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Are rice (*Oryza sativa* L.) phosphate transporters regulated similarly by phosphate and arsenate? A comprehensive study

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Abstract Rice is one of the most important staple foods worldwide, but it often contains inorganic arsenic, which is toxic and gives rise to severe health problems. Rice plants take up arsenate As(V) via the phosphate transport pathways, though it is not known how As(V), as compared to phosphate, modifies the expression of phosphate transporters (PTs). Therefore, the impact of As(V) or phosphate (Pi) on the gene expression of PTs and several Pi signaling regulators was investigated. Rice plants were grown on medium containing different As(V) or Pi concentrations. Growth was evaluated and the expression of tested genes was quantified at different time points, using quantitative RT-PCR (qPCR). The As and P content in plants was determined using inductively coupled plasma mass spectrometry (ICP-MS). As(V) elicited diverse and opposite responses of different PTs in roots and shoots, while Pi triggered a more shallow and uniform transcriptional response in several tested genes. Only a restricted set of genes, including *PT2, PT3, PT5* and *PT13* and two SPX-MFS family members, was particularly responsive to As(V). Despite some common reactions, the responses of the analyzed genes were predominantly ion-specific. The possible reasons and consequences are discussed.

Keywords Arsenic · Arsenate · Phosphate transporters · Rice (*Oryza sativa*) · Gene regulation · Growth inhibition

Introduction

The Food and Agricultural Organization of the United Nations used the slogan "Rice is Life" to classify rice as one of the most important staple foods worldwide (FAO 2004). However, commercially available rice often contains inorganic arsenic (Williams et al. 2005; Meharg et al. 2009), which can cause severe health problems including cancer (NRC 2001). The situation is especially dramatic in Southeast Asia, where As-contaminated ground water is used both for drinking and for irrigating rice fields (Kinniburgh and Smedley 2001; Meharg 2004; Saha and Ali 2007). Also in regions where drinking water does not significantly contribute to arsenic toxicity, ingestion of contaminated rice may cause elevated genotoxic effects (Banerjee et al. 2013). Traditionally, rice is grown on continuously flooded, anoxic paddy fields (Liesack et al. 2000; Spanu et al. 2012), where mobile As mainly prevails as arsenite As(III) (Marin et al. 1993; Smedley and Kinniburgh 2002). As(III) uptake into plants, especially rice, has been intensively studied (Bienert et al. 2008; Meharg and Jardine 2003; Ma et al. 2008). However, the presence of arsenate As(V) in paddy soil and its subsequent uptake into rice should not be underestimated. As(III)-oxidizing bacteria convert As(III) to As(V) in oxic and anoxic environments (Oremland and Stolz 2003), while chemical As(III)

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oxidation occurs in oxic soils, created by the application of new irrigation techniques (Xu et al. 2008) that could lead to decreased As accumulation in rice grains (Somenahally et al. 2011; Spanu et al. 2012; Sarkar et al. 2012). More importantly, the presence of phosphate (Pi), a constituent of fertilizers, increases As(V)-bioavailability in reducing and oxidizing environments, as it competes for sorption sites on minerals (Jain and Loeppert 2000; Dixit and Hering 2003) and mobilizes mineral-bound As(V) (Manning and Goldberg 1996; Jain and Loeppert 2000; Gao and Mucci 2003; Liu et al. 2004).

The interrelation between As(V) and Pi is also very important biologically, as they compete with each other for uptake, translocation and physiological activity in plants (Zhao et al. 2009). As(V) enters plants via phosphate transporters (PTs) (Ullricheberius et al. 1989; Macnair and Cumbes 1987; Wu et al. 2011; Meharg and Macnair 1990), which exhibit either a low or high affinity to Pi. Low-affinity PTs are constitutively expressed and function at external Pi concentrations in the millimolar range, while high-affinity PTs are often induced upon Pi starvation and operate at Pi concentrations as low as a few micromoles (Liu et al. 1998; Raghothama 1999, 2000; Muchhal and Raghothama 1999; Chen et al. 2008). Several PTs responsible for Pi acquisition have been identified in various plant species, including rice (Muchhal et al. 1996; Paszkowski et al. 2002; Muchhal and Raghothama 1999). On the basis of their structural similarity, these transporters have been assigned to the PHT1 family of PTs (Liu et al. 2011), which consists of 13 members in rice, OsPT1 (PT1) through OsPT13 (PT13) (Paszkowski et al. 2002). Time- and tissue-specific expression patterns of the different PTs indicate that the transporters play distinct roles in the Pi transport pathway (Muchhal and Raghothama 1999; Liu et al. 2011; Ai et al. 2009). This was also supported by the functional characterization of several rice PTs (Ai et al. 2009; Paszkowski et al. 2002; Sun et al. 2012; Jia et al. 2011).

Phosphate Transporters are complexly regulated, with several genes identified in rice so far. Under Pi-limited conditions, the MYB family transcription factor OsPHR2 (PHR2) positively regulates some *PT* genes, whereas the ubiquitin-conjugating E2 enzyme OsPHO2 (PHO2) negatively regulates Pi uptake and translocation (Zhou et al. 2008; Chiou and Lin 2011; Wu et al. 2011; Hu et al. 2011). PHR2-mediated Pi starvation signaling can be suppressed by OsSPX1 (SPX1), which is itself up-regulated in *PHR2* overexpressing plants, and thus, participates in a negative feed-back loop of *PHR2* regulation (Liu et al. 2010; Wang and Duan 2009). OsPHO1;2 (PHO1;2) is involved in loading Pi into the xylem and is regulated by its *cis*-natural antisense transcript (hereafter referred to as PHO1;2anti) (Secco et al. 2010). OsPHF1 (PHF1) participates in the correct localization of PTs to the plasma membrane (Chen et al. 2011), while OsSPX-MFS1 (SPX-MFS1) and SPX-MFS2 are involved in the re-mobilization of Pi in leaves (Lin et al. 2010; Secco et al. 2012; Wang et al. 2012). To what extent the expression of these regulators is responsive to As(V) remains to be elucidated.

Along with an enhanced production of phytochelatins, the suppression of the Pi/As(V) uptake system has been considered to be a major mechanism of As(V) tolerance in plants (Hartley-Whitaker et al. 2001; Schmoger et al. 2000; Meharg and Macnair 1990, 1992). High external Pi concentrations were shown to decrease the uptake of As(V) in different plants (Tu and Ma 2003; Mkandawire et al. 2004; Tu et al. 2004; Wang and Duan 2009). A higher resistance to As was also shown for the *Arabidopsis* mutants *pht1;1-3*, which exhibits lower Pi/As(V) uptake rates (Catarecha et al. 2007), and *phf1*, which is defective in Pi transport (Gonzalez et al. 2005). However, the high As(V) tolerance of the *Arabidopsis* mutant *ars1* was correlated to an increase in Pi uptake and hence, plant phosphorus status, even though the intracellular As concentration remained comparable to that in wild type (Lee et al. 2003). This indicates that different mechanisms mediate Pi acquisition and As resistance. Catarecha et al. (2007) further showed that As(V) repressed several Pi-starvation-induced genes in *Arabidopsis* roots, though the effect was not quite comparable to that of Pi. To analyze the contribution of PTs to As accumulation in more detail, Wu et al. (2011) characterized rice mutants that were either defective in *PHF1* or overexpressed the phosphate transporter *PT8* or the transcription factor *PHR2*. The overexpression of both *PHR2* and *PT8* resulted in increased Pi and As concentrations in the roots and shoots of hydroponically grown rice plants. In the *PHR2* overexpressor, however, the increase in Pi accumulation matched the increase in As accumulation, whereas As accumulation was considerably higher than that of Pi in the *PT8* overexpressor (Wu et al. 2011). This indicates that, despite the chemical similarity between both ions, some PTs can, to some extent, discriminate between them. Whether they react specifically to either one of these ions at the transcriptional level is not yet clear.

In fact, our knowledge as to the extent to which As(V) can substitute for Pi in different plant cellular processes is very limited. In their recent review, Tawfik and Viola (2011) summarized the available data concerning the promiscuity of several Pi-utilizing enzymes towards As(V). It appears that enzymes generally show a low degree of discrimination between binding Pi and As(V). However, most reported data refer to in vitro binding assays exploiting only a few enzymes (Kline and Schramm 1993; Kish and Viola 1999; Silva et al. 2011). Moreover, apart from their similar physicochemical properties, As(V) and Pi possess distinct characteristics, such as the hydrolytic instability of their esters or the lower reduction potential of As(V),

which can determine their intracellular fate (Tawfik and Viola 2011). Taking into account that the HA phosphate transport system is tightly regulated by Pi availability (Lee et al. 1990; Furihata et al. 1992; Schachtman et al. 1998), the question arises whether As(V) is able to induce similar responses when substituting for Pi in the signaling pathway. In this study, a comprehensive analysis of the transcriptional responses of the rice *PHT1* gene family to different concentrations of Pi and As(V) was performed. We also analyzed the expression levels of genes involved in Pi signaling and showed that, despite some common reactions, the responses of the analyzed genes were predominantly ion-specific.

Materials and methods

Seed germination and seedling growth experiments

Oryza sativa L. cv. Gladia rice seeds were obtained from the Agricultural Research Council in Vercelli, Italy. As(V) (Na_2HAsO_4) and P_i ($Na_2HPO_4 \times 2H_2O$) stock solutions of appropriate concentrations were prepared anoxically in butyl stoppered vials (100 % v/v N_2 atmosphere) by dissolving the applicable salt in deoxygenated MilliQ water. For each treatment, 100–115 rice seeds were glued husk-down onto microscopic glass slides, using a medical adhesive (Secure Medical Adhesive, Factor II, B-400, USA), and placed in glass tanks (Fig. S1a and insert). Seeds were germinated in 200 ml sterile MilliQ water supplemented with 0 μM, 1 μM (0.075 mg l^{-1}), 13.3 μM (1 mg l^{-1}), 50 μM (3.75 mg l^{-1}) and 500 μM (37.5 mg l^{-1}) As(V). For the As(V) and Pi competition experiment, seeds were grown in the presence of either 100 μM As(V) (7.5 mg l^{-1} As) or 100 μM Pi (3.1 mg l^{-1} P) or a combination of both. The number of germinated seeds was determined after incubation for 9 days at 25 ± 2 °C, at a light intensity >700 lx for 12 h day^{-1}. Root and shoot lengths were measured to the millimeter from the germination site to the tip (Fig. S1b). Data are presented as means ± SD and in % relative to the control.

Experimental setup for gene expression analysis

Rice plants were germinated in 0.5 ml plastic tubes filled with 1/10 Murashige and Skoog (MS) medium and 0.6 % phyto-agar (Duchefa). Seeds were stratified for 1 day at 4 °C, transferred to light conditions (23 °C, humidity 60 %, 13 h light) and covered with cling film until germination. The plastic tubes with cut-off tips were placed in plastic boxes containing 1/10 MS medium (Fig. S1c,d), whereby ~20 seedlings of similar height were selected and placed in one box. Plants undergoing all treatments were grown for 3 weeks in total. For the long-term treatment, 2-week-old plants were grown for 7 days on fresh 1/10 MS medium with addition of 10 or 100 μM As(V) or Pi (Fig. S1e), while for the short-term exposure, 10 μM As(V) or Pi were added to the medium 16 h prior to harvesting (Fig. S1f). Negative controls without additional supplement of either As(V) or Pi were conducted for each time frame and concentration. The increase in root and shoot elongation was determined for long-term treated plants. For this purpose, the length of the shoots and the longest root of the same plant were measured at the beginning and at the end of the treatment; the data for 20–25 plants are presented as mean ± SD.

For Pi quantification in the growth medium, 0.5 ml-samples were analyzed by HPLC (DX-120, Dionex). At the end of the experiment roots and shoots of all plants from one treatment were pooled, ground in liquid nitrogen (3 times, for 30 s each) and stored at −80 °C. The experiment was repeated three times.

Gene expression analysis

RNA was extracted with the RNeasy Plant Mini kit (Quiagen) from ~80 mg shoot and root tissue. An additional DNA digestion step was applied to ensure the absence of genomic DNA. Using the Experion™ RNA StdSens system (BIO-Rad), RNA with a quality index of ≥7 was used for cDNA synthesis with the Invitrogen kit using the SuperScriptIII reverse transcriptase.

For cDNA amplification, the PerfeCta qPCR SuperMix or PerfeCta SYBR Green SuperMix (Quanta Biosciences, distributed by VWR) were used. When necessary, the corresponding probe (Universal ProbeLibrary Single Probes, Roche) was added according to the manufacturer's instructions. All primer sequences are listed in Tables S1 and S2. qPCRs were run in the Bio-Rad CFX384 Real Time PCR system (Bio-Rad) and the relative gene expression levels were analyzed with the CFX manager software (Version 1.1; Bio-Rad). The expression levels of each gene were normalized to the expression of the ubiquitin-like protein SMT3 (NM_001051742). At least three technical replicates per treatment per target gene were performed. For each gene, values of the corresponding controls were set to one, and those from different treatments are represented as relative fold changes compared to the control. The data were collected from three independent experiments and are presented as mean values ± SE. Only genes with a fold change greater than twofold were considered as differentially expressed.

Total As quantification and As speciation in plant material

For quantification of total As and P in root and shoot tissue, liquid-N_2-ground plant material was dried at 60 °C.

20–70 mg material was digested overnight in 2 ml 65 % HNO_3 at room temperature. The samples were boiled for 2 h, followed by the addition of 2 ml ≥30 % H_2O_2 and boiling until brown gasses stopped evolving. Samples were diluted in MilliQ water before As and P quantification by ICP atomic emission spectrometry (concentrations >0.1 mg l^{-1}, CIROS, Spectro A.I.) or for lower concentrations by ICP-MS (Agilent Technologies 7700 Series). The limit of quantification for arsenic by the ICP-MS method was 0.2 μg l^{-1}. For As species determination in root and shoot tissue, 20–50 mg dry plant material was extracted for 2 h in 10 ml 10 mM phosphoric acid at 150 rpm and room temperature. The material was filtered through Whatman #1 filter paper before the redox species arsenate and arsenite were analyzed with LC–ICP–MS. Details of the speciation method are described elsewhere (Daus et al. 2008).

Results

Growth responses of *O. sativa* L. cv. Gladia to As(V) and Pi

Since the sensitivity of rice plants to As treatment tightly correlates with genotypic variations (Marin et al. 1992; Abedin and Meharg 2002; Geng et al. 2006; Abbas and Meharg 2008), we initially analyzed the physiological responses of the *O. sativa* L. cv. Gladia variety to different As(V) concentrations. For this purpose, seed germination and early root and shoot elongation were quantified 9 days after incubation and growth in liquid media containing different As(V) concentrations (Fig. 1). A significant decrease in seed germination of 22 ± 2 % compared to that of untreated plants was observed at 500 μM As(V) (Fig. 1a), whereas 10 mM As(V) repressed seed germination completely (data not shown). However, shoot and especially root elongation were already significantly reduced at much lower As(V) concentrations (Fig. 1b, c). With increasing As(V) concentrations, root elongation was repressed more drastically than shoot growth. In the presence of 50 μM As(V), root length was only 18 ± 3 %, while shoot length still constituted about 54 ± 2 % of that of untreated control seedlings, and 500 μM As(V) completely repressed the growth of roots but not of shoots (Fig. 1b, c).

Next, we investigated whether Pi can compensate the effect of As(V) on young rice seedling growth (Table 1). The presence of 100 μM Pi alone caused a significant increase in shoot and root length compared to that of control plants growing without Pi. In contrast, 100 μM As(V) completely abolished root growth and substantially reduced shoot elongation, which is comparable to the results observed in the previous experiment. Equimolar concentrations (100 μM) of As(V) and Pi resulted in partially rescued root growth (26 ± 4 % of untreated roots) and increased shoot length (73 ± 7 % of untreated shoots), when compared to treatment with 100 μM As(V) and even 50 μM As(V) alone (Table 1; Fig. 1b, c). Thus, the presence of external Pi positively influenced the growth of seedlings exposed to As(V). The increased seedling growth caused by the addition of 100 μM Pi alone indicated that endosperm storage capacities did not provide sufficient amounts of P for an optimal early growth of the seedlings.

To avoid Pi starvation effects and thus better evaluate Pi–As(V) interrelations in the plant, we spiked the growth medium with a basal amount of 125 μM Pi in all further experiments. During the first 2 weeks of seedling growth, the Pi concentration in the medium dropped from 125 μM to approximately 8–10 μM. Then, the plants were grown for another 7 days on fresh 1/10 MS medium (containing 125 μM Pi and referred to as control) supplemented with 0, 10 or 100 μM Pi or As(V) (see Fig. S1e for experimental setup). The additional Pi did not significantly influence plant growth, whereas the addition of As(V) inhibited shoot and root growth in a concentration dependent manner (Fig. 1d–g; Table 2). Similarly to the previous results, root elongation was inhibited to a higher degree than shoot growth. At 10 μM As(V), root length decreased by 38 % compared to that of plants grown without As (Table 2). No root elongation was detected in plants grown on 100 μM As(V), although the viability assay through staining of roots with fluorescein diacetate/propidium iodide (Ishikawa and Wagatsuma 1998) demonstrated that the root cells were alive (data not shown).

Tissue-dependent expression of *PT* genes and their response to As(V) and Pi

The Gladia phosphate transporter genes *PT2, PT4* and *PT8* showed the highest transcript abundance among all *PT* genes (data not shown). In consistence with previous reports (Liu et al. 2011; Paszkowski et al. 2002), transcripts of *PT2, PT3, PT6, PT9* and *PT10* were preferentially expressed in roots (Fig. 2), with *PT9* and *PT10* being hardly detectable in shoots. *PT1, PT4, PT5* and *PT8* were more or less equally expressed in roots and shoots, while *PT7* and *PT12* were expressed in shoots at much higher levels than in roots (Fig. 2). Even though higher *PT6* transcript levels were detected in roots, the overall expression of *PT6* was very low in the analyzed plant material, which is in agreement with previous reports showing that *PT6* is strongly activated by Pi starvation (Ai et al. 2009). Since non-Pi-starvation conditions were used in the present experiment, *PT6* seems to be repressed and was, therefore, excluded from further analysis. *PT11* transcripts were not detectable in any of the treated plants, which supports other studies demonstrating that *PT11* is specifically expressed

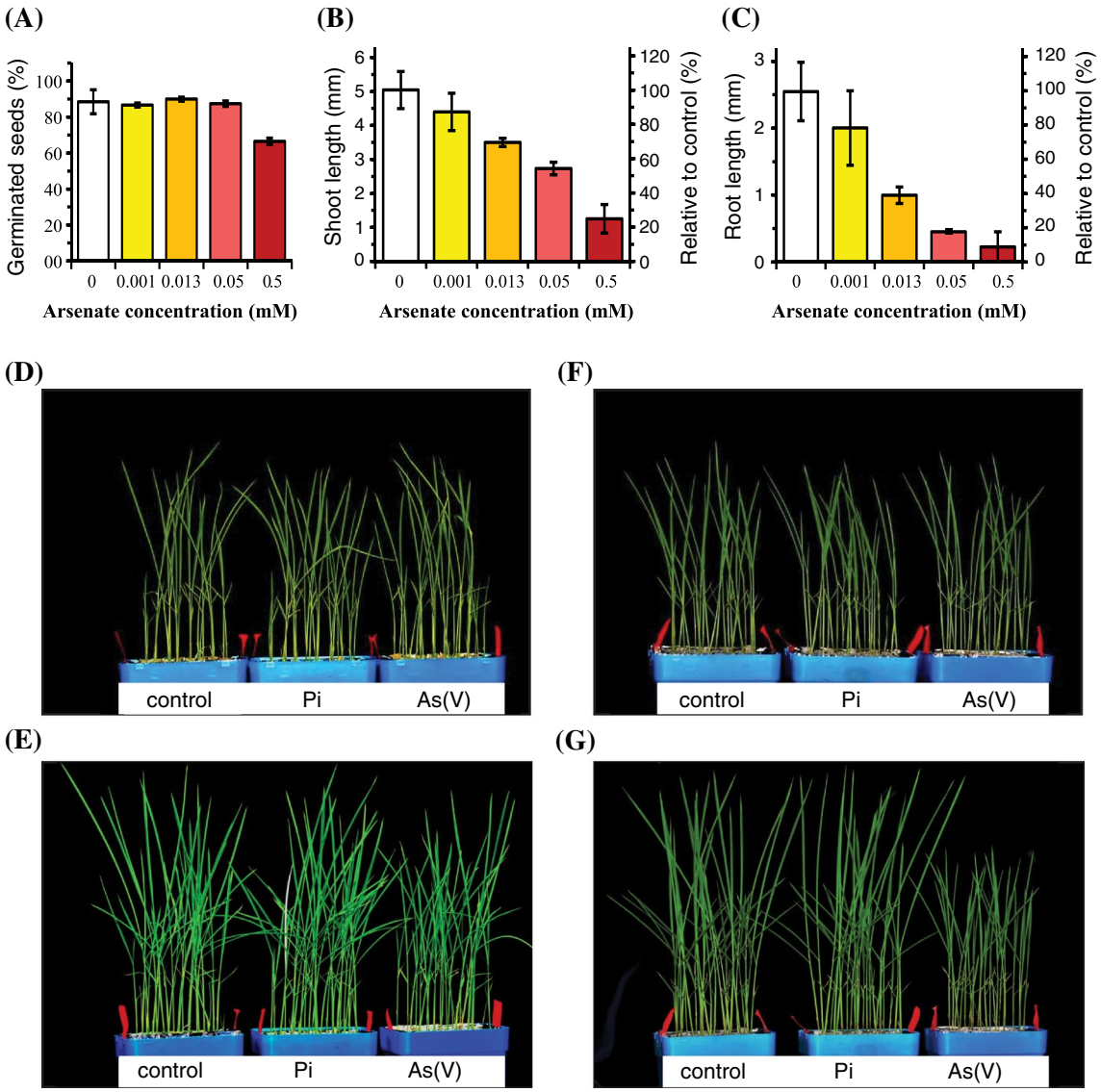

Fig. 1 Physiological effect of increasing As(V) concentration on seed germination and growth of *O. sativa* L. seedlings. Seedlings were grown in water supplemented with 0, 0.001, 0.013, 0.05, 0.5 mM As(V). The number of germinated seeds in % (**a**) and the average length in mm of shoots (**b**) and roots (**c**) per As(V) concentration are depicted. **d**–**g** The effect of As(V) and Pi on *O. sativa* growth after 7 day incubation for the gene expression analysis experiment. Plant phenotype prior to and after 1 week of 10 (**d** and **e**, respectively) or 100 μM (**f** and **g**, respectively) As(V) or Pi exposure

only in stamen (Liu et al. 2011) or in roots upon symbiosis with arbuscular mycorrhiza (Paszkowski et al. 2002).

The addition of As(V) to the growth medium induced a broad range of PT transcriptional responses (Fig. 3). In roots, *PT2* and *PT3* were strongly inhibited by 10 and 100 μM As(V) treatment. In contrast, *PT13* was induced by both As(V) concentrations, and *PT5* and *PT10* only by 100 μM As(V) (Fig. 3a). The expression of *PT1, PT4, PT8* and *PT12,* was not influenced by treatment with any As(V) concentration, whereas the expression of *PT7* and *PT9* strongly fluctuated, being oppositely regulated by a particular As(V) concentration in some experimental repeats, which led to high values of SE.

In shoots, higher transcript levels of *PT1, PT5* and *PT8* were observed upon 100 μM As(V) treatment (Fig. 3b). Expression of *PT2* and *PT12* was slightly reduced in shoots of As(V)-treated plants.

Our next question was whether the *PT* genes would respond similarly to treatment with the same concentration of Pi compared to As(V). Generally, the responses of PTs to 1 week Pi treatment were of a much lower amplitude and more uniform than responses to As(V) treatment.

Table 1 Mean shoot and root lengths of *O. sativa* L. seedlings, germinated and grown for 9 days in the absence or presence of either As(V) or Pi or a combination of both

As(V)/Pi (μM)	Shoot length (cm)		Root length (cm)	
	Mean ± SD	% of control	Mean ± SD	% of control
0/0	3.9 ± 1.4	100	2.0 ± 1.0	100
0/100	4.6 ± 1.6	118***	2.5 ± 1.2	121**
100/0	2.3 ± 1.1	59***	0	0***
100/100	2.9 ± 1.0	73***	0.5 ± 0.3	26***

Mean shoot and root lengths are given in cm and in % of the control group (no As(V) or Pi in the growth solution)

SD standard deviation

Asterisks indicate whether the mean of the control group is significantly different to the mean of a treatment group using the unpaired *t* test

* $p < 0.05$

** $p < 0.01$

*** $p < 0.001$

In roots, the transcript levels of *PT3* and *PT9* decreased by half upon Pi addition, and a tendency towards decreased *PT10* expression was observed at the higher Pi concentration (Fig. 3a). The expression of all other *PT* genes in roots was not substantially altered by increased external Pi concentration.

In shoots, treatment with 100 μM Pi resulted in up-regulated transcript levels of several PTs, including *PT1, PT3*, *PT5* and *PT7* (Fig. 4b). An increased expression of these genes in shoots upon Pi treatment was observed in each experimental repeat, even though the difference in transcript levels was not statistically significant due to repeat-dependent variations.

In summary, Pi application triggered a different transcriptional response in most *PT* genes compared to As(V) treatment. Moreover, the responsiveness to As(V) was comparatively stronger in roots than in shoots, whereas the expression of more genes in shoots changed upon Pi addition. To determine whether the observed differences in *PT* gene expression were caused by a specific sensitivity to As(V) or Pi, or were merely due to different plant-internal As and P contents or changes in As redox state, we analyzed the spatial distribution of As and P in roots and shoots of the rice plants.

Accumulation of As and P in rice plants

The concentration of P in shoots of 3-week-old plants prevailed at about 8 mg g^{-1} dry shoot material and did not differ under the different treatments (Table 3). P concentrations in roots were generally between 4 and 5 mg g^{-1} dry root material, but decreased to 2.6 ± 0.1 mg g^{-1} in plants grown for 7 days with supplementation of 100 μM As(V).

To analyze the spatial distribution of As(V) in the plants, we quantified the total As content and the inorganic As species in roots and shoots. Since both As species are comparatively labile and can easily be converted to each other upon extraction, we used 10 mM phosphoric acid to maximally preserve the extracted As species (Daus et al. 2002). The total As content was almost 100 times higher in roots than in shoots, consisting of approximately 20 and 60 % As(V), respectively (Table 3). Even though the ratio of As(V) to As(III) was lower in roots than in shoots, the absolute amount of As(V) in roots was still substantially higher than in shoots. The external concentration of As(V) did not affect the ratio of As(V) to As(III) in roots and shoots. The total As content in shoots was similar in plants grown on 10 and 100 μM As(V), whereas the roots of plants grown on 100 μM As(V) contained twice as much As as those grown on 10 μM As(V). Thus, rice roots accumulated a

Table 2 Increment in mean shoot and root length of *O. sativa* L. plants over 7 days growth period

Treatment	Shoot length (cm ± SD)				Root length (cm ± SD)			
	Start	Final	Increment	% of control	Start	Final	Increment	% of control
Control (125 μM Pi)	11.9 ± 3.9	23.6 ± 4.4	11.6 ± 2.9	100	7.6 ± 2.4	12.9 ± 2.4	5.3 ± 2.6	100
+10 μM As(V)	12.3 ± 4.1	22.1 ± 5.1	9.8 ± 2.1	84*	8.8 ± 2.3	12.6 ± 2.2	3.8 ± 1.3	72*
+100 μM As(V)	12.3 ± 4.1	16.5 ± 5.1	4.3 ± 2.3	37***	9.0 ± 2.1	9.0 ± 2.1	0	0***
+100 μM Pi	11.9 ± 3.9	23.6 ± 4.4	11.9 ± 1.6	102	9.2 ± 2.3	14.3 ± 3.0	5.2 ± 1.9	97

The 2 week-old plants were grown for 7 days on 1/10 MS-medium with supplementation of As(V) or Pi as indicated

The increment of shoot and root length before and after 7 days of growth is given in cm and in % to the control group (no additional Pi or As(V) in the growth medium)

SD standard deviation

Asterisks indicate whether the mean of the control group is significantly different to the mean of a treatment group using the unpaired *t* test

* $p < 0.05$

** $p < 0.01$

*** $p < 0.001$

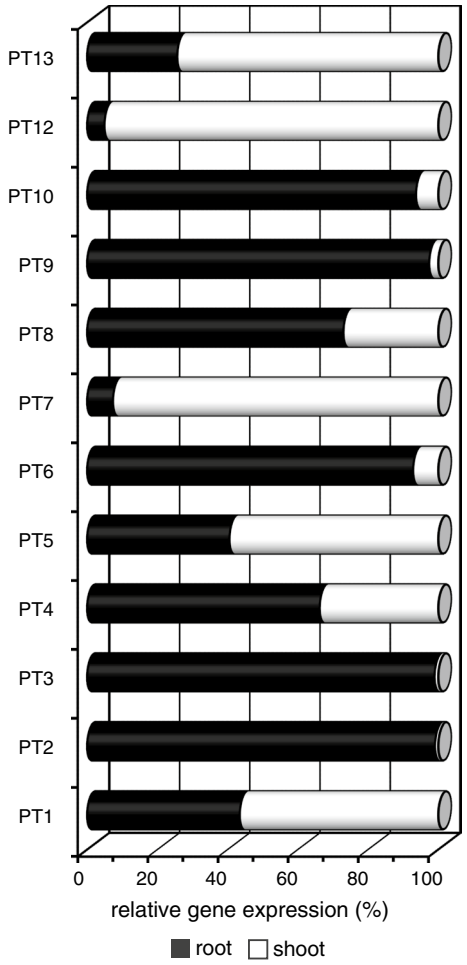

Fig. 2 Relative transcript accumulation (in %) of different PTs in *O. sativa* roots and shoots. The relative transcript accumulation was quantified in roots (*black*) and shoots (*white*), when no As(V) or additional Pi was added to the growth medium

large amount of As, which was mainly present as As(III). The detoxification of As(III) is associated with an increased synthesis of phytochelatins and hence causes the exhaustion of the intracellular thiol pool, and induces oxidative stress (Requejo and Tena 2005; Ahsan et al. 2008; Tripathi et al. 2013; Huang et al. 2012). To minimize the effect of As(III) as an oxidative stressor on the expression of the tested genes, we performed short-term treatment experiments (see Fig. S1f for experimental setup).

Phosphate transporters responses to short-term As(V) and Pi treatments

The concentration of 10 μM Pi or As(V) was chosen for the short-term treatment in order to avoid the accumulation of a higher amount of As(III). The response of most PTs to short-term As(V) treatment in roots was weaker than that observed for the 1 week As(V) treatment (Fig. 4a). Only *PT5* was induced in roots in all three experimental repeats indicating that short-term and long-term responses may be mediated by different mechanisms. In shoots, *PT1*, *PT3* and *PT13* were up-regulated to a different extent in all repeats (Fig. 4b). Transcript levels of all other PTs in shoots and roots were not affected by exposure to As(V). In contrast to that of As(V), the increase in Pi concentration did not significantly affect the expression of *PT* genes (Fig. 4). Therefore, similarly to the 1 week treatment results, there was a distinct difference between the *PT* gene responses to short-term As(V) and Pi exposures.

The amount of P quantified in the plant material revealed no difference between short-term and long-term treatment (Table 3). Also, the speciation of As in shoots was comparable in plants grown on 10 μM As(V) for 16 h and 7 days. The total amount of As was only slightly lower in shoots under short-term treatment. Roots, however, accumulated 2.5 times less As when exposed to As(V) for 16 h compared to 7 days exposure. As(V) fraction comprised approximately 50 % of total As in roots (Table 3), and was thus, essentially higher than after the 7 day treatment.

The expression of genes involved in Pi signaling

Taking into account the observed differential regulation of *PT* genes, we next analyzed the effect of Pi and As(V) on upstream regulatory elements involved in phosphate acquisition. The expression of the transcription factor *PHR2*, a central integrator of Pi transcriptional responses (Chiou and Lin 2011; Zhou et al. 2008), was not considerably different in treated and non-treated tissues (Figs. 5, 6). The same was true for the expression of *SPX1* and *PHO2*, the negative regulators of Pi starvation responses (Zhou et al. 2008; Wang et al. 2009). The expression of *PHO2* was higher in roots than in shoots, whereas *PHR2* and *SPX1* were expressed in both tissues at comparable levels (Fig. 7).

Neither 1 week nor short-term Pi treatment caused significant changes in *PHF1, PHO1;2* and *PHO1;2anti* transcript levels (Figs. 5, 6). However, the *PHO1;2anti* transcript level was increased and *PHF1* decreased in roots upon 1 week As(V) treatment (Fig. 5a). The expression of *PHO1;2*, which was manifold higher in roots than in shoots (Fig. 7), remained mostly unchanged (Figs. 5, 6).

In consistence with previously published data (Lin et al. 2010), the two SPX domain-containing genes, SPX-MFS1 and SPX-MFS2, were predominantly expressed in shoots (Fig. 7). In roots, the transcript level of SPX-MFS2 was down-regulated by 1 week exposure to 100 μM Pi, whereas SPX-MFS1 expression was not substantially changed (Figs. 5b, d, 6). However, SPX-MFS1 was strongly up-regulated by short-term As(V) treatment in roots, and to a lesser extent in shoots, by both short-term and 1 week treatments (Figs. 5c, 6). In contrast, SPX-MFS2 was

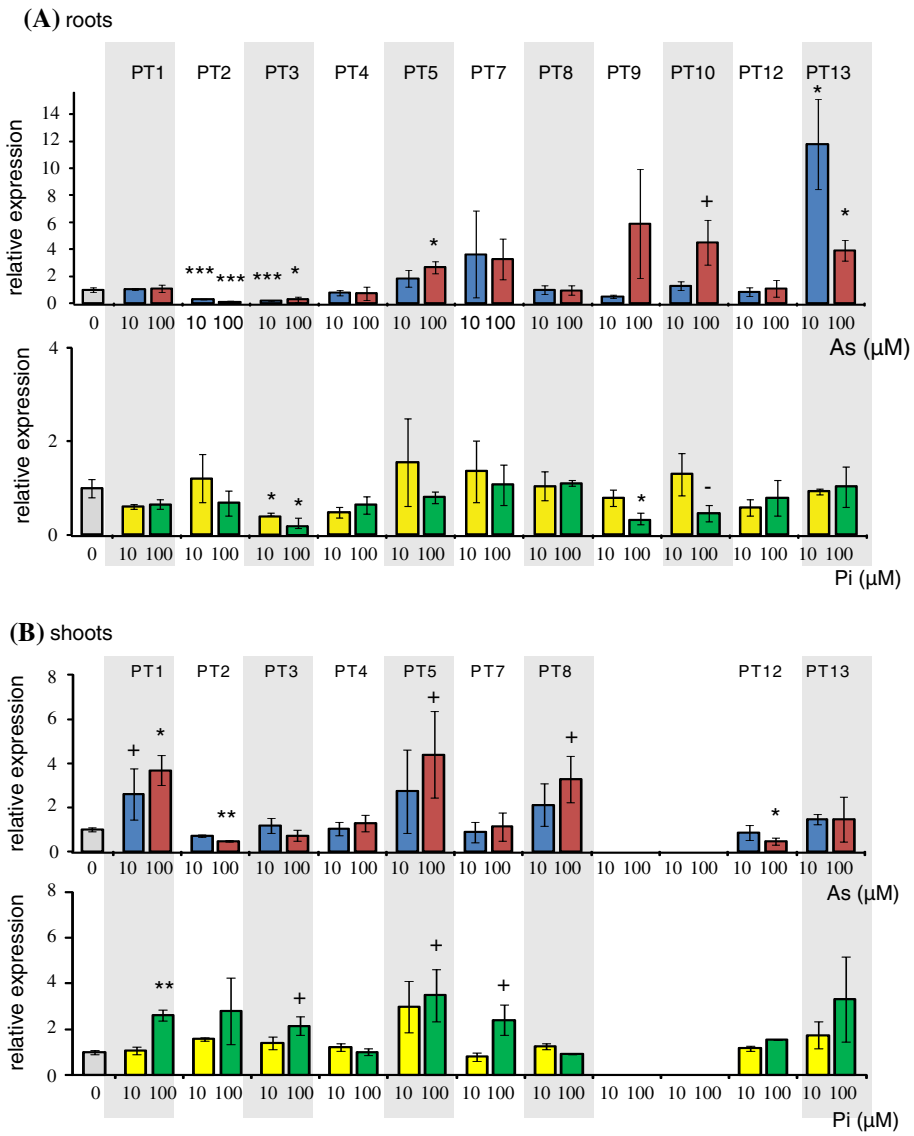

Fig. 3 Transcript accumulation levels of different PTs in *O. sativa* roots and shoots after 7 days of As(V) or Pi exposure. Two-week old plants were grown on 1/10 MS medium with no additional As(V) or Pi (*grey*) or with an addition of 10 µM As(V) (*blue*), 100 µM As(V) (*red*), 10 µM Pi (*yellow*) or 100 µM Pi (*green*) for 7 days. The transcript accumulation was quantified in roots (**a**) and shoots (**b**). Transcript accumulation levels are given in relative fold expression to the control (*grey*). The means of three biological repeats for treated and control groups were compared using the *t* test. A *single asterisk* indicates a significant difference ($p < 0.05$), while *two asterisks* depict a very significant difference ($p < 0.01$). *Plus* Expression level increased in all three experimental repeats, but $p > 0.05$. *Minus* Expression level decreased in all three experimental repeats, but $p > 0.05$

predominantly down-regulated in roots and in shoots by As(V) addition (Figs. 5a, c, 6).

In conclusion, most of the regulatory genes analyzed here responded rather weakly to the treatment with As(V), and even less to that with Pi. Only the expression of SPX-MFS1, which has recently been shown to play a key role in the control of Pi homeostasis in leaves (Wang et al. 2012), was strongly induced by As(V) treatment, whereas SPX-MFS2 was repressed.

Discussion

The exposure of plants to elevated As in the environment causes severe disorders in plant growth and development (Abedin and Meharg 2002; Meharg and Zhao 2012). The ability to tolerate As greatly differs between different plant species and ecotypes (Marin et al. 1992; Abedin and Meharg 2002; Geng et al. 2006; Abbas and Meharg 2008). Although *O. sativa* L. cv. Gladia seed germination was not impaired by the presence of low As(V) concentrations, seedling growth was already reduced in the presence of 1 µM As(V), which is comparable to the As concentrations found in aquifer and sediment pore water (Smedley and Kinniburgh 2002). We used this wet season rice variety, which is non-tolerant to As, to decipher the interrelations of As(V) and Pi in the transcriptional regulation of the *PHT1* family transporters, i.e. the genes responsible for delivering both ions into the plant.

Trade-off between Pi uptake and As(V) toxicity in *O. sativa* L. cv. Gladia

The observed positive effect of Pi supplementation on the growth of As(V)-treated rice plants is in agreement with

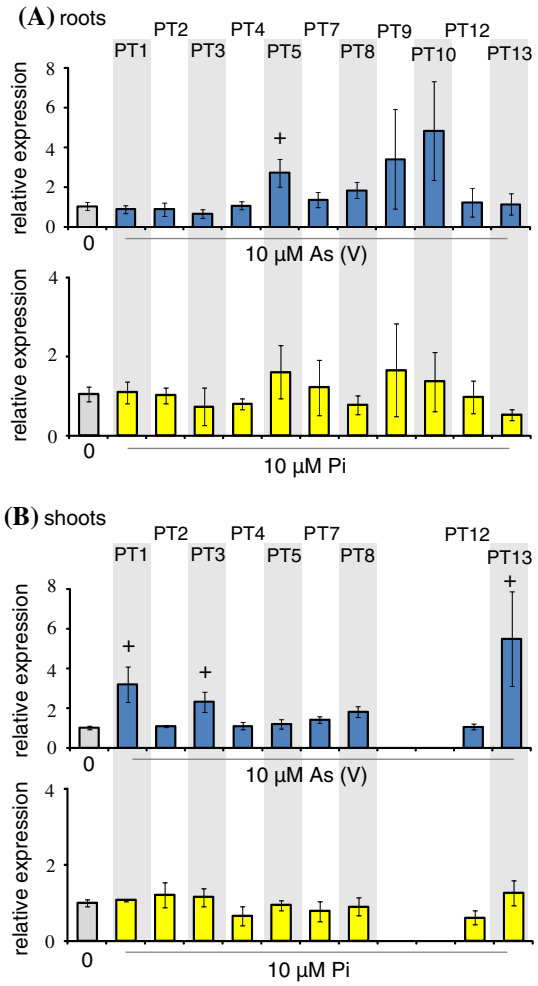

Fig. 4 Transcript accumulation levels of different PTs in *O. sativa* roots and shoots after 16 h of either As(V) or phosphate treatment. Three-week old plants were grown on 1/10 MS medium without (*grey*) or with supplementation of 10 µM As(V) (*blue*) or 10 µM Pi (*yellow*) for 16 h. The transcript accumulation was quantified in roots (**a**) and shoots (**b**). Transcript accumulation levels are given in relative fold expression to the control (*grey*). Statistical analysis was performed as indicated in Fig. 3

previous studies (Jankong et al. 2007; Swarnakar and Mukherji 2005). Yet even a Pi concentration (125 µM) ten-times higher than As(V) (10 µM) could not completely rescue the phenotype of As(V)-affected plants. Since both As(V) and Pi use the same transport system, one might expect a decreased Pi uptake due to direct competition of both ions for the transporters. However, no difference in plant P content was detected in the shoots and roots of untreated and 10 µM As(V)-treated plants. Moreover, the tissue-specific P content in these plants was comparable to that found in plants grown with higher Pi, indicating that the intracellular P status was actively maintained at these steady-state levels by the plant. Thus, it is not likely that the observed growth sensitivity of the plant to 10 µM As(V) was due to limiting P content. Since the roots accumulated a high level of As, most of which was in the reduced form, the observed negative effect might be, at least partially, caused by As(III). The reduced form of As is highly toxic, is capable of binding to sulfhydryl groups and interferes with the cellular redox equilibrium and multiple metabolic processes (Requejo and Tena 2005; Ahsan et al. 2008; Hartley-Whitaker et al. 2001; Kitchin and Wallace 2006). The reduction of As(V) to As(III), with subsequent binding to phytochelatins and sequestration to root cell vacuoles, was reported for different plants and is considered to be a major As detoxification pathway (Zhao et al. 2009; Meharg and Hartley-Whitaker 2002; Moore et al. 2011; Finnegan and Chen 2012). This concurs with the much lower level of As in shoots compared to roots and, correspondingly, the lower responsiveness of the shoot growth to As(V) exposure.

At comparable As(V) and Pi concentrations in the growth medium (~100 µM), As(V) appears to directly compete for the phosphate transport system, as only half the amount of P is accumulated in the roots compared to control plants. Thus, the observed drop in the intracellular P content together with the high-energy demanding processes of As(III) detoxification might mainly contribute to the complete inhibition of root growth under 100 µM As(V) treatment.

Interestingly, the P content in shoots was not significantly reduced in plants grown with 100 µM As(V). Moreover, the As concentration was much lower in shoots than in roots and only slightly higher than that in plants grown with 10 µM As(V). Hence, the strong inhibition of shoot growth in plants exposed to 100 µM As(V) is probably not directly caused by intracellular shoot As and P concentrations but results from a root-derived systemic signal. Uroic et al. (2012) showed that As(V) significantly affected the metabolite profile of xylem sap and greatly inhibited the production of sap by roots of cucumber plants. A decreased sap flow, and hence nutrient availability, could have negatively influenced the growth of the rice shoots.

Effect of external Pi on *PT* gene expression

It is generally known that the cytoplasmic phosphorus concentration is maintained at a constant level in spite of external Pi fluctuations (Raghothama 1999 and references therein). This homeostasis is partially achieved by reallocating the excessive Pi to vacuoles and back to the cytoplasm when required (Raghothama 1999). The determination of the P content in Gladia plants showed that intracellular P remained unaltered in plants grown with or without additional Pi, indirectly indicating that the vacuolar storage capacity was saturated and that the P homeostasis was maintained by regulating Pi uptake and efflux (Mimura 1995). It has been shown that, in Pi-deficient conditions,

Table 3 Total P and As in shoots and roots of *O. sativa* L. exposed to As(V) or Pi for either 7 days or 16 h

Treatment	Content in shoots (mg g^{-1} dry material)			Content in roots (mg g^{-1} dry material)		
	P	As	As speciation	P	As	As speciation
7 days						
Control (125 μM Pi)	8.18 ± 0.54	n.d.		4.53 ± 0.21	n.d.	
+10 μM Pi	8.31 ± 0.18	n.d.		5.02 ± 0.32	n.d.	
+10 μM As(V)	8.57 ± 0.72	0.020 ± 0.003	62.7 % As(V) 37.3 % As(III)	4.36 ± 0.21	1.280 ± 0.150	19.2 % As(V) 80.8 % As(III)
+100 μM Pi	9.59 ± 0.64	n.d.		5.00 ± 0.36	n.d.	
+100 μM As(V)	8.85 ± 0.14	0.027 ± 0.003	58.8 % As(V) 41.2 % As(III)	2.61 ± 0.13*	2.438 ± 0.274	25.2 % As(V) 74.8 % As(III)
16 h						
Control (125 μM Pi)	8.35 ± 0.48	n.d.		4.59 ± 0.20	n.d.	
+10 μM Pi	8.25 ± 0.41	n.d.		5.59 ± 0.52	n.d.	
+10 μM As(V)	7.77 ± 0.28	0.017 ± 0.002	64.1 % As(V) 35.9 % As(III)	3.94 ± 0.58	0.506 ± 0.110	49.1 % As(V) 50.9 % As(III)

As speciation in the tissue is given in % of the total As

Data are presented as mean values ± SD

n.d. not detected

* Significant difference to control, $p < 0.05$

plants enhance Pi uptake rates by up-regulating *PT* genes resulting in an increased number of transporter molecules (Liu et al. 1998; Leggewie et al. 1997; Paszkowski et al. 2002; Smith et al. 1997; Chen et al. 2008; Muchhal and Raghothama 1999). Our results confirm that P homeostasis is also maintained under Pi-sufficient conditions and that its maintenance is achieved, at least partially, by the differential expression of *PT* genes. However, not all PTs responded similarly to an increase in external Pi concentration. Most *PT* genes in Gladia plants were expressed at a steady level, independently of changes in Pi concentration in the medium. Only some PTs, such as *PT3, PT9* and *PT10* in roots and *PT1*, *PT3*, *PT5* and *PT7* in shoots, were characterized by altered transcript levels. The differential responsiveness of *PHT1* family members to Pi deficiency in rice has been shown in other reports. However, different subsets of *PT* genes have been identified as Pi starvation-inducible in different studies. For example, Zhou et al. (2008) showed an increased expression of *PT5*, *PT7*, *PT9* and *PT12* in roots of plants grown at low Pi (1 mg l^{-1}) compared to those grown at high Pi (10 mg l^{-1}), while *PT2*, *PT3* and *PT6* have been reported to be Pi starvation-inducible in other studies (Ai et al. 2009; Wang et al. 2009; Paszkowski et al. 2002). In fact, *PT6* appears to be the most sensitive to external Pi as it was already repressed in Gladia plants grown in control medium. This is in accordance with Ai et al. (2009), who showed that *PT6* was strongly induced in Pi-deficient conditions and was primarily involved in the acquisition of Pi upon starvation. Furthermore, microarray analysis of the roots of two rice varieties deprived of Pi revealed that the induction of particular *PT* genes depended on the plant genotype and treatment duration (Li et al. 2010). Taken together, these data indicate that *PT3* in roots appears to be responsive to a wide range of Pi concentrations, whereas changes in the expression of other PTs are either restricted to specific genotypes or are only responsive to a limited range of Pi concentrations.

Since the root system mediates Pi acquisition in plants, and most *PT* genes are preferentially expressed in roots, data concerning *PT* gene regulation in shoots are limited. However, the translocation of Pi to the shoots and within the green tissue is indispensable for the maintenance of P homeostasis and plant growth and development. Depending on Pi availability, plant leaves can function either as a sink (under sufficient Pi conditions) or as a source (on Pi starvation) for Pi (Raghothama 1999), which indicates the importance of the regulation of the phosphate translocation system. Our findings suggest that several genes, including *PT1, PT3*, *PT5* and *PT7*, are involved in controlling the redistribution of Pi in shoots under Pi-sufficient conditions. Remarkably, most of these genes are either preferentially expressed in shoots (*PT7*) or at comparable levels in root and shoot tissues (*PT1* and *PT5*). In consistence with our results, higher transcript levels of *PT5* were found in shoots of rice plants grown at high compared to low external Pi levels (Zhou et al. 2008). Also, the recent molecular characterization of *PT1* revealed its important role in Pi uptake and translocation in Pi-replete rice (Sun et al. 2012). Thus, *PT1* and *PT3*, which is more strongly expressed in roots, may contribute to long-distance Pi transport, whereas *PT5*

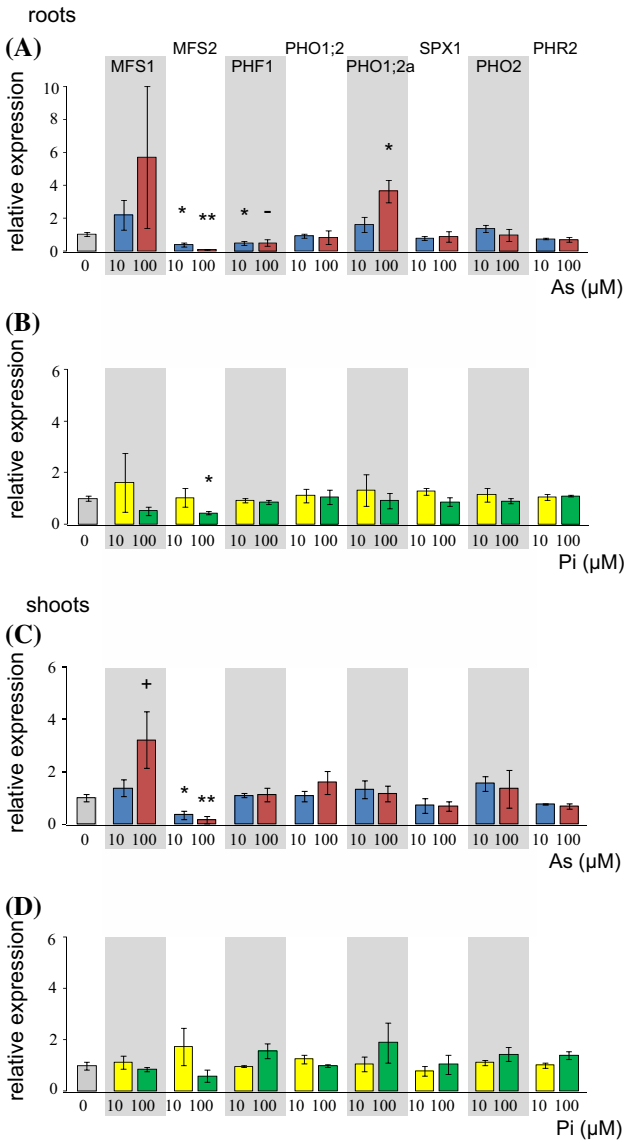

Fig. 5 Transcript accumulation levels of genes involved in phosphate acquisition in *O. sativa* roots and shoots after 7 days of either As(V) or phosphate treatment. Two-week old plants were grown on 1/10 MS medium without (*grey*) or with supplementation of either 10 μM (*yellow*) or 100 μM Pi (*green*), or 10 μM (*blue*) or 100 μM As(V) (*red*) for 7 days. The transcript accumulation was quantified in roots (**a**) and shoots (**c**) of As(V)-exposed plants as well as in roots (**b**) and shoots (**d**) of phosphate-exposed plants. Transcript accumulation levels are given in relative fold expression to the control (*grey*). Statistical analysis was performed as indicated in Fig. 3

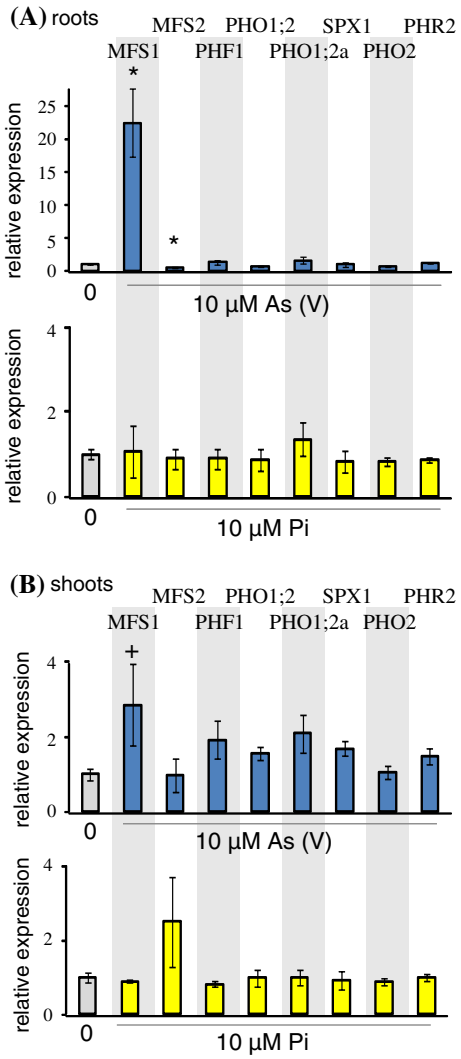

Fig. 6 Transcript accumulation levels of genes involved in phosphate acquisition in *O. sativa* roots and shoots after 16 h of either As(V) or Pi treatment. Three-week old plants were grown on 1/10 MS medium without As(V) or Pi (*grey*) or in the presence of either 10 μM As(V) (*blue*) or Pi (*yellow*) for 16 h. The transcript accumulation was quantified in roots (**a**) and shoots (**b**). Transcript accumulation levels are given in relative fold expression to the control (*grey*). Statistical analysis was performed as indicated in Fig. 3

and *PT7* probably play a more important role in maintaining P homeostasis in shoots.

Whereas increasing external Pi concentrations affected the expression of some *PT* genes, no substantial change was observed in the expression of genes potentially involved in *PT* transcriptional regulation. The similar *PHR2* transcript levels observed in control and Pi-treated Gladia plants are in line with previous reports of constitutive expression of *PHR2* (Zhou et al. 2008). The activity of PHR2, which is involved in the regulation of at least *PT2* and *PT9* (Liu et al. 2010; Zhou et al. 2008), is negatively regulated by *SPX1* (Liu et al. 2010). The latter gene was shown to be induced by Pi deprivation (Liu et al. 2010), but remained unchanged in response to an increase of external Pi under Pi non-starvation conditions, i.e. in the present experiment. This suggests that distinct mechanisms are involved in the regulation of PTs operating during acute P demand and during maintenance of P homeostasis in non-starvation conditions.

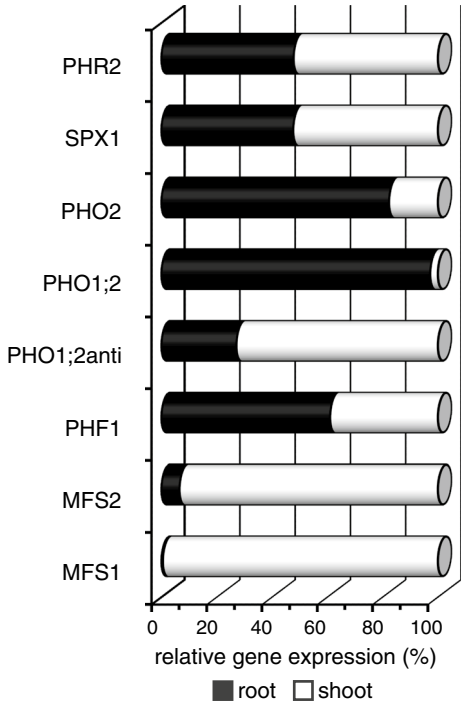

Fig. 7 Relative transcript accumulation (in %) of phosphate acquisition regulatory genes in *O. sativa* roots and shoots. The relative transcript accumulation was quantified in roots (*black*) and shoots (*white*) when no As(V) or additional Pi was added to the growth medium

Arsenate versus phosphate in *PT* gene regulation

To our knowledge, this study has been the first to consider the dynamics of the expression of different *PT* genes in response to treatment with comparable amounts of As(V) and Pi. Since Pi and As(V) are structurally homologous and compete for Pi binding sites, it is essential to set up appropriate experimental conditions to properly evaluate their interrelations. The enhancement of Gladia seedling growth by the supplementation of Pi (see Table 1) suggests that, when deprived of the medium, Pi is the rate-limiting factor for seedling growth. Subsequently, Pi deficiency might (1) benefit As(V) in its competition with Pi for binding sites and (2) induce starvation-related stress responses. Addition of 125 μM Pi to the growth medium appeared to satisfy the plant's demand for P, as an additional 10 or 100 μM Pi did not significantly affect seedling growth. In addition, the expression of most *PHT1* family genes was verified in the roots and shoots of plants grown under these conditions, which was a prerequisite for the study of their regulation.

Surprisingly, despite of the structural similarities of both ions, most *PT* genes, especially in roots, responded differently to the application of As(V) compared to Pi. While the additional Pi elicited a down-regulation of several root *PT* genes, treatment with As(V) caused a divergent regulation of specific *PT* genes in roots. Among them, *PT2* and *PT3* were repressed and *PT5*, *PT10* and *PT13* up-regulated by exposure to As(V). Only *PT3* was also down-regulated in roots by increased external Pi concentration, though the inhibition caused by As(V) was stronger. The repression of *PT2* and *PT3* in roots appears to be a general response to As(V) exposure, since it was also reported for rice grown for 1 week with 13.3 μM As(V) in the absence of Pi (Norton et al. 2008). Similarly, *PT2* was down-regulated in roots of rice plants grown for 10 days with 250 μM As(V) (Chakrabarty et al. 2009). Since the growth medium used in the latter experiment contained 625 μM Pi, *PT3* was probably not expressed and, therefore, its repression by As(V) could not be observed.

Unlike those in Pi-treated plants, some of the *PT* genes in roots of As(V)-treated plants were either up-regulated or showed a tendency towards increased expression. So far, only *PT5* has been shown to be up-regulated in response to As(V) (Huang et al. 2012). Since the authors used a short-term treatment, it is not surprising that the expression of the other *PT* genes did not change in their experiment. *PT5* was the only differentially expressed *PT* gene in roots of short-term treated Gladia plants. However, it is not fully understood why the induction of particular *PT* genes by As(V) was not reported in other studies (Norton et al. 2008; Chakrabarty et al. 2009). Here, perhaps the genotype of the analyzed plants, or the As(V) and Pi concentrations used, played a major role in the regulation of *PT* genes, which caused, for example, a strong up-regulation of PT expression by Pi starvation (Norton et al. 2008) or a repression by high external Pi (Chakrabarty et al. 2009).

Despite the substantial progress that has been achieved in understanding Pi starvation-dependent gene regulation, little is yet known about the molecular mechanisms controlling *PT* gene responses to Pi availability. It is accepted that plants adopt two strategies for monitoring Pi availability: local signaling, which depends on the external Pi concentrations, and systemic signaling, which is determined by the P status of the whole plant (Chiou and Lin 2011). While local signaling controls root architecture, Pi uptake, and hence *PT* gene expression, is regulated systemically (Chiou and Lin 2011) and references therein). However, the nature of the systemic signal(s) is not yet resolved. Pi itself is thought to directly act as a systemic signal, since the repression of the responses to Pi starvation, including gene expression, was demonstrated using a non-metabolizable, structural Pi analog known as phosphite (Yuan and Liu 2008; Chiou and Lin 2011). One may further speculate that Pi can be recognized by specific regulatory proteins which directly govern the activity of corresponding Pi responsive promoters. In this case, the substitution of As(V) for Pi in the signaling pathway would lead to similar transcriptional responses. However, the possibility cannot be excluded that the transcription of Pi-sensitive genes, including PTs,

might also be regulated via the sensing of some Pi-utilizing molecules, for example ATP/ADP. In such a case, due to chemical differences in some of the P- and As-containing molecules, As(V) would not cause a response similar to Pi, but rather an opposite one like that observed for Pi starvation. It was shown that As(V) ester bonds are characterized by very fast hydrolysis in aqueous solution, which would lead to the rapid decomposition of As(V) esters, resulting in futile cycles (Moore et al. 1983; Baer et al. 1981). Additionally, As(V) in AMP-As(V) or ADP-As(V) molecules are more readily reducible to As(III) than inorganic As(V) (Nemeti et al. 2010), thus even further depleting the ATP/ADP pools. Our results demonstrated rather divergent PT responses to As(V) exposure, which do not completely fit either hypothesis. Whereas *PT3* was repressed by treatment with either ion, *PT9* and *PT10* were regulated oppositely. Moreover, the transcript levels of *PT2*, *PT5* and *PT13* were only affected in As(V)-treated roots, suggesting a higher complexity of PT responsiveness to As(V). Most probably, the observed pattern of *PT* gene expression in roots is caused by several factors, including the accumulation of As(III). The reduced As(III) species did not only prevail in roots after 1 week As(V) treatment, but comprised half of the total As in roots exposed to As(V) for 16 h. Interestingly, the induction of *PT5* observed in Gladia roots upon short-term As(V) treatment was also reported for a Nipponbare variety upon 6 h treatment with As(III) (Yu et al. 2012). This further suggests that the observed changes in PT expression in roots are, at least partially, caused by As(III).

Intriguingly, most of the As-responsive PT and regulatory genes were regulated differently in rice roots and in shoots. Only *PT5* was induced upon 1 week As(V) exposure in both tissues. Moreover, *PT1*, *PT5* and *SPX-MFS2* in shoots were regulated in a similar way by As(V) and Pi, and only *PT12* responded to both treatments antagonistically. Taking into account that the proportion of As(V) was comparatively higher in shoots, one cannot exclude the possibility that the observed similar responses of these genes to Pi and As(V) treatment might be a consequence of As(V) mimicking the effect of Pi on gene regulation. Still, the majority of tested genes was regulated distinctly by both ions suggesting that, although As(V) can be recognized by specific Pi-utilizing proteins in vitro, the fate of both ions differs in the plant cells.

Compared to roots, Gladia shoots accumulated much less As, even when they were grown on 100 μM As(V) for 1 week. Apart from a sequestration of As in vacuoles of root cells, the low As content in shoots could partially result from a strong repression of *PT2* and *PT3*, a significant down-regulation of *PHF1*, and an induction of *SPX-MFS1,* which was recently shown to play a key role in controlling Pi homeostasis in leaves (Wang et al. 2012). According to Zhu et al. (2006), some PTs have a higher affinity for Pi than for As(V), which might ensure higher rates of Pi uptake and translocation at comparable external concentrations of both ions. Thus, the differential responses of particular *PT* genes to As(V) might contribute to the maintenance of steady P levels but reduced As accumulation in shoots being developed as an evolutionary adaptation against As(V) stress. The comparative analysis of the K_{max} and V_{max} of Pi and As(V) transport mediated by these PTs will further enhance our understanding of As responses in plants.

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