the plant journal

SEB SOCIETY FOR EXPERIMENTAL BIOLOGY

The Plant Journal (2010) doi: 10.1111/j.1365-313X.2010.04170.x

OsSPX1 suppresses the function of OsPHR2 in the regulation of expression of *OsPT2* and phosphate homeostasis in shoots of rice

Fang Liu[2,†], Zhiye Wang[1,†], Hongyan Ren[1], Chenjia Shen[1], Ye Li[3], Hong-Qing Ling[3], Changyin Wu[2], Xingming Lian[2,*] and Ping Wu[1,*]

[1]*State Key Laboratory of Plant Physiology and Biochemistry, College of Life Science, Zhejiang University, Hangzhou 310058, China,*

[2]*National Key Laboratory of Crop Genetic Improvement, National Center of Plant Gene Research (Wuhan), Huazhong Agricultural University, Wuhan 430070, China, and*

[3]*State Key Laboratory of Plant Cell and Chromosome Engineering, Institute of Genetics and Developmental Biology, Chinese Academy of Sciences, Beichen West Road 1, Chaoyang District, Beijing 100101, China*

Received 11 December 2009; revised 17 January 2010; accepted 27 January 2010.

[*]For correspondence (fax +86 571 88206617; e-mail clspwu@zju.edu.cn; or xmlian@mail.hzau.edu.cn).

[†]These authors contributed equally to this work.

SUMMARY

Phosphate (Pi) homeostasis in plants is required for plant growth and development, and is achieved by the coordination of Pi acquisition, translocation from roots to shoots, and remobilization within plants. Previous reports have demonstrated that over-expression of *OsPHR2* (the homolog of *AtPHR1*) and knockdown of *OsSPX1* result in accumulation of excessive shoot Pi in rice. Here we report that OsPHR2 positively regulates the low-affinity Pi transporter gene *OsPT2* by physical interaction and upstream regulation of *OsPHO2* in roots. OsPT2 is responsible for most of the OsPHR2-mediated accumulation of excess shoot Pi. OsSPX1 suppresses the regulation on expression of *OsPT2* by OsPHR2 and the accumulation of excess shoot Pi, but it does not suppress induction of *OsPT2* or the accumulation of excessive shoot Pi in the *Ospho2* mutant. Our data also show that OsSPX1 is a negative regulator of OsPHR2 and is involved in the feedback of Pi-signaling network in roots that is defined by OsPHR2 and OsPHO2. This finding provides new insight into the regulatory mechanism of Pi uptake, translocation, allocation and homeostasis in plants.

Keywords: *Oryza sativa* L., OsPT2, OsSPX1, Pi homeostasis, Pi signaling.

INTRODUCTION

Maintenance of phosphate (Pi) homeostasis in plants is essential for plant growth and development, and is achieved by coordination of acquisition of Pi from soils, translocation of Pi from roots to shoots, and remobilization of internal Pi (Poirier and Bucher, 2002). In yeast (*Saccharomyces cerevisiae*), the regulatory mechanism of Pi homeostasis driven by the phosphate signal transduction (PHO) pathway is thoroughly understood (Pinson *et al.*, 2004). However, full knowledge regarding the regulatory mechanism of Pi homeostasis in plants is still lacking.

AtPHR1, a transcription factor with a MYB domain, is a key regulator in the Pi-signaling pathway in Arabidopsis (Rubio *et al.*, 2001). Over-expression of *AtPHR1* leads to an increased concentration of Pi in the shoot tissues, together with induction of a range of Pi-starvation induced genes that encode Pi transporters, phosphatases and RNase (Nilsson *et al.*, 2007). Schachtman and Shin (2007) proposed a possible regulatory system downstream of AtPHR1, whereby *miR399* (a PHR1 target) reciprocally regulates the gene *PHO2* at the post-transcriptional level (Fujii *et al.*, 2005; Bari *et al.*, 2006; Chiou *et al.*, 2006). *miR399*-mediated *PHO2* cleavage is controlled by *IPS1*, a non-coding RNA, through target mimicry (Franco-Zorrilla *et al.*, 2007). PHO2 functions as a ubiquitin-conjugating E2 enzyme (UBC24), and loss of function of PHO2 leads to accumulation of excess shoot Pi (Aung *et al.*, 2006; Bari *et al.*, 2006). This regulatory system is

conserved in plants (Bari *et al.*, 2006). Two homologues of AtPHR1 in rice, OsPHR1 and OsPHR2, have been identified as being involved in the Pi-signaling pathway, and over-expression of *OsPHR2* results in accumulation of excess shoot Pi and a phenotype similar to that of *pho2* mutants under abundant Pi conditions (Zhou *et al.*, 2008; Wang *et al.*, 2009a).

Phosphate (Pi) is taken up by plant roots through Pi transporters, and Pi transporter activity must be adjusted to changing intracellular and extracellular Pi levels. A total of 13 putative high-affinity Pi transporters (OsPT1–13) have been identified in the rice genome (Goff *et al.*, 2002). Of them, at least seven (*OsPT1*, *OsPT2*, *OsPT3*, *OsPT6*, *OsPT9*, *OsPT8* and *OsPT10*) are primarily expressed in roots (Paszkowski *et al.*, 2002). The functions of OsPT2 and OsPT6 have been described recently (Ai *et al.*, 2009). Both are responsive to Pi-starvation signaling. OsPT6 is a high-affinity Pi transporter that is expressed in the epidermis, cortex, phloem and xylem in roots, and the mesophyll in leaf. OsPT2 is a low-affinity Pi transporter that is expressed in the root stele and leaf phloem and xylem. Based on its tissue-specific expression pattern, OsPT2 is assumed to function in translocation of stored Pi in rice. Two high-affinity transporter genes in Arabidopsis, *Pht1;8* and *Pht1;9*, are most likely responsible for most of the *pho2* phenotype, as RNAi-mediated inhibition of their expression in the *pho2* background suppresses the accumulation of excess shoot Pi (Bari *et al.*, 2006). However, little is known regarding the Pi transporters and regulators responsible for Pi homeostasis downstream of AtPHR1 and its rice homolog OsPHR2.

Recently, Hurlimann *et al.* (2009) reported that the SPX domain of two low-affinity Pi transporters in yeast, Pho87 and Pho90, limits the phosphate uptake velocity (V_{max}), suppresses phosphate efflux, and affects regulation of the phosphate signal transduction pathway through physical interaction with the regulatory protein Spl2. Based on these findings, they suggest that the SPX domain inhibits a low-affinity phosphate transporter. A set of SPX domain genes in Arabidopsis and rice has been shown to be involved in the Pi-signaling pathway downstream of PHR1 (Duan *et al.*, 2008; Wang *et al.*, 2009a,b). OsSPX1 is positively regulated by OsPHR2 at the transcriptional level, and suppresses the accumulation of excess shoot Pi in rice when Pi is abundant (Wang *et al.*, 2009a,b). These reports suggest that the regulatory effect of the SPX domain on Pi signaling and Pi homeostasis may be conserved in yeast and plants.

In this paper, we provide genetic and molecular evidence that *OsPT2* is up-regulated by OsPHR2 through physical interaction, and by regulation of OsPHR2 through OsPHO2. OsPT2 is responsible for most of the OsPHR2-mediated accumulation of excess shoot Pi. OsSPX1 suppresses the induction of *OsPT2* by OsPHR2, and consequently the accumulation of excess shoot Pi. OsSPX1 is also a negative regulator in a feedback network of Pi signaling defined by OsPHR2 and OsPHO2. Our findings provide new insight into the regulatory mechanisms of Pi uptake, translocation and homeostasis in plants.

RESULTS

Over-expression of *OsPT2* results in accumulation of excess shoot Pi

It has been reported that OsPT2 is a low-affinity Pi transporter, and knockdown of *OsPT*2 expression by RNA interference significantly decreased both the uptake and long-distance transport of Pi from roots to shoots (Ai *et al.*, 2009). To determine the function of OsPT2 in Pi uptake and translocation, two independent transgenic lines over-expressing *OsPT2* (Figure 1a,b) were used to determine Pi concentrations in shoots and roots in solution cultures. Under Pi-sufficient conditions (10 mg Pi L^{-1}), the transgenic plants over-expressing *OsPT2* (*PT2(O)*) showed leaf toxic symptoms and growth retardation similar to that of *OsPHR2*-over-expressing plants (*PHR2(O)*) and rice *pho2* mutants (*pho2*) (Figure 1c) (Zhou *et al.*, 2008; Wang *et al.*, 2009a,b). The Pi concentration in the shoots of the transgenic plants was increased approximately fourfold, but was only increased approximately two to five fold in roots compared with wild-type plants (Figure 1e). Under Pi-deficient conditions (0.5 mg Pi L^{-1}), no significant difference in the concentration of Pi was found in either shoots or roots between wild-type and *PT2(O)* (Figure 1e). The biomass of *PT2(O)* plants was greatly decreased under Pi-sufficient conditions, but this was only alleviated under Pi-deficient conditions (Figure 1f). These results indicate that over-expression of *OsPT2* increases Pi uptake and translocation of Pi from roots to shoots, resulting in the accumulation of excess shoot Pi under abundant Pi conditions. Under low Pi conditions, no significant difference in the concentration of Pi was found in either shoot or root between wild-type and *PT2(O)* (Figure 1e), but the *PT2(O)* plants were smaller than wild-type (Figure 1d). This may be due to the low affinity of OsPT2 (Ai *et al.*, 2009) and the constitutive over-expression of *OsPT2* driven by the 35S promoter, which may have negative effects on plant growth.

OsPT2 is positively regulated by OsPHR2 and its product is responsible for most of the OsPHR2-mediated accumulation of excess shoot Pi

Semi-quantitative real-time PCR and quantitative real-time analysis showed that *OsPT2* is positively regulated in the roots of plants over-expressing *OsPHR2* (designated *PHR2(O)*) (Figure 2a,c). Because over-expression of *OsPT2* results in accumulation of excess shoot Pi, we reasoned that OsPT2 may be responsible for the OsPHR2-mediated accumulation of excess shoot Pi. To confirm this, plants over-expressing *OsPHR2* in the repressed *OsPT2* background were developed by crossing a *PHR2(O)* plant (Zhou *et al.*, 2008) and a T-DNA insertional *pt2* mutant (http://signal.

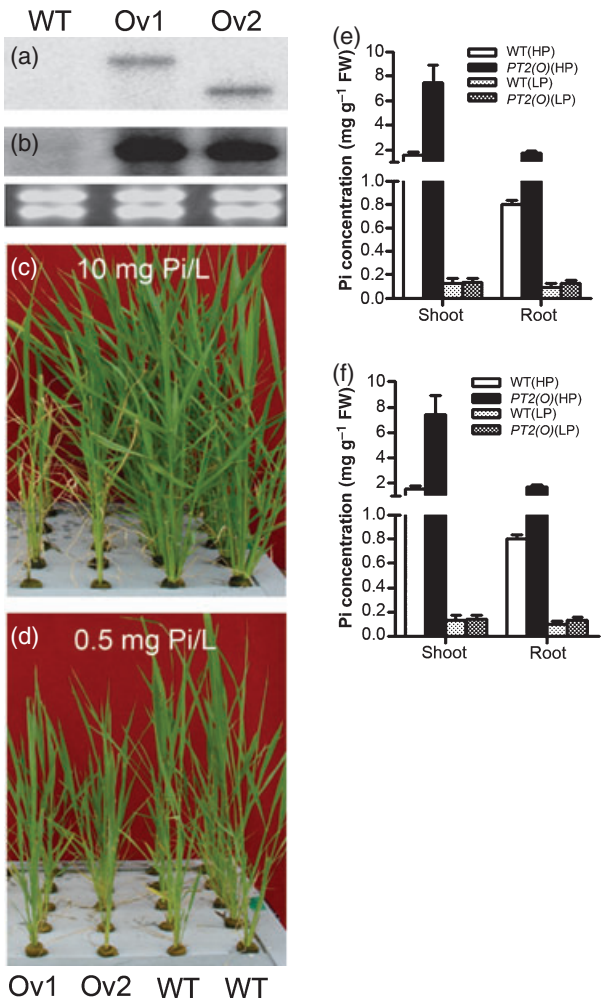

Figure 1. Phenotype, Pi concentration and Pi uptake rate of transgenic plants over-expressing *OsPT2* (Ov1 and Ov2) and wild-type (WT) plants.
(a) Southern blot analysis of WT, Ov1 and Ov2 plants using the GUS gene as a probe. Genomic DNA (5 μg) was digested using *Hin*dIII and separated on an agarose gel.
(b) Northern blot analysis of WT, Ov1 and Ov2 plants using the *OsPT2* coding sequence as a probe. The ethidium bromide-stained gel is shown at the bottom.
(c, d) Growth of WT, Ov1 and Ov2 under Pi-sufficient (10 mg Pi/l) and Pi-deficient (0.5 mg Pi L^{-1}) conditions for 40 days.
(e, f) Pi concentrations and biomass of shoots and roots of WT and Ov1 plants. HP, high Pi, LP, low Pi.

salk.edu/cgi-bin/RiceGE), and designated *PHR2(O)/pt2*. The T-DNA insertion at −569 bp of the *OsPT2* promoter greatly reduces the transcript level of *OsPT2*, and represses its response to Pi starvation in roots (Figure S1). The Pi concentration in the shoots of *PHR2(O)/pt2* plants under Pi-sufficient conditions was decreased by about 70% compared with that in *OsPHR2(O)* plants (Figure 2d), indicating that OsPT2 is responsible for most of the OsPHR2-mediated accumulation of excess shoot Pi. At upstream of T-DNA insertion site, there is only one PHR1-specific binding sequence (P1BS) at between −346 and −338 bp upstream of the ATG of the *OsPT2* (Figure S1). It was concluded that the T-DNA insertion interferes with binding of OsPHR2 to the *cis*-element or that the *cis*-element alone is not sufficient for OsPT2 to respond to Pi-starvation signaling.

OsPT2 is not responsible for most of the OsPHO2-mediated accumulation of excessive shoot Pi

PHO2/UBC24 acting downstream of PHR1 negatively regulates the accumulation of shoot Pi in Arabidopsis and rice (Aung *et al.*, 2006; Bari *et al.*, 2006; Wang *et al.*, 2009a,b). Our results showed that the transcript level of *OsPT2* is increased in the roots of the *pho2* mutant (Figure 2b,c). To determine the role of OsPT2 in the accumulation of excessive shoot Pi in the *pho2* mutant, the double mutant *pho2/pt2* was developed for Pi concentration analysis by crossing the *pho2* mutant (Wang *et al.*, 2009a,b) and the *pt2* mutant. In contrast to *PHR2(O)/pt2*, the Pi concentration in the shoots of the double mutant *pho2/pt2* was only decreased by about 30% compared with that in the *pho2* mutant (Figure 2d). Severe growth retardation of the double mutant was observed, as in the *pho2* mutant, under Pi-sufficient conditions (Figure 2f). These results indicate that, although *OsPT2* is reciprocally regulated by OsPHR2 and OsPHO2, their mode of action on Pi transporters leading to accumulation of excess shoot Pi is different.

OsPHR2 physically regulates the expression of *OsPT2*

The observation that repression of *OsPT2* results in a greater decrease in the shoot Pi concentration of *PHR2(O)* plants and only a partial decrease in the shoot Pi concentration in the *pho2* mutant suggests that OsPHR2 may have a direct impact on the expression of *OsPT2* in addition to its action through *OsPHO2*. OsPHR2 is a homolog of AtPHR1, encoding a member of the MYB family (Zhou *et al.*, 2008). AtPHR1 regulates a number of Pi starvation-induced genes by binding a P1BS *cis*-element (GNATATNC) (Rubio *et al.*, 2001). The promoter of *OsPT2* contains a P1BS *cis*-element between -346 and -338 bp upstream of the ATG of the *OsPT2* gene. Co-expression of *OsPT2* and *OsPHR2* was observed under both Pi-sufficient and -deficient conditions (Figure 3a). Physical interaction of OsPHR2 with the promoter of *OsPT2* was indicated by electrophoretic mobility shift assay (EMSA) using the 174 bp sequence between −267 and −441 bp, including the *cis*-element (Figure 3b), and a modified Hybri-Zap two-hybrid system (Yuan *et al.*, 2008) (Figure 3c). Taken together, the results indicate that OsPHR2 can physically interact with *OsPT2* through the *cis*-element, and this may be the major pathway for OsPHR2-mediated accumulation of excessive shoot Pi dependent on OsPT2.

OsSPX1 suppresses the function of OsPHR2 in regulation of *OsPT2* expression in roots

It has been reported that OsSPX1 negatively regulates accumulation of shoot Pi under Pi-sufficient conditions

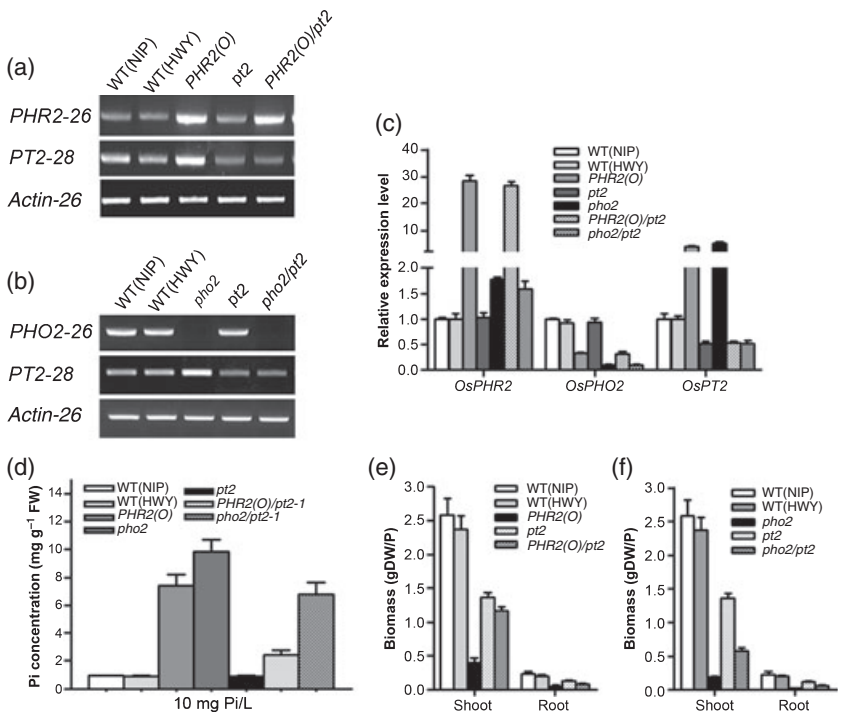

Figure 2. Expression of *OsPHR2*, *OsPHO2* and *OsPT2*, shoot Pi concentration and dried biomass measurements for transgenic plants over-expressing *OsPHR2* (*PHR2(O)*), the *pho2* mutant, *PHR2(O)*/*pt2* plants and the double mutant *pho2*/*pt2*.

(a) Semi-quantitative real-time PCR analysis for expression of *OsPHR2* and *OsPT2* in roots of plants under Pi-supplied conditions (10 mg Pi L^{-1}). WT(NIP), wild-type Nipponbare; WT(HWY), wild-type Hwayoung; *PHR2(O)*, plants over-expressing *PHR2*; *pt2*, *pt2* mutant; *PHR2(O)*/*pt2*, plants over-expressing *PHR2* in the *pt2* mutant background.

(b) Semi-quantitative real-time PCR analysis for expression of *OsPHO2* and *OsPT2* in roots of plants under Pi-supplied conditions (10 mg Pi L^{-1}). NIP, wild-type Nipponbare; HWY, wild-type Hwayoung; *pho2*, *pho2* mutant; *pt2*, *pt2* mutant; *pho2*/*pt2*, double mutant of *pho2* and *pt2*.

(c) Quantitative real-time PCR analysis for expression of *OsPHR2*, *OsPHO2* and *OsPT2* in roots of plants.

(d) Shoot Pi concentrations of various plants under Pi-sufficient conditions (10 mg Pi L^{-1}).

(e, f) Dried biomass of the shoots and roots of 50-day-old plants under Pi-sufficient conditions (10 mg Pi L^{-1}). The primers for the genes tested are listed in Tables S1 and S2. The data are the means of three replications. The bars represent SD.

(Wang *et al.*, 2009a,b). To determine whether OsSPX1 is a negative regulator involved in the PHR2-mediated accumulation of shoot Pi, transgenic plants that simultaneously over-express *OsPHR2* and *OsSPX1*, designated *PHR2(O)*/*SPX1(O)*, were developed (Figure 4a,c). Data from solution culture experiments indicated that the Pi concentration in the shoots of *PHR2(O)*/*SPX1(O)* plants was reduced to the level of wild-type plants (Figure 4d), and up-regulation of *OsPT2* by PHR2 was also repressed in the *PHR2(O)*/*OsSPX1(O)* plants (Figure 4a,c). The results indicate that OsSPX1 has a counteracting effect on the up-regulation of *OsPT2* in roots mediated by *OsPHR2* over-expression and the accumulation of excess shoot Pi under abundant Pi.

The quantitative real-time PCR results showed that *OsSPX1* expression is increased in the *pho2* mutant, indicating negative feedback regulation of OsPHO2 on *OsSPX1*. Reciprocally, *OsPHO2* expression is increased in *SPX1(O)* (Figure 4c), which indicates a counteracting effect of OsSPX1 on PHR2 because over-expression of *OsPHR2* represses the expression of *OsPHO2*. To confirm the counteracting effect of OsSPX1 on OsPHR2, a pot soil experiment with three replications was performed under Pi-sufficient conditions (60 mg Pi/kg soil). Severe growth retardation of *PHR2(O)* plants was observed, but this was rescued in *PHR2(O)*/*SPX1(O)* plants (Figure 5a). Excessive shoot Pi accumulation in *PHR2(O)* plants, but was reduced to the level of wild-type plants in *PHR2(O)*/*SPX1(O)* plants (Figure 5b). Because over-expression of *OsPHR2* can mimic Pi-starvation signaling under Pi-supplied conditions (Zhou *et al.*, 2008), the expression patterns of Pi starvation-induced genes, including *OsIPS1* (Hou *et al.*, 2005), *OsmiR399d* and *j* primary transcripts (Zhou *et al.*, 2008), *OsSQD2* and *OsPAP10* (Wang *et al.*, 2006), were investigated in the shoots and roots of the plants to determine the counteracting effect of OsSPX1 on Pi-starvation signaling driven by over-expression of *OsPHR2*. The results showed that up-regulation of Pi-starvation induced genes in the shoots and roots of *PHR2(O)* plants was reduced in the *SPX1(O)* background (Figure 5b,c). Taken together, these results indicate that OsSPX1 counteracts the function of OsPHR2 in shoot Pi accumulation and Pi signaling.

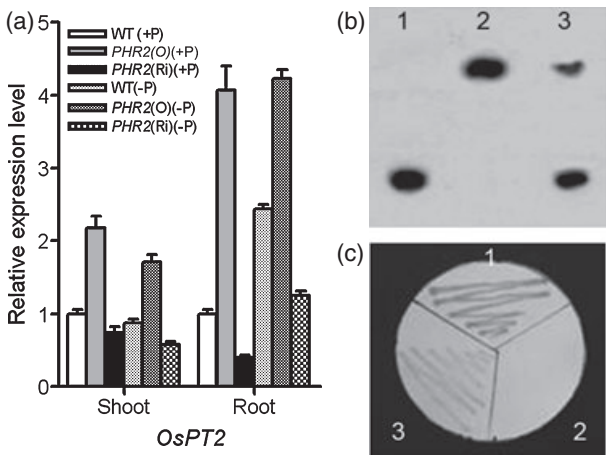

Figure 3. Physical regulation of *OsPT2* by OsPHR2.
(a) Quantitative real-time PCR analysis of the co-expression pattern of *OsPT2* with *OsPHR2* under Pi-sufficient (10 mg Pi L^{-1}) and Pi-starvation (0 mg Pi/l) conditions.
(b) Electrophoretic mobility shift assay (EMSA) to test binding of OsPHR2 to the promoter of *OsPT2* using 832 bp of the promoter of *OsPT2* including a P1BS *cis*-element between −346 and −338 bp. Lane 1, biotin-labeled promoter fragments of *OsPT2* (20 fmol) (−832 bp); lane 2, biotin-labeled promoter fragments of *OsPT2* (20 fmol) (−800 bp) plus OsPHR2 protein (500 ng); lane 3, OsPHR2 protein (500 ng) and a 200-fold molar excess of unlabeled *OsPT2* promoter fragment plus biotin-labeled promoter fragments (20 fmol).
(c) Regulation of *OsPT2* by OsPHR2 in yeast cells (Yuan *et al.*, 2008). (1) Positive control: pAD-*AtbHLH38*/pBD-*FIT*-P_{IRT1}::*GUS*; (2) negative control: pAD-protein/pBD-promoter::*GUS*; (3) pAD-OsPHR2/pBD-P_{PT2}::*GUS*.

OsSPX1 does not counteract the effect of OsPHO2 on regulation of expression of *OsPT2*, but is involved in a feedback Pi-signaling network in roots defined by OsPHR2 and OsPHO2

To determine whether OsSPX1 is involved in the regulation of expression of *OsPT2* downstream of OsPHO2 and accumulation of excess shoot Pi, plants with over-expression of *OsSPX1* in the *pho2* mutant background, designated *pho2*/*SPX1(O)*, were developed by crossing *pho2* mutant plants and *SPX1(O)* plants. Quantitative and semi-quantitative real-time PCR analyses showed that up-regulation of *OsPT2* in the *pho2* mutant was not repressed in *pho2*/*SPX1(O)* plants, and consequently accumulation of excess shoot Pi and retardation of plant growth was not alleviated compared with the *pho2* mutant (Figure 4b–d,f). The results indicate that OsSPX1 does not counteract the function of OsPHO2 in regulation of expression of *OsPT2* and the accumulation of excess shoot Pi.

OsSPX1 and *OsIPS1* were up-regulated in the roots, but not the shoots, of the *pho2* mutant (Figure 6), suggesting that OsSPX1 may be a negative regulator involved in a feedback Pi-signaling pathway defined by OsPHR2 and OsPHO2. Because *miR399* controls inorganic phosphate (Pi) homeostasis by regulating *PHO2* post-transcriptionally, we developed transgenic plants over-expressing *miR399k* to investigate the expression pattern of *OsSPX1*. Under Pi-sufficient conditions, the toxic symptom and excessive accumulation of shoot Pi were observed in the transgenic plants (Figure S2A–C). Quantitative real-time PCR analysis showed that *OsPHO2* was repressed in both shoots and roots of the transgenic plants, but *OsSPX1* was only up-regulated in the roots (Figure 6). These results indicate that OsSPX1, as a negative regulator, is involved in a feedback Pi-signaling network in roots defined by OsPHR2 and OsPHO2.

DISCUSSION

OsPT2 is responsible for most of the OsPHR2-mediated accumulation of excess shoot Pi

It has been reported that OsPT2 is a low-affinity Pi transporter (Ai *et al.*, 2009). Repression of *OsPT2* due to a T-DNA insertion in its promoter severely inhibits plant growth in solution and soil under Pi-sufficient conditions (Figure S1), confirming that OsPT2 is an important Pi transporter in rice. Three lines of evidence indicate that the low-affinity Pi transporter OsPT2 is responsible for most of the OsPHR2-mediated accumulation of excess shoot Pi under abundant Pi conditions: (i) over-expression of *OsPT2* results in accumulation of excess shoot Pi and the same phenotype as *PHR2(O)* plants and *pho2* mutants under abundant Pi (Figure 1) (Zhou *et al.*, 2008; Wang *et al.*, 2009a,b), (ii) *OsPT2* is co-expressed with *OsPHR2* and can physically interact with OsPHR2 (Figure 3), and (iii) repression of *OsPT2* suppresses the accumulation of excess shoot Pi in the *PHR2(O)* background (Figure 2).

OsPT2 is also up-regulated in the roots of the *pho2* mutant. Because OsPHR2 negatively regulates *OsPHO2* (Figure 2) (Bari *et al.*, 2006), it was assumed that repression of *OsPT2* would reduce the excessive shoot Pi in the *pho2* mutant as it does in *PHR2(O)* plants. However, our data showed that accumulation of excessive shoot Pi in the double mutant *pho2*/*pt2* was less decreased compared with that in *PHR2(O)*/*pt2* plants (Figure 2), suggesting that Pi transporters other than OsPT2 are responsible for most of the OsPHO2-mediated accumulation of excess shoot Pi. It has been reported that over-expression of the two high-affinity transporter genes *Pht1;8* (At1g20860) and *Pht1;9* (At1g76430) is probably responsible for most of the *pho2* phenotype in Arabidopsis (Bari *et al.*, 2006). Phylogenetic analysis indicates that the protein sequences encoded by *OsPT9* and *OsPT10* are closely related to those encoded by *Pht1;8* and *Pht1;9* (Paszkowski *et al.*, 2002). The transcript levels of *OsPT9* and *OsPT10* in the shoots and roots of *PHR2(O)* and *pho2* mutant plants were investigated under Pi-sufficient conditions. The data showed that *OsPT9* and *OsPT10* were up-regulated in the roots and shoots of *pho2* mutant plants, but were only up-regulated in the roots of *PHR2(O)* plants (Figure S3). The results suggest that OsPT9 and OsPT10 may play important roles in the OsPHO2-mediated accumulation of shoot Pi.

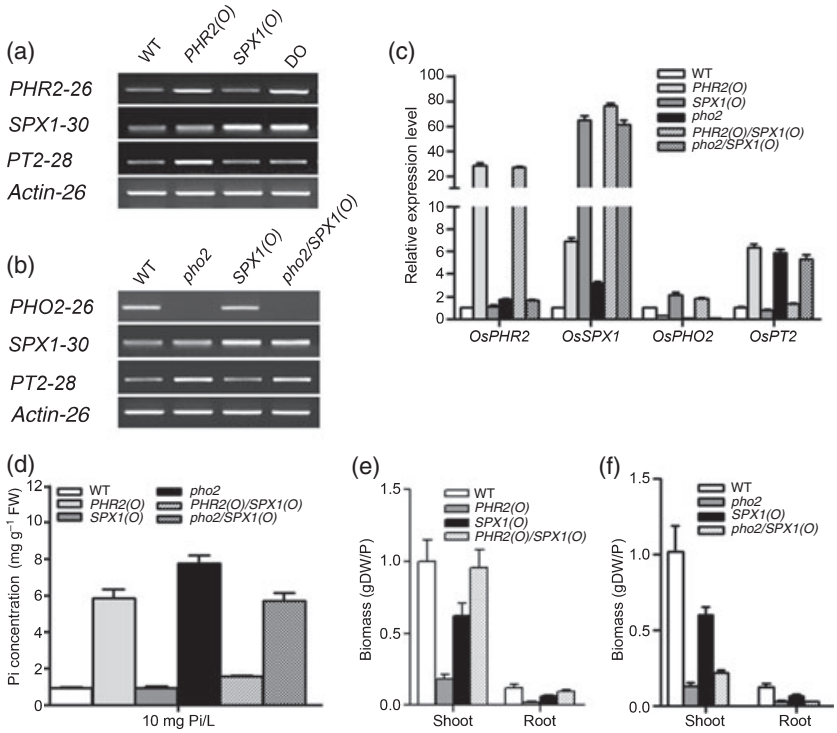

Figure 4. Expression of *OsPHR2*, *OsSPX1*, *OsPHO2* and *OsPT2*, shoot Pi concentration and dried biomass measurements for transgenic plants over-expressing *OsPHR2* (*PHR2(O)*) or over-expressing *OsSPX1* (*SPX1(O)*), the *pho2* mutant, plants simultaneously over-expressing *OsPHR2* and *OsSPX1* (*PHR2(O)*/*SPX1(O)*), and the *pho2* mutant in the *SPX1(O)* background (*pho2*/*SPX1(O)*).
(a) Semi-quantitative real-time PCR analysis for expression of *OsPHR2*, *OsSPX1* and *OsPT2* in roots of plants under Pi-supplied conditions (10 mg Pi L^{-1}). WT, wild-type Nipponbare; DO, over-expression of both *OsPHR2* and *OsSPX1*.
(b) Semi-quantitative real-time PCR analysis for expression of *OsPHO2*, *OsSPX1* and *OsPT2* in roots of plants under Pi-supplied conditions (10 mg Pi L^{-1}). WT, wild-type Nipponbare; *pho2*, *pho2* mutant; *pho2*/*O*, *pho2* mutant in the *SPX1(O)* background.
(c) Quantitative real-time PCR analysis for expression of *OsPHR2*, *OsSPX1*, *OsPHO2* and *OsPT2* in roots of plants under Pi-supplied conditions (10 mg Pi L^{-1}).
(d) Shoot Pi concentrations of various plants under Pi-sufficient conditions (10 mg Pi L^{-1}).
(e, f) Dried biomass of shoots and roots of 30-day-old plants under Pi-sufficient conditions (10 mg Pi L^{-1}). The data are the means of three replications. The bars represent SD.

OsSPX1 suppresses the regulation on expression of *OsPT2* by OsPHR2 and accumulation of shoot Pi

OsSPX1 negatively regulates the accumulation of shoot Pi and is positively regulated by OsPHR2 (Wang *et al.*, 2009a,b). In this paper, we provide genetic evidence that OsSPX1 is a negative regulator that suppresses the up-regulation of *OsPT2*, and consequently the accumulation of shoot Pi in the *PHR2(O)* background. The transcript level of *OsPT2* in roots and the shoot Pi concentration of *PHR2(O)*/*SPX1(O)* plants were similar to those of wild-type plants (Figures 4 and 5). It has been reported that the SPX domain in the yeast low-affinity Pi transporters Pho87 and Pho90 inhibits the transporters through physical interaction with the regulatory protein Spl2 (Hurlimann *et al.*, 2009). The repression effect of OsSPX1 on *OsPT2* in the *PHR2(O)* background also occurs through an unknown regulator(s). Alignment analysis of the SPX domains in OsSPX1 and the yeast low-affinity Pi transporters Pho87, Pho90 and Pho91 indicated that the amino acid identities of the N-terminal SPX domain in OsSXP1 with those in Pho87, Pho90 and Pho91 are only 11, 8 and 11%, respectively (Figure S4). Analysis of the secondary structure of SPX domains in ScPHO87, ScPHO90, ScPHO91 and OsSPX1 showed a relatively simple protein structure of the SPX domain in OsSPX1 compared with those in ScPHO87, ScPHO90 and ScPHO91(Figure S4B). These data suggest that the SPX domain is of evolutionary variation and may have distinct functions in yeast low-affinity Pi transporters and rice SPX domain proteins. No SPX domain is found in the low-affinity Pi transporter OsPT2. The function of low-affinity Pi transporters in higher plants, at least OsPT2, includes transport and translocation of Pi from roots to shoots. Therefore, the low-affinity Pi transporters may have been evolved from different gene families in yeast and higher plants.

OsSPX1 is a negative regulator that is involved in a feedback network of Pi signaling defined by OsPHR2 and OsPHO2 in roots

The Pi-signaling pathway defined by AtPHR1 regulates several Pi starvation-induced genes to allow plants to adjust to external and internal Pi levels, and has been thoroughly

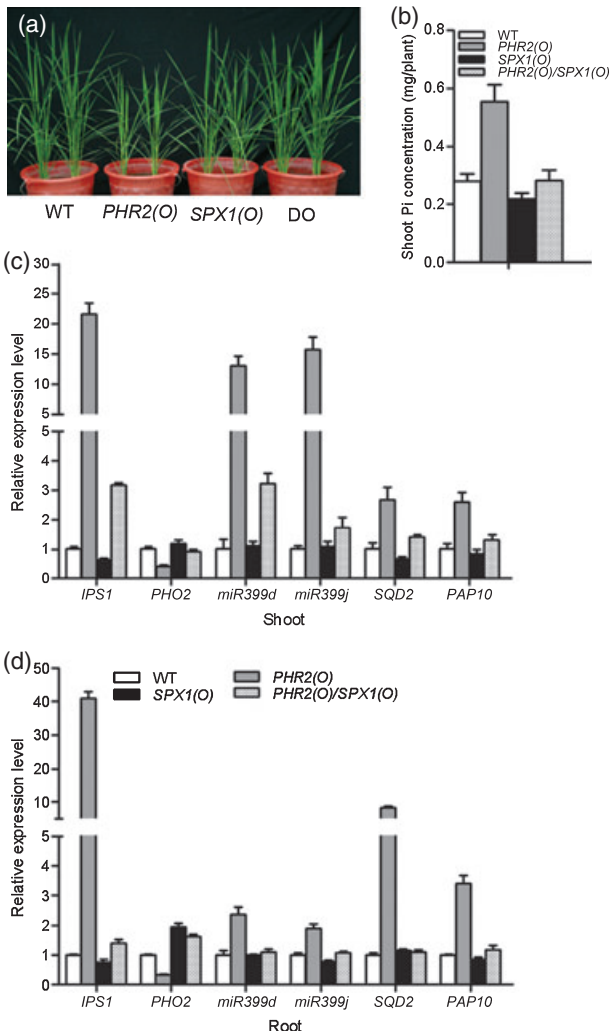

Figure 5. Growth performance and shoot Pi concentration of WT (Nipponbare), *PHR2(O)*, *SPX1(O)* and *PHR2(O)/SPX1(O)* plants in a pot experiment.
(a) Sixty-day-old plants of wild-type (WT), *PHR2(O)*, *SPX1(O)* and *PHR2(O)/SPX1(O)* (DO) under Pi level with 60 mg Brad-I Pi.
(b) Shoot Pi concentration of the various plants.
(c, d) Quantitative real-time PCR analysis for Pi-starvation induced genes in shoots (c) and roots (d) of WT, *PHR2(O)*, *SPX1(O)* and *PHR2(O)/SPX1(O)* plants. The data are the means of three replications. The bars represent SD. The primers for the genes tested are listed in Tables S1 and S2.

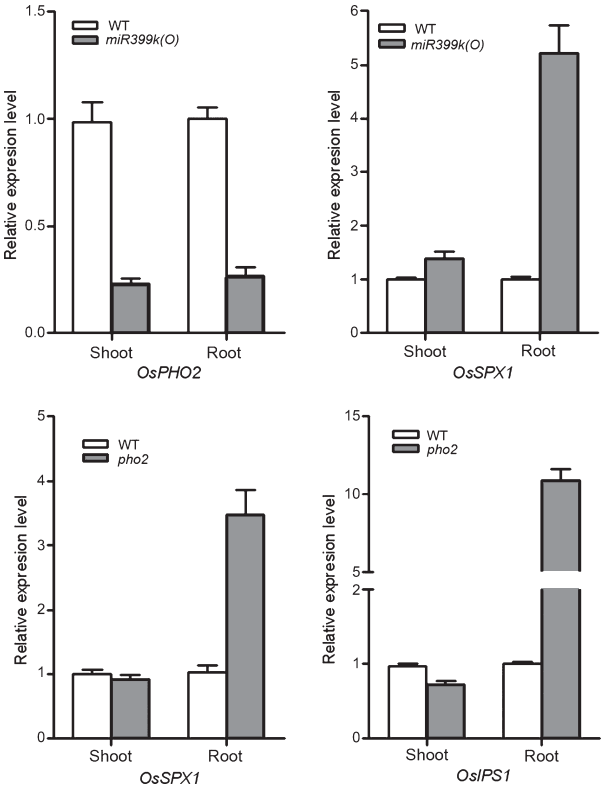

Figure 6. Quantitative real-time PCR analysis for *OsSPX1*, *OsIPS1* and *OsPHO2* in the shoots and roots of wild-type (WT) plants, the *pho2* mutant and transgenic plants over-expressing *miR399k*.
The data are the means of three replications. The bars represent SD. The primers for the genes tested are listed in Table S2.

reviewed (Schachtman and Shin, 2007). Several Pi-starvation induced genes are up-regulated by over-expression of *OsPHR2* under Pi-supplied conditions (Zhou *et al.*, 2008), and this up-regulation is repressed by over-expression of *OsSPX1* in both shoots and roots (Figure 5). These results suggest that OsSPX1 is involved in the OsPHR2-mediated Pi-signaling pathway as a negative regulator in both shoots and roots. However, the present results also showed that *OsSPX1* is only up-regulated in the roots of the *pho2* mutant and transgenic plants over-expressing *miR399k*, which represses the expression of *OsPHO2* in both shoots and roots (Figure 6). It has been demonstrated that, downstream of AtPHR1, *PHO2* is suppressed by *miR399* through long-distance movement of miR399s from shoots to roots, which is crucial to enhance Pi uptake and translocation under Pi deficiency (Aung *et al.*, 2006; Bari *et al.*, 2006; Lin *et al.*, 2008; Pant *et al.*, 2008). *miR399*-mediated *PHO2* cleavage is controlled by *IPS1*, a non-coding RNA, through target mimicry in roots (Franco-Zorrilla *et al.*, 2007). It was found that both *OsIPS1* and *OsSPX1* are up-regulated in the roots of the *pho2* mutant and the transgenic plants over-expressing *miR399k* (Figure 6). Therefore, in addition to the counteracting effect of OsSPX1 on OsPHR2, OsSPX1 is involved in a feedback Pi-signaling network defined by OsPHR2 and OsPHO2 in roots, regulating Pi uptake, translocation and Pi homeostasis in shoots, and mostly dependent on the low-affinity Pi transporter OsPT2, as outlined in Figure 7.

Although determination of the feedback regulation defined by OsPHR2, OsPHO2 and OsSPX1 in roots provides new insight into the sophisticated regulatory mechanism for Pi uptake, translocation and homeostasis in plants, further work on proteins is required to confirm the functions of the genes in the regulatory network. In addition, exploration of how SPX1 suppresses the function of PHR1 and the biological significance of this feedback regulation may

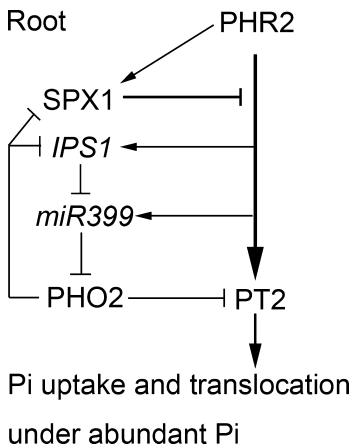

Figure 7. Proposed model for regulation of the low-affinity Pi transporter *OsPT2* and the feedback Pi-signaling network defined by OsPHR2, OsSPX1 and OsPHO2 in roots under abundant Pi.

provide an approach for molecular breeding of plants with more efficient Pi uptake and assimilation. It will be also interesting to explore the factors whose alteration results in changes in OsPHR2-mediated accumulation of excess shoot Pi, and their interaction with OsSPX1.

EXPERIMENTAL PROCEDURES

Hydroponic and pot experiments

Hydroponic experiments were performed using standard rice culture solution (10mg Pi/l) (Yoshida *et al.*, 1976). The experiments were performed in a growth room under a photosynthetic photon flux density of approximately 200 μmol photons m^{-2} sec^{-1} with a 16 h light (30°C)/8 h dark (22°C) photoperiod and 70–80% relative humidity. Experiments in soil were performed with three replications in a greenhouse using clay soil collected from a rice field on an experimental farm in Zhejiang Province, China (pH 5.8, water/soil 1:1), with two Pi levels: 60 mg and 30 mg Pi kg^{-1} soil, as determined using the Bray-I method (Bray and Kurtz, 1945). Each pot contained 15 kg of air-dried soil and two plants.

Construction of over-expression vectors and development of transgenic plants

The coding sequence of *OsPT2* was amplified using Nipponbare genomic DNA as the template and primers 5′-GGGGA GCTCGCTTATAACTTTGCAGCTTGAGG-3′ (forward) and 5′-GGG CTGCAGGGGAAAGTTCACAAAATCTCACA-3′ (reverse), which were designed based on a previously published sequence (GenBank accession number AF536962) (Paszkowski *et al.*, 2002). The PCR product was cleaved using *Sac*I and *Pst*I, and ligated into the pCAMBIA1301S vector (http://www.cambia.org/), driven by the CaMV 35S promoter, with a nopaline synthase terminator (Tnos). The constructs were transformed into the *japonica* rice cultivar Hejiang 19 (HJ19) by an *Agrobacterium tumefaciens*-mediated transformation method (Hiei *et al.*, 1994).

pt2 mutant identification

A T-DNA insertional mutant line was obtained from a library (http://signal.salk.edu/cgi-bin/RiceGE). Two primers flanking the T-DNA borders and one primer specifically for T-DNA were used to confirm the insertional site. To determine the expression of *OsPT2* in the mutant, real-time PCR analysis was performed using primers designed based on the gene sequence.

Development of genetic materials

Plants with over-expression of *OsPHR2* (*PHR2(O)*) under the repression of *OsPT2* background or in the *OsSPX1* over-expression background were developed by crossing *PHR2(O)* plants (Zhou *et al.*, 2008) and the *pt2* mutant or *PHR2(O)* plants and *SPX1(O)* plants (Wang *et al.*, 2009a,b), and designated *PHR2(O)*/*pt2* and *PHR2(O)*/*SPX1(O)*. The double mutant *pho2*/*pt2* was developed by crossing *pho2* and *pt2* mutant plants. Plants with *SPX1(O)* in the *pho2* mutant background were developed by crossing *pho2* mutant and *SPX1(O)* plants. F_3 lines identified from the related F_2 lines were used for the experiments.

Semi-quantitative and quantitative real-time PCR

Total RNA was extracted using Trizol reagent (Invitrogen, http://www.invitrogen.com/) according to the manufacturer's instructions. First-strand cDNAs were synthesized from total RNA using SuperScript III reverse transcriptase (Invitrogen). Semi-quantitative real-time PCR was performed using a pair of gene-specific primers. Quantitative real-time PCR was performed using a SYBR *PremixEx Taq* kit (TaKaRa Biomedicals, http://www.takara-bio.com/) or the universal probe library (UPL) and a LightCycler480 probes master kit on a LightCycler480 machine (Roche Diagnostics, http://www.roche.com), according to the manufacturer's instructions. Triplicate quantitative assays were performed on each cDNA sample. The relative expression was calculated using the formula $2^{-\Delta(\Delta Cp)}$. All primers used for the semi-quantitative and quantitative real-time PCR are listed in Tables S1 and S2.

Southern and Nothern blotting analysis

Rice DNA isolation, digestion, electrophoresis, blotting and hybridization for Southern blotting were perfomed as described previously (Zhou *et al.*, 2008), using the coding sequence of the GUS gene as the hybridization probe. For Northern blotting analysis, total RNA was isolated from leaves using Trizol reagent (Invitrogen) according to the manufacturer's instructions. Gene-specific probes were labeled with ^{32}P-dCTP using a random primer kit (Invitrogen) and hybridized to the RNA blots. The probes were 491 bp long, and were amplified by PCR using the following primers: 5′-CTTCTTCTTCGCCAACTTCG-3′ (left) and 5′-AGAAACCCCACAAATCCACA-3′ (right).

Electrophoretic mobility shift assay (EMSA)

The coding region of *OsPHR2* was amplified using primers 5′-TTTGGTACCATGGAGAGAATAAGCACCAAT-3′ (added *Kpn*I site underlined) and 5′-TTTGTCGACTCTGTCACCTGATTCTGTTTG-3′ (added *Sal*I site underlined), and subcloned into the His ×6 expression vector pET29b (Promega, http://www.promega.com/) using *Kpn*I and *Sal*I. Recombinant protein produced in *Escherichia coli* was purified using an Ni-NTA column, according to the manufacturer's instructions (Qiagen, http://www.qiagen.com/). Purified protein concentrations were determined using a dye binding assay with bovine serum albumin as the standard (Bio-Rad, http://www.bio-rad.com/). The 832 bp promoter of *OsPT2* was obtained by PCR amplification using the primers 5′-TTTGCATGCATA-GCTTTGTCACTGCCCAGC-3′ and 5′-CTTGCATGCCTGCAGGTC-GACGATTCGATCC-3′ (added *Sph*I site underlined). The promoter was then digested using *Sph*I. Deletion derivatives of the promoter fragment were labeled with biotin as described by the manufacturer (Pierce, Rockford, IL). Three reactions were performed as described in Figure 3. Samples were incubated in a reaction buffer containing

1 μg of poly(dI-dC), 50 mM KCl, 10 mM Tris/Cl pH 7.5 and 1 mM dithiothreitol at 37°C for 20 min, electrophoresed on a 4% polyacrylamide gel for 3 h at 15 V cm^{-1} at 4°C, and detected as described by the manufacturer (Pierce).

Analysis of regulation of *OsPT2* by OsPHR2 in yeast cells

The effect of OsPHR2 on expression of *OsPT2* in yeast cells was investigated as described by Yuan *et al.* (2008). A 2000 bp segment of the *OsPT2* promoter with 5′ *Bam*HI and 3′ *Sal*I sites were integrated into pCambia1391 (http://www.cambia.org/). The GUS expression cassette with the *OsPT2* promoter and CaMV terminator (OsPT2::*GUS*::T_{CaMV}) was cut from the pCambia1391 derivative using *Bam*HI/*Not*I, blunt-ended, and integrated into pBD-GAL4 to generate yeast expression plasmid pBD-GAL4-P_{PT2}::*GUS*. The three plasmids pAD-*AtbHLH38*/pBD-*FIT*-P_{IRT1}::*GUS* (positive control) (Yuan *et al.*, 2008), pAD-protein/pBD-promoter::*GUS* (negative control) and pAD-*OsPHR2*/pBD-P $_{PT2}$::*GUS* were introduced into yeast strain YRG-2. A β-glucuronidase (GUS) assay in yeast cells was performed as described by Yuan *et al.* (2008). In brief, yeast clones grown on plates with histidine were transferred to nitrocellulose paper, and lysed by three repeated freeze/thaws in liquid nitrogen for 10 s. The thawed paper was soaked with GUS staining buffer for 3 h as described by Jefferson *et al.* (1987).

Measurement of Pi concentration and Pi uptake rate

For Pi concentration measurements, fresh samples (approximately 0.5 g) were used for determination of Pi concentration as previously described (Zhou *et al.*, 2008). Soil Pi concentrations were determined by the Bray-I method (Bray and Kurtz, 1945). The Pi uptake rate was measured based on the rate of depletion of the nutrient from solution over 24 h. Thirty-day-old plants were used. Before measurement, the plants were moved into a solution culture without Pi for 3 days, then transferred to a pot with four plants per liter of fresh solution (6 mg Pi L^{-1}). A 1 ml aliquot of solution was removed from each pot at 4, 8 and 24 h time points for phosphorus concentration analysis by phosphomolybdenum blue reaction. The roots of the plants in each pot were harvested and oven dried, and the Pi uptake rate was calculated as depletion of the Pi in the solution per gram of dried root biomass.

Statistical analysis

Data were analyzed by ANOVA using the SAS 9.0 program (SAS Institute Inc., http://www.sas.com). Duncan's method was used for multiple mean comparisons of Pi concentration and plant dried biomass.

ACKNOWLEDGEMENTS

We are grateful to Dr K.G. Raghothama (Department of Horticulture and Landscape Architecture, Purdue University, West Lafayette, IN) for constructive suggestions and comments on this work. This research is supported by the National Basic Research and Development Program of China (grant number 2005CB120900), the Special Program of Rice Functional Genomics of China (grant number 2006AA10A102), and the Science and Technology Department of Zhejiang Province, China.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article:

Figure S1. Identification of a T-DNA insertional *OsPT2* mutant.

Figure S2. Quantitative real-time PCR analysis for *OsPHO2*, *OsSPX1* and *OsPT2* in transgenic plants over-expressing *OsmiR399k*.

Figure S3. Quantitative real-time PCR analysis for *OsPT9* and *OsPT10* in plants over-expressing *OsPHR2* (*PHR2(O)*) and *pho2* mutant seedlings.

Figure S4. Multiple alignment of the SPX domains of ScPho87, ScPho90, ScPho91 and OsSPX1.

Table S1. Primers used for semi-quantitative RT-PCR.

Table S2. Primers used for quantitative RT-PCR.

Please note: As a service to our authors and readers, this journal provides supporting information supplied by the authors. Such materials are peer-reviewed and may be re-organized for online delivery, but are not copy-edited or typeset. Technical support issues arising from supporting information (other than missing files) should be addressed to the authors.

REFERENCES

Ai, P., Sun, S., Zhao, J. *et al.* (2009) Two rice phosphate transporters, OsPht1;2 and OsPht1;6, have different functions and kinetic properties in uptake and translocation. *Plant J.* **57**, 798–809.

Aung, K., Lin, S.I., Wu, C.C., Huang, Y.T., Su, C.L. and Chiou, T.J. (2006) *pho2*, a phosphate overaccumulator, is caused by a nonsense mutation in a microRNA399 target gene. *Plant Physiol.* **141**, 1000–1011.

Bari, R., Datt Pant, B., Stitt, M. and Scheible, W.R. (2006) PHO2, microRNA399, and PHR1 define a phosphate-signaling pathway in plants. *Plant Physiol.* **141**, 988–999.

Bray, R.H. and Kurtz, L.T. (1945) Determination of total, organic, and available forms of phosphorus in soils. *Soil Sci.* **59**, 39–45.

Chiou, T.J., Aung, K., Lin, S.I., Wu, C.C., Chiang, S.F. and Su, C.L. (2006) Regulation of phosphate homeostasis by microRNA in Arabidopsis. *Plant Cell*, **18**, 412–421.

Duan, K., Yi, K., Dang, L., Huang, H., Wu, W. and Wu, P. (2008) Characterization of a sub-family of Arabidopsis genes with the SPX domain reveals their diverse functions in plant tolerance to phosphorus starvation. *Plant J.* **54**, 965–975.

Franco-Zorrilla, J.M., Valli, A., Todesco, M., Mateos, I., Puga, M.I., Rubio-Somoza, I., Leyva, A., Weigel, D., Garcia, J.A. and Paz-Ares, J. (2007) Target mimicry provides a new mechanism for regulation of microRNA activity. *Nat. Genet.* **39**, 1033–1037.

Fujii, H., Chiou, T.J., Lin, S.I., Aung, K. and Zhu, J.K. (2005) A miRNA involved in phosphate-starvation response in Arabidopsis. *Curr. Biol.* **15**, 2038–2043.

Goff, S.A., Ricke, D., Lan, T.-H. *et al.* (2002) A draft sequence of the rice genome (*Oryza sativa* L. ssp. japonica). *Science*, **296**, 92–100.

Hiei, Y., Ohta, S., Komari, T. and Kumashiro, T. (1994) Efficient transformation of rice (*Oryza sativa* L.) mediated by *Agrobacterium* and sequence analysis of the boundaries of the T-DNA. *Plant J.* **6**, 271–282.

Hou, X.L., Wu, P., Jiao, F.C., Chen, H.M., Yu, J., Song, X.W. and Yi, K.K. (2005) Regulation of the expression of *OsIPS1* and *OsIPS2* in rice via systemic and local Pi signalling and hormones. *Plant Cell Environ.* **28**, 353–364.

Hurlimann, H.C., Pinson, B., Stadler-Waibel, M., Zeeman, S.C. and Freimoser, F.M. (2009) The SPX domain of the yeast low-affinity phosphate transporter Pho90 regulates transport activity. *EMBO Rep.* **10**, 1003–1008.

Jefferson, R.A., Kavanagh, T.A. and Bevan, M.W. (1987) GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. *EMBO J.* **6**, 3901–3907.

Lin, S.I., Chiang, S.F., Lin, W.Y., Chen, J.W., Tseng, C.Y., Wu, P.C. and Chiou, T.J. (2008) Regulatory network of microRNA399 and PHO2 by systemic signaling. *Plant Physiol.* **147**, 732–746.

Nilsson, L., Muller, R. and Nielsen, T.H. (2007) Increased expression of the MYB-related transcription factor, *PHR1*, leads to enhanced phosphate uptake in *Arabidopsis thaliana*. *Plant Cell Environ.* **30**, 1499–1512.

Ogawa, N., Noguchi, K.I., Sawai, H., Yamashita, Y., Yompakaee, C. and Oshima, Y. (1995) Functional domains of Pho81p, an inhibitor of Pho85p protein kinase, in the transduction pathway of Pi signals in *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **15**, 997–1004.

Pant, B.D., Buhtz, A., Kehr, J. and Scheible, W.R. (2008) microRNA399 is a long-distance signal for the regulation of plant phosphate homeostasis. *Plant J.* **53**, 731–738.

Paszkowski, U., Kroken, S., Roux, C. and Briggs, S.P. (2002) Rice phosphate transporters include an evolutionarily divergent gene specifically activated

in arbuscular mycorrhizal symbiosis. *Proc. Natl Acad. Sci. USA*, **99**, 13324–13329.

Pinson, B., Merle, M., Franconi, J.M. and Daignan-Fornier, B. (2004) Low affinity orthophosphate carriers regulate PHO gene expression independently of internal orthophosphate concentration in *Saccharomyces cerevisiae*. *J. Biol. Chem.* **279**, 35273–35280.

Poirier, Y. and Bucher, M. (2002) In *The Arabidopsis Book* (Somerville, C.R. and Meyerowitz, E.M., eds). Rockville, MD: American Society of Plant Biologists, pp. 1–35.

Rubio, V., Linhares, F., Solano, R., Martin, A.C., Iglesias, J., Leyva, A. and Paz-Ares, J. (2001) A conserved MYB transcription factor involved in phosphate starvation signaling both in vascular plants and in unicellular algae. *Genes Dev.* **15**, 2122–2133.

Schachtman, D.P. and Shin, R. (2007) Nutrient sensing and signaling: NPKS. *Annu. Rev. Plant Biol.* **58**, 47–69.

Wang, X., Yi, K., Tao, Y., Wang, F., Wu, Z., Jiang, D., Chen, X., Zhu, L. and Wu, P. (2006) Cytokinin represses phosphate-starvation response through increasing of intracellular phosphate level. *Plant Cell Environ.* **29**, 1924–1935.

Wang, C., Ying, S., Huang, H., Li, K., Wu, P. and Shou, H. (2009a) Involvement of OsSPX1 in phosphate homeostasis in rice. *Plant J.* **57**, 895–904.

Wang, Z., Hu, H., Huang, H., Duan, K., Wu, Z. and Wu, P. (2009b) Regulation of OsSPX1 and OsSPX3 on expression of *OsSPX* domain genes and Pi-starvation signaling in rice. *J. Integr. Plant Biol.* **51**, 663–674.

Yoshida, S., Forno, D.A., Cock, J.H. and Gomez, K.A. (1976) *Laboratory Manual for Physiological Studies of Rice*, 3rd edn. Manila, The Philippines: International Rice Research Institute.

Yuan, Y., Wu, H., Wang, N., Li, J., Zhao, W., Du, J., Wang, D. and Ling, H.Q. (2008) FIT interacts with AtbHLH38 and AtbHLH39 in regulating iron uptake gene expression for iron homeostasis in Arabidopsis. *Cell Res.* **18**, 385–397.

Zhou, J., Jiao, F., Wu, Z., Wang, X., He, X., Zhong, W. and Wu, P. (2008) OsPHR2 is involved in phosphate-starvation signaling and excessive phosphate accumulation in shoots of plants. *Plant Physiol.* **146**, 1673–1686.

