

Genome-wide identification and expression profiling analysis of the SPX gene family in *Setaria italica*

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Abstract

Background

In plant adaptation to adversity, phosphorus is pivotal and exerts a beneficial influence on both yield and quality formation in foxtail millet. Proteins containing SPX domains are essential for the maintenance of phosphate ion (Pi) balance in plants. However, the mechanisms that control phosphorus deficiency in foxtail millet are not yet fully understood.

Results

Through genome-wide analysis, we identified 15 members of the millet *SPX* gene family, which were categorized into four subfamilies (*SPX*, *SPX-MFS*, *SPX-EXS*, and *SPX-RING*) according to their structural characteristics. The genes encoding proteins with SPX domains were found on six out of the 9 chromosomes. Enrichment for multiple biotic and abiotic stresses, as well as hormone responses, was observed in the promoter regions of *SiSPXs*. Analysis of transcriptome data (RNA-seq) revealed diverse expression patterns among 15 genes encoding SPX-domain-containing proteins. Under low phosphate (Pi) stress, specific members, such as *SiSPX3*, and *SiSPX9*, showed a pronounced response.

Conclusions

The *SPX* gene primarily regulates phosphorus homeostasis and transport in foxtail millet by binding to the signal molecule InsP₈. This study enhanced the comprehension of the millet *SPX* gene family, thereby advancing future biological investigations of *SiSPX* genes in millet. Additionally, it offers a selection of potential candidate genes for functional research on phosphate ion (Pi) nutrition in millet.

Background

The SPX domain (PF: 03105) is named according to the initials of yeast gpa1 (SYG1), phosphatase 81 (PHO81), and heterotrophic and polytropic retrovirus receptor 1 (XPR1) [3, 4]. According to the domain, the *SPX* family can be divided into four different subfamilies, namely *SPX*, *SPX-RING*, *SPX-MFS* and *SPX-EXS*. Previous studies have confirmed that *SPX* family genes are essential for phosphorus signal transduction and maintenance of phosphorus homeostasis in plants. SPX protein has been confirmed to exist in Arabidopsis (20), rice (15), and maize (33) [5, 6, 7].

The *SPX* subfamily represents the first type. Receptor SPX proteins perceive inositol polyphosphate (InsP) as a phosphorus signal, which is crucial for plant growth, energy metabolism, and stress resistance. Arabidopsis and rice contain four and six SPX protein genes, respectively (*AtSPX1-AtSPX4* and *OsSPX1-OsSPX6*) [8]. OsPHR2 binds to cis regulatory element (P1BS) dimerization under low phosphorus conditions, activating phosphorus activation reactive protein, thereby enhancing phosphorus absorption and utilization [9]. In Arabidopsis, the nuclear protein AtSPX1 functions as a Pi-dependent inhibitor, competitively inhibiting the regulatory factor PHR1's recognition of P1BS elements [10]. The *SPX-EXS* subfamily, is known as the *PHO1* gene in Arabidopsis and rice. PHO1 transporters primarily facilitate phosphorus movement from root epi-dermal cells to the xylem and back to the roots via the phloem [11]. Arabidopsis contains ten *AtPHO1* homologous genes, *AtPHO1*; *H1-H10*, predominantly expressed in root, leaf, stem, and flower vascular tissues. *AtPHO1* overexpression triggers phosphate influx from vacuoles to the cytoplasm and efflux from cells, leading to xylem accumulation and secretion levels 100 times higher than in wild-

type Arabidopsis [12, 13, 14]. In alfalfa, *MtPHO1.1* and *MtPHO1.2* exhibited wide expression in the nodule area [15], while maize's four *ZmPHO1* genes are all induced by low-phosphorus conditions [16].

The *SPX-MFS* subfamily, known as the *VPT* gene in Arabidopsis. This gene maintains phosphorus homeostasis in plants under adequate phosphorus conditions, with vacuoles storing up to 70% of cellular phosphates. The Arabidopsis AtVPT transporter primarily facilitates phosphorus storage in vacuoles, transferring inorganic phosphorus from the cytoplasm to vacuoles and regulating cellular Pi stability. The *vpt1* mutant exhibits severely compromised phosphorus accumulation and heightened sensitivity to high phosphorus toxicity. Conversely, their overexpression leads to increased phosphate accumulation in plants, thereby impacting plant growth [17]. The *SPX-RING* subfamily represents the fourth type, is known as the *NLA* gene in Arabidopsis and rice. The *NLA* encodes a E3 ubiquitin ligase, which facilitates the ubiquitination of phosphorus transporters within the plasma membrane. In Arabidopsis, *AtNLA* (Nitrogen limitation adaptation, NLA) is regulated by *miR827* under low-phosphorus conditions. The primary cause of leaf senescence in *atnla* mutant plants is phosphorus toxicity, a phenotype that can be rescued by *AtPHT1* and *AtPHT1;1* [18].

Millet is one of the World's oldest crops and a model plant for stress-responsive biology and genetics research. Phosphorus is an essential nutrient for plant growth and development. Phosphorus, as a component of nucleic acids, phospholipids and primary metabolites in plants, is crucial in maintaining the structure of cells and exerting the function of cells. It directly restricts most of the physiological reactions in plants, such as photosynthesis, respiration, metabolic reactions, and glycolysis. The normal growth of foxtail millet requires sufficient phosphorus, the role of *SPX* gene family mainly lies in the collection, transport, distribution of phosphorus and plays an important role in Pi signaling pathway. Therefore, it is meaningful to perform genome-wide identification and characterization of the *SPX* domain protein gene family of foxtail millet.

In this study, we identified 15 protein genes containing the *SPX* domain, which belong to four subfamilies (*SPX*, *SPX-EXS*, *SPX-MFS*, *SPX-RING*). In addition, we systematically analyzed 15 protein genes containing *SPX* domain, including gene structure, phylogenetic relationship, conserved motif, chromosome distribution, gene collinearity and expression pattern prediction. This result allows us to clearly understand how they play a potential key role in maintaining phosphorus balance. Therefore, this study preliminarily revealed the relationship between *SiSPX* gene family members and low phosphorus stress response, thereby establishing a foundation for further investigation into their function and mechanism in low phosphorus response.

Methods

Identification of *SPX*-domain-containing proteins in Foxtail Millet.

Obtained from the foxtail millet database are the genome, proteome, coding sequence, and GFF annotation files for this species [19]. The *SPX* peptide sequences originating from Arabidopsis and rice were downloaded and subsequently utilized as queries in a BLASTP search against the foxtail millet proteomes, employing an E-value threshold of 1×10^{-5} [20]. Subsequently, download Hidden Markov Model (HMM) via PF: 03105 of *SPX* domain, HMM-based search program Hmmer3 performs HMM search on foxtail millet protein sequences [21, 22]. Following BLASTP screening, the candidate gene sequences were further refined based on the *SPX* gene family's key features, resulting in the final identification of *SiSPX* genes in foxtail millet. The physical and chemical properties, including molecular weight, isoelectric point, and hydrophilicity, of the *SiSPX* peptide sequence were analyzed using ExPASy Proteomics [23].

Analysis and classification of SiSPX genes in Foxtail Millet through phylogenetics

The multiple sequence alignment of SPX protein sequences from Arabidopsis, rice, and foxtail millet was carried out using MAFFT [24]. The alignment results were employed in MEGA11.0 software to construct a phylogenetic tree using the neighbor-joining algorithm and 1000 bootstrap replicates [25]. Finally, the iTOL online tools were employed to enhance the phylogenetic tree's visual appearance [26].

Analysis of gene structure and conserved motifs in SiSPX genes of Foxtail Millet The candidate genes' structures were visualized using GSDS2.0 [27]. Utilizing the Multiple Expectation Maximization for Motif Elucidation suite, conserved motifs were identified with a maximum parameter value of 5. These motifs displayed an optimal width spanning from 6 to 200 residues, each demonstrating an E-value below 1×10^{-10} [28].

Analysis of cis-regulatory elements and chromosomal distribution of SiSPX genes in foxtail millet

The TBtools software was employed to retrieve the promoter sequences (2000 bp upstream of the transcription start site) of *SiSPX* genes, which were then analyzed for cis-regulatory elements using the PlantCARE database [29]. Subsequently, the analysis results were graphed with the use of R scripts.

Expression Pattern Analysis of *SiSPX* Genes in Foxtail Millet

To identify the expression characteristics of the *SPX* gene in various tissues, Jigu 13 (a laboratory millet material) was used as the test material, with its roots, stems, leaves, and ears at the filling stage being collected for RNA extraction. These were then reverse transcribed into cDNA. The levels of *SPX* gene expression in these tissues were evaluated using real-time fluorescent quantitative PCR, with comparisons made to the expression levels in the root. Millet RNA was isolated utilizing a polysaccharide polyphenol plant total RNA extraction kit (Tiangen, Beijing), followed by cDNA synthesis using the PrimeScript™ II 1st Strand cDNA Synthesis Kit (Perfect Real Time). The reaction procedure involved a two-step method: initial denaturation at 95 °C for 3 minutes, followed by 40 cycles of denaturation at 95 °C for 5 seconds, annealing at 60 °C, and extension for 30 seconds. The $2^{-\Delta\Delta C_t}$ method was employed to determine the relative expression of the target gene.

Response of *SiSPX* genes to low P in Foxtail Millet

Jigu 13 was grown in pots, with two fertilization treatments: CK (120 mg/kg of N, 100 mg/kg of P_2O_5 , 70 mg/kg of K_2O) and LP (120 mg/kg of N, 0 mg/kg of P_2O_5 , 70 mg/kg of K_2O), each with three biological replicates. RNA was extracted from above-ground and underground parts of Jigu 13 for reverse transcription. *SiSPX* relative expression levels were quantified using qPCR, following the method described in 2.5ds. The study examined the expression profiles of *SiSPX* family members across diverse tissues subjected to low phosphorus stress. Data analysis was performed using SPSS 19, while OriginPro 2021 software was used for mapping.

Results

Identification of the SiSPX genes in *Setaria italica*

Through the utilization of HMM search and multiple rounds of BLASTP analysis, genes exceeding a significance threshold of $E < 10^{-5}$ were pinpointed within the entire genome. A total of 15 *SPX* gene candidates were discovered in the foxtail millet genome and denoted as *SiSPX1-SiSPX15* based on their chromosomal locations (Table 1). These genes were divided into four subfamilies based on conserved domain differences (Fig. 1). The *SPX* subfamily,

containing only one SPX domain, has six members (*SiSPX3/8/9/13/14/15*). The *SPX-EXS*, *SPX-MFS*, and *SPX-RING* subfamilies have three (*SiSPX2/7/10*), four (*SiSPX1/4/6/11*), and two members (*SiSPX2/5*). The *SiSPX* proteins display a protein sequence length that varies from 252 (*SiSPX15*) to 849 (*SiSPX7*) amino acids. Their molecular weights range from 28.22 (*SiSPX15*) to 97.22 (*SiSPX7*) kDa, and their theoretical pI values range from 4.91 (*SiSPX3*) to 9.84 (*SiSPX14*). Eight *SiSPX* proteins were acidic, and seven were alkaline. Signal peptides were predicted in three of the 15 SPX proteins. The average hydrophobicity index ranged from -0.660 to 0.241.

Table 1
Analysis of physicochemical properties of SPX genes in the foxtail millet genome

Gene ID	Gene Name	Chromosome Location	Signal Peptide Prediction	Number of Amino Acids (aa)	Molecular Weight (kD)	Isoelectric Point (pI)	GRAVY
SETIT_016511mg	<i>SiSPX1</i>	Chr1:34468314–34473622	-	696	77.73	6.25	0.180
SETIT_016343mg	<i>SiSPX2</i>	Chr1:41039587–41045398	-	802	90.00	9.51	0.001
SETIT_018026mg	<i>SiSPX3</i>	Chr1:4369196–4371337	+	282	31.37	4.91	-0.582
SETIT_032524mg	<i>SiSPX4</i>	Chr2:37705406–37708296	-	655	72.71	6.25	0.241
SETIT_030542mg	<i>SiSPX5</i>	Chr2:48008454–48011476	-	321	36.10	8.75	-0.168
SETIT_005968mg	<i>SiSPX6</i>	Chr4:1240707–1247249	+	699	77.58	7.48	0.183
SETIT_005850mg	<i>SiSPX7</i>	Chr4:17829122–17833674	-	849	97.22	9.18	-0.133
SETIT_006987mg	<i>SiSPX8</i>	Chr4:34331493–34335790	-	302	33.86	6.71	-0.542
SETIT_002654mg	<i>SiSPX9</i>	Chr5:24684447–24685331	-	253	28.67	5.39	-0.261
SETIT_000294mg	<i>SiSPX10</i>	Chr5:6365034–6369978	+	844	94.40	9.08	-0.139
SETIT_009523mg	<i>SiSPX11</i>	Chr7:27771624–27778529	-	696	78.01	6.10	0.215
SETIT_036461mg	<i>SiSPX12</i>	Chr9:10010452–10015528	-	342	39.19	7.43	-0.304
SETIT_037151mg	<i>SiSPX13</i>	Chr9:1499434–1501982	-	252	28.60	5.24	-0.660
SETIT_039108mg	<i>SiSPX14</i>	Chr9:20299892–20301516	-	261	29.11	9.84	-0.232
SETIT_037147mg	<i>SiSPX15</i>	Chr9:42960333–42961402	-	252	28.22	5.47	-0.238

Phylogenetic analysis and sequence similarity of *SiSPX* genes

For the assessment of the evolutionary relationships of SiSPX proteins in foxtail millet, Arabidopsis, and rice, a phylogenetic tree was constructed through the application of the neighbor-joining (NJ) method (Fig. 2). This tree was based on the full-length sequences of 15 SiSPX proteins alongside other SPX proteins from the three species. The *SPX* members formed four distinct groups: *SPX*, *SPX-EXS*, *SPX-MFS*, and *SPX-RING*. The distribution of *SPX* subfamily proteins was highly asymmetrical across the species. For example, six *SiSPXs*, four *OsSPXs*, and three *AtSPXs* were classified in the *SPX* subfamily. In the *SPX-MFS* subfamily, there were three *OsSPXs*, four *SiSPXs*, and three *AtSPXs*. The *SPX-EXS* subfamily included three *SiSPXs*, 11 *AtSPXs*, and three *OsSPXs*. The *SPX-RING* subfamily had two *OsSPXs*, two *SiSPXs*, and two *AtSPXs*. The findings indicate that the *SPX* gene family displays a combination of high conservation and diversity across various plant species.

Analysis of gene structures and conserved motifs

We analyzed the gene structure and conserved motifs using NCBI-CDD and MEME databases, respectively. We found that within the same subgroups, SiSPXs exhibited similar gene structures and conserved motifs (Fig. 3). The *SPX-RING* subfamily had the fewest motifs, while the *SPX-EXS* subfamily had the most. In addition to *SPX* domains, the *SPX* family includes three specific domains: EXS, MFS, and RING. Among the 15 *SiSPXs*, three members contained the EXS domain, four contained the MFS domain, and two contained the RING domain. These differences in gene structure and conserved motifs likely reflect the diverse biological functions of *SiSPXs*.

Cis-Regulatory Elements in *SiSPX* Gene Promoters

Cis-elements play a crucial role as molecular switches in the transcriptional control of genes involved in plant development and responses to abiotic stress. In order to explore the regulatory mechanisms of *SiSPX* genes in the growth and development of foxtail millet, we analyzed the 2000 bp promoter sequences located upstream of the translation start codon of 15 *SiSPXs* obtained from foxtail millet genome sequences using the PlantCARE database. We have identified 10 types of cis-acting elements, which are divided into three distinct groups: plant hormone-responsive elements (auxin, gibberellin, abscisic acid), stress-responsive elements (low temperature and light stress), and others (Fig. 4). Among the *SiSPX* genes, the distribution of cis-acting elements was notably uneven. In accordance with earlier studies, each *SiSPX* gene harbored a minimum of one cis-acting element associated with phytohormones [30]. Over three types of phytohormone-responsive elements were identified in the promoters of the majority of *SiSPX* genes, except for *SiSPX2*, *SiSPX4*, *SiSPX5*, *SiSPX6*, and *SiSPX10*. Among the different types of cis-acting elements, PHR is found exclusively in three genes: *SiSPX3*, *SiSPX9*, and *SiSPX11*. Notably, *SiSPX9* has the highest number of PHR elements, with six in its promoter region, while *SiSPX3* and *SiSPX11* contain three and four, respectively. These findings indicate that *SiSPX* genes are involved in tissue development and may responsive to low phosphorus stress.

Chromosomal Distribution and Synteny Analysis of the *SiSPX* Genes

An analysis of chromosomal localization indicated that the 15 *SiSPX* genes are unevenly distributed among the six chromosomes of foxtail millet (Fig. 5). Chr1 and Chr4 each host three *SiSPX* genes, comprising 40% of the total. Chr2 and Chr5 each contain two genes (26.67%), while Chr7 has the least distribution, with only one *SiSPX* gene.

In order to explore the evolutionary mechanisms of *SiSPX* genes in foxtail millet, a collinear map was created, incorporating data from foxtail millet and three representative plant species: two monocots (rice and maize) and one dicot (Arabidopsis). Figure 6 illustrates that only one pair of collinear *SPX* genes was identified between Arabidopsis and foxtail millet, likely due to their distant phylogenetic relationship as a dicotyledon and monocotyledon,

respectively. Among monocotyledonous plants, a total of 16 pairs of collinear *SPX* genes were detected in the comparison between foxtail millet and maize, while the highest number of collinear *SPX* genes (19 pairs) was observed in the comparison between foxtail millet and rice.

Analysis of *SiSPX* Gene Expression Patterns across Various Tissues

Through qRT-PCR analysis, the expression patterns of *SiSPX* genes across different tissues of millet, such as roots, stems, leaves, and spikes, were investigated. The results (Fig. 7) indicated that 15 genes were expressed across these tissues under normal growth conditions. *SiSPX3*, *SiSPX4*, *SiSPX9*, and *SiSPX11* showed high expression in the roots, while *SiSPX5*, *SiSPX6*, *SiSPX8*, *SiSPX15*, and *SiSPX10* were highly expressed in the stems. In the leaves, *SiSPX12*, *SiSPX13*, and *SiSPX14* had the highest expression levels. *SiSPX1*, *SiSPX2*, and *SiSPX7* were most highly expressed in the spikes. Most *SiSPX* genes exhibited low relative expression in the leaves, thereby suggesting functional differences based on expression patterns.

Expression analysis of *SPX* family genes in different millet tissues treated with low phosphorus

In order to examine how low phosphorus levels affect the growth and development of *Setaria italica*, millet specimens were exposed to varying doses of phosphate fertilizer. Root length was considerably shorter when exposed to low phosphorus conditions as opposed to normal Pi supply. The expression levels of 15 *SiSPX* family members were assessed by qRT-PCR in the above-ground (stem, leaf, spike) and underground (root) parts of millet under low Pi treatment. In the above-ground parts (Fig. 7), *SiSPX1/2/3/4/5/6/8/9/11/12* were significantly up-regulated in stems under low phosphorus treatment. In leaves, *SiSPX1/2/3/4/6/9/11* were significantly up-regulated, while *SiSPX5/7/8/10* were down-regulated. In spikes, *SiSPX3/9/10/11* were significantly up-regulated, and *SiSPX1/6* were almost not expressed. In the underground part (Fig. 8), except for *SiSPX5*, which was significantly down-regulated, all other genes in the roots showed an up-regulation trend, with *SiSPX3/4/9/11* being significantly up-regulated.

In conclusion, under low-P treatment, the same gene may perform different functions in different tissues, responding uniquely to P level signals. *SiSPX3*, *SiSPX4*, *SiSPX9*, and *SiSPX11* were up-regulated in both above-ground and underground parts, with *SiSPX3* and *SiSPX9* significantly up-regulated in roots, stems, leaves, and spikes. These genes likely play a critical role in responding to low-P stress.

Discussion

In response to low-Pi tolerance, plants develop biochemical and physiological adaptations to enhance Pi absorption and utilization. The adaptive changes are intricately controlled by Pi signaling networks, with the SPX domain family genes playing a significant role in regulating the phosphorus signaling network. Millet, a significant crop in China, has experienced declining yields due to reduced planting areas and insufficient soil phosphorus levels. Therefore, understanding the biological functions of SPX proteins can clarify how *Setaria italica* adapts to low-Pi conditions. *Setaria italica* has fewer *SPX* genes compared to wheat (46), maize (33), and Arabidopsis (20). Environmental changes that took place during evolution are the probable cause of the variations seen among species. The relationships between orthologs and paralogs suggest that gene family diversity occurred both pre- and post- the divergence of monocots and dicots. Additionally, Mutations in regulatory regions, including promoters and coding sequences, might have caused varied expression patterns among duplicated genes. Research has demonstrated the

significant involvement of *SPX* family genes in modulating phosphate signaling networks in Arabidopsis, rice, and maize. However, the function and evolution of *SiSPX*s have not been reported. In this study, a total of 15 *SiSPX* genes were identified and divided into 4 subfamilies. The basic member distribution is *SPX* (6), *SPX-EXS* (3), *SPX-MFS* (4), *SPX-RING* (2).

Within the *SPX* subfamily, the distinguishing feature is the presence of an SPX protein domain at the N-terminus and the lack of a C-terminus domain. This family of proteins is responsible for regulating phosphate uptake and mobilization in plants [31]. The identification of six *SPX* family members (*SETIT_018026mg*, *SETIT_006987mg*, *SETIT_002654mg*, *SETIT_037151mg*, *SETIT_039108mg*, *SETIT_037147mg*) was made in the genome of *Setaria italica*. With the exception of *OsSPX5*, which contains two exons and one intron, the gene structure of the *SPX* family genes is comparable between rice and Arabidopsis, comprising three exons and two introns [32]. In the *SPX* family of *Setaria italica*, four members (*SETIT_018026mg*, *SETIT_006987mg*, *SETIT_037151mg*, and *SETIT_039108mg*) exhibit a gene structure similar to that of Arabidopsis. Upon closer examination, it was found that the gene structure of *SETIT_002654mg* and *SETIT_037147mg* bore similarities to *OsSPX5*. The *SPX* family genes in *Setaria italica* exhibited diverse expression patterns, with approximately half showing significant expression levels in root tissue.

The *SPX-EXS* subfamily is characterized by the presence of an SPX protein domain at the N-terminus and an EXS protein domain at the C-terminus. It may facilitate Pi uptake in the xylem or contribute significantly to the signaling cascade for Pi deficiency from roots to shoots. [33]. There are 11 genes belonging to the *SPX-EXS* family in *Arabidopsis thaliana*, which are *AtPHO1-AtPHO1*; h10. It has been shown that these genes are involved in Pi loading into xylem and displayed various expression patterns in Arabidopsis. [34]. Three genes, namely *OsPHO1*;1, *OsPHO1*;3, and *OsPHO1*;2, from the *SPX-EXS* family in rice, play a vital role in Pi translocation from roots to shoots [35]. Various expression patterns were observed among the members, with strong expression detected in roots for two specific members, namely *OsPHO1*;1 and *OsPHO1*;2. The *Setaria italica* genome harbors three members of the *SPX-EXS* family, designated as *SiSPX2*, *SiSPX7*, and *SiSPX10*. The expression patterns of the *SPX-EXS* family in *Setaria italica* closely resemble those observed in Arabidopsis and Rice patens. Notably, one member (*SETIT_016343mg*) displays dominant expression in root tissues.

The *SPX-MFS* subfamily, is characterized by the presence of an N-terminal SPX domain and a C-terminal MFS domain. This family is responsible for phosphate remobilization within leaf tissues. *SPX-MFS* family members exist in Arabidopsis (3) and rice (4). The expression function is different in different crops. For example, in Arabidopsis, it acts as a vacuolar Pi (vac-Pi) importers, The expression patterns of *AtPHT5*;1 and *AtPHT5*;3 were similar, being present in the majority of tissues, while *AtPHT5*;2 was confined to guard cells, vascular tissue, and pollen [36]. The *OsSPX-MFS* genes in rice function as vacuolar phosphate efflux transporters, facilitating the efflux of Pi from the vacuole to the cytoplasm. These genes, namely *OsSPX-MFS1*, *OsSPX-MFS2*, and *OsSPX-MFS3*, are expressed across various plant parts including leaves, stems, roots, flowers, and ears. Notably, the expression level of *OsSPX-MFS3* is significantly higher compared to *OsSPX-MFS1* and *OsSPX-MFS2* [37]. In *Setaria italica*, four members of the *SPX-MFS* family were identified, yet their functions remain unclear. In *Setaria italica*, the four *SPX-EXS* family genes were found to be expressed in different tissues, including the root, stem, leaf, and spike. Notably, one member of this family, *SETIT_009523mg*, displayed similarities to *OsSPX-MFS3* by exhibiting significantly higher expression levels across various tissues.

The *SPX-RING* subfamily, the fourth type, is distinguished by the presence of an N-terminal SPX domain and a C-terminal RING protein domain. Not only was it related to nitrogen limitation responses, but it was also involved in phosphate homeostasis [38]. In Arabidopsis, there is only one member, named *NLA*. In roots and stems, the expression of *AtNLA* surpasses that in seedlings, flowers, and leaves. *OsNLA1* and *OsNLA2* are both present in rice,

with *OsNLA1* serving a pivotal function in the regulation of Pi homeostasis. The expression of *OsNLA1* in roots and stems is higher than that in seedlings, and flowers. Comprising two members, the *SPX-RING* family in foxtail millet requires further examination of its function in Pi homeostasis. The expression of two *SPX-RING* family members (*SETIT_030542mg* and *SETIT_036461mg*) was detected in roots, stems, leaves, and spikes, although the levels were not notably high.

Conclusions

The millet genome contains a total of 15 genes that encode proteins with SPX domains. Various expression patterns were observed for all 15 *SiSPX* genes in different tissues under Pi stress conditions, as indicated by the study results. *SiSPX3* and *SiSPX9* showing high sensitivity to low Pi stress. The induction of this phenomenon in millet may be significantly mediated by the signaling molecule InsP8. In conclusion, these findings enhance our comprehension of the millet *SPX* gene family and offer potential some genes for further investigation into phosphate nutrition in *Setaria italica*.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

All data pertinent to this study are openly accessible, with detailed information on the databases, websites, and software utilized provided in the article for interested researchers. Supplementary Data includes: S1-Protein sequences of SPXs from plants, S2-Sequences of primers used in the study, S3-Sequence alignment of SiSPXs, S4-CDS sequences of the *SiSPX* gene family, and S5-Protein sequences of the *SiSPX* gene family.

Competing interests

The authors declare that they have no competing interests.

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Author contributions

CM.M. and HJ.G. were responsible for the design and composition of the manuscript, and they also supervised the study, and contributed equally to this work. Valuable input to this study was provided by C.W., FR.Y. and B.D. All the authors have reviewed and consented to the final published version of the manuscript.

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Figures

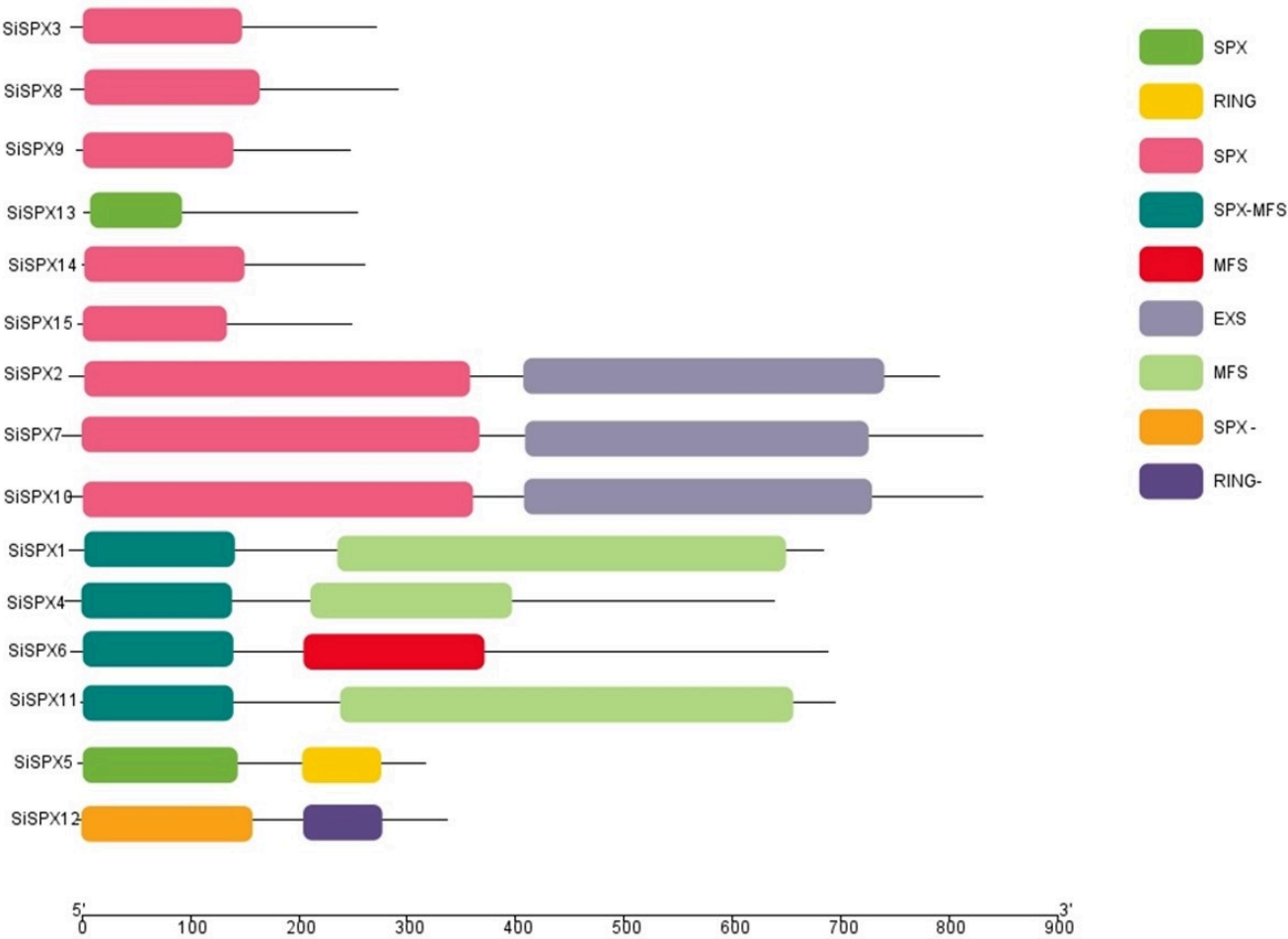


Figure 1

SPX gene domains in the foxtail millet genome.

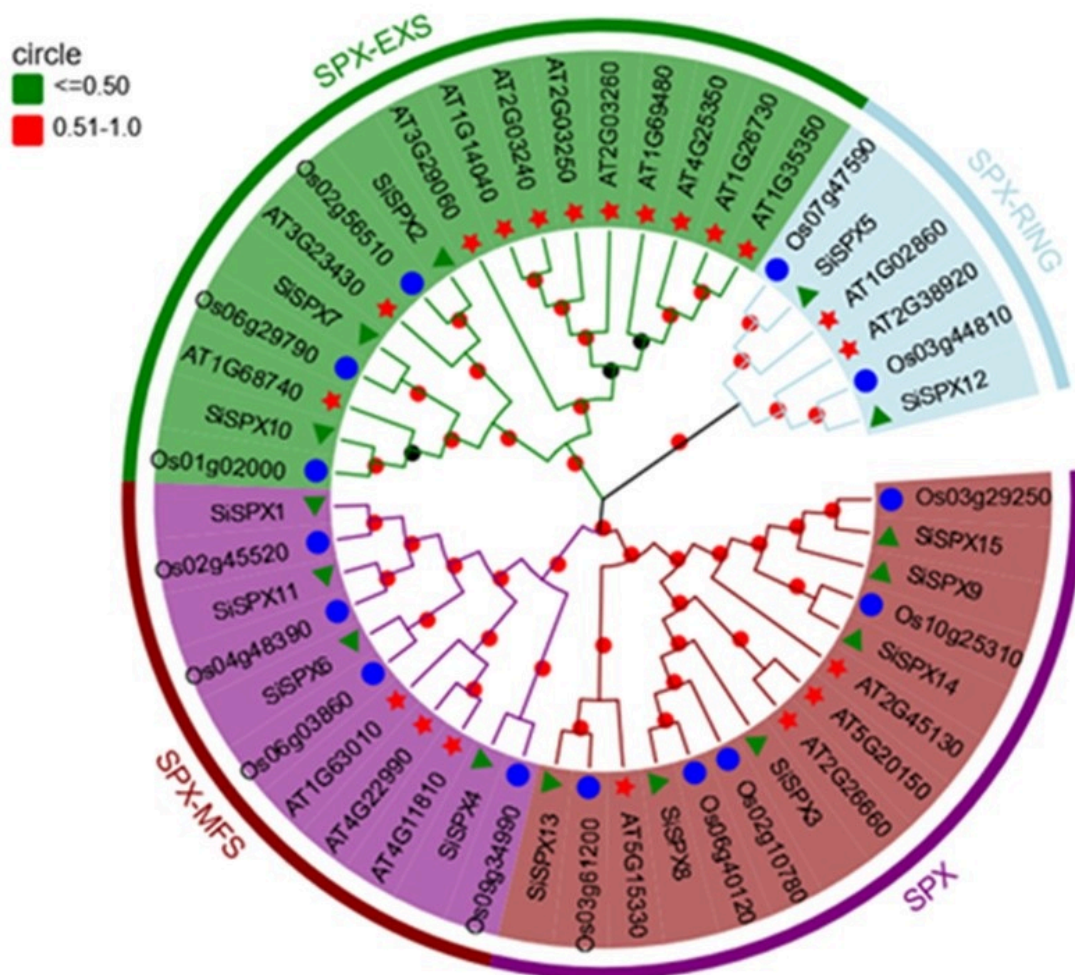


Figure 2

Phylogenetic analysis of the SPX-domain-containing protein genes in *Setaria italica*, *Arabidopsis thaliana*, and *Oryza sativa*. The phylogenetic tree was constructed with the neighbor-joining (NJ) algorithm and 1000 bootstrap replicates. Different colors indicate the different subfamilies of SPX-domain-containing proteins.

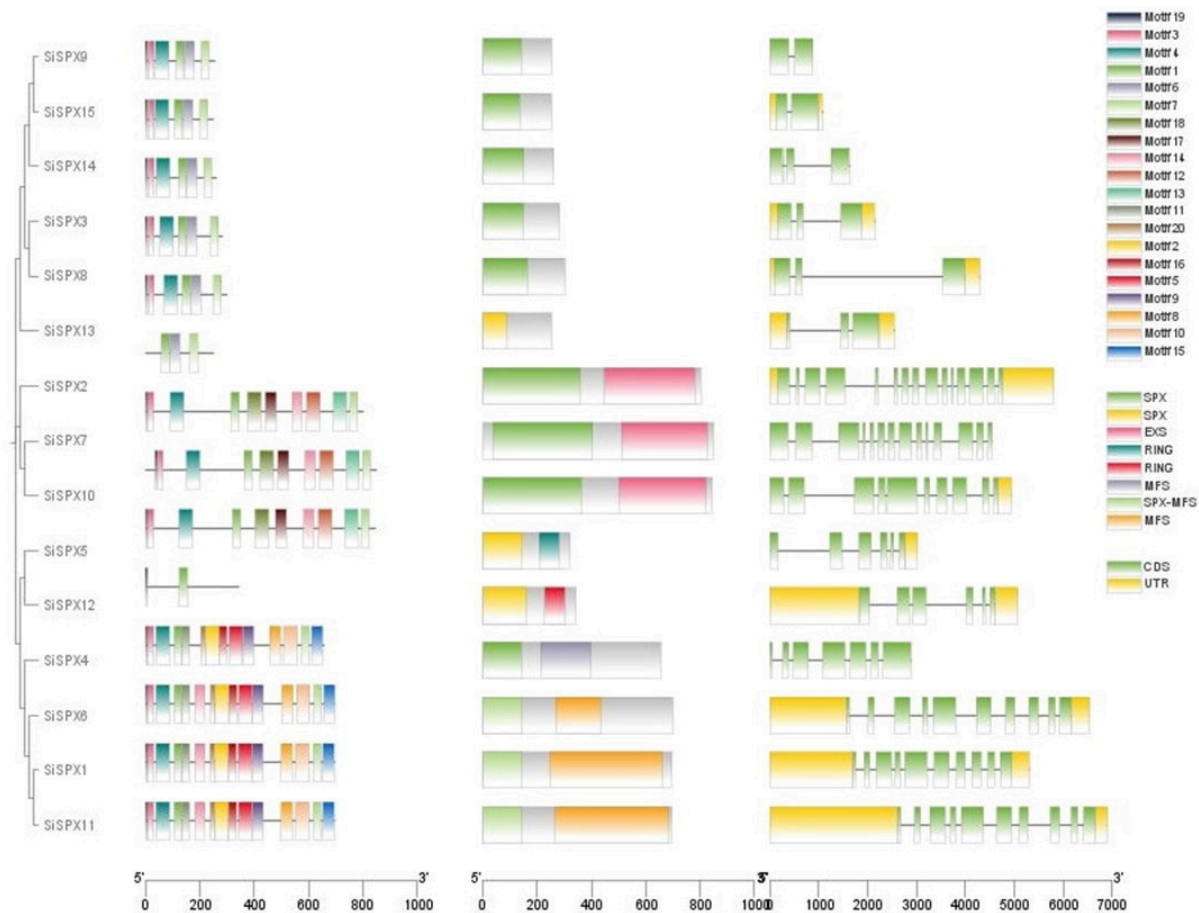


Figure 3

Gene structure and conserved motif analysis of SiSPX proteins. From left to right: Phylogeny tree, motif patterns, domain and gene structure analysis. Coding sequence (CDS) and untranslated region (UTR) are represented by different colored boxes. Conserved motifs in the SiSPX proteins are represented by different colored boxes.

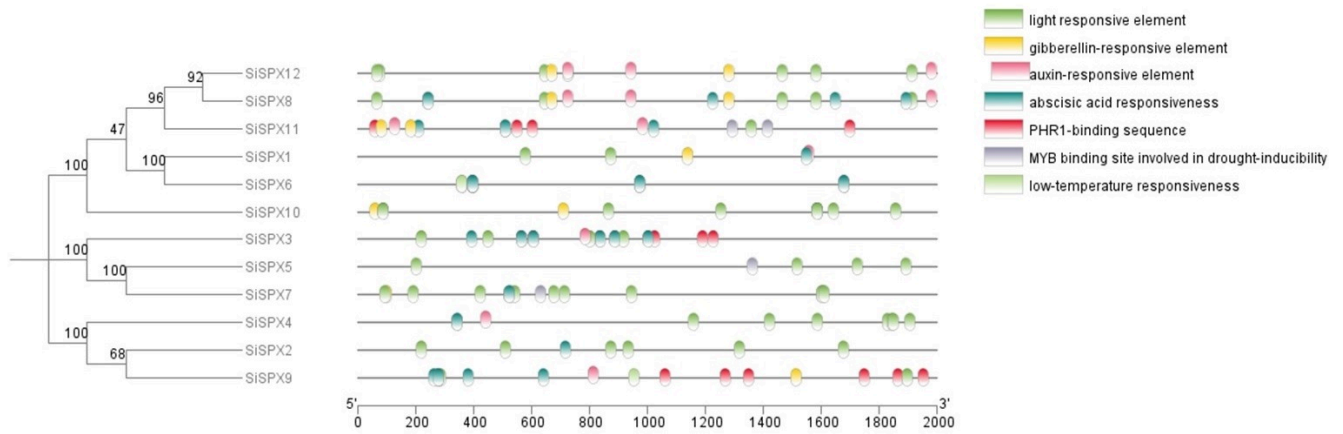


Figure 4

Cis-regulatory elements in the SiSPX family gene promoters.

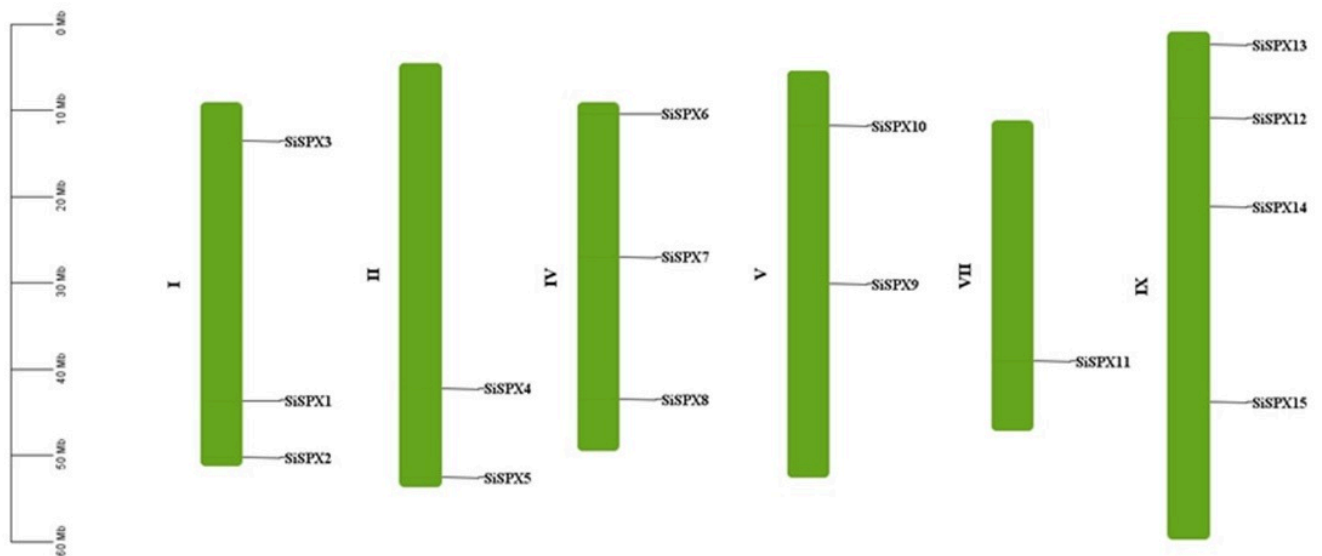


Figure 5

SPX gene location in foxtail millet genome.

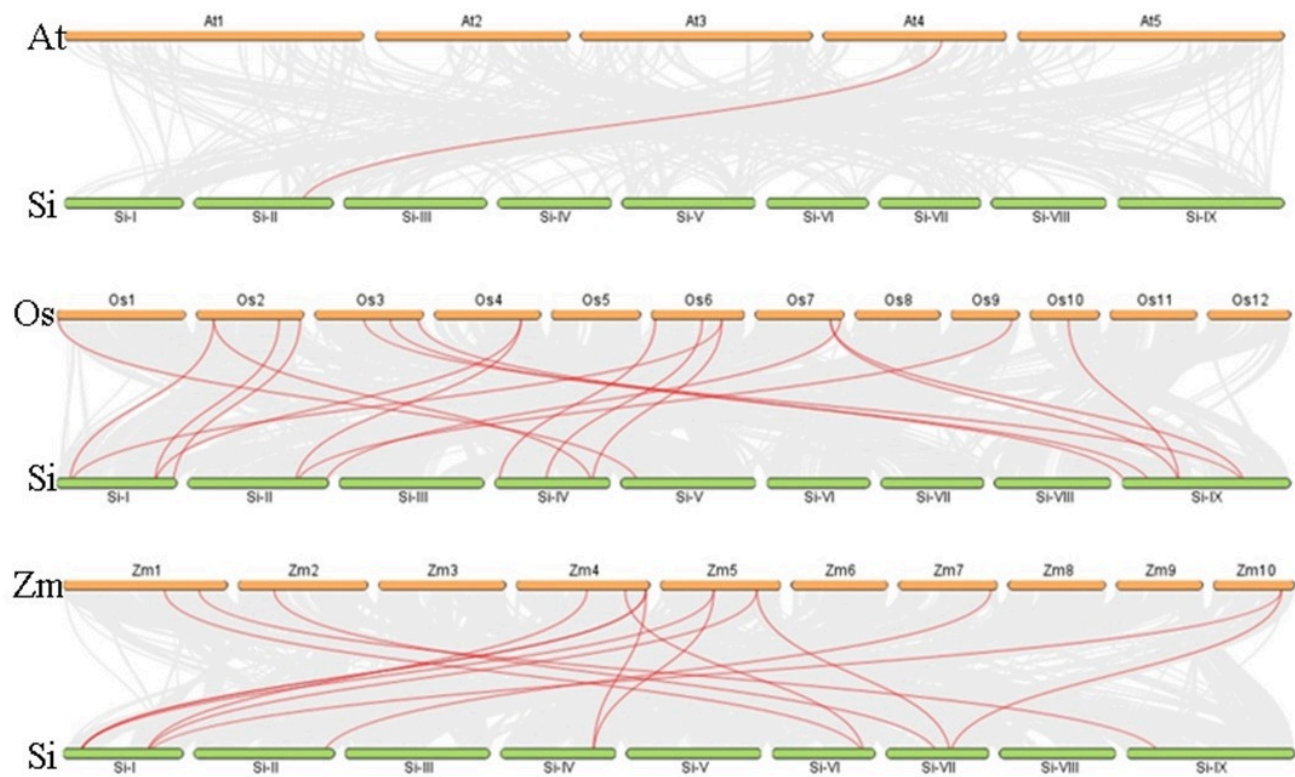


Figure 6

Synteny analyses of the SiSPX genes between *Setaria italica* and three representative plant species. Gray lines on the background indicate the collinear blocks within *Setaria italica* and other plant genomes; red lines highlight the syntenic *Setaria italica* SiSPX gene pairs.

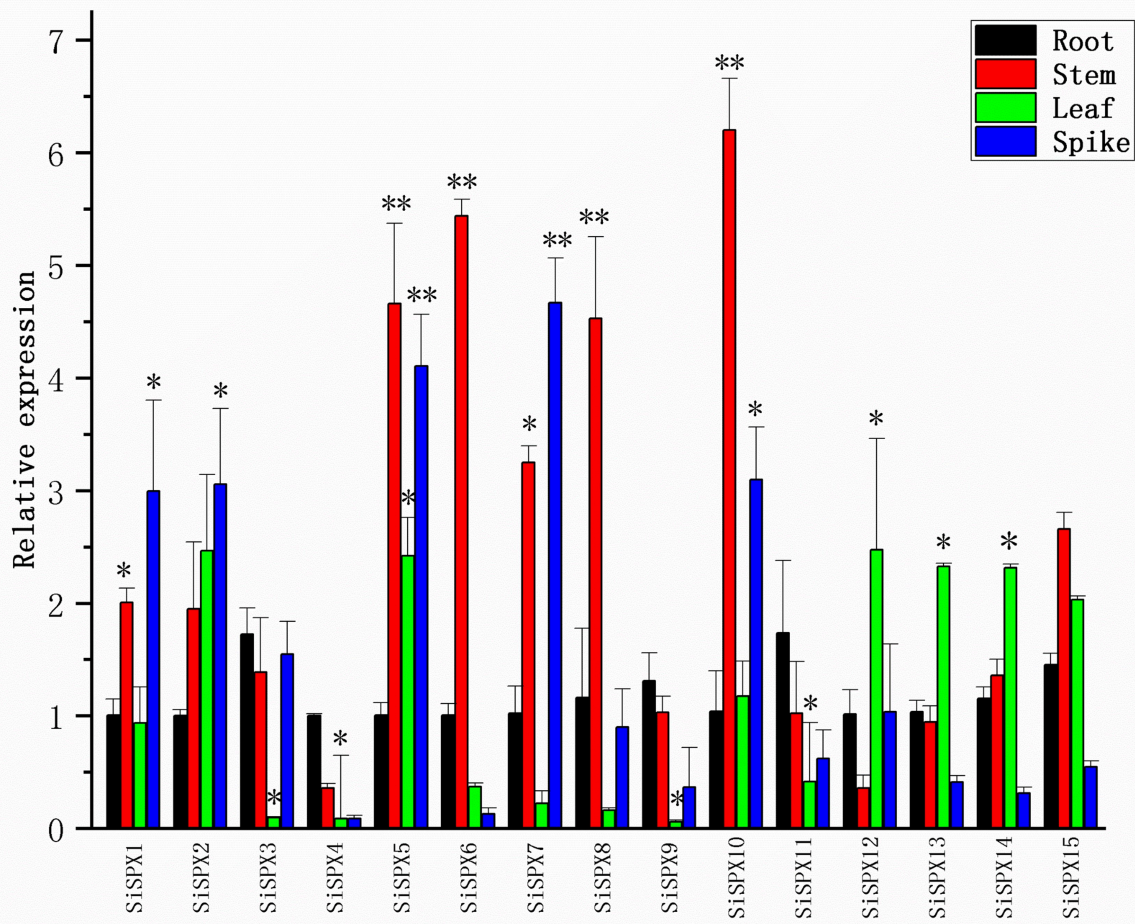


Figure 7

Quantitative RT-PCR analysis of SiSPX family genes expression in various tissues (spike, leaf, stem, and root tissues).

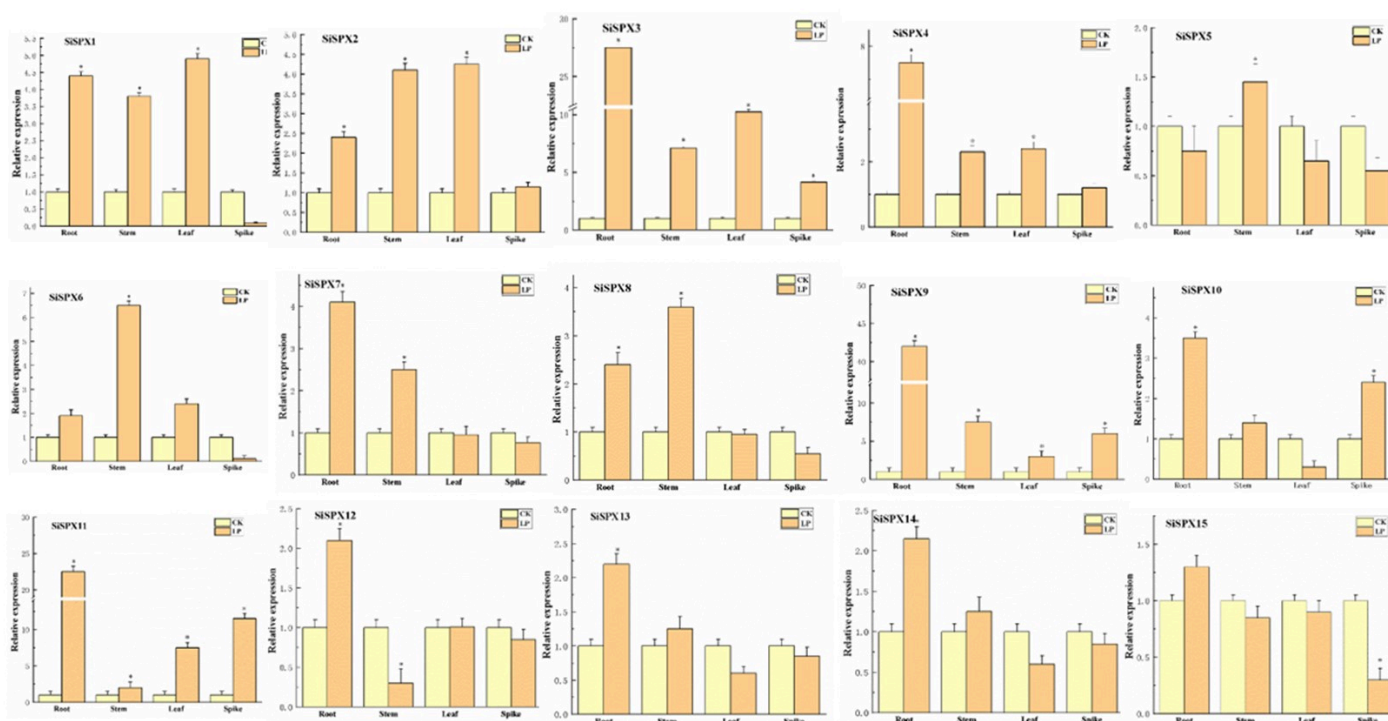


Figure 8

Expression patterns of SiSPX family genes in *Setaria italica*. Quantitative RT-PCR analysis of SiSPX family genes under low-phosphorus treatment in various tissues (spike, leaf, stem, and root tissues).

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