



Genome-Wide Identification and Expression Analysis of *SPX* Domain-Containing Gene Subfamily in Response to Phosphorus-Solubilizing Bacteria in Apple (*Malus domestica*)

Mehmet Kural¹ · Ali Kiyak² · Selman Uluisik^{1,3} · Ersin Atay^{1,3}

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Abstract

Phosphate is one of the major elements that significantly affects fruit yield and quality. The aim of the study was to determine whether using phosphorus-solubilizing bacteria could produce high-quality apple nursery trees. Five different treatments were tested on a “Granny Smith” apple cultivar that was grafted onto an M.9 rootstock. These were 100% P, 50% P, 50% P + *Bacillus megatarum* (plant growth promoting rhizobacteria, PGPR), 0%P, and 0%P + PGPR. The study also identified the *SPX* gene family, which is essential for plant growth and development and responds to phosphorus (P) stress. A total of 72 *SPX* genes were identified in different plant species based on structural and phylogenetic analysis. The apple genome contains seven different *SPX* genes distributed on five of the 17 chromosomes. Gene structure and motif analysis showed that *SPX* genes show a relatively conserved exon/intron arrangement and motif composition in five different species: apple, strawberry, peach, apricot, and grape. Protein–protein network analysis showed that *SPX* proteins are closely related to proteins involved in P metabolism in apple. The digital expression profiles of *MdSPX* genes among 47 apple tissues were characterized to provide insight into their potential functions. RT-qPCR revealed that the expression level of all *MdSPXs* was significantly downregulated in 50% P + PGPR treatments, indicating that 50% P combined with PGPR is effectively taken up by the plant, saving it from Pi starvation. These results not only confirm the key role of *MdSPXs* in Pi homeostasis and the Pi signaling pathway but also clarify the importance of Pi-solubilizing bacteria in plant nutrition.

Keywords Apple · Genome wide · Phosphate · Phosphorus · *SPX*

Introduction

Plant growth and development are largely regulated by 17 macro/micro-nutrients, with phosphorus (P) being a critical micronutrient (Pandey et al. 2021). While P is typically

abundant (0.02–0.5%) in soils (Walpola and Hettiarachchi 2020), only a minute fraction of it (< 1%) is in a dissolved form. This limited solubility contributes to substantial yield losses, and the continuous depletion of P in modern agriculture poses a significant threat to global food security (Bünemann 2015). To enhance P uptake efficiency, it is essential to identify key root traits, genes, or beneficial microbial associations involved (Bilen and Turan 2022). Plants have developed strategies to facilitate inorganic phosphate (Pi, the major form of P taken up by plants) uptake and remobilization to cope with reduced availability (Jain et al. 2012). Plants undergo phenotypic changes to boost Pi uptake, such as developing a larger root system and excreting organic anion (Abel et al. 2002). Pi starvation also causes a wide range of molecular responses, with regulation occurring at both transcriptional and posttranscriptional levels (Han et al. 2022). Phosphate Starvation Response1 (PHR1), a MYB transcription factor, is the central regulator in the PSRs (Pi starvation responses) regulatory system in plants via the transcriptional regulation of *P starvation-induced (PSI)* genes

Key Message

- 50%P+PGPR could be potential treatment to save apple plant from phosphate starvation.

✉ Selman Uluisik
suluisik@mehmetakif.edu.tr

¹ Department of Sustainable Agriculture and Food Systems, Graduate School of Natural and Applied Sciences, Burdur Mehmet Akif Ersoy University, Burdur, Turkey

² Scientific, Technology Application and Research Center, Burdur Mehmet Akif Ersoy University, Burdur, Turkey

³ Burdur Food Agriculture and Livestock Vocational School, Burdur Mehmet Akif Ersoy University, 15030 Burdur, Turkey

by binding to their promoters through the *cis*-element PHR1-binding sequence (P1BS; GNATATNC), and regulating their expression, including several *PSR* genes, microRNA399 and *SPX* genes (Bari et al. 2006). *PSR* genes are involved in physiological and biochemical responses to increase P absorption and utilization, including root system architecture remodeling, secretion of organic acids, Pi transport and remobilization, reduction in photosynthesis, and starch and anthocyanin biosynthesis in leaves (López-Arredondo et al. 2014). Although knowledge of Pi uptake and the Pi-signaling system has increased recently at the molecular level, including protein-encoding genes and non-coding RNA genes (Han et al. 2022; Zhao et al. 2022), the molecular network underlying plant adaptation to Pi starvation and/or satisfaction is still to be elucidated.

The *SPX* proteins, which contain only the conserved N-terminal hydrophilic *SPX* domain (Pfam: F03105), are found in all vascular plants and could potentially serve as a new group of Pi transporters (Kumar et al. 2019). In plants, *SPX*-domain-containing proteins are classified into four subfamilies based on the presence of C-terminal extra domains: *SPX*, *SPX-EXS*, *SPX-MFS*, and *SPX-RING* (Secco et al. 2012). *SPX* members were first discovered in rice and *Arabidopsis thaliana* (Duan et al. 2008; Wang et al. 2009). A growing number of *SPX* genes have been discovered in plants, including wheat (*Triticum aestivum*) (Kumar et al. 2019), soybean (*Glycine max* L.) (Yao et al. 2014), tomato (*Solanum lycopersicum*) (Li et al. 2021), *Brassica napus* (Du et al. 2017), and apple (*Malus domestica*) (Xie et al. 2023). Studies on plants like *Z. mays*, *B. napus*, *G. max*, and *P. masoniana* have shown that Pi deficiency strongly induces most *SPX* subfamily members (Du et al. 2017; Wang et al. 2021b). For example, *AtSPX1* and *AtSPX3* in *Arabidopsis*, 9 out of 46 *TaSPXs* genes in wheat, and *MdSPX1*, *MdSPX2*, *MdSPX4*, and *MdSPX5* genes in apple were significantly expressed in response to Pi deficiency (Duan et al. 2008; Kumar et al. 2019; Xie et al. 2023). In maize, *ZmSPX4.1* and *ZmSPX4.2* are strongly upregulated under low P stress, indicating their involvement in the P stress response (Xiao et al. 2021). In addition to its crucial role in P metabolism, the *SPX* gene family critically regulates plant growth and development. For example, in tomato, eight *SPX* domain-containing genes were identified, and half of those were strongly expressed in root tissue. One member (Solyc08g060920) was expressed in the bud, flower, root, mature green fruit, breaker fruit, and breaker + 10_fruit (Li et al. 2021). Downregulation of *OsSPX1* disrupted normal anther and pollen development in rice and resulted in semi-male sterility and low grain yield (Zhang et al. 2016). These studies indicate the possible functions of *SPX* genes involved in plant and fruit development.

P deficiency affects around 50% of agricultural ecosystems worldwide and is a critical limiting factor for plant development (Etesami 2020). The use of P fertilizers has increased globally to address this issue, but 70% of the

soluble P fertilizer in the soil rapidly combines with cations, turning into an insoluble form (Skinner and Attiwill 1981). The adaptation of plant roots to low dissolved P concentrations in the soil varies depending on the species and genotype (Beroueg et al. 2021). Plants with extensive root systems benefit more from P as they can spread over a wider soil area (Lynch and Brown 2008). Apple, one of the world's most widely produced fruits alongside tomato, banana, watermelon, grape, and orange, is a highly profitable crop (Nielsen and Nielsen 2003; FAO 2024). However, characterized by a species-specific low rooting density, apples exhibit a limited capacity to effectively utilize P (Nielsen and Nielsen 2003). P fertilization of apple trees improves vigor, flowering, yield, fruit quality, and storability (Wojcik and Wojcik 2007). Therefore, enhancing the absorption and utilization of P by apple trees is of great importance, both ecologically and economically. PSB (phosphate-solubilizing bacteria) are a group of PGPR that dissolve unusable inorganic P in the soil, making it useful for plant growth (Walpola and Hettiarachchi 2020). For instance, applications of PSB have been shown to increase peanut yield, protein content in peanut seeds (Wang et al. 2021a, b, c), grain yield, biological yield, and total number of stems in rice (Bakhshandeh et al. 2015) and increase yield parameters in sunflower (Shahid et al. 2015). Therefore, investigating what role PGPR will play in the development of the apple plant is one of the main objectives of this study.

The primary hypothesis of this study suggests that enhancing the quality of apple nursery trees is achievable by reducing chemical phosphorus use by at least 50% in soils with sufficient P and incorporating PSB. To support this hypothesis, we identified *SPX* gene family members in apple and four other fruit species, unraveling the evolutionary history of *SPX* family genes through comprehensive analyses of phylogeny, conserved domains, selective pressures, syntenic relationships, and gene duplication events. Furthermore, our study delved into the expression patterns of *SPX* genes using transcriptome data from various apple tissues. We assessed the expression levels of these genes in apple plants treated with chemical P fertilizer and PSB through quantitative-real time qRT-PCR analysis. Additionally, we sought to understand how the P nutrition levels of nursery trees treated with PSB would impact the quality of the trees.

Materials and Methods

Identification of *SPX* Subfamily Genes in the Apple Genome

To identify *SPX*-domain-containing proteins in the *Malus domestica* Borkh. genome, the Hidden Markov Model (HMM) profiles of the *SPX* (IPR004331) domain were

downloaded from the InterPro (<https://www.ebi.ac.uk/interpro/>) database (Paysan-Lafosse et al. 2023) and used as the query to search against the apple genome in the GDR (<https://www.rosaceae.org/>) (Jung et al. 2019) using the HMMER 3.0 with the threshold of E -value $< 1e-5$. Non-redundant putative protein sequences were manually checked with the InterPro (<https://www.ebi.ac.uk/interpro/>) and the SMART (<http://smart.embl-heidelberg.de/>) (Letunic & Bork 2021) databases. Protein sequences with a low degree of similarity, poor domain coverage, and different functional annotations were filtered, and seven *SPX* genes were detected in the apple genome. The same procedure was used to search for *SPX* genes in *Prunus persica*, *Fragaria vesca*, *Vitis vinifera*, and *Prunus armeniaca*, and 11, 15, 13, and 4 genes were detected, respectively. The *SPX* genes of *Arabidopsis thaliana*, *Hordeum vulgare*, *Zea mays* and, *Oryza sativa* were downloaded from the Phytozome database (<https://phytozome-next.jgi.doe.gov/>) (Goodstein et al. 2012).

Basic Sequence Characterization of SPX Proteins in Five Different Species

The physicochemical characteristics of SPXs, including protein length, isoelectric point (pI), molecular weight (MW), instability index, aliphatic index, and GRAVY were predicted by ExPASy ProtParam (<https://web.expasy.org/protparam/>) (Wilkins et al. 1999). Protein subcellular localizations of SPXs were predicted with the Plant-mPLoc server (<http://www.csbio.sjtu.edu.cn/bioinf/plant-multi/>) (Chou and Shen 2010).

Phylogenetic Evolution, Gene Structure Analysis, and Promoter Sequence Analysis of SPX Proteins

The full-length protein sequences of all predicted 72 *SPX* protein sequences were used to perform multiple sequence alignment, and the phylogenetic tree was performed using MEGA 11.0.13 with the maximum likelihood method (ML), a bootstrap of 1,000 replications, and the Jones–Taylor–Thornton (JTT) model (Kumar et al. 2018). The iTOL v.6 (<https://itol.embl.de/>) (Letunic and Bork 2021) server was used to view and annotate the phylogenetic tree. The Gene Structure Display Server 2.0 (<http://gsds.gao-lab.org/>) (Hu et al. 2015) was used for the prediction of the exon and intron structures.

The conserved motifs of SPXs were determined by MEME (<http://meme-suite.org/tools/meme>) (Bailey et al. 2015), with the number of motifs set to 5 and the order of site distribution being zero or one occurrence per sequence. Conserved motif distribution was visualized by TBtools

(Chen et al. 2020). The PlantCARE database (Rombauts et al. 1999) was used to extract 2000 bp sequences upstream of the transcription start codon of *SPX* genes, and *cis*-element compositions were visualized using Gene Structure View in TBtools (Chen et al. 2020).

The nonsynonymous (Ka) and synonymous (Ks) substitution rates and the Ka/Ks value between homologous gene pairs were calculated using the PAL2NAL server (<http://www.bork.embl.de/pal2nal/>) (Suyama et al. 2006). The Ks values were used to calculate the divergence time with the following formula: $T = Ks/2r$ ($r = 1.5 \times 10^{-8}$) (Koch et al. 2000).

Chromosomal Localization and Collinearity Analysis of SPX Genes

To identify the synteny of *SPX* family genes among five different species, information on syntenic blocks was downloaded from the GDR (<https://www.rosaceae.org/>) database (Jung et al. 2019). Synteny analysis was generated and visualized in a globe plot using the program Circos in the TBtools (Chen et al. 2020).

Transcriptome Expression Pattern and Protein–Protein Interaction of MdSPX Genes

To determine the expression patterns of *MdSPX*s, the transcriptome data for 47 different apple tissues were obtained from the Apple Multi-Dimensional Omics Database (AppleMDO) (Da et al. 2019). The TBtools software was used to transform normalized log2 values and generate the heat map (Chen et al. 2020).

To further investigate the interactions of proteins related to members of the *MdSPX* family, the STRING 11.5 protein–protein interaction database (<https://string-db.org/>) was used. To reveal ontological information about all proteins interacting with *MdSPX*, all the proteins were analyzed in the UniProt protein database (<https://www.uniprot.org/>).

Networks between proteins were analyzed using Cytoscape, overlapping networks were removed, and the network was rearranged with a yFiles circular layout and visualized with Cytoscape (version 3.9.1) software (Shannon et al. 2003).

Gene ontology (GO) and Functional Annotation of MdSPX Genes

Functional annotation of the *MdSPX* genes was predicted using PANNZER 2 online software (<http://ekhidna2.biocenter.helsinki.fi/sanspanz/>) (Törönen et al. 2018), and the functional categories were visualized using the bar chart generator tool (<http://www.bioinformatics.com.cn/>).

Plant Materials and Treatments

In this study, a “Granny Smith”/M.9 rootstock-cultivar combination was used. Bench grafts of this combination were planted in a nursery plot in March 2022 at Burdur Mehmet Akif Ersoy University, Turkey. The soil properties and monthly weather data during the experiment are given in Table S2 and Fig. S1. The study was set up in a randomized block design with 3 replications, and 10 plants were included in each replication. Trees were irrigated daily when tensiometers (Irrometer, Inc., Model SR, Riverside, CA, USA) at 30 cm depth showed -20 kPa. (Fig. S2).

The experiment consisted of a chemical phosphorus fertilizer dose and the use of PGPR (plant growth promoting rhizobacteria, here *Bacillus megaterium*, phosphorus-solubilizing bacteria) (Table S3). Nitrogen (N) (17 g/tree) and potassium (K_2O) (7 g/tree) determined by the soil analysis results were applied in the same way in all treatments. The fertilization program was applied based on soil analysis, in which 100% P was considered control treatment. In 50% P+PGPR and 0% P+PGPR treatments, the first application of *B. megaterium* was applied by dipping the root system in a bacterial suspension for 20 min before planting. The second and third applications were applied to the soil surface at 1-month intervals, with 250 ml of bacterial solution per tree to maintain the bacterial population.

Tree Quality and Leaf Nutrients

The total leaf area was determined when seasonal growth ceased in November 2022. For the total leaf area calculations, the leaf numbers of all trees were counted, and the leaf area of 20 leaves for each replication (i.e., $20 \times 3 = 60$ leaves/treatment) was measured using the ImageJ software (<https://imagej.nih.gov/ij/>). In December 2022, tree height and trunk diameter (15 cm above the graft union) were measured.

After lifting 3 trees for each replication, the root systems were separated from the soil by pressured water, kept in a room at 23 °C for one week, and then dried in an oven at 70 °C for 72 h. After this period, the root part and shoot system were separated and placed in an oven at 70 °C for 72 h to dry, then weighed with an electronic scale.

Leaf samples were collected from three randomly selected plants from each treatment and control in August 2022. The leaves were rinsed with tap water, 1 M HCl, and distilled water and dried on absorbent papers at room temperature for 1 h. They were then placed in an oven at 45 °C for 24 h and then at 70 °C for 24 h until stable weights were obtained. Samples were then grounded up for the macro- and micronutrient analyses. P content was analyzed spectrophotometrically using the vanadate-yellow colorimetric method (Genesys 10S VIS, Thermo Scientific, USA). Potassium (K), calcium (Ca), magnesium (Mg), iron (Fe), copper

(Cu), manganese (Mn), and zinc (Zn) contents were determined with a spectrometer (ICE 3000 Series, Thermo Scientific, USA). The total nitrogen (N) was determined using the Kjeldahl method with a distillation unit (Hanon K9840, Jinan Hanon Instruments, China). Nutrient contents in leaf tissues were expressed on a dry mass basis. When the *F*-test was significant in one-way analysis of variance (ANOVA), means were separated using the Least Significant Difference (LSD) test.

Total RNA Extraction and qRT-PCR Analysis

Leaf samples were collected from three randomly selected plants from each treatment and control and used for total RNA extraction. The RNA was isolated using the GeneJET Plant RNA Purification Kit (Thermo Scientific) and reverse-transcribed into cDNA using the VitaScript™ FirstStrand cDNA Synthesis Kit (Procomcure Biotech). The primers for selected SPX genes were designed using the tool (<https://www.ncbi.nlm.nih.gov/tools/primer-blast/>). Details of the primers used for the qRT-PCR are given in Table S4. qRT-PCR experiments were conducted on the BioRad CFX96 (BioRad Laboratories) using SYBR® Green Master Mix (BioRad). The reaction mixture contained 5 µL of SYBR® Green Master Mix, 0.5 µL forward and reverse primers, 1 µL of template cDNA, and 3 µL sterile water. Reaction conditions were as follows: 95 °C for 5 min, 35 cycles of 5 s at 95 °C, 30 s at 60 °C, and 30 s at 72 °C. The results are presented as the means of three biological replicates with two technical replicates each. *MdActin* was used as an internal reference gene, and the $2^{-\Delta\Delta C_t}$ method was used to calculate the relative gene expression (Livak and Schmittgen 2001), and the difference was analyzed by the LSD test of single-factor ANOVA ($p < 0.05$).

Results

Identification and Characterization of SPX Genes in Five Different Species

To identify all the SPX gene members in five different species (apple, peach, strawberry, apricot, and grape), the HMM profile of the SPX gene domain (IPR004331) was used as queries to search against the genome databases of five species. Then, the candidate MdSPX members were further confirmed if they contained the SPX domain by using online programs InterPro (<https://www.ebi.ac.uk/interpro/>) and the SMART (<http://smart.embl-heidelberg.de/>) databases. After removing the redundant and false protein sequences, a total of 51 SPX genes were identified, with 7 in apple, 11 in peach, 15 in strawberry, 4 in apricot, and 14 in grape (Table S1). Identified MdSPXs were filtered by removing

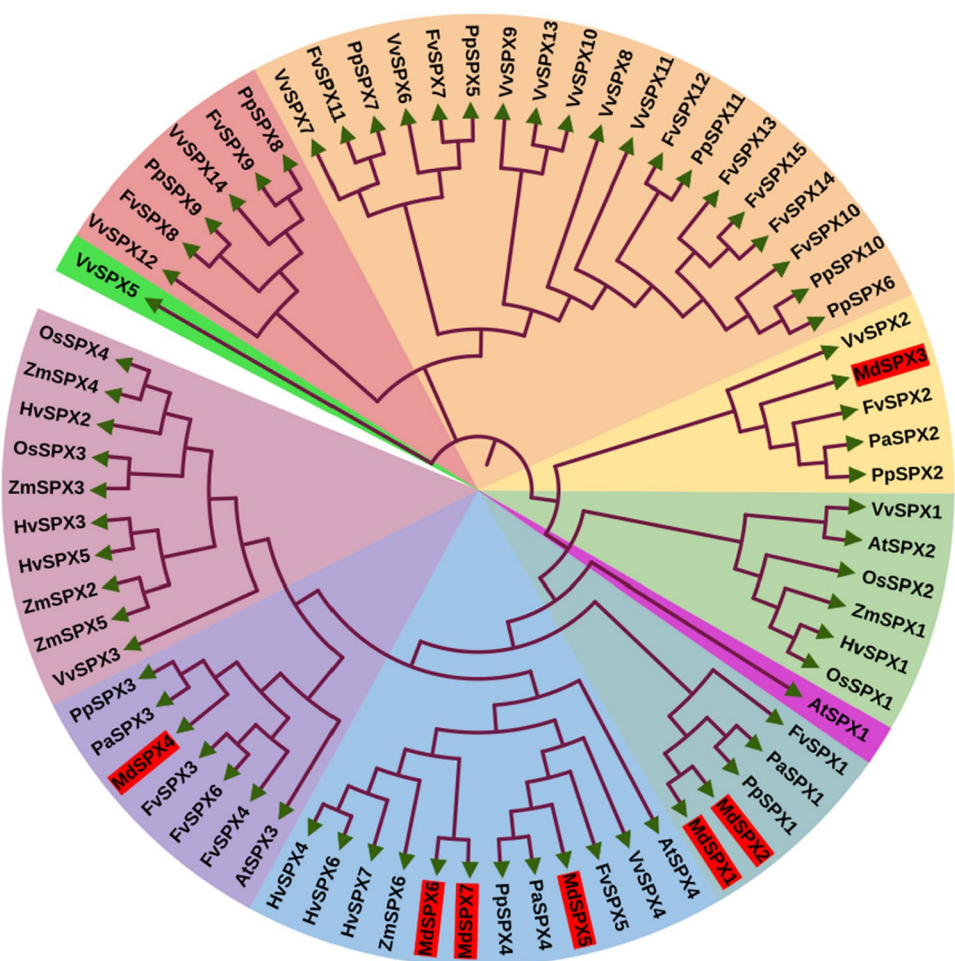
redundant sequences and different transcripts of the same gene. The basic information for the *MdSPXs*, including their amino acid sequence, molecular weight (MW), amino acid length, isoelectric point (pI), gene name and accession number, chromosomal location, aliphatic index, GRAVY, and putative location, is listed in Table S1. The protein length ranged from 142 aa (MdSPX7) to 903 aa (PpSPX8), with an average of 530 amino acids. The molecular weight (MW) varied from 16.3 kDa (MdSPX7) to 96.1 kDa (FvSPX13), with an average of 60.6 kDa. The isoelectric point (pI) ranged from 4.89 (MdSPX5) to 9.53 (VvSPX8) with an average of 7.41. The instability index ranged from 20.36 (MdSPX7) to 54.69 (FvSPX1) with an average of 42.1, and 14 of the predicted SPX proteins were considered stable proteins, while others were unstable. The aliphatic index, which ranged from 72.6 to 115.28 with an average of 90.8, indicated the thermal stability of SPX proteins. The GRVAY values of SPX proteins ranged from -0.652 (FvSPX2) to 0.392 (VvSPX12), with positive GRAVY values for 7 SPX proteins and negative GRAVY values for the remaining 44. Using the Plant-mPLOC server for subcellular localization analysis, detected SPX proteins were located in the cell

membrane, chloroplast, nucleus, mitochondria, and vacuole; also, the corresponding number of members is 19, 4, 2, 19, and 7, respectively.

Phylogenetic Analysis, Gene Structure, and Conserved Motifs of the SPX Genes

72 SPX protein sequences, 51 from five different fruit species, 4 from *Arabidopsis*, 4 from rice, 7 from barley, and 6 from maize, were aligned with MUSCLE, and then, a rootless phylogenetic tree was created with MEGA software (11.0.13) according to the Maximum Likelihood Estimation (MLE) method. 72 SPX proteins are divided into 9 subfamilies, which are shown in different colors (Fig. 1). MdSPX1, MdSPX2, and MdSPX3 are only in the yellow and green subfamilies with studied species, while MdSPX4 is in the purple subfamily with *Arabidopsis* and studied species. Interestingly, MdSPX6 and MdSPX7 are located very closely in barley, maize, and rice in the blue group, suggesting that MdSPX6 and MdSPX7 may be involved in similar functional roles as monocot SPX homologs. Likewise, MdSPX5 is in the blue group

Fig. 1 The maximum likelihood phylogenetic tree of SPX family members *Arabidopsis thaliana*, *Oryza sativa*, *Hordeum vulgare*, and five different species, *Malus domestica* (Md), *Fragaria vesca* (Fv), *Prunus persica* (Pp), *Vitis vinifera* (Vv), and *Prunus armeniaca* (Pa). The nine subfamilies are displayed in different colors. MdSPX genes are indicated by the red background



together with AtSPX4, PpSPX4, PaSPX4, FvSPX5, and VvSPX4.

Analysis of intron and exon structures provides important clues for the evolutionary and functional diversification of gene family members. To obtain more information about the gene structures of *SPX* genes belonging to the different five studied species, exon–intron structures were analyzed with the GSDS server (Fig. 2). The number of exons is highly divergent, ranged from 1 (*MdSPX1*–*MdSPX7*) to 15 (*FvSPX7*, *VvSPX6*, *PpSPX5*), and 23 members of the *SPX* genes had three exons. While *MdSPX2*–*3*–*4*–*5* had three exons and two introns, it has been determined that *MdSPX1* and *MdSPX7* do not contain both exons and introns. To summarize, *SPX* genes show a complex gene structure with varying intron positions and lengths within the analyzed species, which may indicate the functional diversity of *SPX* genes.

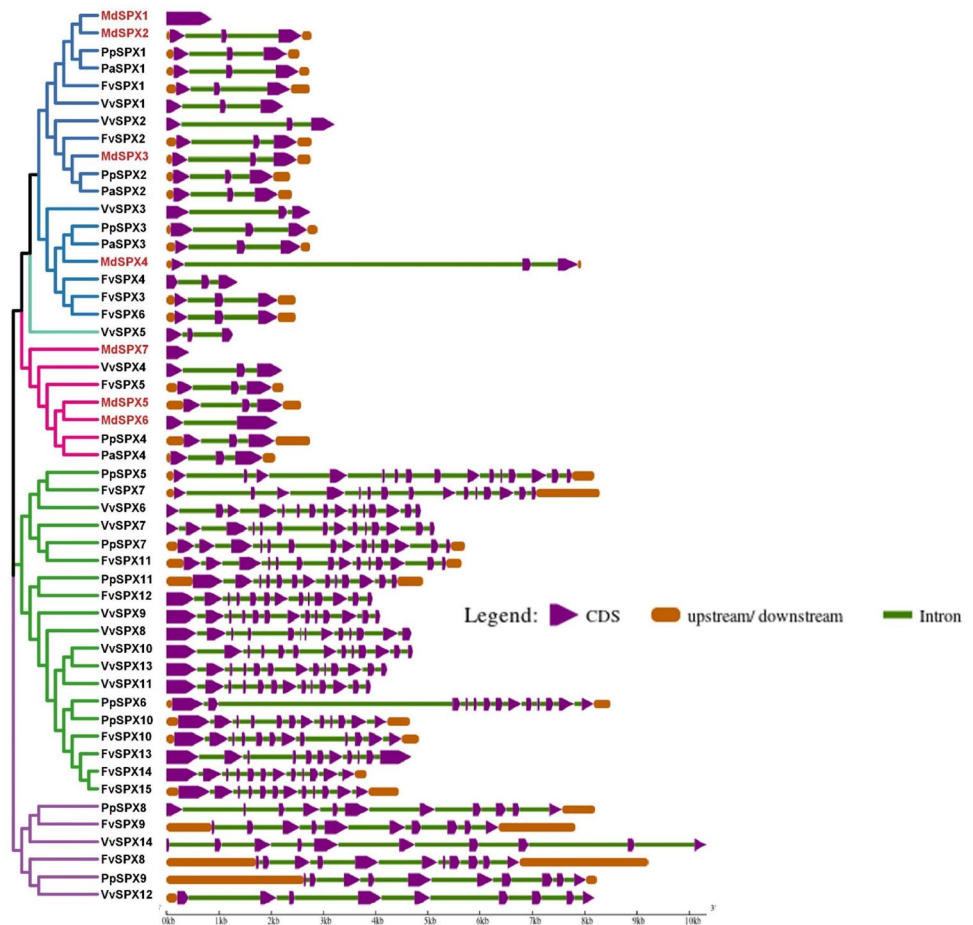
To further demonstrate the structures of the *SPX* proteins, the MEME analysis was used to verify the classification of the conserved motifs in 51 *SPX* family members. Sequence logos and sequences of five motifs are shown (Fig. 3). Proteins in the same group exhibited similar pattern distributions due to evolutionary conservation. Some motifs (1 and 4) were present in all groups, suggesting that these two

motifs might be important and may be responsible for their common function, while other motifs, for example, 2, 3, and 5, were observed only in the green subgroup.

Cis-Acting Regulatory Element Analysis

2000-bp upstream promoter regions of *SPX* genes were scanned using the PlantCARE database. A total of 39 *cis*-acting elements involved in different metabolic pathways were detected in the promoter regions of the *SPX* family genes (Fig. 4). These elements were classified as light-sensitive (3-AF1 binding, Box4, CATT-motif, GATA, G-Box, GT1, I-box, L-box, TCCC), hormone-sensitive (ABRE, TGACG, TGACG, AuxRR, GARE, P-box, TCA, CGTCA), environmental stress-related (LTR, MBS, ARE, W-box, WUN-motif), developmental processes related (O2-site, as1, CAT-box, HD-Zip-1), promoter-associated (A-box, CAAT-box, TATA-box), and site-binding elements (MBSII). The results suggest that differences in *cis*-acting elements and transcription factors binding to these elements in *SPX* genes may be responsible for the differential expression of these genes during developmental stages and under different stresses.

Fig. 2 The exon–intron structure of 51 *SPX* genes belongs to five different species. On the left side of the figure is a phylogenetic tree. CDS regions were indicated by a purple wedge, intron regions by a green bar, and UTR regions by an orange double-sided wedge



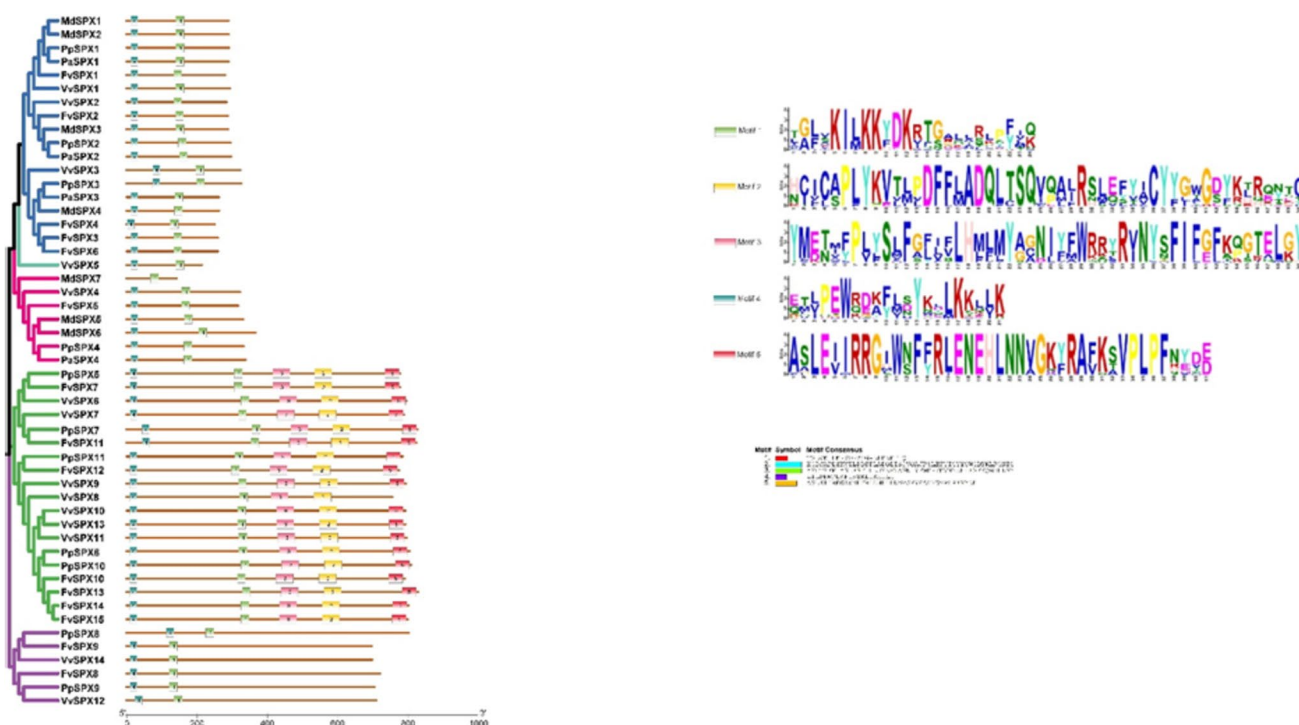


Fig. 3 Conserved protein sequences and motif patterns of the 51 *SPX* genes belonging to five different species. Five motifs (1–5) were identified and indicated by different colors. The color boxes represent the

conserved motif, while the black lines represent the non-conserved sequences. On the left side of the figure is the phylogenetic tree

Chromosomal Location and Synteny Analysis of *SPX* Gene Family in Rosaceae

The chromosomal distributions of 51 *SPX* genes in five different Rosaceae species were also analyzed, and 51 genes were randomly distributed across 30 different chromosomes (Fig. 5). Genome-wide duplication is an important evolutionary event that takes place throughout the evolution of plants through tandem and segmental duplication. We performed all-to-all BLASTP between the genomes of *M. domestica* (Md), *P. persica* (Pp), *P. armeniaca* (Pa), *V. vinifera* (Vv), and *F. vesca* (Fv) to better understand the evolution of *SPX* family genes. We also compared protein-coding genes with their own genomes by BLASTP (e-values $< 1e^{-10}$) and scanned genomes with MCScanX (Wang et al. 2012a, b) for possible collinear blocks within and between the genomes of different species. All intra and inter-genomic synteny relationships of *SPX* genes were visualized with TBtools (Chen et al. 2020). A total of 21 collinear gene pairs were identified from 51 *SPX* genes belonging to five different genus. We observed large interspecies collinear blocks between five species, which indicates a high level of macrosynteny. Based on genomic comparisons, two homolog gene pairs were detected in *M. domestica* and one in *V. vinifera*, and one in *F. vesca*. Four orthologous gene pairs were found

between Pp and Pa, Pp, and Fv, respectively. There were two orthologous gene pairs between Md and Pp, or Pa. The number of orthologous gene pairs between Md and Pp, Md and Fv, Md and Vv, and Pp and Vv was one. The results of the collinearity analysis of the *SPX* family genes were compatible with the phylogenetic analysis (Fig. 1).

Selective Pressure Analysis of *SPX* genes in Five Rosaceae Species

In the evolutionary process, harmful mutations can be eliminated by negative (purifying) selection, and advantageous mutations can be accumulated and passed on to future generations by positive (Darwinian) selection (Starr et al. 2003). The Ka/Ks ratio estimates selection pressure, with Ka/Ks < 1 indicating purifying selection, Ka/Ks = 1 indicating neutral selection, and Ka/Ks > 1 indicating positive selection. In five species, 14 *SPX* family gene paralogs were found, with Ka/Ks values ranging from 0.008 to 0.55, indicating purifying selection (Table 1).

Based on the Ks values of duplication blocks, the divergence times were estimated. The results of the analysis showed that the divergence times of the *SPX* genes in the five species date back to 1429.32 MYA, indicating that these paralogs are relatively ancient.

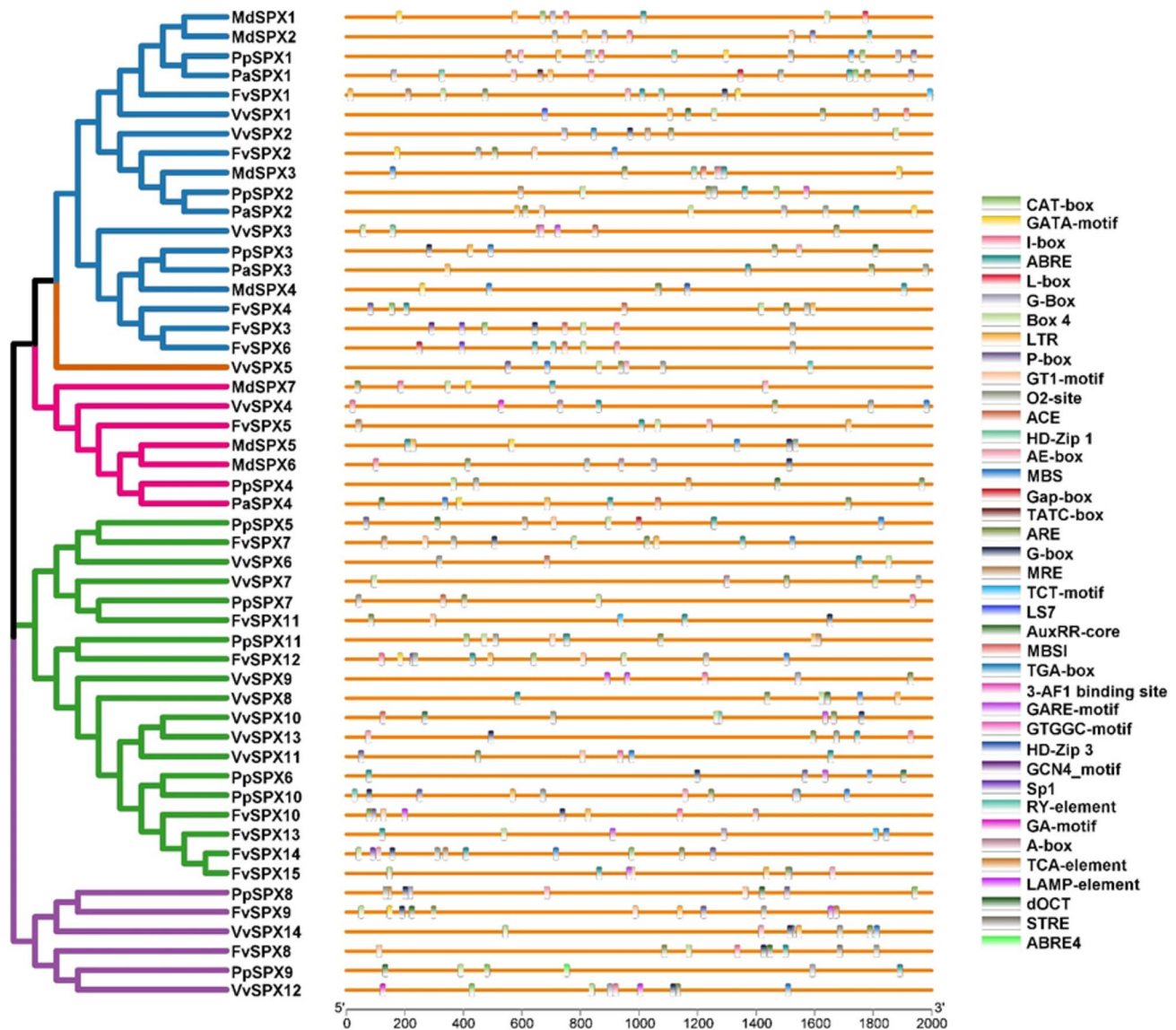


Fig. 4 Cis-acting element analysis of 51 *SPX* gene promoters in the five different species. On the left side of the figure is the phylogenetic tree

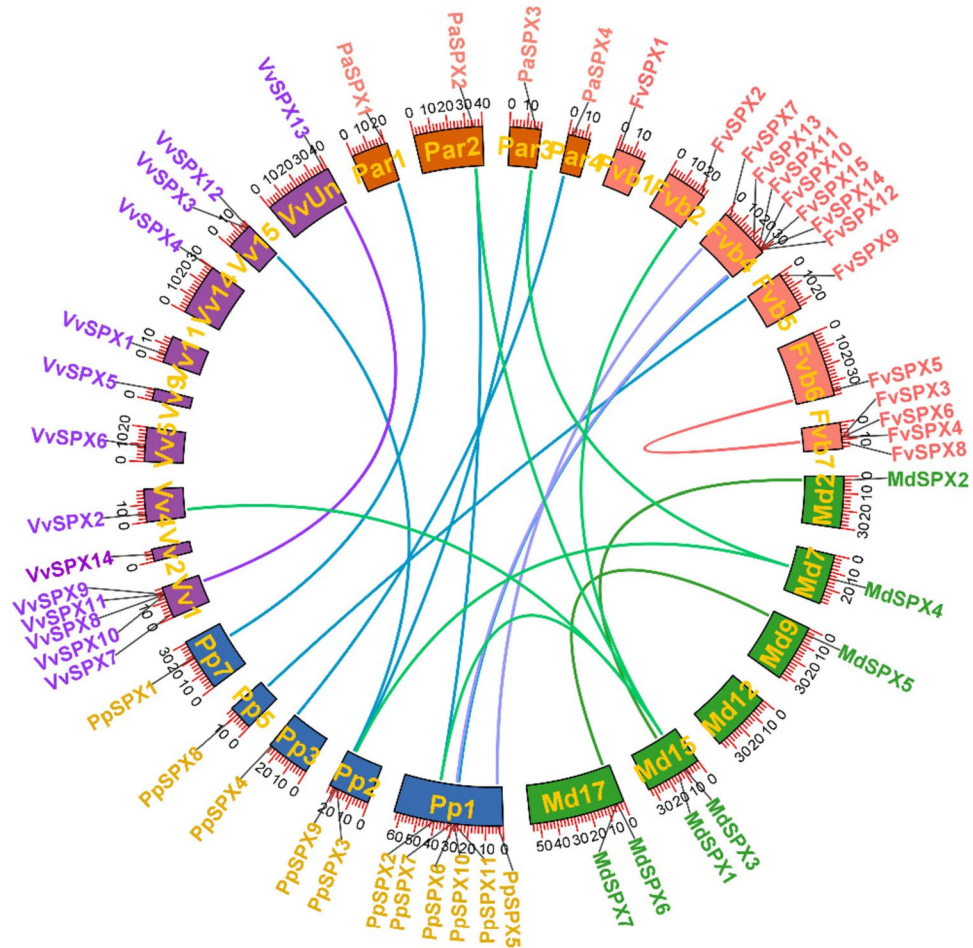
Gene Ontology (GO) Annotation Analysis of SPX Proteins

Gene Ontology is a comprehensive analysis that structures information about the functions of genes in a form suitable for computational analysis to support modern biological research. The PANNZER server was used to study the specific functions of SPX proteins in five species. As shown in Fig. 6, a total of 15 biological processes, 10 molecular functions, and nine cellular components were identified. For biological processes, cellular response to phosphate starvation (GO:0016036), positive regulation of cellular response to phosphate starvation (GO:0080040), response to oxidative stress (GO:0006979), cellular oxidant detoxification (GO:0098869), cellular response to cold (GO:0070417),

phosphate ion homeostasis (GO:0055062), phosphate ion transport (GO:0006817), inorganic anion transmembrane transport (GO:0098661), cell wall biogenesis (GO:0042546), transmembrane phosphate ion transport from cytosol to vacuole (GO:1905011), response to stimulus (GO:0050896), transmembrane transport (GO:0055085), cellular response to hypoxia (GO:0071456), inositol phosphate-mediated signaling (GO:0048016), and polyphosphate biosynthetic process (GO:0006799) were identified.

SPX genes are involved in various cell processes such as oxidative stress, cold stress response, cellular hypoxia, cell wall biogenesis, and phosphate metabolism. For molecular function, peroxidase activity (GO:0004601), heme binding (GO:0020037), inositol hexakisphosphate binding

Fig. 5 Chromosome distribution and collinear synteny analysis of 51 *SPX* genes among the five species. The chromosomes of each species were shown in different colors as circular. The positions of the *SPX* genes were marked with a short black line on the chromosome. Syntenic gene pairs are linked by different colored lines



(GO:0000822), phosphate ion transmembrane transporter activity (GO:0015114), inositol monophosphate 3-phosphatase activity (GO:0052832), inositol monophosphate

4-phosphatase activity (GO:0052833), inositol monophosphate 1-phosphatase activity (GO:0008934), galactoside 2-alpha-L-fucosyltransferase activity (GO:0008107), zinc ion

Table 1 Ka/Ks analysis and estimate of the absolute dates for the divergence events between the *SPX* homologs of five different species

Gene1	Gene2	Ka	Ks	Ka/Ks	Purifying selection	Divergence time (Mya)
MdSPX1	MdSPX2	0.0652	0.3115	0.20931	Yes	10.38
MdSPX5	MdSPX6	0.0726	0.2873	0.252698	Yes	9.58
PpSPX6	PpSPX10	0.0284	0.087	0.326437	Yes	2.90
PpSPX5	PpSPX7	0.3774	8.8642	0.042576	Yes	295.47
PpSPX8	PpSPX9	0.1598	1.0193	0.156774	Yes	33.98
FvSPX1	FvSPX2	0.2477	2.0122	0.123099	Yes	67.07
FvSPX8	FvSPX9	0.1675	1.1813	0.141793	Yes	39.38
FvSPX14	FvSPX15	0.0193	0.0346	0.557803	Yes	1.15
FvSPX7	FvSPX11	0.3768	24.9701	0.01509	Yes	832.34
VvSPX1	VvSPX2	0.2076	2.1126	0.098268	Yes	70.42
VvSPX6	VvSPX7	0.3765	42.8797	0.00878	Yes	1429.32
VvSPX10	VvSPX13	0.0738	0.1467	0.503067	Yes	4.89
VvSPX12	VvSPX14	0.1358	0.831	0.163418	Yes	27.70
PaSPX1	PaSPX2	0.2512	1.3747	0.182731	Yes	45.82

binding (GO:0008270), transmembrane transporter activity (GO:0022857) were found. For cellular components, nucleus (GO:0005634), membrane (GO:0016020), cytosol (GO:0005829), Golgi apparatus (GO:0005794), cell periphery (GO:0071944) trans-Golgi network (GO:0005802), plant-type vacuole membrane (GO:0009705), plant-type vacuole (GO:0000325), and organelle sub-compartment (GO:0031984) were identified. To summarize, MdSPX proteins are thought to be specialized to take part in different pathways of cell metabolism.

Protein–Protein Interaction Analysis Between MdSPX Proteins

Protein network interaction analysis is an important step toward a better understanding of the rich biological functions of proteins and the dynamic regulatory networks between various biomolecules. Therefore, to explore potential interactions between MdSPX members and other proteins in apple, we conducted a protein–protein interaction analysis in the UniProt and STRING databases.

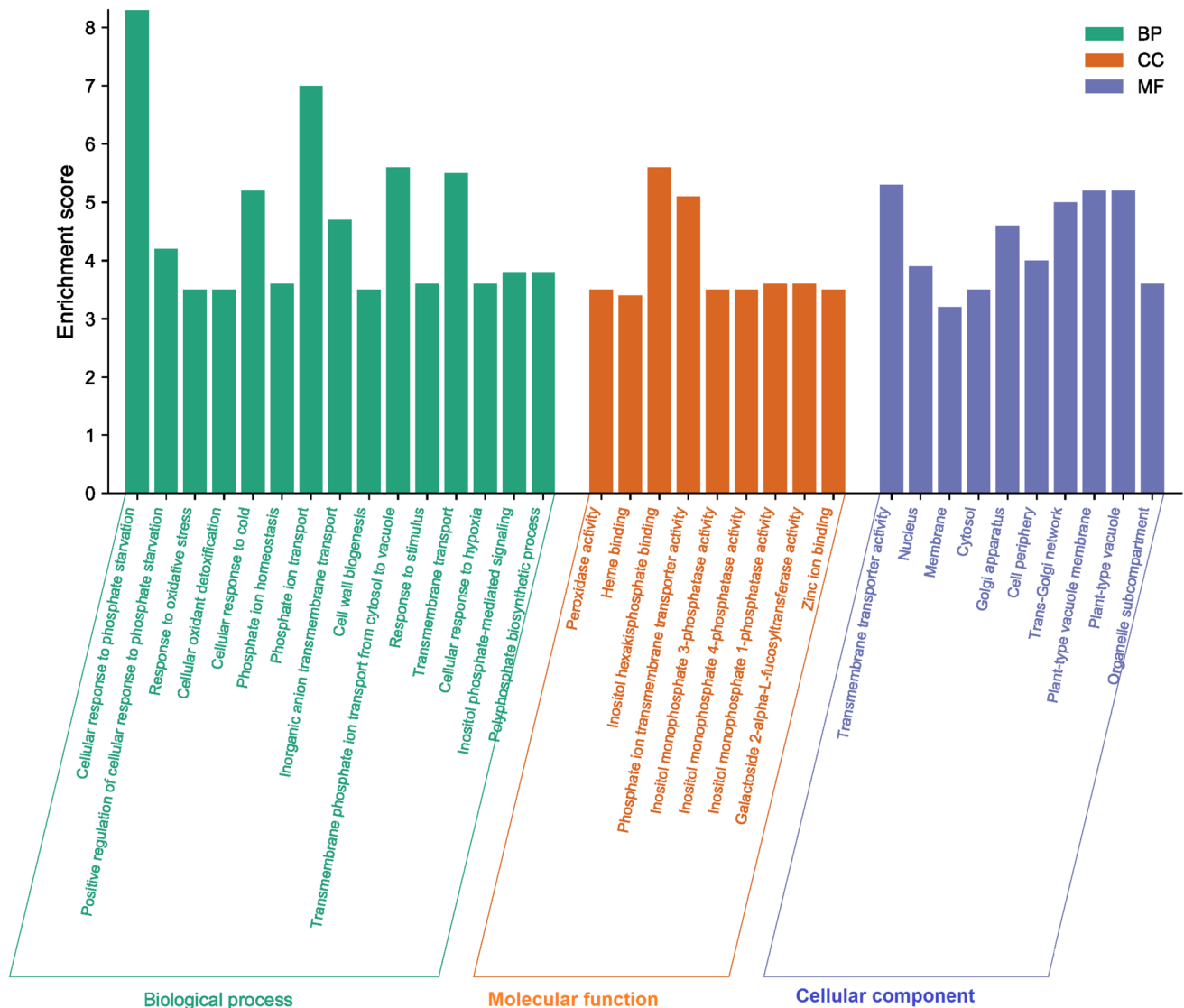


Fig. 6 Gene ontology (GO) analysis of the SPX proteins. The SPX amino acid sequences were annotated using the local PANNZER (Protein ANnotation with Z-score) server

According to the analysis, we identified two distinct clusters of 44 proteins and 174 interacting protein branches. Interestingly, these clusters consisted of six MdSPX (1–2–4–5–6–7) proteins and only one MdSPX3 protein (Fig. 7). The results show that MdSPX family proteins have functional and physical associations with many proteins involved in biological processes, including phosphate ion detection (MdSIZ1-like, DNA binding transcription factor activity (MdPHR1-LIKE), photoreactive repair (MdDNA photolyase), protein phosphorylation (MdPAP14), phospholipid metabolic process (MdLPP) phosphatidylinositol-mediated signaling (MdMINP1), metal ion binding (MdPAP22), protein secretion (MdSEC12-like-1), D-xylose transmembrane transport (MdxylE-like-2), iron-sulfur cluster biosynthesis (MdNUBPL-like), phosphate-containing compound metabolic process (MdPPA1), and cellular response to phosphate starvation (MdSQD2). In addition, MdSPX3 has been found to directly interact with the MdYABBY3 protein, which promotes adaxial cell identity and regulates the initiation of embryonic shoot apical meristem (SAM) development (Stahle et al. 2009), suggesting that MdSPX3 is involved in the development of these tissues.

Expression Patterns of MdSPX Genes in Different Tissues

To dissect the expression patterns of *MdSPX* genes in various tissues/organs, developmental stages, and biotic stress treatment, a total of 47 expression profiles were obtained from

the Apple Multi-Dimensional Omics Database (AppleMDO) (Fig. 8) (Da et al. 2019). As can be clearly seen from the heat-map, *MdSPX6* and *MdSPX7* are not expressed in all 47 tissues/organs and developmental stage profiles. *MdSPX1,2,3,4* and 5 genes are expressed in at least one profile. Among them, *MdSPX3* is expressed in all plant tissues, developmental stages and under biotic stress treatments, and it shows the highest expression level in pollen tissues, suggesting that this gene may be involved in pollen development and/or fertility. *MdSPX1* and *MdSPX2* are highly expressed in response to biotic stress treatment, especially post-infection by *Venturia inaequalis*, indicating their potential role in response to a/biotic stress conditions. These results show that most *MdSPX* genes have dynamic expressions in different tissues/organs, developmental stages, and stress-responsive mechanisms. The data could be used to investigate the functional diversity of apple *MdSPX* genes.

Tree Quality and Leaf Nutrients

The P nutrition and quality attributes of trees were assessed for various treatments. The mean leaf-P content values ($\% \pm \text{sd}$) were 0.1631, 0.1414, 0.1375, 0.1348, and 0.1250 for 50%P + PGPR, 100%P, 50%P, and 0%P, followed by 0%P + PGPR, respectively. However, no meaningful differences were found regarding leaf-P content among treatments (Fig. 9a). Treatments had a significant impact on tree height. 100%P showed higher values than 50%P, 0%P, and

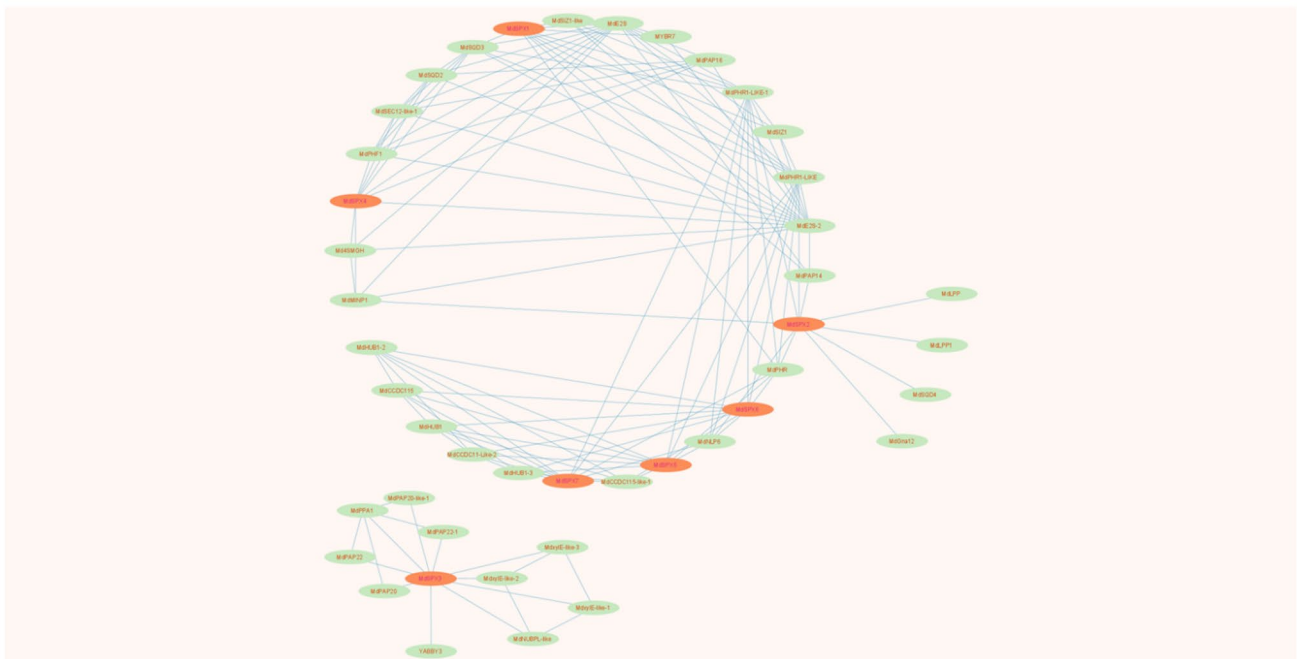


Fig. 7 The protein–protein interaction network between MdSPXs and other apple proteins. The MdSPX proteins are in orange, and the other apple proteins are in green

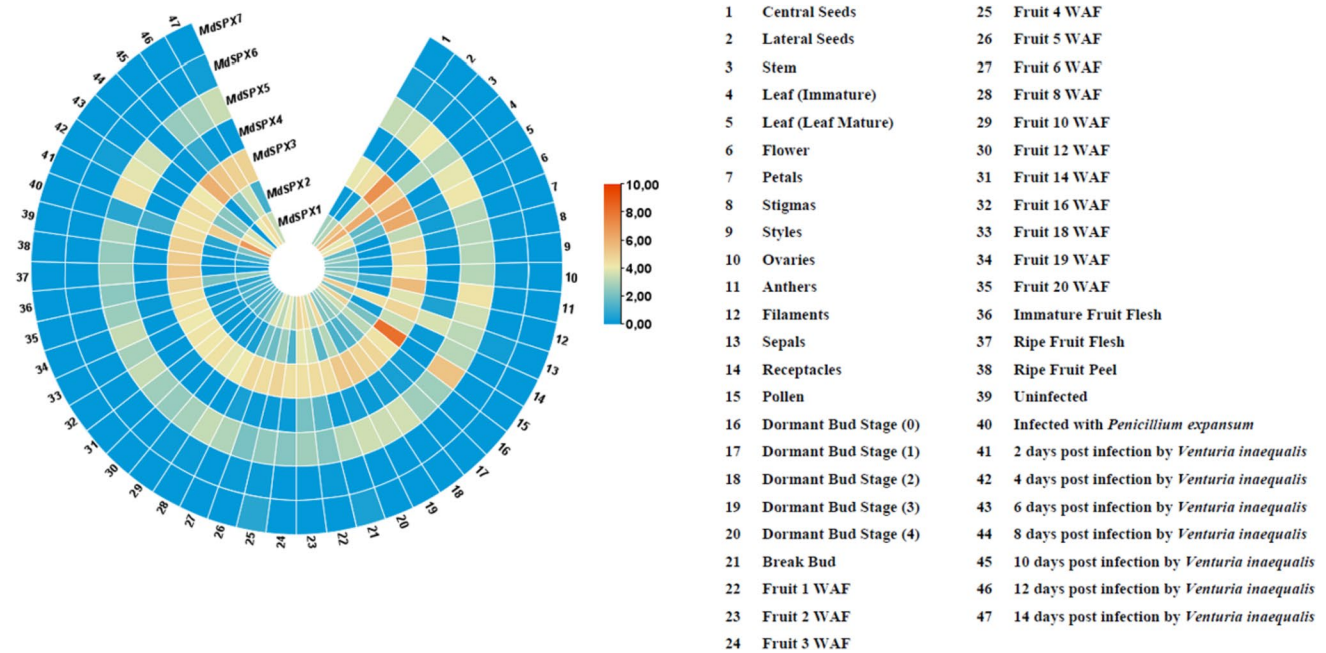


Fig. 8 Expression profiles of *MdSPX* genes in different tissues/organs, developmental stages, and biotic stress infections. The names of tissues/organs and development stages are indicated on the right. WAF: week after full bloom. The FPKM values were transformed by log2

0%P+PGPR, although it was not significantly different from 50%P+PGPR (Fig. 9b). The treatment effect was similar for trunk diameter (Fig. 9c). Differences in total leaf area were found between 100%P and other treatments (Fig. 9d). Dry shoot weight (Fig. 9e) and dry root weight (Fig. 9f) were relatively higher in 100% P and 50% P + PGPR, although they were not significantly different from other treatments. Other nutrient element results of the samples, except P, are given in Table S5.

Expression Analysis of *MdSPX* Genes Under Different P and PGPR Treatments

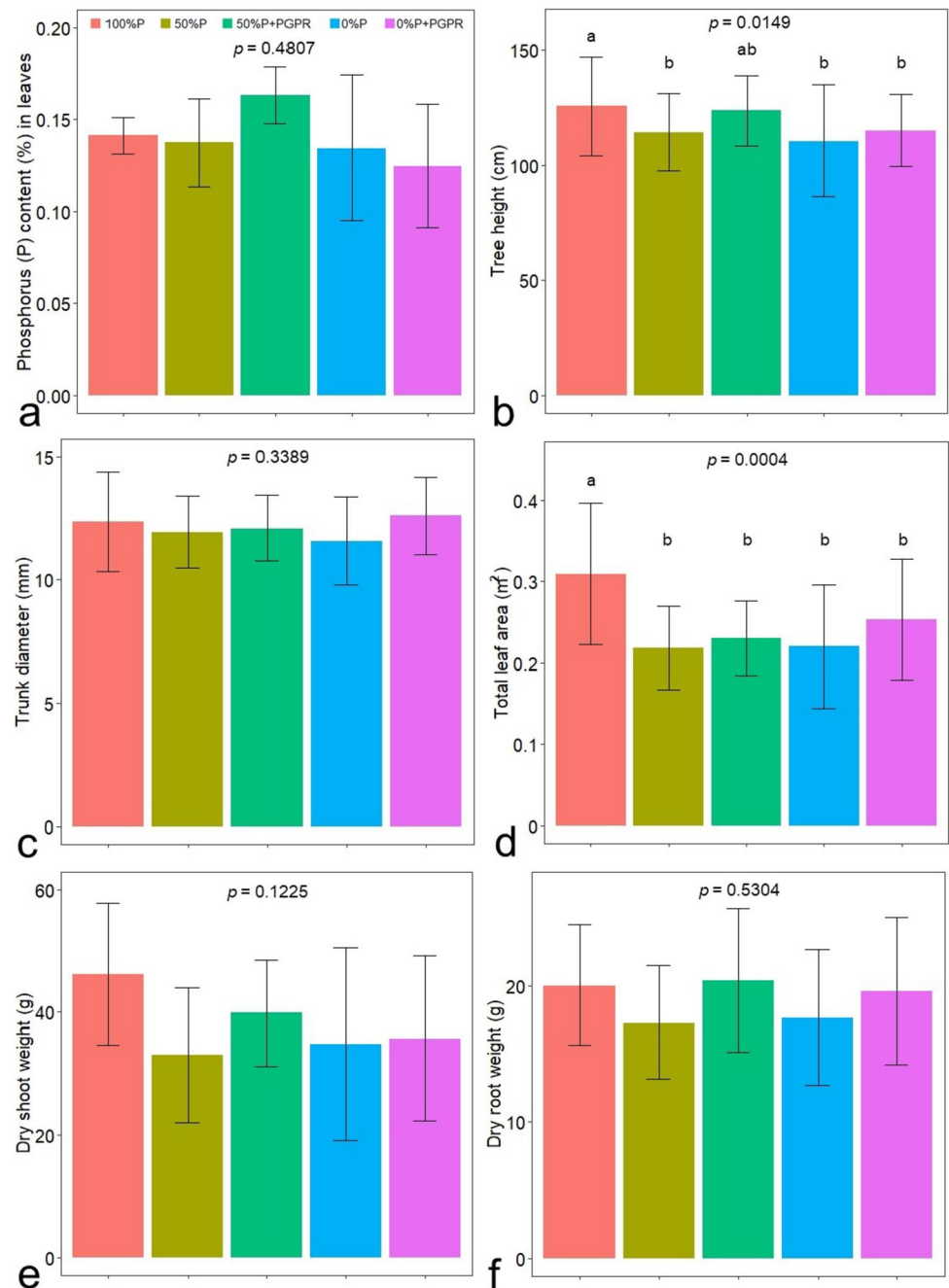
The expression of seven *MdSPX* genes under different P and PGPR treatments was analyzed by qRT-PCR (Fig. 10). *MdSPX2* was the most expressed gene under 50% P and 0% P treatments, indicating that P starvation is at its maximum level in these treatments. However, the expression of *MdSPX2* and other six genes (*MdSPX1,3,4,5,6,7*) was strongly downregulated in 50%P+PGPR treated plants, showing that 50% P combined with PGPR is effectively taken up by the plant, saving it from P starvation. Expression patterns very similar to this result were observed in all other *MdSPX* genes with different expression levels. These results suggested that *MdSPX* genes show their involvement in the P signaling pathways through induced gene expression patterns.

Discussion

P is an essential macronutrient for plant growth and a critical component of various structural and biological pathways (White and Brown 2010). Plants have to adjust their gene expression levels to facilitate phosphorus uptake, distribute it to other organs, or cope with Pi deficiency. In plants, SPX domain-containing proteins can be divided into four families, namely SPX family, SPX-X family, SPX-MFS family, and SPX-RING, respectively (Secco et al. 2012). *SPX* genes have been extensively studied in a number of plants and have been reported to participate in metabolism and localize in different parts of the cell (Zhou et al. 2015). Until today, 69 *SPX* genes in *B. napus* (Du et al. 2017), 46 in *T. aestivum* (Kumar et al. 2019), 33 in *Z. mays* (Xiao et al. 2021), 10 in *G. max* (Yao et al. 2014), 19 in *S. lycopersicum* (Li et al. 2021), and 10 in *P. massoniana* (Wang et al. 2021a, b, c) have been identified. The *Malus domestica* genome encodes 25 *SPX* domain-containing proteins spanning all four families, including *MdSPX*, *MdSPX-EXS*, *MdSPX-MFS*, and *MdSPX-RING* (Xie et al. 2023). *MdSPX1-6* subfamily genes identified by Xie et al. (2023); however, an extra gene, *MD17G1052100*, was extracted in this study from the apple genome and named *MdSPX7*, which is equivalent number of *SPX* subfamily genes to that in tomato and maize (Li et al. 2021; Xiao et al. 2021).

To understand the molecular evolution of the *SPX* gene family in different species, in this study we identified 7, 11,

Fig. 9 Effects of treatments on **a** phosphorus (P) content in leaves, **b** tree height, **c** trunk diameter, **d** total leaf area, **e** dry shoot weight, and **f** dry root weight. Different letters indicate significant differences at $p < 0.05$. The values shown are means $\pm$ standard deviation (sd)

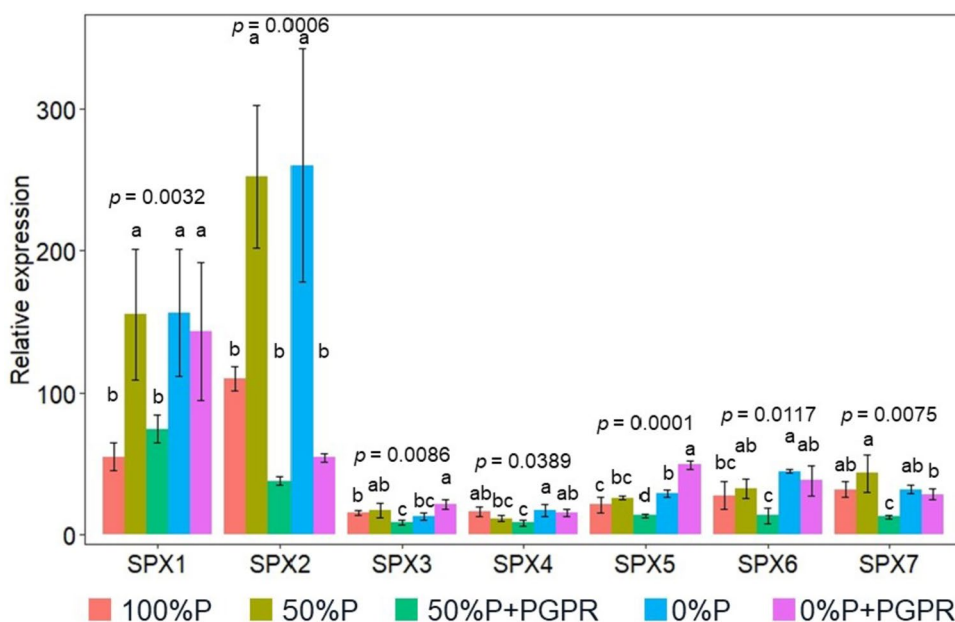


15, 14, and 4 *SPX* genes from *M. domestica*, *P. persica*, *F. vesca*, *V. vinifera*, and *P. armeniaca*, respectively (Table S1). The difference in *SPX* gene numbers in these five species suggested that a gene duplication or deletion event occurred in the evolutionary process. Based on phylogenetic analysis with *SPX* homologs from *Arabidopsis*, rice, and different species, 72 *SPX* genes can be divided into 8 subfamilies (Fig. 1). As expected, the majority of *MdSPX* genes were localized together with those from *SPX* genes belonging to *P. persica* and *P. armeniaca*, which indicates that the *SPX* gene family is conserved in the studied species. Interestingly, *MdSPX6* and

MdSPX7 fall into the same group as monocot plants, barley, and maize. This suggested that *SPX* proteins differed after monocots and dicots split out from a common ancestor.

Genes in the same subfamily may exhibit different exon-intron structures, but having similar conserved protein motifs may indicate that these genes have similar biological functions (Fig. 2). The *MdSPX* subfamily genes have a similar gene structure with three exons and two introns between *Arabidopsis*, rice, and tomato, except for *OsSPX5*, which possessed two exons and one intron (Li et al. 2021). Interestingly, *MdSPX1* and *MdSPX7* were found to lack both

Fig. 10 Expression analysis of seven *MdSPX* genes under 0%P+PGPR, 50%P, 50%P+PGPR, 0%P, and 100%P treatments. Different letters indicate significant differences at $p < 0.05$. The values shown are means $\pm$ sd



exons and introns, and this may indicate that *SPX* genes are specialized for different gene structures or even different functional roles in different species. In this study, we identified five different conserved protein motifs in the *SPX* genes of different Rosaceae species, and the first and fourth motifs of these motifs are common to all subfamilies, suggesting that they may be important for the common function of *SPX* genes in the studied species (Fig. 3).

To investigate the evolutionary relationships between different species of the *SPX* gene family, we selected five species and performed synteny analyses (Fig. 5). As a result, *MdSPX* genes showed very strict orthologous correlations with other selected Rosaceae species. Therefore, we assume that syntenic correlations among *SPX* members may be linked to the evolutionary differentiation of species. Consequently, intersections of syntenic *SPX* members between different species may be valuable for conducting relevant research on the evolution of the *SPX* gene family (Table 1). At the level of molecular evolution, positive selection retains amino acid mutations that enhance adaptation, while negative selection removes amino acid mutations that impair adaptation (Fetterman et al. 2008). The positive selection analysis of *MdSPX* implies that these genes were subjected to strong positive selection in the evolutionary process and evolved under the influence of purifying selection. The promoter regions of 51 analyzed *SPX* genes exhibited more than one *cis*-element type involved in various functions, such as light-sensitive, hormone-sensitive, environmental stress-related elements, developmental processes-related elements, and abiotic and biotic stress responses (Fig. 4). This has also been explored in prior studies that *SPX* ABA, salicylic acid (SA), jasmonic acid (JA), and their combination

significantly changed the expression of all *SPX* genes in *Arabidopsis* (Nezamivand-Cheghini et al. 2021). Forty-three promoter elements were identified in the *MdSPX* gene family (Xie et al. 2023), of which environmental stress elements related to plant resistance to abiotic stresses such as low-temperature and drought were identified, supported by our *cis*-elements prediction analysis. The presence of many *cis*-acting elements that mediate responses to different environmental stresses and developmental processes suggests that they play a role in different biological processes.

Gene ontology is an analysis used to describe the putative functional contributions and functions of genes among living organisms. Gene ontology analysis results showed that *SPX* genes may be involved in biological processes such as oxidative stress response, cellular response to cold, cell wall biogenesis, and cellular response to hypoxia, as well as phosphate metabolism (Fig. 6). Furthermore, GO showed molecular functions where *SPX* genes participate in peroxidase activity, hem binding, phosphate ion transmembrane transporter activity, and zinc ion binding-related processes.

The expression profiling of *MdSPX* in different tissues and developmental stages showed that at least one *MdSPX* gene was expressed in various tissues, except *MdSPX6* and *MdSPX7*. Although we did not include it in this study, tomato *Solyc08g060920* (Li et al. 2021), which we know to be the ortholog of *MdSPX3*, is actively expressed in all tissues and developmental stages of fruit. Therefore, *MdSPX3* might be involved in different developmental processes and stresses beyond phosphate starvation response. Down-regulation of *OsSPX1*, a rice *SPX* domain gene, caused a reduction of seed-setting rate and filled grain number, suggesting these genes may be related to pollen fertility and

male gametophyte development (Zhang et al. 2016). In our study, the expression pattern analysis shows that *MdSPX2* (in sepals), *MdSPX3* (in stigmas, ovaries, anthers, and pollen), and *MdSPX5* (in pollen) showed higher expression potential, suggesting that these *MdSPXs* might have essential roles in pollen development and fruit yield. Pi starvation strongly induced expression of *AtSPX1* and *AtSPX3* with distinct dynamic patterns, implying that these genes play positive roles in plant adaptation to phosphate starvation (Duan et al. 2008). Based on this, *MdSPX1* and 2, which fall in the same location in phylogenetic tree and specifically expressed in leaves, might be key players in maintaining Pi homeostasis in the leaves, and function in plant tolerance to phosphorus starvation.

All studies conducted so far were based on Pi starvation and it was shown that the expression of *SPX* genes is induced under these conditions. However, this study, unlike previous descriptions of similar studies, investigated the effect of P dissolved by phosphate-solubilizing bacteria on plant nutrition and the expression of *MdSPX* genes. In other words, this study investigated how more P delivered to the plant affects the plant at the morpho-physiological and molecular level. In fact, although the capacity for Pi uptake is limited due to its low rooting density in apple, 100% P and 50% P+PGPR applications, which contain relatively high Pi, positively affected the nursery tree quality criteria (Nielsen and Nielsen 2003). In this study, a relatively high P amount in plant leaves was reached with PGPRs applied to half of the recommended P amount (50%P+PGPR), according to the soil analysis results. The relatively low leaf phosphorus content obtained in 0%P and 50%P treatments indicates that the available phosphorus in the soil of the trial area is insufficient. However, it was determined that there was 43 ppm of phosphorus in the soil of the trial area (TableS1). This ratio shows that there was enough phosphorus in the soil. However, alkaline soil (pH=8.06) of the study area may have fixed phosphorus. Plants cannot uptake phosphorus forms because they are fixed at high levels by iron and aluminum in acidic soils (Whitelaw 2000) and by calcium carbonate in alkaline soils and, therefore, cannot be dissolved (Gyaneshwar et al. 1998). The relatively low leaf phosphorus content in the 0%P+PGPR treatment indicates that even if there is sufficient phosphorus in the soil, the applied PGPR alone is insufficient for P nutrition, and that chemical phosphorus fertilization is important. Especially in 50%P+PGPR, the better plant phosphorus nutrition and tree quality can be attributed to the fact that bacteria dissolve at least some of the applied chemical phosphorus without being fixed in the soil, and in addition, the undissolved phosphorus in the soil is dissolved by bacteria and becomes available to plants. Plants cannot benefit from undissolved phosphorus (Kour et al. 2021). In addition, chemical phosphorus applied to the soil as fertilizer quickly turns into unavailable forms that plants cannot use (Gudino-Gomezjurado et al. 2022), and

the majority (75–90%) cannot be used by plants (Podile and Kishore 2006; Walpola and Hettiarachchi 2020).

This result was strongly supported by qRT-PCR that 50%P+PGPR substantially decreased the expression of all *MdSPX* genes, especially *MdSPX1* and *MdSPX2*. It was previously confirmed that the SPX-PHR1 complex might respond to the presence of P, and PHR1 could subsequently control the expression of PSI genes (Maeda et al. 2018). Supporting this, we also found an interaction of *MdSPX1-2-4-5-6-7* with *MdPHR1* and *MdSIZ1*. In the light of previous studies, a possible explanation for this might be that *MdSPX-MdPHR1* and—*MdSIZ1* interactions are able to regulate the expression of PSI genes, thereby maintaining a balance of intercellular Pi contents in apple, in this case under Pi excess conditions. This is the reason that the most important point of this study can be considered a reverse confirmation of previous studies. For example, *OsSPX1*, 3 and 4 were significantly induced after Pi starvation treatment in rice (Wang et al. 2012a, b). In maize, most *SPX* subfamily genes were highly induced by P deficiency, except for *ZmSPX3*, which had the highest homology with *OsSPX4* and *AtSPX4* (Xiao et al. 2021). In a recent study in apple, *MdSPX1*, *MdSPX2*, *MdSPX4*, and *MdSPX5* were significantly upregulated in response to P deficiency (Xie et al. 2023). In the same study, only *MdSPX3* showed a downregulation under N supply and P deficiency condition among six *MdSPXs*. Notably, in our study, *MdSPX3* and *MdSPX4* responded to P satisfaction less strongly than *MdSPX1*, *MdSPX2*, and *MdSPX5-6-7*. In *A. thaliana*, comparable outcomes showed that *AtSPX1/3* was strongly induced while *AtSPX2* was poorly raised in response to the P deficient condition (Duan et al. 2008). In this study, a promising finding is that PGPR application enabled the soluble phosphate to pass into the plant and solved the Pi deficiency problem.

These results provide further support for the hypothesis that if *SPX* genes are induced under Pi starvation, they will conversely be downregulated if the plant is able to acquire sufficient amounts of phosphate, as has been shown experimentally. In order to understand these results in more detail, it is necessary to study how the expression of all genes in the Pi signaling mechanism changes in the presence of phosphate-solubilizing bacteria.

Conclusion

Effective usage of phosphate-solubilizing bacteria is a promising tool to maintain the sustainable fertility of soil and improve plant growth. The biggest feature that distinguishes this study from previous studies was that, while all studies in the literature investigated the response of plants to P deficiency, this study investigated how a bacterium that dissolves phosphate affects the P status in the plant and the plant's response to it. In this study, we applied half of the recommended P in soil analysis with PGPR. The results showed that the quality of apple nursery trees could

be increased by reducing the use of chemical phosphorus by 50% in soils containing sufficient phosphorus but by using appropriate phosphorus-solubilizing bacteria. We supported these results by the fact that increased *MdSPX* gene expression was effectively downregulated by increasing the level of available P in the apple nursery plants. Obviously, this study needs to be further improved by using different field locations with different soil properties and understanding the molecular regulatory mechanisms by using different Pi transporter genes.

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1007/s11105-024-01443-8>.

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Author Contributions MK, AK, SU, and EA designed the research; MK and EA contributed to the growth of the plant material; AK performed bioinformatic analysis; SU performed RNA isolation and RT-qPCR. All authors contributed writing—review and editing.

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Data Availability The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Competing Interests The authors declare no competing interests.

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