, presumably as the neutral boric acid $B(OH)_3$, in boron limited conditions (Takano et al., 2006). OsNIP2;1 (Lsi1) is a major pathway for uptake of Si (as $Si(OH)_4$), a beneficial nutrient that is required in large amounts by rice (Tanaka et al., 2008). Several other group II and III NIPs are also Si permeable but their physiological function remains unclear (Ma et al., 2008a).

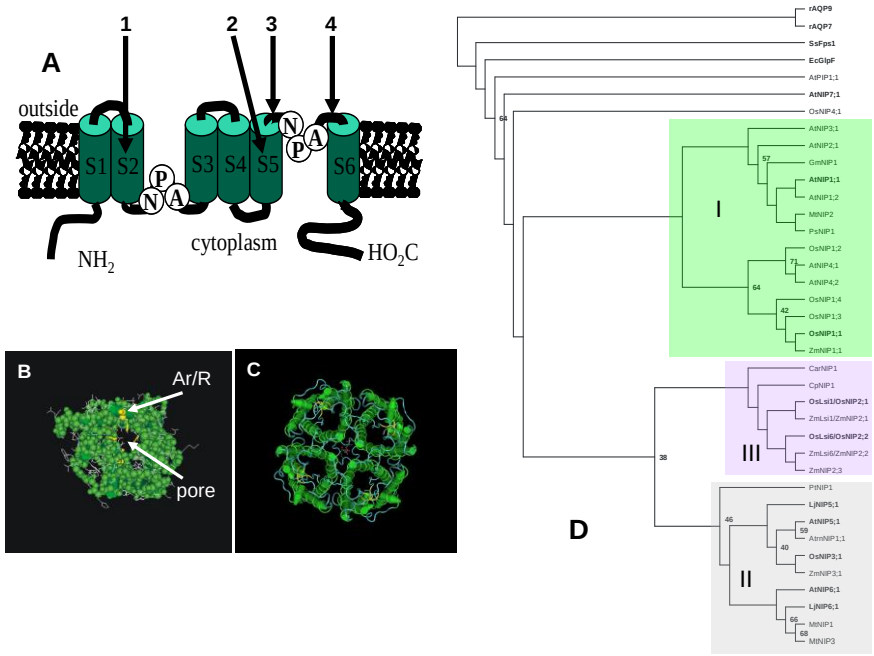


Figure 2.1 Generalised structure of aquaporin and the phylogenetic tree of plant NIPs

(A) Generalised secondary structure of an aquaporin monomer with 6 transmembrane domains (S1-S6) and 2 canonical NPA motifs between S2 and S3 and S5 and S6. The arrows 1-4 point to the approximate positions of residues that make up the Ar/R (aromatic/arginine) region that is essential for channel selectivity. **(B)** Each monomer forms a pore (arrow) with the Ar/R residues (in yellow) forming the narrowest part of the channel pore. **(C)** Four monomers form a functional aquaporin. Views in (B) and (C) are perpendicular to the membrane from the outside. **(D)** Phylogenetic tree of plant NIPs. At, *Arabidopsis thaliana*; Os, *Oryza sativa*; Zm, *Zea mays*; Mt, *Medicago truncatula*; Cp, *Cucubrita pepo*; Nal, *Nicotiana alata*; Lj, *Lotus japonicus*; Pt, *Pinus taeda;* Atrn, *Atriplex nummularia*; Car, *Cicer arietinum*; Gm, *Glycine max*; Ps, *Pisum sativum*; Ec, *Escherichia coli*; Ss, *Saccharomyces cerevisiae* (figures reproduced from Ali et al.,(2009)).

To assess the possibility that NIPs form arsenic transporters, several groups recently studied the physiological and transport characteristics of these proteins. Homozygous T-DNA lines for the three group II *A. thaliana* NIPs (NIP5;1, NIP6;1 and NIP7;1) showed that loss of function in NIP7;1 led to plant tolerance to As^{III} but not As^{V} whereas arsenic

tolerance in *Atnip5;1* and *Atnip6;1* mutants was not affected significantly (Isayenkov and Maathuis, 2008). Total arsenic concentration in *Atnip7;1* was reduced and heterologous expression of this protein increased As^{III} sensitivity in the Δ*fps1* yeast strain that lacks the arsenite uptake aquaglyceroporin FSP1. Transport assays showed that expression of AtNIP7;1 also increased short term As^{III} uptake in yeast.

In rice, the Os NIP2;1 was previously shown to provide a silicon uptake pathway and is therefore also referred to as Lsi1 (Ma et al., 2006). Loss of function in NIP2;1 not only affects silicon accumulation but resulted in greatly reduced arsenite influx into roots compared with the wild-type rice. Heterologous expression of OsNIP2;1 in *Xenopus laevis* oocytes showed that OsNIP2;1 has a high As^{III} conductivity but did not transport As^{V}. Loss of function in another group II NIP (OsNIP2;2) did not significantly change plant arsenic levels, in spite of As^{III} transport capacity in oocytes, presumably because of a very low in planta expression level of this isoform.

Further work, based primarily on heterologous expression of NIPs in yeast, confirmed the As^{III} transport capacity of AtNIP7;1 and OsNIP2;1 but in addition showed that all group II NIPs are capable of As^{III} transport. Indeed, several of the NIPs derived from *A. thaliana, Lotus japonicus* and rice were capable of mediating bidirectional As^{III} transport which could have implications for their *in planta* role (Bienert et al., 2008b, Isayenkov and Maathuis, 2008).

Although suggested by some authors, the idea that only 'metalloid' NIPs have significant As^{III} permeability and therefore a role in As^{III} uptake and distribution in plants has been shown to be too narrow. A forward genetics screen with *A. thaliana* identified the group I NIP1;1 as a determinant of As^{III} tolerance (Kamiya et al., 2009). Expression of AtNIP1;1 in oocytes showed As^{III} transport in these cells as did its close homologue AtNIP1;2, another member of the group I NIPs. Work with rice provided similar insights for group I NIPs from this species with OsNIP1;1 and OsNIP3;1 both capable of mediating As^{III} uptake in oocytes, albeit significantly less so for OsNIP3;1 (Ma et al., 2008a). In total, these recent findings suggest that all NIP aquaporins may have some permeability to As^{III} but that specific isoforms are more important than others where plant arsenic tolerance is concerned, depending on expression levels and tissue localisation.

It has already been demonstrated that the expression of NIPs sensitises yeast when exposed to As^{III} and leads to moderate resistance to As^{V}, suggesting their role in bidirectional transport of As^{III}. Here we investigated the NIP loss of function mutants in *Arabidopsis* (*nip5;1*, *nip6;1* and *nip7;1*) for their role in As efflux, translocation and sequestration at both cellular and intact plant levels. The selection of these mutants (*nip5;1*, *nip6;1* and *nip7;1*) for the present studies were based on few assumptions. Firstly, all the three mutants were already reported to be involved in metalloid transport and secondly the expression of the genes to the plasma membrane, which is desirable in terms of efflux from the cytosol.

2.2 Material and Methods

2.2.1 Chemicals and consumables

The laboratory chemicals used in the present study were purchased from different suppliers including Bio-Rad (UK), Sigma (UK), Fischer Scientific (UK) and Micherey Nigel (UK). The consumables used were purchased from StarLab (UK) and Eppendorf (UK). The reaction kits for DNA and RNA extraction, Gel purification, minipreps and midi preps were purchased from Micherey Nigel (UK), Fermentas (UK) and Qiagen UK.

2.2.2 Genomic DNA extraction

The CTAB method with some modifications was used for the genomic DNA extraction. About 200 mg plant leaf tissue was ground to powder using liquid nitrogen and mixed with 500 µl of pre-warmed (65^{o}C) CTAB extraction buffer (2 % CTAB, 1.4 M NaCl, 100 mM Tris-HCl (pH 8) and 20 mM Na-EDTA) and incubated at 65^{o}C in water for 30-60 minutes. After heating and vortexing the mixture, 300 µl of chloroform:isoamylalcohol (24:1) was added to the mixture. The mixture was vigorously shaken and centrifuged at 3000 rpm for 5 minutes. The supernatant was collected into new sterilised eppendorf tubes and the DNA was precipitated by adding 2 volumes of 96% ethanol and 4% of 3M Na-acetate. For the DNA precipitation the mixture was left at room temperature for 15 minutes. The DNA pellet was collected via centrifugation for 10 minutes at 13000 rpm. The DNA was then washed with 70 % ethanol, dried at room temperature and diluted in 50 µl of sterilised water.

2.2.3 RNA extraction and cDNA synthesis

RNA was extracted using the standard protocol from Qiagen-RNeasy kits (UK). For the complementary DNA synthesis, mRNA was used as a template using the enzyme reverse transcriptase isolated from retroviruses. Reverse transcriptase can add dNTPs to the 3' end of the RNA such as the poly A tail of mRNA to which a short oligo dT primer is hybridised. Complementary DNA was synthesised using SuperScript II reverse transcriptase enzyme (Invitrogen, UK). The cDNA synthesis was started using 2 µg of RNA, adding 1µl oligo dT primer and making the final volume to 12 µl by adding sterilised water. The mixture was denatured by heating at 70°C for 10 min and quickly chilled on ice. After a brief centrifugation, 6 µl of 5x First Strand Buffer, 2 µl of 0.1 M DTT and 1 µl of dNTPs were added to the mixture. The mixture was then incubated at 42°C for 2 minutes. In the last step, 1 µl of SuperScript RT II enzyme was added for cDNA synthesis at 42°C for 1 hour. The enzyme was inactivated at 70°C for 15 minutes. The synthesised cDNA was stored at -20°C.

2.2.4 Oligonucleotides

All the primers used in the present study were purchased from Eurofins-MWG (UK) and Sigma (Germany). Stock solutions of 100 pmol (picomole) from the HPLC-purified primers were prepared in sterilised eppendorf tubes and stored at -20^{0}C.

2.2.5 PCR analyses

PCR analyses were carried out by preparing a mixture of 5 µl of Flexi buffer, 2 µl of $MgCl_2$, 1 µl of dNTPs (10 mM of each deoxyribonucleotide triphosphates), Gotaq-polymerase (5U/ µl), 2 µl of DNA and 12.5 µl of water to make the final volume to 25 µl. PCR was performed in a Mastercycler machine (Eppendorf, UK) using the gene specific primers with the following program: 5 min initial denaturation at 94^{o}C followed by 30 cycles (denaturation for 30 sec at 94^{o}C, annealing at 55-57^{o}C for 30 sec and extension for 1-2 min). The PCR products were run on an agarose gel (1.2%).

2.2.6 Gel electrophoresis

Gel electrophoresis was performed to analyse the size of the DNA (nucleic acids). The electrophoresis was performed in horizontal agarose gel (1.2 % agarose with 0.1µg/ml sybr safe). As a marker, 2-Log DNA ladder (New England Biolabs) was included with samples for the estimation of size and concentration of bands. After migration, the

samples were visualised using a UV transilluminator (DR-45M, 230 V, 50 Hz and 9 W) at 302 nm. For the documentation of the gels, the SEIKOSHA VP- 1500 together with Alphamanager-2000 was used. For cloning, the desired fragment was excised using a sterile scalpel while viewing on the transilluminator.

2.2.7 Plant material and characterisation of mutants

The *Arabidopsis* loss of function mutants in NIPs (*nip5;1*, *nip6;1* and *nip7;1*) in Columbia (Col-0) ecotype background were isolated from T-DNA lines generated at NASC. The loss of function in *Atnip5;1* (At4g10380, salk_012572), *Atnip6;1* (At1g80760, salk_046323) and *Atnip7;1* (At3g06100, salk_057023) mutants were identified by PCR for the presence of T-DNA. PCR analyses were performed using genomic DNA with gene specific primers or forward/reverse of gene specific primer in combination with lba primers (Table 2.1). After identification at DNA level, the mutants were then analysed at cDNA level using semiquantitative RT-PCR with cDNA primers (Table 2.2).

Table 2.1 List of primers for characterisation of *Arabidopsis* mutants in NIPs (*nip5;1*, *nip6;1* and *nip7;1*) using genomic DNA

Primers used to detect T-DNA in genomic DNA of the control (wild type) and loss of function mutants in *Arabidopsis* NIPs (*nip5;1*, *nip6;1* and *nip7;1*) using PCR.

Gene ID	Primer sequence	Tm	Product size
Nip5;1(2287)_LP	5'-TCCTAGCTCCATTTTCGTTTTC-3'	59.74	1182 bp
Nip5;1(2287)_RP	5'-CTCCAAGTGTGACGTAAACCC -3'	59.51	1182 bp
Nip6;1(6323)_LP	5'-TGTGCTTGTAAAGTTGGGAGC-3'	60.30	1250 bp
Nip6;1(6323)_RP	5'-GCAATCGTTGTAATCGCTAGC-3'	59.89	1250 bp
Nip7;1(7023)_LP	5'-TGTCAAAACTCCGATTATGGC-3'	59.95	1116 bp
Nip7;1(7023)_RP	5'-AGTGTGTGTGGGGTCATAAGC-3'	59.91	1116 bp
Lba	5'-TGGTTCAGGTAGTGGGCCATC-3'	62.25	

Table 2.2 List of primers for characterisation of *Arabidopsis* mutants in NIPs (*nip5;1*, *nip6;1* and *nip7;1*) using cDNA

Primers used to detect gene expression in control (wild type) and loss of function mutants in *Arabidopsis* NIPs (*nip5;1*, *nip6;1* and *nip7;1*) using semiquantitative RT-PCR

Gene ID	Primer sequence	Tm	Product size
NIP5;1-For	5'-GGTAATGGTGATGGCTCCTC-3'	57.83	0.894 bp
NIP5;1-Rev	5'-TTAACGACGAAAGCTCCTAAC-3'	55.00	0.894 bp
NIP6;1-For	5'-GATTCGAAGGGAAGAGGAATG-3'	58.27	0.694 bp
NIP6;1-Rev	5'-TGGACCCAGTGTTCTTACAGG-3'	58.49	0.694 bp
NIP7;1-For	5'-GCACGGTCAAGAGTAGTCGAC-3'	58.40	0.741 bp
NIP7;1-Rev	5'-TGTCAAAACTCCGATTATGGC-3'	58.49	0.741 bp

2.2.8 Seed sterilisation and growth conditions

To analyse the growth of *Arabidopsis* loss of function mutants on plates, the seeds were surface sterilised with 90% ethanol for 5 minutes followed by washing with 40% bleach for 5 minutes and then rinsed five times with sterilised distilled water. The medium containing 0.5 MS (Murashige and Skoog, 1962) macro and micro nutrients was used for plant growth on plates. The medium was prepared from 10x stock solutions of macro and micro nutrients (Sigma) adding 0.8% agar. As^{III} (As_2O_3) and As^{V} (KH_2AsO_4) were added to the medium in different concentrations (10 µM As^{III} and 160 µM As^{V}) each before autoclaving. After stratification the seeds were placed on control and treatment plates which were sealed with micropore tape. The plates were placed vertically in racks in a growth room (16h light, 8h dark; photon flux density ~ 65 µmole per m^2 per second, 20-25^{o}C day/night) for three weeks. The growth of plate-grown seedlings is expressed as average fresh weight per plant (%).

2.2.9 Total arsenic and arsenic speciation analyses

Arsenic content was determined by HPLC-ICP-MS (Agilent LC1100 series and Agilent ICP-MS 7500ce, Agilent Technologies, Santa Clara, CA, USA). For the efflux analyses

3 week old plants were exposed to 5µM As^{V} for 24 hours. Samples from the exposure solutions were collected after 24 hours for speciation analyses. For this, the roots and shoots of these plants were rinsed with deionised water and roots were then placed in the desorption solution containing 1 mM K_2HPO_4, 0.5 mM, $Ca(NO_3)_2$ and 5 mM MES-pH 5.6 for 30 minutes to remove the apoplastic As. The root and shoot tissues were weighed and ground to powder in liquid nitrogen and phosphate buffer solution (2 mM NaH_2PO_4, 0.2 mM Na_2- EDTA, pH- 6). The extracts were filtered twice using filter papers and 0.45 µm syringe filters. The samples of plant tissues were analysed for As speciation using HPLC-ICP-MS as described previously (Xu et al., 2007, Li et al., 2009).

2.2.10 Protoplast viability assays

Protoplasts from *Arabidopsis* loss of function mutants and wild type were isolated according to Maathuis et al., (1998). The protoplasts were incubated in different concentrations of As^{III} and As^{V} for 24 hours under continuous light. The viability of protoplasts was scored at 0 and 24 hours time points using Evan's blue staining dye to assess protoplast survival.

2.2.11 Statistical analyses

Where appropriate, data were analysed using statistical tests using SPSS (v.17) software. Significance levels were at $p < 5\%$ where indicated. The analysis of variance was carried out using one way or two ANOVA tests. Error bars in figures represent standard errors.

2.3 Results

2.3.1 Characterisation of loss of function mutants

The loss of function mutants in *Arabidopsis* NIPs (*nip5;1*, *nip6;1* and *nip7;1*) were characterised both at DNA and cDNA levels using the T-DNA primers (not shown) and cDNA primers (Table 2.1 and 2.2). The data for the cDNA analyses using RT-PCR is shown in the results only. The cDNA was prepared as mentioned in the methods. The quality and transcript levels of the cDNA was analysed using actin and gene specific primers for all the mutants (*nip5;1*, *nip6;1* and *nip7;1*) and wild type. The characterisation of the mutants are shown and explained in the figure legends.

2.3.1.1 Characterisation of *Atnip5;1*

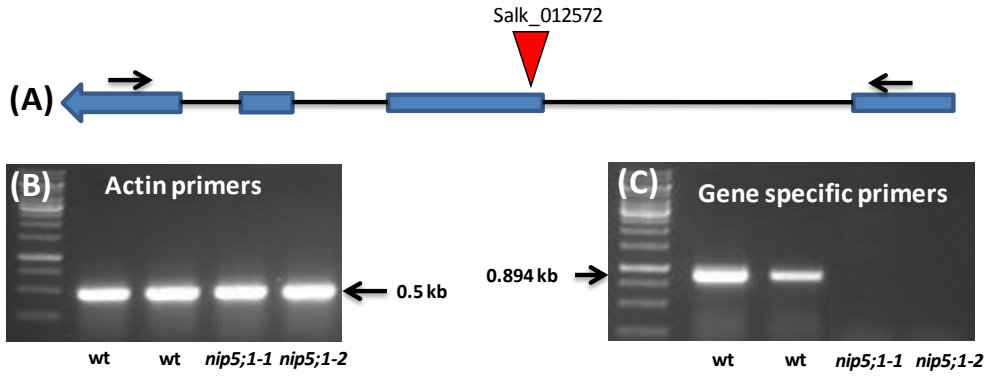


Figure 2.2 Characterisation of *Atnip5;1* using RT-PCR

Position of T-DNA and primers (A), expression analyses of wild type and *Atnip5;1* (two plants) cDNA using semiquantitative RT-PCR with actin (B) and gene specific primers (C). The cDNA quality was fine for the mutants and wild type as shown by the PCR with actin primers. The arrows show the band size for both actin (0.5 kb) and gene specific primers (0.894 kb). The gel shows no expression of the gene for *nip5;1-1* and *nip5;1-2* (two individual plants) compared to wild type.

2.3.1.2 Characterisation of *Atnip6;1*

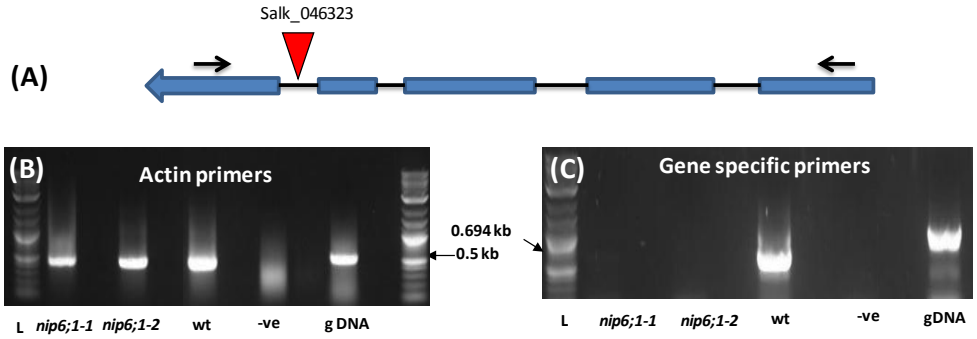


Figure 2.3 Characterisation of *Atnip6;1* using RT-PCR

Position of T-DNA and primers (A), expression analyses of *Atnip6;1* (two plants) and wild type cDNA with water (-ve) and genomic DNA (+ve) as controls using semiquantitative RT-PCR with actin (B) and gene specific primers (C). The cDNA quality was fine for the mutants and wild type as shown by the PCR with actin primers. The arrows show the band size for both actin (0.5 kb) and gene specific primers (0.694 kb). The gel shows no expression of the gene for *nip6;1-1* and *nip6;1-2* (two individual plants) compared to wild type and expression with wild type gDNA using the same set of primers.

2.3.1.3 Characterisation of *Atnip7;1*

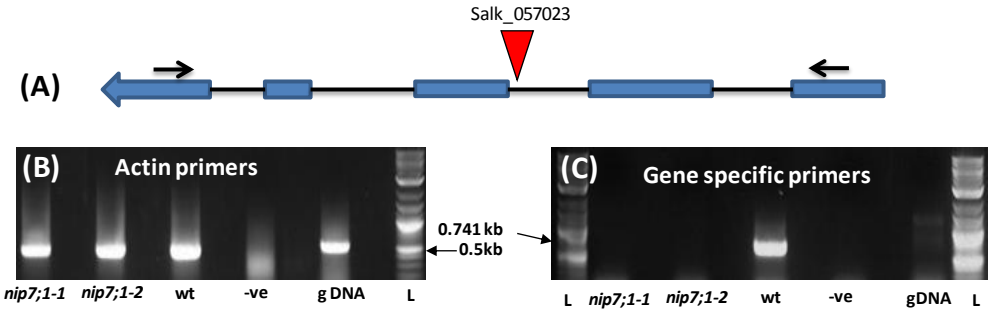


Figure 2.4 Characterisation of *Atnip7;1.* using RT-PCR

Position of T-DNA and primers (A), expression analyses of *Atnip7;1* (two plants) and wild type cDNA with water (-ve) and genomic DNA (+ve) as controls using semiquantitative RT-PCR with actin (B) and gene specific primers (C). The cDNA quality was fine for the mutants and wild type as shown by the PCR with actin primers. The arrows show the band size for both actin (0.5 kb) and gene specific primers (0.741 kb). The gel shows no expression of the gene for *nip7;1-1* and *nip7;1-2* (two individual plants) compared to wild type and expression with wild type gDNA using the same set of primers.

2.3.2 Growth analyses of mutants

For the growth analyses, sixteen seeds of each genotype were grown on plates containing As^{III}, As^{V} and control without As. No significant differences in average fresh weight were observed for the loss of function mutant (*nip5;1* and *nip6;1*) compared to wild type (figure 2.5). The loss of function in NIP7;1 has already been reported to have better growth than wild type on plates containing As^{III}, which suggested its involvement in As^{III} uptake (Isayenkov and Maathuis, 2008). However, I included this genotype for rest of the experiments.

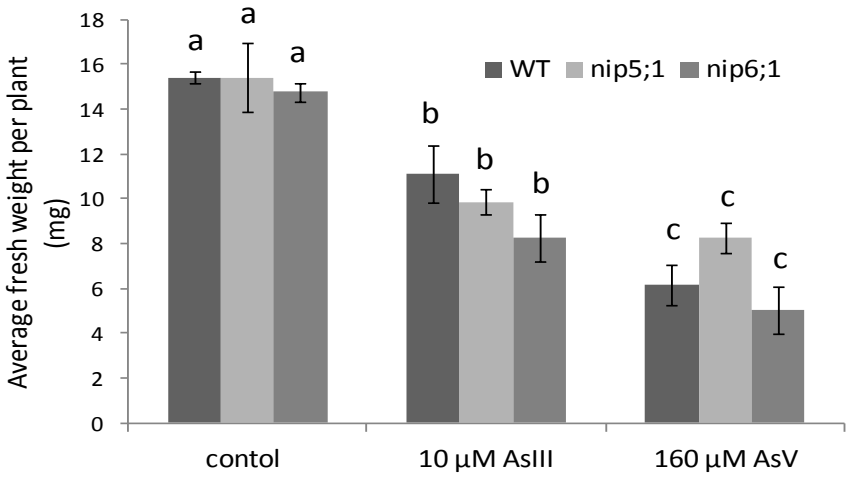


Figure 2.5 Growth analyses of *Arabidopsis* mutants in NIPs and wild type on plates exposed to 10 µM As^{III} and 160 µM As^{V}

Growth of *Arabidopsis* loss of function mutants in NIPs (*nip5;1* and *nip6;1*) and wild type on ½ MS medium with As^{III} and As^{V} and control having no As. The data are expressed as average fresh weight per plant (mg). *Atnip7;1* was not included for the growth analyses on plates. Data are from three independent assays, values are means ± SD, and different letters on the bars indicate significant differences between genotypes at $P < 0.05$ level.

2.3.3 As^{III} efflux analyses and shoot:root partitioning of total As

The loss of function mutants in *Arabidopsis* NIPs (*nip5;1*, *nip6;1* and *nip7;1*) were analysed for their efflux capacity by exposing the seedlings to 10 µM As^{V} for 24 hours. The nutrient medium collected after 24 hours and mixed with PBS (phosphate buffer solution) for analyses using HPLC-ICP-MS. The data were plotted as As^{III} efflux (as % of As^{V} uptake) relative to wild type (%). The data showed that the loss of function mutants *Atnip5;1* (80 %) and *Atnip6;1* (60 %) released significantly less As^{III} into the external medium compared to wild type (100 %). However the *Atnip7;1* mutant released significantly more (10 %) As^{III} into the external medium compared to wild type (figure 2.6). The results showed that *NIP5;1* and *NIP6;1* might have a role in As^{III} efflux,

whereas *NIP7;1* did not appear to play any role in As^{III} efflux but may be involved the sequestration and translocation of As^{III}. NIP5;1 and NIP6;1 seem to play a role in As^{III} efflux, the double knock out for these mutants may be developed and analysed for their role in As efflux.

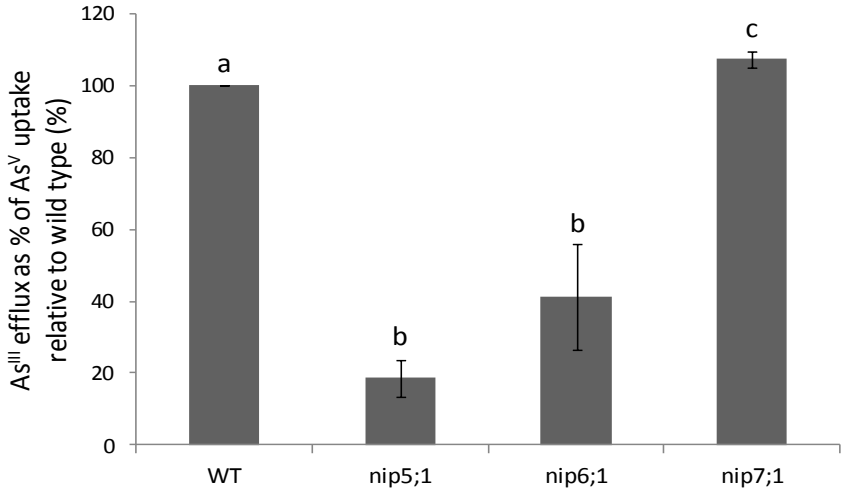


Figure 2.6 As^{III} efflux (as % of As^{V} uptake) analyses for *Arabidopsis* mutants in NIPs and wild type exposed to 10 µM As^{V} for 24 hours

As^{III} efflux (as % of As^{V} uptake) relative to wild type (%) for the loss of function mutants (*nip5;1*, *nip6;1* and *nip7;1*). Data are from three independent assays, values are means ± SD, and different letters on the bars indicate significant differences between genotypes at $P < 0.05$ level.

The seedlings used for the efflux experiment were also analysed for total As concentration in the roots and shoots tissue. The data were plotted relative to wild type (%). There were significant differences for total As concentration in shoots, where

Atnip6;1 had a significantly higher total As concentration in the shoots compared to wild type. *Atnip5;1* had a significantly lower total As concentration in shoots compared to wild type. No significant differences in total As concentration in shoots were observed for *Atnip7;1* compared to wild type (figure 2.7). In roots, the total As concentration for the mutants was not significantly different compared to wild type (figure 2.8).

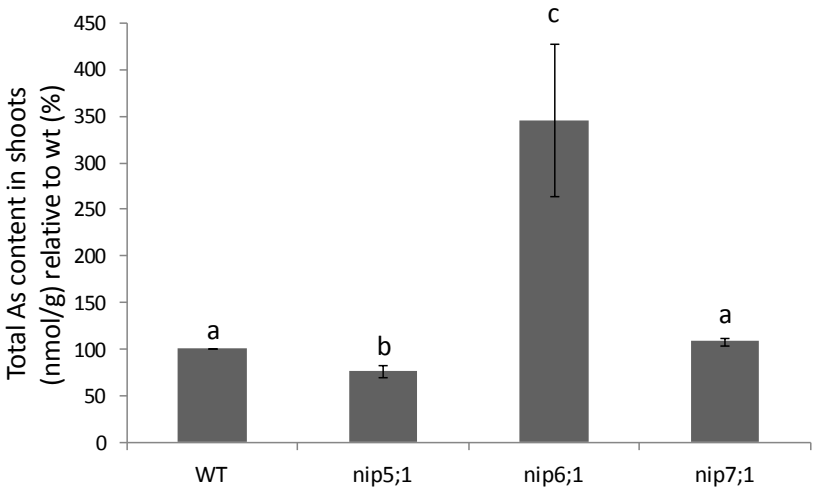


Figure 2.7 Total As concentration in shoots for *Arabidopsis* mutants in NIPs and wild type exposed to 10 µM As^V for 24 hours

Total As concentration in the shoots (nmol/g) for wild type and loss of function mutants. Data are from three independent assays, values are means ± SD, and different letters on the bars indicate significant differences between genotypes at $P < 0.05$ level.

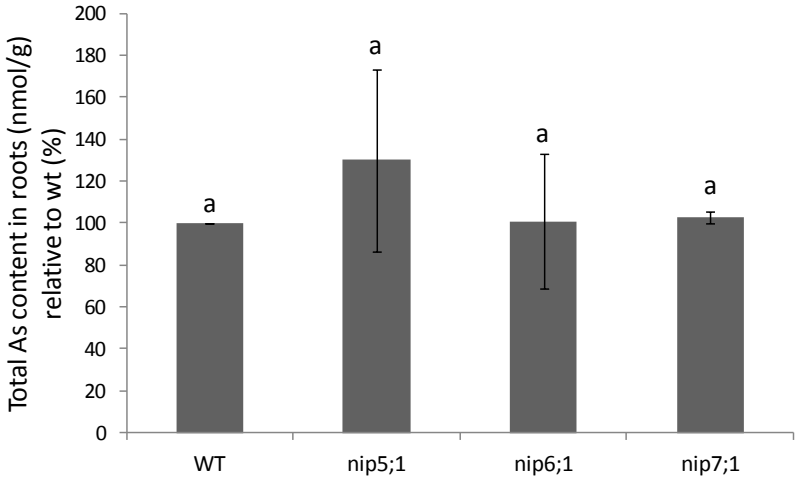


Figure 2.8 Total As concentration in roots for *Arabidopsis* mutants in NIPs and wild type exposed to 10 µM As^V for 24 hours

Total As concentration in roots for the wild type and loss of function mutants. Data are from three independent assays, values are means ± SD, and different letters on the bars indicate significant differences between genotypes at $P < 0.05$ level.

The shoot:root partitioning ratio showed that among the loss of function mutants only *Atnip6;1* had a significantly higher concentration of total As concentration in the shoots compared to wild type (figure 2.8).

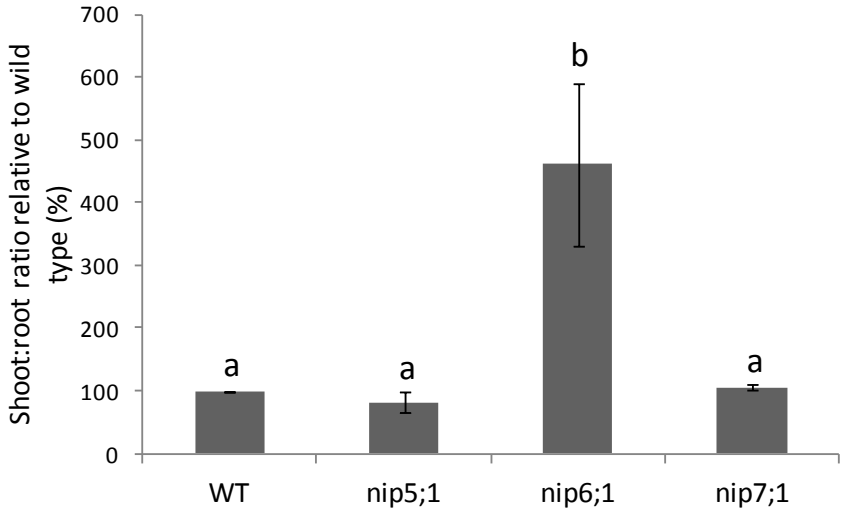


Figure 2.9 Shoot:root partitioning ratio for *Arabidopsis* mutants in NIPs and wild type exposed to 10 µM As^V for 24 hours

The shoot:root ratio of total As for the loss of function mutants and wild type. Data are from three independent assays, values are means ± SD, and different letters on the bars indicate significant differences between genotypes at $P < 0.05$ level.

2.3.4 Protoplast viability assays

Protoplasts are cells without a cell wall that largely carry out their normal physiological functions. As such, protoplasts have been proven to be a useful tool to compare cellular properties with those of intact plants. The effect of As on the protoplasts isolated from the mutant lines offers another system to see how individual cells with loss of function in a particular gene respond to As. Evans blue is a non toxic, water soluble pigment used to test cell viability (Kanai and Edwards, 1973, Gaff and Okong'O-Ogola, 1971, Kawai et al., 1998, Huang et al., 1986, Evans and Bravo, 1983). Living cells with a semi

permeable membrane do not allow the stain to pass through; however dead cells allow the dye to permeate to the cytoplasm through a damaged plasma membrane and the dye can therefore be used to test viability (Crippen and Perrier, 1974, Turner and Novacky, 1974, Kanai and Edwards, 1973, Tenhaken et al., 1995).

At 3.5 mM As^{III}, protoplasts from *Atnip5;1* were significantly more sensitive compared to protoplasts from the other mutants and wild type. Protoplasts from *Atnip7;1* showed more tolerance to As^{III} (3.5 mM and 7 mM) compared to the other mutants and wild type. However, all the mutants were significantly more compared to wild type only at 190 mM As^{V} (figure 2.9).

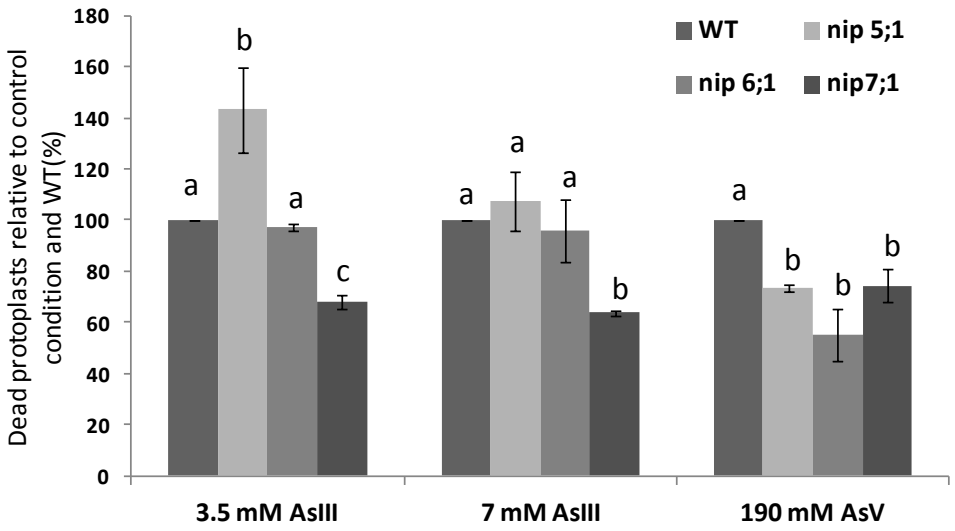


Figure 2.10 Percentage of non viable protoplasts for *Arabidopsis* mutants in NIPs and wild type exposed to As^{III} and As^{V}

Percentage of dead protoplasts derived from the loss of function mutants and wild type relative to the control condition and wild type by exposing to media containing 3.5 mM As^{III}, 7 mM As^{III} and 190 mM As^{V} for 24 hours. Data are from three independent assays, values are means ± SD, and different letters on the bars indicate significant differences between genotypes at $P < 0.05$ level.

2.4 Discussion

In recent times, aquaglyceroporins from bacteria, mammals and yeast were shown to be involved in the transport of metalloids like As^{III} and Sb^{III}. In an attempt to analyse if the members of the NIPs, a sub family of plant aquaporins can have the same function, NIPs from *Arabidopsis*, rice and *Lotus japonicus* were expressed in yeast for functional characterisation. Expression of AtNIP5;1, AtNIP6;1, OsNIP2;1, OsNIP3;2, LjNIP5;1 and LjNIP6;1 in yeast made it more sensitive to As^{III} and Sb^{III} suggesting a role of these NIPs in metalloid transport. It was concluded that the transport of metalloids is a conserved feature of NIPs (Bienert et al., 2008a). Based on these results, we analysed different loss of function mutants in *Arabidopsis* NIPs for their role in As uptake/efflux/translocation/sequestration. These mutants were analysed both at cellular and seedling levels.

2.4.1 Loss of function mutant *nip5;1*

NIP5;1 has been reported to be involved in the uptake of the essential nutrient boron (Takano et al., 2006). Based on the hypothesis that aquaporins are responsible for the uptake of metalloids (arsenic, silicon and boron), *nip5;1* was analysed for its role in As transport. No significant differences in growth on plates were observed for the mutant compared to wild type (figure 2.5). *Atnip5;1* seedlings were also analysed for their role in As^{III} efflux. The loss of function mutation in *nip5;1* resulted in significantly reduced As^{III} efflux compared to wild type when exposed to As^{V} (figure 2.6). This suggests that NIP5;1 may be involved in the efflux of As^{III} from the cytosol. The data for the total As concentration in root and shoot tissues revealed that shoots of *nip5;1* had significantly lower total As concentration compared to wild type, however there was no significant difference observed for the total As concentration in roots (figure 2.7 and 2.8). The shoot:root partitioning ratio showed no significant difference in the concentration of total As available in the shoots for *nip5;1* compared to wild type (figure 2.8). Lower efflux and retention of more As in the roots of the mutant suggest that NIP5;1 may have roles in As^{III} efflux and loading to the xylem. At the cellular level, the protoplasts from *nip5;1* were more sensitive to As^{III} compared to wild type at lower concentrations (3.5 mM As^{III}), which could mean that the As^{III} accumulation in the cytosol has a toxic effect on protoplasts (figure 2.10).

2.4.2 Loss of function mutation *nip6;1*

NIP6;1 has been reported to be involved in the uptake of boron (Tanaka et al., 2008) and is another potential candidate to be involved in the transport of other metalloids. No growth phenotype was observed for *Atnip6;1* on the plates compared to wild type (figure 2.5). When exposed to As^{V}, the mutant released significantly less As^{III} to the external medium, suggesting the NIP6;1 might have a role in the efflux of As^{III} (figure 2.6). The total As in the roots has no significant differences compared to wild type. However, a significantly higher concentration of total As was present in the shoots for *nip6;1* compared to wild type (figure 2.7 and 2.8). In addition, the shoot:root partitioning ratio also showed a significantly higher concentration of total As in the shoots of *nip6;1* compared to wild type (figure 2.8). The data suggest that *nip6;1* might have a role in the vacuolar sequestration of As. The loss of function resulted in decreased efflux and vacuolar deposition of As^{III} with no significant accumulation of As in the roots, which is mobilised towards the apoplast in the shoots. The data at the cellular level showed no significant differences in tolerance compared to wild type on both concentrations of As^{III} (3.5 mM and 7mM). However, the protoplasts showed significant tolerance compared to wild type on As^{V} (190 mM) (figure 2.9). In all, no phenotype was observed for *nip6;1* compared to wild type both at the cellular level (except on As^{V}) and regarding growth on plates. As suggested from the efflux data, it would be interesting to know the total As component in roots and shoots tissue of the seedlings grown on plates. The effect of mutation in NIP6;1 might not be showing a phenotype in terms of tolerance at the cellular or whole plant level but may affect As^{III} efflux to the external medium and retention of As in the roots.

2.4.3 Loss of function mutant *nip7;1*

NIPs are reported to be involved in the bidirectional transport of As^{III}. In *Arabidopsis*, NIP7;1 has been reported to be involved in As^{III} uptake (Isayenkov and Maathuis, 2008, Bienert et al., 2008b). Based on the assumption that NIP7;1 might be involved in the efflux of As^{III}, *Atnip7;1* was analysed for its role in As^{III} efflux. The data from the efflux analyses showed that *nip7;1* released significantly more As^{III} to the external medium compared to wild type (figure 2.6). Based on these results, NIP7;1 might have no or little role in As^{III} efflux. No significant differences in total As for roots and shoots were observed for *nip7;1* compared to wild type (figure 2.7 and 2.8). This suggests that NIP7;1 might have a role in the vacuolar sequestration or translocation, as the cytosolic

As^{III} is directed towards the apoplast, which resulted in higher As^{III} efflux. At the cellular level, *Atnip7;1* showed significantly more tolerance compared to wild type, both on As^{III} (3.5 M and 7 mM) and AsV (190 mM). The loss of function in NIP7;1 would mean low uptake of As^{III} and hence more tolerance as shown by the data from protoplasts, however there was more efflux of As^{III} which negates that assumption. The difference in results at the cellular level and intact seedling level suggests that the loss of function in NIP7;1 alters As release from the symplast differently at the cellular and tissue level. At the cellular level, there were no differences in the protoplast efflux of *nip7;1* compared to wild type (as mentioned in section 4.3.6). But the efflux was significantly higher at the plant level for *nip7;1* compared to wild type (figure 2.6). This suggests that the release of As^{III} is manipulated differently at the cellular and plant levels.

NIPs offer another group of potential candidates involved in As transport. The putative roles of NIP isoforms could help in understanding the mechanisms of plant As transport in more details. In summary, the results showed that NIP5;1 and NIP6;1 may have role in As^{III} efflux. Also, *Atnip6;1* contains a higher concentration of As in the shoots, suggesting that this NIP isoform may be involved in the vacuolar sequestration of As. In addition, *Atnip7;1* showed more efflux, suggesting a role in translocation or vacuolar sequestration. The As^{III} released into the external medium by *nip5;1* (80 %) and *nip6;1* (60 %) was lower compared to wild type (100 %), this suggests that these isoforms might have main role in As efflux due to a significant decrease in the efflux, which can be more useful for plants if exposed for a long time or the overexpression might be of more interesting in relation to As efflux. However, *nip7;1* showed significantly higher As efflux but the difference was only 10 % compared to wild type. This suggests that this specific isoform has a limited role in As partitioning to the shoots but does play roles in the uptake, sequestration or translocation of As.

At the cellular level, the mutants showed different levels of tolerance from more sensitive (*Atnip5;1*) to more tolerant (*Atnip7;1*). The overall results showed that the loss of function in any mutant resulted in an As related phenotype, suggesting that NIPs are contributing to various aspects of As transport in *Arabidopsis*. It will be interesting to analyse double or even triple mutants to see if there are additive or synergistic effects. Furthermore, the NIP isoforms involved in the As efflux and sequestration can be overexpressed to help lower accumulation of As in the seed. The efflux would result in

the release of cytosolic As^{III} to the external medium and the sequestration will increase the accumulation of As^{III} in the roots with lower amounts available for translocation to the shoots and grain.

NIPs are reported to be involved in the uptake of essential nutrients like boron and silicon (Takano et al., 2006, Tanaka et al., 2008). Knocking any of them out can therefore result in the deficiency of a specific plant nutrient. On the other hand if one of the NIP isoforms needed to be overexpressed, this might result in the excess of a particular nutrient which can also be toxic to the cells. The overexpression may also disturb trafficking of these channels, as different NIPs might work in co ordination. The differences in results at the cellular and plant levels might be due to the regulation of these NIPs differently at cellular and plant levels, rather the phenotype shown at the tissue level might be a collective effect. NIPs might be active in specific stress conditions and the activity of some isoforms can be up and down regulated accordingly. In addition, the cell specific gene expression and localisation are the other two factors responsible for the physiological function of NIPs. The results from the present study showed that NIPs are playing different roles in As transport in *Arabidopsis* when exposed for a short time to As^{V}, however the results might be different if the plants are exposed to different As species or for a long duration such as exposure in the paddy fields.

Chapter 3

3 Expression of the yeast arsenite transporter *ScACR3* in rice

3.1 Introduction

Rice is the second most consumed cereal in the world and the major source of calories for half of the world population (Fageria, 2007). Rice is of much concern for plant scientists in relation to As poisoning. It is mostly grown in south Asian countries where the As contamination of drinking water is above the WHO permissible limits of 10 µg/l (WHO, 2001). The contaminated water used for irrigation tends to mobilise As into the rice grain. Rice is the main source of dietary As for populations not exposed directly to contaminated water (Meharg et al., 2009).

Compared to other cereals, rice is more efficient in As uptake and translocation to the shoots (Su et al., 2010, Williams et al., 2007) due to the anaerobic reduced conditions in the paddies (Takahashi et al., 2004, Xu et al., 2008). The populations in south Asian countries exposed to As contaminated water have a significant (~50%) intake of As through consumption of rice (Mondal and Polya, 2008, Ohno et al., 2007). For example, the average rice intake in Bangladesh is as much as 450 g/day for a 60 kg adult (Duxbury and Zavala, 2005).

Market surveys showed that rice grain contains relatively high levels of As compared to other crops (Schoof et al., 1999, Williams et al., 2007). Thus, the consumption of As contaminated rice is potentially a serious health problem. It is therefore important to understand the As transport mechanisms/pathways in rice and other crops, which will be helpful to reduce the food chain contamination.

The inorganic forms of As are more toxic compared to the organic forms. There are reports that As found in the vegetative parts and the grain of rice is mostly inorganic with relatively low levels of dimethyl arsinic acid (DMA) and monomethyl arsonic acid (MMA) (Williams et al., 2007, Lombi et al., 2009). There are significant yield losses due to As phytotoxicity and its prevalence in the vegetative parts offer another route to the food chain if fed to animals (Azizur Rahman et al., 2008, Panaullah et al., 2009).

In plants, arsenate (As^{V}) is taken up by the plant roots through phosphate transporters (Wu et al., 2011, Ullrich-Eberius et al., 1989), while As^{III} enters the root via a subgroup of plant aquaporins, the NIPs (nodulin like intrinsic proteins) (Isayenkov and Maathuis, 2008, Kamiya et al., 2009, Ma et al., 2008b). In *Arabidopsis thaliana*, two of the NIP aquaporins AtNIP1;1 and AtNIP7;1 are involved in As^{III} uptake (Isayenkov and

Maathuis, 2008, Kamiya et al., 2009). In rice, the silicon transporter Lsi1 (OsNIP2;1) is involved in the uptake of As^{III} (Ma et al., 2008b). The rice Lsi1 has also been reported to be involved in the uptake of MMA^{V} and DMA^{V}, with the loss of function in *lsi1* resulting in 80% and 50% decrease in uptake of MMA^{V} and DMA^{V} respectively (Li et al., 2009).

The general mechanism of As detoxification is the reduction of As^{V} to As^{III} via the reductases identified in many plant species including *Arabidopsis (Bleeker et al., 2006), H. lanatus (Dhankher et al., 2006), P. vittata* (Ellis et al., 2006) and rice (Duan et al., 2007). The As^{III} produced due to reduction is complexed with PCs and sequestered to the vacuole via the recently identified vacuolar transporters AtABCC1 and AtABCC2 (Song et al., 2010) or released to the external medium via unknown transporters.

As yet no specific efflux pathway in plants has been identified for the removal of cellular As^{III}. However, in rice the Lsi2 (silicon/arsenite efflux carrier) has been shown to mediate efflux of As^{III} from roots to the xylem (Ma et al., 2008b). In addition, there are reports that NIPs may be capable of bidirectional transport of As^{III} in yeast, and this suggests that NIPs may also be carrying out As^{III} efflux in plants (Bienert et al., 2008a, Isayenkov and Maathuis, 2008). Recently, another group of the aquaporins has been reported to be able to transport As. The expression of rice plasma membrane intrinsic proteins OsPIP2;4, OsPIP2;6, and OsPIP2;7 in *Xenopus laevis* oocytes resulted in increased As^{III} uptake. Also, the overexpression of OsPIP2;4, OsPIP2;6, and OsPIP2;7 in *Arabidopsis* resulted in higher As^{III} tolerance (Mosa et al., 2012).

Arsenical resistance has been reported in many organisms ranging from prokaryotes to mammals. ACR3, an antiporter localised in the plasma membrane, extrudes cellular As^{III} from the yeast cytosol, whereas in bacteria a similar function is catalysed by the ArsAB or ArsB (Ghosh et al., 1999, Wysocki et al., 1997). The yeast ACR3 belongs to the BART (bile/arsenite/riboflavin transporter) super family (Mansour et al., 2007). ACR3 homologues have been identified mostly in prokaryotes and fungi but the first ACR3 gene was found in the bacterium *Bacillus subtilis* (Sato and Kobayashi, 1998, Takemaru et al., 1995). ACR3 homologues have been identified in mosses (*Physcomitrella patens*), ferns (*Ceratopteris richardii*), lycophytes (*Selaginella moellendorffii*) and gymnosperms (*Welwitschia milabilis* and *Picea sitchensis*). However ACR3 like genes were not found in angiosperms such as *Arabidopsis*, rice,

maize etc (Indriolo et al., 2011). It will be difficult to answer the question why ACR3 has lost from the angiosperms. This may be linked to the dependency of these plants on insects and animals for their pollination. On the other hand the As hyperaccumulation in *Pteris vittata* may be used in defense to avoid eating by animals and insects (Tripathi et al., 2012, Rathinasabapathi et al., 2007).

Recently, heterologous expression of ACR3 in rice has been reported, where the transgenics had 30% higher As^{III} efflux and a lower As concentration in the roots (30%), shoots (42%) and grain (Duan et al., 2011).

The topology of yeast ACR3 showed that it consists of 10 transmembrane domains with 8 integral transmembrane spans and 2 integral spans. The Acr3p with 48.5 k-Da protein size consists of 404 amino acids, about 64.1% of which are residues in the membrane. ACR3 contains putative sites for phosphorylation (4 for protein kinase C and 4 for casein kinase II) and 3 potential sites for N-glycosylation (figure 3.1) (Wysocki et al., 1997, Klein et al., 1985).

ACR3 has been reported to have specificity for As^{III} (Wysocki et al., 1997, Ghosh et al., 1999, Sato and Kobayashi, 1998, Fu et al., 2009), however in addition to As^{III} it can transport Sb^{III} (Maciaszczyk-Dziubinska et al., 2010).

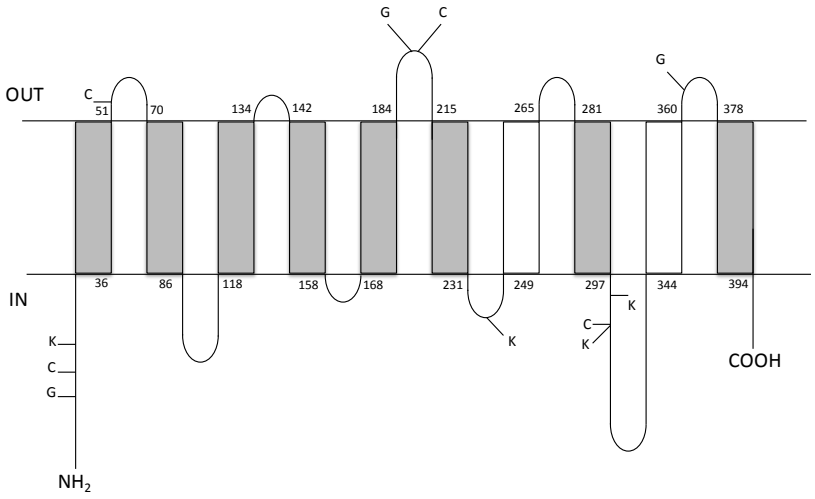


Figure 3.1 Predicted topology of *ScACR3*

Predicted topology of the yeast ACR3 antiporter, showing ten transmembrane domains, out of which 8 (1-6, 8 and 10) are transmembrane spans and 2 (7 and 9) are integral spans. Putative sites for N-glycosylation (G), phosphorylation by protein kinase C (C) and casein kinase II (K) are indicated (Wysocki et al., 1997)

Several strategies have been proposed to reduce the As accumulation in rice grain. However, modern molecular biological tools are useful in modifying the genetic makeup by incorporating desired genes like those involved in the As efflux, reduction and vacuolar sequestration. The aim here was to assess, if the heterologous expression of the yeast ACR3 antiporter can have a positive effect on plant growth and to study whether this would increase the efflux of As.

3.2 Materials and Methods

3.2.1 Plant material

The rice (*Oryza sativa*) sub group japonica cv Nipponbare was used in the present study.

3.2.2 Chemicals, consumables and oligonucleotides

For the chemicals and consumables see Chapter 2 (section 2.2.1). The sequences for the oligonucleotides are listed in the relevant sections.

3.2.3 Microorganisms

The *Agrobacterium* strain *AGl1* was used in the present study for transformation with ACR3. For the multiplication and purification of DNA, the *E. coli* strain DH5α was used as recipient. For the multiplication of microorganisms different culture media were used: *E. coli* cells were grown in LB (Luria-Bertani) medium (Peptone, Yeast extract, NaCl, pH 5.5) at 200 rpm in a 37^{o}C shaker incubator or on solid LB medium (as above with 0.8 % w/v bacto-agar) with antibiotics for selection. *Agrobacterium tumefaciens* cells were grown in YEP medium (yeast extract and bacto-peptone) with rifampicin (100 mg/µl) and kanamycin (50 mg/µl) for selection at 28^{o}C in an incubating shaker at 200 rpm. However, for the selection of recombinant agrobacteria, tetracycline (25 mg/µl) was also added to the growth medium.

3.2.4 Plasmid vectors

The binary vector pGreen (0179) and pSoup were used in the present study for rice transformation (figure 3.2). Both vectors were obtained from John Innese Research Centre, UK. For making the transformed binary vector construct containing ACR3, cDNA was synthesized from yeast RNA and the full *ACR3* coding sequence was amplified using primers ACR3-For gcctcgagATGTCAGAAGATCAAAAAAGT and ACR3-Rev gccccgggATTTCTATTGTTCCATATAT. ACR3 was cloned into the 35S promoter cassette of pART7 between *XhoI* and *SmaI* restriction sites. The fragment of pART7 containing the 35S-ACR3 cassette was subcloned into the T-DNA region of the binary vector pGreen0179 using NotI.

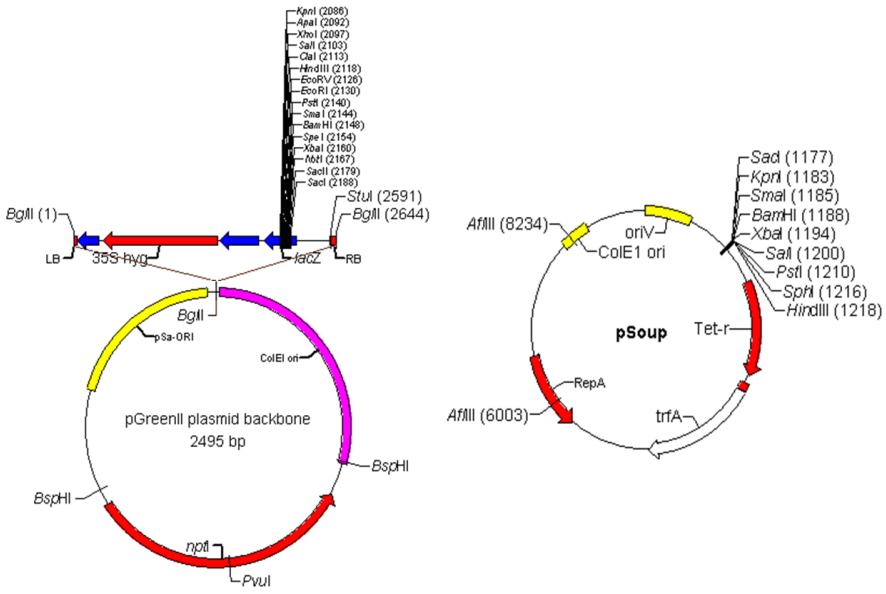


Figure 3.2 pGreen/pSoup based vectors used for cloning of ACR3 and rice transformation

Binary vectors used for cloning of ACR3 and rice transformation. pGreen and pSoup contain kanamycin and and tetracycline as selectable markers respectively.

3.2.5 *Agrobacterium* mediated transformation of rice with ACR3

3.2.5.1 Rice transformation

Mature seeds of japonica rice cultivar Nipponbare were used for transformation using *Agrobacterium* strain *AGL1* based on procedures described by Nishimura et al., (2007). The chemical composition of the media that were used in the present study is described in table 3.1.

Table 3.1 Media composition used for rice transformation

.Composition	N6D (mg/l)	2N6-AS (mg/l)	AAM (mg/l)	MS- NK (mg/l)	MS- HF (mg/l)
Macronutrient components					
KNO_3	2,830	2,830		1,900	1,900
NH_4NO_3				1650	1650
$(NH_4)_2SO_4$	463	463			
$MgSO_4.7H_20$	185	185	250	370	370
$CaCl_2.2H_20$	166	166	150	440	440
$NaH_2PO_4.2H_20$			150		
KH_2PO_4	400	400		170	170
KCl			3,000		
Micronutrient components					
Na_2EDTA	37.3	37.3		37.3	37.3
$FeSO_4.7H_20$	27.8	27.8		27.8	27.8
Fe-EDTA			40		
$MnSO_4.\ 4H_20$	4.4	4.4	10	22.3	22.3
$ZnSO_4.\ 7H_20$	1.5	1.5	2	8.6	8.6
$CuSO_4.5H_20$			0.025	0.025	0.025
$CoCl_2.7H_20$			0.025	0.025	0.025
KI	0.8	0.8	0.75	0.83	0.83
H_3BO_3	1.6	1.6	3	6.2	6.2
$Na_2MoO_4.2H_2O$			0.25	0.25	0.25
Organic components					
Casamino Acid	300	300	500	2,000	
Glycine	2	2	7.5	2	2
L-Arganine			176.7		
L-Proline	2,878				
L-Glutamine			900		
L-Aspartic Acid			300		
myo-Inositol	100	100	100	100	100
Nicotinic Acid	0.5	0.5	1	0.5	0.5
Pyridoxine HCl	0.5	0.5	1	0.5	0.5
Thiamine HCl	1	1	10	0.1	0.1
Phytohormones					
2,4-D	2	2			
NAA				0.02	
Kinetin				2	
Acetosyringone		10~20	10~20		
Carbon source					
Sucrose	30,000	30,000	68,500	30,000	30,000
Sorbitol				30,000	
Glucose		10,000	36,000		
PH	5.8	5.2	5.2	5.8	5.8

3.2.5.2 Plasmid construct for rice transformation

Rice calli were transformed using the binary vectors pGreen (pG0179) and pSoup (figure 3.2). pG0179 is a pGreen based vector containing the hygromycin resistance gene for the selection of transformants under the 35S promoter from cauliflower mosaic virus. For replication in *Agrobacterium* it needs a helper plasmid pSoup, which contains a tetracycline resistance gene for selection (figure 3.2).

3.2.5.3 Seed sterilisation

For sterilisation, the rice seeds were dehusked manually. The seeds were first sterilised using 70% ethanol for 1 minute, then using 2.5 % sodium hypochlorite with a drop of tween-20 for 15 minutes and followed by washing five times using sterilised distilled water.

3.2.5.4 Callus induction

The sterilised seeds were inoculated on N6D medium under continuous light (3.5 klux) for 3 weeks at 30^{o}C. Calli were formed after 3-4 weeks (figure 3.3a). The seeds contaminated with fungi during the callus formation were removed and the uncontaminated seeds were transferred to fresh N6D medium. The loose embryogenic units formed in the periphery of the calli formed from the seeds were removed by rolling and placing them on fresh N6D medium for an additional two weeks. This generated the embryogenic units used for transformation. The selection of the embryogenic units (actively growing calli with yellowish white colour, spherical, compact and around 2 mm in size) is reported to be the key for efficient transformation.

3.2.5.5 Co-cultivation of calli

Three days before co-cultivation, the *Agrobacterium* was grown for 3 days at 28^{o}C in AB medium with suitable antibiotics (tetracycline for pSoup, rifampicin for agrobacteria and kanamycin for pGreen) (figure 3.3b). The bacterial cells were collected and resuspended in 30 ml of AAM medium containing acetosyringone (1000X-AS) with an OD of around 1. The embryogenic units were flooded in the bacterial suspension for 5 minutes with moderate shaking. The bacterial culture was then removed and the calli blotted dry using sterile filter paper. The calli were placed on 2N6-AS (co-cultivation medium) for 3 days in the dark at 28^{o}C. *Agrobacterium* growth is often a problem, therefore the contaminated calli were washed in sterilised water containing antibiotic timentin for the removal of the excess agrobacteria.

3.2.5.6 Selection and regeneration of transformed calli

The calli were placed on N6D-S medium containing hygromycin for 3-4 weeks under continuous light (3.5 Klux) at 30^{o}C. The calli were regularly checked and washed if contaminated. The resistant calli were then placed on shooting medium (MS-NK) with specific antibiotic (hygromycin). One plant was regenerated from a single embryogenic unit, which represents one transgenic line. Six embryogenic units were place on a single plate for shoot regeneration. The plates were incubated again at 30^{o}C under continuous light (4.5 Klux) for 4 weeks. The regenerated shoots were then transferred to the MS-HF medium for root regeneration (figure 3.3c). Usually some roots can be seen even at the earlier stage. The produced seedlings were transferred to a growth chamber (figure 3.3d).

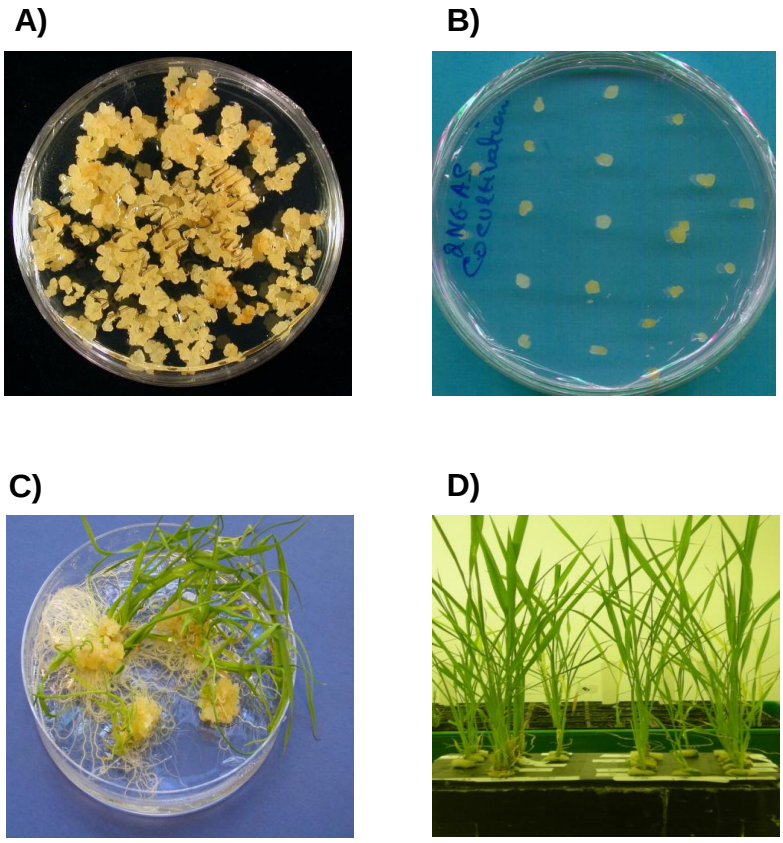


Figure 3.3 Different stages of rice transformation

Callus induction on N6D medium (A), co cultivation of calli with *Agrobacterium* (B), root regeneration on MS-HF medium (C) and seedlings grown in hydroponics for recovery.

3.2.6 PCR analyses of the transgenic lines

The transgenic lines obtained via selection were analysed for the presence of the transgene. DNA extracted from the leaf tissue as mentioned in Chapter 2 (section 2.2.2) of the transgenic lines was used as a template for the PCR. PCR mix was prepared in a 50 µl tube by mixing 5 µl of Flexi buffer, 2 µl of $MgCl_2$, 1 µl of dNTPs (10 mM of each deoxyribonucleotide triphosphates), Gotaq-polymerase (5U/ µl), 2 µl of DNA and 12.5 µl of water to make the final volume to 25 µl. PCR was performed in a Mastercycler machine (Eppendorf, UK) using the gene specific primers for ACR3 (For - ATGTCAGAAGATCAAAAAAGT and Rev- ATTTCTATTGTTCCATATAT with the following program: 5 min initial denaturation at 94^{o}C followed by 30 cycles (denaturation for 30 sec at 94^{o}C, annealing at 57^{o}C for 30 sec and extension for 1 min and 20 sec). The PCR products were run on an agarose gel (1.2%).

3.2.7 Identification of homozygous lines and qRT-PCR analyses

Hygromycin resistant primary transformants were selfed and homozygous lines identified in the T3 generation. Three homozygous lines ACR-R1, ACR-R2 and ACR-R3 were identified. The cDNA from the transgenic lines was prepared as mentioned in chapter 2 (2.2.3) and used as template for the qRT-PCR to analyse the transcript level of ACR3. The quantitative analyses were carried out in triplicate using SYBR Green master mix in an ABI 7300 sequence detection system. The amplicon of the rice actin gene was used as a control to normalise the data. The analyses showed that ACR-R2 had two times higher expression of the transgene compared to ACR-R1 and ACR-R3 (figure 2.4).

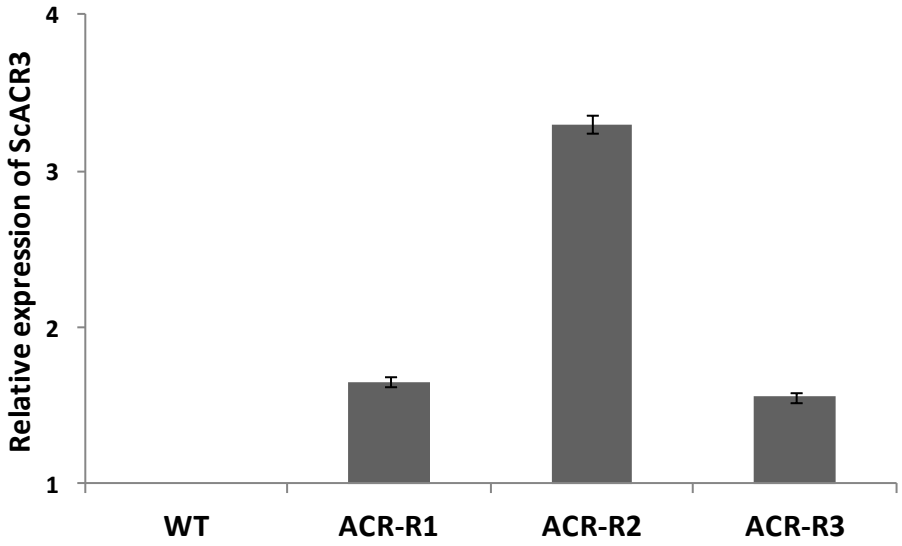


Figure 3.4 Relative expression of *ScACR3* in transgenic rice lines

qRT-PCR showing the relative transcript levels of ScACR3 in transgenic rice lines. PCR was carried out using the RNA isolated from 3 transgenic lines. The rice actin transcript level was used to normalise the transcript level of ACR3. The PCR analyses were performed in triplicate.

3.2.8 Growth conditions

Rice seeds were geminated on tera green, kept for five days in the dark at 28^{o}C and 90% relative humidity. The germinated seedlings were transferred to hydroponic medium in 2 litre boxes containing the growth medium. The growth medium consists of macronutrients (1.25 mM KNO_3, 0.5 mM $Ca(NO_3)_2.4H_2O$, 0.5 mM $MgSO_4.7H_2O$, 42.5 µM Fe-EDTA and 0.625 mM KH_2PO_4) and micronutrients (0.16 µM $CuSO_4.5H_2O$, 0.38 µM $ZnSO_4.5H_2O$, 1.8 µM $MnSO_4.H_2O$, 45 µM H_3BO_3, 0.015 µM $(NH_4)_2MO_7O_{24}.4H_2O$ and 0.01 µM $CoCl_2$) as described by Miyamoto et al., (2001). The hydroponic boxes were placed in the growth chamber (16h light/8h dark; photon flux density ~ 300 µmole per m^2 per second; 20-25^{o}C day/night; 60 % relative humidity). The growth medium was changed every three days. Three week old seedlings were used for different physiological experiments.

3.2.9 Growth assays

The transgenic lines (ACR-R1, ACR-R2 and ACR-R3) and wild type were analysed for their As tolerance in hydroponic (5 µM As^{III} or 50 µM As^{V}) boxes containing 2L of growth medium (composition as described above). Three week old seedlings were used for different physiological experiments. Five plants of each genotype were used for each treatment and experiments were replicated three times. RGRs (relative growth rates) were calculated according Poorter and Garnier (1996).

3.2.10 Total arsenic and arsenic speciation analyses

For the efflux analyses, the transgenic lines (ACR-R1, ACR-R2 and ACR-R3) and wild type were exposed to 20 µM As^{V} for 24 hours. The total As concentration and As species were analysed as mentioned in chapter 2 (section 2.2.9).

3.2.11 Subcellular localisation of ACR3

In order to determine the subcellular localisation of ACR3 in rice, *ACR3* was subcloned into pART7/EYFP. The ACR3 coding sequence was amplified using primers gcctcgagATGTCAGAAGATCAAAAAAGT and gccccgggATTTCTATTGTTCCATATAT carrying respectively *XhoI* and *SmaI* restriction sites (underlined). *ACR3::YFP* was transiently expressed in rice protoplasts according to Abel and Theologis (1994), and the ACR3 localisation was imaged by laser scanning confocal microscopy (Zeiss LSM5 Meta). In order to confirm the plasma membrane localisation of ACR3, protoplasts were cotransformed with pART7-ACR3-EYFP and the plasma membrane marker AtPIP2A-CFP (Cutler et al., 2000). Protoplasts expressing ACR3-YFP and/or the fluorescent marker were analysed using confocal microscopy.

3.2.12 Protoplast viability assays

The effect of As on protoplasts isolated from the transgenic lines offers another useful and quick system to see how individual cells containing the transgene respond to As. Protoplasts from the transgenic lines ACR-R1, ACR-R2, ACR-R3 and wild type were isolated from the etiolated rice seedlings according to Bart et al., (2006). The protoplasts were incubated in different concentrations of As^{III} (5 mM) and As^{V} (5 mM) under continuous light for 24 hours. The fluorescent nuclear staining dye 'Hoechst' was used for the identification of living and dead cells after 24 hours of incubation using

fluorescence microscopy. The dye permeates through the nuclear membrane of dead cells and binds to the double helix of the DNA. The dead cells will fluoresce blue under UV (380 nm) light. The number of dead cells was counted and expressed as a percentage of the total number of cells at the beginning of the experiment.

3.2.13 Xylem sap analyses of transgenic lines

Three week old seedlings of transgenic lines (ACR-R1, ACR-R2 and ACR-R3) and wild type were exposed to 50 µM As^{V} in hydroponics for three days before collecting the xylem sap. Shoots from the seedlings were removed by a sharp blade 20 mm above the root/shoot junction and transferred to a pressure chamber (Digital plant water potential apparatus, EL540-300). Xylem sap was collected for 30 minutes by applying an osmotic pressure greater than that of the external solution. The sap collected was mixed with PBS and analysed using HPLC-ICP-MS (Agilent LC1100 series and Agilent ICP-MS 7500ce, Agilent Technologies, Santa Clara, CA, USA).

3.2.14 Statistical analyses

Statistical analyses were done as described in chapter 2 (section 2.2.11).

3.3 Results

3.3.1 Growth of transgenic rice lines in hydroponics

Transgenic lines ACR-R1, ACR-R2, ACR-R3 and wild type (non transgenic control) seedlings were analysed in hydroponic medium containing As^{III} (5 µM), As^{V} (50 µM) and control without As. No significant differences in tolerance were observed for the transgenic lines ACR-R1, ACR-R2 and ACR-R3 compared to wild type on the medium containing As^{III} or As^{V}. However, a growth advantage for the transgenic line ACR-R2 was observed compared to ACR-R1, ACR-R3 and wild type both on media containing As^{III} or As^{V} (figure 3.5).

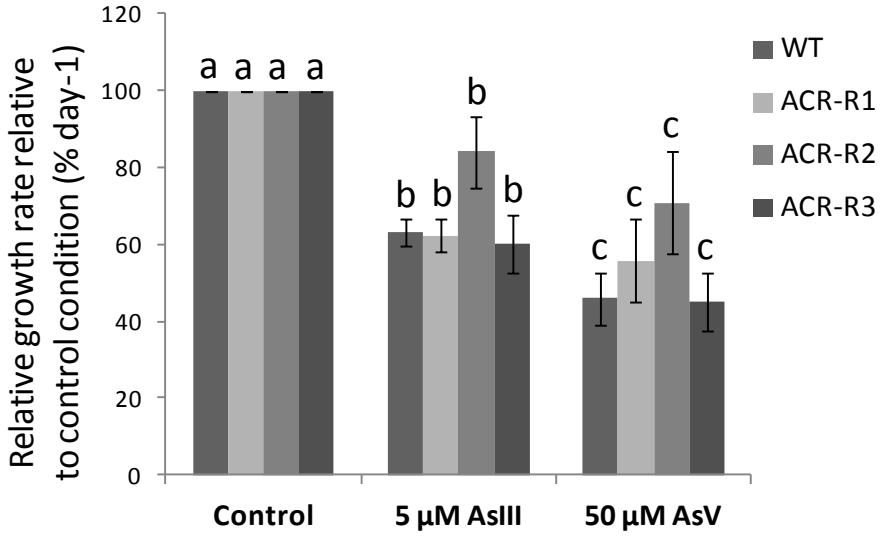


Figure 3.5 Relative growth rate for transgenic rice lines and wild type in hydroponics exposed to 5 µM As^{III} and 5 µM As^{V}

Relative growth rate relative to control condition (% day^{-1}) after 14 days for the transgenic lines ACR-R1, ACR-R2, ACR-R3 and wild type in hydroponics containing 5 µM of As^{III}, 50 µM of As^{V} and control having no As. Data are from three independent assays, values are means ± SD, and different letters on the bars indicate significant differences between genotypes at $P < 0.05$ level.

3.3.2 As^{III} efflux analyses and shoot:root partitioning of As for the transgenic rice lines

The transgenic lines ACR-R1, ACR-R2, ACR-R3 and wild type were exposed to 20 µM As^{V} for 24 hours in the hydroponic culture. To study how the transgene affects the capacity of plants to efflux cytosolic As^{III} to the external medium and the root-shoot partitioning of As, both the nutrient medium and the As concentration in the root and shoot tissue were analysed.

The data from the efflux analyses showed that the transgenic lines ACR-R1, ACR-R2 and ACR-R3 released a significantly higher fraction of As^{III} (as % of As^{V} uptake) to the external medium compared to wild type (figure 3.6). The data therefore suggest that ACR3 is involved in the efflux of As^{III} to the external medium.

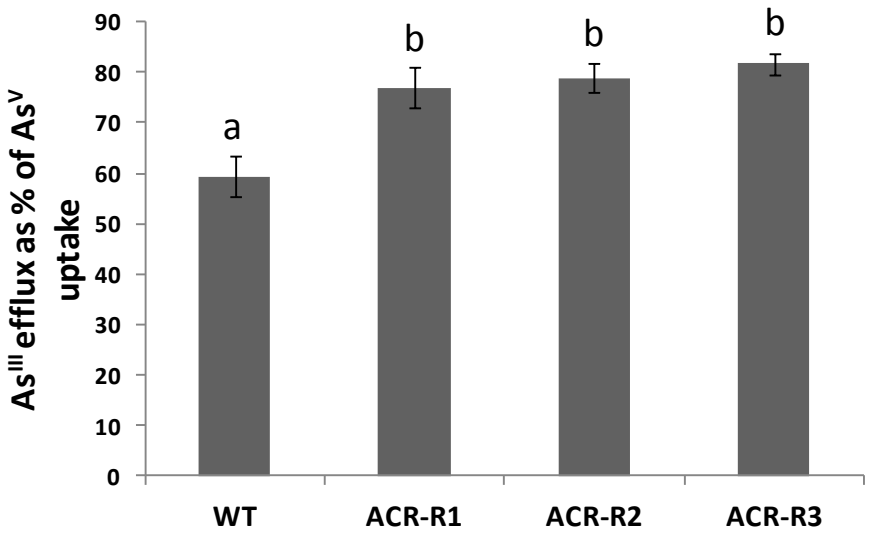


Figure 3.6 As^{III} (as % of As^{V} uptake) efflux analyses for transgenic rice lines and wild type exposed to 20 µM As^{V} for 24 hours

As^{III} efflux (as % of As^{V} uptake) for the transgenic lines ACR-R1, ACR-R2, ACR-R3 and wild type. Data are from three independent assays, values are means ± SD, and different letters on the bars indicate significant differences between genotypes at $P < 0.05$ level.

The total As concentration in shoots and roots tissue of the seedlings used for the efflux analyses was also analysed. The data show that the transgenic lines ACR-R1, ACR-R2 and ACR-R3 had significantly higher total As concentration in the shoots compared to wild type (figure 3.7a). The total As concentration in the roots was significantly lower for ACR-R2 and ACR-R3 compared to wild type, however no significant difference in total As concentration was observed for ACR-R1 compared to wild type (figure 3.7b).

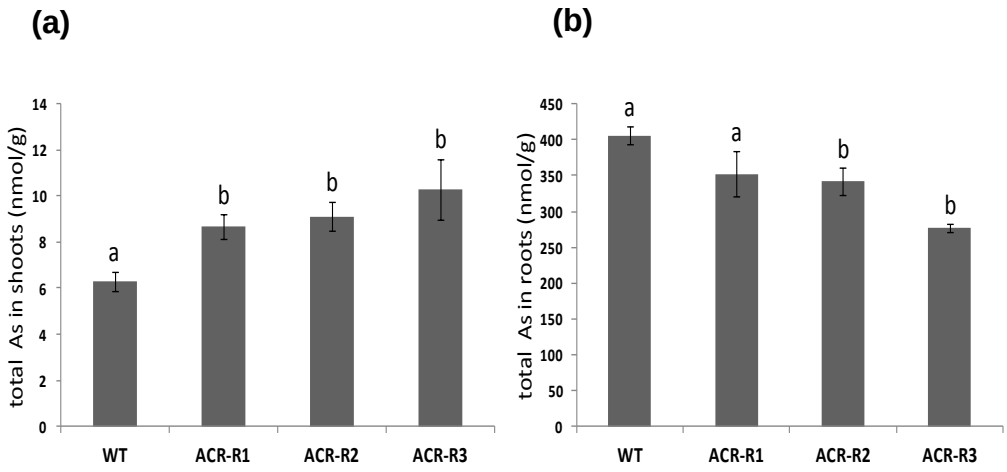


Figure 3.7 Total As concentration in shoots and roots for transgenic rice lines and wild type exposed to 20 µM As^V for 24 hours

Total As in the shoots (nmol/g) (7a) and roots (nmol/g) (7b) for the transgenic lines ACR-R1, ACR-R2, ACR-R3 and wild type. Data are from three independent assays, values are means ± SD, and different letters on the bars indicate significant differences between genotypes at $P < 0.05$ level.

The total As concentration in shoots and roots tissue was also analysed to assess the shoot:root partitioning. The data show that the transgenic lines had a significantly higher total As concentration in the shoots (figure 3.8). The data from the transgenic lines therefore suggest that, in addition to efflux, ACR3 also alters the translocation of As from root to shoot.

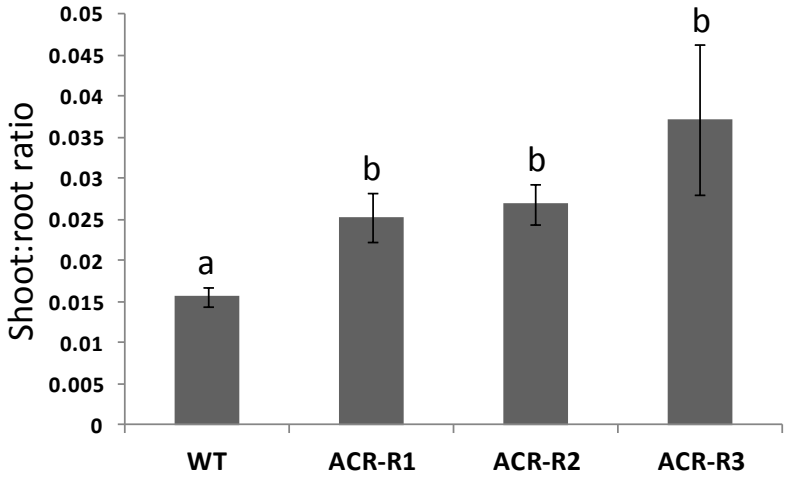


Figure 3.8 Shoot:root concentration ratio for transgenic rice lines and wild type exposed to 20 µM As^V for 24 hours

The shoot:root concentration ratio of total As for the transgenic lines ACR-R1, ACR-R2, ACR-R3 and wild type. Data are from three independent assays, values are means ± SD, and different letters on the bars indicate significant differences between genotypes at $P < 0.05$ level.

3.3.3 Xylem sap analyses of transgenic rice lines

The analyses of the xylem sap collected from the transgenic lines and wild type exposed to 20 µM As^V showed that the transgenic lines ACR-R1, ACR-R2 and ACR-R3 had a significantly higher fraction of As^{III} concentration in the xylem sap compared to wild type (figure 3.9). The data suggest that the expression of ACR3 resulted in higher translocation of As^{III} or efflux towards the xylem.

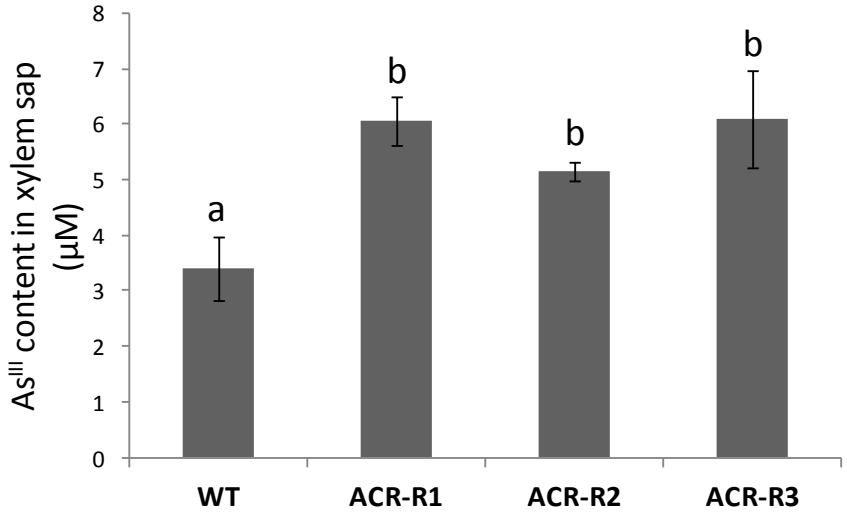


Figure 3.9 As^{III} concentration in xylem sap for transgenic rice lines and wild type exposed to 20 µM As^{V} for 24 hours

As^{III} concentration (µM) in the xylem sap of the transgenic lines ACR-R1, ACR-R2, ACR-R3 and wild type. Data are from three independent assays, values are means ± SD, and different letters on the bars indicate significant differences between genotypes at $P < 0.05$ level.

3.3.4 Total As concentration in seed and husk of the transgenic rice line

Wild type and transgenic line ACR-R3 were exposed to As^{III} for 4 weeks for growth analyses and were then grown to maturity on control medium. The seed and husk collected from the transgenic line and wild type were ground and digested using HNO_3. The total As concentration was measured using ICP-MS. The data showed that both the seed and husk from the transgenic line ACR-R3 had significantly lower total As concentration compared to wild type (figure 3.10).

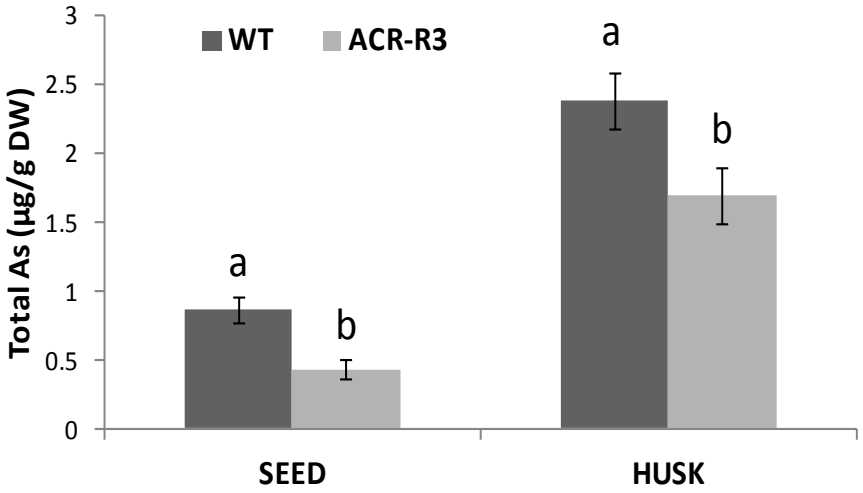


Figure 3.10 Total As concentration in seed and husk for the transgenic rice line and wild type exposed to 10 µM As^{III}

Total As concentration in seed and husk of wild type and the transgenic line ACR-R3. Data are from three independent assays, values are means ± SD, and different letters on the bars indicate significant differences between genotypes at $P < 0.05$ level.

3.3.5 Protoplast viability assays

No significant differences in tolerance were observed on the control medium among transgenic lines and wild type. However, protoplasts from the transgenic lines ACR-R1, ACR-R2 and ACR-R3 showed significantly increased tolerance compared to wild type both on the media containing As^{III} (5 mM) and As^{V} (5 mM) (figure 3.11).

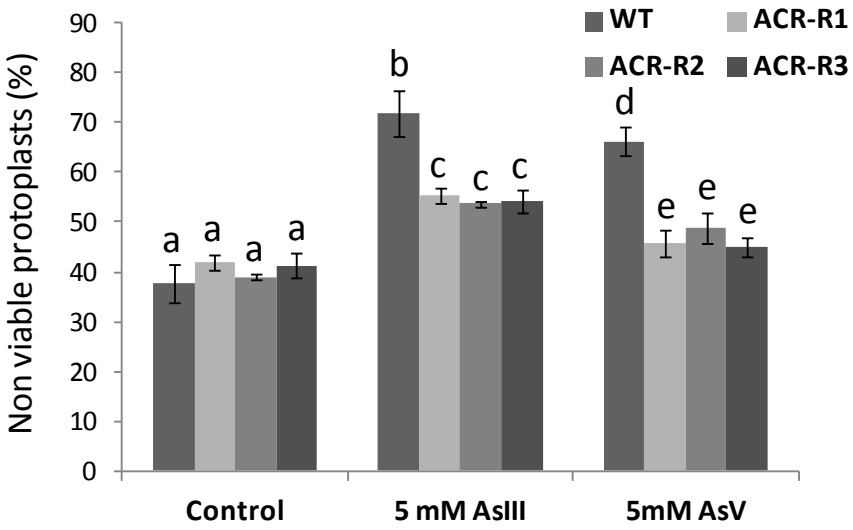


Figure 3.11 Percentage of non viable protoplasts for transgenic rice lines and wild type when exposed to As exposed to 5 mM As^{III} and 5 mM As^{V}

Percentage of non viable protoplasts derived from transgenic lines ACR-R1, ACR-R2, ACR-R3 and wild type exposed to media containing 5 mM As^{III} and 5 mM As^{V} for 24 hours. Data are from three independent assays, values are means ± SD, and different letters on the bars indicate significant differences between genotypes at $P < 0.05$ level.

3.3.6 Localisation of ACR3 in rice protoplasts

To study the putative cellular function and localisation of ACR3, protoplasts from rice were transformed with pART7-ACR3-EYFP. Figure 3.12A shows a YFP signal localised at the periphery of the protoplast suggesting that ACR3 is expressed in the plasma membrane. In order to confirm the plasma membrane localisation of ACR3, protoplasts were co-transformed with the plasma membrane marker, aquaporin AtPIP2A-CFP (Cutler et al., 2000) (figure 3.12B). The merge picture shows that the

signals from EYFP and CFP overlap each other, which suggests that ACR3 is localised in the plasma membrane (figure 3.12C).

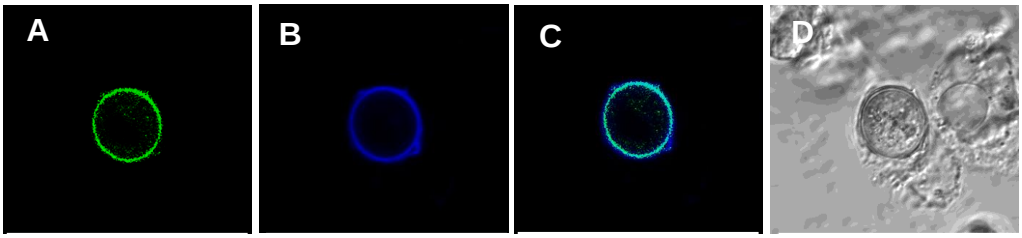


Figure 3.12.Localisation of *ScACR3* in rice protoplasts

Plasma membrane localisation of the ACR3 transporter in rice protoplasts. Fluorescence images (left) and bright field (right) of the protoplasts show ACR3-EYFP expression in the plasma membrane with AtPIP2A-CFP as plasma membrane marker. Bar = 10 µm.

3.4 Discussion

Understanding the mechanisms of As transport in plants would help in devising strategies to limit As in food crops, such as rice which is the major source of food chain contamination. Compared to other cereals like wheat and barley, rice is more efficient in As uptake which ends up in the grain (Su et al., 2010, Williams et al., 2007, Bogdan and Schenk, 2008).

Several strategies have been adapted to limit the entry of As into the rice grain, the most important of these will be to limit or stop the entry of As into the plant. However, due to the similarity in chemical structure between As species and essential nutrients, it will be difficult to completely stop the entry of As at the dispense of essential nutrients such as phosphate and silicate. However, there is a possibility to identify variant transporters which are selective/discriminative against As (Zhao et al., 2010b). The important step in limiting As entry to the plants will be to manipulate it at the root level. Increasing the rate of cellular sequestration, and efflux to the external medium also will help in decreasing the As load on the plants.

In plants, so far no dedicated system has been identified for As efflux. As^{III} efflux has been reported in many plant species (Xu et al., 2007, Su et al., 2010, Zhao et al., 2010a), however the molecular components of this pathway are unknown. To test whether heterologous systems are capable of increasing As extrusion, and as such improve plant tolerance to As toxicity, we cloned the yeast ACR3 As antiporter which mediates As^{III} efflux from the yeast cytosol (Ghosh et al., 1999). ACR3 was expressed in rice under the control of a strong constitutive promoter to test whether this affected plant growth in the presence of As.

Growth analyses of the transgenic lines in hydroponics showed no significant differences in relative growth rate compared to wild type, both in media containing As^{III} or As^{V} (figure 3.5). However, the trend shows that transgenic line ACR-R2 grows better compared to wild type and other transgenic lines in media containing As^{III} or As^{V}. The reason for the better growth might be the ACR3 expression level of ACR-R2, which is double for this line compared to other lines (figure 3.4).

The transgenic lines expressing ACR3 showed higher efflux compared to the control line. The data showed that 60 % of the As^{V} taken up by the wild type roots has been converted to As^{III} and was released to the external medium, however the efflux capacity of the transgenic lines ACR-R1, ACR-R2 and ACR-R3 was around 20 % higher, a significant increase compared to wild type (figure 3.6). This shows that the expression of ACR3 resulted in higher efflux in the transgenic lines. In comparison, a similar study reported that rice transgenic lines expressing ACR3 had 30% higher As^{III} efflux compared to wild type plants when exposed to 10 µM As^{V} (Duan et al., 2011).

The shoot and root tissues of the plants used for the efflux experiment were also analysed for total As content. The analyses showed that a significantly higher fraction of total As was found in the shoots of transgenic lines ACR-R1, ACR-R2 and ACR-R3 compared to wild type (figure 3.7a). The reverse pattern was observed for total As concentration in the root tissue, where the transgenic lines ACR-R2 and ACR-R3 had a significantly lower total As concentration compared to wild type. However, the transgenic line ACR-R1 had no significant difference in root total As concentration compared to wild type (figure 3.7b). Higher As concentration in the shoots and a reduction in the roots of the transgenic lines suggest that ACR3 may be involved in the translocation of As to the shoots. This assumption is also supported by the shoot:root concentration ratio, which is also significantly higher for the transgenic lines compared to wild type (figure 3.8).

Interestingly, it was reported in a previous study that the ACR3 expressing rice lines had 30 % lower As concentration in the shoots (Duan et al., 2011). The transgenic lines were exposed to low (5 µM) and high (40 µM) As^{III} for 24 hours. After 24 hours, the roots, shoots and xylem sap of these seedlings were analysed for total As concentration. Under low As^{III} treatment, the roots of the transgenic lines had significantly lower (around 28 %) As concentration. The shoots also had a lower As concentration but not significantly different. While under higher As^{III} treatment, the roots (23%) and shoots (42%) had significantly lower As concentration compared to wild type. The xylem saps from the transgenic lines were significantly lower (20 %) compared to wild type.

The growth showed by the rice transgenic lines had significantly higher As concentration compared to wild type, it did not show any toxicity symptoms within measured range of 0.015-0.025, although significant but the partitioning was within the range as reported earlier (Su et al., 2010). Though ACR3 might have role in the translocation of As^{III} but it did not show any significant effect on the phenotype in the previous and current studies when exposed to As^{V} for 24 hours, however the effect might be different if exposed for a long time duration.

The contradictory results might be due to the differences in ACR3 expression levels between lines of both studies. The relative expression of ACR3 reported by Duan et al., (2011) for the transgenic lines were around 20 times higher compared to the non transgenic control, while in our studies the relative expression were 3-4 times higher

compared to wild type. This might be the reason for a higher efflux and lower translocation towards the shoots and grain as reported by Duan et al., (2011). Higher expression of ACR3 might lead to higher As^{III} efflux from the cytosol to the external medium in parallel to the vacuolar sequestration. The differences in the results may be due to different experimental procedures and growth stages of the plants. A question arises here if the ACR3 localises to the plasma membrane as in our study, the efflux must be towards the xylem as well due to the constitutively expressed promoter (35S). Unfortunately, Duan et al., (2011) did not provide any data on the localisation of ACR3, which might be useful to explain these contradictory results.

One of the transgenic lines, ACR-R3 had been grown to maturity in control medium for seed setting. The seed and husk of the transgenic line had significantly lower total As concentration compared to wild type (figure 3.10). The possible explanation could be that the As^{III} available in the phloem might be directed in two ways, one towards the seed, and the recirculation towards the lower parts of the shoots due to the efflux activity provided by ACR3 (figure 3.13). It will be interesting to analyse the seed for the As species, this will give a clearer idea about the role of ACR3 in relation to seed As^{III} accumulation. These plants were treated with As^{III} for four weeks only and then grown on the control medium. Unfortunately, the total As concentration data for the root and shoot tissues of these plants are not available due to lack of time, mostly because the regeneration time of the transgenic rice proved to be extremely long. These information could have given us a clue about the translocation and distribution of As to the seed. The results might be different if the plants were treated with As till maturity/seed setting.

In rice, both roots and shoots are the sites for the As^{V} reduction. However, roots are the primary site where the As^{III} load in the cytosol is directed to the xylem as observed in many plant species (Duan et al., 2007, Zhao et al., 2009). In order to confirm the notion that ACR3 is involved in the efflux of As^{III} towards the xylem, As^{III} concentration in the xylem sap of the transgenic lines and wild type was analysed. The data showed that the xylem sap in the transgenic lines ACR-R1, ACR-R2 and ACR-R3 contains 20-40 % more As^{III}, a significantly higher fraction compared to wild type (figure 3.9). These results are in contrast with a recent study on ACR3 expression in rice, where the transgenic lines had 30 % lower As^{III} concentration in the xylem sap compared to wild

type (Duan et al., 2011). This might be due to higher efflux of As^{III} leaving smaller amounts for translocation towards the xylem.

Similarly at the cellular level, protoplasts from transgenic lines ACR-R1, ACR-R2 and ACR-R3 showed significantly increased tolerance compared to wild type both in media containing As^{III} and As^{V}. Tolerance shown by the protoplasts might be provided by the exclusion of cytosolic As^{III} through the ACR3 efflux system, which localises to the plasma membrane as in yeast (figure 3.11). At the cellular level the tolerance was much more pronounced than at the whole plant level. This is possibly due to efflux being solely directed to the external medium without interactions with other cells/tissue. The whole plant phenotype is difficult to explain due to complex interactions between different cells or tissue. To minimise these interactions, it will be interesting to analyse the efflux capacity using the transgenic protoplasts. Unfortunately, this was not carried out due to limited numbers of transgenic seed.

In summary, the transgenic plants expressing ACR3 showed tolerance at the cellular level but no growth phenotype was observed using intact plants. ACR3 localises to the plasma membrane as in yeast (figure 3.12) and appears to catalyse the same function of As^{III} efflux in rice as in yeast (Ghosh et al., 1999). The viability shown at the cellular level might be difficult to explain at the whole plant level, as there are higher level of interactions between different cells/tissues at the whole plant level or the local differences in ACR3 functioning might be preventing the overall net positive effect. Efflux of As could result in the accumulation of As in the apoplast, which might affect the overall net effect of ACR3 at the plant level. However, the short term As^{III} efflux analyses with seedlings showed that the transgenic lines had significantly higher efflux of As^{III} and translocation to the shoots. The availability of a significantly higher fraction of As^{III} in the xylem also provides proof of the efflux activity provided by ACR3 in the transgenic lines. The total As concentration data from one of the transgenic lines showed that the As^{III} may be redirected towards the shoot via phloem resulting in lower As in the husk and seed (figure 3.13). It is desirable to have higher efflux of the cytosolic As^{III} as we observed in this study but the increased translocation to the shoots is not desirable, since shoot As may ultimately end up in the grain. To increase the As efflux without increased translocation, an alternative approach could be the use of tissue specific promoters for example for roots.

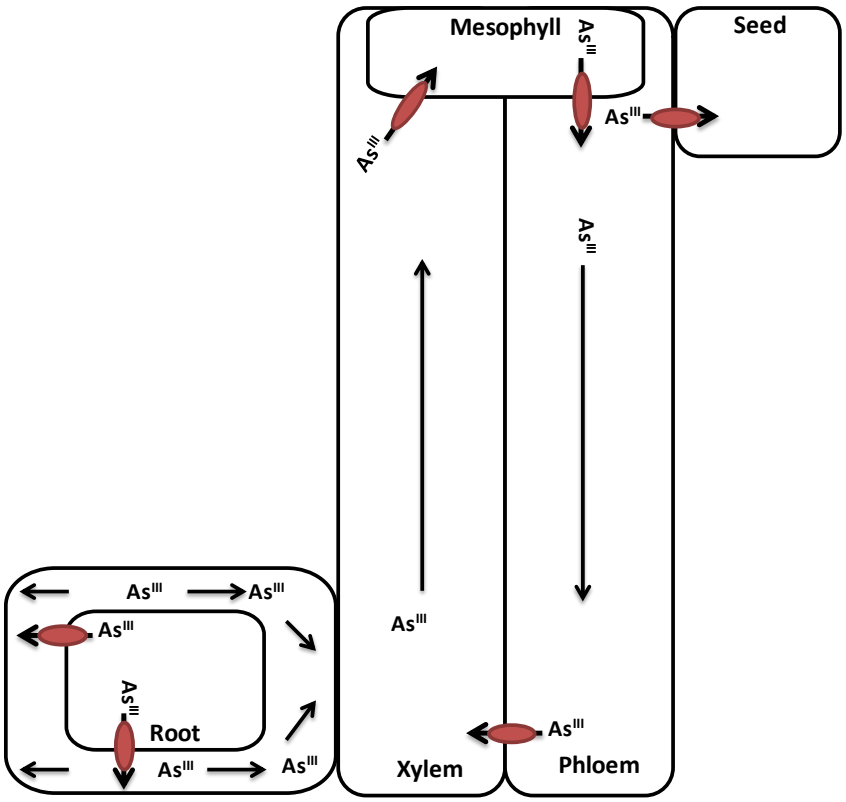


Figure 3.13 Putative functions of *ScACR3* in rice

ACR3 is localised to the plasma membrane and involved in As^{III} efflux to the apoplast, which is directed either to the external medium or towards the xylem for translocation to the shoots. As^{III} is mobilized to the seed and lower parts of the plant via phloem with the food prepared in the mesophyll tissue.

Chapter 4

4 Expression of the yeast arsenite transporter *ScACR3* in *Arabidopsis thaliana*

4.1 Introduction

Arsenic (As), a metalloid occurring ubiquitously in nature, is a potent carcinogen. Arsenic poisoning has arisen as a global health concern to many populations around the globe particularly to those who are either drinking As contaminated water or consuming the food contaminated with As (Tripathi et al., 2007a). The problem is alarming in the South Asian countries with rice as a major food component in their diet, particularly if it is grown in contaminated soils or water (Norton et al., 2012).

Significant progress has been made in the last two decades understanding the mechanisms of arsenic transport in plants. The transporters involved in the uptake of inorganic As has been identified in plants. Among plants, the aquaporins from bothe Arabidopsis and rice are mainly responsible for the uptake of metalloids including As.

Prokaryotes (bacteria) and some eukaryotes (yeast) have a well established system for As detoxification. General mechanisms for As detoxification are common; arsenate (As^{V}) is taken up by phosphate transporters and is reduced to As^{III}, which is either complexed with phytochelatins (PCs) and sequestered to the vacuole or removed from the cytosol by Acr3p in yeast while in bacteria As^{III} is either volatilised to the environment through sequential methylation or efflux through ArsAB (Dey et al., 1994, Kuroda et al., 1997, Wysocki et al., 1997, Tripathi et al., 2007b) . ACR3 is a plasma membrane transporter involved in As^{III} efflux from the yeast cytosol, which works as an As^{III}/H^{+} antiporter (Maciaszczyk-Dziubinska et al., 2010, Wysocki et al., 1997). The overexpression of ACR3 in yeast resulted in higher tolerance to As^{III}, while a null mutation in ACR3 resulted in the loss of function and sensitivity to both As^{V} and As^{III} (Wysocki et al., 1997). In plants, yeast ACR3 homologues have been identified in the hyperaccumulator fern *Pteris vittata*, where they are involved in the vacuolar sequestration of complexed As^{III} (Indriolo et al., 2011). Recently, the expression of ACR3 in rice resulted higher efflux of As^{III} and less movement towards the shoots and grain (Duan et al., 2011).

So far no efflux pathway for As^{III} efflux has been identified in *Arabidopsis*. However in rice, the Lsi2 (silicon/arsenite efflux carrier) has been shown to mediate efflux of As from roots to the xylem (Ma et al., 2008a). In addition, there are reports that NIPs may be capable of bidirectional transport of As^{III} in yeast, and this suggests that NIPs may

also be carrying out As^{III} efflux in plants (Bienert et al., 2008a, Isayenkov and Maathuis, 2008).

A main focus of research into plant arsenic resistance is to reduce the mobilisation of As into the food chain. This could be possible by modifying plants to have reduced uptake of As, higher efflux of cytosolic As^{III} to the vacuole or the external medium and lower translocation to the seed. Engineering plants with desirable genes offers another alternative to the conventional breeding methods and to improve plant tolerance to different biotic and abiotic stresses. Among plants, *Arabidopsis thaliana* has been used for the expression of non plant genes, due to its short life cycle and ease of transformation. In this chapter, we will discuss how the heterologous expression of the yeast efflux transporter ACR3 affects growth and As tolerance of *Arabidopsis thaliana* wild type and *Atinp7;1*. AtNIP7;1 has been reported to be involved in the uptake of As^{III} and the loss function in *Atnip7;1* resulted in increased growth compared to wild type. The idea was to analyse, if the expression of ACR3 in this back ground can have an additive/synergistic effect in relation to As tolerance (Isayenkov and Maathuis, 2008). The study will also add to the information how the expression of ACR3 can interact with other genes in the loss of function mutant back ground compared to its effect on the wild type.

4.2 Materials and Methods

4.2.1 Plant material and growth conditions

Arabidopsis thaliana wild type (Col-0) and *nip7;1* seeds were grown on F2+S (Levington, UK) soil, placing the trays at 4^oC for stratification for 24-48 hours. The trays were then transferred to the growth room (16h light/8h dark; photon flux density ~ 65 µmole per m^2 per second, 20-25^oC day/night) for 10 days. For culturing plants in hydroponics, two week old seedlings were transferred to culture boxes containing 1 litre of nutrient medium as mentioned in chapter 3 (section 3.2.8).For growth analyses of plate-grown plants, the seeds were sterilised and grown on the plates containing ½ MS medium with As^{III} and As^V as mentioned in Chapter 2 (section 2.2.7).

4.2.2 Cloning of ACR3 and *Arabidopsis* transformation

The information about the cloning of ACR3 into the binary vector has been mentioned in chapter 3 (section 3.2.4). *Agrobacterium* strain *GV3101* was cotransformed with the

binary vector pGreen0179:35S:ACR3 and pSoup by electroporation. *Agrobacterium* cells were grown in LB for 24 h at 28°C to the stationary phase. The cells were collected and resuspended in transformation medium (5% sucrose, 0.02% Triton X-100) to an OD_{600} of 0.8. Plants with primary inflorescence stems of about 3-5 cm in length were selected for transformation and were dipped into the bacterial suspension (Clough and Bent, 1998) for 2 minutes and transferred to the greenhouse. Seeds from plants transformed with *Agrobacterium* were harvested and screened on selection plates containing hygromycin (20 mg/µl).

4.2.3 Identification of homozygous lines and qRT-PCR analyses

Hygromycin resistant primary transformants were selfed and homozygous lines identified in the T3 generation. Two homozygous lines each in the wild type background (ACR-W1 and ACR-W2) and *nip7;1* background (ACR-N1 and ACR-N2) were identified. The transcript level of ACR3 was analysed using qRT-PCR. The quantitative analyses were carried out in triplicate using SYBR Green master mix in an ABI 7300 sequence detection system. cDNA from wild type and transgenic lines was prepared as mentioned in Chapter 2 (section 2.2.3) and used as a template for qRT-PCR. The amplicon of the *Arabidopsis* actin gene was used as a control to normalise the data. The analyses showed that ACR-W1 had 6 times higher expression of the transgene compared to ACR-W2 and three times compared to ACR-N1 and ACR-N2.

4.2.4 Growth assays

The transgenic lines and wild type were analysed for their As tolerance both on ½ MS plates (10 µM As^{III} and 160 µM As^{V}) and in hydroponics (10 µM and 15 µM As^{III} or 160 µM and 200 µM As^{V}). Briefly, seeds were surface sterilised and sown on ½ MS medium with or without As^{III} or As^{V} (12 seeds of each genotype) for four weeks. Plate growth experiments were replicated six to seven times and growth was measured as average fresh weight/plant relative to the control condition (%). For the hydroponic culture, two week old seedlings from soil were transferred to hydroponic medium (composition as described above) with or without As^{III} and As^{V} for an additional three weeks. Five plants of each genotype were used for each treatment and experiments were replicated three times. RGR (relative growth rate) was calculated according to Poorter and Garnier (1996).

4.2.5 Total arsenic and arsenic speciation analyses

For the efflux analyses, the transgenic lines (ACR-W1, ACR-W2, ACR-N1 and ACR-N2) and the corresponding control lines (WT and *nip7;1*) were exposed to 5 µM As^V for 24 hours. The total As concentration and As species were analysed as mentioned in Chapter 2 (section 2.2.9).

4.2.6 Subcellular localisation of ACR3

In order to determine the subcellular localisation of ACR3 in planta, we subcloned *ACR3* into pART7/EYFP. The ACR3 coding sequence was amplified using primers gcctcgagATGTCAGAAGATCAAAAAAGT and gccccgggATTTCTATTGTTCCATATAT carrying respectively *XhoI* and *SmaI* restriction sites (underlined). *ACR3::YFP* was transiently expressed in *Arabidopsis* protoplasts according to Abel and Theologis (1994), and the ACR3 localisation was imaged by laser scanning confocal microscopy (Zeiss LSM5 Meta). In order to confirm the plasma membrane localisation of ACR3, protoplasts were cotransformed with pART7-ACR3-EYFP and the plasma membrane marker AtPIP2A-CFP (Cutler et al., 2000). Protoplasts expressing ACR3-YFP and/or the fluorescent marker were analysed using confocal microscopy.

4.2.7 Protoplast viability assays

Protoplasts from transgenic and wildtype *Arabidopsis* lines were isolated according to Maathuis et al.,(1998). The protoplasts from transgenic lines and wild type were exposed to As^{III} (3.5 mM and 7 mM) and As^V (190 mM) for 24 hours under continuous light and scored for viability at 0 and 24 hours by using the Evan's blue staining assay to assess cell survival. The % of dead protoplasts relative to control condition was determined using a microscope.

4.2.8 Protoplast As efflux assays

Protoplasts from transgenic lines (ACR-W1, ACR-W2, ACR-N1 and ACR-N2) and the corresponding control lines (WT and *nip7;1*) were isolated according to Maathuis et al., (2008). Protoplasts (0.6 million per genotype) were exposed to protoplast incubation medium containing 2 mM As^V for 2 hours under continuous light. The protoplasts were then collected by centrifugation and washed three times with protoplast incubation medium (0.6 M Mannitol, 4 mM MES, 4 mM KCl and 3 mM $CaCl_2$) to remove the As^V.

The protoplasts were then exposed to protoplast incubation medium without As^{V} for 6 hours. After exposure, 1 ml of medium was collected at time points, 0, 3 and 6 hours followed by the addition of PBS and filtration. The protopasts were also collected and disrupted using sonication and filtered for As sepeciation analyses. The As^{III} efflux (%) for both time points (3 and 6 hours) was measured relative to the total As^{III} available in the protoplasts.

4.2.9 Statistical analyses

Statistical analyses were done as described in chapter 2 (section 2.2.11).

4.3 Results

4.3.1 Growth of *Arabidopsis* seedlings on plates

Transgenic lines (ACR-W1, ACR-W2, ACR-N1 and ACR-N2) with the corresponding control lines (WT and *nip7;1*) were analysed by growing seedlings on ½ MS medium with or without As^{III} (8 µM) and As^{V} (160 µM). On the medium containing As^{III}, the transgenic line ACR-W1 showed significant tolerance ($p<0.05$) compared to wild type and the other transgenic line ACR-W2. Similarly, ACR-W1 showed significant tolerance on media containing As^{V} (figure 4.1). The ACR3 transgenic lines in the *nip7;1* background also showed significant tolerance compared to both the background lines on media containing As^{III} (8 µM) and As^{V} (160 µM) (figure 4.1).

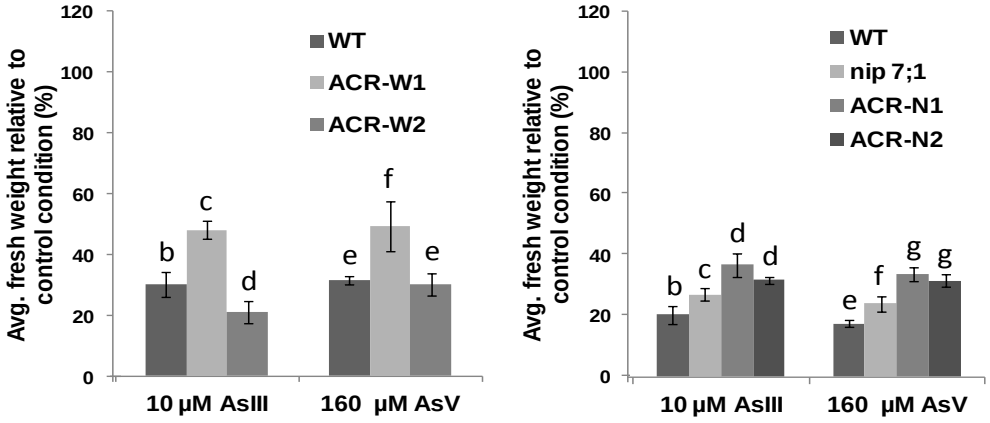


Figure 4.1 Growth analyses of *Arabidopsis* transgenic and control lines on plates 10 µM As^{III} and 160 µM As^{V}

Growth of the transgenic lines (ACR-W1, ACR-W2, ACR-N1 and ACR-N2) with the corresponding control lines (WT and *nip7;1*) on ½ MS medium with As^{III} and As^{V}. The data are expressed as average fresh weight per plant relative to the control condition (%). Data are from six independent assays, values are means ± SD, and different letters on the bars indicate significant differences between genotypes at $P < 0.05$ level.

4.3.2 Growth of *Arabidopsis* mature plants in hydroponics

The transgenic lines (ACR-W1, ACR-W2, ACR-N1 and ACR-N2) with the corresponding control lines (WT and *nip7;1*) were also analysed in hydroponics. Three week old plants were transferred to control medium with or without As^{III} (10 µM and 15 µM) and As^{V} (160 µM and 200 µM). Phosphate and As^{V} share the same uptake pathway. To have the same uptake rate, the medium was supplemented with equal amounts of As^{V} and phosphate (KH_2PO_4). Supplementing medium only with As^{V} has resulted in toxicity to the seedlings, which might be due to low phosphate uptake needed for energy requirements. To avoid the toxic effects on seedlings, medium was supplemented with equal amounts of As^{V} and phosphate.

Relative growth rates were calculated after a 14 day period in the different treatments. The transgenic line ACR-W1 showed significantly ($p<0.05$) better growth both on media containing As^{III} (15 µM) and As^{V} (160 µM and 200 µM) compared to ACR-W2 and wild type (figure 4.2). The transgenic lines in *nip7;1* background ACR-N1 and ACR-N2 showed significantly ($p<0.05$) higher tolerance compared to both *nip7;1* and

wild type (figure 4.3), on the media containing As^{III} (15 µM) and As^{V} (160 µM and 200 µM) (figure 4.3).

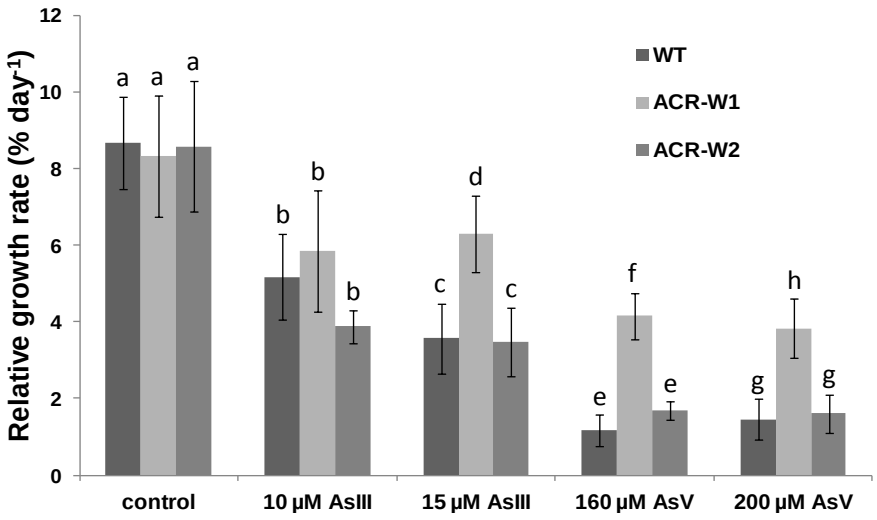


Figure 4.2 Relative growth rate for *Arabidopsis* transgenic and control lines in hydroponics exposed to As^{III} (10 µM and 15 µM) and As^{V} (160 µM and 200 µM)

Relative growth rate (% day^{-1}) after 14 days for the transgenic lines (ACR-W1 and ACR-W2) and wild type in hydroponics containing two concentrations of As^{III}, As^{V} and control having no As. Data are from three independent assays, values are means ± SD, and different letters on the bars indicate significant differences between genotypes at $P < 0.05$ level.

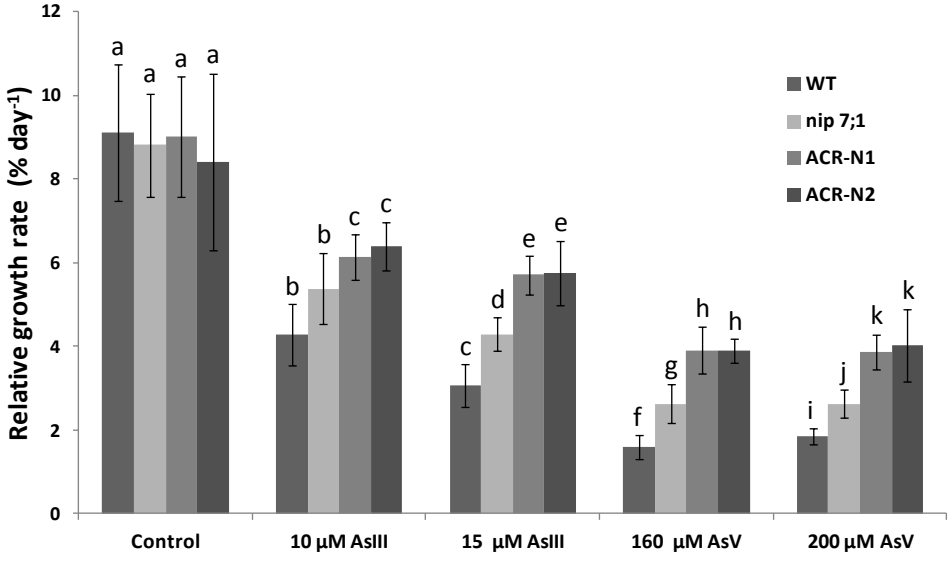


Figure 4.3 Relative growth rate for *Atnip7;1* transgenic and control lines in hydroponics exposed to As^{III} (10 µM and 15 µM) and As^{V} (160 µM and 200 µM)

Relative growth rate (% day^{-1}) after 14 days for the transgenic lines (ACR-N1 and ACR-N2) and *nip7;1* in hydroponics containing two concentrations of As^{III}, As^{V} and control having no As. Data are from three independent assays, values are means ± SD, and different letters on the bars indicate significant differences between genotypes at $P < 0.05$ level.

4.3.3 As efflux analyses of *Arabidopsis* seedlings

The transgenic lines and wild type were exposed to As^{V} for 24 hours in hydroponic culture. The analyses of the nutrient medium after this period and the tissue (roots and shoots) As concentration s will show the capacity of plants to efflux cytosolic As^{III} to the external medium and how the root-shoot partitioning of As is affected.

4.3.3.1 As^{III} efflux (as % of As^{V} uptake)

The data in figure 4.4 show that 56 percent of the As^{V} taken up by the wild type was converted to As^{III} and released to the external medium. However this efflux capacity was significantly (*p<0.05*) higher for the transgenic lines ACR-W1 and ACR-W2, which suggests that ACR3 increases As^{III} efflux. A similar pattern of efflux was observed for the transgenic lines in the *nip7;1* background (ACR-N1 and ACR-N2) compared to *nip7;1*, although the differences were not significant.

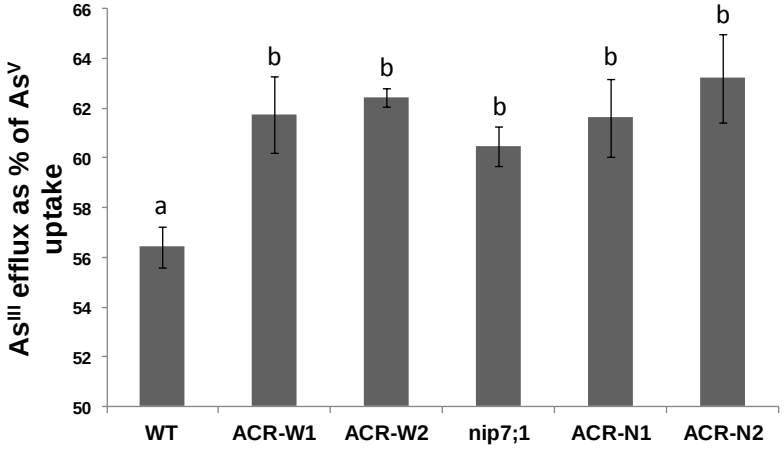


Figure 4.4 As^{III} efflux (as % of As^{V} uptake) analyses for *Arabidopsis* transgenic and control lines exposed to 10 µM As^{V} for 24 hours

As^{III} efflux (as % of As^{V} uptake) for the transgenic lines (ACR-W1, ACR-W2, ACR-N1 and ACR-N2) and the corresponding control lines (WT and *nip7;1*). Data are from three independent assays, values are means ± SD, and different letters on the bars indicate significant differences between genotypes at $P < 0.05$ level.

4.3.3.2 Total As concentration in roots and shoots

The plants used for the efflux analyses were also analysed for the total As concentration in roots and shoots tissue. Although the differences were not significant the trend showed that the transgenic lines ACR-W1 and ACR-W2 had a higher total As

concentration in the shoots compared to wild type. One of the transgenic lines in the *nip7:1* background, ACR-N1 showed a significantly higher total As in the shoots compared to *nip7:1* and ACR-N2, whereas the transgenic line ACR-N2 had significantly less total As in the shoots compared to *nip7;1* and ACR-N1 (figure 4.5 a).

The total As concentration in roots for the transgenic lines ACR-W1 and ACR-W2 was lower compared to wild type, however the differences were not significant. No significant differences in total As concentration of roots were observed for the transgenic lines in the *nip7;1* background, ACR-N1 and ACR-N2 compared to *nip7;1* (figure 4.5 b).

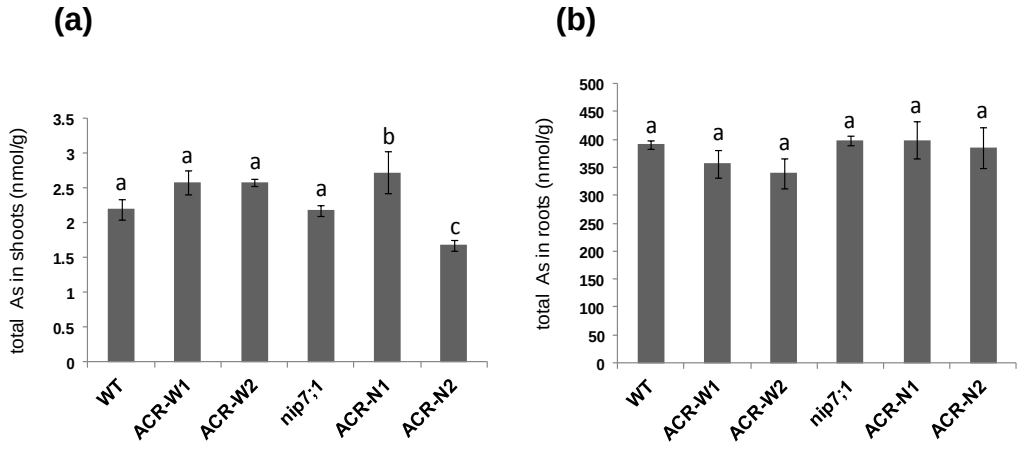


Figure 4.5 Total As concentration in shoots and roots for *Arabidopsis* transgenic and control lines exposed to 10 µM As^V for 24 hours

Total As in the shoots (nmol/g) (5a) and roots (5b) for the transgenic lines (ACR-W1, ACR-W2, ACR-N1 and ACR-N2) with the corresponding control lines (WT and *nip7;1*). Data are from three independent assays, values are means ± SD, and different letters on the bars indicate significant differences between genotypes at $P < 0.05$ level.

4.3.3.3 Shoot:root partitioning

The total As concentrations in the roots and shoots were also analysed to assess shoot:root partitioning. The data (figure 4.6) showed that a significantly higher concentration of total As was translocated to the shoots in the transgenic lines ACR-W1 and ACR-W2 compared to wild type. No significant differences were observed in shoot:root partitioning for the transgenic lines ACR-N1 and ACR-N2 compared to *nip7;1*. The data from the transgenic lines in the wild type background suggest that ACR3 may be involved in the translocation of As.

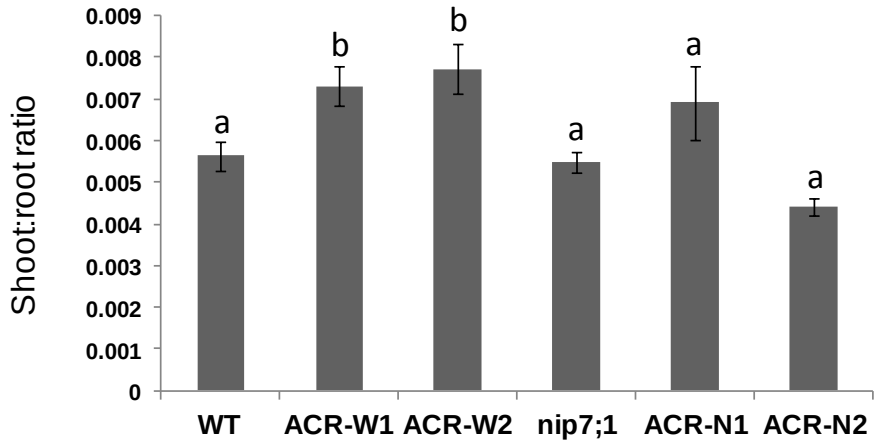


Figure 4.6 Shoot:root partitioning ratio for *Arabidopsis* transgenic and control lines exposed to 10 µM As^V for 24 hours

The shoot:root ratio of total As for the transgenic lines (ACR-W1, ACR-W2, ACR-N1 and ACR-N2) with the corresponding control lines (WT and *nip7;1*). Data are from three independent assays, values are means ± SD, and different letters on the bars indicate significant differences between genotypes at $P < 0.05$ level.

4.3.3.4 As^{III} concentration in plant tissue (% of total As)

The fraction of As^{III} and As^{V} available in the root and shoot tissues of the transgenic lines and the corresponding control lines was also determined. The data revealed that the transgenic lines ACR-W1 and ACR-W2 contain more As^{III} (% of total As) in the shoots and less in the roots compared to wild type, but only significant for ACR-W2 roots (figure 4.7a and figure 4.7b). Low levels of As^{III} content (as a % of total As) in the roots and higher concentrations in the shoots suggest that ACR3 may be involved in the translocation of As^{III}. However, no significant differences in As^{III} content in roots and shoots (as % of total As) were observed for the transgenic lines ACR-N1 and compared to *nip7;1* except for ACR-N2 containing significantly lower As^{III} in shoots (figure 4.7a and figure 4.7b). The differences found here were not significant (except for ACR-W2 roots and ACR-N2 shoots) and very small, this may not be biologically important as the plants did not show any toxicity symptoms within the range (around 1%) of As^{III} translocation. However, no significant differences for ACR-W2 roots and ACR-N2 shoots compared to the corresponding control might be due to the internal differences for these genotypes. ACR3 expression may result in pronounced differences if exposed for a long duration.

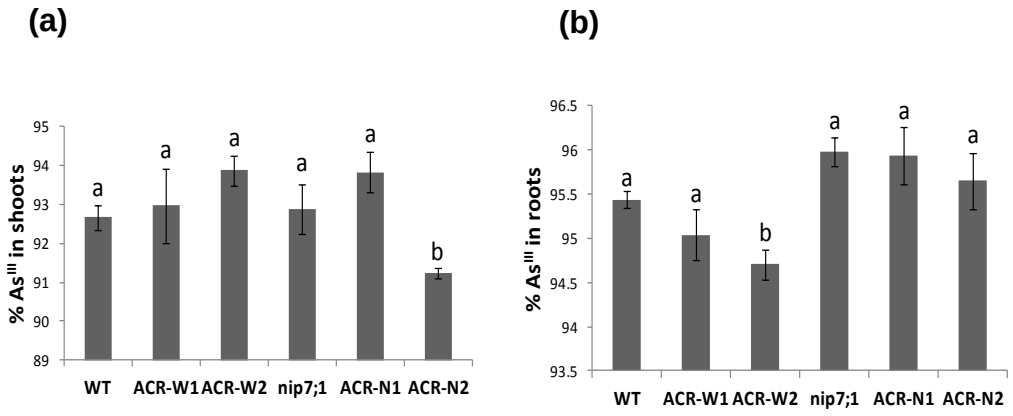


Figure 4.7 Percentage of As^{III} concentration in shoots and roots for *Arabidopsis* transgenic and control lines exposed to 10 µM As^{V} for 24 hours

As^{III} content in shoots (% of total As) (7a) and roots (7b) for the transgenic lines (ACR-W1, ACR-W2, ACR-N1 and ACR-N2) with the corresponding control lines (WT and *nip7;1*). Data are from three independent assays, values are means ± SD, and different letters on the bars indicate significant differences between genotypes at $P < 0.05$ level.

4.3.4 Localisation of *ScACR3* in rice protoplasts

To study the putative cellular function and localisation of ACR3, protoplasts from *Arabidopsis* were transformed with pART7-ACR3-EYFP. Figure 4.8A shows a YFP signal localised at the periphery of the protoplast suggesting that ACR3 is expressed in the plasma membrane. In order to confirm the plasma membrane localisation of ACR3, protoplasts were co-transformed with the plasma membrane marker, aquaporin AtPIP2A-CFP (Cutler et al., 2000) (figure 4.8B). The merge picture showed that both signals from EYFP and CFP overlap each other, which suggests that ACR3 is localised in the plasma membrane (figure 4.8C)

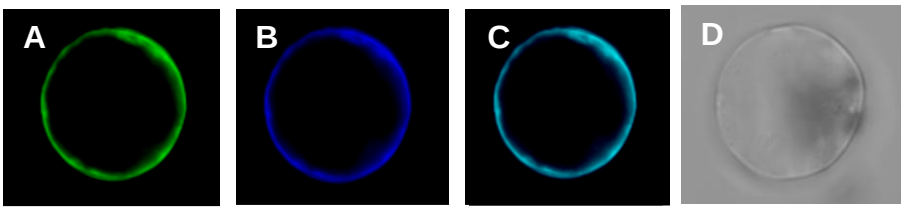


Figure 4.8 Localisation of *ScACR3* in *Arabidopsis* protoplasts

Plasma membrane localisation of ACR3 transporter in *Arabidopsis thaliana* protoplasts. Bright field (right) and fluorescence (left) images of the protoplasts show ACR3-EYFP expression in the plasma membrane with AtPIP2A-CFP as plasma membrane marker. Bars = 10 µm.

4.3.5 Protoplast viability assays

Protoplasts from the transgenic lines (ACR-W1, ACR-W2, ACR-N1 and ACR-N2) showed more tolerance to As^{III} (3.5 mM and 7 mM) and As^{V} (190 mM) compared to the

non transgenic controls (WT and *nip7;1*) (Figure 4.9). Significant tolerance was shown by the protoplasts from the transgenic lines compared to control lines after 24 hours exposure in media containing As^{III} and As^{V}.

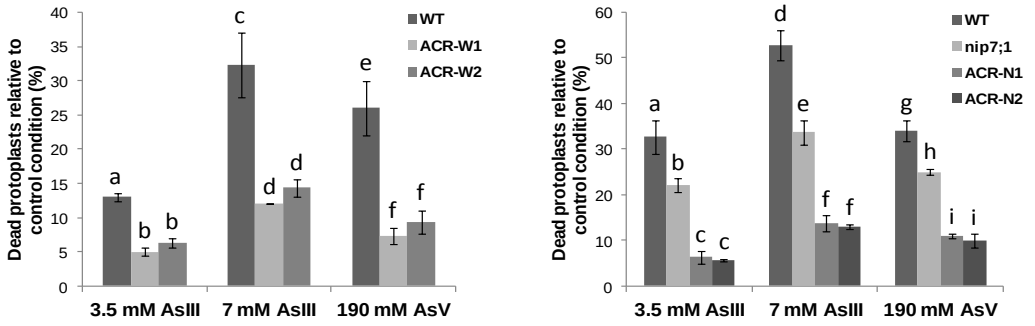


Figure 4.9 Percentage of non viable protoplasts for transgenic and control lines when exposed to As exposed to As^{III} (3 mM and 7 mM) and As^{V} (190 mM)

Percentage of dead protoplasts derived from the transgenic lines (ACR-W1, ACR-W2, ACR-N1 and ACR-N2) with the corresponding control lines (WT and *nip7;1*) relative to the control condition by exposing to the media containing 3.5 mM As^{III}, 7 mM As^{III} and 190 mM As^{V} for 24 hours. Data are from three independent assays, values are means ± SD, and different letters on the bars indicate significant differences between genotypes at $P < 0.05$ level.

4.3.6 Protoplast As efflux assays

The results from the protoplast viability study suggest that the transgenic protoplasts may be more tolerant due to the efflux provided by ACR3. To further confirm this, protoplasts from the transgenic and control lines were exposed to 2 mM of As^{V} for 2 hours. The As^{III} efflux was analysed by placing the protoplasts in As free medium and collecting samples at 3 and 6 hour time points. The transgenic lines ACR-W1 and ACR-W2 showed significantly higher As^{III} efflux for both time points compared to wild type. However, the transgenic lines ACR-N1 and ACR-N2 showed significantly reduced As^{III}

efflux compared to *nip7;1* for both time points (figure 4.10). The total As^{III} and As^{V} concentration in the protoplasts varied among the transgenics and control lines. Transgenic lines ACR-N1 and ACR-N2 in the *nip7;1* background and wild type had significantly higher As^{III} content in the protoplasts compared to the other transgenic lines ACR-W1, ACR-W2 and *nip7;1*. However, the transgenic lines in the *nip7;1* background had significantly higher As^{V} concentration in the protoplasts compared to wild type, ACR-W1, ACR-W2 and *nip7;1* (figure 4.11). The other factors which can affect the results are the exposure time and As species. If the protoplasts were exposed to As^{III}, the results might be different as the uptake of As^{V} is affecting the overall net efflux. Considering the total As concentration, the results were different as mentioned by Ali et al., (2012), where the transgenic lines had higher efflux of total As compared to their corresponding control lines.

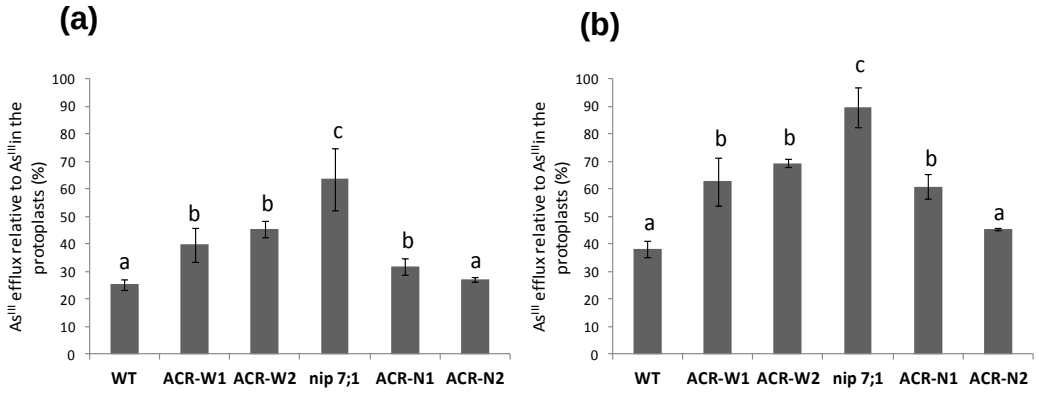


Figure 4.10 Percentage of As^{III} efflux relative to the total As^{III} in the protoplasts for *Arabidopsis* transgenic and control lines exposed to 2 mM As^{V}

Percentage of As^{III} efflux relative to total As^{III} available in the protoplasts after 3 (10a) and 6 (10b) hours, for the transgenic lines (ACR-W1, ACR-W2, ACR-N1 and ACR-N2) and the corresponding control lines (WT and *nip7;1*). Data are from three independent assays, values are means ± SD, and different letters on the bars indicate significant differences between genotypes at $P < 0.05$ level.

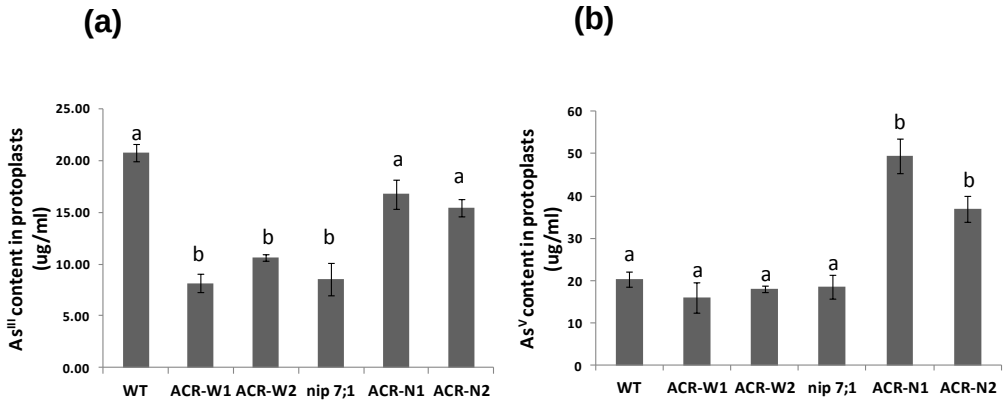


Figure 4.11 As^{III} and As^{V} concentration in the protoplasts for *Arabidopsis* transgenic and control lines exposed to 2 mM As^{V}

As^{III} (a) and As^{V} (b) concentration in the protoplasts of the transgenic lines (ACR-W1, ACR-W2, ACR-N1 and ACR-N2) with the corresponding control lines (WT and *nip7;1*). Data are from three independent assays, values are means ± SD, and different letters on the bars indicate significant differences between genotypes at $P < 0.05$ level.

4.4 Discussion

Microbes have membrane transporters for the efflux of cellular As^{III} to the external medium consisting of ArsAB in *E. coli* and Acr3p in yeast (Ghosh et al., 1999, Wysocki et al., 1997). No comparable efflux transporters have been identified so far in plants that mediate extrusion of As into the apoplast, although vacuolar sequestration has been reported to be one of the main strategies adapted by plants to cope with As (and other metals/metalloids). Recently it was reported that the ABC transporters AtABCC1 and AtABCC2 are involved in the vacoular deposition of complexed A^{III} in *Arabidopsis* (Song et al., 2010).

To test whether heterologous systems are capable of increasing As extrusion, and as such improve plant tolerance to As toxicity, we cloned the yeast ACR3 As antiporter which mediates As^{III} efflux from the yeast cytosol (Ghosh et al., 1999). ACR3 was expressed in *Arabidopsis thaliana* under the control of a strong constitutive promoter to

test whether this affected plant growth in the presence of As. A recent study where the yeast ACR3 was expressed in rice showed that it increased As^{III} efflux from the roots and resulted in lower As concentration in the roots, shoots and grain (Duan et al., 2011).

Growth analyses with *Arabidopsis* seedlings and mature plants showed that the transgenic line ACR-W1 had significantly higher tolerance, both on media containing As^{III} and As^{V} compared to wild type plants. A similar pattern of growth was observed for ACR-W1 in hydroponics in media containing As^{III} and As^{V}. The difference in growth was especially pronounced when using higher concentrations of As^{III} or As^{V}. The tolerance of ACR-W1 on plates and in hydroponics suggests that somehow this transgenic line excludes cytosolic As^{III} to reduce the cellular toxicity level. In contrast to ACR3-W1, the transgenic line ACR-W2 showed a reduced growth phenotype both on plates and in hydroponics. One possible reason for this apparent contradiction could be the ACR3 expression level, which was 6 times higher in ACR-W1. The expression level of ACR3 in ACR-W2 may not be sufficient to confer increased tolerance to this line.

The transgenic lines in the *nip7;1* background, ACR-N1 and ACR-N2 showed significantly better tolerance both on plates and in hydroponics (figure 4.1 and 4.3) compared to *nip7;1* and wild type. The transgenic lines ACR-N1 and ACR-N2 have around two times higher levels of ACR3 expression compared to the ACR-W2 genotype. Though this is still significantly lower than in ACR-W1, it may be sufficiently high to result in a detectable tolerance phenotype.

As^{III} efflux has been reported in many plant species (Xu et al., 2007, Su et al., 2010, Zhao et al., 2010a). However the molecular components of this pathway are unknown. The transgenic lines in the wild type background showed higher efflux compared to the control line. However, the transgenic lines in the *nip7;1* background do not show any significantly greater efflux compared to *nip7;1* (figure 4.4). AtNIP7;1 has been reported to be involved in the uptake of As^{III} and NIPs (aquaporins) may be involved in the bidirectional transport of As^{III} (Isayenkov and Maathuis, 2008, Bienert et al., 2008b). The loss of function in *nip7;1* would therefore suggest lower efflux and uptake of As^{III}, so the As^{III} produced due the reduction of As^{V} would remain in the cytoplasm. Yet there was more efflux in the *nip7;1* compared to wild type, which was further increased by the presence of ACR3 in the transgenic lines. As efflux from *nip7;1* control protoplasts was similar to that of wild-type protoplasts, whereas, curiously, As efflux from *nip7;1*

plants was significantly greater than that from wild-type plants. This suggests that the loss of function in NIP7;1 alters As release from the symplast, but does so in a cell- and ⁄or tissue-dependent manner.

ACR3 may efflux As^{III} to the apoplast where it may accumulate without reaching the external medium. Such a process could explain why a larger fraction of As^{III} was present in the roots of the transgenic lines ACR-N1 and ACR-N2 and *nip7;1* compared to wild type (figure 4.7b). The differences were not very high but may be the exposure for a long time could have an overall effect on the phenotype. However, the transgenic lines in the wild type background ACR-W1 and ACR-W2 had a higher fraction of As^{III} in the shoots and lower in the roots compared to wild type (figure 4.7a). Again these differences were not very high but just as a notion, the presence of ACR3 might have resulted in this difference which may increase once exposed for a long duration. In comparison, rice transgenic lines expressing ACR3 had 30 % higher As^{III} efflux compared to wild type plants when exposed to 10 µM As^{V} (Duan et al., 2011).

The tissue analyses of the plants used for the efflux analyses showed a significantly higher fraction of total As in the shoots of the transgenic lines ACR-W1 and ACR-W2 compared to wild type. However, no significant differences in total As concentration were observed for the roots and shoots of the transgenic lines ACR-N1 and ACR-N2 compared to *nip7;1* (figure 4.6). The data suggest that ACR3 may be involved in the translocation of As but more so in transgenic lines in the wild type background. Interestingly, in rice expressing ACR3 about 30 % lower As concentrations were found in the shoots (Duan et al., 2011). The other explanation could be the mechanism how the ACR3 activity is regulated in both species. In addition, the differences in the root structure and uptake pathways could be the other reasons of the differences in translocation. The specificity of ACR3 for As^{III} and interaction with other transporters/NIPs could also affect the translocation.

At the cellular level, protoplasts from all transgenic lines showed significantly more tolerance compared to the corresponding control lines, both in media containing As^{III} and As^{V}. Tolerance shown by the protoplasts might be provided by the exclusion of cytosolic As^{III} through the ACR3 efflux system, which is localised in the plasma membrane as in yeast (figure 4.8). For further confirmation of this notion, the As^{III}

efflux activity of the protoplasts from the transgenic lines was analysed. Protoplasts from the transgenic lines ACR-W1 and ACR-W2 showed significantly higher efflux compared to wild type, which suggests that ACR3 provides tolerance via efflux of As^{III}. However, the transgenic lines in the *nip7;1* background showed significantly lower As^{III} efflux compared to the control line. This intriguing result might be due to significantly higher concentrations of As^{III} and As^{V} that were found in the protoplasts of ACR-N1 and ACR-N2 (figure 4.11a and 4.11b). High cytosolic levels of As could impair the ACR3 pump activity and so reduce the efflux of As^{III}. For example, the higher amount of As^{V} found in ACR-N1 and ACR-N2 protoplasts might negatively affect ATP formation which is necessary to energise ACR3 activity. It could also be explained by a negative effect of the *nip7;1* mutation on ACR3 function under these conditions.

The tolerance shown by the protoplasts was similar irrespective of the expression levels of the transgene in the transgenic lines. This shows that responses at the cellular level can be quite different from those observed with intact plants. The reduced growth phenotype for ACR-W2 plants indicates that, although there is increased efflux of As^{III} as is shown by the protoplast assays, this does not benefit overall growth of intact plants (see figure 4.1 and 4.2). The latter could be caused by increased accumulation of As in the apoplast in this genotype, particularly in sensitive tissues, which may result in larger toxicity to the plants.

In summary, the present study showed that ACR3 expression resulted in increased As tolerance in *Arabidopsis* both at cellular and intact plant levels. After heterologous expression, ACR3 localised to the plasma membrane as in yeast (Ghosh et al., 1999) and appears to catalyse the same function of As^{III} efflux in *Arabidopsis*. This was confirmed by the experiments at the cellular level, which showed that protoplasts from the transgenic lines were more tolerant to As and had a higher efflux of As^{III} compared to the control lines.

The growth phenotype observed at the seedling or mature plant level can also be explained by the efflux of As^{III}, which somehow releases the cytosolic As^{III} to the apoplast, which is directed towards the xylem or the outside medium to reduce the cellular toxicity levels. Efflux is important to remove the cytosolic As^{III} at the root level and as such limit the entry of As^{III} into the seed by decreasing the translocation. Clearly this did not occur in our study with ACR3. However, an increased translocation to the

shoot as observed in the transgenic ACR3 lines could be potentially used for the phytoremediation. Other approach that may further improve efflux to the external medium is the use of root specific promoters instead of the constitutive promoter CaMV 35-S. The root specific expression of ACR3 will help in manipulating the As species mostly in the roots through efflux and sequestration. This may lower the translocation of As to the shoots and grain.

5 Final Conclusions

5.1 Arsenic toxicity problem

Metalloids have different roles in biological systems, the effects of these can be either useful (e.g. silicon and boron) or harmful to cells (e.g. arsenic). Arsenic is the most toxic among all the metalloids and harmful to humans, animals and plants. Once in the cell, As has either to be metabolised to less toxic forms or must be removed for the cell (Bienert et al., 2008a).

Humans are exposed to As either by using As contaminated water or through the food chain. Among cereals rice accumulates a higher fraction of As in the grain compared to other cereals and it is therefore one of the main pathways of dietary intake (Williams et al., 2005). The reduced conditions in paddy soils and the use of As contaminated water are the main factors for As accumulation in this species. However, there are many more crops that suffer from As toxicity and there is therefore a great need to reduce As in the food chain to ensure food safety. To achieve this, the molecular mechanisms of how plants deal with As need to be clarified which include As transport mechanisms, the subject of this thesis.

The As detoxification system has been fully characterised in prokaryotes (bacteria) and unicellular eukaryotes (yeast), and relies heavily on As^{III} efflux and the relevant membrane transporters have been well characterised (Bhattacharjee et al., 2008). The question whether As^{III} efflux is an important component of arsenic tolerance in plants has yet to be answered definitively but several studies have shown that plants release As^{V} and As^{III} into the external medium (Zhao et al., 2009). As^{III} efflux occurs soon after plants are exposed to arsenate but the mechanistic details of this process are unclear (Xu et al., 2007). Aquaporins offer another group of potential system of As^{III} bidirectional transport in mammals, bacteria and yeast. A subgroup of plant aquaporins, NIPs (nodulin like intrinsic proteins), has also been shown to participate in the bidirectional transport of As^{III}.

The expression of ACR3 *in planta* has added to the knowledge that how a gene from non plant organisms can be functionally analysed in plants. In a broader context, this study demonstrates that genes from micro organisms can be utelised in plants for abiotic stress tolerance. The co transformation of plants with ACR3 and other genes involved in the sequestration of As can be further analysed if it can add more tolerance to the plants.

Tissue specific expression of the desired genes would be another factor need consideration.

In a similar way, the aquaporins can also be useful tools in plant As tolerance. Other members of the aquaporin family can also be analysed. Similarly, crosses among different aquaporins can also be useful to be analysed. There are still some missing links in understanding the As transport mechanisms in plants like xylem loading, phloem unloading and accumulation in grain are still unclear and need further research (Zhao et al., 2010c).

5.2 ACR3 expression in rice and *Arabidopsis*

ACR3 expression has resulted in increased tolerance for the transgenic rice lines. The tolerance shown was both at cellular and seedling levels. ACR3, a plasma membrane localised antiporter was reported to be involved in the efflux of As^{III} from the yeast cytosol (Ghosh et al., 1999). As reported in yeast, ACR3 also localises to the plasma membrane, both in *Arabidopsis* and rice protoplasts as reported in the present study.

The expression of ACR3 has resulted in a phenotype at the cellular level, however the phenotype at the seedling level was not pronounced. I was unable to perform experiments at different concentrations and conditions for the toxicity studies due to limited number of transgenic seed. In a recent study, Duan et al., (2011) reported the expression of yeast ACR3 in rice has resulted in increased tolerance in the transgenic lines. Both under low and high As^{III} treatments, the roots and shoots tissue of the transgenic lines had lower As concentration compared to wild type. I do not have any data for the total As concentration in root and shoots of the transgenic lines used for the growth analyses. Duan et al., (2011) reported a significantly lower As^{III} concentration in the xylem sap for the transgenic lines compared to wild type, however my results indicated that transgenic lines had significantly higher concentration of As^{III} in the xylem, which was opposite to the earlier study reported.

The only reason could be the expression level of ACR3, which was about 20 times higher compared to wild type in the study conducted by Duan et al., (2011). However, I observed only three times expression of the transgene compared to wild type. The higher expression resulted in higher efflux leaving lower amounts of As for translocation to the shoots. Duan et al., (2011) did not provide any information on the

localisation of ACR3 in rice. Indeed this information could be helpful in comparison of the two results.

Extrusion of cellular As^{III} to the external medium is the basic pathway adapted by microbes and plants as detoxification mechanisms (Zhao et al., 2009). As efflux has been reported in many plant species but no specific transporters in plants have been identified which is involved in As^{III} efflux. Significantly higher efflux of As^{III} was observed for the transgenic lines compared to wild type when exposed to As^{V}. These results on As^{III} efflux were in agreement with previous studies conducted in rice, wheat, tomato and *Holcus lanatus* (Logoteta et al., 2009, Zhao et al., 2009).

Rice was reported to have lower As^{III} efflux and higher translocation to the shoots and grain. The authors of this study reported an efflux in rice (63%), barley (74%) and wheat (86%). The expression of ACR3 resulted in an increased efflux in the transgenic lines (80 % for all transgenic lines) compared to wild type (60 %). In the study reported by Duan et al., (2011), the efflux was in range of 60-80 % compared to wild type (50 %). Compared to other cereals like wheat and barley, rice had significantly lower As^{III} efflux. The expression of ACR3 has resulted in the increase efflux (80 %) compared to wild type. The findings from the present study were in agreement with already reported study by Duan et al., (2011), who observed a similar increase in As^{III} efflux for the transgenic lines compared to wild type (50 %).

The results for the data of total As concentration in the grain was in contradiction to the earlier reported study. Although, I did not expose the plants to As until seed filling as reported previously. Organic arsenical like DMA was the main specie found in the rice grain. It is not clear whether the DMA is translocated to the seed from the roots or the inorganic forms are methylated to organic forms in the shoots. Both studies have focussed on the total As concentration in the seed, rather it will be interesting to see which particular As species were present in the seed of the transgenic lines.

Arabidopsis has been used by many plant scientists for functional analyses of non plant genes due to ease of transformation and short life cycle. The expression of ACR3 has resulted in tolerance both at cellular and seedling levels. Many genes have been reported in *Arabidopsis*, that participate in As uptake like AtNIP1;1 and AtNIP7;1 (Isayenkov and Maathuis, 2008, Kamiya et al., 2009). However, no efflux transporter has been

identified in *Arabidopsis*. Some of the genes from bacteria have been expressed and analysed in *Arabidopsis*. Although, the expression in transgenic lines was about 1000 times higher compared to control but still there was translocation to the shoots. Based on the assumption of higher ACR3 expression in transgenic rice in earlier study, there should be more efflux and lower translocation in *Arabidopsis*. Having higher expression and pronounced translocation, the differences from the study in rice by Duan et al., (2011) might be due to genotypic differences between monocot (rice) and dicot (*Arabidopsis*), which include differences in root structure and As uptake pathways.

The functional analyses of ACR3 *in planta* has resulted in increased tolerance in both studies reported and suggested that genes from non plant organisms can be useful in plant tolerance against biotic and abiotic stresses. However, tissue specific expression must be considered for any specific purpose.

5.3 *Arabidopsis* NIPs and As transport

Among the plant aquaporins, NIPs showed a broad range of subcellular localisation, suggesting their role in different cellular compartments and at different growth stages (Maurel et al., 2008). In *Arabidopsis*, so far the NIPs have been reported to be involved in the uptake of essential nutrients like boron and harmful element like arsenic. The metalloid transport can be a unique character for the transport by NIPs. The results obtained from the study on different NIPs suggest their role in different pathways. However, there was no clear phenotype from different experiments conducted. The change in experimental conditions like growth on plates with higher concentrations of As and the analyses at the cellular level will be of greater value as there are complex interactions at the tissue level. Based on the results, there was no clear function of any specific isoforms but a kind of multifunctional role. This suggests that these NIPs might interact with each other at cellular level but due to lower expression do not display a pronounced phenotype. It will be interesting to analyse the double/triple knock outs among different NIPs in a similar way, which will give us an idea about the interactions among them.

5.4 The use of transgenic technology for As tolerance

Many genes and gene products are involved in As uptake, reduction, efflux, complexation, sequestration, translocation and seed accumulation. The advances in molecular biological techniques make it possible to transform/express genes of interest from other organisms. Attempts have been made to modify the genetic makeup of plants by silencing/expressing (overexpress) genes involved in As tolerance.

From the tolerance point of view, efforts have been focused on the vacuolar accumulation or efflux of cytosolic As available in the root cells. The accumulation of As in roots would decrease the translocation towards the shoots, which is the main strategy used by plants to reduce the cellular toxicity when exposed to a metalloids/stress.

The reduction of As^{V} and the manipulation of cytosolic As^{III} are the main factors affecting plant As tolerance (Bleeker et al., 2006). The cytosolic As^{III} forms complexes with PCs and is sequestered into the vacuole through the recently identified transporters AtABCC1 and AtABCC2, yet there may be accumulation of inorganic As^{III} in the vacuole through other, unknown transporters (Song et al., 2010, Tripathi et al., 2007a). Increasing the PCs status of the cell could increase As^{III}-PC complexes available for vacuolar deposition. The overexpression of phytochelatin synthase (AtPCS1) in *Arabidopsis* resulted in greater As tolerance with 20-100 times increase in biomass and increased sensitivity to cadmium (Li et al., 2004). Similarly, the overexpression of γ-glutamylcysteine synthetase from *E.coli* in *Arabidopsis* also resulted in more tolerance to As with more PCs and GSH (Li et al., 2005). However, the stimulation of PCs would not be effective until there is enough As^{III} available in the cytosol. Silencing the arsenate reductase (AtACR2) in *Arabidopsis* resulted in greater accumulation of As in the shoots and lower concentrations in the roots of the transgenics due to lower reduction of As^{V} (Dhankher et al., 2006). However, further research is needed to identify and genetically manipulate the transporters involved in the xylem loading, phloem unloading and grain filling.

Due to the recent advances in the vectors and transformation efficiency, the *Agrobacterium* mediated transformation has been used in generating transgenics in many plant species including rice and *Arabidopsis*.

The transformation of *Arabidopsis* with transgenes is rather simple and less time consuming due to the short life cycle of this plant compared to rice, which takes around 9 months to generate the transgenic lines. The *Agrobacterium* strain *AGL1* containing the ACR3 gene from yeast was used to transform rice calli (nipponbare). *Agrobacterium* mediated transformation is one of the easy and effective methods of rice transformation; however there are some public concerns over the use of GM crops. So far limited literature is available on the possible adverse effects of GM food/crops on human health and environment. There is a need to study the possible negative impacts of GM food/crops, these should be investigated for toxicity, allergic effects, fate of the inserted gene and changes in the nutritional value of the food after genetic modification (Domingo, 2011, Malarkey, 2003). The other concern is about outcrossing of conventional crops with GM crops, which can also have and indirect effect on human health.

5.5 Proposed model and roles of ACR3 *in planta* based on the present study

The expression of ACR3 affected the As tolerance both in rice and *Arabidopsis* (chapter 3 and chapter 4). The putative roles of ACR3 *in planta* are summarised in Figure 5.1. The data from transient expression of ACR3 both in rice and *Arabidopsis* protoplasts showed that ACR3 is localised to the plasma membrane. The experiments at the cellular level using protoplasts in both rice and *Arabidopsis* showed tolerance due to the efflux provided by ACR3 efflux system. The As^{III} released to the apoplast is directed towards the external medium or to the xylem through the plasmodesmatal (and symplastic) connections between the root cells. Higher fraction of As^{III} in xylem was found for trasgenic rice lines compared to wild type as mentioned in chpater 3 (section 3.3.3). Once in the xylem, As^{III} is translocated towards the shoots. In the mesophyll cells, the photosynthetic machinery remobilised As with the food perapred through phloem to the seed and other parts of the plant.

The data for total As concentration in one of the transgenic rice lines showed that the seed and husk of the transgenic line has lower total As concentration compared to wild type. This suggests that As^{III} may be remobilised to the shoots via the phloem or there may be a higher fraction of total As concentration in the husk due to constititive expression which may have resulted in more transpiration and lower the amounts of As

in the seed. However, the putative role of ACR3 in seed filling is still unclear and needs further analyses.

In both rice and *Arabidopsis*, ACR3 expression was under a constititive promotor (35S). This may have resulted in the expression of ACR3 in cells where not desirable. The efflux provided by ACR3 system may also have resulted in As accumulation in the apoplastic and translocation to the shoots and grain apart from the efflux to the external medium. The translocation of As to the upper parts (shoots and grain) of the plant is not desirable particularly in rice, the alternative will be to express ACR3 under a tissue specfic promotor (roots) for more efflux and lower translocation. The constitutive expression of ACR3 can be potentially employed for the phytoremediation purposes in contaminated sites.

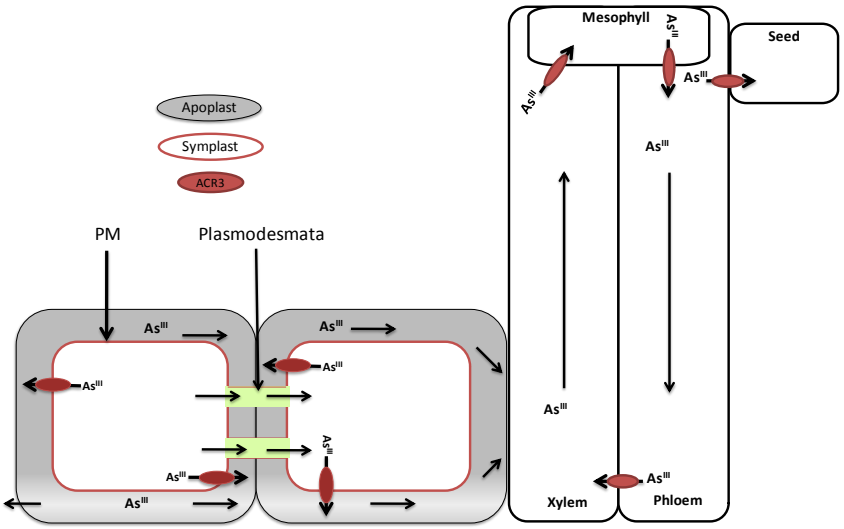


Figure 5.1 Putative roles of *ScACR3 in planta*.

ACR3 is localised to the plasma membrane and involved in As^{III} efflux to the apoplast, which is directed either to the external medium or towards the xylem for translocation to the shoots. As^{III} is mobilized to the seed and lower parts of the plant via phloem.

5.6 Proposed model and roles of NIPs *in planta* based on the present study

NIPs, a subgroup of plant aquaporins offers another potential group of transporters involved in the bidirectional transport of As^{III}. The heterologous expression of different NIP isoforms (from rice, *Arabidopsis* and *Lotus japonicus*) in yeast and *Xenopus laevis* oocytes resulted in the sensitised cells. Although some of the NIP isoforms (AtNIP1;1, atNIP7;1, OsNIP2;1 and OsNIP3;2) have been reported to play a role in As^{III} uptake using the plant growth assays.

Loss of function mutants in *Arabidopsis* NIPs were analysed for their role in As uptake/ efflux/sequestration/translocation. The data suggest that different NIP isoforms are playing many putative roles in As transport. AtNIP7;1 has been reported to be involved in the uptake of As^{III} (Isayenkov and Maathuis, 2008). The data from our experiments showed that *nip7;1* has no role in As^{III} efflux, but may be involved in the sequestration or translocation of As. The other NIP isoforms (*nip5;1* and *nip6;1*) may have role in As^{III} efflux.

The overall results showed that the loss of function in any of the NIP isoforms has resulted in an As related phenotype, suggesting that NIPs are contributing to various aspects of As transport in *Arabidopsis* (figure 5.2). This suggests that these and other NIP isoforms might work together in co-ordination and might be regulated under specific cellular conditions such as metalloid or any other stress. Double/triple knock out mutants in NIPs can be generated via breeding the isoforms involved in As^{III} efflux. This will also add to the knowledge of their co coordinated roles and regulation. Further research focuses the modification of selectivity filter (Ar//R) in NIPs which are selective against As.

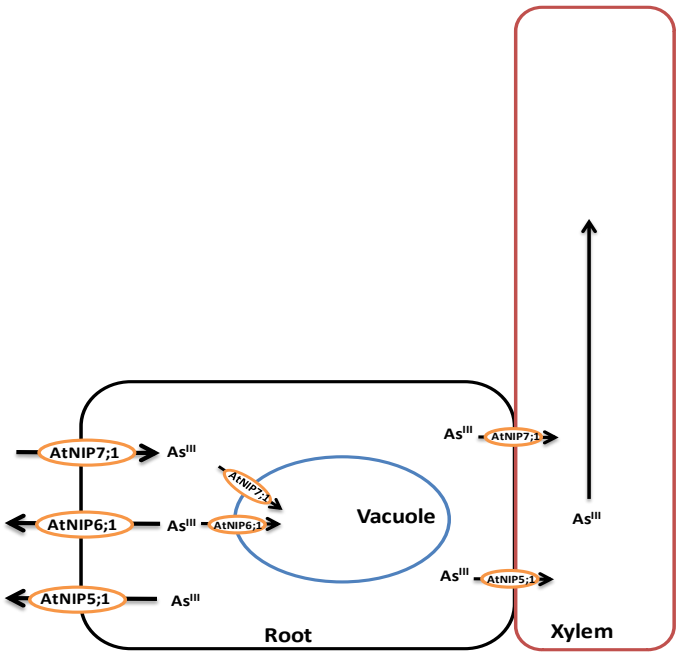


Figure 5.2 Putative roles of *Arabidopsis* NIPs in As transport

The NIP isoforms in the present study showed to have effect on different aspects of As transport in *Arabidopsis*. On the basis of results obtained during this and previous studies it can be concluded that NIPs are involved in As^{III} uptake (NIP7;1), efflux (NIP5;1 and NIP6;1), vacuolar sequestration (NIP6;1 and NIP7;1) and xylem loading of As^{III} (NIP5;1 and NIP7;1).

5.7 Functional analysis of plant expression libraries using yeast heterologous system

The cDNA expression libraries from *Arabidopsis* and rice were screened using the yeast heterologous system. The yeast strains used in the study did not show any tolerant/sensitive growth phenotype. This suggests the absence of any new proteins in the plant expression libraries that may be involved in As transport. There might be putative new proteins with a lower expression which may affect the As accumulation in the yeast cells. Other yeast mutant strains (*pdr12*Δ and *fps1*Δ) and cDNA libraries (*Pteris vittata*) can also be utelised for functional analyses.

5.8 Future outlook

Chronic arsenic poisoning is a major health concern to large sections of the global population and food consumption is one of the biggest contributors to human arsenic exposure. The main strategies to limit the entry of As into the food chain would be the use of non contaminated water for irrigation and soil remediation for growing crops, however it will be more costly and time consuming. Alternative approaches would be to use the knowledge about As transport in plants and manipulate differnet pathways to reduce As uptake in crops.

The details about As transport in plants are mostly understood, however there are still many missing links in the complete detoxification pathway. The transgenic approach could be used to engineer (express/overexpress) genes (PDR12 from yeast, ArsB and ArsM from bacteria, and some of the NIP isoforms (*nip5;1* and *nip6;1*) as suggested from our studies) that are responsible for As removal, reduction and vacoular sequestration (YCF1 from yeast anf AtABCC1 and AtABCC2) from non plant organisms. Transporters involved in the uptake of As have been identified, but the information available in terms of As efflux is not sufficient.

To manipulate As in the roots, varients in both NIPs (aquaporins) and phosphate tansporters should be identified which are selective aginst As^{III} and As^{V} respectively. Once in the root, there should be enough PCs and GSH to form complexes with As^{III} for vacuolar sequestration via the recently identified transporters AtABCC1 and AtABCC2. However, there is need to identify the transporters involved in the sequestration of inorganic As^{III}, which will further decrease the As load in the cells. Little information is

available on how As is loaded into the xylem and translocated to the shoots. Similarly, the mechanisms of As tansport to the grain via phloem are still unclear. As methylation has been reported in bateria and fungi, do plants have this strategy to convert the toxic forms of As to relatively less toxic forms and if there is methylation, is there any volatilisation or loss of As throuogh gutation/transpiration are the issues need further research.

Beside these future issues, there are few other aspects which might be helpful in limiting As entry into the grain and into the food chain. The rice cultivars having low As uptake and translocation to the shoots and grain should be grown in the As contaminated areas. Both genetic and environmental factors are also important as some of the cultivars might need acclimatisation to the new environment. The use of As free water for irrigation is another alternative, which is not feasible in those areas which geogenically has higher As in their aquifers and the alternative will be to modify plants. High redox potential could decrease the reduction of As^{V} and hence lower the quantities of As^{III} for mobilisation to the grain. Flooding of the paddy fields leads to the mobilisation of As^{III} produced by the environmental factors in the reduced conditions. Rice can be grown in soil and irrigated regularly to reduce the uptake of As^{III}.

Appendix

Appendix A

Screening of cDNA libraries from rice (*Oryza sativa*) and *Arabidopsis thaliana* to identify (new) transporters involved in As transport

Introduction

Saccharomyces cerevisiae (baker's yeast) has proved to be a unicellular eukaryotic model for cellular and molecular biological studies (dos Santos et al., 2012). Yeast is used more commonly these days as a heterologous expression system due to the ease of transformation and the availability of deletion mutants. Different yeast mutants defective in specific transporters are available, which can be used to discover, analyse and characterise plant transporters with different roles.

Among eukaryotes, *S. cerevisiae* serves as a model system for arsenic resistance (figure.1). As^{V} and As^{III} enter the yeast cell through phosphate transporters (Pho84 and Pho87) and aquaglyceroporins (Fps1) respectively (Tripathi et al., 2007a, Wysocki et al., 2001). Removal of As from the yeast cytosol can occur via two pathways: in one pathway via Acr3p, a plasma membrane transporter involved in extrusion of As^{III} from the cytosol, while in the second pathway, the As^{III} available in the cytosol is complexed with glutathione which is sequestered into the vacuole by an ABC-type transporter, YCF1 (yeast cadmium factor 1) (Ghosh *et al*., 1999). In *Saccharomyces cerevisiae* three ACR (arsenic compounds resistance) genes located on chromosome XVI are involved in As sensing, As^{V} reduction and As^{III} extrusion (figure. 1). *ACR1* produces a transcription factor which induces *ACR2* to produce arsenate reductase converting As^{V} to As^{III}. In the last step As^{III} is extruded from the cytosol by Acr3p.

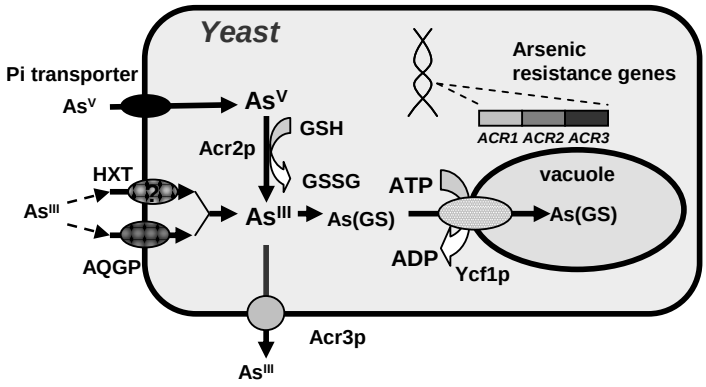


Figure 1 Arsenic transport in yeast

In yeast, uptake of arsenate is facilitated by Pho87 type phosphate transporters, and arsenite is taken up mainly via the aquaglyceroporin Fps1p but may also enter cells through hexose permeases (HXTs). The reduction of arsenate to arsenite in yeast cells is carried out by the Acr2p arsenate reductase via glutathione reduction (GSH to GS). Removal of cytoplasmic As^{III} can occur through conjugation to glutathione ($As(GS)_3$) which is sequestered into vacuoles by the ABC transporter Ycf1p, or through extrusion of As^{III} via the plasma membrane carrier Acr3p (figure reproduced from (Ali et al., 2009).

Different yeast mutant strains are available in relation to As tolerance. The yeast transporters involved in the efflux (Acr3p) or vacoular sequestration (Ycf1p) of As are of much interest to plant biologists because they can be used to search for orthologs in plants. Yeast mutants have been utilised for the functional analyses of aquaporins from rice, *Arabidopsis* and *Lotus japonicus* with respect to their role in As tolerance (Bhattacharjee et al., 2008). In addition, cDNA libraries from plants can be used to search for any growth complementation, for example the cDNA library from the As hyperaccumulator *Pteris vittata* has been screened using the yeast strain *acr3*Δ lacking the As^{III} efflux mechanism ACR3. The growth complementation in the mutant line resulted in the identification of two plant ACR3 homologues (*ACR3* and *ACR3;1*) involved in the vacoular sequestration of complexed As^{III} (Indriolo et al., 2011).

To identify if new transporters exists in other plants species involved in As transport, cDNA libraries from rice (*Oryza sativa*) and *Arabidopsis thaliana* were screened using the yeast strains *acr3*Δ lacking the As^{III} efflux mechanism ACR3 and *ycf1*Δ lacking the tonoplast ABC transporter YCF1. These strains were transformed with the plant

libraries and yeast was assayed for change in growth when exposed to different levels of As^{V} and As^{III}.

Material and Methods

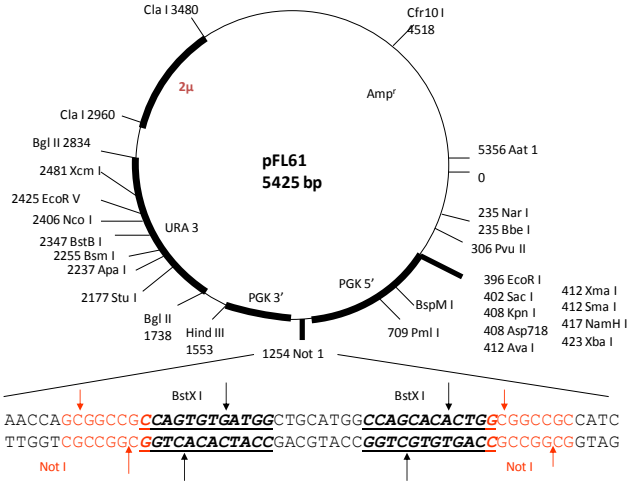
Chemicals and consumables

For the chemicals and consumables see Chapter 2 (section 2.2.1).

Yeast strains and expression vectors

Two yeast mutant strains were used in the present study: *acr3*Δ (ade, leu, ura, trp) is based on the parental strain WT303 (ade, leu, ura, trp, his) and *ycf1*Δ (mat, his, leu, met) which is based on the parental strain BY4741 (mat, his, ura, leu, met). The cDNA library from *Arabidopsis* was already available in our lab stocks, it was cloned into the yeast expression vector pFL61 (figure 2a). The rice (*Oryza sativa*) cDNA library was provided by Dr Toshihiro Kumamaru from Japan and cloned into vector pYES2 (figure 2b).

(a)



(b)

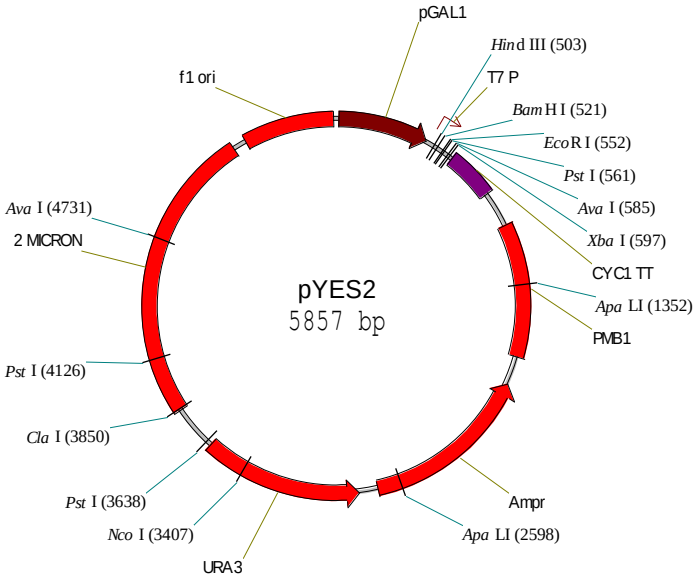


Figure 2 Maps of (a) pFL61 and (b) pYES2 vectors used for cloning of cDNA libraries from *Arabidopsis* and rice respectively.

Yeast media and growth

For the maintenance of yeast culture, YPD (yeast extract/peptone/glucose) medium was used. Untransformed yeast was grown on SD (synthetically defined) medium containing glucose, ammonium sulphate, yeast nitrogen base without amino acids and supplemented with leucine, methionine, histidine and uracil. For the selection of transformed yeast SC drop out medium (galactose, ammonium sulphate, yeast nitrogen base without ammonium sulphate, drop out medium without uracil) was used. The yeast plates and liquid cultures were grown at 30^{o}C.

Yeast transformation

Yeast was transformed using the protocol from Breeden Lab, Fred Hutchinson Cancer Research Centre. The protocol starts with the inoculation of a single colony in liquid medium overnight at 30^{o}C. The OD was measured in the morning and to get the desired OD of 0.3, the culture was incubated for an additional 3-4 hours. The pellet was washed after centrifugation in 25 ml of sterilised water and then resuspended in 2 ml of 1xLiAc/0.5xTE solution. The culture mixture was incubated for 10 minutes. After incubation, the yeast culture (100 µl) was mixed with salmon sperm DNA (10 µl), DNA (15 µl) and 1xLiAc/1xTE/40 % PEG mix (700 µl), incubated for 30 minutes at 30^{o}C. DMSO (80µl) was added to the mixture before heat shock at 42^{o}C for 15 minutes. The mixture was resuspended in 0.5 ml of 1xTE and 100-200 µl incubated on different plates at 30^{o}C.

Results and Discussion

The yeast mutants were first grown on SD-Control medium (figure 3A) and different concentrations of As^{III} and As^{V} to identify putative As related phenotypes (figure 3B and 3C). *acr3*Δ and *ycf1*Δ showed severe growth reduction in the presence of 1 mM As^{III}.

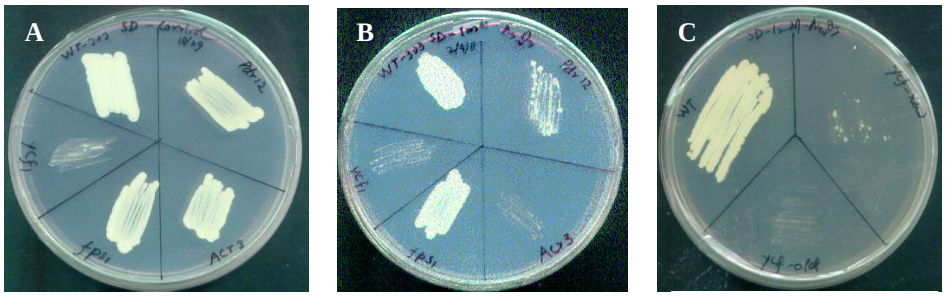


Figure 3 Yeast strains grown on SD medium (A), As related phenotypes for yeast mutants were observed on SD-1 mM As^{III} for *acr3*Δ (B) and *ycf1*Δ (C).

Yeast transformation with cDNA libraries from *Arabidopsis* or *Oryza sativa* did not result in any growth complementation. No growth tolerant phenotype was observed for any of the yeast strains with both cDNA libraries; therefore we also tested if the expression of plant cDNA libraries can result in increased sensitivity of these strains. For this, the transformed yeast colonies for both yeast strains were grown on lower concentrations of As^{III}, As^{V} and control medium by replica plating. No growth sensitive phenotype was observed for any of the yeast strains used on lower concentrations compared to control plates.

In summary, no growth tolerant/sensitive phenotype was observed in any yeast strain with both expression libraries suggesting the absence of putative new transporters/proteins involved in As transport. There can be proteins produced by the expression libraries; however the expression might not be strong enough to show a phenotype. It would be interesting to analyse the As concentration of the transformed

colonies compared to control colonies (transformed with empty vector) to see if there are any differences in As accumulation. Yeast strains like *pdr12*Δ and *fps1*Δ can also be used to see if there is any pronounced growth complementation. The other reason for absence of phenotypes might be the age and expression capacity of the libraries to truly represent the whole genome. It would also be interesting to functionally analyse a cDNA library from the As hyperaccumulator *Pteris vittata* for putative new transporters using the yeast heterologous system.

Abbreviations

As	Arsenic
As^{III}	Arsenite (As_2O_3)
As^{V}	Arsenate $(AsO_4)^{-3}$
At	*Arabidopsis thaliana*
Col	Columbia
WT	Wild type
T-DNA	Transferred DNA
NIPs	Nodulin-26like intrinsic proteins
Ar/R	Aromatic/Arginine
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
cDNA	Complementary DNA
PCR	Polymerase chain reaction
qRT-PCR	Quantitative reverse transcriptase PCR
bp	Base pair
dNTP	Deoxyribonucleotide triphosphates
EDTA	Ethylenediaminetetraacetic acid
E.Coli	*Escherichia coil*
HPLC	High performance liquid chromatography
ICP-MS	Inductively coupled plasma mass spectrometry
PBS	Phosphate buffer solution
GSH	Glutathione
PCs	Phytochelatins
ACR3	Arsenical compound resistance 3
EYFP	Enhanced yellow fluorescent protein
CFP	Cyan fluorescent protein

LB	Luria bertani
YPD	Yeast peptone dextrose medium

Gene annotations

Uppercase	Referring to the protein e.g. ACR3
Uppercase	Italicised referring to the gene e.g. *NIP7;1*
Lowercase	Italicised referring to a mutant form of the gene e.g. *nip7;1*

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