

RESEARCH

Open Access



Mining key genes associated with phosphorus deficiency through genome-wide identification and characterization of cucumber *SPX* family genes

Jialin Li^{1†}, Linyue Hu^{1†}, Qianqian Luan^{3†}, Jingdan Zhang¹, Xueru Feng¹, Hongmei Li¹, Zenghui Wang^{2*} and Wenxing He^{1*}

Abstract

Background Proteins harboring the SPX domain are crucial for the regulation of phosphate (Pi) homeostasis in plants. This study aimed to identify and analyze the entire *SPX* gene family within the cucumber genome.

Results The cucumber genome encompassed 16 *SPX* domain-containing genes, which were distributed across six chromosomes and categorized into four distinct subfamilies: *SPX*, *SPX-MFS*, *SPX-EXS* and *SPX-RING*, based on their structure characteristics. Additionally, gene duplications and synteny analysis were conducted for *CsSPXs*, revealing that their promoter regions were enriched with a variety of hormone-responsive, biotic/abiotic stress and typical P1BS-related elements. Tissue expression profiling of *CsSPX* genes revealed that certain members were specifically expressed in particular organs, suggesting essential roles in cucumber growth and development. Under low Pi stress, *CsSPX1* and *CsSPX2* exhibited a particularly strong response to Pi starvation. It was observed that the cucumber cultivar Xintaimici displayed greater tolerance to low Pi compared to black-spined cucumber under low Pi stress conditions. Protein interaction networks for the 16 *CsSPX* proteins were predicted, and yeast two-hybrid assay revealed that *CsPHR1* interacted with *CsSPX2*, *CsSPX3*, *CsSPX4* and *CsSPX5*, implying their involvement in the Pi signaling pathway in conjunction with *CsPHR1*.

Conclusion This research lays the foundation for further exploration of the function of the *CsSPX* genes in response to low Pi stress and for elucidating the underlying mechanism.

Keywords Cucumber, *SPX* family, Pi starvation, *PHR1*, Regulatory networks

[†]Jialin Li, Linyue Hu and Qianqian Luan contributed equally to this work.

*Correspondence:
Zenghui Wang
374141645@qq.com

Wenxing He

163.hwx@163.com

¹School of Biological Science and Technology, University of Jinan, Jinan 250022, China

²Shandong Institute of Pomology, Tai'an, Shandong 271000, China

³Gansu Agricultural Engineering Technology Research Institute, Lanzhou 730000, China



Introduction

Phosphorus (P) is a fundamental element for the growth and development of plants, required in considerable quantities [1]. It is implicated in a multitude of metabolic pathways, playing a pivotal role not only in the structure of biofilms and nucleic acids, but also in essential processes such as photosynthesis, modulation of enzyme activities, respiration, signal transduction, energy metabolism, and carbon assimilation [2–4]. Additionally, phosphorus plays an essential role in enhancing plant resistance. For instance, under low-temperature stress, phosphorus enhances the levels of phospholipids and soluble sugars in plants, thereby sustaining normal physiological functions. A deficiency in phosphorus can severely compromise the growth and development as well as yield of different crops [5–8]. Given the dynamic fluctuations of phosphorus levels in the surrounding environment, cellular mechanisms must engage to preserve internal phosphorus homeostasis, ensuring optimal survival. To achieve this balance, organisms must detect variations in phosphorus concentrations both within and outside the system and initiate adaptive measures. These include modulating gene expression, metabolism, and tissue morphology to acclimate to phosphorus deficiency [9–12]. Recent studies have identified numerous genes at the molecular level that are involved in the plant's response to low Pi levels, collectively constituting the phosphorus signaling regulatory pathway in plants. Proteins containing the SPX domain are widely involved in the regulation of phosphorus signaling networks [13, 14], and the name SPX domain is derived from the acronyms of the three proteins in which it was first identified: the yeast SYG1 protein [15], the PHO81 protein [16], and the human XPR1 protein [17]. Typically, the SPX domain is located at the N-terminus of proteins and consists of approximately 180 amino acids. In yeast, the SPX domains of Pho87 and Pho90 regulate the activity of Pi uptake and influence the regulation of the Pi signaling pathway [18]. In plants, SPX domain-containing proteins are essential for Pi homeostasis and signaling transduction [19], and the *SPX* family genes are divided into four subfamilies based on the presence of other conserved domains. Among them, proteins containing only the SPX domain are referred to as the *SPX* subfamily [20]; those with an additional MFS (Major Facility Superfamily) domain at the C-terminus, in addition to the N-terminal SPX domain are denoted as the *SPX-MFS* subfamily; the *SPX-RING* subfamily also contains the RING (Really Interesting New Gene) domain at the C-terminus; and the *SPX-EXS* subfamily contains the EXS (ERD1/XPR1/SYG1) domain at the C-terminus [11]. Specific roles in Pi homeostasis and signaling have been ascribed to several of these genes [21, 22]. In *Arabidopsis* and rice, *SPX1* and *SPX2* have been demonstrated to negatively regulate the

phosphorus starvation response by inhibiting PHR1 or PHR2 activity [23, 24]. With the exception of *SPX4*, the remaining *SPX* subfamily genes are induced in both aerial parts and roots in response to phosphorus deficiency [20]. The *SPX-EXS* subfamily, also recognized as PHO1 in eukaryotes, is predominantly expressed in the protoxylem cells of plant roots and is responsible for loading the phosphorus absorbed by the roots into the xylem for transport to aerial parts [25]. The *Atpho1* mutants exhibit increased phosphorus concentration in the root, decreased the phosphorus concentration in the stem, and delayed the development of aerial parts [26, 27]. Overexpression of *OsSPX-MFS3*, a low-affinity phosphorus transporter that facilitates the efflux of phosphorus from the vacuole to the cytosol in conjunction with proton movement, results in a reduced concentration of Pi in rice tissue vacuoles [28]. In transgenic *Arabidopsis*, overexpression of the *CoSPX-MFS3* gene can improve tolerance to low Pi by increasing biomass and organic acid content [21]. Two members of the *SPX-RING* family, also known as *NLA*, have been identified in both *Arabidopsis* and rice, with both being linked to the Pi response and involved in Pi homeostasis [29, 30].

The systematic categorisation of gene family members within the genome constitutes an initial step in exploring biological queries related to species characteristics, and establishes a solid evidence base for subsequent studies of gene function and genetic transformation. Currently, *SPX* family genes have been extensively studied in *Arabidopsis*, rice, rape, soybean, maize and wheat, with 20 *AtSPXs*, 15 *OsSPXs*, 69 *BnaSPXs*, 9 *GmSPXs*, 33 *ZmSPXs* and 46 *TaSPXs* identified [11, 14, 20, 31–33]. However, a comprehensive systematic analysis of the *SPX* gene family in cucumber has not been conducted, and the expression patterns of *SPX* genes in different cucumber cultivars under low phosphorus remain unreported. In this study, we identified a total of 16 *CsSPX* genes and performed a suite of bioinformatics analyses for these family members, including gene structures, conserved domains, phylogenetic relationships, synteny analysis, *cis*-acting elements, and protein interaction network maps. Furthermore, we also demonstrated the interactions between *CsSPX* proteins and *CsPHR1*. This study provided an initial foray into the molecular functions of the *CsSPX* gene family and will lay the foundation for in-depth biological exploration of this pivotal gene family in cucumber.

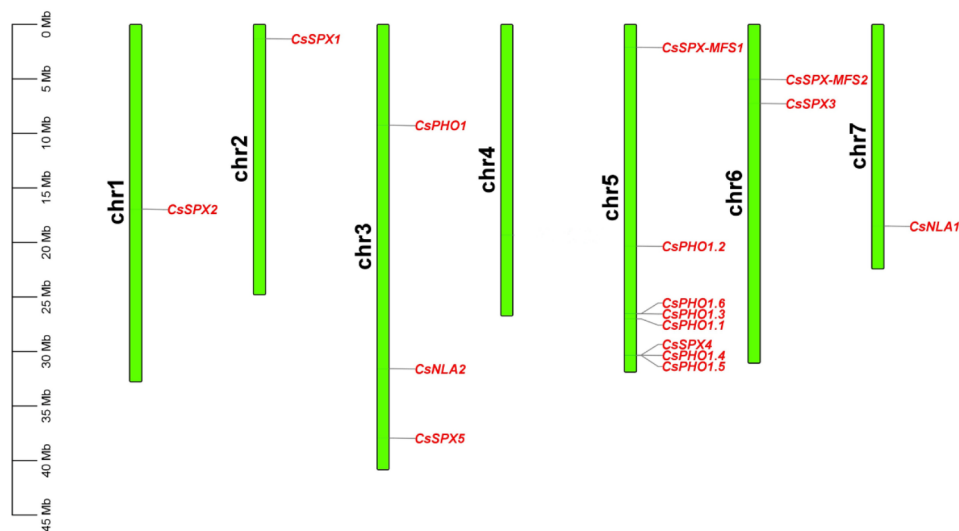
Results

Identification of *SPX* genes in cucumber

Employing a BlastP search within the cucumber genome database, we identified 16 *CsSPX* genes utilizing 20 *Arabidopsis* *SPX* proteins as the reference sequences. The authenticity of these cucumber *SPX* genes was corroborated using the Pfam database (<http://pfam.janelia.org/>)

Table 1 Description of cucumber SPX family genes

Gene ID	Gene name	Location	Molecular weight (kD)	Amino acid length (aa)	pI
CsaV3_2G002890	CsSPX1	Chr2: 1309249–1313288	33.39	290	5.13
CsaV3_1G030190	CsSPX2	Chr1: 16941555–16944650	32.56	286	6.19
CsaV3_6G009000	CsSPX3	Chr6: 7249890–7252124	28.58	246	6.54
CsaV3_5G038190	CsSPX4	Chr5: 30321807–30327195	37.00	325	4.97
CsaV3_3G046460	CsSPX5	Chr3: 37933215–37937774	30.61	263	5.24
CsaV3_3G011910	CsPHO1	Chr3: 9232360–9241503	88.78	767	9.39
CsaV3_5G033820	CsPHO1.1	Chr5: 27001449–27006958	95.80	825	9.30
CsaV3_5G025280	CsPHO1.2	Chr5: 20322929–20327860	92.16	800	9.31
CsaV3_5G033010	CsPHO1.3	Chr5: 26530988–26536992	95.33	830	9.11
CsaV3_5G038200	CsPHO1.4	Chr5: 30327522–30331226	90.77	785	9.11
CsaV3_5G038220	CsPHO1.5	Chr5: 30338753–30344652	91.01	784	9.34
CsaV3_5G032990	CsPHO1.6	Chr5: 26522669–26527656	70.20	609	8.51
CsaV3_5G003300	CsSPX-MFS1	Chr5: 2084005–2092264	77.99	696	6.16
CsaV3_6G005890	CsSPX-MFS2	Chr6: 5022224–5033149	77.49	694	6.68
CsaV3_7G029190	CsNLA1	Chr7: 18489593–18493659	35.73	313	8.95
CsaV3_3G038390	CsNLA2	Chr3: 31576664–31580636	33.93	297	6.77

**Fig. 1** Distribution of *CsSPX* family genes on cucumber chromosomes

and SMART website (<http://smart.embl-heidelberg.de/>) [34]. Based on their homologs in *Arabidopsis*, the *CsSPX* family genes were designated as follows: five genes were classified as members of the *SPX* subfamily, seven as *SPX-EXS* subfamily members, two as *SPX-MFS* subfamily members, and two as *SPX-RING* subfamily members. Table 1 summarised the gene ID, molecular weight, amino acid length, and isoelectric points (pI) for the 16 *CsSPX* genes. The amino acid sequences of the 16 *CsSPX* proteins ranged from 246 aa (*CsSPX3*) to 830 aa (*CsPHO1.3*), with predicted molecular weights spanning 28.58 kD to 95.80 kD. All members of the *SPX* and *SPX-MFS* subfamilies exhibited a pI below 7, denoting their acidic nature, while all members of the *SPX-EXS* and *SPX-RING* subfamilies, except *CsNLA2*, presented a pI above 7, suggesting a basic character (Table 1). Figure 1

illustrated the chromosomal distribution of *CsSPX* family genes, with 16 *SPX* genes from cucumber effectively mapped onto six chromosomes. Only one gene was present on chromosomes 1, 2 and 7, while chromosome 6 had two genes. Chromosome 3 contained three genes and chromosome 5 had the highest number of genes - eight genes (Fig. 1).

Analysis of conserved motifs and gene structures in *SPX* gene family

To establish preliminary evolutionary relationships of cucumber *SPX* proteins, we conducted a detailed comparative analysis of phylogenetic trees, conserved motifs, and gene structures of 16 *CsSPX* and 20 *AtSPX* proteins (Fig. 2). Figure 2A presented a phylogenetic categorization of the 36 *SPX* proteins into quartets of evolutionary

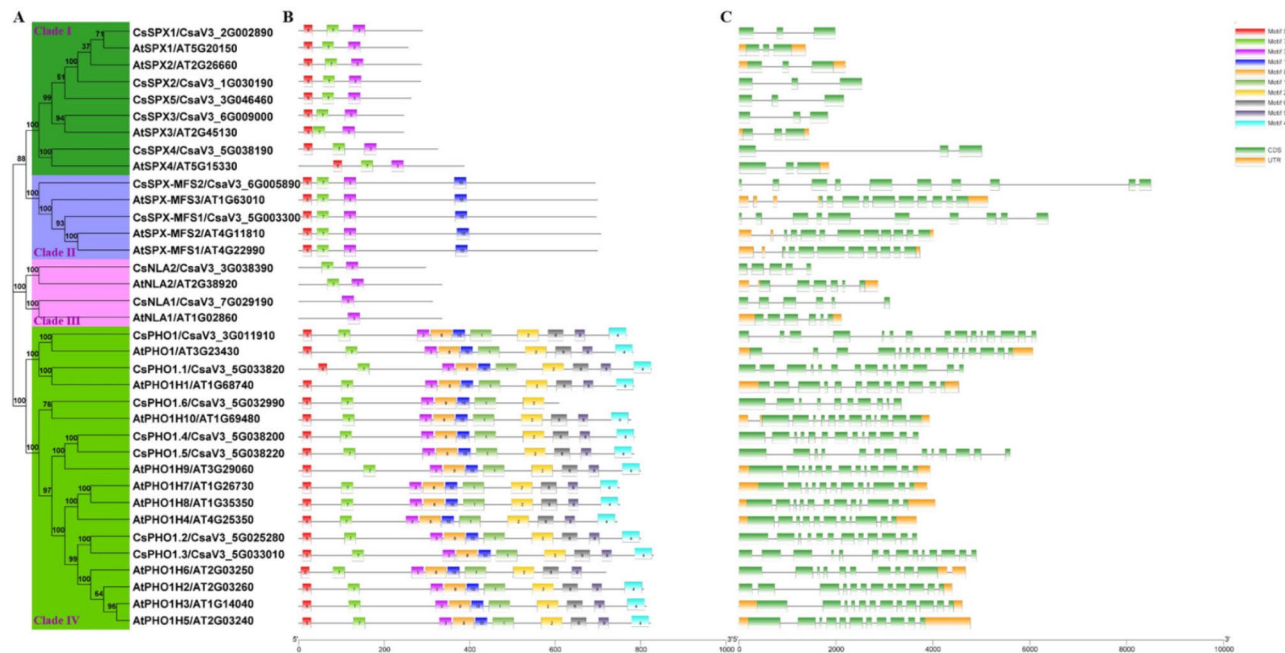


Fig. 2 Phylogenetic and structural analyses of *SPX* genes in cucumber and *Arabidopsis*. **(A)** A phylogenetic tree was constructed using MEGA 7.0, containing 16 *CsSPX* and 20 *AtSPX* protein sequences, which were grouped into four distinct clades. **(B)** Conserved motifs in *SPX* proteins were predicted using MEME software, with the 10 identified motifs depicted in Fig. S1. **(C)** The gene structure of *SPX*s was illustrated, providing insights into their exon-intron organization

clusters - Clade I, Clade II, Clade III, and Clade IV. These clades were aligned with the *SPX*, *SPX-MFS*, *SPX-RING*, and *SPX-EXS* subfamilies, respectively. Each clade was composed of both *CsSPX* and *AtSPX* genes, with members within each subfamily exhibiting a high degree of evolutionary conservation and sequence homology.

To gain insights into the evolution and function of gene family members, a scrutiny of their gene architectures is essential. The gene structures of the *CsSPX*s were visualized using the Gene Structure Display Server (GSDS), revealing a clear pattern of structural homology within each clade (Fig. 2C). Similar gene structure patterns were observed within the same clade. The *SPX* subfamily members, out of the 36 *SPX* genes, possessed only three exons. In contrast, the *SPX-MFS* subfamily exhibited a more intricate structure, with the number of exons ranging from nine to ten. The *SPX-RING* subfamily consisted of five to six exons, and the *SPX-EXS* subfamily contained ten to fifteen exons (Fig. 2C). This variation in gene architecture across subfamilies might reflect divergent functional specializations and evolutionary adaptations.

To enhance our understanding of the structural diversity of *SPX* proteins and predict their roles, we utilized MEME for predicting the number and composition of conserved motifs in *AtSPX* and *CsSPX* proteins (Fig. 2B). Ten distinct motifs were identified (Fig. S1), with proteins within the same clade exhibiting

analogous motif distribution patterns. All *SPX* proteins contained Motif 3, indicating that this motif was specific to *SPX* family genes and might be related to their shared function. Certain motifs, namely 1, 2, 4, 6, 8, and 9, were exclusively present in all members of Clade IV subfamily and not in other Clades. This observation further validated the accuracy of the subfamily classification. Clade IV possessed the most motifs, totaling 10, while the other three subfamilies had only 1–3 motifs in total (Fig. 2B). Therefore, the roles of these motifs warranted further investigation to comprehend the functioning of these proteins.

In general, closely related *SPX* genes in the phylogenetic tree exhibited similar conserved motifs and gene structures, suggesting that proteins within the same subfamily might share similar functions.

Analysis of the evolutionary relationships of *SPX* proteins across various plant species

To further evaluate the evolutionary relationships among the *SPX* gene family in multiple plant species, we aligned 147 *SPX* protein sequences using CLUSTALW, encompassing 16 *CsSPX*, 18 *SISPX*, 15 *OsSPX*, 33 *ZmSPX*, 46 *TaSPX*, and 20 *AtSPX* from six distinct species. Subsequently, we employed the Neighbor-Joining (NJ) method implemented in MEGA 7 to construct a phylogenetic tree, which resulted in the division of proteins into four subfamilies, with *SPX-EXS* subfamily containing the highest number

of proteins (Fig. 3; Table S1). Distinct evolutionary clades were apparent for monocotyledonous species (*Oryza sativa*, *Zea mays*, and *Triticum aestivum*) and dicotyledonous species (*Arabidopsis*, *Cucumis sativus*, and *Solanum lycopersicum*). The members of *SPX-EXS* and *SPX-MFS* subfamilies exhibited evident species differentiation, indicating that *SPX-EXS* and *SPX-MFS* proteins might have varying biological functions in divergent plant species. The observed phylogenetic divergence between monocots and dicots was likely the result of a combination of factors, including functional differentiation, environmental adaptation, genomic divergence, and developmental regulation. These differences highlighted the complexity of plant evolution and the intricate ways in which plants have adapted to their environments over time. Furthermore, we separately compared the evolutionary relationship of *SPX* gene family in *Cucumis sativus* and *Cucurbita moschata*, both within the same family Cucurbitaceae. The findings indicated that genes from the same genera might exhibit a closer phylogenetic relationship

(Fig. S2). Overall, a phylogenetic analysis could offer valuable insights into the evolution and function of family genes.

Synteny analysis of *SPX* genes in tomato, *Arabidopsis* and cucumber

Gene duplication is a significant driving force in genome and genetic system evolution [35]. The expansion of plant gene families is generally attributed to segmental duplication and tandem duplication [36]. The duplication of *CsSPXs* was revealed by analyzing the syntenic regions through the MCscanX software. The cucumber genome was found to encompass 231 segmental duplication blocks and 1468 tandem duplication gene pairs (Table S2). Our synteny analysis identified four segmental duplication gene pairs within the cucumber *SPX* gene family (*CsSPX1/CsSPX2*; *CsSPX1/CsSPX5*; *CsPHO1.4/CsPHO1.6*; *CsSPX-MFS1/CsSPX-MFS2*), while no instances of tandem duplication gene pairs were detected (Fig. 4A; Table S2).

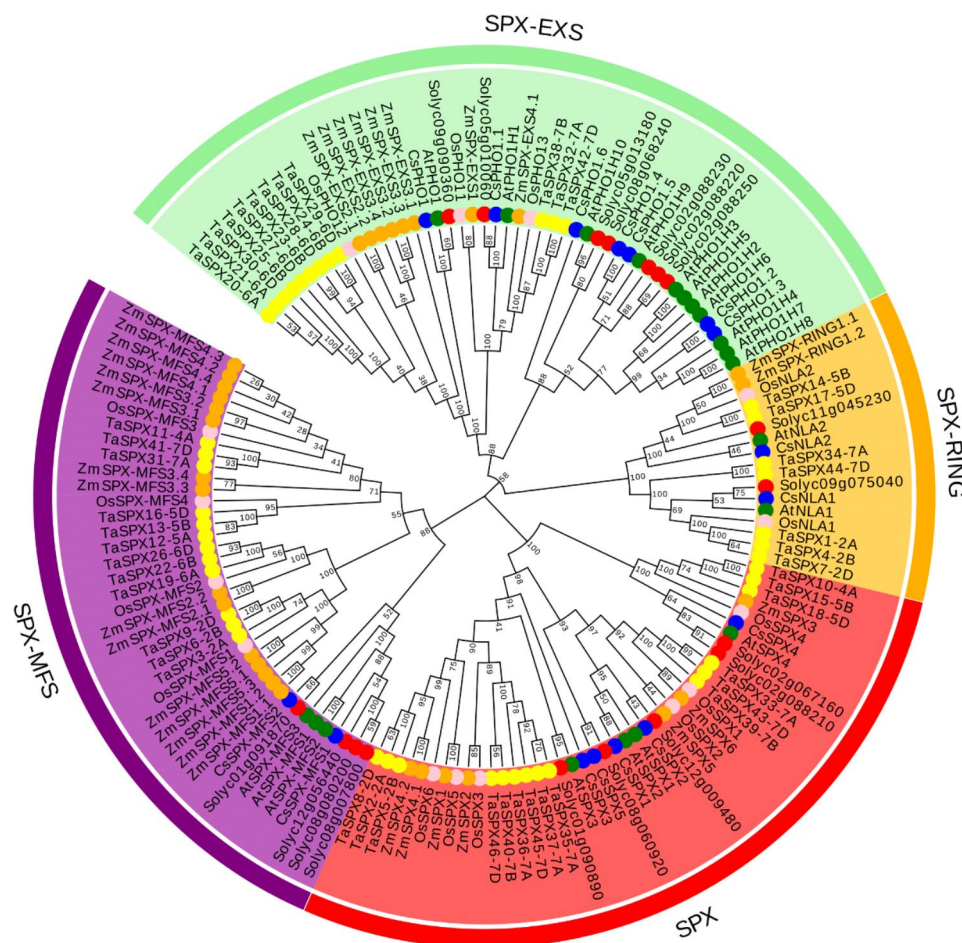


Fig. 3 Phylogenetic tree composed of 147 *SPX* proteins belonging to *Cucumis sativus*, *Solanum lycopersicum*, *Arabidopsis thaliana*, *Triticum aestivum*, *Oryza sativa*, and *Zea mays*. Colour-coded stars indicated *SPX* proteins from distinct species

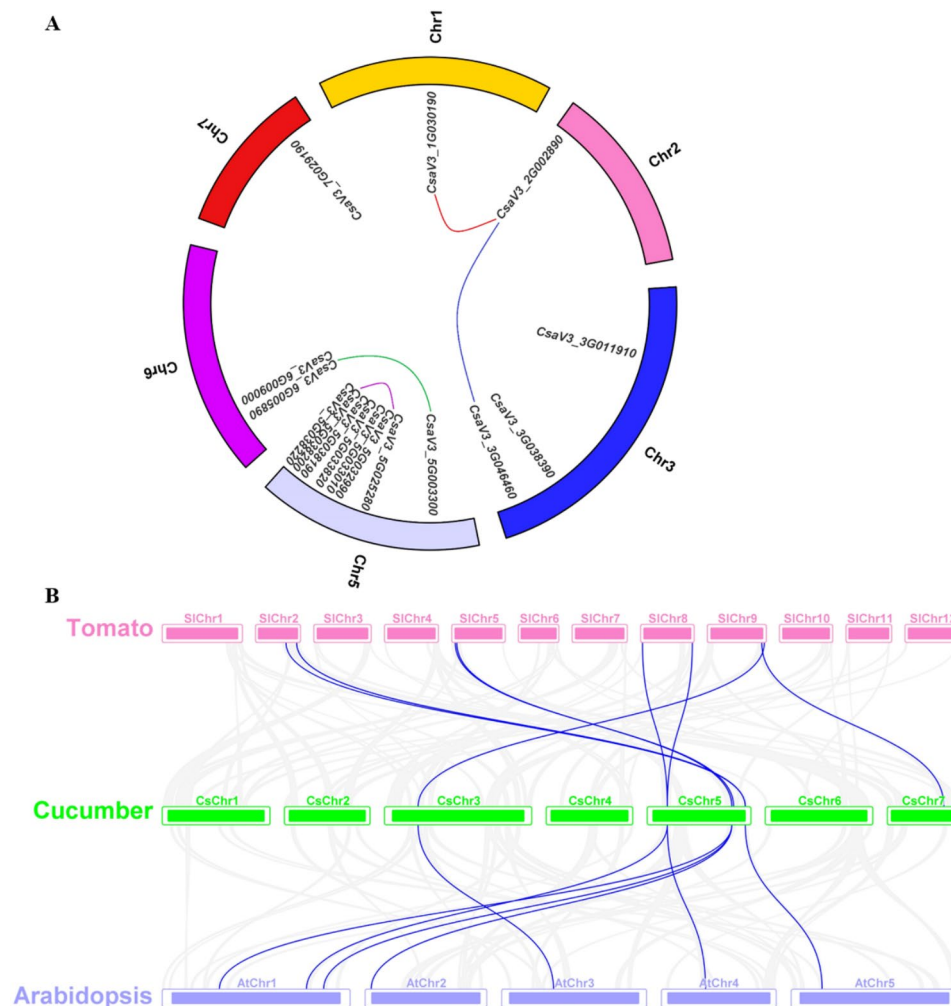


Fig. 4 Duplication and synteny analysis of *CsSPX* genes. **(A)** *CsSPX* gene duplication events between chromosomes. Different colored lines connected distinct segmental duplication gene pairs. **(B)** Synteny analysis of *SPX* genes between cucumber and *Arabidopsis* and tomato revealed collinear blocks of the *SPX* gene within the three genomes, as denoted by the blue lines

The syntenic maps of tomato, cucumber and *Arabidopsis* were compared to elucidate the phylogenetic mechanism of the cucumber *SPX* family (Fig. 4B; Table S3). Seven *CsSPX* genes in cucumber showed collinearity with *SPX* genes in tomato and *Arabidopsis*. Our findings suggested that the *CsPHO1.6* gene was linked to more than two syntenic gene pairs between cucumber and *Arabidopsis*. Furthermore, *CsSPX4* and *CsSPX-MFS1* genes were also associated with two syntenic gene pairs between cucumber and tomato, indicating that these *SPX* genes might play a pivotal role in the evolutionary process. Additionally, we discovered that specific collinear pairs were present between cucumber and both *Arabidopsis* and tomato, as exemplified by *CsSPX4*, *CsPHO1* and *CsSPX-MFS1* (Fig. 4B; Table S3). This highlighted that these orthologous pairs could have already existed prior to the ancestral divergence.

Analysis of *cis*-elements in *CsSPX* genes

Cis-elements embedded within gene promoters are important for regulating gene expression. We utilized PlantCARE and PlantPAN 3.0 software to predict the *cis*-elements present in the 2-kb upstream region of the transcription start site of the *CsSPX* genes and classified them into three types: biotic/abiotic-related stress response elements, growth and development response elements, and hormone response elements (Figs. 5 and 6; Table S4). The stress-related elements encompassed light response components, such as the G-box, ATCT-motif, GT1-motif, TCT-motif, I-box, Box4, GATA-motif, ARE and Sp1, as well as low temperature response element LTR, drought response elements including MBS, MYC, DRE core and TC-rich repeats, and damage response elements WRE3. Our findings indicated that the promoters of several

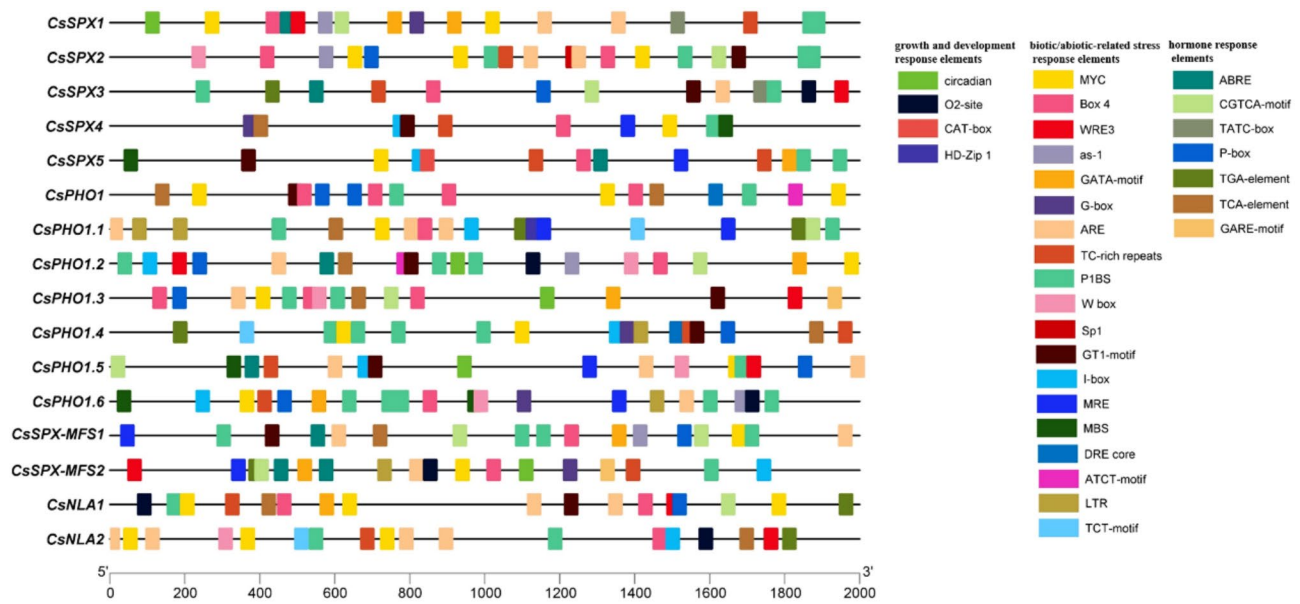


Fig. 5 Depiction of *cis*-elements in *CsSPX* gene promoters. They were grouped into three main categories: biotic and abiotic stress responses, growth and developmental responses and hormonal responses

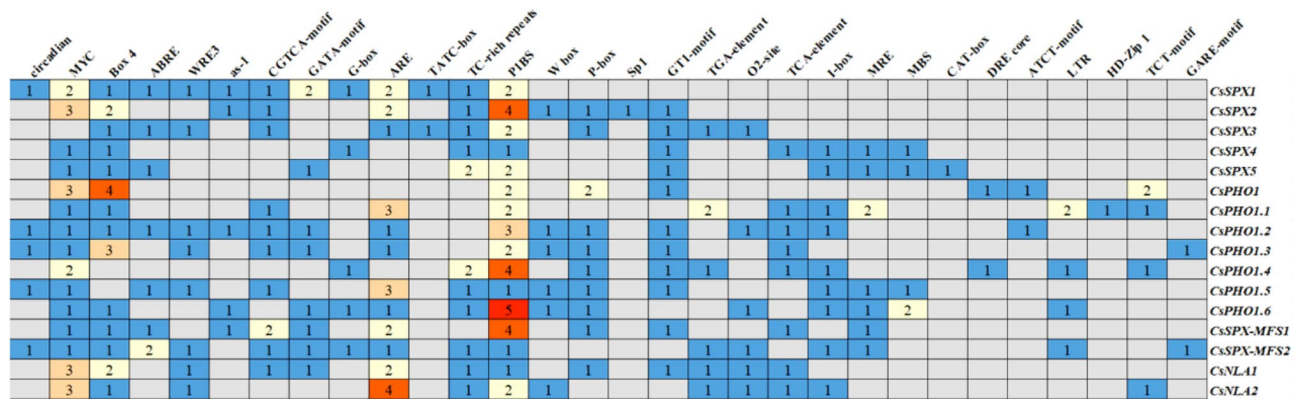


Fig. 6 Statistical information on *cis*-elements in *CsSPX*s. The numerical values within the boxes correspond to the count of each type of *cis*-element

CsSPX genes contained an abundance of W box elements, which implied that these genes might be targeted by WRKY transcription factors. Promoters also frequently contained elements associated with developmental processes, including the O2-site, circadian, CAT-box and HD-Zip 1. Furthermore, seven hormone elements relating to phytohormones were identified (Figs. 5 and 6; Table S4). *SPX* genes have crucial functions in maintaining Pi homeostasis, and their promoters contain P1BS elements (PHR1 binding site) that are vital for responding to Pi starvation. The promoters of all *CsSPX* genes exhibited a pronounced enrichment of P1BS elements, with multiple copies of such elements present, suggesting that *CsPHR1* might regulate these genes under Pi-deficient conditions.

Predicting the interaction network of *CsSPX* proteins

Protein interaction network analysis has proven to be an effective method for studying gene function. Protein interaction networks were predicted through a STRING online software query of 16 *CsSPX* protein sequences. The analysis of the *CsSPX* protein interaction network revealed that several *CsSPX* proteins interacted with each other. For instance, *CsPHO1.1* was predicted to engage in potential interactions with *CsSPX3*, *CsSPX-MFS2* and *CsSPX5* as illustrated in Fig. 7. The PHR proteins are recognized as essential transcription factors in the plant phosphorus regulatory network, playing a pivotal role in modulating signaling pathways during Pi starvation. PHR1 or PHL1 typically interacts with proteins that have SPX domains

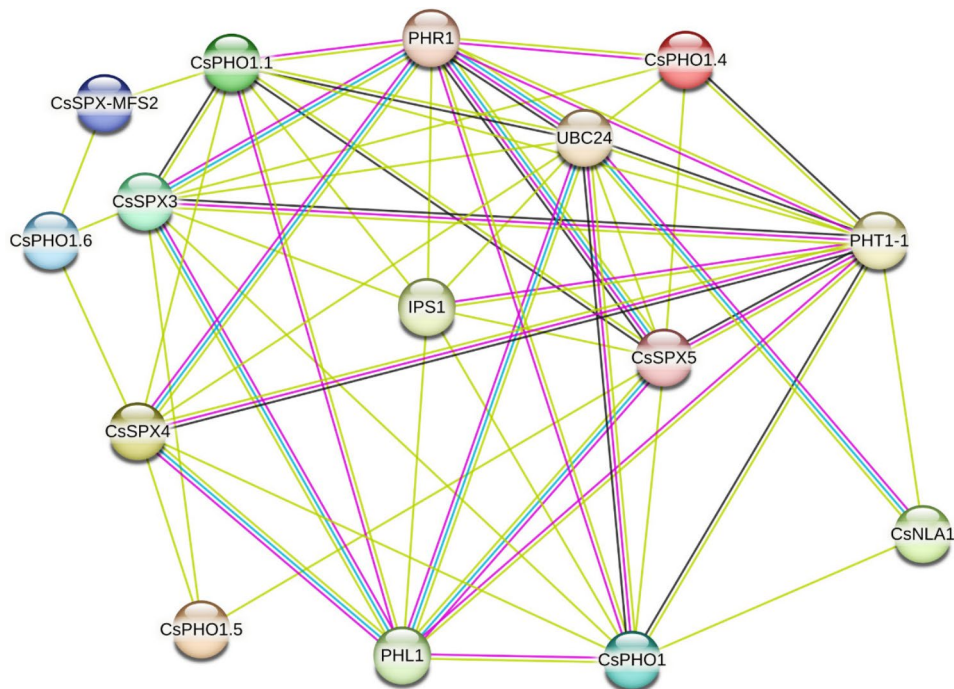


Fig. 7 CsSPX protein interaction network. The STRING online software was chosen to build the network. The various lines of different colours represented diverse types of evidence used to predict the interaction network

in a Pi-dependent way to control transcription. The observed interactions between several CsSPX proteins and Pi starvation response (PHR1 and PHL1) proteins were discovered, further emphasising the significance of SPXs in sustaining phosphorus homeostasis. We also found that IPS1 interacted with certain CsSPX proteins. Additionally, inorganic phosphate transporter 1–1 (PHT1-1), as a high-affinity transporter for external inorganic phosphate, had also been identified to interact with numerous CsSPX proteins (Fig. 7). Moreover, UBC24, a ubiquitin-conjugating enzyme E2 24, which negatively regulates the protein abundance of PHF1 and PHT1s under Pi sufficient conditions by facilitating the degradation of PHT1 proteins at the endomembrane, was also identified within the interaction network. The protein association network delineated in this study provided a valuable resource for future investigations into the molecular mechanisms underlying phosphorus regulation in plants.

CsSPXs interact with CsPHR1

The protein interaction network showed that CsPHR1 interacted with numerous CsSPX proteins. Yeast two-hybrid experiments were conducted to ascertain whether CsPHR1 interacts with CsSPXs. The BD-CsPHR1 bait construct was co-transformed with each of the recombinant plasmids (AD-CsSPX1 through AD-CsSPX5) into yeast cells. The growth of yeast cells was then observed on SD/-Trp-Leu-His-Ade medium with X-a-gal.

Figure 8 illustrated that CsPHR1 interacted with CsSPX2, CsSPX3, CsSPX4 and CsSPX5, while not interacting with CsSPX1.

Expression patterns of 16 CsSPX genes in different tissues of cucumber

To examine the expression patterns of the 16 CsSPX genes in various cucumber tissues, we analyzed their expression levels based on publicly available transcriptomic data from different tissues of cucumber (female, leaf, male, ovary_fertilized, ovary, ovary_unfertilized, root, stem, tendril_base and tendril) [37]. The results revealed that four of the five genes in the SPX subfamily were expressed in all tissues (FPKM>0), except for CsSPX5 (Fig. 9; Table S5). Notably, the CsPHO1 and CsPHO1.1 genes, which belong to the SPX-EXS subfamily, displayed markedly higher expression levels in root tissues, suggesting their vital roles in regulating phosphorus homeostasis and translocation in roots. The CsSPX-MFS1 and CsSPX-MFS2 genes were also expressed in all tissues, but preferentially in female and roots. The CsNLA1 of the SPX-RING subfamily exhibited significantly higher expression levels in tendrils than in other tissues, while CsNLA2 was highly expressed in female and male, indicating potential key roles in phosphorus regulatory network in floral tissues (Fig. 9; Table S5). To further validate the reliability of the transcriptomic data, we conducted an independent assessment of the 16 CsSPX gene expression across a range of cucumber tissues, including roots,

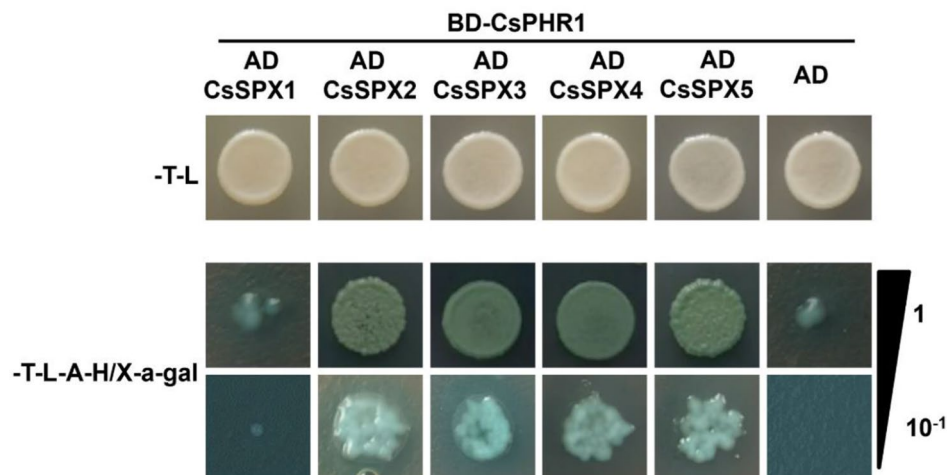


Fig. 8 Yeast two-hybrid assays validation of CsPHR1 interactions with CsSPX proteins

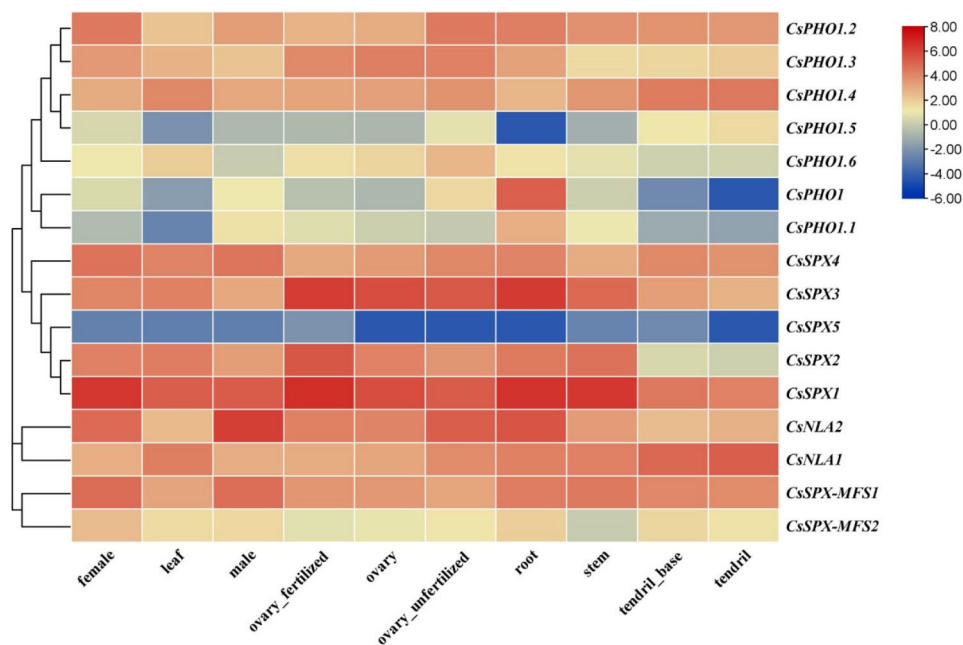


Fig. 9 Tissue-specific expression of *CsSPX* genes. An analysis of the expression levels of the *CsSPX* genes in nine different tissues of cucumber 9930, utilizing publicly available transcriptomic data

stems, leaves, male flowers, ovaries and tendrils, employing qRT-PCR. The expression profiles obtained from this analysis corroborated the patterns observed in the transcriptomic dataset, as depicted in Fig. S3.

Assessment of differential cucumber cultivar responses to low pi stress

The hydroponic experiments revealed that under normal Pi conditions, there was no significant growth disparity between the Xintaimici and black-spined cucumber cultivars. However, the shoot weight of both cucumber cultivars decreased under low Pi stress, but black-spined cucumber was more sensitive to low Pi

stress, with significant changes in root weight in black-spined cucumber, but not in Xintaimici (Fig. 10). Some *CsSPX* subfamily genes were highly induced by low Pi stress, with *CsSPX1* and *CsSPX2* being particularly responsive (Fig. S4). Notably, we found that the expression levels of *CsSPX1*, *CsSPX2* and *CsPHR1* in Xintaimici cucumber were significantly higher than those in black-spined cucumber under low Pi stress (Fig. S5). This differential expression pattern underscored the pivotal role of *CsSPX1* and *CsSPX2* in the acclimation to phosphorus deficiency, potentially contributing to the superior Pi stress tolerance exhibited by the Xintaimici cucumber.

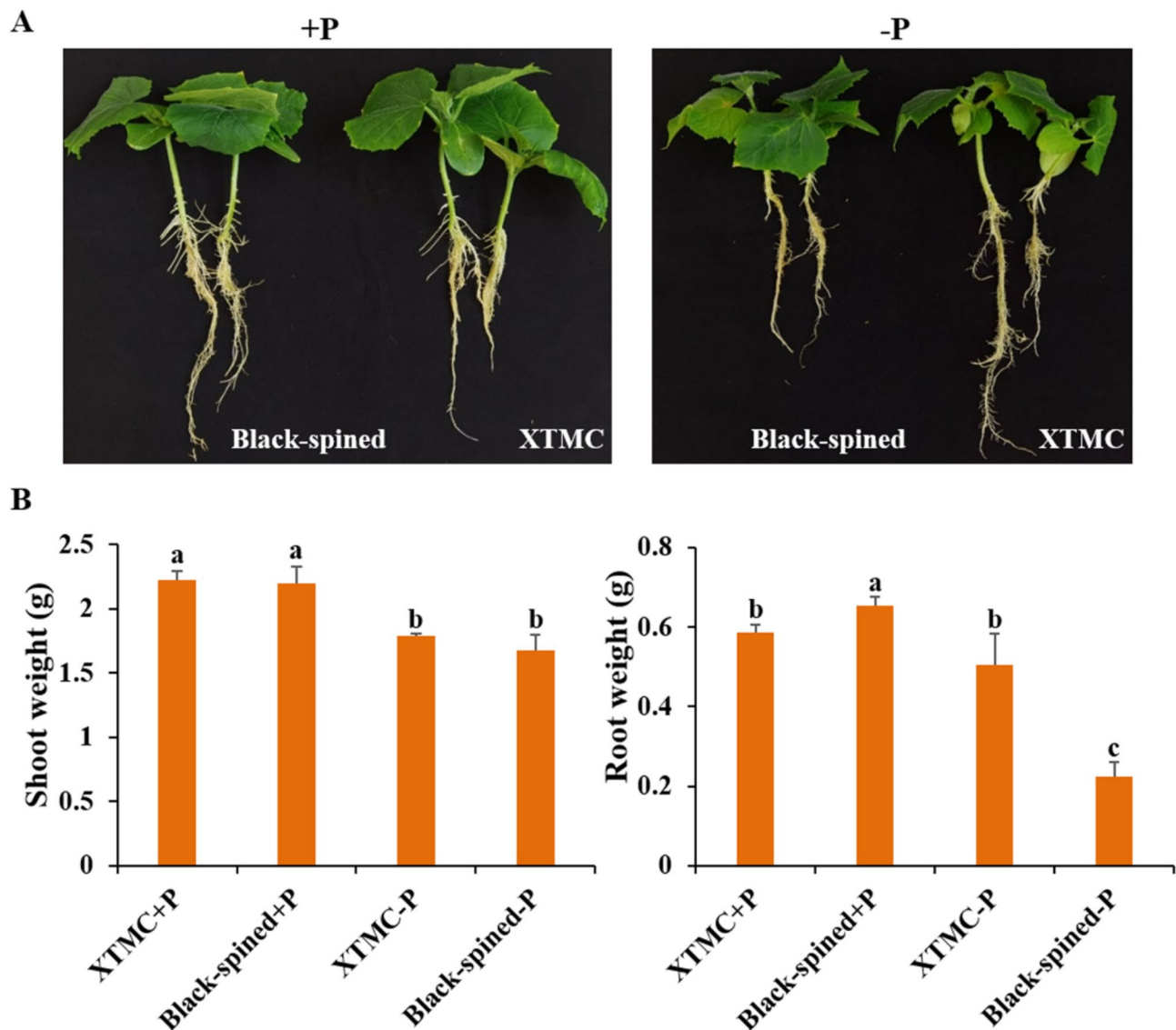


Fig. 10 Phenotypic disparities of Xintaimici and black-spined cucumber cultivars subjected to low Pi stress. Phenotypic observations were made on Xintaimici and black-spined cucumber after 10 days of normal and low Pi stress growth conditions

Discussion

Phosphorus (Pi) is a critical mineral element that is indispensable for plant growth and development, playing a pivotal role in many metabolic pathways of plants, such as enzymatic reaction, sugar metabolism, photosynthesis and energy metabolism [2]. Physiological and metabolic processes in plants responding to phosphorus deficiency rely on a sophisticated and intricate regulatory network of phosphorus signaling within their systems [38]. With the advancements in molecular biology research techniques, a greater number of transcription factors and phosphorus starvation-responsive genes involved in plant phosphorus signaling regulatory pathways have been identified, significantly enhancing our understanding of the phosphorus signaling network and their roles

in plant adaptation to Pi limitation. Studies have shown that WRKY transcription factors have been implicated in the regulation of Pi-starvation responses [12]. In addition, one of the well-studied transcription factors is PHR1 (Phosphate Starvation Response 1), which plays a pivotal role in the regulation of Pi homeostasis in *Arabidopsis thaliana*. PHR1 binds to the promoters of target genes, such as those encoding for high-affinity phosphate transporters, and regulates their expression in response to Pi availability [23]. Another key player in Pi signaling is the SPX domain-containing family of proteins, which have been the focus of recent investigations into the modulation of Pi signaling in model plants such as *Arabidopsis*, maize, wheat and rice. Despite these advances, the functional and evolutionary studies of *CsSPXs* genes in

cucumber remain underexplored. In this study, 16 *CsSPX* genes were identified, which were classified into four distinct subfamilies based on their structural and sequence homology (Fig. 2; Table 1). Within multi-genic families, gene structure and motif patterns can provide valuable insights into the genetic relationships and potential functional redundancies among family members [39, 40]. Figure 2 illustrated that SPXs exhibited a close similarity in gene structure and motif composition within the same clade. Genes with analogous structures and conserved motifs typically share comparable functions. Cucumber SPX proteins were categorized into several functional clades in *Arabidopsis*, providing valuable insights into gene function of *CsSPX*. The construction of a comprehensive phylogenetic tree, encompassing 147 SPX proteins from six species, allowed us to infer the evolutionary relationships and diversification of *CsSPX* genes in cucumber relative to other plant species (Fig. 3). Compared to the *SPX* family genes present in wheat, maize, and rice, the *SPX* family genes found in cucumber were distinctly separated into different evolutionary branches. Furthermore, the evolutionary relationships between members of the *SPX-EXS* and *SPX-MFS* subfamilies significantly differed between species, implying that the roles of these two subfamily proteins may differ across different plants.

As in other species, the promoters of the *CsSPX* gene were found to harbor a diverse array of *cis*-elements associated with biotic and abiotic-related responses, as well as hormone signaling [32, 33]. Studies have shown that *SPX* family genes can be regulated by WRKY transcription factors. For instance, the *AtPHO1* gene can be regulated by WRKY6 and WRKY42 through targeting the W-box element of its promoter [12]. In our analysis, six *CsSPX* genes were identified to contain W-box elements within their promoter regions, suggesting possible regulation by WRKY transcription factors (Figs. 5 and 6). The P1BS (GNATATNC) element, known for its involvement in the response to Pi deficiency, is enriched in the promoters of many *PSR* genes [38, 41, 42]. For instance, the P1BS elements are prevalent in the promoter regions of 18 *ZmSPX* genes and 31 *BnaSPX* genes [14, 32]. In this study, all *CsSPX* genes contained varying amounts of P1BS elements in their promoter regions (Figs. 5 and 6), indicating a conserved regulatory mechanism in response to Pi deficiency in cucumber. The activity of a promoter region is directly influenced by the position, quantity, and flanking sequence of all elements. The diversity of these elements implied a complex regulatory landscape for *CsSPXs* genes, potentially involving multiple associated transcription factors.

The expression analysis of *CsSPXs* genes under varied durations of low Pi treatment indicated that these genes exhibit diverse responses to Pi stress. Notably,

CsSPX1, *CsSPX2* and *CsPHR1* exhibited diverse expression patterns in Xintaimici and black-spined cucumber, leading to dissimilar alterations in root weight (Fig. 10; Fig. S4; Fig. S5). Several studies have demonstrated that under low Pi conditions, certain *SPX* subfamily genes are up-regulated, such as *AtSPX1* and *AtSPX2* [23]. In our study, the *CsSPX* subfamily gene members were markedly induced when cucumber lacked Pi, which was consistent with the results of previous studies [20, 43].

Utilizing the STRING online software, we predicted the protein interaction networks for the 16 *CsSPX* protein sequences, revealing potential interactions between several *CsSPX* proteins and *CsPHR1*. *AtPHR1* is known to play a role in Pi homeostasis by interacting with *SPX* domain-containing proteins under low Pi conditions [23]. The Y2H assay results demonstrated that *CsSPX2*, *CsSPX3*, *CsSPX4* and *CsSPX5* exhibited interaction with *CsPHR1*, whereas *CsSPX1* displayed no interaction with *CsPHR1*, similar to that found in maize and rice. This indicated that the regulatory network for responding to Pi stress in cucumber was relatively conserved, providing a foundation for further functional studies of these interactions and their implications for phosphorus metabolism in plants.

The differential expression of *CsSPX* genes in response to Pi stress and their interactions with *CsPHR1* underscored the complexity of the phosphorus signaling network in cucumber. These findings not only contribute to our understanding of how cucumber adapts to Pi limitation but also have implications for crop improvement. By identifying key regulatory genes and their interactions, we can potentially manipulate the expression of these genes to enhance phosphorus use efficiency and improve crop yield under Pi-deficient conditions. Future research should focus on functional validation of these candidate genes and exploring their roles in the broader context of plant phosphorus nutrition. Additionally, comparative studies across a wider range of plant species will provide further insights into the evolution and diversification of phosphorus signaling pathways, which could inform strategies for the development of plants with improved tolerance to Pi deficiency.

Conclusions

In this study, a total of 16 *CsSPX* genes were identified in the cucumber genome and systematically classified into four distinct subfamilies. A detailed analysis of gene duplications, phylogenetic relationships, *cis*-elements, gene structures and conserved motifs was carried out to comprehensively describe the biological characteristics of these *CsSPX* genes. The expression analysis of the 16 *CsSPXs* during various stages of Pi stress revealed that *CsSPXs* have crucial involvement in the response to Pi

starvation. Particularly, *CsSPX1* and *CsSPX2* exhibited a pronounced response to low Pi stress conditions. Certain SPXs might have functions under Pi starvation through interaction with PHR1. In essence, this study has significantly advanced our understanding of the cucumber *SPX* gene family, offering a substantial foundation for forthcoming biological explorations into the realm of *SPX* genes.

Methods

Identifying *CsSPX* Genes in cucumber

Using 20 *Arabidopsis* SPX proteins as query sequences, Blastp search was performed on cucumber genome database to screen for members of the *CsSPX* gene family. Subsequently, the Pfam database (<http://pfam.xfam.org/>) was utilized to further refine the search for *CsSPX* genes by employing the Hidden Markov Model (HMM) SPX domain (PF03105). Verification of all candidate genes was carried out by employing the Pfam and SMART (<http://smart.embl-heidelberg.de>) tools. Through this systematic approach, 16 *CsSPX* genes were meticulously selected from the cucumber genome and designated based on their evolutionary relationship to *AtSPXs*.

Alignment of multiple sequences and analysis of their phylogeny

Multiple alignments of SPX proteins from 20 *AtSPXs*, 15 *OsSPXs*, 33 *ZmSPXs*, 18 *SISPXs*, 16 *CsSPXs* and 46 *TaSPXs* were conducted using CLUSTALW to elucidate their evolutionary relationships across different species. The obtained results were used to develop a phylogenetic tree through the neighbour-joining (NJ) method in MEGA 7 [44], with support from 1000 bootstrap replicates to ascertain the robustness of the branching patterns. The phylogenetic tree was subsequently visualized and refined using the EvolView tool (<http://www.evolgenius.info>) [45]. The tree was further categorized into four distinct subfamilies, aligning with the known characteristics of the *SPX* gene family, to provide a clearer understanding of the functional and evolutionary diversification within this protein family.

Investigation of gene structures and conserved motifs within *CsSPX* and *AtSPX* family members

We extracted the DNA and cDNA sequences of all *CsSPX* and *AtSPX* genes from the genomes of cucumber and *Arabidopsis*, respectively. The Gene Structure Display Server (GSDS: <http://gsds.cbi.pku.edu.cn/>) [46] was employed to analyze and illustrate the architectural composition of these genes. Additionally, we identified conserved motifs in all *CsSPX* and *AtSPX* amino acid sequences by the MEME tool (<http://meme-suite.org/index.html>). TBtools was used to visualize the results.

Physicochemical properties and chromosome location analysis

The amino acid lengths, molecular weights and isoelectric points (pI) of the 16 *CsSPX* protein sequences were predicted using the ExPASy online website (<http://web.expasy.org/protparam/>). The chromosomal allocation of all *CsSPXs* genes within the cucumber genome was meticulously mapped using TBtools [47], utilizing physical location data extracted from the cucumber genome database.

Gene duplication analysis

The gene duplication events were analyzed using the Multiple Collinearity Scan toolkit (MCScanX) with the default parameters [48]. Syntenic analysis maps were generated using TBtools to display the syntenic relationships of orthologous *SPXs* in cucumber, tomato and *Arabidopsis*.

Identifying *cis*-elements in the promoters of *CsSPX* genes

Sequences of 2 kb upstream of the transcription start site for the 16 *CsSPX* genes were obtained from the cucumber genome database with the TBtools. Next, we uploaded the entire set of sequences to the PlantCARE (<http://bioinformatics.psb.ugent.be/webtools/plantcare/html/>) and PlantPan3 databases (http://plantpan.itps.ncku.edu.tw/plantpan4/promoter_analysis.php) for the purposes of identifying and characterizing *cis*-elements [49]. The collation and visualisation of the results was then performed by TBtools.

Creating protein association networks with STRING

We uploaded the amino acid sequences of the 16 *CsSPX* to the online STRING software (version 11.5; <http://string-db.org>) and selected "*Arabidopsis thaliana*" as the reference organism. We constructed the network by selecting proteins with the highest interaction scores after conducting a BLAST analysis. Proteins lacking interactions with other entities were excluded from the network. We manually transferred the functional annotations from the BLAST results. The names of PHR1, IPS1, PHL1, PHT1-1 and UBC24 proteins were provided automatically by the system, streamlining the process of network annotation and analysis.

Yeast two-hybrid (Y2H) assays

Recombinant plasmids AD-*CsSPX1*, AD-*CsSPX2*, AD-*CsSPX3*, AD-*CsSPX4*, AD-*CsSPX5* and BD-*CsPHR1* were prepared for Y2H assays by cloning the full-length *CsPHR1* coding sequence (CDS) into the bait vector pGBKT7 and the CDSs of *CsSPX1*, *CsSPX2*, *CsSPX3*, *CsSPX4* and *CsSPX5* into the prey vector pGADT7. Y2H assays were performed in accordance with the manufacturer's instructions with yeast strain AH109 (Clontech).

Yeast cells were grown at 28 °C on synthetic dropout medium lacking Trp and Leu (SD/-Trp-Leu). To screen interactions, colonies were transferred to medium lacking Trp, Leu, His and adenine (SD/-Trp-Leu-His-Ade) containing X-a-gal. A negative control experiment was carried out using empty vector pGADT7 [50]. Table S6 provided the primers that were used in this study.

Plant growth conditions and low pi stress treatments

The black-spined cucumber utilized in this study, characterized by its black spines, is preserved by our laboratory. Seeds of Xintaimici and black-spined cucumber cultivars were germinated on moist filter paper in an incubator at 28 °C for 1 day. Then, the germinated seeds were sown into the substrate mixture, and after 10 days, the seedlings were gently washed to remove any residual soil, and then transferred to nutrient solution containing whole nutrients for hydroponics. Following two days of equilibration, the nutrient solution was exchanged with the full nutrient treatment containing KH_2PO_4 1 mM (+P) and the full nutrient treatment containing KH_2PO_4 10 μM low Pi treatment (-P), respectively. Root samples were subsequently collected at 0 h, 3 h, 6 h, 12 h, 24 h and 48 h and immediately frozen in liquid nitrogen for semi-quantitative analysis.

RNA extraction and qRT-PCR analysis

Total RNA was isolated from the designated samples using a plant RNA extraction kit (TianGen, Beijing, China). The isolated RNA was then reverse transcribed into cDNA utilizing the PrimeScript® 1st Strand cDNA Synthesis Kit (Takara, Japan). The qRT-PCR was executed according to the previously outlined method [50]. The expression levels were normalized against the internal control, the cucumber β -actin gene. Each analysis was conducted with three biological replicates to ensure experimental reliability.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s12870-024-05436-3>.

Supplementary Material 1
Supplementary Material 2
Supplementary Material 3
Supplementary Material 4
Supplementary Material 5
Supplementary Material 6
Supplementary Material 7

Acknowledgements

Not applicable.

Author contributions

JL, WH and ZW conceived and designed the experiments. JL, LH, QL, JZ, XF and HL performed the experiments. JL analyzed the data and wrote the manuscript. WH and ZW revised the manuscript. All authors have read and approved this manuscript.

Funding

This work was supported by fundings from the National Natural Science Foundation of China (32202484 and 32102310), the Natural Science Foundation of Shandong Province (ZR2022QC008), Doctoral Fund project of Jinan University (XBS2104), and Longyuan Youth Innovation and Entrepreneurship Talents (Individual) Project of Gansu Province (Gan Guitong [2023] No. 20). The funds played no role in study design, data analysis, and manuscript preparation.

Data availability

The data that support the results are included within the article and its additional files. Other relevant materials are available from the corresponding authors on reasonable request.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

Received: 23 October 2023 / Accepted: 18 July 2024

Published online: 24 July 2024

References

- Chen L, Liao H. Engineering crop nutrient efficiency for sustainable agriculture. *J Integr Plant Biol*. 2017;59(10):710–35.
- Chiou TJ, Lin SI. Signaling network in sensing phosphate availability in plants. *Annu Rev Plant Biol*. 2011;62:185–206.
- Michigami T, Kawai M, Yamazaki M, Ozono K. Phosphate as a signaling molecule and its sensing mechanism. *Physiol Rev*. 2018;98(4):2317–48.
- Satheesh V, Tahir A, Li J, Lei M. Plant phosphate nutrition: sensing the stress. *Stress Biol*. 2022;2(1):16.
- Lipsett J. The phosphorus content and yield of grain of different wheat varieties in relation to phosphorus deficiency[J]. *Aust J Agric Res*. 1964; 15.
- López-Arredondo DL, Leyva-González MA, González-Morales SI, López-Bucio J, Herrera-Estrella L. Phosphate nutrition: improving low-phosphate tolerance in crops. *Annu Rev Plant Biol*. 2014;65:95–123.
- Khan F, Siddique AB, Shabala S, Zhou M, Zhao C. Phosphorus Plays Key roles in regulating plants' physiological responses to Abiotic stresses. *Plants (Basel)*. 2023;12(15):2861.
- Razzaque MS. Phosphate toxicity: new insights into an old problem. *Clin Sci (Lond)*. 2011;120(3):91–7.
- Liu TY, Huang TK, Yang SY, Hong YT, Huang SM, Wang FN, Chiang SF, Tsai SY, Lu WC, Chiou TJ. Identification of plant vacuolar transporters mediating phosphate storage. *Nat Commun*. 2016;7:11095.
- Park BS, Seo JS, Chua NH. NITROGEN LIMITATION ADAPTATION recruits PHOSPHATE2 to target the phosphate transporter PT2 for degradation during the regulation of *Arabidopsis* phosphate homeostasis. *Plant Cell*. 2014;26(1):454–64.
- Secco D, Wang C, Arpat BA, Wang Z, Poirier Y, Tyerman SD, Wu P, Shou H, Whelan J. The emerging importance of the SPX domain-containing proteins in phosphate homeostasis. *New Phytol*. 2012;193(4):842–51.
- Stefanovic A, Ribot C, Rouached H, Wang Y, Chong J, Belbahri L, Delessert S, Poirier Y. Members of the *PHO1* gene family show limited functional redundancy in phosphate transfer to the shoot, and are regulated by phosphate deficiency via distinct pathways. *Plant J*. 2007;50(6):982–94.
- Nezamivand-Cheginini M, Ebrahimie E, Tahmasebi A, Moghadam A, Eshghi S, Mohammadi-Dehchesmeh M, Kopriva S, Niaz A. New insights into the

- evolution of *SPX* gene family from algae to legumes; a focus on soybean. *BMC Genomics*. 2021;22(1):915.
14. Xiao J, Xie X, Li C, Xing G, Cheng K, Li H, Liu N, Tan J, Zheng W. Identification of *SPX* family genes in the maize genome and their expression under different phosphate regimes. *Plant Physiol Biochem*. 2021;168:211–20.
 15. Spain BH, Koo D, Ramakrishnan M, Dzudzor B, Colicelli J. Truncated forms of a novel yeast protein suppress the lethality of a G protein alpha subunit deficiency by interacting with the beta subunit. *J Biol Chem*. 1995;270(43):25435–44.
 16. Huang S, Jeffery DA, Anthony MD, O'Shea EK. Functional analysis of the cyclin-dependent kinase inhibitor Pho81 identifies a novel inhibitory domain. *Mol Cell Biol*. 2001;21(19):6695–705.
 17. Battini JL, Rasko JE, Miller AD. A human cell-surface receptor for xenotropic and polytropic murine leukemia viruses: possible role in G protein-coupled signal transduction. *Proc Natl Acad Sci U S A*. 1999;96(4):1385–90.
 18. Hürliemann HC, Pinson B, Stadler-Waibel M, Zeeman SC, Freimoser FM. The SPX domain of the yeast low-affinity phosphate transporter Pho90 regulates transport activity. *EMBO Rep*. 2009;10(9):1003–108.
 19. Zhang J, Zhou X, Xu Y, Yao M, Xie F, Gai J, Li Y, Yang S. Soybean SPX1 is an important component of the response to phosphate deficiency for phosphorus homeostasis. *Plant Sci*. 2016;248:82–91.
 20. Duan K, Yi K, Dang L, Huang H, Wu W, Wu P. Characterization of a sub-family of *Arabidopsis* genes with the SPX domain reveals their diverse functions in plant tolerance to phosphorus starvation. *Plant J*. 2008;54(6):965–75.
 21. Chen J, Han X, Liu L, Yang B, Zhuo R, Yao X. Genome-wide detection of *SPX* Family and Profiling of *CoSPX-MFS3* in regulating low-phosphate stress in Tea-Oil Camellia. *Int J Mol Sci*. 2023;24(14):11552.
 22. Wang C, Huang W, Ying Y, Li S, Secco D, Tyerman S, Whelan J, Shou H. Functional characterization of the rice *SPX-MFS* family reveals a key role of *OsSPX-MFS1* in controlling phosphate homeostasis in leaves. *New Phytol*. 2012;196(1):139–48.
 23. Puga MI, Mateos I, Charukesi R, Wang Z, Franco-Zorrilla JM, de Lorenzo L, Irigoyen ML, Masiero S, Bustos R, Rodríguez J, Leyva A, Rubio V, Sommer H, Paz-Ares J. SPX1 is a phosphate-dependent inhibitor of PHOSPHATE STARVATION RESPONSE 1 in *Arabidopsis*. *Proc. Natl. Acad. Sci. Unit. States Am*. 2014; 111: 14947–14952.
 24. Wang Z, Ruan W, Shi J, Zhang L, Xiang D, Yang C, Li C, Wu Z, Liu Y, Yu Y, Shou H, Mo X, Mao C, Wu P. Rice SPX1 and SPX2 inhibit phosphate starvation responses through interacting with PHR2 in a phosphate-dependent manner. *Proc Natl Acad Sci U S A*. 2014;111(41):14953–8.
 25. Hamburger D, Rezzonico E, MacDonald-Comber Petétot J, Somerville C, Poirier Y. Identification and characterization of the *Arabidopsis PHO1* gene involved in phosphate loading to the xylem. *Plant Cell*. 2002;14(4):889–902.
 26. Poirier Y, Thoma S, Somerville C, Schiefelbein J. Mutant of *Arabidopsis* deficient in xylem loading of phosphate. *Plant Physiol*. 1991;97(3):1087–93.
 27. Ribot C, Wang Y, Poirier Y. Expression analyses of three members of the *AtPHO1* family reveal differential interactions between signaling pathways involved in phosphate deficiency and the responses to auxin, cytokinin, and abscisic acid. *Planta*. 2008;227(5):1025–36.
 28. Wang C, Yue W, Ying Y, Wang S, Secco D, Liu Y, Whelan J, Tyerman SD, Shou H. Rice SPX-Major Facility Superfamily3, a vacuolar phosphate Efflux Transporter, is involved in maintaining phosphate homeostasis in Rice. *Plant Physiol*. 2015;169(4):2822–31.
 29. Kant S, Peng M, Rothstein SJ. Genetic regulation by NLA and microRNA827 for maintaining nitrate-dependent phosphate homeostasis in *Arabidopsis*. *PLoS Genet*. 2011;7(3):e1002021.
 30. Yue W, Ying Y, Wang C, Zhao Y, Dong C, Whelan J, Shou H. OsNLA1, a RING-type ubiquitin ligase, maintains phosphate homeostasis in *Oryza sativa* via degradation of phosphate transporters. *Plant J*. 2017;90(6):1040–51.
 31. Yao Z, Tian J, Liao H. Comparative characterization of GmSPX members reveals that GmSPX3 is involved in phosphate homeostasis in soybean. *Ann Bot*. 2014;114(3):477–88.
 32. Du H, Yang C, Ding G, Shi L, Xu F. Genome-wide identification and characterization of SPX Domain-Containing members and their responses to phosphate Deficiency in *Brassica napus*. *Front Plant Sci*. 2017;8:35.
 33. Kumar A, Sharma M, Gahlaut V, Nagaraju M, Chaudhary S, Kumar A, Tyagi P, Gajula MNVP, Singh KP. Genome-wide identification, characterization, and expression profiling of *SPX* gene family in wheat. *Int J Biol Macromol*. 2019;140:17–32.
 34. Letunic I, Doerks T, Bork P. SMART 6: recent updates and new developments. *Nucleic Acids Res*. 2009;37:D229–232. (Database issue).
 35. Das Laha S, Dutta S, Schäffner AR, Das M. Gene duplication and stress genomics in Brassicas: current understanding and future prospects. *J Plant Physiol*. 2020;255:153293.
 36. Cannon SB, Mitra A, Baumgarten A, Young ND, May G. The roles of segmental and tandem gene duplication in the evolution of large gene families in *Arabidopsis thaliana*. *BMC Plant Biol*. 2004;4:10.
 37. Li Z, Zhang Z, Yan P, Huang S, Fei Z, Lin K. RNA-Seq improves annotation of protein-coding genes in the cucumber genome. *BMC Genomics*. 2011;12:540.
 38. Wu P, Shou H, Xu G, Lian X. Improvement of phosphorus efficiency in rice on the basis of understanding phosphate signaling and homeostasis. *Curr Opin Plant Biol*. 2013;16(2):205–12.
 39. Boudet N, Aubourg S, Toffano-Nioche C, Kreis M, Lecharny A. Evolution of intron/exon structure of DEAD helicase family genes in *Arabidopsis*, *Caenorhabditis*, and *Drosophila*. *Genome Res*. 2001;11(12):2101–14.
 40. Babenko VN, Rogozin IB, Mekhedov SL, Koonin EV. Prevalence of intron gain over intron loss in the evolution of paralogous gene families. *Nucleic Acids Res*. 2004;32(12):3724–33.
 41. Zhou J, Jiao F, Wu Z, Li Y, Wang X, He X, Zhong W, Wu P. *OsPHR2* is involved in phosphate-starvation signaling and excessive phosphate accumulation in shoots of plants. *Plant Physiol*. 2008;146(4):1673–86.
 42. Pant BD, Pant P, Erban A, Huhman D, Kopka J, Scheible WR. Identification of primary and secondary metabolites with phosphorus status-dependent abundance in *Arabidopsis*, and of the transcription factor PHR1 as a major regulator of metabolic changes during phosphorus limitation. *Plant Cell Environ*. 2015;38(1):172–87.
 43. Wang ZY, Hu H, Huang HJ, Duan K, Wu ZC, Wu P. Regulation of OsSPX1 and OsSPX3 on expression of OsSPX domain genes and Pi-starvation signaling in rice. *J Integr Plant Biol*. 2009;51:663–74.
 44. Li J, Li H, Quan X, Shan Q, Wang W, Yin N, Wang S, Wang Z, He W. Comprehensive analysis of cucumber C-repeat/dehydration-responsive element binding factor family genes and their potential roles in cold tolerance of cucumber. *BMC Plant Biol*. 2022;22(1):270.
 45. Li J, Song C, Li H, Wang S, Hu L, Yin Y, Wang Z, He W. Comprehensive analysis of cucumber *RAV* family genes and functional characterization of *CsRAV1* in salt and ABA tolerance in cucumber. *Front Plant Sci*. 2023;14:1115874.
 46. Guo AY, Zhu QH, Chen X, Luo JC. GSDS: a gene structure display server. *Yi Chuan*. 2007;29:1023–6.
 47. Chen C, Chen H, Zhang Y, Thomas HR, Frank MH, He Y, Xia R. TBtools: an integrative Toolkit developed for interactive analyses of big Biological Data. *Mol Plant*. 2020;13(8):1194–202.
 48. Wang Y, Tang H, DeBarry JD, Tan X, Li J, Wang X, et al. MCScanX: a toolkit for detection and evolutionary analysis of gene synteny and collinearity. *Nucleic Acids Res*. 2012;40:e49.
 49. Li J, Wang T, Han J, Ren Z. Genome-wide identification and characterization of cucumber *bHLH* family genes and the functional characterization of *Csb-HLH041* in NaCl and ABA tolerance in *Arabidopsis* and cucumber. *BMC Plant Biol*. 2020;20(1):272.
 50. Li J, Luan Q, Han J, Zhang C, Liu M, Ren Z. CsMYB60 directly and indirectly activates structural genes to promote the biosynthesis of flavonols and proanthocyanidins in cucumber. *Hortic Res*. 2020;7:103.

Publisher's Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.