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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.

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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 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 (Catarecha 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

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detoxification in the nematode *Caenorhabditis elegans* (Broeks et al., 1996; Schwartz et al., 2010), the protozoan *Leishmania tarentolae* (Papadopoulou 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 μmol m^{-2} s^{-1}. Seedlings were grown on plates containing half-strength Murashige and Skoog (MS) salts (Murashige and Skoog, 1962) with 1% (w/v) 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 days and subsequently transferred to MS media lacking Pi for an additional 2 days. 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). 20 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 4–6 replicates using the $2^{-\Delta\Delta Ct}$ 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 $2^{-\Delta Ct}$ rather than $2^{-\Delta\Delta Ct}$ 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 *Nco*I 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 *Xho*I and *Pst*I 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 *Pst*I and *Bam*HI restriction sites (*pBS::YCF1*). The *Nco*I and *Bam*HI 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 2 days. The *PHT1* sequence was modified using overlap extension PCR (Ho et al., 1989) to eliminate an internal *Nco*I 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 *Nco*I and *Hind*III 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 *Bsp*HI and *Kpn*I restriction sites. A second *Kpn*I 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 SalI 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

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