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Pan-genome-wide identification and evolutionary analysis of SPX gene family reveal distinct phosphorus adaptation regulatory strategies in foxtail millet

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Abstract

Background: Phosphorus (P) is an essential macronutrient, and SPX domain-containing proteins are central regulators of phosphate homeostasis in plants. Foxtail millet (Setaria italica) is renowned for its exceptional nutrient-use efficiency under low-fertility soils; however, its SPX gene family remains largely uncharacterized at the genome level. A pan-genome-wide investigation is therefore required to reveal the evolutionary and regulatory basis of phosphorus adaptation in this agronomically important cereal.

Results: In this study, we conducted a comprehensive pan-genomic analysis of 114 diverse germplasm accessions of foxtail millet, and identified 1646 SiSPX genes. These genes were phylogenetically classified into four distinct subfamilies, SPX, SPX-EXS, SPX-MFS, and SPX-RING. Evolutionary analyses revealed pervasive purifying selection (Ka/Ks < 1) across the entire family, while lineage-specific expansions within the SPX-MFS and SPX-RING subfamilies suggested adaptive diversification during domestication. Cis-regulatory element profiling uncovered subfamily-specific enrichment of P1BS motifs, with SPX subfamily members harboring up to five Pi-starvation-responsive elements. Under low-P stress, root-specific SiSPX-MFS genes, notably SiSPX-MFS2, exhibited progressive upregulation, correlating with stress duration, while core SPX subfamily members (SiSPX2/3/6/13) showed significant root-

specific induction. Weighted gene co-expression network analysis (WGCNA) further implicated these genes in transcriptional modules involving key transcription factors such as NAC, WRKY, and bHLH, highlighting their synergistic roles in P signaling. Interestingly, the SPX-RING subfamily displayed a notable absence of P1BS elements but a significant enrichment of MYB-binding motifs, suggesting alternative regulatory strategies for P adaptation.

Conclusions: This study provides the first pan-genomic resource for foxtail millet SPX genes, deciphers their functional divergence in P adaptation, and identifies promising targets for enhancing crop resilience and phosphorus-use efficiency in nutrient-limited agricultural systems.

Keywords: Foxtail millet; SPX domain-containing proteins; Pan-genome; Phosphorus

1.Introduction

Phosphorus (P) is an indispensable macronutrient for plant growth and development, serving as a fundamental constituent of nucleic acids, phospholipids, and energy transfer molecules in cultivated plants [1]. In agricultural ecosystems, inorganic phosphate (Pi) is predominantly sequestered within vacuolar storage compartments and actively participates in fundamental physiological processes, including photosynthetic energy transduction, hormonal signaling, and post-translational regulation [2]. The acquisition and systemic distribution of Pi, mediated by root absorption and vascular transport, are precisely orchestrated through intricate, multilayered regulatory mechanisms operating at both transcriptional and post-transcriptional levels [3, 4, 5]. At the core of phosphorus homeostasis in crops are SPX domain-containing proteins, a highly conserved family of regulators that govern Pi acquisition, allocation, and intricate signaling networks in staple crop species [6, 7, 8]. The SPX domain, initially characterized in plant systems, possesses dual ligand-binding capabilities crucial for precise phosphorus sensing. It exhibits low-affinity interactions with Pi ions while simultaneously maintaining high-affinity binding to inositol pyrophosphate signaling molecules via conserved basic residues [8, 9]. This distinctive molecular architecture positions SPX-containing proteins as central phosphorus status sensors and signaling

hubs, orchestrating nutrient responses in coordination with developmental programs across various crop species.

SPX domain-containing proteins are broadly categorized into four functional subgroups based on their distinct domain architectures: SPX (single domain), SPX-EXS, SPX-MFS, and SPX-RING [9, 10]. Comparative genomic analyses have revealed differential expansion patterns across species, with Arabidopsis encoding four SPX members (*AtSPX1-AtSPX4*) and rice possessing six homologs (*OsSPX1-OsSPX6*). Molecular studies have demonstrated that these SPX proteins physically interact with PHR1/2 transcription factors under phosphate-replete conditions [11, 12, 13]. This molecular interaction allows SPX proteins to negatively regulate phosphorus signaling through competitive inhibition, where the SPX domain sterically hinders PHR binding to the promoters of phosphate starvation-induced (PSI) genes, thereby repressing their expression under sufficient Pi availability [3, 13]. Notably, Arabidopsis *AtSPX1* and *AtSPX3* exhibit specialized regulatory roles, forming SPX-PHR protein complexes that directly repress the transcriptional activation of phosphate-responsive genes [3, 12].

The SPX-EXS protein family is characterized by an EXS structural domain containing two transmembrane helices, functioning as a phosphate (Pi) exporter during plant responses to low-phosphorus stress [14]. This protein family additionally regulates the subcellular localization and functional activation of phosphate transporter 1 (*PHO1*) [14]. Genetic evidence demonstrates that the SPX-EXS family plays essential functions in three critical phosphorus management processes, rhizospheric phosphate acquisition, vascular translocation, and tissue-specific partitioning [15]. *PHO1,* a core SPX-EXS member, localizes to the Golgi apparatus in root tissues, where it mediates long-distance phosphorus signaling through root-to-shoot coordination under Pi-deficient conditions [14]. The C-terminal EXS domain of PHO1 governs two mechanistically distinct processes, facilitating cellular phosphate efflux and maintaining protein localization within Golgi/trans-Golgi networks [14].

The SPX-MFS protein family exhibits phylogenetic conservation with yeast vacuolar phosphate transporters such as ScVTC4 and ScPHO91. In plants, SPX-MFS members have been identified as tonoplast-localized phosphate transporters in model

species *Arabidopsis* and *Oryza sativa*, where they perform essential functions in vacuolar phosphate sequestration [16, 17]. These membrane-integral proteins mediate intracellular phosphate translocation through vesicular trafficking pathways [18]. Recent advances demonstrate that all three OsSPX-MFS paralogs in rice function as vacuolar phosphate influx transporters via endocytic mechanisms, displaying functional redundancy while maintaining distinct regulatory specializations [19].

The SPX-RING subfamily is characterized by a zinc finger domain that facilitates participation in E3 ubiquitin ligase complexes. Experimental evidence in *Arabidopsis* reveals that SPX-RING-mediated protein interactions critically regulate phosphate transport dynamics. A prominent example is the Nitrogen Limitation Adaptation (NLA) protein, which orchestrates ubiquitination-dependent degradation of plasma membrane-resident *PHT1* phosphate transporters through its SPX-RING architecture [20]. This post-translational regulatory mechanism coordinates plant phosphate homeostasis in a nitrogen-dependent manner, thereby establishing metabolic crosstalk between essential nutrient pathways [21].

The SPX gene family has been extensively characterized in model plants (*Arabidopsis thaliana*, *Oryza sativa*) and major crops (*Triticum aestivum*, *Glycine max*, *Brassica napus*), establishing foundational knowledge through phylogenetic, molecular, and functional analyses [3, 5, 22, 23, 24, 25, 26]. These investigations have generated essential genomic resources for functional validation studies and genetic engineering applications. Despite these advancements, foxtail millet (*Setaria italica*), domesticated in northern China over five millennia ago [27], with exceptional nutrient utilization efficiency and abiotic stress tolerance under low-fertility conditions [28], lacks notably systematic genome-wide investigation of its SPX gene family. Therefore, we performed pan-genomic analysis of 114 foxtail millet accessions, systematically identifying and isolating SPX domain-containing genes for multi-dimensional computational characterization. Our integrated approach included presence-absence variation (PAV) profiling, gene structure elucidation, phylogenetic reconstruction, conserved motif identification, repetitive element analysis, promoter *cis*-element prediction, evolutionary selection pressure assessment, and transcriptomic response profiling

under low-phosphorus stress. This comprehensive investigation provides unprecedented insights into the molecular architecture and functional diversification of SPX genes in foxtail millet, establishing a robust theoretical framework for future mechanistic studies and genetic improvement strategies targeting this agronomically important gene family.

2. Materials and methods

2.1. Pan-genomic Identification and Characterization of SPX Genes in Foxtail Millet

To comprehensively identify *SPX* gene family members in foxtail millet (*Setaria italica*), we used genetic resources including, (1) the reference genome "Yugu1"; (2) 35 wild accessions, 40 landraces, and 35 modern cultivars obtained from Setaria-db (www.setariadb.com/millet) [29]; and (3) wild "A10" and cultivars "Zhanggu" and "*xiaomi*", each with complete genome assemblies and annotations. SPX domain (PF03105) identification was performed using HMMER 3.0 with stringent parameters (E-value threshold ≤1e-5), followed by systematic validation through Pfam (http://pfam.xfam.org) and SMART (http://smart.embl-heidelberg.de) domain architecture analyses. Candidate sequences were subjected to BLASTP alignment against the Setaria-db database, applying the homology criteria by Tong [30], ≥90% sequence coverage and ≥95% protein similarity. The longest transcript variant was selected as representative for each gene locus. Gene presence-absence patterns were categorized as core (present in all accessions), softcore (≥90% prevalence), shell (≥10% prevalence), and cloud (<10% prevalence), following established pan-genome classification standards.

2.2. Physicochemical Properties and Subcellular Localization Prediction of SiSPX Proteins

To characterize the basic properties of foxtail millet SPX proteins, we analyzed 32 representative genes. Molecular weight and theoretical pI were determined using the ExPASy website (https://web.expasy.org/protparam/). Subcellular localization prediction was analyzed with Cell-PLoc 2.0 (http://www.csbio.sjtu.edu.cn/bioinf/Cell-

PLoc-2/).

2.3. Gene Structure, Phylogenetic Analysis, and Cis-regulatory Element Identification

Conserved protein motifs within SPX sequences were predicted using MEME Suite (v.5.5.1) with default parameters, followed by visualization of domain architectures and gene structures through TBtools (v.1.108) [31]. For cross-species comparison, experimentally validated SPX protein sequences from *Arabidopsis thaliana* (AT), *Oryza sativa* (OS), and *Zea mays* (ZM) were retrieved from UniProt (https://www.uniprot.org/) and aligned against foxtail millet SPX homologs using MAFFT (v7.508) with L-INS-i algorithm [32]. A maximum-likelihood phylogenetic tree was subsequently constructed using FastTree (v2.1.11) under Jones-Taylor-Thornton (JTT) substitution model [33]. Upstream regulatory regions (2,000 bp proximal to transcription start sites) were extracted from SPX genomic sequences using TBtools. These promoter sequences were subjected to *cis*-element prediction via PlantCARE database [34] with subsequent visualization of regulatory motifs implemented in TBtools.

2.4. Gene Duplication and Selection Pressure Analysis (Ka/Ks)

Protein sequences from 114 foxtail millet genomes were subjected to pairwise BLASTP alignment (E-value ≤1e-5) using NCBI BLAST+ (v2.13.0), retaining the top five significant hits per query sequence for subsequent analysis. Gene duplication events were classified using MCScanX (v1.1) with default parameters [35], employing syntenic block information and chromosomal coordinates. Tandem duplication events were defined as paralog pairs (E-value ≤1e-5) residing within 10 adjacent genes on the same chromosome. Segmental duplications were identified as homologous gene pairs located in collinear genomic blocks, indicative of whole-genome duplication (WGD) events. Evolutionary selection pressures were quantified through calculation of non-synonymous (Ka) and synonymous (Ks) substitution rates using the NJ method implemented in Ka/Ks Calculator (v2.0), with results visualized through ggplot2 (v3.4.2) in R Studio.

2.5. Mutation load analysis

Whole genome SNP data of 1844 germplasm accessions including 630 wild, 829 landrace and 385 cultivars were obtained from the Foxtail millet Database (www.setariadb.com/millet), and the identified variants in the *SPX* gene region of foxtail millet were filtered and categorized using the reference genome Yugu1 as a mapping tool and visualized with the R package.

2.6. Gene expression analysis and co-expression network analysis

To investigate the regulatory responses of SiSPX family members to phosphorus, we utilized transcriptome data from the landrace "Longzhao Nuo" (L37), previously identified by our research team, to assess the expression patterns of these genes. The plants were cultivated in Hogland complete nutrient solution, with phosphorus concentrations adjusted using KH_2PO_4 to establish conditions of normal phosphorus supply (NP, 0.25 mM) and low phosphorus stress (LP, 0.0025 mM). Root tissues were sampled at three distinct time points (1 day, 4 days, and 7 days) following the imposition of low phosphorus stress, with sequencing reads aligned to the "*xiaomi*" cultivar reference genome (v1.6) using the same pipeline. Raw reads were quality-filtered using Trimmomatic (v0.39) with parameters. Gene expression levels were quantified as Transcripts Per Million (TPM) using StringTie (v2.2.1). Phosphorus stress-responsive co-expression networks were constructed through Weighted Gene Co-expression Network Analysis (WGCNA, v1.72) in R package, integrating transcriptomic profiles with phenotypic measurements (root architecture parameters, biomass accumulation) and physiological indices (phosphatase activity, Pi concentration). This integrative approach enabled the systematic identification of hub genes coordinating *SiSPX* interactions with phosphorus transport and assimilation pathways.

2.7. Quantitative Real-Time PCR (qRT-PCR) Analyses

The expression levels of 6 differentially expressed genes (DEGs) were detected using qRT-PCR. Total RNA was extracted using Trizol reagent according to the manufacturer’s protocol (TaKaRa, Dalian, Liaoning, China). The primer-BLAST online tool from the National Center for Biotechnology Information (NCBI) was used to design gene-specific primers for each gene (Table S1). Reverse transcription was performed according to the instructions of Genstar's cDNA first strand synthesis kit.

After diluting the reverse transcription product 10 times, TB Green was prepared under the product name Takara® Premix Ex Taq™ Real time fluorescence quantitative PCR using the RR820A kit. Actin was used as the internal control and the relative expression level of each assessed gene was calculated by using the $2^{-\triangle\triangle Ct}$ method.

3. Result

3.1. Identification of SiSPX Pan-gene Family Members

Based on the presence of the classic SPX domain, a total of 1646 SiSPX genes were identified from 114 foxtail millet accessions. Following alignment of their protein sequences, these genes were classified into four subfamilies: SPX, SPX-MFS, SPX-EXS, and SPX-NLA (RING) (Figure 1-A). Among cultivars, C12, C25, and C29 harbored the highest number of SPX genes (16), whereas C22 harbored the lowest (13). Among landraces, L36 possessed the most SPX genes (17), whereas L05, L08, L27, L30, L31, and L35 possessed the fewest (13). Among wild accessions, A10 had the highest number (23), whereas Q1, Q10, and Q12 had the lowest (12), representing an almost twofold difference. Statistical analysis across different material sources and the four subfamilies indicated that the average total number of SPX family genes in cultivars, landraces, and wild species was 14.65, 14.25, and 14.48, respectively, with no significant difference observed among the three groups. Based on their prevalence across all accessions, 1238 genes were categorized as softcore, 375 as shell, 33 as cloud, and no core genes were identified. Statistical analysis of gene proportion categories and domain architectures revealed that none of the softcore genes contained the SPX-NLA domain, and none of the shell genes contained the SPX-MFS and SPX-EXS domains (Figure 1-C). Based on the protein sequence similarity of these genes, they were classified into 32 homologous genome genes, named according to their contained domains and chromosomal positions (Figure 1-D, Table S2).

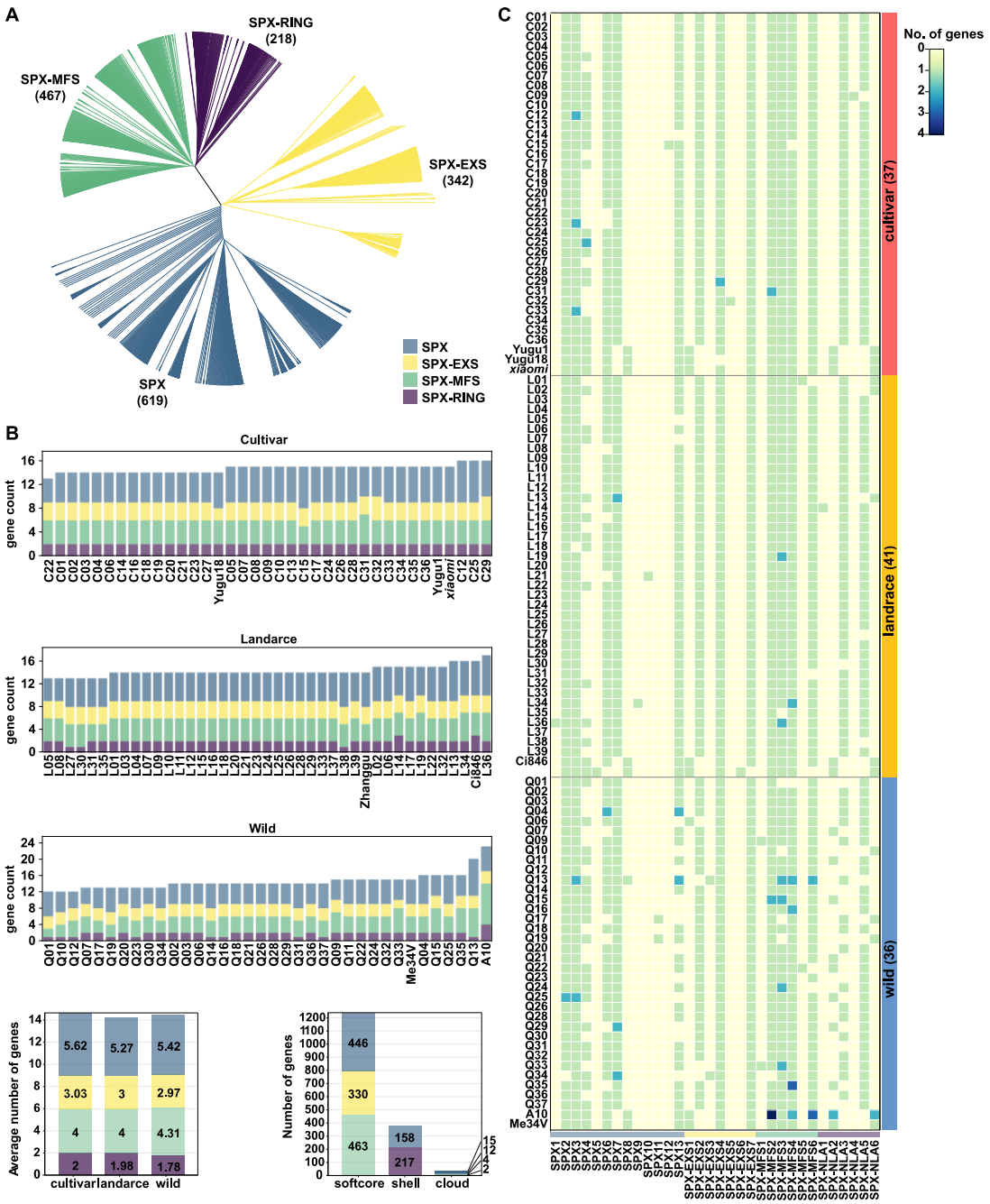


Figure 1 Analysis of members of the SPX pan-gene family in foxtail millet. (A) Phylogenetic tree of 1646 SiSPX family member proteins. (B) Number of genes in different subfamilies from three material sources. (C) Presence and absence of 1646 SiSPX genes in 114 materials.

3.2. Physicochemical Properties and Subcellular Localization Prediction of SiSPX Proteins

The identified 32 *SiSPX* genes were phylogenetically classified into four subfamilies: SiSPX, SiSPX-MFS, SiSPX-EXS, and SiSPX-RING. Chromosomal mapping revealed their distribution across six foxtail millet (*Setaria italica*)

chromosomes (excluding Chr3, 6, and 8), demonstrating non-random genomic organization. Encoded proteins exhibited substantial structural diversity, ranging from 176 to 1,122 amino acids (19.97-126.96 kDa) with isoelectric points spanning 4.78-9.91. Physicochemical analyses predicted moderate instability indices (34.17-61.91) and variable aliphatic indices (73.55-110.91), indicating differential thermostability among subfamily members. Hydropathicity profiling revealed distinct hydrophobicity patterns: *SiSPX-EXS1* and other SPX-EXS subfamily members exhibited positive Grand Average of Hydropathicity (GRAVY) values (>0), classifying them as hydrophobic proteins, whereas all other SiSPX proteins were hydrophilic (GRAVY <0). Subcellular localization predictions demonstrated subfamily-specific compartmentalization: SiSPX and SiSPX-MFS members localized to vesicular membranes (tonoplast), while SiSPX-RING proteins showed nuclear targeting (Table S3). This compartmental specialization aligns with functional annotations of respective subfamilies in phosphate transport and signaling.

3.3. Structural and Functional Characterization of SiSPX genes

Comprehensive domain architecture analysis of the 32 SiSPX proteins revealed subfamily-specific structural conservation, characterized by highly conserved domain sizes and positional arrangements among members within each subgroup (Figure 2). This structural uniformity suggests conserved biological functions across subfamily members, potentially manifesting as coordinated gene expression patterns, shared interaction partners, and common regulatory mechanisms mediated by identical transcription factors. MEME Suite (v5.5.1) analysis identified 15 conserved motifs across the SPX family, exhibiting distinct subfamily-specific distribution patterns (Supplementary Table S4). The SiSPX-EXS subfamily exhibited the highest motif complexity, containing up to nine motifs per protein including three exclusive signatures (Motifs 2, 5, 6). *SPX-MFS* members universally possessed Motifs 11, 14, and 15, while sharing Motif 13 with *SiSPX-NLA4*. SPX-RING proteins featured a unique identifier (Motif 10). Notably, Motif 1 (29 aa) served as a universal signature present in all SiSPX family members. The conserved correlation between gene structure (intron-exon architecture) and motif organization strongly supported phylogenetic

relationships, with identical subfamily members clustering together in evolutionary analyses. This structure-function congruence reinforces the evolutionary conservation of SPX-mediated regulatory mechanisms in foxtail millet.

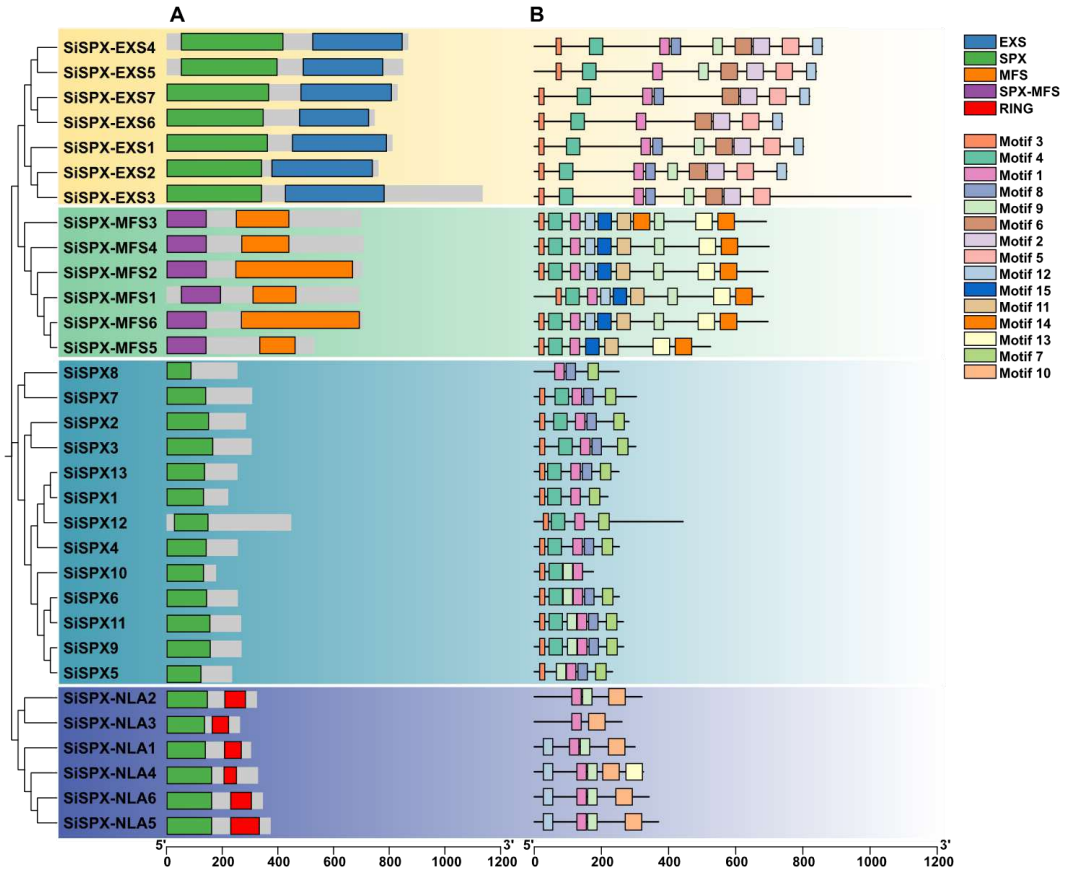


Figure 2 Structural and motif analyses of SiSPX. (A) Comparative analysis of SPX domains. (B) Comparative analysis of SPX motif.

3.4. Phylogenetic Analysis of SPX Proteins in Foxtail Millet and Other Crops

Comparative analysis of 32 SiSPX proteins with 65 orthologs from *Arabidopsis thaliana* (20 AtSPXs), *Zea mays* (33 ZmSPXs), and *Oryza sativa* (12 OsSPXs) resolved four evolutionarily distinct subfamilies (Figure 3), consistent with homology-based classification (Supplementary Table S2). The SPX-RING subfamily represented the smallest cluster, while the SPX subfamily constituted the largest group, demonstrating differential expansion across lineages. Protein length conservation was observed within subfamilies across species, with SPX-EXS proteins containing the longest sequences (176-1,122 aa) and SPX members the shortest (176-425 aa). Phylogenetic topology revealed clear divergence between monocot (*Setaria italica*, *Z. mays*, *O. sativa*) and

dicot (*A. thaliana*) lineages, particularly within SPX-MFS and SPX-EXS subfamilies (Figure 3). This species-specific clustering suggests functional diversification of these transporter subfamilies during plant evolution.

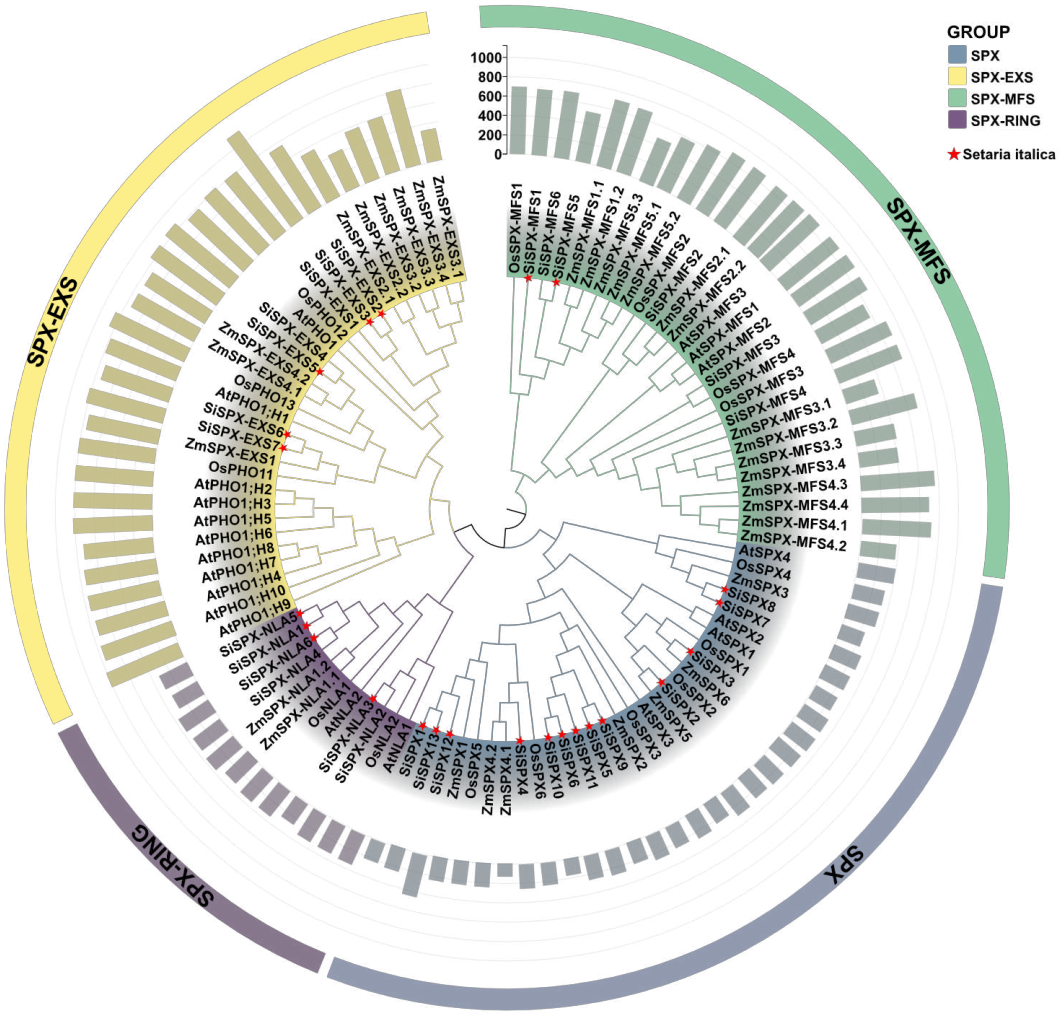


Figure 3 Phylogenetic tree of SPX encoded by genes from *Arabidopsis*, *Oryza sativa*, *Zea mays* and *Setaria italica* pangenome.

3.5. Analysis of Promoter Cis-regulatory Elements of SiSPX Genes in Foxtail Millet

To elucidate which transcription factors or other cis-elements interact with SiSPX family members and consequently influence gene transcriptional activity, we analyzed potential cis-elements in the promoter region of SiSPX genes (2000 bp upstream of the start codon) using the PlantCARE database. Most of the cis- elements of the SiSPX genes can be broadly categorized into four groups, transcription factors, plant growth and development, phytohormone-related, and stress-related (Figure 4). These include a

large number of light-responsive (ACE, Sp1, GT1-motifs, G-boxes, MRE), MeJA-responsive (TGA CG-motifs, GCTCA-motifs), ABA (ABRE)-responsive elements, as well as other hormone responsiveness (growth hormone, gibberellic acid, salicylic acid), meristematic tissue-expression elements (CAT-box), biological response elements (low-temperature response, anaerobic induction, drought inducibility), and Pi starvation-responsive elements (P1BS) (Figure 4). These findings suggest that SPX genes may be induced by a variety of environmental conditions and hormones. Although many SiSPX genes are induced by Pi starvation, detailed information on their regulation by specific transcription factors remains limited.

Analysis of the SiSPX promoter identified a large number of typical P1BS elements in the upstream regions of 12 SPX subfamily genes, SPX-MFS subfamily genes (SiSPX-MFS2 and SiSPX-MFS3), SPX-EXS subfamily genes (SiSPX-EXS4, SiSPX-EXS6, and SiSPX-EXS7), and SPX-RING (SiSPX-NLA4). These are highly conserved cis-acting elements with an approximate palindromic sequence length of 8 bp and are responsive to Pi stress. It is noteworthy that the number of P1BS elements was not identical among these 18 genes, with a maximum of five and a minimum of one (Figure 4).

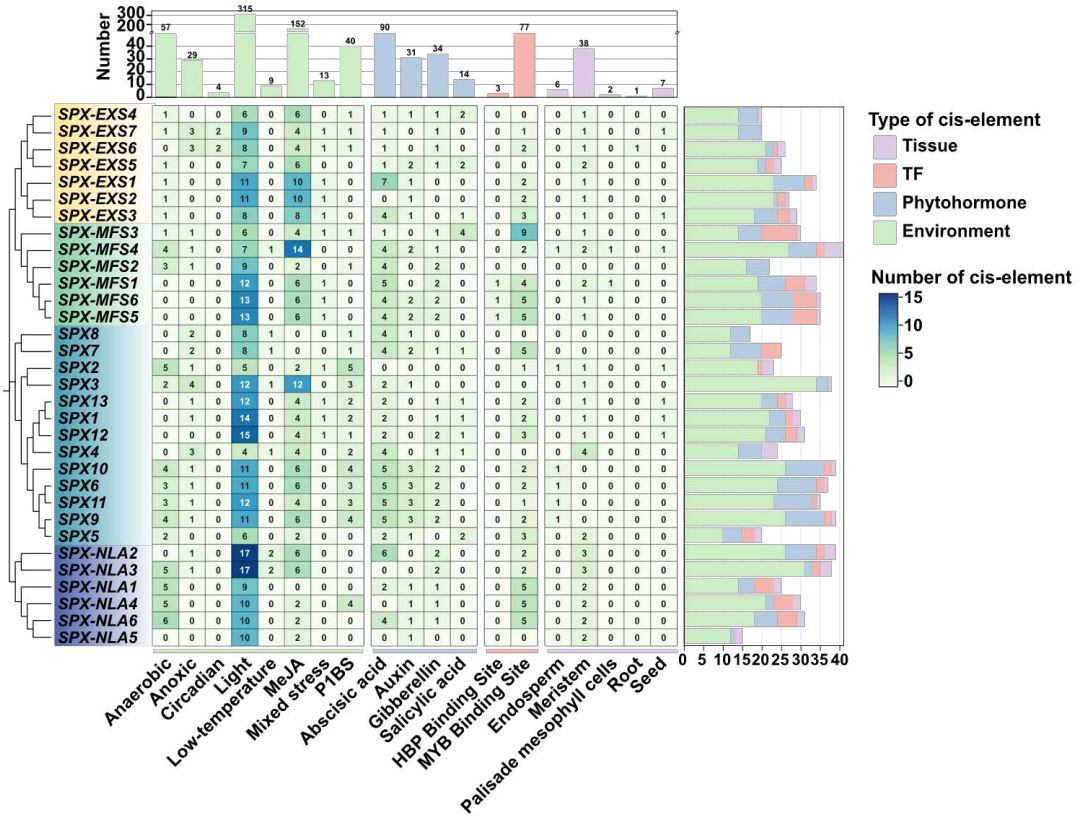


Figure 4 Prediction of cis-acting elements of the promoter of the *SPX* genes in foxtail millet.

3.6. Gene Duplication Analysis

To explore the amplification patterns of paralogous genes, we conducted a comprehensive analysis of all duplication events involving SPX genes in foxtail millet. Our findings demonstrated that a total of 103 samples contained 154 pairs of tandemly duplicated genes. Specifically, some samples harbored up to 10 pairs of tandemly duplicated genes, whereas others contained only a single pair. Additionally, we identified 231 pairs of segmentally duplicated genes, with the majority of varieties exhibiting only two pairs, namely SiSPX2/SiSPX3 and SiSPX-MFS2/SiSPX-MFS6. It is hypothesized that these genes have consistently fulfilled core biological functions crucial for species survival throughout the domestication process of foxtail millet. We selected eight representative samples from cultivated, landrace, and wild to visually represent the duplication events of their SiSPX genes (Figure 5 A). Furthermore, our analysis revealed that although certain genes were initially classified as segmentally duplicated gene pairs, some were subsequently grouped into the same homologous gene cluster due to their sequence coverage or p-value meeting the standard threshold for

homologous genes (Table S5). These results indicate that the majority of foxtail millet samples maintain low gene copy numbers to mitigate the adaptive costs associated with dosage imbalance, while a minority of individuals explore the potential for new functionalization through gene amplification.

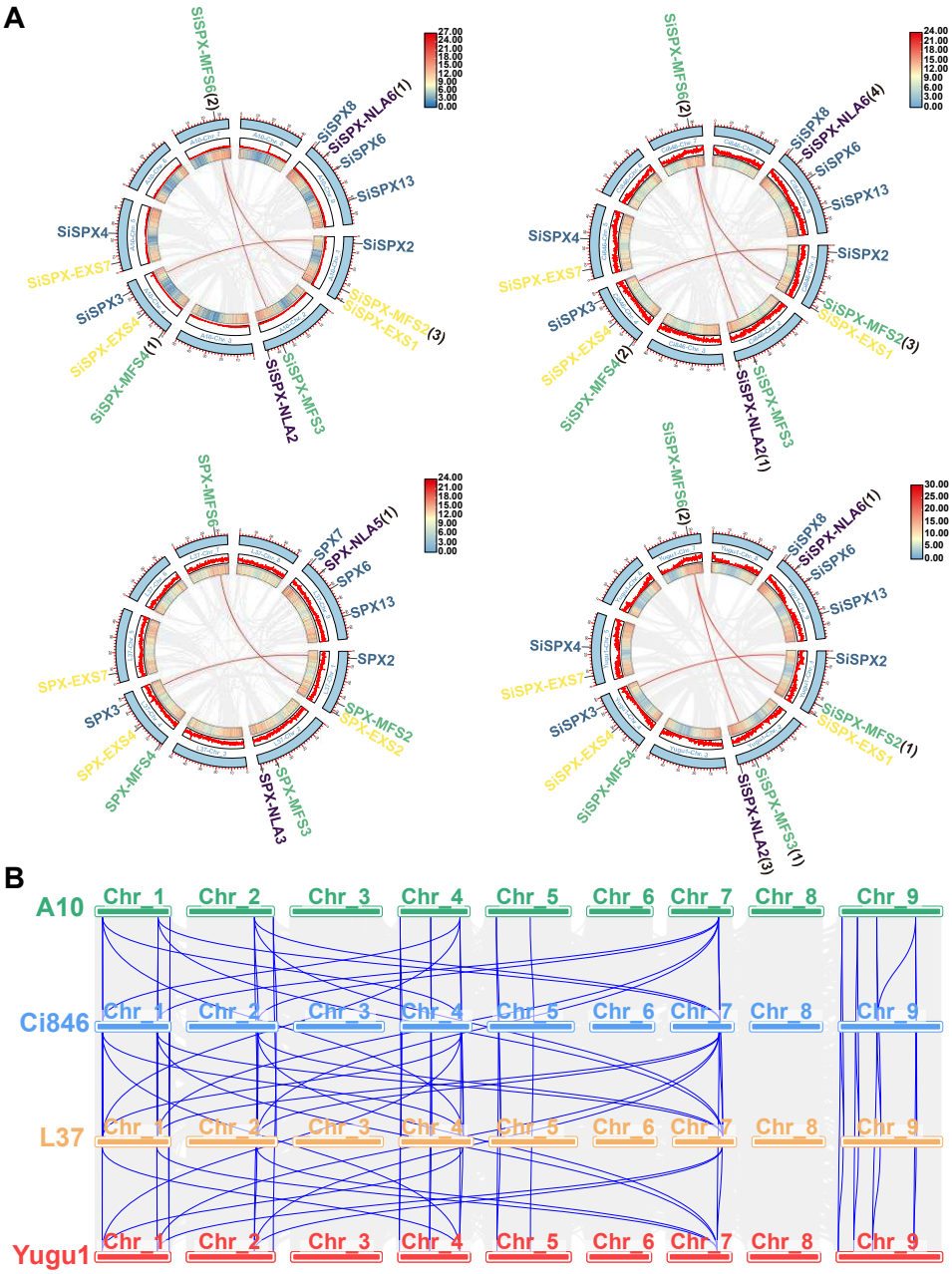


Figure 5 Analysis of SiSPX gene duplication events. (A) represent the gene duplication analysis of four varieties, namely "A10", "Ci846", "L37" and "Yugu1", respectively. From the inner circle to the outer circle, they are segmental duplication genes (red line), gene density within chromosomes (heatmap, line chart), physical location of each SiSPX gene, and tandem duplication gene pairs (black numbers).

3.7. Gene Selection Pressure Analysis (Ka/Ks) and Mutation Load Analysis of SPX Genes in Foxtail Millet Accessions

Evolutionary selection pressures were further quantified through Ka/Ks analysis, excluding cloud genes due to incomplete data availability. The majority of *SiSPX* genes exhibited Ka/Ks ratios between 0 and 1, consistent with strong purifying selection during domestication. Subgroup-specific patterns emerged, SPX-RING members (excluding *SiSPX-NLA5*) universally displayed Ka/Ks < 1, while *SPX-EXS* exhibited more divergent selection pressures. Strikingly, *SiSPX-EXS4* showed elevated Ka/Ks ratios compared to its subgroup counterparts, which clustered near Ka/Ks = 0. These findings suggest evolutionary conservation of *SPX-RING* genes across domesticated varieties, contrasting with localized positive selection observed in SPX-EXS. Within the SPX subgroup, *SiSPX8* uniquely underwent purifying selection, while sporadic members displayed Ka/Ks >1, indicative of potential neofunctionalization events (Figure 6A, Table S6).

Genome-wide mutation load analysis across 1,844 foxtail millet accessions revealed limited polymorphism within the SPX gene family, with only 15 *SiSPX* genes containing detectable single nucleotide polymorphisms (SNPs). Eight variant categories were identified: intron variants, splice region variants, missense variants, synonymous variants, upstream/downstream gene variants, and 5'/3' UTR variants, with upstream variants exhibiting particularly high prevalence. *SiSPX-MFS4* emerged as the most polymorphic locus, demonstrating both broad distribution across accessions and the richest variant diversity, predominantly involving 3' UTR and 5' UTR modifications. Despite comparable variant type richness, *SiSPX-MFS6* showed exclusive wild accession specificity. In contrast, *SiSPX4* and *SiSPX6* displayed moderate polymorphism (6 and 4 variant types respectively) but maintained wide germplasm distribution. Notably, wild accessions harbored the highest diversity of variant types across all *SiSPX* genes (Figure 6B).

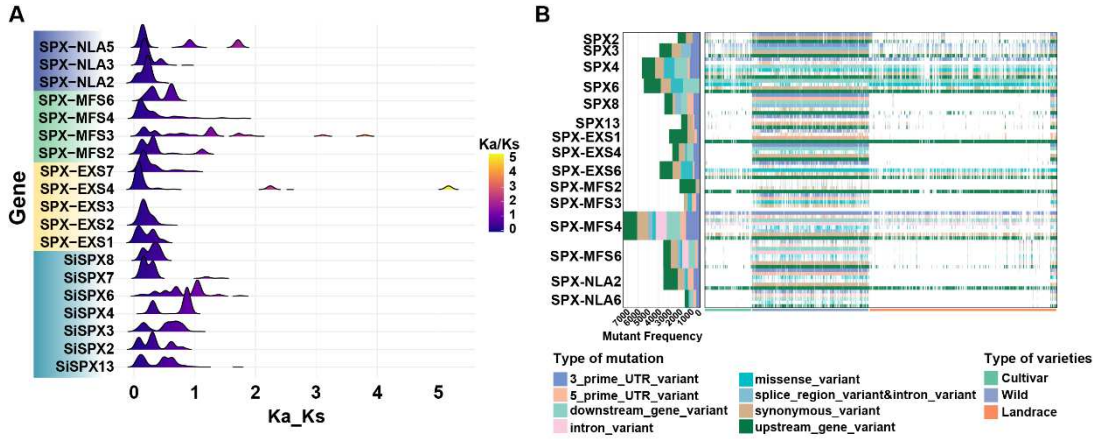


Figure 6 Selection pressure analysis. (A) The distribution of Ka/Ks for SPX gene in 114 samples. (B) Waterfall plot of the variation burden of SPX genes in the 1848 foxtail millet accessions.

3.8. Gene Expression and Co-expression Network Analysis under Phosphorus Stress

To investigate the responsiveness of SiSPX genes to phosphorus deficiency, a controlled low-phosphorus stress assay was implemented on foxtail millet landrace L37. Transcriptional profiling under low-P conditions revealed distinct expression patterns across SPX subgroups. In the SPX-MFS subgroup, *SiSPX-MFS3* and *SiSPX-MFS6* maintained consistently low expression levels without significant alterations, while *SiSPX-MFS4* exhibited constitutive high expression independent of phosphorus status. Notably, *SiSPX-MFS2* displayed progressive upregulation in L37 roots, reaching 1.8-fold and 2.3-fold increases at days 4 and 7 post-stress, respectively (Fig. 7A). The SPX subgroup demonstrated pronounced stress responsiveness, with *SiSPX2*, *SiSPX3*, *SiSPX6*, and *SiSPX13* showing significant root-specific induction in L37 ($P<0.01$), exhibiting 3.1- to 5.4-fold expression elevation by day 7. Contrastingly, SPX-EXS members displayed divergent regulation: *SiSPX-EXS4* maintained basal expression levels across treatments, while *SiSPX-EXS7* showed stable root expression unaffected by phosphorus limitation in L37 but significant aerial tissue downregulation (2.1-fold decrease). This differential tissue-specific regulation suggests a potential role for *SiSPX-EXS7* in modulating phosphorus allocation between root and shoot systems.

To further investigate the interaction network among SiSPX genes under phosphorus stress conditions, we integrated the transcriptomic data of L37 with its

physiological traits and performed a weighted gene co-expression network analysis (WGCNA). The network analysis demonstrated that, apart from the interactions among SPX genes themselves, a multitude of transcription factors were also identified to interact with SPX genes, encompassing bHLH, bZIP, GRAS, MYB, NAC, WRKY, among others. Notably, MYB transcription factors were the most abundant (15), whereas AP2 and C3H exhibited only a single interaction each, with *SiSPX-EXS4* and *SiSPX6*, respectively. *SiSPX-MFS2* displayed the highest number of interacting transcription factors (12), in contrast to *SiSPX6*, which had the lowest (8). Additionally, *Seita.5G286200* and *Seita.8G057800*, belonging to the MYB family, showed the most extensive interactions with SPX genes, implying that these genes may collaboratively respond to phosphorus stress alongside the SPX genes.

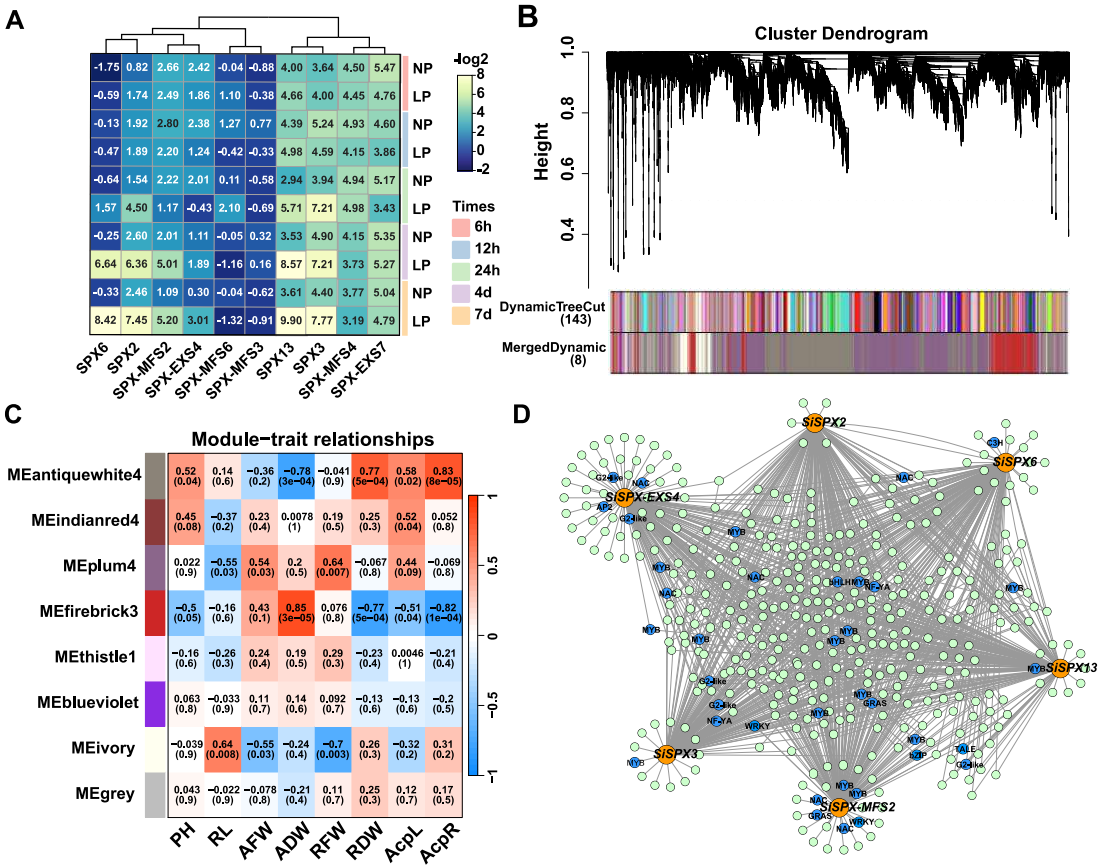


Figure 7 Expression of SPX genes in foxtail millet after phosphorus stress treatment. A indicate the expression profiles of the *SiSPX* gene family in L37 under phosphorus stress treatment, respectively, B/C/D indicate the WGCNA and gene-interaction network maps analyzed in L37 under phosphorus stress.

3.9. Validation of SiSPX Gene Expression by qRT-PCR

As illustrated in Figure 8, we employed qRT-PCR technology to examine the expression patterns of *SiSPX2*, *SiSPX3*, *SiSPX6*, *SiSPX13*, *SiSPX-EXS7* and *SiSPX-MFS2* under varying phosphorus treatment conditions in L37. The qRT-PCR findings were generally in alignment with the RNA-seq results, revealing a notable upregulation in the expression levels of most genes under low-phosphorus conditions at multiple time points (Figure 8). The consistent differential expression of SiSPX genes across different materials and tissues suggests their multifaceted and crucial roles in plant growth and development.

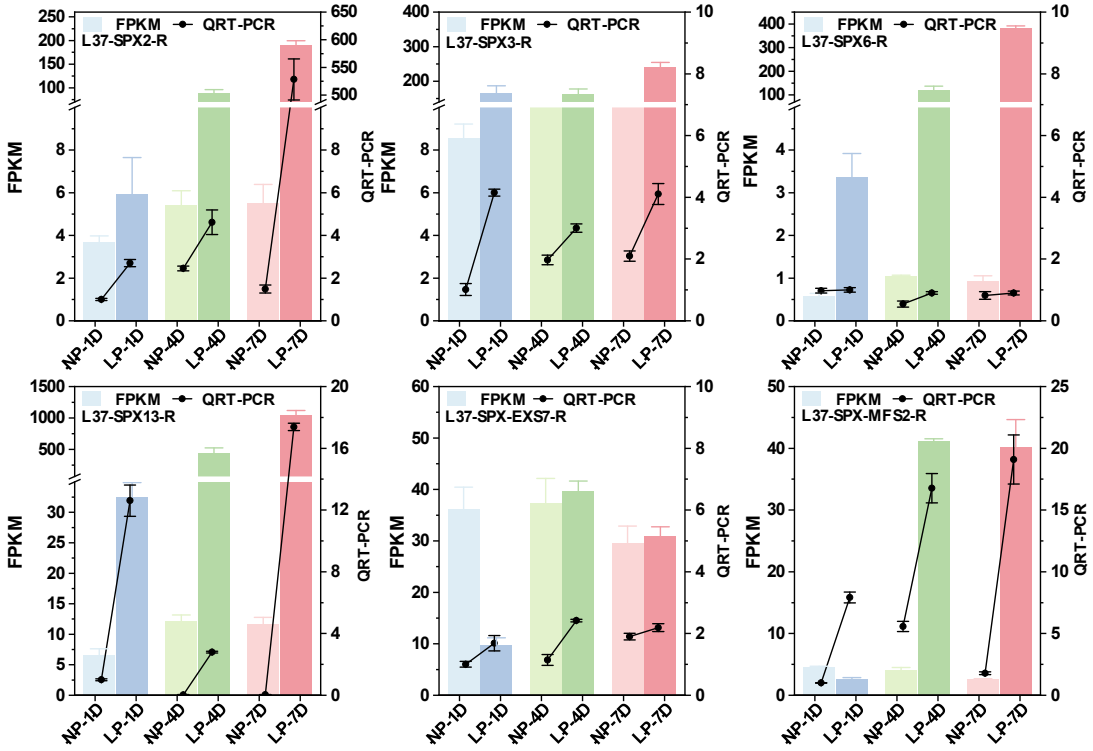


Figure 8 Expression detection of *SiSPX2*, *SiSPX3*, *SiSPX6*, *SiSPX13*, *SiSPX-EXS7* and *SiSPX-MFS2* by qPCR.

4. Discussion

SPX domain-containing genes are well-established regulators of phosphorus signaling in model plants such as Arabidopsis and rice [3, 36]. However, a systematic investigation of this gene family in foxtail millet (*Setaria italica*), a resilient cereal renowned for its nutrient efficiency, has been notably lacking. Using pan-genomics, this study analyzed 114 diverse foxtail millet accessions. This approach revealed a

remarkable 1646 *SiSPX* genes – far exceeding the 15 members typically identified in the single reference genome – and provided unprecedented insights into their structural diversity, evolutionary dynamics, and expression patterns under phosphate stress. Our pan-genomic resource thus unveils the comprehensive landscape of the *SiSPX* family, effectively capturing genetic variations inaccessible through traditional single-genome studies.

In this study, statistical analysis of various gene families and material sources indicates that the total gene copy number of the SPX family remains generally stable throughout the domestication process of different varieties, without significant expansion or contraction, suggesting that this family exhibits core conservation essential for plant survival and functional maintenance. Phylogenetic and collinearity analyses demonstrate that SPX genes in foxtail millet share higher homology with those in rice, whereas there are notable differences exist in the number of members between foxtail millet and Arabidopsis thaliana. This implies that these variations in family members among different species may stem from environmental changes during evolution, and the diversity among gene family members likely emerged after the divergence of monocots and dicots

The SPX subfamily plays a pivotal role in mediating phosphorus absorption and transport in plants. Comparative analysis revealed distinct subfamily sizes across species: while Arabidopsis possesses four SPX members [3] and rice harbors six [4], our pan-genomic study identified 13 *SiSPX* genes in foxtail millet, representing a significant expansion. Structurally, these SiSPX proteins exhibit shorter amino acid sequences compared to other subfamilies, a feature that may confer enhanced structural flexibility, potentially facilitating rapid responses to environmental fluctuations. Evolutionarily, the consistent Ka/Ks ratios < 1 across this subfamily indicate strong purifying selection, suggesting highly conserved biological functions. Crucially, promoter analysis demonstrated that 10 out of 13 *SiSPX* genes contain P1BS elements, which are key cis-regulatory motifs responsive to phosphate starvation. Notably, all SPX subfamily members except *SiSPX5* harbored P1BS elements, exhibiting the highest abundance among all subfamilies. This molecular signature aligns with

functional observations: under low-P stress, *SiSPX2*, *SiSPX3*, *SiSPX6* and *SiSPX13* displayed significant and progressive upregulation specifically in root tissues of both cultivars. This root-specific induction pattern mirrors the behavior of core orthologs in model systems. In Arabidopsis, *AtSPX1/3* are strongly induced and positively regulate the phosphate starvation response (PSR), with *AtSPX3* exerting negative feedback on *AtSPX1* [5, 3, 37], while *AtSPX2* shows minimal induction [3, 5]. Similarly, in rice, *OsSPX1/2/3/5/6* (but not *OsSPX4*) are induced upon Pi deficiency in roots and shoots [3, 5, 11, 36, 37, 38, 39]. The pronounced induction of *SiSPX2/3/6/13* correlates with their enrichment in P1BS elements, positioning these genes as central regulators orchestrating the phosphorus stress response in foxtail millet.

The SPX-EXS subfamily exhibits conserved functions yet divergent evolutionary trajectories across plant species. In Arabidopsis, 11 members display varied expression patterns [41], while rice maintains three members with distinct spatial regulation—notably *OsPHO1;1* and *OsPHO1;2* showing root-predominant expression critical for phosphate transport [42]. This root-specific functional conservation also extends to maize homologs [25]. Central to this subfamily, *PHO1* orchestrates phosphorus translocation from roots to shoots through vesicular trafficking in both Arabidopsis and rice [6, 43, 44]. In foxtail millet, our pan-genomic analysis uncovers distinctive characteristics of the SPX-EXS subfamily. SiSPX-EXS proteins possess significantly extended amino acid sequences compared to other subfamilies, suggesting potential domain acquisitions enabling functional specialization. Subcellular localization predictions strongly support plasma membrane targeting (probability >92%), aligning with their putative role in transmembrane phosphorus transport. Strikingly, *SiSPX-EXS7* exhibits constitutive high expression in roots under normal phosphorus conditions but undergoes significant downregulation (4.1-fold at 72 h) under low-P stress—a response divergent from canonical phosphorus starvation induction. This unique regulatory behavior correlates with its novel N-terminal motif architecture containing three MYB-binding elements, a feature absent in other subfamily members. Furthermore, elevated Ka/Ks ratios (>1.2) across multiple accessions indicate recurrent positive selection acting on *SiSPX-EXS7*, suggesting adaptive evolution driving

neofunctionalization for enhanced environmental stress adaptation, potentially through acquired roles in nutrient signaling cross-talk.

The SPX-MFS protein family represents a major class of vacuolar phosphate transporters in plants. While Arabidopsis possesses three SPX-MFS members and rice contains four, both functioning in intracellular phosphate partitioning [17, 45]. Notably, our pan-genomic analysis uncovered six *SPX-MFS* homologs in foxtail millet. This expansion includes four softcore and two cloud genes, with cloud members exhibiting distinctive enrichment in MYB-binding elements (5 motifs per protein) and complex motif architectures. Such regulatory diversification suggests an enhanced capacity to coordinate phosphate homeostasis with stress adaptation pathways. Functionally, *SiSPX-MFS4* displays constitutive high expression in both aerial and root tissues under low-P stress, mirroring the role of *OsSPX-MFS3* in vacuolar phosphate efflux [45] and supporting its involvement in systemic phosphorus redistribution. Crucially, *SiSPX-MFS2* demonstrates progressive upregulation in roots correlating with stress duration, indicating specialized functions in maintaining rhizospheric phosphate stability during prolonged deficiency.

The SPX-RING subfamily comprises only two conserved members in both Arabidopsis and rice, functioning as key regulators of phosphorus homeostasis through ubiquitin-mediated mechanisms [1, 46, 47, 48]. In comparison, our pan-genomic analysis reveals a striking expansion to six SPX-RING members in foxtail millet. Despite their low prevalence across accessions, these genes exhibit distinctive molecular characteristics, extended polypeptide sequences contrast with minimal motif complexity, making them the simplest among SPX subfamilies. Evolutionary analyses indicate strong functional constraint ($Ka/Ks << 1$) across most members, suggesting highly conserved core cellular roles. Crucially, promoter architecture displays a unique regulatory shift, the complete absence of P1BS elements, typically associated with phosphate starvation responses, contrasts with a significant enrichment of MYB-binding motifs. This divergent cis-regulatory landscape implies alternative transcriptional control mechanisms, warranting further investigation into their specialized roles in phosphorus adaptation.

Through WGCNA analysis, we identified interaction networks between members of the SPX gene family and multiple transcription factors. Subsequent homology searches in rice and Arabidopsis revealed that nine of these transcription factors have homologs previously implicated in root development or phosphorus (Pi) stress responses. Specifically, the AP2 family homolog OsSAE1, which co-expressed with SiSPX-EXS4, exhibited delayed seed germination and reduced root length during germination and early seedling stages in mutant lines [49] The MYB family homolog OsPHR3, co-expressed with SiSPX3 and SiSPX13, showed attenuated primary root hair growth in loss-of-function mutants, whereas overexpression lines demonstrated enhanced tolerance to low Pi stress while maintaining normal growth under Pi-replete conditions [50]. Under Pi-sufficient conditions, overexpression of MYB family homologs co-expressed with SiSPX3, SiSPX13, SiSPX-EXS4, and SiSPX-MFS2 resulted in elevated Pi accumulation in stems and upregulated expression of Pi starvation-responsive genes in OsPHR4-overexpressing plants, ultimately promoting root hair elongation [51]. These findings suggest coordinated expression of these genes may collectively regulate foxtail millet root responses to low Pi stress.

5. Conclusion

This study presents the comprehensive pan-genomic characterization of the SPX gene family in foxtail millet, integrating multi-omics data from 114 diverse accessions. Our analysis successfully identified 1646 SiSPX genes, including previously uncharacterized "cloud genes", thereby significantly expanding the known genetic repertoire beyond single-reference genome limitations. We elucidated subfamily-specific evolutionary trajectories, revealing adaptive expansions within the SPX-MFS and SPX-EXS subfamilies during domestication, alongside a unique regulatory innovation in SPX-RING members marked by the absence of canonical P1BS elements and a prominent enrichment of MYB-binding motifs. Functionally, under phosphate deficiency, we delineated a hierarchical regulatory framework, core P1BS-containing SiSPX genes (e.g., SiSPX2/3/6/13) exhibited robust root-specific induction, while SiSPX-MFS transporters demonstrated tissue-partitioned specialization in phosphate

retention and systemic redistribution. Furthermore, co-expression networks involving key transcription factors (bHLH, MYB, NAC, WRKY) were identified, collectively orchestrating phosphorus reallocation. These findings provide a mechanistic framework for understanding phosphorus adaptation in foxtail millet, offering invaluable genetic targets for enhancing phosphorus-use efficiency and crop resilience in nutrient-limited agricultural systems.

Supplementary Information

The online version contains supplementary material available at

Additional file 1: Table S1. Detailed information on primers used for qPCR analysis.

Additional file 2: Table S2. Count of SiSPX genes in foxtail millet pangenome.

Additional file 3: Table S3. Basic physicochemical properties, Secondary structure prediction and subcellular localization of SPXs in *Setaria italica*.

Additional file 4: Table S4. Detailed information on the identified motifs in foxtail millet pangenome.

Additional file 5: Table S5. Detailed summary of SiSPX genes duplication.

Additional file 6: Table S6. Ka/Ks analysis of duplication SiSPX genes.

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Authors' contributions

BL conceived and designed the research, performed the bioinformatics analyses, analysis the data, visualization and drafted the manuscript; DX, ZJ and JY planted the crops and collected materials; ZS, CR and QL participated in experimental design and provided technical guidance; GX and YH supervised the research and modified the manuscript. All authors read and approved the final manuscript.

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Data availability

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no conflicts of interest.

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