**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 TTCTTCTACCTCC-3'; reverse, 5'-TCACATGATGTGGTCTATCTGTATC-3'; for *GmSPX1* forward, 5'-ATGAAATTCGGGAAGAGCCTGAGC-3'; reverse, 5'-TTACTTGGCTGCTTGTTCAG-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'-CTCTTTTCAATT TTCTTCTACCTCC-3'; reverse, 5'-TCACATGATGTGGTCTATCTGTATC-3'; for *GmSPX1* forward, 5'-ATGAAATTCGGGAAGAGCCTGAGC-3'; reverse, 5'-TTACTTGGCTGCTTGTTCAG-3'. 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SPX1 ATG upstream sequence fragment was named ProS1 (product of GmSPX1): ProS1-F: 5'-TGTACATCGTAGCCTATCTAGTG-3', ProS1-R: 5'-TCCGTTAGCAAGGGAAATAA 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'-CGAGCTCTGTACGTCGTAAGCCTATCT-3', Pro1E-R: 5'-CCGCTCGAGGGAGGATTTGTT-3', with an amplified fragment size of 562 bp. Pro2E-F: 5'-CGAGCTCTGTACGTCGTAAGCCTATCT-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'-TCCCCCAGGATGAATGAGAACAAA-TG-3', AD-R: 5'-CCGCTCGAGTTGGCTTGGAGA-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.: A1310799) 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.
**P1BS involved in tolerance to phosphate starvation**

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).

<table>
<thead>
<tr>
<th>PHR</th>
<th>AtPHR1</th>
<th>OsPHR1</th>
<th>OsPHR2</th>
<th>GmPHR1</th>
<th>GmPHR2</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>AtPHR1</strong></td>
<td>1.000</td>
<td>0.093</td>
<td>0.093</td>
<td>0.053</td>
<td>0.073</td>
</tr>
<tr>
<td><strong>OsPHR1</strong></td>
<td>1.000</td>
<td>0.077</td>
<td>0.044</td>
<td>0.100</td>
<td></td>
</tr>
<tr>
<td><strong>OsPHR2</strong></td>
<td>1.000</td>
<td>0.067</td>
<td>0.075</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>GmPHR1</strong></td>
<td>1.000</td>
<td></td>
<td>0.044</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>GmPHR2</strong></td>
<td>1.000</td>
<td></td>
<td></td>
<td></td>
<td>1.000</td>
</tr>
</tbody>
</table>

**Table 1. Sequence Identity Matrix of PHR.**

**P1BS cis-acting regulatory element**

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.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Species</th>
<th>Sequence</th>
<th>Position</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>AtlIPS</td>
<td>Arabidopsis thaliana</td>
<td>GCATATTC</td>
<td>-598</td>
<td>(Martín et al., 2000)</td>
</tr>
<tr>
<td>AtACP5</td>
<td>Arabidopsis thaliana</td>
<td>GAATATCC</td>
<td>-290</td>
<td>(Del Pozo et al., 1999)</td>
</tr>
<tr>
<td>AtPT1</td>
<td>Arabidopsis thaliana</td>
<td>GTATATCC</td>
<td>-200</td>
<td>(Muchhal et al., 1996)</td>
</tr>
<tr>
<td>At4</td>
<td>Arabidopsis thaliana</td>
<td>GCATATCC</td>
<td>-245</td>
<td>(Burleigh and Harrison, 1999)</td>
</tr>
<tr>
<td>RNS1</td>
<td>Arabidopsis thaliana</td>
<td>GTATATCC</td>
<td>-188</td>
<td>(Bariola et al., 1994)</td>
</tr>
<tr>
<td>PAP1</td>
<td>Arabidopsis thaliana</td>
<td>GGATATAC</td>
<td>-149</td>
<td>(Haran et al., 2000)</td>
</tr>
<tr>
<td>TPS1</td>
<td>Lycopersicum esculentum</td>
<td>GCATATCC</td>
<td>-551</td>
<td>(Liu et al., 1997)</td>
</tr>
<tr>
<td>Mt4</td>
<td>Medicago truncatula</td>
<td>GCATATCC</td>
<td>-230</td>
<td>(Burleigh and Harrison, 1997)</td>
</tr>
<tr>
<td>OsPT2</td>
<td>Oryza sativa</td>
<td>Unknown</td>
<td>-346</td>
<td>(Liu et al., 2010)</td>
</tr>
<tr>
<td>LaSAP1</td>
<td>Lupinus albus</td>
<td>GNATATNC</td>
<td>-160</td>
<td>(Zinn et al., 2009)</td>
</tr>
</tbody>
</table>

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 P1 regulatory network, we used the yeast one-hybrid method. We first conducted site-directed mutagenesis (GGATATTC mutated into GGAATATTC) 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 mutagenesis, 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.

**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).

**ACKNOWLEDGMENTS**

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**Supplementary material**

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P1BS involved in tolerance to phosphate starvation


