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Heterologous expression of the yeast arsenite efflux system ACR3 improves *Arabidopsis thaliana* tolerance to arsenic stress

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Summary

- Arsenic contamination has a negative impact on crop cultivation and on human health. As yet, no proteins have been identified in plants that mediate the extrusion of arsenic. Here, we heterologously expressed the yeast (*Saccharomyces cerevisiae*) arsenite efflux transporter ACR3 into Arabidopsis to evaluate how this affects plant tolerance and tissue arsenic contents.
- ACR3 was cloned from yeast and transformed into wild-type and *nip7;1* Arabidopsis. Arsenic tolerance was determined at the cellular level using vitality stains in protoplasts, in intact seedlings grown on agar plates and in mature plants grown hydroponically. Arsenic efflux was measured from protoplasts and from intact plants, and arsenic levels were measured in roots and shoots of plants exposed to arsenate.
- At the cellular level, all transgenic lines showed increased tolerance to arsenite and arsenate and a greater capacity for arsenate efflux. With intact plants, three of four stably transformed lines showed improved growth, whereas only transgenic lines in the wild-type background showed increased efflux of arsenite into the external medium. The presence of ACR3 hardly affected tissue arsenic levels, but increased arsenic translocation to the shoot.
- Heterologous expression of yeast ACR3 endows plants with greater arsenic resistance, but does not lower significantly arsenic tissue levels.

Introduction

Arsenic (As) is toxic to all forms of life and is a potent carcinogen, which poses a risk to human health (Tripathi *et al.*, 2007; Ali *et al.*, 2009; Zhao *et al.*, 2010a). In general, the inorganic forms (As^{III} and As^{V}) are more prevalent than the organic forms in terrestrial environments, and also more toxic, although this depends on the exact As species. Arsenite (As^{III}) has an affinity for sulfhydryl groups found in cysteine residues and, as such, affects protein structure and function. Arsenate (As^{V}) is a phosphate analogue which can substitute for phosphate at binding sites and can be incorporated into ATP (Meharg & Hartley-Whitaker, 2002).

Humans are exposed to As mainly from drinking water and via the food chain. It has been reported that *c.* 150 million people are exposed to water containing As above the World Health Organization (WHO) recommended limit of 0.01 ppm (Nordstrom, 2002; Brammer & Ravenscroft, 2009). Dietary intake is especially problematic amongst populations with high rice consumption, because this cereal accumulates relatively large amounts of As (Zhao *et al.*, 2010a).

In plants, As^{V} is taken up by phosphate transporters (Ullrich-Eberius *et al.*, 1989; Wu *et al.*, 2011), whereas As^{III} enters roots via a subgroup of aquaporins, the nodulin-like intrinsic proteins (NIPs) (Isayenkov & Maathuis, 2008; Ma *et al.*, 2008; Kamiya *et al.*, 2009). In *Arabidopsis thaliana*, two specific NIP isoforms (AtNIP1;1 and AtNIP7;1) have been shown to be involved in the uptake of As^{III} (Isayenkov & Maathuis, 2008; Kamiya *et al.*, 2009). In rice, the silicon transporter Lsi1 (OsNIP2;1) is a major uptake pathway for As^{III} (Ma *et al.*, 2008).

Although some clarity exists about the uptake mechanisms of As^{V} and As^{III} in plants, it largely remains an open question whether plants contain dedicated As efflux systems. This is in stark contrast with prokaryotes, unicellular eukaryotes and mammals: in bacteria, the ArsAB operon encodes As efflux transporters in the form of antiporters and ABC transporters to pump As^{III} out of the cell; in yeast, the ACR3 antiporter removes As^{III} from the cytosol to the external medium; and the ABC transporter MRP2 is believed to deliver complexed As^{III} into the bile of mammals for subsequent secretion via the faeces (Wysocki *et al.*, 1997; Rosen, 2002; Maciaszczyk-Dziubinska *et al.*, 2011).

As yet, no specific As efflux pathway has been identified in plants, but several studies have shown that plants release As^{V} and As^{III} into the external medium (Xu *et al.*, 2007; Zhao *et al.*, 2010b). Recent work on NIPs has shown that many may be capable of bidirectional transport (Bienert *et al.*, 2008; Isayenkov &

Maathuis, 2008; Zhao *et al.*, 2010b), and thus, in certain conditions, may contribute to As removal from the symplast, but the physiological relevance of this process is unknown.

To assess whether As efflux in plants can be augmented, and whether this has positive effects on plant growth, we decided to heterologously express *ScACR3* from the yeast *Saccharomyces cerevisiae* in *A. thaliana*. ScACR3 is a plasma membrane-located, H^+ gradient-driven antiporter, which forms the primary As^{III} efflux mechanism in yeast (Wysocki *et al.*, 1997; Maciaszczyk-Dziubinska *et al.*, 2011). No ACR3 homologues have been found in higher plants but, interestingly, the As hyperaccumulator fern *Pteris vittata* expresses several ACR3-type proteins, which are expressed at the tonoplast and mediate the vacuolar sequestration of As^{III} (Indriolo *et al.*, 2010). We found that heterologous expression of *ScACR3* improved plant tolerance in response to As^{III} and As^{V}, at both the cellular and whole plant level. ACR3 activity increased As efflux to the external medium and affected the root–shoot partitioning of As.

Materials and Methods

Plant material and growth conditions

Arabidopsis thaliana (L.) Heynh wild-type (Col-0) seeds were grown on F2 + S (Levington, UK) soil, after stratification. The trays were then transferred to growth rooms (20 : 23°C night : day temperature, 16 : 8 h light : dark cycle, 65 μmol s^{-1} m^{-2}) for 10 d. For the hydroponics culture, 2-wk-old seedlings were transferred to 1-l boxes with nutrient medium consisting of 0.09 mM $(NH_4)_2SO_4$, 0.05 mM KH_2PO_4, 0.05 mM KNO_3, 0.03 mM K_2SO_4, 0.06 mM $Ca(NO_3)_2$, 0.07 mM $MgSO_4$, 0.11 mM Fe-EDTA, 4.6 μM H_3BO_3, 1.8 μM $MnSO_4$, 0.3 μM $ZnSO_4$, 0.3 μM $CuSO_4$, pH 5–5.6.

For plate growth analyses, 12 seeds of each genotype were placed on half-strength Murashige and Skoog medium agar plates. The plates were placed vertically in a growth room with conditions as described above, and growth was maintained for 3 wk. To test exposure to As, wild-type and transgenic plants were grown on plates supplemented with 10 μM As^{III} or 160 μM As^{V} concentrations that reduced plant growth by 50–75%. Experiments were replicated six to seven times and growth was measured as the average fresh weight per plant relative to the control condition (%). For the hydroponic culture, 3-wk-old plants were transferred to medium (composition as described above) with or without As^{III} (10 or 15 μM) and As^{V} (160 or 200 μM). Five plants of each genotype were used for each treatment and the experiments were replicated three to four times. The relative growth rate (RGR) was measured according to Poorter & Garnier (1996) across a 14-d period.

Cloning of ACR3 and *Arabidopsis* transformation

cDNA was synthesized from yeast (*S. cerevisiae*) total RNA and the full-length *ACR3* coding sequence was obtained using ACR3-For (gcctcgagATGTCAGAAGATCAAAAAAGT) and ACR3-Rev (gccccgggATTTCTATTGTTCCATATAT) primers. *ACR3* was cloned into the 35S promoter cassette of pART7 between *Xho*I and *Sma*I restriction sites, and the pART7 35S-ACR3 cassette was then subcloned into the binary vector pGreen0179 using *Not*I. *Agrobacterium* strain *GV3101* was cotransformed with the binary vector pGreen0179:35S:ACR3 and pSoup by electroporation. *Agrobacterium* culture was used to transform *A. thaliana* Col-0 and *A. thaliana* Col-0 *nip7;1*, which carries a loss of function in AtNIP7;1 (Isayenkov & Maathuis, 2008), according to the protocol by Clough & Bent (1998).

Identification of homozygous lines and reverse transcription-polymerase chain reaction (RT-PCR) analysis

Hygromycin-resistant primary transformants were selfed and homozygous lines were identified in the T3 generation via segregation analysis. Two homozygous lines in the wild-type background were isolated (ACR3-W1 and ACR3-W2) and two in the *nip7;1* background (ACR3-N1 and ACR3-N2). Quantitative RT-PCR analyses on 2–3-wk-old seedlings showed that ACR3 expression in ACR3-W1 was *c.* five times higher than in ACR3-W2 and *c.* three times higher than in lines ACR3-N1 and ACR3-N2 (data not shown).

Total As and As speciation analyses

Tissue and medium As concentrations were determined by inductively coupled plasma-mass spectrometry (ICP-MS) (Agilent ICP-MS 7500ce; Agilent Technologies, Santa Clara, CA, USA). Plant tissues (roots and shoots) were collected from hydroponically grown plants. The roots were washed with ice-cold buffer (1 mM K_2HPO_4, 0.5 mM $Ca(NO_3)_2$ and 5 mM Mes, pH 5.6) to remove apoplastic As. Roots and shoots were dried at 70°C for 48 h and the dry weight was noted. Tissues were digested using HNO_3/$HClO_4$ (70 : 30%, v/v) employing a heating block or a CEM MARS5 microwave (CEM, Buckingham, UK).

Arsenite efflux assays were performed as described previously (Xu *et al.*, 2007; Li *et al.*, 2009). Briefly, 3-wk-old plants were exposed to 5 μM As^{V} for 24 h. Samples from the solutions were collected after 24 h for the analysis of As species. The roots and shoots of the plants were rinsed with deionized water. The roots were then placed in ice-cold washing buffer for 30 min. Root and shoot tissues were ground to powder in liquid nitrogen for As extraction (Xu *et al.*, 2007). The procedure was tested for extraction and conversion artefacts using As-free *Arabidopsis* plants, spiked with either arsenate or arsenite standard, which followed the same extraction and analytical procedure (Li *et al.*, 2009). Arsenic speciation was determined using high-performance liquid chromatography (HPLC)-ICP-MS (Agilent LC1100 series and Agilent ICP-MS 7500ce; Agilent Technologies) (Li *et al.*, 2009).

Protoplast assays

Protoplasts from transgenic and wild-type *Arabidopsis* shoots were isolated according to Maathuis *et al.* (1998). Protoplasts

were incubated in buffer (0.6 M mannitol, 4 mM Mes/KOH, pH 5.5, 4 mM KCl and 3 mM $CaCl_2$) and exposed to As^{III} (3.5 or 7 mM) and As^{V} (190 mM), concentrations that produced comparable cell death rates, for 24 h under continuous light, and scored for viability at 0 and 24 h using Evan's blue stain to assess cell survival. The percentage of dead protoplasts relative to the control condition was determined using a microscope and counting chamber, and experiments were replicated four to five times with independently isolated batches of protoplasts.

To assess protoplast As extrusion capacity, protoplasts (*c.* 6×10^5) from control and ACR3-expressing plants were incubated in 4 ml of buffer supplemented with 2 mM As^{V} and loaded for a period of 2 h. Protoplasts were washed three times in As-free buffer and resuspended in 4 ml of fresh buffer. The efflux capacity is expressed as the total As measured in the external buffer solution after 3 and 6 h. Experiments were replicated four to five times with independently isolated batches of protoplasts.

Subcellular localization of ACR3

In order to determine the subcellular localization of ACR3 *in planta*, *ACR3* was fused to *EYFP* by cloning it into pART7/EYFP. The *ACR3* coding sequence was amplified using primers gcctcgagATGTCAGAAGATCAAAAAAGT and gccccg ggATTTCTATTGTTCCATATAT carrying *Xho*I and *Sma*I restriction sites (italic), respectively. *ACR3::YFP* was transiently expressed in *Arabidopsis* protoplasts according to Bart *et al.*, (2006), and the ACR3 localization was imaged by laser scanning confocal microscopy (Zeiss LSM5 Meta).

Results

ScACR3 expresses in plant plasma membranes

Before studying any physiological effects, it is necessary to establish whether the yeast-derived ACR3 protein expresses in plant cells and, if so, in which cellular compartment. For this purpose, we prepared (C-terminal) ACR3–yellow fluorescent protein (YFP) fusions. Transient expression in protoplasts from *Arabidopsis* showed that the YFP signal localized at the periphery of the cell (Fig. 1b). This suggests that, as is the case in yeast, ACR3 is most probably localized to the plasma membrane. To confirm the plasma membrane localization of ACR3, protoplasts were co-transformed with a plasma membrane marker, the aquaporin AtPIP2;1 (Santoni *et al.*, 2006), fused to the reporter cyan fluorescent protein (CFP) (Fig. 1c). Combining the YFP and CFP signals shows that the images for PIP2;1 and ACR3 overlap (Fig. 1d), providing proof that in plants, too, ACR3 localizes to the plasma membrane. Parallel experiments with N-terminal YFP fusions produced similar plasma membrane expression of ACR3 (data not shown).

Although absent from higher plants, it has been reported recently that ACR3-type transporters are present in the fern *Pteris vittata* (Indriolo *et al.*, 2010). In this plant, ACR3-type transporters reside in the tonoplast. We therefore tested for the presence of the yeast ACR3 in the tonoplast by determining whether there was any fluorescence apparent in the released vacuoles after osmotic disruption of ACR3–YFP protoplasts (Maathuis, 2011). No fluorescence signal was detected in the vacuolar compartment (data not shown).

ACR3 expression improves tolerance to As at the cellular level

To assess how plant growth is affected by the heterologous expression of the yeast transporter ACR3, stably transformed Arabidopsis wild-type plants were generated. We also transformed the loss-of-function mutant *nip7;1* with *ScACR3* as this genotype has been shown previously to be more tolerant to As^{III} (Isayenkov & Maathuis, 2008). In each of these two backgrounds, several transgenic lines were identified. Two lines in the wild-type background (ACR-W1 and ACR-W2) and two lines in the nip7;1 background (ACR-N1 and ACR-N2) were used for further experimentation.

Whole-plant phenotypes can be difficult to interpret because of large degrees of cell specialization and complicated interactions at the cell, tissue and organ level. We therefore first evaluated how ACR3 affects cellular properties. To this purpose, we used protoplasts, cells without cell walls that carry out most normal physiological functions. To study the effect of ACR3 expression at the cellular level, protoplasts isolated from the various genotypes were exposed to As^{III} and As^{V}. We found that very high concentrations of As were required to induce significant cell death (based on Evans blue staining) within 24 h. Protoplasts

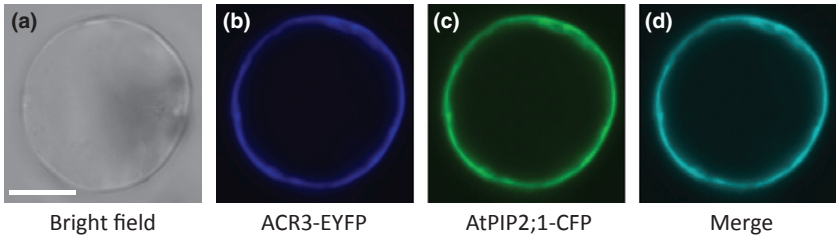

Fig. 1 Yeast ACR3 localizes to the plasma membrane. Protoplasts from *Arabidopsis thaliana* were transiently cotransformed with ACR3–yellow fluorescent protein (YFP) reporter fusions (ACR3–EYFP) and the plasma membrane marker PIP2;1 (PIP2;1–CFP). (a) Bright field image of protoplast. (b) EYFP fluorescence signal showing expression at the cell periphery. (c) Cyan fluorescent protein (CFP) fluorescence signal derived from the marker PIP2;1–CFP. (d) After signal deconvolution, the YFP and CFP signals were merged, showing that the ACR3 expression pattern overlaps with that of PIP2;1. Bar, 10 μm.

from all transgenic lines showed more tolerance to As^{III} (3.5 and 7 mM) and As^{V} (190 mM) relative to the nontransgenic controls (Fig. 2).

To assess whether the increased tolerance of transgenic protoplasts was linked to ACR3 efflux activity, we measured As efflux from protoplasts that were preloaded by exposure to As^{V} (2 mM) for 2 h. Fig. 3 shows that transgenic protoplasts in the wild-type background released more As than control protoplasts, measured after both 3 h and 6 h. Transgenic protoplasts in the *nip7;1* background did not show significant changes in As efflux when measured after 3 h, but did show significantly higher concentrations in the external medium after 6 h.

In combination, these data provide good evidence that the expression of ACR3 improves cell tolerance to As, and that this is likely to be a result of increased As efflux.

ACR3 expression affects tolerance to As in intact plants

To assess how plant growth is affected by the heterologous expression of the yeast transporter ACR3, the growth of seedlings and mature plants was followed in the presence of various concentrations of As^{III} and As^{V}. Fig. 4(a) shows the relative fresh weights of plants grown on agar plates. In the presence of As^{III} (10 μM), wild-type growth dropped by *c.* 70% compared with control conditions, whereas that of ACR-W1 was reduced by only *c.* 55%. However, the growth of ACR-W2 was depressed

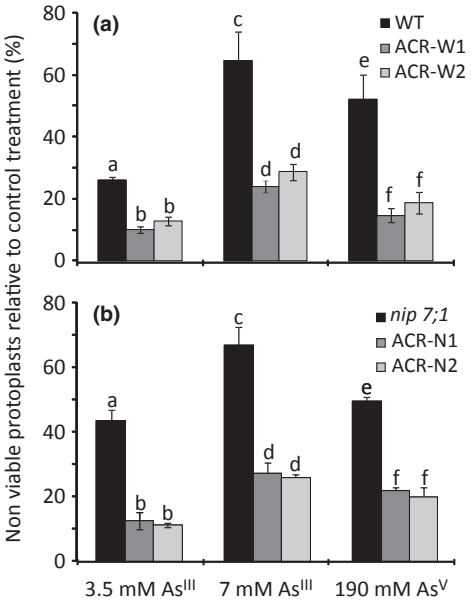

Fig. 2 Transgenic protoplasts are more tolerant to As^{III} and As^{V}. Protoplasts isolated from *Arabidopsis thaliana* nontransgenic control plants (wild-type (WT) and *nip7;1*) and from ACR3-expressing lines (ACR-W1, ACR-W2, ACR-N1 and ACR-N2) were exposed to As^{III} or As^{V} for 24 h. (a) The proportion of viable WT control and transgenic protoplasts was determined at the end of exposure using Evans blue as a vitality stain. (b) The same as (a) for *nip7;1* control and transgenic protoplasts. Data are from five to six independent assays, values are means ± SD, and different letters on the bars indicate significant differences at $P < 0.05$ between genotypes for each treatment using a one-way ANOVA test.

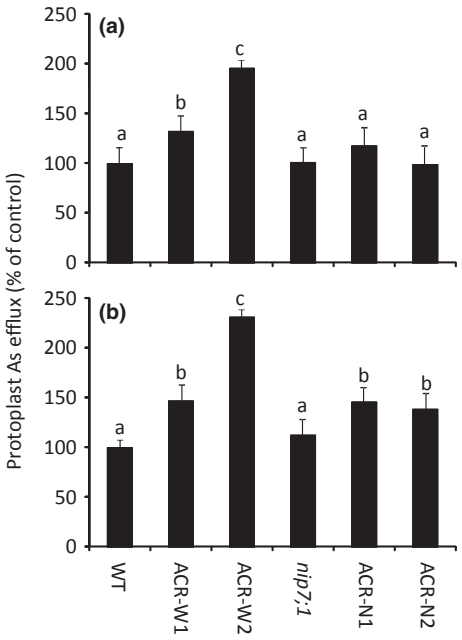

Fig. 3 Arsenic efflux in nontransformed and transgenic protoplasts. Protoplasts isolated from *Arabidopsis thaliana* nontransgenic control plants (wild-type (WT) and *nip7;1*) and from ACR3-expressing lines (ACR-W1, ACR-W2, ACR-N1 and ACR-N2) were exposed to As^{V} for 2 h, transferred to As-free buffer, and total As in the external medium was measured after 3 and 6 h. (a) Arsenic levels in the external medium after 3 h, relative to that found for wild-type protoplasts (actual flux: 24.2 ng 10^6 protoplasts^{-1} h^{-1}). (b) Arsenic levels in the external medium after 6 h, relative to that found for wild-type protoplasts (actual flux: 14.3 ng 10^6 protoplasts^{-1} h^{-1}). Data are from three to four independent assays, values are means ± SD, and different letters on the bars indicate significant differences at $P < 0.05$ using a one-way ANOVA test.

even further than that of the wild-type. At 160 μM, As^{V} caused similar levels of growth retardation as with 10 μM As^{III} and, in this condition, the growth of line ACR-W1 was again significantly better than that of the wild-type, whereas the growth of line ACR-W2 was comparable with that of the wild-type. Fig. 4(b) shows that the *nip7;1* mutant grows better than wild-type plants in the presence of As^{III} and As^{V}, as reported previously (Isayenkov & Maathuis, 2008). The expression of ACR3 in this background further increased the tolerance for both line ACR-N1 and ACR-N2, but to a lesser extent than that seen for line ACR-W1.

The growth pattern observed on agar plates was largely repeated when plants were grown to maturity in hydroponic conditions. RGRs of lines ACR-W1 and both lines ACR-N1 and ACR-N2 were significantly higher than those of their respective wild-type and *nip7;1* controls (Fig. 5), particularly when exposed to As^{V} (160 or 200 μM). Again, the relative enhancement in the growth of ACR-N lines was less pronounced than that observed for line W1 in the same growth conditions.

Heterologous expression of ScACR3 increases As efflux

To investigate the possible mechanism by which ACR3 affects whole-plant As tolerance, As concentrations of roots and shoots were quantified and As efflux to the external medium was

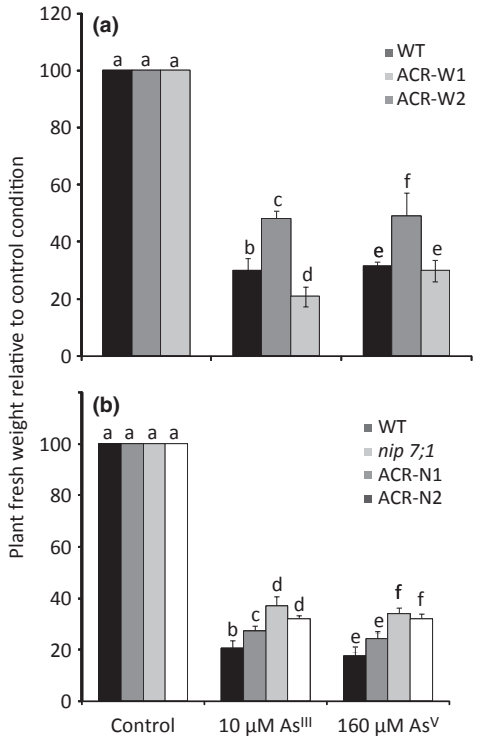

Fig. 4 ACR3 expression affects Arabidopsis seedling growth. Growth of *Arabidopsis thaliana* wild-type (WT) (a) and nip7;1 (b) control lines and two lines expressing the yeast ACR3 efflux system in the WT background (lines ACR-W1 and ACR-W2) and nip7;1 background (lines ACR-N1 and ACR-N2). Plants were grown on agar plates from seed on medium without (control) and with As^{III} (10 μM) or As^{V} (160 μM) for 2 wk. Fresh weight was measured and is expressed relative to the control condition. Data are from three to four independent assays, values are means ± SD, and different letters on the bars indicate significant differences between genotypes at $P < 0.05$ for each treatment using a one-way ANOVA test.

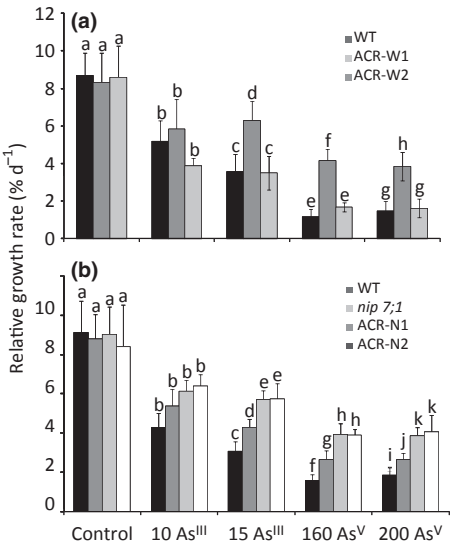

Fig. 5 ACR3 expression affects the growth of mature Arabidopsis. The relative growth rates (RGRs) of *Arabidopsis thaliana* wild-type (WT) (a) and *nip7;1* (b) control lines and two lines expressing the yeast ACR3 efflux system in the WT background (lines W1 and W2) and nip7;1 background (lines W1 and W2). Two-week-old plants were grown in hydroponic culture for 2 wk in medium without (control) and with As^{III} (10 or 15 μM) or As^{V} (160 or 200 μM). Data are from three to four independent assays, values are means ± SD, and different letters on the bars indicate significant differences between genotypes at $P < 0.05$ for each treatment using a one-way ANOVA test.

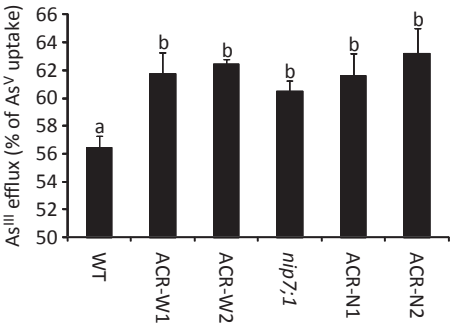

Fig. 6 ACR3 expression increases As^{III} efflux. As^{III} efflux as a percentage of As^{V} uptake for *Arabidopsis thaliana* control and transgenic lines. Plants were exposed to short-term (24-h) As^{V} treatment and the appearance of As^{III} in the medium was determined. Data are from three independent assays, values are means ± SD, and different letters on the bars indicate significant differences at $P < 0.05$ between genotypes using a one-way ANOVA test. WT, wild-type.

measured. Plants were exposed to a relatively low level (5 μM) of As^{V} for 24 h, after which As^{III} and As^{V} were measured in both plants and the external medium. The production of As^{III} in the nutrient solution has been shown to be the result of As^{III} efflux by plant roots following As^{V} uptake and reduction in the root cells (Xu *et al.*, 2007). Fig. 6 shows that nontransgenic Arabidopsis plants recycle a large proportion (*c.* 56%) of As^{V} that enters the symplast into the external medium as As^{III}, a phenomenon which has also been observed in other plants (Xu *et al.*, 2007; Li *et al.*, 2009; Liu *et al.*, 2010). However, in the transgenic lines in the wild-type background, this efflux capacity was significantly higher than in the control line. Although a similar trend was observed for control and transgenic lines in the *nip7;1* background, the efflux increases in lines ACR-N1 and ACR-N2 were not significant. Overall, these data suggest that the presence of ACR3 increases the efflux of As to the external medium.

Most of the As (92–95%) found in roots and shoots was in the form of As^{III}. Fig. 7(a) shows that the roots of transgenic lines in the wild-type background had reduced levels of As. In the *nip7;1* control and associated transgenic lines, root As levels were comparable. In shoots, three of four transgenic lines showed a higher total As concentration (Fig. 7b), whereas that in line ACR-N2 was considerable lower. Although these changes in tissue levels were moderate, they altered significantly the shoot : root distribution of As within plants (Fig. 7c), with three of four transgenic lines showing a large increase in the partitioning ratio. The results imply that, in addition to augmented As efflux (Fig. 6), ACR3 promotes the transfer of As^{III} from root to shoot tissue (Fig. 7c).

Discussion

Arsenic is toxic to all living organisms, including plants. Consequently, agricultural production is inhibited in areas in which As

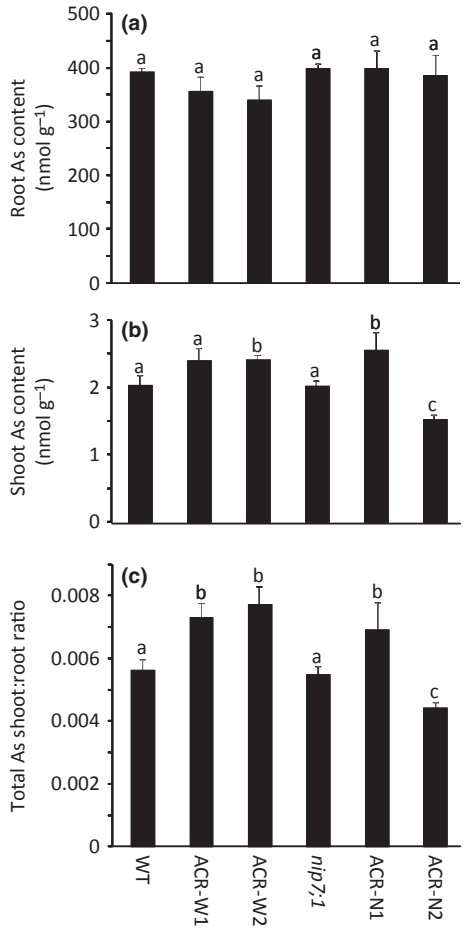

Fig. 7 Total As in root and shoot tissue is altered in ACR3-expressing plants. *Arabidopsis thaliana* plants were exposed to 5 μM As^V for 24 h, after which As concentrations in roots (a) and shoots (b) were measured for control plants (wild-type (WT) and *nip7;1*) and transgenic lines expressing ACR3 (ACR3-W1, ACR3-W2, ACR3-N1 and ACR3-N2). The shoot : root ratio of total As contents is shown in (c). Data are from three to four independent experiments, values are means ± SD, and different letters on the bars indicate significant differences at $P < 0.05$ between genotypes using a one-way ANOVA test.

toxicity is prevalent, such as South-East Asia (Panaullah *et al.*, 2009). Crop plants, especially rice, are also an important route for the transfer of environmental As to humans (Meharg *et al.*, 2009; Zhao *et al.*, 2010a). A lower overall As load in plants is therefore likely to reduce As-induced growth inhibition and would also limit potential human exposure.

The toxicity of As manifests itself mainly in the cytoplasm and, to achieve a reduction in As in this compartment, As influx must be restricted, its efflux must be increased or a mixture of the two will be required. Cytoplasmic efflux can be directed to the vacuole or the apoplast. Recently, the molecular identity of several tonoplast transporters has been reported which are involved in the vacuolar sequestration of As: the ABC transporters AtABCC1 and AtABCC2, which mediate the vacuolar deposition of As^{III}–phytochelatin complexes (Song *et al.*, 2010), and antiport mechanisms similar to the yeast ACR3, which sequester free As^{III} in the vacuoles in lower plants (Indriolo *et al.*, 2010). As is the case for most harmful substances, the majority of symplastic As remains in the root, and vacuolar sequestration is therefore ultimately limited by the relatively small volume of the root. Not surprisingly, plants possess many clearly defined transporters in root cells for the movement of harmful substances to the apoplast (for a review, see Meharg, 2005). For the removal of cytoplasmic As to the external medium, no dedicated mechanisms have been identified in plants. Nevertheless, As^{III} efflux has been reported in many plant species (Xu *et al.*, 2007; Su *et al.*, 2010, Zhao *et al.*, 2010b), but the physiological relevance of this process remains to be assessed. In an attempt to increase As efflux, we used a transgenic approach based on the heterologous expression in Arabidopsis of a well-characterized yeast As^{III} efflux system, ScACR3.

ACR3 increases As tolerance in cells and intact plants

Protoplasts from all transgenic lines showed greater viability and As extrusion relative to their respective controls. Thus, at the cellular level, ACR3 expression improves tolerance, presumably through larger As efflux, in all transgenics. In intact plants, three of four of the Arabidopsis lines that expressed the yeast ACR3 under the control of a strong promoter showed better growth in the presence of As stress, whether in the form of As^V, which is rapidly reduced to As^{III} in the cytosol, or in the form of As^{III} (Figs 4, 5). In addition, the As efflux capacity of transgenic plants was increased significantly in transgenic lines in the wild-type background, but not in transgenic lines in the *nip7;1* background (Fig. 6). These findings show that the presence of ACR3 can positively affect plant tolerance, but the level of tolerance may vary depending on the transgenic line, and may be based on different underlying mechanisms.

The overall growth phenotype is probably the result of multiple aspects, and so there may be several factors that contribute to the above-described variation. For example, increased efflux would lower the cytoplasmic As content and, as such, reduce As toxicity and promote growth. However, constitutive As efflux could lead to local apoplastic accumulation around tissues and cells that are particularly sensitive, and thus have a negative impact on systemic tolerance. Enhanced As^{III} efflux could increase As loading into the xylem vessels in roots, resulting in increased translocation of As^{III} from roots to shoots (Fig. 7). Altered distribution between tissues could either benefit or harm growth and tolerance. For example, protoplasts from line ACR3-W2 showed better As resistance and higher As efflux, but intact ACR3-W2 plants did not show any improvement in growth, irrespective of growth method and As stress. Indeed, occasionally, this line performed worse than the wild-type controls (Fig. 4a). Of all the transgenic lines, line ACR3-W2 showed the lowest ACR3 expression level (*c.* five- and two-fold lower, respectively, than lines ACR3-W1 and ACR3-N; Supporting Information Fig. S1). This could affect expression patterns or cause local differences in ACR3 functioning, preventing an overall net positive impact of ACR3 function in intact plants. In ACR3-W2 protoplasts, As extrusion is into an 'infinite' external medium, and higher level interactions between different compartments, cells or tissues do not occur.

The transgenic lines ACR-N1 and ACR-N2 showed a growth advantage, but no significant increases in As efflux in intact plants. The latter may be a result of an already greater As efflux in *nip7;1* plants relative to wild-type plants (Fig. 6). As it has been suggested that NIPs are bidirectional with respect to their As transport (Bienert *et al.*, 2008; Isayenkov & Maathuis, 2008), a loss of function would be expected to reduce As^{III} release, but 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 (Fig. 6). 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. The observation that As efflux in the *nip7;1* transgenic plants was not increased significantly suggests that either improved tolerance of these plants relates to phenomena other than As efflux or that efflux is in fact higher in these plants, but the extruded As is largely retained in the apoplast.

ACR3 alters the partitioning of As to root and shoot tissue

In yeast, ACR3 has been shown to be a plasma membrane-localized As^{III} efflux system. Our localization study showed that, in plants, too, the yeast ACR3 is localized in the plasma membrane (Fig. 1). In combination with the increased As^{III} efflux in transgenic protoplasts (Fig. 3) and from roots of several transgenic lines (Fig. 6), it is likely that ACR3 in plants functions in a similar manner as in yeast, that is, the removal of cytoplasmic As^{III} to the apoplast. The lower total As in roots of some transgenic lines is also in agreement with this notion. However, the reduction in total tissue As levels was moderate, which may point to retention of extruded As in the apoplast. However, there are also other processes affected by ACR3 activity: the increased shoot As levels suggest that ACR3 expression enhances As translocation from root to shoot. In general, excess (heavy) metals and metalloids are prevented from entering the shoot, possibly to protect photosynthetic tissues. For As, this strategy is obvious from the very low (*c.* 0.005) shoot : root ratios that are obtained for total As (Fig. 6), although these would increase after more prolonged exposure to As. The expression of ACR3 in xylem parenchyma cells could contribute to xylem As^{III} loading and, as such, alter the shoot : root ratio. The fact that lines ACR-W1, ACR-W2 and ACR-N1 not only have increased shoot : root ratios, but also show better growth, suggests that, within the measured range of *c.* 0.005–0.008, increased movement of As to the shoot is not detrimental to overall tolerance, possibly because ACR3 expression in shoot tissue contributes to apoplastic deposition of shoot As.

Conclusions

The present study shows that the expression of the yeast ACR3 arsenite export system in Arabidopsis improves tolerance at both the cellular and whole-plant level. The underlying mechanism is a result, at least in part, of greater As^{III} efflux into the apoplast and/or external medium, which detoxifies the cytoplasm. Interestingly, a recent study in which ScACR3 was expressed in rice (Duan *et al.*, 2011) also showed that ScACR3 increases efflux. Unfortunately, these authors did not show any growth data, and hence it is not clear whether any increased rice tolerance was achieved, but tissue levels of As were reduced, as was the level of As in the grain for two of three overexpressing lines. Duan *et al.* (2011) found a greater increase in As efflux in transgenics than reported here, but no change in root–shoot partitioning. Thus, the effects of the heterologous expression of ScACR3 depend on many factors, including plant species, developmental stage and tissue complexity, but, overall, the use of well-characterized As transporters from bacteria and fungi could be a useful tool for the future improvement of crops. This approach would enhance the growth potential of crops in environments in which As is present at toxic levels, but further fine tuning may be required, for example in the form of tissue-specific promoters, to avoid potentially harmful effects, such as greater root to shoot translocation of As. The latter may lead to increased As accumulation in grain, which is undesirable for food crops. However, increased root to shoot translocation provides a means to increase shoot As content, which could greatly benefit phytoremediation applications.

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References

Ali W, Isayenkov SV, Zhao FJ, Maathuis FJM. 2009. Arsenite transport in plants. *Cellular and Molecular Life Sciences* **66**: 2329–2340.

Bart R, Chern M, Park CJ, Bartley L, Ronald P. 2006. A novel system for gene silencing using siRNAs in rice leaf and stem-derived protoplasts. *Plant Methods* **2**: 13.

Bienert GP, Thorsen M, Schüssler MD, Nilsson HR, Wagner A, Tamás MJ, Jahn TP. 2008. A subgroup of plant aquaporins facilitates the bidirectional diffusion of $As(OH)_3$ and $Sb(OH)_3$ across membranes. *BMC Biology* **6**: 26–33.

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

Clough SJ, Bent AF. 1998. Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant Journal* **16**: 735–743.

Duan G, Kamiya T, Ishikawa S, Arao T, Fujiwara T. 2011. Expressing ScACR3 in rice enhanced arsenite efflux and reduced arsenic accumulation in rice grains. *Plant and Cell Physiology* **53**: 154–163.

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

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

Kamiya T, Tanaka M, Mitani N, Ma FJ, Maeshima M, Fujiwara T. 2009. NIP1;1, an aquaporin homolog, determines the arsenite sensitivity of *Arabidopsis thaliana*. *Journal of Biological Chemistry* **284**: 2114–2120.

Li RY, Ago Y, Liu WJ, Mitani N, Feldmann J, McGrath SP, Ma JF, Zhao FJ. 2009. The rice aquaporin Lsi1 mediates uptake of methylated arsenic species. *Plant Physiology* **150**: 2071–2080.

Liu WJ, Wood BA, Raab A, McGrath SP, Zhao FJ, Feldmann J. 2010. Complexation of arsenite with phytochelatins reduces arsenite efflux and

translocation from roots to shoots in *Arabidopsis*. *Plant Physiology* **152**: 2211–2221.

Ma JF, Yamaji N, Mitani N, Xiao XY, McGrath SP, Zhao FJ. 2008. Transporters of arsenite in rice and their role in arsenic accumulation in rice grain. *Proceedings of the National Academy of Sciences, USA* **105**: 9931–9935.

Maathuis FJM. 2011. Vacuolar two pore K^+ channels act as vacuolar osmosensors. *New Phytologist* **191**: 84–91.

Maathuis FJM, May ST, Graham W, Moone HC, Trimmer PB, Bennett MJ, Sanders D, White PJ. 1998. Cell-marking in *Arabidopsis thaliana* and its application to patch clamp studies. *Plant Journal* **15**: 843–851.

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

Meharg AA. 2005. Mechanisms of plant resistance to metal and metalloid ions and potential biotechnological applications. *Plant and Soil* **274**: 163–174.

Meharg AA, Hartley-Whitaker J. 2002. Arsenic uptake and metabolism in arsenic resistant and nonresistant plant species. *New Phytologist* **154**: 429–432.

Meharg AA, Williams PN, Adomako E, Lawgali YY, Deacon C, Villada A, Cambell RCJ, Sun G, Zhu YG, Feldmann J *et al.* 2009. Geographical variation in total and inorganic arsenic content of polished (white) rice. *Environmental Science and Technology* **43**: 1612–1617.

Nordstrom DK. 2002. Public health – worldwide occurrences of arsenic in ground water. *Science* **296**: 2143–2145.

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

Poorter H, Garnier E. 1996. Plant growth analysis: an evaluation of experimental design and computational methods. *Journal of Experimental Botany* **47**: 1343–1351.

Rosen BP. 2002. Transport and detoxification systems for transition metals, heavy metals and metalloids in eukaryotic and prokaryotic microbes. *Comparative Biochemistry and Physiology. Part A. Molecular & Integrative Physiology* **133**: 689–693.

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

Song W, Park J, Mendoza-Cozatl DG, Suter-Grotemeyer M, Shim D, Hoertensteiner S, Geisler M, Weder B, Rea PA, Rentsch D *et al.* 2010. Arsenic tolerance in *Arabidopsis* is mediated by two ABCC-type phytochelatin transporters. *Proceedings of the National Academy of Sciences, USA* **107**: 21187–21192.

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

Tripathi RD, Srivastava S, Mishra S, Singh N, Tuli R, Gupta DK, Maathuis FJM. 2007. Arsenic hazards: strategies for tolerance and remediation by plants. *Trends in Biotechnology* **25**: 158–165.

Ullrich-Eberius C, Sanz A, Novacky AJ. 1989. Evaluation of arsenate- and vanadate-associated changes of electrical membrane potential and phosphate transport in *Lemna gibba* G1. *Journal of Experimental Botany* **40**: 119–128.

Wu ZC, Ren HY, McGrath SP, Wu P, Zhao FJ. 2011. Investigating the contribution of the phosphate transport pathway to arsenic accumulation in rice. *Plant Physiology* **157**: 498–508.

Wysocki R, Bobrowicz P, Ulaszewski S. 1997. The *Saccharomyces cerevisiae* ACR3 gene encodes a putative membrane protein involved in arsenite transport. *Journal of Biological Chemistry* **272**: 30061–30066.

Xu XY, McGrath SP, Zhao FJ. 2007. Rapid reduction of arsenate in the medium mediated by plant roots. *New Phytologist* **176**: 590–599.

Zhao FJ, Ago Y, Mitani N, Li RY, Su YH, Yamaji N, McGrath SP, Ma JF. 2010b. The role of the rice aquaporin Lsi1 in arsenite efflux from roots. *New Phytologist* **186**: 392–399.

Zhao FJ, McGrath SP, Meharg AA. 2010a. Arsenic as a food-chain contaminant: mechanisms of plant uptake and metabolism and mitigation strategies. *Annual Review of Plant Biology* **61**: 535–559.

Supporting Information

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

Fig. S1 ScACR3 transcript levels.

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