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Enhanced arsenate reduction by a CDC25-like tyrosine phosphatase explains increased phytochelatin accumulation in arsenate-tolerant *Holcus lanatus*

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Summary

Decreased arsenate [As(V)] uptake is the major mechanism of naturally selected As(V) hypertolerance in plants. However, As(V)-hypertolerant ecotypes also show enhanced rates of phytochelatin (PC) accumulation, suggesting that improved sequestration might additionally contribute to the hypertolerance phenotype. Here, we show that enhanced PC-based sequestration in As(V)-hypertolerant *Holcus lanatus* is not due to an enhanced capacity for PC synthesis as such, but to increased As(V) reductase activity. Vacuolar transport of arsenite-thiol complexes was equal in both ecotypes. Based on homology with the yeast As(V) reductase, Acr2p, we identified a Cdc25-like plant candidate, HlAsr, and confirmed the As(V) reductase activity of both HlAsr and the homologous protein from *Arabidopsis thaliana*. The gene appeared to be As(V)-inducible and its expression was enhanced in the As(V)-hypertolerant *H. lanatus* ecotype, compared with the non-tolerant ecotype. Homologous ectopic overexpression of the At*ASR* cDNA in *A. thaliana* produced a dual phenotype. It improved tolerance to mildly toxic levels of As(V) exposure, but caused hypersensitivity to more toxic levels. Arabidopsis *asr* T-DNA mutants showed increased As(V) sensitivity at low exposure levels and enhanced arsenic retention in the root. It is argued that, next to decreased uptake, enhanced expression of Hl*ASR* might act as an additional determinant of As(V) hypertolerance and As transport in *H. lanatus*.

Keywords: arsenate tolerance, arsenate reductase, CDC25, phytochelatin synthase, *Holcus lanatus*, vacuolar transport.

Introduction

During the past century, anthropogenic activities such as mining, land irrigation, the use of arsenic based pesticides, manufacturing and waste disposal have locally led to severe arsenic contamination (Sandberg and Allen, 1975). Inorganic arsenate [As(V)] and arsenite [As(III)] are the predominant plant-available forms of arsenic in oxic and anoxic soils, respectively (Haswell *et al.*, 1985; Onken and Hossner, 1995; Porter and Peterson, 1977). As(V) is a chemical analogue of phosphate and is taken up into plant roots by the high-affinity phosphate uptake system (Asher and Reay, 1979). Once inside the plant, As(V) can exert toxicity itself, e.g. through phosphate substitution in phosphorylation reactions, or after reduction to As(III), which is more toxic than As(V) due to its high affinity for protein thiols (Jocelyn, 1972). Arsenite is taken up at considerable rates as well (Abedin *et al.*, 2002), mainly via aquaglyceroporins (Meharg and Jardine, 2003; Wysocki *et al.*, 2001).

Plant populations from strongly As(V)-enriched soils have been shown to exhibit substantially enhanced levels of As(V) tolerance (De Koe and Jacques, 1993; Macnair and Cumbes, 1987; Rocovich and West, 1975). In most of the plant species investigated thus far, As(V) hypertolerance appeared to be associated with decreased As(V) uptake through suppression of the high-affinity phosphate uptake system (Bleeker *et al.*, 2003; Meharg, 1994; Meharg and Macnair, 1992a). Nevertheless, when growing in heavily As-contaminated

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soil, hypertolerant plants accumulate arsenic at considerable concentrations in their tissues (De Koe, 1994). The apparent absence of damage presupposes the existence of effective plant-internal sequestration machinery. Suppression of the high-affinity phosphate uptake system is doubtlessly the major genetic determinant of As(V) hypertolerance in *Holcus lanatus* (Meharg and Macnair, 1992a). However, there seem to be additional determinants with smaller but still considerable effects (Macnair *et al.*, 1992). It is conceivable that these so-called 'modifiers' are involved in the control of the plant-internal As(V) detoxification machinery (Meharg, 1994; Meharg and Hartley-Whitaker, 2002).

Paradoxically, the first step in As(V) detoxification, i.e. reduction to As(III), is toxifying as such, because As(III) is more cytotoxic than As(V) (Bertolero *et al.*, 1987). Nevertheless, As(V) reduction is essential for further sequestration. Only As(III) can form complexes with low molecular metal-binding thiolic peptides, such as glutathione (GSH) or, in plants and several fungi, phytochelatins (PCs). In addition, transporters that efflux arsenic from the cytosolic compartment, either into the vacuole or out of the cell, use As(III) as a substrate, usually in the form of a triglutathione complex, As(III)-$(GS)_3$ (Gosh *et al.*, 1999; Rosen, 2002). In plants and a number of fungi, As(III) may be transported into the vacuole as a PC complex as shown for cadmium (Cd) PC complexes (Salt and Rauser, 1995). In cells, As(V) will be non-enzymatically reduced by GSH. However, this is a relatively slow process. Bacteria and yeasts possess effective As(V) reductases, which have been shown to be essential for As(V) tolerance (Bobrowicz *et al.*, 1997; Gladysheva *et al.*, 1994; Ji *et al.*, 1994). In *Aspergillus* sp., enhanced As(V)-inducible As(V) reduction has been proposed to be involved in As(V) hypertolerance in the P37 strain (Cánovas *et al.*, 2003). The only eukaryotic As(V) reductase known so far (Acr2p) belongs to the 'rhodanese/Cdc25-phosphatase superfamily' (Bordo and Bork, 2002). Plant genomes contain homologues of the yeast As(V) reductase gene, *Acr2*, suggesting that enzyme-catalyzed As(V) reduction occurs in plants as well. The latter has been recently confirmed by Duan *et al.* (2005), who demonstrated GSH-dependent As(V) reductase activity in root extracts of *Pteris vittata*, an arsenic hyperaccumulating fern. The enzyme involved appeared to be induced by plant exposure to As(V).

Phytochelatins are metal-binding peptides with the general structure $(\gamma\text{-Glu-Cys})_n$-Gly, where $n = 2$–11 (Grill *et al.*, 1985). They are synthesized from GSH by a γ-Glu-Cys transpeptidase, called phytochelatin synthase (PCS; Grill *et al.*, 1989). Phytochelatin synthase-deficient *Arabidopsis thaliana* is hypersensitive to Cd, mercury (Hg) and As(V), showing that PCs are absolutely required for As(V) tolerance in this species (Ha *et al.*, 1999; Howden *et al.*, 1995). Also, inhibition of PC synthesis by buthionine sulfoximine (BSO) almost completely abolished tolerance to As(V) in *H. lanatus*, both in a non-metallicolous (NM) ecotype with normal As(V) sensitivity and a metallicolous (M) ecotype with hypertolerance to As(V) (Hartley-Whitaker *et al.*, 2001; Schat *et al.*, 2002). In addition, when grown at their individual 50% root growth inhibition (50% effect concentration, EC_{50}) exposure levels, As(V)-hypertolerant *H. lanatus* ecotypes exhibited elevated root-internal PC-thiol to As molar ratios, as compared with NM ecotypes (Hartley-Whitaker *et al.*, 2001). Also, in *Cytisus striatus*, As(V) hypertolerance was associated with enhanced PC chain length (Bleeker *et al.*, 2003). These findings suggest that, next to decreased As(V) uptake, enhanced PC-based sequestration plays a role in As(V) hypertolerance in these species. If the capacity for PC synthesis itself would be rate-limiting in arsenic sequestration, then As(V) hypertolerance should be associated with enhanced As(III) tolerance, because only As(III) can activate PCS, bind to PCs (Maier *et al.*, 2003) and form the As(III)-GS_3 complex, the high-affinity substrate for PCS (Vatamaniuk *et al.*, 2000).

Enhanced As(V)-induced PC accumulation, such as found in As(V)-hypertolerant *H. lanatus* (see above), can be explained either by an increased capacity for PC synthesis as such, or by enhanced As(V) reduction, which triggers PCS activity. Enhanced As(V) reduction could result from elevated expression of an As(V) reductase, or from increased cellular GSH levels, particularly because GSH is not only the major non-enzymatic As(V) reductant in cells (Jocelyn, 1972), but also the electron donor in enzyme-catalyzed As(V) reduction (Gladysheva *et al.*, 1994; Mukhopadhyay *et al.*, 2000).

In this study, we aim to assess the potential role for enzymatic As(V) reduction in PC-based arsenic sequestration and As(V) tolerance in *H. lanatus*. To this end, we compared responses to As(V) and As(III) in an NM and an As(V)-hypertolerant ecotype of this species. To reveal if enhanced PC-based sequestration [i.e. the formation and compartmentalization of As(III)-PC] does contribute to As(V) hypertolerance, we measured As(III)-induced PC accumulation and As(III) tolerance in both ecotypes. Also, the kinetic parameters of PCS activity have been compared *in vitro*, using crude root protein extracts. To assess the possible role of differential compartmentalization, we also compared the uptake of free and GSH-complexed As(III) in energized tonoplast-enriched membrane vesicles. Because these experiments clearly demonstrated that enhanced PC accumulation in the hypertolerant ecotype is a mere consequence of an enhanced rate of As(V) reduction, we compared As(V) reductase activities in crude root extracts. Next, the closest homologue of the yeast *Acr2* As(V) reductase gene in *H. lanatus* was cloned. The expression of this gene (Hl*ASR*) was established in both ecotypes by Northern blot analysis. To show As(V)-reductase activity of this type of plant proteins, we constructed a his-tagged version of Hl*ASR* and the Arabidopsis homologue (At*ASR*) and tested the purified proteins for As(V)-reductase activity.

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To assess the role for As(V)-reductase activity in As(V) tolerance, we established the dose-response curves for As(V)-imposed root growth inhibition and arsenic (As) accumulation in *A. thaliana asr* T-DNA insertion mutants, as well as in lines ectopically overexpressing the gene.

Results

Arsenate hypertolerance does not confer arsenite tolerance

To assess As(V) and As(III) tolerance, the root elongation response under arsenic exposure was determined for both NM and M *H. lanatus* under different phosphate supply rates (Figure 1a,b). As expected the M ecotype exhibited enhanced As(V) tolerance (Figure 1a). Furthermore, phosphate (P) supply severely affected As(V) toxicity in NM plants at low external As(V), but only at high As(V) in the M ecotype. Arsenate uptake was higher in NM than in M and, in conformity with root growth performance, suppressed by increased P supply exclusively at low external As(V) in the NM ecotype and at high As(V) in the M ecotype (Figure 2a).

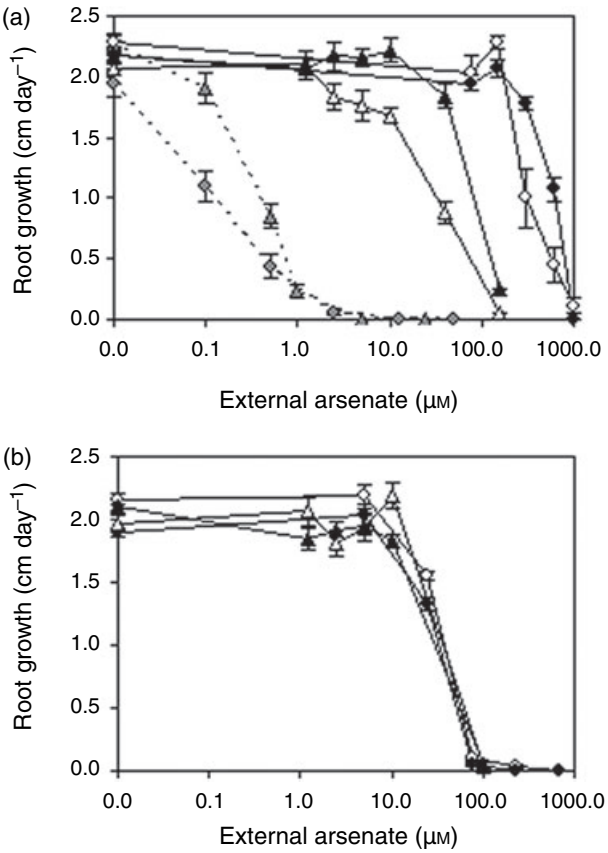

Figure 1. Maximum root growth (cm day^{-1}) of *Holcus lanatus* (mean ± SE, $n = 12$) exposed to (a) arsenate [As(V)] and (b) arsenite [As(III)] for 5 days. Non-metallicolous (NM; triangle) and metallicolous (M; circle) plants at 10 (open symbols) and 100 μM P supply (closed symbols) and 250 μM L-buthioninesulfoximine (dotted lines) are presented.

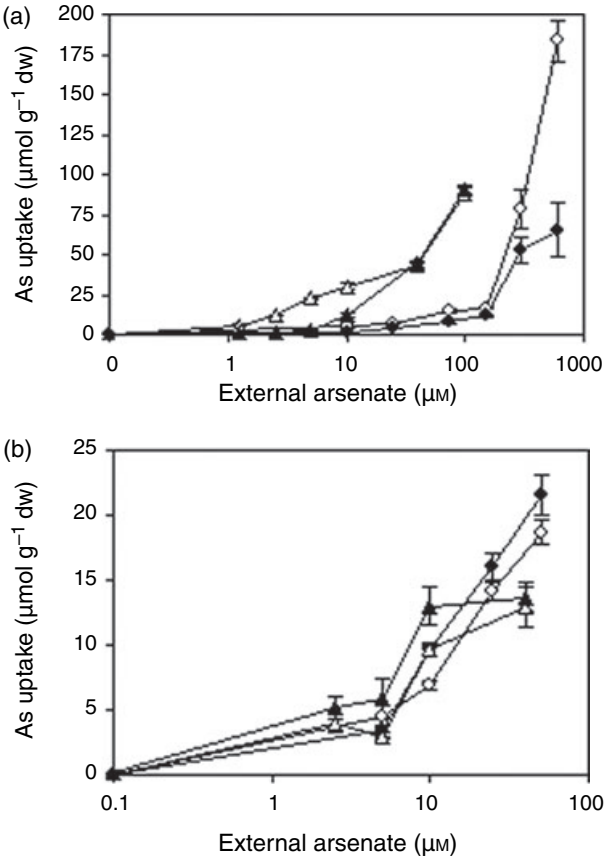

Figure 2. Plant arsenic uptake (μmol g^{-1} root dw) of *Holcus lanatus* (mean SE, $n = 5$) exposed to (a) arsenate [As(V)] and (b) arsenite [As(III)] for 5 days. Non-metallicolous (NM; triangle) and metallicolous (M; circle) plants at 10 (open symbols) and 100 μM P supply (closed symbols) are presented.

Addition of BSO fully abolished PC accumulation (data not shown) and resulted in hypersensitivity in the presence of As(V) concentrations otherwise not affecting root growth (Figure 1a). Remarkably, the M plants were more sensitized than the NM plants ($P < 0.01$), in spite of the lower As(V) uptake in the former ecotype.

Arsenate hypertolerance in the M ecotype did not confer enhanced As(III) tolerance. The inferred EC50 for As(III) were not different between ecotypes and unaffected by P-supply levels (Figure 1b). Also, As(III) uptake was not significantly different between NM and M plants and was independent of P supply (Figure 2b).

Enhanced arsenate reductase activity explains enhanced phytochelatin accumulation

The kinetics of PC synthesis and enzymatic As(V) reduction in crude root protein extracts and the *in planta* PC accumulation patterns under As(V) and As(III) exposure were compared with identify the rate limiting step in PC accumulation. The *in vitro* kinetic analysis demonstrated no differences

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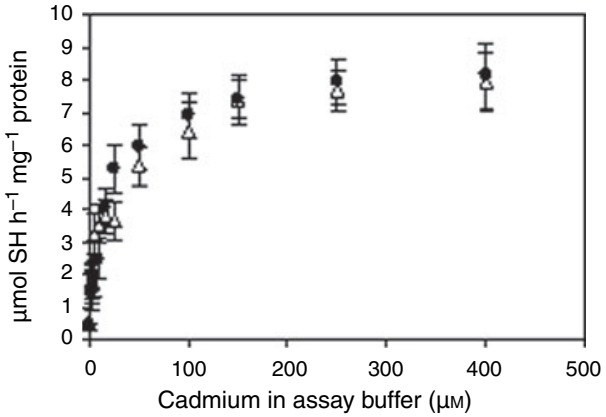

Figure 3. Extractable activity of phytochelatin synthase (PCS; µmol PC-SH h^{-1} mg protein^{-1}) as a function of Cd in the assay buffer, in crude root protein extracts of non-metallicolous (NM; triangle) and metallicolous (M; circle) *Holcus lanatus* (mean ± SE, $n = 5$).
Plants were grown in non-metal amended solution.

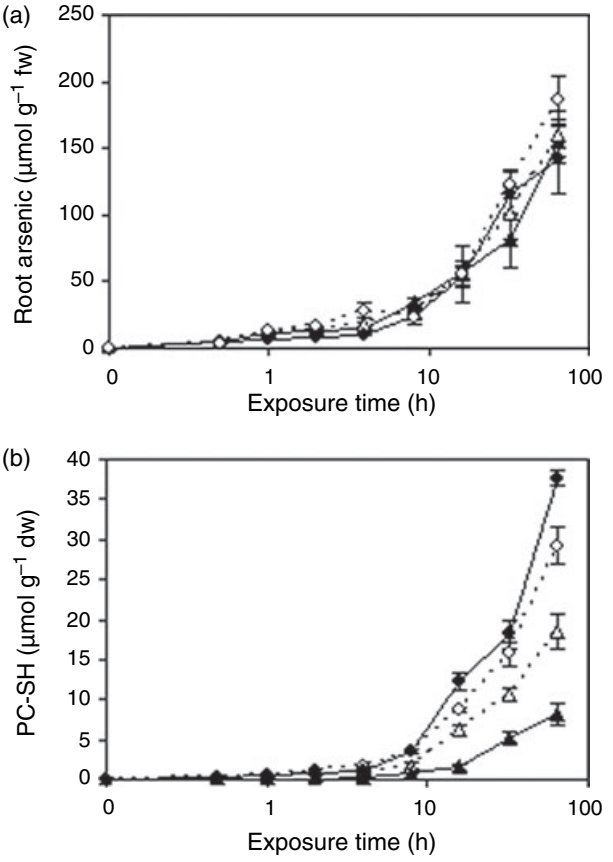

Figure 4. (a) Root As (µmol g^{-1} fw) and (b) total phytochelatin (PC) concentration in roots (µmol PC-SH g^{-1} dw) as a function of exposure time (h) to the individual 50% effective concentrations (EC_{50}) of arsenate [As(V); closed symbols] and an estimated equivalent internal arsenite [As(III); open symbols] in non-metallicolous (NM; triangle) and metallicolous (M; circle) *Holcus lanatus* (mean ± SE, $n = 5$).

between ecotypes in the PCS enzymatic capacity, both with regard to the maximum velocity (V_{max}) and the 50% activation concentration for Cd (Figure 3).

Assuming that the As(V) uptake rates in NM and M plants would be similar at equal degrees of root growth inhibition, total PC accumulation was determined in plants exposed to their respective intrapolated EC50 of As(V). Also, plants were exposed to As(III) levels expected to yield uptake rates comparable to those for As(V) at EC50. Arsenic accumulated at very similar rates in both ecotypes, indeed (Figure 4a). Overall, in the M plants, As(V) and As(III) induced similar degrees of PC accumulation (Figure 4b). In the NM plants, on the other hand, PC accumulation was much lower under As(V) exposure than under As(III) exposure, even though the root-internal arsenic concentrations were comparable (As species × ecotype interaction, $P < 0.001$). Additionally, under As(V) exposure, the formation of longer-chain PCs (PC3 and PC4) started much earlier in the M plants (data not shown). Where substantial concentrations of PC3 could not be detected until 16 h upon exposure in the NM plants, the compound was formed already after 0.5 h in M plants. Moreover, throughout the experiment, considerable PC4 accumulation was measured in M plants only. Under non-exposed conditions, cellular levels of GSH were 40% higher in the M plants: NM, 1.15 µmol g^{-1} dw; and M, 1.84 µmol g^{-1} dw. However the difference disappeared within 4 h under arsenic exposure: NM, 1.12 µmol g^{-1} dw; and M, 0.97 µmol g^{-1} dw.

To test the possibility that differential PC accumulation is due to differential vacuolar transport of thiol-complexed As(III), As(III) accumulation into tonoplast vesicles isolated from NM and M plants was investigated. Although As(III)-PC is doubtlessly the dominant form of thiol-complexed As in plants (Raab *et al.*, 2004), we used GSH-complexed As(III), As(III)-GS_3, as a homologue, because purified PCs were not available in sufficient quantities. The isolation yielded 68% pure tonoplast membrane material forming 69% right-side-out vesicles, as determined by ATP hydrolysis assays for both ecotypes (data not shown). Fluorescence quenching showed the build-up and maintenance of a membrane potential and a pH gradient upon addition of 2 mM magnesium (Mg) ATP. When supplied as As(III)-GS_3, arsenic was accumulated in MgATP-energized vesicles at equal rates in the vesicles isolated from NM and M plants (12.9 ± 0.42 and 14.0 ± 0.79 µmol g^{-1} vesicle protein, respectively; mean ± SE, $n = 7$). In the absence of MgATP, there was no detectable arsenic accumulation. No effects on the pH gradient, nor on the membrane potential were observed when As(III)-GS_3 was supplied. Free As(III) was taken up at a much lower rate (2.8 ± 1.25 µmol g^{-1} protein in both ecotypes). Charge-neutral, ATP-dependent and ΔpH-independent transport of the complex suggests transport via an ATP-binding

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cassette(-)type (ABC) transporter indeed. Conceivably, free As(III) travelled via aquaglyceroporin channels, as shown for plasma-membrane uptake (Meharg and Jardine, 2003). However, the net uptake was too low to allow further characterization of the mechanism.

The above results suggest that differential *in planta* PC accumulation rates result from differential As(V) reduction and neither from differential PCS capacity nor from differential vacuolar transport of thiol-complexed As(III). Therefore, the enzymatic As(V) reduction rates in crude protein extracts, measured as the As(III)-GS_3 formation, were compared (spontaneous reduction by GSH appeared to be negligible). In both ecotypes, the As(V) reductase activity appeared to increase after exposing plants to As(V) ($P < 0.001$). Compared at similar rates of As(V) uptake, the As(V) reductase activity was threefold higher in M plants than in NM plants, whereas the constitutive levels differed less (Table 1), resulting in a significant arsenic × ecotype interaction effect ($P < 0.001$). However, when expressed as 'fold regulation', the ecotype effect remained significant ($P < 0.001$), but the arsenic × ecotype interaction became insignificant, suggesting a 'proportional' As(V)-imposed regulation in both ecotypes.

Table 1 *In vitro* arsenate [As(V)] reduction measured as arsenite [As(III)]–GS_3 formation (mmol g^{-1} h^{-1}) under As(V) incubation in crude root extracts of non-metallicolous (NM) and metallicolous (M) *Holcus lanatus* and wild type (WT) and T-DNA (SALK_143282) and T2 35S:At*ASR Arabidopsis thaliana* lines after 20 min of incubation at 37°C (mean ± SE; $n = 5$); nm = not measured

	0 μM	50% effective concentration (EC50)
Holcus lanatus		
Non-metallicolous (NM)	6.09 ± 1.24	11.06 ± 1.32
Metallicolous (M)	9.99 ± 0.85	33.02 ± 2.41
Arabidopsis thaliana		
Wild type (WT)	4.48 ± 0.37	6.72 ± 0.59
T-DNA	4.52 ± 0.34	4.29 ± 0.26
35S:*AtASR*	9.98 ± 0.67	nm

Reduction of arsenate by a plant Acr2p-like protein

In silico analysis revealed significant homology between the only known As(V)-reductase in eukaryotes, *Saccharomyces cerevisiae* Acr2p and a number of 'rhodanese/Cdc25-like' plant proteins (Figure 5). Therefore, we tested the effect of trinitrobenzenesulfonic acid (TNBS), an inhibitor of rhodanese activity (Prieto *et al.*, 1997), on As(V) reductase activity in both ecotypes. In both cases, reductase activity was approximately 90% inhibited by 10 mM TNBS, suggesting that a rhodanese-like protein might be responsible indeed (data not shown).

To further determine the role of this protein in As(V) hypertolerance in *H. lanatus*, we isolated a full-length cDNA by PCR (see Experimental procedures. Sequence analysis identified a 131-amino-acid, rhodanese/Cdc25-like protein (HlAsr), showing 60.3% amino acid identity with the Arabidopsis protein (Figure 5). No apparent relatives with significant homology to the corresponding Arabidopsis gene, At*ASR*, were present within the Arabidopsis genome. However, the rice genome contained two Asr-like homologues. The $HC(X)_5R$ motif representing the catalytic centre for As(V) reduction in Acr2p (Mukhopadhyay and Rosen, 2001) appears to be conserved in all of them (Figure 5). Primary structure analysis (PSORT, SOSUI) predicted both AtAsr as well as HlAsr to be stable, soluble proteins, located in the plant mitochondrial matrix or in the cytoplasm.

A Northern blot analysis was performed to establish whether HlAsr could be responsible for the enhanced As(V)

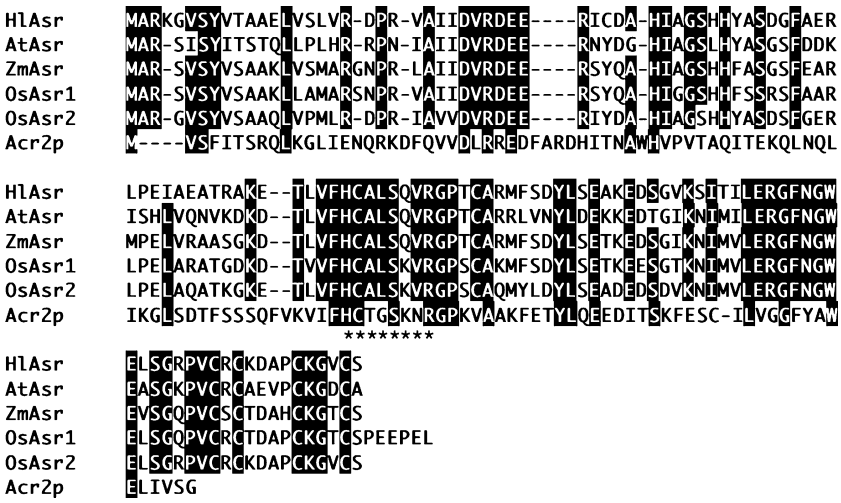

Figure 5. Deduced amino acid sequence alignment showing the position of the consensus $HC(X)_5R$ motif (stars) and *Holcus lanatus* (Hl), *Arabidopsis thaliana* (At), *Zea mays* (Zm), *Oryza sativa* (Os).

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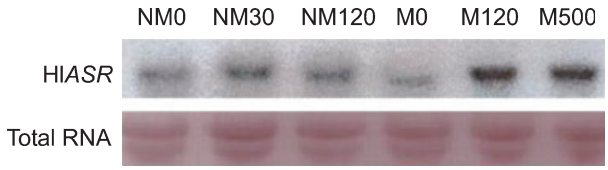

Figure 6. Northern blot analysis of *Holcus lanatus* HI*ASR* gene expression in the absence of arsenate [As(V)] and under comparable levels of As(V) stress for non-metallicolous (NM) and metallicolous (M) *H. lanatus* root after 1-day exposure.
The lower panel shows total RNA as a loading control.

reduction in the M ecotype of *H. lanatus*. The blots contained root RNA of unexposed plants and from plants exposed to different As(V)-exposure levels including the EC50 levels of both ecotypes (Figure 6). The At*ASR* probe used for Northern hybridization appeared to be specific for one gene only, because Southern analysis revealed that the gene was single copy in both NM and M plants (data not shown). Three independent Northern blots all showed the same expression pattern. Transcription of HI*ASR* was induced by As(V) exposure in roots of NM and M. When both were compared at EC50 exposure and at equal concentrations in the growth medium, the gene appeared to be more expressed in the M ecotype.

In order to confirm As(V) reductase activity of the protein, HI*ASR* and the Arabidopsis homolog At*ASR* were expressed in *Escherichia coli* as a fusion protein with an N-terminal His_6-tag and purified by Ni^{2+} affinity chromatography. Spot blotting revealed a limited number of fusion protein fractions. The As(V) reductase activity from the pRSET-HI*ASR* and pRSET-At*ASR* protein fractions were compared with the empty vector fractions. The pRSET-At*ASR* and pRSET-HI*ASR* protein fractions exhibited a 20- to 30-fold increased *in vitro* As(V) reductase activity respectively, compared with the corresponding empty vector fractions (Table 2), demonstrating that both proteins are As(V) reductases indeed.

Arsenate tolerance, accumulation and reduction phenotypes in Atasr T-DNA and 35S:AtASR lines

To reveal a role for At*ASR* in arsenic metabolism *in vivo*, we tested At*asr* T-DNA insertion mutant lines as well as transgenic lines ectopically expressing At*ASR* for As(V) tolerance, arsenic accumulation in the root and shoot, and As(V) reductase activity. Two homozygous T2 lines derived from each of the two available T-DNA insertion mutants were tested against wild type (WT) for root growth under As(V) exposure in separate experiments. Both experiments revealed a significant plant type effect and a significant plant type × exposure level interaction (Table 3). A posteriori comparison of means, using Tukey's method, revealed root growth was significantly inhibited in the T2 lines as compared with WT plants ($P < 0.05$), though exclusively at the lowest As(V) exposure level. Significant differences between the T2 lines were not observed, and the SALK lines and the GABI-Kat lines showed very similar phenotypes (Figure 7a,b).

Ectopic expression of the At*ASR* gene under the 35S promoter in Arabidopsis produced a dual tolerance phenotype (Figure 7c). The three independent 35S:At*ASR* lines tested expressed the gene 20- to 30-fold higher than WT. Under As(V) exposure, they performed better than WT plants, though only at relatively low external As(V) exposure levels; whereas at higher exposure levels, the ectopic-expression lines clearly exhibited hypersensitivity. Statistical analysis revealed significant plant type effects and a significant plant type × exposure level interaction (Table 3). Tukey's a posteriori test demonstrated significant differences ($P < 0.05$) at the 3-, 9- and 81-mM exposure levels between, on the one hand, the three 35S lines and, on the other hand, the WT plants. At the 27-mM exposure level, two of the 35S lines differed significantly from the controls. There were no significant differences between the three 35S lines.

Table 2 *In vitro* enzymatic arsenate [As(V)] reduction as arsenite [As(III)]–GS_3 formation (mmol g^{-1} h^{-1}) in different eluate fractions collected by FPLC in pRSET-A vector, empty or containing the At*ASR*/HI*ASR* gene

Eluate fraction	pRSET empty	pRSET-At*ASR*	pRSET-HI*ASR*
no. 25	0.40	0.74	1.89
no. 26	0.27	0.47	1.42
no. 27	1.07	20.95	32.30
no. 28	0.94	1.62	4.18

Table 3 Two-way ANOVA tables for root elongation in *Arabidopsis thaliana* under exposure to different arsenate [As(V)] concentrations in: (A) wild type (WT) and two T2 At*asr* T-DNA insertion mutant lines (GABI-Kat, ID 772G06); (B) WT and two T2 At*asr* T-DNA insertion mutant lines (SALK_143282); and (C) WT and three T2 35S:At*ASR* lines

Source	d.f.	MS	Fs	*P*-value
A				
Plant type	2	5.46	6.43	<0.01
Exposure level	3*	33.46	38.02	<0.001
Interaction	6	3.29	3.74	<0.01
Error	132	0.88		
B				
Plant type	2	5.76	4.43	<0.05
Exposure level	3*	29.75	22.88	<0.001
Interaction	6	3.86	2.96	<0.01
Error	132	1.30		
C				
Plant type	3	24.92	28.64	<0.001
Exposure level	3*	32.77	37.67	<0.001
Interaction	9	3.36	3.86	<0.01

Untransformed values have been analysed.
*To avoid inhomogeneity of variances, the two highest exposure levels have been excluded from the ANOVA.

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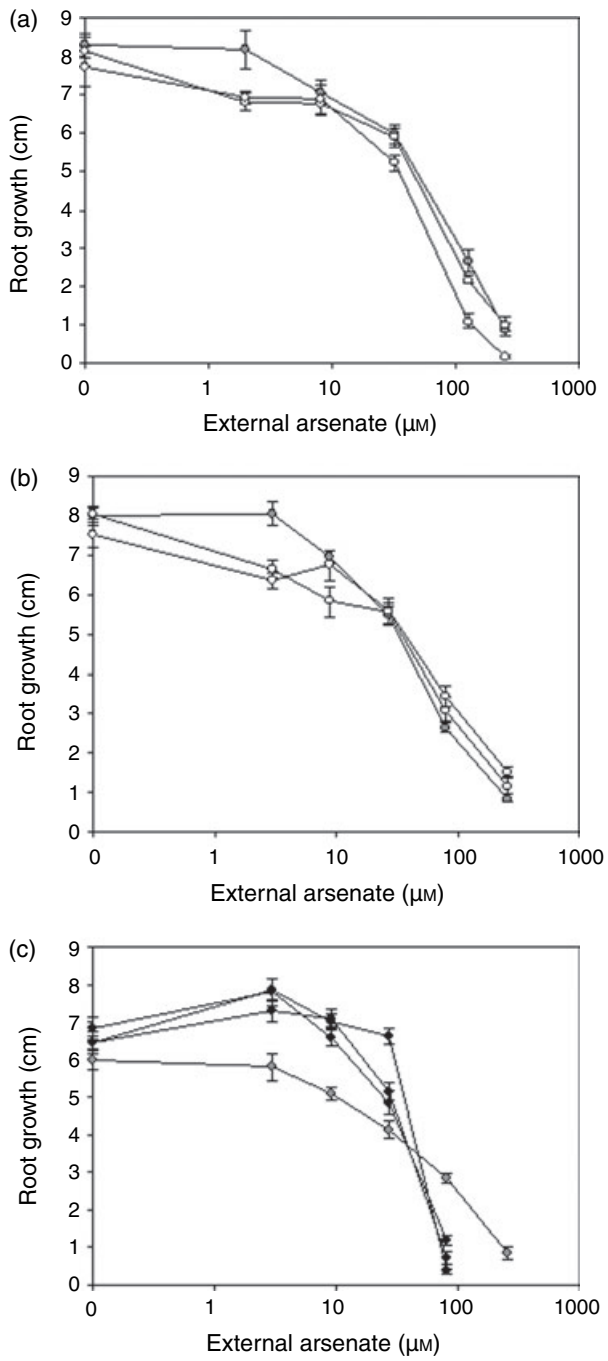

Figure 7. Maximum root growth (cm) of *Arabidopsis thaliana* (mean ± SE, $n = 12$) exposed to arsenate for 5 days. Wild type (WT; grey circles).
(a) Two T2 SALK (143282) T-DNA insertion lines (open circles).
(b) Two T2 GABI-Kat (ID 772G06) T-DNA insertion lines (open circles).
(c) Three T2 35S:At*ASR* transgenic lines (black circles).

The arsenic concentrations in roots and shoots were measured in a single experiment in one of the 35S lines, one of the T2 GABI-Kat T-DNA insertion lines and in WT (Table 4). Statistical analysis demonstrated a significant plant type effect, but no plant type × exposure interaction for shoot arsenic concentration (Table 5). As demonstrated by Tukey's test, at all the As(V) exposure levels the shoot arsenic concentrations were significantly lower ($P < 0.05$) in the T-DNA line than in wild-type plants and in the 35S line. There were no significant differences between the 35S line and the wild-type plants. Total arsenic uptake on a whole plant basis and root arsenic concentrations were not significantly different between the plant types.

The As(V) reductase activities in root protein extracts were measured in one SALK T2 *asr* T-DNA insertion line and in one 35S line. Arsenate reductase activity in the T-DNA line did not differ from WT in non-exposed plants (Table 1). Arsenate exposure significantly increased As(V) reductase activity in WT, but not in the T-DNA line [As(V) × plant type, $P < 0.05$], suggesting that At*ASR* is induced by As(V).

Table 4 Arsenate [As(V)] uptake and arsenic in shoot and root (μmol g^{-1} dw) in wild type (WT) Arabidopsis, an *asr* T-DNA (SALK_143282) insertion mutant line and a 35S:At*ASR* transgenic line, over a range of As(V) for 5 days ($n = 5 \pm$ SE)

	Uptake	Shoot	Root
Wild type (WT)			
0	0.08 ± 0.02	0.01 ± 0.00	0.04 ± 0.01
3	3.11 ± 0.26	0.05 ± 0.01	2.72 ± 0.21
9	6.74 ± 0.89	0.18 ± 0.05	5.49 ± 0.59
27	53.64 ± 4.74	0.46 ± 0.04	51.61 ± 4.72
81	74.74 ± 3.95	1.20 ± 0.41	69.47 ± 4.42
35S:At*ASR*			
0	0.01 ± 0.01	0.01 ± 0.00	0.02 ± 0.00
3	4.02 ± 0.53	0.05 ± 0.01	3.69 ± 0.51
9	6.73 ± 0.55	0.07 ± 0.01	9.06 ± 3.06
27	41.68 ± 7.82	0.35 ± 0.06	36.93 ± 6.31
81	93.11 ± 7.28	1.27 ± 0.38	86.04 ± 6.27
T-DNA			
0	0.03 ± 0.01	0.00 ± 0.00	0.01 ± 0.00
3	1.53 ± 0.05	0.01 ± 0.00	1.45 ± 0.05
9	6.50 ± 0.25	0.03 ± 0.01	6.29 ± 0.25
27	25.06 ± 3.07	0.10 ± 0.01	24.39 ± 3.10
81	68.08 ± 13.73	0.23 ± 0.06	65.88 ± 13.66

Arsenate uptake was calculated as the total plant arsenic burden per gram of root DW.

Table 5 Two-way ANOVA table for shoot arsenic concentrations in an At*ars* T2 T-DNA insertion mutant line (GABI-Kat ID 772G06), a T2 35S:At*ARS* line and in wild type (WT) of *Arabidopsis thaliana* exposed to a series of arsenate [As(V)] concentrations

Source	d.f.	MS	Fs	*P*-value
Plant type	2	2.61	59.30	<0.001
Exposure level	3	4.95	112.41	<0.001
Interaction	6	0.04	1.01	NS
Error	48	0.04		

Values have been log-transformed prior to analysis (NS, not significant).

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Ectopic expression of At*ASR* in Arabidopsis indeed led to enhanced enzymatic As(V) reduction capacity (Table 1).

Discussion

Arsenate uptake and tolerance

In all organisms, As(V) enters the cell via the phosphate transport system (Meharg and Macnair, 1991; Willsky and Malamy, 1980; Yompakdee *et al.*, 1996). As a consequence, As(V) will compete with phosphate for uptake carriers and for binding sites within the plants. Elevated phosphate supply improves As(V) tolerance through suppressing As(V) uptake (Meharg and Macnair, 1992a), or plant-internal competition, such as demonstrated in the Arabidopsis *ars1* mutant with an enhanced phosphate uptake phenotype (Lee *et al.*, 2003). For grasses, it was shown that As(V) hypertolerance is genetically correlated with reduced uptake, through downregulation of the high-affinity phosphate transporters (Meharg and Macnair, 1992b). Segregation analysis in *H. lanatus* backcrosses revealed the involvement of one single gene and one or more modifiers influencing the levels of As(V) hypertolerance (Macnair *et al.*, 1992). The results of this study confirm that the differences in root growth responses to As(V) between M and NM *H. lanatus*, are largely explained by differential As(V) uptake. Also, the patterns of toxicity alleviation by high phosphate supply seem to be largely in line with competitive inhibition of As(V) uptake (compare Figures 1a and 2a).

Arsenate reduction and sequestration

Phytochelatin-based sequestration is considered to be essential for As(V) tolerance in plants. First, under As(V) exposure hypertolerant ecotypes exhibited higher PC-thiol to As ratios compared with non-tolerant ecotypes (Hartley-Whitaker *et al.*, 2001; Schat *et al.*, 2002). Secondly, both hypertolerant and non-tolerant plants exhibited hypersensitivity to As(V) when PC accumulation was abolished by BSO treatment (Hartley-Whitaker *et al.*, 2002; Schat *et al.*, 2002). Thirdly, Ha *et al.* (1999) found that the Arabidopsis *cad1-3* mutant, which is lacking functional PCS, has an As(V)-hypersensitivity phenotype.

Vacuolar transport of metal PC complexes, mediated by an ABC-type transporter, HMT1, has been shown to be essential for tolerance to Cd in *Schizosaccharomyces pombe* (Ortiz *et al.*, 1995). There is evidence of Cd-triglutathione complex (Cd-GS_3) and Cd-PC transport over the tonoplast in *Avena sativa* by an ABC transporter (Salt and Rauser, 1995). Here, we showed that GSH-complexed As(III) is effectively transported across the tonoplast, most likely by an ABC transporter, suggested by the MgATP-dependency and the absence of associated transport of protons or charge. However, As(III)-GS_3 transport across the tonoplast was equal in NM and M plants, suggesting that an enhanced vacuolar compartmentalization capacity is not responsible for increased PC accumulation and does not contribute to hypertolerance in M *H. lanatus*. It may be argued that As(III)-PCs or the mixed As(III)-GS-PC2 complex are the dominant forms of thiol-complexed As(III) in plants (Raab *et al.*, 2004) and that the tonoplast transport kinetics of these complexes might differ from those of As(III)-GS_3. This may be true, but it is unlikely, in view of the structural homology of the complexes, that there would be complex-specific differences between NM and M plants.

Our observations here confirm the previously observed hypersensitivity under BSO treatment and the higher PC to As ratios in the M ecotype under As(V) treatment. However, it can be excluded that an increased capacity for PC synthesis as such would contribute to the superior As(V) tolerance in the M ecotype, because we did not observe any co-tolerance to As(III). In addition, the higher PC accumulation rate in M was neither explained by an enhanced PCS capacity, as shown by the *in vitro* assay, nor by altered vacuolar transport of thiol-complexed As(III). One might argue that we should have used As(III) instead of Cd as an activator in our PCS assay; however, indications of intraspecific variation in metal specificity for PCS activation have never been reported to our knowledge. On the other hand, our results provide strong evidence that the As(V) reduction capacity is the rate-limiting step in As(V)-induced PC accumulation. At equal rates of influx, As(V) and As(III) induced similar PC accumulation rates in the M plants, whereas As(III) was a much better inducer than As(V) in the NM ecotype. Because the PCS capacities were equal in both ecotypes, this can only be explained by a faster As(V) reduction. In addition, faster As(V) reduction could explain the early onset of longer-chain PC synthesis in the M plants, because longer-chain PCs are produced from shorter ones (Hayashi *et al.*, 1991). It is generally believed that longer-chain PCs contribute to the efficiency of cellular detoxification due to a higher metal binding capacity and formation of more stable complexes (Matsumoto *et al.*, 1990).

In bacteria, yeast and most likely in fungi as well, enzymatic As(V) reduction is a prerequisite for extrusion or compartmentalization, because efflux carriers predominantly transport As(III) or As(III)-GS_3 complexes (Cánovas *et al.*, 2003; Rosen, 2002). No plant As(V) reductases have been identified thus far. Although non-enzymatic reduction by GSH has been the suggested mechanism in plants (Delnomdedieu *et al.*, 1994), in our As(V) reductase assay we found that at least 90% of the reduction capacity in root protein extracts was enzymatic. Moreover, the enzymatic reduction capacity was inducible by As(V) and was enhanced in the M plants compared with the NM plants, which explains the elevated PC accumulation in the M ecotype. Faster reduction could also explain the stronger As(V) hypersensitivity reaction in M under BSO treatment, due to a more rapid accumulation of free As(III) in the cytosol.

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Asr-mediated arsenate reductase activity

Our results obtained with the recombinant Asr proteins from *Holcus* and Arabidopsis clearly show that Asr is capable of catalysing As(V) reduction, using GSH as an electron donor. Ectopic overexpression of the *ASR* gene in Arabidopsis resulted in a strongly enhanced As(V) reduction capacity of root protein extracts, and Arabidopsis *asr* T-DNA insertion mutant lines were lacking a detectable As(V)-inducible As(V) reductase capacity. Moreover, the gene appeared to be As(V)-inducible in *Holcus* and the mRNA expression patterns did match the As(V) reductase capacity patterns. Taken together, these results strongly suggest that Asr acts as an As(V)-inducible As(V) reductase in plants, such as shown for its homologue in yeast, the only eukaryotic As(V) reductase known thus far (Bobrowicz *et al*., 1997; Mukhopadhyay *et al*., 2000). The well conserved phosphatase $HC(X)_5R$ motif is most probably responsible for As(V) reduction, such as shown for yeast Acr2p (Bordo and Bork, 2002; Mukhopadhyay and Rosen, 2001). The cysteine residue may account for various sulphur transfer reactions, including GSH oxidation (Bordo and Bork, 2002). However, it is unlikely that As(V) reduction would be the primary function of Asr. Recently, the AtAsr protein has been characterized as a CDC25 dual-specificity tyrosine phosphatase capable of activating cycline-dependent kinases in Arabidopsis. Thus, its primary function might lie in cell cycle regulation, such as shown for the human CDC25 phosphatases with the same catalytic motive and largely similar secondary and tertiary structural features (Landrieu *et al*., 2004a,b). However, in the absence of As(V) exposure, the *asr* T-DNA insertion lines did not show any macroscopic phenotype for growth rate or growth habit. In any case, our results strongly suggest that Asr is responsible for at least a significant part the As(V) reduction capacity in As(V)-exposed plants and that it strongly affects the plant-internal arsenic transport.

Arsenate reduction and tolerance

By analysis of 35S:At*ASR* and At*asr* T-DNA insertion lines, we showed that At*ASR* expression influenced the As(V) tolerance level in Arabidopsis. Disruption of the gene caused some loss of As(V) tolerance, though only at relatively low exposure levels. Overexpression enhanced tolerance at low concentrations but caused hypersensitivity at higher concentrations of As(V). Enhanced tolerance at low concentrations points to improved detoxification through reduction and subsequent sequestration. Hypersensitivity at higher concentrations should be expected to occur as soon as the rate of cellular As(III) accumulation exceeds the PC-based sequestration capacity. In conformity with this explanation, Dhankher *et al.* (2002) found As(V) hypersensitivity upon expressing a bacterial As(V) reductase (ArsC) in the above-ground parts of *A. thaliana*. This hypersensitivity reaction could be overcome by simultaneous expression of *E. coli* γ-glutamylcysteine synthetase. However, only testing over an extended range of As(V) exposure levels reveals the dual phenotype found here. The tolerance phenotypes of the Arabidopsis lines observed in this study are subtle. Root elongation appeared to be more sensitive to As(V) than total plant biomass or shoot performance. Particularly at the lower exposure levels, there were no obvious effects of As(V) on shoot performance, possibly because of the low rate of arsenic translocation to the shoot. We did not observe any shoot phenotype for arsenic tolerance in the 35S lines, nor in the T-DNA lines. Therefore, it seems that the role for Asr in As(V) tolerance is apparent, but limited.

Because total As(V) uptake was unaltered, the significantly decreased arsenic shoot concentrations in the At*ars* T-DNA line may be taken to suggest that arsenic is transported from the root to the shoot in the reduced form, at least in part. This is in agreement with results obtained with *P. vittata*, in which enzymatic As(V) reduction is largely confined to the roots, whereas virtually all of the shoot arsenic is present as As(III) (Duan *et al*., 2005). In addition, based on a study of arsenic transport in the Arabidopsis *pho1* and *pho2* mutants, which are defective in the regulation of phosphate translocation, it was concluded that arsenic is mainly translocated as As(III) (Quaghebeur and Rengel, 2004). On the other hand, we did not observe significantly enhanced arsenic translocation rates in the 35S:At*ASR* lines. In *H. lanatus* however, arsenic translocation to the shoot was significantly higher in the M ecotype than in the NM ecotype (PMB, unpublished results).

The question is, does enhanced expression of Hl*ASR* in the M ecotype contribute to As(V) hypertolerance or not? First, Hl*ASR* is As(V)-inducible like At*ASR* in Arabidopsis. Secondly, the slope of the dose-response curves for As(V)-imposed root growth inhibition is much steeper in M plants than in NM plants (Figure 1a), at least at low phosphate supply, comparable to the difference between the 35S:At*ASR* and the wild-type phenotype in Arabidopsis. It is likely that As(V) exposure in the natural environment of the M plant coincides with the highest no-effect concentration in our experiment. Thus, improving tolerance by increased reductase capacity, even when this would occur at the expense of loss of tolerance to higher concentrations, would be expected to be favoured by natural selection. However, we can not exclude that the difference in slope might be in part explained by differential concentration dependent As(V) uptake rates. Conclusive evidence can only be provided by knocking out Hl*ASR* in *H. lanatus*. However, transformation of *H. lanatus* was not successful thus far.

In conclusion, our data suggest that, as long as the capacity limits of the PC-based detoxification machinery are not exceeded, increased enzymatic As(V) reduction can contribute to As(V) tolerance. Enhanced reduction, due to increased *ASR* expression, explains the higher PC accumulation rate associated with naturally selected As(V) hyper-

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tolerance in *H. lanatus* mine populations. Increased *ASR* expression might contribute to As(V) hypertolerance in these populations, although decreased uptake is doubtlessly the major determinant.

Experimental procedures

Plant material, experimental conditions and design

We used an *H. lanatus* L. ecotype from an uncontaminated site (NM; Exeter University campus, Exeter, UK) and an ecotype from an arsenic-copper contaminated site (M; Gawton United mine, Devon, UK). Both *H. lanatus* and *A. thaliana* (ecotype Columbia) were grown in 1-l polyethylene pots in nutrient solution composed of 1.5 mM KNO_3, 0.4 mM $Ca(NO_3)_2$, 100 μM $NH_4H_2PO_4$, 0.25 mM $MgSO_4$, 20 μM Fe(Na)-EDTA, 1 μM KCl, 25 μM H_3BO_3, 2 μM $MnSO_4$, 2 μM $ZnSO_4$, 0.1 μM $CuSO_4$ and 0.1 μM $(NH_4)_6Mo_7O_{24}$ in demineralized water buffered with 2 mM 2 morpholinoethanesulfonic acid, pH 5.5, adjusted with KOH. Solutions were renewed weekly and plants were grown in a growth chamber (20/15°C day/night; light intensity, 200 μE m^{-2} s^{-1}, 14 h day^{-1}; relative humidity (RH) 75%). For half of the *H. lanatus* plants, $NH_4H_2PO_4$ concentrations were lowered to 10 μM under As(V) stress.

Arsenate tolerance was assessed from root elongation throughout 5 days of exposure. The EC50 was inferred from the dose-response curve. For each treatment, four plants of either of the ecotypes or expression lines were placed in 1-l pots containing nutrient solution as described above, with three replicate pots per treatment. After 1.5 weeks of pre-culture, plants were exposed to a range of Na_2HAsO_4 concentrations. Roots were stained with active coal powder and rinsed with demineralized water prior to exposure (Schat and Ten Bookum, 1992). *Holcus lanatus* clones of both ecotypes were additionally grown in nutrient solution on a range of As(V) in the presence of 250 μM L-buthioninesulfoximine (BSO), an inhibitor of γ-glutamylcysteine synthetase and, consequently, of PC synthesis (Reese and Wagner, 1987). Five days after exposure, maximum root growth, i.e. the length of the longest unstained root segment, was recorded.

Arsenic uptake and translocation were determined in root and shoot material of five individual plants per treatment. Root material was carefully rinsed with demineralized water. Arsenic was determined by digesting 50–100 mg of oven-dried plant material in 2 ml 37% (v/v) HCl: 65% (v/v) HNO_3 (1:4, v/v) in Teflon cylinders for 7 h at 140°C, after which the volume was adjusted to 5 ml with demineralized water. Arsenic was determined on a flame atomic spectrophotometer (Perkin Elmer 2100; Perkin-Elmer Nederland, Nieuwerkerk a/d Yssel, the Netherlands), coupled to an MHS-10 hydride system (Waters Nederland, Etten-Leur, the Netherlands). Blanks of reagents and standardized reference material (SRM) were included in all analytical schemes (Buffalo River sediment, SRM 2704).

In planta *phytochelatin accumulation*

In planta accumulation of PCs was studied as a function of exposure time. Unrooted tillers of *H. lanatus* were grown for 2 weeks. Per treatment, four tillers were placed in 1-l nutrient solution, with three replicate pots per treatment. After pre-treatment, solutions were amended with the individual EC50 of As(V) or As(III) levels expected to yield comparable uptake. At time intervals after the start of arsenic exposure, five plants were harvested for PC analysis. From the same batch, five plants were harvested for determination of total root As concentration, as described above.

Roots for PC analysis were rinsed with demineralized water, blotted with tissue paper and processed immediately. Phytochelatins were extracted by homogenising 0.5–1 g fresh material in 1.98 ml of 6.3 mM diethylenetriaminepentaactic acid (DTPA) with 0.1% trifluoric acid (TFA), using mortar, pestle and quarz sand. *N*-acetyl-L-cysteine (NAC) was added during homogenization as an internal standard. The homogenates were centrifuged for 15 min at 15 000 ***g*** at 4°C. The supernatant was filtered through a Costar Spin-X centrifuge tube (Corning-Costar, Cambridge, MA, USA) with a nylon filter (0.22 μm). Derivatization was carried out according to Sneller *et al.* (2000). Four hundred and fifty μl of 200 mM 4-(2-hydoxyethyl)piperzanine-1-propanesulphonic acid (HEPPS) buffer, pH 8.2, containing 6.3 mM DTPA, was mixed with 10 μl of 25 mM monobromobimane. To this mixture, 250 μl of the filtered sample was added and derivatization was carried out for 30 min at 45°C in the dark. The reaction was stopped by the addition of 300 μl of 1 M methanesulphonic acid. Samples were stored at 4°C in the dark prior to analysis. Reverse phase liquid chromatography (RPLC) analysis was performed as described in Sneller *et al.* (2000). Phytochelatins were separated on a Nova-Pak C_{18} column (6 nm, 4 μm, 3.9 × 300 mm, Waters catalogue no. 11695) at 37°C and were eluted with a slightly concave gradient of methanol and water both containing 0.1% TFA, with fluorescence detection. Depending on sulfhydryl concentrations, 5–50 μl of derivatized sample was injected. The total analysis time was 70 min. Peaks were identified by overlay with a profile of known thiol composition.

Enzyme activity assays

For the *in vitro* PCS analysis, 2-week-old root material was harvested, rinsed in demineralized water and 1–1.5 g was immediately homogenized in 5 ml ice cold 30 mM Tris-HCl, 10 mM β-mercaptoethanol and 0.5 g polyvinylpyrrolidone at 0°C using mortar, pestle and quartz sand. The homogenate was centrifuged at 15 000 ***g*** at 4°C for 10 min. The supernatant was loaded on a Sephadex G-25 gel filtration column (PD-10; Pharmacia, Amersham Biosciences, Roosendaal, the Netherlands) and proteins were eluted with 0.4 M Tris-HCl (pH 8.0 at 37°C), 10 mM β-mercaptoethanol at 4°C. Protein concentrations in the extract were determined with bovine serum albumin as a standard after Peterson (1983). Because the stability of the As(III)-GS_3 complex is not optimal at pH 8.0, Cd was used as an activator in the enzyme assay. The enzyme incubation mixture contained 900 μl protein extract, 50 μl 200 mM GSH and 50 μl 0.5–5 mM $CdSO_4$. The final pH was adjusted to 8.0 with 10 μl 1 M KOH. Incubation was performed for 2 h at 37°C and arrested by adding 100 μl 5 M HCl. Samples were filtered through a Costar Spin-X centrifuge tube (0.22 μm) and PC concentrations were directly determined by RPLC with post-column derivatization with Ellmans's reagent [(5,5′-dithiobis)2-nitrobenzoic acid, DTNB] as described by De Knecht *et al.* (1994). Thiols were separated on a Waters Nova-Pak C18 column (catalog no. 36920) at 37°C, using a linear gradient of acetonitril and water, both acidified with 0.1% (v/v) TFA. The column effluent was derivatized with DTNB at pH 7.8, in a Waters RXN 1000 coil (Waters Nederland), with an Eldex post-column pump (Eldex Laboratories, Napa, CA, USA). Absorbance at 412 nm was monitored by a Waters 996 PDA detector (Waters Nederland). Of the derivatized sample 10–50 μl was injected and total analysis time was 40 min.

Arsenate-reductase activity was measured by determining the concentrations of As(III)-GS_3 and As(III)-PC at regular time intervals (these compounds elute at the same retention time), using the RPLC method described above. Values were corrected for spontaneous reduction by GSH, estimated from controls with GSH, but without protein extract. Activity was determined with purified recombinant Arabidopsis Asr protein, as well as in crude root extracts of both

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H. lanatus and *A. thaliana*. For plant extracts, 2-week-old root material was harvested and processed immediately as described before. Protein elution was done using 0.4 M Tris HCl (pH 7.2 at 37°C) at 4°C from which any reducing agent was omitted this time. Throughout the experiment, extracts were kept under argon. The enzyme incubation mixture contained 900 μl protein extract, 50 μl 200 mM GSH and 50 μl 1 M Na_2HAsO_4. After 10 min of incubation at 37°C, reductase activity was measured by RPLC and post-column DTNB derivatization as described previously. Additionally, prior to incubation, aliquots of protein extract were pre-incubated at 4°C with 5–10 mM TNBS, an inhibitor of rhodanese activity (Prieto *et al.*, 1997). Residual enzyme activity was measured as previously described.

Tonoplast isolation and transport assays

Tonoplast isolation and transport of As(III)-GS_3 and free As(III) was characterized by direct filtration assays and fluorescence spectroscopy on tonoplast vesicles, isolated from the roots of both NM and M *H. lanatus* using the procedures described in Chardonnens *et al.* (1999). Fluorescence quenching of Acridine Orange (AO) and Oxonol-V was used to monitor the build-up and maintenance of the membrane potential and the pH gradient of the vesicles on a luminescence spectrometer (SLM Aminco Bowmand; Thermo Electron Cooperation, Waltham, MA, USA). Purity of the vesicle fraction was evaluated using inhibitors of specific proton ATPases (KNO_3, Na-molybdate, Na_3VO_4). The percentage of right-side-out vesicles was estimated in an ATP-hydrolysis assay using spectrophotometric measurements of phosphate as described by Murphy and Riley (1962). After allowing the build-up of the maximum proton gradient, uptake of As(III)-GS_3 or free As(III) was measured in a direct filtration assay (Chardonnens *et al.*, 1999). A correction was made for non-specific binding to the membranes. Arsenic concentrations were determined as described above.

RNA and DNA blot analysis

Total RNA of 2-week-old root material exposed for 3 days to 0, 30 and 120 μM As(V) in the case of NM *H. lanatus* and 0, 120 and 500 μM As(V) in the case of M plants was extracted as described by Van Tunen *et al.* (1988). Ten micrograms RNA was separated by gel-electrophoresis on a 1.3% agarose gel and vacuum blotted onto a Hybond N^+ nylon membrane (Amersham Biosciences) in 20 × SSC according to standard procedures. RNA isolation and blotting was independently repeated three times. A cDNA PCR fragment representing the complete *H. lanatus* Asr gene (Hl*ASR*) was used as a hybridization probe. The full-length cDNA was isolated in three steps. First, a PCR was performed on genomic DNA with degenerate primers based on sequence homology between multiple plant *ASR* genes (Asr1, 5′-AARGANCANBTNGTNTTYCAYTGYGC-3′; Asr2, 5′-YTCCCANCCRTTRAANCCNCKYTC-3′). Based on the sequence, a specific forward primer (HlAsrd1, 5′-TTYCAYTGYGCCCTCAGCCAG-3′) was designed that in combination with an oligo dT primer (5′-ATTCTAGAGGCCGAGGCGGCCGACATG-T13-VN) yielded the complete 3′ end of the gene. To isolate the 5′ end, adaptors (5′-CTAATACGACTCACTATAGGGCTCGAGCGGCCGCCCGGGCAGGT-3′ annealed to 5′-ACCTGCCC-3′) were ligated to blunt digested genomic DNA (*Sma*I, *Hin*CII, *Eco*RV *Pml*I, *Stu*I). A nested PCR was performed with the following primers HlAsr6 (5′-GCACCTGGCTGAGGGCGCA-3′) and HlAsr7 (5′-TCTTGCACAAGTTGGACCACGC-3′) in combination with prim1 (5′-CTAATACGACTCACTATAGGGC-3′) and nest1 (5′-TCGAGCGGCCGCCCGGGCAGGT-3′). The sequenced product now covered the complete Hl*ASR* gene allowing for a PCR with specific primers (HlAsrdf, 5′-GGCCGGAGATGGCGAGGAAAG-3′; HlAsrdr, 5′-CGCCAATAAGGTATGCAGTTTAC-3′) on *H. lanatus* root cDNA. For RNA blot hybridization, the membrane was pre-hybridized in solution containing 7% SDS, 0.5 M sodium phosphate (pH 7.2), 1 mM EDTA (pH 8.0) and 1% bovine serum albumine and consequently hybridized with a ^{32}P-labelled probe. After an overnight incubation at 65°C, the membrane was washed twice in 1 × SSC, 0.1% SDS at 65°C and exposed on a Phosphor Imager screen (Syngene, Cambridge, UK).

For DNA blot analysis, approximately 1 μg genomic DNA was digested with *Hin*dIII, *Xba*I or *Eco*RI and separated by gel-electrophoresis using a 1% agarose-trisbase-acetic acid-EDTA gel. DNA was blotted overnight onto Hybond N+ nylon membrane according to standard procedures and was cross-linked in 0.5 M NaOH. Blot hybridization was carried out as described for RNA blot hybridization using the Holcus ^{32}P-labelled probe.

Protein expression

The Arabidopsis rhodanese/CDC25-like gene (At*ASR*) was amplified by RT-PCR using total root RNA as a template with primers: AtAsrd1, 5′-GGGGATCCGTTTGGAGAGATATGGGGAGAAGC-3′; and AtAsrd2, 5′-GGGAATTCGTTTAGGCGCAATCGCCCTTGCAAG-3′. Hl*ASR* was amplified using primers HlAsrdf and HlAsrdr. To express both At*ASR* and Hl*ASR* as a his-tagged fusion, the amplification products were digested with *Bam*HI and *Eco*RI and subsequently ligated in *Bam*HI/*Eco*RI digested pRSET-A (Invitrogen, Breda, the Netherlands). These constructs, as well as an empty pRSET vector were transformed into *E. coli* BL21-AI competent cells. Clones were inoculated in Luria Bertani Broth (LB) containing 250 μg ml^{-1} ampicillin overnight at 37°C. A 1:100 dilution of overnight cultures was grown in 1 l LB till mid-log phase at 37°C, after which the temperature was lowered to 30°C and 1 mM isopropyl-3-D-thiogalactopyranoside (IPTG) and 0.2% L-arabinose were added. Induction was allowed for 3 h, after which optical density at 600 nm was determined. Cells were harvested by centrifugation, frozen in liquid nitrogen and stored at −80°C. From an aliquot, the expression of the At*ASR* gene was confirmed by Western analysis using the T7.Tag horseradish peroxidase antibody Conjugate directed against the leader peptide (Novagen, Madison, WI, USA). For enzyme purification, cells were resuspended in 30 ml argon flushed extraction buffer containing 20 mM Tris-Cl (pH 8.0), 0.7 M NaCl, 1% Trition-X100, 10 mM imidazol, 1 mM PMSF, 1 μM leupeptine, 5 μg ml^{-1} DNase and 5 μg ml^{-1} RNase and passed through a French press three times. The lysate was centrifuged at 20 000 ***g*** for 30 min at 4°C and the supernatant was passed over a 22-μm filter before being loaded onto an $NiSO_4$ saturated HiTrap chelating HP column, packed with 5 ml chelating Sepharose™ High Performance (Amersham Biosciences). The column had been pre-equilibrated with 50 ml elution buffer A [20 mM Tris-Cl (pH 8.0), 0.7 M NaCl, 10% glycerol, 10 mM imidazol]. His-tagged AtAsr was eluted from the Ni column using an FPLC chromatography system, with a gradient of argon flushed elution buffers A and B [20 mM Tris-Cl (pH 7.0), 0.7 M NaCl, 10% glycerol, 500 mM imidazol]. Two-millilitre fractions were collected and checked for activity in a spot blot using the T7.Tag antibody. Positive fractions from empty pRSET, pRSET-At*ASR* and pRSET-Hl*ASR* were compared in an As(V)-reductase activity assay as described above.

Analysis of Arabidopsis asr T-DNA and 35S:ASR lines

T-DNA insertions in the At*ASR* gene (At5G03455.1) were obtained from the Arabidopsis Stock Center (SALK_143282; T-DNA start position 863366) and from GABI-Kat (line ID 772G06; T-DNA start position 863262). To confirm the presence of the T-DNA inserts in

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the At*ASR* gene, leaf DNA was used in a PCR reaction containing primers directed against the T-DNA and At*ASR* gene: IBb1 (5′-GCGTGGACCGCTTGCTGCAACT-3′) or FISH1LB (5′CTGGGAATGG-CGAAATCAAGGCATC-3′) and AtAsrd2. Finally, sequence analysis confirmed the SALK T-DNA insertion to be in the second intron, while the GABI-Kat T-DNA insertion was positioned in the second exon of At*ASR*. Of both insertion mutants, two T2 lines were tested for As(V) tolerance in a root growth assay as described earlier.

Ectopic expression of the Arabidopsis At*ASR* gene was achieved by ligation of At*ASR* between the CaMV35S promoter and the nopaline synthase polyadenylation signal. This construct was PCR amplified using primers CaMV-*Xba* and *Nos–Eco*, cut with *Eco*RI and *Xba*I and inserted into the binary vector pFLUAR-100 which contains a napin-GFP cassette as a transformation marker. After sequence analysis, this construct was introduced into *Agrobacterium tumefaciens* after which transformation of *A. thaliana* was performed by the floral dip method (Clough and Bent, 1998). Transformants were isolated through visual screening of seeds under a fluorescence microscope. Northern analysis was performed to determine the mRNA expression level of the transgene. At*ASR* expression was 20–30 times higher compared with the background expression. Three T2 expression lines were selected and analysed for root growth inhibition under As(V) stress, as described earlier.

Additionally, one T2 SALK-insertion mutant line, one T2 expression line and a WT line were selected and analysed for *in vitro* rate of As(III)-GS_3 formation in a crude protein extract and tested for As(V)-uptake and translocation.

Statistics

Statistic analysis was performed using two- or three-way ANOVA, followed by Tukey's test for comparison of individual means, using the statistical program SPSS 10.1.0 for Windows (SPSS Inc. 2000, Chicago, IL, USA). When necessary, values were log-transformed prior to analysis.

Sequence data from this article have been deposited with the European Molecular Biology Laboratory/GenBank data libraries under accession number AY704470. GenBank accession numbers for other sequences mentioned are: *S. cerevisiae* Acr2p, AAS56379; *A. thaliana* Asr protein (AtAsr), AAO39886/At5G03455; *Zea mays* Asr, AY108501; *Oryza sativa* Asr1, AAP54884; *O. sativa* Asr2, AAN62781.

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References

Abedin, M.J., Feldmann, J. and Meharg, A.A. (2002) Uptake kinetics of arsenic species in rice plants. *Plant Physiol.* **128**, 1–9.

Asher, D.J. and Reay, P.F. (1979) Arsenic uptake by barley seedlings. *J. Plant Physiol.* **6**, 459–466.

Bertolero, F., Pozzi, G., Sabbioni, E. and Saffiotti, U. (1987) Cellular uptake and metabolic reduction of pentavalent to trivalent arsenic as determinants of cytotoxicity and morphological transformation. *Carcinogenesis*, **8**, 803–808.

Bleeker, P.M., Schat, H., Vooijs, R., Verkleij, J.A.C. and Ernst, W.H.O. (2003) Mechanisms of arsenate tolerance in *Cytisus striatus*. *New Phytol.* **157**, 33–38.

Bobrowicz, P., Wysocki, R., Owsianik, G., Goffeau, A. and Ulaszewski, S. (1997) Isolation of three contiguous genes, *ASR1*, *ASR2* and *ASR3*, involved in resistance to arsenic compounds in the yeast *Saccharomyces cerevisiae*. *Yeast*, **13**, 819–828.

Bordo, D. and Bork, P. (2002) The rhodanese/Cdc25 phosphatase superfamily. *EMBO Rep.* **3**, 741–746.

Cánovas, D., Mukhopadhyay, R., Rosen, B.P. and De Lorenzo, V. (2003) Arsenate transport and reduction in the hyper-tolerant fungus *Aspergillus* sp. P37. *Environ. Microbiol.* **5**, 1087–1093.

Chardonnens, A.N., Koevoets, P.L.M., Van Zanten, A., Schat, H., Verkleij, J.A.C. and Ernst, W.H.O. (1999) Properties of enhanced tonoplast zinc transport in naturally selected zinc tolerant *Silene vulgaris*. *Plant Physiol.* **120**, 779–785.

Clough, S.J. and Bent, A.F. (1998) Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. *Plant J.* **16**, 735–743.

De Knecht, J.A., Van Dillen, M., Koevoets, P.L.M., Schat, H., Verkleij, J.A.C. and Ernst, W.H.O. (1994) Phytochelatins in cadmium-sensitive and cadmium-tolerant *Silene vulgaris*: chain length distribution and sulfide incorporation. *Plant Physiol.* **104**, 255–261.

De Koe, T. (1994) *Agrostis castellana* and *Agrostis delicatula* on heavy metal and arsenic enriched sites in NE Portugal. *Sci. Total Environ.* **145**, 103–109.

De Koe, T. and Jacques, N.M. (1993) As(V) tolerance in *Agrostis castellana* and *Agrostis delicatula*. *Plant Soil*, **151**, 185–191.

Delnomdedieu, M., Basti, M.M., Otvos, J.D. and Thomas, D.J. (1994). Reduction and binding of arsenate and dimethylarsinate by glutathione: a magnetic resonance study. *Chem. Biol. Interact.* **90**, 139–155.

Dhankher, O.M., Li, Y., Rosen, B.P., Shi, J., Salt, D., Senecoff, J.F., Sashti, N.A. and Meagher, R.B. (2002) Engineering tolerance and hyperaccumulation of arsenic in plants by combining arsenate reductase and γ-glutamylcysteine synthetase expression. *Nat. Biotech.* **20**, 1140–1145.

Duan, G.-L., Zhu, Y.-G., Tong, Y.-P., Cai, C. and Kneer, R. (2005) Characterization of arsenate reductase in the extract of roots and fronds of Chinese Brake Fern, an arsenic hyperaccumulator. *Plant Physiol.* **138**, 461–469.

Gladysheva, T.B., Oden, K.L. and Rosen, B.P. (1994) Properties of the arsenate reductase of plasmid R773. *Biochemistry*, **33**, 7288–7293.

Gosh, M., Shen, J. and Rosen, B.P. (1999) Pathways of As(III) detoxification in *Saccharomyces cerevisiae*. *Proc. Natl Acad. Sci. USA*, **96**, 5001–5006.

Grill, E., Winnacker, E.-L. and Zenk, M.H. (1985) Phytochelatins: the principal heavy-metal complexing peptides of higher plants. *Science*, **230**, 674–676.

Grill, E., Loeffler, S., Winnacker, E.-L. and Zenk, M.H. (1989) Phytochelatins, the heavy metal-binding peptides of plants, are synthesized from glutathione by a specific γ-glutamylcysteinedipeptyl transpeptidase (phytochelatin synthase). *Proc. Natl Acad. Sci. USA* **86**, 6838–6842.

Ha, S.B., Smith, A.P., Howden, R., Dietrich, W.M., Bugg, S., O'Connell, M.J., Goldsbrough, P.B. and Cobbett, C.S. (1999) Phytochelatin synthase genes from Arabidopsis and the yeast *Schizosaccharomyces pombe*. *Plant Cell*, **11**, 1153–1163.

Hartley-Whitaker, J., Ainsworth, G., Vooijs, R., Ten Bookum, W., Schat, H. and Meharg, A.A. (2001) Phytochelatins are involved in differential arsenate tolerance in *Holcus lanatus*. *Plant Physiol.* **126**, 299–306.

Hartley-Whitaker, J., Woods, C. and Meharg, A.A. (2002) Is differential phytochelatin production related to decreased arsenate influx in arsenate tolerant *Holcus lanatus*? *New Phytol.* **155**, 219–225.

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Haswell, S.J., O'Neill, P. and Bancroft, K.C.C. (1985) Arsenic speciation in soil-pore waters from mineralised and unmineralised areas of South–West England. *Talanta*, **32**, 69–72.

Hayashi, Y., Nakagawa, C.W., Mutoh, N., Isobe, M. and Goto, T. (1991) Two pathways in the biosynthesis of cadystins (γ-EC)$_n$G in the cell-free system of the fission yeast. *Biochem. Cell Biol.* **69**, 115–121.

Howden, R., Andersen, C.R., Goldsbrough, P.B. and Cobbett, C.S. (1995) A cadmium sensitive, glutathione-deficient mutant of *Arabidopsis thaliana*. *Plant Physiol.* **107**, 1067–1073.

Ji, G., Garber, E.A.E., Armes, L.G., Chen, C.M., Fuchs, J.A. and Silver, S. (1994) Arsenate reductase of *Staphylococcus aureus* plasmid pI258. *Biochemistry* **33**, 7294–7299.

Jocelyn, P.C. (1972) *Biochemistry of the SH Group*. London: Academic Press.

Landrieu, I., da Costa, M., De Veylder, L. *et al.* (2004a) A small CDC25 dual-specificity tyrosine-phosphatase isoform in *Arabidopsis thaliana*. *Proc. Natl Acad. Sci. USA*, **101**, 13380–13385.

Landrieu, I., Hassan, S., Sauty, M., Dewitte, F., Wieruszeski, J.M., Inze, D., De Veylder, L. and Lippens, G. (2004b) Characterization of the *Arabidopsis thaliana Arath*;CDC25 dual-specificity tyrosime phosphatase. *Biochem. Bioph. Res. Co.* **322**, 734–739.

Lee, D.A., Chen, A. and Schroeder, I. (2003) *ars1*, an Arabidopsis mutant exhibiting increased tolerance to arsenate and increased phosphate uptake. *Plant J.* **35**, 637–646.

Macnair, M.R. and Cumbes, Q. (1987) Evidence that arsenic tolerance in *Holcus lanatus* L. is caused by an altered phosphate uptake system. *New Phytol.* **107**, 387–394.

Macnair, M.R., Cumbes, Q. and Meharg, A.A. (1992) The genetics of arsenate tolerance in Yorkshire Fog, *Holcus lanatus* L. *Heredity*, **69**, 325–335.

Maier, T., Yu, C., Küllertz, G. and Clemens, S. (2003) Localization and functional characterization of metal-binding sites in phytochelatin synthases. *Planta*, **218**, 300–308.

Matsumoto, Y., Okada, Y., Min, K.-S., Onosaka, S. and Tanaka, K. (1990) Amino acids and peptides XXVII. Synthesis of phytochelatin-related peptides and examination of their heavy metal-binding properties. *Chem. Pharm. Bull.* **38**, 2364–2368.

Meharg, A.A. (1994) Integrated tolerance mechanisms – constitutive and adaptive plant – responses to elevated metal concentrations in the environment. *Plant Cell Environ.* **17**, 989–993.

Meharg, A.A. and Hartley-Whitaker, J. (2002) Arsenic uptake and metabolism in arsenic resistant and non-resistant plant species. *New Phytol.* **154**, 29–43.

Meharg, A.A. and Jardine, L. (2003) Arsenite transport into paddy rice (*Oryza sativa*) roots. *New Phytol.* **157**, 39–44.

Meharg, A.A. and Macnair, M.R. (1991) Uptake, accumulation and translocation of arsenate in arsenate tolerant and non-tolerant *Holcus lanatus* L. *New Phytol.* **117**, 225–231.

Meharg, A.A. and Macnair, M.R. (1992a) Suppression of the high-affinity phosphate-uptake system – a mechanism of arsenate tolerance in *Holcus lanatus* L. *J. Exp. Bot.* **43**, 519–524.

Meharg, A.A. and Macnair, M.R. (1992b) Genetic correlation between arsenate tolerance and the rate of influx of arsenate and phosphate in *Holcus lanatus* L. *Heredity*, **69**, 336–341.

Mukhopadhyay, R. and Rosen, B.P. (2001) The phosphatase C(X)$_5$R motif is required for catalytic activity of the *Saccharomyces cerevisiae* Asr2p arsenate reductase. *J. Biol. Chem.* **276**, 34738–34742.

Mukhopadhyay, R., Shi, J. and Rosen, B.P. (2000) Purification and characterization of Asr2p, the *Saccharomyces cerevisiae* arsenate reductase. *J. Biol. Chem.* **275**, 21149–21157.

Murphy, J. and Riley, J.P. (1962) A modified single solution method for the determination of phosphate in natural waters. *Anal. Chim. Acta*, **27**, 31–36.

Onken, B.M. and Hossner, L.R. (1995) Plant uptake and determination of arsenic species in soil solutions under flooded conditions. *J. Environ. Qual.* **24**, 373–381.

Ortiz, D.F., Ruscitti, T., MacCue, K.F. and Ow, D.W. (1995) Transport of metal-binding peptides by HMT1, a fission yeast ABC-type vacuolar membrane protein. *J. Biol. Chem.* **270**, 4721–4728.

Peterson, G.L. (1983) Determination of total protein. *Methods Enzymol.* **91**, 95–121.

Porter, E.K. and Peterson, P.J. (1977) Arsenic tolerance in grasses growing on mine waste. *Environ. Pollut.* **14**, 255–265.

Prieto, J.L., Pérez-Catiñeira, J.R. and Vega, J.M. (1997) Thiosulfate reductase from *Chlamydomonas*. *J. Plant Physiol.* **151**, 385–389.

Quaghebeur, M. and Rengel, Z. (2004) Arsenic uptake, translocation and speciation in *pho1* and *pho2* mutants of *Arabidopsis thaliana*. *Physiol. Plant.* **120**, 280–286.

Raab, A., Feldmann, J. and Meharg, A.A. (2004) The nature of arsenic phytochelatins complexes in *Holcus lanatus* and *Pteris cretica*. *Plant Physiol.* **134**, 1113–1122.

Reese, R.N. and Wagner, G.J. (1987) Effect of buthionine sulfoximine on Cd-binding peptide levels in suspension-cultured tobacco cells treated with Cd, Zn, or Cu. *Plant Physiol.* **84**, 574–577.

Rocovich, S.E. and West, D.A. (1975) Arsenic tolerance in a population of the grass *Andropogon scoparius* Michx. *Science*, **188**, 263–264.

Rosen, B.P. (2002) Biochemistry of arsenic detoxification. *FEBS Lett.* **529**, 86–92.

Salt, D.E. and Rauser, W.E. (1995) MgATP-dependent transport of phytochelatins across the tonoplast of oat roots. *Plant Physiol.* **107**, 1293–1301.

Sandberg, G.R. and Allen, I.K. (1975) A proposed arsenic cycle in an agronomic ecosystem. *ACS Symp. Ser.* **7**, 124–147.

Schat, H. and Ten Bookum, W.M. (1992) Genetic control of copper tolerance in *Silene vulgaris*. *Heredity*, **68**, 219–229.

Schat, H., Llugany, M., Vooijs, R., Hartley-Whitaker, J. and Bleeker, P.M. (2002) The role of phytochelatins in constitutive and adaptive heavy metal tolerances in hyperaccumylator and non-hyperaccumuylator metallophytes. *J. Exp. Bot.* **53**, 2381–2392.

Sneller, F.E.C., Van Heerwaarden, L.M., Koevoets, P.L.M., Vooijs, R., Schat, H. and Verkleij, J.A.C. (2000) Derivatization of phytochelatins from *Silene vulgaris* induced upon exposure to arsenate and cadmium: comparison of derivatization with Ellmans's reagent and monobromobimane. *J. Agric. Food Chem.* **48**, 4014–4019.

Van Tunen, A.J., Koes, R.E., Spelt, C.E., Van der Krol, A.R., Stuitje, A.R. and Mol, J.N. (1988) Cloning of the two chalcone flavonone isomerase genes from *Petunia hybrida*: coordinate light-regulated and differential expression of flavonoid genes. *EMBO J.* **7**, 1257–1263.

Vatamaniuk, O.K., Mari, S., Lu, Y.P. and Rea, P.A. (2000) Mechanism of heavy metal ion activation of phytochelatin (PC) synthase: blocked thiols are sufficient for PC synthase-catalyzed transpeptidation of glutathione and related thiol peptides. *J. Biol. Chem.* **275**, 31451–31459.

Willsky, G.R. and Malamy, M.H. (1980) Effect of arsenate on inorganic phosphate transport in *Escherichia coli*. *J. Bacteriol.* **144**, 366–374.

Wysocki, R., Chery, C.C., Wawrzycka, D., Van Hulle, M., Cornelis, R., Thevelein, J.M. and Tamas, M.J. (2001) The glycerol channel Fps1p mediates the uptake of arsenite and antimonite in *Saccharomyces cerevisiae*. *Mol. Microb.* **40**, 1391–1401.

Yompakdee, C., Bun-ya, M., Shikata, K., Ogawa, N., Harashima, S. and Oshima, Y. (1996) A putative new membrane protein, Pho86p, in the inorganic phosphate uptake system of *Saccharomyces cerevisiae*. *Gene*, **171**, 41–47.

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