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Genome-wide analysis of the family 10 plant pathogenesis-related proteins in *Pyrus bretschneideri* and functional analysis of *PbrMLP* for *Colletotrichum fructicola* resistance

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Abstract

Pathogenesis-related (PR) genes are key regulators of plant adaptation responses to biotic and abiotic stresses. Family 10 PRs (PR10s, also known as major latex proteins) are usually induced by pathogens and environmental stresses. However, the evolutionary trajectory and functional divergence of the PR10 gene family in Chinese white pear (*Pyrus bretschneideri* 'Dangshan Suli') remain uncharacterized. The presence of 61 PR10s was detected across six Rosaceae species. The PR10 gene family was classified into two distinct groups by employing phylogenetic analysis and the taxonomic criteria of model plants. Interspecies synchrony revealed an ancient origin of the PR10 family in the six Rosaceae species, with 18 synchronic gene pairs. The expansion and evolution of the PR10 family were driven by various types of gene duplication events, with whole-genome duplication (WGD) being the primary mechanism. A candidate *Colletotrichum fructicola* (*C. fructicola*) resistance gene *Pyrus bretschneideri* major latex-like proteins (*PbrMLP*) belonging to the PR10 family was screened through transcriptomics and qRT-PCR. In addition, *PbrMLP*-silenced pear seedlings were more sensitive to *C. fructicola* than the controls. These results showed that *PbrMLP* is a candidate gene vital for anthracnose resistance in pears. These findings offer novel insights into the molecular mechanisms underlying the resistance to pear anthracnose infection in Rosaceae species and identify potential target genes for developing disease-resistant cultivars through genetic engineering.

Keywords Pathogenesis-related protein family 10, Plant defense response, Chinese white pear, Pear anthracnose, *Colletotrichum fructicola*

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Introduction

Pear is a perennial deciduous tree belonging to Rosaceae. The fruits are sweet, juicy, and nutritious. After grapes and apples, pears are the third largest temperate fruit crop in the world and are widely planted across six continents (Wu et al. 2018). The main producing areas include China, the USA, Italy, Argentina, and Spain. Pear anthracnose is a prominent disease in the major pear-producing regions of China, causing substantial economic losses due to its negative impact on both yield and quality (Zhang et al. 2015; Li et al. 2022). However, the severe occurrence of pests and diseases, coupled

with the lack of effective prevention measures and control technology, constitutes an impediment to the development of China's pear industry (Zhang et al. 2015; Li et al. 2022). Since 2007, the occurrence of pear anthracnose in China has increased acutely, and has spread rapidly to many varieties in the significant pear-producing areas, such as Anhui, Jiangsu, and Shandong (Li et al. 2021; Fu et al. 2019). Pear anthracnose causes fruit rotting, which mainly occurs in the middle and late stages of fruit growth (Jiang et al. 2014). *Colletotrichum fructicola* (*C. fructicola*) can cause diseases in several woody plants, such as mango, citrus, strawberry, and other fruit trees, which sharply reduces the crop yield.

Biotic and abiotic stresses significantly disrupt plant development and agricultural production. To cope with these challenges, plants develop sophisticated acclimation and defense mechanisms to regulate stress responses. Pathogenesis-related (PR) proteins are critical to plant defense and can be induced and expressed efficiently during biotic (Jiang et al. 2015) and abiotic stresses (Farrakh et al. 2018; Rawat et al. 2017; Wang et al. 2019). PR proteins are also associated with plant disease resistance. To date, 17 distinct families of PR proteins have been detected in a diverse range of plants, denoted as PR1–PR17 (Sels et al. 2008). These families are widespread in flowering plants and are essential components of the plant defense system (Liu and Ekramoddoullah 2006). PR1 is not only involved in disease resistance but also plays a pivotal role in regulating normal plant growth and development, as well as the response to hormone induction and abiotic stress. PR2s act as β -1,3-endoglucanases, which can catalyze the degradation of glucan, a component of fungal cell walls. PR3, PR4, and PR11 possess chitinase activity, which inhibits the growth of fungi by hydrolyzing chitin in the fungal cell wall (Bertini et al. 2009). PR5s are sweet-like proteins involved in systemic acquired resistance (SAR) and biotic stress responses. PR6s play a role in the resistance responses to nematodes and herbivorous insects (Laluk and Mengiste 2011). PR7s demonstrate intracellular protease activity and contribute to the lysis of the cell walls of pathogenic bacteria. PR8s have lysozyme activity and can directly interact with bacterial pathogens. PR9s are peroxidases that strengthen the plant cell walls against pathogenic bacteria by catalyzing the biosynthesis of lignin and suberin (Passardi et al. 2004). The defensin PR12, the thionine PR13, and the transfer protein PR14 demonstrate broad antibacterial and antifungal effects (Pomar et al. 2004; Thomma et al. 2002). PR15 and PR16 are typical monocot protein families with superoxide dismutase activities that can directly or indirectly produce H_2O_2 and induce plant disease resistance (Donaldson et al. 2001; Hu et al. 2003). PR17s have a zinc protease activity. Although these studies are relatively extensive

and in-depth, the PR10s are relatively less studied. Therefore, it is imperative to investigate the production and accumulation of PR10 in response to both biotic and abiotic stresses in plants.

PR10 represents one of the largest and most crucial families among the 17 PRs identified in plants. Several PRs have been identified and functionally characterized. In contrast, there is a paucity of knowledge regarding the PR10 gene family, specifically its role in plant immunity. PR10s, also known as major latex-like proteins (MLPs) (Fujita and Inui 2021), were first identified in opium poppy latex and are found in a wide range of plants. MLPs are structurally distinct from other PRs, although they were initially described as PR1s. PR10 was first isolated and identified from parsley in 1988. The essential feature of PR10 is a conserved glycine-rich domain (GxG-GxGxxK) with a P-loop, which affects its nuclease activity. Several years later, parsley PR1 and PR2, asparagus AoPR1, and bean PvPR1 and PvPR2 were collectively referred to as “PR10 proteins” (Langrish et al. 1992). PR10 is a vital defense-related protein in plants (Souza et al. 2017). It is ubiquitous in both monocots and dicots and is the most prominent PR gene family (Sinha et al. 2020; van Loon et al. 2006). The expression of PR10 responded to both pathogens and abiotic stresses.

PR10s play a pivotal role in plant growth, development, and response to both biotic and abiotic stresses. A part of the PR10 protein can hydrolyze RNA and the CaPR10 of pepper has RNase activity, which is closely related to its antiviral function (Choi et al. 2012). Phosphorylation may regulate the RNase activity (Park et al. 2004). *Vitis pseudoreticulata* pathogenesis-related 10.2 (VpPR10.2) plays a potentially pivotal role in the defense mechanism against grape downy mildew (He et al. 2013). The cold stress-induced expression of PpPR10 occurs in peaches, which accumulates in a large amount in the bark and xylem in winter, helping trees resist or recover from abiotic and biotic stresses. In *Arabidopsis* and tobacco, the contents of PR10 elevated markedly under drought stress (Xu et al. 2014; Kole et al. 2015).

In summary, PR10 primarily participates in plant responses to both biotic and abiotic stresses, and its RNase activity is the key to exerting antibacterial activity. PR10 assumes a pivotal role throughout the entire life cycle of plants. PR10 is also involved in plant resistance to certain diseases. Pear anthracnose is an extremely grave disease of pears. PR10 may be a potential pear anthracnose disease-resistance gene and is crucial for the molecular breeding of pears in the future. There are only a few reports concerning the resistance of pears and other Rosaceae plants to anthracnose. The healthy development of the plants of Rosaceae and in other industries should be ensured to clarify the disease resistance of

pears and other Rosaceae fruit trees. This study explored the function of *PR10*, focusing on its role in disease resistance and providing a scientific basis for the prevention and control of anthracnose in pears.

Materials and methods

Plant materials and bacterial strains

The Chinese white pear (*Pyrus bretschneideri* ‘Dangshan Suli’) seedlings were cultivated in a greenhouse under the controlled conditions of 75% relative humidity and 25°C under a 16 h/8 h light/dark photoperiod at the Pear Center of the Nanjing Agricultural University, Nanjing, Jiangsu, China. The *Agrobacterium tumefaciens* GV3101 strain was cultured in LB media at 28°C for virus-induced gene silencing (VIGS) and virus-inoculation experiments. The *C. fructicola* strain was identified and preserved at the Plant Protection Institute of the Jiangsu Academy of Agricultural Sciences, Nanjing, China. Subsequently, the plants were cultured at 25°C in an incubator in the dark at the Pear Center.

Sequence identification

The genome sequence of *P. bretschneideri* was acquired from the Pear Genome Project (<http://peargenome.njau.edu.cn/>) (Wu et al. 2013). The genome sequences of apple (*Malus domestica*) (Daccord et al. 2017), Shanxi wild pear (*P. betulifolia*) (Dong et al. 2020), strawberry (*Fragaria vesca*) (Li et al. 2019) and Japanese apricot (*Prunus mume*) (Zhang et al. 2012) were obtained from the Rosaceae Genome Database (<http://www.rosaceae.org/>). The genomic data of Peach (*Prunus persica*) (Verde et al. 2013) were downloaded from the Joint Genome Institute (JGI) (<http://www.jgi.doe.gov/>). The Conserved Domain Database (CDD) (<https://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml>) and the SMART online platform (<http://smart.embl-heidelberg.de/>) were employed to identify the presence of the Bet v I allergen domain and validate the accuracy of the findings.

Phylogenetic analysis

The multiple sequences of *PR10* gene families from six Rosaceae species and *Arabidopsis* were aligned using MUSCLE (<https://www.drive5.com/muscle/>) (Edgar 2004) and online by employing MAFFT (<https://mafft.cbrc.jp/alignment/server/index.html>). A maximum likelihood (ML) phylogenetic tree was constructed with 1000 bootstrap replicates utilizing IQ-TREE (<http://www.cibiv.at/software/iqtree>) to facilitate comparison of the results (Trifinopoulos et al. 2016). All *PR10* genes were named based on their positions in the phylogenetic tree.

Analysis of gene structure, functional domains, and conserved motifs

The *PR10* gene structure was analyzed using the Gene Structure Display Server (GSDS 2.0) provided by Peking University’s Computational Biology Institute (<http://gsds.cbi.pku.edu.cn/>) (Hu et al. 2015). The functional domains were annotated utilizing CDD. The MEME tool (<http://meme-suite.org/>) and TBtools with default settings were employed to comprehensively analyze the conserved motifs (Chen et al. 2020; Bailey et al. 2006).

Chromosomal location, synteny analysis, and Ka/Ks calculation

The chromosome position of *PR10* in six Rosaceae species was ascertained by annotating the genome of each species. Synteny among the six Rosaceae genomes was initially analyzed using a methodology akin to that used for Plant Genome Duplication Database (PGDD, <http://chibba.agtec.uga.edu/duplication/>). A subsequent blastp (<https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE=Proteins>) search identified the homologous gene pairs across multiple genomes. Next, the MCScanX software was utilized for collinearity analysis and the results were visualized with TBtools (Qiao et al. 2019). The Ka/Ks value was computed using TBtools (Chen et al. 2020).

Analysis of cis-acting elements in the promoter region

The region 2,000 bp upstream of the gene transcription start site is the promoter region. The promoter region is defined as the region 2,000 bp upstream of the gene transcription start site. The *cis*-acting elements within the gene promoters were analyzed using the PlantCARE web tool (<http://bioinformatics.psb.ugent.be/webtools/plantcare>).

Expression pattern and analysis of genes post *C. fructicola* infection

The transcriptome data from pears infected with *C. fructicola* is yet to be released. The transcriptome data mentioned above were visualized using TBtools (Chen et al. 2020). RNA was extracted from pear leaves using an RNA extraction kit (Nanjing Vazyme Biotech Co., Ltd, Nanjing, China) and subsequently reverse transcribed into cDNA using a reverse transcription kit. The primers specific to the candidate genes were designed based on information obtained from NCBI (<https://www.ncbi.nlm.nih.gov/>) (Table S3). The qRT-PCR experiments were conducted using the SYBR Master Mix reagent (CW BIO, Jiangsu, China) on a Lightcycler 480 system (Roche,

Basil, Switzerland). Data were analyzed using the $2^{-\Delta\Delta C_t}$ method.

Subcellular localization

The 35S-*PbrMLP-GFP* fusion vector was constructed and subsequently introduced into *A. tumefaciens* GV3101 cells. The tobacco leaflets were injected with these cells and incubated for 24 h in the dark, followed by 48 h under light. An LSM800 confocal microscope (Zeiss, Oberkochen, Germany) was utilized for image acquisition, and the ZEN 3.1 software (Zeiss) was used for image analysis.

VIGS

VIGS was conducted following established protocols (Zhang et al. 2022). The full-length cDNA sequence of the gene was used for primer design. A ~300 bp sequence was selected, and sites for the restriction endonucleases *EcoRI* and *BamHI* were introduced at both ends. The gene fragment was PCR-amplified and inserted into the cloning site of the Tobacco Rattle Virus-based vector (TRV2). The constructed vector was subsequently employed to transform *A. tumefaciens* strain GV3101 cells. The cell culture was then adjusted to an OD₆₀₀ of 1.0. The buffer solution consisted of 10 mM MgCl₂, 10 mM 2-Morpholinoethanesulphonic acid (MES), and 200 mM acetosyringone at pH 5.6. *Agrobacterium* culture and buffer were mixed in a ratio of 1:1 v/v and shaken slowly for 4 h in the dark at room temperature. The above mixture was injected into the leaves of pear seedlings. The treated plants were then transferred to a growth chamber specifically designed for optimal pear seedling growth. The injected pear seedlings were dark-treated for 1 day and then grown in the greenhouse for 2 weeks. After a fortnight, the relevant uninfected leaves were collected from each plant and subjected to genomic PCR and qRT-PCR analysis.

C. fructicola infection and analysis of the physiological indices

C. fructicola was cultured on potato glucose agar (PDA) media for about 1 week, and the agar was used to inoculate pear leaves. The inoculated leaves were then cultured in the dark and sampled. Trypan blue staining was carried out next to determine the extent of cell death in the pear leaves after treatment. H₂O₂ levels, superoxide dismutase (SOD) activity, and peroxidase (POD) activity were ascertained by kits bought from the Nanjing Jiancheng Institute of Bioengineering (Nanjing, China).

Statistical analysis

The data are presented as the mean ± standard error (SE) obtained from a minimum of three independent replicates of each representative experiment. The data were analyzed using Duncan's multiple range tests and Student's *t*-test by employing SPSS 22 (IBM, Chicago, IL, USA), with statistical significance set at *P* < 0.05, *P* < 0.01, and *P* < 0.001.

Results

Identification of PR10s in six Rosaceae species

A hidden Markov model (HMM) search was conducted using the HMM profile of the Bet v I allergen domain (PF00407) and blastp utilizing the MLP protein sequences from *Arabidopsis* to search for PR10 proteins in the six rosaceous plants, *P. bretschneideri*, *M. domestica*, *P. betulifolia*, *F. vesca*, *P. persica*, and *P. mume*. The accuracy of the results was validated by employing CDD and SMART to detect the presence of the Bet v I allergen domain. A total of 268 candidate *PR10* family genes were identified in the six Rosaceae plants (Fig. 1). The number of genes identified in Maloideae was significantly greater than that in other rosaceous plants due to a unique whole-genome duplication (WGD) event that occurred ~30–45 million years ago (Mya), in pear and Maloideae. The lengths of the encoded proteins ranged from 97 (FvH4_4g32801.t1) to 760 (GWHPAAYT014441) amino

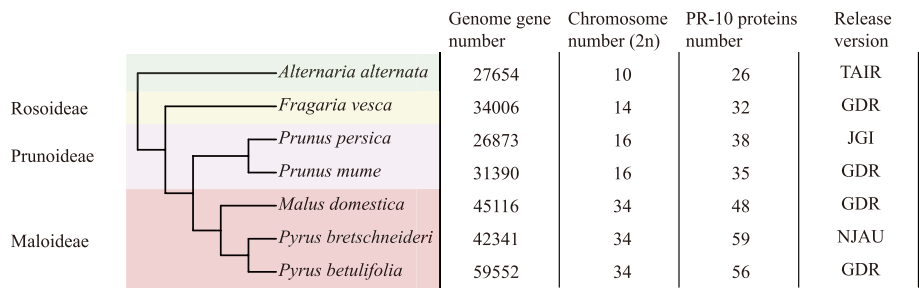


Fig. 1 Genome information and the number of the family 10 plant pathogenesis-related proteins (PR10s) identified in six Rosaceae species (*Pyrus bretschneideri*, *Malus domestica*, *Pyrus betulifolia*, *Fragaria vesca*, *Prunus persica*, and *Prunus mume*)

acids, with an average length of 167 amino acids. The sequence information can be found in Table S1.

Phylogenetic analyses of the PR10 family

The amino acid sequences of the PR10s from the six species were used to construct a phylogenetic tree utilizing the ML method, with *Arabidopsis* serving as the

outgroup (Fig. 2). An analysis of the phylogenetic tree taxonomically classified the 206 PR10s in two distinct subgroups, 1 and 2 (Fig. 2). Notably, all *Arabidopsis* PR10s were assigned to only group 1.

The PR10 AT1G70890 from *Arabidopsis* reduced Abscisic Acid (ABA) sensitivity during seed germination, while the AT1G70890-transgenic *Arabidopsis* plants were

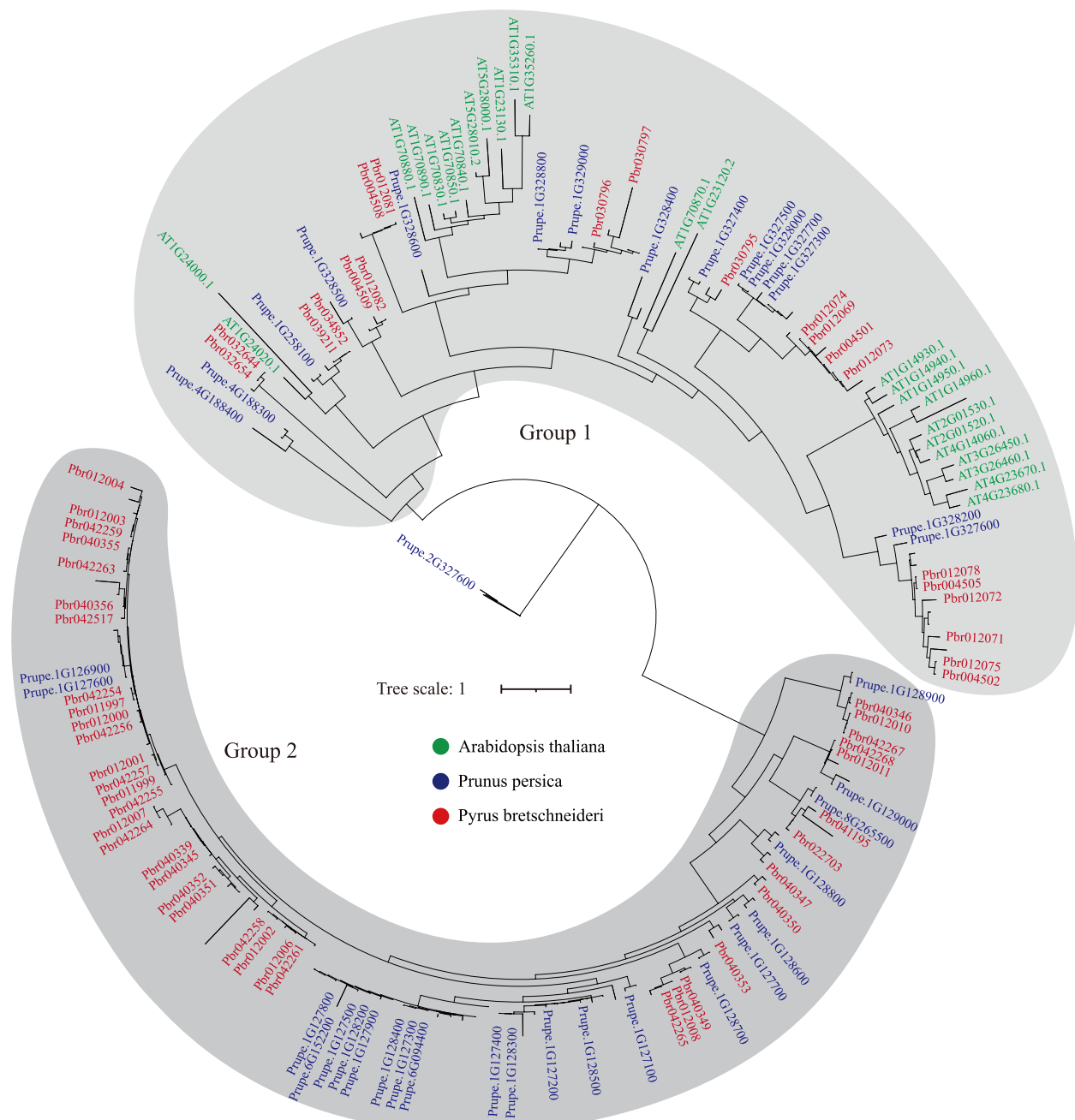


Fig. 2 Phylogenetic analysis of the family 10 plant pathogenesis-related proteins (PR10s) from *Arabidopsis* and six Rosaceae plants (*Pyrus bretschneideri*, *Malus domestica*, *Pyrus betulifolia*, *Fragaria vesca*, *Prunus persica*, and *Prunus mume*)

drought tolerant, indicating that *AT1G70890* was sensitive to drought stress (Wang et al. 2016). The branching relationship of the phylogenetic tree revealed that the two *PR10*s, *Pbr030796* and *Pbr030797*, in Chinese white pear were more closely related to *AT1G70890*. Therefore, it was speculated that *Pbr030796* and *Pbr030797* may confer drought stress resistance. Additionally, two more *PR10*s in *Arabidopsis*, *AT2G01520* and *AT2G01530*, were induced by *cis*-cinnamic acid to facilitate vegetative growth and prolong the onset of flowering (Guo et al. 2011). The phylogenetic tree indicated that the *PR10*s *Pbr012078* and *Pbr004505* of Chinese white pear were

more closely related to *AT2G01520* and *AT2G01530*. Therefore, it was speculated that *Pbr012078* and *Pbr004505* were functionally similar to those described above.

Analyses of the conserved motifs and domains of the PR10s

The phylogenetic relationships among the *PR10*s of 59 Chinese white pears were clarified by constructing a neighborhood joining (NJ) phylogenetic tree (Fig. 3). The findings classified the protein sequences into two distinct categories: groups 1 and 2 (Fig. 3a).

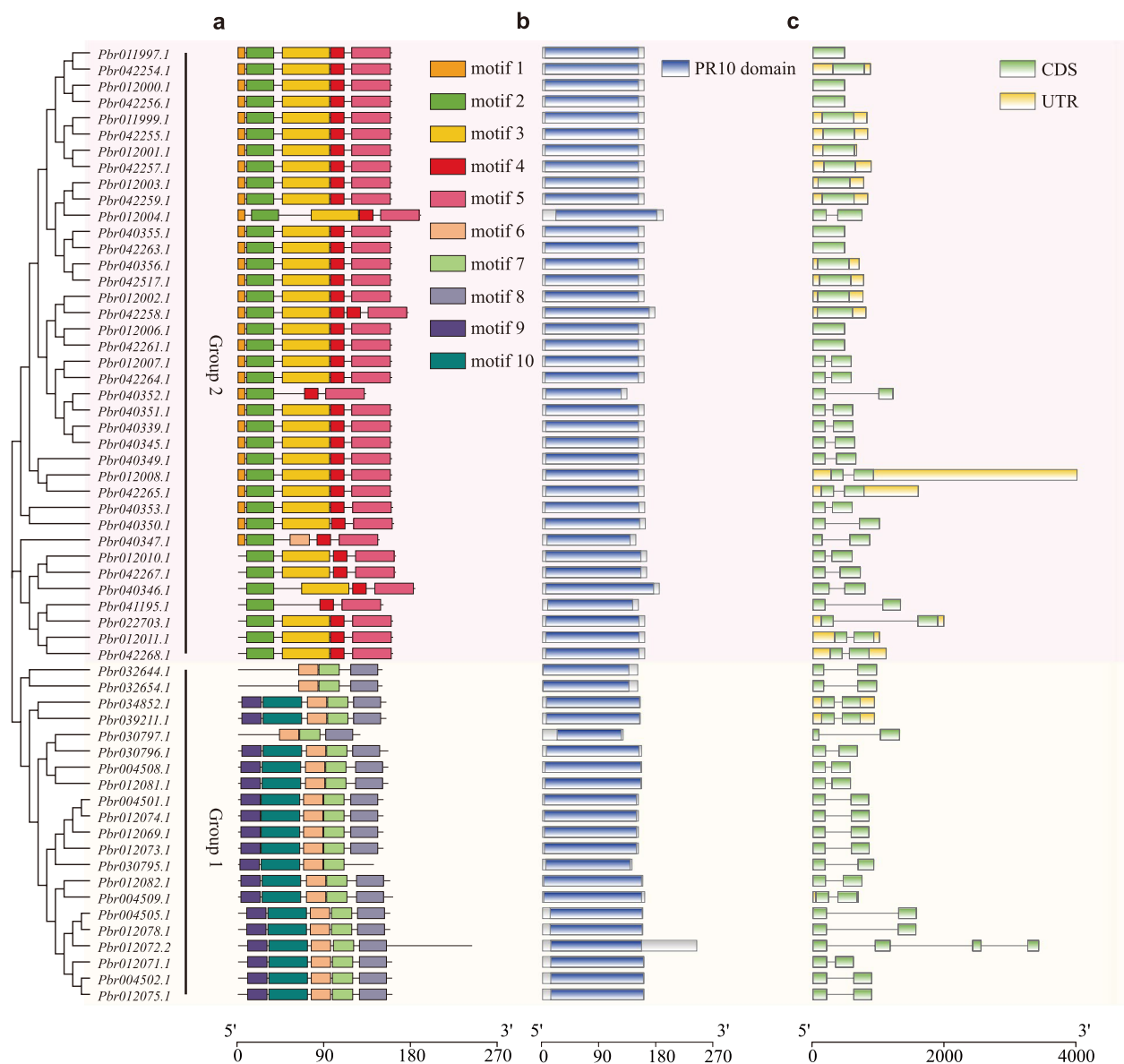


Fig. 3 Schematics of the conserved motifs (a), functional domains (b), and gene structure (c) of the family 10 plant pathogenesis-related proteins (*PR10*s) in pear

The open reading frames (ORFs) exhibited intact functional domains (Fig. 3b). Exon/intron structure analysis revealed that of the 59 *PbrPR10*s, the coding sequences (CDSs) of 41 (~69%) were disrupted by introns (Fig. 3c). Among these, 18 had two exons, 40 had three exons, and *Pbr102072.2* had three exons. These discrepancies may have arisen from alterations in gene duplication events. Previous investigations on apples have shown that a majority of the *MdMLPs* exhibit a two-exon structure, which was consistent with *PbrPR10*, suggesting that these genes are evolutionarily conserved (Yuan et al. 2020). The genes clustered in the same subgroup exhibited similar exon/intron structures, albeit with varying intron lengths.

The motifs were identified by searching Motif-based sequence analysis tools (MEME), and the sequence logos of motif 1–motif 10 were drawn to predict the structural features of the *PbrPR10* protein and to identify the conserved amino acid residues (Fig. 3a). The results demonstrated a high degree of conservation in the structure of the *PbrPR10* protein family. Among the 59 *PbrPR10*s, 46 had five motifs, eight had four motifs, and four had three motifs. However, *Pbr042258.1* had six motifs. Compared with the other proteins in Group 1, *Pbr042258.1* had one more motif, motif 4. In addition, the motif components of Groups 1 and 2 varied markedly. Most Group 1 proteins consisted of motif 1–motif 5, and most Group 2 proteins consisted of motif 6–motif 10.

Chromosomal localization and collinearity analysis of the *PR10* family

The duplication of genes plays a pivotal role in the expansion of the gene family and represents the most prominent characteristic of the plant genome structure. Chromosomal localization and gene collinearity analysis were performed on the chromosomes of the six Rosaceae plants to obtain a deeper understanding of the amplification of the Rosaceae *PR10* gene family, chromosomal localization, and gene collinearity (Fig. 4). The collinearity investigations identified 5, 4, 6, 1, 1, and 1 gene pairs in the six species. The expansion of the *PR10* family in the Maloideae genomes can be attributed to a recent WGD event.

Duplicate type, synteny, and Ka/Ks of *PR10* genes in pear

The evolutionary phase of WGD is usually estimated using the rate at synonymous sites (Ks) (Lemoine et al. 2007; Jun et al. 2009; Xu et al. 2018). The most recent WGD in pears was detected at 30–45 Mya ($K_s=0.15–0.3$). The genes generated by WGD had higher Ka/Ks values than those reduced by other replication methods. The direction and magnitude of the selective pressure can be inferred from the Ka/Ks ratio. Positive selection

is indicated when $K_a/K_s > 1$, neutral evolution is identified when $K_a/K_s = 1$, and purifying selection occurs when $K_a/K_s < 1$ (Starr et al. 2003). The findings illustrated that in each species, the Ka/Ks ratios for all paralogous *PR10* gene pairs were consistently < 1 (Fig. 5, Table S2), indicating that purifying selection has facilitated the expansion of the *PR10* gene family within Rosaceae.

The potential role of *PR10*s in the response of pears to infection by *C. fructicola*

The possible response of *PR10*s to *C. fructicola* inoculation was identified by simulating inoculation and *C. fructicola* inoculation and generating a heatmap to express the differences (Fig. 6a). The putative functions of *PR10*s in response to *C. fructicola* inoculation and their expression patterns were determined by investigating the expression levels of the 59 *PbrPR10*s. Notably, the two classes of *PR10*s showed dramatically varied expression patterns. The expression of Group 2 proteins was markedly upregulated immediately after inoculation and peaked at 12 or 24 h post-infection (hpi). However, that of the Group 1 proteins peaked when not inoculated and then gradually reduced.

In Group 2, the peaks of *Pbr012006.1* and *Pbr042261.1* were relatively more pronounced at 24 hpi, while those of *Pbr040352.1* and *Pbr040353.1* were at 12 hpi. At 48 hpi, the degree of downregulation of *Pbr040347.1* and *Pbr042264.1* was relatively obvious. In Group 2, *PbrMLP* was primarily focussed on, which exhibited an upregulated expression at 12 hpi and peaked at 24 hpi. The expression levels also enhanced at 24–48 hpi. This observation suggests that *PbrMLP* may confer resistance to *C. fructicola*. In Group 1, the protein expression level was relatively high in the non-inoculated group and gradually diminished after inoculation. The expression levels of *Pbr004505.1*, *Pbr030797.1*, and *Pbr012073.1* were relatively high in non-inoculated plants. At 48 hpi, the expression levels of *Pbr030796.1* and *Pbr012071.1* were relatively low.

In addition, the expression of six genes in plants inoculated with *C. fructicola* was verified by transcriptomic data and qRT-PCR (Fig. 6c). At 72 hpi, the relative expression of *Pbr004509.1* and *Pbr012072.2* in Group 1 reduced, while that in Group 2 elevated. Notably, that of *Pbr040356.1* in Group 2 enhanced.

A promoter is a DNA sequence that can be recognized by RNA polymerase and transcription factors and specifically combined with it, thereby determining the correct and efficient initiation of transcription of a gene. To gain further insights into the mechanisms underlying the transcriptional regulation of *PR10* in pear plants, PlantCARE (<http://bioinformatics.psb.ugent.be/webtools/plantcare>) was utilized to analyze the promoter, aiming

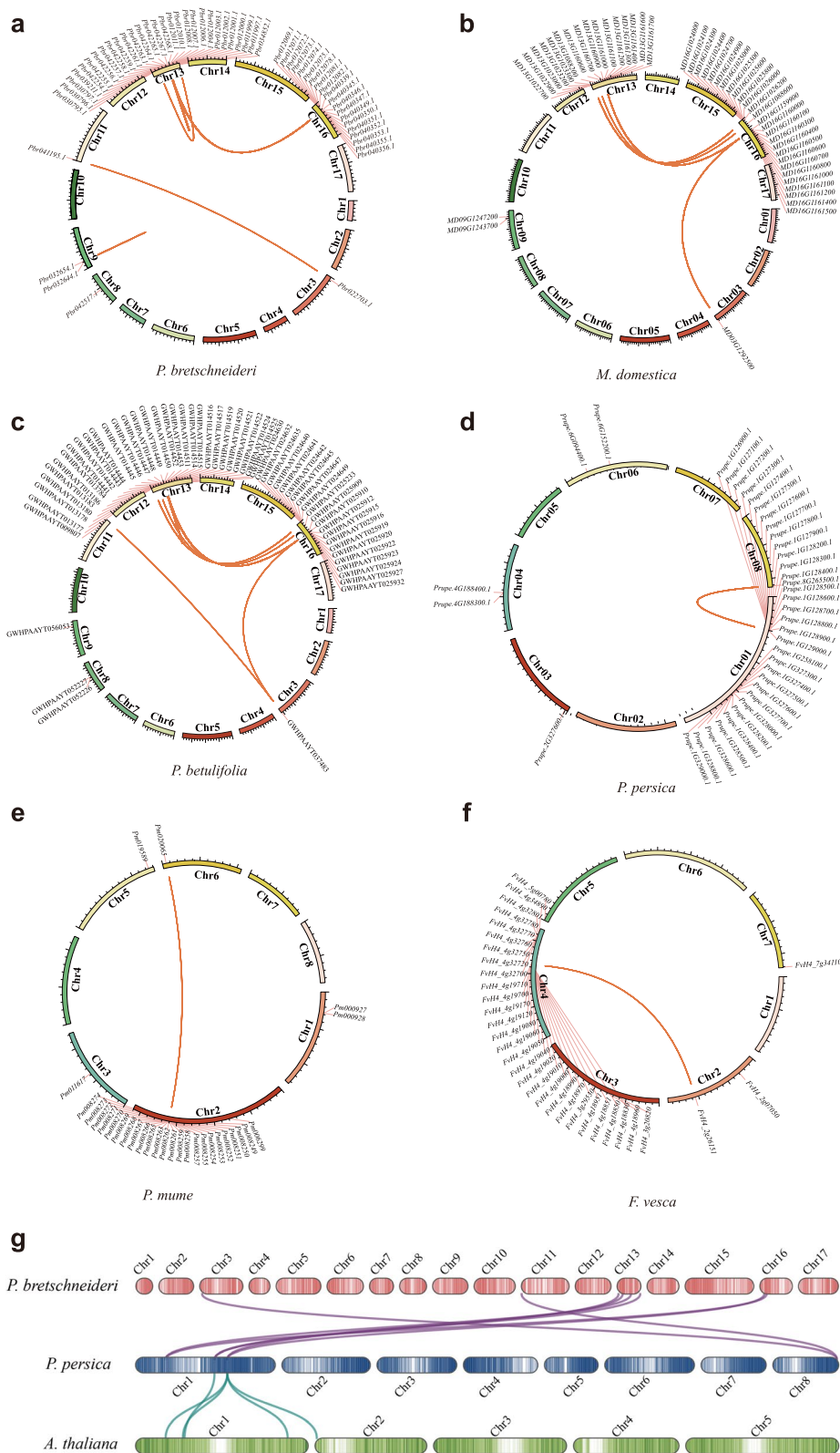


Fig. 4 Gene location and collinearity analysis of the family 10 plant pathogenesis-related protein (PR10) gene family. **a** *Pyrus bretschneideri*. **b** *Malus domestica*. **c** *Pyrus betulifolia*. **d** *Prunus persica*. **e** *Prunus mume*. **f** *Fragaria vesca*. The lines indicate collinear gene pairs with syntenic relationships in the different gene families. **g** Synteny of the PR10s

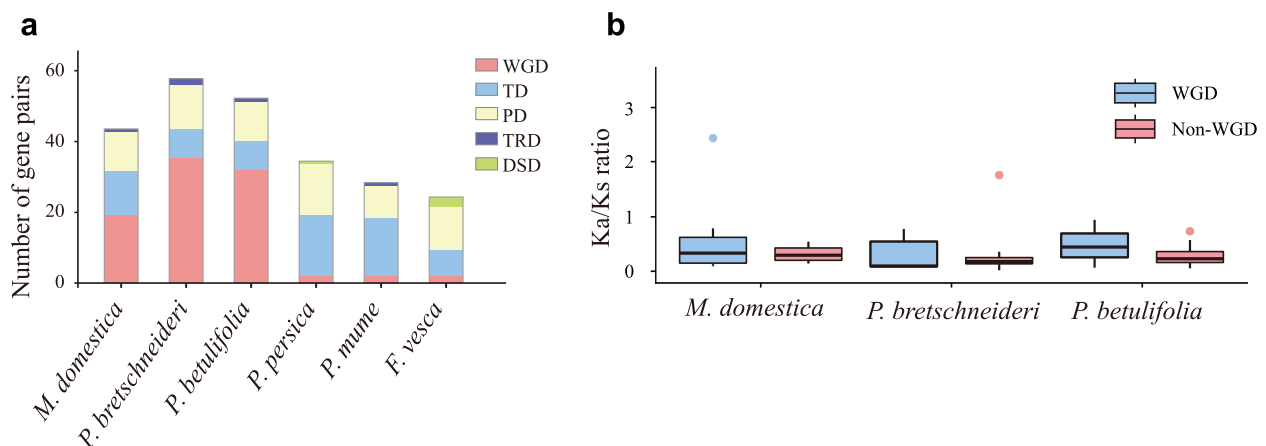


Fig. 5 Gene pairs and collinearity analysis of the *PR10* gene family in six Rosaceae species (*Pyrus bretschneideri*, *Malus domestica*, *Pyrus betulifolia*, *Fragaria vesca*, *Prunus persica*, and *Prunus mume*). **a** Number of gene pairs. **b** Ka/Ks ratio. WGD, whole-genome duplication; TD, tandem duplication; PD, proximal duplication; TRD, transposed duplication; DSD, dispersed duplication

to identify and characterize the *cis*-regulatory elements (Fig. 6b). These elements can be categorized into five main groups: salicylic acid-responsive, low temperature-responsive, methyl jasmonate-responsive defense and stress-responsive, and v-myb avian myeloblastosis viral oncogene homolog (MYB) binding site elements induced by drought. The results revealed that the content of the methyl jasmonate responsive element (TGACG/CGTCA motif) was the greatest, which is relevant to drought induction. The downstream MYB binding site elements were subsequently detected. A substantial proportion of these genes exhibited *cis*-elements associated with stress response, including motifs such as drought responsive elements (MBSs) and low-temperature responsive elements (LTRs).

Subcellular localization of *PbrMLP*

Subcellular localization can reveal the specific localization of the target protein in the cell and provides a more direct understanding of the gene expression process at the cellular level. In this study, we constructed a fusion vector of *PbrMLP* and green fluorescent protein (GFP), *35S-PbrMLP*-GFP; subsequently, these genes were transferred into small leaf tobacco leaves via an *Agrobacterium*-mediated approach. The injected lobular tobacco plants were subjected to darkness for 24 h and light for 48 h, after which the leaf slices were observed under a laser confocal microscope. Compared with the leaves in the control group injected with the empty GFP vector containing *Agrobacterium*, those cells expressing the *35S-PbrMLP*-GFP fusion protein exhibited green fluorescence in the nucleus, cytoplasm, and cell membrane. Therefore, it can be postulated that *PbrMLP* may be localized to these organelles (Fig. 7).

Transient silencing of *PbrMLP* in pear seedlings

To determine whether *PR10* is needed for pear disease resistance, we used a VIGS system to silence *PbrMLP*. The role of *PbrMLP* in the defense response against pathogens was investigated by employing VIGS to suppress its expression in pear leaves temporarily. The purpose of this study was to explore further the role of *PR10* in pear anthracnose resistance. After 14 days post-injection, leaves were collected from the newly grown VIGS-silenced and non-silenced plants. The effectiveness of the VIGS procedure was confirmed through qRT-PCR (Fig. 8b). These plants were selected for further analysis and inoculated with *C. fructicola*. After 1 week, the size of each lesion was measured. The leaves inoculated with TRV2-*PbrMLP* bearing *Agrobacterium* enlarged (Fig. 8a). Trypan blue staining revealed remarkably higher cell death in the TRV2-*PbrMLP* group than in the TRV control group. The H_2O_2 levels in the silenced leaves were evidently greater than that in the control check (CK) leaves (Fig. 8c), while the opposite was observed in POD and SOD activities (Fig. 8d–e). These results indicated that *PbrMLP* is critical for resistance to anthracnose in pears.

Discussion

PRs are a class of plant proteins that are induced and expressed in response to disease progression. PRs play a pivotal role in plant growth, development, and response to both biotic and abiotic stresses. PRs are divided into 17 families, with the *PR10* family having nuclease or synthetase activities. The expression of *PR10* can be induced under the conditions of biotic stress, such as those involving infection by viruses, bacteria, and fungi, as well as under abiotic stress.

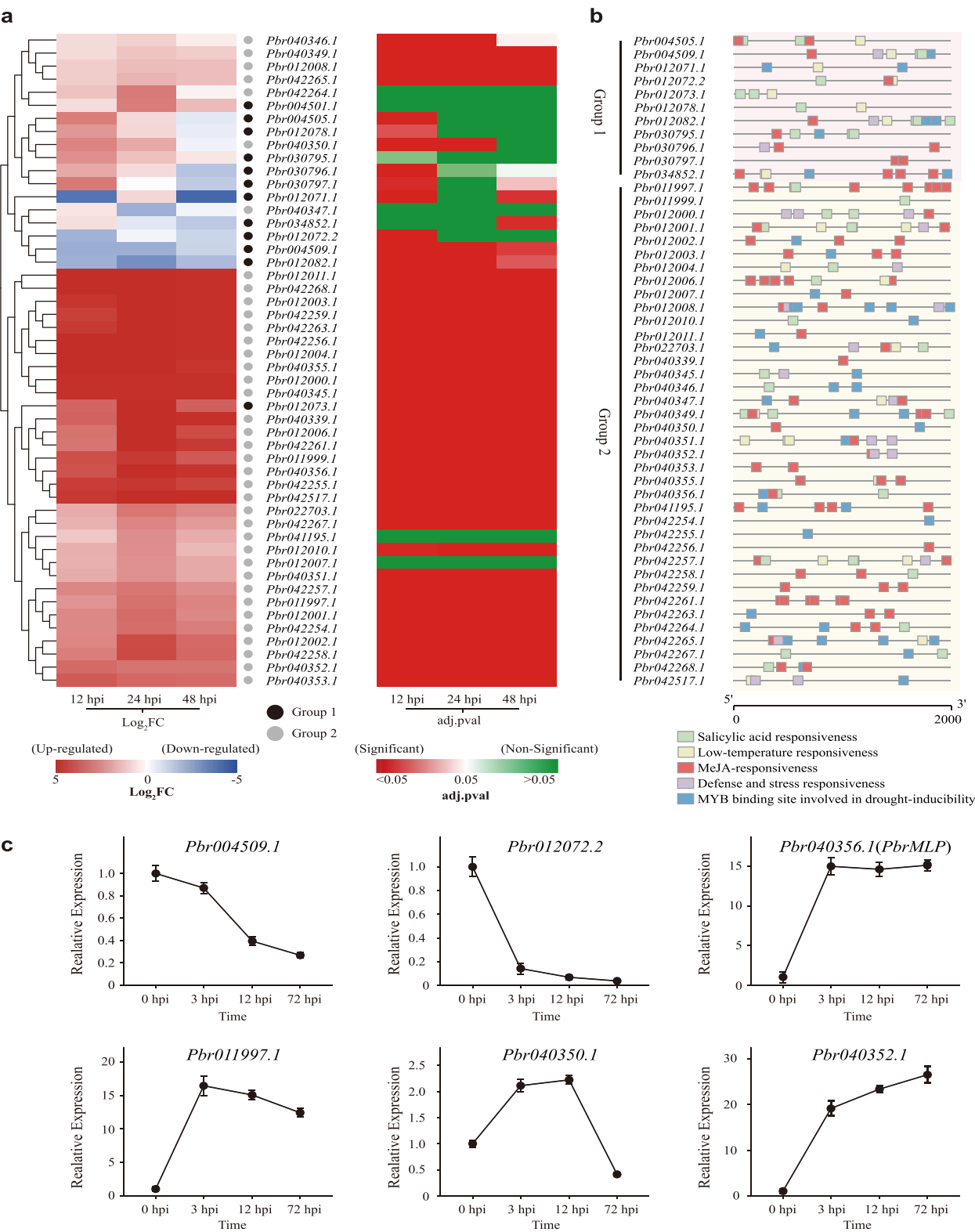


Fig. 6 Alterations in the expression patterns and functional prediction of family 10 PRs (PR10s, also known as major latex proteins) in pears. **a** Changes in the expression of PR10s in pear after inoculation of *Colletotrichum fructicola*. **b** The distribution of cis-acting elements in the promoters of the PR10 regulators. **c** qRT-PCR analysis of six genes at different time of inoculation of *Colletotrichum fructicola*. MYB, v-myb avian myeloblastosis viral oncogene homolog; hpi, hour post infection; Log₂ FC, Log₂ Fold Change; adj.pval, adjusted *p*-value

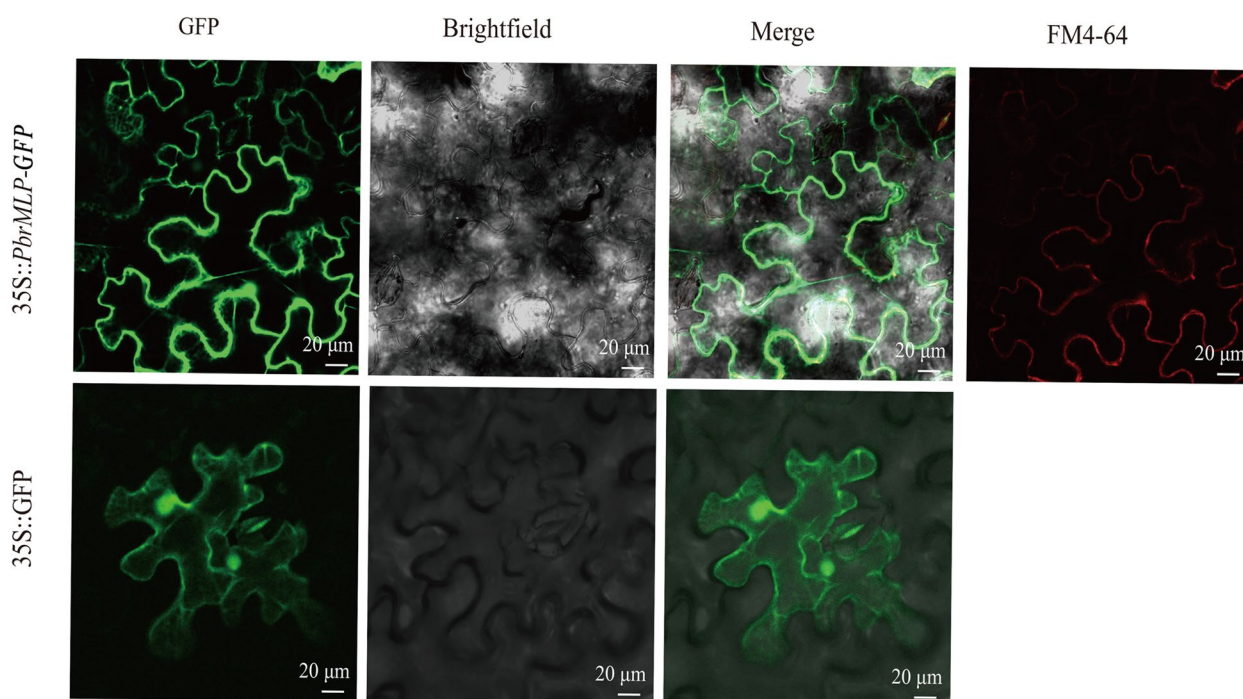


Fig. 7 Subcellular localization of the *Pyrus bretschneideri* major latex-like protein (*PbrMLP*). The fusion vector 35S::PbrMLP-GFP and empty vector (35S::GFP) were transiently expressed in tobacco leaf epidermal cells

PR10 can be induced by biotic stress and participates in the defense response. *WpPR10-3.1* of western white pine (*Pinus monticola*), when expressed in *Escherichia coli*, conferred non-specific resistance to a broad spectrum of pathogens. In soybean (*Glycine max*), *GmPR10* was upregulated by *Phytophthora sojae*, and *PR10*-transgenic tobacco and soybean plants exhibited an improved resistance to *Phytophthora sojae*. The maize the family 10 plant pathogenesis-related protein (*ZmPR10*) and *ZmPR10.1* genes were overexpressed in *Arabidopsis thaliana*, leading to resistance against the *Pseudomonas syringae* pv. tomato DC3000 strain (Xie et al. 2010). *VpPR10.2* of the Chinese wild-type grape *Vitis pseudoriculata*, when expressed in *V. vinifera*, a European grape susceptible to downy mildew, the transgenic plants revealed a markedly elevated resistance to *Plasmopara viticola* (He et al. 2013).

The *PR10* gene family encodes a group of proteins that play pivotal roles in defense and stress responses. Their diverse functions in abiotic stress and disease tolerance have been identified in many species (Fujita and Inui 2021). The expression of the *SmPR10* in willow was induced by salt, thereby enhancing salt tolerance (Han et al. 2017). In rice, the RSOsPR10 protein is induced under abiotic stress and in the roots of blast-infected plants by activating the jasmonic acid signaling pathway. The *ThPR10* plays a crucial role in the response of *Salix*

tomentosa to abiotic stress and is regulated through the ABA-dependent signaling pathway (Zhang et al. 2010). The expression of the *PR10-3.1* homolog was elevated after methyl jasmonate treatment, indicating that it also plays a role in the abiotic stress response of conifers (Liu et al. 2017). *PgPR10-2* in *Panax ginseng* is expressed explicitly in the roots and can improve salt tolerance (Lee et al. 2012).

As a constituent of the PR family, *PR10* is a protein with a conserved structure and multiple functions, and the phenomenon mentioned above assumes a pivotal role in bolstering plant resilience against environmental stresses. Several reports are available on the classification, physicochemical properties, and biological functions of *PR10*s. The two *PR10*s *AT2G01520* and *AT2G01530* in *Arabidopsis* can promote vegetative growth and delay flowering via induction by *cis*-cinnamic acid (Guo et al. 2011). The clade relationships depicted in the phylogenetic tree indicated that the two *PR10* members *Pbr012078* and *Pbr004505* in Chinese white pears and *Arabidopsis* are more closely related. Therefore, it was hypothesized that the two *PR10* members in Chinese white pears, *Pbr012078* and *Pbr004505*, which promote vegetative growth and delay flowering, can also be induced by *cis*-cinnamic acid. *AT1G70830* and *AT1G70890* in *Arabidopsis* root tips were upregulated under gravitational and mechanical stimuli (Kimbrough et al. 2004; Lytle

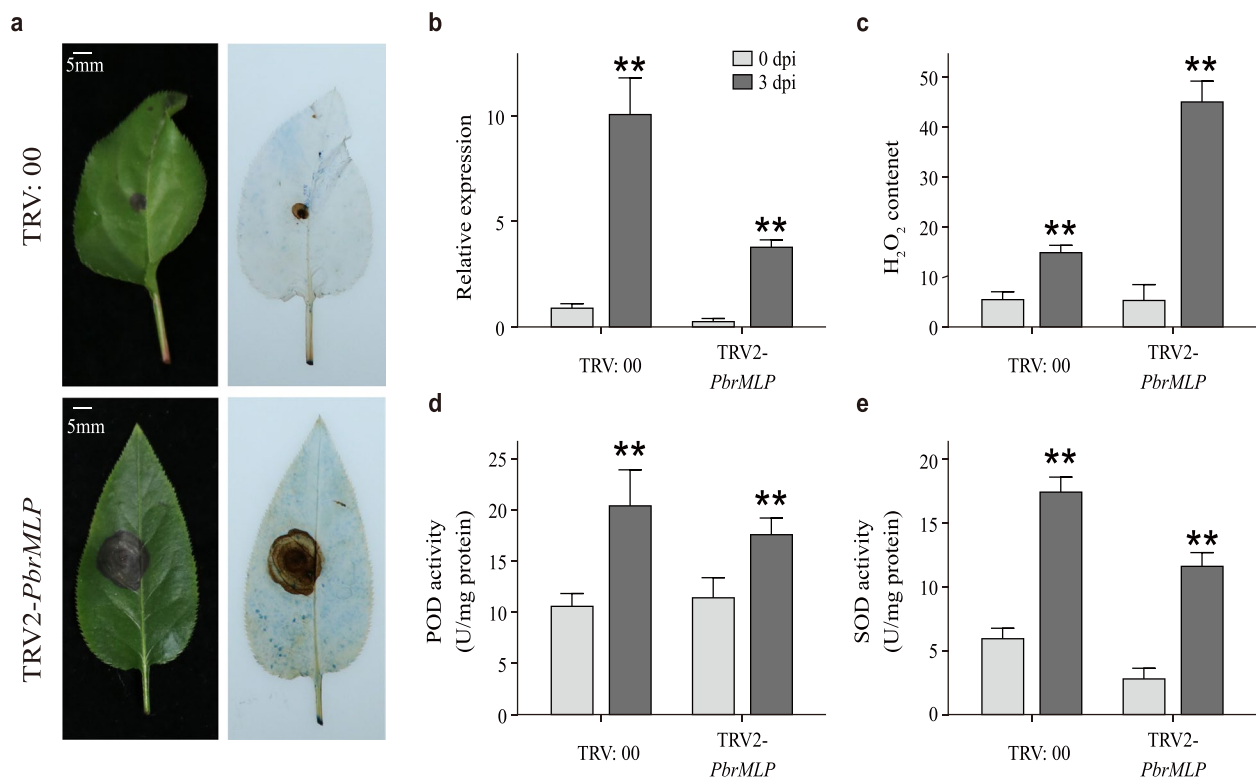


Fig. 8 Silencing of the major latex protein (*PbrMLP*) reduced the resistance to pear anthracnose in *Pyrus bretschneideri*. **a** Leaf phenotype and Trypan blue staining after 1 week of inoculation of *Colletotrichum fructicola*. **b** Relative expression of *PbrMLP* in the control and virus-induced gene silencing (VIGS) leaves before and after inoculation of *Colletotrichum fructicola*. **c–e** H₂O₂ contents (**c**), peroxidase (POD) activity (**d**) and superoxide dismutase (SOD) activity (**e**) in the control and VIGS leaves before and after inoculation of *Colletotrichum fructicola*. (***P* < 0.01; *n* = 3–12). TRV, tobacco rattle virus

et al. 2009). Therefore, based on the branching relationship identified in the phylogenetic tree, it was speculated that the expression of the two PR10s of the Chinese white pear, *Pbr030796* and *Pbr030797*, may also be upregulated by the same stimuli.

Gene duplication promotes morphological diversity in a species and plays a pivotal role in driving species evolution (Zhang 2003; Flagel and Wendel 2009; Panchy et al. 2016). The gene duplication events can generally be classified into five distinct types: WGD, tandem duplication (TD), proximal duplication (PD), dispersed segmental duplication (DSD), and transposed duplication (TRD) (Qiao et al. 2015). WGD simultaneously replicates all nuclear genes of an organism, generating a substantial number of duplicated genes. WGD represents a prominent characteristic of the evolutionary dynamics of plant genomes (Moghe and Shiu 2014; Michael and VanBuren 2015; Salman-Minkov et al. 2016). WGDs lead to a 90% increase in the number of genes in the *Arabidopsis* family. An unequal intersection results in TD, which is frequently associated with inversion events (Freeling 2009; Hahn 2009). Additionally, two gene duplications result in

the formation of closely clustered proximal gene pairs on chromosomes, with only a few intervening genes (Wang et al. 2012). The amplification of the ethylene response element binding regulation APETALA2/ethylene-responsive element binding factors (AP2/ERF) and *WRKY* gene families are most likely attributed to segmental and tandem repeats, which may be the leading force for AP2/ERF and *WRKY* expansion (Du et al. 2012; Guo et al. 2014). Protein–protein interactions and those involving multiple genetic factors, such as gene signatures, expression levels, and alternative splicing events, are the two main processes for the preservation of the duplicated genes (Du et al. 2012; Guo et al. 2012; Grishkevich and Yanai 2014; McGrath et al. 2014). The two gene families, the Al-activated malate transporter and heat shock transcription factors, were amplified by WGD and DSD (Xu et al. 2018; Qiao et al. 2015). Among the six Rosaceae members, the number of *PR10*s identified varied, the main reason was that apples, pears, and other species in the apple subfamily underwent additional WGDs. This study found that apple, Chinese white pear, and Shanxi wild pear exhibited the greatest number of *PR10*s with WGDs

and DSDs, unlike the other three species, indicating that DSD and WGD are majorly involved in the amplification of the *PR10* gene family in these three species. WGDs are divided into two subtypes: old and recent. Previous studies have shown that pears, apples, and strawberries of Rosaceae experienced the same ancient hexaploidy event as dicots 140 Mya, and a WGD event occurred. Pears and apples underwent another WGD event ~30–45 Mya, and they differentiated ~0.54–21.5 Mya (Wu et al. 2013). Thus, because of these events, apples, Chinese white pears, and birch-leaf pears had a large proportion of WGDs in *PR10*, while strawberries, peaches, and plums had DSDs. The estimation of the evolutionary timing of WGD or SD events primarily relies on the *Ks* value. In this study, the WGD-derived genes of peaches, strawberries, and plum blossoms belonging to Rosaceae had *Ks* values > 1. Such a result further suggested that these genes only experienced the ancient hexaploidization event common to dicots 140 Mya but not the more recent WGD event and also that these genes were copied and preserved in much older WGD events (Shulaev et al. 2011; Zhang et al. 2012; Verde et al. 2013).

Conclusions

Here, we identified 268 candidate *PR10*s across six Rosaceae species. The *PR10*s were divided into two significant branches based on a phylogenetic tree. The *PR10* levels were amplified in the six species. Only a limited number of gene pairs exhibited *Ka/Ks* values > 1, while the majority underwent rigorous purification selection processes. Finally, *PbrMLP* was transiently silenced in pear leaves *via* VIGS technology, inoculated with *C. fructicola*, and qRT-PCR analysis was performed. *PbPbMLP* was recognized as a candidate *PR10* gene conferring resistance to pear anthracnose. The findings presented here establish a foundation for further investigations into the molecular mechanism underlying this resistance.

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1007/s44281-024-00037-4>.

Supplementary Material 1: Table S1. Overview of the *family 10 plant pathogenesis-related protein (PR10)* gene family in six Rosaceae species.

Supplementary Material 2: Table S2. *Ka/Ks* and duplicate type analysis of the duplicated gene pairs in the *major latex proteins (PbrMLPs)*.

Supplementary Material 3: Table S3. Primers were designed using the Primer Premier 6 software (<https://primer-premier.software.informer.com/6.0/>).

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Authors' contributions

SZ and XH designed experiments. ZS and CH performed all bioinformatics analysis. ZS performed all molecular biology experiments. ZS, CH, QQ, CL, HD, XW, KQ and ZX analyzed all data. SZ, XH and ZS wrote the manuscript. All authors have read and approved the final version of the manuscript.

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Availability of data and materials

Data will be shared upon request by the readers.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests, no financial or nonfinancial interests.

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