

Identification and expression analysis of the SQUAMOSA promoter-binding protein (SBP)-box gene family in *Prunus mume*

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Abstract SQUAMOSA promoter-binding protein (SBP)-box family genes encode plant-specific transcription factors that play crucial roles in plant development, especially flower and fruit development. However, little information on this gene family is available for *Prunus mume*, an ornamental and fruit tree widely cultivated in East Asia. To explore the evolution of SBP-box genes in *Prunus* and explore their functions in flower and fruit development, we performed a genome-wide analysis of the SBP-box gene family in *P. mume*. Fifteen SBP-box genes were identified, and 11 of them contained an miR156 target site. Phylogenetic and comprehensive bioinformatics analyses revealed that different groups of SBP-box genes have undergone different evolutionary processes and varied in their length, structure, and motif composition. Purifying selection has been the main selective constraint on both paralogous and orthologous SBP-box genes. In addition, the sequences of orthologous SBP-box genes did not diverge widely after the split of *P. mume* and *Prunus persica*. Expression analysis of *P. mume* SBP-box genes revealed their diverse spatiotemporal expression patterns. Three duplicated SBP-box

genes may have undergone subfunctionalization in *Prunus*. Most of the SBP-box genes showed high transcript levels in flower buds and young fruit. The four miR156-nontargeted genes were upregulated during fruit ripening. Together, these results provide information about the evolution of SBP-box genes in *Prunus*. The expression analysis lays the foundation for further research on the functions of SBP-box genes in *P. mume* and other *Prunus* species, especially during flower and fruit development.

Keywords Genome-wide analysis · SBP-box genes · *Prunus mume* · Phylogenetic relationship · Expression analysis

Introduction

Prunus mume, an important ornamental and fruit tree belonging to the Rosaceae, has been cultivated in East Asia for more than 3000 years. This species has prominent ornamental characteristics such as colorful corollas, a pleasant fragrance, and various types of flowers (Sun et al. 2013). Its fruit is economically valuable, as it can be preserved or processed into wine and juice. The fruit products are considered to be beneficial to human health. Despite its importance, the molecular mechanisms that regulate its flower and fruit development are still largely unknown. SQUAMOSA promoter-binding protein (SBP)-box genes encode plant-specific transcription factors that control the expression of genes involved in floral organ identity determination. Therefore, SBP-box genes affect floral transition and flower development (Klein et al. 1996; Gandikota et al. 2007). These genes also play roles in fruit development and ripening after fertilization (Manning et al. 2006). To provide insights into the molecular mechanisms of flower

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and fruit development in *Prunus* genus and other Rosaceae trees, it is essential to conduct a genome-wide analysis of these genes in *P. mume* and to elucidate their roles in flower and fruit development.

SBP-box genes are defined by a conserved region of approximately 76 amino acids designated as the SBP domain (Cardon et al. 1999; Klein et al. 1996). This domain features two zinc-binding sites (one is C3H or C4, and the other is C2HC), and it is essential for both DNA-binding and nuclear localization (Birkenbihl et al. 2005; Yamasaki et al. 2004). SBP proteins bind to the *cis*-element sequence TNCGTACAA in the promoter region of *SQUAMOSA* (Cardon et al. 1999). SBP-box genes were initially identified in *Antirrhinum majus* and were shown to play roles in regulating the expression of *SQUAMOSA* in early flower development (Klein et al. 1996). Since then, many SBP-box genes have been identified in other plants. The first functionally characterized *Arabidopsis* SBP-box gene was the *SQUAMOSA PROMOTER BINDING PROTEIN Like-3* gene (*SPL3*), which is involved in floral transition. *SPL3* also plays roles in the development of the inflorescence and floral organs (Gandikota et al. 2007). There are 16 SBP-box genes in the *Arabidopsis* genome (Birkenbihl et al. 2005), and they have diverse functions in development, especially in reproduction. For example, *SPL8* affects pollen sac development and plays a positive role in GA-mediated anther development (Unte et al. 2003; Zhang et al. 2007). In addition, *SPL8* and other *SPL* genes are expressed in the developing gynoecium, and they redundantly control the development of the female reproductive tract (Xing et al. 2013). *SPL2*, 9, 10, 11, and 15 have been shown to be involved in the shoot maturation and morphological changes during the reproductive phase (Schwarz et al. 2008; Shikata et al. 2009). Moreover, the distribution of trichomes during flowering is temporally regulated by *SPL3*, 9, 10, and 13 (Yu et al. 2010). *SPL7* is a central regulator for copper homeostasis because it activates the transcription of multiple genes involved in copper homeostasis (Yamasaki et al. 2009).

Several members of the SBP-box family have been functionally characterized in plants other than *Arabidopsis* and have been shown to have diverse functions in development. For instance, the two SBP-box genes *BpSPL1* and *CRR1* (*Copper Response Regulator 1*) were identified from *Betula pendula* and *Chlamydomonas*, respectively (Kropat et al. 2005; Lännpää et al. 2004). The *BpSPL1* protein binds to the promoter region of *BpMADS5*, a homolog of *Arabidopsis FRUITFULL* in *B. pendula*. The *CRR1* protein recognizes and binds to the GTAC core sequence in the promoters of *CYC6* and *CPX1*. These results indicate that apart from regulating the expression of floral meristem identity genes (*SQUAMOSA* and *API*), SBP-box genes also regulate the expression of other genes, and thus, have

diverse functions in plant development. Recent studies have shown that SBP-box genes play essential roles in fruit development and ripening. For instance, there are 19 SBP-box genes in the rice genome, and more than half of them are predominantly expressed in young panicles (Xie et al. 2006). *OsSPL16* has been shown to play roles in determining rice grain size, shape, and quality (Miura et al. 2010; Wang et al. 2012). In tomato, the SBP-box gene *Colorless Non-Ripening* (*CNR*) is critical for fruit ripening, and an epigenetic mutation in the promoter of this gene was shown to inhibit fruit ripening (Manning et al. 2006). A recent study showed that some miR156-targeted SBP-box genes regulate ovary and early fruit development in tomato (Silva et al. 2014).

MicroRNAs (miRNAs) are small, non-coding RNAs (22–24 nucleotide) that play crucial regulatory roles in development in plants and animals. They bind to the target sites of mRNAs for transcript cleavage or translational repression. About half of the target genes of miRNAs encode transcription factors. SBP-box genes are potential targets of miR156/157 family members (Bartel 2004; Hou et al. 2013; Rhoades et al. 2002). For example, it has been reported that 11 of the 19 SBP-box genes are targets of miR156 in rice (Xie et al. 2006). Similarly, more than half of the putative SBP-box genes in tomato, apple, and grape contain miR156 target sites (Hou et al. 2013; Li et al. 2013; Salinas et al. 2012). The target sites are located both in the coding region and in the 3'-untranslated region (3' UTR) (Hou et al. 2013; Li et al. 2013).

Although genome-wide analyses have been performed on SBP-box genes in *Arabidopsis*, rice, and other species (Hou et al. 2013; Li and Lu 2014; Li et al. 2013; Riese et al. 2007; Yang et al. 2008), a systematic analysis of this gene family has not been conducted for the genus *Prunus*, which includes many important ornamental and fruit trees. Also, little is known about the functions and expression patterns of SBP-box genes in *Prunus* flower and fruit development or about the evolution of SBP-box genes in related species, especially in two species belonging to the same genus. In this study, we first identified 15 SBP-box genes in the *P. mume* genome and then conducted comprehensive bioinformatics analyses of gene structure, phylogeny, conserved motifs, miRNA target sites, and chromosomal location. The driving forces for the evolution of orthologous genes in two *Prunus* species were also determined. Finally, we investigated the expression patterns of *P. mume* SBP-box genes in various organs, and in flowers and fruit at different developmental stages. The results of this study provide an overview of the organization of the SBP-box family in *P. mume* and other *Prunus* species. These results lay the foundation for further functional analyses of SBP-box genes in *P. mume* and further our understanding of the roles of SBP-box genes in regulating flower and fruit development.

Materials and methods

Identification and annotation of SBP-box genes in *P. mume*

The genome database of *P. mume* was downloaded from the *P. mume* genome project (<http://prunusmumegenome.bjfu.edu.cn/>). A Hidden Markov Model was constructed from the SBP-domain profile (PF03110) downloaded from the Pfam database (<http://pfam.janelia.org/>) and was used to search the *P. mume* genome database using HMMER software. The e-value threshold was 0.1 as recommended in the HMMER user's guide (Finn et al. 2011). All of the obtained protein sequences were submitted to Interpro (<http://www.ebi.ac.uk/interpro/>) to confirm the presence of the SBP domain. Sequences without the SBP domain were discarded from further analyses. The nomenclature of the putative SBP-box genes was according to their gene IDs. *Arabidopsis* SBP-box genes were downloaded from TAIR using the accession numbers reported by Yang (Yang et al. 2008). *P. persica* SBP-box genes were downloaded from the Plant Transcription Factor Database (<http://planttfdb.cbi.pku.edu.cn/index.php>, Jin et al. 2014).

Phylogenetic and sequence analyses

Multiple sequence alignment of SBP-box protein sequences from *Arabidopsis*, *P. mume* and *P. persica* was performed using ClustalX 2.0 with default parameters (Larkin et al. 2007), and then refined manually. Phylogenetic trees were constructed using MEGA 5.1 software using the neighbor-joining (NJ) method with 1000 bootstrap replications. The full-length protein sequences of *P. mume* SBP-box genes were analyzed using the MEME online tool (<http://meme.nbcr.net/meme/intro.html>) to identify conserved motifs. The parameters were as follows: number of repetitions, any; maximum number of motifs, 20; minimum motif width, 6 and maximum motif width, 80. The identified motifs were annotated by SMART (<http://smart.embl-heidelberg.de/>). Sequence logos were created using the Weblogo online application (<http://weblogo.threeplussone.com/>). Structural information for SBP-box genes was obtained from the *P. mume* and *P. persica* genome project. Diagrams of exon–intron structures were produced using Gene Structure Display Server 2.0 (<http://gsds.cbi.pku.edu.cn/index.php>).

Chromosomal location and miR156 target site prediction

The chromosomal locations of SBP-box genes were assessed according to *P. mume* and *P. persica* genome data. MapDraw

V2.1 was used to construct gene location diagrams (Liu and Meng 2003). To predict miR156 target sites, full-length *PmSBPs* nucleic acid sequences (including introns and UTRs) were analyzed using the psRNATarget online tool (<http://plantgrn.noble.org/psRNATarget/?function>).

Calculation of synonymous (Ks) and nonsynonymous (Ka) substitutions per site

Orthologous genes in *P. mume* and *P. persica* were identified from the phylogenetic tree and sequence similarity. Paralogous genes were inferred from the phylogenetic tree and duplicated blocks. Pairwise alignments of homologous nucleotide sequences were performed using the RevTrans 1.4 Server, which aligned the nucleotide sequences with the corresponding protein sequences as guides (Wernersson and Pedersen 2003). Ks and Ka values were estimated using K-estimator 6.1 V (Comeron 1999).

Plant materials

Plant materials for expression analyses were collected from *P. mume* ‘Sanlun Yudie’ at the Beijing Jiufeng International Plum Blossom Garden, Beijing, China (40°07'N, 116°11'E). The samples included various tissues (root, stem, leaf, sepal, petal, stamen, pistil, exocarp and mesocarp, endocarp, and seed), flower buds at different developmental stages (from flower primordium formation to anther and ovule development stages) and fruit at different developmental stages (from fruit set to ripening). The development stages of flower buds were identified from paraffin sections. Fruit at various developmental stages (FrS1–FrS6) was sampled at 10, 30, 60, 100, 125, and 140 days after blooming. All samples were collected, frozen in liquid nitrogen, and stored at –80 °C until RNA extraction.

Expression analyses

Total RNA was extracted from samples using TRIzol reagent (Invitrogen, USA) following the manufacturer's instructions. RNA was treated with RNase-free DNase (Promega, USA) to remove residual genomic DNA. First-strand cDNA was synthesized from 2 µg total RNA using a TIANScript First Strand cDNA Synthesis Kit (Tiangen, China) according to manufacturer's instructions. Real-time RT-PCR was conducted using the PikoReal real-time PCR system (Thermo Fisher Scientific, Germany). Reactions were carried out in a 20-µl volume containing 0.5 µl cDNA, 400 nM each primer (Online resource 1), and 10 µl SYBR Premix Ex Taq II (Takara, China) with the following conditions: 30 s at 95 °C, 40 cycles of 5 s at 95 °C, and 30 s at 60 °C. The specificity of the amplicon for each primer pair was verified by melting curve analysis. All real-time

Table 1 SBP-box gene family in the *Prunus mume* genome

Name	Gene ID	Locus	Strand	Length ^a	MW (kDa) ^b	Introns
PmSBP1	Pm002693	Pa1:20969892:20973764	+	383	41.11	2
PmSBP2	Pm007020	Pa2:20851318:20853843	–	480	52.58	3
PmSBP3	Pm007035	Pa2:20960217:20961741	–	316	34.59	2
PmSBP4	Pm008092	Pa2:30819676:30826337	–	816	91.52	9
PmSBP5a	Pm008474	Pa2:33454254:33467634	–	1027	114.22	9
PmSBP5b	Pm008475	Pa2:33461309:33467634	–	1034	114.85	9
PmSBP6	Pm010075	Pa3:2404865:2406231	+	161	18.53	1
PmSBP7	Pm011381	Pa3:10704691:10705520	+	162	18.45	1
PmSBP8	Pm014154	Pa4:10035344:10036476	+	189	21.59	1
PmSBP9	Pm016138	Pa4:23333197:23334845	+	389	42.99	2
PmSBP10	Pm017777	Pa5:14035545:14037080	+	327	36.22	2
PmSBP11	Pm017778	Pa5:14045497:14048386	–	425	46.82	2
PmSBP12	Pm022881	Pa7:116896:121543	–	1070	118.28	9
PmSBP13	Pm024528	Pa7:12867067:12869465	–	403	44.26	2
PmSBP14	Pm025028	Pa7:15633103:15635711	+	551	60.26	2
PmSBP15	Pm030597	scaffold56:960704:963861	+	488	54.05	2

^a The length of *P. mume* SBP-box proteins

^b Molecular weight

RT-PCR experiments were performed with three biological replicates, and each replicate was analyzed in triplicate. The relative expression levels were calculated using the $2^{-\Delta\Delta C_t}$ method, with the protein phosphatase 2A (*PP2A*) gene of *P. mume* as the reference gene.

Results

Identification and chromosomal location of SBP-box genes in *P. mume*

To identify the SBP-box genes in *P. mume*, we conducted HMMER searches against the *P. mume* protein database using the SBP-domain profile PF03110 as the search query. Initially, 16 protein sequences were obtained. However, two of them were located at the same genome locus (Pm008474 and Pm008475) and were the results of alternative splicing of one gene. They were named PmSBP5a and 5b, and only one form (PmSBP5b) was included in the following analyses (Table 1). The Interpro analysis revealed that all 15 sequences contained an SBP-domain. Therefore, the *P. mume* genome contained at least 15 SBP-box genes. These genes were designated as *PmSBP1* to *PmSBP15*, with *PmSBP5* represented by two forms (Table 1, Online resource 2). As shown in Table 1, the PmSBP proteins varied greatly in their length and molecular weight. The protein length ranged from 161 to 1070 amino acids and the molecular weight from 18.45 to 118.28 kDa.

The conserved domains of PmSBP proteins were identified by Interpro software and aligned by ClustalX 2.0. All

15 SBP-box proteins contained the complete SBP domain; the domain consisted of 76 amino acids and had two zinc-binding sites and one bipartite nuclear localization signal (Fig. 1). All SBP domains contain two zinc fingers. The first zinc finger was C3H for all of the SBPs except for PmSBP4; the first zinc finger of PmSBP4 was C4. The second finger of all SBPs was CCHC.

Based on the *P. mume* genome data, we located 14 SBP-box genes on chromosomes. One gene was located on an anchored scaffold. The SBP-box genes were unevenly distributed across the *P. mume* genome (Fig. 2). There were four SBP-box genes located on the longest chromosome, Chr 2, and none on Chr 6 and Chr 8. The gene distribution pattern was similar in *P. persica* (Online resource 3). Five *P. persica* SBP-box genes were located on Chr 1, the longest chromosome in *P. persica*, and none was located on Chr 8. Three *P. mume* SBP-box genes, *PmSBP 3*, *9*, and *13*, were located in duplicated blocks. Their orthologous genes in *P. persica* (*ppa023657 m*, *ppa007202 m*, and *ppa006611 m*; Fig. 3) were also located in duplicated regions. None of the *P. mume* SBP-box genes was tandemly duplicated, but the *P. persica* genome contained one tandemly duplicated pair of SBP-box genes (*ppa000690 m* and *ppa000792 m*).

Phylogenetic analysis

To examine the phylogenetic relationships of SBP-box genes between *Prunus* and *Arabidopsis*, we constructed a phylogenetic tree based on multiple sequence alignments of *P. mume*, *P. persica*, and *Arabidopsis* SBP-box proteins (Fig. 3). The 48 SBP-box genes formed nine groups

Fig. 1 Multiple sequence alignment and sequence logo of the *P. mume* SBP-box domain. Multiple sequence alignment was performed using DNA-MAN. The two conserved zinc finger and NLS are indicated. The sequence logo was obtained from Weblogo online software. The overall height of the *stack* indicates the sequence conservation at that position

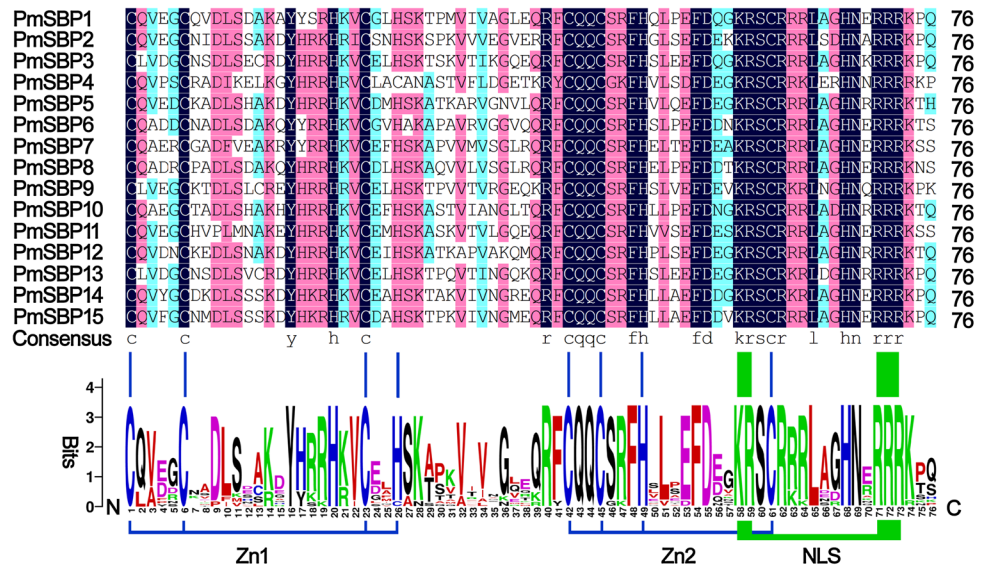
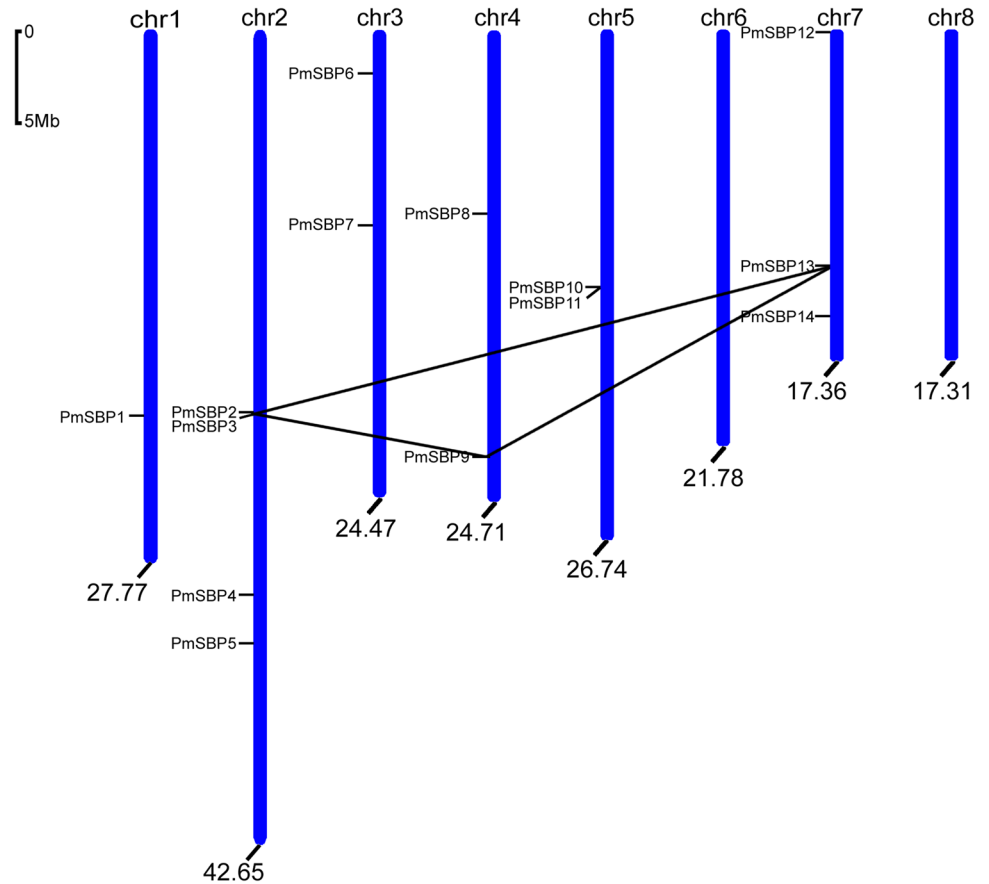


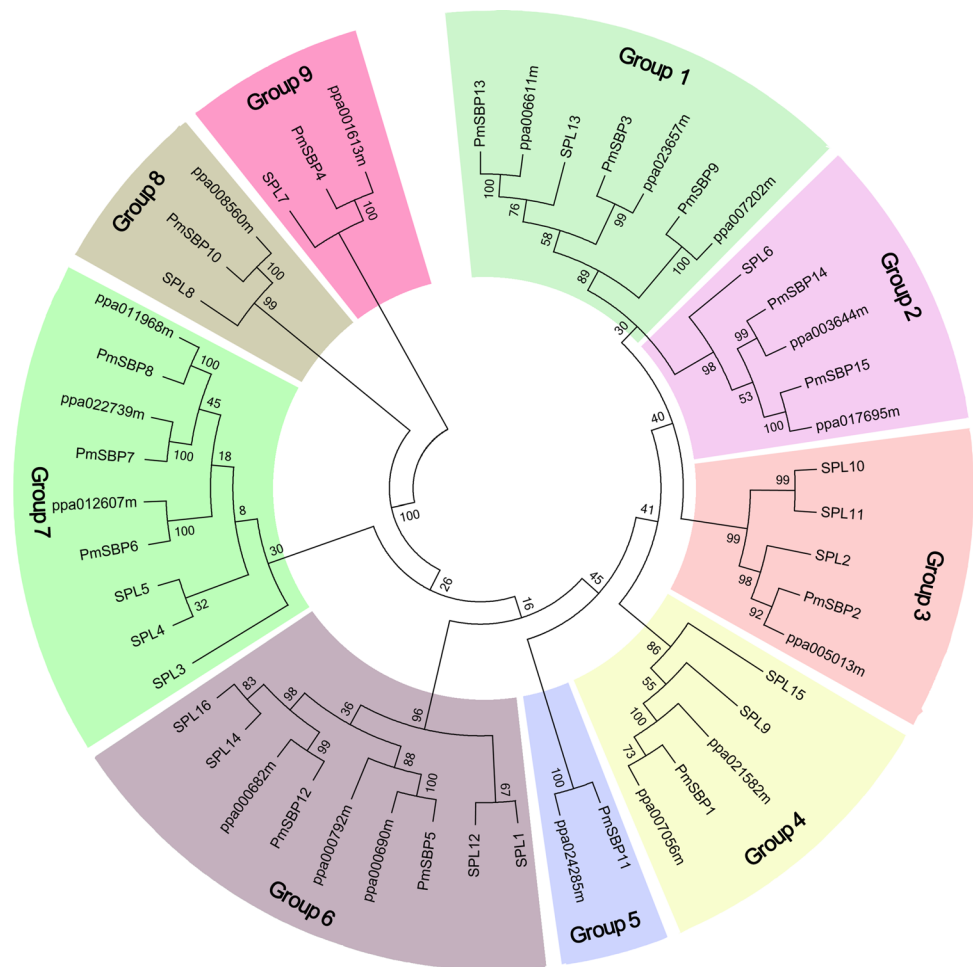
Fig. 2 Chromosomal locations of SBP-box genes on *P. mume* chromosomes. The scale refers to a 5-Mb chromosomal distance. Segmentally duplicated genes are linked by *black lines*



in the unrooted phylogenetic tree. The *Prunus* SBP-box genes were distributed in all nine groups and grouped with their *Arabidopsis* counterparts in eight groups (Fig. 3). Group 5 contained genes only from *P. mume* and *P. persica*, indicating that this *Prunus*-specific subfamily had emerged after the divergence of *Prunus* and *Arabidopsis*.

Every *P. mume* SBP-box gene except for *PmSBP1* and 5 had an orthologous gene in *P. persica*; both *PmSBP1* and 5 had two orthologous genes in *P. persica*. This phenomenon suggested that most SBP-box genes existed before the divergence of *P. mume* and *P. persica* and the orthologous genes of *PmSBP1* and 5 duplicated after their divergence to

Fig. 3 Unrooted phylogenetic tree of SBP-box genes from *Prunus* and *Arabidopsis*. The tree was generated using MEGA 5.1 with the neighbor-joining method. Multiple sequence alignment of the SBP-box proteins was performed using clustal X2.0. Numbers above branches indicate bootstrap value



give rise to two new paralogs. Some *Arabidopsis* SBP-box genes in groups 1, 2, and 4 had two or more *Prunus* paralogs, indicating that *Prunus* SBP-box genes duplicated and diversified after the divergence of *Arabidopsis* and *Prunus*. Each *P. mume* SBP-box gene in groups 8 and 9 had one orthologous gene in *P. persica* and one in *Arabidopsis*. The dendrogram suggested that different subfamilies may have undergone different evolutionary processes, and some subfamilies underwent species-specific evolutionary processes after speciation.

Gene structure analysis and miR156 target site prediction

The exon–intron structures of the *Prunus* SBP-box genes were generated based on their corresponding coding sequences and genome sequences (Fig. 4). To assess the structural diversity among different groups, we constructed an unrooted phylogenetic tree using *P. mume* and *P. persica* SBP proteins. The classification of the *Prunus* SBP-box genes corresponded to the phylogenetic groups described above. The number of introns differed among the

different groups. Group 7 genes contained only one intron while genes in groups 6 and 9 had nine introns. Genes in the other groups had two to four introns. Most of the *Prunus* SBP-box genes within a group shared a similar exon–intron composition. For example, all genes in group 4 had two introns, all those in group 7 had only one intron, and all those in group 3 had three introns. However, the exon–intron structure of some genes differed from that of other members of the same group. For instance, all group 1 members had two introns except for *ppa007202 m*, which had another short intron next to the last exon, resulting in three introns in total.

Eleven miR156 family members (Pmu-miR156a-k) have been identified in *P. mume* (Wang et al. 2014). Multiple sequence alignments of the *P. mume* SBP-box genes and reverse complement sequences of Pmu-miR156 showed that 11 *PmSBPs* contained sequences complementary to the Pmu-miR156 mature sequences, with a maximum of one to three mismatches (Fig. 5). The miR156 target sites were located in the coding regions in eight genes (*PmSBP1*, 2, 3, 9, 11, 13, 14, and 15), but in the 3' UTR in group 4 genes (*PmSBP6*, 7, and 8), like in their orthologous genes

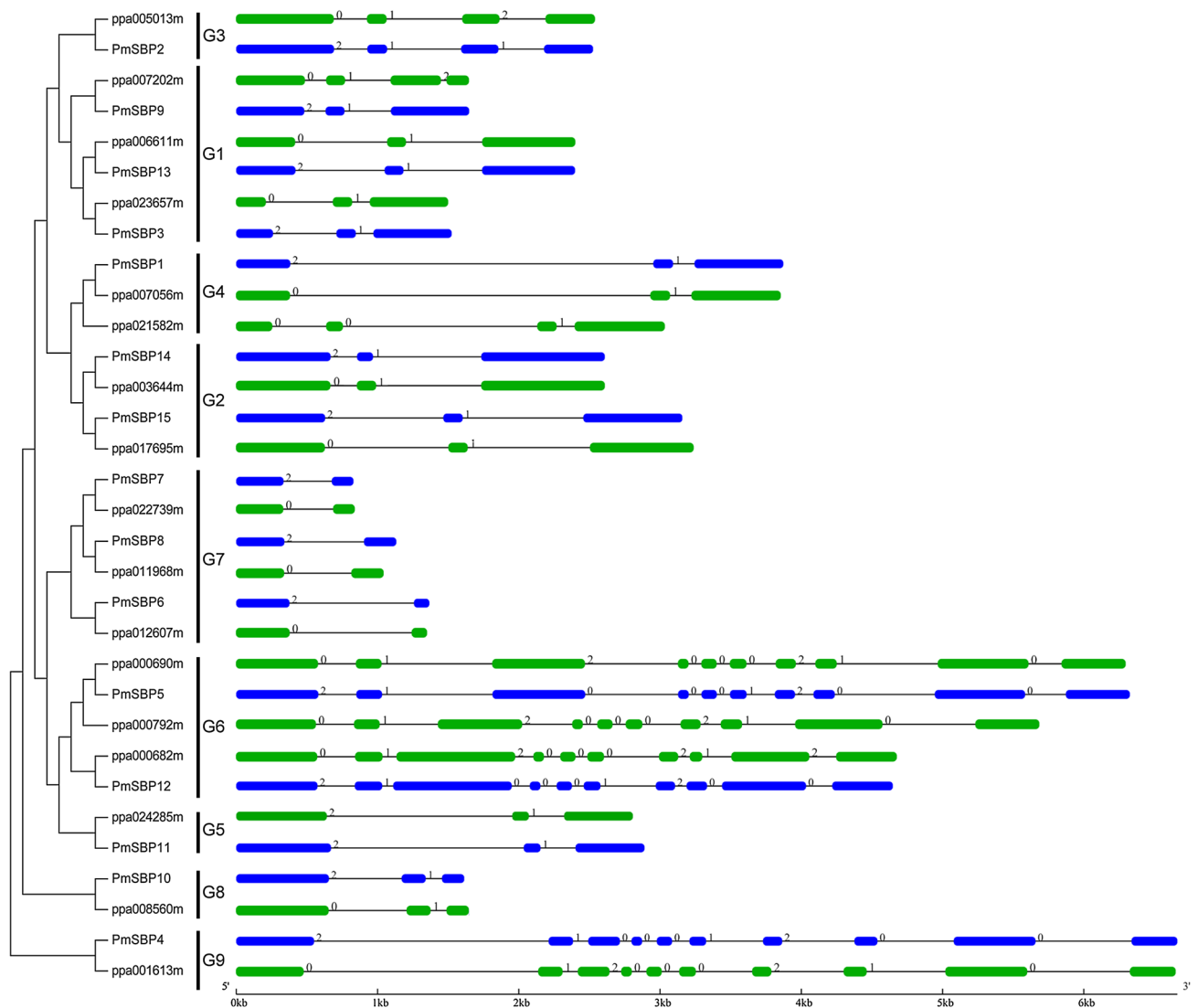


Fig. 4 Exon-intron structures of *P. mume* and *P. persica* SBP-box genes. Phylogenetic tree was constructed based on the *P. mume* and *P. persica* SBP-box proteins. Exons of *P. mume*, exons of *P. persica*, and

introns are indicated by blue rounded rectangles, green rounded rectangles, and black lines, respectively. The scale represents the lengths of the genes, exons, and introns (color figure online)

(*SPL3*, 4 and 5) in *Arabidopsis* (Guo et al. 2008). All genes belonging to a group (including *Prunus* and *Arabidopsis* genes) contained the miR156 target site, and the target site locations of group members were similar. This result suggested that the miR156 target sites existed before the divergence of *Prunus* and *Arabidopsis* and were retained during the gene duplication and diversification process.

Conserved motif analysis

To examine conserved sequences other than the SBP-domain and to assess the composition and distribution of conserved sequences in *P. mume* SBP-box proteins, we used the online MEME tool to identify motifs in the 15 *P. mume* SBP-box proteins. The number of motifs varied

greatly among the *P. mume* SBP-box genes (Fig. 6); there were 12 in PmSBP5 but only 1 in each of PmSBP6, 7, and 8.

As shown in Fig. 6, only motif 1, which specifies the SBP-domain, was present in all sequences, indicating that no common domain except the SBP existed in the 15 *P. mume* SBP-box genes. Members of the same group usually had similar motif composition. Some groups had unique motifs; for example, both PmSBP14 and 15 (members of group 2) contained motifs 16, 5, 1, 8, 3, and 6 and the distribution of the motifs was similar. Motifs 4, 12, and 20 were restricted to group 6 while motif 16 was unique to group 2. Some motifs were shared by different groups. For instance, motif 5 was present in some members of groups 1, 2, 3, 4, 5, and 6. Motif 3 was encoded by a conserved sequence

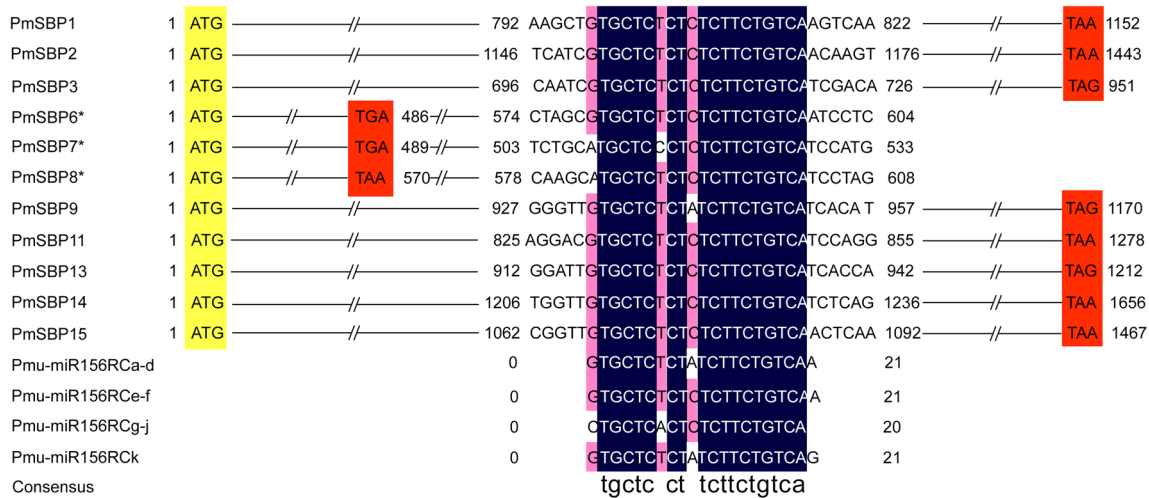


Fig. 5 Multiple sequence alignment of Pmu-miR156 complementary sequences with *P. mume* SBP-box genes. Multiple sequence alignment was performed using DNAMAN software. Numbers indicate the position of the nucleotide. *Yellow block* highlights the start

codons while *red blocks* highlight the stop codons. Genes bearing miR156 target site in the 3'UTR are marked with *asterisks* (color figure online)

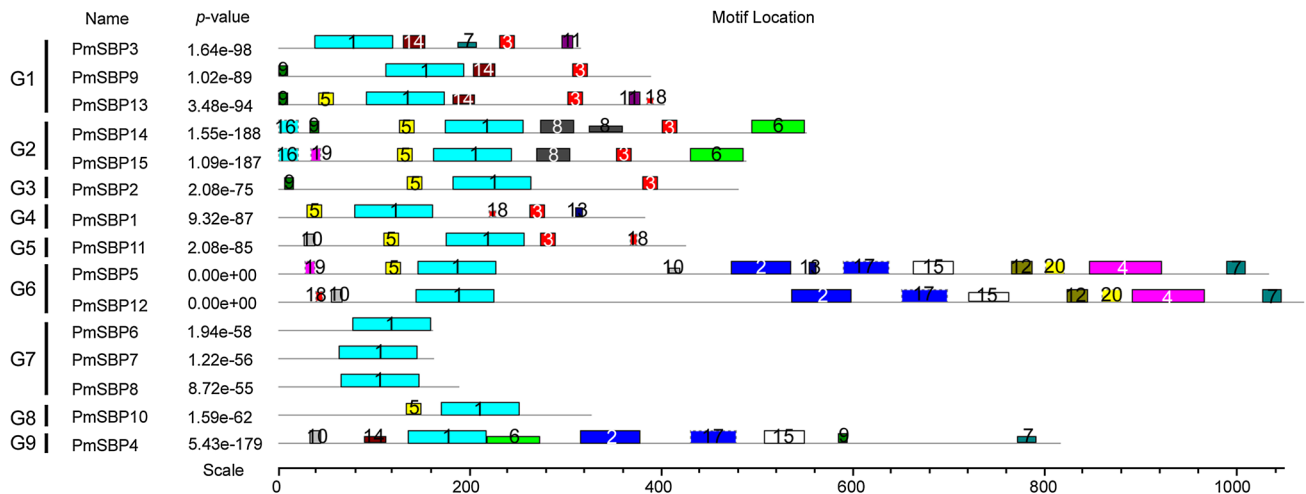


Fig. 6 Motif distribution of *P. mume* SBP-box genes. Twenty motifs were identified by MEME online tool. Each motif is represented by a *colored block* with a number. The lengths and positions of the blocks

are consistent with the lengths and positions of motifs in the protein sequences (color figure online)

that contained the miR156 target site. This sequence was shared by all of the miR156-targeted genes with the target site located in the coding region (*PmSBP1*, 2, 3, 9, 11, 14, and 15).

Driving forces for divergence of SBP-box genes in *Prunus*

To explore the degree of sequence diversification and determine which selection pressures drove the evolution of SBP-box genes after the split of *P. mume* and *P. persica*, we calculated the Ka, Ks, and Ka/Ks for each of the 15 orthologous gene pairs and 8 paralogous gene pairs. As a

general rule, a Ka/Ks ratio of >1 implies positive selection, a ratio of 1 may indicate neutral evolution, and a ratio of <1 implies purifying selection. All the Ka/Ks ratios of orthologous genes were <1 (Table 2), suggesting that SBP-box genes have evolved mainly under purifying selection after the divergence of *P. mume* and *P. persica*. The Ka/Ks ratios of the eight paralogous gene pairs were also <1. This result indicated that purifying selection was also involved in driving gene diversification after duplication events. In general, the values of Ka and Ks were smaller for orthologous gene pairs than for paralogous gene pairs; therefore, the degree of divergence was smaller for orthologous sequences than for paralogous sequences. We also calculated the Ka, Ks,

Table 2 Ka, Ks, and Ka/Ks ratios for orthologous and paralogous SBP-box genes

Gene pair	Total			SBP domain			Out of SBP domain		
	Ka	Ks	Ka/Ks	Ka	Ks	Ka/Ks	Ka	Ks	Ka/Ks
PmSBP1-ppa007056m	0.0093	0.0310	0.30	0	0.0132	0.00	0.0117	0.0351	0.33
PmSBP2-ppa005013m	0.0137	0.0270	0.51	0.0118	0	–	0.0141	0.0317	0.44
PmSBP3-ppa023657m	0.0179	0.0307	0.58	0.0118	0.0135	0.87	0.0200	0.0350	0.57
PmSBP4-ppa001613m	0.0081	0.0220	0.37	0	0	0.00	0.0089	0.0244	0.36
PmSBP5- ppa000690m	0.0334	0.0904	0.37	0	0.0135	0.00	0.0361	0.0962	0.38
PmSBP6-ppa012607m	0.0144	0.0416	0.35	0.0068	0.0433	0.16	0.0212	0.0400	0.53
PmSBP7-ppa022739m	0.0080	0.0462	0.17	0	0.0290	0.00	0.0152	0.0614	0.25
PmSBP8-ppa011968m	0.0179	0.0715	0.25	0	0.1027	0.00	0.0299	0.0505	0.59
PmSBP9-ppa007202m	0.0208	0.0435	0.48	0.0120	0.0760	0.16	0.0232	0.0367	0.63
PmSBP10-ppa008560m	0.0067	0.0660	0.10	0	0.0291	0.00	0.0088	0.0773	0.11
PmSBP11-ppa024285m	0.0066	0.0440	0.15	0	0.0687	0.00	0.0081	0.0413	0.20
PmSBP12-ppa000682m	0.0058	0.0388	0.15	0	0.0274	0.00	0.0062	0.0394	0.16
PmSBP13- ppa006611m	0.0045	0.0299	0.15	0	0.0134	0.00	0.0056	0.0332	0.17
PmSBP14-ppa003644m	0.0079	0.0293	0.27	0.0067	0.0134	0.50	0.0081	0.0292	0.28
PmSBP15-ppa017695m	0.0138	0.0210	0.66	0.0117	0	–	0.0142	0.0247	0.57
ppa007056m-ppa021582m	0.0905	0.2336	0.39	0.0052	0.1011	0.05	0.1140	0.2682	0.43
ppa000690m-ppa000792m	0.0910	0.2496	0.36	0.0326	0.0727	0.45	0.0928	0.2593	0.36
PmSBP3- PmSBP9	0.6994	1.3388	0.52	0.1471	0.8835	0.17	0.9678	1.5600	0.62
PmSBP3- PmSBP13	0.3624	1.2844	0.28	0.0769	0.8497	0.09	0.8497	1.8119	0.47
PmSBP9- PmSBP13	0.6134	1.3742	0.45	0.1322	0.8996	0.15	0.8343	1.6081	0.52
ppa023657m-ppa007202m	0.5922	1.5112	0.39	0.1553	0.9258	0.17	0.9296	2.2017	0.42
ppa023657m- ppa006611m	0.4986	1.5511	0.32	0.0786	0.8487	0.09	0.7361	1.9741	0.37
ppa007202m- ppa006611m	0.5395	1.3739	0.39	0.1382	1.0853	0.13	0.8088	1.5825	0.51

and Ka/Ks ratios for sequences inside and outside the SBP domain. As shown in Table 2, the Ka values of most orthologous SBP domains were zero or near zero, suggesting that the amino acid sequences of orthologous SBP domains were less diverged. For most gene pairs, the Ka/Ks ratios were smaller for the SBP domain than for the regions outside the SBP domain. These results implied that the SBP domain was under stronger purifying selection and evolved more slowly than did the region outside the SBP domains.

Expression profiles of *P. mume* SBP-box genes in different organs

To elucidate the potential roles of *P. mume* SBP-box genes in organ development, the expression profiles of the 15 SBP-box genes in ten organs (root, stem, leaf, four whorls of flower, and three parts of fruit) were determined by quantitative RT-PCR. There were diverse transcription patterns of the *PmSBPs* among the different organs (Fig. 7). Out of the ten organs analyzed, more than four organs contained *PmSBPs* transcripts, indicating that some *P. mume* SBP-box genes play roles in the development of different organs. Nine genes (*SBP1*, 2, 3, 4, 5, 8, 12, 13, and 14) were expressed in all organs analyzed, while six genes

(*SBP6*, 7, 9, 10, 11, and 15) showed organ-specific expression patterns.

Further analysis of the expression patterns of the *PmSBPs* in each group showed that some genes in a group exhibited similar expression patterns or had specific predominant-expression organ. For example, transcripts of the two genes in group 6, *PmSBP5* and 12, were detected in all organs at relatively stable levels. Although three genes in group 7 (*PmSBP6*, 7, and 8) showed different expression patterns, all of them were strongly expressed in reproductive organs (flower and fruit) but weakly expressed in vegetative organs (root, stem, and leaf). In contrast, the expression patterns of three duplicated genes (*PmSBP3*, 9, and 13) differed in the flower. *PmSBP3* and 13 transcripts were detected in all floral organs, with the highest transcript levels in the sepal and pistil, respectively. However, no *PmSBP9* transcripts were detected in the petal. Some genes in different groups had the same predominantly expressed organs. For example, *PmSBP1*, *PmSBP7*, *PmSBP10*, and *PmSBP15*, which belonged to four different groups, were predominantly expressed in the pistil. *PmSBP2*, *PmSBP6*, *PmSBP8*, and *PmSBP9* were predominately expressed in the exocarp and mesocarp, indicating they play roles in fruit development.

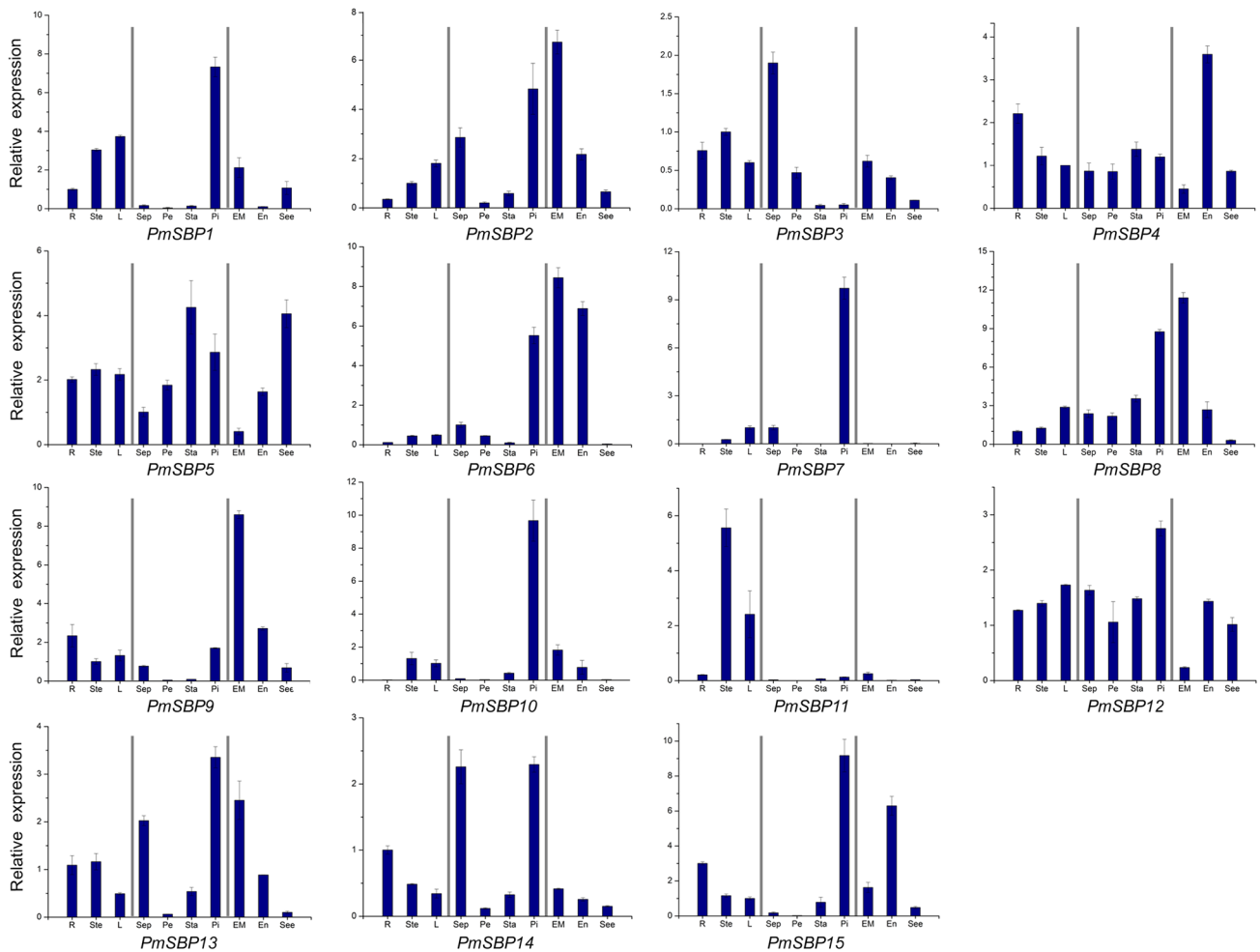


Fig. 7 Expression profiles of *P. mume* SBP-box genes in different organs. Total RNA was extracted from the root (R), stem (Ste), leaf (L), sepal (Sep), petal (Pe), stamen (Sta), pistil (Pi), exocarps and mesocarps (EM), endocarps (En), and seed (Se). The expression levels of the SBP-box genes were assessed by quantitative real-time RT-

Expression patterns of *P. mume* SBP-box genes during flower bud differentiation and fruit development

Previous studies have revealed that SBP-box genes play key roles in flower and fruit development (Cardon et al. 1997; Manning et al. 2006). To investigate their roles in *P. mume* flower and fruit development, we conducted quantitative real-time RT-PCR analyses to determine the relative expression levels of the 15 *PmSBPs* during flower bud differentiation and fruit development. Based on analyses of paraffin sections (Online resource 4), we divided the flower bud differentiation of *P. mume* into six stages: flower primordium formation (FIS1), sepal initiation (FIS2), petal initiation (FIS3), stamen initiation (FIS4), pistil initiation (FIS5), and anther and ovule development (FIS6). Samples were collected at each of the six stages. The fruit development process was also divided into six stages (FrS1–FrS6,

PCR. The *Protein Phosphatase 2A (PP2A)* gene of *P. mume* was used as reference gene. Three independent biological replicates were performed, and each replicate was measured in triplicate. The error bars show the standard deviation of the results of three technical replicates

online resource 5), corresponding to 10, 30, 60, 100, 125, and 140 days after blooming. FrS1–FrS3 represented the fruit development stage and FrS4–FrS6 the fruit ripening stage.

All of the genes except for *PmSBP11* showed high expression levels in the flower bud (Fig. 8), indicating that most *PmSBPs* played important roles in flower bud differentiation. The *PmSBPs* could be classified into four expression clusters according to their transcriptional patterns. Cluster one included five genes (*PmSBP6*, *11*, *12*, *14*, and *15*) that showed steady expression levels during flower bud differentiation. Cluster two comprised three genes (*PmSBP1*, *9*, and *13*) that were up-regulated during flower differentiation. The gene in cluster three (*PmSBP3*) showed decreased transcript levels after the second stage. The genes in cluster four (*PmSBP2*, *4*, *5*, *7*, *8*, and *10*) showed high transcript levels at some stages and low transcript levels

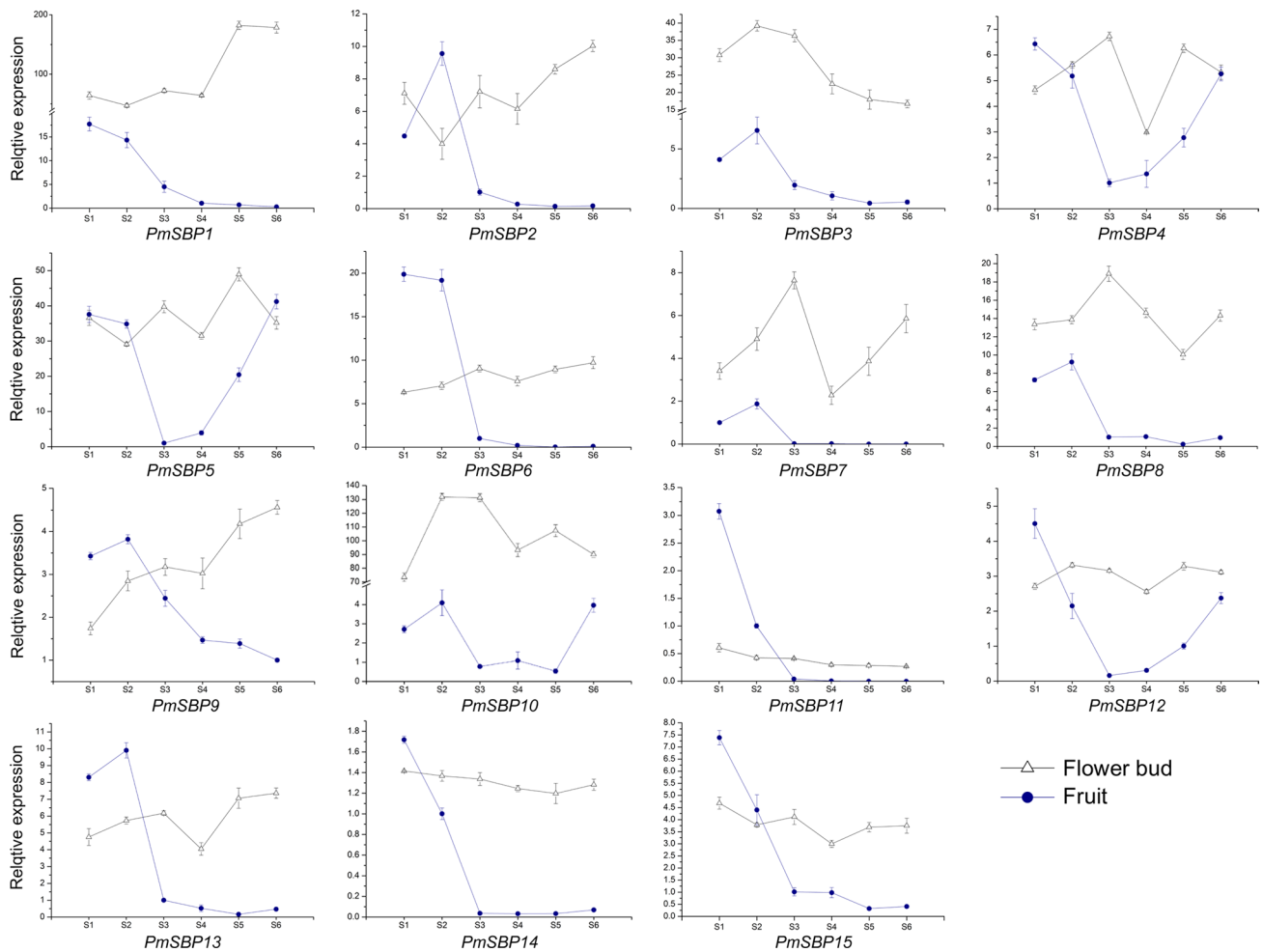


Fig. 8 Expression patterns of *P. mume* SBP-box genes during flower bud and fruit development. S1–S6 indicate development stages (stage1–stage6) of flower bud and fruit. White triangles and navy circles represent the expression levels of SBP-box genes in flower bud

at other stages. This “wave” pattern of transcript accumulation during flower bud differentiation may indicate that they had roles in the development of specific organs.

Generally, the expression levels of *PmSBPs* were higher in the fruit development stages (FrS1–FrS3) than in the fruit ripening stages (FrS4–FrS5). As shown in Fig. 8, 11 genes (*PmSBP1*, 2, 3, 6, 7, 8, 9, 11, 13, 14, and 15) showed high transcript levels in the fruit development stages and then decreased transcript levels during fruit ripening. Among them, six genes (*PmSBP2*, 3, 7, 8, 9 and 13) showed the highest transcript levels at the FrS2 stage while five genes (*PmSBP1*, 6, 11, 14, and 15) showed the highest transcript levels at the FrS1 stage. *PmSBP7* transcripts could not be detected after the FrS3 stage, and *PmSBP6* and 11 transcripts could not be detected after the FrS4 stage, indicating that they did not play a role in fruit ripening. In contrast, four genes (*PmSBP4*, 5, 10, and 12) were

and fruit, respectively. The error bars show the standard deviation of the results of three technical replicates. All expression levels of genes were normalized against *PmPP2A* expression

down-regulated during the fruit development stages but up-regulated during the fruit ripening stages.

Discussion

SBP-box genes in *P. mume* and their evolution

SBP-box genes are plant-specific transcription factors that evolved before the divergence of green algae and the ancestor of land plants (Cardon et al. 1999; Guo et al. 2008). The number of SBP-box genes in the genome varies among land plants. For instance, there are 27 SBP-box genes in apple, 28 in *Populus trichocarpa*, and 15 in *Castor Bean* (Li and Lu 2014; Li et al. 2013; Zhang and Ling 2014). In this study, we identified 15 SBP-box genes in the *P. mume* genome. In the phylogenetic tree of SBP-box genes from

two *Prunus* species and *Arabidopsis*, the genes formed nine groups, one of which was specific to *Prunus*. This classification was consistent with gene structure and motif composition. Genes within a group shared a similar length, structure, motif distribution, and miR156 target site location. However, there were variations in gene length, structure, and motif composition among the different groups. For example, group 6 genes contained nine introns and were longer than 4500 bp, while group 7 genes contained only one intron and were shorter than 2000 bp (Fig. 4). Therefore, the classification and evolution of SBP-box genes might be closely related to their structural divergence and diversification.

The phylogenetic analysis revealed that each of the two *P. mume* SBP-box genes, *PmSBP1* and *PmSBP5*, had two orthologous genes in *P. persica* (Fig. 3). This result indicated that the orthologous genes of *PmSBP1* and 5 in *P. persica* duplicated after the divergence of *P. mume* and *P. persica*, whereas *PmSBP1* and 5 did not. In fact, the *PmSBP5* orthologs ppa000690 m and ppa000792 m arose from tandem duplication. Different gene duplication rates resulted in different numbers of SBP-box genes in the genomes of these two *Prunus* species. As indicated in Fig. 3, group 1 contained one *Arabidopsis* SBP-box gene, three *P. mume* genes, and three *P. persica* genes. Both the *P. mume* genes and the *P. persica* genes were derived from segmental duplication, while the *Arabidopsis* gene in this group had not undergone a duplication event. It has been reported that *SPL10* and *11* were tandemly duplicated genes and that *SPL4/5*, *SPL1/12*, and *SPL10/11* were located in segmental duplication regions (Hou et al. 2013; Yang et al. 2008). However, their homologs in *Prunus* have not undergone a duplication event. These results indicate that genes in different groups showed different duplication histories, and some showed a species-specific expansion pattern and duplication rate. The present SBP-box genes in *Prunus* and *Arabidopsis* may have originated from a few ancestral genes that existed before the divergence of *Prunus* and *Arabidopsis*, via several different duplication events.

A previous study reported that the SBP-domain of paralogous genes has been under purifying selection while some regions outside the SBP domain have been under positive selection or relaxed purifying selection (Yang et al. 2008). However, the selection pressures during the evolution of orthologous SBP-box genes are still unclear. In this study, we calculated K_a , K_s , and K_a/K_s values for 15 orthologous gene pairs from *P. mume* and *P. persica*. The results indicated that purifying selection has played an important role in the evolution of orthologous SBP-box genes. Both the SBP-domain and the outside region have been under purifying selection. The K_a and K_s values implied that there was less sequence divergence between two *Prunus* orthologous

genes than between two paralogous genes, because the K_a and K_s values of orthologous gene pairs were nearly zero, substantially lower than those for paralogous gene pairs (Table 2). Purifying selection is the selective removal of harmful variants and is largely responsible for maintenance of functions (Du et al. 2013; Peng et al. 2012). Thus, based on these results, we concluded that the functions of the SBP-box genes did not change substantially after the split of *P. mume* and *P. persica*. These results suggest that the functions of orthologous SBP-box genes have been maintained during the evolution of the *Prunus* genus.

Expression patterns of SBP-box genes in *P. mume*

The expression pattern of a gene is often correlated with its function (Li and Lu 2014). We determined the expression profiles of the 15 *P. mume* SBP-box genes in different organs, during flower bud differentiation and fruit development by quantitative real-time RT-PCR. The results indicated that *P. mume* SBP-box genes may be involved in the development of diverse organs, because the 15 genes showed distinct expression patterns among different organs. The expression profiles of *P. mume* SBP-box genes were similar to those of their orthologs in *Arabidopsis*, suggesting that their function has been conserved. For example, *SPL2*, *10*, and *11*, the *PmSBP2* orthologs in *Arabidopsis*, were expressed in all tissues including roots, juvenile leaves, adult leaves, cauline leaves, inflorescence stems, flowers, and fruits, with low expression levels in vegetative organs (Shikata et al. 2009). Similarly, *PmSBP2* transcripts were detected in all organs analyzed, with low transcript levels in the root, leaf, and stem. It was reported that *SPL2*, *10*, and *11* control morphological changes and shoot maturation in the reproductive phase (Shikata et al. 2009). Therefore, *PmSBP2* may play roles in morphogenesis or shoot development in *P. mume*. A recent study showed that *SPL8* together with other miR156-targeted *SPL* genes controlled gynoecium patterning and that *SPL8* was highly expressed in the inflorescence and silique (Xing et al. 2013). Likewise, there were high transcript levels of *PmSBP10*, the ortholog of *SPL8*, in the pistil and fruit, implying that it may play a role in gynoecium development.

The duplicated genes in *P. mume* showed different expression patterns. As shown in Fig. 7, the expression patterns of three group 1 members (*PmSBP3*, *9*, and *13*) that arose from segmental duplication events were different from each other. Also, their expression profiles varied considerably during flower bud differentiation (Fig. 8). Previous studies have shown that duplicated genes can be expressed differently. We also observed a divergence in expression profiles between duplicated MADS-box genes (Li et al. 2005; Xu et al. 2014). The different expression patterns of *PmSBP3*, *9*, and *13* indicated that there is also

divergence in expression patterns between duplicated SBP-box genes. The chromosome location and phylogenetic analyses indicated that the ancestor of *PmSBP3*, *9*, and *13* underwent a segmental duplication event to give rise to these three genes, while its ortholog in *Arabidopsis* (*SPL13*) did not. According to the AtGenExpress Visualization Tool (<http://www.weigelworld.org/resources/microarray/AtGenExpress/>) (Schmid et al. 2005), the transcript levels of *SPL13* were high in the flower and leaf, moderate in the root, and low in the seed. Although it was highly expressed in the flower, the transcript levels varied widely among the different floral organs. There were high transcript levels in the sepal, petal, and pistil, but very low transcript levels in the stamen and mature pollen. Therefore, the ancestral gene of *PmSBP3*, *9*, and *13* should have a similar expression pattern to that of *SPL13*. It appears that the three duplicated genes had partial functions of their ancestral gene. As shown in Fig. 7, their expression patterns were similar to those of their ancestral gene in the vegetative organs and seed. However, in the floral organs, the combination of their expressions was equal to that of their ancestral gene. Previous studies on shifts in expression patterns have suggested that duplicated regulatory genes may undergo subfunctionalization (where both copies retain partial function of their ancestral gene and their total functions represent that of their ancestral gene) and/or neofunctionalization (one copy gains a new function while the other copy retains the origin function). In either case, the surviving duplicates will be under strong purifying selection pressure (Duarte et al. 2006; Lynch and Conery 2000). The expression profiles and Ka/Ks ratios of *PmSBP3*, *9*, and *13* indicate that they might have undergone subfunctionalization after the duplication events and that purifying selection has driven the evolution and expression divergence of the three duplicates.

The *PmSBPs* were expressed during flower bud differentiation, implying that most of these genes play roles in flower development. All of them showed relatively high transcript levels in the flower bud, except for *PmSBP11* (Fig. 8). *PmSBP11* belongs to group 5, a *Prunus*-specific group, and has no orthologous gene in *Arabidopsis*. It might play roles in vegetative organs and in the development of young fruit, because its transcripts were detected in the root, leaf, stem, and fruit at early developmental stages (Figs. 7, 8). Previous studies have shown that an SBP-box gene *CNR* control fruit ripening in tomato (Karlova et al. 2013; Manning et al. 2006). A recent study showed that miR156-targeted SBP-box genes also regulate early fruit development in tomato (Silva et al. 2014). The expression profiles of the *P. mume* SBP-box genes suggested that they play roles in fruit development in both the early and ripening stages. All 15 genes showed high transcript levels in fruit at stages 1 and 2 and were down-regulated

continuously during the early development process. Only four genes were up-regulated after stage 3 (Fig. 8). These findings indicate that all SBP-box genes are involved in fruit development; most play roles in early fruit development, while four play roles in fruit ripening.

Different expression patterns of miR156-targeted and -nontargeted SBP-box genes in *P. mume*

Previous studies confirmed that more than half of the miRNA targets are transcription factors such as SBP, MYB, ARF, HD-zip, TCP, and AP2 (Aukerman and Sakai 2003; Martín-Trillo and Cubas 2010; Rhoades et al. 2002). Consistent with previous studies, 11 of the 15 *P. mume* SBP-box genes contained putative miR156 target sites located in the coding region or the 3' UTR. These two locations of miR156 target sites have also been observed in SBP-box genes in other plants (Arazi et al. 2005; Guo et al. 2008; Riese et al. 2007; Sun et al. 2013), suggesting that they were present in the ancestors of SBP-box genes. The interaction between miR156 and SBP-box genes is a conserved and ancient regulatory mechanism that has been retained during evolution.

The miR156-targeted genes showed organ-specific or somewhat organ-specific expression patterns, whereas all of the miR156-nontargeted genes except for *PmSBP10* were expressed ubiquitously and constitutively in all organs examined (Fig. 7). Similar expression patterns were reported for grape (Hou et al. 2013). This phenomenon suggests that miR156 may regulate the organ-specific expression patterns of SBP-box genes. In addition, there were different expression patterns among the SBP-box genes regulated by miR156. For example, there were high transcript levels of *PmSBP6* in the pistil and fruit, while *PmSBP7* showed high transcript levels in the pistil but was not detected in fruit. This indicates that different miR156-targeted genes are regulated differently or that a specific miR156-targeted gene is regulated by specific miR156 member. Thus, in further research, it will be important to investigate how specific miR156 members regulate specific SBP-box genes. The results of such studies will further our understanding of the regulatory mechanisms of miRNAs and their target genes.

As mentioned above, four SBP-box genes were up-regulated during fruit ripening (Fig. 8). All of them were miR156-nontargeted genes, and all of the miR156-targeted genes were down-regulated during fruit ripening. These findings indicated that the miR156-nontargeted genes play more important roles than miR156-targeted genes in fruit ripening. In tomato, the mRNA of *CNR* is degraded by miR156 during fruit ripening (Karlova et al. 2013). Thus, the down-regulated expression patterns of miR156-targeted SBP-box genes during *P. mume* fruit ripening may result

from miR156-guided mRNA cleavage. This indicates that miR156 plays crucial roles in fruit development and ripening. In further research, it will be of great importance to investigate the mechanisms of miR156 and SBP-box genes in regulating fruit ripening.

In conclusion, this study is the first genome-wide analysis of the SBP-box gene family in *Prunus*. Our results showed that some SBP-box genes have shown species-specific expansion patterns and that their expression patterns have diverged after duplication events. Purifying selection has been the main driving force for the evolution of *Prunus* SBP-box genes, not only for paralogous genes, but also for orthologous genes. Based on the Ka and Ks values and the expression patterns of SBP-box genes, we conclude that the sequences of orthologous SBP-box genes have not diverged widely after the split of *P. mume* and *P. persica* and that the functions of orthologous genes may be conserved among different species. Three *P. mume* SBP-box genes may have undergone subfunctionalization after duplication. These findings lay the foundation for future research on the evolution of SBP-box genes in plants. Analyses of the different expression patterns of the miR156-targeted SBP-box genes may help to elucidate the regulatory mechanism of miRNAs and their target genes. The different expression patterns of miR156-targeted and -nontargeted genes during the fruit ripening process indicate that miR156-nontargeted genes play more important roles during fruit ripening than miR156-targeted genes and that miR156 may have an important regulatory function during fruit development. Thus, it is essential to perform functional analyses of miR156-nontargeted genes in fruit ripening in the future. The expression analysis of SBP-box genes in flowers and fruit at different developmental stages enables us to investigate the molecular mechanisms that regulate flower and fruit development in *P. mume* and other species.

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