Molecular characterization of *PSEUDO RESPONSE REGULATOR* family in Rosaceae and function of *PbPRR59a* and *PbPRR59b* in flowering regulation

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Abstract

Background *PSEUDO RESPONSE REGULATOR* (*PRR*) genes are essential components of circadian clock, playing vital roles in multiple processes including plant growth, flowering and stress response. Nonetheless, little is known about the evolution and function of PRR family in Rosaceae species.

Results In this study, a total of 43 *PRR* genes in seven Rosaceae species were identified through comprehensive analysis. The evolutionary relationships were analyzed with phylogenetic tree, duplication events and synteny. *PRR* genes were classified into three groups (PRR1, PRR5/9, PRR3/7). The expansion of PRR family was mainly derived from dispersed and whole-genome duplication events. Purifying selection was the major force for PRR family evolution. Synteny analysis indicated the existence of multiple orthologous *PRR* gene pairs between pear and other Rosaceae species. Moreover, the conserved motifs of eight PbPRR proteins supported the phylogenetic relationship. *PRR* genes showed diverse expression pattern in various tissues of pear (*Pyrus bretschneideri*). Transcript analysis under 12-h light/ dark cycle and constant light conditions revealed that *PRR* genes exhibited distinct rhythmic oscillations in pear. PbPRR59a and PbPRR59b highly homologous to AtPRR5 and AtPRR9 were cloned for further functional verification. PbPRR59a and PbPRR59b proteins were localized in the nucleus. The ectopic overexpression of PbPRR59a and PbPRR59b significantly delayed flowering in Arabidopsis transgenic plants by repress the expression of *AtGI*, *AtCO* and *AtFT* under long-day conditions.

Conclusions These results provide information for exploring the evolution of *PRR* genes in plants, and contribute to the subsequent functional studies of *PRR* genes in pear and other Rosaceae species.

Keywords Pear, PSEUDO RESPONSE REGULATOR, Circadian clock, Flowering time, Gene expression, Functional analysis

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Background

The circadian clock system of plants is an endogenous timing mechanism derived from adaptation to day/ night cycles of the environment during the long evolutionary process. Through the circadian clock system, plants are able to sense and predict the dynamics of environmental changes and complete critical growth and development processes at the right time [1, 2]. In recent decades, numerous studies have found that physiological activities, such as stomatal movement, leaf motion, flower opening and closure, hypocotyl elongation, photoperiodic flowering, photosynthesis, stress resistance and immune response, exist circadian rhythms and are regulated by circadian clock [3–8]. The central oscillator of clock system is composed of transcriptional-translational feedback loops, in which *CCA1* (*CIRCADIAN CLOCK ASSOCIATED 1*), *LHY* (*LATE ELONGATED HYPOCOTYL*), *PRRs* (*PSEUDO RESPONSE REGULA-TORs*), *ELF3* (*EARLY FLOWERING 3*), *ELF4*, *LUX* (*LUX ARRHYTHMO*) and other clock-related genes are play critical roles [9]. Not only in Arabidopsis (*Arabidopsis thaliana*), more and more clock-related genes involved in key agricultural traits have been discovered and utilized [10, 11].

In 1995, the core clock mutant, *toc1*, was identified in Arabidopsis [12]. In 2000, *AtTOC1* (*TIME OF CAB EXPRESSION 1*, also called *PRR1*) was isolated, and the first feedback loop of a plant central oscillator was established between *AtCCA1/LHY* and *AtTOC1* in 2001 [13]. Subsequently, four *PRR* genes (*AtPRR3*, *AtPRR5*, *AtPRR7*, and *AtPRR9*) were characterized to participate in the formation of other feedback loops [14–16]. As core components of the circadian clock, five *PRR* genes show robust rhythmic expressions with a near 24 h period. From dawn to evening, the transcriptional levels of *AtPRR9/ AtPRR7/AtPRR5/AtPRR3/AtPRR1* accumulate sequentially and peak 2 h to 3 h intervals [17]. Arabidopsis *prr* mutants display altered phenotypes, such as late flowering, decreased growth, and increased drought and cold stress resistance [18–21]. *CO* (*CONSTANT*) is the key element of photoperiodic flowering, and circadian clock genes control the dynamics of CO from multiple levels such as transcription and translation [22]. PRR9, PRR7, and PRR5 promote flowering by activating *CO* expression during the daytime [21], or by interacting with CO proteins to mediate its accumulation [23].

Members of the *PRR* gene family contribute greatly to the clock system and serve as vital components. PRRs regulates gene expression by forming protein complexes, and integrates information such as light and temperature in the clock system [24, 25]. Given the influence of clock systems on photoperiodic responses such as flowering, *PRR* genes are found to have mutants that alter flowering timing or affect photoperiodic sensitivity [11, 24, 25]. *PRR* homologous genes have also been identified in other plant species, including monocotyledons such as rice (*Oryza sativa*) [26], maize (*Zea mays*) [27], wheat (*Triticum aestivum*) [28] and cotton (*Gossypium hirsutum*) [29], and dicotyledons such as soybean (*Glycine max*) [30], rose (*Rosa chinensis*) [31] and *Chrysanthemum morifolium* [32]. These PRR proteins have two highly conserved structures with a psREC (pseudo receiver) domain at the N-terminus and a CCT (CONSTANS, CO-like, and TOC1) motif at the C-terminus. Based on phylogenetic relationships and similarities to Arabidopsis proteins, the PRR family is divided into three clades, including PRR1, PRR3/7 and PRR5/9 [25]. Circadian researches have gradually shifted to more valuable crops, and in-depth studies on *PRR* gene function have been carried out in a variety of crop species [10, 11]. For example, BvBTC1 (BOLT-ING TIME CONTROL 1), which belongs to the PRR family, interacts with BvBBX19 (B-BOX 19) to achieve CO function, inducing beet flowering by regulating downstream target gene *BvFT1* (*FLOWERING LOCUS T 1*) and *BvFT2* [33, 34]. In soybean, *GmPRR3a* and *GmPRR3b* are involved in photoperiodic flowering, and *GmPRR3b* have been selected for geographic expansion during domestication [35, 36]. In addition, *OsPRR37* in rice [37], *SbPRR37* in sorghum (*Sorghum bicolor*) [38], and *HvPRR3* in barley (*Hordeum vulgare*) [39] play an important role in controlling flowering time.

Members of the *PRR* gene family have been identified in many plants [33–39]. However, few studies have explored these genes and their functions in fruit crops. Rosaceae contains several economically valuable fruit crops, representing species such as pear (*Pyrus bretschneideri*), apple (*Malus domestica*), peach (*Prunus persica*), strawberry (*Fragaria vesca*), Japanese apricot (*Prunus mume*), sweet cherry (*Prunus avium*) and black raspberry (*Rubus occidentalis*). Moreover, the genome of these plants has been sequenced and released. Pear is widely cultivated worldwide, and flowering is the basis for the formation of pear fruit. As noted previously, *PRR* genes involved in flowering regulation, but the molecular characterizations of the *PRRs* in pear and other Rosaceae species have not been studies in detail. In this study, we identified 43 members of PRR family from seven Rosaceae species, and systematically analyzed their phylogenetic relationship, classification, conserved domain, chromosomal location, and evolutionary history. Furthermore, the expression patterns, subcellular localization, and flowering phenotypes of PbPRRs were conducted. We found that PbPRR59a and PbPRR59b may be involved in circadian and photoperiodic responses and influence flowering time. The

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results of this work provide a basic for further elucidating the functions of PRRs in Rosaceae species.

Results

Genome-wide identification and classification of *PRR* members in seven Rosaceae species

To identify the members of PRR family in the genomes of seven Rosaceae species, five PRR (AtPRR1/ AtPRR3/ AtPRR5/ AtPRR7/ AtPRR9) protein sequences of Arabidopsis were used as query for BLASTP search. The transcripts of the same gene were removed, and the conserved domains of the sequences were identified by NCBI CD-Search tool to obtain the final PRR members. In this study, 43 *PRR* candidate genes from seven Rosaceae species were confirmed, including eight in pear, eight in apple, nine in peach, four in strawberry, four in Japanese apricot, five in sweet cherry, and five in black raspberry (Fig. 1 and Table S1). The 43 putative proteins, like the known PRRs, have the N-terminal psREC domain and

the C-terminal CCT domain, with less conserved intermediate sequences (Fig. 1).

To investigate the evolutionary relationships of PRR members in these representative species of Rosaceae, a phylogenetic tree was generated using all protein sequences. These *PRR* homologous genes were categorized into group I (PRR1)、group II (PRR5/9) and group III (PRR3/7) according to their similarity with Arabidopsis (Fig. 1). *PRR* members of different species were distributed in all three groups. Phylogenetic analysis showed that *PRR* genes of pear and apple were clustered in the same clade indicating their close relationships.

Furthermore, the genetic characteristics of each *PRR* were analyzed, including the position of the gene on a chromosome, protein sequence length, protein molecular weight, and isoelectric point (Table S1). The length of majority PRR proteins were between 500 and 800 amino acids. The protein molecular weight of 43 PRR proteins ranged from 53.32 to 115.57 kDa, and the
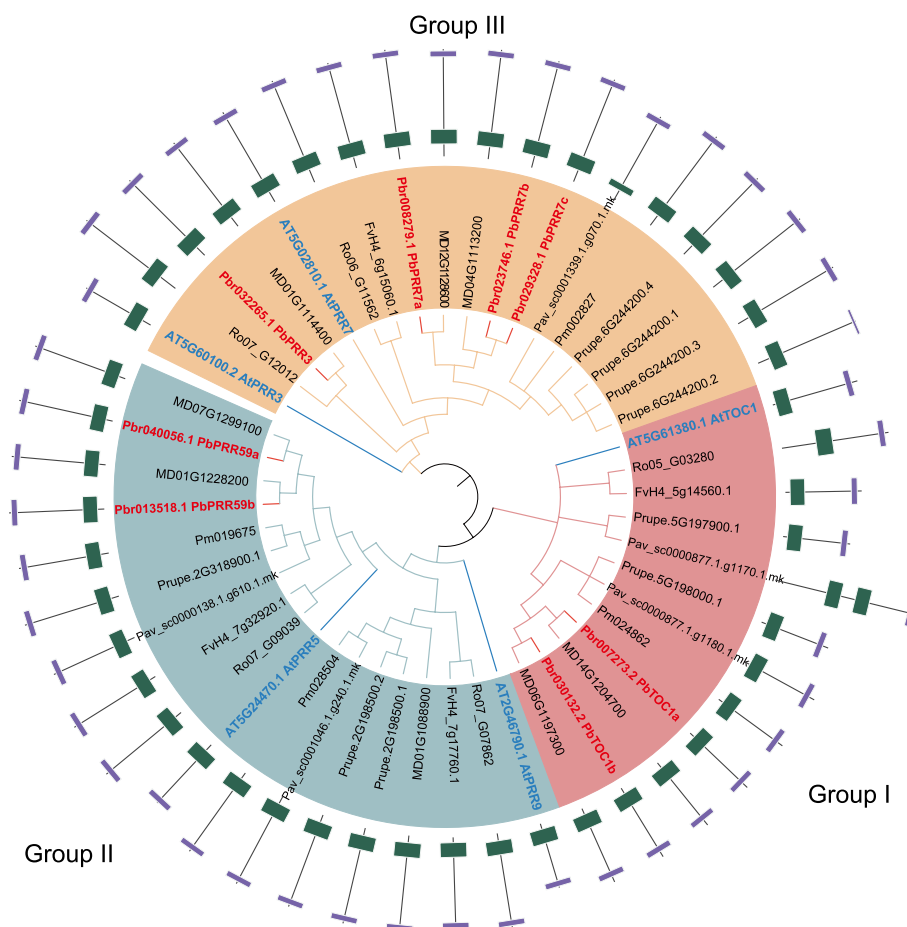

Fig. 1 Phylogenetic relationships and conserved domains of the PRR proteins in the seven Rosaceae species and Arabidopsis. The pink, blue, and orange blocks represent groups I, II, and III respectively. Green and purple rectangles outside the circle represent psREC and CCT conserved domains, respectively

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isoelectric point ranged from 5.16 to 8.74. Of the 43 genes, 40 genes were randomly distributed on chromosomes. In details, six of the eight *PRR* genes were anchored onto five of the 17 chromosomes in pear, and two were located on the scaffolds. In Japanese apricot, chromosomes 1, 5, and 7 each harbored a *PRR* gene, and the remaining one was located on scaffold1366. Eight *PRR* genes were mapped onto six chromosomes in apple, and nine *PRR* genes were mapped onto three chromosomes in peach. In strawberry, two genes were located on chromosome 7, and one gene was mapped onto each of chromosome 5 and 6. Five *PRR* genes were distributed on Chr2, Chr5 and Chr6 in sweet cherry. Five *PRR* genes were distributed on Chr5, Chr6 and Chr7 in black raspberry.

Gene duplication events and synteny analysis of *PRR* gene family in seven Rosaceae species

Gene duplication events have contributed to gene family evolution and gene function diversification [40]. Gene duplication events of the PRR family in seven Rosaceae species were analyzed using DupGen_finder pipeline software [41]. A total of 51 duplicated gene pairs were found that were assigned to four common events, including DSD (dispersed duplication), WGD (whole-genome duplication), TD (tandem duplication), and TRD (transposed duplication), while PD (proximal duplication) were not detected (Fig. 2). DSD-derived gene pairs existed in all seven Rosaceae species, and the number accounted for 56.86% (29 gene pairs) of all pairs. Except strawberry and Japanese apricot, some gene pairs of the other five
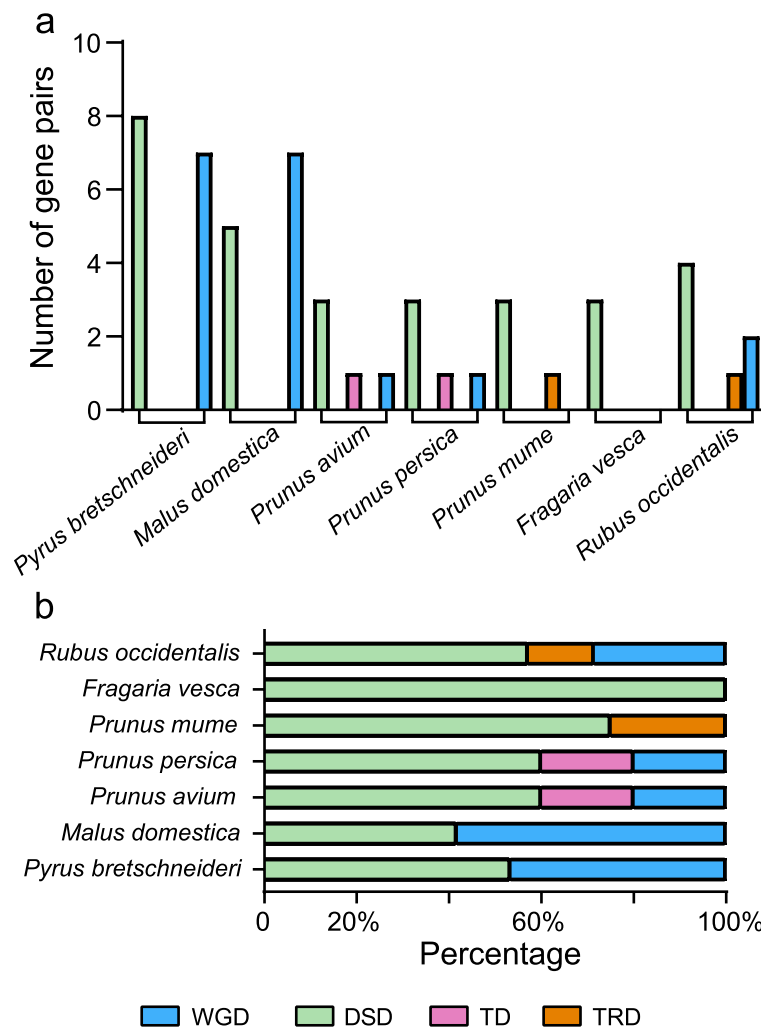

Fig. 2 Gene duplication events of the PRR family in the seven Rosaceae species. Statistical results of the number (**a**) and percentage (**b**) of gene pairs driven by different types of duplication events in each species. Duplication events include DSD (dispersed duplication, green), WGD (whole-genome duplication, blue), TD (tandem duplication, pink), and TRD (transposed duplication, orange), each represented by a different colored bar

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species were derived from WGD event, accounting for 35.29% (18 gene pairs) of the total (Table S2). Only DSD- and WGD-derived gene pairs were found in pear and apple, suggesting that they might contribute to the evolution of the corresponding PRR family. Peach and sweet cherry had the same modes of gene duplication events, consisting of DSD (3 gene pairs), TD (1 gene pair), and WGD (1 gene pair). DSD was identified as the only driving force for PRR family in strawberry. In addition, one pair of duplicated gene in Japanese apricot and one in black raspberry were derived from TRD event (Fig. 2 and Table S2).

In molecular evolution, Ks (synonymous substitution rate) and Ka (non-synonymous substitution rate) are fundamental parameters for estimating selection tendencies between homologous genes, where Ks is generally used to understand the timing of evolutionary divergence [42, 43]. In this study, the Ks values of WGD-derived gene pairs ranged from 0.02 to 2.58 in pear, 0.17 to 2.93 in apple, and 1.43 to 1.97 in black raspberry (Table S3). The Ks value of one WGD-derived gene pair in peach was 1.31, and one in sweet cherry was 1.35. Previous study has shown that the pear genome has experienced two rounds of WGD events, occurring at 30–45 million years ago (Ks=0.15–0.30) and ~140 million years ago (Ks=1.50–1.80) [44]. In detail, the Ks values of four WGD-derived gene pairs in pear were between 0.15 and 0.30, indicating that the divergent time of these *PbPRR* paralogs were during the recent WGD event. Ka/Ks ratio greater than one or less than one represents different selection pressure. Moreover, the ratios of all *PRR* duplicated gene pairs in seven Rosaceae species were less than one, suggesting that purifying selection was the main driving force for PRR family evolution in Rosaceae species (Table S3).

Paralogous *PRR* gene pairs in seven Rosaceae species were analyzed above, and further orthologous *PRR* gene pairs between pear and other six species were identified by McScanx [45]. Excluding the *PRR* genes located on scaffolds, 49 gene pairs had syntenic relationships (Fig. 3). The number of *PRR* gene pairs between pear and apple was the highest with 12 pairs, followed by 10 pairs between pear and black raspberry. In addition, there were 8, 7, 6, and 6 interspecies syntenic gene pairs between pear and strawberry, sweet cherry, peach and Japanese apricot. The results suggested that *PRR* genes in seven Rosaceae species had strong homology and conserved relationship.

Conserved motif and gene structure analysis of *PRR* genes in pear

To comprehension the characteristics and functions of *PRR* genes in pear, conserved motif and gene structure were performed using MEME and GSDS online software. Eight *PbPRR* genes were named based on *AtPRR* genes in phylogenetic tree. The results of MEME analysis showed that PbPRR and AtPRR proteins in the same group had similar motif composition, which supported the phylogenetic relationship (Fig. 4a). Motifs 1, 3, and 4 were present in all PbPRR proteins, which represented the conserved pseudo receiver domain at the N-terminus. Motif 2 were ubiquitous in all members and identified as the CCT domain. Whereas group I only had motifs 1–4, group II and III had more diverse motifs. For example, each member of group II and III contained motif 6. Except for AtPRR9 and PbPRR3, motif 9 was detected in most group II and III proteins. In addition to the motifs shared with AtPRR7, four PbPRRs in group III possessed special motifs 7 and 8, suggesting that they may have functional divergence.

The results of gene structure analysis showed that all *PRR* members contain multiple exons and introns (Fig. 4b). In group I, both *PbTOC1a* and *PbTOC1b* had six exons, the same number as *AtTOC1*. The exon number of *PbPRR59a* (8) and *PbPRR59b* (8) were more than *AtPRR5* (6) and *AtPRR9* (7) in group II, while *PbPRR3* (8) was less than *AtPRR3* (9) in group III. Despite smaller fluctuations in exon number, the same groups still had similar gene structures. Taken together, these evidences indicated that some members of the PbPRR family have conservative functions similar to AtPRRs, and others may have different roles.

Expression analysis of *PbPRR* genes

To obtain more information about the biological functions of PbPRRs, gene expression patterns were analyzed using qRT-PCR assay. First, we examined the expression levels of eight *PbPRR* genes in vegetative and reproductive organs, including root, stem, leaf, leaf bud, flower bud, petal, anther and ovary (Fig. 5). The relative expression levels of *PbTOC1a* and *PbTOC1b* were higher in root and ovary than in other organs. *PbPRR59a* and *PbPRR59b* had a similar expression pattern with extremely high levels in root and stem. The transcription levels of three *PbPRR7* homologous genes were higher in vegetative organs, and lower in reproductive organs except petal. Unlike the other seven *PbPRR* genes which exhibited a predominant expression in root, more *PbPRR3* transcripts were accumulated in leaf bud.

In Arabidopsis, *PRR* genes are key components of circadian clock [9], and increasing evidence shows that the expression pattern of *PRR* genes in other species also present rhythmic diurnal oscillation nearly 24 h [27, 29, 30]. Eight *PbPRR* genes were clearly detected in the pear leaves, which are the primary site for sensing diurnal changes. Next, we examined the expression
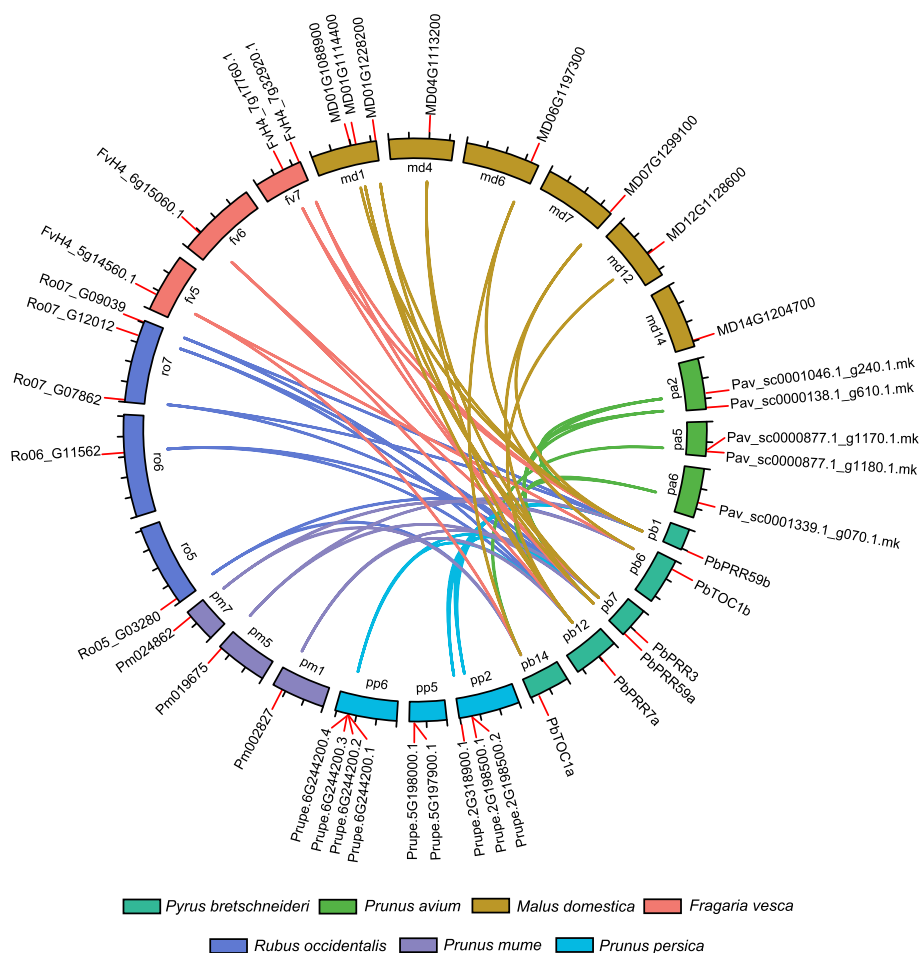
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Fig. 3 Syntenic relationships of *PRR* gene pairs between pear and six Rosaceae species. The curved boxes of different colors represent the chromosomes of the seven species, and the curved lines of corresponding colors represent the *PPR* gene pairs between the corresponding species and pear. The short red lines outside the circles represent the approximate locations of *PRR* genes

changes of *PbPRR* genes in pear leaves under 12-h light/12-h dark condition (Fig. 6). All *PbPRR* genes exhibited obvious rhythmic diurnal patterns during a light/ dark cycle. Transcription levels of *PbTOC1a* and *PbTOC1b* dropped steeply after dawn (zeitgeber time 5; ZT 5), gradually rose throughout the day, and rapidly accumulated to peak before dawn (ZT 21). In contrast, transcripts of the remaining *PbPRR* genes peaked at 9 h (ZT 9) after exposure to light. For example, both *PbPRR59a* and *PbPRR59b* remained low expression levels in the morning and evening, with higher detected only in the afternoon (ZT 9- ZT 13).

To investigate the clock properties of *PbPRR* genes, we examined their expression profiles in pear leaves under constant light conditions. For three days, *PbPRR* genes except *PbPRR3* still showed stable patterns of rhythmic expression, implying that they were clock-related genes (Fig. 7). The dynamics of the first day were similar to the 12-h light/12-h dark condition, with *PbPRRs* peaking at

the corresponding time point. On the second and third days, they maintained the same rhythm, but the expression period was longer and the expression amplitudes were reduced due to the continuous light treatment.

Subcellular localization of PbPRR59a and PbPRR59b proteins

In Arabidopsis, PRR proteins are localized in the nucleus and act as transcription factors to regulate gene expression [17, 46]. To further study potential functions of PbPRR59a and PbPRR59b, we detected their subcellular localization in epidermal cells of tobacco leaves. The full-length coding regions of PbPRR59a and PbPRR59b were used to construct fusion proteins with GFP (green fluorescent protein), respectively. Transient expression assay showed that the fluorescent signals of PbPRR59a-GFP and PbPRR59b-GFP fusion proteins were observed only in the nucleus (Fig. 8).

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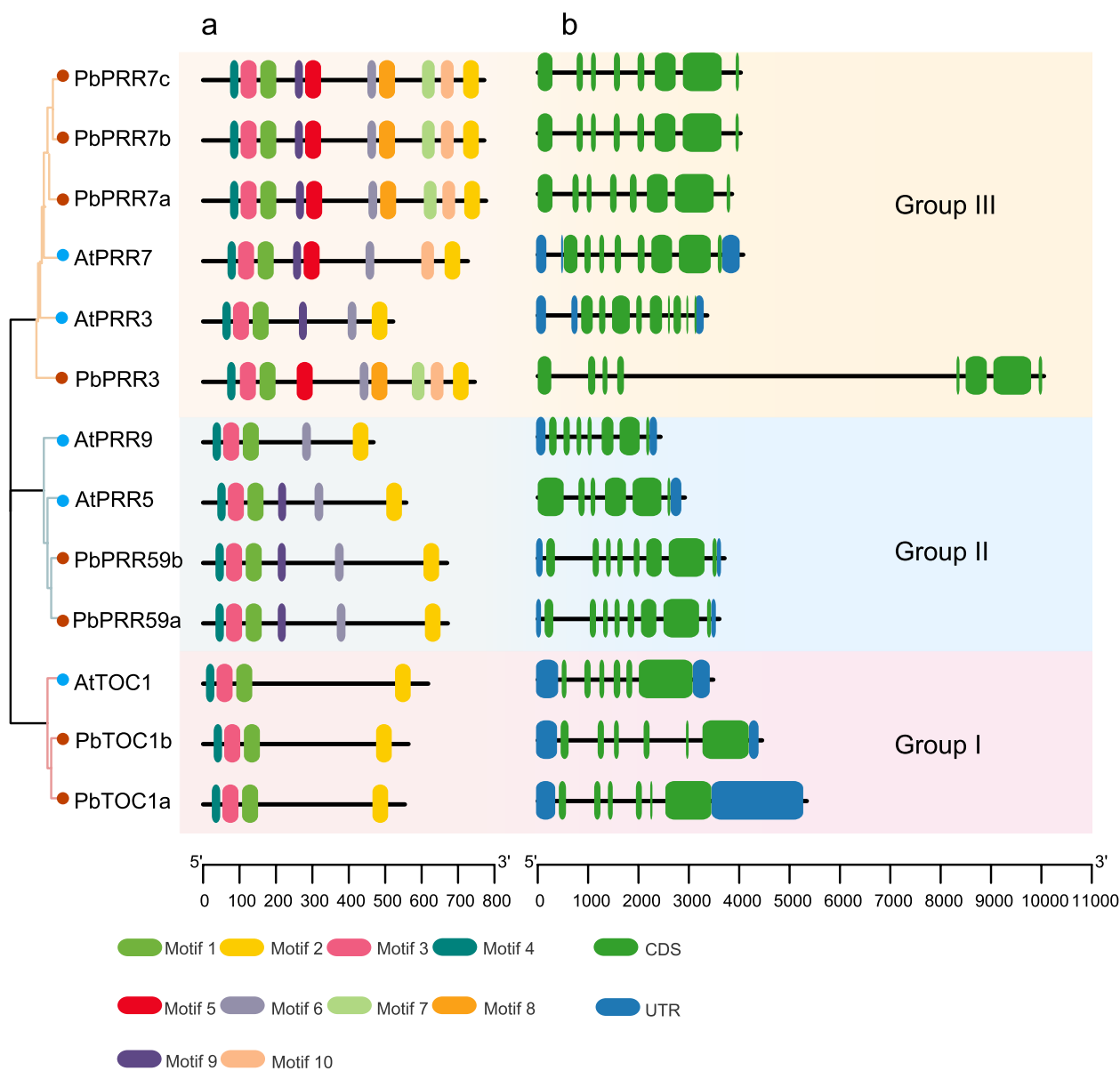
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Fig. 4 Conserved motif and gene structure analysis of PRR family in Arabidopsis and pear. **a** Conserved motif compositions of PbPRR and AtPRR proteins. Ten motifs identified by MEME are represented by rounded rectangles of different colors. **b** Exon–intron structures of *PbPRR* and *AtPRR* genes. Blue rounded rectangles, green rounded rectangles, and black lines represent UTRs (untranslated region), exons and introns, respectively. The members of PbPRR and AtPRR are marked with red and blue dots, respectively. The scales represent the length of the protein and genomic sequence, respectively

Ectopic overexpression of PbPRR59a and PbPRR59b delayed flowering in Arabidopsis under long-day conditions

Because the stable genetic transformation system of pear has not been established to observe the flowering phenotype, heterologous transgenic materials of Arabidopsis were used to explore the influence of genes on flowering. The full-length coding sequences of *PbPRR59a* and *PbPRR59b* were placed into constitutive CaMV 35S promoter-driven vectors, and these constructs were introduced into wild-type Arabidopsis (Col-0), respectively. The PbPRR59a and PbPRR59b independent transgenic lines were identified through semi-quantitative RT-PCR analysis and participated in subsequent phenotypic observation (Fig. 9b).

Cultured from seed under long-day (16-h light/8-h dark) conditions, PbPRR59a and PbPRR59b overexpression (OE) transgenic plants exhibited a delayed flowering

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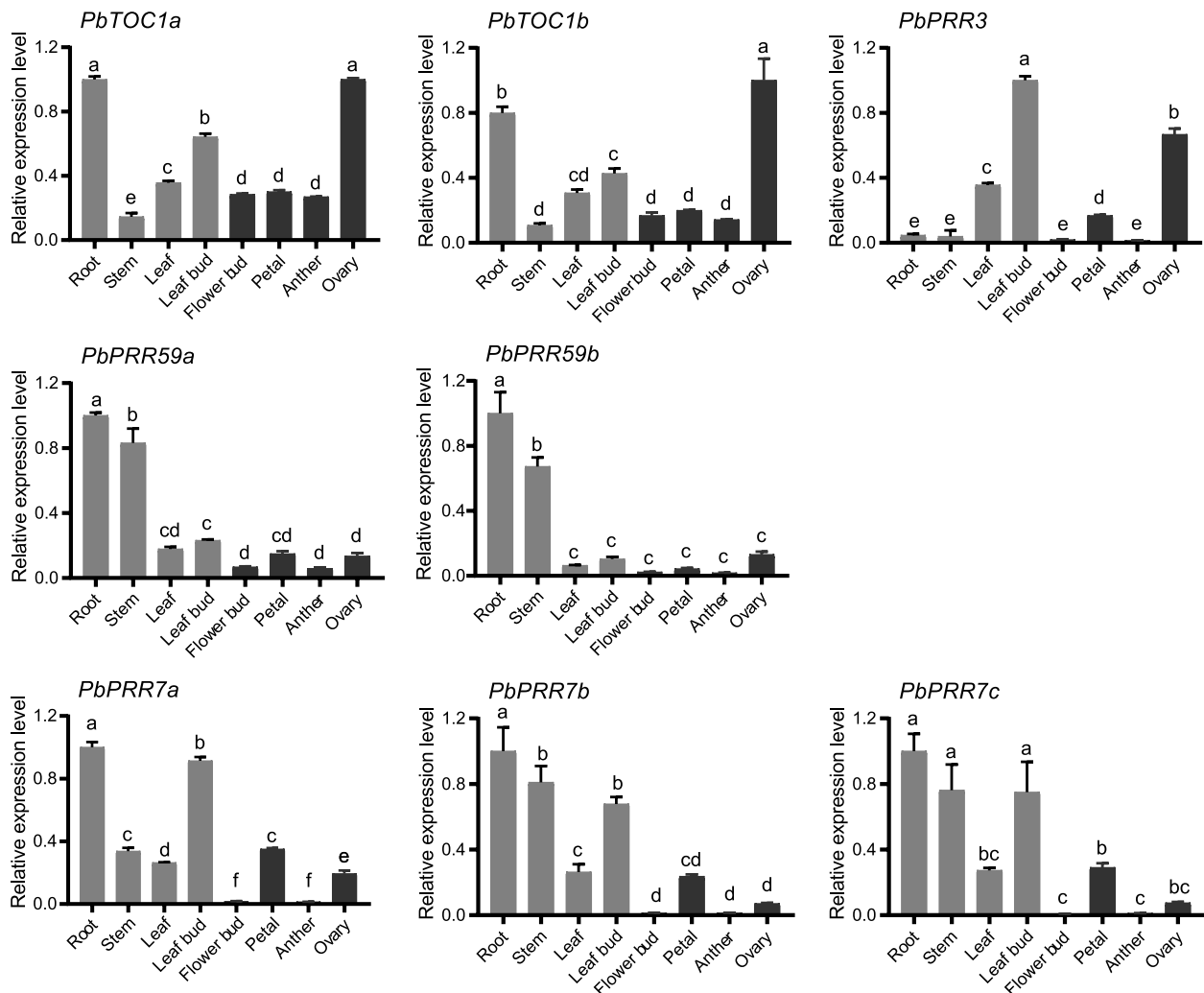
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Fig. 5 Expression analysis of *PbPRR* genes in different tissues. The gray columns represent vegetative organs including root, stem, leaf, and leaf bud, while the black columns represent reproductive organs including flower bud, petal, anther, and ovary. The relative expression was calculated using *PbUBQ* as the reference gene. The maximum expression of each gene was set to "1" for standardized calculation. Data are means of three replicates with standard error. Different letters above the columns represent significant differences ($P < 0.05$) using one-way ANOVA Tukey's multiple range tests

phenotype compared to the empty vector control plants (Fig. 9a). We measured the physiological indexes of these plants, including the time of the first flower opening after bolting and the corresponding number of rosette leaf. In detail, both PbPRR59a-OE and PbPRR59b-OE lines took more than 35.70 days to open their first flower on average, compared to 32.79 days for control plants (Fig. 9c). Simultaneously, the mean number of rosette leaf also increased significantly in transgenic lines. To further investigate the underlying cause of PbPRR59a and PbPRR59b on flowering, the expression levels of endogenous flowering time-related genes in different lines were examined by qRT-PCR assay. The transcripts of these key genes, including *AtGI*, *AtCO* and *AtFT*, were significantly

reduced in both PbPRR59a and PbPRR59b transgenic lines than in control plants (Fig. 9d). The results implied that PbPRR59a and PbPRR59b might negatively regulate flowering time by inhibiting the expression of flowering related genes.

Discussion

The PRR family is a small gene family that belongs to a clade of CCT family and is conserved in plants [47]. *PRR* genes are involved in maintaining the stable operation of circadian clock and play important roles in transition from vegetative growth to reproductive growth in plants [24, 25]. However, detailed research on the PRR family of Rosaceae plants is still scarce. In this study, we
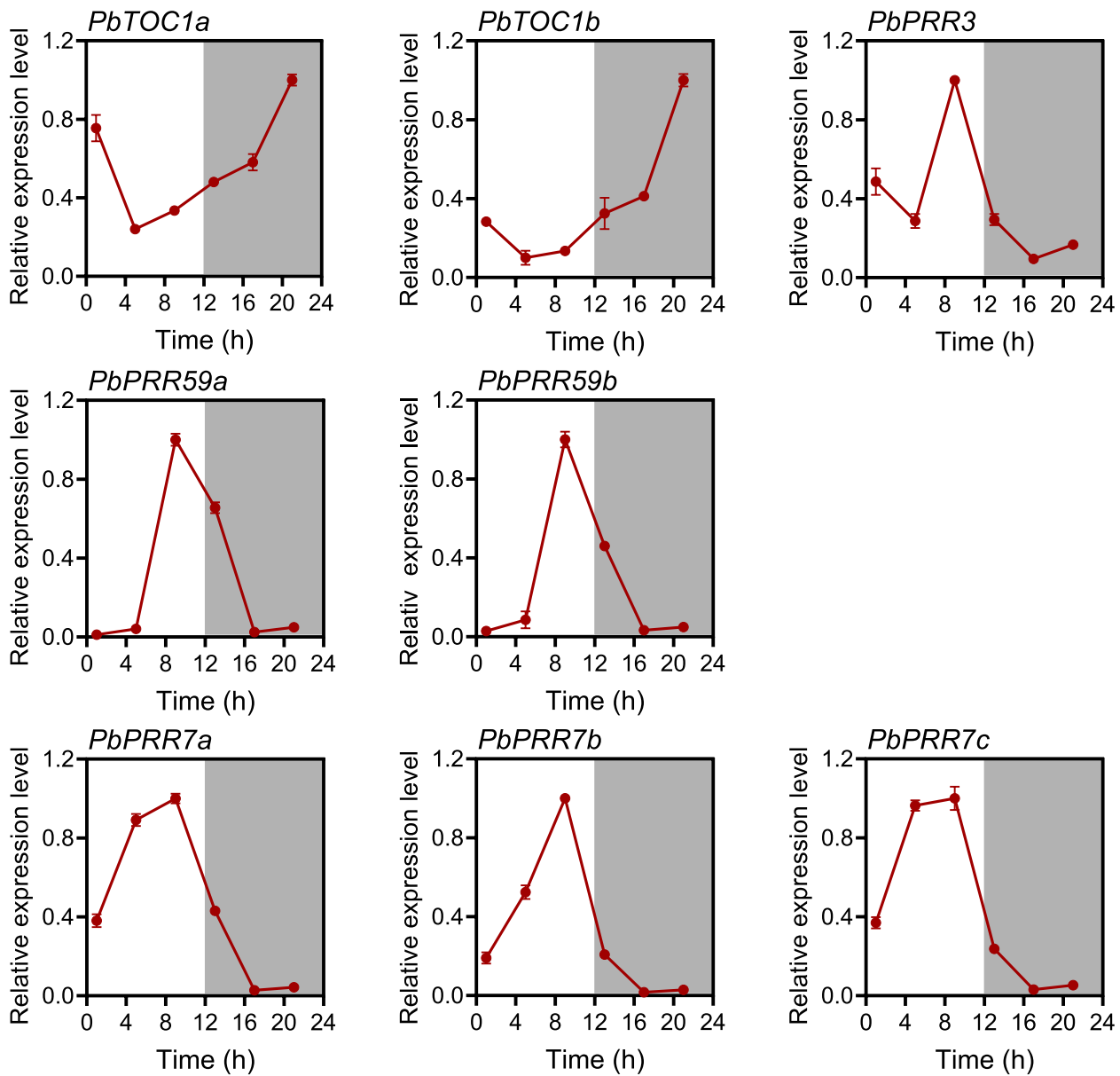
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Fig. 6 Expression patterns of *PbPRR* genes under 12-h light/12-h dark conditions. The initial exposure to light is defined as zeitgeber time zero (ZT 0). Starting from ZT1, leaf samples were collected 4 h intervals in the given 24 h. White and gray backgrounds indicate light and dark treatment, respectively. The relative expression was calculated using *PbUBQ* as the reference gene. The maximum expression of each gene was set to "1" for standardized calculation. Data are means of three replicates with standard error

identified 43 *PRR* genes from seven Rosaceae species and analyzed their evolutionary relationships through phylogenetic tree, duplication event, and syntenic relationship. Moreover, the gene structure, conserved domain and expression profile of eight *PRR* genes in pear were investigated. Transgenic plants were used to verify that PbPRR59a and PbPRR59b may be involved in flowering regulation as repressors.

Genome-wide identification and characterization of PRR family in more than twenty species has proved

that *PRR* genes are well conserved in plant evolution [24, 27–30, 48, 49]. At least one PRR member is present in the original single-celled green plant (e.g. *Chlamydomonas reinhardtii*), in the liverwort (e.g. *Marchantia polymorpha*) and moss (e.g. *Physcomitrella patens*), in the fern (e.g. *Selaginella moellendorffii*), and in the gymnosperm (e.g. *Picea abies*) and angiosperms [24, 49]. The numbers of *PRR* genes are often greater in angiosperms than in other plants [25]. In this study, the PRR families of seven Rosaceae species
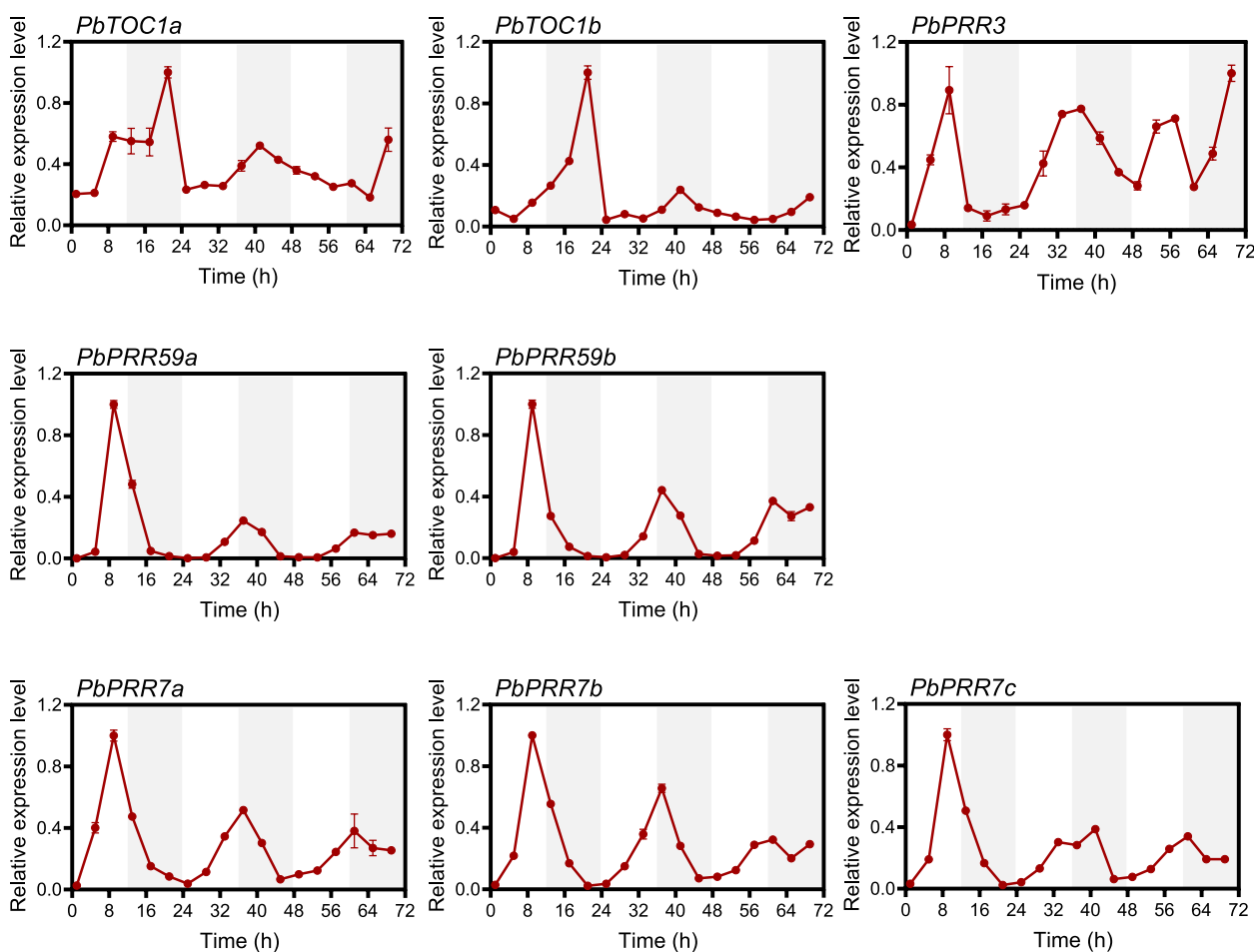
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Fig. 7 Expression patterns of *PbPRR* genes under constant light conditions for three days. The initial exposure to light is defined as zeitgeber time zero (ZT 0). Starting from ZT1, leaf samples were collected 4 h intervals in the given 72 h. White and light gray backgrounds indicate the alternation of subjective day and night. The relative expression was calculated using *PbUBQ* as the reference gene. The maximum expression of each gene was set to "1" for standardized calculation. Data are means of three replicates with standard error

were investigated, with the number of genes distributed between 4 and 9, which is close to that of many reported angiosperms, such as Arabidopsis (5), rose (5) [31], and poplar (7) (*Populus trichocarpa*) [50]. The PRR1 (TOC1) clade of the PRR family contains only one gene in most plants, such as dicotyledonous representative Arabidopsis [17] and monocotyledonous representative rice [26]. However, two *PRR1* homologous genes were found in four of the seven Rosaceae species, all of which were gene pairs. Gene and genome duplication are major forces driving gene family expansion and biodiversity. The contribution of five duplication events (WGD, TD, PD, TRD and DSD) varies across species [41]. *PbTOC1a* and *PbTOC1b* in pear were WGD-derived gene pair, which were also present in apple, while the two homologs of *PRR1* in peach and sweet cherry were both TD-derived. In addition to Rosaceae species, additional gene copies of *PRR1* have also been

found in soybeans and maize [27, 30], and the potential functions of these homologous genes that have been retained in the long evolutionary process need to be further elucidated.

As previously reported in other species [25], the 43 *PRR* genes identified in Rosaceae were well divided into three groups, all of which had N-terminal and C-terminal conserved domains. The characteristics of PbPRR and AtPRR protein sequences showed that the motif composition in the same group were similar, while were distinctly among groups. The divergence of the PRR group holds potential important implications for understanding evolution of clock and its adaptation to environmental cues. Evolutionary studies suggest that PRR1 group was the first to diverge and then split the PRR5/9 group and the PRR3/7 group [51]. The early divergence of PRR1 could date back before the divergence of moss from higher plant lineages. *PRR1* orthologs in *Chlamydomonas reinhardtii*,
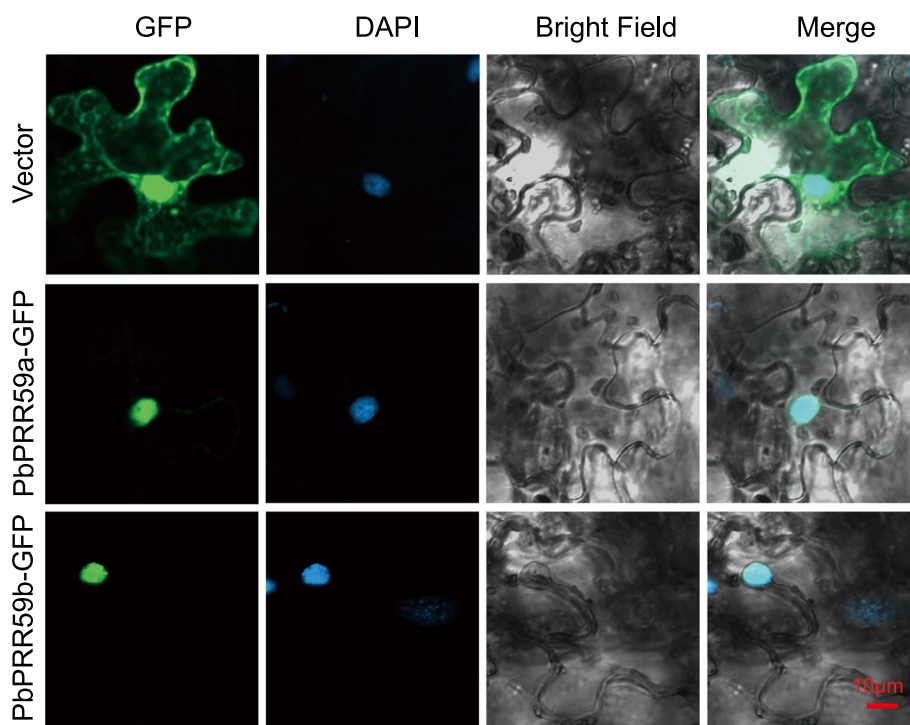
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Fig. 8 Subcellular localization of PbPRR59a and PbPRR59b proteins in tobacco epidermal cells. Green represents GFP signal from the GFP-fusion proteins. Blue represents DAPI fluorescence as the nuclear localization marker. Merge represents superimposed GFP, DAPI and bright field images. Vector represents the control using 35S: GFP empty vector. Bar = 10 μm

Chlorella variabilis and *Ostreococcus tauri* [24, 51] supports the hypothesis that the *PRR1* orthologs might be the original members of PRR family. In this study, conserved motif analysis revealed that motifs 6 and 9 are present in the PRR5/9 and PRR3/7 groups, but not in the PRR1 group. This finding indicates a distinct divergence between PRR1 group and other PRR groups. Understanding these divergences might provide insights into the functional evolution and specialization of the PRR family in response to environmental adaptations. Recent analysis revealed that PbPRR5/9 and PbPRR3/7 clades appear before differentiation in monocotyledonous and dicotyledonous plants, but the expansion of the two groups happened independently in two types of plants [24]. Analysis of PRR3/7 and PRR5/9 gene expansions using chromosomal synteny suggests that the PRR3/7 clade expansion in monocots is associated with the ρ (rho) polyploidy event, while the PRR5/9 clade duplication happened earlier. In eudicots, the expansion of PRR3/7 and PRR5/9 genes is linked to the γ (gamma) polyploidy event. However, the presence of PRR5/9 genes in water lilies, which diverged before the eudicot-monocot split, indicates that the PRR5/9 duplication in eudicots may have occurred before the γ polyploidy event. The sequence identity and positional orthology suggest that PRR5/9 genes in water lilies are more similar to monocot genes, indicating a

complex evolutionary history [24]. In pear genome, PbPRR5/9 clade had two genes as a duplicated gene pair, and PbPRR3/7 clade had four genes that are identified six gene pairs. However, the occurrence time of these gene pairs varied widely, and further understanding the evolutionary history of PRR family in different plants is valuable.

Since plants cannot choose suitable living environment through movement like animals, they must adjust their physiological activities to adapt to periodic changes in the external environment. In Arabidopsis, five *PRR* genes, as key components of circadian clock, involve in regulating various physiological activities, and their transcription levels oscillate regularly throughout the day [9]. In this study, the expression of eight *PbPRR* genes had strong diurnal rhythm, which in the same group exhibit a similar pattern. In Arabidopsis, the expression peaks of five *PRR* genes appear sequentially from morning to evening [17]. The transcription levels of *PRR* genes in maize show clear diurnal oscillation pattern within 24-h cycle. Starting at dawn, they peaks occur approximately three hours apart, with *ZmPRR73*, *ZmPRR37*, *ZmPRR95*, *ZmPRR59*, and *ZmPRR1* arranged in order of their peak times [27]. However, eight *PbPRR* genes in pear appeared only two peaks, *PbTOC1a* and *PbTOC1b*
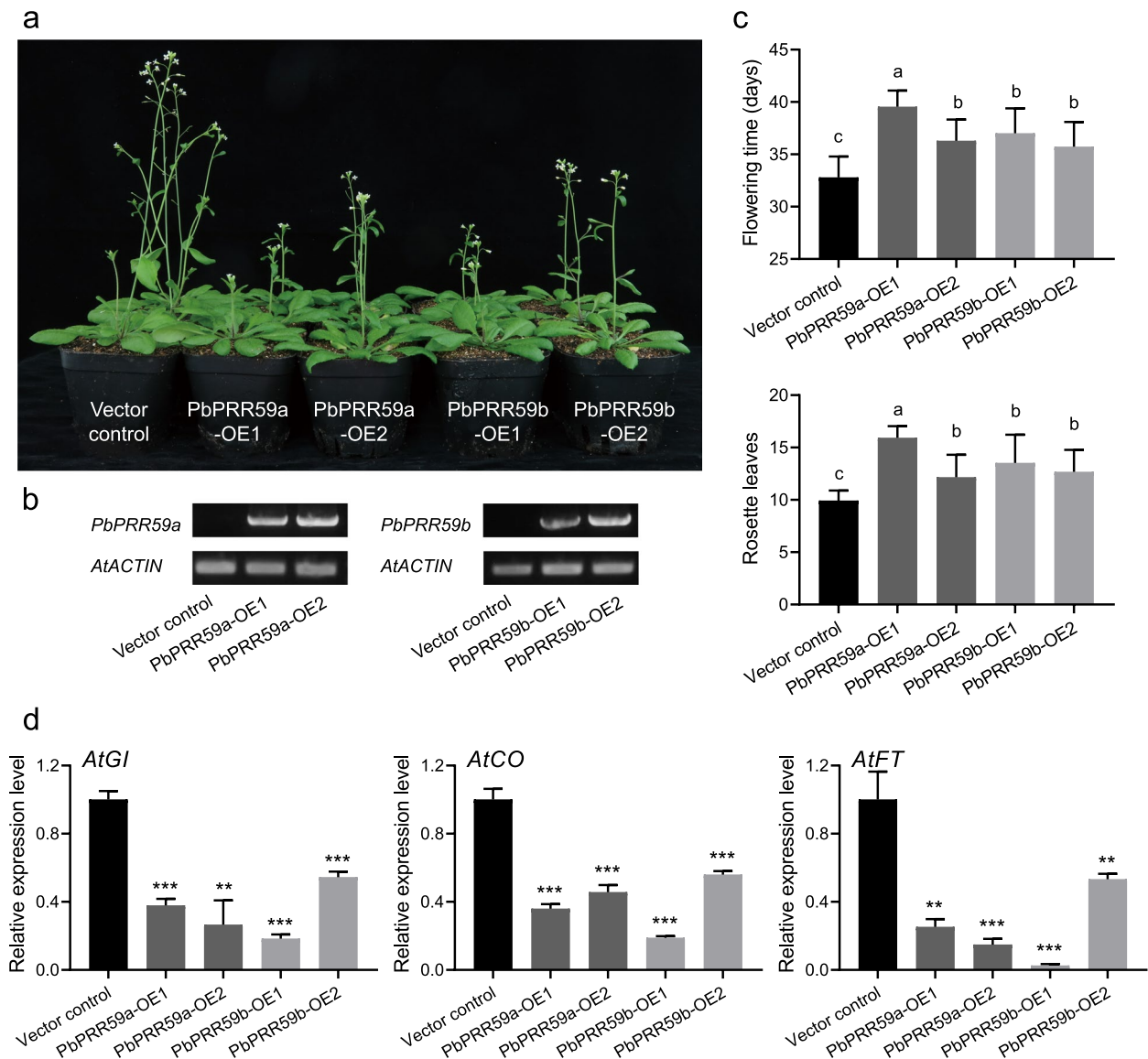
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Fig. 9 Overexpression of PbPRR59a and PbPRR59b delayed flowering in Arabidopsis under LD conditions. **a** Flowering time phenotypes of vector control plants, PbPRR59a- and PbPRR59b-overexpression transgenic lines under long-day conditions (16-h light/8-h dark). **b** Identification of PbPRR59a and PbPRR59b transgenic lines using semi-quantitative RT-PCR and gene-specific primers shown in table S4. **c** Days to first flowering and number of corresponding rosette leaf of transgenic lines and control plants. Data are means with standard error (n ≥ 13). Different letters above the columns represent significant differences ($P < 0.05$) using one-way ANOVA Tukey's multiple range tests. **d** Transcript levels of *AtGI*, *AtCO*, and *AtFT* in PbPRR59a and PbPRR59b transgenic lines. Data are means of three replicates with standard error. The relative expression levels were normalized to *AtACTIN*, and the maximum expression of each gene was set to "1". ** indicates $P < 0.01$ and *** indicates $P < 0.001$ using Student's *t*-tests compared with vector control plants

peaking before dawn (ZT 21), and the remaining six genes peaking at ZT 9. These results speculate that *PbPRR* genes may have distinctive functions at these phases. The rhythmic feature of circadian clock genes is that they autonomously maintain periodic oscillations under free-running conditions, such as constant light [52]. In addition to *PbPRR3*, the remaining seven *PbPRR* genes all have this feature, indicating that they

are clock related-genes. Notably, the circadian clock senses changes in the external photoperiod and adjusts itself to synchronize with it [53]. The diurnal expression trend of *PbPRR* genes has been displayed by analyzing leaf transcriptome data under different photoperiodic conditions [54]. Combining three photoperiods, including 12-h light/12-h dark, 16-h light/8-h dark (long-day) and 8-h light/16-h dark (short-day), we found that the

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change of light signal had different effects on *PbPRR* genes. For example, compared with the other two conditions, the peak time of *PbTOC1b* and *PbPRR7a/b/c* is significantly earlier under short-day conditions, while the peak time of *PbPRR59a/b* under long-day conditions is delayed. Similar diurnal patterns have been reported in crops such as maize [27]. Compared with the long-day conditions, the peak time of *ZmPRR1-1* and *ZmPRR1-2* is three hours earlier under short-day conditions. Different from the effect of photoperiod in pear on the peak time of *PRR* genes, the expression levels of *GmPRRs* in soybean under long-day conditions was higher than that under short-day conditions [30]. As clock-related and photoperiod-responsive genes, PbPRR may participate in the cooperative regulation of pear growth and development, and their potential functions need to be further explored.

Various functions of *PRR* genes in different plants have been identified, including flowering, hypocotyl growth and other developmental processes, as well as response to abiotic stresses such as cold, drought and salt [20, 21, 34, 55–57]. *ppd-H1* mutant causes late flowering under long-day condition. Ppd-H1 is identified as HvPRR37 in barley, and the expressions of *HvCO* and *HvFT* decrease in *ppd-H1* mutant [58]. In the past 20 years, similar researches have been reported in rice, sorghum, wheat, soybean, with functional genes clustered in the PRR3/7 clade [35, 37, 38, 59]. These *PRR* genes in crops exhibit photoperiodic sensitivity and play critical roles in flowering time regulation, leading to alter of yield and growth areas. Based on the pervasive impact of the circadian clock systems on crops, more studies have been devoted to improving agricultural traits by modifying clock-related genes [10]. Therefore, it is of great significance to explore the biological functions of *PRR* genes in pear, especially in flowering related to fruit. We focused on the performance of *PbPRR59a* and *PbPRR59b* genes in flowering regulation. They are highly conserved with AtPRR5, including sequence characteristics, expression patterns, and subcellular localization. AtPRR5 shows opposite regulatory effects in Arabidopsis and rice, promoting flowering of Arabidopsis and delaying flowering of rice [21, 60]. In this study, overexpression of both PbPRR59a and PbPRR59b could inhibit the flowering of transgenic Arabidopsis, indicating that the role of homologous genes in PRR5/9 clade may vary in different species. The current study shows that the complexity of circadian clock network tends to increase during plant evolution. Further studies will elucidate the specific role and molecular mechanism of *PbPRR* genes in pear flowering, and lay a foundation for the construction of pear circadian clock system.

Conclusions

In this study, 43 *PRR* genes were identified from seven Rosaceae species, and phylogenetic tree, duplication events and synteny analyses were performed. A more comprehensive analyses, including expression patterns, subcellular localization, and biological functions, were conducted for *PbPRR* genes. Our findings revealed that *PbPRR* genes had stable rhythms as clock-related genes. Notably, overexpression of PbPRR59a and PbPRR59b delayed flowering in transgenic Arabidopsis plants. Overall, our results shed light on the involvement of potential pear clock genes PbPRR59a and PbPRR59b in flowering regulation. Circadian clock has great potential and value in agricultural production, and the molecular mechanism of *PbPRR* genes in regulating pear flowering will be comprehensively and deeply studied in the future, so as to provide the basis for high-quality and efficient pear industry.

Methods

Plants materials

For expression experiments, materials were collected from pear trees (*Pyrus bretschneideri* cultivar 'Dangshan Suli') growing in experimental orchard at Nanjing Agricultural University in Nanjing, China. Leaf buds and flower buds were collected in March. Buds ready for flowering were picked to collect the petals, anthers, and ovaries separately. Seeds used in this study were harvested from pear trees. The sprouted and rooted pear seedlings were cultured in a growth chamber to collect roots, stems and leaves. The ambient condition of the chamber was photoperiod 12-h light/12-h dark, 100 μmol m^{-2} s^{-1} light, temperature 22 °C, and 65% relative humidity. Diurnal expression experiment was carried out when pear seedlings grew for 30 days under the same condition. The time when the light turns on is defined as zeitgeber time zero (ZT 0). Under 12-h light/12-h dark conditions, leaf samples were collected at ZT 1, ZT 5, ZT 9, ZT 13, ZT 17, and ZT 21 time points in one day, respectively. There were three replicates at each sampling point, each consisting of three randomly selected seedlings with a total of nine leaves. At the same time, some seedlings were transferred to constant light conditions. Under constant light conditions, leaf samples were collected at each corresponding time point (ZT 1, ZT 5, ZT 9, ZT 13, ZT 17, and ZT 21) for three consecutive days. Each experiment had three biological replicates. All samples were frozen immediately in liquid nitrogen and stored at -80 °C.

Arabidopsis and tobacco (*Nicotiana benthamiana*) plants were cultured in a similar chamber with photoperiod 16-h light/8-h dark. Murashige and Skoog (MS) medium with hygromycin was used to screen Arabidopsis transgenic seedlings. The statistics of flowering phenotype and gene expression analysis were measured in T3 homozygous transgenic lines.

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Gene identification

The genome information of pear (*Pyrus bretschneideri*) was acquired from the Pear Genome Project (http://gigadb.org/dataset/100083) [44]. The genome information of apple (*Malus domestica*) and peach (*Prunus persica*) were acquired from Phytozome (https://phytozome-next.jgi.doe.gov). The genome information of strawberry (*Fragaria vesca*), black raspberry (*Rubus occidentali*), and sweet cherry (*Prunus avium*) were acquired from the Genome Database for Rosaceae (https://www.rosaceae.org/). The genome information of Japanese apricot (*Prunus mume*) was acquired from the *Prunus mume* Genome Project [61] (https://github.com/lileiting/prunusmumegenome).

Protein sequences of five PRR members in Arabidopsis (*Arabidopsis thaliana*) were download from TAIR (http://www.Arabidopsis.org/). To identify the PRRs in the above seven Rosaceae species, AtPRR proteins served as query to perform BLAST in TBtools software with default parameters [62]. The candidate protein sequences were blast in NCBI database to confirm. The redundant and incomplete sequences were manually removed. Basic information of PRR proteins were analyzed using ProtParam online tool (https://web.expasy.org/protparam/), including number of amino acids, molecular weight and theoretical isoelectric point.

Phylogenetic analysis

ClustalW in MEGA7.0 software was used to perform multiple sequence alignment of PRR protein sequences from Arabidopsis and Rosaceae species. Next, the phylogenetic tree of above PRR proteins was constructed by maximum likelihood method in MEGA7.0 software, and the parameters were set to bootstrap value 1000 and Partial deletion 95% [63]. iTOL online tool (Interactive Tree Of Life, https://itol.embl.de/) was used to visualize and annotate the phylogenetic tree [64].

Evolution and synteny analysis

DupGen_finder (https://github.com/qiao-xin/DupGen_finder) was used to identify PRR paralogous gene pairs and corresponding duplication events in the genome of each Rosaceae species [41]. KaKs_caculator 2.0 software combined with Nei-Gojobori method was used to calculate Ka, Ks and ratio of duplicated gene pairs [65]. The synteny analysis between pear and six Rosaceae species was performed according to a method similar to the Plant Genome Duplication Database (http://chibba.agtec.uga.edu/duplication/) [66]. The potential orthologous gene pairs were identified by DIAMOND software [67], and the syntenic chains were identified by MCScanX software [45]. The final syntenic relationships were visualized using TBtools software.

Conserved motif and gene structure analysis

Conserved motifs of PRR proteins were predicted using MEME (Multiple Em for Motif Elicitation) online tool (http://meme-suite.org/tools/meme) [68]. The number of motifs was changed to 10, leaving the other parameters as default. Gene structures of *PRR* genes were plotted using GSDS (Gene Structure Display Server) online tool (http://gsds.gao-lab.org/) [69]. These results were integrated using TBtools software.

RNA extraction and quantitative real-time PCR (qRT-PCR)

The Plant Total RNA Isolation Kit (FOREGENE, Chengdu, China) was used to extract total RNA from pear and Arabidopsis frozen tissues [70]. The OneStep gDNA Removal and cDNA Synthesis SuperMix (TransGen Biotech, Beijing, China) was used to reverse transcribe 1 µg total RNA [71]. All operations were performed according to the instructions. qRT-PCR was performed using SYBR Green I Master Mix (Roche, Germany) on a Roche LightCycler 480 II. *PbUBQ* (*POLYUBIQUITIN*) was as the internal reference gene for pear and *AtACTIN* was as the internal reference gene for Arabidopsis. They were applied to calculated relative expression according to the $2^{-\Delta ct}$ method [71]. Three biological replicates in each experiment were measured. All qRT-PCR experiments followed MIQE guidelines [72]. Gene-specific primers for selected genes are listed in Table S4.

Subcellular localization analysis and plant transformation

The full-length coding sequence of *PbPRR59a* and *PbPRR59b* without the termination codon were respectively inserted into pCAMBIA1300 vector, which was driven by the CaMV (cauliflower mosaic virus) 35S promoter and fused with a GFP protein [73]. The recombinant plasmids and empty vector plasmid were respectively transformed into *Agrobacterium tumefaciens* GV3101 cells, and then injected into tobacco leaves. The subcellular localization assay was performed according to a published protocol [74]. DAPI (Thermo Fisher Scientific, US) is a nucleic acid stain to visualize nuclei. The fluorescence in tobacco epidermal cells was detected with a confocal laser scanning microscope LSM800 (Zeiss, Germany). To generate PbPRR59a and PbPRR59b overexpression transgenic plants, the corresponding *Agrobacterium* strains were transformed into Arabidopsis accession Columbia (Col-0) using the floral dip method [75]. The empty vector was also transformed into Arabidopsis Col-0 as the control plant. All transgenic lines were screened on MS medium containing hygromycin, and confirmed by semi-quantitative RT-PCR analysis with primers shown in Table S4.

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Abbreviations

PRR	PSEUDO RESPONSE REGULATOR
CCA1	CIRCADIAN CLOCK ASSOCIATED 1
LHY	LATE ELONGATED HYPOCOTYL
ELF3	EARLY FLOWERING 3
LUX	LUX ARRHYTHMO
TOC1	TIMING OF CAB EXPRESSION 1
CO	CONSTANS
BTC1	BOLTING TIME CONTROL 1
GI	GIGANTEA
psREC	pseudo receiver
CCT	CONSTANS, CO-like, and TOC1
FT	FLOWERING LOCUS T
DSD	dispersed duplication
WGD	whole-genome duplication
TD	tandem duplication
TRD	transposed duplication
PD	proximal duplication
ZT	zeitgeber time
GFP	green fluorescent protein
OE	overexpression
qRT-PCR	quantitative real-time PCR

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12864-024-10720-5.

> Supplementary Material 1: Table S1. Characteristics of the PRR family members in the seven Rosaceae species. Table S2 Numbers of *PRR* gene pairs from different duplication events in seven Rosaceae species. Table S3 Nonsynonymous substitution rates (Ka) and synonymous substitution rates (Ks) calculation of duplicated *PRR* gene pairs in seven Rosaceae species. Table S4 The primers used in the assays.
>
> Supplementary Material 2: Fig. S1. Original gels for Fig. 9b. The red rectangular box represents the cropped gels.

Availability of data and materials

All data used in this study are included in this article and additional flies. All genome sequence used in this study are publicly available in: Arabidopsis (http://www.Arabidopsis.org/), Chinese white pear (http://gigadb.org/dataset/100083), apple and peach (https://phytozome-next.jgi.doe.gov), strawberry, black raspberry and sweet cherry (https://www.rosaceae.org/), Japanese apricot (https://github.com/lileiting/prunusmumegenome).

Declarations

Ethics approval and consent to participate

All methods in this research were carried out in accordance with relevant guidelines. No specific permits are required for sample collection in this study.

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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