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Comparative characterization of *GmSPX* members reveals that *GmSPX3* is involved in phosphate homeostasis in soybean

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• **Background and Aims** Proteins containing the SPX (SYG1/Pho81/XPR1) domain are vital components in the phosphorus (P) signalling pathway, and regulate phosphate (Pi) homeostasis in plants. However, the characteristics and functions of GmSPX members in soybean (*Glycine max*) remain largely unknown.

• **Methods** BLAST searching revealed nine *GmSPX* members in the soybean genome. Subsequently, expression patterns of *GmSPX* members were investigated in various tissues of soybean grown in nutrient solution or sand culture through quantitative real-time PCR (qPCR) analysis. Sub-cellular localization of GmSPX was examined via transient expression of *35S:GmSPX-GFP* in epidermal cells of onion (*Allium cepa*). Finally, soybean transgenic composite plants were generated to study *GmSPX3* functions.

• **Key Results** Nine GmSPX members were identified, which were classified into three groups based on phylogenetic analysis. Diverse responses of *GmSPX* members to deficiencies of nutrients (nitrogen, phosphorus, potassium and iron) or inoculation of arbuscular mycorrhizal fungi and rhizobia were observed in soybean. In addition, variations of sub-cellular localization of GmSPX members were found. Among them, GmSPX3, GmSPX7 and GmSPX8 were localized in the nuclei, and the other GmSPX members were confined to the nuclei and cytoplasm. The nuclear-localized and Pi starvation responsive-gene, *GmSPX3*, was functionally analysed in soybean transgenic composite plants. Overexpression of *GmSPX3* led to increased P concentrations in both shoots and roots in the high-P treatment, and increased transcription of seven Pi starvation-responsive genes in soybean hairy roots.

• **Conclusions** Taken together, the results suggest that *GmSPX3* is a positive regulator in the P signalling network, and controls Pi homeostasis in soybean.

Key words: Expression pattern, phosphate starvation, Pi homeostasis, phosporus signalling, soybean, *Glycine max*, SPX domain, GmSPX members.

INTRODUCTION

Phosphorus (P) is a macronutrient and major constituent in plant cells that participates in multiple physiological and biochemical processes. However, available P is commonly limited in soils because P is easily fixed by soil particles into unavailable forms (Vance *et al.*, 2003; Richardson *et al.*, 2009). As a result, low P is often a major limitation for crop growth, especially in tropical and sub-tropical regions where P fixation occurs widely (Graham and Vance, 2000; Hardarson and Atkins, 2003). Although application of P fertilizers helps plants to cope with soil P deficiency, it is a partial solution, because >80 % of applied P fertilizers are wasted and cause environment pollution due to low P acquisition and utilization efficiency in crops (Richardson *et al.*, 2009). Furthermore, rock phosphate is a non-renewable P resource estimated to be exhausted within the next 50 years (Cordell *et al.*, 2009). Therefore, future sustainable agricultural development will require improved P acquisition and utilization efficiency in crop varieties along with optimized field P management (Tian *et al.*, 2012).

Cumulative results suggest that plants have developed a wide range of adaptive strategies to improve P acquisition and utilization efficiency, such as optimized root morphology and architecture, increased root exudation (e.g. protons and organic acids) and

enhanced internal and secreted acid phosphatase (APase) activities (Liang *et al.*, 2010; Chen *et al.*, 2011; Péret *et al.*, 2011; Qin *et al.*, 2012; Robinson *et al.*, 2012; Taghipour and Jalali, 2012; Tian *et al.*, 2012). These physiological and morphological responses are co-ordinately regulated in plants by an elaborate P signalling network in which the major components include phosphate (Pi), sugar, phytohormone, microRNAs (e.g. miR399) and transcription factors (Chiou and Lin, 2011). Among the transcription factors, a well-characterized R2R3 MYB member, PHR1 or PHR1-like, is generally considered as the central regulator of Pi starvation-responsive genes (Bari *et al.*, 2006; Nilsson *et al.*, 2007; Valdés-López *et al.*, 2008; Zhou *et al.*, 2008; Chiou and Lin, 2011). Overexpression of *AtPHR1* in arabidopsis (*Arabidopsis thaliana*) or *OsPHR2* in rice (*Oryza sativa*) resulted in excessive P accumulation in shoots (Nilsson *et al.*, 2007; Zhou *et al.*, 2008). Similarly, excessive P accumulation in arabidopsis shoots was also observed with overexpression of *miR399* (Bari *et al.*, 2006). Through genetic and molecular analysis, it has been determined that miR399 and PHO2 are downstream of PHR1 in the P signalling pathway that tightly regulates Pi acquisition and mobilization (Bari *et al.*, 2006; Chiou and Lin, 2011).

The complexity of the P signalling pathway in plants has been highlighted with identification and characterization of proteins

containing the SPX (Syg1, Pho81 and Xpr1) domain in this kingdom. Proteins containing the SPX domain were first isolated in yeast, and named after the suppressor of yeast gpa1 (Syg1), yeast phosphatase (Pho81) and the human xenotropic and polytropic retrovirus receptor 1 (Xpr1) (Secco *et al.*, 2012*b*). In yeast, most proteins containing the SPX domain, such as two low affinity transporters Pho87 and Pho90, have been well characterized as being involved in regulating Pi homeostasis (Secco *et al.*, 2012*b*). In plants, there are four families containing the SPX domain, SPX-EXS, SPX-MFS, SPX-RING and SPX (Hamburger *et al.*, 2002; Stefanovic *et al.*, 2007; Lin *et al.*, 2010; Secco *et al.*, 2010, 2012*a*; Kant *et al.*, 2011; Wang *et al.*, 2012). Recently, it has been well documented that proteins only containing the SPX domain play a vital role in the P signalling pathway in model plants, including arabidopsis and rice (Duan *et al.*, 2008; C. Wang *et al.*, 2009; Z. Wang *et al.*, 2009; Liu *et al.*, 2010; Yang *et al.*, 2011; Wu *et al.*, 2013). In arabidopsis, a positive regulatory role for *AtSPX1* and a negative role for *AtSPX3* have been suggested (Duan *et al.*, 2008). In rice, the *SPX* family contains six members, namely *OsSPX1*, *OsSPX2*, *OsSPX3*, *OsSPX4*, *OsSPX5* and *OsSPX6* (C. Wang *et al.*, 2009; Z. Wang *et al.*, 2009; Liu *et al.*, 2010). Among these, *OsSPX1*, *OsSPX3*, *OsSPX4* and *OsSPX5* have been proposed to be negative regulators of transcription of several Pi starvation-responsive genes (C. Wang *et al.*, 2009; Z. Wang *et al.*, 2009; Liu *et al.*, 2010; Wu *et al.*, 2013; Lv *et al.*, 2014; Shi *et al.*, 2014). Furthermore, it has been demonstrated that OsSPX4 could interact with OsPHR2, and thus antagonize OsPHR2 activity in regulation of its downstream gene expression (Lv *et al.*, 2014). Recently, three Pi starvation-responsive *SPX* members have been cloned and characterized in bean (*Phaseolus vulgaris*), in which PvSPX1 is found to be a positive regulator in the P signalling pathway (Yao *et al.*, 2014). However, genome-wide analysis of SPX members in other crops, especially in legume crops, remains largely unknown.

Soybean (*Glycine max*) is a major oil and food crop, as well as an exceptionally good protein source for humans and animals. As a major legume crop, soybean requires a relatively high amount of P because it can form nodules with rhizobia to fix atmospheric nitrogen, which is a high-P-demanding process (Chen *et al.*, 2011; Qin *et al.*, 2012). Adaptive strategies of soybean to P deficiency have been well characterized, including development of a shallower root system, an increase in root exudation (e.g. malate and protons), enhancement of APase activity and formation of symbiosis with arbuscular mycorrhizal (AM) fungi (Shen *et al.*, 2006; Liu *et al.*, 2008; Z. Wang *et al.*, 2009; Wang *et al.*, 2010; Chen *et al.*, 2011; Li *et al.*, 2012; Liang *et al.*, 2013). Furthermore, complex molecular responses of soybean to Pi starvation have been revealed through suppression subtraction hybridization, along with microRNA sequence and proteomic analysis (Guo *et al.*, 2008; Chen *et al.*, 2011; Qin *et al.*, 2012; Xu *et al.*, 2013). Recently, with the release of the soybean genome, genome-wide analysis of several gene families as related to P acquisition and utilization (e.g. *GmPAP* and *GmPT* genes) has been studied (Li *et al.*, 2012; Qin *et al.*, 2012; Li *et al.*, 2013). However, key regulators in the soybean P signalling pathway remain to be identified. In the present study, nine soybean *GmSPX* members were identified and subsequently examined for sub-cellular localization and expression patterns in response to nutrient deficiencies. Furthermore, the functions

of *GmSPX3* were analysed in transgenic soybean composite plants, which revealed that GmSPX3 might be involved in positively controlling expression of several Pi starvation-responsive genes and Pi homeostasis in soybean.

MATERIALS AND METHODS

Identification and bioinformatics analysis of GmSPX *genes in soybean*

Using the amino acid sequence of PvEF191350 in common bean (Tian *et al.*, 2007) as a query sequence, BLAST searches were conducted to yield nine *GmSPX* genes in the soybean (*Glycine max*) genome at the Phytozome website (http://www.phytozome.net). Furthermore, BLAST searches were performed separately using the nine *GmSPX* sequences as query sequences at the same website, and no more SPX genes were identified. The *GmSPX* genes were named *GmSPX1*–*GmSPX9* according to their positions on the soybean chromosomes.

The molecular weight of the proteins was predicted by the ExPASY web server (http://www.expasy.org/). The numbers of exons and introns, the first exon length and total exon length were extracted separately from the Phytozome website. The sub-cellular localization of GmSPXs was predicted on the WoLF PSORT server (http://wolfpsort.org/).

A multiple alignment of GmSPX amino acid sequences was constructed using the ClustalX program. A phylogenetic tree of the SPX family based on alignment of the entire protein sequences obtained from ClustalX was constructed using the Neighbor–Joining method in the MEGA 4·1 program, with the complete-deletion option selected for dealing with gaps or missing data in sequences.

Plant materials and treatments

Seeds of soybean genotype HN66 were surface sterilized for 1 min using 10 % (v/v) H_2O_2 and then germinated in the dark on germination paper moistened with quarter-strength modified nutrient solution for 7 d as described previously (Li *et al.*, 2012). After germination, seedlings were grown in full-strength nutrient solution for 10 d when the first trifoliate leaves were fully developed, and then were treated with two P levels (−P, 5 μM KH_2PO_4; +P, 500 μM KH_2PO_4) as described previously (Li *et al.*, 2012). Roots, stems, leaves, flowers, young pods and seeds were harvested separately as described by Li *et al.* (2012). Nutrient solution was well aerated and its pH was maintained between 5·8 and 6·0.

For other nutrient deficiency stress experiments, seedlings with the first fully developed trifoliate leaves were transplanted into the nutrient solution lacking nitrogen (N), potassium (K) or iron (Fe). Control plants were grown in full-strength nutrient solution. Leaves and roots were harvested separately at 14 d after initiating treatments. For the N deficiency (−N) experiment, KNO_3 and $Ca(NO_3)_2$ were replaced by K_2SO_4 and $CaCl_2$, respectively. For the K deficiency (−K) experiment, KNO_3 and KH_2PO_4 were replaced by $Ca(NO_3)_2$ and $NH_4H_2PO_4$, respectively. For the Fe deficiency (−Fe) experiment, Fe-EDTA was withdrawn from the nutrient solution. Nutrient solutions were well aerated and the pH was maintained between 5·8 and 6·0.

To test for effects of AM fungal inoculation on expression of GmSPX, soybean seeds were surface sterilized and then germinated in sand culture with (+M) or without (−M) *Glomus mosseae* spores and mycelium. After 7 d, seedlings were irrigated by low-P nutrient solution as described previously (Li *et al.*, 2012). Roots were harvested for RNA extraction after 50 d.

For expression analysis of GmSPX in nodules, soybean seeds were surface sterilized and then germinated on germination paper as above. After 7 d, seedlings were inoculated with rhizobia, *Bradyrhizobium* sp. BXYD3, and then grown in the nutrient solution containing 50 μM NH_4NO_3 supplied with 5 μM (−P) or 500 μM (+P) KH_2PO_4. After 30 d, nodules were harvested to extract RNA for further analysis.

All expression analysis samples were stored at −80 °C prior to RNA extraction. Four biological replicates were included in all of the experiments.

RNA extraction and quantitative real-time PCR analysis

Total RNA was isolated from different tissues of soybean plants using RNAiso Plus reagent (TaKaRa, Japan) following the manufacturer instructions. RNA samples were treated with *DNase* I (TaKaRa, Japan) to avoid amplification from genomic DNA. The first-strand cDNA was synthesized from 2 μg of total RNA using MMLV reverse transcriptase and oligo(dT) according to the kit protocol (Promega, USA). First-strand cDNA was used for SYBR Green Premix EX Taq^{TM}- (TaKaRa, Japan) monitored quantitative real-time PCR (qPCR) analysis. The qPCR analysis was performed using a Rotor-Gene 3000 system (Corbett Research, Australia) with initial denaturation at 95 °C for 1 min, followed by 40 cycles of 95 °C for 15 s, 58–60 °C for 15 s and 72 °C for 30 s. The soybean housekeeping gene *EF-1α* (X56856) was used as an endogenous control to normalize the samples. The primer pairs used for qPCR analysis are listed in Supplementary Data, Table S1. All gene expression analysis experiments included four biological replicates.

Sub-cellular localization analysis

The coding regions of the nine *GmSPX* members without stop codons were ligated separately into the transient expression vector (*pBEGFP*) and fused with the *GFP* (green fluorescent protein) gene as described previously (Liang *et al.*, 2010). All of the *35S:GmSPX-GFP* fusion constructs, as well as the *35S:GFP* empty vector control were transiently expressed in onion epidermal cells using the Bio-Rad biolistic PDS/1000 system (http://www.bio-rad.com/). Epidermal cells were observed by the green fluorescence of the GmSPX–GFP protein using a fluorescence microscope (LEICA, DM5000B, Germany) as described previously (Liang *et al.*, 2010). The GFP fluorescence was imaged using a LEICA DFC 480 camera. The onion epidermal cells were plasmolysed by treating them with 30 % sucrose solution.

GmSPX3 *overexpression in soybean hairy roots*

The coding region of *GmSPX3* was cloned into the pTF101-OX vector. The *OX-GmSPX3* or empty vector construct was transformed into *Agrobacterium rhizogenes* strain K599, which infected soybean to produce composite plants with transgenic hairy roots as described previously (Guo *et al.*, 2011). Composite plants were grown in full-strength nutrient solution until the emerged hairy roots were approx. 10 cm long, when they were transplanted separately into nutrient solution supplied with 5 μM KH_2PO_4 (−P) or 500 μM KH_2PO_4 (+P) for 14 d. Nutrient solution was well aerated and the pH was maintained between 5·8 and 6·0. One independent transgenic line originating from one transgenic hairy root was considered as one biological replicate. Four biological replicates were included in this experiment. Roots were harvested separately after 14 d in P treatments. The fresh weight and P concentration of each transgenic line was determined separately. A small portion of hairy roots were also harvested for qPCR analysis.

Determination of dry weight and P concentration

Hairy roots and shoots were kept in an oven at 75 °C until completely dried. The dry weight of hairy roots and shoots from the empty vector control and *OX-GmSPX3* transgenic lines was analysed. Then the hairy roots and shoots were ground for P concentration analysis using the phosphorus–molybdate blue colour reaction as described previously (Murphy and Riley, 1962). Four biological replicates were included in all the experiments.

Transcripts of downstream genes in transgenic soybean hairy roots

To analyse expression patterns of genes downstream of *GmSPX3* in the soybean P signalling pathway, total RNA was extracted from the transgenic hairy roots grown under normal P conditions. Subsequently, qPCR was conducted to analyse the expression of seven *GmSPX3* downstream genes. All qPCR primers (Supplementary Data Table S1) were designed according to the sequences from GenBank (http://www.ncbi.nlm.nih.gov/genbank) for *GmPT2* (FJ814696) and *GmALMT1* (EU586178), or from Phytozome (http://www.phytozome.net) for *GmHAD1-6* (Glyma08g20810), *GmPAP21* (Glyma10g08300), *GmPT7* (Glyma10g33030), *GmIPS1* (Glyma13g21070) and *GmRNS1* (Glyma01g05840).

RESULTS

Identification and characterization of the GmSPX *family genes in soybean*

A total of nine putative *GmSPX* members were identified in the soybean genome database (http://www.phytozome.net) through BLAST searching. General information on the *GmSPX* members is summarized in Table 1. The *GmSPX* members were unevenly distributed on soybean chromosomes 1, 4, 10, 13, 17 and 20 (Table 1). Based on their chromosomal positions, the nine *GmSPX* members were named *GmSPX1*, *GmSPX2*, *GmSPX3*, *GmSPX4*, *GmSPX5*, *GmSPX6*, *GmSPX7*, *GmSPX8* and *GmSPX9*. Open reading frames of the *GmSPX* members ranged from 753 to 936 bp in length, which were predicted to encode proteins 250–311 amino acids in length and 29·1–35·2 kDa in molecular weight. Sub-cellular localization of GmSPX members predicted on the WoLF PSORT website showed that GmSPX6, GmSPX7 and GmSPX8 might reside in the nucleus, GmSPX2, GmSPX3, GmSPX4, GmSPX5 and GmSPX9 might localize in the cytoplasm, and GmSPX1 might be located in the chloroplast (Table 1).

TABLE 1. *General information for the nine* GmSPX *members*

Gene locus	Proposed name	No. of exons/introns	First/total exon length (bp)	No. of amino acids	Predicted size (kDa)	Sub-cellular location
Glyma01g33170	*GmSPX1*	4/3	249/852	283	32·2	chlo: 5·0
Glyma04g07160	*GmSPX2*	3/2	243/753	250	29·1	cyto: 12·0
Glyma04g19450	*GmSPX3*	4/3	126/870	286	32·9	cyto: 8·0
Glyma06g07260	*GmSPX4*	2/1	437/888	296	29·1	cyto: 12·0
Glyma10g40820	*GmSPX5*	3/2	244/754	289	33·5	cyto: 11·0
Glyma13g05570	*GmSPX6*	3/2	285/936	311	35·2	nucl: 5·5
Glyma13g23580	*GmSPX7*	3/2	282/855	284	32·6	nucl: 8·0
Glyma17g12340	*GmSPX8*	4/3	110/753	295	33·5	nucl: 8·0
Glyma20g26490	*GmSPX9*	3/2	86/861	270	31·3	cyto: 9·0

The gene locus, exon or intron length, and protein size were extracted and analysed from the Phytozome website (http://www.phytozome.net) and the ExPASY web server (http://www.expasy.org/).

Chlo: chloroplast; cyto: cytoplasm; nucl: nucleus.

Numbers after organelles are scores from the WoLF PSORT prediction; a higher score indicates a greater possibility of the protein localization.

To examine the similarity and identify the conserved domain among GmSPX members, a multiple sequence alignment was constructed. Results showed that the amino acid sequences of all GmSPX members were similar to each other (Supplementary Data Fig. S1). The nine GmSPX members contained a highly conserved region (i.e. the SPX domain) in the N-terminal region, which could be further divided into three sub-domains (Supplementary Data Fig. S1).

A phylogenetic tree was constructed to examine evolutionary relationships among SPX family members in soybean, arabidopsis, rice, *Medicago truncatula*, common bean (*Phaseolus vulgaris* L.), *Capsella rubella*, *Eutrema salsugineum* and rapeseed (*Brassica napus* L.). Phylogenetic analysis demonstrated that the SPX family in plants could be classified into three groups, and that group I could be further subdivided into two groups (Fig. 1). All GmSPX members belonged to group I, except for GmSPX1 and GmSPX6, which belonged to group II and III, respectively. Subdivision of group I placed GmSPX3, GmSPX7 and GmSPX8 into sub-group I-1, along with PvSPX1 and PvSPX2 from bean, OsSPX1 and OsSPX2 from rice, AtSPX1 and AtSPX2 from arabidopsis, MtXP_003609335 from *M. truncatula*, and EsXP_006409728 and EsXP_006400582 from *E. salsugineum* (Fig. 1). The remaining soybean members, GmSPX2, GmSPX4, GmSPX5 and GmSPX9 were clustered in sub-group I-2 with PvSPX3 from bean and MtXP_003588760 from *M. truncatula* (Fig. 1).

Sub-cellular localization of GmSPX members

To determine the sub-cellular localization of GmSPX members, GmSPX–GFP fusion proteins were transiently expressed in onion epidermal cells. The results showed that GmSPX3, GmSPX7 and GmSPX8 were confined to the nuclei of onion epidermal cells (Fig. 2), suggesting that they might be nuclear proteins. Furthermore, signals of GFP fused with GmSPX1, GmSPX2, GmSPX4, GmSPX5, GmSPX6 and GmSPX9 were detected in many areas in onion epidermal cells, including the cytoplasm and nucleus (Fig. 2).

Tissue-specific responses of GmSPX to P deficiency

Expression patterns of the nine *GmSPX* genes were investigated at two P levels in various soybean tissues, including young leaves, stems, roots, flowers, pods and seeds (Fig. 3). Under P-replete conditions, transcripts of *GmSPX* genes were detected in all the tested tissues, except that negligible expression levels were observed for *GmSPX4* in seeds and for *GmSPX5* in roots (Fig. 3). Even so, *GmSPX* genes exhibited diverse expression levels among the tissues. For example, *GmSPX6* and *GmSPX8* had similar expression levels among all the tissues (Fig. 3), whereas *GmSPX2*, *GmSPX7* and *GmSPX9* had the highest expression levels in flowers (Fig. 3), and *GmSPX1* was expressed preferentially in seeds (Fig. 3).

Diverse responses of *GmSPX* members to P deficiency were further demonstrated in the various soybean tissues (Fig. 3). All *GmSPX* members, except *GmSPX6*, were significantly regulated by Pi starvation in at least one tissue. Six *GmSPX* transcripts were significantly increased by P deficiency in four or more tissues, with *GmSPX1*, *GmSPX5* and *GmSPX6* being the exceptions (Fig. 3). Significant increases in transcription in all tissues in response to P deficiency were observed only for *GmSPX8*. Finally, reduced transcription in response to P deficiency was only found for *GmSPX1* in seeds and for *GmSPX5* in both flowers and seeds (Fig. 3).

Responses of GmSPX transcripts to other nutrient deficiencies

Expression patterns of *GmSPX* genes in both leaves and roots were further studied under deficiencies of N, K or Fe. Similar to responses to P deficiency, transcripts of most *GmSPX* genes in leaves were increased by N, K and Fe deficiencies, except that suppressed transcripts were observed for *GmSPX7* and *GmSPX8* under K deficiency conditions, and for *GmSPX5* under Fe deficiency conditions (Fig. 4). More diverse responses of *GmSPX* genes were observed in roots under N, K and Fe deficiency conditions (Fig. 4). Under N deficiency conditions, transcription levels of *GmSPX1*, *GmSPX2*, *GmSPX3* and *GmSPX6* in roots were enhanced, while expression of *GmSPX5* was reduced by >2 fold, and expression of the other *GmSPX* genes remained unchanged (i.e. expression changes <2-fold) (Fig. 4). In the K-deficient treatment, expression levels of *GmSPX2* and *GmSPX5* in roots increased >2-fold, and transcripts of *GmSPX1*, *GmSPX3* and *GmSPX9* were reduced >2-fold (Fig. 4). Under Fe deficiency conditions, only *GmSPX8* expression was increased by >2-fold in roots, and reduced transcripts were found for most
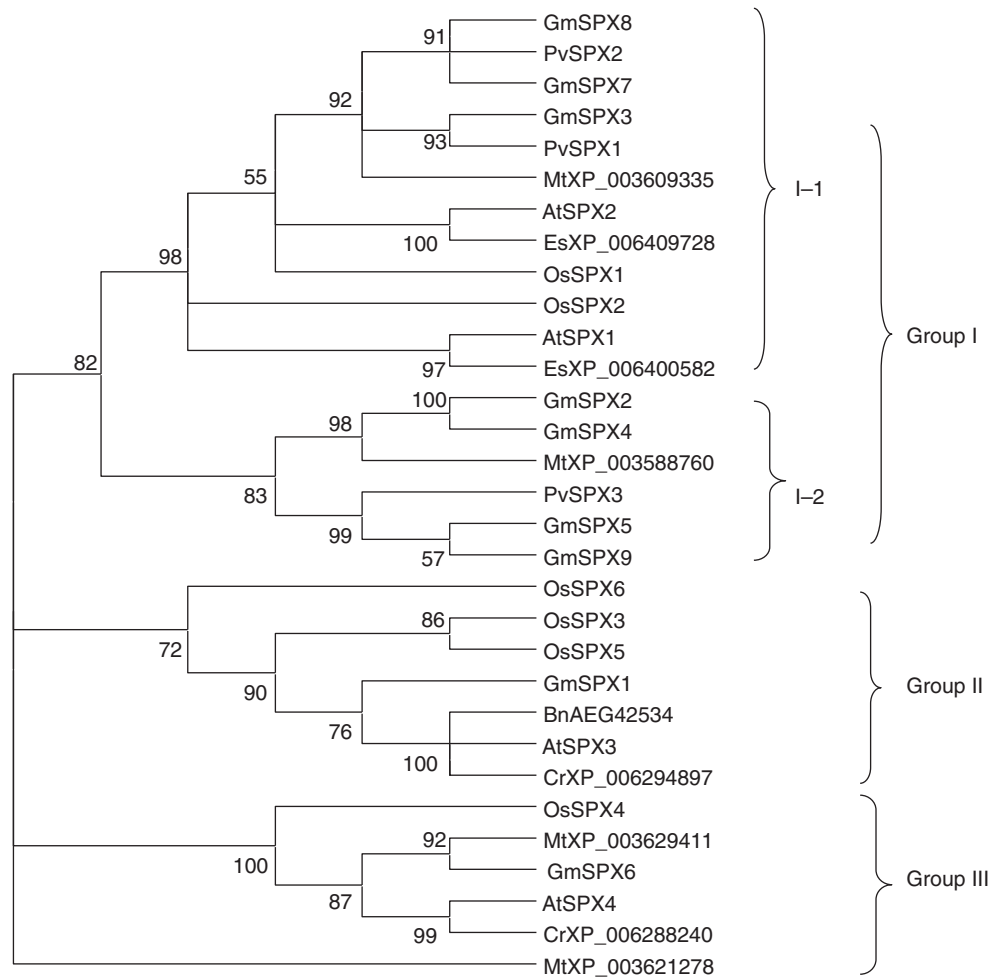
FIG. 1. Phylogenetic analysis of SPX proteins in plants. The phylogenetic tree was created in the Mega 4·1 program. The GenBank accession numbers of the protein or gene locus are listed as follows: AtSPX1 (At5g20150); AtSPX2 (At2g26660); AtSPX3 (At2g45130); AtSPX4 (At5g15330); GmSPX1 (Glyma01g33170); GmSPX2 (Glyma04g07160); GmSPX3 (Glyma04g19450); GmSPX4 (Glyma06g07260); GmSPX5 (Glyma10g40820); GmSPX6 (Glyma13g05570); GmSPX7 (Glyma13g23580); GmSPX8 (Glyma17g12340); GmSPX9 (Glyma20g26490); OsSPX1 (Os03g0343400); OsSPX2 (Os02g10780); OsSPX3 (Os10g25310); OsSPX4 (Os03g61200); OsSPX5 (Os03g29250); OsSPX6 (Os07g42330); PvSPX1 (EF191350); PvSPX2 (EG594307); PvSPX3 (EG594308). The first two letters of each protein label represent the abbreviated species name, followed by the GenBank accession number. At, *Arabidopsis thaliana*; Gm, *Glycine max*; Os, *Oryza sativa*; Mt, *Medicago truncatula*; Pv, *Phaseolus vulgaris*; Bn, *Brassica napus*; Cr, *Capsella rubella*; Es, *Eutrema salsugineum*.

GmSPX genes, especially *GmSPX1*, *GmSPX2*, *GmSPX3*, *GmSPX4* and *GmSPX9* (Fig. 4).

GmSPX *transcripts in mycorrhizal roots and nodules*

Soybean can form symbiotic associations with rhizobia and AM fungi, which can influence nutrient (e.g. P and N) status in soybean. Therefore, expression patterns of *GmSPX* genes in mycorrhizal roots and nodules were also investigated. Expression levels of *GmSPX5* were significantly enhanced by AM fungal infection under low P conditions (Fig. 5A). Changes in transcription of the other *GmSPX* genes were <2-fold, compared with those without AM fungi inoculation, suggesting that their expression levels were not significantly affected by AM infection at a low P level (Fig. 5A).

In soybean nodules, expression levels of most *GmSPX* genes, except *GmSPX1* and *GmSPX6,* were up-regulated by Pi starvation (Fig. 5B). Most notably, *GmSPX3*, *GmSPX4* and *GmSPX9*

transcripts increased considerably (Fig. 5B). In contrast, a suppressed or <2-fold change in transcription was observed for *GmSPX1* and *GmSPX6* in nodules under Pi starvation, respectively (Fig. 5B).

Functional analysis of GmSPX3 in soybean composite plants

Since GmSPX3 exhibited high sequence homology to and had similar properties (e.g. increased transcription under Pi starvation, nuclear localization) to those reported for OsSPX1 and AtSPX1, the functions of *GmSPX3* were further evaluated through analysis of soybean transgenic composite plants with *GmSPX3*-overexpressing hairy roots. Increased expression of *GmSPX3* in transgenic hairy roots was verified through qPCR analysis (Supplementary Data Fig. S2). Soybean transgenic composite plants were grown in nutrient solution at two P levels. After 14 d, the fresh weight and P concentration were separately determined. Under P deficiency conditions, the fresh
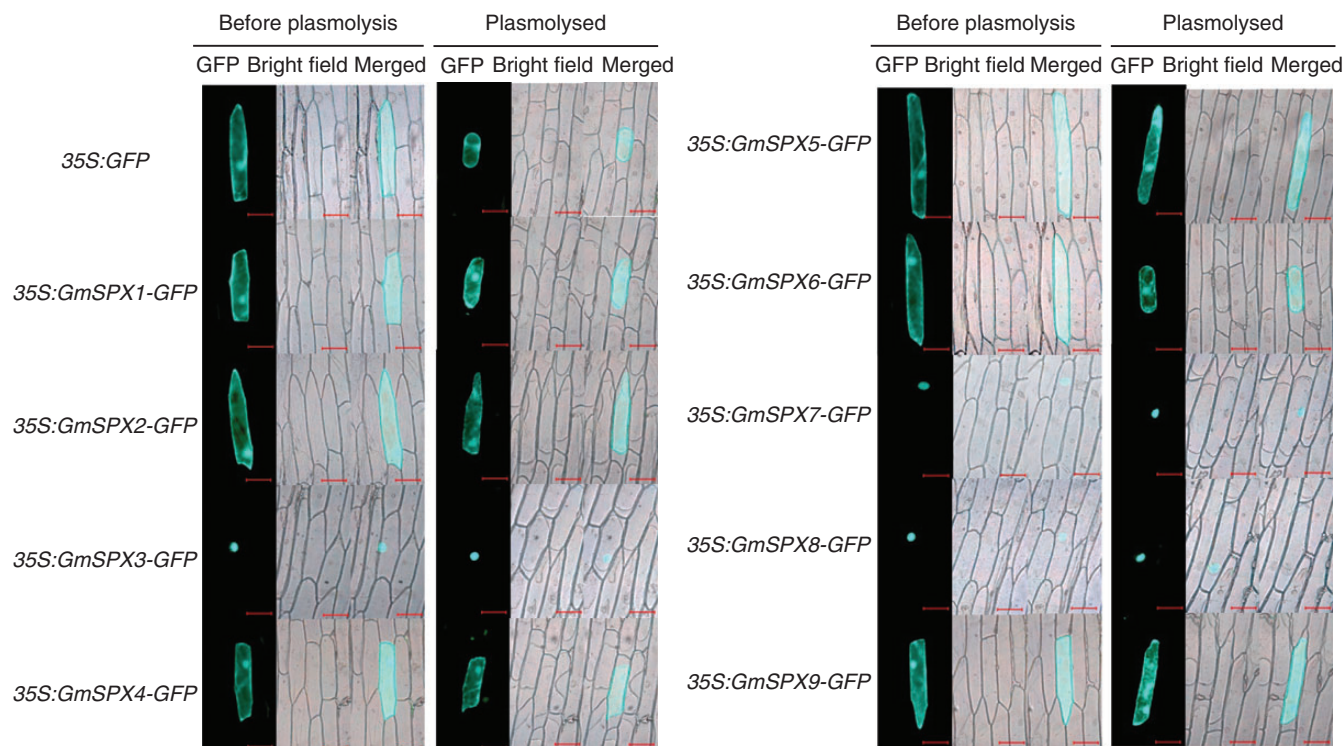
FIG. 2. Sub-cellular localization of GmSPX in onion epidermal cells. The first row shows the localization of the empty vector control before and after plasmolysis, followed by localization of 35S:GmSPX–GFP constructs before and after plasmolysis. Cells were observed by green fluorescence of GFP in the control and of GmSPX–GFP protein constructs in the remaining panels. Scale bars = 100 μm.

weight of CK (i.e. soybean hairy roots transformed with the empty vector) and OX (i.e. soybean hairy roots overexpressing *GmSPX3*) lines was similar (Fig. 6). However, under P-sufficient conditions, the fresh weight of the OX line was significantly lower than that in the CK line (Fig. 6). In contrast, higher P concentrations were observed in shoots and roots of the OX line under P-sufficient conditions (Fig. 6), suggesting that overexpression of *GmSPX3* could increase P accumulation in both shoots and roots in soybean.

GmSPX3 participates in the soybean P signalling network

In order to demonstrate the regulatory roles of *GmSPX3* in the soybean P signalling network, transcription levels of seven Pi starvation-responsive genes were analysed in OX and CK transgenic hairy roots. The seven genes comprised a ribonuclease gene (*GmRNS1*), *GmIPS1*, two Pi transporters (*GmPT2* and *GmPT7*), a protein phosphatase (*GmHAD1-6*), a malate transporter (*GmALMT1*) and a purple acid phosphatase (*GmPAP21*). The qPCR results showed that overexpressing *GmSPX3* led to significantly increased transcription of all the seven tested genes in the transgenic hairy roots (Fig. 7). Among them, compared with those in CK lines, the expression levels of three genes in *GmSPX3*-overexpressing soybean hairy roots were increased by >2-fold, but <8-fold, namely *GmPT2*, *GmIPS1* and *GmHAD1-6* (Fig. 7). However, transcripts of the remaining genes were increased by >20-fold, i.e. *GmPT7*, *GmALMT1*, *GmRNS1* and *GmPAP21* (Fig. 7). These results strongly suggest that GmSPX3 might positively regulate the transcription levels of Pi starvation-responsive genes in soybean.

DISCUSSION

Critical roles in the P signalling network for proteins containing the SPX domain have been well documented in model organisms, including yeast, arabidopsis, bean and rice (Duan *et al.*, 2008; C. Wang *et al.*, 2009; Z. Wang *et al.*, 2009; Liu *et al.*, 2010; Chiou and Lin, 2011; Secco *et al.*, 2012*a*; Lv *et al.*, 2014; Shi *et al.*, 2014; Yao *et al.*, 2014). However, genome-wide analysis of SPX family members in other crops remains scarce. The release of the soybean genome sequence provided an opportunity to characterize *GmSPX* members and to dissect their potential roles in soybean adaptation to P deficiency. In this study, structure, sub-cellular localization and expression patterns of *GmSPX* members were analysed in soybean for the first time. Furthermore, positive regulatory roles of GmSPX3 in the P signalling pathway were evaluated.

In total, nine GmSPX members were identified in the soybean genome by blastp searches on the Phytozome website (Table 1). Through phylogenetic analysis, SPX members in plants can be divided into three groups. Most SPX members in the tested species belong to group I, which can be further classified into two sub-groups (Fig. 1). In subgroup I-1, three GmSPX members, GmSPX3, GmSPX7 and GmSPX8, were grouped with AtSPX1 from arabidopsis, OsSPX1 from rice and PvSPX1 from bean; all of them have been well documented to be involved in plant P signalling (Duan *et al.*, 2008; C. Wang *et al.*, 2009; Z. Wang *et al.*, 2009; Liu *et al.*, 2010; Yao *et al.*, 2014). Furthermore, GmSPX3, GmSPX7 and GmSPX8 were localized in the nucleus (Fig. 2), the same as AtSPX1, OsSPX1 and PvSPX1 (Duan *et al.*, 2008; Z. Wang *et al.*, 2009; Yao
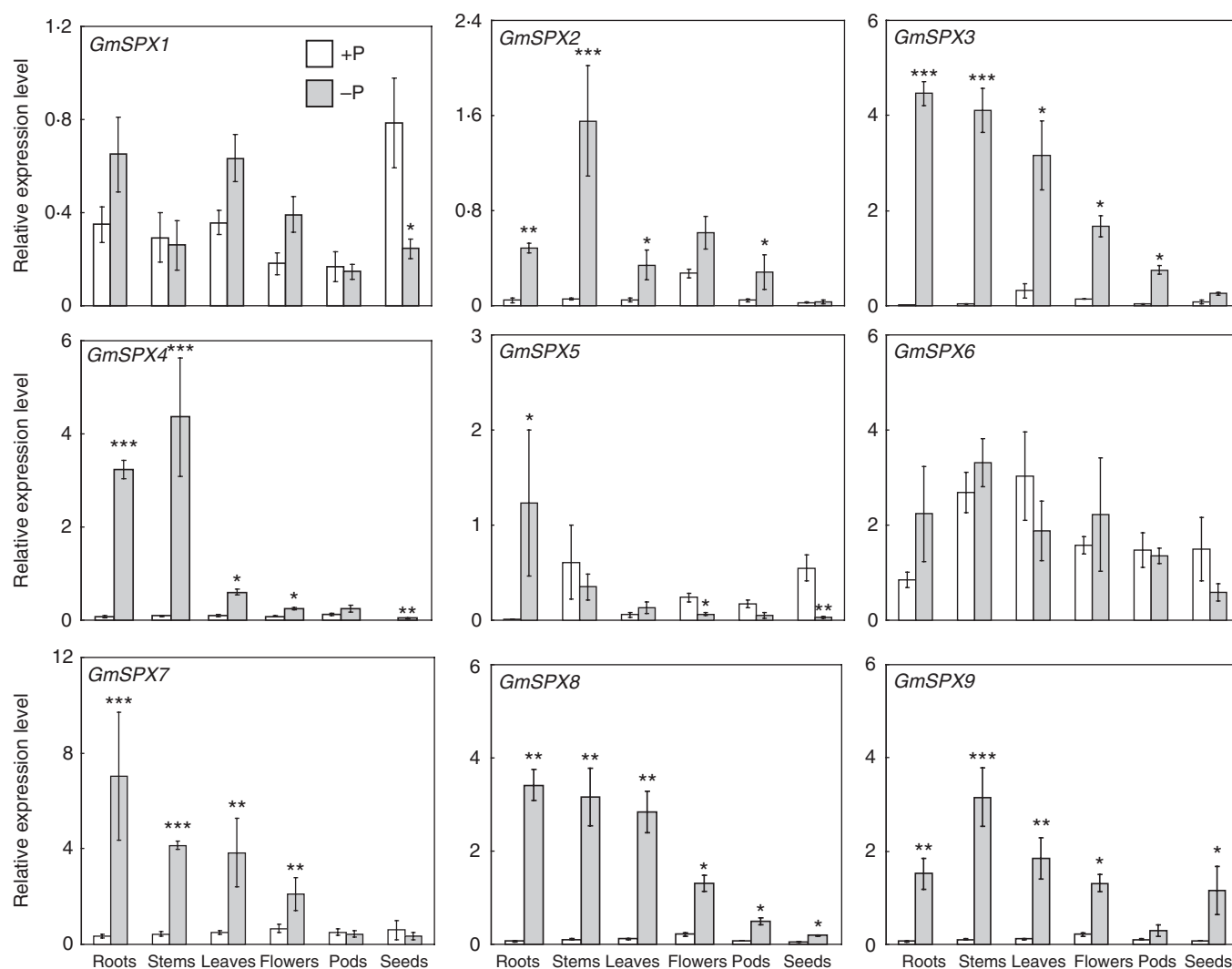
FIG. 3. Expression levels of *GmSPX* members in various tissues of soybean under high-P (+P) and low-P (−P) conditions. Asterisks indicate significant differences in *GmSPX* gene expression levels between high-P and low-P conditions. *$0.01 < P < 0.05$; **$0.001 < P < 0.01$; ***$P < 0.001$.

et al., 2014), which further suggests that these three GmSPX members might play regulatory roles in soybean P signalling similar to those of AtSPX1, OsSPX1 and PvSPX1 in their respective genomes (Fig. 2). Other GmSPX members were found in many locations in onion epidermal cells, including the cytoplasm and nucleus (Fig. 2). This suggests that GmSPX members might fulfil diverse functions throughout the cell. It has been suggested that the nuclear-localized AtSPX1 and inter-cellular membrane-localized AtSPX3 play contrasting regulatory roles in arabidopsis P signalling (Duan *et al.*, 2008). A similar pattern requiring further characterization is possible in soybean.

With the advent of next-generation sequencing, transcriptomics analysis has been performed in various tissues of soybean, and transcripts of *GmSPX* members have been detected (Libault *et al.*, 2010; Severin *et al.*, 2010). Consistent with these reports, transcripts of all *GmSPX* members were found among the tested tissues of soybean (Fig. 3). However, expression levels of several *GmSPX* members differed from previous results. For example, preferential expression in flowers was

observed for *GmSPX2*, *GmSPX3*, *GmSPX7* and *GmSPX9* at high P levels (Fig. 3), which contrasted with results from previous transcriptomics analyses (Libault *et al.*, 2010; Severin *et al.*, 2010). The inconsistencies might be caused by different experimental materials, growth conditions or techniques.

Responses of *GmSPX* members to P deficiency were examined in a variety of soybean tissues. In this study, transcripts of most *GmSPX* genes were significantly increased by P deficiency in more than four tissues (Fig. 3). However, no response to P deficiency was observed for *GmSPX6* in any tested tissue, or for *GmSPX1* in any of the tested tissues except seeds (Fig. 3). Similar expression patterns of *SPX* members were found in arabidopsis, rice and bean. In arabidopsis, P deficiency resulted in increased transcription of *AtSPX1*, *AtSPX2* and *AtSPX3*, but reduced transcription of *AtSPX4* in shoots and roots (Duan *et al.*, 2008). In rice, expression of five *OsSPX* members (i.e. *OsSPX1*, *OsSPX2*, *OsSPX3*, *OsSPX5* and *OsSPX6*) was upregulated by P deficiency, while no response was observed for *OsSPX4* (Z. Wang *et al.*, 2009). Although genome-wide analysis
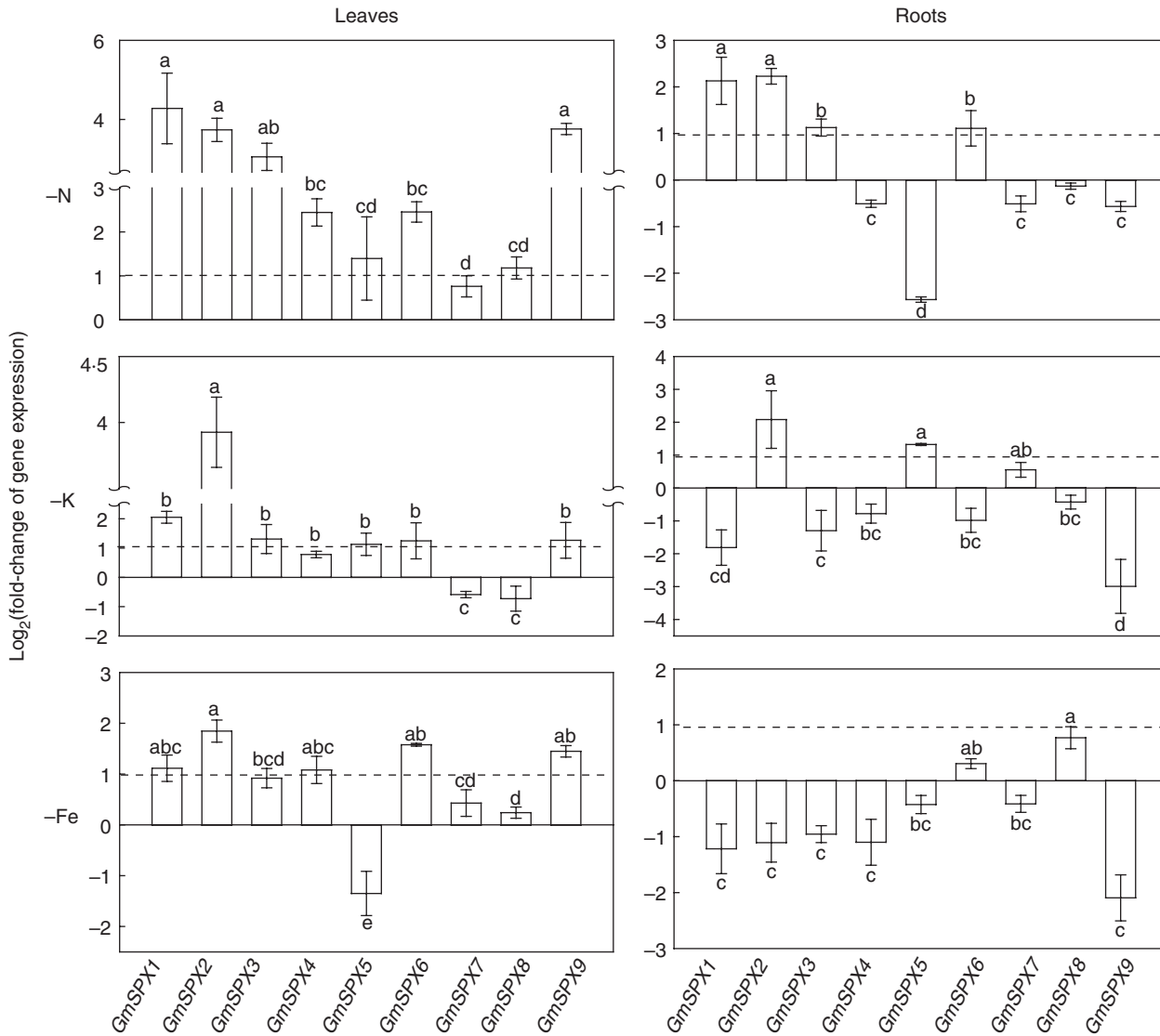
FIG. 4. Expression patterns of *GmSPX* in response to nutrient deficiency in leaves and roots. Soybean seedlings with the first fully developed trifoliate leaves were transplanted separately to nutrient solution lacking N (−N), K (−K) or Fe (−Fe). Control plants (CK) were those grown in full-strength solution. Leaves and roots were harvested separately after 14 d of the treatments. The fold change of the expression of *GmSPX* genes between nutrient-deficient and control treatments was calculated as $\log_2$ (nutrient deficiency/CK). Data in the figure are the mean of four replicates with the standard error. Dotted lines indicate the $\log_2(2)$ scale. Different letters represent significant differences for *GmSPX* transcripts at 0·05 levels.

of the responses of *PvSPX* members to Pi starvation have not been studied in bean, at least three *PvSPX* members were upregulated by Pi starvation (Yao *et al.*, 2014). These results suggest that the expression patterns of *SPX* members in responses to P deficiency might be conserved among plant species, and diverse functions might be present among SPX members. Interestingly, several SPX members exhibiting no upregulated response to Pi starvation were classified into group III, including GmSPX6 in soybean, AtSPX4 in arabidopsis and OsSPX4 in rice (Fig. 1), which strongly suggests conservation of functions among these SPX members in plants that warrants further clarification.

In addition to P deficiency responses, genome-wide analysis of *GmSPX* responses to other nutrient deficiencies was first

examined in plants. The results showed that expression of most *GmSPX* members was significantly affected by N, K or Fe deficiency, particularly in leaves (Fig. 4). This suggests that transcriptional responses of *GmSPX* members to P deficiency are non-specific, and that interactions are possible between pathways involved in soybean adaptation to P, N, Fe or K deficiency. Although cross-talk between these nutrients has been studied and recognized, the critical components remain to be characterized (Coello and Polacco, 1999; Wang *et al.*, 2002; Misson *et al.*, 2005). Recently, it has been documented that a protein containing the SPX domain, AtNLA, could control Pi homeostasis in a nitrate-dependent manner in arabidopsis (Kant *et al.*, 2011), which indicates that proteins containing the SPX domain might
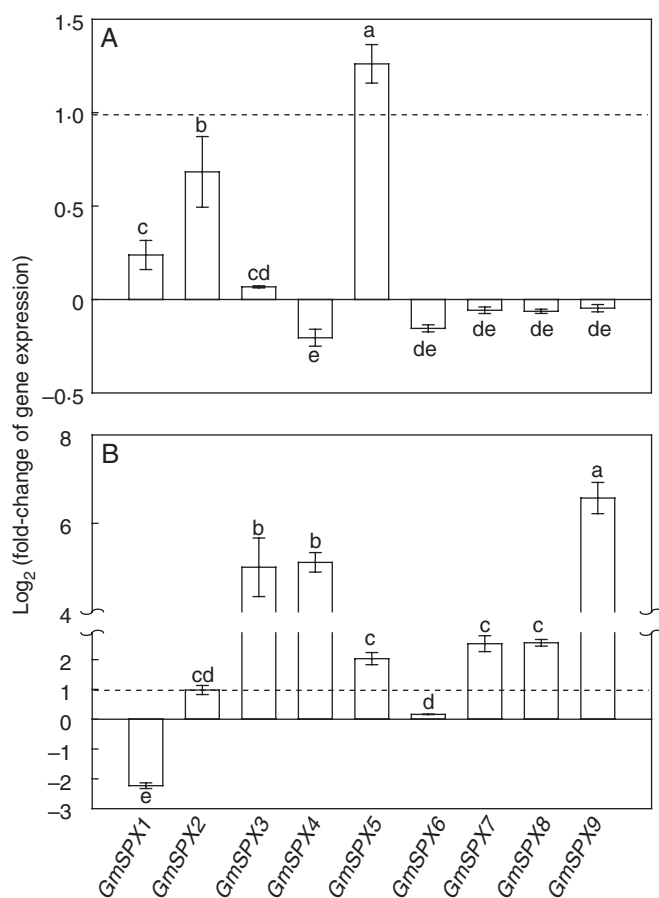
Fɪɢ. 5. Expression patterns of *GmSPX* in mycorrhizal roots and nodules. (A) Fold change of the expression of *GmSPX* in roots inoculated with AM fungi (+AM) relative to roots without AM fungi inoculation (−AM) was calculated as $\log_2$ (+AM/−AM). (B) Fold change of the expression of *GmSPX* in nodules formed in low-P (−P) treatment relative to those in high-P (+P) treatment was calculated as $\log_2$ (−P nodules/+P nodules). Data in the figure are the mean of four replicates with the standard error. Dotted lines indicate the $\log_2(2)$ scale. Different letters represent significant differences for *GmSPX* transcripts at 0·05 levels.

be important for dissecting interactions among the nutrients in plants in the future.

Soybean can form nodules with rhizobia, which fix atmospheric N and provide N resources for soybean growth. However, biological N fixation is a high energy consuming process, which is easily inhibited by P deficiency (Chaudhary *et al.*, 2008; Hernández *et al.*, 2009; Chen *et al.*, 2011; Qin *et al.*, 2012). Therefore, it has been documented that one adaptive strategy of soybean nodules to P deficiency is to maintain Pi homeostasis through increased transcription of a Pi transporter gene, *GmPT5*, in nodules (Qin *et al.*, 2012). In this study, we found that transcripts of seven of nine *GmSPX* members increased considerably in nodules exposed to P deficiency (Fig. 5B). Since it has been well documented that *AtSPX3*, four *OsSPX* members (i.e. *OsSPX1*, *OsSPX3*, *OsSPX4* and *OsSPX5*) and *PvSPX1* participate in regulating Pi homeostasis in plants, it can be hypothesized that some GmSPX members are involved in regulating Pi homeostasis

in soybean nodules. Another typical symbiotic association for soybean is to develop symbiotic relationships with AM fungi, which affect plant Pi acquisition and utilization (Wang *et al.*, 2010). Recently, it has been documented that inoculation of AM fungi significantly increases transcription of a group of genes in soybean, such as purple APases (*GmPAP*) and expansin (*GmEXPB*) genes (Li *et al.*, 2012; Li *et al.*, 2013). Strangely, most *GmSPX* members exhibited no response to inoculation with AM fungi, except for *GmSPX5* (Fig. 5A). Since regulatory roles for SPX in P signalling have been well documented in rice and arabidopsis, it is possible *GmSPX5* might be involved in regulating a group of AM fungal symbiosis-related genes in soybean.

Among all the GmSPX members, GmSPX3 was most similar to PvSPX1, AtSPX1 and OsSPX1 in sequence homology, Pi starvation responses and nuclear localization. Therefore, we selected *GmSPX3* as the candidate for further functional analysis in soybean. Composite soybean plants with *GmSPX3*-overexpressing hairy roots were generated. In this study, seven Pi starvation-responsive genes were significantly upregulated in the *GmSPX3*-overexpressing roots, implying that GmSPX3 is a positive regulator in the soybean P signalling network (Fig. 7). Regulatory roles for SPX family members in P signalling have also been identified in arabidopsis, rice and bean. Duan *et al.* (2008) found that *AtSPX1* overexpression in arabidopsis enhances the transcription of several Pi starvation-induced genes (e.g. *AtACP5*, *AtPAP2* and *AtRNS1*), indicating a positive regulatory role for *AtSPX1*. Similarly, a positive regulatory role for PvSPX1 has also been suggested in bean (Yao *et al.*, 2014). On the other hand, regulatory roles for SPX members appear to vary among plant species, because OsSPX1, OsSPX3, OsSPX4 and OsSPX5 negatively regulate expression levels of several Pi starvation-induced genes in rice (C. Wang *et al.*, 2009; Liu *et al.*, 2010; Lv *et al.*, 2014; Shi *et al.*, 2014). These results imply that the molecular mechanisms underlying the regulatory roles of SPX members might be different between monocot and dicot plants, which requires further study. Consistent with increased transcription of two Pi transporter genes, *GmPT2* and *GmPT7*, in *GmSPX3*-overexpressing hairy roots, the P concentration in shoots and roots of *GmSPX3*-overexpressing composite plants was significantly higher than that in control lines under high-P conditions (Fig. 6). This suggests that *GmSPX3* plays a regulatory role in Pi absorption and translocation in soybean. The involvement of *SPX* members in Pi homeostasis has also been demonstrated in rice, in which suppression of *OsSPX1* results in P overaccumulation in both leaves and roots under high P conditions (C. Wang *et al.*, 2009; Liu *et al.*, 2010). However, the molecular mechanisms of SPX regulation of P acquisition and translocation need to be further clarified.

In summary, *GmSPX* members were systematically characterized with respect to gene structure, sub-cellular localization, tissue-specific expression patterns and responses to nutrient (i.e. N, P, K and Fe) deficiencies. Furthermore, the functions of *GmSPX3* involved in the P signalling pathway and Pi homeostasis in soybean have been elucidated. These results provide not only comprehensive information on properties of *GmSPX* members in soybean, but also the impetus to understand further the molecular mechanisms underlying soybean adaptation to P deficiency.
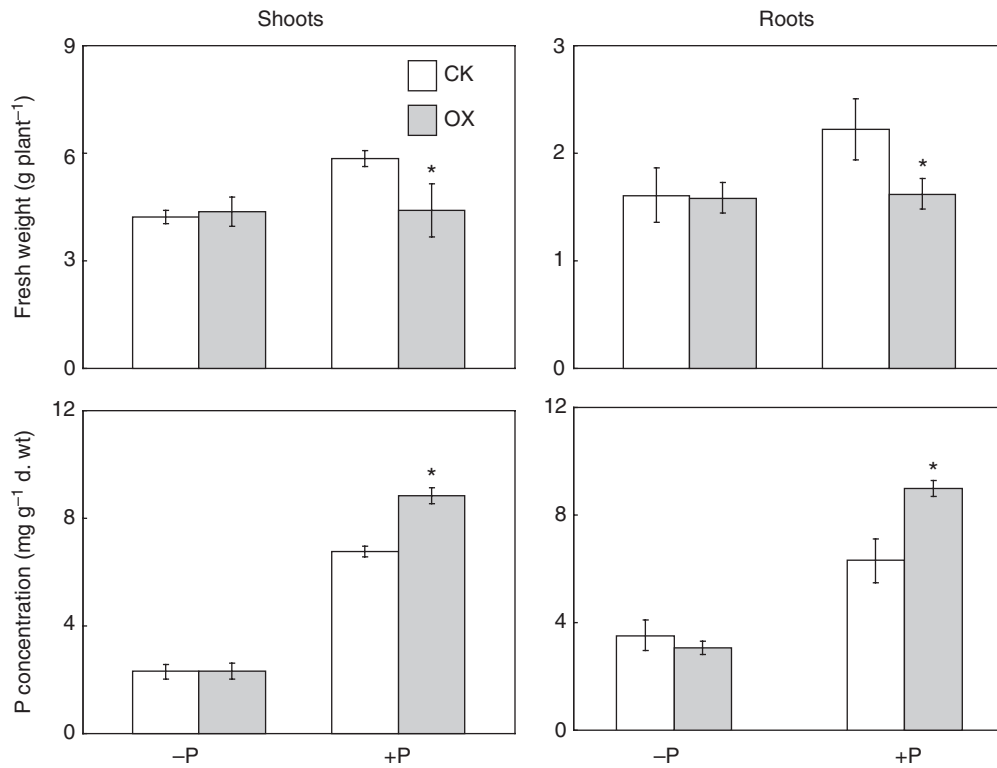
FIG. 6. Fresh weight and P concentration in composite soybean plants. Fresh weight (top) and phosphorus concentration (bottom) in shoots and roots. Composite soybean plants with transgenic hairy roots were grown in full-strength nutrient solution for 7 d prior to transfer into nutrient solution containing 5 μM (−P) or 500 μM (+P) phosphorus. After 14 d in P treatments, shoots and roots were harvested separately for analysis. CK represents soybean hairy roots transformed with the empty vector; OX indicates transgenic soybean hairy roots overexpressing of *GmSPX3*. Each bar is the mean of four replicates with the standard error. Asterisks indicate significant differences between OX and CK within the same P treatments at 0·05 levels.
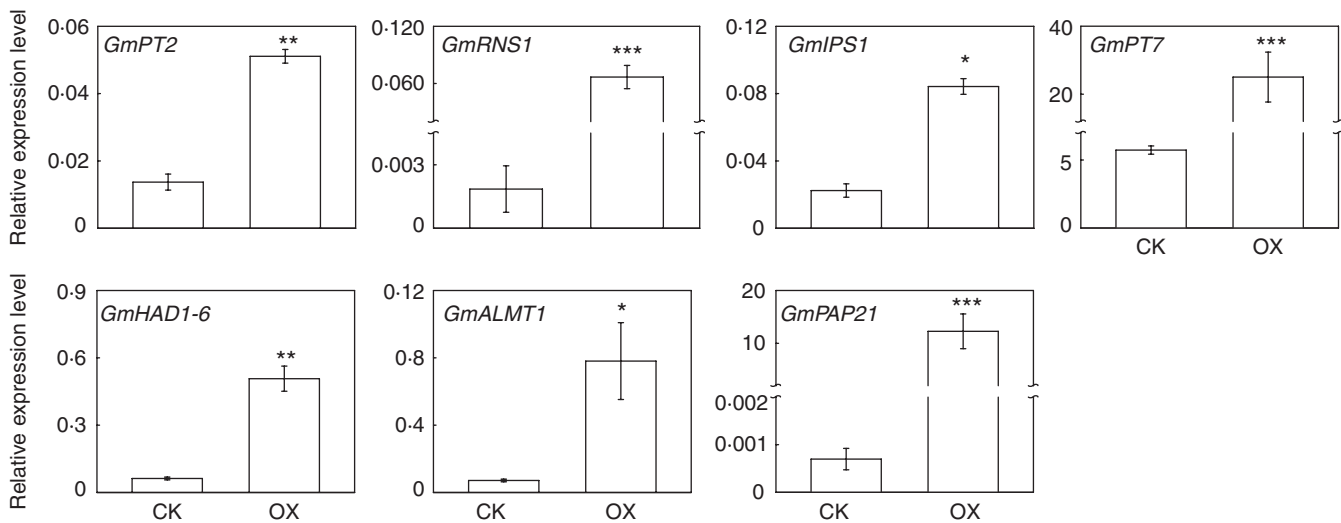

FIG. 7. Transcription of seven Pi starvation-responsive genes in CK and *GmSPX3*-overexpressing transgenic hairy roots. Soybean composite plants were grown in nutrient solution containing 500 μM (+P) phosphorus for 21 d. Transcripts of the seven genes in hairy roots were determined by qPCR analysis. CK represents soybean hairy roots transformed with the empty vector; OX indicates transgenic soybean hairy roots overexpressing *GmSPX3*. Each bar is the mean of four replicates with the standard error. Asterisks indicate significant differences in downstream gene expression between OX and CK. $*0·01 < P < 0·05$; $**0·001 < P < 0·01$; $***P < 0·001$.

SUPPLEMENTARY DATA

Supplementary data are available online at www.aob.oxford journals.org and consist of the following. Table S1: primer sequences used for quantitative real-time PCR. Table S2: primer sequences used for transient expression and *GmSPX3* overexpression. Figure S1: multiple alignment of the GmSPX

family members in soybean. Figure S2: expression of *GmSPX3* in transgenic soybean hairy roots.

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