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Identification of the Calcineurin B-like gene family and gene expression patterns in response to low temperature stress in *Prunus mume*

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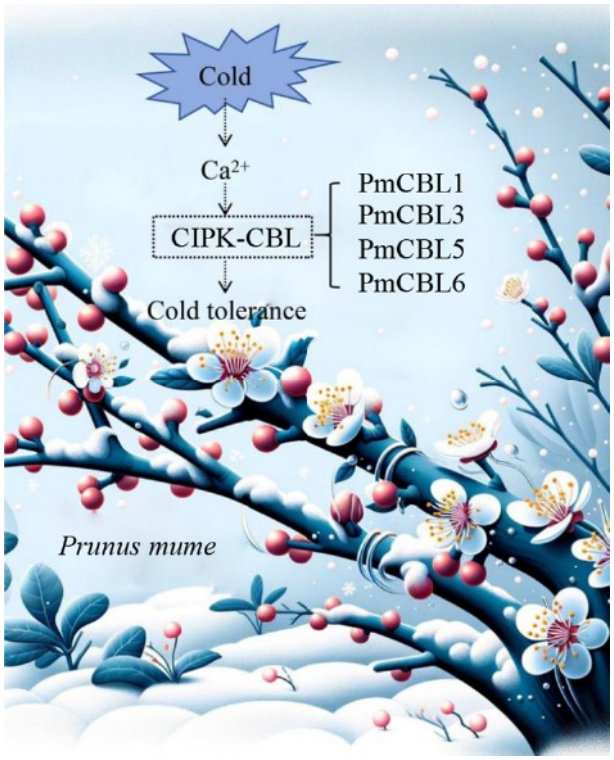
In Brief

This study identified six *PmCBL* genes, including key genes *PmCBL1*, *PmCBL3*, *PmCBL5*, and *PmCBL6*, involved in the response to cold stress in *Prunus mume*. These findings provide valuable genetic resources for enhancing cold resistance in *P. mume* through breeding programs.

Graphical abstract



Highlights

- For the first time, six *PmCBL* genes had been comprehensively and systematically identified in *P. mume*.
- The PmCBL gene family responded to cold stress, and *PmCBL1/3/5/6* were identified as potential key genes involved in regulating cold tolerance in *P. mume*.

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Identification of the Calcineurin B-like gene family and gene expression patterns in response to low temperature stress in *Prunus mume*

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Abstract

The CBL gene family is an important family in the Ca^{2+} mediated signal transduction pathway in plants and plays a crucial role in plant stress responses and growth development. However, research on the response of members of the *Prunus mume* CBL gene family to low temperature stress remains scarce. In this study, we systematically analyzed the protein physicochemical properties, chromosome localization, phylogenetic evolution, gene structure, conserved domains, cis-acting elements, and gene expression patterns in response to low temperature stress of members of the *P. mume* CBL gene family using bioinformatics tools. Six *PmCBL* gene family members were identified in the *P. mume* genome. Phylogenetic trees were constructed, revealing three subfamilies named Group I, Group II, and Group III. In the *P. mume* gene family, *PmCBL4* and *PmCBL5* were paralogous genes. The members of the *P. mume* CBL gene family were unevenly distributed on three chromosomes. The CBL encoding protein, the number of isoelectric points (pI), the number of introns and exons of the six gene families were different. Analysis of the upstream 700 bp promoter sequences of the *P. mume* CBL gene family revealed the presence of various types of cis-acting elements involved in non-biological stress responses. Among the six identified genes, each gene exhibited different expression patterns in response to low temperature. Among them, the up-regulated expression of *PmCBL5* was the largest, and the expression of *PmCBL1*, *PmCBL3* and *PmCBL5* showed the up-regulated trend. These results indicated that *PmCBL1, PmCBL3, PmCBL5*, and *PmCBL6* were key genes involved in the response of *P. mume* to low temperature stress. This study provided comprehensive and systematic analysis of the *P. mume* CBL gene family members and identified key genes involved in the response to low temperature stress, thereby providing genetic resources for molecular breeding programs aimed at enhancing cold resistance in *P. mume*.

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Introduction

In plants, Ca^{2+} is an integral element in several stress related signallings. During environmental stresses, the cytosolic Ca^{2+} concentration tends to rise rapidly, which is further co-ordinate with EF-hand domain-containing proteins[1]. Low temperature stress is an environmental challenge that severely limits the geographical distribution and survival rate of certain perennial plants[2]. Nowadays, some plants have evolved specific and effective molecular mechanisms to resist cold damage, and many functional genes involved in cold response have been identified in plants, with some of these genes being closely associated with Ca^{2+}. Stomatal immunity is regulated by pathogen-associated molecular patterns (PAMPs)- and abscisic acid (ABA)-triggered signalling in different ways. Cytoplasmic Ca^{2+} signature in the guard cells plays a vital function in stomatal immunity, but the mechanism of Ca^{2+} import is unknown. In the study of the mechanism of PAMPs triggering stomatal closure, it was found that four types of CNGCs (CNGC2, 5, 6, 9, and 12) were enriched in *Arabidopsis thaliana* guard cells and involved in ABA induced cytoplasmic Ca^{2+} oscillations. However, only some inactive stomatal movements indicate that other Ca^{2+} channels (possibly OSCAs) are actively involved in Ca^{2+} mediated stomatal immunity. In addition, CDPKs mediated phosphorylation of CNGC6 also indicates the existence of alternative pathways. However, the interrelationships between various Ca^{2+} channels and their mechanisms of simultaneous activation during stomatal defense need to be validated in future research. In addition, ROS induced Ca^{2+} signaling is not affected in cells, indicating the function of these channels downstream of the ROS pathway[3]. Ca^{2+} signaling represents a universal transduction signal in plants, mediated by a complex network of Ca^{2+} binding proteins, many of which function as Ca^{2+} sensors and act on downstream responses[4]. Calcium-dependent protein kinases (CDPKs) form functional complexes with CBL-interacting protein kinases (CIPKs), enabling plants to respond to various environmental signals and regulate ion fluxes[5]. The CBL-CIPK complex plays a crucial role in signal transduction pathways, where Ca^{2+} serves as a second messenger, particularly in the regulation of ion transporter activities in response to non-biological signals[6]. In poplar trees, the PeCBL/PeCIPK complex has been identified and shown to play a role in Na^+/K^+ homeostasis[7].

Over the past few decades, numerous CBL-CIPK complexes have been shown to be involved in signal transduction under non-biological stress. The CBL-CIPK signaling network system, composed of CBLs and their target proteins CIPKs (CBL-interacting protein kinases), plays a significant role in plant responses to various abiotic stresses such as drought, salinity, and low temperature[8–11]. The plant CBL gene family typically consists of 10 members and was initially discovered in *A. thaliana*, but has since been successfully isolated from various crops such as *Zea mays*, *Populus alba*, *Gossypium sp*, and *Oryza sativa*[11–14]. Numerous studies have shown that calcineurin B like proteins (CBL) play an important role in plant stress response. Recent studies on *Setaria italica* have found that *SiCBL3* is widely involved in the response of *S. italica* seedlings to various abiotic stress conditions, such as PEG, salt, high temperature, low temperature, and ABA; *SiCBL*3 is highly expressed during normal heading and filling stages, and is extensively induced under drought stress during the jointing, heading, and filling stages of *S. italica*[15]. Studying the gene structure, distribution, and expression characteristics of *CBL* genes in plant genomes is of great significance in gaining a deeper understanding of their roles in growth, development, and stress responses. The members of the CBL gene family show a strong conservation in their structural features, with most CBL family members containing 7~8 introns, and the positions of these introns within the coding region are relatively fixed[14,16]. The differences in domain structures among different CBL proteins may contribute to their varying abilities to bind calcium ions, providing a foundation for CBLs to sense changes in Ca^{2+} concentration triggered by various stimulus signals[17]. In recent years, *CBL* genes from multiple species have been successfully isolated and functionally validated[18–24]. In *A. thaliana*, *AtCBL1* and *AtCBL9* are localized to the plasma membrane, and both genes show expression throughout development, although their expression levels are relatively weak in roots. *AtCBL5* is also a calcium signaling component located on the plasma membrane, and it exhibits high-level expression in leaves[18,19]. Numerous studies have demonstrated that *CBLs* exhibit different expression patterns in response to various stressors and during growth and development processes. For example, *AtCBL1* is strongly induced by low temperature but is not influenced by exogenous ABA stress[18,20], while *AtCBL9* is induced by ABA stress[19]. Although significant progress has been made in the study of *CBL* genes, most research has been limited to a few plant species, and the majority of *CBL* genes remain unisolated and functionally characterized.

P. mume is an important ornamental woody plant. It is native to the Sichuan-Yunnan-Tibet region and mainly distributed in the Yangtze River Basin (China). To date, research on freezing resistance in *P. mume* has primarily focused on the ICE-CBF signaling pathway, while studies on the upstream Ca^{2+} signaling pathway have been relatively limited. Building upon previous studies on the response of *CIPK* gene family to low temperature stress, this research focused on identifying and investigating the response to low temperature stress of CBL protein family members that interact with CIPK proteins. The use of bioinformatics analysis methods, that is, based on existing bioinformation databases and resources, the use of mature bioinformatics tools to solve bioinformatics problems. In this study, all CBL gene family members were identified based on the whole genome sequence information of *P. mume*. The number of *CBL* genes, gene structure, evolution, and expression patterns under low temperature stress were analyzed at the genome level, providing insights into the biological functions of these family members. Additionally, this study provides an important theoretical basis for the breeding of cold resistant *P. mume* varieties.

Materials and methods

Plant materials

The *P. mume* cultivar 'Zao Lve' was used as a plant material. The 'Zao Lve' variety, after years of cold domestication, could survive and bloom when grown in open fields in North China. For the experiment, two-year-old grafted seedlings of the 'Zao Lve' variety were selected and subjected to low-temperature treatment.

Identification of the *P. mume* CBL gene family

The data for the *P. mume* gene family in this study were obtained from the *P. mume* database, including the *P. mume* gff annotation file and *P. mume* CDS encoding protein sequences. The CBL gene family *A. thaliana* was derived from the Uniprot gene database (www.uniprot.org)[25]. The CBL domain files for each gene member of the obtained *A. thaliana* gene family were determined and downloaded using PFAM (https://precisionflange.com/)[26]. To identify the members of the *P. mume* CBL gene family, the ten published *A. thaliana* CBL gene family members were used as query genes. The hmmsearch plugin in the hmmer software was utilized for the search (e-value set at 10^{-5}, with other parameters set to default values) to screen for CBL homologous sequences from the *P. mume* gene family members. The results were then searched against the *A. thaliana* family in the String database, and members of the *P. mume* CBL gene family with a similarity greater than 50% were selected.

Gene characteristic analysis and protein physicochemical property prediction

Based on the identified sequence IDs, the GXF Select tool in TBtools was used to extract the annotation information of the identified sequences. This allowed us to obtain the chromosome numbers and location information of the *P. mume* CBL gene family members. The six CBL family members were then named based on their respective location information. The ExPASy-Protparam online tool (https://web.expasy.org/protparam) was used to analyze the physicochemical properties of the gene family members. It involved amino acid count statistics and predicted protein properties such as molecular weight (MW), theoretical isoelectric point (pI), aliphatic index, instability index, and grand average of hydropathicity (GRAVY). The SignalP v6.0 online tool was used to predict signal peptides, while the TMHMM v2.0 online tool was utilized to predict transmembrane helices. The Cell-PLoc v2.0 online tool (www.csbio.sjtu.edu.cn/bioinf/Cell-PLoc-2) was employed for subcellular localization prediction.

Phylogenetic analysis of the *P. mume CBL* gene

In this study, the phylogenetic tree of *P. mume* CBL gene family was analyzed by Neighbor-Joining Algorithm method[27]. The MEGA 7.0 (7170509-X86_64) software was used for phylogenetic analysis of the gene family. The protein sequences of CBLs from *A. thaliana*, *O. sativa*, *Vitis vinifera* and *Nicotiana*

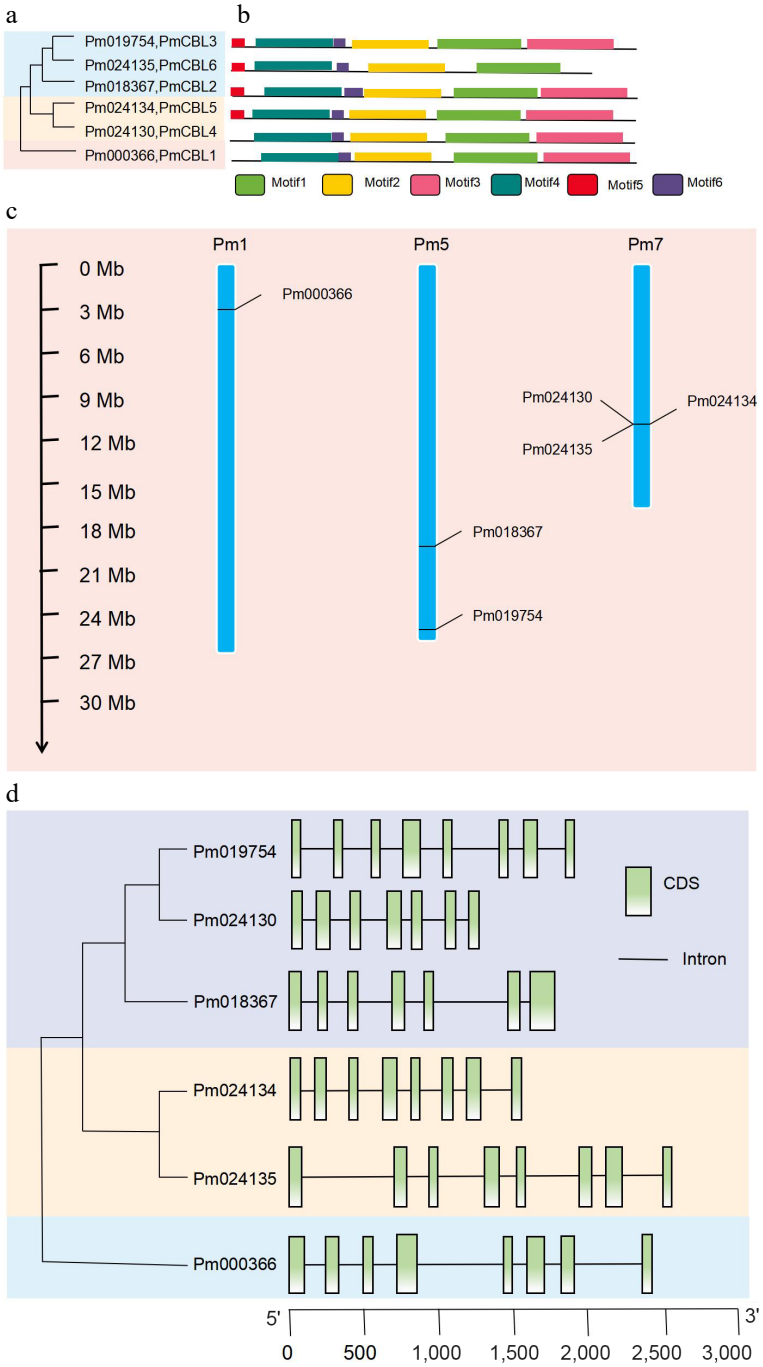

Fig. 1 Phylogenetic analysis, protein motif structure, chromosome localization, and gene structure analysis of *PmCBL* genes. (a) Phylogenetic analysis of *PmCBL* genes. (b) Protein motif structure of *PmCBL* genes. (c) Chromosome localization analysis of *PmCBL* genes. (d) Gene structure analysis of *PmCBL* genes.

Table 1. Members of the *P. mume* PmCBL gene family and their main molecular characteristics and information.

Gene name	Gene ID	Chromosome	Position	Subcelllar localization	CDS (bp)	Intron
PmCBL1	Pm000366	Chr01	2287536~2289774	Cell membrane	2,418	5
PmCBL2	Pm018367	Chr05	17870110~17871915	Cell membrane	1,805	4
PmCBL3	Pm019754	Chr05	25555186~25557079	Cell membrane	1,893	5
PmCBL4	Pm024130	Chr07	10584318~10585570	Cell membrane	1,252	4
PmCBL5	Pm024134	Chr07	10621981~10623545	Cell membrane	1,564	5
PmCBL6	Pm024135	Chr07	10622516~10628186	Cell membrane	2,474	5

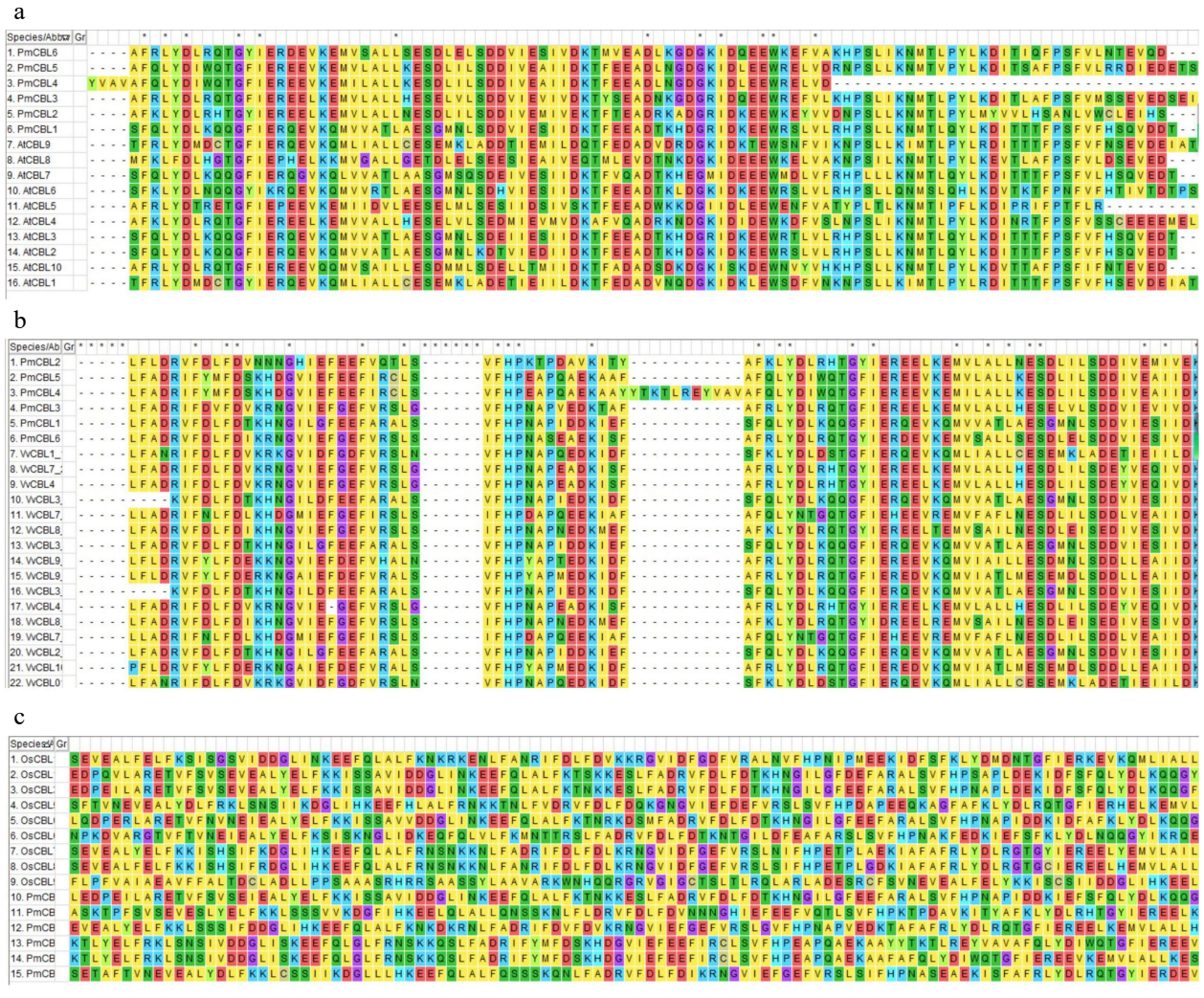

Fig. 2 Protein amino acid sequence comparison diagram. (a) Protein amino acid sequence comparison diagram between *P. mume* and *A. thaliana*. (b) Protein amino acid sequence comparison diagram between *P. mume* and *V. vinifera*. (c) Protein amino acid sequence comparison diagram between *P. mume* and *O. sativa*.

Table 2. Physicochemical properties of *P. mume CBL* gene family members.

Protein name	Gene ID	Number of amino acids	Molecular weight	Thereotical pI	Instability index	Signal peptide
PmCBL1	Pm000366	226	26052.71	4.82	49.06	NO
PmCBL2	Pm018367	217	24903.52	5.14	35.97	NO
PmCBL3	Pm019754	212	24429.82	4.73	46.65	NO
PmCBL4	Pm024130	186	21665.75	4.6	40.14	NO
PmCBL5	Pm024134	213	24720.17	4.57	40.98	NO
PmCBL6	Pm024135	218	25144.71	4.86	46.47	NO

tabacum were obtained from the Uniprot database (www.uniprot.org)[25]. The MUSCLE program in the software was used for multiple sequence alignment of the selected CBL protein sequences with their corresponding *A. thaliana* homologous protein sequences (using default parameters). The aligned results were subjected to phylogenetic analysis using the Neighbor-Joining (NJ) method, with the number of differences as the computational model and pairwise deletion as the gap handling option. Bootstrap support was set to 500 for tree reliability assessment. The resulting phylogenetic tree was visualized and beautified using the Evolview online tool (www.evolgenius.info/evolview/#/)[28].

Chromosomal localization of *P. mume CBL* genes

The identified *CBL* genes were assigned to their corresponding positions on the *P. mume* genome. The location information of each gene on the chromosomes was obtained using the online tool MG2C (http://mg2c.iask.in/mg2c_v2.1/) to generate a chromosomal localization map.

Protein alignment and conserved motif analysis of *P. mume* CBL proteins

Multiple sequence alignment of the selected CBL protein sequences was performed using the Clustal W program in MEGA software (with default parameters). The MEME online

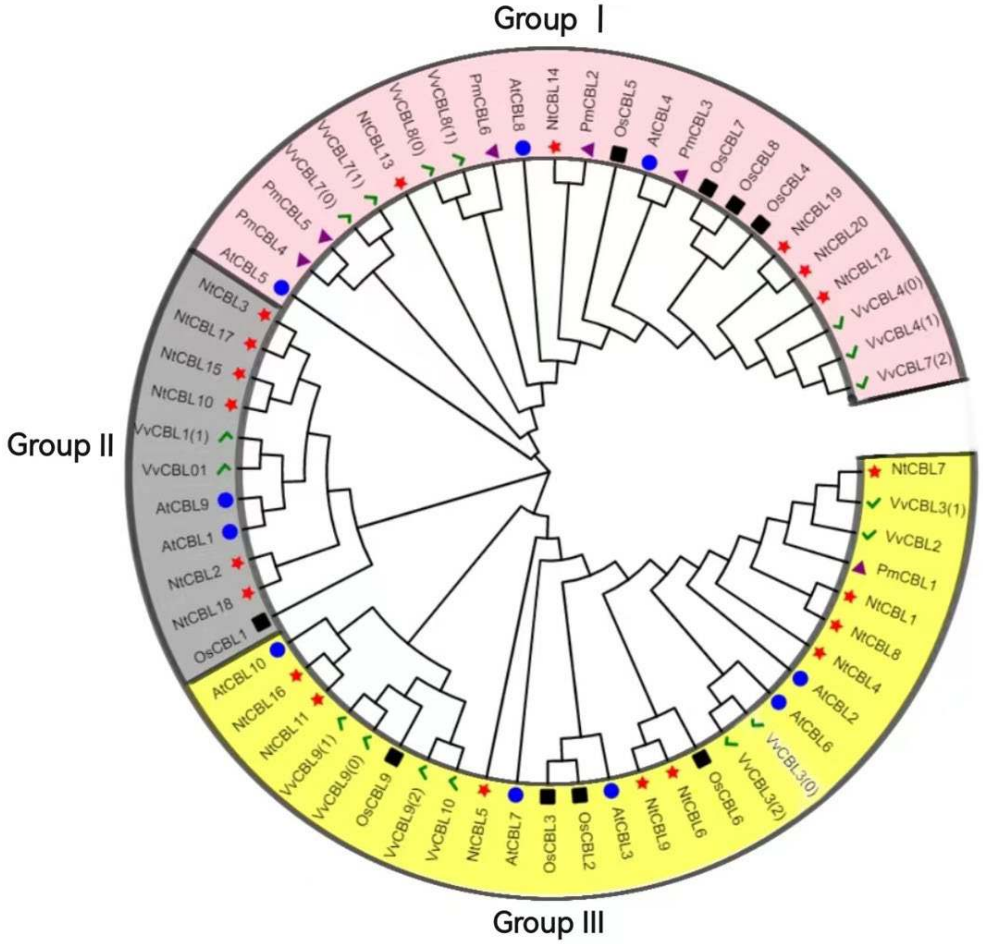

Fig. 3 Phylogenetic tree of *P. mume*, *A. thaliana*, *O. sativa*, *N. tabacum* and *V. vinifera*. The green checkmark represents *V. vinifera*, the black square represents *O. sativa*, the blue circle represents *A. thaliana*, the red star represents *N. tabacum*, and the purple triangle represents *P. mume*.

tool (https://meme-suite.org/meme/) was utilized for motif analysis to predict conserved motifs and domains present in the protein sequences. Based on the identified *P. mume* CBL gene family with six gene members, the Gene Structure View (Advanced) tool in TBtools software was used along with the downloaded *P. mume* annotation information gff file from the *P. mume* database to analyze the gene structure of the *P. mume* CBL gene family.

Analysis of cis-acting elements in the *P. mume* CBL gene family

In order to analyze the potential cis-acting elements in the promoter sequences of the *P. mume* CBL gene family, the upstream 700 bp promoter sequence regions were downloaded in bulk from the ATG start codon. The obtained promoter sequences were then analyzed for cis-acting elements using the PlantCARE online tool (http://bioinformatics.psb.ugent.be/webtools/plantcare/html)[29]. The resulting Tab file was opened in an Excel spreadsheet, and the desired cis-acting elements were filtered. Finally, the Simple BioSequence Viewer function in TBtools software was used to visualize and plot the identified cis-acting elements.

Expression analysis of *PmCBLs* genes

In order to further investigate the expression patterns of *PmCBL* genes, We collected transcriptomic expression profile data from different tissues (roots, stems, leaves, buds, and fruits) and during the natural overwintering process (November, December, January, and February) in *P. mume*[30].

Low temperature treatment of plant materials and detection of relative expression levels

Seedlings cultivated under long-day conditions (16 h light/8 h dark) at 24 °C were used to investigate the effect of *PmCBL* genes on cold response. The seedlings were incubated at 4 °C, mimicking a cold environment. One-year-old branches that underwent 4 °C treatment were selected for sampling at various time points including 0, 1, 4, 6, 12, and 24 h. Total RNA was extracted from the samples using a suitable method. First-strand cDNA synthesis was performed using the TIANScript First Strand cDNA Synthesis Kit (Tiangen, China). qRT-PCR was performed using the PikoReal Real-Time PCR System (Thermo Fisher Scientific, CA, USA) and SYBR Premix ExTaq TM (TaKaRa, Dalian, China). The reaction was carried out in a 10 μL system, which included 5 μL of SYBR Premix ExTaqII, 0.25 μL each of forward and reverse primers, 0.5 μL of cDNA, and 3 μL of ddH_2O. The PCR program consisted of 40 cycles at 95 °C for 30 s, 95 °C for 5 s, and 60 °C for 40 s, followed by a final extension at 60 °C for 30 s. Three replicates were performed for each sample. The internal reference gene used was *PP2A* from *P. mume*. The relative expression levels of *PmCBL* genes were

Table 3. Analysis of cis-acting elements in the *P. mume* CBL gene family members.

Gene	*PmCBL1*	*PmCBL2*	*PmCBL3*	*PmCBL4*	*PmCBL5*	*PmCBL6*
Gene ID	Pm000366	Pm018367	Pm019754	Pm024130	Pm024134	Pm024135
CAAT-box	3	4	6	2	4	4
CGTCA-motif	2	1	1	2	1	
TGACG-motif	2	1	1	2	1	
TATA-box	4	4	4	11	8	
GT1-motif	1					
TCCC-motif	1					
GATA-motif	1			1	1	
ARE		1			1	
G-box				1	1	
MBS				1		1
TGA-element						1
A-box					1	1
I-box		1				
O_2-site			1	1	1	2
LAMP-element			1			
ABRE				1	1	
TCA-element					1	

CAAT-box was a common cis-acting element in the promoter and enhancer regions. CGTCA-motif/TGACG-motif was cis-acting regulatory elements involved in MeJA response. TATA-box was a core promoter element located around the transcription start site (-30). GT1-motif was a light-responsive element. TCA-element was a cis-acting element involved in salicylic acid response. TGA-element was an element involved in auxin response. ABRE was a cis-acting element involved in abscisic acid response. A-box was a cis-acting regulatory element. ARE was a cis-acting element required for anaerobic induction. G-box was a cis-acting element involved in light response. O_2-site/MBS was cis-acting regulatory elements involved in zein protein metabolism regulation. TCCC-motif/GATA-motif/I-box/LAMP-element was part of light-responsive elements.

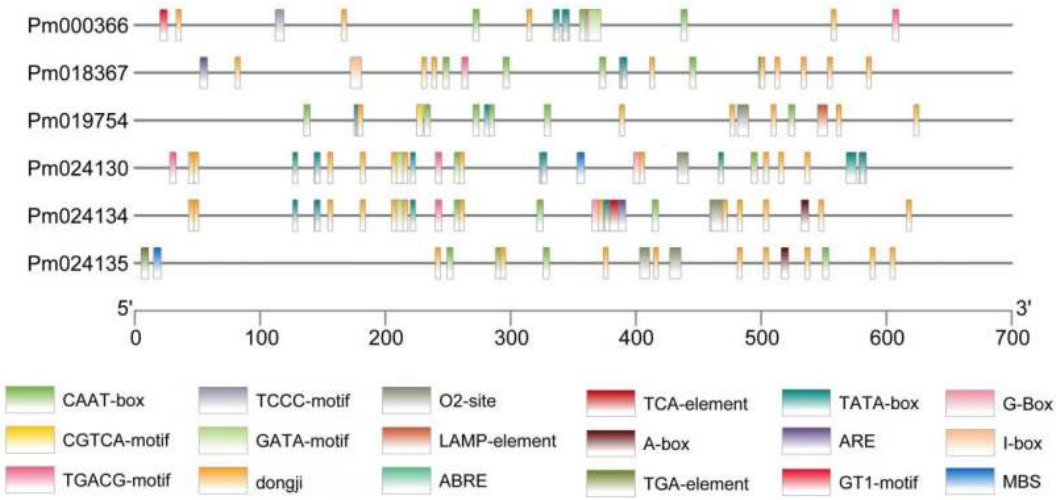

Fig. 4 Analysis of cis-acting elements in *PmCBL* genes.

calculated using the $2^{-\Delta\Delta CT}$ method. Statistical analysis with standard deviation was performed on the final data.

Results

Basic characteristics of gene family members and results of the alignment and analysis of conserved motifs in *P. mume* CBL proteins

Through bioinformatics analysis methods, a total of six CBL gene family members were identified (Fig. 1a) and named as *PmCBL1*~*PmCBL6* (Table 1). These six *CBL* genes were unevenly distributed on the chromosomes. *PmCBL1* was located on chromosome 1, *PmCBL2* and *PmCBL3* were located on chromosome 5, and *PmCBL4*, *PmCBL5*, and *PmCBL6* were located on chromosome 7. In the PmCBL family, the coding sequence (CDS) of *PmCBL6* was the longest, comprising 2,475 bp. *PmCBL1* had the second-longest CDS with 2,418 bp, followed by *PmCBL3* with 1,893 bp, *PmCBL2* with 1,805 bp, *PmCBL5* with 1,564 bp, and *PmCBL4* with the shortest CDS of 1,252 bp. The subcellular localization prediction of these six genes was all on the cell membrane. Through analysis of conserved protein domains, it was found that *PmCBLs* contain six conserved motifs (Motif 1~6) (Fig. 1b). Specifically, *PmCBL2, PmCBL3, PmCBL5,* and *PmCBL6* contained all six motifs (Motif 1~6), while *PmCBL1* and *PmCBL4* contained Motif 1~4 and Motif 6 but did not contain Motif 5. From the results of protein multiple sequence alignment among *P. mume*, *A. thaliana*, *V. vinifera*, and *O. sativa*, it could be observed that *P. mume* showed higher homology with *A. thaliana* and *V. vinifera*, while the homology with *O. sativa* was relatively lower (Fig. 2).

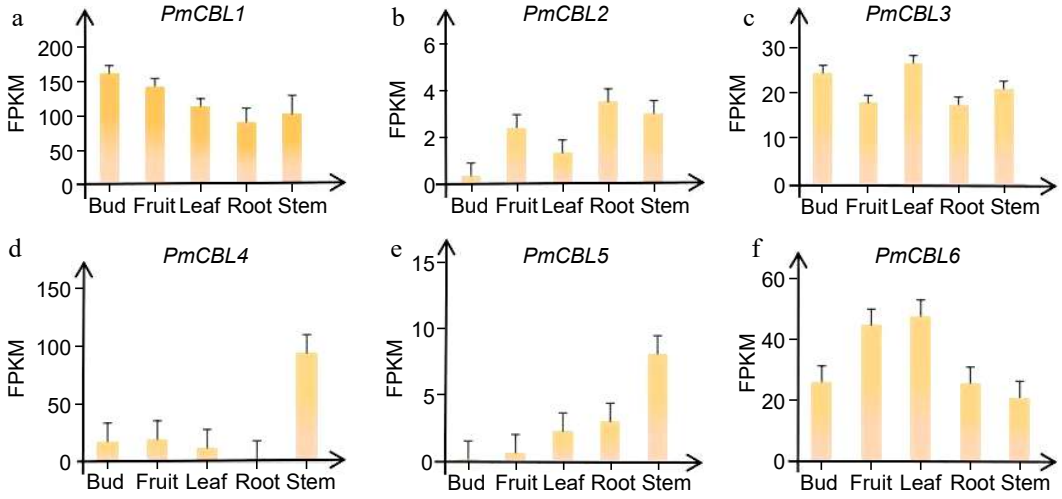

Fig. 5 Expression patterns of *PmCBL* genes in different tissue parts of *P. mume*.

Physicochemical property analysis of the *P. mume* CBL gene family

The physicochemical properties of the six CBL homologous sequences were analyzed (Table 2). The results showed that the amino acid (aa) numbers of the PmCBL protein sequences ranged from 186 (PmCBL4) to 226 (PmCBL1), with molecular weights (MV) ranging from 21.66 kDa (PmCBL4) to 26.05 kDa (PmCBL1). The theoretical isoelectric points (pI) ranged from 4.57 to 5.14. The instability index values ranged from 35.97 to 49.06, with only PmCBL1 having an instability index below 40, indicating high stability of CBL protein. The grand average of hydropathicity (GRAVY) values for PmCBL1 ~ PmCBL6 were all negative, indicating that these six proteins were hydrophilic. The aliphatic index values ranged from 90.09~96.96, indicating that all six proteins were lipophilic. None of the six gene family members contained signal peptides or transmembrane helices.

Evolutionary relationship analysis of the CBL gene family in *P. mume*

To understand the evolutionary relationship between members of the *P. mume* CBL gene family, a phylogenetic tree was constructed using the selected six *PmCBL* genes, 17 *OsCBLs* genes from *O. sativa*, 16 *VvCBLs* genes from *V. vinifera*, *20 NtCBLs* genes from *N. tabacum* and 10 *AtCBLs* genes from the model plant *A. thaliana* (Fig. 3). The results of the evolutionary analysis showed that the CBL gene family could be divided into three groups, each containing among them, *P. mume* Group I included *PmCBL2, PmCBL3, PmCBL4, PmCBL5*, and *PmCBL6*. Group III consisted of *PmCBL1*. In Group I, *PmCBL4* and *PmCBL5* were paralogous gene pairs. This suggested that the *CBL* gene in *P. mume* underwent expansion, expansion and replication. Some genes in *AtCBLs, NtCBLs, VvCBLs* and *PmCBLs* could be considered as orthologous gene pairs, such as *AtCBL4* and *PmCBL3, NtCBL14* and *PmCBL2, VvCBL8* and *PmCBL6*. The discovery of orthologous gene pairs suggests the existence of ancient *CBL* genes before *P. mume* and *A. thaliana, N. tabacum, V. vinifera* were also similar.

Analysis of gene structure and chromosome localization of the *P. mume* CBL gene family

Further analysis of the gene structure of the *P. mume CBL* gene family revealed that the six *PmCBL* genes share a similar overall gene structure (Fig. 1d). Specifically, *PmCBL3* had eight exons separated by introns. *PmCBL4* had seven exons with approximately equal sizes separated by introns. *PmCBL2* had six exons separated by introns, with two exons being relatively far apart. *PmCBL5* had eight exons separated by introns, and the sizes of these eight exons were roughly similar with a similar distance between each pair of exons. *PmCBL6* had eight exons separated by introns, with some exons having larger inter-exon distances. *PmCBL1* also had eight exons separated by introns, and similarly, some exons had larger inter-exon distances.

Based on the results from the MG2C online tool (Fig. 1c), the six identified *P. mume CBL* genes were found to be located on three chromosomes. *PmCBL2* and *PmCBL3* were located on one chromosome, *PmCBL4, PmCBL5*, and *PmCBL6* were located on another chromosome, and *PmCBL1* was located on a separate chromosome.

Analysis of cis-acting elements in the *P. mume CBL* genes

In order to further investigate the potential functional roles of *PmCBL* genes, the 700 bp sequence upstream of the start codon of each *PmCBL* gene was extracted as its promoter region. Cis-acting element analysis was performed on this region, focusing on important elements that had been extensively studied and were associated with plant growth and development, as well as stress responses. (Table 3, Fig. 4) The results showed that a total of 17 cis-acting element types responsive to plant hormones and stress were identified, and there were differences in the types and quantities of elements among different genes. Among them, all genes contained common cis-acting elements (CAAT-box) in their promoter and enhancer regions, with slight differences in the number of elements ranging from 2~6. *PmCBL3* had the highest number of six (CAAT-box elements) , while *PmCBL4* had the lowest two. Two cis-acting regulatory elements involved in MeJA response (CGTCA-motif and TGACG-motif) were found, and five genes contained both of these elements, with an equal number in each gene. Five genes contained the core promoter element (TATA-box) located around the transcription start site −30, namely *PmCBL1* ~ *PmCBL5*, but there was a large variation in the number of elements among the members, ranging from 4~11. *PmCBL1* had one light-responsive element (GT1-motif), while the other genes did not contain it. There were four

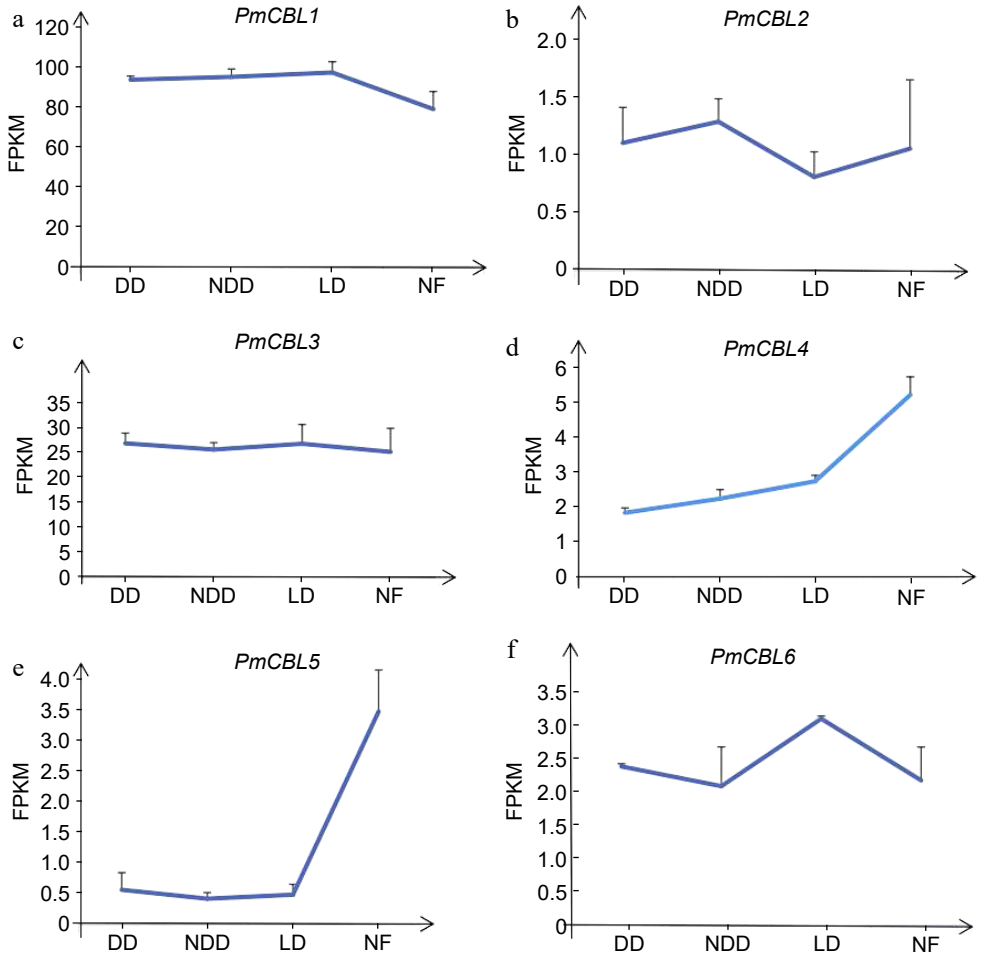

Fig. 6 The expression pattern of *PmCBLs* genes during overwintering. DD, November; NDD, December; LD, January; NF, February.

light-responsive elements identified: *PmCBL1*, *PmCBL4*, and *PmCBL5* each contained one GATA-motif, *PmCBL2* contained one I-box, *PmCBL1* contained one TCCC-motif, and *PmCBL3* contained one LAMP-element. Two cis-acting regulatory elements involved in zein protein metabolism regulation were found: O2-site and MBS. *PmCBL3*, *PmCBL4*, and *PmCBL5* each contained one O2-site, *PmCBL6* contained two O2-sites, and *PmCBL4* and *PmCBL6* each contained one MBS. One cis-acting regulatory element involved in light response (G-box) was identified, with *PmCBL4* and *PmCBL5* each containing one. One cis-acting regulatory element required for anaerobic induction ARE was found, with *PmCBL2* and *PmCBL5* each containing one. One cis-acting regulatory element (A-box) involved in cis-element regulation was found, with *PmCBL5* and *PmCBL6* each containing one. One cis-acting element involved in abscisic acid response ABRE was found, with *PmCBL4* and *PmCBL5* each containing one. One cis-acting element involved in auxin response (TGA-element) was found in *PmCBL6*. One cis-acting element involved in salicylic acid response (TCA-element) was found in *PmCBL4*.

Gene expression of the *P. mume* CBL gene family

By analyzing transcriptome data from different parts of the *P. mume*, tissue-specific expression patterns were observed in different members of the *P. mume* CBL gene family (Fig. 5). *PmCBL1* showed higher expression levels in flower buds, *PmCBL2* exhibited higher expression levels in roots, *PmCBL3* and *PmCBL6* had higher expression levels in leaves, and *PmCBL4* and *PmCBL5* showed higher expression levels in stems. From this, it could be observed that *PmCBL3* and *PmCBL6* had similar expression patterns, while *PmCBL4* and *PmCBL5* had similar expression patterns. This suggested that they belonged to the same Group I subfamily.

By analyzing the transcriptome data of *P. mume* during wintering, the wintering process of *P. mume* could be divided into three stages: the early stages of overwintering, which was November (DD); midwinter, which was December (NDD); late overwintering which was January (LD); and naturalness, which was February (NF). The expression patterns of the *P. mume* CBL gene family could be obtained (see Fig. 6), with different genes showing different expression patterns. *PmCBL1* showed an upregulation trend in all three stages compared to the NF stage, reaching its peak in the LD stage, with expression levels 1.23 times higher than the NF stage. *PmCBL2* exhibited both upregulation and downregulation trends in the three stages compared to the NF stage. It reached its maximum expression level in the NDD stage, being 1.22 times higher than the NF stage. However, it reached its minimum expression level in the LD stage, showing a downregulation of 1.3 times compared to the NF stage. Overall, *PmCBL2* displayed a downregulation trend. *PmCBL3* showed an upregulation trend in all three stages compared to the NF stage, with the highest expression level observed in the DD stage, being 1.06 times higher than the NF

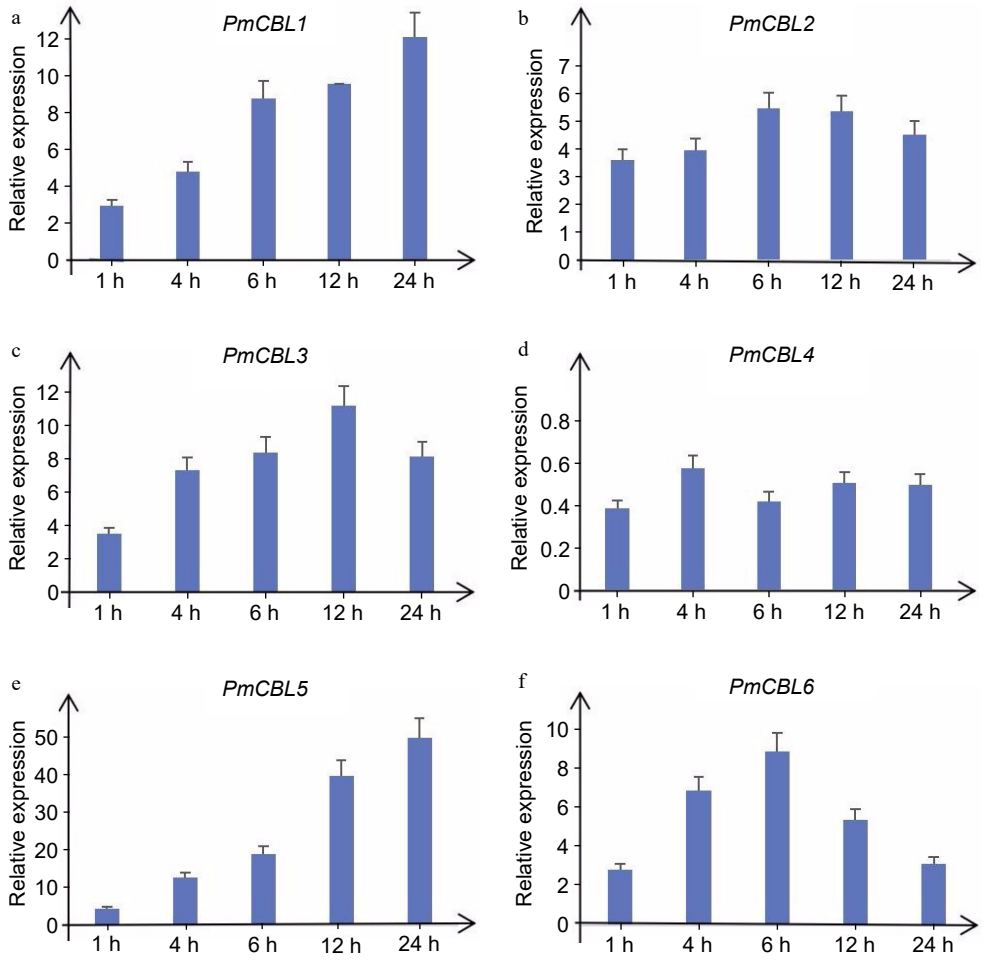

Fig. 7 Expression levels of *PmCBL* genes under 4 °C treatment.

stage. *PmCBL4* showed a downregulation trend in all three stages compared to the NF stage, with the minimum expression level observed in the DD stage, showing a downregulation of 1.20 times compared to the NF stage. *PmCBL5* exhibited a downregulation trend relative to the NF stage, reaching its minimum expression level in the NDD stage, showing a downregulation of 8.28 times compared to the NF stage. *PmCBL6* showed an upregulation trend relative to the NF stage, with an upregulation of 1.42 times compared to the NF stage. *PmCBL4* and *PmCBL5* were known to had significant roles in the wintering process of *P. mume*.

Quantitative analysis of gene expression

To further investigate the expression patterns of *PmCBL* genes in response to low temperature stress, qRT-PCR experiments were conducted to examine the expression levels of *PmCBLs* under cold treatment. During the treatment at 4 °C, the expression levels of *PmCBL* genes showed an upregulation or downregulation trend over a 24-h time course (Fig. 7). *PmCBL1, PmCBL2, PmCBL3, PmCBL5,* and *PmCBL6* showed an upregulation trend in their expression levels. Among them, *PmCBL1* and *PmCBL5* exhibited the highest expression levels at 24 h, being 12.18-fold and 50.11-fold higher than the pre-treatment levels, respectively. *PmCBL2* and *PmCBL6* reached their maximum expression levels at 6 h, with a 5.50-fold and 8.90-fold increase, respectively, compared to the pre-treatment levels. *PmCBL3* showed the highest expression level at 12 h, which was 11.22-fold higher than the pre-treatment level. *PmCBL4* showed a downregulation trend compared to the pre-treatment levels.

Discussion

Currently, members of the *CBL* gene family have been identified in various vegetables, fruits, and cereal crops. The CBL gene family has been extensively studied in various plant species. In *V. vinifera*, a total of eight members of the CBL gene family were found[31]. Similarly, in *O. sativa*, 10 members of the CBL gene family were identified[32]. *A. thaliana*, known for its significance in plant research, has also revealed the presence of 10 CBL gene family members[11]. *Solanum lycopersicum*, commonly known as tomato, exhibited 13 characterized members of the CBL gene family[33]. Remarkably, *Triticum aestivum*, or wheat, stands out with the largest number of identified CBL gene family members, totaling 68[34]. Additionally, *CBL* gene family members have also been identified in *Algae*, *Racomitrium canescens*, *Pteridophyta*, and gourd plants. In this study, a bioinformatics approach was employed to obtain the structural domain files of the *CBL* gene family and perform an HMMER SEARCH. Through Pfam database, a total of six *P. mume* CBL gene family members were obtained. These findings shed light on the diversity and complexity of the CBL gene family across different plant species.

The composition of introns and exons can reflect the evolutionary relationships within a gene family. It has been found that *CBL* genes in dicot model plants such as *A. thaliana* and poplar contained 6~7 introns. In the six identified *P. mume CBL* gene family members, all of them contained introns, with a ranged of 7~8 introns and minimal variation in the numbers. This suggested that introns in *P. mume CBL* genes might be more active during the evolutionary process compared to plants like *A. thaliana* and poplar. Generally, early-stage plant evolution tends to exhibit a higher enrichment of introns compared to later stages[35], with a higher rate of intron loss than gain[36]. To further explore the evolutionary relationships, phylogenetic trees were constructed using CBL proteins from *A. thaliana*, *V. vinifera*, *O. sativa*, *N. tabacum* and *P. mume*. The CBL proteins from these five species can be divided into three subfamilies, which may have evolved from different ancestral sequences. The six *CBL* genes of *P. mume* were distributed in Group I and Group III. In Group I, homologous gene pairs of *P. mume* and homologous gene pairs of *P. mume* and other species had been found. The identification of homologous gene pairs not only provides insights into the duplication and diversification processes within *P. mume*'s own genome but also sheds light on the phylogenetic relationships between *P. mume* and other species. This discovery highlights the intricate mechanisms underlying the preservation and replication of genetic information, as well as the evolutionary connections that exist among different organisms.

Low temperature conditions, also known as cold stress, pose a significant threat to plant growth. Freezing stress impedes the growth of most plants and poses a great risk to the cultivation of many perennial woody plants. Previous studies have found that *T. aestivuml* grown under normal temperature conditions are killed at freezing temperatures of approximately −5 °C. However, if the species undergoes cold acclimation, it can survive at temperatures as low as −20 °C[37]. In previous research on *Pyrus*, it is found that under low temperature (4 °C) stress, the expression levels of *PbCBL2, PbCBL4,* and *PbCBL8* were upregulated, *PbCBL1* and *PbCBL3* exhibited downregulation in expression under low-temperature stress[38]. Additionally, in previous studies on *V. vinifera*, except for *VvCBL5*, which shows a significant downregulation expression trend under low temperature stress, all seven *VvCBL* genes show a significant upregulation expression trend[39]. In this study, the *P. mume* gene database was utilized to obtain the sequences of *CBL* gene family members. These sequences were used as probes for expression analysis, enabling the investigation of expression characteristics. The results from real-time fluorescence quantitative PCR demonstrated that the expression patterns of the six *P. mume PmCBLs* varied under low-temperature stress. This variation may be attributed to the involvement of *PmCBLs* in regulating the signaling pathways associated with low-temperature responses. During the 4 °C treatment, the expression levels of *PmCBL* genes showed an upregulation or downregulation trend over a 24 h time period. *PmCBL1*, *PmCBL2*, *PmCBL3*, *PmCBL5*, and *PmCBL6* exhibited upregulation in expression, among which *PmCBL1*, *PmCBL3*, *PmCBL5* and *PmCBL6* showed the most significant upregulation, suggesting their crucial roles in regulating *P. mume* response to low-temperature stress. Through comparative analysis of *P. mume*, *V. vinifera*, and *Pyrus,* it can be seen that they have certain similarities in response to low temperature stress.

Cold-responsive genes were cloned, and functional analysis was performed using the whole genome. The exact role of the c-repeat/DRE binding factor (CBF/DRE) in cold tolerance was studied in *P. mume*[25,40]. After cold treatment, the expression levels of *PmCBLs* showed either upregulation or downregulation, but these genes exhibited differential expression levels as shown in Fig. 6. These expression patterns, similar to their homologs, suggest that *PmCBLs* might play important roles in cold response. To date, numerous studies have demonstrated the important role of plant *CBL* genes in plant stress responses. For instance, *AtCBL1* in *A. thaliana* can be strongly induced by non-biological stresses such as low temperature and injury but is not influenced by exogenous ABA[18,20]. On the other hand, *AtCBL9* plays a role in both the ABA signaling pathway and ABA biosynthesis pathway and is primarily involved in the stress response of *A. thaliana* during the seedling stage[19]. *ZmCBL4* in maize can significantly enhance salt tolerance in transgenic *A. thaliana*[13]. These findings highlight the significance of *CBL* genes in mediating plant responses to various stressors. Due to the signaling crosstalk among different stresses, multiple Ca^{2+} signals can be generated even under the same stress conditions. In addition, different CIPK target proteins may bind to the same sensor, and there may be functional redundancy between different *CBL* genes, making the entire CBL-CIPK signaling pathway complex and diverse. In order to gain a deeper understanding of the CBL-CIPK signaling pathway and how *PmCBLs* interacts with target proteins to activate downstream responses in response to low temperature stress, it is necessary to further explore other *CBL* genes.

Through transcriptomic data analysis, the expression patterns of CBL proteins can be obtained. Previous studies have shown that *N. tabacum* CBL proteins exhibit tissue-specific expression patterns. *NtCBL13* and *NtCBL14* share similar expression patterns, with low expression levels in all tissues except for roots where they are expressed. On the other hand, *NtCBL6*, *NtCBL8*, *NtCBL7*, *NtCBL4*, *NtCBL5*, *NtCBL1*, and *NtCBL9* exhibit similar expression patterns with high expression levels in various tissues, except *NtCBL9* which shows lower expression levels in mature roots. The remaining NtCBL proteins have higher expression levels in flower tissues. Overall, NtCBL proteins are abundantly expressed in flowers, leaves, and roots[41]. According to the research conducted on *P. mume*, the gene expression of *PmCBLs* varies in different tissue parts of *P. mume* (Fig. 6). *PmCBL1* showed higher expression levels in flower buds, *PmCBL2* exhibited higher expression levels in roots, *PmCBL3* and *PmCBL6* showed higher expression levels in leaves, and *PmCBL4* and *PmCBL5* display higher expression levels in stems. The gene expression pattern analysis revealed that the expression levels of *PmCBL* genes were relatively high in stems. The differential expression levels of genes in different tissue parts might be a result of biological evolution. For *N. tabacum*, *NtCBL6*, *NtCBL8, NtCBL7, NtCBL4, NtCBL5, NtCBL1,* and *NtCBL9* share similar expression patterns and belong to the Group A subfamily of the evolutionary system, while the remaining NtCBL proteins belong to the Group B subfamily[41]. As for *P. mume*, *PmCBL3,* and *PmCBL6* exhibit similar expression patterns, and *PmCBL4* and *PmCBL5* also showed similar expression patterns, indicating that they belong to the Group I subfamily. Therefore, it can be observed that higher homology between genes leads to more similar gene expression patterns.

Conclusions

To summarize, we conducted a genome-wide identification of the *P. mume* CBL gene family for the first time. We identified six *CBL* genes, among which *PmCBL1*, *PmCBL3*, *PmCBL5,* and *PmCBL6* showed the most significant upregulation, suggesting their crucial roles in regulating *P. mume* response to low-temperature stress. Therefore, this study indicated that *PmCBLs* might play a key role in enhancing freezing tolerance and winter hardiness in *P. mume* by modulating responses to low-temperature stress. This study contributed valuable genetic resources that could be utilized for the molecular breeding of cold-resistant *P. mume*.

Author contributions

The authors confirm contribution to the paper as follows: study conception and design: Li P, Zhang Q; data collection and analysis platform: Liu H, Hao L; data analysis and draft manuscript preparation: Liu H; provided help with the experiments: Zhang X, Zhang Y; manuscript revision: Wang H, Wang J, Liu Z, Zhang Q, Li P. All authors reviewed the results and approved the final version of the manuscript.

Data availability

All data generated or analyzed during this study are included in this published article.

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Conflict of interest

The authors declare that they have no conflict of interest.

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