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Hijacking membrane transporters for arsenic phytoextraction

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Abstract

Arsenic is a toxic metalloid and recognized carcinogen. Arsenate and arsenite are the most common arsenic species available for uptake by plants. As an inorganic phosphate (Pi) analog, arsenate is acquired by plant roots through endogenous Pi transport systems. Inside the cell, arsenate is reduced to the thiol-reactive form arsenite. Glutathione (GSH)-conjugates of arsenite may be extruded from the cell or sequestered in vacuoles by members of the ATP-binding cassette (ABC) family of transporters. In the present study we sought to enhance both plant arsenic uptake through Pi transporter overexpression, and plant arsenic tolerance through ABC transporter overexpression. We demonstrate that *Arabidopsis thaliana* plants overexpressing the high-affinity Pi transporter family members, *AtPht1;1* or *AtPht1;7*, are hypersensitive to arsenate due to increased arsenate uptake. These plants do not exhibit increased sensitivity to arsenite. Co-overexpression of the yeast ABC transporter *YCF1* in combination with *AtPht1;1* or *AtPht1;7* suppresses the arsenate-sensitive phenotype while further enhancing arsenic uptake. Taken together, our results support an arsenic transport mechanism in which arsenate uptake is increased through Pi transporter overexpression, and arsenic tolerance is enhanced through YCF1-mediated vacuolar sequestration. This work substantiates the viability of coupling enhanced uptake and vacuolar sequestration as a means for developing a prototypical engineered arsenic hyperaccumulator.

Keywords

arsenate; phytoremediation; yeast cadmium factor 1; phosphate transporter; glutathione; ABC transporter

1. Introduction

Long-term human exposure to arsenic has been linked to an increased incidence of several cancers and non-cancer skin pathologies (Akter et al., 2005; Duker et al., 2005; Smith et al., 1992). Unfortunately, environmental levels of arsenic exceeding safe standards set by the WHO and EPA are found at many locations worldwide [e.g. India (Patel et al., 2005), Bangladesh (Smith et al., 2000), China (Xia and Liu, 2004), Vietnam (Berg et al., 2001), Australia (Smith et al., 2003), and the U.S. (Durant et al., 2004; Erickson and Barnes, 2005; Welch et al., 2000)]. Concerned over the health issues associated with arsenic pollution, we

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seek to develop strategies for extracting arsenic from contaminated water and soil (Meagher et al., 2007).

Arsenic is present in the majority of aerobic soils as the toxic oxyanion arsenate (AsO_4^{-3}). Due to its chemical similarity to inorganic phosphate (Pi; PO_4^{-3}), arsenate gains access to plant cells by hijacking endogenous Pi transport systems (Abedin et al., 2002; Meharg and Macnair, 1990; Wang et al., 2002). Due to the essential roles Pi plays in growth and development, plants have evolved a variety of responses to facilitate uptake under Pi-limiting conditions (Raghothama, 1999; Yang and Finnegan, 2010). One such response is the induction of high-affinity Pi transporter expression (Muchhal et al., 1996; Shin et al., 2004). The mobilization of phosphorus from mature shoot tissues to actively growing tissues is another Pi starvation response (Jeschke et al., 1997; Mudge et al., 2002; Nagarajan et al., 2011), which may be achieved in part through regulation of Pi transporter expression. The Arabidopsis genome encodes nine high-affinity Pi transporters (Pht1;1 – Pht1;9, hereafter called PHT1 – PHT9), eight of which are induced in roots under Pi-deficient conditions (*PHT1-5* and *PHT7-9*; Mudge et al., 2002). While the relative contribution of the nine Arabidopsis PHT family members to arsenic uptake is not yet known, PHT1 and PHT4 have both been implicated in arsenate transport. *pht1* null mutants are moderately arsenate tolerant, while *pht1 pht4* double mutants are significantly arsenate tolerant, indicating that arsenic uptake is compromised by mutations in these Pi transporters (Shin et al., 2004). Plants overexpressing *PHT1*, on the other hand, have been shown to be arsenate-sensitive (Catarcha et al., 2007). In the present study, we sought to increase plant arsenic uptake through overexpression of the high-affinity Arabidopsis Pi transporters *PHT1* (*PHT1ox*) and *PHT7* (*PHT7ox*).

Several members of the ATP-binding cassette (ABC) transporter family have been implicated in the transport of arsenic across membranes. For example, *HsABCC1/HsMRP1* over-expression has been linked to arsenic resistance in a tumor-derived cell line (Vernhet et al., 1999). Furthermore, HsABCC1 homologs contribute to arsenic detoxification in the nematode *Caenorhabditis elegans* (Broeks et al., 1996; Schwartz et al., 2010), the protozoan *Leishmania tarentolae* (Papadopolou et al., 1994), and the yeast *Saccharomyces cerevisiae* (Song et al., 2003; Guo et al., 2012). Whereas HsABCC1 is found at the plasma membrane, the ABC transporter Yeast Cadmium Factor 1 (YCF1) has been localized to the vacuolar membrane in both yeast (Wemmie and Moye-Rowley, 1997) and transgenic Arabidopsis plants (Song et al., 2003). Several YCF1 homologs in plants have been found at the tonoplast as well (Jaquinod et al., 2007; Klein et al., 2006; Liu et al., 2001). More recently, two transporters in Arabidopsis, AtABCC1 and AtABCC2, were shown to contribute to arsenic tolerance via vacuolar sequestration of As(III)-phytochelatin conjugates (Song et al., 2010). While plasma membrane ABC transporters are involved in substrate extrusion, vacuolar ABC transporters are involved in substrate sequestration. Although both cellular extrusion and vacuolar sequestration of arsenic are viable mechanisms for arsenic detoxification, only vacuolar sequestration would result in the combination of increased arsenic resistance and accumulation. In the present study we sought to increase plant arsenic resistance through overexpression of yeast YCF1 (*YCF1ox*).

Coupling two intuitively mutually exclusive activities - increased arsenic uptake and increased arsenic tolerance - is essential to engineering a plant appropriate for arsenic phytoremediation. Toward this end, we created transgenic Arabidopsis plants overexpressing Arabidopsis Pi transporters (*PHT1ox* or *PHT7ox*) either alone or in combination with *YCF1ox*. Analysis of the transgenic plants revealed that overexpression of *PHT1*, *PHT7*, or *YCF1* enhanced plant arsenic accumulation. Furthermore, coupling *PHT*- and *YCF1*-overexpression conferred both enhanced arsenic tolerance and accumulation on transgenic plants. Our results support the viability of coupling enhanced uptake with

enhanced vacuolar sequestration as a means for developing a prototypical engineered arsenic hyperaccumulator.

2. Materials and methods

2.1 Plant growth and treatments

For all experiments, wild-type *Arabidopsis thaliana* (ecotype Columbia) and transgenic seeds were surface-sterilized and grown under a long-day photoperiod (16 h light / 8 h dark) at 22°C with an average light intensity of 60 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Seedlings were grown on plates containing half-strength Murashige and Skoog (MS) salts (Murashige and Skoog, 1962) with 1% (wt/vol) sucrose (MS plates) for the duration indicated. For assaying arsenate exposure phenotypes and quantifying transgene abundance, plants were grown on MS plates supplemented with 1.2% (vertically-oriented) or 1.0% (horizontally-oriented) agar (Caisson). Otherwise, MS media was solidified with agar (Type E, Sigma) concentrations of 0.5% for horizontally oriented plates and 0.9% for vertically oriented plates. To generate Pi-starved tissue for *PHT1* and *PHT7* cDNA amplification, seedlings were grown in liquid MS media on a rotary shaker for 10 d and subsequently transferred to MS media lacking Pi for an additional 2 d. For seedling treatments, sodium arsenite, sodium arsenate, or buthionine sulfoximine (Sigma) was added to plant media after autoclaving at the indicated concentration from an aqueous, sterile stock solution.

2.2 Reverse Transcription-PCR

RNA was isolated using the Spectrum Plant Total RNA Kit (Sigma), except for transgene quantification experiments, which utilized the Qiagen RNeasy plant mini kit (Qiagen). One microgram of RNA was DNase treated using RQ1 RNase-free DNase (Promega), and complementary DNA synthesis was performed with an oligo(dT)₂₀ primer using the Superscript III First-Strand Synthesis System for RT-PCR (Invitrogen). Twenty μL reaction volumes containing 2 μM each of sense and antisense primers, 4 μL of 20-fold diluted cDNA, and 10 μL SYBR Green PCR Master Mix (Applied Biosystems) were used for quantitative RT-PCR (qRT-PCR) experiments using an Applied Biosystems 7500 Real-Time PCR instrument. The *ACTIN2* transcript was used as the endogenous control, and relative expression levels for each *Arabidopsis* Pi transporter were calculated based on four to six replicates using the $2^{-\Delta\Delta\text{Ct}}$ method of relative quantification (Livak and Schmittgen, 2001). These data were normalized to transcript abundance in untreated control tissues. The transcript abundance of *YCF1*, *PHT1* or *PHT7* in overexpression lines relative to wild-type plants was expressed as $2^{-\Delta\text{Ct}}$ rather than $2^{-\Delta\Delta\text{Ct}}$ since the *YCF1* transcript is not detected in wild-type *Arabidopsis*.

2.3 Cloning and expression of YCF1, PHT1, and PHT7 in plants

The 4548 nucleotide *YCF1* coding sequence was amplified in two pieces from genomic DNA isolated from the BY4742 *S. cerevisiae* parental strain produced by the Saccharomyces Genome Deletion Project (http://www-sequence.stanford.edu/group/yeast_deletion_project/deletions3.html) and assembled using overlap extension PCR (Ho et al., 1989). The 5' half of *YCF1* (2.6 Kb) was modified during PCR to eliminate an internal *NcoI* site, using the primers YCF1-XhNcIS, YCF1-NcMutN, YCF1-NcMutS, and YCF1-PstIN (all primer sequences are shown in Table S1). This 2.6 Kb fragment was cloned into pBluescript KS via the *XhoI* and *PstI* restriction sites (*pBS::YCF1-2.6*). The 3' half of *YCF1* (2.0 Kb) was amplified using the primers YCF1-PstIS and YCF1-ScBmN, and cloned into *pBS::YCF1-2.6* using the *PstI* and *BamHI* restriction sites (*pBS::YCF1*). The *NcoI* and *BamHI* restriction sites flanking *YCF1* were used to subclone the *YCF1* coding sequence into a constitutive vegetative *ACTIN2* promoter/terminator cassette (*A2pt*) (described in Kandasamy et al., 2002), within the binary vector pCambia-1300 (*pCambia::A2pt::YCF1*).

The *PHT1* and *PHT7* cDNAs were amplified from total RNA extracted from 12-day-old wild-type Arabidopsis seedlings that had been starved for Pi for two days. The *PHT1* sequence was modified using overlap extension PCR (Ho et al., 1989) to eliminate an internal *NcoI* site, with the primers PHT1_S1, PHT1_A894, PHT1_S863, and PHT1_A1575noHA. A single set of flanking primers was used to amplify the constructed sequence (PHT1_flank_S and PHT1_flank_A) prior to cloning into an *ACTIN2* promoter/terminator cassette (Kandasamy et al., 2002) via *NcoI* and *HindIII* restriction sites within the binary vector pCambia-1300 (*pCambia::A2pt::PHT1*). The *PHT7* sequence was amplified in 3 pieces and modified using overlap extension PCR (Ho et al., 1989) to eliminate internal *BspHI* and *KpnI* restriction sites. A second *KpnI* site was eliminated via point mutation in the PHT7_S1 primer. In addition, a serine codon was inserted following the methionine start to maintain in-frame cloning via a *NcoI* restriction site. The primers used for amplification were: PHT7_S1, PHT7_A876, PHT7_S845, PHT7_A1073, PHT7_S1045, and PHT7_A1608noHA. A single set of flanking primers was used to amplify the constructed sequence (PHT7_flank_S and PHT7_flank_A) prior to cloning into the *ACTIN2* promoter/terminator cassette via *NcoI/BspHI* and *Sall* restriction sites within the binary vector pCambia-1300 (*pCambia::A2pt::PHT7*).

Wild-type Arabidopsis plants were transformed independently with each membrane transporter overexpression construct using *Agrobacterium*-mediated transformation (Clough and Bent, 1998). PCR analysis of genomic DNA from hygromycin-resistant plants using one vector-specific and one gene-specific primer was used to verify the presence of the correct transgene within each plant line. The segregation of hygromycin resistance was used to identify T3 plant lines homozygous for each construct. *YCF1* and *PHT1* or *PHT7* co-overexpression plants were generated through cross-fertilization between appropriate transgenic plants.

2.4 Arsenic accumulation

Plant samples were swirled for 2 min in 0.01 M HCl, followed by two rinses in deionized water to remove residual arsenic-containing media from tissue surfaces (Aldrich et al., 2003). The samples were prepared for elemental analysis based on methods described in Li et al., 2004. Samples were dried for 3d at 60° C. Prior to arsenic analysis, dried plant tissue was digested overnight with 7 parts nitric acid : 1 part perchloric acid, diluted with water, and passed through a 0.45 micron syringe filter unit. Arsenic levels in these samples were determined using ICP-OES (Chemical Analysis Laboratory, Center for Applied Isotope Studies, University of Georgia).

2.5 Glutathione depletion experiments

Seedlings were germinated on MS plates, oriented vertically. 5-day old seedlings were transferred to a new MS plate supplemented with 250 μ M BSO or 100 μ M sodium arsenate, or both chemicals. At the time of transfer, root tips were marked with a dot on the bottom of the Petri plate. Following three additional days of growth, Image J was used to measure the length of root growth in pixels from the dot to the new location of the root tip (Abramoff et al., 2004).

2.6 Quantification of thiol content

Sulfhydryl groups were quantified in seedlings exposed to BSO, arsenate, or both (as described above), using Ellman's Reagent [DTNB, (Ellman, 1959), Pierce Protein Research Products, Thermo Fisher Scientific] based on a method modified from Lee et al (2003). Approximately 100 mg of plant tissue was ground in liquid nitrogen and resuspended in 300 μ L 1 M NaOH and 1 mg/L NaBH₄. After a 5 min spin at 13,000 x g at 4° C, the supernatant was moved to a new tube, and 50 μ L of concentrated HCl was added. Samples were

vortexed briefly and spun at 13,000 x g for 5 min at room temperature. Eighty μL of supernatant was transferred to a new tube containing 1 mL of reaction buffer (0.1 M sodium Pi pH 8.0, containing 5 mM EDTA) and 20 μL of 4 mg/mL Ellman's reagent. Samples were incubated for 15 min at 30° C, at which time the absorbance at A_{412} nm was recorded relative to blanked control with reaction buffer.

2.7 Statistics

Where indicated, statistical significance was determined using Student's t-Test ($p < 0.05$), and standard deviations or standard errors are shown in the bar graphs.

3. Results

3.1 Selection of Pi transporters for overexpression

We assayed the transcript abundance of the Arabidopsis high-affinity Pi transporter (PHT) family in shoot and root tissues of arsenic-exposed seedlings. Observing increased *PHT* transcript abundance in response to arsenate may deem one or more PHT proteins worthy of targeting for enhancing arsenate acquisition. However, no *PHT* transcripts were induced in shoots or roots of plants exposed to 150 μM sodium arsenate for 21 days, but rather, *PHT8* transcript abundance decreased 5.6-fold in roots (Fig. 1; *PHT3* and *PHT6* transcripts were consistently below the limit of detection in our assays). Since arsenate exposure did not implicate any PHTs in arsenate acquisition from the substrate by roots, we selected one vegetative (*PHT1*) and one reproductive (*PHT7*) Pi transporter for constitutive overexpression in wild-type Arabidopsis plants, based on their contrasting root and floral RNA expression levels, respectively (Mudge et al., 2002; Fig. 2).

3.2 Generation of transgenic Arabidopsis overexpressing PHT and/or YCF1

We cloned the coding sequences of each of *PHT1*, *PHT7*, and *YCF1* into binary vectors for plant expression, under the control of the constitutive, vegetative *ACTIN2* promoter and terminator sequences (*A2pt*; Kandasamy et al., 2002). Agrobacterium-mediated transformation was used to independently introduce the *A2pt::PHT1* (*PHT1ox*), *A2pt::PHT7* (*PHT7ox*), and *A2pt::YCF1* (*YCF1ox*) constructs into wild-type Arabidopsis. Plant lines homozygous for a single insertion of each transgene were identified by hygromycin resistance of the T3 generation, and used to generate *PHT1oxYCF1ox* and *PHT7oxYCF1ox* co-overexpressing plant lines through crossing. Overexpression of the *YCF1*, *PHT1*, and *PHT7* transgenes in the lines selected for further study were verified using quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR; Fig. 3). Transcript levels from each transgene were lower in the co-overexpression lines as compared to the parental, single transformants, although expression of the endogenous *ACT2* gene was unaffected, as determined via qRT-PCR analysis (data not shown). This could be the result of partial silencing of the *ACT2* promoters driving transgene expression, but not of the endogenous *ACT2* promoter, through small RNA-dependent promoter methylation (Eamens et al., 2008). Indeed, endogenous promoters appear less sensitive to this co-suppression silencing mechanism than transgene promoters (Okano et al., 2008).

3.3 Growth phenotypes of transgenic lines on arsenate

Initial screening of segregating *YCF1ox* lines in the presence of arsenate (200 μM) demonstrated that *YCF1*-overexpression conferred arsenate tolerance on 30-day-old Arabidopsis seedlings (Fig. 4A), presumably due to vacuolar sequestration of arsenic via the transport activity of *YCF1*. In contrast, assaying the growth of several independent *PHT1ox* and *PHT7ox* lines in the presence of arsenate (150 μM) demonstrated that *PHT*-overexpression conferred arsenate sensitivity on 30-day-old Arabidopsis seedlings (Fig. 4B), presumably due to elevated arsenate uptake. *YCF1ox* and *PHTox* co-overexpression lines

were generated based on the hypothesis that the in planta activities of these transporters would complement each another. Indeed, overexpression of the *YCF1* transgene in a *PHTox* background rescued the arsenate-sensitivity of the *PHTox* lines (Fig. 4B). Based on visual screening for the best growth on arsenate, lines *A2pt::PHT1-16 x A2pt::YCF1-16* (*PHT1oxYCF1ox*) and *A2pt::PHT7-8 x A2pt::YCF1-16* (*PHT7oxYCF1ox*) were selected for further study (Fig. 4A,B). Whole-seedling fresh weights of 30-day-old plants grown on 150 μ M arsenate were measured to quantify the arsenate tolerance/sensitivity of the various transgenic lines. Both *PHToxYCF1ox* co-expression lines accumulated at least 2.5-fold more biomass than the wild type, and approximately 4-fold more biomass than each corresponding single *PHTox*-expressor (Fig. 4C). Separating root and shoot fresh weights indicated that root growth in the co-expressors is enhanced to a greater extent than shoot growth relative to wild type (Supplementary material, Fig. S1). This may be linked to a change in root morphology in plants containing a *PHT1ox* or *PHT7ox* transgene (Supplementary material, Fig. S2), which could be the result of altered Pi uptake/homeostasis and/or increased arsenate accumulation (see below). Notably, relative to wild-type plants, *PHT1ox* and *PHT7ox* plants were not hypersensitive to arsenite (AsO_3^{-3}), the electrochemically reduced form of arsenate (Supplementary material, Fig. S3).

3.4 Arsenic accumulation of the transgenic lines

The arsenic content of seedlings grown in the presence of 150 μ M sodium arsenate for 21 days was measured using Inductively Coupled Plasma - Optical Emission Spectrometry (ICP-OES; Fig. 5). Both *PHT1ox* and *PHT7ox* plants accumulated significantly higher arsenic levels than wild-type plants ($p < 0.05$), consistent with increased arsenate acquisition in these lines. Similarly, *YCF1ox* plants contained significantly higher As levels than wild type ($p < 0.05$), consistent with previously reported vacuolar metal-sequestering activities of YCF1 in yeast cells (Ghosh et al., 1999) and transgenic Arabidopsis (Guo et al., 2012). Whereas *YCF1ox*, *PHT1ox*, and *PHT7ox* lines contained 125% to 135% higher arsenic levels relative to wild type, *PHToxYCF1ox* co-expressors accumulated significantly higher arsenic levels (~150%; $p < 0.05$).

3.5 Glutathione depletion differentially affects the arsenate-sensitivity of the transgenic lines

Arsenate exposure induces glutathione (GSH) synthesis in plants, and in so doing, increases in planta sulfhydryl levels (Li et al., 2004; Schat et al., 2002; Srivastava et al., 2009). The synthetic amino acid buthionine sulfoximine (BSO), on the other hand, inhibits GSH synthesis (Griffith and Meister, 1979), depleting in planta sulfhydryl levels. We quantified the effect of GSH depletion by BSO treatment on root arsenate-sensitivity of *PHTox*, *YCF1ox*, and *PHToxYCF1ox* plants. Five-day old seedlings were exposed to 250 μ M BSO, 100 μ M arsenate, or both chemicals for 3 days, and the change in primary root elongation was measured (Fig. 6A). The root growth of transgenic plants was not significantly altered by BSO, relative to wild-type plants ($p < 0.05$). In contrast, root growth of all transgenic lines was more sensitive to arsenate compared to wild type ($p < 0.05$), which may reflect the increased arsenate accumulation in these lines (Fig. 5). This was surprising based on our data from 30-day-old plants grown on 150 μ M arsenate, in which the *PHToxYCF1ox* co-expressors exhibited increased root biomass relative to wild type (Supplementary material, Fig. S1). This apparent discrepancy likely reflects a difference in root morphology (Supplementary material, Fig. S2; see Discussion). Root growth of *PHTox* seedlings was more sensitive to arsenate or the combination of arsenate and BSO than *YCF1ox* seedlings ($p < 0.05$; Fig. 6A). Also, depletion of GSH magnified root arsenate sensitivity in all *PHTox* and *PHToxYCF1ox* lines, as compared to *YCF1ox*, with the *PHToxYCF1ox* co-expressors being less sensitive than lines overexpressing only *PHT1* or *PHT7* ($p < 0.05$; Fig. 6A). Together these results highlight the importance of GSH in compensating for the increased

cytotoxic effects of elevated arsenic uptake in the *PHTox* and *PHToxYCF1ox* lines, and that although GSH is also required for the arsenic-transporting activity of YCF1, the level of GSH depletion achieved by 250 μ M BSO exposure was not enough to eliminate the beneficial effect of *YCF1* overexpression. However, increasing the concentrations of arsenate and BSO to 150 μ M and 500 μ M, respectively, did eliminate the beneficial role of YCF1 (Supplementary material, Fig. S4).

To further characterize the role of GSH and other thiols in the arsenate-responses of the transgenics, we quantified free sulfhydryl levels in all genotypes exposed to 250 μ M BSO, 100 μ M arsenate, or both chemicals, using Ellman's reagent (Fig. 6B). As expected, all genotypes contained significantly elevated free sulfhydryl levels in the presence of 100 μ M arsenate relative to exposure to 250 μ M BSO ($p < 0.05$). Intermediate free sulfhydryl levels were observed in all genotypes when plants were treated with both 100 μ M arsenate and 250 μ M BSO. This result is consistent with the contrasting effects of arsenate [stimulatory (Li et al., 2006)] and BSO [inhibitory (Griffith and Meister, 1979)] on cellular thiol peptide levels. Notably, thiol peptide levels of *YCF1ox* plants were comparable to those of wild-type plants in the presence of arsenate. In contrast, the *PHToxYCF1ox* plants contained higher free sulfhydryl levels in the presence of arsenate than plants overexpressing *YCF1* alone, but did not have elevated thiol levels relative to *PHTox* plants in the presence of BSO and arsenate ($p < 0.05$; Fig. 6B). Thus, the enhanced root growth seen in the *PHToxYCF1ox* lines grown under these conditions, relative to *PHTox* lines, (Fig. 6A) is not linked to a direct de novo increase in free sulfhydryl levels, but is likely due to enhanced vacuolar sequestration activity of YCF1.

4. Discussion

The form of phosphorus available to plant roots for uptake and assimilation is inorganic phosphate (Pi). Unfortunately, one of the most common environmental forms of the toxic metalloid arsenic is the Pi analog, arsenate. Due to the chemical similarity between Pi and arsenate, arsenate is absorbed by plant roots through the Pi transport system (Abedin et al., 2002; Meharg and Macnair, 1990; Wang et al., 2002). Hence, suppression of high-affinity Pi transport systems seems to be a common mechanism for acquired arsenic tolerance (Meharg and Macnair, 1992). This is consistent with our observation of decreased *PHT8* transcripts in roots of arsenate-treated plants (Fig. 1). Similarly, Catarecha et al (2007) reported repression of the Pi transporter *PHT1* in Pi-starved plants (3d) exposed to arsenate (8h).

Because arsenate has been shown to enter plants via the Pi transport system, and *pht1 pht4* double mutants in Arabidopsis exhibit increased tolerance to arsenate (Shin et al., 2004), we reasoned that over-expression of high-affinity Pi transporters in Arabidopsis would lead to plants with increased arsenate uptake. Based on the tissue localization of members of the Arabidopsis *PHT* family (Mudge et al., 2002), we chose one vegetatively expressed (*PHT1*) and one reproductively expressed (*PHT7*) Pi transporter for our studies. Our qRT-PCR studies on seedlings grown under Pi-replete conditions confirmed the vegetative expression previously seen for *PHT1* and floral expression for *PHT7* (Fig. 2). We chose to include both a vegetative and a reproductive PHT in our study in case either of these two classes of Pi transporters has evolved an ability to discriminate between arsenate and Pi.

Catarecha et al (2007) demonstrated that overexpression of *PHT1* (*35S::PHT1*) conferred increased arsenate sensitivity on plants. Herein, we showed that overexpression of the Pi transporters *PHT1* (*A2pt::PHT1*; *PHT1ox*) or *PHT7* (*A2pt::PHT7*; *PHT7ox*) conferred arsenate sensitivity (Fig. 4), as well as enhanced arsenic accumulation (Fig. 5). We also demonstrated that the *PHTox* plants are not sensitive to the closely related, but chemically reduced oxyanion, arsenite (Supplementary material, Fig. S3). This result is consistent with

previously published reports that arsenite is not taken up through the Pi transport system in plants (Abedin et al., 2002; Bienert et al., 2008; Isayenkov and Maathuis, 2008). Under our conditions, *PHT7ox* plants appeared marginally more sensitive to arsenate than *PHT1ox* plants (Supplementary material, Fig. S1, Fig. 4, and Fig. 6A), but accumulated about the same quantity of arsenic (Fig. 5). Since *PHT7* transcript abundance was greater in *PHT7ox* plants relative to *PHT1* in corresponding *PHT1ox* plants, the expression level of a given *PHT* may impact root biomass in a dosage-dependent manner by modulating root morphology, which does not directly correlate with arsenic accumulation. Additional evidence for this is seen with the *PHToxYCF1ox* co-expressors (see below).

Yeast *YCF1* was originally identified for its role in vacuolar cadmium sequestration in yeast (Li et al., 1996; Szczypka et al., 1994), and subsequently shown to transport GSH conjugates of not only cadmium, but also mercury (Gueldry et al., 2003) and arsenic (Ghosh et al., 1999). Overexpression of *YCF1* (*35S:YCF1*) was shown to confer both enhanced resistance to, and accumulation of, lead and cadmium on Arabidopsis plants (Song et al., 2003). Recently, Guo et al. (2012) confirmed increased cadmium accumulation and tolerance of transgenic Arabidopsis overexpressing *YCF1* (*35S:YCF1*), and also showed increased arsenic accumulation and tolerance in these plants. Similarly we demonstrated that overexpression of *YCF1* (*A2pt::YCF1*) conferred enhanced arsenate resistance (Fig. 4) and accumulation (Fig. 5) to Arabidopsis plants.

By coupling *YCF1ox* with overexpression of *PHT1* or *PHT7*, we showed further enhancement of arsenate tolerance and accumulation (Fig. 4 and Fig. 5). Although both shoot and root fresh weights of 30-day-old *PHToxYCF1ox* co-expressors grown on 150 μ M arsenate were greater than wild type, as well as *YCF1ox* and *PHTox* single expressors (Supplementary material, Fig. S1 and Fig. 4), short-term primary root growth assays of young seedlings showed decreases in the *PHToxYCF1ox* lines compared to wild type (Fig. 6A). This likely reflects a difference in root morphology that correlates with *PHTox*. Specifically, compared to wild type, all transgenic lines containing a *PHT*-overexpression transgene have relatively shorter primary roots, but greater proliferation of lateral roots, when grown in the presence of arsenate (Supplementary material, Fig. S2). This is consistent with the transgenics overexpressing *PHT1* or *PHT7* having relatively slower primary root growth in the seedling stage (Fig. 6A), but having greater total root mass at maturity, relative to wild type (Fig. 4C and Supplementary material, Fig. S1). It is possible that increased arsenate acquisition due to *PHT*-overexpression alters root system architecture (RSA), and that the addition of *YCF1ox* to *PHT*-overexpressors can compensate for the increased arsenic accumulation, ultimately leading to greater root proliferation, but it cannot reestablish the normal RSA program. This is further supported by comparing the arsenic accumulation and root biomass of all the *PHT* transgenic lines. In comparing the single *PHT*-overexpressors, *PHT7* expression is 1.75-fold higher than *PHT1* expression (Fig. 3), whereas *PHT1ox* plants have 1.3-fold higher root biomass than *PHT7ox* plants (Supplementary material, Fig. S1). Similarly, in the *PHToxYCF1ox* co-overexpression lines, *PHT7* expression is 2.6-fold higher than *PHT1* (Fig. 3), and the root biomass of *PHT1oxYCF1ox* plants is 1.3-fold higher than that of *PHT7oxYCF1ox* plants (Supplementary material, Fig. S1). In contrast, the accumulation of arsenic is not statistically different between *PHT1ox* and *PHT7ox* or between *PHT1oxYCF1ox* and *PHT7oxYCF1ox*. This suggests that root biomass is more tightly linked to the level of *PHT* expression than arsenic accumulation. A threshold level of *PHT* expression appears sufficient for enhanced arsenic accumulation when co-expressed with *YCF1*, but root biomass may be more affected by specific *PHT* expression levels in a way not directly related to arsenate uptake capacity, but perhaps through a more fundamental impact on RSA.

Depletion of GSH by BSO magnified root arsenate sensitivity in all plants overexpressing *PHT1* or *PHT7* (Fig. 6A). This highlights the importance of GSH in plant arsenic tolerance. In addition to bringing in more arsenate than wild type, we show that *PHTox* plants also require a higher intracellular GSH concentration than wild type to support plant growth in the presence of elevated arsenic levels. GSH plays an important role in arsenic detoxification by binding the reduced oxyanion arsenite, to create the arsenite:trisglutathione substrates for ABC transporters such as YCF1 (Ghosh et al., 1999). Hence, the arsenic-transporting activity of YCF1 depends on GSH. The *PHToxYCF1ox* co-expressors were less sensitive to the combination of arsenate and BSO than *PHTox* alone (Fig. 6A). Also, the *PHToxYCF1ox* co-expressors contained lower or comparable sulfhydryl levels relative to the *PHTox* lines under these conditions. Together these results suggest that YCF1 activity is less dependent on de novo GSH synthesis than the ability to tolerate increased arsenate acquisition resulting from *PHT*-overexpression. However, because YCF1-mediated arsenic transport activity requires GSH, further depletion of GSH by BSO should phenocopy the *PHTox* root arsenate sensitivity in the *PHToxYCF1ox* lines. Indeed, we demonstrated that in the presence of higher arsenate and BSO concentrations (150 μ M and 500 μ M, respectively) the arsenate sensitivity of *PHTox* lines was phenocopied in *PHToxYCF1ox* lines (Supplementary material, Fig. S4).

In conclusion, we demonstrate that Arabidopsis plants overexpressing *PHT1* or *PHT7* are hypersensitive to arsenate, due to increased arsenic uptake. These plants do not exhibit increased sensitivity to externally supplied arsenite. Co-overexpression of yeast YCF1 with the *PHT1* or *PHT7* Pi transporters both enhances *PHTox*-mediated arsenic accumulation, and alleviates *PHTox*-induced arsenate sensitivity. Taken together, our results support an arsenic transport mechanism in which arsenate uptake is enhanced through Pi transporter overexpression, while arsenic resistance is enhanced through YCF1-mediated vacuolar sequestration.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Appendix A. Supplementary material

Supplementary material associated with this article can be found in the online version.

Highlights

Overexpressing Pht1 family transporters increases arsenate uptake in Arabidopsis.

Overexpressing *YCF1* in Arabidopsis increases arsenic tolerance and accumulation.

Co-expressing Pht1 and *YCF1* further enhances arsenic tolerance and accumulation.

Fig. 1.

Arsenic has a minor impact on the transcript levels of Arabidopsis high-affinity phosphate transporter genes. A phylogenetic tree depicting the relationship of the Arabidopsis PHT family members was prepared in Paup 4.0 using a heuristic method based on distance, after aligning full-length amino acid sequences in ClustalW. The tree was rooted using PHT8 as the out-group, and bootstrapped 1000 times. Bootstrap values are indicated at nodes where applicable. Shoot and root transcript levels for each *PHT* from 21-day-old seedlings grown in the presence (black bars) or absence (gray bars) of 150 μ M arsenate are presented relative to the untreated shoot transcript levels for each corresponding *PHT*. Values are the means of four to six replicates, and error bars indicate standard deviation. Asterisks indicate *PHT* transcript levels that differ significantly between arsenic-treated and untreated tissues ($p < 0.05$). ND, not detected.

Fig. 2. Tissue-specificity of *PHT1* and *PHT7* transcript abundance. *PHT1* and *PHT7* transcript levels in shoot, root, and flower tissues of wild type Arabidopsis are presented relative to the corresponding transcript level in shoot tissue. Values, displayed on logarithmic plots, are the means of four to six replicates, and error bars indicate standard deviation.

Fig. 3. Confirmation of transgene overexpression in transgenic seedlings. *YCF1*, *PHT1*, and *PHT7* transcript levels in whole seedlings of the genotypes indicated are presented relative to the transcript abundance of the *ACTIN2* endogenous control gene for each corresponding genotype. Values are the means of four to six replicates, and error bars indicate standard deviation. ND, not detected.

Fig. 4.

Arsenate phenotypes of transgenic *Arabidopsis* plants overexpressing *PHT* and/or *YCF1*. (A) Wild-type (WT) and transgenic *Arabidopsis* seedlings segregating for the *YCF1*-overexpression transgene were screened for resistance on 200 μ M sodium arsenate for 30 days. Lines 16, 20, and 32 represent three independent *YCF1*-overexpression lines. (B) Wild-type (WT) and homozygous transgenic lines were screened for phenotypes on 150 μ M sodium arsenate for 30 days. Representative images of two independent *PHT1*- and *PHT7*-overexpression lines and their corresponding hybrids with *YCF1* (line 16) are shown. (C) Fresh weights of seedlings grown as in panel B were quantified. Error bars indicate standard deviation, $n > 60$.

Fig. 5.

Arsenic accumulation in transgenic *Arabidopsis* plants overexpressing *PHT* and/or *YCF1*. Seedling arsenic content of wild-type (WT) and transgenic plants was determined by ICP-OES following 21 days of growth on 150 μ M sodium arsenate. The percent of arsenic accumulation relative to arsenic levels in wild-type plants (100) is noted on each bar. Error bars reflect the standard error, $n > 5$.

Fig. 6.

The impact of glutathione depletion on arsenate-sensitivity of *Arabidopsis* plants overexpressing *PHT* and/or *YCF1*. Root elongation (A) and seedling sulfhydryl levels (B) were quantified for eight-day old wild-type (WT) and transgenic plants grown for three days in the presence of 250 μ M BSO, 100 μ M sodium arsenate (AsV), or both chemicals. Error bars indicate standard error (A) or standard deviation (B), $n = 5$.