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Research

Functional characterization of the rice *SPX-MFS* family reveals a key role of *OsSPX-MFS1* in controlling phosphate homeostasis in leaves

Chuang Wang[1,2]*, Wei Huang[1]*, Yinghui Ying[1], Shuai Li[1], David Secco[2,3], Steve Tyerman[4], James Whelan[2,3] and Huixia Shou[1,2]

[1]State Key Laboratory of Plant Physiology and Biochemistry, College of Life Sciences, Zhejiang University, Hangzhou, 310058, China; [2]Joint Research Laboratory in Genomics and Nutriomics, College of Life Sciences, Zhejiang University, Hangzhou, 310058, China; [3]Australian Research Council Centre of Excellence in Plant Energy Biology, University of Western Australia, Crawley 6009, WA, Australia; [4]Australian Research Council Centre of Excellence in Plant Energy Biology, School of Agriculture, Food and Wine, Waite Research Institute, University of Adelaide, PMB1, Glen Osmond, SA 5064, Australia

Author for correspondence:
Huixia Shou
Tel: +86 0571 88206133
Email: huixia@zju.edu.cn

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Summary

- Proteins possessing the SPX domain are found in several proteins involved in inorganic phosphate (Pi) transport and signalling in yeast and plants. Although the functions of several SPX-domain protein subfamilies have recently been uncovered, the role of the SPX-MFS subfamily is still unclear.
- Using quantitative RT-PCR analysis, we studied the regulation of *SPX-MFS* gene expression by the central regulator, *OsPHR2* and Pi starvation. The function of *OsSPX-MFS1* in Pi homeostasis was analysed using an *OsSPX-MFS1* mutant (*mfs1*) and *osa-miR827* overexpression line (*miR827*-Oe). Finally, heterologous complementation of a yeast mutant impaired in Pi transporter was used to assess the capacity of *OsSPX-MFS1* to transport Pi.
- Transcript analyses revealed that members of the *SPX-MFS* family were mainly expressed in the shoots, with *OsSPX-MFS1* and *OsSPX-MFS3* being suppressed by Pi deficiency, while *OsSPX-MFS2* was induced. Mutation in *OsSPX-MFS1* (*mfs1*) and overexpression of the upstream *miR827* (*miR827-Oe*) plants impaired Pi homeostasis in the leaves. In addition, studies in yeast revealed that *OsSPX-MFS1* may be involved in Pi transport.
- The results suggest that *OsSPX-MFS1* is a key player in maintaining Pi homeostasis in the leaves, potentially acting as a Pi transporter.

Introduction

Phosphorus (P) is an essential nutrient element for growth and development in all living organisms (Filippelli, 2008). Although P is abundant in the Earth's crust, it is often present in forms not available to plants. Plants adapt to low inorganic phosphate (Pi) by expansion of the root system to accelerate soil exploration, increasing high-affinity Pi uptake capacity, by adjusting metabolism to maintain intracellular Pi homeostasis, and by induction and secretion of phosphatases and organic acids to mobilize Pi from organic matter (Ticconi & Abel, 2004; Rouached *et al.*, 2010). Intensive research has defined a Pi starvation gene regulatory network that involves various developmental, physiological and metabolic pathways (Schachtman & Shin, 2007; Rouached *et al.*, 2010; Chiou & Lin, 2011). In Arabidopsis, the central regulator of this network is *PHR1* and its homologue gene *PHR1-LIKE 1* (*PHL1*), both of which regulate the majority of the *Phosphate Starvation Induced* (*PSI*) genes by binding to the *PHR1* binding sequences (*P1BS*, GNATATNC) in the promoters of *PSI* genes (Rubio *et al.*, 2001; Bustos *et al.*, 2010). Mutation or overexpression of *PHR1* changes the expression of a number of *PSI* genes and the physiological responses to Pi starvation in Arabidopsis (Nilsson *et al.*, 2007; Bustos *et al.*, 2010). In rice, overexpression of *OsPHR2*– the orthologue of *AtPHR1* – resulted in changes in root architecture, increased Pi uptake and induction of *PSI* genes (Zhou *et al.*, 2008; Liu *et al.*, 2010), indicating that a similar regulatory network exists in rice. Several other transcription factors, including *WRKY75*, *ZAT6*, *bHLH32*, *MYB62* and *WRKY6* in Arabidopsis, and *OsPTF1* and *OsMYB2P-1* in rice, have also been reported to be involved in Pi starvation responses (Yi *et al.*, 2005; Chen *et al.*, 2007; Devaiah *et al.*, 2007a,b, 2009; Chen *et al.*, 2009; Dai *et al.*, 2012).

The SPX domain (named after proteins **S**YG1/**P**HO81/**X**PR1) is found in several Pi transporters or signalling proteins in yeast and plants(Secco *et al.*, 2012a,b). In yeast, the SPX domain

*These authors contributed equally to this work.

of PHO87 and PHO90, two SPX domain-containing low affinity Pi transporters, has been shown to be required for proper phosphate uptake velocity, phosphate efflux and a phosphate signal transduction, through direct interaction with SPL2 (Hurlimann *et al.*, 2009; Ghillebert *et al.*, 2011; Secco *et al.*, 2012b). In plants, SPX domain-containing proteins can be divided into four classes based on the presence of additional domains in their predicted protein structure (Supporting Information Fig. S1). Class 1 proteins have no other conserved domains except the SPX domain (Duan *et al.*, 2008; Wang *et al.*, 2009) and have already been described as being key players of phosphate homeostasis. In rice the best characterized member of this class, *OsSPX1* has been shown to suppress Pi uptake and the phosphate starvation signal pathway via a negative feedback regulation (Wang *et al.*, 2009; Liu *et al.*, 2010). The second class of SPX domain-containing proteins is associated with a C-terminal EXS domain (named after **E**RD1, **X**PR1 and **S**YG1) and constitutes the PHO1 family, involved in phosphate transport and signalling from the root to the shoot in Arabidopsis and rice (Hamburger *et al.*, 2002; Stefanovic *et al.*, 2007; Secco *et al.*, 2010). Class 4 proteins have a RING-type zinc finger domain which may regulate protein degradation (Peng *et al.*, 2007). A recent study in Arabidopsis showed the involvement of one member, *AtNLA*, in maintaining phosphate homeostasis in a nitrate-dependent manner (Peng *et al.*, 2007; Yaeno & Iba, 2008; Kant *et al.*, 2011). The last subgroup of SPX domain-possessing proteins is the SPX-MFS (Major Facility Superfamily) family. The MFS proteins are the largest group of secondary membrane transporters in the cell, and can function as uniporters, symporters and antiporters with a broad range of substrates (Pao *et al.*, 1998). Interestingly, among the many different MFS families, the phosphate transporter family *PHT1* possesses the MFS domain. In rice the SPX-MFS family constitutes four members, with *SPX-MFS1* and *SPX-MFS2* being the targets of the Pi starvation-induced *osa-miR827* (Lin *et al.*, 2010). In addition, in the same study Lin and co-workers, focusing on two members of the family, *SPX-MFS1* and *SPX-MFS2*, demonstrated that they were differentially regulated by Pi starvation and that they were preferentially expressed in the leaves, more precisely in the mesophyll and parenchyma cells surrounding the xylem. However, no function was associated with these two genes. Based on the function of the MFS domain, one interesting hypothesis would be that some of the SPX-MFS family members could be involved in mediating Pi transport and thus controlling shoot Pi homeostasis.

In order to test this hypothesis, we characterized the molecular response of all the members of the *SPX-MFS* family, as well as the response of *osa-miR827* under different Pi conditions. In addition, we confirmed that *osa-miR827,* and consequently its two target genes *SPX-MFS1* and *SPX-MFS2,* are under the control of the transcription factor *OsPHR2*. Using the *osa-miR827* overexpression and *OsSPX-MFS1* mutant plants, we demonstrated that *OsSPX-MFS1* is involved in controlling Pi homeostasis in leaves and that *OsSPX-MFS1* can transport Pi in yeast.

Materials and Methods

Plant materials and conditions of growth

The *Oryza sativa* L. *japonica* variety of Nipponbare was used for all physiological experiments and rice transformations. Hydroponic experiments were conducted using a modified rice culture solution (Yoshida *et al.*, 1976) 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 $MgSO_4$, 0.25 mM $NaSiO_3$, 0.009 mM $MnCl_2$, 0.075 μM $(NH_4)_6Mo_7O_{24}$, 0.019 μM H_3BO_3, 0.155 μM $CuSO_4$ and 0.152 μM $ZnSO_4$ with 0.125 mM EDTA-Fe (II). pH of the solution was adjusted to 5.5. Rice plants were grown in growth chambers with a 12-h photoperiod (200 μmol photons m^{-2} s^{-1}) and a day : night temperature of 30 : 22°C after germination. Humidity was controlled at 60% RH (relative humidity). For phenotype and Pi concentration measurements, seeds were germinated and grown under HP (10 mg l^{-1} Pi) or LP (0.1 mg l^{-1} Pi) conditions for 3 wk.

Construction of vectors and plant transformation

The binary vector pTF101.1 was modified by the introduction of the maize ubiquitin promoter and a nos terminator (Zheng *et al.*, 2010). DNA fragments including the precursor fold-back structure of *osa-miR827* gene were amplified from rice genomic DNA. The purified PCR product was inserted into pTF101.1-ubi. The resultant vector was used for *Agrobacterium*-mediated rice transformation as described previously (Nishimura *et al.*, 2006). The primers used for the amplification are listed in Table S1.

For the GUS construct, the 3.8 kb region upstream ATG of *OsSPX-MFS1* was amplified from genomic DNA using primers shown in Table S1. The 5' primers were extended to add *Hin*dIII restrict sites and 3' primers were extend to add *Bam*HI restrict sites. pBI101.3 was modified in our laboratory by introducing the GUS plus sequence of pCAMBIA1305.1 between *Bam*HI and *Sac*I, and hereafter referred to as pBIGUS-plus. The PCR product of *OsSPX-MFS1* promoter was digested by *Hin*dIII and *Bam*HI. The purified fragments were introduced into pBIGUS-plus liberalized by *Hin*dIII and *Bam*HI. The resultant vector was identified by restriction analysis and sequenced for confirmation. Transgenic lines were made with resulted plasmid by *agrobacterium*-mediated rice transformation.

Measurement of 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 (Wang *et al.*, 2009). Briefly, 50 μg of fresh tissue was homogenized with 50 μl of 5M 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 3 : 1 ratio and analysed 20 min afterwards. The absorption

values for the solution at 650 nm were determined using a Spectroquant NOVA60 spectrophotometer (Merck, Darmstadt, Germany). Pi concentration was calculated from a standard curve generated with varying concentrations of KH_2PO_4.

GUS histochemical analysis

Histochemical GUS staining was performed as described previously (Jefferson *et al.*, 1987). In detail, plant tissues harvested from GUS transgenic lines were immediately submerged in GUS staining solution and placed in a vacuum for 10 min. The samples were incubated overnight in darkness at 37°C. Chlorophyll was removed by submerging the tissue in 70% ethanol. Plant material was placed on glass slides and visualized using a stereoscope. The GUS staining solution contained 100 mM sodium phosphate buffer, pH 7.0, Na_2EDTA 10 mM, $K_3[Fe(CN_6)]$ 1 mM, $K_4[Fe(CN_6)]$ 1 mM, TritonX-100 0.5% (v/v), methanol 20% (v/v), and 0.5 mg ml^{-1} 5-bromo-4chloro-3-indolyl-β-D-glucuronic acid (X-gluc).

Identification of the homozygous *mfs1* mutant

The *TOS17* insertion mutant of *OsSPX-MFS1*, *mfs1*, was obtained from the Rice Genome Resource Center, Japan (RGRC). The homozygous mutant plants were identified using two PCR reactions as instructed at the website of RGRC (http://www.rgrc.dna.affrc.go.jp/index.html). The primers that were used for the two PCR reactions are listed in Table S1.

RNA isolation, cDNA preparation and quantitative real-time PCR

Total RNA was extracted from plant samples using RNeasy mini kits (Qiagen; http://www.qiagen.com) according to the manufacturer's recommendations. First-strand cDNAs were synthesized from total RNA using SuperScript II reverse transcriptase (Invitrogen). Quantitative real-time PCR was performed using SYBR Premix Ex Taq™ (Perfect Real Time) Kit (TaKaRa Biomedicals, Tokyo, Japan) on a LightCycler480 machine (Roche), according to the manufacturer's instructions. The amplification program for SYBR Green I was performed at 94°C for 10 s, 58°C for 10 s, and 72°C for 10 s. Triplicate quantitative assays were performed on each cDNA sample. The ACTIN cDNA is an endogenous control that is used to normalize the samples. Relative expression level was calculated by the formula $2^{-\Delta(\Delta Cp)}$. All the primers that were used for the qRT-PCR are given in Table S1. Three biological experimental repeats were performed for all PCR reactions.

Yeast Pi transport and growth assay

The open reading frame of *OsSPX-MFS1* was cloned into yeast expression vector pAG426GPD-ccdB (http://www.addgene.org/14156/). These constructs were transformed into the growth defect of the yeast Pi transport-deficient strain PAM2 (Martinez & Persson, 1998). Transformed yeast was grown in SD(-Ura) medium containing 0.22 mM Pi and 25 mM Na-citrate buffer pH 4.5 to an OD_{600} of 1. Cells were harvested and washed with water then suspended in fresh medium to an OD_{600} of 0.2 and incubated at a 30°C shaker. OD_{600} was monitored as a measurement of growth each 3 h.

Results

Structural analysis of the rice *OsSPX-MFS* family

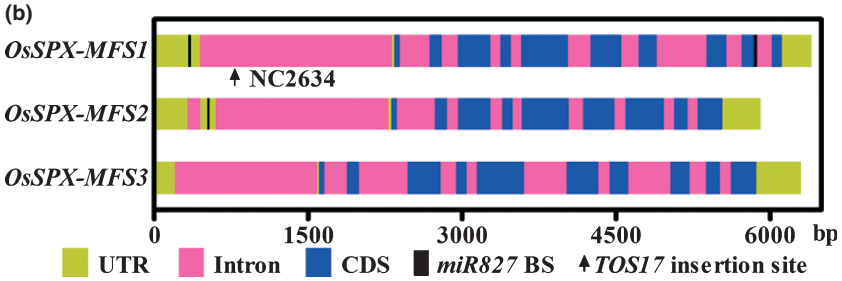
In plants, SPX domain-containing proteins are clustered into four groups, based on the presence of additional domains in the C-terminal region. The four families of SPX domain-possessing proteins are designated after their domains, namely the SPX, the SPX-EXS (ERD1, XPR and SYG1), the SPX-RING (Really Interesting New Gene) and the SPX-MFS (Major Facilitator Superfamily) families (Fig. S1). Among the 15 SPX domain-containing proteins found in the rice genome, the SPX-MFS family is composed of four putative genes, referred to as *SPX-MFS1* to *SPX-MFS4* (Table S2). However, no full length cDNA or EST sequences could be found for *SPX-MFS4* (Loc_Os09g34990), therefore the following study only focused on the other three genes, *OsSPX-MFS1* (Loc_Os04g48390), *OsSPX-MFS2* (Loc_Os02g45520) and *OsSPX-MFS3* (Loc_Os06g03860). Protein identity and similarity analysis showed that OsSPX-MFS1 and OsSPX-MFS2 were highly similar with 93.4% and 82.7% of protein identity and similarity, respectively (Fig. 1a). Analysis of the gene structures revealed that the *OsSPX-MFS* genes have very similar DNA sequence structure with 10 introns and 11 exons including a large intron (> 1.3 kb) in the 5'UTR region (Fig. 1a). The predicted *osa-miR827* binding sites are located in the 5'UTR regions of both *OsSPX-MFS1* and *OsSPX-MFS2* genes, but not in *OsSPX-MFS*3 (Fig. 1b). Unlike most of the MFS proteins, which possess 12 transmembrane regions, the SPX-MFS proteins have 10 predicted transmembrane domains, with the SPX domain being predicted to be in the opposite cytoplasm space (Fig. 1c).

Expression of *OsSPX-MFS* genes and *osa-miR827* in response to Pi starvation

In order to gain insight into the function of the rice *SPX-MFS* genes, quantitative RT-PCR (qRT-PCR) was performed on RNA samples extracted from 3-wk-old rice seedlings grown at different Pi conditions. Under Pi-sufficient conditions, transcript analysis showed that the three members of the *SPX-MFS* family were expressed mainly in the leaves (Fig. S2). Furthermore, phosphate availability in the media differentially regulated the expression of the *SPX-MFS* transcripts. While the expression of *SPX-MFS1* was already low at 5 mg l^{-1} Pi, and decreased further at lower Pi concentration, the expression of *OsSPX-MFS3* was only suppressed below 0.5 mg l^{-1} Pi supply (Fig. 2a). By contrast, the transcripts of *OsSPX-MFS2* and *osa-miR827* were greatly induced by Pi starvation (Fig. 2a). Similar responses to Pi starvation were observed in the roots (Fig. S3A). To further characterize the effect of Pi starvation on the expression of

(a)

	OsSPX-MFS1	OsSPX-MFS2	OsSPX-MFS3
OsSPX-MFS1		82.4	75.2
OsSPX-MFS2	93.4		73.2
OsSPX-MFS3	87.4	86.4	



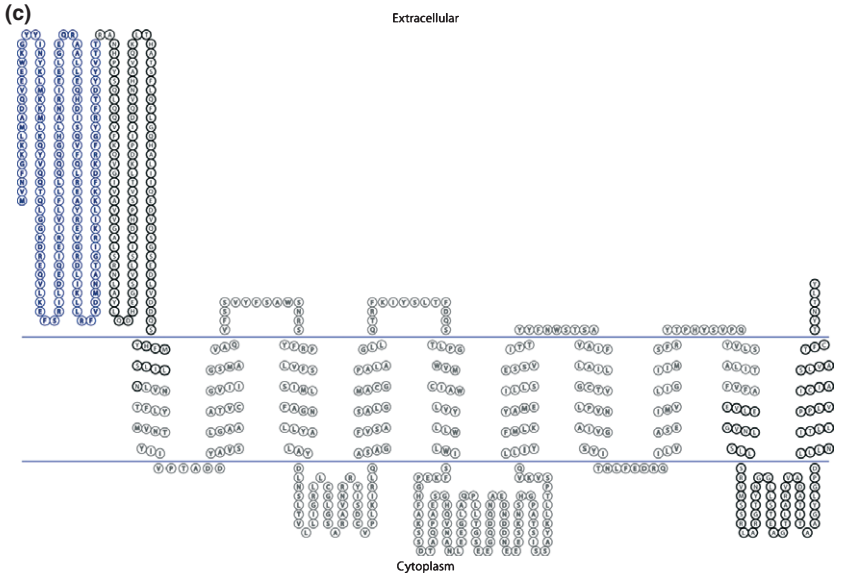


Fig. 1 Structures of SPX-MFS proteins and *SPX-MFS* genes. (a) Identity/similarity matrix for OsSPX-MFS proteins. Amino acid similarity and identity are indicated as light blue and pink, respectively. (b) Gene structures of *OsSPX-MFS1*, *OsSPX-MFS2* and *OsSPX-MFS3*. 5′ and 3′ UTR are shaded yellow, CDS is shaded blue and introns are shaded pink. The *miR827* binding site (miR827 BS) is marked in black. Arrows show *TOS17* insertion site. UTR, untranslated region; CDS, coding sequence. (c) Predicted topology profile of OsSPX-MFS1 was made and displayed using the TMHMM and Topo2 software (http://www.sacs.ucsf.edu/TOPO2/). SPX domain and MFS domain were highlighted by blue and grey bold amino acids, respectively.

the members of the *SPX-MFS* genes, a time course experiment was performed as follows: 10-d-old seedlings grown under sufficient Pi condition were transferred to Pi-deficient conditions for 1, 2, 3, 5, 7 and 10 d, before being re-supplied with Pi for 1 d. The three transcripts of *SPX-MFS* genes showed a slow response to Pi starvation, reaching their maximum or minimum levels of expression after 10 d of Pi starvation (Fig. 2b). Interestingly, the expression levels of *SPX-MFS2*, *SPX-MFS3* and *osa-miR827*, but not *SPX-MFS1,* were quickly reversed after 1 d of Pi re-supply (Fig. 2b). Similar patterns of expression were observed in the roots (Fig. S3B).

OsPHR2 regulates the expression of osa-miR827, OsSPX-MFS1 and OsSPX-MFS2

In order to determine if the *osa-miR827-SPX-MFS1/2* pathway is under the control of *OsPHR2*, the central transcription factor positively regulating Pi homeostasis in rice (Rubio *et al.*, 2001; Zhou *et al.*, 2008; Bustos *et al.*, 2010), we compared the expression levels of *osa-miR827*, *OsSPX-MFS1*, *OsSPX-MFS2* and *OsSPX-MFS3* using the previously reported *OsPHR2* overexpression plants (Zhou *et al.*, 2008). Analysis of the promoter region of *osa-miR827* showed the presence of the *P1BS cis*-elements, the binding site of *OsPHR2* (Fig. S4). Under sufficient Pi conditions, the transcript abundance of *pri-miR827* was increased eight-fold in O*sPHR2* overexpression plants compared to WT plants, in the leaves (Fig. 3). Accordingly, the targets of *osa-miR827, OsSPX-MFS1* and *OsSPX-MFS2,* were downregulated in the *OsPHR2* overexpression plants compared to WT (Fig. 3). The expression *OsSPX-MFS3,* which is not a target of *osa-miR827*, was unaffected by the overexpression of *OsPHR2*.

Mutation in *OsSPX-MFS1* alters Pi re-mobilization in rice

In order to investigate the function of the *SPX-MFS* family, we searched different rice genomic databases for publicly available mutants. While no mutants could be found for *OsSPX-MFS2* and *OsSPX-MFS3,* a *TOS17* transposon insertion mutant of *OsSPX-MFS1*, *mfs1*, was found, with the transposon being inserted in the first intron of the gene (Fig. 1a). Homozygous

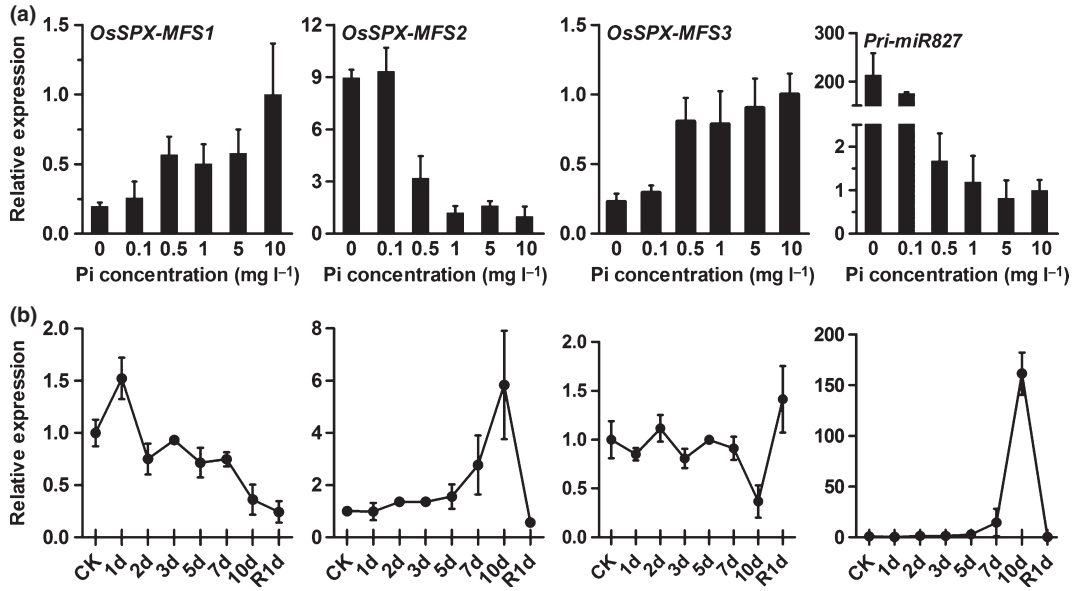


Fig. 2 Expression pattern of *OsSPX-MFS1*, *OsSPX-MFS2*, *OsSPX-MFS3* and *osa-miR827* in the leaves in response to phosphate (Pi) starvation. (a) Effects of varying Pi concentrations on the expression of *SPX-MFS* and *osa-miR827* transcripts. Rice (*Oryza sativa*) seeds were geminated and grown on nutrient solutions with different Pi concentrations for 3 wk. Leaves were sampled separately for analysis. (b) Effects of Pi starvation duration on the expression of *SPX-MFS* and *osa-miR827* transcripts. Seeds were geminated and grown on full nutrient solution (10 mg l^{-1} Pi) for 10 d before being transferred to Pi-deficient conditions (0.1 mg l^{-1}, T = 0 d) for the desired period of time (from 0 to 10 d). Leaves were sampled at different time following treatment (from 0 to 10 d). After 10 d of Pi starvation, plants were re-supplied with Pi for 1 d. All data are means of three biological replicates with error bars indicating SD. CK, Pi sufficient condition; R1d, 10 d Pi starvation followed by 1 d Pi sufficient supplement. Expression of *OsACTIN* was used as the internal control.

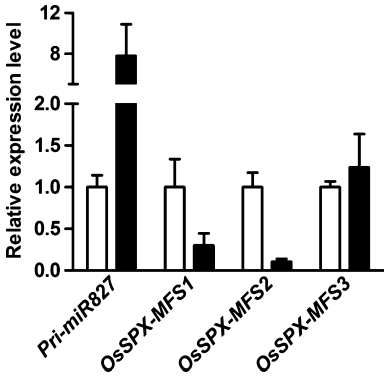


Fig. 3 Expression pattern of *Pri-miR827*, *OsSPX-MFS1*, *OsSPX-MFS2* and *OsSPX-MFS3* in WT (open bars) and *OsPHR2* overexpression (closed bars) plants. Rice (*Oryza sativa*) seeds were geminated and grown on full nutrient solution for 3 wk. Total RNA was extracted from the leaves and used for qRT-PCR. All data are means of three biological replicates with error bars indicating SD. Expression of *OsACTIN* was used as the internal control.

mutants were identified by PCR (data not shown). Analysis by qRT-PCR showed that the *mfs1* is a knock-down mutant, with decreased *SPX-MFS1* transcript levels compared to WT, both under Pi-sufficient and -deficient conditions (Fig. 4a). However, the expression levels of *OsSPX-MFS2* and *OsSPX-MFS3* were unaffected by the mutation in *SPX-MFS1* (Figs 4, S4). In addition to the *mfs1* mutant, transgenic plants overexpressing *osa-miR827* were generated for the study. DNA fragments including the precursor fold-back structure of *osa-miR827* were amplified and inserted into the binary vector pTF101.1 under the control of a maize *Ubiquitin-1* promoter. The resulting construct was used for rice transformation. Among the different *osa-miR827* overexpressing lines obtained, three lines – *osa-miR827*-Oe1, *osa-miR827*-Oe2 and *osa-miR827*-Oe3 – were analysed and showed increased *pri-miR827* transcript levels compared to WT under both Pi-sufficient and -deficient conditions (Fig. 4d). Overexpression of *osa-miR827* greatly decreased the expression of *OsSPX-MFS1* under both Pi-sufficient and -deficient conditions, and that of *OsSPX-MFS2* under Pi-deficient conditions (Fig. 4a,b), which is consistent with reported results (Lin *et al.*, 2010). The expression of *OsSPX-MFS3* was not influenced by the alteration of *osa-miR827*. These expression patterns were similar in both leaves and roots under all the tested conditions (Fig. S5).

The Pi concentrations of roots and shoots were assessed in plants grown in Pi-sufficient (10 mg Pi l^{-1}; HP, high phosphorus) and -deficient (0.1 mg Pi l^{-1}; LP, low phosphorus) conditions for 3 wk. Interestingly, Pi concentration in leaves of the *miR827-Oe* lines and *mfs1* mutant was significantly higher than that of the WT plants (Fig. 5a,b). In the roots, Pi concentration was not significantly different between wild-type (WT) and transgenic mutant plants under HP and LP conditions (Fig. 5a,b).

We further examined the involvement of *SPX-MFS1* in regulating Pi homeostasis in the leaves by analysing Pi concentration in individual leaves of WT, *osa-miR827* overexpression and *mfs1* plants grown for 2 wk under Pi-sufficient conditions. At this age, the seedlings have five leaves. The Pi concentrations of the second to fifth leaves were measured. As already observed, the Pi concentration in the second leaf of *mfs1* was higher than that in the second leaf of WT plants, with more than four-fold difference (Fig. 5c). Similar differences were observed in all the old leaves,

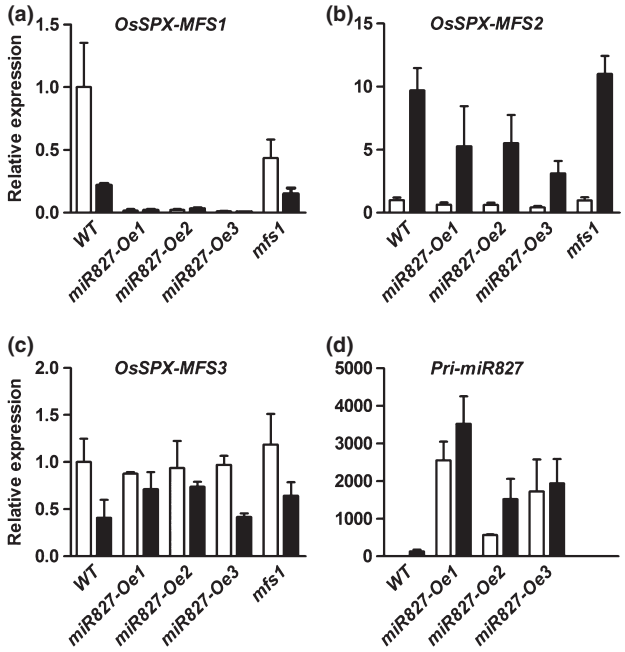


Fig. 4 Expression analysis of*OsSPX-MFS1* (a), *OsSPX-MFS2* (b), *OsSPX-MFS3* (c) and *Pri-miR827* (d) in *osa-miR827* overexpression lines and *msf1*. Rice (*Oryza sativa*) seeds were geminated and grown on nutrient solutions with high phosphate (Pi) concentration (HP, open bars; 10 mg l^{-1}) and low Pi concentration (LP, closed bars; 0.1 mg l^{-1}) for 3 wk. Total RNA was extracted from the leaves and used for qRT-PCR. All data are means of three biological replicates with error bars indicating SD. Expression of *OsACTIN* was used as the internal control. WT, wild- type; Oe1/2/3, *osa-miR827* overexpression lines; *mfs1*, *OsSPX-MFS1* mutant line NC2634.

with Pi concentration being more than thrice and twice as high as WT in the third and fourth leaves of *mfs1*, respectively (Fig. 5c). Interestingly, in the youngest leaf, no significant differences were observed in Pi concentration. A similar phenomenon was observed in the *osa-miR827* overexpression plants also, although to a lesser extent.

Spatial expression of *OsSPX-MFS1*

As changed expression of *OsSPX-MFS1* altered Pi homeostasis, we studied the expression pattern of *OsSPX-MFS1* in the various tissues. A 3.8 kb promoter region upstream ATG of *OsSPX-MFS1* gene, which includes 5'UTR and the first intron, was amplified and fused to GUS reporter gene to make an *OsSPX-MFS1*:GUS construct. As expected, the transgenic plants showed strong staining in all the leaves tested and weak staining in the roots under Pi-sufficient conditions (Fig. 6a,e). Interestingly, we also observed a high expression of *OsSPX-MFS1* in the base of leaf sheath (Fig. 6b,d), collar (Fig. 6c) and root junction (Fig. 6e), indicating a function of *OsSPX-MFS1* in these tissues.

Effect of the mutation of *OsSPX-MFS1* on phosphate signalling

In order to examine whether or not *OsSPX-MFS1* played a role in Pi starvation signalling, the transcript abundance of several root PSI genes was determined. Upon Pi starvation, all the tested PSI genes, including *OsSPX1*, *OsPAP10a*, *OsIPS1*, *OsACP1*, *OsPT2*, *OsPT3*, *OsPT7* and *OsPT8* were upregulated in WT, *osa-miR827* overexpression and *mfs1* plants (data not show). However, the extent of induction of the PSI genes by Pi starvation was attenuated in the *osa-miR827* overexpression and *mfs1* plants (Fig. 7). Indeed, under Pi-deficient conditions, genes such as *IPS1* and *PT3*, were five and three times less induced in the *mfs1* mutant than in the WT plants, respectively.

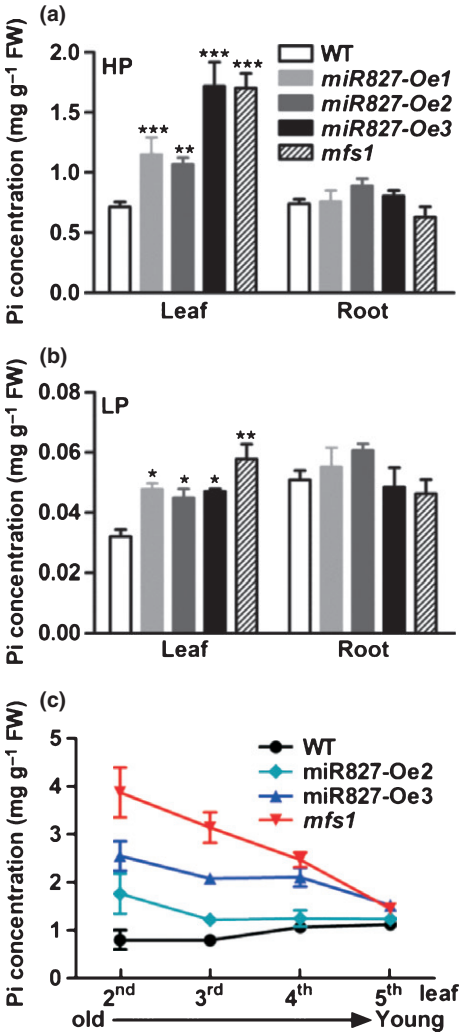


Fig. 5 (a) Phosphate (Pi) concentration of the second leaves and roots in 3-wk-old *osa-miR827* overexpression and *OsSPX-MFS1* mutant rice (*Oryza sativa*) plants grown on nutrient solution with high Pi concentration (HP, 10 mg l^{-1}). (b) Pi concentration of the second leaves and roots in 3-wk-old *osa-miR827* overexpression and *mfs1* mutant plants grown on nutrient solution with low Pi concentration (LP, 0.1 mg l^{-1}) for 3 wk. (c) Pi concentration in different leaves of the WT, *osa-miR827* overexpression and *mfs1* mutant plants. Seeds were geminated and grown on HP or LP nutrient solutions for half a month. Individual leaves were sampled for measurements. All data are means of three biological replicates with error bars indicating SD. Significant differences are indicated: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$. WT, wild- type; Oe1/2/3, *osa-miR827* overexpression lines; *mfs1*, *OsSPX-MFS1* mutant line NC2634.

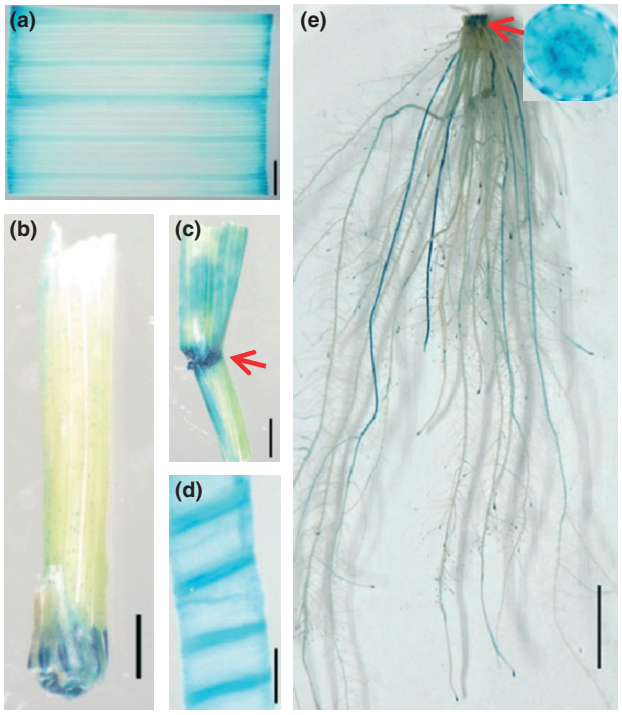


Fig. 6 Spatial expression of GUS reporter gene under the control of the *OsSPX-MFS1* promoter. (a) Rice (*Oryza sativa*) leaf; (b) stem with leaf sheath; (c) leaf collar (red arrowhead); (d) base leaf sheath; (e) roots (red arrowhead shows root junction). Bars: (a–d) 2 mm; (e) 1 cm.

Complementation of a yeast Pi-transport mutant

In order to test the possibility that *OsSPX-MFS1* could function as a Pi transporter, a functional complementation approach of a yeast strain (PAM2) lacking the two high-affinity Pi uptake transporters was undertaken (Martinez & Persson, 1998). To do so, the full length cDNA sequence of *OsSPX-MFS1* was amplified by RT-PCR and cloned into a yeast expression vector, before being transformed in the PAM2 yeast strain. The growth rate of cells expressing *OsSPX-MFS1* as well as the empty vector was monitored under low external Pi, in a pH-specific manner. While no significant differences of growth rate between cells expressing the empty vector or *OsSPX-MFS1* could be observed at pH 6 and pH 7, the cells expressing *OsSPX-MFS1* had a significantly higher growth rate at pH 4 and pH 5, compared to the one expressing the empty vector (Fig. 8).

Discussion

The SPX domain is found at the N-terminal region in a variety of proteins often involved in Pi signalling or transport (Secco *et al.*, 2012a,b). In this work, we studied the involvement of the rice SPX-MFS family in phosphate homeostasis. The rice genome has three validated genes possessing both the SPX and MFS domains. Proteins containing the MFS domain are the largest group of secondary membrane transporters, transporting small solutes, including sugar and Pi (Pao *et al.*, 1998; Abramson *et al.*, 2004; Lemieux *et al.*, 2004). Interestingly, the structure of SPX-MFS proteins is very similar to the three yeast low-affinity Pi transporters, namely PHO87, PHO90 and PHO91, containing a C-terminal transporter activity domain and an N-terminal SPX domain (Hurlimann *et al.*, 2007, 2009). The SPX domain in the three yeast low-affinity Pi transporters is proposed to act as an external Pi sensor, thereby regulating Pi starvation signalling responses in yeast (Pinson *et al.*, 2004). Under Pi-deficient conditions, SPL2 (Suppress the temperature-sensitive phenotype of the *plc1Δ*2) downregulates the transport activity of PHO87 and PHO90 by direct physical interaction via the SPX domain (Hurlimann *et al.*, 2009). Reduction of the low-affinity Pi transporter activity results in a decrease of internal phosphate content, which induces the central transcription factor *PHO4* and its downstream high-affinity Pi transporters *PHO84* (Wykoff *et al.*, 2007). In this study, we show that *OsSPX-MFS1* can complement the growth defect of the PAM2 yeast strains, depleted of both high-affinity Pi uptake transporters, suggesting that *OsSPX-MFS1* may have Pi transporter activity. In addition, this activity appears to be pH dependent, having an optimum activity at pH 4.0. Consistently, mutation of *OsSPX-MFS1* or overexpression of *osa-miR827* resulted in impaired Pi homeostasis in the leaves, with older leaves overaccumulating Pi. These results contradict those from Lin *et al.* (2010) where no phenotype could be detected with overexpression of *osa-miR827*. However, in our study Pi concentrations of individual leaves were measured, enabling the identification of subtle changes in Pi

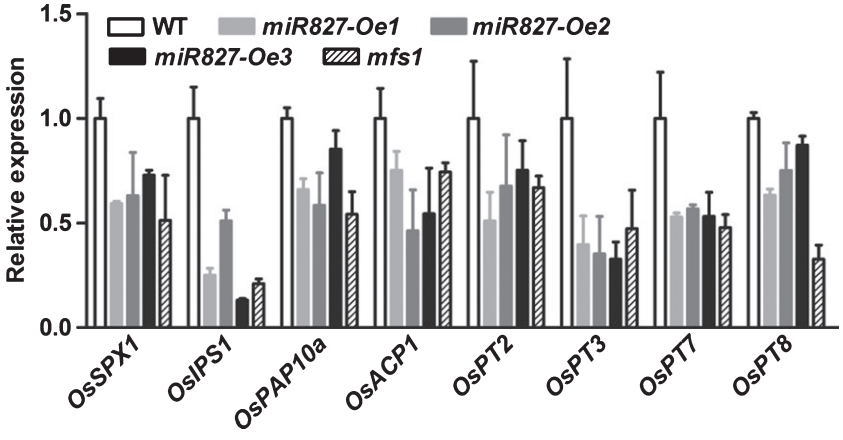


Fig. 7 Expression of phosphate (Pi) starvation-induced genes in *mfs1* mutant plants under low-Pi (0.1 mg l^{-1}) conditions. Rice (*Oryza sativa*) seeds were germinated and grown on nutrient solutions with low Pi concentration (0.1 mg l^{-1}) for 3 wk. Total RNA was extracted from the roots and used for qRT-PCR. All data are means of three biological replicates with error bars indicating SD. Expression of *OsACTIN* was used as the internal control.

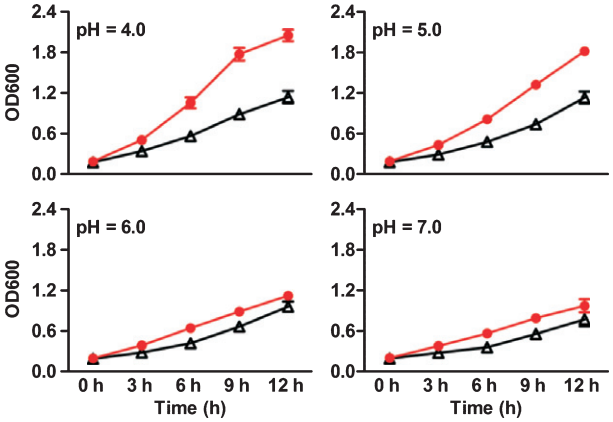


Fig. 8 Complementation of a yeast high-affinity phosphate (Pi) transport mutant by *OsSPX-MF1*. Yeast PAM2 cells harbouring either an empty expression vector (control; triangles) or *OsSPX-MF1* (circles) gene constructs were grown in synthetic dextrose -Ura medium to the logarithmic phase, OD600 ≈ 1.0 and then suspended to OD600 = 0.2 in fresh SD medium containing 0.22 mM Pi and 25 mM Na-Citrate buffer with indicated pH, incubated at 30°C. OD600 was monitored as a measurement of growth.

concentration, which may not be detected when assessing whole shoot Pi concentration as in the case of Lin *et al.* (2010). In addition, the *osa-miR827* overexpressing plants have a weaker phenotype than the *mfs1* mutant, accumulating intermediate concentrations of Pi in old leaves, potentially explaining the lack of phenotype of the osa-miR827 overexpressing lines reported by Lin *et al.* (2010).

Several miRNAs, such as *miR399*, *miR447*, *miR778*, *miR827* and *miR2111*, have already been demonstrated to be induced upon Pi starvation (Pant *et al.*, 2009; Hsieh *et al.*, 2009). Among them, *miR399* has been the most intensively studied, revealing that the *miR399-PHO2* pathway is conserved in both Arabidopsis and rice, and is under the control of PHR, the central transcription factor maintaining Pi homeostasis (Bari *et al.*, 2006; Liu *et al.*, 2010). *miR827* exists in numbers of plant species and has already been demonstrated to be specifically induced by Pi starvation (Lu *et al.*, 2008; Hsieh *et al.*, 2009; Pang *et al.*, 2009; Xue *et al.*, 2009; Zhang *et al.*, 2009). Our work, as well as the study of Lin *et al.* (2010), showed that the rice *osa-miR827* is preferentially expressed in the leaves, is greatly induced by Pi starvation and regulates the expression of *OsSPX-MFS1* and *OsSPX-MFS2*, and that they are all expressed in the same cells, mainly around the vascular bundles and in parenchyma cells associated with xylem (Lin *et al.*, 2010). Furthermore, this pathway, similarly to the *miR399-PHO2* pathway, is under the control of the central transcription factor *OsPHR2*. The Arabidopsis orthologue of *osa-miR827*, *ath-miR827*, is also upregulated by Pi starvation, but is preferentially expressed in the roots. In addition *ath-miR827* was shown to target a single gene, *AtNLA*, recently described to be involved in regulating Pi homeostasis in a nitrate-dependent manner, by regulating the expression of a high-affinity Pi transporter *PHT1.1* and its traffic facilitator, *PHF1* (Kant *et al.*, 2011). Indeed, *AtNLA* is a member of the SPX-RING family, and encodes E3 ubiquitin ligase, involved in protein degradation. In Arabidopsis, the *miR399-PHO2* and *miR827-NLA* pathways are thought to be coordinated in order to allow fine-tuning of the expression of *PHT1.1* and *PHF1* (Kant *et al.*, 2011). Therefore, *ath-miR827* and *osa-miR827,* via *NLA1* and *SPX-MFS1,* are involved in regulating leaf Pi homeostasis, by negatively regulating the expression of different Pi transporters.

Under Pi-limiting conditions, overexpression of *osa-miR827* or mutation in *OsSPX-MFS1*, despite having similar Pi concentrations as WT plants in roots, showed a reduction in the level of induction of some PSI genes in roots, compared to WT. Because *OsSPX-MFS1* may function as a transporter, it is unlikely that *OsSPX-MFS1* directly regulates expression of PSI genes. Rather, the reduction of induction observed for some PSI genes, could be explained by higher Pi concentration in leaves detected in the *mfs1* mutants and *osa-miR827* overexpressing plants, compared to WT. It is suggested that OsSPX-MFS proteins may localize on tonoplast and regulate Pi storage in parenchyma cells. Under Pi starvation conditions, the cells will mobilize Pi stored in vacuoles, which will attenuate the phosphate starvation response of the cell (Pratt *et al.*, 2009). Thus, reducing the level of expression of *OsSPX-MFS1* may increase the Pi concentration in cytoplasm, which in turn could suppress the expression of PSI genes. Interestingly, while the residual level of expression of *OsSPX-MFS1* is higher in the *mfs1* mutants than the *osa-miR827* overexpressing plants, the Pi concentration in old leaves is higher in the *mfs1* mutants than the *osa-miR827* overexpressing plants. In addition, the level of expression of *OsSPX-MFS2* is downregulated in the *osa-miR827* overexpressing lines, compared to WT and *mfs1.* Thus, it is tempting to hypothesize that *OsSPX-MFS1* and *OsSPX-MFS2* could have opposite functions, fine-tuning Pi concentration in leaves. This hypothesis is also strengthened by the expression profiles of *OsSPX-MFS1* and *OsSPX-MFS2*, being oppositely regulated by Pi availability. In addition OsSPX-MFS proteins are predicted to be localized on the tonoplast, thus OsSPX-MFS members could be involved in regulating vacuolar Pi concentration. Indeed in higher plants, vacuoles are thought to have dual roles as both sink and source for Pi, making them a key component in buffering Pi fluctuations due to external Pi supply or metabolic changes.

In summary, under Pi starvation, *OsSPX-MFS1* and *OsSXP-MFS2* are negatively regulated by the *osa-miR827/OsPHR2* pathway. Mutation of *OsSPX-MFS1* resulted in disturbed Pi homeostasis in the leaves, associated with altered responses of some of the PSI genes. Heterologous complementation studies also revealed that *OsSPX-MFS1* could mediate Pi transport in yeast.

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Supporting Information

Additional supporting information may be found in the online version of this article.

Fig. S1 Modular structure of rice proteins encompassing the SPX domain.

Fig. S2 Expression analysis of *OsSPX-MFS1*, *OsSPX-MFS2* and *OsSPX-MFS3* in leaves and roots.

Fig. S3 Expression analysis of *OsSPX-MFS1*, *OsSPX-MFS2*, *OsSPX-MFS3* and *osa-miR827* under different Pi starvation treatments.

Fig. S4 Nucleotide sequence of the promoter and gene region of *osa-miR827*.

Fig. S5 Expression analysis of*OsSPX-MFS1* (A), *OsSPX-MFS2* (B), *OsSPX-MFS3* (C) and *Pri-miR827* (D) in *osa-miR827* over-expression lines and *msf1* in the roots.

Table S1 Sequences of the primer sets used in the study

Table S2 General information of *SPX-MFS* genes in rice

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