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REGULAR ARTICLE

Influences of phosphorus starvation on *OsACR2.1* expression and arsenic metabolism in rice seedlings

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Abstract The effects of phosphorus (P) status on arsenate reductase gene (*OsACR2.1*) expression, arsenate reductase activity, hydrogen peroxide (H_2O_2) content, and arsenic (As) species in rice seedlings which were exposed to arsenate after −P or +P pretreatments were investigated in a series of hydroponic experiments. *OsACR2.1* expression increased significantly with decreasing internal P concentrations; more than 2-fold and 10-fold increases were found after P starvation for 30 h and 14 days, respectively. *OsACR2.1* expression exhibited a significant positive correlation with internal root H_2O_2 accumulation, which increased upon P starvation or exposure to H_2O_2 without P starvation. Characterization of internal and effluxed As species showed the predominant form of As was arsenate in P-starved rice root, which contrasted with the +P pretreated plants. Additionally, more As was effluxed from P-starved rice roots than from non-starved roots. In summary, an interesting relationship was observed between P-starvation induced H_2O_2 and *OsACR2.1* gene expression. However, the up-regulation of *OsACR2.1* did not increase arsenate reduction in P-starved rice seedlings when exposed to arsenate.

Keywords Arsenate reductase · Arsenic species · Hydrogen peroxide · Phosphatase · Phosphate · Rice (*Oryza sativa* L)

Introduction

Arsenic (As), a group A carcinogen (Smith et al. 1992), is commonly found in rice (*Oryza sativa* L.) at higher grain concentrations than in other cereals, such as wheat (*Triticum aesticum* L.) and barley (*Hordeum vulgare* L.) (Williams et al. 2007). Being a phytotoxin, As can also impair crop yields and degrade arable lands (Heikens 2006). Although rice is often grown in sub-oxic conditions where arsenite prevails, a significant amount of arsenate (the oxidized form of inorganic As) can exist in paddy rhizospheres, due principally to oxygen release from rice roots (Chen et al. 2008). Rice agro-ecosystems can be contaminated by As mainly by irrigation with As-tainted groundwater, mineral exploration, processing industries, and as a result of As pesticide use (Zhu et al. 2008a). When rice is grown in paddy soils which did not exceed global background As levels, internal plant As concentrations in rice straw and grain can still be high in comparison with other cereal crops (Williams et al. 2007).

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In contrast, phosphorus (P), the chemical analogue of As, is one of 17 essential elements required for plant growth (Bieleski 1973; Raghothama 1999), and often the overriding nutritional limitation in modern cereal farming (Runge-Metzger 1995; von Uexküll and Mutert 1995). It is estimated that crop yields on around 30–40% of the world's arable land are limited by P availability (Runge-Metzger 1995; von Uexküll and Mutert 1995). Sensitivity to arsenate in rice seedlings is closely associated with P nutrition; even cultivars found to be susceptible to arsenate can be made more resistant by raising levels of intracellular P (Geng et al. 2006). Therefore, arsenate-induced toxicity in rice can be more prevalent in situation where As contamination is found coexisting with low available P. Further understanding of the complex relationship between As and P is needed to improve agronomic methods and rice yield.

The initial step in arsenate metabolism, in all organisms studied so far, is reduction to arsenite. Arsenate reductase is one of the key enzymes involved in this transformation (Rosen 2002; Duan et al. 2005; Ellis et al. 2006; Dhankher et al. 2006). It is believed that this enzyme plays a crucial role in determining not only *in planta* As speciation but also translocation, and can affect the plants ability to withstand As-induced stress. Recently, two genes (*OsACR2.1* and *OsACR2.2*) in rice encoding arsenate reductase were cloned and characterized (Duan et al. 2007). Upon arsenate exposure, *OsACR2.1* expression was demonstrated to be much higher than *OsACR2.2*, resulting in much greater enzyme activity. Therefore, OsACR2.1 is thought to be more important than OsACR2.2 as arsenate reductase in rice. In addition to reducing arsenate, OsACR2.1 exhibits phosphatase activity. Phosphatase activities are commonly increased when internal levels of P are depleted (Duff et al. 1991; del Pozo et al. 1999; Baldwin et al. 2001; Vance et al. 2003). However, how *OsACR2.1* expression is regulated when rice is P limited still needs to be determined.

P deficiency and As exposure result in H_2O_2 accumulation in a number of plants, including rice (Schachtman and Shin 2007; Shin et al. 2005; Geng et al. 2006; Mascher et al. 2002). H_2O_2 is a signaling molecule in plants (Shin and Schachtman 2004), which has been shown to be involved in lots of different chemical messenger pathway responses, especially those relating to tissue damage and/or potassium deprivation (Shin and Schachtman 2004; Joo et al. 2001; Orozco-Cárdenas et al. 2001). Therefore, it is essential to investigate whether H_2O_2 is the medium connecting P deficiency and *OsACR2.1* expression.

In order to understand how P status and H_2O_2 affect *OsACR2.1* gene expression and how this influences As metabolism within rice seedlings, a number of experiments were conducted to unravel these questions.

Materials and methods

Plant growth

Rice (*Oryza sativa* L. cv Jiahua 1) seeds were sterilized in 10% H_2O_2 solution for 15 min followed by thorough washing with de-ionized water. The seeds were germinated in moist perlite. After 3 weeks, uniform seedlings were transplanted to PVC pots (7.5 cm diameter and height 14 cm, one plant per pot) containing 500 ml 1/6 strength macronutrients solution and 1/4 strength micronutrients solution. After 1 week, 1/3 strength macronutrients solution and 1/2 strength micronutrients solution was used. The nutrient solution composition was the same as that used by Duan et al. (2007), which contained 1.3 mM KH_2PO_4, and was renewed every 2 days. The plants were cultivated in a growth room at a 14-h light period (260–350 μE m^{-2} s^{-1}); 28/20°C day/night; 60% relative humidity. All the experiments were carried out with three replicates.

Plant treatments

Plants for P, arsenate reductase and phosphatase activity analysis

Nine pots of 7-week-old rice plants were treated with P-free (−P) nutrient solution (KCl was added instead of KH_2PO_4) for 0, 7 and 14 days. When the plants were harvested, they were 9 weeks old and were thoroughly washed with tap water, and then with de-ionized water; adhering water was then removed with filter paper. For arsenate reductase and phosphatase activity analysis, root and shoot materials were frozen in liquid nitrogen, crushed to powder and stored in liquid nitrogen for enzyme activity analysis. For P

concentration analysis, root and shoot materials were oven-dried at 70°C for 2 days, and dry weight was determined.

Plants for OsACR2.1 expression and H_2O_2 content analysis

Twenty-one pots of 6-week-old rice plants were treated with −P nutrient solution for short-term starvation (0, 6 and 30 h) and long-term starvation (0, 3, 7 and 14 days). The plants were harvested and kept in liquid nitrogen for H_2O_2 content analysis, RNA extraction and real-time PCR.

Plants for H_2O_2 exposure

Twelve pots of 8-week-old rice plants were treated with 0, 0.5, 1 and 2 mM H_2O_2 for 6 h. The concentrations of H_2O_2 and treatment time were chosen based on the results described by Shin and Schachtman (2004). The plants were harvested and kept in liquid nitrogen for H_2O_2 content analysis, RNA extraction and real-time PCR.

Plants for total As and As speciation analysis in plants and efflux solution

Twelve pots of 5-week-old rice plants were selected for this experiment. Six pots were pretreated with P-free nutrient solution for 14 days, and then exposed to 20 μM arsenate in P-free culture for 48 h (−P treatment). The other 6 pots of rice plants were cultivated in normal nutrient solution for 14 days, and then exposed to 20 μM arsenate in normal nutrient solution for 48 h (+P treatment). For total As and As speciation analysis, plants were harvested quickly and kept in −80°C; for efflux experiment, plants were washed quickly with ice-cold phosphate buffer (0.5 mM $Ca(NO_3)_2$ $4H_2O$, 5.0 mM MES, 1.0 mM K_2HPO_4 $3H_2O$), and then put into 25-ml normal nutrient solution for efflux experiment. Accumulative efflux time was 0.33, 0.67, 1, 5, 10, 30, 60, 90, 120 and 180 min.

Determination of H_2O_2 and protein concentrations

For the H_2O_2 assay, fresh roots (2.0 g) were homogenized in refrigerated acetone (2.0 ml). The reaction mixture was precipitated by Ti $(SO_4)_2$ and ammonia, and the pellet was dissolved in 2.0 M H_2SO_4 and the absorbance was read at 415 nm (Mulherjee and Choudhuri 1983). Protein contents were determined according to Bradford (1976), using Coomassie Brilliant Blue G-250 (Sigma) as dye and albumin (Bovine V; Sigma) as a standard.

Enzyme assays

Arsenate reductase activity was assayed using the coupled enzymatic reaction described by Duan et al. (2005). The assay was performed in 50 mM MOPS/MES buffer (pH 6.5), containing 1.5 mM NADPH, 1 unit yeast (*Saccharomyces cerevisiae*) glutathione reductase (Sigma), 1 mM GSH, 10 mM sodium arsenate, and protein extracts were added as indicated, in a total volume of 1.5 ml. All the measurements were performed at 37°C. NADPH oxidation was monitored by using a U-3010 spectrophotometer (Hitachi, Tokyo, Japan). The amount of NADPH oxidized was calculated using a molar extinction coefficient of 6,200 M^{-1} cm^{-1}. Control samples were added to correct for NADPH consumption independent of sodium arsenate.

Phosphatase activity was assayed at 37°C with 20–30 mg rice root protein extracts with the indicated amounts of p-nitrophenyl phosphate (pNPP) in 0.1 M MOPS/MES buffer, pH 6.5 (Zhou et al. 2006). The assay was initiated by the addition of pNPP, and the rate of hydrolysis was estimated from the increase in absorption at 405 nm. Each value was corrected for nonenzymatic pNPP hydrolysis. The phosphatase activity were analyzed by using an extinction coefficient for nitrophenol of 18,000 M^{-1} cm^{-1}.

Analysis of total As and P concentrations

Oven-dried plant materials were digested in nitric acid on a heating block (Digestion Systems of AIM500; A. I. Scientific, Australia); the temperature was at 100°C for 1 h and then at 120°C for 60 h. Reagent blank and standard reference (GBW07605, from the National Research Center for Standard Materials, China) were used to verify the precision of analytical procedures. The concentrations of P were measured by inductively coupled plasma optical emission spectrometer (ICP-OES, Optima 2000 DV; Perkin-Elmer, USA). The concentrations of As were measured by an atomic fluorescence spectrometry (AF-610A; Beijing Ruili Analytical Instrument, Beijing, China).

As speciation analysis

Samples of 0.2 g were weighed into 50-ml extraction vessels, and then steeped in 10 ml of 1% HNO_3 and left overnight, heated to 95°C in a microwave accelerated reaction system (CEM Microwave Technology, USA) for 30 min, and then filtered. To minimize speciation, transformation samples were kept dark, on ice and run within a few hours of extraction. Spikes revealed any transformation of As species was minimal.

Arsenic speciation was assayed by high performance liquid chromatography-inductively coupled plasma-mass spectrometry (HPLC-ICP-MS) (7500a Agilent Technologies) as detailed in (Zhu et al. 2008b). Chromatographic columns were obtained from Hamilton and consisted of a precolumn (11.2 mm, 12–20 μm) and a PRP-X100 10-μm anion-exchange column (250×4.1 mm). The mobile phase consisted of 8 mM diammonium hydrogenphosphate ($(NH_4)_2HPO_4$) and 8 mM ammonium nitrate (NH_4NO_3), adjusted to pH 6.2 using ammonia. Total As in the extract solutions was determined by ICP-MS.

RNA extraction and cDNA synthesis

Total RNA was isolated from each rice root using the TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. Concentrations of RNA were measured using a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies). The integrity of RNA samples were assessed by agarose gel electrophoresis. cDNA was synthesized from 5 μg of total RNA after DNase treatment (Invitrogen), using 200 U of MMLV Reverse Transcriptase (Invitrogen) and oligo-$(dT)_{18}$ as a primer, in 20-μl reactions, as described in Sambrook et al. (1989).

Real-time PCR analysis

UBQ5 was used as internal control (Jain et al. 2006). Relative quantitative RT-PCR was performed in a 25-μl reaction volume, containing 1 μl cDNA, 200 nM each gene-specific primers, and SYBR Premix ExTaq (Takara Bio) using Bio-Rad iQ5. The primer sequences are given in Table 1. All the PCRs were performed under the following conditions: 94°C for 30 s, 40 cycles of 94°C for 10 s, 58°C for 30 s and 72°C for 30 s. The specificity of amplification was verified by melting curve analysis (60–95°C) after 40 cycles. The products were run on a gel to check the specificity of the primers. Three biological replicates for each sample were used for real-time PCR and three technical replicates were analyzed for each biological replicate.

Statistical analysis

Analysis of variance (ANOVA) on concentrations of nutrients and enzyme activities were performed using SPSS 11.5 for windows. Curve fitting was performed using Sigma Plot 9.0 (Jandel Scientific, Erkrath, Germany).

Results

Plant growth and P concentrations

Dry weights and P concentrations were analyzed in plants under the treatments of 0-, 7- and 14-day P starvation. No significant difference was found in dry weights of rice roots and shoots after 7-day P starvation. However, a significant decrease in shoot dry weight was found after P starvation for 14 days (Table 2, $P<0.05$). Internal P concentrations in rice

Table 1 Primer sequences used for real-time PCR analysis

Gene name	Accession no.[a]	Primer sequences [b]	Amplicon lenghth (bp)
OsACR2.1	AY860059	^{345}5′- GGAAGAATCAGGAACAAAGAACATC-3′ ^{496}5′- CAGGAAGCGAGTAGAGTTACAAC-3′	152
UBQ5	AK061988	^{506}5′-ACCACTTCGACCGCCACTACT-3′ ^{574}5′- ACGCCTAAGCCTGCTGGTT-3′	69

[a] Full-length cDNA accession number

[b] Forward (upper line) and reverse (lower line) primer sequences along with their position (number given as superscript on the left) on the full-length cDNA sequence

Table 2 Biomass and phosphorus concentrations in rice roots and shoots after P starvation for 0, 7 and 14 days

Treatments	Biomass (g)		P concentrations (mg g^{-1} DW)	
	Root	Shoot	Root	Shoot
−P 0 days	0.28±0.03 a	0.92±0.02 a	9.12±0.39 a	12.24±0.45 a
−P 7 days	0.29±0.01 a	0.86±0.05 a	3.70±0.24 b	6.23±0.41 b
−P 14 days	0.25±0.01 a	0.72±0.02 b	2.46±0.19 c	3.20±0.19 c

Data are means ± SE (n=3). Treatments with the different letter s(a, b, c) are significantly different (P<0.05)

roots and shoots decreased significantly with increasing P starvation time, by 50% and 75% after P-starvation for 7 and 14 days, respectively (Table 2, P<0.05).

Arsenate reductase and phosphatase activities

Arsenate reductase and phosphatase activities were determined in root extracts deprived P for 0, 7 and 14 days. After 7 days of P starvation, no significant difference was found in arsenate reductase and phosphatase activities; while after 14 days of P starvation, activities of both enzymes increased significantly (Fig. 1; P<0.05).

OsACR2.1 expression and H_2O_2 accumulation in response to P starvation

Relative *OsACR2.1* transcription levels and H_2O_2 accumulation were determined after short-term (6 and 30 h) and long-term (3, 7 and 14 days) P starvation. Both *OsACR2.1* expression and H_2O_2 accumulation increased with increasing P starvation time (Fig. 2A), but only slightly before 7 days and rapidly after 7 days. *OsACR2.1* expression increased 3-fold and 10-fold after 7- and 14-day P starvation, respectively. H_2O_2 accumulation increased 1.5-fold and 4-fold after 7- and 14-day P starvation, respectively. There was a significant positive linear correlation between *OsACR2.1* expression and H_2O_2 accumulation in rice roots (r=0.9681, P<0.0001) (Fig. 2B).

OsACR2.1 expression in response to exogenous H_2O_2 accumulation

H_2O_2 accumulation and relative *OsACR2.1* expression increased significantly with increasing H_2O_2 concentrations in the nutrient solutions (Fig. 3B; P< 0.05). H_2O_2 accumulation and *OsACR2.1* expression in the roots treated with 2 mM H_2O_2 were 5-fold and 13-fold higher, respectively, than that of the control (0

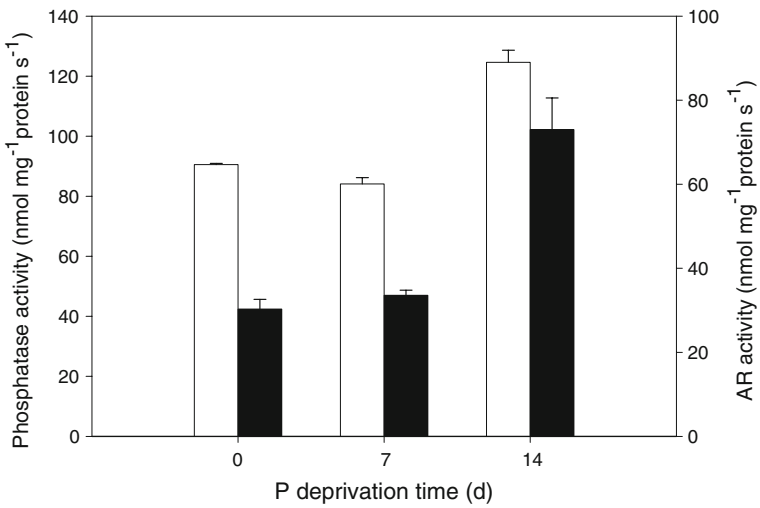

Fig. 1 Arsenate reductase (*AR*) (*black column*) and phosphatase (*open column*) activity in rice roots after 0, 7 and 14 days of P starvation. Data are means ± SE (n=3)

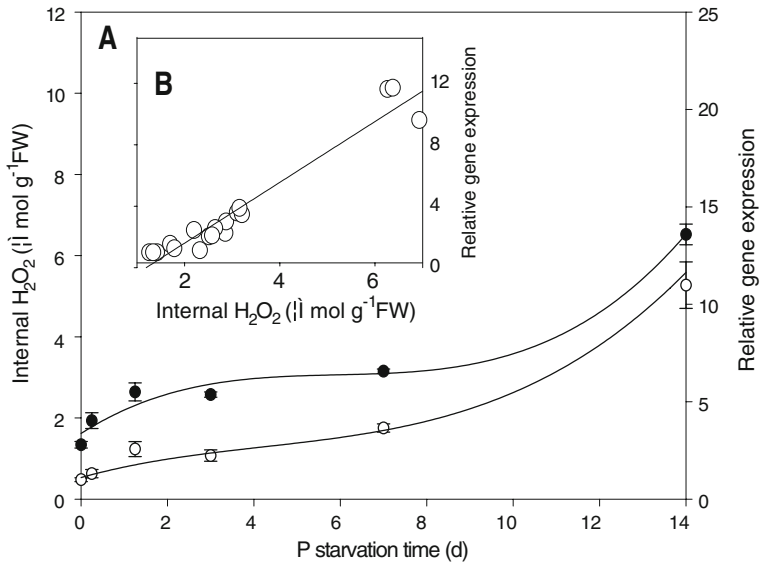

Fig. 2 The relationship between P starvation, H_2O_2 accumulation and *OsACR2.1* expression in rice roots. A: *OsACR2.1* expression (*open circles*) and H_2O_2 accumulation (*filled circles*) in rice roots after P starvation. B: Correlation between H_2O_2 accumulation and O*sACR2.1* expression (r=0.9681, P< 0.0001). Data are means ± SE (n=3)

mM H_2O_2). *OsACR2.1* expression and H_2O_2 accumulation in rice roots exhibited a significant positive linear correlation (r=0.9350, P<0.0001; Fig. 3A).

As concentrations and species in plants and efflux solutions

Total As concentrations in rice shoots and the whole plants were significantly increased by pretreatment with P-free nutrient solution. However, no significant difference was found in total As concentrations in roots when rice plants were pretreated with or without P (Table 3).

In rice seedlings, As was present mainly as arsenite and arsenate; no organic As was found. After P starvation for 14 days, arsenite concentration decreased by 58% in the rice roots, from 508 μg g^{-1} in non-starved plants to 214 μg g^{-1} in P-starved plants; percentage of arsenite in total As also decreased significantly after P starvation, 95% in non-starved plants and 51% in P-starved plants (Fig. 4). For all

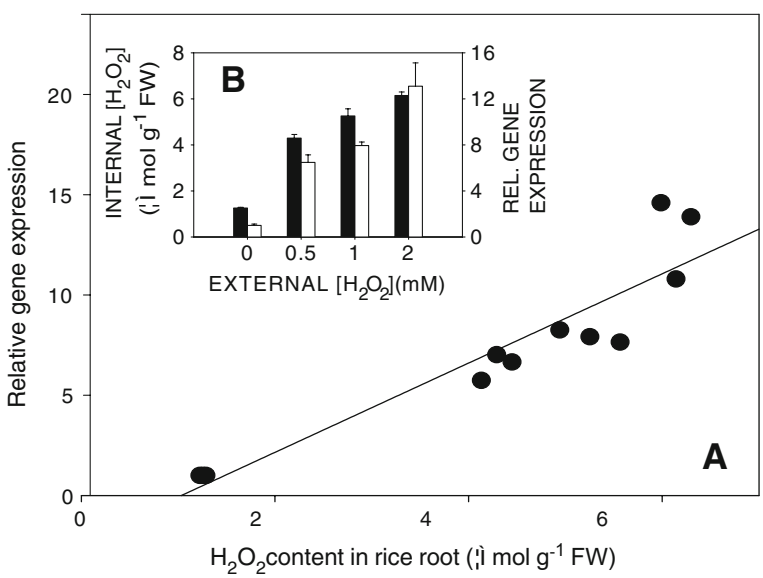

Fig. 3 **A** Correlation between H_2O_2 content and relative *OsACR2.1* expression in rice roots (r=0.9350, P<0.0001). **B** H_2O_2 accumulation (*black column*) and *OsACR2.1* expression (*white column*) in rice roots after exposure to 0, 0.5, 1 and 2 mM H_2O_2 for 6 h. Data are means ± SE (n=3)

Table 3 Total arsenic concentrations in rice roots and shoots which were exposed to 20 μM arsenate for 48 h after pretreatment with −P or +P nutrient solutions for 14 days

Treatments	As (μg g $^{-1}$ DW)		
	Root	Shoot	Whole plant
−P	475.30±45.17 a	110.10±10.55 a	210.35±16.15 a
+P	554.60±36.07 a	34.81±4.31 b	145.15±13.47 b

Data are means ± SE (n=3). Treatments with the different letter s(a, b) are significantly different (P<0.05)

samples, the average extraction efficiency of 1% nitric acid was 88±2% and the average column recovery was 88±3%.

The amount of As effluxed from P-starved plant roots was higher than that from the non-starved ones (Fig. 5A). In the efflux solution, the predominant species were arsenate (76%) from P-starved plants, and arsenite (86%) from non-starved plants, no organic As was found (Fig. 5B). The species of effluxed As was in good agreement with As species in plant roots (Fig. 4).

Discussion

Cellular P levels decreased in rice seedlings starved of P for 7 days; however, this decrease was not sufficient to impact on the phosphatase activity (Table 2 and Fig. 1). The effect of P starvation became significant only after P supply was withheld for 2 weeks. In this study, P starvation for 2 weeks resulted in a 40% increase in the phosphatase activity, whilst only moderately impacting on plant biomass (Table 2 and Fig. 1). After 2 weeks of P starvation, P concentrations of both rice roots and shoots were still above the critical internal P-deficiency concentrations for rice (Fageria 1976), suggesting that the plants were not suffering from severe P-deficiency stress.

It is unknown why the arsenate reductase activity and *OsACR2.1* expression were stimulated during prolonged P starvation. OsACR2.1 exhibits dual functions as a phosphatase and arsenate reductase (Duan et al. 2007). AtACR2 (also named as Arath or CDC25) has been shown to be an arsenate reductase (Dhankher et al. 2006), in addition to being a small CDC25 dual-specificity tyrosine-phosphatase isoform; the latter functions by dephosphorylating both Thr-14 and Tyr-15 residues on the cyclin-dependent kinases to release free P (Landrieu et al. 2004). OsACR2.1 is a homolog of AtACR2, sharing 52.5%

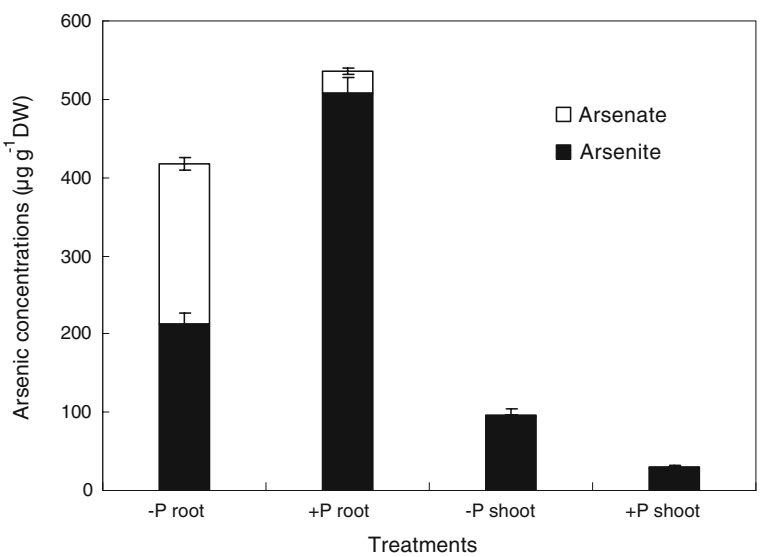

Fig. 4 Arsenate and arsenite concentrations in rice roots and shoots which were exposed to 20 μM arsenate for 48 hours after pretreatment with −P or +P nutrient solutions for 14 days. Data are means ± SE (n=3)

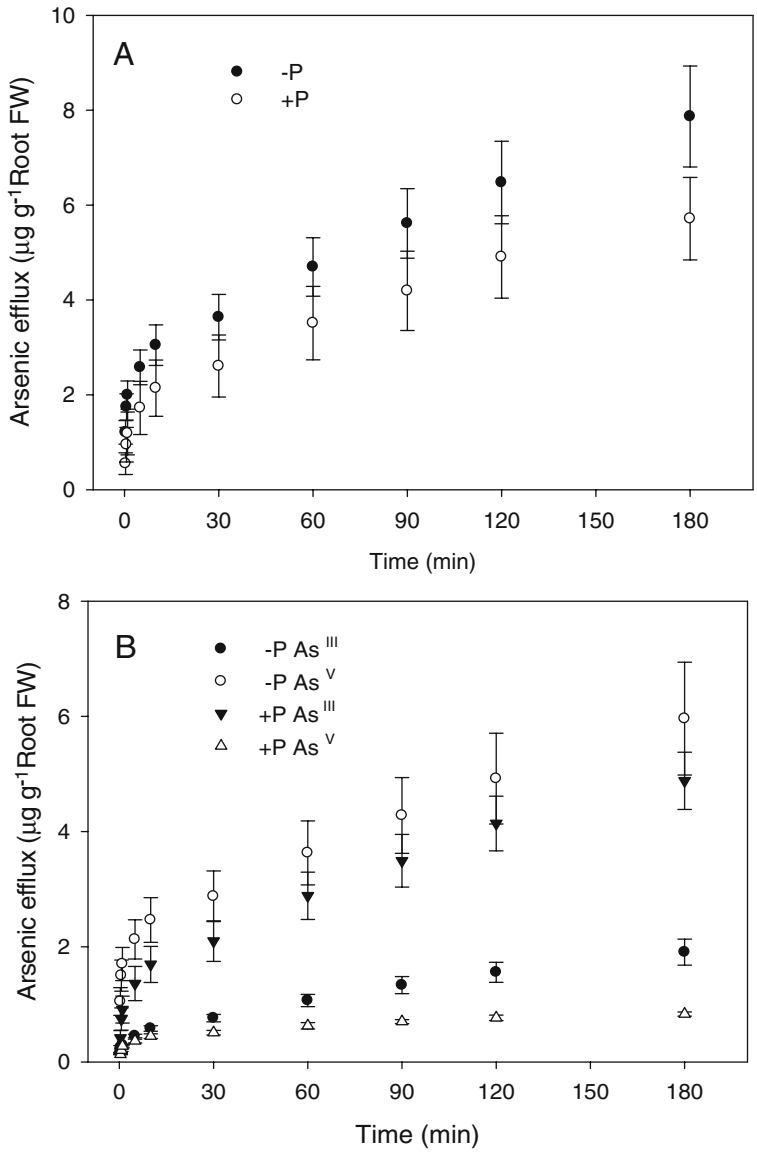

Fig. 5 Arsenic efflux to +P nutrient solution of rice roots which were exposed to 20 μM arsenate for 48 h after pretreatment with −P or +P nutrient solutions for 14 days. **A** Total arsenic efflux. **B** Arsenate (As^{V}) and arsenite (As^{III}) efflux. Data are means ± SE (n=3)

similarity with AtACR2 and containing the active site motif $HC(X)_5R$ with the superfamily of phosphotyrosine protein phosphatases (PTPases), and has been shown to exhibit phosphatase activity (Duan et al. 2007; Landrieu et al. 2004). Therefore, rice may utilize OsACR2.1 as a phosphatase during critical periods in response to P-deficiency stress.

The number of chemical messengers available for plants to induce developmental responses is small. Therefore, many signaling pathways may utilize the same messengers (Bennett et al. 2005). H_2O_2 has been shown to play a role as a signal molecule in plants (Bailey-Serres and Mittler 2006), regulating not only numerous gene expressions directly (Alvarez et al. 1998; Desikan et al. 2000), but also acting as a second messenger for the induction of defense genes (Orozco- cárdenas et al. 2001). Shin and Schachtman (2004) found that potassium deprivation increased H_2O_2 accumulation and that H_2O_2 was involved in K^+ signaling. This study shows a close relationship between P starvation-induced H_2O_2 and *OsACR2.1* gene expression (Fig. 2B) in rice roots. Whether the association is strictly causative is not confirmed, although it is still of interest as it has never been documented before (to the best of the authors' knowledge). Manipulation of internal H_2O_2 level

resulted in further association with gene expression, supporting our first observations (Fig. 3). Therefore, H_2O_2 could possibly act as a signaling molecular controlling *OsACR2.1* expression in rice roots. Further experiments are underway with H_2O_2 mutants to further test this hypothesis.

As cellular P levels decrease, homeostasis mechanisms try to readdress the nutrient balance by increasing the expression of P transporters and subsequently P uptake. This can lead inadvertently to greater arsenate absorption (Meharg and Hartley-Whitaker 2002). Short-term uptake experiments (data not shown) with rice seedlings starved of P for 2 weeks showed higher As accumulation than P-replete plants when exposed to arsenate. In the presence of P, arsenate uptake can be strongly suppressed because of competition for the same membrane transporter (Abedin et al. 2002). In this study, no significant difference was observed in root As concentrations between P-starved rice plants which were exposed to arsenate without P and those of P-replete plants exposed to arsenate in the presence of 1.3 mM P. However, a significant difference was observed in shoot As concentration between the different P status rice plants, with shoot levels in the −P treatment being ~3 times higher (Table 3; $P<0.05$).

Rice plants starved of P may suffer from As phytotoxicity more than the P-replete plants. This may explain why As concentration in the −P rice roots was not significantly elevated compared with +P roots because of a decreased As uptake as a result of phytotoxicity. Another possible reason is the uptake of arsenite. Xu et al. (2007) showed that plant roots mediated rapid transformation of arsenate in hydroponic medium. After P-replete rice were exposed to 10 μM arsenate for 24 h, 11% of the As in the nutrient solution was found to be in the form of arsenite. In this study, P-replete plants were exposed to 20 μM arsenate for 48 h and more arsenite could possibly be found in the growth medium compared with the result of Xu et al. (2007). As arsenite enters plants via glycerol transporter, its uptake is not limited by the amount of P present (Meharg and Jardine 2003).

Internal damage caused by As is governed by the plant's ability to improve the propensity of As to be bound to reduced glutathione and/or phytochelatins, as well as intracellular compartmentation. This is dependent on the amount and type of As binding ligands available, as well as the extent and rapidity of the reduction of arsenate to arsenite (Meharg and Hartley-Whitaker 2002). Xu et al. (2007) showed that As reduction was rapid in 20-day-old rice exposed to arsenate. It was expected that P-starved rice would reduce arsenate more effectively than P-replete plants because of the increased arsenate reductase activity and *OsACR2.1* expression after a prolonged period of P starvation (Figs. 1 and 2). However, the data of root As speciation revealed that the proportion of arsenate in the P-starved rice was far higher than that in the +P rice (Fig. 4). The data of root As speciation was consistent with in the speciation pattern of As effluxed by roots, with the P-starved rice effluxing predominantly arsenate whilst the P replete plants releasing mainly arsenite (Fig. 5B).

As the arsenate reductase activity or *OsACR2.1* expression was not measured in this experiment after −P or +P rice were exposed to As, it is uncertain if arsenate induced arsenate reductase activity in the P-replete rice to levels exceeding that of the P-starved plants. *OsACR2.1* expression and arsenate reductase activity have been shown previously to be higher in −P rice compared with +P plants when exposed to As (Duan et al. 2007); although in that study a shorter duration of P starvation was used. In a similar experiment with wheat, no significant differences were observed in root arsenate reductase activity between the +P and −P treatments (Wang et al. 2007). Arsenate reduction may also be mediated by non-enzymatic reactions, such as reduction by glutathione (Delnomdedieu et al. 1994). Therefore, it is possible that non-enzymatic reactions or some other arsenate reduction pathways were active in reducing arsenate in the +P rice but failed after P starvation for a prolonged period.

In conclusion, this study highlights the complexity of the As–P relationship in rice. For the first time, we demonstrate a possible relationship between H_2O_2 and *OsACR2.1* gene expression in rice roots as well as some interesting rice root As speciation findings resulting from the prolonged starvation of P.

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