The paralogous SPX3 and SPX5 genes redundantly modulate Pi homeostasis in rice

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Abstract

The importance of SPX-domain-containing proteins to phosphate (Pi) homeostasis and signalling transduction has been established in plants. In this study, phylogenetic analysis revealed that OsSPX3 and OsSPX5 (SPX3/5) are paralogous SPX genes (SYG1/Pho81/XPR1) in cereal crops. SPX3/5 are specifically responsive to Pi starvation at both the transcriptional and post-transcriptional levels. Similar tissue expression patterns of the two genes and proteins were identified by in situ hybridization and the transgenic plants harbouring SPX3pro-SPX3-GUS or SPX5pro-SPX5-GUS fusions, respectively. Both SPX3/5 are localized in the nucleus and cytoplasm in rice protoplasts and plants. SPX3/5 negatively regulate root-to-shoot Pi translocation with redundant function. The data showed that the Pi-starvation-accumulated SPX3/5 proteins are players in restoring phosphate balance following phosphate starvation. In vitro and in vivo protein–protein interaction analyses indicated that these two proteins can form homodimers and heterodimers, also implying their functional redundancy. Genetic interaction analysis indicated that SPX3/5 are functional repressors of OsPHR2 (PHR2), the rice orthologue of the central regulator AtPHR1 for Pi homeostasis and Pi signalling. These results suggest that the evolution of the additional redundant paralogous SPX genes is beneficial to plants recovering Pi homeostasis after Pi starvation by PHR2 pathway.

Key words: Negative regulation, Oryza sativa L., Pi homeostasis, protein interaction, SPX proteins.

Introduction

The maintenance of phosphate (Pi) homeostasis in plants is crucial for plant growth and development. Pi homeostasis is determined by Pi acquisition through the root system, loading to the xylem, translocation between root and shoot, and remobilization of internal Pi, which is achieved by coordination of different Pi transporters underlying an elaborate Pi-signalling network comprising local and systemic machineries (see review by Chiou and Lin, 2011; Wu et al., 2013).

The hydrophilic SPX domain (SYG1/Pho81/XPR1) is found at the N-termini of various proteins, particularly signal transduction proteins (Barabote et al., 2006). Increasing evidence shows that the proteins containing the SPX domain are key players controlling a set of processes involved in Pi homeostasis (Hamburger et al., 2002; Duan et al., 2008; Wang et al., 2009a; Lin et al., 2010; Liu et al., 2010, 2012; Kant et al., 2011). In plants, SPX-domain-containing proteins can be divided into four classes depending on the presence of additional protein domains. Proteins exclusively harbouring the SPX domain are referred to as SPX proteins and fall into class I, with four members in Arabidopsis and six members in rice (Duan et al., 2008; Secco et al., 2012). Phylogenetic analysis has revealed three evolutionary clades of these SPX proteins: clade I contains SPX1 and SPX2, clade III contains SPX4, and clade II contains SPX3 in Arabidopsis (AtSPX3) and three paralogous proteins in rice, designated SPX3, SPX5, and SPX6 (Secco et al., 2012).

Transcriptional and histochemical analyses have shown that all of the SPX genes, with the exception of SPX4, are...
responsive to Pi starvation (Duan et al., 2008; Wang et al., 2009b). The various subcellular localizations of the SPX proteins in Arabidopsis and rice have been described (Duan et al., 2008; Wang et al., 2009b), with results suggesting that the different SPX proteins may play distinct roles in Pi signalling and homeostasis processes.

In Arabidopsis, the expression of AtSPX1–4 genes is reduced to different extents in the phr1 mutant under Pi starvation (Duan et al., 2008), indicating that these genes are downstream of the central regulator AtPHR1 for Pi signalling. Overexpression of AtSPX1 upregulates Pi-starvation-induced (PSI) genes, such as ACP5, PAP2, and RNS1, suggesting a potential transcriptional regulation role of AtSPX1 in Pi starvation. Repression of AtSPX3 alters the response of PSI genes to Pi starvation, resulting in total phosphorus higher in shoots and lower in roots (Duan et al., 2008).

In rice, OsSPX1 (SPX1), similar to AtSPX3, is involved in negative regulation to adjust the expression of several PSI genes under Pi-limited conditions (Wang et al., 2009a). Genetic analysis has demonstrated that SPX1 counteracts the function of OsPHR2 (PHR2), a rice orthologue of AtPHR1 as a central Pi-signalling regulator (Rubio et al., 2001; Zhou et al., 2008; Liu et al., 2010), inducing expression of the low-affinity Pi transporter gene PtPT2, which plays a major role in Pi translocation and accumulation (Ai et al., 2009). The function of other SPX proteins in rice, however, has not been yet characterized.

In this study, a phylogenetic tree was generated from alignments of the protein sequences of all of the SPX proteins in representative dicotyledonous plants and cereal monocotyledonous crops. The results show that three paralogous SPX proteins, SPX3, SPX5, and SPX6, have evolved in cereal crops. The data reveal that SPX3 and SPX5 can form homodimers and/or heterodimers. SPX3 and SPX5 play roles in maintaining cellular Pi homeostasis when plants are exposed to an external change in Pi, and this implies a more controllable regulation system for crops to adapt to environmental Pi variations. In addition, genetic interaction analyses indicate that SPX3 and SPX5 are repressors of PHR2 function. This finding increases the understanding of the integrated regulation of SPX domain proteins in Pi homeostasis and signalling in plants.

Materials and methods

Plant materials and growth conditions

The spx3 T-DNA insertional mutant (ALNE05) (japonica cv. Nipponbare) was obtained from the CIRAD database (Centre de Cooperation International en Recherche Agronomique pour le Developpement; http://orygenesdb.cirad.fr/). The spx3 T-DNA insertion site at the second exon was determined by sequencing analysis (Supplementary Fig. S3, available at JXB online). The primers for the identification of the spx3 mutant are listed in Supplementary Table S1. The hydroponic experiments were performed using rice solution culture (Zhou et al., 2008). Rice plants were grown in a greenhouse with a 12/12 light/dark cycle (200mol m⁻² s⁻¹ photon density) at 30/22 °C after germination. Humidity was controlled at approximately 60%.

Cloning of SPX5 and SPX6

Total RNA was extracted from the rice seedling roots after 7 d of Pi-deficient treatment using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). SMART RACE cDNA Amplification Kit (Clontech, USA) was used for rapid amplification of cDNA ends (RACE); the primers for RACE-PCR are provided in Supplementary Table S1. The PCR products were gel purified and subcloned into the pGEM-T Easy vector (Promega) for sequencing.

Construction of the phylogenetic tree

Amino acid sequences of the tested species SPX proteins were retrieved from GenBank (http://www.ncbi.nlm.nih.gov/Genbank). The phylogenetic tree was constructed using MEGA 5.10 (Tamura et al., 2011) based on the neighbour-joining method with parameters of Poisson correction model, pairwise deletion, and bootstrap (1000 replicates; random seed).

In situ hybridization and GUS histochemical analyses

In situ hybridization was performed as described previously (Zhao et al., 2009). The primer pairs SPX3 insitu-F/SPX3 insitu-T7-R, and SPX5 insitu-F/SPX5 insitu-T7-R were used to amplify the anti-sense templates of SPX3 and SPX5. The primers SPX3 insitu-SP6-F/SPX3 insitu-R, and SPX5 insitu-SP6-F/SPX5 insitu-R were used to amplify the sense templates of SPX3 and SPX5. For development of SPX3pro-SPX3-GUS and SPX5pro-SPX5-GUS transgenic plants, the Nipponbare genomic DNA fragments containing the coding region of SPX3 or SPX5 and approximately 2.9-kb promoter sequence were amplified with primer pairs SPX3-GUS-F/SPX3-GUS-R and SPX5-GUS-F/SPX5-GUS-R, and cloned into the binary vector GUS-pBI101.3 by In-fusion kit (Clontech), respectively. The GUS constructs were introduced into rice plants (Nipponbore) using the Agrobacterium-mediated transformation. Histochemical GUS analysis was performed as described previously (Jefferson et al., 1987). The primers are listed in Supplementary Table S1.

Development of the transgenic plants

The transgenic plant with knockdown of SPX3 was developed using RNA interference (RNAi). A 260-bp fragment of SPX3 in sense and antisense orientations was inserted into both sides of the second intron of the maize NIR1 gene, and the fragment was subcloned into the 35S-pCAMBIA1300-mod vector. For development of transgenic plants with overexpression of SPX3 and SPX5 (SPX3/5), full-length SPX3/5 were cloned into the 35S-pCAMBIA1300-mod vector. These constructs were transformed into rice using the Agrobacterium-mediated transformation. The primers are listed in Supplementary Table S1.

Yeast two-hybrid assay

The yeast two-hybrid assay was performed using the Matchmaker GAL4 Two-Hybrid System3 (Clontech), following the manufacturer’s protocol. To construct SPX3-pGADT7 (AD-SPX3), SPX3-pGBK T7 (BD-SPX3), AD-SPX5, and BD-SPX5 vectors, full-length coding sequences of SPX3 and SPX5 were amplified by primer pairs SPX3-YF/SPX3-YR and SPX5-YF/SPX5-YR with adapters (Supplementary Table S1) for NdeI-BamHI and NdeI-EcoRI digestion sites, and cloned into pGBK T7 and pGADT7, respectively. The vectors were cotransformed into yeast strain AH109 with the resulting constructs and plated onto synthetic medium lacking Trp, Leu, His and Ade (SD/-Trp–Leu–His–Ade) or SD/-Trp–Leu. Plates were photographed after incubation at 30 °C for 3–5 d.

Coimmunoprecipitation assay

To construct the 35S-SPX3-MYC, 35S-SPX5-MYC, 35S-SPX3-FLAG, and 35S-SPX5-FLAG vectors, full-length coding sequences
of SPX3 and SPX5 were amplified by primer pairs SPX3-MYC-F/SPX3-MYC-R, SPX5-MYC-F/SPX5-MYC-R, SPX3-FLAG-F/SPX3-FLAG-R, and SPX5-FLAG-F/SPX5-FLAG-R (Supplementary Table S1), and were inserted into the modified pCAMBIA1300–35S-MYC vector (Hong et al., 2012) or 35S-FLAG vector (Menon et al., 2005), respectively. The resulting constructs were transiently expressed in tobacco leaves (Nicotiana benthamiana) by Agrobacterium tumefaciens EHA105 infiltration. The leaves were harvested after 2 d and lysed with a buffer (50 mM Tris, 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 1 mM PMSF, 100 μM MG132 (Sigma–Aldrich), and 1× protease inhibitor cocktail (Roche)). Lysates were incubated for 4 h with anti-FLAG antibody-conjugated beads (Sigma) at 4 °C. The beads were then washed three times and solubilized in 15 μl SDS sample buffer. Samples were analysed by 12% SDS-PAGE and immunoblotted as described in immunoblot analyses. Each immunoblot was incubated with the appropriate primary antibody (1:250; mouse anti-FLAG monoclonal or mouse anti-MYC monoclonal, Sigma) in 1× TBST for overnight at 4 °C. Membranes were developed using peroxidase-conjugated secondary antibody (anti-mouse IgG, Sigma), and proteins were detected by chemiluminescence using an ECL-detecting reagent according to the manufacturer’s protocol (Thermo Scientific).

Bimolecular fluorescence complementation assays

For the production of bimolecular fluorescence complementation (BiFC) vectors, the full-length coding sequences of OsSPX3/5 were amplified using the primer pairs SPX3–2YN-F/SPX3–2YN-R and SPX5–2YN-F/SPX3–2YN-R (Supplementary Table S1), and cloned into p2YN or p2YC as a fusion with the N-terminal or C-terminal fragment of YFP, resulting in SPX3–2YN-F/SPX3–2YN-R (Supplementary Table S1). The resulting constructs were transiently expressed in tobacco leaves by A. tumefaciens EHA105 infiltration. BiFC experiments were performed as described previously (Yang et al., 2007). YFP fluorescence was observed and photographed by confocal microscopy (LSM 510 META, Zeiss, Germany) at 48–72 h after infiltration.

RNA isolation, reverse-transcription PCR, and qRT-PCR

RNA isolation and quantitative real-time PCR (qRT-PCR) were performed as described previously (Liu et al., 2010). qRT-PCR for quantification of mature miR399 was performed following a published protocol (Varkonyi-Gasic et al., 2007). The primers for reverse-transcription PCR and qRT-PCR analyses are listed in Supplementary Table S1.

Measurements of Pi concentration and Pi uptake ability in plants

Measurement of Pi concentration, biomass, Pi uptake ability, and distribution in plants were performed as described previously (Zhou et al., 2008; Wu et al., 2011).

Results

SPX3, SPX5, and SPX6 are paralogous genes in crops

Six SPX genes (SPX1–6) have been identified in rice that are induced by Pi starvation with the exception of SPX4 (Wang et al., 2009b). The current data further showed that SPX3, SPX5, and SPX6 are specifically induced by Pi starvation, which was remarkably reduced in the phr2 mutant (Supplementary Fig. S1; Chen et al., 2011). The full-length cDNA sequences of SPX1–4 are available in the Rice Genome database (http://rice.plantbiology.msu.edu/). To generate a rooted phylogenetic tree for all of the SPX proteins, full-length cDNA of SPX5 and SPX6 was cloned using RACE assays based on the conserved SPX domain sequence in the rice genome (Rice Genome Annotation Project). The deduced full-length cDNA sequence of SPX5 (GenBank accession number KF267997) consists of 1090 nucleotides, containing an open reading frame of 744-bp for 247 amino acids, a 72-bp 5′-untranslated region, and 274-bp 3′-untranslated region; SPX6 (GenBank accession number KF267998) consists of 1045 nucleotides, containing an open reading frame of 705-bp for 234 amino acids, a 116-bp 5′-untranslated region, and a 239-bp 3′-untranslated region. The gene structure analysis showed two exons and one intron in SPX5 and SPX6, respectively (Fig. 1A).

A phylogenetic tree was generated from alignments of the sequences of the six SPX proteins in dicotyledonous plants, monocotyledonous cereal crops, and the ancestral genome Chlamydomonas reinhardtii. The dicotyledonous plants included Arabidopsis, Chinese cabbage (Brassica rapa), and...
grape (*Vitis vinifera*), and monocotyledonous cereal crops included rice (*Oryza sativa* L.), maize (*Zea mays*), and sorghum (*Sorghum bicolor*). Banana (*Musa acuminate*), a monocotyledonous herb of the order Zingiberales and a sister group to the well-studied Poales including cereals (D’Hont et al., 2012), was also analysed. The phylogenetic analysis showed that the six SPX proteins fall into three clades (Fig. 1B). Clades I and III contain SPX1 and SPX2, and SPX4, respectively, with a more divergent evolutionary relationship between dicotyledonous and monocotyledonous plants. Clade II contains SPX3, SPX5, and SPX6 proteins, which fall into three subgroups in monocotyledonous crops, while SPX3 is only present in the dicotyledonous plants *Arabidopsis* and grape. Chinese cabbage and banana contain two copies of SPX3 as sister pairs. The results indicate that the three paralogous genes (SPX3, SPX5, and SPX6) are evolved in cereal crops.

**Subcellular localization and expression patterns of SPX3 and SPX5**

This work examined the subcellular localization of the paralogous proteins in rice protoplasts and rice plants. The similar subcellular localizations of SPX3 and SPX5 (SPX3/5) in both nucleus and cytoplasm were visualized using the fusions of SPX3-GFP and SPX5-GFP (Supplementary Fig. S2). Because of the same subcellular localization of SPX3/5, this study focused on the functional relationship of these two SPX proteins.

The tissue expression patterns of SPX3/5 were investigated using *in situ* hybridization with probes specific to SPX3 or SPX5 (Supplementary Table S1). The roots of 15-d-old plants treated with Pi starvation for 7 d had an overlapped hybridization signal of SPX3/5 in root epidermis, exodermis, and the sclerenchymal layer. The hybridization signal was also detected for SPX3 in cortex and endosperm (Fig. 2A–F). Under Pi starvation, the specific hybridization signals of SPX3/5 were present in mesophyll and phloem in the vascular bundles (Fig. 2G–L). The signal was highly specific and did not appear in leaf and root tissues when using control sense probes. To determine the expression patterns of SPX3/5 proteins, this work developed transgenic plants harbouring the SPX3pro-SPX3-GUS or SPX5pro-SPX5-GUS fused gene. Histochemical analyses of GUS activity in the transgenic plants grown under Pi-supplied (200 µM Pi) and Pi-deficient conditions showed

**Fig. 2.** Expression patterns of SPX3/5 in roots and leaf blades indicated by *in situ* hybridization. (A–F) *In situ* hybridization on cross-sections of primary roots with SPX3 or SPX5 antisense probes (B and E); negative control with sense probes (A and D); and detailed antisense signals (C and F). (G–L) *In situ* hybridization on cross-sections of leaf blades with SPX3 or SPX5 antisense probes (H and K); negative control with sense probes (G and J); and detailed antisense (I and L). Roots and leaves were sampled from 20-d-old seedlings treated with Pi starvation for 7 d. Ab, abaxial; Ad, adaxial; Co, cortex; Ep, epidermis; Ex, exodermis; Mes, mesophyll; Mtx, metaxylem; Ph, phloem; Sc, sclerenchyma layer; St, stele; Xy, xylem. Bars, 100 µm.
that SPX3/5 were induced under deficient Pi with consistent patterns of their transcripts, as indicated by in situ hybridization. The same expression patterns of SPX3/5 were observed in roots and leaf blades (Fig. 3A–T). GUS activity was also detected in leaf sheath, node, stem vascular bundle, hull, and anther (Fig. 3U–Af). The same general expression patterns of SPX3/5 suggest that they may have similar functions.

**SPX3/5 redundantly modulate Pi homeostasis**

This work isolated a spx3 mutant (Nipponbare, ALNE05) from the CIRAD T-DNA insertion library (Supplementary Fig. S3A–C). Because the spx5 mutant is unavailable, the transgenic plants (Nipponbare) with repression of SPX5 were developed using RNAi (Supplementary Fig. S3D, G).

**Fig. 3.** Tissue expression patterns of SPX3 and SPX5 indicated by expression SPX3pro-SPX3-GUS and SPX5pro-SPX5-GUS fusions in the transgenic plants. (A–P) SPX3 and SPX5 expression in root tips (A, E, I, M) and lateral roots (B, F, J, N) under +P (200 μM Pi) or –P conditions (0 μM Pi); bars, 200 μm and SPX3 and SPX5 expression in cross-sections of primary roots (C, G, K, O) and lateral roots (D, H, L, P) under +P or –P conditions; bar, 20 μm. (Q–T) SPX3 and SPX5 expression patterns in cross-section of leaf blade under +P (Q, S) or –P (R, T) conditions; bar, 20 μm. (U–Af) GUS staining on hull (X, Ad), stigma (Y, Ae), anther (Z, Af), and cross-sections of leaf sheath (U, Aa), stem (V, Ab), and node (W, Ac). Bars, 50 μm (U–Z, Aa, Ad–Af; 400 μm (V, W, Ab, Ac).
The plants with repression of \textit{SPX5 (RiSPX5)} under the \textit{spx3} mutant background were developed by a cross between the homozygous \textit{spx3} mutant and plants harbouring a single copy of a plasmid containing the \textit{SPX5-RNAi} vector (designated \textit{spx3/RiSPX5}). The wild-type plants (Nipponbare), \textit{spx3} mutants, two independent lines of \textit{RiSPX5} plants (\textit{RiSPX5-1} and \textit{RiSPX5-3}), and \textit{spx3/RiSPX5} plants were used for cellular Pi concentration and Pi signalling analyses in hydroponic cultures. Under both high Pi (200 \text{M} \text{Pi}) and low Pi (20 \text{M} \text{Pi}) conditions, no significant phenotypic difference between wild-type plants and \textit{spx3} mutants or \textit{RiSPX5} plants was observed (data not shown), while the growth of \textit{spx3/RiSPX5} plants was significantly inhibited compared to wild-type plants (Fig. 4A–D). A significantly higher shoot Pi concentration was observed in \textit{spx3/RiSPX5} plants compared to wild-type plants, but not in \textit{spx3} mutants or \textit{RiSPX5} plants (Fig. 4E). No statistically significant difference was observed in root Pi concentration between wild-type and \textit{spx3/RiSPX5} plants under either Pi condition (Fig. 4G). The significantly higher Pi-uptake ability and shoot-to-root ratio of Pi in \textit{spx3/RiSPX5} plants compared to wild-type plants were confirmed by \textsuperscript{33}P-labelled Pi uptake and concentration ratio of shoots to roots (Fig. 4F, H). These results indicated the redundant negative effect of SPX3 and SPX5 on Pi homeostasis.

\textit{SPX3} and \textit{SPX5} negatively regulate Pi signalling

The redundant negative effect of \textit{SPX3} and \textit{SPX5} on Pi signalling was indicated by the expression of the Pi-starvation-responsive genes \textit{IPS1}, \textit{miR399}, \textit{PT2}, \textit{PHO2}, \textit{SPX6}, \textit{miR827}, \textit{PAP10}, and \textit{SQD2}. Significantly different transcript accumulation levels of the tested genes in \textit{spx3/RiSPX5} plants grown under Pi-sufficient (200 \text{M} \text{Pi}) condition were detected compared with wild-type, \textit{spx3} mutant, and \textit{RiSPX5} plants (Fig. 5A, B). Under Pi deficiency for 10 d, however, no significant difference in Pi signalling between wild-type and \textit{spx3/RiSPX5} plants was observed (data not shown). Given that both transcripts and proteins of \textit{SPX3/5} are induced by Pi starvation and that induced protein levels were maintained for a longer time than the induced transcript levels by recovery of Pi after Pi starvation (Supplementary Fig. S4), it was hypothesized that the induced proteins might play a role in Pi signalling. To investigate this possibility, this work tested the reduction of Pi-starvation signalling in a Pi-recovery time course after Pi starvation for 10 d. The elapsed time of reduction of Pi-starvation signalling after Pi recovery in \textit{spx3/RiSPX5-3} plants was prolonged compared to wild-type plants in roots (Fig. 5C–F), and the similar trend was observed in shoots (data not shown), confirming that \textit{SPX3/5} proteins are involved in a negative regulation of Pi signalling.

\textit{SPX3} and \textit{SPX5} are repressors of function of PHR2

Previous reports have demonstrated that \textit{SPX1} is a repressor of \textit{PHR2} function (Wang et al., 2009a; Liu et al., 2010). To determine whether \textit{SPX3/5} have the similar function, this work first developed \textit{SPX3}-overexpressing and \textit{SPX5}-overexpressing plants. Two independent lines with

\textsuperscript{33}P-labelled Pi uptake and concentration ratio of shoots to roots (Fig. 4F, H). These results indicated the redundant negative effect of SPX3 and SPX5 on Pi homeostasis.
overexpression of SPX3 or SPX5, as confirmed by Southern blotting (Supplementary Fig. S3E, F, and H), were used for the hydroponic experiments with high Pi (200 µM Pi) and low Pi (20 µM Pi) levels. Similar to SPX3 overexpressors, overexpression of SPX5 inhibited plant growth under both Pi levels (Fig. 6A, B; Supplementary Fig. S5; Wang et al., 2009b). Significantly lower shoot and higher root Pi concentrations were observed in the overexpressors compared with the wild-type plants under both Pi levels (Fig. 6C, D). These results indicate the similar function of SPX3 and SPX5 in root-to-shoot Pi translocation. Then, the transcript accumulation levels of these PSI genes downstream of PHR2 were tested in the plants with overexpressed SPX3/5. Compared with wild-type plants, a significant reduction of IPS1, miR399, PT2, and SPX6, and increase of PHO2 in shoots and roots under the Pi-supplied conditions were observed (Fig. 6E–H).

This work developed plants simultaneously overexpressing SPX3 and PHR2 or SPX5 and PHR2 through crossing between the plants with overexpressed PHR2 (Zhou et al., 2008) and overexpressed SPX3 or SPX5 plants (designated OxPHR2/OxSPX3 and OxPHR2/OxSPX5). As with SPX1, overexpression of SPX3 or SPX5 completely reduced excessive Pi accumulation in the shoots (Fig. 7A). The upregulation of IPS1, miR399, and PT2, and the reduction of PHO2 driven by PHR2 overexpression, was repressed by overexpression of SPX3/5 in roots (Fig. 7B), and the similar results were observed in shoots (data not shown). The results indicated that SPX3/5 are the repressors of PHR2.

Protein interaction between SPX3 and SPX5

The redundancy and the overall similar tissue expression patterns of SPX3/5 led to the hypothesis that the two proteins might interact with each other. Yeast two-hybrid assays were performed to explore this idea. The results showed that SPX3/5 can form homodimers as well as heterodimers in yeast cells (Fig. 8A). To verify the interactions, this work performed coimmunoprecipitation assays using transiently expressed proteins of SPX3-MYC or SPX3-FLAG, and SPX5-MYC or SPX5-FLAG in tobacco leaves. The coimmunoprecipitation assays for the combinations of fusions showed results consistent with those in yeast cells (Fig. 8B). In vivo interactions between SPX3 and SPX5, or with themselves, were also indicated by BiFC assays in tobacco leaves (Fig. 8C). Taken together, the findings suggest that SPX3/5 can form homodimers and heterodimers in vitro and in vivo, which supports the concept of their redundant effect, and implies a sophisticated regulation in modulating Pi homeostasis and signalling.

Discussion

This work reports that SPX3/5 evolved in cereal crops redundantly modulate Pi homeostasis and signalling. The data indicate that the SPX proteins act as repressors of PHR2, the rice orthologue of AtPHR1, which is a central regulator of Pi signalling and homeostasis (Rubio et al., 2001; Bustos et al., 2010). A previous report described SPX1 as repressing PHR2 by counteracting PHR2 function (Liu et al., 2010).
The functional similarity of SPX3/5 to SPX1 found in this study indicates an integrated negative regulation system of Pi signalling and homeostasis by SPX proteins in crops. The role
of SPX1 in negative regulation of PHR2 has been reported (Liu et al., 2010), and the current findings provide evidence that the negative regulation of Pi homeostasis and signalling involves different SPX proteins. The evolution of the additional redundant paralogous SPX genes in crops implies a more controllable regulation system for crops to adapt to environmental Pi variations.

**SPX3 and SPX5 are paralogous genes evolved in cereal crops**

Four and six SPX proteins have been identified in Arabidopsis and rice, respectively, designated as AtSPX1–AtSPX4 and OsSPX1–OsSPX6 (SPX1–6) (Duan et al., 2008; Wang et al., 2009b). These SPX proteins are grouped into three evolutionary clades (Secco et al., 2012). This study cloned the full-length SPX3 and SPX6 through RACE assays and analysed the phylogenetic relationship of the six SPX proteins in dicotyledonous and cereal monocotyledonous plants to investigate whether the two additional SPX proteins in rice had evolved in other crops. The rooted phylogenetic tree generated from alignments of the SPXs in the tested plants and the ancestral genome C. reinhardtii indicated that SPX3, SPX5, and SPX6 are paralogous genes in cereal crops (Fig. 1). There were two copies of SPX3 and SPX4 and three SPX1 copies in banana. Three rounds of whole-genome duplications in the Musa lineage, independently of those previously described in the Poales lineage, appear to have occurred, and the earliest duplication in the Musa lineage is not shared with Poaceae (D’Hont et al., 2012). The three copies of paralogous SPX1 and two copies of SPX3 and SPX4 in the Musa imply these independent whole-genome duplications. The classical models predict that the most likely fate of duplicate gene pairs is degeneration into a pseudogene or loss from the genome because of the vagaries of chromosomal remodelling, locus deletion, or point mutation (in a process known as nonfunctionalization; Prince and Pickett, 2002). The results indicate that the paralogous genes SPX3, SPX5, and SPX6 might have been maintained in cereals during their whole-genome duplication (Tang et al., 2010; Wang et al., 2011) but not in dicotyledonous plants. The redundancy of the paralogous SPX3/5 as repressors of the central regulator of PHR2 of Pi homeostasis and signalling presented in this study implies that a more controllable response to Pi starvation evolved in crops.

**SPX3/5 redundantly modulate root-to-shoot Pi translocation**

Both Pi inadequacy and Pi excess are detrimental to plant growth. Therefore, Pi homeostasis is required for plant growth and development, and the regulatory mechanism of Pi homeostasis is conserved in many plant species (Chiou and Lin, 2011). The current data showed that SPX3/5 redundantly modulate root-to-shoot Pi translocation, as the following evidence supports: (1) spx3/RisSPX5 plants, but not the spx3 mutant or the plants with knockdown of SPX5, displayed increased shoot Pi accumulation, higher Pi uptake activity, and Pi translocation from root-to-shoot; (2) overexpression of SPX3/5 suppressed root-to-shoot translocation of Pi; and (3) SPX3/5 can form homodimers and heterodimers (Figs. 4, 6, and 8).
SPX3/5 were expressed in root epidermal cells, including epidermis, exodermis, and the sclerenchyma layer (Figs. 2 and 3). From this expression pattern, it was expected that SPX3/5 would be involved in Pi uptake. The transgenic plants with overexpressed SPX3/5, however, showed decreased root-to-shoot Pi translocation, indicating a main function of SPX3 and SPX5 in Pi translocation from root to shoot. The genetic interaction analysis indicated that overexpression of SPX3 and SPX5 completely rescued the excessive shoot Pi accumulation and the upregulation of PSI genes driven by overexpression of PHR2 (Fig. 5). PHR2 directly upregulates PT2, and mutation of PT2 almost completely rescues the excessive shoot Pi accumulation caused by overexpression of PHR2 (Liu et al., 2010), indicating that the PHR2-driven excessive accumulation of shoot Pi is largely attributable to upregulation of PT2. Tissue expression patterns show that PT2 is predominantly expressed in root stele and the sclerenchyma layer and in leaf mesophyll and phloem in the vascular bundle (Ai et al., 2009). The overlapped expression patterns of SPX3/5 and PT2 and the negative regulation of transcript accumulation levels of PT2 by SPX3/5 may, at least partially, explain the function of SPX3 and SPX5 in Pi translocation.

The central pathway of Pi signalling and homeostasis under the control of the partially redundant transcription factors AtPHR1/AtPHL1 (PHR1-like) is conserved in plants (Rubio et al., 2001; Duan et al., 2008; Valdés-López et al., 2008; Zhou et al., 2008; Wang et al., 2009a, 2013; Bustos et al., 2010; Ren et al., 2012). The non-coding RNAs IPS1 and miR399 are specifically responsive to Pi starvation as the targets of AtPHR1. miR399 targets the mRNA of PHO2 and directs the cleavage of PHO2 mRNA, which encodes a ubiquitin-conjugating E2 enzyme (Aung et al., 2006; Bari et al., 2006). Reciprocally, IPS1 represses miR399 through a mechanism termed ‘target mimicry’, interfering with miR399 targeting of PHO2 mRNA (Franco-Zorrilla et al., 2007). Loss of function of PHO2 leads to excessive shoot Pi accumulation. PHO1 mediates Pi loading to the xylem and consequently controls the root-to-shoot Pi translocation (Poirier et al., 1991; Hamburger et al., 2002; Secco et al., 2010). Recently, PHO2-dependent degradation of PHO1 as a function of cellular Pi concentration was described (Liu et al., 2012). miR399, PHO2, and PHO1 form a branch of the Pi-signalling network downstream of AtPHR1. The current data showed that transcript accumulation levels of PHO2 are significantly suppressed in spx3/RiSPX5 plants and conversely in plants with overexpressed SPX3/5 (Figs. 5 and 6). Thus, the alteration of transcript accumulation levels of PHO2 regulated by SPX3/5 through their negative effect on PHR2 can be inferred. This finding increases the understanding of SPX domain proteins in regulated Pi homeostasis and signalling under the control of PHR2 in rice.

SPX3 and SPX5 are involved in fine-tuning Pi signalling

When plants are exposed to Pi starvation, a large proportion of the transcriptional activation and repression as an integral part of the Pi-starvation response is under the control of the partially redundant transcription factors AtPHR1 and AtPHL1 (PHR1-like) (Bustos et al., 2010). This genome-wide reprogramming of genes has been suggested to inhibit plant growth, but not as a direct consequence of Pi deficiency (Rouached et al., 2011). The current data showed that SPX3/5 are positively responsive to Pi starvation largely under the control of PHR2 and that their proteins accumulated under Pi starvation (Supplementary Figs. S1 and S4). Overexpression of SPX3/5 and genetic interaction analysis indicated that SPX3/5 are repressors of PHR2 function (Figs. 6 and 7). Thus, it was expected that double repression of SPX3/5 would enhance Pi-starvation signalling. Under Pi-supplied conditions, significant upregulation of PSI genes downstream of PHR2 in spx3/RiSPX5 plants was found (Fig. 5); however, the expected enhanced Pi signalling under Pi starvation was not. SPX6, as the paralogous gene of SPX3/5, may play a compensatory role (Figs. 5 and 6), and SPX1 RNAi lines are correlated with increased transcript accumulation levels of some PSI genes (Wang et al., 2009a), so it is speculated that SPX3/5 might be involved in a fine-tuned regulation of Pi signalling together with other SPX proteins. This reasoning is supported by the results that Pi-starvation-accumulated SPX3/5 transcripts played a negative role in reduction of Pi-starvation signalling in a time course of Pi recovery after Pi starvation (Fig. 5). The Pi-starvation-induced transcripts of SPX3/5 were rapidly suppressed by Pi recovery over 12 h while the induced protein levels were maintained for a longer time after Pi recovery (Supplementary Fig. S4). This result suggests that, following reduction of Pi-starvation-induced expression of SPX3/5, the Pi-starvation-accumulated SPX3/5 proteins still played a negative role in Pi-starvation signalling. This negative role should be beneficial to plants in recovering Pi homeostasis after Pi starvation.

**Supplementary material**

Supplementary data are available at *JXB* online.

**Supplementary Fig. S1.** SPX3/5/6 are specifically induced by Pi starvation largely under the control of PHR2.

**Supplementary Fig. S2.** Subcellular localization of SPX3/5.

**Supplementary Fig. S3.** Isolating the spx3 mutant and development of the transgenic plants.

**Supplementary Fig. S4.** Time course of transcript and protein levels of SPX3/5 during Pi supply after Pi starvation.

**Supplementary Fig. S5.** Biomass of shoots and roots of SPX3/5-overexpressed plants.

**Supplementary Fig. S6.** qRT-PCR analysis for transcript accumulation levels of PHR2 and SPX3 in OxPHR2/OxSPX3-1 and of PHR2 and SPX5 in OxPHR2/OxSPX5-1 plants.

**Supplementary Table S1.** Primers used in this study.

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