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P1BS, a conserved motif involved in tolerance to phosphate starvation in soybean

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ABSTRACT. Available phosphate (Pi) is a major limiting factor for plant growth, development, and productivity. Phosphate starvation response 1 (PHR1) is a binding dimer that binds to an imperfect palindromic sequence. PHR1-binding sequences (GnATATnC) exist in the promoter of Pi starvation-responsive structural genes, indicating an effect occurring downstream in the Pi starvation signaling pathway. These sequences are referred to as PHR1-binding site (P1BS) structures. In this study, the sequences of *GmPHR1* and *GmSPX1* from *Glycine max* (L.) Merr. soybean were determined and analyzed. We found that *GmPHR1* is an MYB-related transcription factor. In addition, *GmSPX1* contained a P1BS structure, which is an important *cis*-regulatory motif in the phosphate signaling pathway. We found that *GmPHR1* can physically interact with *GmSPX1* through the *cis*-element, which may be a major pathway for the *GmPHR1*-mediated Pi starvation stress response. Thus, the P1BS structure in the Pi starvation signaling pathway is an important *cis*-regulatory motif that improves the tolerance to low phosphorus conditions in soybean.

Key words: *Cis*-regulatory motif; Soybean; *GmPHR1*; *GmSPX1*; Phosphate starvation

INTRODUCTION

Phosphorus (P) is an essential macronutrient in plants. It plays a key role in growth and metabolism, and is an indispensable element in nucleic acids, phospholipids, and ATP. Pi availability is limited in most soils worldwide, particularly in tropical and subtropical areas (Vance et al., 2003; Wang et al., 2010). However, in many natural and agricultural ecosystems, plants often face conditions in which Pi concentrations in the soil solution rarely exceed 2 mM and cellular Pi concentrations are greater than 10 mM. Thus, plants must absorb Pi into the roots against a steep concentration gradient (Mimura, 1999; Raghothama, 2000).

To cope with low-phosphate conditions, plants have evolved tightly controlled mechanisms to maintain phosphate homeostasis, including the development of lateral roots and root hairs as well as more dramatic root structures such as proteoid and dauciform roots, the secretion from roots of phosphatases and organic acids, and the induction of high-affinity and some low-affinity Pi transporters (phosphate transporter, PT). Many plants have also established symbiotic associations with mycorrhizal fungi that aid in Pi acquisition. A variety of adaptive strategies has evolved in plants to alleviate or help plants overcome Pi deficiency. The implementation of these strategies requires changes in the expression profiles of hundreds of genes. The extent and complexity of the network of regulatory genes necessary to sense Pi status and regulate the deployment of these adaptive strategies has been examined previously. The network components identified thus far include transcription factors, SPX sub-family proteins, non-coding RNAs, and protein modifiers, including proteins involved in SUMOylation, phosphorylation, dephosphorylation, and protein translocation. However, the regulatory mechanism of Pi homeostasis in plants is not well-understood.

In yeast (*Saccharomyces cerevisiae*), the regulatory mechanism of Pi homeostasis driven by the phosphate signal transduction pathway, has been widely examined (Pinson et al., 2004). In the white lupin (*Lupinus albus*), the expression of *LaSAP1* (acid phosphatase) in phosphorus deficiency proteoid roots depends on DNA located from -465 to -345 base pairs (bp) 5' of the ATG start codon and that the PHR1 binding site (P1BS) element located at -160 bp, and contributes to regulatory control (Zinn et al., 2009). However, knowledge of the regulatory mechanism of Pi homeostasis in plants remains lacking. The promoters of 3 white lupin genes that are upregulated in P-deficient proteoid roots (*LaSAP1*, *LaPT*, and *LaMATE*) also contain P1BS elements (Zinn et al., 2009). The *Arabidopsis thaliana* transcription factor phosphate starvation response 1 (*AtPHR1*) is a well-characterized member of the MYB family and shares homology with the *Chlamydomonas reinhardtii* phosphate starvation response 1 (*CrPSR1*) gene (Rubio et al., 2001). The DNA motif to which PHR1 binds, the P1BS element (GnATATnC), is present in promoters of a number of genes from several plant species. The barley (*Hordeum vulgare*) phosphate transporter (*HvPht1*;*1*) contains 3 P1BS elements. Expression of *HvPht1*;*1* in transgenic rice *(Oryza sativa*) requires a single P1BS element in addition to 195 bp of promoter DNA located immediately upstream of the transcriptional start site (Schünmann et al., 2004).

Although several transcription factors, such as *AtPHR1* (*OsPHR2*), *OsPTF1*, *AtBHLH32*, *AtWRKY75*, *AtZAT6*, and *AtMYB62*, have been reported to be involved in the adaptation of plants to low-P conditions and were shown to regulate the expression of a subset of Pi starvation-inducible genes (Rubio et al., 2001; Yi et al., 2005; Zhou et al., 2008; Devaiah et al., 2007a,b, 2009), only the PHR1-binding *cis*-acting element P1BS (GnATATnC) was found to be involved in the activation of Pi starvation-inducible genes, including phosphate transport-

ers (Rubio et al., 2001; Schünmann et al., 2004).

AtPHR1 is an MYB domain transcription factor and a key regulator of the Pi signaling pathway in *Arabidopsis* (Rubio et al., 2001). Overexpression of *AtPHR1* leads to increased Pi concentrations in aerial parts of the plant, which induces the expression of a series of Pi starvation response genes, including PT, phosphatase, and RNase (Nilsson et al., 2007). There are 2 genes that are homologous to *AtPHR1* in rice, including *OsPHR1* and *OsPHR2*. These genes are involved in the signal transduction pathway of Pi; over-expression of *OsPHR2* causes the above-ground parts of the plant to excessively accumulate Pi, and there were similar phenotypes with its mutant phenotype (pho2) in high phosphorus conditions (Zhou et al., 2008; Wang et al., 2009). *OsPHR2* is homologous to the *AtPHR1* gene; its protein product belongs to the MYB family (Zhou et al., 2008). *AtPHR1* combines with one type of P1BS *cis*-element (GnATATnC), resulting in altered expression of various low-phosphorus responsive genes (Rubio et al., 2001). This suggests the presence of a regulatory region for *GmSPX1* expression at -178 to -200 bp upstream of the start ATG. The *GmPHR1* and P1BS element may interact, and there is a P1BS *cis*-element in upstream of *OsPT2* (Liu et al., 2010). Although numerous recent studies have examined Pi uptake and the Pi-signaling system (Fujii et al., 2005; Bari et al., 2006; Chiou et al., 2006; Shin et al., 2006; Chen et al., 2007; Devaiah et al., 2007a,b), the molecular network underlying plant adaptation in Pi starvation remain unknown.

In this study, we cloned a number of related genes, such as *PHRs* and *SPXs* (*Syg1*, *Pho81*, and *XPR1*). We identified a *cis*-element which in the upstream region of *GmSPX1* and used bioinformatics to analyze the structure of these PHRs and SPXs. In addition, we demonstrated that *GmPHR1* can physically interact with *GmSPX1* through the *cis*-element, which may be the major pathway for *GmPHR1* repression responses to phosphate starvation in soybean.

MATERIAL AND METHODS

Plant materials and growth conditions

Soybean (*G. max* L.) 'Gantai' was used to isolate the *GmPHR1* and *GmSPX1* genes. Three-week-old seedlings grown at 28/23°C with a 16/8 h (light/dark) photoperiod in an artificial climate box. Leaves were harvested then stored at -70°C until RNA extraction.

Gene cloning

Using the *AtPHR1* and *AtSPX1* nucleotide sequence (Accession no. AJ310799 and AT5G20150.1) as the query, a BALSTN (Altschul et al., 1990) search was conducted on the web page of the phytozome9.1 (http://www.phytozome.net/search.php?show=blast) to identify sequences containing *AtPHR1* orthologs in the soybean genomic database. Two pairs of primers were used for polymerase chain reaction amplification: forward, 5'-CCTTTCCAATT TTCTTCTACCTTCC-3'; reverse, 5'-TCACATGATGTGGTCTATCTGTATC-3'; for *GmSPX1* forward, 5'-ATGAAATTCGGGAAGAGCCTGAGC-3'; reverse, 5'-TTACTTGGCTGCTTGT TCCAG-3'. Full-length cDNA was amplified from the root cDNA template using the primers at the end of the cDNA sequence and then cloned into the pMD-19 Simple T vector (Takara, Shiga, Japan) for sequencing. The M1E-F primer had the following sequence: 5'-CTCTTTTTA GGGAGATTCCAACAATACC-3', with the underline indicating the mutation site. The M1E-R primer had the following sequence: 5'-TATATTTGGGGTTTTATTCCTCTCTC-3'. The *Gm-*

SPX1 ATG upstream sequence fragment was named ProS1 (product of GmSPX1): ProS1-F: 5'-TGTCATCGTAGCCTATCTAGTTG-3', ProS1-R: 5'-TCCGTTAGCAAAGGGAAAATAA C-3', with an amplified fragment size of 724 bp. The primer design included 1 or 2 P1BS *cis*-acting element fragments (named Pro1E and Pro2E): Pro1E-F: 5'-CGAGCTCTGTCATCGT AGCCTATCT-3', Pro1E-R: 5'-CCGCTCGAGGGAGGGTATTGTT-3', with an amplified fragment size of 562 bp. Pro2E-F: 5'-CGAGCTCTGTCATCGTAGCC TATC-3', Pro2E-R: 5'-CCGCTCGAG TCCGTTAGCAAAG-3', with an amplified fragment size of 740 bp. Designed primers for the open reading frame of *GmPHR1* were named AD-F and AD-R. AD-F: 5'-TCCCCCGGGATGAATGAGAACAAA-3', AD-R: 5'-CCGCTCGAGTTGGCTTGGAG A-3', with an amplified fragment size of 1299 bp. The fragment was subcloned into the vector pGADT7 AD.

Sequence analysis

Sequence analysis was conducted using the ANTHEPROT (Deléage et al., 2001), Lasergene version 7.0.1, and the DNAMAN version 6.0.3.93 software. We used ScanProsite to scan the protein sequences to identify patterns stored in the PROSITE database (Sigrist et al., 2010). The ScanProsite tools are available on the ExPaSy Molecular Biology of Geneva (Switzerland) website (http://expasy.org/tools/scanprosite/). Multiple sequence alignment was conducted using ClustalW (Thompson et al., 1994).

RNA isolation and cDNA synthesis

Total RNA was extracted using the Total RNA Plant Extraction Kit (Tiangen, Beijing, China) according to the manufacturer protocol. First-strand cDNA was synthesized using the PrimeScript first-strand cDNA Synthesis Kit (Takara) according to the manufacturer instructions.

Yeast one-hybrid assays

Pro1E and Pro2E fragment was cloned into the pAbAi vector, transformed into the yeast strain Y1HGold, and screened on synthetic defined (SD)/-uracil plate. The transformed yeast strain was measured to determine the minimum Aureobasidin A (AbA) inhibitory concentration. The pGADT7 AD plasmids were transformed into Y1HGold pAbAi::Pro1E strains and screened on an SD/-Leu plate. Positive single colonies were confirmed on an SD/-Leu-AbA plate.

RESULTS

Genes cloning and sequence analysis of GmPHR1 and GmSPX1 in soybean

To determine the molecular mechanisms underlying the responses to phosphate starvation in soybean, we cloned soybean genes likely to be involved in this process. Because the whole soybean genome sequencing was completed in 2008 (Schmutz et al., 2010), we used the *AtPHR1* (Accession no.: AJ310799) and *AtSPX1* (Accession no.: AT5G20150.1) nucleotide sequences in a query of BALSTN (Altschul et al., 1990) on the phytozome web page (http://www.Phytozome.net/search.php?Show=blast) to identify sequences containing *AtPHR1* and *AtSPX1* orthologs in the soybean genomic database, respectively, which were named *Gm-*

PHR1 and *GmSPX1*. Based on the sequence obtained, specific primers for each sequence for the full-length candidate genes were designed. *GmPHR1* is 1521 bp and contains an open reading frame encoding a 427-amino acid polypeptide (Figure 1A). *GmSPX1* is 868 bp and contains an open reading frame encoding a 295-amino acid polypeptide (Figure 1B). Sequence identity matrix of PHR (Table 1) shared a lower degree of similarity with other species. However, *GmPHR1* and *GmSPX1* were highly conserved (Figure 1) compared with other species. The P1BS element played a role in the expression of a number of P-deficiency responsive genes in the white lupin (*Lupinus albus*).

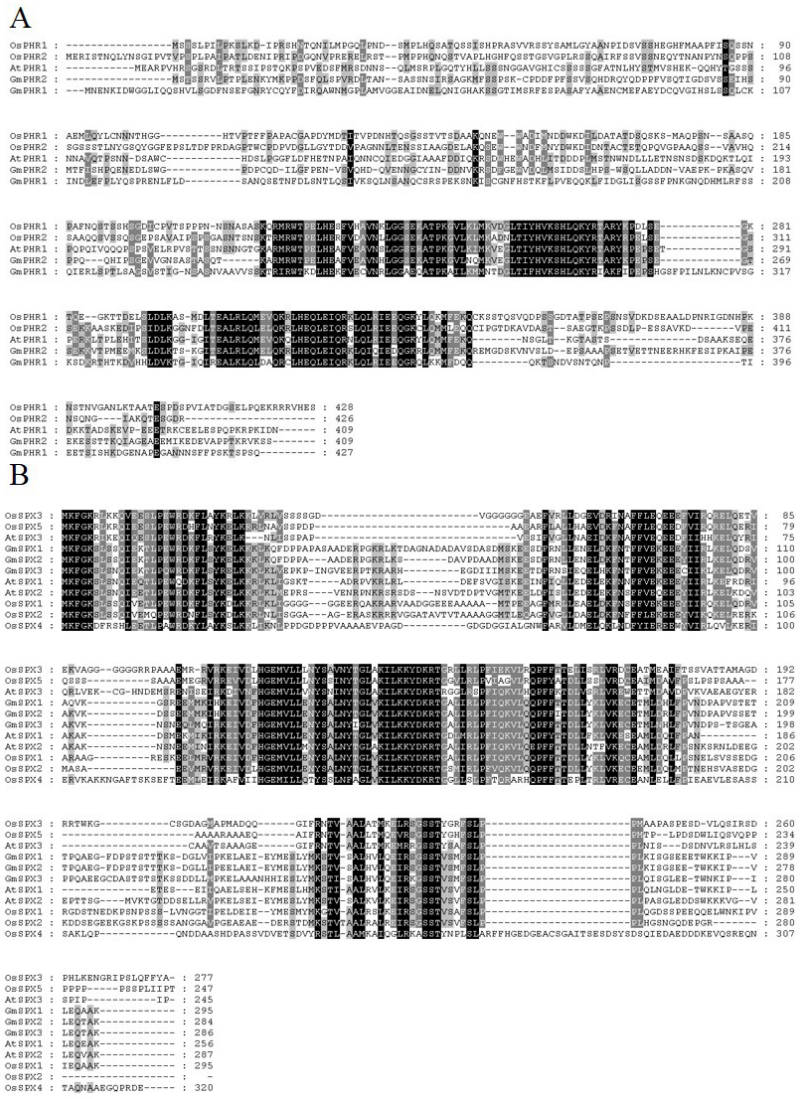

Figure 1. Alignment of amino acid sequences. (**A**) Alignment of amino acid sequences of *GmPHR1*, *GmPHR2*, and other plant PHR protein. (**B**) Alignment of amino acid sequences of *GmSPX1-3* and other plant SPX protein.

Table 1. Sequence Identity Matrix of PHR.

	AtPHR1	OsPHR1	OsPHR2	GmPHR1	GmPHR2
AtPHR1	1.000	0.093	0.093	0.053	0.073
OsPHR1		1.000	0.077	0.044	0.100
OsPHR2			1.000	0.067	0.075
GmPHR1				1.000	0.044
GmPHR2					1.000

P1BS cis-acting regulatory element

Located in the nucleus, *AtPHR1* can bind as a dimer to the PHR1 binding site via its MYB domain (P1BS, sequence GnATATnC), which is a motif present in the promoters of crucial Pi-responsive genes (Rubio et al., 2001; Schünmann et al., 2004; Bustos et al., 2010; Nilsson et al., 2010; Oropeza-Aburto et al., 2012). In *Arabidopsis*, the expression of *AtSPX1* genes was reduced to different degrees in phr1 mutants in a low-phosphorus environment, indicating that this protein is a part of the phosphate-signaling network involving PHR1 (Duan et al., 2008). *OsPHR2* over-expression and *OsSPX1* knockdown resulted in accumulation of excessive shoot concentrations of Pi in rice. *OsSPX1* is a negative regulator of *OsPHR2* and is involved in the feedback of Pi-signaling network in roots defined by *OsPHR2* (Liu et al., 2010).

In this study, we used the primers ProS1-F and ProS1-R to amplify root cDNA and obtained a 724-bp fragment containing 2 P1BS *cis*-elements (GnATATnC) (Figure 2), but they were not consistent with other reported sequences (Table 2).

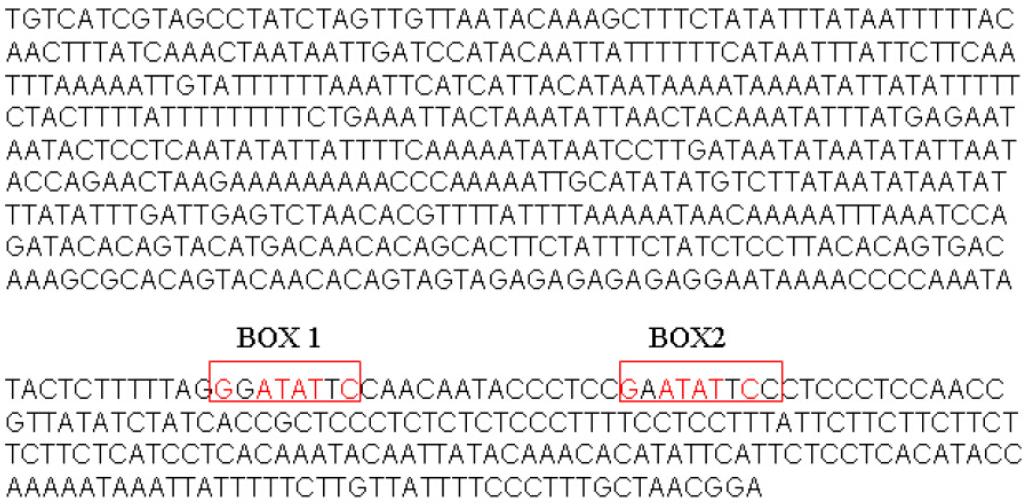

Figure 2. P1BS *cis*-acting elements of *GmSPX1*.

Table 2. Sequences related to the PHR1-binding site found at the upstream region of phosphate starvation responsive genes from several plant species.

Gene	Species	Sequence	Position	Reference
AtIPS1	*Arabidopsis thaliana*	GCATATTC	-598	(Martín et al., 2000)
AtACP5	*Arabidopsis thaliana*	GAATATCC	-290	(Del Pozo et al., 1999)
AtPT1	*Arabidopsis thaliana*	GTATATCC	-200	(Muchhal et al., 1996)
At4	*Arabidopsis thaliana*	GCATATTC	-245	(Burleigh and Harrison, 1999)
		GTATATGC	-782	
RNS1	*Arabidopsis thaliana*	GTATATAC	-188	(Bariola et al., 1994)
PAP1	*Arabidopsis thaliana*	GGATATAC	-149	(Haran et al., 2000)
TPSI1	*Lycopersicum esculentum*	GCATATCC	-551	(Liu et al., 1997)
Mt4	*Medicago truncatula*	GCATATCC	-230	(Burleigh and Harrison, 1997)
OsPT2	*Oryza sativa*	Unknown	-346	(Liu et al., 2010)
LaSAP1	*Lupinus albus*	GNATATNC	-160	(Zinn et al., 2009)

GmPHR1 can physically interact with GmSPX1 through cis-element repression in response to phosphate starvation in soybean

An electrophoretic mobility shift assay was used to verify the interaction between AtPHR1 and the P1BS element in the *AtIPS* promoter region (Rubio et al., 2001). Similar results showed that *OsPHR1* and the P1BS element in the *OsPT2* promoter region interacted with each other (Liu et al., 2010).

To confirm the importance of P1BS acting as a key *cis*-regulatory motif and to gain further insight into the role of the downstream gene *GmPHR1* in the Pi regulatory network, we used the yeast one-hybrid method. We first conducted site-directed mutagenesis (GGATATTC mutated into GGAGATTC) and constructed the pAbAi::Pro1E vector (Figure 3 and **Figure S1**).

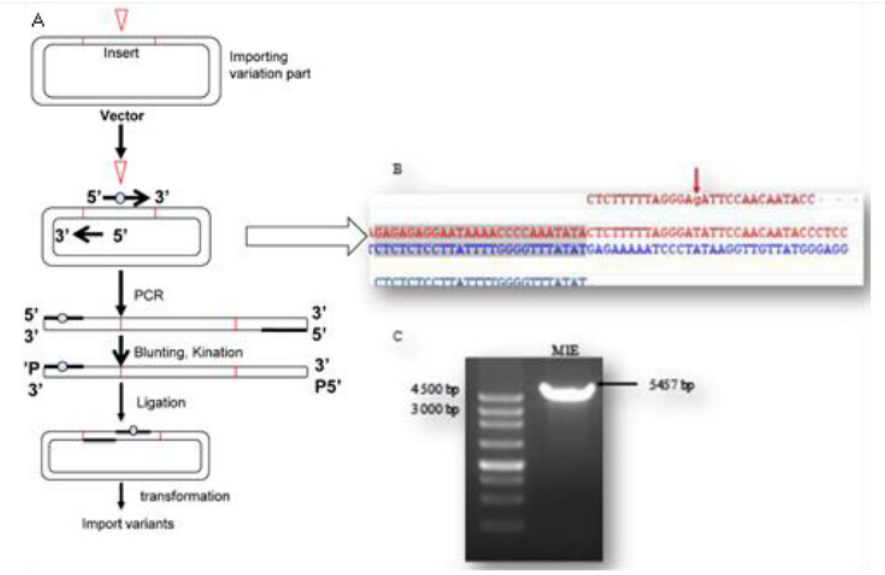

Figure 3. Construction of M1E vector. (**A**) Principle and flowchart of site-directed mutagenesis. (**B**) Site of mutagensis, as shown with arrow. (**C**) *pAbAi::Pro1E* as template amplicated with M1E-F and M1E-R. The production was 5457bp.

The results suggested that pAbAi::Pro1E and pAbAi::Pro2E as well as the positive control pAbAi::p53 grew on an SD/-Leu,AbA plate, but the site-directed mutagenesis carriers with the *cis*-acting element M1E-P1BS did not grow on the SD/-Leu or SD/-Leu,AbA plate (Figure 4). The results showed that the transcription factor *GmPHR1* and P1BS element -178 and -200 bp upstream of the *GmSPX1* region may interact with each other, thereby regulating Pi starvation signaling in soybean.

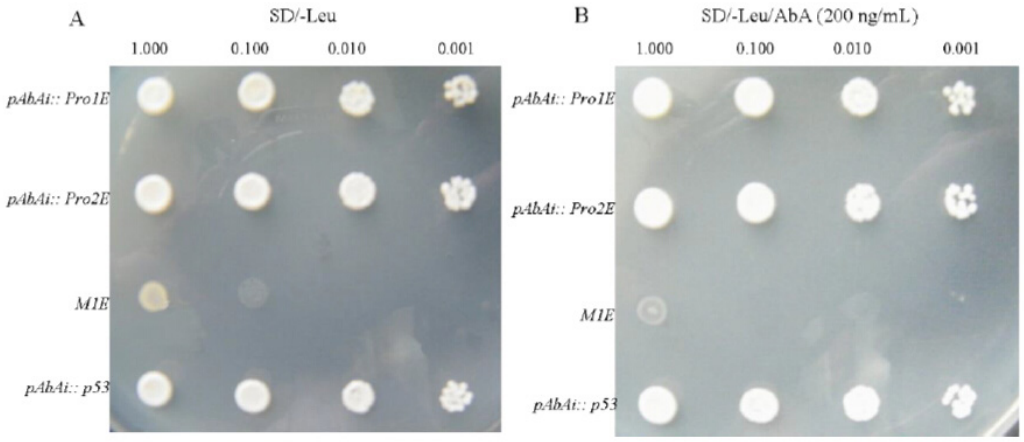

Figure 4. Physical interaction of GmPHR1and P1BS. A single clone on the SD/-Leu plate was picked, and then cultured with YPDA liquid medium. Cultures grew to densities OD_{600nm} 1.0 and diluted with fresh medium to an OD_{600nm} of 0.100, 0.010, and 0.001. Spot of 3-µL culture on the SD/-Leu (**A**) and SD/-Leu/AbA (200 ng/mL) plate (**B**).

DISCUSSION

The PHR1 protein contains an MYB domain and a coiled-helical domain, which belong to the MYB-CC subtype family of the MYB superfamily. Therefore, PHR1 binds to the imperfect palindromic sequence (GnATATnC) in a dimer. PHR1 is one of the most widely studied transcription factors and encodes an MYB domain-containing protein that binds to a short DNA motif commonly found in the promoters of several Pi starvation-induced genes (Rubio et al., 2001). Interestingly, these genes included P1BS structure, indirectly demonstrating a PHR transcriptional repression response (Bustos et al., 2010). PHL1 (PHR1-LIKE, PHR1 with a high homology in the amino acid sequence) when removing the N-terminal 2 proteins and the electrophoretic mobility shift assay results revealed interactions with P1BS (Bustos et al., 2010).

Arabidopsis AtSPX1, a possible ortholog of rice *OsSPX1*, showed 52-fold increased induction under Pi starvation conditions (Bari et al., 2006). Recently, the AtSPX family was identified to be involved in regulating the Pi pathway. The expression levels of *AtSPX1* and *AtSPX3* were strongly induced by Pi starvation; *AtSPX2* was weakly induced and *AtSPX4* was suppressed. The AtSPX family may be a part of the phosphate signaling pathway controlled by PHR1 and SIZ1 (Duan et al., 2008). In rice, our real-time reverse transcription-polymerase chain reaction analysis showed that most OsSPX genes were significantly upregulated under cold stress (Zhao et al., 2009). *OsSPX1* has been reported to be involved in phosphate homeostasis, and plays an essential role during the phosphate-starvation signaling pathway in

rice (Wang et al., 2009). Through data mining and test mining, we identified 3 OsSPX genes (OsSPX1, OsSPX2, and OsSPX3) acting as Pi starvation response genes (Wang et al., 2006). Furthermore, we found through data mining that certain other cold stress-response genes were upregulated by Pi deficiency in plants (Zhao et al., 2009), such as genes for phosphoenolpyruvate carboxylase kinase, glycerol-3-Pi transporter, Pi:H^+symporter, glycerophosphoryl diester phosphodiesterase, inorganic pyrophosphatase, 1,2-diacylglycerol, and 3-β-galactosyltransferase. Most of these genes are related to the signal transduction pathway of Pi starvation, and they may work together with OsSPX genes in the cross-talk between cold stress and Pi starvation. In *Arabidopsis*, *AtSPX1* was induced by Pi starvation, and over-expression of *AtSPX1* increased the transcript levels of Pi-responsive genes, such as *ACP5*, *RNS1*, and *PAP2* (Duan et al., 2008). PHO2 encodes an ubiquitin-conjugating E2 enzyme as a key gene regulating Pi signaling pathways. *Arabidopsis* pho2-1 mutants were used to investigate whether low phosphate levels trigger cold acclimatization of photosynthetic carbon metabolism (Hurry et al., 2000). *AtSIZ1* encodes SUMO E3 ligase as a key controller of Pi starvation-dependent responses; it is a negative regulator of Pi starvation signaling and functions in tolerance to freezing and other stresses (Miura et al., 2005, 2007a,b).

Over-expression of *OsSPX1* enhanced cold tolerance and affected gene expression of Pi starvation-related genes during cold stress in *Arabidopsis* (Zhao et al., 2009). All of these functions are related to the P1BS structure, based on important *cis*-regulatory motifs in the Pi starvation signaling network (Bustos et al., 2010). We chose the promoter of *IPS1* (a specific Pi starvation-induced gene) and *RNS1* (a gene involved in the trauma response). For the 2 P1BS structures in the promoters of *IPS1*, we created single and double mutant. Our results indicated that P1BS plays a critical role in the Pi starvation response. Both PHR1 and P1BS were specific for low phosphorus stress (Bustos et al., 2010).

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Supplementary material

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