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Differential expression of genes encoding phosphate transporters contributes to arsenic tolerance and accumulation in shrub willow (*Salix* spp.)

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ABSTRACT

Studies of arsenate and phosphate uptake by plants in hydroponic and soil systems indicate a common transport mechanism via the phosphate transporters (PHTs) due to structural similarity of the anions. Typically, the presence of phosphate decreases plant uptake and translocation of arsenate in hydroponic solution. This study quantified arsenic (As) uptake related to the presence of phosphorus in an As-tolerant willow (*Salix viminalis* × *Salix miyabeana*) and an As-sensitive willow (*Salix eriocephala*). Addition of phosphate resulted in greater As accumulation than in treatments without phosphate in both genotypes, although the tolerant genotype accumulated more As than the sensitive one. Expression of genes for two putative high-affinity phosphate transporters, *PHT1;3* and *PHT1;12*, were up-regulated in both willow genotypes upon addition of As, but to a greater extent in the As-sensitive genotype. Expression of a third putative transporter, *PHT1;4*, was greater in the As-sensitive genotype but was not up-regulated as a result of As addition in either genotype. Leaves of the As-tolerant willow genotype contained greater concentrations of γ-glutamylcysteine (γEC) than the sensitive genotype, although this was not due to differential expression of the *γ-glutamylcysteine synthetase* (*γECS*) gene. The results suggest that increased expression of *PHT1* upon exposure to As in an As-sensitive genotype contributes to rapid toxicity. Our data suggest that although detoxification capacity may be different between genotypes, the differences are not due to up-regulation of γECS or phytochelatin synthase.

1. Introduction

The historic use of lead arsenate as a pesticide in horticultural and agricultural applications has left significant acreage of contaminated soil throughout the northeastern United States. Due to the strong affinity of lead arsenate to bind to iron (Fe) and manganese (Mn) on soil particle surfaces, decontamination and removal can be difficult (Peryea, 1998). Many of these contaminated sites are no longer used for commercial agriculture and stand fallow or have been developed exposing soil particles to surface and groundwater, allowing arsenic (As) pollution to spread and potentially contaminate sources of drinking water (Woolson, 1983). Phytoremediation could be used to remove residual As and to restore the productivity of fallow orchards and farm land, which are currently contaminated with As, to regulatory limits (Robinson et al., 2007). Arsenic is also a common contaminant of mine tailings that cover extensive areas of the US; this land could be used for the production of biofuel feedstocks, if those plants can tolerate the toxin. Fast-growing shrub willow (*Salix* spp.), a proven feedstock crop for bioenergy and biofuels production (Volk et al., 2006), has also been shown to have other valuable economic properties, including phytoremediation capabilities (Greger and Landberg, 1999; Kuzovkina and Quigley, 2005; Watson et al., 2003b). Using a locally grown source of biomass, ideal for growing on fallow land within the northeastern United States, would provide an opportunity to restore contaminated land and promote rural economic development.

Abbreviations: Cys, Cysteine; GSH, Glutathione; HPLC, High pressure liquid chromatography; ICP-OES, Inductively couple plasma-optical emission spectrometry; P_{Pre}, Pretreatment phosphorus; PAR, Photosynthetically active radiation; PC, Phytochelatins; PCS, Phytochelatin synthase; PHT, Phosphate transporters; qRT-PCR, Quantitative reverse transcriptase-polymerase chain reaction; γEC, gamma-glutamylcysteine; γECS, gamma-glutamylcysteine synthetase.

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Experimental evidence suggests that plants uptake arsenate using phosphate transporters (PHT), due to their similar size and charge. However, reaction kinetics have shown that phosphate is highly favored over arsenate (Sadiq, 1997; Ullrich-Eberius et al., 1989; Wang et al., 2002). Plants have both high- and low-affinity phosphate transporters, PHT1 and PHT2 respectively. High-affinity transporters operate when the concentration of phosphate in the substrate solution is in the micromolar range; conversely, low-affinity transporters operate when phosphate concentrations are in the millimolar range (Muchhal et al., 1996). Annotation of genome sequences indicates that *Arabidopsis thaliana* has nine PHT1 (Mudge et al., 2002); whereas *Populus* spp. putatively has 12 PHT1 transporters (DOE JGI Poplar database, annotation). We hypothesize equal numbers of *Populus* and *Salix* PHT genes due to the salicoid specific whole-genome duplication (Tuskan et al., 2006).

Of the high-affinity transporters in *A. thaliana*, only *AtPHT1;6* was not expressed in roots while all other transporters were, additionally *AtPHT1;2*, *8*, and *9* were expressed exclusively in roots (Mudge et al., 2002; Poirier and Bucher, 2002). To date there are no studies of tissue-specific expression of poplar *PtrPHT1* genes. Studies in several species demonstrated greater *PHT* transcript abundance following phosphate starvation (Leggewie et al., 1997; Muchhal et al., 1996; Ribot et al., 2008) and down-regulation upon resupply of phosphate (Catarecha et al., 2007; Ribot et al., 2008). Muchhal and Raghothama (1999) further demonstrated that increased *PHT* transcript accumulation resulted in greater protein levels and correspondingly higher rates of phosphate transport (Raghothama, 1999). Meharg and Macnair (1992) proposed that As-tolerant species (or cultivars) suppress expression of the PHTs, thus limiting arsenate influx and slowing uptake to a rate at which intracellular detoxification occurs before toxicity. Prior experiments have shown that root As uptake responds differentially to phosphate addition depending on the growth medium of the experiment. In hydroponic experiments, the presence of phosphate decreased arsenate uptake as the anions compete for uptake through PHTs (Fitz and Wenzel, 2002). Experiments using soil demonstrated that phosphate addition increased root As uptake through competitive interference for soil particle sorption sites, thereby creating a larger plant available pool (Fitz and Wenzel, 2002; Peryea and Kammereck, 1997).

Following uptake, arsenate (AsO_3^{2-}) is converted to arsenite (AsO_2^{2-}) by arsenate reductase (ARS; Dhankher et al., 2006). Intracellular detoxification occurs through a mechanism that includes binding of arsenite to phytochelatins (PC; low molecular weight, enzymatically synthesized thiol peptides) and sequestration into the vacuole (Grill et al., 1985). A peptide bond forms between the γ-carbon of glutamate (Glu) and cysteine (Cys) by the cytosolic enzyme γ-glutamylcysteine synthetase (γECS) thereby forming γ-glutamylcysteine (γEC; Buchanan et al., 2000). The production of γEC was identified as the rate limiting step for PC production in poplar (Arisi et al., 1997), indicating that γECS is a critical enzyme in As detoxification (Dhankher, 2005). Studies show that increased synthesis of thiol compounds and phytochelatins contribute to arsenic tolerance (Dhankher et al., 2002; Li et al., 2006; Liu et al., 2010). However, very little is known about how the genes involved in detoxification (*γECS*, *phytochelatin synthase* [*PCS*], etc.) are regulated and induced by As stress (Sung et al., 2009).

This work expands on that of Purdy and Smart (2008), who used a 4-week +P pretreatment (+P_{Pre}) followed by a 3-week treatment period with −P/−As, −P/+As, and +P/+As treatments and four *Salix* genotypes. They demonstrated that As uptake was greater in +P/+As treatments than in −P/+As treatments in As-tolerant and As-sensitive willows, a result that contrasts with results from other studies with various plant species. We expand upon that work by characterizing the effects of phosphorus starvation pretreatments expected to up-regulate *PHT1* genes prior to As addition. Our first objective was to compare As sequestration between an As-tolerant and As-sensitive willow genotype. We tested the hypothesis that an As-tolerant willow hybrid, *Salix viminalis* × *S. miyabeana*, suppresses expression of putative *PHT1* genes in roots to a greater extent than the As-sensitive willow, *Salix eriocephala*, upon exposure to arsenate. Our second objective was to quantify gene expression relative to treatment exposure to P, As, and willow genotype. Finally, we wanted to understand the role of thiol metabolism in willow relative to As exposure. The goals of this project were to further understand the relationship between arsenate uptake and phosphate availability and determine the role of thiol metabolism in As tolerance by examining metabolites and the differential expression of *PHT1*, *γECS*, and *PCS*.

2. Materials and methods

2.1. Plant growth conditions

Dormant 1-year-old field-grown stem cuttings (25 cm) of *S. viminalis* × *S. miyabeana*, genotype ID 99202–011 and *S. eriocephala*, genotype ID 00X-026-082 were used for all experiments and analysis. These specific *S. viminalis* × *S. miyabeana* and *S. eriocephala* genotypes were selected based upon previous work that identified them as As-tolerant and As-sensitive, respectively (Purdy and Smart, 2008). For hydroponic culture, willow cuttings were supported by inserting them into rubber stoppers with a hole bored in the center. Each cutting and rubber stopper were pressed into a hole cut in the lid of a 6 L plastic bucket (Yankee Containers, New Haven, CT) or into the mouth of a 1 L plastic bottle. Quarter-strength Hoagland's solution was modified based on treatment requirements and adjusted to pH 6.2 (Hoagland and Arnon, 1950; Watson et al., 2003a). The final concentration of P in +P treatments was 500 μM. Equivalent moles of ammonium formate (NH_4HCO_2) replaced the ammonium phosphate in −P treatments, while still maintaining a pH of 6.2. Arsenic was added as sodium arsenate dibasic heptahydrate to a final concentration of 250 μM ($Na_2H_2AsO_4{\cdot}7H_2O$, Alfa Aesar, Ward Hill, MA; 98–102% purity). This concentration was chosen based on Purdy and Smart (2008) who demonstrated that both genotypes could survive that concentration.

2.2. Long-duration hydroponics experiment

Sixty-four 6 L-buckets were evenly divided between two growth chambers (Model GRC-40, BioChambers Inc., Winnipeg, Canada). Relative humidity was 40%; daytime (14 h) and nighttime (10 h) temperatures were 24 and 22 °C; with an average PAR of 540 μmol m^{-2} s^{-1} at the floor of the chamber. The factors and levels (in parentheses) of the complete factorial experimental design with 16 treatment combinations were: two willow genotypes (*S. viminalis* × *S. miyabeana*, genotype ID 99202-011; and *S. eriocephala*, genotype ID 00X–026–082); two pretreatment solutions (P absent, −P_{Pre}; and P present, +P_{Pre}); two P treatments (P absent, −P; and P present, +P); and two As treatments (As absent, −As; and As present, +As). The design was replicated four times in a randomized complete block design.

Solutions were replaced weekly with new Hoagland's solution with a pH of 6.2, reducing the effect of changes in the solution due to metabolic activities. Bud break occurred on the third day after establishment. Beginning on day 9, stem length from each broken bud was measured every 3 days and the cumulative length averaged per bucket to normalize for any variation in bud break. The pretreatment period of growth constituted the first 4 weeks of the experiment (Stoltz and Greger, 2002), after which the pretreatment solutions were replaced with the treatment solutions

(+P/−As, −P/−As, +P/+As, −P/+As) and maintained for 3 additional weeks.

Sixteen leaf disks (8 mm diameter) were collected per plant and immediately submerged in an extraction buffer of 6.3 mM diethylenetriamenepenta acetate (Aldrich) dissolved in 1N NaOH and 0.1% v:v trifluoroacetic acid (Fluka, 99% purity). Samples and buffer were stored frozen at −20 °C. Thiol-containing peptides were assayed using a highly sensitive and reproducible HPLC method developed by Minocha et al. (2008) involving the pre-derivation of the column with monobromobimane. As reported in Subsection 3.1.1, plants within specific treatment combinations died prior to harvest and were not sampled for thiols; therefore, thiol data was analyzed as a one-way ANOVA instead of within the factorial treatment structure.

Upon harvest, plants were separated into leaves, stems, cutting, and roots which were dried in an oven at 65 °C for 3 d, weighed, then milled (Wiley mill, 20 mesh). Tissue was digested in acid in a microwave digester (CEM MARS 5, Matthews, NC) with the following parameters: 1200 W, 800 PSI, and 200 °C maximum temperature. Digested samples were analyzed using inductively coupled plasma-optical emission spectrometry (ICP-OES; Perkin-Elmer Optima 3300 DV, Norwalk, CT). The detection limit of the instrument for As was 5 $\mu g\,\mu L^{-1}$. Subsamples of roots were collected for RNA extraction, but those failed to yield sufficient quantity of usable RNA.

2.3. Short-duration hydroponics experiment

Based on transpiration data from the long-duration hydroponics experiment, and in an effort to reduce generation of As waste solution, 1 L plastic bottles were used in an experiment to study gene expression. Growth chamber conditions were modified resulting in continuous light with a PAR of 470 $\mu mol\,m^{-2}\,s^{-1}$.

This experiment was a $2 \times 2 \times 2 \times 3$ incomplete factorial with willow genotype, P treatment, and As treatment as in the long-duration hydroponic experiment. Additionally, there were three sampling times (4, 12, and 48 h) and a control group ($t = 0$ h) harvested before the treatment period began after a 3 weeks pre-treatment period. Plants were exposed to the same P solution (−P or +P) throughout the duration of the experiment. There were four replicates in a completely randomized block design. Plants were grown for 3 weeks in 0.25× +P or −P modified Hoagland's solution (pH 6.2). After 3 weeks, roots were collected from four replicate +P and −P plants as time 0 h controls. Arsenic was added in the same manner and at the same concentration as the long-duration hydroponics experiment producing the following treatments: +P/−As, −P/−As, +P/+As, and −P/+As. Roots were harvested for RNA extraction from four replicates of each treatment at 4, 12, and 48 h.

2.3.1. RNA extractions and cDNA synthesis

RNA was extracted from root tissue and DNase treated using the Spectrum™ Plant Total RNA Kit (Sigma–Aldrich) according to the manufacturer's instructions. The synthesis of first-strand cDNA was performed using Superscript® III First Strand Synthesis System (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol using random hexamers for amplification. A total of 8 μg of DNase treated RNA was used for each reaction.

2.3.2. Cloning of partial cDNAs

Degenerate primers were designed for the amplification of gene fragments coding for PHT, γECS, and PCS. Primers were designed in conserved regions of each gene (Pulley, 2008). All PCR reactions were performed using an iCycleriQ™ thermocycler (Bio-Rad Laboratories, Hercules, CA). The reaction conditions for *γECS* fragment amplification were 5 min at 95 °C; 39 cycles of 30 s at 95 °C, 30 s at 60 °C, and 65 s at 72 °C; 5 min at 72 °C; ending with a 4 °C hold in the thermocycler. Amplification of *PHT* and *PCS* gene fragments differed in that the annealing temperature was 40 °C, elongation lasted 105 s, and 35 amplification cycles were used. All PCR fragments were cloned into the pCR 2.1-TOPO® vector and were used to transform One Shot® TOP10F' chemically competent *Escherichia coli* according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). Plasmid DNA was isolated from screened colonies using alkaline lysis followed by silica guanidine thiocyanate miniprep purification (Carter and Milton, 1993). The cloned inserts of plasmids were sequenced at the Cornell University Life Sciences Core Laboratories Center (Ithaca, NY).

2.3.3. Sequence analysis

All partial cDNAs (GenBank: HQ228347–HQ228363) for each gene of interest were analyzed with BioEdit 7.0 (Hall, 1999). BLAST searches were performed at the NCBI server (http://ncbi.nlm.nih.gov/BLAST) and the DOE JGI Poplar database, version 1.1 (http://genome.jgi-psf.org/poplar/poplar.home.html). ClustalW multiple alignments were performed with BioEdit 7.0 (Hall, 1999). One sequence for both *γECS* and *PCS* were found. Three distinct sequences were found for the multigene PHT family and named according to the homologous poplar sequence.

2.3.4. Expression analysis by probe-based real-time quantitative PCR

Primers and dual-labeled probes (Tables S1 and S2) were designed according to corresponding sequences identified in *Salix* spp. PCR amplification was done in a volume of 50 μL and contained 1 μg of either cDNA template or the plasmid standard in 1× IQ Supermix (Bio-Rad Laboratories) and corresponding probe and primer pairs (Tables S1 and S2). Final primer concentration was 1 μM; probe concentration was 250 nM for all genes except γECS and PHT1;4 which had a probe concentration of 500 nM. Expression assays were optimized for probe and primer concentrations; standard curves for all assays were fit to a linear model with R^2 values above 0.90 and PCR efficiencies ranged from 90% to 120%. Amplification specificity of the primers was verified by melt-curve analysis. All real-time PCR reactions were performed using an iCycleriQ™ (Bio-Rad Laboratories). After the collection of well factors the reaction protocol was as follows: 3 min at 95 °C; 45 cycles of 10 s at 95 °C and 60 s at 60 °C. The relative abundance of transcripts was normalized to time zero of *S. eriocephala* in the −P/−As treatment. Ratios of gene expression for each gene of interest were calculated using the Pfaffl method (Pfaffl, 2004). Each data represents the average of three experimental replicates except in *S. eriocephala*: −P/+As/4 h where there were four samples, and the following treatment combinations that only had two available samples, as the third was lost: *S. viminalis* × *S. miyabeana*: −P/−As/0 h, −P/+As/4 h, +P/+As/4 h, +P/−As/48 h, and +P/+As/48 h; *S. eriocephala*: −P/−As/12 h, −P/+As/48 h, and +P/−As/12 h.

2.4. Statistical analyses

For the long-duration hydroponics experiment, a one-way analysis of variance (ANOVA) test was performed in SAS (PROC GLM; version 9.1.3, Cary, NC). Approximated factors and interactions were analyzed as contrasts because the factorial treatment structure was lost to missing cells. No data collected from dead plants were included in statistical analysis. For the short-duration hydroponics experiment, a four-way ANOVA test was performed in SAS. Type III error was used to evaluate contrasts from both experiments.

Phytochelatin and precursor thiol concentrations from the long-duration hydroponics experiment were analyzed within one-way multi- and univariate ANOVAs (PROC GLM; SAS), where multivariate analysis included all seven thiol-containing compounds

Table 1
Mean arsenic concentration and content in four tissues from two shrub willow genotypes grown in long-term hydroponic culture in the presence (+, 500 μM) or absence (−) of P over a 4-week pretreatment (P_{Pre}) followed by a 3-week treatment (+/−P). Hydroponic solution contained 250 μM As ($n = 4$).

	S. viminalis × *S. miyabeana*				*S. eriocephala*			
	$+P_{Pre}$		$-P_{Pre}$		$+P_{Pre}$		$-P_{Pre}$	
	+P	−P	+P	−P[a]	+P	−P[a]	+P	−P[a]
As concentration (mg kg^{-1})								
Leaf	66.8	22.8	29.0	*26.4*	20.3	*28.6*	25.4	*84.2*
Stem	34.6	20.4	17.4	*97.0*	16.8	*63.6*	26.4	*244*
Cutting	100	45.2	23.5	*39.2*	58.5	*78.2*	22.0	*55.1*
Root	3170	1800	3110	*214*	2380	*266*	392	*230*
As content (mg plant^{-1})								
Leaf	1.45	0.35	0.12	*0.06*	0.34	*0.15*	0.03	*0.18*
Stem	0.56	0.22	0.06	*0.10*	0.11	*0.07*	0.01	*0.12*
Cutting	1.11	0.37	0.17	*0.24*	0.50	*0.35*	0.08	*0.22*
Root	20.3	7.57	9.76	*0.31*	9.00	*0.33*	0.33	*0.20*
Total	23.42	8.51	10.11	*0.71*	9.95	*0.90*	0.45	*0.72*
Percent deposition (%)								
Leaf	6.2%	4.1%	1.2%	na	3.4%	na	6.7%	na
Stem	2.4%	2.6%	0.6%	na	1.1%	na	2.2%	na
Cutting	4.7%	4.3%	1.7%	na	5.0%	na	17.8%	na
Root	86.7%	89.0%	96.5%	na	90.5%	na	73.3%	na

[a] All plants within the treatment died from As exposure during the 3-week treatment period, thus values are represented in italics and underlined.

quantified. The multivariate data was partitioned between seven eigenvectors. Eigenvalues, a composite of the seven thiol concentrations, were calculated for each sample for each eigenvector then graphed along the first and second eigenvectors. Due to uneven sampling, Type III error was used to evaluate contrasts. All contrasts were assessed at α of 0.05.

3. Results

3.1. Long-duration hydroponics experiment

3.1.1. Clonal differences to experimental treatments

Stem length was measured as a non-destructive assessment of growth throughout the course of the experiments. On the last day of the pretreatment in both hydroponic experiments, stem length (a biomass proxy) was greater for *S. viminalis* × *S. miyabeana* than for *S. eriocephala*, and for each genotype the $+P_{Pre}$ plants had longer stems than $-P_{Pre}$ plants ($P < 0.0001$, Fig. S1). These genotypes were selected due to their differential As sensitivity; coincidentally, stem biomass was significantly higher in *S. viminalis* × *S. miyabeana* than in *S. eriocephala* in the field and in controlled environments (Fig. S2; KD Cameron, personal communication). Arsenic addition resulted in the death after 7–14 days of all the plants in the following treatment combinations: *S. viminalis* × *S. miyabeana*: $-P_{Pre}$/−P, *S. eriocephala*: $+P_{Pre}$/−P, and *S. eriocephala*: $-P_{Pre}$/−P.

3.1.2. Arsenic uptake and tissue deposition

Arsenic deposition as a percent of total As uptake was greatest in the roots. Total plant As content varied with pretreatment and treatment level P availability (Table 1). *S. eriocephala* −P plants wilted in response to As addition within 1 d regardless of pretreatment P status. *Salix viminalis* × *S. miyabeana* plants in the $-P_{Pre}$/−P treatment wilted following 6 d of As exposure. All of the plants that initially wilted died by the end of the 3-week As exposure. Arsenic uptake was determined even for tissue from dead plants; however, these results were not included in statistical analyses. Data are presented on the assumption that there was little change in dry matter from the time of plant death until the day the experiment was terminated when all tissue was collected and dried (a period of approximately 3 d). Arsenic uptake in the roots was 27-fold greater in *S. eriocephala* plants that received P during the pretreatment and treatment period, compared to those that only received P during the treatment period (Table 1).

S. viminalis × *S. miyabeana* plants grown in $+P_{Pre}$ primarily accumulated As in the roots, with 87% and 89% in the +P and −P treatments, respectively. The +P treatment resulted in greater As content in all tissues and the presence of P always resulted in greater As uptake in the roots, exemplified by mean As content of 20.3 mg in roots of the $+P_{Pre}$/+P plants compared to 7.6 mg in those of the $+P_{Pre}$/−P treatment (Table 1). The $-P_{Pre}$/+P plants contained 9.8 mg As in roots representing 96% of total As uptake.

3.1.3. Phosphorus uptake

Within any 2 × 2 × 2 treatment combination of the long-duration hydroponics experiment, P content and concentration were always greater in plants grown in +P than −P, although to a lesser extent in $+P_{Pre}$ than $-P_{Pre}$ (Table 2). Addition of As in the presence of P resulted in lower P content and concentration in the roots of both genotypes with either P pretreatment than in the absence of As (Table 2). *S. eriocephala* was able to accumulate greater concentrations of P in the roots than *S. viminalis* × *S. miyabeana*.

3.1.4. Phytochelatin accumulation

Phytochelatins with γEC chains two to five repeats long (PC_2–PC_5), as well as precursor thiol-containing compounds Cys, γEC, and glutathione (GSH) were quantified from leaf samples at the end of the long-duration hydroponics experiment by HPLC (Table 3). An unknown compound co-eluted with γEC in 12 of the 28 samples from *S. viminalis* × *S. miyabeana*, but not for *S. eriocephala*. Multivariate analysis of the seven concentrations resulted in the first and second eigenvectors explaining 79.8% and 9.4% of the variation in the data respectively (Fig. 1). Samples within each genotype factor grouped closely together indicating that *S. viminalis* × *S. miyabeana* and *S. eriocephala* thiol profiles were significantly different. Specifically, the thiol profile of *S. eriocephala* plants contained higher concentrations of GSH and PC_4. The As treatment factor also grouped *S. eriocephala* samples together where plants exposed to As had thiol profiles with higher Cys and PC_3 concentrations than plants grown in the absence of As.

Concentration of thiol compounds differed by willow genotype (Table 3). In univariate contrasts, *S. viminalis* × *S. miyabeana* had significantly greater concentrations of γEC ($P < 0.0001$) and PC_5 ($P < 0.0001$); while *S. eriocephala* had significantly greater

Table 2
Mean tissue P concentration and content in four tissues from two shrub willow genotypes grown in hydroponic culture in the presence (+, 500 μM) or absence (−) of P over a 4-week pretreatment (P_{Pre}) and 3-week treatment (P), also in the presence (250 μM) or absence of As over 3-week treatment period ($n = 4$).

	S. viminalis × *S. miyabeana*								*S. eriocephala*							
	$+P_{Pre}$				$-P_{Pre}$				$+P_{Pre}$				$-P_{Pre}$			
	−As		+As		−As		+As		−As		+As		−As		+As	
	+P	−P	+P	−P	+P	−P	+P	−P[a]	+P	−P	+P	−P[a]	+P	−P	+P	−P[a]
P concentration (mg kg^{-1} dry weight)																
Leaf	3060	1200	2720	1400	4590	721	2460	*1160*	3520	1400	1960	*4910*	8930	750	1020	*959*
Stem	2490	633	2330	1700	3230	318	1580	*727*	2290	809	1790	*4060*	4120	498	1550	*951*
Cutting	1390	451	1370	1140	1010	182	597	*219*	2060	776	2070	*1980*	1860	238	657	*309*
Roots	6840	1680	6160	3460	9320	709	4230	*780*	11,800	2550	7210	*3170*	10,500	653	1190	*684*
P content (mg plant^{-1})																
Leaf	71.0	25.8	54.4	20.8	67.5	2.10	10.6	*2.78*	73.0	32.7	32.3	*24.3*	61.3	1.90	1.43	*2.03*
Stem	56.6	12.6	46.1	17.6	38.0	0.49	5.93	*0.76*	21.6	7.92	11.5	*4.18*	8.37	0.36	0.56	*0.52*
Cutting	16.1	5.73	13.9	9.23	9.14	1.28	4.29	*1.37*	18.8	7.15	17.2	*8.55*	9.82	1.11	2.54	*1.25*
Roots	74.8	20.4	39.2	13.7	70.5	2.38	13.3	*1.13*	94.8	24.6	27.7	*4.02*	28.3	0.99	1.00	*0.64*
Total	218	64.5	153	61.3	185	54.8	34.1	*5.94*	208	72.4	88.7	*41.1*	107	4.36	5.53	*4.44*
Percent deposition (%)																
Leaf	32.5%	40.0%	35.4%	33.9%	36.5%	33.6%	31.1%	na	35.1%	45.2%	36.4%	na	56.9%	43.6%	25.9%	na
Stem	25.9%	19.5%	30.0%	28.7%	20.5%	7.8%	17.4%	na	10.4%	10.9%	13.0%	na	7.8%	8.3%	10.1%	na
Cutting	7.4%	8.9%	9.0%	15.0%	4.9%	20.5%	12.6%	na	9.0%	9.9%	19.4%	na	9.1%	25.5%	45.9%	na
Roots	34.2%	31.6%	25.5%	22.3%	38.1%	38.1%	39.0%	na	45.5%	34.0%	31.2%	na	26.3%	22.7%	18.1%	na

[a] Indicates all plants within the treatment died from As exposure during the 3-week treatment period, thus all values are represented in italics and underlined.

concentrations of Cys ($P < 0.0001$), GSH ($P < 0.0001$), PC_2 ($P = 0.0376$), and PC_4 ($P < 0.0001$). Concentrations of γEC ($P = 0.0004$) and GSH ($P = 0.0005$) were greater in plants grown without As, while concentrations of Cys ($P = 0.9847$) and PC_4 ($P = 0.1933$) were not significantly different by As treatment.

Varying patterns of thiol accumulation were observed relative to P treatments. For GSH, the four-way interaction between the factors was significant ($P < 0.0001$), as well as all three-way interactions, except the interaction between pretreatment, P, and As ($P = 0.4627$). The pattern of interactions suggests that the genotype factor, in particular the constitutively high concentration of GSH in *S. eriocephala*, was important in these significant interactions. Mean GSH concentration was 60% lower in the absence of P compared to the presence of P in *S. viminalis* × *S. miyabeana*. For γEC, the three-way interaction between genotype, pretreatment, and As was significant ($P = 0.0261$). The absence of P in the pretreatment resulted in greater final leaf γEC concentrations in both willow genotypes, with more γEC in *S. viminalis* × *S. miyabeana* than in *S. eriocephala*. Treatment phase exposure to As resulted in lower γEC concentrations, presumably as it was used for PC production faster than it could be replaced. Conversely, high PC_2 concentration in *S. eriocephala* exposed to As was critical for the significant three-way interaction between genotype, pretreatment, and As ($P < 0.0001$); where As addition resulted in doubling of PC_2 in *S. viminalis* × *S. miyabeana* and 50-fold greater PC_2 in *S. eriocephala* leaves.

3.2. Short-duration hydroponics experiment

3.2.1. Differential expression of PHT1;3, PHT1;12, and γECS

Gene expression assays were to be performed using root tissue from the long-duration hydroponics experiment. However, RNA isolations were unsuccessful from this tissue; therefore, the

Table 3
Mean thiol-containing compound concentrations (nmol g^{-1} FW) in leaves from two shrub willow genotypes grown in hydroponic culturein the presence (+, 500 μM) or absence (−) of Pover a 4-week pretreatment (P_{Pre}) and 3-week treatment (P), also in the presence (250 μM) or absence of As over 3-week treatment period. Plants that died during As treatment were not sampled (−). Thiol compound abbreviations include: cysteine, Cys; γ-glutamylcysteine, γEC; glutathione, GSH; phytochelatin 2, PC_2; phytochelatin 3, PC_3; phytochelatin 4, PC_4; phytochelatin 5, PC_5 ($n = 4$).

	$+P_{Pre}$				$-P_{Pre}$			
	−As		+As		−As		+As	
	+P	−P	+P	−P	+P	−P	+P[a]	−P
S. viminalis × *S. miyabeana*								
Cys	4.23	2.71	3.00	2.87	4.18	1.19	3.94	–
GSH	34.2	15.4	33.1	13.9	25.6	8.10	25.5	–
γEC	35.4	27.3	27.7	22.2	56.9	57.9	43.0	–
PC_2	0.84	0.84	1.79	1.48	0.83	0.85	1.72	–
PC_3	6.96	6.57	7.20	10.2	7.07	7.05	6.23	–
PC_4	0.36	0.71	1.24	0.00	0.00	1.65	0.40	–
PC_5	24.1	17.0	20.2	27.0	24.2	8.85	29.5	–
S. eriocephala								
Cys	4.72	6.65	6.40	–	7.42	11.3	10.4	–
GSH	117	121	94.3	–	144	147	142.0	–
γEC	22.1	30.3	21.3	–	30.5	35.6	16.3	–
PC_2	0.00	0.00	4.37	–	0.10	0.96	5.81	–
PC_3	5.15	6.41	8.96	–	5.10	9.39	11.0	–
PC_4	2.14	2.13	2.28	–	1.88	2.52	2.26	–
PC_5	3.90	4.83	8.72	–	5.55	5.11	6.26	–

[a] For *S. eriocephala*, $n = 2$.

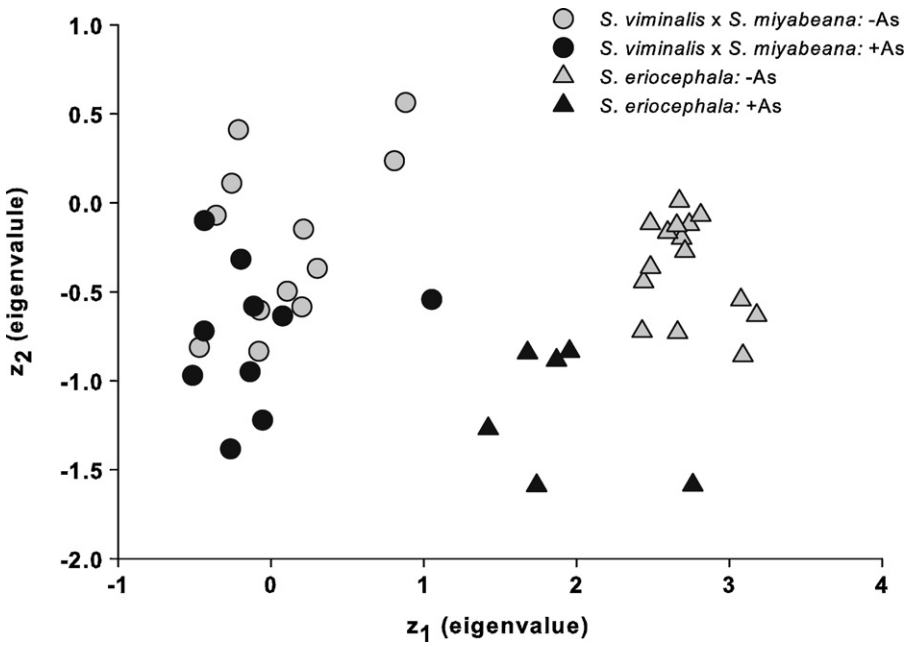

Fig. 1. The first (*x*-axis) and second (*y*-axis) eigenvectors generated through factor analysis for seven thiol-containing peptides from leaves of *S. viminalis* × *S. miyabeana* (circles) and *S. eriocephala* (triangles) grown in hydroponic culture without As (grey) or with As (black).

short-duration hydroponic experiment was developed for the purpose of studying expression profiles of *PHT1;3*, *PHT1;4*, *PHT1;12*, *γECS*, and *PCS*. Gene expression was assayed by qRT-PCR in roots collected immediately prior to As addition (time 0) and at three short-term time points (4, 12, and 48 h) following the addition of As in hydroponic culture in which P status (+P or −P) was consistent prior to and during As treatment. *PHT1;3* mRNA accumulation was significantly greater ($P = 0.0002$) in *S. eriocephala* than in *S. viminalis* × *S. miyabeana* across all treatments and time points (Fig. 2). *PHT1;3* mRNA accumulation in the −P treatments was significantly greater in +As than in −As treatments for *S. eriocephala* and *S. viminalis* × *S. miyabeana*. Greater *PHT1;3* mRNA accumulation upon the addition of As was also observed in +P treatments, but to a lesser extent than under −P/+As conditions. Accumulation of

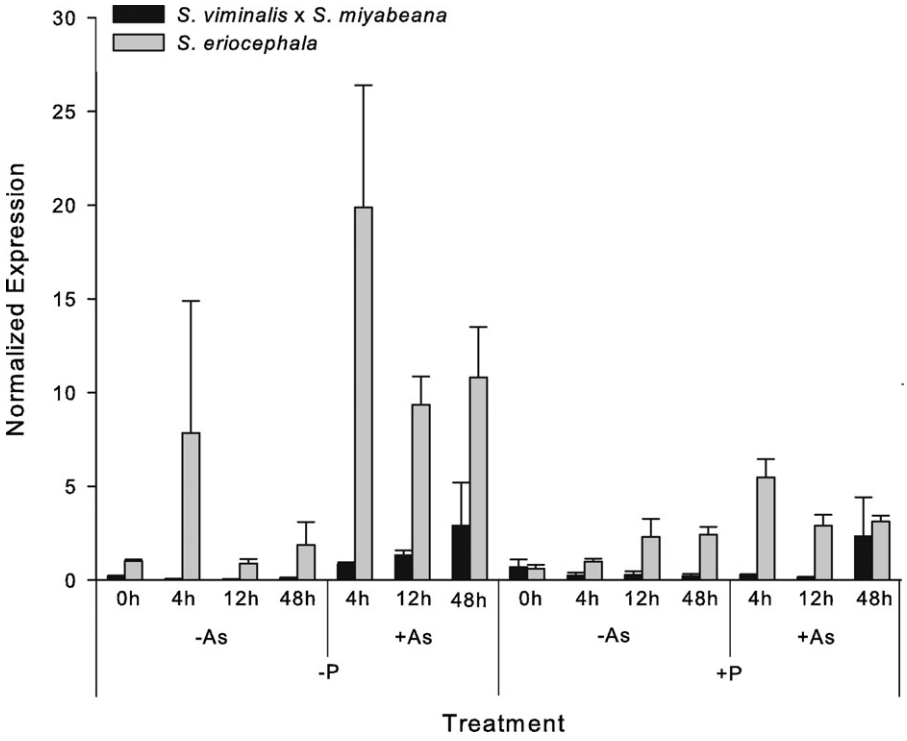

Fig. 2. Ratio of high-affinity phosphate transporter 3 (*PHT1;3*) to the mean expression of *actin* and *elongation factor* 1α in *S. viminalis* × *S. miyabeana* (black) and *S. eriocephala* (grey). All data were normalized to $t = 0$ h *S. eriocephala*: −P/−As treatment using the Pfaffl method. Treatments included the presence (+) or absence (−) of phosphorus (P) or arsenic (As); plants were grown for 3 weeks in −P or +P solution before transfer to final nutrient solution and harvest following 0, 4, 12, or 48 h. Hydroponic solution contained 500 μM P and 250 μM As in appropriate solutions.

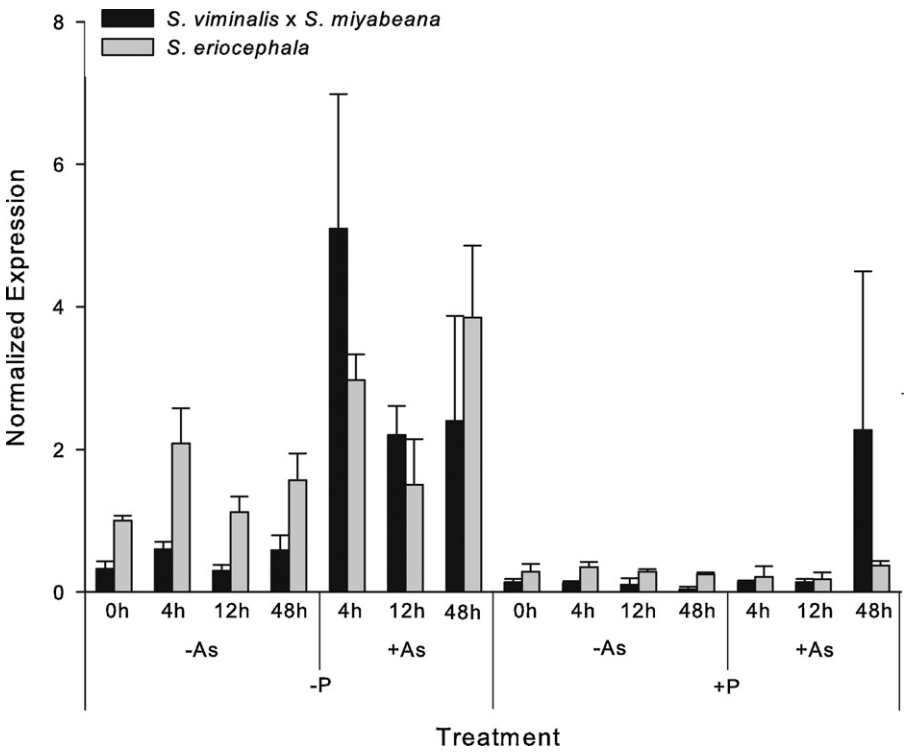

Fig. 3. Ratio of high-affinity phosphate transporter 12 (*PHT1;12*) to the mean expression of *actin* and *elongation factor* 1α in *S. viminalis* × *S. miyabeana* (black) and *S. eriocephala* (grey). All data were normalized to t = 0 h *S. eriocephala*: −P/−As treatment using the Pfaffl method. Treatments included the presence (+) or absence (−) of phosphorus (P) or arsenic (As); plants were grown for 3 weeks in −P or +P solution before transfer to final nutrient solution and harvest following 0, 4, 12, or 48 h. Hydroponic solution contained 500 μM P and 250 μM As in appropriate solutions.

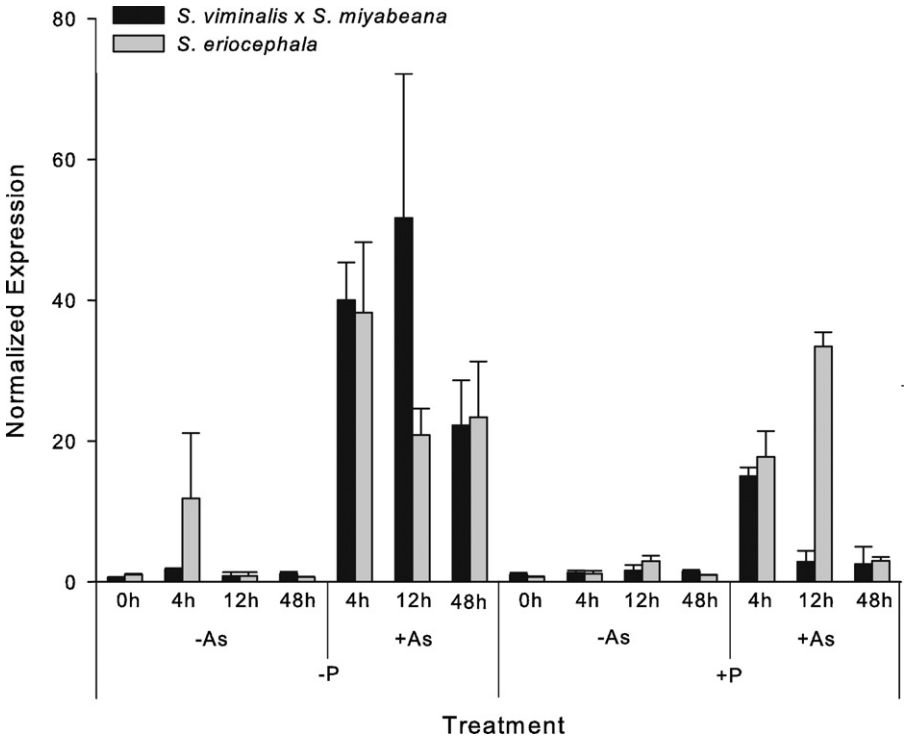

Fig. 4. Ratio of γ-*glutamylcysteine synthetase* to the mean expression of *actin* and *elongation factor* 1α in *S. viminalis* × *S. miyabeana* (black) and *S. eriocephala* (grey). All data were normalized to t = 0 h *S. eriocephala*: −P/−As treatment using the Pfaffl method. Treatments included the presence (+) or absence (−) of phosphorus (P) or arsenic (As); plants were grown for 3 weeks in −P or +P solution before transfer to final nutrient solution and harvest following 0, 4, 12, or 48 h. Hydroponic solution contained 500 μM P and 250 μM As in appropriate solutions.

PHT1;12 mRNA was not significantly different between genotypes ($P = 0.8034$). However, significant differences were independently observed between the P and As treatments (each $P < 0.0001$; Fig. 3). *PHT1;12* mRNA levels were greater in −P treatments than in +P, and greatest expression was observed under −P/+As conditions. Upon addition of As, *γECS* transcript abundance was significantly greater in plants grown without P than with P for both willow genotypes (Fig. 4). In *S. viminalis* × *S. miyabeana* (As-tolerant), in the presence of As, *γECS* mRNA was on average 10-fold greater in the absence of P, compared to the presence of P across all time points. In *S. eriocephala* (As-sensitive), *γECS* mRNA was 2- and 7-fold greater in −P treated plants, compared to +P, after 4 and 48 h exposure to As, respectively; but, this trend was not consistent at the 12 h time point. While there were contrasts in *γECS* mRNA levels based on P addition, there was not a significant difference in expression between the genotypes ($P = 0.7156$), although the overall F test was significant ($P < 0.0001$).

4. Discussion

Phosphate transporters play a crucial role in the transport of As into plant cells. Due to the structural similarity of arsenate and phosphate, arsenate can bind to phosphate transporters and be carried across the plasma membrane. Thus, rates of As uptake can be dependent on the abundance of phosphate transporters regulated by intercellular phosphate (Rubio et al., 2001). The main goal of this study was to better understand how availability of As and P affects the expression of phosphate transporter genes and correspondingly, the uptake and sequestration of As, in genotypes that display contrasting patterns of As accumulation and sensitivity.

4.1. Arsenic tolerance by willow genotypes

The experimental results presented expand upon previous characterization of *S. viminalis* × *S. miyabeana* as As-tolerant and *S. eriocephala* as As-sensitive (Purdy and Smart, 2008). In an As-spiked potting mix experiment, 2.5 times more As (187 mg kg^{-1} compared to 75 mg kg^{-1}) was required to achieve a 39% reduction in biomass of *S. viminalis* × *S. miyabeana* than *S. eriocephala* (Fig. S3). Furthermore, in the 187 As treatment in potting mix, mean As accumulation in *S. viminalis* × *S. miyabeana* plants was 9.3-fold greater in aboveground tissues than in *S. eriocephala* across comparable P treatments (Table S3). The addition of P to the 187 As treatment in potting mix alleviated the effect of As on biomass production, resulting in greater As uptake in *S. viminalis* × *S. miyabeana*, but not for *S. eriocephala* (Fig. S3). A similar result was observed in the long-duration hydroponics experiment, where in the presence of P there was no significant effect of As addition on aboveground biomass for *S. viminalis* × *S. miyabeana*, while there was significantly less biomass accumulation for *S. eriocephala* treated with As. While data from *S. eriocephala* plants that died were not statistically compared to living *S. viminalis* × *S. miyabeana* in respective treatments, an additional line of evidence for differential tolerance was that in similar treatment conditions, *S. viminalis* × *S. miyabeana* survived the stressful −P/+As treatment where *S. eriocephala* did not.

4.2. Effect of P addition on As uptake

In the long-duration hydroponics experiment, the addition of P resulted in greater root and leaf As concentration and content than in the absence of P in the As-tolerant willow genotype, as previously observed for willow (Purdy and Smart, 2008). This phenomenon contrasts with hydroponics experiments using other species, in which P addition resulted in less As uptake due to competition for transporters (Meharg and Macnair, 1991a; Sneller et al., 1999; Wang et al., 2002). We suspect that the phosphate taken up during the pretreatment phase in the *S. viminalis* × *S. miyabeana*: $+P_{Pre}$/−P treatment mitigated the deleterious effects of As by maintaining adequate cellular energy levels allowing those plants to remain metabolically active and continue to accumulate As over the duration of the experiment, as was discussed by Meharg and Hartley-Whitaker (2002). Under conditions of P starvation, the addition of As was likely to have a much more dramatic effect on cellular metabolism due to deplete pools of phosphorylated primary metabolites (Schachtman et al., 1998). A similar result was observed in P-starved *Holcus lanatus* exposed to arsenate for 3 days, where As continued to accumulate in roots over time, particularly in tolerant plants, while sensitive plants died within 2 days of arsenate exposure in the presence of low levels or no phosphorus (Meharg and Macnair, 1991b). While over a longer (3 days) exposure to As, the addition of phosphate ameliorated the toxicity of As in sensitive plants, keeping them viable and allowing greater uptake of As (Meharg and Macnair, 1991b; Pigna et al., 2009).

Another effect of P starvation was to up-regulate *PHT1* high-affinity phosphate transporters, which would result in increased capacity for As uptake. Even though there was no P supplied to the plants in the hydroponic solution, it is necessary to recognize that the willow cutting has P reserves for new growth in the early stages of the experiments. Following the 3-week pretreatment in the short-duration experiment, there was little difference in *PHT1;3* mRNA levels between +P and −P plants at time 0 (Fig. 2), but *PHT1;12* expression at time 0 was significantly greater in −P plants than in +P treated plants (Fig. 3). In the absence of P, the expression of *PHT1;3* and *PHT1;12* was up-regulated in both *Salix* genotypes immediately upon exposure to As, but not in the presence of P. In contrast, *PHT1;1* expression in *A. thaliana* was down-regulated following 8 h arsenate exposure in the absence of P (Catarecha et al., 2007). One explanation for an increase in transporters in +As treatments may be that plants sense a relative decrease in P concentration, and thus up-regulate *PHT1* expression. A similar response was observed for the up-regulation of sulfate transporters upon addition of selenate, presumably due to the competition between these anions for the transporters and perceived lower concentration of sulfate (Berken et al., 2002).

4.3. Differences in PHT expression, As tolerance, and content

Following long-term exposure to P starvation, but prior to the addition of As, *PHT1;3*, *PHT1;4*, and *PHT1;12* transcript abundance was greater in *S. eriocephala* than in *S. viminalis* × *S. miyabeana* (Figs. 2, 3 and S4). In the $+P_{Pre}$/+P/−As treatment, *S. eriocephala* root P content was 1.2% of root biomass with a shoot: root P ratio of 0.27. Conversely in the same treatment, the root biomass of *S. viminalis* × *S. miyabeana* plants contained 0.7% P with a shoot: root P ratio of 0.41. Addition of As in $+P_{Pre}$/+P treatments resulted in less P uptake as a percentage of biomass, with 46% less P in *S. eriocephala* and 18% less P in *S. viminalis* × *S. miyabeana*. In the $-P_{Pre}$/+P/+As treatments, *S. viminalis* × *S. miyabeana* accumulated significantly greater P (concentration and content) than *S. eriocephala*. In the absence of P and As stress, *S. eriocephala* appeared to have greater capacity for P uptake, displaying higher root P concentrations and allocating more P to roots than *S. viminalis* × *S. miyabeana*. The greater overall expression of *PHT1* in the *S. eriocephala* genotype may reflect greater P and As uptake rate and capacity relative to the *S. viminalis* × *S. miyabeana* genotype, which we would expect to have lower rates of As uptake. Our data suggest that As-tolerant varieties have constitutively lower *PHT1* expression, an alternative, but complimentary, hypothesis to that of Meharg and Macnair (1992), who suggested that phosphate transporters were suppressed upon As exposure.

S. eriocephala plants starved for P in the pretreatment ($-P_{Pre}$) and treatment (−P) phases displayed greater aboveground As content

and concentration at the time of their death than the +P treatment plants that survived for several more days. The rapid uptake and translocation of As to stems and leaves in the absence of P competition in hydroponic culture was likely to have overwhelmed the cellular detoxification capacity and contributed to the death of the −P treatment plants relatively soon after exposure. In other hydroponic experiments with no or low P, the non-tolerant *H. lanatus* genotypes were stressed and died within 1 or 4 d of As exposure, while tolerant genotypes survived the treatment period (Meharg and Macnair, 1990; Quaghebeur and Rengel, 2003). Lower rates of arsenate influx due to lower *PHT1* expression in *S. viminalis* × *S. miyabeana* may have allowed for sufficient As detoxification resulting in greater As uptake over an extended exposure time.

4.4. Detoxification capacity between genotypes

A critical factor determining variation in As tolerance in many species of non-hyper-accumulating plants is the capacity of the detoxification mechanisms, primarily phytochelatins and the regulation of their biosynthesis particularly involving the reaction catalyzed by γECS. Over-expression of *γECS* in *A. thaliana* resulted in significantly greater concentrations of γEC, GSH, and PC_2 than in WT in roots of plants grown in 100 μM sodium arsenate as compared to controls (Li et al., 2006). Further, the *γECS* over-expressing line was more tolerant of arsenate than WT (Li et al., 2006). Similar results were obtained using *Brassica juncea* (L.) Czern., where independent lines over-expressing *γECS* and glutathione synthetase (*GSHS*) displayed increased concentrations of GSH and PC_2–PC_4 and greater tolerance to arsenate in seedlings grown for 9 d in 53 μM arsenate (Reisinger et al., 2008).

In our study, concentrations of γEC were significantly greater in *S. viminalis* × *S. miyabeana* than in *S. eriocephala* (Table 3). The greater γEC concentration under As stress observed in *S. viminalis* × *S. miyabeana* compared to *S. eriocephala* may confer some of the observed As tolerance as the larger precursor pool enables GSH and PC synthesis. Thiol profiles strongly distinguished the As-tolerant (*S. viminalis* × *S. miyabeana*) and As-sensitive (*S. eriocephala*) genotypes in a multivariate analysis of seven thiol molecules (Fig. 1). The distinct thiol profiles were apparent in the presence or absence of As in a 7-week hydroponics experiment. Total phytochelatin concentration increased 34% and 79% upon As addition in the As-tolerant and As-sensitive willow genotypes, respectively. The results in this study provide more evidence that thiol metabolism actively contributes to As tolerance and supports prior studies suggesting that enhancing γEC and PC biosynthesis improves As tolerance (Guo et al., 2008; Liu et al., 2010; Sung et al., 2009), although different types of PC can be responsible for detoxification in different species (Schulz et al., 2008). Despite differences observed in metabolite accumulation between the two willow genotypes, *γECS* mRNA accumulation was not significantly different between them. However, upon As addition, *γECS* mRNA levels were significantly up-regulated in both willow genotypes, suggesting that *γECS* expression plays a role in detoxification (Fig. 4). Furthermore, there were no significant differences in the expression of *PCS* ($P = 0.0668$) by genotype or treatment (Fig. S5). Based on our results, we suspect that the As tolerance observed in *S. viminalis* × *S. miyabeana* was a result of slower As influx, due to an overall lower activity of high-affinity phosphate transporters, combined with greater As detoxification capacity.

5. Conclusions

Total As accumulation was greater in plants grown continuously with P than in −P treated plants. Arsenic accumulation over the duration of the experiment was greater in *S. viminalis* × *S. miyabeana* than in *S. eriocephala*. Additionally, As toxicity in *S. eriocephala* plants in the long-duration hydroponics experiment provided further evidence of As sensitivity of this genotype compared to *S. viminalis* × *S. miyabeana*. Phosphorus clearly plays a role in ameliorating As toxicity, with improved growth rates and overall biomass accumulation. Lower levels of expression of phosphate transporters may result in slower As uptake allowing for sufficient detoxification. Thiol metabolite accumulation associated with the As detoxification pathway provided evidence for differences in detoxification capacity associated with the sensitivity of the two willow genotypes tested. Overall, a better understanding into the mechanisms of As tolerance in willow was achieved through these experiments, which may be applied in the breeding and selection of varieties with improved capabilities for As phytoremediation in the field.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.envexpbot.2011.07.008.

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