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Evolution of the *SPX* gene family and its role in the response mechanism to low phosphorus stress in self-rooted apple stock

Zenghui Wang[1], Xiaowen Zhang[2], Xuemei Yang[1], Haixia Tang[1], Lijuan Feng[1], Yanlei Yin[1*] and Jialin Li[2*]

Abstract

Background Phosphorus plays a key role in plant adaptation to adversity and plays a positive role in the yield and quality formation of apples. Genes of the *SPX* domain-containing family are widely involved in the regulation of phosphorus signalling networks. However, the mechanisms controlling phosphorus deficiency are not completely understood in self-rooted apple stock.

Results In this study, 26 members of the apple *SPX* gene family were identified by genome-wide analysis, and further divided into four subfamilies (*SPX*, *SPX-MFS*, *SPX-EXS*, and *SPX-RING*) based on their structural features. The chromosome distribution and gene duplications of *MdSPXs* were also examined. The promoter regions of *MdSPXs* were enriched for multiple biotic/abiotic stresses, hormone responses and typical P1BS-related elements. Analysis of the expression levels of 26 *MdSPXs* showed that some members were remarkably induced when subjected to low phosphate (Pi) stress, and in particular *MdSPX2*, *MdSPX3*, and *MdPHO1.5* exhibited an intense response to low Pi stress. *MdSPX2* and *MdSPX3* showed significantly divergent expression levels in low Pi sensitive and insensitive apple species. Protein interaction networks were predicted for 26 MdSPX proteins. The interaction of MdPHR1 with MdSPX2, MdSPX3, MdSPX4, and MdSPX6 was demonstrated by yeast two-hybrid assay, suggesting that these proteins might be involved in the Pi-signaling pathway by interacting with MdPHR1.

Conclusion This research improved the understanding of the apple *SPX* gene family and contribute to future biological studies of *MdSPX* genes in self-rooted apple stock.

Keywords Self-rooted apple stock, Expression patterns, *SPX* family, Phosphate starvation, Regulatory networks

*Correspondence:
Yanlei Yin
yylei66@sina.com
Jialin Li
bio_lijl@ujn.edu.cn
[1]Shandong Institute of Pomology, Tai'an 271000, Shandong, China
[2]School of Biological Science and Technology, University of Jinan, Jinan 250022, China

Background

Phosphorus (P) is an essential macronutrient for all living organisms. Although phosphorus is abundant in the environment, it is limited due to its low dispersal rate, uptake by the soil, and conversion to organophosphorus by microorganisms. The lack of phosphorus in the soil greatly reduces the growth of plants, and has become the main constraint of agricultural production [1, 2].

Phosphorus plays a key role in plant adaptation to adversity and plays a positive role in the yield and quality formation of apples [3, 4]. According to the soil nutrient conditions in many apple producing areas, it is found that the apple orchards in China are mostly established in the mountains with barren soil layer, water and nutrient shortage and complex climate Hill field, most of the soil has a very low effective P content [5, 6]. In the process of apple cultivation, the lack of effective phosphorus in the soil will delay the growth of trees, form stiff seedlings, delay flowering and maturity, lead to falling flowers and fruits, hinder sugar transportation, and affect the yield and quality of apples. Good root morphology and physiological characteristics are the basis for the absorption and utilization of phosphorus nutrients and adaptation to soil low phosphorus environment [7]. Therefore, the selection of low phosphorus resistant apple stocks is of great significance for the sustainable development of apples.

The *SPX* gene family involved in many phosphate (Pi) signalling pathways and the SPX domain was first identified in a yeast *gpa1* inhibitor (SYG1), a cyclin-dependent kinase inhibitor (PHO81) and human xenotropic and polytropic retrovirus receptor 1 (XPR1) [8, 9, 18]. Based on the presence of additional domains, SPX proteins can be further classified into four subfamilies: SPX proteins, SPX-EXS proteins, SPX-MFS proteins, and SPX-RING proteins [10–12]. The AtSPXs, CoSPXs, GmSPXs, PeSPXs, BnaSPXs, TaSPXs, OsSPXs, and ZmSPXs have been characterized [13–20]. PHOSPHATE RESPONSE 1 (PHR1) as a key regulator of transcriptional responses to Pi starvation, is a transcription factor with an MYB domain and a coiled-coil domain binding to a P1BS *cis*-element, which was identified in *Arabidopsis thaliana* [21]. The certain SPX domain-containing proteins regulated the transcriptional activity of *PHR1*. Under Pi deficiency, the content of cellular inositol pyrophosphate decreases, releasing PHR1 from the SPX1 and PHR1 complex to bind to the promoter of the *PSI* genes, thereby increasing genes transcription [22–25].

Classifying gene family members in the genome in mining biological problems related to species traits is the first step, laying a solid foundation for subsequent gene function studies and genetic transformation studies. However, the mechanisms controlling phosphorus deficiency are not clear in self-rooted apple stock. In this study, a bioinformatics analysis of *SPX* gene family members was performed in the apple genome, including chromosomal locations, phylogenetic relationships, gene structures, gene duplication, and *cis*-element analysis. We further proved that MdSPX2, MdSPX3, MdSPX4, and MdSPX6 physically interacted with MdPHR1. This study revealed the molecular function of *SPX* gene in apples, which can provide a theoretical basis for the breeding of low phosphorus resistance, and has great significance for the sustainable development of the apple industry.

Results

Identification and chromosomal locations of apple *SPX* genes

Using 20 *Arabidopsis* SPX proteins as search sequences, a total of 26 apple *SPX* genes were identified by BlastP search in the *Malus×domestica* reference genome database. The accuracy of the apple *SPX* genes was verified based on Pfam database (http://pfam.janelia.org/) and SMART website (http://smart.embl-heidelberg.de/) [26]. The *MdSPX* family genes were named based on their homologs in *Arabidopsis* as follows: six genes belonged to the *SPX* subfamily, the *SPX-EXS* subfamily contained 12 genes, three members belonged to the *SPX-MFS* subfamily, and five members belonged to the *SPX-RING* subfamily. The gene ID, molecular weight, amino acids length and the isoelectric points (pI) of the 26 *MdSPX* genes were summarized in Table 1. The amino acids lengths of the 26 MdSPX proteins ranged from 262aa (*MD07G1115200*) to 835aa (*MD00G1081000*), and the predicted molecular weights varied from 30.21 kD to 96.25 kD. All proteins of these four subfamilies except *MdSPX1*, *MdSPX2*, *MdSPX4*, *MdSPX5*, *MdSPX6*, *MdSPX-MFS1*, *MdSPX-MFS2*, and *MdNLA3* had a pI greater than seven, indicating that these proteins belonged to basic proteins (Table 1). The chromosomal distribution of the *MdSPX* family genes was shown in Fig. 1. Among the 26 genes in apple, 21 genes were successfully mapped onto 13 chromosomes of apple, and 5 *SPX-EXS* subfamily genes were mapped onto Chr0. Chromosomes 3, 4, 6, 7, 12 and 14 contained only one gene, chromosomes 2, 9, 11, 13, 15 and 17 contained two genes, while chromosome 16 contained three genes (Fig. 1).

Conserved motif and gene structure analysis of *SPX* gene family

To preliminarily resolve the phylogenetic relationships of apple SPX proteins, we comparatively analysed the phylogenetic trees, conserved motifs and gene structures of 26 MdSPX and 20 AtSPX in detail (Fig. 2). As shown in Fig. 2A, the phylogenetic tree classified these 26 SPX proteins into four clades, which were named Clade I, Clade II, Clade III, and Clade IV corresponding to the *SPX-RING* subfamily, *SPX-MFS* subfamily, *SPX* subfamily, and *SPX-EXS* subfamily, respectively. Each clade contains

Table 1 Description of *Malus domestica* SPX family genes

Gene ID	Gene name	Location	Molecular weight (kD)	Amino acid length (aa)	pI
MD02G1031100	*MdSPX1*	Chr02:2446537–2,449,320	33.12	289	6.20
MD15G1124700	*MdSPX2*	Chr15:9047428–9,050,194	33.20	288	5.13
MD07G1115200	*MdSPX3*	Chr07:13857569–13,865,493	30.21	262	7.73
MD09G1054300	*MdSPX4*	Chr09:3612656–3,615,230	36.91	331	4.89
MD17G1052000	*MdSPX5*	Chr17:4091091–4,093,213	41.08	366	5.39
MD15G1172700	*MdSPX6*	Chr15:13456527–13,459,263	33.06	289	5.74
MD13G1216800	*MdPHO1*	Chr13:20718527–20,723,714	57.78	497	9.27
MD16G1222100	*MdPHO1.1*	Chr16:22212605–22,222,240	90.13	778	9.13
MD13G1049800	*MdPHO1.2*	Chr13:3542062–3,548,264	92.42	798	9.27
MD16G1050800	*MdPHO1.3*	Chr16:3627571–3,632,983	95.03	820	9.22
MD16G1068000	*MdPHO1.4*	Chr16:4775222–4,779,764	91.99	799	9.43
MD00G1200800	*MdPHO1.5*	Chr00:47531487–47,534,671	80.65	700	9.35
MD00G1081000	*MdPHO1.6*	Chr00:16188231–16,191,412	96.25	835	9.36
MD00G1081300	*MdPHO1.7*	Chr00:16225026–16,228,305	95.62	829	9.44
MD09G1054400	*MdPHO1.8*	Chr09:3615917–3,620,025	90.32	783	9.39
MD00G1081200	*MdPHO1.9*	Chr00:16198380–16,203,765	52.47	461	9.21
MD00G1081400	*MdPHO1.10*	Chr00:16228664–16,232,575	90.17	778	9.15
MD17G1052300	*MdPHO1.11*	Chr17:4116056–4,120,188	89.75	780	9.08
MD04G1068900	*MdSPX-MFS1*	Chr04:9395159–9,401,627	78.72	704	6.36
MD06G1069900	*MdSPX-MFS2*	Chr06:16904023–16,918,731	75.03	670	6.58
MD02G1195400	*MdSPX-MFS3*	Chr02:18714848–18,719,643	74.71	674	7.90
MD03G1287000	*MdNLA1*	Chr03:36622212–36,624,655	36.29	316	9.05
MD14G1045700	*MdNLA2*	Chr14:4357625–4,361,254	36.07	332	9.15
MD12G1046800	*MdNLA3*	Chr12:5372221–5,375,491	38.00	333	6.78
MD11G1313600	*MdNLA4*	Chr11:42597648–42,600,287	38.05	316	7.78
MD11G1313200	*MdNLA5*	Chr11:42577417–42,579,672	36.19	316	8.88

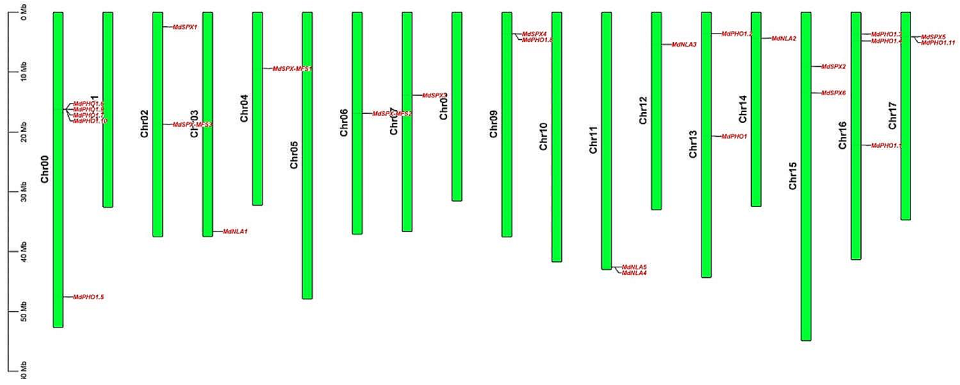

Fig. 1 Distribution of *MdSPX* family genes on apple chromosomes

MdSPX and *AtSPX* genes, and the proteins in the same subfamily were highly related.

For the evolutionary and functional analysis of gene family members, analysing gene structures could provide critical clues. To clarify the exon/intron structure of the *SPX* genes, we visualised their gene structures using GSDS (Fig. 2C). Similar gene structure patterns were presented in the same clade. Of the 26 *SPX* genes, members of the *SPX-RING* subfamily contained only six exons, whereas the number of exons ranged from nine to ten in the *SPX-MFS* subfamily, eight to fifteen in the *SPX-EXS* subfamily, and two to three in the *SPX* subfamily (Fig. 2C).

To further resolve the structural diversity of the SPX proteins and predict their functions, we used MEME to predict the number and composition of conserved motifs in AtSPX and MdSPX proteins (Fig. 2B). Ten different motifs were identified (Fig. S1). Similar motif distribution patterns were found among proteins belonging to the same clade. Motif 4 was present in all SPX proteins, suggesting that this motif was a characteristic motif specific to *SPX* family genes and might be related to the common

function of SPX proteins. Some specific motifs were only present in specific clade as follows motifs 1, 3, 5, 7, 9, and 10 were only included in all members of Clade IV subfamily and not in other subfamilies, further confirming the accuracy of the subfamily division. Clade IV was the subfamily with the most motifs, containing 10 motifs, whereas the other three subfamilies contained only 1–3 motifs (Fig. 2B). Thus, the functions of these motifs need to be further investigated to understand how these proteins function.

Overall, the *SPX* genes that were closely related in evolutionary terms in the phylogenetic tree had similar conserved motifs and gene structures, indicating that proteins within the same subfamily may exhibit similar functions.

Phylogenetic analysis of SPX proteins in different plant species

To further explore the evolutionary relationships of *SPX* gene family members, we performed a CLUSTALW alignment of 157 SPX protein sequences, consisting of 26 MdSPX, 20 AtSPX, 46 TaSPX, 32 ZmSPX, 18 SlSPX, and 15 OsSPX from six different species, and constructed a phylogenetic tree using the NJ method of MEGA 7. These proteins were divided into four subfamilies (Fig. 3), of which *SPX-EXS* was the subfamily containing the most proteins. Monocotyledonous (*Triticum aestivum*, *Zea mays*, and *Oryza. Sativa*) and dicotyledonous species (*Solanum lycopersicum*, *Malus domestica*, and *Arabidopsis*) were clearly divided into distinct evolutionary clades. The members of the *SPX-EXS* and *SPX-MFS* subfamilies showed clear species differentiation, implying that SPX-EXS and SPX-MFS proteins might have different biological functions in distinct plants. In general, phylogenetic analysis could provide reference for the evolution and function of family genes.

Gene duplication analysis of *MdSPXs*

To assess the genomic distribution and duplication of the *MdSPX* genes, we analysed the syntenic regions of the *MdSPX* genes using MCscanX software to examine the duplication of *MdSPXs*. As shown in Table S2, 2814 tandemly duplicated gene pairs and 11,473 segmental duplication blocks were found in the apple genome, respectively. Only one tandemly duplicated gene pair (*MdPHO1.7/MD00G1081300* and *MdPHO1.10/MD00G1081400*) was found in the *MdSPX* gene family (Fig. 4; Table S2). Moreover, we also identified seven pairs of segmental duplication events of *SPX* genes in apple as follows *MdSPX1* and *MdSPX6*; *MdSPX4* and *MdSPX5*; *MdPHO1.8* and *MdPHO1.4*; *MdPHO1* and *MdPHO1.1*; *MdPHO1.2* and *MdPHO1.3*; *MdPHO1.8* and *MdPHO1.11*; *MdNLA2* and *MdNLA3* (Fig. 4; Table S2).

Cis-acting regulatory analysis of *MdSPX* genes

Cis-elements in promoters of genes play an essential role in the overall regulation of gene expression. There is increasing evidence that genes with similar expression patterns may share the same *cis*-elements. The *cis*-elements in the 2-kb upstream of the transcription start site of *MdSPX* genes were predicted using PlantCARE and PlantPAN 3.0 software (Table S3), and three main categories were classified, namely, biotic/abiotic-related stress, growth and development response-elements, and hormone response-elements (Fig. 5; Table S3). Biotic/abiotic-related stresses included light response elements (G-box, GT1-motif, I-box, Box4, GATA-motif, ARE and Sp1), low temperature response element (LTR), drought response elements (MBS, MYC, DRE core and TC-rich repeats) and damage response element (WRE3). We also found that W box elements were extensively present in several *MdSPX* gene promoters, suggesting that these genes might be regulated by WRKY transcription factors. Development response-elements (such as O2-site, CAT-box and RY-element) were also commonly contained in promoters. In addition, six hormone elements related to phytohormones (such as salicylic acid, methyl jasmonate and abscisic acid) were recognised. *SPX* genes play key roles in Pi homeostasis, and the P1BS elements (PHR1 binding site) in their promoters are essential in responding to Pi starvation. P1BS elements were notably enriched in the promoters of all *MdSPX* genes except *MdPHO1.9* and *MdPHO1.7* and multiple copies of P1BS elements were detected in these gene promoters, implying that these genes might be regulated by MdPHR1 under Pi deficiency.

Expression of *MdSPX* genes under low pi condition

In this study, the self-rooted apple stock superior '12−2' and 'M9T337' were screened for low Pi stress treatments. The results of our hydroponic experiments showed that the length of adventitious root, shoot height, and the number of adventitious root in 'M9T337' changed significantly under low Pi stress treatments but exhibited no significant changes in '12−2' plants (Fig. 6A, B). The total Pi concentration, SPAD values, and anthocyanin levels in '12−2' were higher than that in 'M9T337' under low-Pi conditions (Fig. 6C), indicating that 'M9T337' was a Pi-sensitive genotype and that '12−2' was a Pi-tolerant genotype. The expression patterns provide essential clues for understanding the regulatory role of *SPX* genes. A number of *MdSPX* family genes were highly induced under low Pi stress condition, among which the expression levels of *MdSPX2*, *MdSPX3*, and *MdPHO1.5* genes showed strong up-regulation in response to low Pi stress (Fig. 7).

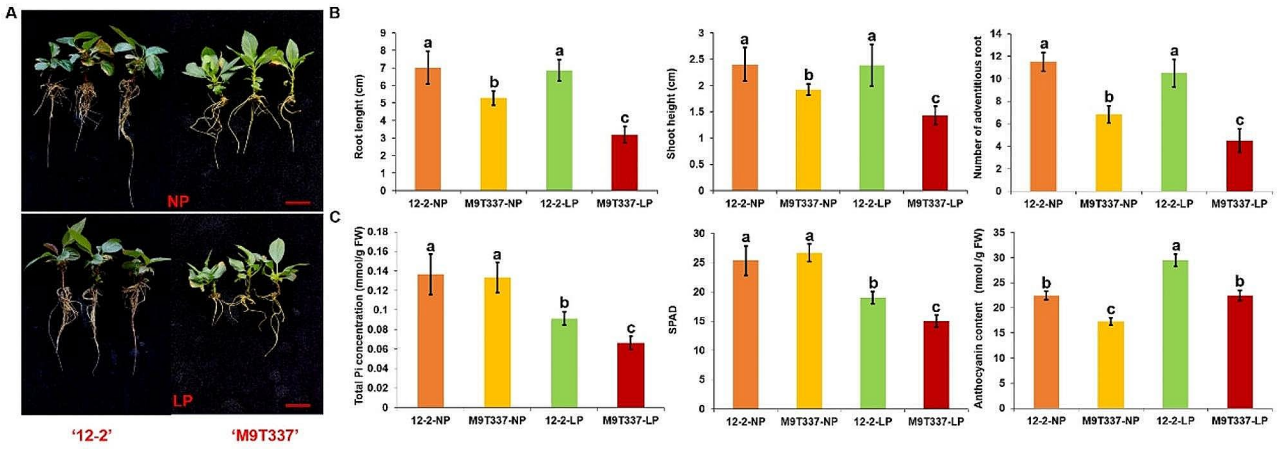

Fig. 6 Phenotypic differences between '12 – 2' and 'M9T337' plants under phosphorus starvation. (**A**) Phenotypic observations of '12 – 2' and 'M9T337' after 21 days of normal (NP: 1 mmol) and phosphorus deficiency stress (LP: 10 µmol). Scale bars = 1 cm. (**B**) The root length, shoot height, and the number of adventitious root between '12 – 2' and 'M9T337' plants were measured on the 21 days after normal Pi and Pi deficiency stress treatments. (**C**) The total Pi concentration, SPAD values, and anthocyanin levels between '12 – 2' and 'M9T337' plants were measured on the 21 days after normal Pi and Pi deficiency stress treatments. Samples with different letters are significantly different: $P < 0.05$ (Fisher's LSD mean separation test)

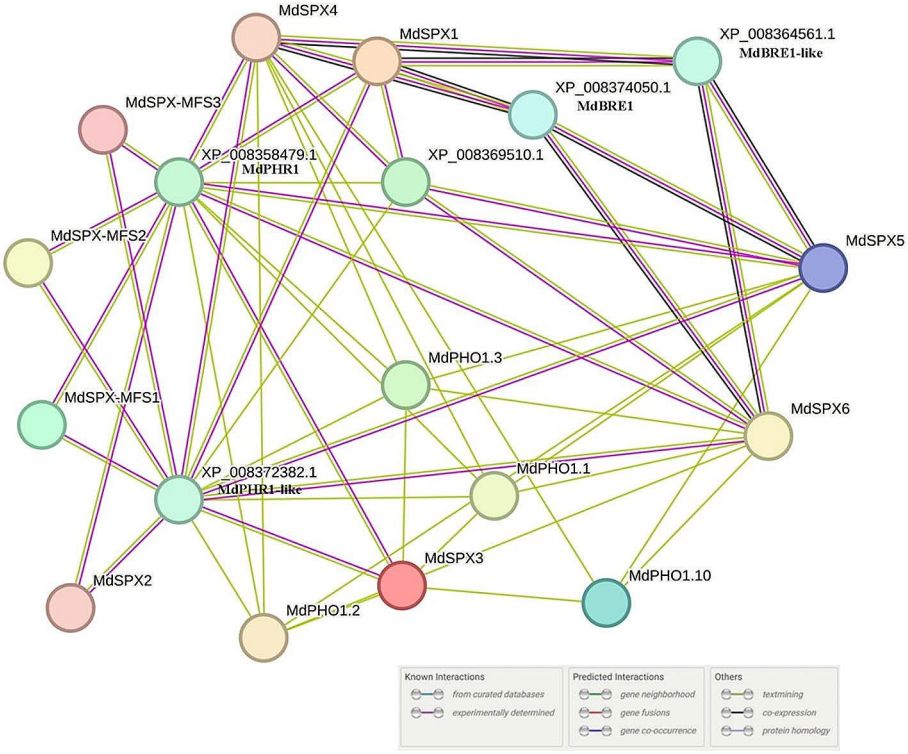

Fig. 8 Protein interaction network of MdSPXs. The network was predicted by the online software STRING. The different coloured lines represented the different types of evidence for the prediction of the interaction network

Prediction of the MdSPX protein interaction network

The analysis of protein interaction network has proven to be an effective approach to study gene function. The STRING online software was used to predict the protein interaction network queried with 26 MdSPX protein sequences. MdSPX protein interaction network analysis showed that several MdSPX proteins interacted with each other, for example MdPHO1.3 might interact with MdSPX3, MdSPX4, MdSPX5 and MdSPX6 (Fig. 8). As an important transcription factor in plant phosphorus regulatory network, PHR protein plays a key role in signal transduction regulation under Pi starvation induction.

PHR1 normally interacts with proteins containing the SPX domains in a Pi-dependent manner to regulate transcription. We found that phosphate starvation response (MdPHR1 and MdPHR1-like) proteins interacted with several MdSPX proteins, further highlighting the importance of SPX in maintaining phosphorus homeostasis. MdBRE1 and MdBRE1-like, the E3 ubiquitin pathway proteins, were also identified to interact with several MdSPX proteins (Fig. 8). In addition, the deoxyribodipyrimidine photo-lyase (XP_008369510.1), an enzyme that catalyzes chemical reactions, was present in the MdSPX protein interaction network. The predicted protein association network provides a meaningful reference for further studies.

MdSPXs physically interact with MdPHR1

The protein interaction network revealed that MdPHR1 interacted with several MdSPX proteins. To verify whether MdPHR1 interacted with MdSPXs, yeast two-hybrid experiments were carried out. The recombinant plasmids AD-MdSPX1, AD-MdSPX2, AD-MdSPX3, AD-MdSPX4, AD-MdSPX5, and AD-MdSPX6 were co-transformed with BD-MdPHR1 in yeast cells, and yeast cells was observed on SD/-Trp-Leu-His-Ade medium with X-a-gal. As shown in Fig. 9, MdPHR1 was found to interact with MdSPX2, MdSPX3, MdSPX4, and MdSPX6, but not with MdSPX1, MdSPX5. To demonstrate biological significance of MdSPX and MdPHR1 interaction, we found that the expression levels of *MdSPX2*, *MdSPX3*, *MdPSI1*, and *MdPSI2* in '12−2' were higher than that in 'M9T337' in response to low Pi stress (Fig. S2), indicating that these genes might play key role in the Pi-signaling pathway in self-rooted apple stock.

Discussion

Physiological and metabolic processes in response to phosphorus deficiency in plants depend on a complex and fine-grained phosphorus signalling regulatory network in their bodies [27]. With the development of molecular biology research techniques, more and more transcription factors and phosphorus starvation-responsive genes in plant phosphorus signalling regulatory pathway have been identified, which in turn has greatly contributed to the understanding of the phosphorus signalling network. Genes of the *SPX* domain-containing family are widely involved in the regulation of phosphorus signalling networks. Over the past years, the role of *SPX* family genes in regulating the Pi signal network has been studied in *Arabidopsis*, wheat, rice and maize. However, functional and evolutionary research on *MdSPXs* has been less reported. In this study, we identified a total of 26 *MdSPX* genes and classified them into four subfamilies (Fig. 2; Table 1). Gene structure and motif composition can provide valuable information on the genetic relationships of multi-gene families [28, 29]. As shown in Fig. 2, *SPXs* shared similar gene structure and motif composition within the same group. Similar functions are usually shared by genes with similar structures and conserved motifs. MdSPX proteins were clustered into several *Arabidopsis* functional clades, offering useful guidance for understanding *MdSPX* gene function.

To explore the evolutionary relationship of *MdSPX* family genes with *SPX* genes from other species, we constructed a phylogenetic tree of 157 SPX proteins across six species and broadly grouped them into four subfamilies (Fig. 3). The apple *SPX* family genes were clearly classified into different evolutionary clades compared to the *SPX* family genes in *Triticum aestivum*, *Zea mays*, and *Oryza Sativa*, and the evolutionary relationships of the *SPX-EXS* and *SPX-MFS* subfamily members

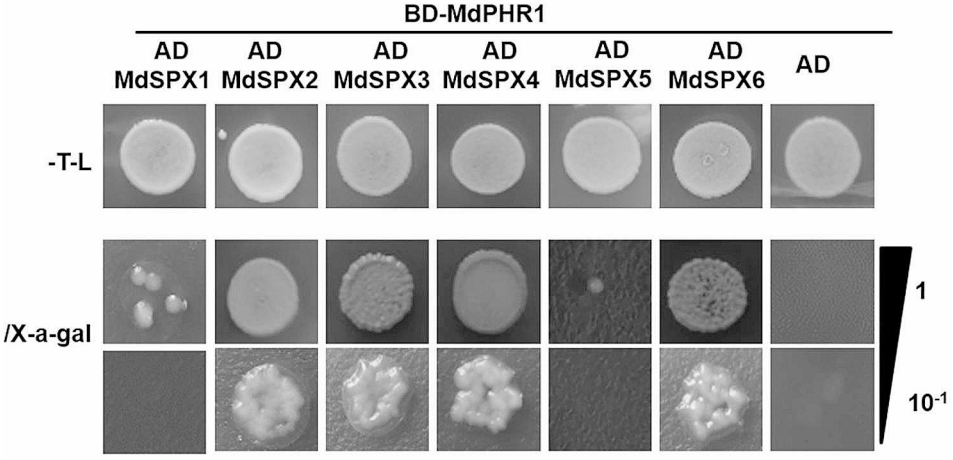

Fig. 9 Yeast-two-hybrid assays validating the interaction of MdPHR1 with the MdSPX proteins. SD, Synthetic defined; X-a-Gal, 5-bromo-4-chloro-3-indolyl-a-D-galactopyranoside

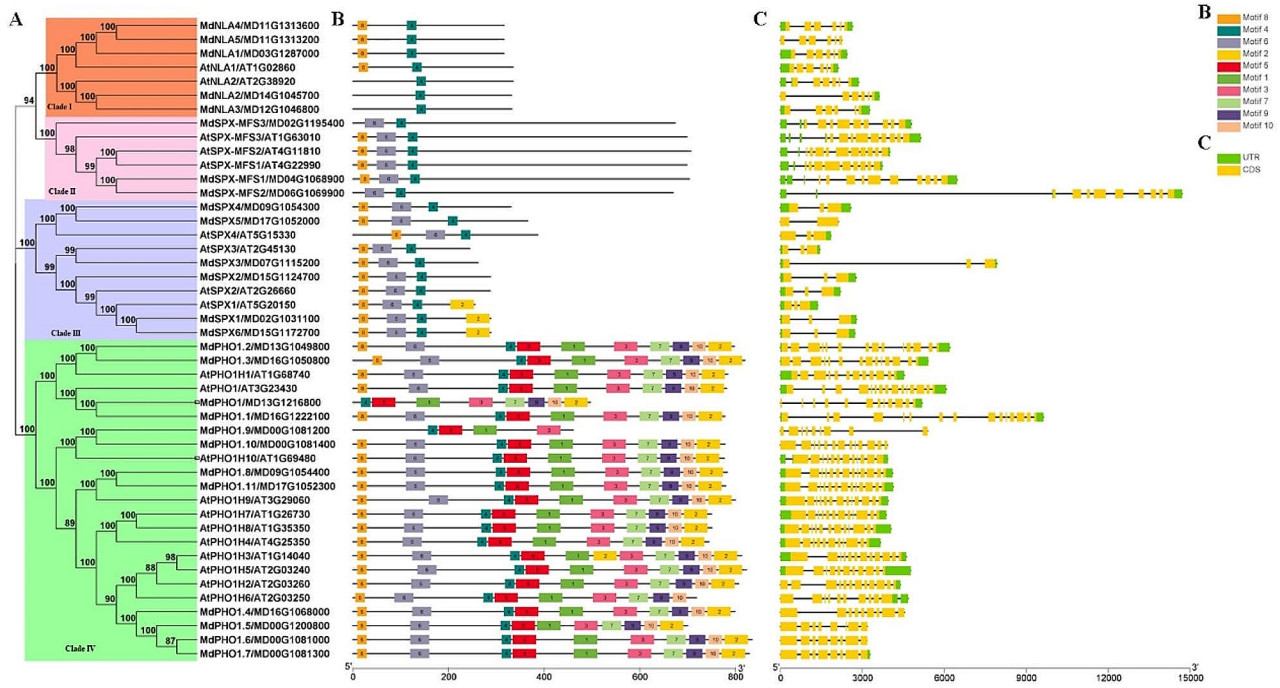

Fig. 2 Phylogenetic relationships, conserved motifs and gene structure analysis of apple and *Arabidopsis SPX* genes. (**A**) A phylogenetic tree of 26 MdSPX and 20 AtSPX protein sequences was generated using MEGA 7.0 and clustered into four branches. (**B**) Prediction of conserved motifs in SPX proteins was performed by MEME software. The corresponding 10 conserved sequences were referred to Figure S1. (**C**) Gene structure of *SPXs*.

varied markedly among species, suggesting that these two subfamily proteins may have different roles in different plants. Gene duplication events have been shown to play an important role in the speedy expansion and evolution of gene families. Overall, 2814 tandem duplication gene pairs and 11,473 segmental duplication blocks were observed in apple genome (Table S2). In this study, only one segmental duplication events and seven tandem duplication gene pairs were identified in the *MdSPX* family by MCScanX (Fig. 4; Table S2).

Similar to other species, the *MdSPX* gene promoters included numerous biotic/abiotic-related and hormone-response elements [17]. It has been shown that WRKY transcription factors can regulate the expression of *SPX* members. For example, both WRKY6 and WRKY42 can regulate the transcription of the *AtPHO1* gene by binding to the W-box element [30]. We identified a total of 18 *MdSPX* genes with W-box elements in their promoter regions, indicating that these genes may be regulated by WRKY transcription factor (Fig. 5; Table S3). P1BS (GNATATNC) is a known *cis*-acting element in response to Pi deficiency and clusters at the promoters of a lot of *PSR* genes [27, 31, 32]. For example, 18 *ZmSPX* genes contain P1BS elements in their promoter regions and 31 *BnaSPX* genes contain P1BS elements in their promoter regions [16, 18]. In our results, except for *MdPHO1.9* and *MdPHO1.7*, the remaining 24 *MdSPX* genes contained different numbers of P1BS in their promoter regions (Fig. 5), predicting that the expression of these *SPX* genes in apple could be similarly induced by Pi deficiency. The position, number, and flanking sequence of all elements within a promoter region directly affects promoter activity. The enrichment of different promoter elements suggests that *MdSPXs* might be regulated by a series of related transcription factors.

Analysis of *MdSPXs* expression under different periods of low Pi treatment showed that several *MdSPX* genes were in response to Pi stress, with different expression patterns in the self-rooted apple stock superior '12−2' (Fig. 7). Many studies have shown that some *SPX* subfamily genes are up-regulated under low Pi, for instance *AtSPX* and *AtSPX2* [33], and we found that most *MdSPX* subfamily genes, except MdSPX1 and MdSPX5, were significantly induced in response to low Pi in apple, a result consistent with earlier studies [13, 34]. *MdSPX2* and *MdSPX3* were significantly expressed in both '12−2' and 'M9T337' plants under low Pi stress condition, but the expression levels of *MdSPX2* and *MdSPX3* in '12−2' under Pi deficient condition were significantly higher than that in 'M9T337', which further suggests the important role of the *SPX* genes in response to low Pi stress.

We also predicted the protein interaction networks of the 26 MdSPX protein sequences queried by STRING online software, which showed that multiple MdSPXs might interact with MdPHR1. AtPHR1 participates in Pi homeostasis by interacting with SPX structural

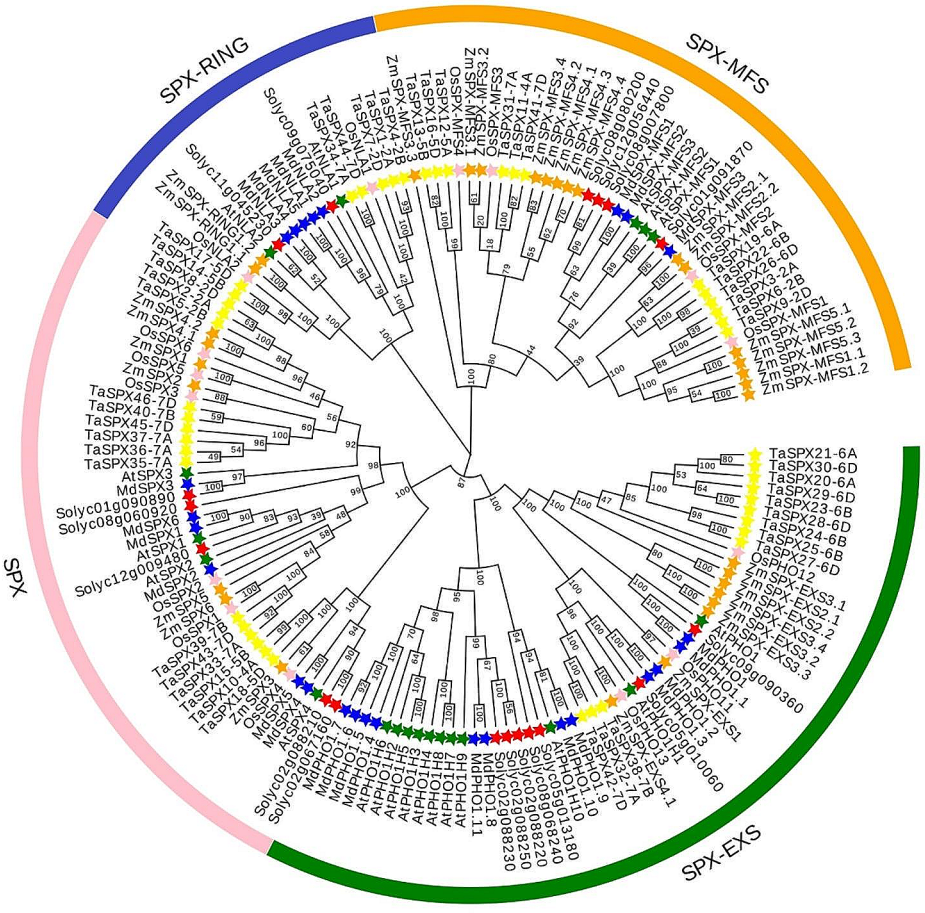

Fig. 3 Phylogenetic tree consisting of 157 SPX proteins from *Arabidopsis thaliana*, *Solanum lycopersicum*, *Zea mays*, *Oryza sativa*, *Malus domestica* and *Triticum aestivum*. SPX proteins from different species were tagged with different coloured stars

domain-containing proteins at low Pi levels [33]. The results of the Y2H assay showed that MdSPX2, MdSPX3, MdSPX4, and MdSPX6 interacted with MdPHR1, while MdSPX1 and MdSPX5 did not interact with MdPHR1, a result similar to that of maize and rice. These findings suggest that a relatively conserved regulatory network for phosphorus stress response exists in self-rooted apple stock.

Conclusions

In the apple genome, 26 *MdSPX* genes were identified and divided into four subfamilies. To fully understand the biological features of these *MdSPX* genes, gene structures, conserved motifs, gene duplications, phylogenetic relationships and *cis*-elements were analysed in detail. The results of the expression of all 26 *MdSPX* genes at different times of Pi stress showed that some *MdSPXs* play key roles in Pi deficient response, especially *MdSPX2*, *MdSPX3*, and *MdPHO1.5*, which were highly responsive to low Pi stress. Some members might function under low Pi by interacting with MdPHR1. In conclusion, these results improved the understanding of the apple *SPX* gene family and contribute to future biological studies of *MdSPX* genes in self-rooted apple stock.

Methods

Identification of *MdSPX* genes in apple

MdSPX gene family members were identified by Blastp search in *Malus×domestica* reference genome database using 20 *Arabidopsis* SPX proteins as query sequences. Additionally, the Hidden Markov Model (HMM) SPX domain (PF03105) in the Pfam database (http://pfam.xfam.org/) were used to search for *MdSPX* genes. All candidate genes were then further verified using the Pfam and SMART (http://smart.embl-heidelberg.de). Twenty-six *MdSPX* genes were finally identified in the apple genome and named in accordance with their phylogenetic relationship to *Arabidopsis SPX* genes.

Multiple sequence alignment and phylogenetic analysis

To investigate the evolutionary relationships of SPX proteins in *Arabidopsis thaliana*, *Solanum lycopersicum*, *Zea mays*, *Oryza sativa*, *Malus domestica* and *Triticum aestivum*, multiple alignments of 20 AtSPXs, 18 SlSPXs, 32

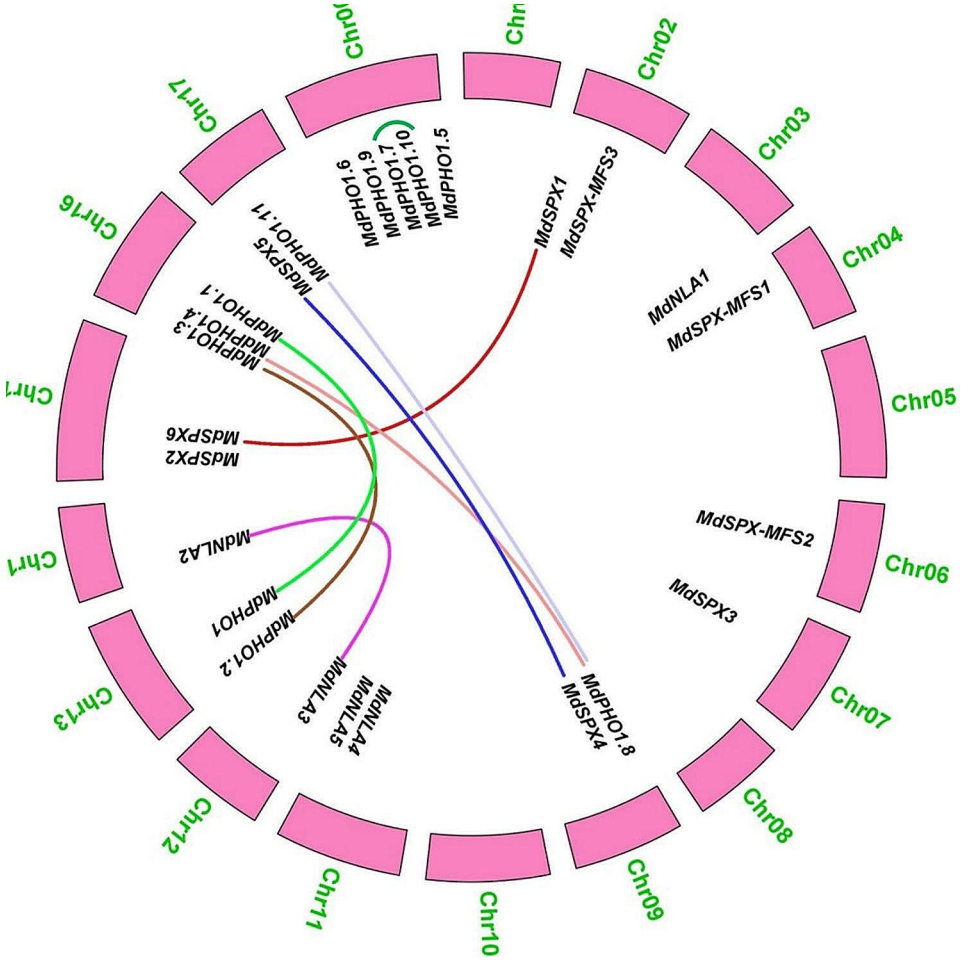

Fig. 4 Analysis of gene duplications between chromosomes for the *MdSPX* genes. Different coloured lines connected segmental duplicated *MdSPX* gene pairs, with a total of seven segmental duplications. Small green lines marked tandemly duplicated gene pair

ZmSPXs, 15 OsSPXs, 26 MdSPXs, and 46 TaSPXs were performed by CLUSTALW, and the obtained results were applied to generate a phylogenetic tree via the neighbour-joining (NJ) method with 1000 bootstrap replicates in MEGA 7. The phylogenetic tree was landscaped using the EvolView tool (http://www.evolgenius.info). It was then classified into four subfamilies based on *SPX* gene family characteristics.

Analysis of gene structure and conserved motifs

The DNA and cDNA sequences of all *MdSPX* and *AtSPX* genes were extracted from the *Malus domestica* and *Arabidopsis* genomes, respectively, and then the structures of the *SPX* genes were characterised using the Gene Structure Display Server (GSDS: http://gsds.cbi.pku.edu.cn/). The MEME motif discovery tool (http://meme-suite.org/index.html) was used for identifying the conserved motif structures of all MdSPX and AtSPX amino acid sequences. Visualization of the results was performed using TBtools.

Physicochemical properties and chromosome location analysis

The 26 MdSPX protein sequences were examined using the online website ExPASy (http://web.expasy.org/protparam/) to predict their amino acid lengths, molecular weights and isoelectric points (pI) [35]. Chromosome position information of apple was downloaded from the GDR database (https://www.rosaceae.org/). The 26 *MdSPX* genes were mapped to their corresponding chromosomes using Mapchart 2.32 software.

Gene duplication analysis

Each of the *MdSPX* genes was mapped to the apple chromosome using TBtools, based on physical positional data from the apple genomic database. The Multiple Collinearity Scan toolkit (MCScanX) was used with default parameters to score gene duplication events [36, 37].

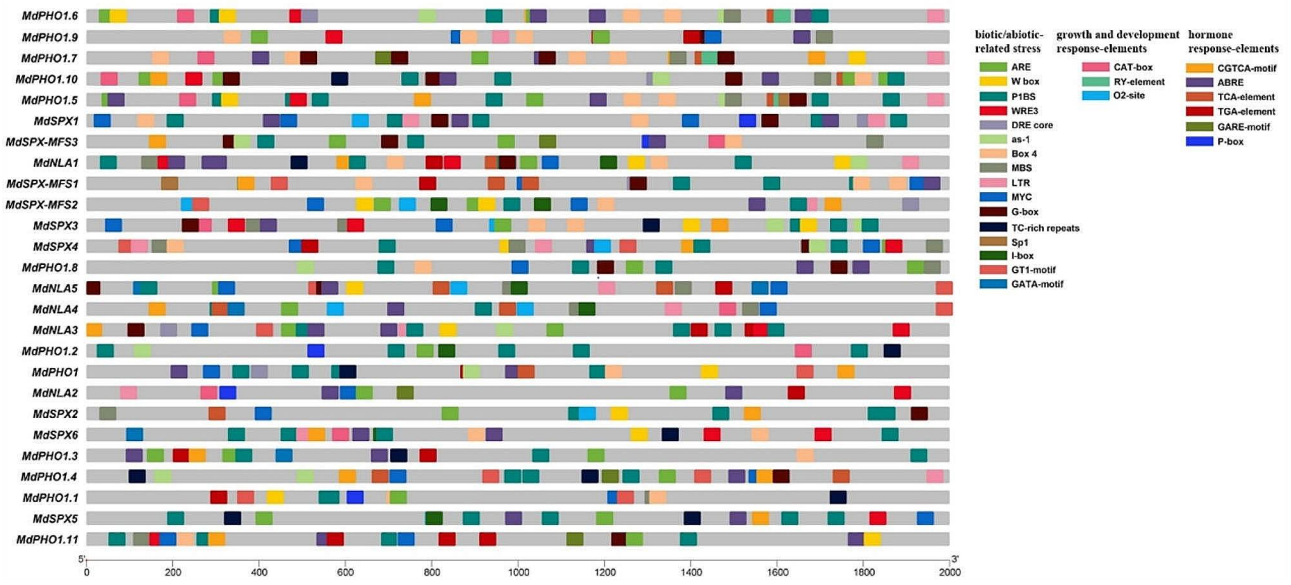

Fig. 5 Display of *cis*-elements in the promoters of the *MdSPX* genes in apple. The different colours represented the corresponding *cis*-element types in the *MdSPX* promoters and were classified into 3 main categories: biotic/abiotic-related stress, growth and development response-elements, and hormone response-elements

Identification of *cis*-elements in the promoters of the *MdSPX* genes

We extracted 2-kb sequences upstream of the transcription start site of the 26 *MdSPX* genes from the *Malus domestica* database using the TBtools. All extracted target sequences were then uploaded to PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/) and PlantPan3 databases (http://PlantPAN.itps.ncku.edu.tw) for *cis*-element analysis. The obtained data were then preliminarily collated and visualised using Tbtools [38].

Plant material, pi stress treatments, and qRT-PCR analysis

The deep-rooted '12−2' (hybrid seedlings of Ralls and *Malus spectabilis*) self-rooted apple stock and 'M9T337' stock, an apple dwarf rootstock were screened. The seedlings of *M. spectabilis* were obtained from the Beijing Botanical Garden (Beijing, China) and those of Ralls and 'M9T337' stock were obtained from Shandong Institute of Pomology. The roots of '12−2' were used for qRT-PCR analysis. The growth conditions used were those described by [39]. Modified Hoagland medium was used in self-rooted apple stock hydroponic cultivation as described by [18]. An RNAprep pure plant kit (Vazyme, Nanjing, China) was used to isolate total RNA from the samples according to the producer's instructions. Reverse transcription was then performed with a *EasyScript*® One-Step gDNA Removal and cDNA Synthesis SuperMix (Trans, Beijing, China). Applied Biosystems 7500 real-time PCR system (Applied Biosystems) was used to perform qRT-PCR reactions using UltraSYBR Mixture (with ROX I; Cwbiotech). The results were standardized with apple *18 S* gene. Three biological replicates were used for each test. Table S4 showed the primers used in this study.

Prediction of protein association networks by STRING

The 26 MdSPX amino acid sequences were uploaded to the online STRING software (http://string-db.org; version 11.5), with the organism selected as " *Malus domestica* ". The highest scoring proteins were selected to construct the network after BLAST analysis. The MdSPXs not interacting with other proteins were deleted. Functional annotations were manually copied from BLAST results. The proteins MdPHR1 (XP_008358479.1), MdPHR1-like (XP_008372382.1), MdBRE1 (XP_008374050.1), and MdBRE1-like (XP_008364561.1) were named based on BLAST results.

Measurement of the total pi concentration and physiological characteristics

To investigate the Pi sensitivity of '12−2' and 'M9T337', we measured the root length, shoot height, and the number of adventitious root of the plants on the 21st day after Pi stress treatments. The chlorophyll content was quantified with a SPAD system [18]. The measurement of the total Pi concentration was performed as described by [40].

Yeast two-hybrid (Y2H) assays

The full-length MdPHR1 CDS was cloned into the bait vector pGBKT7 and the MdSPX1, MdSPX2, MdSPX3, MdSPX4, MdSPX5, and MdSPX6 CDSs were cloned into the prey vector pGADT7 to generate recombinant plasmid AD-MdSPX1, AD-MdSPX2, AD-MdSPX3,

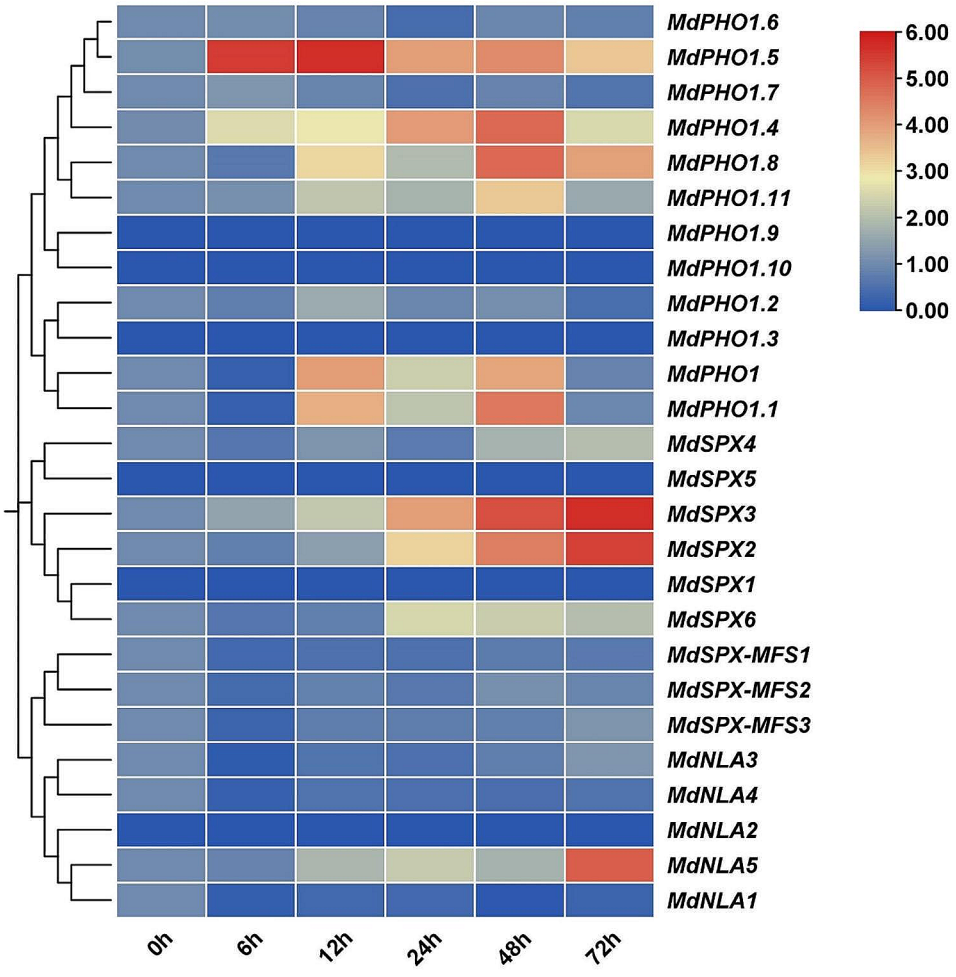

Fig. 7 Expression pattern analysis of 26 *MdSPX* genes in '12 – 2' samples at different times of low Pi treatment (10 μmol). Total RNA was extracted from treated roots. The apple *18 S* gene was used as an internal control, and each experiment included three biological replicates

AD-MdSPX4, AD-MdSPX5, and AD-MdSPX6. Y2H assays were performed using yeast strain AH109 (Clontech) according to the manufacturer's instructions. The yeast cells were plated on medium lacking Trp and Leu (SD/-Trp-Leu) and cultured at 28 °C. For interaction screening, the colonies were transferred into medium lacking Trp, Leu, His and adenine (SD/-Trp-Leu-His-Ade) with X-a-gal. Empty vector pGADT7 was used as negative controls.

Statistical analysis

In this study, the error bars represented the standard error (SE) from at least three biological replicates. The analysis of statistical significance was performed with the student's t-test at $P<0.05$ as described [41].

Abbreviations

Pi	Phosphate
At	*Arabidopsis thaliana*
Md	*Malus domestica*
qRT-PCR	quantitative reverse transcription-PCR
CDS	Coding Sequence
PHR1	PHOSPHATE RESPONSE 1

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12864-024-10402-2.

Supplementary Material 1

Supplementary Material 2

Supplementary Material 3

Supplementary Material 4

Supplementary Material 5

Acknowledgements

Not applicable.

Author contributions

Z.W., Y.Y. and J.L. conceived and designed the experiments. Z.W., X.Z., X.Y., H.T., L.F. and J.L. performed the experiments. Z.W. analyzed the data and wrote the manuscript. Y.Y. and J.L. revised the manuscript. All authors have read and approved this manuscript.

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Data availability
The relevant materials are available from the corresponding authors on reasonable request. The links in this study for the data analyzed are as follows: Pfam database (http://pfam.xfam.org/); SMART (http://smart.embl-heidelberg.de); EvolView tool (http://www.evolgenius.info); Gene Structure Display Server (GSDS: http://gsds.cbi.pku.edu.cn/); MEME motif discovery tool (http://meme-suite.org/index.html); ExPASy (http://web.expasy.org/protparam/); GDR database (https://www.rosaceae.org/); PlantCARE (http://bioinformatics.psb.ugent.be/webtools/plantcare/html/); PlantPan3 databases (http://PlantPAN.itps.ncku.edu.tw); STRING software (http://string-db.org; version 11.5).

Declarations

Ethics approval and consent to participate
All experimental research on plants, including the collection of plant material, comply with relevant institutional, national, and international guidelines and legislation.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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