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Arsenite efflux is not enhanced in the arsenate-tolerant phenotype of *Holcus lanatus*

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Summary

• Arsenate tolerance in *Holcus lanatus* is achieved mainly through suppressed arsenate uptake. We recently showed that plant roots can rapidly efflux arsenite to the external medium. Here, we tested whether arsenite efflux is a component of the adaptive arsenate tolerance in *H. lanatus*.

• Tolerant and nontolerant phenotypes were exposed to different arsenate concentrations with or without phosphate for 24 h, and arsenic (As) speciation was determined in nutrient solutions, roots and xylem sap.

• At the same arsenate exposure concentration, the nontolerant phenotype took up more arsenate and effluxed more arsenite than the tolerant phenotype. However, arsenite efflux was proportional to arsenate uptake and was not enhanced in the tolerant phenotype. Within 2–24 h, most (80–100%) of the arsenate taken up was effluxed to the medium as arsenite. About 86–95% of the As in the roots and majority of the As in xylem sap (*c*. 66%) was present as arsenite, and there were no significant differences between phenotypes.

• Arsenite efflux is not adaptively enhanced in the tolerant phenotype *H. lanatus*, but it could be a basal tolerance mechanism to greatly decrease cellular As burden in both phenotypes. Tolerant and nontolerant phenotypes had a similar capacity to reduce arsenate in roots.

Introduction

Arsenic (As) is a nonessential and highly toxic element to plants. Tolerance (or resistance) to arsenate has been reported in a number of grass or shrub species colonizing As contaminated soil (Porter & Peterson, 1977; Macnair & Cumbes, 1987; Meharg & Macnair, 1991a; Bleeker *et al*., 2003; Arnetoli *et al*., 2008). These studies have shown that the main mechanism of tolerance is decreased arsenate uptake, which is conferred by suppression of the high-affinity phosphate/arsenate transporters in the tolerant ecotype of the grass species *Holcus lanatus* (Meharg & Macnair, 1992c). Genetic studies have shown that a major gene is responsible for the arsenate tolerance in *H. lanatus*, which is linked to arsenate uptake, and that one or more modifiers may influence the level of tolerance (Macnair *et al*., 1992; Meharg & Macnair, 1992a). In addition, it has been found that normal populations of *H. lanatus* are polymorphic for tolerance (Meharg & Macnair, 1992b; Naylor *et al*., 1996).

Despite a restricted arsenate uptake, tolerant plants still accumulate considerable levels of As over their life cycle. This means that mechanisms of internal detoxification are required to underpin the tolerance to cellular As. There is strong evidence that arsenate is detoxified through reduction to arsenite (As(III)), followed by complexation with thiols, especially phytochelatins (PCs) (Sneller *et al*., 1999; Schmöger *et al*., 2000; Schat *et al*., 2002; Raab *et al*., 2005). The As(III)–PC complexes are thought to be sequestered in vacuoles, although there is still no direct evidence for this (Zhao *et al*., 2009). It has been shown that, at the equivalent toxicity level, the arsenate-tolerant ecotype of *H. lanatus* produces more PCs than the As nontolerant ecotype, suggesting an enhanced capacity for detoxification (Hartley-Whitaker *et al*., 2001). Bleeker *et al*. (2006) proposed that, next to decreased uptake, enhanced arsenate reduction by the arsenate reductase HIAsr may contribute to arsenate tolerance in *H. lanatus*. However, they did not quantify As speciation *in planta*.

Exceptions to the tolerance mechanisms mentioned above are the As hyperaccumulating and hypertolerant fern species such as *Pteris vittata* and *Pteris cretica*. These plants have an enhanced arsenate uptake compared with nonhyperaccumulating ferns (Poynton *et al.*, 2004; Caille *et al.*, 2005), an exceedingly high efficiency of As translocation from roots to fronds (Tu & Ma, 2002; Poynton *et al.*, 2004; Caille *et al.*, 2005; Su *et al.*, 2008), little complexation of As(III) with PCs (Zhao *et al.*, 2003; Raab *et al.*, 2004) and sequestration of inorganic As(III) in the vacuoles of frond tissues (Lombi *et al.*, 2002; Pickering *et al.*, 2006).

In microbes, the main mechanism of arsenate tolerance involves reduction of arsenate followed by extrusion (efflux) of arsenite to the external medium (Bhattacharjee & Rosen, 2007). Arsenite efflux is carried out by membrane carrier proteins or pumps such as ArsB or the ArsAB complexes in *Escherichia coli* and Acr3p in yeast (Dey *et al.*, 1994; Wysocki *et al.*, 1997). By this mechanism, the accumulation of As in the cells is decreased without also decreasing the accumulation of phosphate, because the arsenite efflux pumps do not transport phosphate. Recently, Xu *et al.* (2007) presented evidence that roots of tomato (*Lycopersicon esculentum*) and rice (*Oryza sativa*) took up arsenate and effluxed arsenite to the external medium rapidly. The membrane transporter proteins responsible for arsenite efflux in plant roots are not known, but possible candidates include microbial ArsB- or Acr3p-like transporters in plants and some aquaporins channels that may allow bidirectional passage of arsenite (Bienert *et al.*, 2008; Isayenkov & Maathuis, 2008; Zhao *et al.*, 2009).

The findings of Xu *et al.* (2007) raise the question as to whether the rapid arsenite efflux process is also an As detoxification mechanism in plants, as it is in microbes. In the present study, we tested this hypothesis by comparing arsenate uptake and arsenite efflux in the arsenate-tolerant and nontolerant phenotypes of *H. lanatus*. The main objective was to investigate whether arsenite efflux is enhanced adaptively in the tolerant phenotype. This study did not address whether arsenite efflux is a basal and constitutive mechanism of As detoxification because to do so would require testing of plant mutants defective in arsenite efflux only, and these are not yet available. The second objective was to compare As speciation in roots and xylem sap of the two contrasting phenotypes in order to ascertain if they differ in their capacity to reduce arsenate.

Materials and methods

Plant culture

Two phenotypes, arsenate-tolerant (T) and nontolerant (NT), of *H. lanatus* L. were used in this study; both were isolated from the normal, polymorphic population of *H. lanatus* in the Hoopern valley at the University of Exeter, UK. The physiology of the phenotypes has been described in previous investigations (Meharg & Macnair, 1992b, 1993). The use of T and NT phenotypes from the same population has the advantage of reducing problems associated with other genes (those associated with, for example, growth habit and nutrient efficiency) that might be in linkage disequilibrium with the tolerance gene. Before the experiments described here, plants had been maintained in a glasshouse and potted in a general purpose compost for over 4 yr.

Tillers were cultured hydroponically in 40-l vessels with a one-fifth strength modified Hoagland nutrient solution of the following composition: 1.0 mM KNO_3, 1.0 mM $Ca(NO_3)_2$, 0.4 mM $MgSO_4$, 0.1 mM KH_2PO_4, 0.5 μM $MnCl_2$, 3 μM H_3BO_3, 1 μM $(NH_4)_6Mo_7O_{24}$, 0.4 μM $ZnSO_4$, 0.2 μM $CuSO_4$, 20 μM NaFe(III)-EDTA. The pH of the nutrient solution was buffered at 6.0 with 2 mM MES (pH adjusted with KOH). Nutrient solution was aerated continuously and renewed every 3 d. All the experiments were performed in a growth chamber (20°C day : 16°C night temperature; light intensity 500 μmol m^{-2} s^{-1}, 16 h photoperiod per day; relative humidity 70%).

Experiment 1

Tolerance to arsenate was evaluated by measuring root elongation in response to arsenate exposure. After 10 d of preculture, rooted tillers of each phenotype were grown in 1-l pots (four plants per pot) and exposed to increasing concentrations of arsenate (0, 10, 50, 100, 250 μM). Arsenate (Na_2HAsO_4) was added to the basal nutrient solution containing 0.1 mM phosphate and other nutrients as described earlier. Each treatment was replicated fivefold. Before arsenate exposure, roots were stained black with active charcoal powder, followed by rinsing in deionized water (Schat & Ten Bookum, 1992). The increase in root length was recorded 5 d after the start of the assay.

Experiment 2

This experiment was set up to investigate the effect of phosphorus (P) on arsenate uptake and arsenite efflux in the two contrasting phenotypes of *H. lanatus*. Rooted tillers after 12 d preculture were transferred to 1.2-l pots (two plants per pot) and treatments were imposed for 24 h, consisting of 5 μM arsenate (Na_2HAsO_4) with or without 100 μM phosphate. The level of arsenate chosen may be expected from the As concentration in the pore water from contaminated soils. Each treatment was replicated in three pots. Aliquots of 0.5 ml nutrient solution were removed from each pot at 2, 6 and 24 h and diluted with phosphate buffer solution (PBS) containing 2 mM NaH_2PO_4 and 0.2 mM Na_2-EDTA (pH 6.0), which was the eluant solution used for As speciation analysis (see later). After 24 h, the volume of nutrient solution was recorded. Plant shoots were rinsed with deionized water, blotted dry and weighed. Plant roots were rinsed briefly in an ice-cold desorption solution containing 1 mM K_2HPO_4, 0.5 mM $Ca(NO_3)_2$ and 5 mM MES (pH 6.0), and immersed in 1 l of the same solution for 10 min to remove apoplastic As. Fresh root weight was recorded. Shoots

and roots were frozen in liquid nitrogen and ground to fine powder in a mortar and pestle for As analysis.

Experiment 3

This experiment was carried out to compare arsenate uptake, arsenite efflux and As speciation in roots and xylem sap of the T and NT phenotypes of *H. lanatus* in response to a range of arsenate exposures. After 15-d preculture, rooted tillers were transferred to 1-l pots containing 900 ml nutrient solution with different concentrations of arsenate (1, 5, 10 and 25 μM). Each treatment was replicated in three pots. Phosphate was present at 100 μM in all treatments. Aliquots of 0.5 ml of solution were collected at 6 h and 24 h for analysis of As speciation. After 24 h, stems were cut with a sharp blade at *c.* 1 cm above the root system and the cut surfaces rinsed with deionized water. Xylem exudates were collected by pipette for 1 h after decapitation and diluted with PBS. Roots and shoots were harvested as described in Experiment 2 for the analysis of total As. Roots were also analysed for As speciation.

Analytical methods

For analysis of As speciation, aliquots (0.2–0.5 g) of fresh root materials finely ground in liquid nitrogen were extracted with 20 ml PBS for 1 h under sonication. The extracts were filtered through four layers of muslin cloth and then through 0.45-μm filters before analysis of As speciation. Arsenic speciation in nutrient solutions, xylem saps and plant extracts was determined using HPLC-ICP-MS (Agilent LC1100 series and Agilent ICP-MS 7500ce; Agilent Technologies, Santa Clara, CA, USA), as described previously (Xu *et al.*, 2007). Arsenic species (arsenite, arsenate, DMA (dimethylarsinic acid), and monomethylarsonic acid (MMA)) were separated by an anion-exchange As speciation column (Agilent G3154-65001), fitted with a guard column (Agilent G3154-65002). The mobile phase was the PBS solution (2 mM NaH_2PO_4, 0.2 mM Na_2-EDTA, pH 6.0), which was pumped through the column isocratically at 1 ml min^{-1}. The solution from the separation column was mixed continuously with an internal standard solution (germanium, Ge) before being introduced to a concentric nebulizer and a water-jacketed cyclonic spray chamber of the ICP-MS. Signals at *m/z* 75 (As), 35 (Cl) and 72 (Ge) were collected with a dwell time of 500 ms. Possible polyatomic interference of ArCl on *m/z* 75 was removed by the Agilent Octopole Reaction System operating in helium gas mode. The counts of As signal were normalized by those of the internal standard Ge to correct any drift. Arsenic species in the samples were quantified by external calibration curves with peak areas. Analysis of As species was carried out immediately following sample collection or extraction. For each batch of samples, the analysis was completed within 12 h. Samples that were analysed at the beginning of the run were repeated at the end of the run; no changes in As speciation were observed during this period of time.

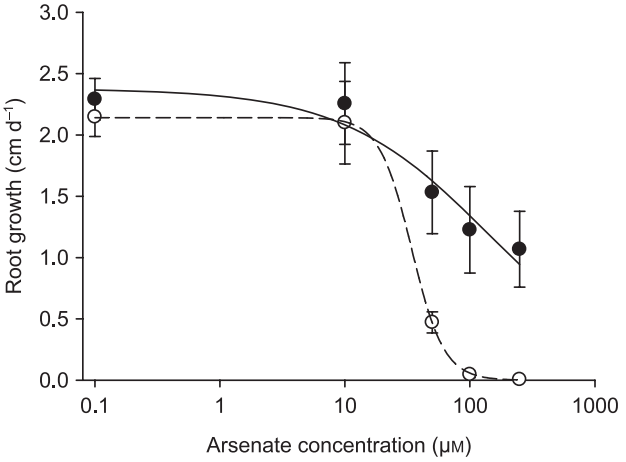

Fig. 1 Inhibition of root growth by arsenate exposure for 5 d in the tolerant (T, closed circles) and nontolerant (NT, open circles) phenotypes of *Holcus lanatus*. Data are means ± SE ($n = 5$). Lines are fitted log-logistic curves. A small value (0.1) was added to the zero arsenate treatment to allow log-transformation.

For analysis of total As concentration, ground plant samples (*c.* 0.5 g FW) were digested in 5 ml high purity HNO_3/$HClO_4$ (85 : 15, v : v). Total As concentrations in the samples were determined by ICP-MS (Agilent 7500ce) operating in the helium gas mode to remove possible interference of ArCl on *m/z* 75. Certified reference materials (IAEA-140/TM seaweed, International Atomic Energy Agency, Vienna, Austria; and NIST1573a tomato leaves, National Institute of Standards and Technology, Gaithersburg, MD, USA) and blanks were included for quality assurance. Repeated analysis of the two certified reference materials gave 42.8 ± 1.53 μg As g^{-1} (mean ± SD) for IAEA-140/TM (certified value 44.3 ± 2.1 μg As g^{-1}) and 0.114 ± 0.016 μg As g^{-1} for NIST1573a (certified value 0.112 ± 0.004 μg As g^{-1}), respectively.

Statistical analysis

The significance of treatment effects was determined by two-way analysis of variance (ANOVA). Where necessary, data were transformed logarithmically to stabilize the variance.

Results

Arsenate tolerance

Figure 1 shows the effect of arsenate exposure on root elongation in the T and NT phenotype of *H. lanatus*. Note that phosphate (100 μM, sufficient for plant growth) was present in the nutrient solution during the experiment. The T phenotype was clearly much more tolerant to arsenate than the NT phenotype. The effect concentration of arsenate that caused a 50% inhibition on root elongation (EC_{50}) was estimated by fitting the dose-response data to a log-logistic curve (Fig. 1). The EC_{50} obtained for the T and NT phenotypes was 142.0 ± 51.5 and 34.3 ± 0.9 μM, respectively.

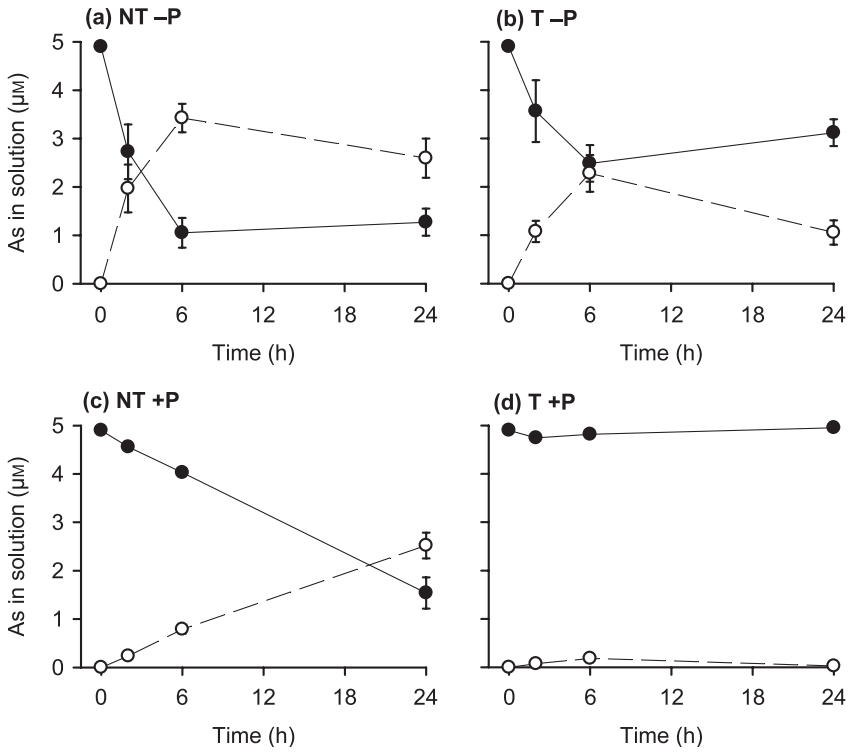

Fig. 2 Arsenic speciation in the nutrient solution during 24-h exposure of *Holcus lanatus* to arsenate as influenced by phosphate supply: (a,c), nontolerant (NT) phenotypes; (b,d) tolerant (T) phenotype; (a,b), no phosphate (–P); (c,d), with 100 μM phosphate (+P). Closed circles, arsenate; open circles, arsenite. Data are means ± SE (n = 3).

Effect of phosphate on arsenate uptake and arsenite efflux

Both T and NT phenotypes were exposed to 5 μM arsenate for 24 h with or without 100 μM phosphate (P). Root biomass was similar between the two phenotypes (P = 0.44), while shoot biomass was 30% larger (P = 0.01) in the NT plants than in the T plants (data not shown).

Arsenic speciation was monitored in the nutrient solution at 0, 2, 6 and 24 h (Fig. 2). In the absence of P, the arsenate concentration in the nutrient solution decreased rapidly in the first 0–6 h, while the concentration of arsenite increased concurrently. The changes from 6 to 24 h were relatively small. The NT phenotype produced significantly (P < 0.001) more arsenite in the nutrient solution than the T phenotype (Fig. 2a,b). After 6 h, 77% and 48% of the As in the nutrient solution was present in the form of arsenite in the NT and T phenotypes, respectively. In the presence of P, striking differences occurred between the two phenotypes (Fig. 2c,d). The NT plants decreased arsenate, and concurrently produced arsenite, in the solution linearly during the 24-h time-course; by 24 h 69% of the initial arsenate had been depleted and 52% was found as arsenite in the solution. By contrast, negligible amounts of arsenate were depleted by the T plants, while negligible amounts of arsenite was produced in the solution.

Arsenate uptake was calculated from the decrease in solution arsenate concentration and arsenite efflux from the appearance of arsenite in the solution, both corrected for transpiration water loss and normalized by root FW. Despite a large phenotypic difference in either arsenate uptake or arsenite efflux, there was no significant difference between the two phenotypes in arsenite efflux as a percentage of arsenate uptake (means over all time-points: NT = 90%, T = 86%, P = 0.33). This percentage was higher after 2–6 h than 24 h. Figure 3a and b show that a linear relationship existed between arsenate uptake and arsenite efflux in the nutrient solution in the two phenotypes over 2–6 h and 24 h, respectively. It is clear that the data from both phenotypes fitted on the same regression line with a slope of 1.00 and 0.85 for 2–6 h and 24 h, respectively.

Total As concentrations in roots and shoots were determined after 24 h exposure (Fig. 4). There were highly significant (P < 0.001) differences between phenotypes and between –P and +P treatments, and significant (P < 0.001) interactions between phenotype and P treatment. The most noticeable difference between the two phenotypes is that +P decreased root and shoot As concentration by 80–90% in the T phenotype, but only by 30–35% in the NT phenotype. In the absence of P the NT plants had a 44% higher As concentration in roots than the T plants, but similar concentrations in the shoots. In the presence of P the NT plants had 10.2- and 3.2-fold higher As concentration in roots and shoots, respectively, than the T plants.

Effect of the arsenate exposure concentration

In Experiment 3, the NT and T plants were exposed to 1–25 μM arsenate in the presence of 100 μM P. Arsenic speciation in the nutrient solution was determined at 6 h and 24 h. The production of arsenite in the nutrient solution increased linearly

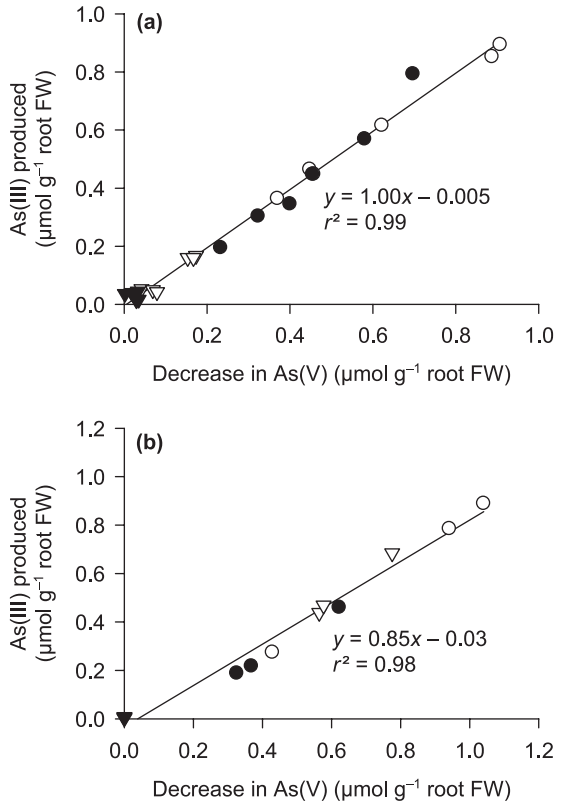

Fig. 3 Relationship between arsenate uptake (measured as the decrease of arsenate in solution) and arsenite efflux (measured as the production of arsenite in solution) over 2–6 h (a) and 24 h (b) in the tolerant (T) and nontolerant (NT) phenotypes of *Holcus lanatus* as influenced by phosphate supply. Closed symbols, T phenotype; open symbols, NT phenotype; circles, no phosphate (–P); triangles, with 100 μM phosphate (+P).

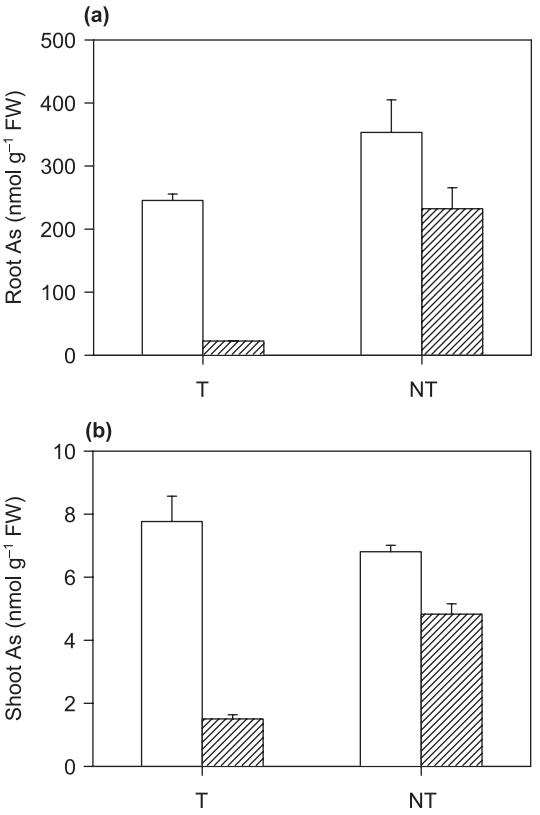

Fig. 4 Arsenic concentrations in roots and shoots of the tolerant (T) and nontolerant (NT) phenotypes of *Holcus lanatus* after a 24-h exposure to an initial 5 μM arsenate with (+P, hatched bars) or without (–P, open bars) 100 μM phosphate. Data are means ± SE (n = 3).

with increasing exposure concentration of arsenate (Fig. 5). The NT plants produced significantly ($P < 0.001$) more arsenite than the T plants. Furthermore, arsenite production by the NT plants increased from 6 h to 24 h, whereas arsenite production by the T plants did not increase from 6 h to 24 h.

An ANOVA showed no significant difference between the two phenotypes in arsenite efflux as a percentage of arsenate uptake (means: NT = 88%, T = 79%, $P = 0.30$), but a significant difference between 6 h and 24 h (means: 6 h = 95%, 24 h = 72%, $P = 0.011$). For both time-points, there was a linear relationship between arsenate uptake and arsenite efflux (Fig. 6); the slope of the regression line was 0.90 and 0.80 for 6 h and 24 h, respectively. Despite the T plants producing less arsenite in the solution than the NT plants, the relationship between arsenate uptake and arsenite production in the solution was essentially the same.

The As speciation in roots was determined. Both the concentrations of arsenite and arsenate in roots increased with increasing arsenate exposure, with the NT plants containing significantly ($P < 0.001$) higher concentrations than the T plants (Fig. 7a,b). However, arsenite was the predominant species in roots, accounting for 86–94% of the total As (Fig. 7c). This percentage increased with the concentration of arsenate the plants were exposed to in the solution ($P < 0.001$), whereas the phenotype difference was not significant ($P = 0.20$). No methylated As species were detected in the root samples. The sum of arsenite and arsenate was in good agreement with the total As determined after acid digestion (mean recovery 113%); hence the pattern for total As concentration in roots (data not shown) was similar to that shown for the individual As species.

For the shoot samples, only the concentration of total As was determined. In both phenotypes, shoot As concentration increased linearly with arsenate exposure (Fig. 7d). There was a significant interaction between phenotype and arsenate exposure, with the NT plants accumulating a significantly ($P < 0.01$) higher As concentration in shoots than the T plants only at the highest exposure concentration (25 μM). Total As in shoots accounted for only a small percentage of the total As uptake: 5.1% and 8.2% in the NT and T plants, respectively ($P < 0.001$). The ratio of shoot to root As concentration was 2.6-fold higher in T (0.145) than in NT (0.056) ($P < 0.001$).

Xylem sap was collected after 24 h exposure to arsenate. On average, arsenite accounted for 66% of the total As in the sap, with the remainder being arsenate (Fig. 8). There was no

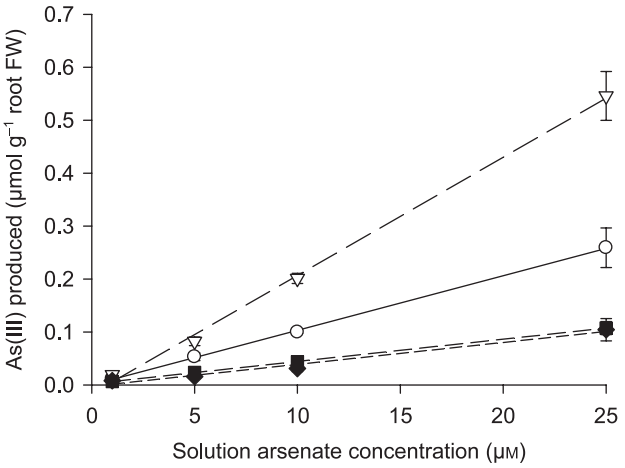

Fig. 5 Arsenite efflux by the tolerant (T) and nontolerant (NT) phenotypes of *Holcus lanatus* in relation to the concentration of arsenate exposure for 24 h. Phosphate was supplied at 100 μM in all treatments. Open circles, NT at 6 h; open triangles, NT at 24 h; closed squares, T at 6 h; closed diamonds, T at 24 h. Data are means ± SE (n = 3).

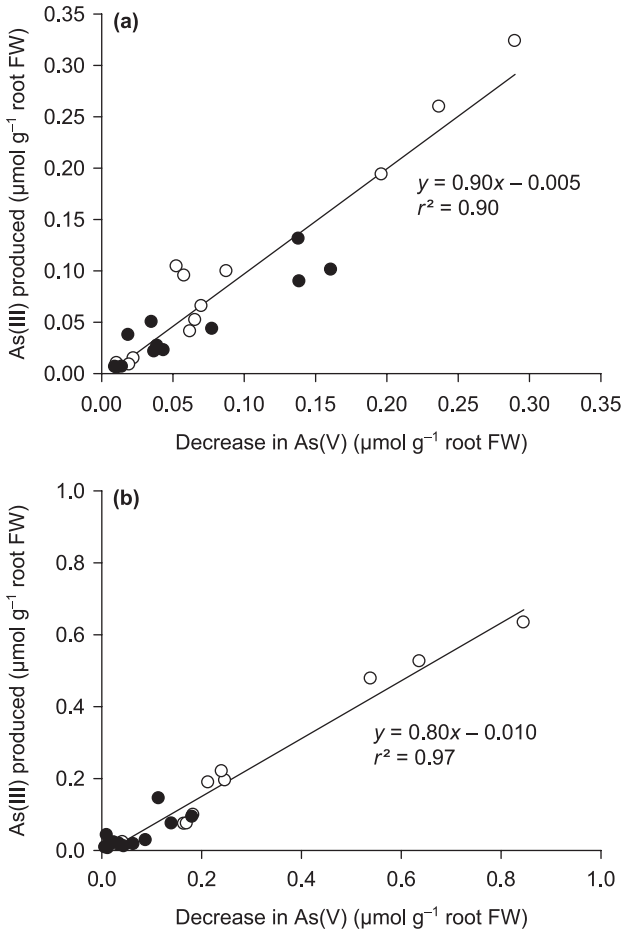

Fig. 6 Relationship between arsenate uptake and arsenite efflux by the tolerant (T, closed circles) and nontolerant (NT, open circles) phenotypes of *Holcus lanatus* across different arsenate treatments at 6 h (a) and 24 h (b) after exposure.

significant (P = 0.68) difference between the NT and T plants in the percentage of arsenite, although the former had significantly higher concentrations of both As species than the latter (P < 0.05 and P < 0.01 for arsenate and arsenite, respectively).

Discussion

Arsenate tolerance is polymorphic in normal populations of *H. lanatus* growing on As uncontaminated sites, with 20–70% of individual plants exhibiting the T phenotype (Meharg & Macnair, 1992b; Naylor *et al.*, 1996). The reasons for such high frequency of the T phenotype remain unclear, but it may suggest a low cost of tolerance (Naylor *et al.*, 1996). At As-contaminated sites, the T phenotype approaches 100% because of the selection pressure of As toxicity. Regardless whether the T phenotype is from As contaminated sites or from normal populations, tolerance in *H. lanatus* is achieved primarily through decreased arsenate uptake (Meharg & Macnair, 1991b, 1992b,c; Bleeker *et al.*, 2006). The results from the present study are consistent with the following model: the T phenotype of *H. lanatus* accumulated significantly less As than the NT phenotype both in the presence or absence of P (Fig. 4) and from a range of arsenate concentrations (1–25 μM) in the medium (Fig. 7). Although arsenate uptake was suppressed by P in both phenotypes, the phenotypic difference was greater in the presence than in the absence of P (Fig. 4). The time-course of arsenate depletion from the medium also showed that the suppression of arsenate uptake by P was greater in the T phenotype than in NT phenotype (Fig. 2). This difference can be explained by a higher affinity of the phosphate/arsenate transporter(s) for arsenate in the NT plants than in the T plants. Indeed, Meharg *et al.* (1994) reported a large difference in the K_m for arsenate in the two phenotypes, 0.025 mM and 0.56 mM for NT and T, respectively. When compared with a similar K_i of *c.* 0.02 mM phosphate, the concentration of P required to inhibit the maximum arsenate influx (V_{max}) by 50%, in both phenotypes (Meharg *et al.*, 1994), it is not surprising that phosphate would have a much stronger inhibitory effect on arsenate uptake in T than in NT plants. Another hypothesis is that the high-affinity phosphate transporter is downregulated in the roots of T plants (Meharg & Macnair, 1992c). This would explain the lower arsenate uptake by T, but not the different potency of P inhibition in the two phenotypes. The exact mechanism responsible for the decreased arsenate uptake by T requires further investigations using molecular approaches.

Consistent with the findings by Xu *et al.* (2007) with tomato and rice, the present study showed a strong efflux of arsenite into the external medium following arsenate uptake by the roots of *H. lanatus* (Figs 2, 5). The amount of arsenite efflux varied between phenotype, P treatment and the concentration of arsenate supplied to the roots. However, this variation was found

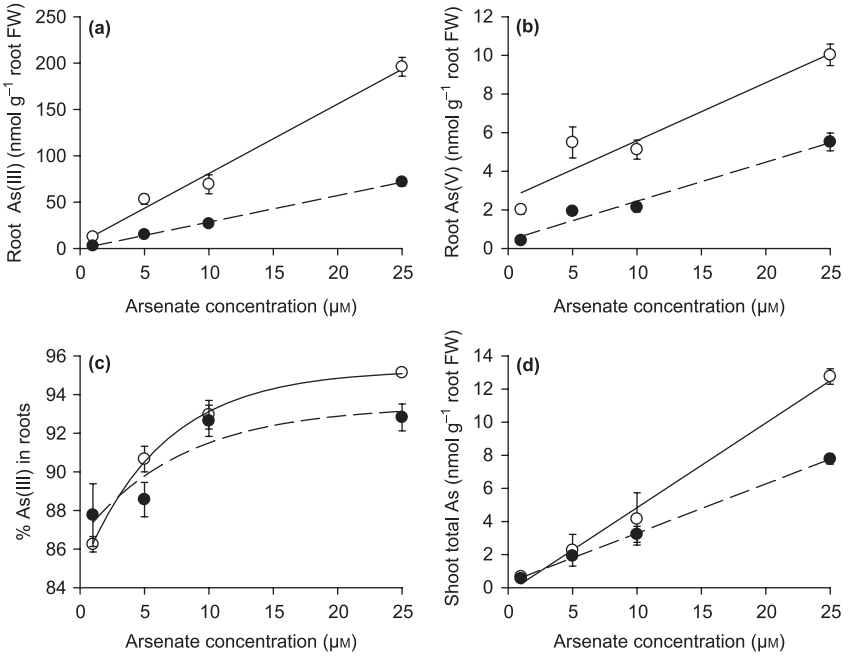

Fig. 7 Concentrations of arsenite (As(III)) (a) and arsenate (As(V)) (b) in roots, the percentage of arsenite in the total extractable As in roots (c) and total As concentration in shoots of the tolerant (T, closed circles) and nontolerant (NT, open circles) phenotypes of *Holcus lanatus* in relation to the concentration of arsenate exposure. Data are means ± SE (n = 3). Linear regression lines are shown in (a), (b) and (d), and hyperbolic curves for (c).

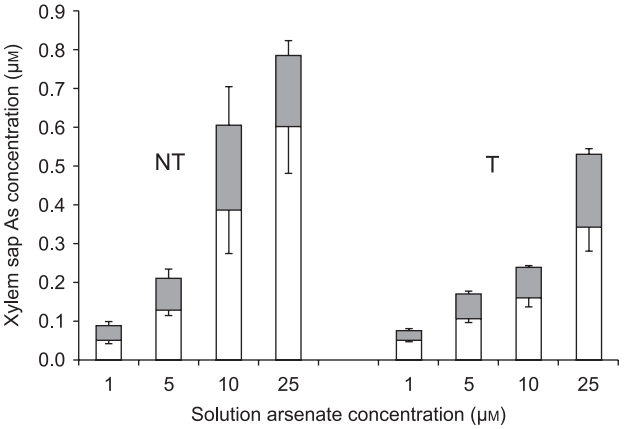

Fig. 8 Arsenic speciation in the xylem sap collected from the tolerant (T) and nontolerant (NT) phenotypes of *Holcus lanatus* in relation to the concentration of arsenate exposure. Closed bars, arsenate; open bars, arsenite. Data are means ± SE (n = 3).

to correlate linearly with the amount of arsenate uptake (Figs 3, 6). The T phenotype took up less arsenate and effluxed less arsenite than the NT phenotype. The presence of P inhibited arsenate uptake and the subsequent arsenite efflux. The slope of the linear regression between arsenite efflux and arsenate uptake provides an estimate of how much arsenite was effluxed as a proportion of arsenate uptake; this varied from 0.8 to 1.0 during the 2–24 h exposure period in the two experiments, indicating a rapid conversion of As species in the medium mediated by plant roots. Previous studies (Xu *et al.*, 2007) showed that microbes and root exudates played little role in the reduction of arsenate to arsenite in the nutrient solution. The fact that the slope was smaller for 24 h than for 2–6 h (Figs 3, 6) can be explained by the reabsorption of arsenite after its efflux. Uptake of arsenite may be mediated by the NIP (nodulin 26-like intrinsic protein) aquaporin channels, which have recently been shown to be permeable to arsenite (Bienert *et al.*, 2008; Isayenkov & Maathuis, 2008; Ma *et al.*, 2008). However, since these aquaporins may mediate bidirectional transport of arsenite dependent on the concentration gradient, whether arsenite is reabsorbed would depend on the concentration gradient of uncomplexed arsenite between the cytoplasm and the external medium. By contrast, efflux carrier proteins similar to microbial ArsB and Acr3p would be able to mediate arsenite efflux against a concentration gradient with the aid of the proton driving force. Accumulation of arsenite was detected in the rhizosphere of sunflower (*Helianthus annuus*) grown in soil (Ultra *et al.*, 2007) and of maize (*Zea mays*) grown in a quartz–goethite system (Vetterlein *et al.*, 2007), suggesting that arsenite efflux also occurs in plants grown in soil or solid media.

The question that was specifically addressed by the present study is whether arsenate-tolerant *H. lanatus* has an enhanced arsenite efflux in addition to the decreased arsenate uptake and increased PC complexation. The answer was negative because both T and NT phenotypes effluxed arsenite as a similar proportion of their respective arsenate uptake (Figs 3 and 6). This suggests no adaptive enhancement of arsenite efflux in the T phenotype. However, it is possible that arsenite efflux represents a basal tolerance mechanism employed by both tolerant and nontolerant plants. Without the strong efflux of arsenite, the amount of As accumulated in plant tissues would have been much higher (by approx. 10-fold), which would then require a much higher capacity of internal detoxification by either PC complexation or vacuolar sequestration. It is possible that arsenite efflux is already so efficient in the NT phenotype that there

is little room for further adaptive improvement. Alternatively, if NIP aquaporins play a major role in both arsenite uptake and efflux, it would be difficult to evolve As tolerance by enhancing their expression or activities. This may explain why there is no enhanced tolerance to arsenite in the metallicolous populations of *Agrostis capillaris* and *H. lanatus*, which are adapted to the high As environment (Porter & Peterson, 1977; Bleeker *et al.*, 2006).

Most of the As in *H. lanatus* roots was in the form of arsenite after exposure to arsenate for 24 h, indicating the efficient reduction of arsenate in the roots. The percentage of arsenite in total As increased with the concentration of arsenate exposure (Fig. 7c), suggesting a mild degree of induction of arsenate reduction. These results are consistent to those of Quaghebeur & Rengel (2003), who compared NT from an uncontaminated site and T from an As contaminated site. They found that the NT phenotype contained a higher proportion of arsenite with regard to the total As in roots than the T phenotype, when both were exposed to the same concentration of arsenate. However, when the percentage of arsenite was plotted against the total As concentration in roots, both T and NT phenotypes appeared to be on the same hyperbolic curve, suggesting that the larger arsenite percentage in NT was a result of its larger arsenate uptake leading to a greater induction of arsenate reduction. In the present study, the percentage of arsenite in total root As was slightly higher in the NT than the T phenotype in the 5–25 μM treatments, although the overall difference was not significant (Fig. 7c). These results and those of Quaghebeur & Rengel (2003) indicate that the two phenotypes have a similar capacity of arsenate reduction in roots, with no adaptive enhancement in the T phenotype of *H. lanatus*. This conclusion is at variance with that of Bleeker *et al.* (2006), who found a higher activity of the arsenate reductase HlAsr in the T phenotype from an As-contaminated site. However, they did not determine *in planta* As speciation. It is possible that there is more than one arsenate reductase enzyme, or that nonenzymatic pathways of arsenate reduction exist (Zhao *et al.*, 2009), which would explain why ecotypic difference in HlAsr activity (Bleeker *et al.*, 2006) might not lead to the expected difference in the capacity of arsenate reduction measured by *in planta* As speciation.

Analysis of As speciation in the xylem sap showed arsenite to be the dominant species of As in *H. lanatus*, accounting for approximately two-thirds of the total As in the sap, with no significant difference between the two phenotypes (Fig. 8). Previous studies of other plant species showed a range of 60–100% arsenite with regard to the total As in the xylem sap in plants exposed to arsenate (Zhao *et al.*, 2009). It thus appears that arsenite is the main form of As transported in xylem. If the transport pathways for arsenate and arsenite are different this could partly explain why the difference in shoot As concentration between the T and NT phenotypes of *H. lanatus* was not as large as that in arsenate uptake by roots (Figs 4 and 7). In rice, transport of arsenite towards the xylem involves the efflux of arsenite mediated by the silicon (Si) transporter Lsi2 (Ma *et al.*, 2008). Whether a similar mechanism operates in other plant species remains to be investigated.

In conclusion, the present study showed a strong arsenite efflux into the growth medium by *H. lanatus* roots following arsenate uptake. This efflux was proportional to arsenate uptake in both the NT and T phenotypes, suggesting no adaptive enhancement in the arsenate-tolerant plants of *H. lanatus*. However, arsenite efflux may be a constitutive mechanism of As tolerance, without which As accumulation in roots would be dramatically elevated. Furthermore, there was no significant difference between the NT and T phenotypes in the capacity of arsenate reduction in roots. Therefore, suppressed arsenate uptake is the key adaptive mechanism of arsenate tolerance in the T phenotype of *H. lanatus*.

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