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Involvement of *OsSPX1* in phosphate homeostasis in rice

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Summary

Arabidopsis thaliana **SPX (SYG/PHO81/XPR1) domain genes have recently been shown to be involved in the phosphate (Pi) signaling pathway. We show here that a rice (*Oryza sativa*) *SPX* gene, *OsSPX1*, is specifically induced by Pi starvation in roots. Suppression of *OsSPX1* by RNA interference resulted in severe signs of toxicity caused by the over-accumulation of Pi, similar to that found in *OsPHR2* (phosphate starvation response transcription factor 2) overexpressors and *pho2* (phosphate-responsive mutant 2). Quantitative RT-PCR showed that expression of *OsSPX1* was strongly induced in *OsPHR2* overexpression and *pho2* mutant plants, indicating that *OsSPX1* occurs downstream of *OsPHR2* and *PHO2*. The expression of 10 genes associated with the phosphate-starvation signal pathways was analyzed. Expression of *OsPT2* (phosphate transporter 2) and *OsPT8* was significantly induced in *OsSPX1*-RNAi (*OsSPX1*-Ri) plants, suggesting that over-accumulation of Pi in *OsSPX1*-Ri plants results from an increase in Pi transport. In contrast, overexpression of *OsSPX1* suppressed the induction of expression by Pi starvation of all 10 phosphate starvation-induced genes tested: *IPS1* (induced by phosphate starvation 1), *IPS2*, *OsPAP10* (purple acid phosphatase 10), *OsSQD2* (sulfoquinovosyldiacylglycerol 2), *miR399d* and *miR399j* (microRNA 399), *OsPT2*, *OsPT3*, *OsPT6* and *OsPT8*. This suggests that OsSPX1 acts via a negative feedback loop to optimize growth under phosphate-limited conditions.**

Keywords: rice, SPX domain, phosphate, homeostasis, suppressor.

Introduction

Phosphorus (P) is a macronutrient that is essential for plant growth and development. Although abundant in the soil, it is often limited for plants because of its low bioavailability (Raghothama, 2000; Vance *et al.*, 2003). To cope with an inadequate supply of phosphate (Pi), plants have evolved adaptive responses that include expansion of the root system to accelerate soil exploration, the adjustment of metabolism to protect intracellular Pi homeostasis, and the induction and secretion of phosphatases and organic acids to mobilize Pi from organic matter (Raghothama, 2000; Rausch and Bucher, 2002; Ticconi and Abel, 2004). Genes encoding proteins involved in responses to Pi starvation, and the mechanism of these proteins, have been intensively studied (Schachtman and Shin, 2007). Mutation in a MYB transcription factor, phosphate starvation response 1 (PHR1), reduces the expression of several phosphate starvation-induced (PSI) genes and results in evident signs of Pi deficiency (Rubio *et al.*, 2001). Arabidopsis PHR1 was found to bind to an imperfect palindromic sequence (GNATATNC) that has been found in the promoter regions of many PSI genes (Franco-Zorrilla *et al.*, 2004; Rubio *et al.*, 2001). A microRNA gene, *miR399*, the expression of which is induced by Pi starvation, is under the control of PHR1. miR399 targets the mRNA of a ubiquitin-conjugating E2 enzyme, UBC24 (PHO2), and reciprocally regulates the expression of *PHO2* at the transcriptional level (Aung *et al.*, 2006; Chiou *et al.*, 2006; Fujii *et al.*, 2005), and consequently regulates several phosphate transporters in Arabidopsis (Bari *et al.*, 2006). Overexpression of *AtPHR1* and *miR399* and mutation in *AtPHO2* led to over-accumulation of Pi in Arabidopsis (Bari *et al.*, 2006; Chiou *et al.*, 2006; Nilsson *et al.*, 2007). A member of the PSI cDNAs of the Mt4/TPS1 family, *IPS1* (induced by phosphate starvation 1), was recently shown to mimic the target of *miR399* and to offset the function of *miR399* (Franco-Zorrilla *et al.*, 2007). Overexpression of *OsPHR2*, an ortholog of *AtPHR1*, resulted in over-accumulation of Pi and

an increase in the transcript abundance of PSI genes (Zhou *et al.*, 2008), suggesting that a similar regulatory network exists in rice.

The SPX domain is a conservative domain found in the N-termini of yeast SYG1 and PHO81, and human XPR1 proteins (Wang *et al.*, 2004). SYG1 and XPR1 have been proposed to be involved in signal transduction via association with G proteins (Battini *et al.*, 1999; Spain *et al.*, 1995). PHO81 from *Saccharomyces cerevisiae* and its homolog NUC2 from *Neurospora crassa* are involved in control of the PHO regulon (Lenburg and O'Shea, 1996; Poleg *et al.*, 1996). PHO1 is a protein that contains SPX and EXS (a conserved domain existing in ERD1/XPR1/SYG1 proteins) domains. Arabidopsis and *Physcomitrella patens PHO1* orthologs have been shown to be involved in the transport and acquisition of Pi (Wang *et al.*, 2004, 2008). The Arabidopsis *pho1* mutant shows signs of Pi deficiency in the shoot (Hamburger *et al.*, 2002; Poirier *et al.*, 1991). In Arabidopsis, 20 SPX domain-containing proteins have been identified (Wang *et al.*, 2004). Sixteen members contain both SPX and EXS domains, while the other four proteins, AtSPX1–AtSPX4, contain a single SPX domain (Duan *et al.*, 2008). Expression of the *AtSPX1* and *AtSPX3* genes is induced by Pi starvation (Duan *et al.*, 2008; Franco-Zorrilla *et al.*, 2005). *AtSPX1* and *AtSPX3* have been proposed to play different roles in the Pi signaling network in Arabidopsis (Duan *et al.*, 2008). Although these studies are informative, they allow only general conclusions to be drawn regarding the functions and the regulatory network of *AtSPX* genes (Duan *et al.*, 2008).

Six SPX proteins have been identified in the rice genome (Duan *et al.*, 2008). In this study, we investigated the effects of rice *SPX1* on the expression of Pi-responsive genes and the acquisition of Pi. Our results showed that *OsSPX1* is specifically induced by Pi starvation. The expression of OsSPX1 inhibits the acquisition of Pi, which is located downstream of *PHR2/PHO2*. Furthermore, we found that overexpression of *SPX1* could suppress the induction of expression of PSI genes by Pi starvation.

Results

OsSPX1 *is induced specifically by phosphorus starvation in roots*

Previous microarray analysis on 7-day-old rice seedlings that were maintained with various levels of phosphate and iron supply showed that expression of *OsSPX1* (locus Os06g40120), a rice ortholog of *AtSPX1*, was upregulated approximately fourfold by Pi starvation in the roots, regardless of the supply of iron (Table S1) (Cheng *et al.*, 2007). To confirm this result, expression of the *OsSPX1* gene under Pi-sufficient (+P, 0.323 mM) or Pi-deficient (–P, no P) conditions was analyzed by Northern blot and quantitative RT-PCR using RNA samples extracted from roots, stems and leaves. The results showed that, under Pi-sufficient conditions, expression of the *OsSPX1* gene was very limited (Figure 1a). When plants were exposed to conditions of low Pi, the expression of *OsSPX1* was induced significantly in roots and stems (Figure 1a and Figure S1). The response of *OsSPX1* expression to Pi starvation coincided with the level of the transcript of the marker gene *OsIPS1*, the expression of which is induced by Pi starvation (Hou *et al.*, 2005). To determine whether upregulation of the *OsSPX1* gene was caused by a universal nutrient stress signal, expression of the *OsSPX1* gene in roots was analyzed in seedlings on which other nutrient stresses were imposed, including deprivation of nitrogen (N), potassium (K), sulfur (S) and iron (Fe). As shown in Figure 1(b), expression of the *OsSPX1* gene was not affected by deficiency of the other nutrients tested.

Kinetin (Kin) and phosphite (Phi) are known to specifically suppress the expression of genes that are involved in responses to Pi starvation (Martin *et al.*, 2000; Ticconi *et al.*, 2001; Varadarajan *et al.*, 2002). To determine whether Kin or Phi had any effect on the expression of *OsSPX1*, 14-day-old plants were transferred to a nutrition solution that contained no Pi, and were supplied with 0.1 μM 6-benzyladenine (6-BA) or 0.323 mM Phi for 5 days. As shown in Figure 1(b), in the presence of Kin or Phi, expression of the *OsSPX1* transcript

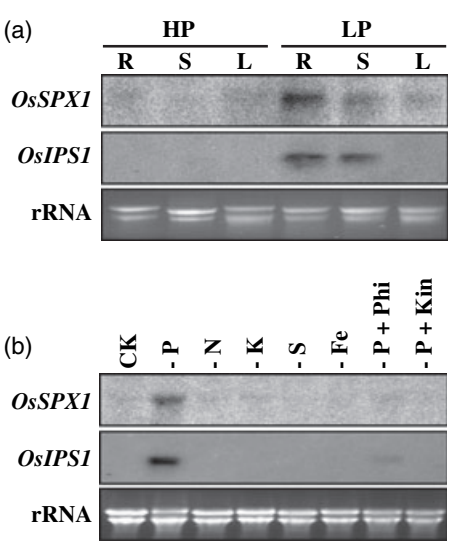

Figure 1. RNA blot analysis of *OsSPX1* and *OsIPS1* under various levels of nutrient supply.
(a) Expression of *OsSPX1* and *OsIPS1* in roots (R), stems (S) and leaves (L). Nipponbare seedlings were grown in nutrient solution to which 0.323 mM Pi (HP) or 0.032 mM Pi (LP) was added, for 30 days prior to sampling.
(b) *OsSPX1* was specifically induced by Pi starvation. Fourteen-day-old seedlings were transferred to a nutrient solution that lacked Pi (–P), nitrogen (–N), potassium (–K), sulfur (–S) or iron (–Fe), or Na_2HPO_4 was replaced by phosphite (–P+0.323 mM Phi) or NaCl plus 0.1 μM 6-BA (–P+0.323 mM NaCl +0.1 μm 6-BA) for 5 days (as described in Experimental procedures). The ethidium bromide gels shown below are the loading control. The RNA gels were probed with a ^{32}P-labeled gene fragment.

was no longer induced by Pi starvation. This response is similar to that of *OsIPS1*, which indicates that *OsSPX1* is involved in signal transduction during Pi starvation.

Generation of transgenic rice showing OsSPX1 *overexpression and suppression*

To characterize the role of *OsSPX1* in Pi homeostasis, we constitutively increased or suppressed the expression of *OsSPX1* in transgenic rice. Transgenic lines were generated by introducing the *OsSPX1* overexpression construct (*OsSPX1*-Oe) or the *OsSPX1* RNA interference construct (*OsSPX1*-Ri) into the *japonica* cultivar Nipponbare via *Agrobacterium*-mediated transformation (Chen *et al.*, 2003). Integration of the transgene cassette was verified by Southern blot analysis using the hygromycin phosphotransferase gene (*hpt*) (data not shown).

Overexpression or suppression of *OsSPX1* in the transgenic plants was confirmed by Northern blot analysis and quantitative RT-PCR. Under Pi-sufficient conditions, the *OsSPX1*-Oe plants accumulated a substantial amount of the transcript, whereas the level of transcript was very low in wild-type (WT) and RNAi roots (Figure 2a,b). Elimination of Pi from the nutrient solution for 5 days increased the expression of *OsSPX1* mRNA in the roots of WT plants nearly 10-fold (Figure 2a,b). Expression of *OsSPX1* was suppressed by introduction of the RNAi construct under both Pi-sufficient and -deficient conditions (Figure 2a,b).

Inhibition of plant growth by overexpression or suppression of OsSPX1

T_3 transgenic seeds that overexpressed or suppressed the *OsSPX1* gene or WT plants were grown in nutrient solution that was supplied with high Pi (HP, 0.323 mM) or low Pi (LP, 0.032 mM). Under both HP and LP conditions, overexpression or suppression of the *OsSPX1* gene led to the inhibition of plant growth compared with the WT plants. The shoot and root biomass of the 30-day-old transgenic plants was significantly lower than that of the WT plants (Figure 3a,c and Table 1).

Necrotic spots were observed in the mature leaves of *OsSPX1*-Ri plants grown under Pi-sufficient conditions (Figure 3b). These signs were not seen when these plants were grown under Pi-deficient conditions (Figure 3d), implying that they may have been caused by toxicity resulting from excessive accumulation of Pi. In response to Pi starvation, plants normally increase root growth to improve their capacity to obtain Pi from the soil (Raghothama, 1999; Ticconi and Abel, 2004). No significant differences were observed between the WT and *OsSPX1* overexpressing or RNAi lines with respect to the shoot/root ratio under either HP or LP conditions (Table 1). Thus, alterations in *OsSPX1* expression do not affect this Pi-starvation response.

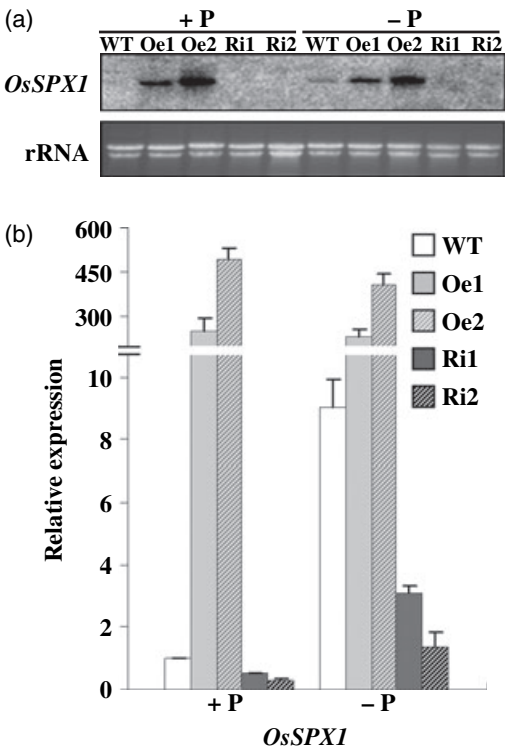

Figure 2. Expression of *OsSPX1* in the roots of the transgenic plants.
Oe1, Oe2, Ri1 and Ri2 represent independent *OsSPX1* overexpressing and RNA interference lines, respectively.
(a) Northern blot of transgenic plants. Fourteen-day-old seedlings were transferred to nutrient solution containing 0.323 mM Pi (+P) or no Pi (–P) for 5 days. RNA was extracted from the roots of the seedlings.
(b) Detection of the expression level of *OsSPX1* in WT and transgenic plants by quantitative RT-PCR. cDNAs were prepared from the RNA isolated as above (a). The expression of rice *ACTIN* was used as the internal control.

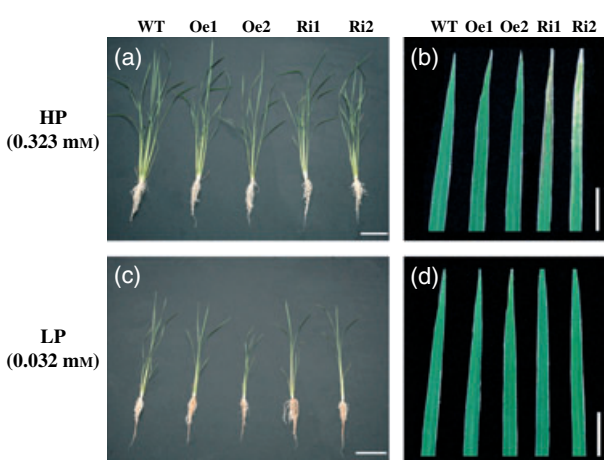

Figure 3. Characterization of the WT and transgenic plants.
Plants were grown in nutrient solution to which 0.323 mM Pi (HP) or 0.032 mM Pi (LP) was added for 30 days.
(a, c) Seedlings of the WT and transgenic plants. Scale bar = 8 cm.
(b, d) Mature leaves of the WT and transgenic plants. Scale bar = 2 cm.

Table 1 Root and shoot biomass of the WT and transgenic plants

	Plant growth (g FW)			Leaf P content		Root P content	
Genotype	Root	Shoot	Root:shoot ratio	Pi content (nmol mg^{-1} FW)	Total *P* (nmol mg^{-1} DW)	Pi content (nmol mg^{-1} FW)	Total *P* (nmol mg^{-1} DW)
HP (0.323 mM Pi)							
WT	2.78 ± 0.30^{a}	8.59 ± 0.62^{a}	0.32 ± 0.01^{bc}	17.42 ± 2.32^{c}	215.7 ± 46.9^{c}	3.45 ± 0.29^{b}	155.5 ± 13.1^{b}
Oe1	1.57 ± 0.41^{bc}	5.16 ± 1.02^{b}	0.30 ± 0.02^{cd}	11.87 ± 3.77^{c}	185.7 ± 14.1^{c}	3.57 ± 0.28^{b}	153.4 ± 23.4^{b}
Oe2	1.16 ± 0.27^{de}	3.99 ± 0.68^{cd}	0.29 ± 0.03^{d}	11.44 ± 3.54^{c}	181.8 ± 39.8^{c}	3.70 ± 0.41^{b}	173.4 ± 14.0^{ab}
Ri1	1.47 ± 0.23^{c}	4.85 ± 0.71^{b}	0.30 ± 0.00^{cd}	33.52 ± 3.03^{b}	318.4 ± 20.8^{b}	5.04 ± 0.59^{a}	196.9 ± 14.3^{a}
Ri2	1.64 ± 0.23^{bc}	4.98 ± 0.59^{b}	0.33 ± 0.02^{bc}	51.16 ± 7.90^{a}	415.6 ± 45.5^{a}	5.42 ± 1.25^{a}	189.5 ± 8.2^{a}
LP (0.032 mM Pi)							
WT	1.87 ± 0.08^{b}	4.53 ± 0.22^{bc}	0.41 ± 0.02^{a}	1.09 ± 0.09^{e}	39.9 ± 1.3^{e}	0.20 ± 0.06^{c}	29.3 ± 10.7^{c}
Oe1	1.38 ± 0.21^{cd}	3.44 ± 0.32^{de}	0.40 ± 0.04^{a}	0.84 ± 0.16^{e}	31.5 ± 4.6^{f}	0.21 ± 0.06^{c}	29.9 ± 4.4^{c}
Oe2	0.84 ± 0.06^{e}	2.42 ± 0.37^{f}	0.35 ± 0.03^{b}	0.64 ± 0.15^{e}	28.0 ± 1.8^{f}	0.25 ± 0.04^{c}	34.3 ± 11.1^{c}
Ri1	1.36 ± 0.44^{cd}	3.24 ± 0.71^{e}	0.41 ± 0.05^{a}	1.83 ± 0.30^{d}	45.5 ± 1.4^{d}	0.25 ± 0.02^{c}	39.5 ± 1.1^{c}
Ri2	1.50 ± 0.21^{c}	3.50 ± 0.31^{de}	0.43 ± 0.03^{a}	1.87 ± 0.16^{d}	52.5 ± 4.1^{d}	0.20 ± 0.02^{c}	37.6 ± 3.0^{c}

Biomass measurements were obtained from the roots and shoots of 30-day-old seedlings of WT and transgenic plants grown in nutrient solution to which 0.323 mM Pi (HP) or 0.032 mM Pi (LP) was added. Five plants per line were measured. Data are means ± SD. Means with different letters are significantly different at $P < 0.05$ (LSD test). FW, fresh weight; DW, dry weight.

Alteration of Pi concentration in OsSPX1 *transgenic plants*

To investigate the effects of *OsSPX1* on Pi homeostasis, we measured the inorganic Pi and total P concentration in mature leaves of main tillers and roots of WT and transgenic plants that had been grown for 30 days under either HP or LP conditions. When grown under HP conditions, the Pi concentration of *OsSPX1*-Ri leaves was significantly higher than that of the WT plants (Table 1). The mean Pi concentrations of *OsSPX1*-Ri1 and *OsSPX1*-Ri2 leaves were 1.924 and 2.937 times as high as that of WT plants, respectively (Table 1). The leaf Pi concentrations of the *OsSPX1*-Oe1 and *OsSPX1*-Oe2 plants were lower, but not statistically significantly so, than that of the WT plants (Table 1). Similarly, the root Pi concentration of *OsSPX1*-Ri plants was significantly higher than those of WT and *OsSPX1*-Oe plants (Table 1). Under LP conditions, the leaf Pi concentration of the *OsSPX1*-Ri plants was significantly higher than that of the WT plants. No significant difference was detected in the root Pi concentration among the *OsSPX1*-Ri, *OsSPX1*-Oe and the WT plants under LP conditions (Table1).

A similar trend was evident for total Pi concentrations in the leaf and root of transgenic plants relative to WT plants (Table 1). Under Pi-sufficient supply conditions, the leaf total P contents of *OsSPX1*-Ri1 and *OsSPX1*-Ri2 were 1.476 and 1.927 times as high as that of the WT plants. In contrast, those in the *OsSPX1*-Oe1 and *OsSPX1*-Oe2 leaves were 86.1% and 84.3% of that in the WT leaves, but the difference was not statistically significant (Table 1). When grown under LP conditions, while the total P contents of the *OsSPX1*-Ri leaves were significantly higher than that of the WT, those of the *OsSPX1*-Oe leaves were significantly lower than that of the WT (Table 1). No significant difference was found in the root total P content of the *OsSPX1*–Ri, *OsSPX1*-Oe and the WT plants under LP conditions (Table 1).

Detailed time-course analysis showed that the Pi concentration of the WT leaves remained constant or slightly increased over the growth period (Figure 4a–d). By contrast, the Pi concentration of the *OsSPX1*-RNAi plants increased rapidly during the growth period (Figure 4a–d). The Pi concentrations of the newly emerged leaves were represented by that of the 3rd leaf at 14 day after germination (DAG) and that of the 4th leaf at 18 DAG. The results showed that the Pi concentrations in the youngest leaves in the *OsSPX1*-Ri plants were significantly higher than those of WT plants (Figure 4c,d). In contrast, the Pi concentrations in the new leaves of the *OsSPX1*-Oe plants were significantly lower than in the WT plants (Figure 4c,d). These results imply that *OsSPX1* suppresses either Pi uptake or the transfer of Pi from the roots to the leaves.

OsSPX1 *occurs downstream of* OsPHR2 *and* OsPHO2

In order to understand the relationship between *OsSPX1* and other members of the Pi-starvation signaling pathway, the relative expression levels of *OsSPX1* in the *pho2* (phosphate-responsive mutant 2) lines *pho2-1* (NE7032), *pho2-2* (NE8536), and *pho2-3* (NE9017) from a Tos17-based insertional mutant library (Miyao *et al.*, 2003) and in an *OsPHR2* overexpression line (Zhou *et al.*, 2008) were examined. Homozygous plants in which a Tos17 retrotransposon was inserted in *OsPHO2* were identified by PCR analysis (Figure S2). Semi-quantitative RT-PCR revealed that expression of *OsPHO2* was not detectable in either leaves (Figure 5a) or roots (data not shown) of all three lines. Similar to the *PHR2* overexpressors (Zhou *et al.*, 2008), the *pho2* lines

accumulated 5–7 times more Pi in their leaves than the WT when grown in soil with sufficient Pi supply, which resulted in toxicity due to excessive Pi in the leaves (Figure 5a,b). This suggests that rice *PHO2* has a similar function to that of its Arabidopsis ortholog.

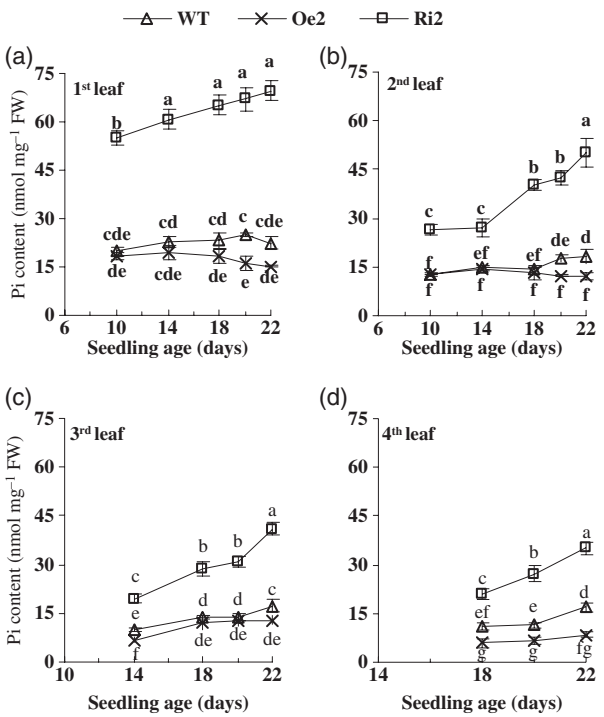

Figure 4. Pi concentration in leaves of the WT and transgenic plants over time.
(a) First leaf. (b) Second leaf. (c) Third leaf. (d) Fourth leaf. Points with different letters are significantly different at $P < 0.05$ (least significant difference test).

The expression of *OsSPX1* in the roots of *PHR2* overexpression and *pho2* mutant plants was markedly increased, indicating that *OsSPX1* is under the control of *OsPHR2* and *OsPHO2*. In contrast, although the expression of *OsSPX1* in the leaves was slightly higher than that in roots, it was not affected by the alteration of *OsPHO2* and *OsPHR2* expression (Figure 5c), implying that leaf expression of *OsSPX1* is not directly influenced by Pi-starvation signals.

To determine whether *OsSPX1* affects the expression of *OsPHO2* reciprocally, expression of *OsPHO2* in the *OsSPX1*-Oe and *OsSPX1*-Ri plants was analyzed. The results showed that alterations of *OsSPX1* expression had no effect on the expression of *OsPHO2* (Figure S3).

Overexpression of OsSPX1 *suppressed the induction of PSI genes by Pi starvation*

The effects of *OsSPX1* on the expression of other genes that respond to Pi starvation were analyzed by quantitative RT-PCR of 14-day-old WT, *OsSPX1*-Oe and *OsSPX1*-Ri seedlings that had been transferred into medium with (+Pi) or without (−Pi) Pi for 5 days. As expected, expression of the tested PSI genes [*OsIPS1* and *OsIPS2* (Hou *et al.*, 2005; Wasaki *et al.*, 2003), *OsPAP10* (purple acid phosphatase 10), *OsSQD2* (sulfoquinovosyldiacylglycerol 2), *miR399d* and *miR399j* (microRNA 399), and the phosphate transporters *OsPT2*, *OsPT3*, *OsPT6* and *OsPT8*] was substantially upregulated by Pi starvation in WT plant roots (Figure 6). Induction of expression of these PSI genes by Pi starvation was suppressed by overexpression of *OsSPX1* (Figure 6), indicating that OsSPX1 plays a role in suppression of the Pi-starvation signal.

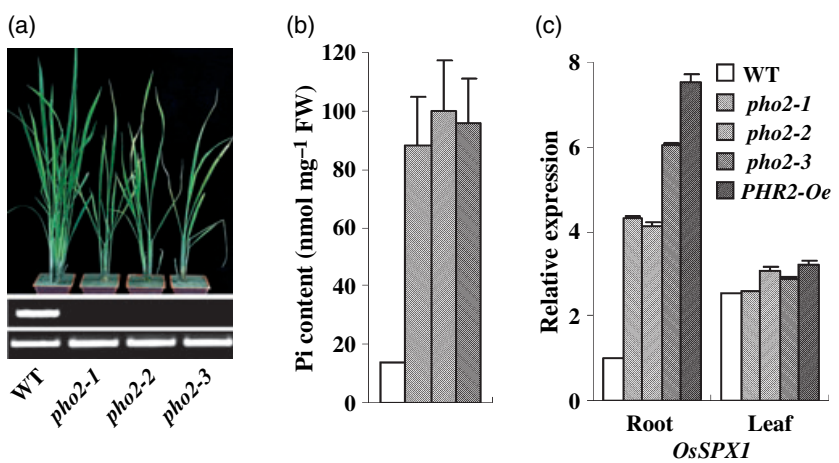

Figure 5. Characterization of the *pho2* mutants.
pho2-1, *pho2-2* and *pho2-3* are three independent Tos17 insertional lines with mutations in the *OsPHO2* gene. The original codes for these lines are NE7032, NE8536 and NE9017, respectively.
(a) Sixty-day-old plants and *PHO2* expression of the WT and *pho2* mutants. RT-PCR of the *OsPHO2* gene and the housekeeping gene *OsACTIN* of the mutant and WT plants is shown below the corresponding plants .
(b) Pi concentration in the mature leaves of the WT and *pho2* mutants. The leaves were collected from 60-day-old plants grown in soil pots.
(c) Expression of *OsSPX1* mRNA in the leaves and roots of the 60-day-old WT, *pho2* mutant and *OsPHR2* overexpression plants. Relative RNA expression was detected by quantitative RT-PCR analysis.

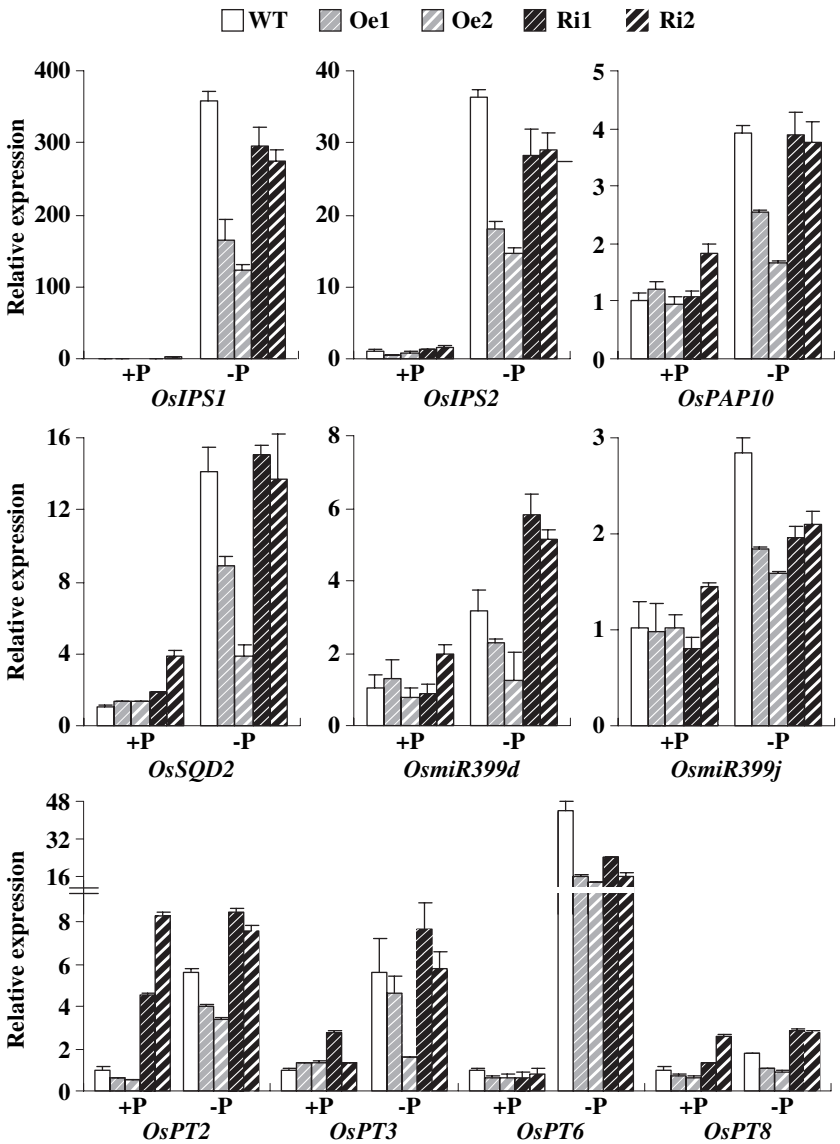

Figure 6. Expression of Pi starvation-induced genes in the WT and *OsSPX1* transgenic plants. Total RNA samples were extracted from roots of seedlings grown in normal nutrient solution for 14 days, followed by treatment with or without P for 5 days.

The induction of PSI genes by Pi starvation in *OsSPX1*-Ri roots relative to that of WT roots varied (Figure 6). While expression of *OsIPS1*, *OsIPS2*, *OsmiR399j* and *OsPT6* in *OsSPX1*-Ri roots was lower than that in the WT roots, expression of *OsmiR399d*, *OsPT2* and *OsPT8* was higher than that in the WT roots (Figure 6). The expression of *OsPAP10*, *OsSQD2* and *OsPT3* in the *OsSPX1*-Ri roots was similar to that of the WT roots (Figure 6). The different effects of under-expression of the *SPX1* gene on different PSI genes are probably due to the fact that a variety of other factors act downstream of OsSPX1 for the induction of at least some PSI genes.

Expression of OsPT2 *and* OsPT8 *was enhanced in OsSPX1-Ri plants*

As shown previously (Table 1 and Figure 4), the Pi concentrations in newly emerged leaves of *OsSPX1*-Ri plants were significantly higher than in those of WT plants. It is likely that the *OsSPX1*-Ri plants were able to obtain more Pi from root uptake than WT plants. The results of quantitative RT-PCR showed that, under Pi-sufficient conditions, expression of the *OsPT2* and *OsPT8* genes in the roots of *OsSPX1*-Ri plants was significantly ($P < 0.05$) increased compared with that in WT plants (Figure 6). This suggests that the suppression of *OsSPX1* may have promoted Pi uptake by stimulating Pi transporters.

Discussion

The identification of SPX domain proteins and their involvement in signaling in response to Pi starvation has significantly increased our understanding of Pi acquisition by plants (Chiou *et al.*, 2006; Duan *et al.*, 2008; Franco-Zorrilla *et al.*, 2007; Stefanovic *et al.*, 2007; Wang *et al.*,

2004). In this study, we investigated the roles of rice *SPX1* on the expression of Pi-responsive genes and the acquisition of Pi. Our results indicate that rice *SPX1* plays a regulatory role in the induction of some Pi transporters in rice, and also that it probably plays a role in Pi homeostasis and/or other functions, as both over- and under-expression lead to growth retardation even under non-limiting Pi conditions.

Both overexpression and suppression of *OsSPX1* resulted in inhibition of plant growth in the T_3 transgenic plants (Figure 3). The backbone vectors used for overexpression or RNAi transformations have been used in a number of previous studies, and no negative effects on the growth of transgenic plants other than the T_0 generation have been observed (Chen *et al.*, 2003; Yi *et al.*, 2005). Thus, it is unlikely that the growth inhibition was caused by integration of T-DNA in the genome. Under normal growth conditions, *OsSPX1* is expressed at a low level in leaves and roots (Figure 1a). While *OsSPX1* expression in roots was induced by Pi deficiency, transcript abundance in the leaves was not affected (Figure 1a). This suggests that OsSPX1 might play roles other than in Pi homeostasis in leaves. Using the constitutive promoter, CaMV 35S, expression of *OsSPX1* in the transgenic plants was increased more than 100-fold (Figure 2b). The high expression of *OsSPX1* in leaves may contribute to the growth disorder observed in the *OsSPX1*-Oe plants. Further studies using transgenic plants with tissue-specific expression would help to clarify this.

While T-DNA insertion mutants of *AtSPX1*, *AtSPX2* and *AtSPX4* showed no detectable phenotypic changes under both Pi-sufficient and -deficient conditions, repression of *AtSPX3* led to an increased concentration of Pi in the leaves and 'hypersensitivity' to Pi starvation (Duan *et al.*, 2008). It has been hypothesized that the repression of *AtSPX3* may inhibit re-allocation of Pi from the shoot to the root (Duan *et al.*, 2008). However, no direct evidence has been provided in support of this hypothesis. We measured the concentrations of Pi and total P in various leaves and in individual leaves over time, and observed a significant increase in the concentration of Pi in leaves in the *OsSPX1*-Ri lines (Table 1 and Figure 4). Suppression of *OsSPX1* increased the expression of Pi transporters *OsPT2* and *OsPT8* in roots (Figure 6), thus it is possible that the high expression level of these two transporters promoted the acquisition of Pi in the *OsSPX1*-Ri plants. Overexpression of *OsSPX1* resulted in a marginal decrease in the leaf Pi contents in the *OsSPX1*-Oe plants, but this was not statistically significant. It is possible the significant growth inhibition caused by overexpression in the *OsSPX1*-Oe plants partially masked the negative effect of SPX1 on Pi uptake.

In Arabidopsis, although the *pho2* mutant contains an excessive amount of Pi, many PSI genes remain activated in the mutant (Bari *et al.*, 2006). The Arabidopsis *SPX1* gene (At5g20150) is one of the genes upregulated in the *pho2* mutant (Bari *et al.*, 2006). In this study, we found that the rice *SPX1* gene is controlled by *OsPHR2*/*OsPHO2* (Figure 5c). The *pho2* mutant mimics the Pi-starvation signal and constitutively activates the expression of downstream genes, including *OsSPX1*, regardless of the Pi status in plants. Pi starvation-responsive genes probably play diverse roles in plant Pi homeostasis. Among the PSI genes are Pi transporters, which have a positive affect on the accumulation of Pi. In contrast, *IPS1* is also a PSI gene, but it negatively affects plant Pi uptake (Franco-Zorrilla *et al.*, 2007). By mimicking targeting of miR399, *IPS1* overexpression results in increased accumulation of the miR399 target, *PHO2* mRNA, and reduced shoot Pi content in Arabidopsis (Franco-Zorrilla *et al.*, 2007). Under the control of phosphate starvation response transcription factors, *SPX1* also acts as a negative factor for the accumulation of Pi in leaves. Thus, it is an interaction between both positive and negative regulators of Pi accumulation that regulates Pi homeostasis under Pi-limiting conditions.

In the presence of sufficient Pi, suppression of *OsSPX1* in transgenic plants induced expression of the transporters *OsPT2* and *OsPT8*, leading to altered Pi uptake in the transgenic plants. Under these conditions, no correlation was observed between the expression of other PSI genes and the extend of OsSPX1 suppression. It is possible that the effect of *OsSPX1* on PSI genes is target-specific. Under Pi-deficient conditions, overexpression of *OsSPX1* suppressed induction of the tested PSI genes (*IPS1*, *IPS2*, *OsPAP10*, *OsSQD2*, *miR399d*, *miR399j*, *OsPT2*, *OsPT3*, *OsPT6* and *OsPT8*) by Pi starvation (Figure 6). This suggests that *OsSPX1* plays roles in suppression of Pi-deficient signaling in rice. Yet *OsSPX1* itself is induced by Pi starvation in roots (Figures 1a and 2b). This apparent paradox strongly suggests that OsSPX1 acts as a negative feedback factor of the PSI signaling pathway in rice. Overexpression and suppression of *OsSPX1* result in a negative growth phenotype under both high- and low-phosphate conditions. This suggests that a runaway phosphate-starvation response might have negative growth consequences. Thus, under phosphate starvation or limitation, plants respond to optimize growth by inducing a number of genes whose expression results in greater uptake and use of phosphate. However, induction of these genes is controlled via a negative feedback loop that involves *OsSPX1*, itself a PSI gene. Thus OsSPX1 is an essential component of the PSI signaling pathway in rice, acting to suppress the PSI signaling pathway and resulting in optimized plant growth.

Experimental procedures

Plant materials and conditions of growth

The *japonica* variety Nipponbare was used for all physiological experiments and rice transformation. Hydroponic experiments were performed using normal rice culture solution containing 1.425 mM NH_4NO_3, 0.323 mM NaH_2PO_4, 0.513 mM K_2SO_4, 0.998 mM $CaCl_2$,

1.643 mM $MgSO_4$, 0.009 mM $MnCl_2$, 0.075 mM $(NH_4)_6Mo_7O_24$, 0.019 mM H_3BO_3, 0.155 mM $CuSO_4$, 0.036 mM $FeCl_3$, 0.070 mM citric acid and 0.152 mM $ZnSO_4$ (Yoshida *et al.*, 1976). Rice plants were grown in growth chambers with a 12 h photoperiod (200 μmol photons m^{-2} sec^{-1}) and a day/night temperature of 30/22°C after germination. Humidity was controlled at approximately 60%. Thirty-day-old seedlings were evaluated for phenotype or sampled so that the concentrations of Pi and total P could be measured. For Northern analysis and RT-PCR analysis of gene expression under nutrient starvation and kinetin and phosphite treatments, 14-day-old seedlings were transferred to the solutions described below for 5 days. For the LP treatment, the concentration of NaH_2PO_4 was reduced to 0.032 mM. In the no Pi (−P), kinetin and phosphite treatments, the NaH_2PO_4 of the hydroponic medium was replaced by NaCl (0.323 mM), NaCl plus 0.1 μM 6-BA, or 0.323 mM phosphite (Sigma, http://www.sigmaaldrich.com/), respectively.

The *pho2* mutant seeds were germinated in the normal culture solution for 7 days and screened for homozygous insertion of Tos17. The verified homozygous mutant seedlings were transferred into pots with soil containing 12.1 mg kg^{-1} Pi.

Construction of overexpression and RNAi vectors, and plant transformation

The binary vector pCAMBIA1300 was modified by introduction of a double CaMV 35S promoter between the *Eco*RI and *Sac*I sites, and a *nos* terminator between the *Pst*I and *Hin*dIII sites. The resultant vector was named pCAMBIA1300-mod. Full-length cDNA of *OsSPX1*, from the 5′ UTR to the 3′ UTR, was amplified from cDNA synthesized from leaf total RNA. The purified PCR product was digested with *Kpn*I and *Xba*I, then inserted into pCAMBIA1300-mod. The resultant vector was verified by restriction analysis and DNA sequencing. For the RNAi construct, a 209 bp fragment of *OsSPX1* in sense and antisense orientations was inserted into both sides of the second intron of the maize *NIR1* gene. The fragment was then inserted into the multiple cloning site of pCAMBIA1300-mod to construct the *OsSPX1*-RNAi vector. The primers used for amplification are given in Table S2. The above constructs were transformed into callus derived from mature seeds of WT via *Agrobacterium*-mediated transformation, as described previously (Chen *et al.*, 2003).

Measurement of total P and Pi concentration in plants

Leaves and roots of the WT and transgenic seedlings from either Pi-sufficient or Pi-deficient treatments were sampled separately. The Pi concentration was measured using the procedure described previously (Delhaize and Randall, 1995). Briefly, 50 μg fresh samples were homogenized with 50 μl of 5 M H_2SO_4 and 3 ml H_2O. The homogenate was transferred to 1.5 ml tubes and centrifuged at 10 000 ***g*** for 10 min at 4°C. The supernatant was collected and diluted to an appropriate concentration. The diluted supernatant was mixed with a malachite green reagent in a 3:1 ratio, and analyzed 30 min afterwards. The absorption values for the solution at 650 nm were determined using a Spectroquant NOVA60 spectrophotometer (Merck, http://www.merck.com.cn). The Pi concentration was calculated from a standard curve generated with varying concentrations of KH_2PO_4.

For total P measurement, the leaves and roots were dried at 80°C to a constant weight. Dried samples (30 μg) were pre-digested in glass tubes with H_2SO_4 for 2 h. The tubes were then heated to 180°C, and 50 μl H_2O_2 was added every 10 min until the solution turned colorless. The digestion was continued another 30 min. The digested solution was cooled to room temperature and the pH was adjusted to 5.0 using 30% NaOH. The solution was diluted to an appropriate concentration and Pi content was analyzed as described above.

Northern blot analysis

Total RNA was extracted from plant samples using TRIzol Reagent (Invitrogen, http://www.invitrogen.com/) according to the manufacturer's recommendations. Total RNA (20 μg) was electrophoretically separated in a denaturing formaldehyde agarose gel and blotted onto a Hybond N^+ nylon membrane (Amersham Pharmacia Biotech, http://www5.amershambiosciences.com/). The full-length *OsSPX1* was amplified and labeled with ^{32}P-dCTP. The membrane was hybridized with the labeled probe and washed at 65°C. The hybridized membrane was exposed to a phosphor imaging screen and screened using a Typhoon-8600 phosphor imager (Amersham Biosciences).

Identification of the homozygous pho2 *mutants*

The *pho2* Tos17 insertion mutants were obtained from the Rice Genome Resource Center, Japan. The homozygous *pho2* mutant plants were identified using two PCR reactions as instructed on the Rice Genome Resource Center website (http://tos.nias.affrc.go.jp/). The primers that were used for the PCR reactions are listed in Table S2.

Semi-quantitative RT-PCR and quantitative real-time PCR

First-strand cDNAs were synthesized from total RNA using SuperScript II reverse transcriptase (Invitrogen). Semi-quantitative RT-PCR was performed using a pair of gene-specific primers (Table S2). The PCR products were loaded on 1.2% agarose gels and photographed using a CCD camera. The levels of gene expression were compared using the densities of the PCR products as the basis. The housekeeping gene *ACTIN* was used as an internal control. Real-time quantitative RT-PCR was performed using a SYBR Premix Ex Taq™ (perfect real time) kit (TaKaRa Biomedicals, http://www.takara.com.cn) on a LightCycler 480 machine (Roche Diagnostics, http://www.roche-applied-science.com), according to the manufacturer's instructions. The amplification program for SYBR Green I was performed at 94°C for 10 sec, 58°C for 10 sec and 72°C for 10 sec. Triplicate quantitative assays were performed on each cDNA sample. The relative level of expression was calculated using the formula $2^{-\Delta(\Delta Cp)}$. All primers used for RT-PCR are given in Table S2.

Statistical analysis

Statistical significance was determined using the SAS program (SAS Institute Inc., http://www.sas.com).

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Supporting Information

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

Figure S1. Expression of *OsSPX1* in leaves and roots of the WT plants.

Figure S2. Position of the Tos17 insertion in the *pho2* mutants, and identification of the homozygous *pho2* mutants.

Figure S3. Expression of *OsPHO2* in *OsSPX1* transgenic plants.

Table S1. Microarray data showing expression of *OsSPX* genes under various conditions of Pi starvation or iron starvation.

Table S2. Sequences of the primer sets used in the study.

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