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Identification and Comparative Analysis of the Rosaceae RCI2 Gene Family and Characterization of the Cold Stress Response in *Prunus mume*

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Abstract: Rare cold inducible 2 (RCI2) proteins are a group of low molecular weight proteins that widely exist in various tissues of plants and play crucial roles in plant growth and development and abiotic stress responses. Genome-wide identification and analysis of *RCI2* have not been documented in Rosaceae plants. Therefore, we identified 23 *RCI2* genes from seven Rosaceae plants, which were classified into three subfamilies. The RoRCI2 protein encodes a highly conserved domain of Pmp3. Three homologous *PmRCI2*s genes from *Prunus mume* were cloned and named *PmRCI2-1*, *PmRCI2-2*, and *PmRCI2-3*. The results of subcellular localization prediction showed that three PmRCI2s localized to membrane structures, and the abscisic acid response element were found to have the largest number in the promoter sequences of *PmRCI2*s. The results of quantitative real-time PCR (qRT-PCR) showed that *PmRCI2-3* was significantly induced by low temperature and highly expressed in stems and buds during the endodormancy stage. Our study improves the understanding of the RCI2 family of Rosaceae plants regarding the cold responses and provides a theoretical basis for the cold-resistant breeding of *P. mume*.

Keywords: *Prunus mume*; RCI2 gene family; phylogenetic analysis; cold stress

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1. Introduction

Temperature is an important limiting factor for plant distribution and growth and development [1]. Low temperatures (cold), especially freezing, can disturb ionic homeostasis by impairing cell membrane permeability, thereby inducing electrolyte leakage [2]. Rare cold inducible 2 (RCI2) proteins are important regulators of temperature-mediated signaling pathways, which are active in maintaining cellular ion homeostasis and plasma membrane potential [3]. The genome-wide identification and analysis of *RCI2* have been accomplished in many model plants [3,4] and horticultural herbaceous crops [5]. However, there are few studies on perennial woody plants, especially in Rosaceae.

RCI2 proteins, also named low-temperature induced proteins 6 (Lti6) or plasma membrane proteins 3 (Pmp3), are present as multigene family encoding proteins in plants [6]. RCI2 family gene transcripts are accumulated in response to cold stress differently in different organs, for example, the *AtRC12B* mRNA accumulates in stems, flowers, and siliques of plants exposed to 4 °C, but not in roots. The transcription of *AtRC12A* showed the highest levels in stems, while the lowest in roots. The *RCI2* genes respond to various abiotic stresses and may affect seed germination and root growth in response to low-temperature stress [7,8]. In both *Arabidopsis thaliana* and *Aeluropus littoralis*, *RCI2* homologs respond to

membrane dehydration, caused by freezing injury, by stabilizing membrane proteins and reducing electrolyte leakage [9]. In *A. littoralis*, *AlTMP1/2* overexpressed tobacco improved tolerance to freezing stress at the seedling stage [10,11], and *AlTMP1* transgenic tobacco plants mostly recovered to normal growth after 2 h at −20 °C [11]. In addition to cold stress, these genes can also enhance the tolerance of transgenic plants to other abiotic stresses, such as heat, salt, and drought. Recently, more and more studies show that transgenic plants overexpressing *RCI2* genes showed high tolerance to abiotic stresses [7,8,12]. C-repeat binding factor/dehydration-responsive element binding (*CBF/DREB1*) transcription factors can regulate some cold-induced genes of *Arabidopsis*. The *OsLti6b* is highly expressed in *CBF1/DREB1b* transgenic rice, suggesting that it may play a role in the signaling pathway downstream of the rice *CBF1/DREB1b* ortholog [13]. Therefore, the *RCI2* gene plays a crucial role in regulating plant growth and development and abiotic stress responses [10].

Prunus mume is an important woody ornamental plant in early spring with an attractive aroma, colorful petals, and diverse petal shapes. However, traditional *P. mume* varieties cannot naturally overwinter on land in the north temperate zone, which seriously limits their economic and ornamental value [14]. Breeding super cold-resistant *P. mume* varieties is an important topic at present; however, the molecular mechanism of *P. mume* responding to low temperatures is still unclear. Here, we identified 23 members of the *RCI2*s family in 7 Rosaceae plants and analyzed their gene sequences, phylogenetic trees, evolutionary relationships, and expression patterns under different low-temperature conditions. The findings of this study provide a basis for a comprehensive understanding of the phylogenetic relationship of the *RCI2*s family in Rosaceae and their functions in response to low-temperature stress in *P. mume*.

2. Materials and Methods

2.1. Plant Materials

The plant materials used for qRT-PCR were collected from the greenhouse of Beijing Forestry University. Healthy plants of *P. mume* "Zao Lve" with the age of two years were selected. Plant material was treated at different temperatures (4, 0, −4, −8 °C) for 4 h and at 4 °C for different durations (0, 1, 3, 5, 7, 9, 11 d), respectively. Annual shoots from five plants with different treatments were collected and mixed for RNA-seq with three biological repeats. All samples were immediately frozen in liquid nitrogen and stored at −80 °C for further usage.

2.2. Plants Genome Resources

The high-quality genome assembly and annotation files of *A. thaliana* (TAIR10.41), *Prunus dulcis* (v4.0), *Prunus salicina* (v2.0), *Prunus armeniaca* (v1.0), *Prunus persica* (v2.0), *Prunus avium* (v1.0.a1), *Rosa chinensis* (v1.0), and *P. mume* (v1.0) were downloaded from Ensembl Plants (https://plants.ensembl.org/index.html, accessed on 22 July 2022) [15] and the Genome Database for Rosaceae (GDR, https://www.rosaceae.org, accessed on 22 July 2022) [16].

2.3. Identification of RCI2s Gene Family

According to the *A. thaliana* RCI2 family protein sequence, the BLAST GUI Wrapper-Two Sequences Files ($E = 10^{-5}$) in TB tools (v1.0987663) software [17] were used to search for RCI2 family members in 7 Rosaceae plants. We used phylogenetic trees, sequence size, and conserved domain calibration to check the genetic members and finally screened out *RCI2*s members of 7 Rosaceae plants. With the aid of a phylogenetic tree, sequence size, and conserved domains, we deleted the false gene members. Molecular weights (MW) and isoelectric points (pI) of the RCI2s family members were calculated using the online tool ExPASy (https://www.expasy.org/, accessed on 23 July 2022), The subcellular localization of 7 Rosaceae plants was predicted using the subcellular localization tool WoLF PSORT (https://wolfpsort.hgc.jp/, accessed on 25 July 2022).

2.4. Gene Structure and Protein Conserved Motif Analysis

For investigating the RCI2 domains of the closely related *P. persica*, *P. mume*, *P. armeniaca*, and *P. salicina* in Prunoideae, the conserved domains of the RCI2s gene family were obtained by NCBI CD-Search (https://www.ncbi.nlm.nih.gov/cdd/, accessed on 23 July 2022) and Pfam (https://pfam.xfam.org, accessed on 23 July 2022). The RCI2 proteins were submitted to MEME (v4.12.0) [18] with settings (-nmotifs 3 -minw 8 -maxw 50, accessed on 23 July 2022) to search for conversed motifs. At the same time, Muscl was used to perform multiple sequence alignments of the full-length RCI2 protein sequences of seven Rosaceae plants. The trimAI (v2.1.3) was used to trim the alignment results, and IQ-tree (v2.1.3) was used to automatically screen amino acid replacement models [19]. Finally, TB tools [17] was used to conduct the tree-structure-motif map.

2.5. Chromosome Location and Synteny Analysis

Gene tandems were analyzed using the Multiple Collinearity Scan Toolkit (MCScanX) in TB tools with the default parameters. McscanX analyzed the synteny of the RCI2s across *A. thaliana*, *P. salicina*, *P. armeniaca*, *P. persica*, and *P. mume*, which were visualized in TB tools. The chromosomal length and location information of *RCI2*s were extracted from the gff and fasta files downloaded from the *P. mume* genome project. TB tools [17] were used to map chromosome distribution of RCI2s family members of *P. mume*.

2.6. Cis-Acting Element Analysis of RCI2 Gene Promoters

A cis-element analysis of the 2000 bp upstream genomic sequences of *PmRCI2*s genes, retrieved from the *P. mume* genome database. was performed by PlantCARE (https://bioinformatics.psb.ugent.be/webtools/plantcare/html/, accessed on 20 August 2022) [20], and these sequences were used as putative promoter sequences (Table S1). Then, we screened the results and visualized them by constructing the element distribution with TB tools [17].

2.7. Phylogenetic Analysis of RCI2s

The protein sequences in eight species (*A. thaliana*, *P. dulcis*, *P. salicina*, *P. armeniaca*, *P. persica*, *P. avium*, *R. chinensis*, and *P. mume*) were aligned by ClustalX [21]. At the same time, we constructed the maximum likelihood tree using IQ-tree (v2.1.3) [22]. Finally, the Chiplot (https://www.chiplot.online/, accessed on 15 July 2022) was used to decorate this phylogenetic tree.

2.8. Expression Pattern of PmRCI2s

Aiming to investigate the expression pattern of *PmRCI2*s involved in tissues development and low-temperature response, raw data (Tables S2–S4) from the RNA-seq were collected from different tissues, different dormancy periods, and natural overwintering conditions of *P. mume*. The different tissues of *P. mume* included flower buds, fruits, leaves, roots, and stems [23]. The different dormancy periods of *P. mume* "Zao Lve" buds overwintering in open fields in Beijing area included EDI (Endodormancy I, November), EDII (Endodormancy II, December), EDIII (Endodormancy III, January), and NF stage (Natural Flush, February) [24]. Using the stem of "Songchun" cultivar as material, the transcriptome data of *PmRCI2*s was acquired in three different seasons (autumn, October; winter, January; spring, March) under natural cold at three different places: Beijing (BJ, 39°54′ N, 116°28′ E), Chifeng (CF, 42°17′ N, 118°58′ E), and Gongzhuling (GZL, 43°42′ N, 124°47′ E). We used TB tools [17] to analyze these data and drew gene expression heatmaps.

In order to investigate the expression pattern of *PmRCI2*s under different cold treatment conditions in *P. mume* "Zao Lve", the grafted annual stems of "Zao Lve" were used. Additionally, whole plants were treated at 4 °C for 0, 1, 3, 5, 7, 9, 11 d, and 4, 0, −4, and −8 °C for 6 h. The culture material at 24 °C was used as the inner group control. RNA extraction was performed on the treated material using RNAprep Pure Plant Plus Kit (TIANGEN, Beijing, China). First-strand cDNA synthesis was performed using a TIANScript

First Strand cDNA Synthesis Kit (Tiangen, Beijing, China) according to the manufacturer's instructions. The Integrated DNA Technologies (IDT, https://sg.idtdna.com, accessed on 23 July 2022) was used to design qRT-PCR primers (Table S5), and TB Green chimeric fluorescence method was used for Real-Time PCR. qRT-PCR was carried out using a PikoReal real-time PCR system (Thermo Fisher Scientific, Waltham, MA, USA). *PmPP2A* gene of *P. mume* was selected as the internal reference gene [25]. The experiment was carried out with three biological replicates and three technical replicates, and the expression level of target genes was calculated by the $2^{-\Delta\Delta Ct}$ method [26].

3. Results

3.1. Identification of Rosaceae RCI2s Gene Family

Three members of the RCI2 family were obtained by BLSATp in *P. mume* genome and named *PmRCI2-1* (*Pm027750*), *PmRCI2-2* (*Pm003262*), and *PmRCI2-3* (*Pm003263*). We also identified members of the *RCI2* family in the genomes of almond (*Prunus dulcis*), plum (*Prunus salicina*), peach (*Prunus persica*), apricot (*Prunus armeniaca*), cherry (*Prunus avium*) and rose (*Rosa chinensis*). The amino acid length of *RCI2* in seven Rosaceae plants was between 55 aa and 149 aa, and the molecular weight (MW) was between 5.85 kDa and 15.74 kDa. *Pav_sc0000749.1_g020.1.mk:mrna* in *P. armeniaca* encoded the RCI2 protein with the largest MW among the seven plants, and the predicted isoelectric point (pI) was 4.09~9.95 (Table 1). Subcellular localization prediction results showed that the *RCI2* genes of seven plants were all located in the membrane structures, of which 39% were located in the plasma membrane, and 57% were located in the vacuole.

Table 1. Basic characteristics of the RCI2 gene family in seven Rosaceae plants.

Species	Gene ID	Basic Features			
		pI	MW/kDa	Length/aa	Prediction of Subcellular Localization
Prunus dulcis	*VVA22294*	7.87	12.37	118	Plasma membrane
Prunus persica	*ONH98753*	5.09	8.53	77	Vacuole
	ONI04115	5.43	6.33	58	Plasma membrane
	ONI04114	4.68	6.01	55	Vacuole
	ONI03135	9.69	7.33	69	Plasma membrane
	ONI24010	7.81	13.94	140	Plasma membrane
Prunus armeniaca	*PARG28267m01*	6.15	14.13	123	Plasma membrane
	PARG02720m01	9.95	12.73	115	Mitochondrion
	PARG18782m01	4.25	5.93	55	Vacuole
Prunus avium	*Pav_sc0001938.1_g430.1.mk:mrna*	4.68	6.01	55	Vacuole
	Pav_sc0001938.1_g440.1.mk:mrna	6.5	6.03	55	Plasma membrane
	Pav_sc0000749.1_g020.1.mk:mrna	8.79	15.74	149	Plasma membrane
	Pav_sc0000582.1_g860.1.mk:mrna	8.86	15.12	136	Plasma membrane
Rosa chinensis	*PRQ17753*	4.75	6.6	64	Vacuole
	PRQ42527	5.82	6.02	55	Vacuole
	PRQ42528	5.93	6.36	58	Vacuole
	PRQ58884	4.25	5.85	55	Vacuole
	PRQ43472	4.09	7.53	71	Vacuole
	PRQ46105	5.01	8.59	79	Vacuole
Prunus mume	*Pm027750*	4.79	8.65	77	Vacuole
	Pm003262	5.43	6.35	58	Plasma membrane
	Pm003263	9.58	15.27	135	Vacuole
Prunus salicina	*evm.model.LG07.71*	4.79	8.65	77	Vacuole

3.2. Gene Structure Analysis and Phylogenetic of RCI2 Genes

Chromosome position analysis showed that three *PmRCI2*s were located in two different chromosomes in the *P. mume* genome, two on chromosome 1 and one on chromosome 8 (Figure 1a). In order to better analyze the gene structure of *RCI2*s in *P. mume*, we analyzed the RCI2s domains of *P. armeniaca*, *P. salicina*, *P. persica*, and *P. mume* (Figure 1b), which were closely related in the Rosaceae Prunoideae. A high degree of similarity in the structure of the RCI2s was found among these four plants. The motif was analyzed by MEME software, and three different motifs were identified (Figure 1b). Except for *ONI03135* in *P. persica*, other *RCI2*s all contained two motifs (Motif 1 and Motif 2), indicating that these two motifs were conserved in the RCI2s family in *P. armeniaca*, *P. salicina,* and *P. mume*. In the four species, motif 3 was generally at the end of the peptide segment and it was only present in clade A of the RCI2s.

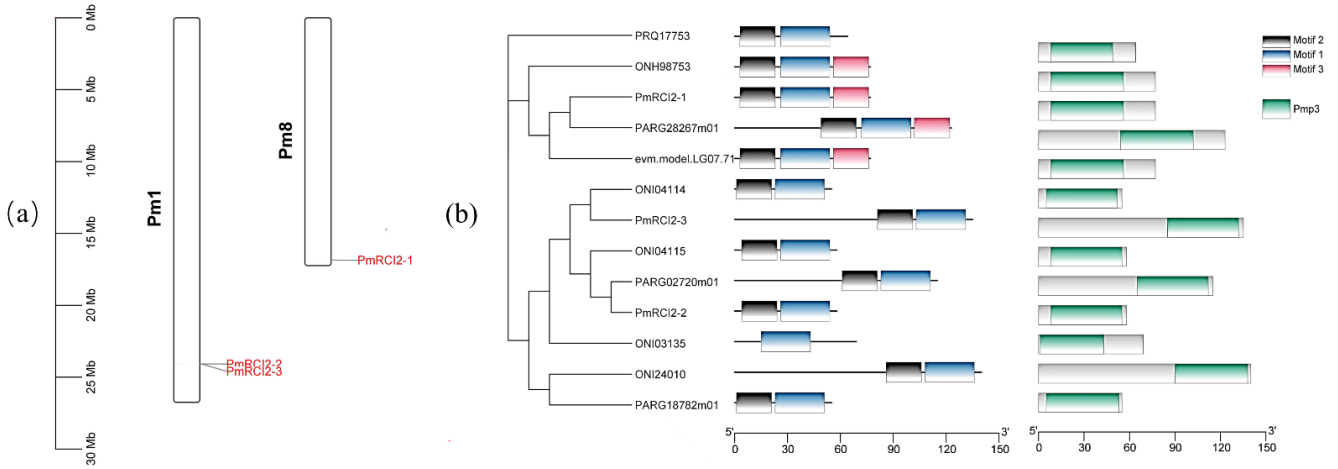

Figure 1. Gene locations and structures of *RCI2*s in Rosaceae. (**a**) Gene locations of *PmRCI2*s on the *P. mume* chromosome. (**b**) The motif composition and conserved domains were identified in RCI2s of *P. mume*, *P. armeniaca*, *P. salicina*, and *P. persica*.

In order to explore the phylogenetic relationship of RCI2s, we used a maximum likelihood method to create a phylogenetic tree of eight plants (Figure 2). The 23 identified RoRCI2 proteins were divided into three distinct clades. Clade A was the largest group and contained the most members (twelve) as compared to clade B containing only eight members (Figure 2). The PmRCI2s were only found in clades A and C, where PmRCI2-1 was homologous to PARG28267 in the *P. armeniaca* and evm.model. LG07.71 in *P. salicina*, PmRCI2-2 was homologous to PARG02720 in *P. armeniaca*, and PmRCI2-3 was homologous to ONI04114 in *P. persica*.

3.3. Analysis of Cis-Elements in the Promoters of PmRCI2s

Identification of cis-elements in the promoter region can help to explore the possible regulatory mechanisms of *PmRCI2*s in *P. mume*. Predicted results of PlantCARE (Table S6) showed that stress response elements were the most abundant (57.65%) among the other elements. multiple number of elements were involved in the light response, such as G-box, GT1-motif, and I-box. They were widely distributed in *PmRCI2*s promoters (Figure 3a,b). Anaerobic induced element (ARE) and MBS, involved in drought inducibility, were found in *PmRCI2-1* and *PmRCI2-2*. Low-temperature response element (LTR) was found only in *PmRCI2-2*. Notably, *PmRCI2-2* only contained some light response elements in stress responses (Figure 3c). In addition, hormone-related elements such as CGTCA-motif, TGACG-motif, P-box, TGA-box, and TCA-element participating in different hormone responses including MeJA, auxin and salicylic acid were found (Figure 3c). All *PmRCI2*s genes had abscisic acid response element (ABRE), and the number of this element

was the largest (Figure 3b), *PmRCI2-2* contained seven ABREs, and *PmRCI2-3* contained six ABREs.

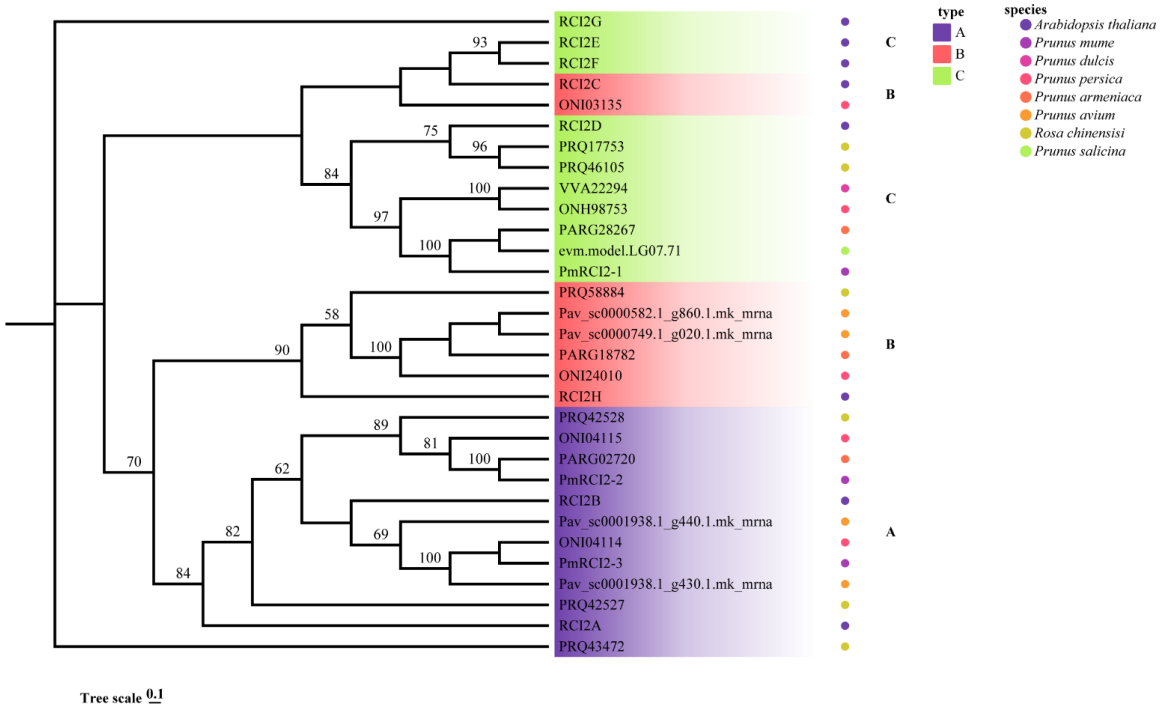

Figure 2. Phylogenetic tree of RCI2s in Rosaceae. The ML phylogenetic tree of RCI2s family in *P. dulcis*, *P. salicina*, *P. persica*, *P. armeniaca*, *R. chinensis*, *P. mume*, and *A. thaliana*.

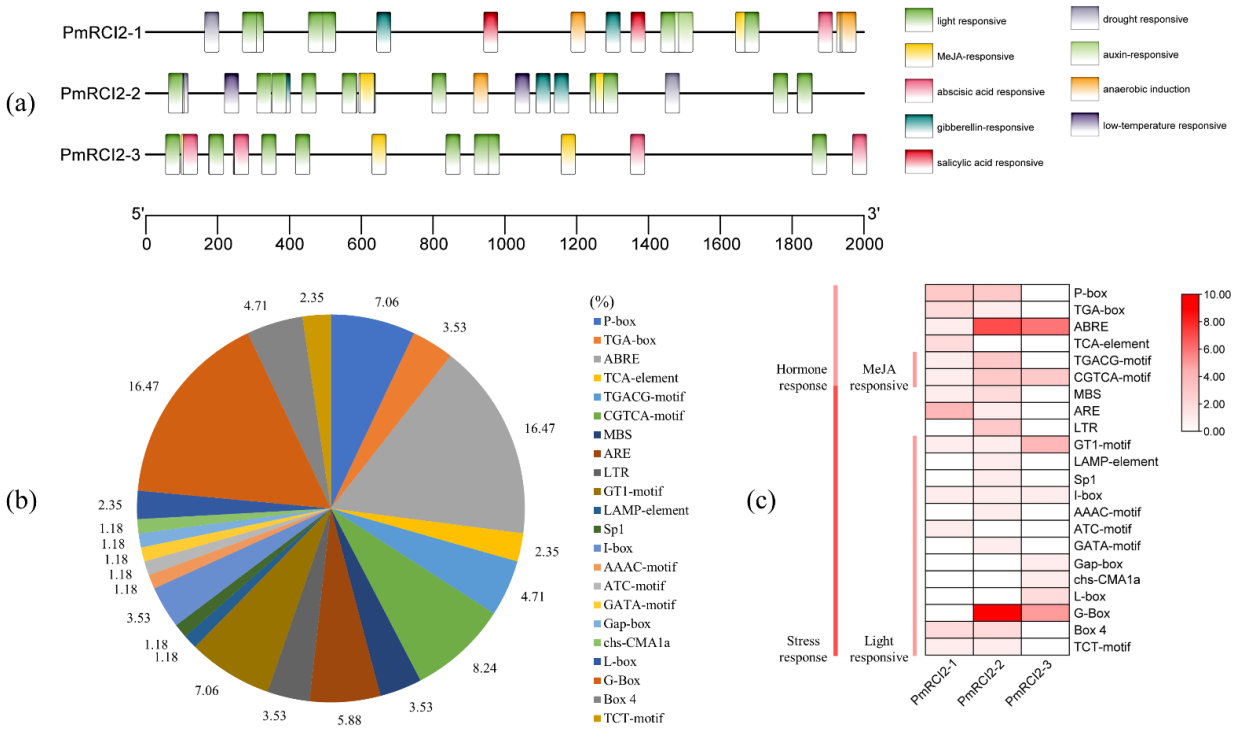

Figure 3. Cis-elements analysis of *PmRCI2*s promoters. (**a**) The distribution of the main cis-elements on promoter of identified *PmRCI2*s gene. (**b**) The proportion (%) of cis-elements in the *PmRCI2*s promoters involved in stress and hormone responses. (**c**) Heatmap of the cis-elements involved in stress responsiveness, and hormone responsiveness.

3.4. Collinear Analysis of RCI2s in Rosaceae and A. thaliana

In order to further analyze the evolution of *RCI2*s in Rosaceae plants, we performed an inter-genome comparison and collinearity analysis of *A. thaliana*, *P. mume*, *P. armeniaca*, *P. salicina* and *P. persica* (Figure 4). There were 4 *RCI2*s homologous gene pairs between *A. thaliana* and *P. mume*. *PmRCI2*s (*PmRCI2-1*, *PmRCI2-2*) had two collinearities in *A. thaliana* (*AT3G05880.1*/*AT1G57550.1*, *AT2G24040.1*/*AT4G30650.1*), one collinearity in *P. persica* (*ONH98753*, *ONI04114*) and in *P. armeniaca* (*PARG28267*, *PARG02720*). In addition, there was only one pair of RCI2 family homologous genes between *P. mume* and *P. persica*, suggesting that RCI2s family was contracted in some Rosacea genomes. *PmRCI2-3* homologous gene could not be found in these species, suggesting that *PmRCI2-3* was a unique gene in *P. mume*, which might be mutated.

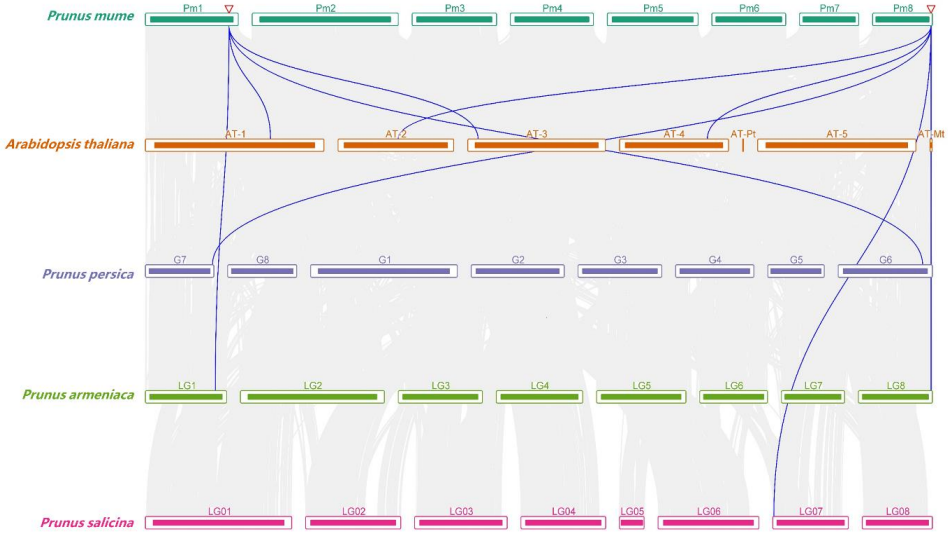

Figure 4. Syntenic analyses of *RCI2*s in *P. mume* and other plants. Blue lines represent syntenic *RCI2* gene pairs.

3.5. Expression Pattern of PmRCI2s under Cold Stress

*PmRCI2*s was expressed differently in different tissues of *P. mume*, such as *PmRCI2-3* had a low expression level in roots, but relatively high expression in stems and buds, and *PmRCI2-2* had a relatively high expression level in fruits and leaves (Figure 5a). The expression level of *PmRCI2-1* and *PmRCI2-2* gradually accumulated and reached the maximum at the NF stage when the bud dormancy was released, and the expression level of *PmRCI2-2* was higher than that of *PmRCI2-1*. On the contrary, the expression level of *PmRCI2-3* gradually upregulated from EDI to NF stage (Figure 5b). Moreover, we examined the expression pattern of "Songchun" cultivar in stems in different seasons at three places in BJ, CF, and GZL. In the same season, the expression levels of the three *PmRCI2*s increased with the decrease in the average temperature in different locations. The variation trend of gene expression of the same gene in different places was different. For example, in Beijing and Chifeng, the expression level of *PmRCI2-1* decreased from autumn to spring, but in Gongzhuling, the expression level of *PmRCI2-1* increased during the cold acclimation period and decreased after the cold acclimation period (from winter to spring). Geographical factors and the cold resistance of the plants may explain differences in gene expression. In addition, we found that the overall expression level of *PmRCI2-3* was higher than that of the other two genes during the cold acclimation period (from autumn to winter) at the same place (Figure 5c).

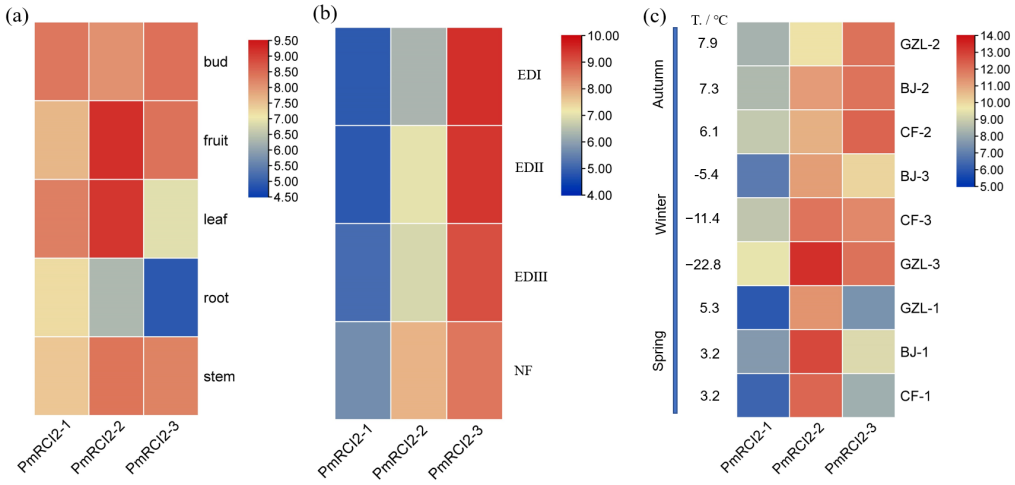

Figure 5. The expression pattern of *PmRCI2*s. (**a**) The expression level of *PmRCI2*s in different tissues of *P. mume*. (**b**) An examination of the expression levels of *PmRCI2*s in flowers of *P. mume* "Zao Lve" during three stages of endodormancy at low temperatures. (**c**) Three regional test sites (Beijing, BJ; Chifeng, CF; Gongzhuling, GZL) were used to collect data on the expression accumulation of *P. mume* "Songchun" stems during three different periods (autumn, winter, and spring). In the same season, the average temperature of different locations is ranked from high to low.

For further understanding the role of *PmRCI2* genes in response to cold stress, the expression level of *PmRCI2* genes under different treatments was detected by qRT-PCR (Figure 6). Under the condition of cold treatment at 4 °C, the expression level of *PmRCI2-3* was significantly higher than that of without cold treatment. The expression level of *PmRCI2-3* increased gradually with the prolonged cold treatment time and reached the maximum value at 9 d. The increase in the expression of *PmRCI2-3* was also significantly higher than the other two genes. Under different temperature treatments, the expression levels of *PmRCI2-1* and *PmRCI2-2* fluctuated to a certain extent but did not show a significant upward or downward trend. However, the expression level of *PmRCI2-3* gradually increased with the severity of cold stress and reached a peak at −8 °C. These results demonstrate that *PmRCI2-3* is significantly induced by cold stress and plays an important role in resisting constant low-temperature conditions.

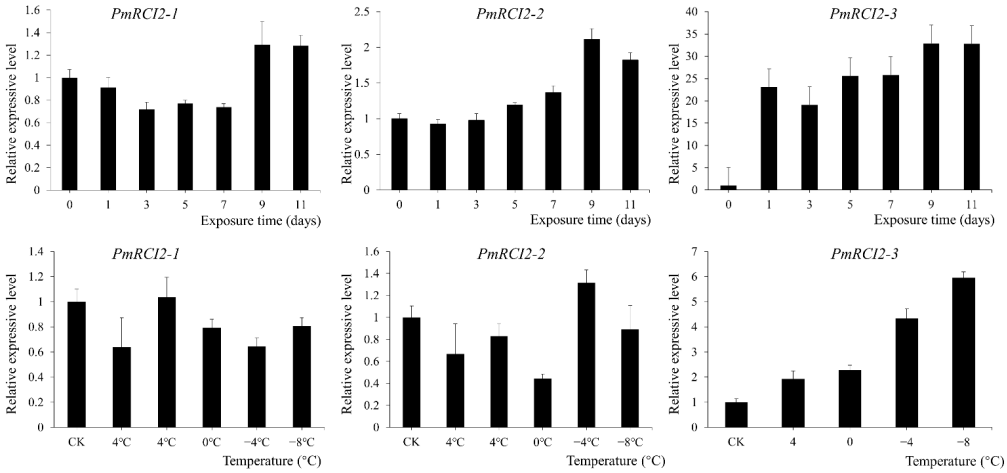

Figure 6. qRT-PCR analysis of *PmRCI2*s in an annual branch of the cultivar "Zao Lve" under low-temperature treatment. The grafted annual stems of "Zao Lve" were treated at 4 °C for 0, 1, 3, 5, 7, 9, 11 d, and 4, 0, −4, and −8 °C for 6 h, the culture material at 24 °C was used as the inner group control.

4. Discussion

Low-temperature stress severely restricts grain production, and China loses 300–500 million tons of rice each year due to cold damage [27]. Low temperatures in early spring and in late spring are the main disastrous weather for agricultural production in the northern temperate zone. Meanwhile, low-temperature stress in early spring is also one of the limiting factors for the growth and distribution of *Prunus* genus. At present, RCI2 family genes have been identified in *A. thaliana* [9], *Oryza sativa* [13], *Cucumis sativus* [5], and other plants, and related studies have shown that *RCI2*s are involved in the biological activities of plants in response to cold stress. In this study, we identified 23 *RCI2* genes from seven Rosaceae plants, of which 3 *PmRCI2*s genes (*PmRCI2-1*, *PmRCI2-2*, *PmRCI2-3*) belonged to *P. mume* (Table 1). Among the four species of *P. armeniaca*, *P. salicina*, *P. persica*, and *P. mume*, *P. salicina* had only one member of the RCI2 gene family, while *P. persica* had the most members, containing five RCI2s. An equal number of RCI2 gene family members were found in *P. armeniaca* and *P. mume*. This is in line with the evolutionary relationship of these four plants, indicating the expansion of the family during the evolution of the *Prunus* genus. In addition, the collinearity analysis showed that *P. salicina*, *P. armeniaca*, and *P. mume* all contained part of the RCI2 gene family members homologous to *AT3G05880.1*/*AT1G57550.1*, *AT2G24040.1*/*AT4G30650.1* of *A. thaliana*, while the *evm.model.LG07.71* of *P. salicina* was homologous to *AT3G05880.1*, suggesting that the genes homologous to *AT3G05880.1*/*AT1G57550.1* in these four plants are all generated by genome amplification (Figure 4).

The *RCI2*s play a role in different membrane structures in the cell [4]. Abiotic stress can affect the normal physiological activities of plants by changing the membrane structure in cells. For instance, heat stress alters thylakoid structure and reduces photosystem activity [28]. In this study, we predicted that *PmRCI2-1* and *PmRCI2-3* were located in the vacuole, whereas *PmRCI2-2* was located on the plasma membrane (Table 1). Plant cell vacuole contains various substances such as inorganic salts, organic acids, and sugars, which play an important role in maintaining intracellular ion homeostasis [29]. Therefore, *PmRCI2-1* and *PmRCI2-3* may have special functions in response to abiotic stresses and maintain normal plant growth and development. Like *OsLti6b* in *O. sativa* [13,30] and *AlTMP2* in *A. littoralis* [10], these genes are also localized in the vacuole and exhibit resistance to cold stress.

Plenty of evidence indicates that salt stress and cold stress are major abiotic stressors that induce *RCI2*s [30,31]. The plant materials used are perennial plants with robust growth, which can respond to cold stress normally; however, in our study, only *PmRCI2-3* was found to have a strong response to low-temperature stress (Figure 6). The expression level of *PmRCI2-3* always maintains a high expression level under long-term low-temperature treatment. The expression level of *PmRCI2-2* increased with the prolongation of the cold treatment time, reached a peak expression in 9 d, and then began to decline. However, the expression trend of *PmRCI2-1* was opposite to that of *PmRCI2-2*, and it increased after 9 d. Under different temperature treatments, the expression level of *PmRCI2-3* gradually increased with the decrease in temperature, while the expression level of *PmRCI2-1* and *PmRCI2-2* showed a trend of first decreasing and then increasing. All three *PmRCI2*s had Pmp3 conserved domains unique to the RCI2s gene family and contained motif 1 and motif 2. Through the analysis of cis-acting elements, no cold-responsive elements were found for *PmRCI2-3* with the highest expression under low-temperature stress (Figure 3). Similar results have been found in cucumber [5], which may be due to the presence of an unknown cold-responsive cis-element in the promoter region.

All three *PmRCI2*s promoter regions contain many abscisic acid response elements (ABRE), which is consistent with the report of a large number of ABRE found in the *RCI2* gene promoters of *Cucumis sativus* [5] and *Brassica napus* [32]. ABA induces second messengers to activate defense responses by generating ROS, and the expression of antioxidant enzyme genes and non-enzymatic defense system genes is also activated by the ABA

signaling-inducible mechanism [33]. This shows that *P. mume* may employ ABA-induced *RCI2*s expression in response to cold stress.

5. Conclusions

Twenty-three *RCI2* genes were identified from seven Rosaceae plants by genome-wide screening. Three *PmRCI2* genes (*PmRCI2-1*, *PmRCI2-2*, *PmRCI2-3*) from *P. mume* were cloned and systematically analyzed. The results of qRT-PCR showed that *PmRCI2-3* is significantly induced by low temperature and highly expressed in stems and buds during endodormancy stage. Our findings provide key candidate genes for cold resistance breeding of *P. mume* and other *Prunus* species.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae8110997/s1. Table S1: The promoter sequence of *PmRCI2*s. Table S2: Expression profiles of *PmRCI2* genes in different tissues. Table S3: Expression profiles of *PmRCI2* genes during the process of flower bud dormancy release. Table S4: Expression profiles of *PmRCI2* genes in different regions and seasons. Table S5: The Primer of qRT-PCR. Table S6: Cis-acting element on the promoter of *PmRCI2*s.

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