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Soybean SPX1 is an important component of the response to phosphate deficiency for phosphorus homeostasis

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ABSTRACT

Phosphate (Pi) homeostasis is required for plant growth and development, but the Pi-signaling pathways in plants still remain largely unknown. Proteins only containing the SPX domain are very important in phosphate (Pi) homeostasis and signaling transduction. In the T-DNA insertion *Arabidopsis* mutant *spx3*, *AtPHT1-4*, *AtPHT1-5*, *AtACP5*, *AtRNS*, and *AtAT4* expression levels were increased under Pi-sufficient condition and low Pi condition compared with WT. Meanwhile, the expression levels of these phosphate starvation genes was inhibited in OXSPX1 and *spx3*/OXSPX1 compared with WT, only under Pi-sufficient condition. These imply that *GmSPX1* may negatively control the transcription of Pi starvation responsive genes indirectly. However, there were no differences between expression levels of these PSI genes in *spx3* and those in WT under −Pi conditions. These facts imply that the negative regulation of *GmSPX1* and *AtSPX3* on PSI genes is depending on Pi concentration. Consistent with this, *GmSPX1* overexpression in the WT and *spx3* decreased the total Pi concentration in plants and changed root hair morphology, suppressing the elongation and number of root hairs compared with the WT and *spx3*. The yeast two-hybrid assays and BiFC assays demonstrated that GmSPX1 could interact with GmMYB48.The qRT-PCR analysis showed that GmMYB48 is a new phosphate starvation induced transcription factor in soybean. Also, *GmSPX1* overexpression led to decreased transcripts of *AtMYB4*, an ortholog of *GmMYB48*, in OXSPX1. Together, these results suggest that *GmSPX1* is a negative regulator in the Pi signaling network of soybean, and the interaction of GmSPX1/GmMYB48 can be considered a potential candidate suppressor.

1. Introduction

As a major constituent of plant cell components, including nucleic acids, membranes and ATP, phosphorus (P) is a macronutrient and plays a crucial role in energy transformation and protein activation. Although P is abundant in many soils, most of the P in the soil is converted to organic compounds or becomes insoluble [1–3]. As a result, very little P is present in ionic forms that are available to plants. Intensive application of chemical fertilizers containing phosphate (Pi) has therefore become a standard agricultural practice to ensure crop productivity [4]. To cope with low nutritional Pi availability, plants have developed a wide spectrum of mechanisms to improve Pi-use efficiency, such as altered morphology, physiology, and biochemical processes [5,6].

At the molecular level, our knowledge of the plant response to Pi starvation has greatly improved with the identification of several key players involved in Pi signaling, which have been well reviewed in recent years [4,7–9]. Using forward and reverse genetics, several important transcription factors in Pi signaling pathways have been identified, including MYB, WRKY, and bHLH family members [10–13]. MYB transcription factors are defined by a highly conserved MYB DNA-binding domain at the N-terminus, and constitute one of the largest transcription factor (TF) families in the plant kingdom [14]. Among MYB TFs, *PHR1*, *MYB62*, *MYB2* and *MYB82* have already been confirmed to be involved in phosphate starvation responses [10,13,15,16]. PHR1, a R2R3-MYB protein, is the central integrator in the transcriptional regulation of phosphate starvation responses [15]. PHR1 plays a crucial role in regulating genes involved in Pi transport and remobilization, anthocyanin biosynthesis, carbohydrate metabolism, and root system architecture (RSA) [15,17–19]. Recent studies have shown that OsSPX1, OsSPX2 and OsSPX4 can interact with OsPHR2 and inhibit its binding activity with the *cis*-element P1BS. The SPX/PHR2 interaction is highly

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Pi-dependent in planta [20,21]. A similar interaction, SPX1/PHR1, was also confirmed in *Arabidopsis* and could negatively regulate Pi signaling [22].

Proteins containing the SPX domain are key players controlling a set of processes involved in maintaining an internal steady state of phosphate ions at the cell level, defined as Pi homeostasis [23–25]. The SPX domain is named after the yeast SYG1 and Pho81 proteins and human XPR1 protein, which contain a conserved domain in their N-terminal peptides [26–29]. In plants, SPX-domain-containing proteins can be divided into four families based on the presence of additional domains in their structure, SPX-EXS, SPX-MFS, SPX-RING and SPX, among which only those containing the SPX domain are referred to as SPX proteins [30–35]. Four members of the SPX family have been found in *Arabidopsis*, named *AtSPX1*, *AtSPX2*, *AtSPX3*, and *AtSPX4*. *AtSPX1* is a positive regulator of some phosphate-starvation-inducible (PSI) genes while *AtSPX3* plays a negative role. Repression of *AtSPX3* by RNA interference led to aggravated Pi-deficiency symptoms, altered P allocation and enhanced expression of a subset of phosphate-responsive genes including *AtSPX1* [23]. However, according to the newest publication, AtSPX1 could inhibit AtPHR1 activity through interaction and then act as negative regulator of some phosphate starvation induced (PSI) genes. Similarly, there are six members of the SPX family in rice, named *OsSPX1*, *OsSPX2*, *OsSPX3*, *OsSPX4*, *OsSPX5* and *OsSPX6* [25,36,37].

In this paper, we cloned the full-length cDNA of *GmSPX1*. Subsequently, analysis of *GmSPX1*'s function showed that it is a negative regulator in the feedback network of Pi signaling. The results of further experimental studies suggested that GmSPX1 might be involved in phosphate starvation responses through interaction with GmMYB48.

2. Materials and methods

2.1. Phylogenetic and gene structure analysis

SPX sequences from *Arabidopsis* (*Arabidopsis thaliana* (L.)) and soybean were identified through a BLAST search of The *Arabidopsis* Information Resource (TAIR, http://www.Arabidopsis.org) and National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/) databases, using *AtSPX* genes as queries [23]. Multiple alignments were prepared using ClustalX 2.0.11. Neighbor-joining phylogenetic trees were generated using the MEGA 5.1 program.

2.2. Plant material and growth conditions

Seeds of soybean genotype Williams 82 were used for gene cloning and expression analysis of *GmSPX1* in response to Pi starva tion. Five-day-old seedlings germinated in vermiculite were transferred to half Hoagland solution for 5 days and then treated with two Pi levels (low Pi, 2.5 μM KH_2PO_4; +Pi, 250 μM KH_2PO_4) for 10 days. A low Pi group re-supplied with Pi for 1 day was called R1d. Leaves and roots were sampled at 0, 1, 5, 10 and 11 days after transfer. All samples were frozen in liquid nitrogen and stored at −80 °C prior to RNA extraction.

Arabidopsis ecotype Columbia-0 (Col-0) was used in this study. The *spx3* T-DNA insertion mutant (SALK_035262C) was obtained from the *Arabidopsis* Biological Resource Center (ABRC). Wild-type (WT), mutant and transgenic seeds were surface-sterilized with chlorine gas produced by adding 3.5 mL 12 N HCl into 100 mL bleach for 3 h in a desiccator. Ten-day-old seedlings were grown in half Murashige and Skoog medium (MS medium) with three Pi levels (-Pi, 0 μM KH_2PO_4; low Pi, 6.25 μM KH_2PO_4 and +Pi, 625 μM KH_2PO_4).

2.3. qRT-PCR

First-strand cDNAs were synthesized from the total RNA extracted from leaves and roots using the HiScript® II Q RT SuperMix for qPCR (Vazyme Biotech, Nanjing, China). Real-time RT-PCR analysis was performed using IQ SYBR Green (Bio-Rad, Hercules, CA, USA) on a CFX96 Touch (Bio-Rad). All reactions were performed under the following conditions: 95 °C for 2 min; 40 cycles of 95 °C for 10 s and 60 °C for 30 s. Triplicate quantitative assays were performed on each cDNA sample. The relative level of expression was calculated using the formula $2^{-\Delta\Delta Ct}$. All primers used for RT-PCR are given in Table S1.

2.4. Over-expression of GmSPX01

Full-length cDNA clones of the *GmSPX1* (Glyma01g33170) gene were obtained from NCBI. An over-expression construct was generated by inserting a full-length *GmSPX1* cDNA fragment into the binary vector pEarleygate103 after the CaMV 35S promoter, using the Gateway Technology with a Clonas II Kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. The specific primers used for cDNA cloning are given in Table S2. The pEarleygate103-*GmSPX1* vector was introduced into *Agrobacterium tumefaciens* strain EHA105 via the freeze–thaw method. The Agrobacterium-mediated floral dip method was used for *Arabidopsis* transformation [38] and transgenic plants were selected on Basta (20 mg L^{-1}) medium.

2.5. Measurements of root hairs

Seedlings were grown on petri dishes under Pi-sufficient conditions for 10 days as described earlier and then transferred to low Pi conditions for another 10 days. The primary root tips were photographed under an OLYMPUS MVX10 stereo microscope with a SPOT-RT digital camera attached to a computer (Olympus, Melville, NY, USA). The total number of root hairs in the region 5 mm from the root tip as well as the lengths of the root hairs in this region was measured using the ImageJ program [39]. Data were recorded from five individual plants from each line per treatment.

2.6. Measurement of P concentration in plants

The transgenic *Arabidopsis* and WT plants were grown in half MS medium with two Pi levels (low Pi, 6.25 μM KH_2PO_4; +Pi, 625 μM KH_2PO_4) for 10 days. Total P content was determined using the Optima™ 2100 DV ICPOES system (PerkinElmer Inc., Waltham, MA, USA) essentially as described previously [40].

2.7. Soybean cDNA library construction and yeast two-hybrid screens

Ten-day-old seedlings were grown in low Pi conditions for 10 days, and then the roots were collected for library construction. High-quality cDNA libraries were constructed using the CloneMiner™ II cDNA Library Construction Kit (Invitrogen). Yeast two-hybrid screens were performed using the Clontech two-hybrid system according to the manufacturer's Yeast Protocols Handbook (Invitrogen). The full-length ORF of *GmSPX1* was cloned into the pGBKT7 vector as bait. *GmMYB48* (Glyma06g00630) was selected from positive clones as a candidate prey.

The CDS of *GmMYB48* was cloned into the pGADT7 vector with *Eco*R I and B*am*H I. The transformants were screened on supplemented synthetic dextrose medium lacking Leu and Trp or on medium lacking Leu, Trp, His and adenine hemisulfate salt with X-α-Gal.