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Does aeration conditions affect arsenic and phosphate accumulation and phosphate transporter expression in rice (*Oryza sativa* L.)?

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Abstract

Widespread contamination of rice with arsenic (As) has revealed a major exposure pathway to humans. The present study aimed to investigate the effects of oxygen in the rhizosphere on phosphate transporter (for arsenate transportation) expressions in four rice genotypes, on As and phosphate accumulation and As speciation. Oxygenation marginally increased root and shoot length. Total As concentrations in rice roots were dramatically reduced following oxygenation compared to stagnant treatments ($p < 0.001$). Oxygenated treatments significantly increased arsenate whilst reducing arsenite concentrations in roots ($p < 0.001$). Root arsenite concentrations were 1.5-2.5 times greater in stagnant than in oxygenated treatments. Total P concentrations in rice roots were dramatically increased following aeration compared to stagnant treatments. The relative abundance of phosphate transporter (inorganic phosphate transporter and phosphate:H⁺ symporter family protein) expressions showed down-regulation in stagnant treatments, particularly for SY-9586, XWX-17, XWX-12 in inorganic phosphate transporter expressions, and XWX-17 in phosphate:H⁺ symporter family protein expression ($p < 0.05$). The relative abundance of phosphate carrier protein expressions were relatively higher than the other phosphate transporters, showing up-regulation in stagnant treatments.

Keywords: Arsenic; Rice; Phosphate; Phosphate transporter

1. Introduction

Arsenic (As) is an environmental contaminant and well documented as a human carcinogen (Zhu et al., 2008E; Wu et al., 2015). Exposure from drinking As-contaminated groundwater has caused thousands of people to develop arsenicosis in parts of Southeast Asia (Meharg et al., 2003; Perry et al., 2011) whilst widespread As contamination of rice and other crops has provided a major exposure pathway to humans via the food chain (EFSA., 2009; Meharg et al., 2003, 2009). Elevated As concentrations in paddy soils has originated from both geological and anthropogenic activities, the latter being the major contributor due to smelting, mining and irrigation using As-contaminated groundwater (Liao et al., 2005; Jia et al., 2014). Due to its inherent physiological characteristics and preference to anaerobic conditions rice is particularly efficient at As uptake and accumulation compared to other crops (Su et al., 2010; Wu et al., 2016). Rice is a staple food consumed by half the world's population, and as an export commodity, rice consumption has posed an increasing threat to human health globally due to contamination by As (Stone et al., 2006; Seyfferth et al., 2014).

Arsenic in rice plants can exist both as inorganic and organic species. Inorganic species, As(V) and As(III), present greater toxicity and bioavailability than organic As (Qu et al., 2014; Wu et al., 2015). Therefore, total As concentration and As speciation should both be taken into consideration for health risk assessment (Novoa et al., 2007; Qu et al., 2014). Arsenic speciation will dictate the uptake pathway into rice (Zhao et al., 2010; Wu et al., 2016). For example, studies have shown that As(III) is taken up through silicic acid transport systems (Ma et al., 2008), whilst As(V), a chemical analog of phosphate, shares the same transporters with phosphate (Meharg and Hartley-Whitaker, 2002; Liu et al., 2004). Abedin et al (2002) revealed that phosphate strongly suppressed As(V) uptake in rice plants. Hu et al (2005) reported that P fertilizer significantly reduced As accumulation in rice roots whilst Wu et al (2015) showed that P addition increased As concentrations in rice shoots. Arsenate is the

predominant As species in aerobic soils and will compete with P for absorption sites and uptake transporters in rice roots (Jiang et al., 2014).

Recent studies have reported that water management has profound influences on As uptake and accumulation in rice plants (Xu *et al.*, 2008; Li *et al.*, 2009; Somenahally *et al.*, 2011; Norton et al., 2013). Takahashi et al (2004) found that As was sequestered on Fe(hydr)oxides when soils were not flooded, but upon flooding, was released into the soil solution due to reductive dissolution of the Fe (hydr)oxides and reduction of As(V) to As(III). Xu et al (2008) reported that aerobic conditions greatly reduced As bioavailability, subsequently reducing As accumulation in rice plants; aerobic conditions reduced the concentration of inorganic As by 2.6-2.9 fold in rice grain compared to flooded treatments, which was in accordance with Norton et al (2013). Li et al (2009) also showed that growing rice aerobically reduced As in rice plants. A field-scale experiment conducted by Somenahally et al (2011) demonstrated that intermittent flooding significantly reduced total As concentrations in the rhizosphere and grain compared to continually flooded conditions.

The objectives of the present work were to evaluate (1), the effects of root aeration on the acquisition of P and biomass production of rice, (2) investigate the changes of Pi transporters (OsPT2, 6 and 11) in rice plants, and (3) investigate the effects of root aeration on the accumulation and transformation of arsenate in rice plants.

2. Materials and Methods

2.1. Rice seedlings

Two hybrid subspecies Xiangfengyou9 ('XFY-9'), Shenyong9586 ('SY-9586') and two indica subspecies Xiangwanxian17 ('XWX-17') and Xiangwanxian12 ('XWX-12'), with radial oxygen losses of 9.55, 10.83, 19.7 and 27.0 $\mu\text{mol O}_2 \text{g}^{-1}$ root dry weight h^{-1} respectively, were chosen for the investigations (Wu *et al.*, 2015). Seeds were obtained from Hunan Agricultural University. The seeds were

germinated in culture dishes on moist filter papers after first being surface sterilized with a 30% H₂O₂ for 15 min. Seeds were then subsequently thoroughly washed with deionized water three times. Germinated rice seedlings were then grown in Kimura B nutrient solution for 2 weeks (Ma *et al.*, 2001).

2.2. *Aeration and Arsenate Treatments*

After 2 weeks growth in the nutrient solution, uniform seedlings (approximately 20 cm) were selected and transplanted into 10-liter plastic vessels (four vessels, twelve plants per vessel) with Kimura B nutrient solution. Initially the nutrient solutions were bubbled with N₂ gas for 24 h to deoxygenate them before use. The deoxygenated nutrient solution contained 0.1% w/v agar, which more closely resembles stagnant conditions of flooded paddy field soil than N₂-flushed solutions alone; dilute agar prevents convective movement within the solution (Wu *et al.*, 2012). The pH of the nutrient solution was maintained with KOH or HCl at approximately 5.8, with the solution renewed once every 5 days. Vessels were placed randomly in a greenhouse (maintained at 25°C during the day and 20°C at night, with 70% relative humidity) and natural light was supplemented with sodium light (1200 Lux, a photoperiod of 12 h light/12 h dark). Seedlings were cultured for a further 60 days. Plants were then transplanted to 2-liter plastic vessels (four plants per vessel) containing Kimura B nutrient solution, with either no arsenic (control) or 4 μM arsenate (Na₂HAsO₄). Half the plants were aerated using an air pump for the entire growth period, while the other half were treated as stagnant as previously described.

Treatments were designated as Stagnant -As (stagnant with no As), Aeration -As (aerated with no As), Stagnant +As (stagnant with arsenate) and Aeration +As (aerated with arsenate). There were three replicates per treatment, with four plants per replicate (vessel). The nutrient solution was renewed every 2 days, and vessels were randomly arranged in the greenhouse and plants were cultured for 10 days.

2.3. *Plant Analysis for Total As and P*

Plants were harvested after 10 days, carefully washed using deionized water, and then divided into roots and shoots. Root length, shoot length and fresh root weight were measured. Then 0.5 g fresh root was collected for RNA extraction. The remaining root and shoot samples were divided equally, and either oven-dried at 70°C to a constant weight for total As determination, or freeze-dried and stored at -20°C prior to total P and As species determination.

For total As determination, 0.5 g sample was weighed into a conical flask (100 ml), and 5 ml concentrated nitric acid added. The samples were left to digest overnight at room temperature (25°C), then placed on an electric hot plate (120°C), until the solution became clear. After digestion, samples were filtered and diluted to 20 ml with deionized water into colorimetric tubes (Wu *et al.*, 2015, 2016). The total As concentration (root and shoot) was determined using HG-AFS (AFS-8230, Beijing Jitian Instruments Co., China) (Shi *et al.*, 2013; Wu *et al.*, 2015). A certified reference material (bush branches and leaves, GBW07603) was used and As recovery ranged from 85.5% to 93.5% (n = 3).

To determine the P concentration, freeze-dried root and shoot samples were mixed and ground using a mill. 0.1 g and 0.5 g sub-samples of roots and shoots were weighed respectively, and digested using 5 ml concentrated sulfuric acid in a 50 ml glass tube on a heating block at 100 °C for 20 min. Tubes were then subsequently placed on a heating block at 380 °C for 2 h. Each digest was diluted, filtered and made to volume (25 ml) with deionized water. Total P in digests was determined using molybdenum blue method (Chen et al., 2013) using a spectrophotometer (UV-1601, Shimadzu, Japan) at a wavelength of 882 nm. A certified reference material (bush branches and leaves, GBW07603) was used and P recovery ranged from 93% to 98% (n = 3).

2.4. *Plant Analysis for As Speciation*

For determination of As species, samples were ground with liquid N₂ to ensure stabilization (Shi *et al.*, 2013; Wu *et al.*, 2015). Milled rice grain (1.0 g) was added to centrifuge tubes (50 ml), and 20 ml nitric acid (1%) added. The samples were then heated to 95°C for 1.5 h. After the samples had cooled to room temperature (25°C), the extracting solution was centrifuged at 5000r/min for 10 min and the supernatant filtered (0.22 µm). Arsenic speciation was determined using HPLC-HG-AFS (HPLC, Shimadzu LC-15C Suzhou Instruments Co., China; HG-AFS, AFS-8230, Beijing Jitian Instruments Co., China) (Shi *et al.*, 2013; Wu *et al.*, 2016).

2.5. RNA Isolation and RT-PCR

The total RNA was extracted from roots using an RNA extraction kit (RNeasy Plant Mini Kit, Qiagen, Germany). Total RNA (500 ng) was used for first-strand cDNA synthesis using SuperScript III Reverse Transcriptase (Invitrogen, USA). One-tenth of the reaction volume was used as the template for phosphate transporters (inorganic phosphate transporter, phosphate carrier protein and phosphate:H⁺ symporter family protein) (Li *et al.*, 2010) and actin (internal control) amplification using PowerUp SYBR Green Master Mix (Life Technologies, USA) for real-time polymerase chain reaction. The three phosphate transporters were selected as they were reported for potential phosphate transportation in indica rice genotypes (Li *et al.*, 2010).

The primer sequences of the different genes were as follows:

inorganic phosphate transporter, 5'-GTACCACCACTGGACGAC-3' (forward) and 5'-AAGTTGGCGAAGAAGAAGG-3' (reverse) (Li *et al.*, 2010);

phosphate carrier protein, 5'-GCGTCAGATTCCTTATACTATG-3' (forward) and 5'-GGATGAGATGCTTGTATGC-3' (reverse);

phosphate:H⁺ symporter family protein, 5'-ACCACTGGACAACGAAAG-3' (forward) and 5'-CGAAGTTGGCGAAGAAGA-3' (reverse) (Li *et al.*, 2010);

Actin, 5'- GACTCTGGTGATGGTGTCAGC-3' (forward) and 5'-GGCTGGAAGAGGACCTCAGG-3' (reverse).

qRT-PCR was carried out in a StepOnePlus instrument (Applied Biosystems, USA) and relative expression normalized against Actin using the comparative CT method recommended by the instrument manufacturer. Experiments were repeated

at least three times for statistical analysis of each individual experimental set. All values in the experiments were expressed as mean \pm SD.

2.6. Data Analysis

All data was analyzed in SPSS 23.0. Figures were created in Origin 9.0.

3. Results and Discussion

3.1. Plant growth

Plant growth parameters root length, root weight and shoot length were measured (Figure 1). There were significant genotypic effects on root length ($P < 0.001$), root weight ($P < 0.001$) and shoot length ($P < 0.001$) of rice plants. The longest root length, 28.5 cm, was from genotype XFY-9 in Stagnant+As treatments, whilst the shortest, 19.8 cm, was from genotype XWX-17 with Stagnant+As treatments. With the exception of genotype XWX-17 in +As treatments ($p < 0.05$), root length was not significantly affected by different aeration treatments. In addition, aeration had significant effects on root length in genotype XWX-12 ($p < 0.05$) in both control and As treatments. Root weight was greatest for genotype XFY-9 in Stagnant+As treatments and lowest in genotype XWX-17 in Aeration-As treatments respectively (Figure 1). Additionally, there were no significant differences between control and +As treatments on root and shoot length and root weight ($p > 0.05$). Root and shoot length and fresh root weight were significantly different between different genotypes, which is in agreement with previous studies (Wu *et al.*, 2015, 2016). However, +As

treatments did not exert any significant difference in root and shoot length and root weight, which was different to other studies (Marin *et al.*, 1993; Abedin *et al.*, 2002b). In other investigations, addition of arsenate had not revealed any significant reductions (Marin *et al.*, 1993; Carbonell *et al.*, 1998; Wu *et al.*, 2015), possibly due to different growing conditions and genotypes. In addition, root length was slightly enhanced by aerated treatments compared with stagnant treatments, which is in agreement with other studies (Comis, 1997; Wu *et al.*, 2012).

3.2. Arsenic accumulation and speciation

Arsenic was undetectable in plants grown in As-free treatments (Figure 2). There were significant genotypic effects on total As concentrations in rice roots ($P < 0.001$) and shoots ($P < 0.001$) with the same treatments. The hybrid genotypes (SY-9586 and XFY-9), with lower ROL, accumulated slightly greater As concentrations in roots than indica genotypes (XWX-12 and XWX-17). Total As concentrations in rice roots were dramatically reduced following aeration, (82.4 to 230.9 mg/kg), compared to stagnant treatments (198.4 to 265.9 mg/kg) ($P < 0.001$). In addition, there were no significant differences in total As concentrations of rice shoots between aeration and stagnant treatments, although aeration slightly reduced total As concentrations in shoots compared to stagnant treatments.

Methylated As species (MMA and DMA) were not detectable in rice roots or shoots in different treatments (Table 1 and 2). Arsenite was the predominant As species in roots, accounting for 39% to 88% of extractable As (the sum of all As species),

except for genotype XWX-12 grown with aeration +As. Arsenate concentrations were undetectable in shoots, with only arsenite detected, even in arsenate treatments. Results indicated that there were genotypic differences in arsenite accumulation in roots and shoots, with hybrid genotypes (SY-9586 and XFY-9) accumulating greater arsenite concentrations in shoots than indica genotypes (XWX-12 and XWX-17). Compared to stagnant treatments, aerated treatments significantly increased arsenate concentrations, but reduced arsenite concentrations in roots ($p < 0.001$). In As treatments, root arsenite concentrations in stagnant treatments were 1.5-2.5-fold greater than that in aerated treatments (Table 1). Genotype XFY-9 in Stagnant+As treatments contained greater arsenite concentrations (165 mg/kg), whilst genotype XWX-12 in Aeration+As treatments contained the lowest arsenite concentrations (30.1 mg/kg). In addition, arsenite concentrations in shoots from stagnant treatments were greater than that from aerated treatments (Table 2). Arsenite concentrations ranged from 7.03 to 36.7 mg/kg in shoots, with the lowest value found in genotype XWX-12 (Aeration +As) and the greatest in genotype XWX-17 (Stagnant +As).

Root and shoot total As were significantly different between different genotypes, with hybrid genotypes, with lower ROL, accumulating greater As than indica genotypes, which is in agreement with previous pot studies (Wu *et al.*, 2015). However, the differences were not significant, which may be due to the increased iron plaque formation in pot experiments sequestering more As on the plaque, and reducing As transportation to rice roots (Wu *et al.*, 2016). Root As concentrations in aerated treatments were significantly lower than stagnant treatments (Figure 2). Aro

et al. (2009) revealed that flooding increased As in rice straw and grains compared with aerobic conditions. Norton *et al.* (2013) also showed that aerobic conditions may decrease grain As content compared with flooded conditions. Furthermore, Hu *et al.* (2015) showed that rice growing in aerobic conditions resulted in 3–16 times lower As accumulations than in flooded conditions.

The dynamics of As speciation under both flooded and aerobic conditions, as well as As accumulation in rice shoots and grains were investigated by Xu *et al.*, (2008); it was observed that As concentrations in the soil solution were 4-16 times greater under flooded conditions, while grain As was 10-15 times greater than the aerobically grown rice. Flooding may reduce redox potential, causing As desorption from soil particles, which greatly increases As bioavailability in both greenhouse (Xu *et al.*, 2008; Hartley *et al.*, 2010) and field studies (Takahashi *et al.*, 2004). In the present hydroponic experiments, decreased As accumulation in aerated treatments may be due to less As transported into rice roots.

The present study demonstrated that arsenite and arsenate were both lower in rice roots and shoots from aerated compared to stagnant conditions (Table 1, 2), especially for root iAs in genotypes XFY-9, XWX-17 and XWX-12, and shoot iAs in SY-9586. Arao *et al.* (2009) also found that the concentration of inorganic As was 2.6-2.9 times greater in grain from flooded treatments than in those from aerobic treatments. In addition, organic As species were undetectable in both roots and shoots, which is in agreement with other studies (Chen *et al.*, 2012; Wu *et al.*, 2012).

3.3. Phosphate accumulation and transporter expression

Phosphate concentrations in rice roots (a) and shoots (b) are shown in Figure 3. The hybrid genotypes (SY-9586 and XFY-9), with lower ROL, accumulated slightly lower P concentrations in roots and shoots than indica genotypes (XWX-12 and XWX-17). Total P concentrations in rice roots were dramatically increased following aeration, (960 to 1616 mg/kg), compared to stagnant treatments (834 to 1188 mg/kg), especially in control and As treatments of genotype SY-9586, in +As of genotype XWX-17 and in controls of genotype XWX-12. In addition, total P concentrations of rice shoots were slightly higher in aerated treatments, especially in +As of genotype SY-9586 and genotype XWX-12 in controls.

The relative abundance of phosphate transporter (inorganic phosphate transporter and phosphate:H⁺ symporter family protein) expressions presented a down-regulation trend in stagnant treatments compared to those that were aerated; being significantly different for SY-9586, XWX-17, XWX-12 in inorganic phosphate transporter expressions, and XWX-17 in phosphate:H⁺ symporter family protein expression ($P < 0.05$) (Figure 4). However, The relative abundance of phosphate carrier protein expressions were relatively higher than the other two phosphate transporters, and presented an up-regulated trend in stagnant treatments, especially in XFY-9 ($p < 0.05$). Furthermore, there were no significant genotypic differences with phosphate transporter expressions, regardless of hybrid or conventional indica genotypes.

Arsenate and phosphate (P) share the same transporters in plants (Chen *et al.*, 2013). The inorganic phosphate transporter, phosphate carrier protein and phosphate:H⁺ symporter family protein were selected in this investigation as their expressions were up-regulated with low P stress in indica rice roots, which show potential for P transportation in indica rice (Li *et al.*, 20010). In the present study, the inorganic phosphate transporter and phosphate:H⁺ symporter family protein expressions were significantly reduced by aeration, leading to a reduction in As accumulation in roots (Figure 4). However, phosphate carrier protein expressions were significantly increased by aeration, stimulating improved root length (Figure 1) and significantly enhancing P accumulation in roots and shoots (Figure 3). The reduced accumulation of total As and arsenite may be due to reduced inorganic phosphate transporter and phosphate:H⁺ symporter family protein expressions, or as a result of competition from P and As for the phosphate carrier protein transporter. Chen *et al.* (2013) also found that the reduced expression of Pi transporters led to reduced arsenate concentrations in plant tissues. Chen *et al.* (2013) found that with the colonization of arbuscular mycorrhizal fungi (AMF), Phosphate transporter OsPT11 increased whereas OsPT2 decreased significantly. The increased expression of OsPT11 was one of the most important factors that led to the significantly higher P concentration in rice tissues, which compensated for the down-regulation of OsPT2. What is more, Rausch *et al.* (2001) found that StPT1 and StPT2 mRNA levels in potato were reduced and StPT3 was significantly induced in cells containing arbuscules. In barley (*Hordeum vulgare*) under AMF colonization, HvPht1;1 and HvPht1;2 genes were

down-regulated while HvPht1;8 was up-regulated and led to lower As uptake and higher P/As molar ratio (Christophersen et al., 2009).

4. Conclusion

The present study aimed to investigate the effects of rhizosphere aeration on phosphate transporter (for arsenate transportation) expressions, and on arsenic accumulation and speciation in two hybrid rice genotypes and two conventional indica genotypes. Aeration marginally increased root length, particularly in genotypes XWX-17 and XWX-12 from both control and As treatments. Total As concentrations in roots were dramatically reduced following aeration compared to stagnant treatments. In addition, there were no significant differences in total As concentrations in shoots between aerated and stagnant treatments, although aeration slightly reduced total As concentrations in shoots. Aerated treatments significantly increased arsenate, whilst reducing arsenite concentrations in roots. Root arsenite concentrations in stagnant treatments were 1.5-2.5-fold greater than in aerated treatments. Total P concentrations in roots were dramatically increased following aeration compared to stagnant treatments, especially in genotype SY-9586 from both control and As treatments, in genotype XWX-17 with +As and in genotype XWX-12 in control treatments. In addition, total P concentrations of shoots were slightly greater in aerated treatments, especially in genotype SY-9586 grown in +As treatments, and in genotype XWX-12 in control treatments. The relative abundance of phosphate transporter expressions also presented a down-regulation trend in

stagnant treatments, especially for SY-9586, XWX-17 and XWX-12 in inorganic phosphate transporter expressions, and XWX-17 in phosphate:H⁺ symporter family protein expression. However, the relative abundance of phosphate carrier protein expressions were relatively higher than the other two phosphate transporters, and presented an up-regulated trend in stagnant treatments. There were also no significant genotypic differences with phosphate transporter expressions, regardless of hybrid or conventional indica genotypes.

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