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Phosphate alleviates arsenate toxicity by altering expression of phosphate transporters in the tolerant barley genotypes

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ABSTRACT

The contribution of the phosphate transporters (PHTs) in uptake of arsenate (As^{5+}) and phosphate (P) is a widely recognized mechanism. Here we investigated how P regulates the uptake of As^{5+} and the subsequent effects on growth and relative expression of PHTs. The study was conducted on 3 barley genotypes differing in As tolerance (ZDB160, As-tolerant; ZDB115, moderately tolerant; ZDB475, As-sensitive) using a hydroponic experiment. There were 3 As^{5+} (0, 10 and 100 μM) and 3 P (0, 50 and 500 μM) levels. The results showed that the negative effect of As stress on plant growth, photosynthesis and cell ultra-structure is As dose and barley genotype dependent, confirming the distinctly genotypic difference in As tolerance. As uptake and accumulation in plant tissues are closely associated with inhibited extent of growth and photosynthesis, with the tolerant genotype ZDB160 having lower As content than other two genotypes. The toxic effect caused by As stress could be alleviated by P addition, mainly due to reduced As uptake. Moreover, the tolerant genotype showed relatively lower expression PHTs than sensitive ones upon exposure to both As stress and P addition, suggesting regulation of PHTs expression is a major mechanism for relative uptake of As and P, in subsequence affecting As tolerance. Moreover, among 6 PHTs examined in this study, the expressions of PHT1.3, PHT1.4 and PHT1.6 showed the marked difference among the three barley genotypes in responses to As stress and P addition, indicating further research on the contribution of phosphate transporters to As and P uptake should be focused on these PHTs.

1. Introduction

Contamination of arsenic (As), which is a highly toxic metalloid and ubiquitous in the environment, has caused a great threat to crop production and human health through the food chain. Currently, the soil As concentration in China ranges from 2.5 to 33.5 mg/kg (He and Charlet, 2013), and some soils have As level over the US Environmental Protection Agency (USEPA) limits of 20 mg/kg in soil. As is toxic to plants, inhibiting growth and synthesis of photosynthetic pigments (Beguma et al., 2016), and inducing oxidative stress (Farooq et al., 2016).

It is well known that in aerobic condition, arsenate (As^{5+}) is a predominant form of As species and is readily taken up by roots via phosphate transporters (PHTs) because of the analogous nature of As^{5+} and phosphate (P). As^{5+} and P have similarly charged oxygen atoms, thermochemical radii with difference by only 4% (Kish and Viola, 1999) and nearly identical acid dissociation constant values. However, some slight differences exist between As^{5+} and P, which could affect the nucleophility of these ions. Furthermore, As^{5+} oxides ($HAsO_4^{2-}$) form analogous esters with P esters (HPO_4^{2-}), however As^{5+} esters are highly unstable in comparison with P esters (Dixon, 1997). It has also been revealed that the enzymes utilizing P can also use As^{5+} as substrate. Faehnle et al. (2004) showed that the enzyme L-aspartate-β-semialdehyde dehydrogenase has an active site which is identical for both As^{5+} and P, with As^{5+} having a lower K_M (1.6 mM) than P (2.9 mM). Therefore it may be assumed that As^{5+} and P should be antagonistic in their effect on the respective ion uptake as well as plant growth. However, little research has been done to verify the antagonistic effect.

PHTs play a pivotal role in the mobilization of As^{5+} into the plant cells due to its structural similarity with P. Like P, As^{5+} can bind to PHTs and thus the uptake of As^{5+} by plant roots is dependent on the relative abundance of P as these two ions are competitive in the binding loci of PHTs. Many PHT genes have been identified based on amino acid homology with yeast PHTs and functionally characterized using yeast mutants lacking high affinity PHTs (Rae et al., 2003). The identified

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PHTs have been classified into 5 families, *viz* PHT1, PHT2, PHT3, PHT4 and PHO, and they are localized in the plasma membrane, chloroplast, mitochondria, Golgi apparatus and plasma membrane, respectively (Guo et al., 2008; Nussaume et al., 2011). In many plants, there are various members of PHT1 family. Thirteen members of PHT1 have been identified in rice (*Oryza sativa*) (Paszkowski et al., 2002), 9 in *Arabidopsis thaliana* (Mudge et al., 2002) and 11 in barley (*Hordeum vulgare*) (Teng et al., 2017). Functional analysis of PHTs have been conducted and the uptake capacity of mutant plants lacking the PHT gene revealed the uptake redundancy of both As^{5+} and P, thus supporting the notion of co-transport of the two ions. WRKY42 over-expressing lines showed a higher As and P content in the roots of Arabidopsis plants (Su et al., 2015), while a rice mutant defective in OsPHF1 (phosphate transporter traffic facilitator1) showed a decrease in P and As^{5+} transport from roots to shoots, whereas transgenic rice over-expressing OsPT8 transporter showed enhanced abilities of P and As^{5+} transport (Wu et al., 2011). However little has been known about the responses of these PHTs to different As^{5+} and P levels in the genotypes differing in As stress tolerance. In addition the studies on the mechanisms underlying the regulation and the specific PHTs involved in As transport in barley are limited, thus much information regarding these concerns is imperative. Furthermore, although it was reported that As-tolerant genotypes suppress expression of PHTs to a greater extent than As-sensitive ones (Meharg and Macnair, 1992), however whether the same mechanism is involved in barley has not yet been explored and the identification of PHTs responsible for As movement in barley has not been done yet.

As far as our knowledge is concerned this study may be the first to explore the simultaneous application of As and P in barley genotypes differing in As tolerance and to detect how various PHTs will respond to As and P addition. The current experiments were conducted with aims of verifying the hypothesis that the expression of PHT genes will be more suppressed in As-tolerant genotypes than in As-sensitive ones in turn helping to unravel information in regards to how the expression of PHTs will affect the plant growth and As uptake. This will help us to identify As-responsive PHTs which will be important for elucidating mechanisms responsible for As tolerance and thus in future engineering of grain crops with less As uptake and accumulation.

2. Materials and methods

2.1. Plant materials

Three barley (*Hordeum vulgare.* L) genotypes (ZDB160, ZDB115 and ZDB475) differing in As tolerance were planted in a greenhouse at Zhejiang University in 2016. According to our previous results the three genotypes were classified as As tolerant, moderately tolerant and As sensitive, respectively (unpublished data). Initially healthy barley seeds were selected, surfaces sterilized with 2% H_2O_2 for 30 min, rinsed with distilled water 3 times and then soaked for 6 h at 25 °C. Thereafter, the seeds were germinated on moistened filter papers in the germination boxes and placed into a growth chamber (22/18 °C, day/night). Uniformly sized 10-days-old seedlings were transplanted into 5 L plastic pots for hydroponic culture. The seedlings were subjected to ½ strength basic nutrient solution (BNS) for 7 d, thereafter full strength BNS was applied in the following proportions (mM): 1 $Ca(NO_3)_2.4H2O$, 1 KCl, 1 $MgSO_4$, 0.25 $NH_4H_2PO_4$, 50 $CaCl_2$, 20 Fe-citrate.nH_2O, 12.5H_3BO_3, 0.5H_2MoO_4, 0.5 $CuSO_4{\cdot}5H_2O$, 2 $MnCl_2{\cdot}4H_2O$, 2 $ZnSO_4{\cdot}7H_2O$ with the pH being adjusted to 5.8 ± 0.1 with HCL/NaOH as required. Continuous aeration was supplied to the nutrient solution by air pumps and the BNS was renewed after every 5 d. At the 3 leaf stage, As^{5+} and P treatments were applied as solutions to the BNS. As^{5+} was administered as a solution of Na_2HAsO_4 in distilled water at concentrations of 0 μM (As0), 10 μM (As1), and 100 μM (As2); PO_4^{3-} was applied as a solution of Na_2HPO_4 in distilled water at concentrations of 0 μM (P0), 50 μM (P1), and 500 μM (P2) to the modified basic nutrient solution, where equivalent moles of $NH_4H_2PO_4$ were replaced with NH_4Cl.

2.2. Measurement of plant growth parameters

At 10 d after treatments, chlorophyll content and photosynthetic parameters were determined on the second fully expanded leaves using a chlorophyll meter (SPAD-502, Minolta) and an infrared analyzer (Li-640 system, Li-COR) on a sunny day from 9 to 12 a.m., with air temperature of 25–28 °C, relative humidity of 50–70%, CO_2 concentration 400 μ mol mol^{-1} and photosynthetic photon flux density (PPFD) of 1000 μmol m^{-2} s^{-1}. Thereafter plants were harvested and immediately the fresh weight, root length and shoot height were measured. Roots were soaked in 20 mM Na_2–ethylenediaminetetraacetic acid (EDTA) for 3 h to remove adhered cations, washed with distilled water and thoroughly rinsed with tap water and blotted on filter paper to remove water.

2.3. Biomass and elemental analysis

For elemental analysis, roots and shoots were separated and were oven dried at 80 °C for 72 h until constant weight and biomass was determined. Arsenic (As) and phosphorus (P) concentrations were determined according to our previous methods (Zvobgo et al., 2015).

2.4. Examination of cell ultra-structure

Fresh samples consisting of the second fully expanded leaves were selected at 10 d after As^{5+} and P treatments and cut into small fragments excluding the midrib. The leaf samples were immediately fixed overnight in 4% glutaraldehyde (v/v) in 0.1 M PBS (sodium phosphate buffer, pH 7.4). Thereafter the samples were washed 3 times in 0.1 M PBS with 15-min interval between each washing, fixed in 1% osmium tetra oxide (OsO_4) for 1 h, followed by subsequent washing in PBS for 3 times, with 10-min interval between each washing. Then the samples were subjected to dehydration in graded series of ethanol (50%, 60%, 70%, 80%, 90%, 95%, and 100%) and absolute acetone with 18-min interval between each wash. The specimens were then infiltrated and embedded in Spur's resin overnight, following heating at 70 °C for 9 h. Finally the specimens were cut into ultra-thin sections (80 nm) and mounted on copper grids for viewing by a transmission electron microscope (JEOL TEM-1230EX, Tokyo, Japan) at an accelerating voltage of 60.0 kV.

2.5. qRT-PCR and gene expression analysis

Fresh plants were harvested and immediately placed in liquid nitrogen and stored in a −80 °C freezer. Total RNA was isolated from 100 mg roots of barley plants exposed to control and As^{5+} and P treatments according to the RNAprep kit (Tiangen) following manufacturers' recommendation. RNA abundance and purity were tested for meeting the requirement. Residual DNA was removed using DNase 1 according to the manufacturer's instructions. The extracted RNA was subsequently used for cDNA synthesis with 100 μM random 6 mers and 50 μM oligo dT primer following the PrimeScript RT Reagent Kit (Perfect Real Time) (Takara, Japan). Three replicates per treatment of cDNA samples were assayed by quantitative real time PCR (qRT-PCR) in the LightCycler 96 (1.1.0.1320) Real-Time PCR system (Roche International Diagnostics system, Switzerland) using the SYBR Green PCR Master Mix (Applied Biosystems). The PCR profile were as follows: initial denaturation 95 °C for 30 s, denaturation 95 °C for 5 s for 40 cycles and annealing at 60 °C for 30 s, followed by steps for Melt-Curve analysis (60 °C −95 °C, 0.5 °C increment for 5 s per step). The barley PHTs (HvPHT) specific primers were designed using Primer-BLAST (www.ncbi.nlm.nih.gov) (Supplementary Table S1) with barley ACTIN (ACT) GenBank ID: AY145451 (Rapacz et al., 2012) as the internal control. Primers were optimized and supplied by Sangon Biotech