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Genome-Wide Analysis of *LEA* Gene Family in *Rosa chinensis* 'Old Blush' and Cold-Induced Expression Patterns in Two Species

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Abstract

Late embryogenesis abundant (LEA) proteins play an essential role in plant growth under various abiotic stresses. In this study, we identified 23 *RcLEA* genes in *Rosa chinensis* 'Old Blush' and subsequently grouped them into eight clades according to phylogenetic relationships and conserved domain features by bioinformatics methods. And conserved protein motifs and gene structure are also analyzed. The cis-regulatory elements of *RcLEA* promoter are enriched with cis-regulatory elements relevant to abiotic stress adaptation. Comparative transcriptomics between two species revealed tissue-specific and cold-induced expression differences, highlighting distinct functional roles of *LEA* genes in growth and abiotic stress tolerance between *Rosa chinensis* 'Old Blush' and *Rosa beggeriana*. Furthermore, Quantitative Real-Time PCR (qRT-PCR validation confirmed divergent cold-responsive expression profiles of *LEA* genes in *R. chinensis* 'Old Blush' compared with the highly cold-tolerant *R. beggeriana* in four *LEA* homologous genes. These findings indicated that *LEA* acts as a cold-response gene in roses and provide foundation to breed cold-tolerant varieties of roses.

Keywords: *LEA* gene family; rose; cold acclimation; expression profiles

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1. Introduction

Abiotic stresses such as drought, salinity, and extreme temperatures disrupt crop growth and cause yield losses and quality decline [1]. Through evolution, plants have acquired molecular, physiological, and biochemical adaptations, prominently the late embryogenesis abundant (LEA) proteins, which respond to low temperature, abscisic acid (ABA), ethylene, drought, and salinity to protect cellular integrity [2–4]. First noted in maturing cotton seeds [5,6], LEA proteins have since been documented in rice [7], maize [8], soybean [9], and many other species. LEA proteins are now recognized as stress-inducible protectors expressed beyond seeds in seedlings, leaves, and roots under drought or salinity [10,11].

LEA proteins constitute a diverse family that can be classified into eight groups based on the Pfam database: LEA1, LEA2, LEA3, LEA4, LEA5, LEA6, dehydrin (DHN), and seed maturation proteins (SMP) [12]. These proteins are typically highly hydrophilic and glycine-rich, a property that contributes to their ability to stabilize cellular structures under stress conditions [13]. Members of different LEA groups have been implicated in a wide range of protective functions, including safeguarding cellular structures during dehydration (LEA1) [14], stabilizing membranes and mediating stress signaling (LEA2) [15], and enhancing drought and salt tolerance through antioxidant regulation (LEA3) [16]. LEA4 enhances tolerance to salt and drought too [17]. Other groups are associated with stress adaptation (LEA5) [18], seed longevity and desiccation tolerance via the glassy state (LEA6) [19], and seed maturation processes [20]. Dehydrins (DHNs) are particularly responsive to diverse abiotic stresses [21]. Drought resistance is enhanced by *AtLEA3-3* in Arabidopsis [22], *WZY3-1* in wheat [23], and *GmPM35* in *Glycine max* [24]. Salt and osmotic stress tolerance are enhanced by *CaDHN5* in pepper [25], *MsLEA4-4* in alfalfa [26], and LEA12^{OR} in wild rice [27]. *ZmDHN15* has been shown to improve cold tolerance in maize. [28], *VamDHN3* in *Vitis amurensis* [29], *AmDHN4* in *Ammopiptanthus mongolicus* [30], *PasLEA3-2*, and Pasdehydrin-3 in *P. armeniaca* L. × *P. sibirica* L. [31].

Roses, being important ornamental plants and pivotal materials in the perfume industry, hold significant cultural and economic value worldwide. The cold resistance of roses can affect their growth, thereby influencing the promotion of their varieties. As the ancestor of modern repeat-flowering roses, *Rosa chinensis* 'Old Blush' endows its descendants with perpetual bloom, compact habit, and vivid color, traits that significantly influenced global horticulture [32]. It serves as a crucial resource for understanding Rosaceae evolution and breeding. In contrast, *Rosa beggeriana*, a cold-resistant wild rose native to Central Asia, holds potential for improving cultivated roses through hybridization [33]. Cold-tolerant germplasm in roses remains limited, and existing cultivars exhibit insufficient hardiness. Our research team has previously conducted systematic studies on *Rosa beggeriana*, including its use in distant hybridization to develop the cold-hardy 'Tianshan' series of rose cultivars, the demonstration of its exceptional freezing tolerance and elucidation of the associated physiological mechanisms, as well as the characterization of phenotypic and genetic diversity of its natural populations in Xinjiang [34–37]. These earlier works establish a solid foundation for the current investigation into its molecular cold-tolerance mechanisms. Although LEA proteins play a notable role in plant low-temperature stress tolerance, their functions within the *Rosa* are still poorly understood.

This study systematically characterized the *LEA* gene family in *R. chinensis* 'Old Blush' by examining gene numbers, structural organization, and motif distribution. In addition, phylogenetic relationships, chromosomal localization, and duplication events were investigated. The cis-acting regulatory elements of LEA members were further predicted based on the available high-quality genome sequence [38]. Expression analysis of *LEA* genes under low-temperature treatment was conducted using transcriptome data to explore their role in cold resistance. Elucidating the cold resistance mechanisms of *LEA* genes in Rosaceae species offers a theoretical foundation for the genetic enhancement and breeding of cold-tolerant rose cultivars.

2. Materials and Methods

2.1. Identification and Characterization of RcLEAs

The reference genome of *R. chinensis* 'Old Blush', along with its annotation, was retrieved from the LIPM database (https://lipm-browsers.toulouse.inra.fr/pub/RchiOBHm-V2/web/fatal/download/download.html, accessed on 1 January 2024). To detect LEA family members, Hidden Markov Model (HMM) profiles corresponding to LEA1 (PF03760),

LEA2 (PF03168), LEA3 (PF03242), LEA4 (PF02987), LEA5 (PF00477), LEA6 (PF10714), DEHYDRIN (PF00257), and SMP (PF04927) domains were obtained from Pfam (http://pfam.xfam.org/, accessed on 1 January 2024) and applied in HMMER v3.3.2 searches (e-value < 1×10^{-5}) [39]. In parallel, 51 Arabidopsis LEA proteins were collected from TAIR (https://www.arabidopsis.org/, accessed on 1 January 2024) and applied as queries in BLASTP (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastp&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome, accessed on 1 January 2024) screening against the *R. chinensis* proteome (cutoff e-value 1×10^{-5}; Table S3). Pfam and NCBI Conserved Domain Database were used to validate candidate proteins for conserved domains subsequently (https://www.ncbi.nlm.nih.gov/cdd/, accessed on 1 January 2024). Physicochemical properties, including sequence length, molecular mass, and theoretical pI, were calculated with the ProtParam tool (https://web.expasy.org/protparam/, accessed on 1 January 2024).

2.2. Gene Structure, Motif, and Phylogenetic Tree Analysis of AtLEAs and RcLEAs

Phylogenetic relationships between 51 *AtLEAs* and 23 *RcLEAs* were established by the neighbor joining (NJ) method and bootstrap analysis (1000 replications) in MEGA11 program [40]. Conserved domains were annotated via NCBI CDD (https://www.ncbi.nlm.nih.gov/Structure/cdd/cdd.shtml, accessed on 1 January 2024), while motifs were identified using MEME (https://meme-suite.org/meme/, accessed on 1 January 2024) [41]. Exon–intron structures of *RcLEA* genes were visualized with TBtools v2.376 [42]. Classification of LEA family members was achieved by integrating domain/motif information with phylogenetic clustering. Cis-regulatory elements within 2000 bp upstream regions were predicted using PlantCARE (https://bioinformatics.psb.ugent.be/webtools/plantcare/html/, accessed on 1 January 2024) [43].

2.3. The Chromosomal Location and Duplication Analysis of RcLEAs

Chromosomal positions of *RcLEA* genes were extracted from genome annotations and mapped using TBtools v2.376. Sequence similarity searches (BLAST v2.16.0, e-value < 1×10^{-5}) were performed to identify tandem and whole-genome duplication events, which were further confirmed with MCScanX v1.0.0 under default parameters [44]. Evolutionary constraints were assessed by calculating Ka/Ks ratios with KaKs_Calculator v3.0 (Table S4) [45].

2.4. Cis-Acting Element Analysis

Promoter sequences spanning 2000 bp upstream of *RcLEA* genes were analyzed using PlantCARE to predict cis-acting elements (http://bioinformatics.psb.ugent.be/webtools/plantcate/html, accessed on 1 January 2024). The distribution of regulatory motifs was illustrated using TBtools.

2.5. Cold Stress Experimental Design and Plant Materials

Annual seedlings of *R. beggeriana* and *R. chinensis* 'Old Blush' obtained by cutting propagation were maintained in a controlled chamber at 24 °C during the day and 18 °C at night under a 10 h light/14 h dark cycle. After a one-week adaptation period, seedlings of both species were subjected to cold treatments. Plants were exposed to 4 °C for 0, 1, 6, and 24 h, followed by −4 °C for 6 h and 24 h for qRT-PCR validation. For *R. beggeriana*, plants were maintained at 4 °C for 0, 1, 6, and 24 h for transcriptome analysis. Each treatment included three independent biological replicates, with 0 h serving as the control. For tissue-specific expression analysis, stem, leaf, root, flower, and fruit tissues of *R. beggeriana* were sampled under normal growth conditions.

2.6. RNA-Seq Data Analysis

Transcriptome data of *R. chinensis* 'Old Blush' (Project accession: PRJNA546486) were downloaded from the SRA database (https://www.ncbi.nlm.nih.gov/sra, accessed on 1 February 2024) [46] to examine the tissue-specific expression profiles of *RcLEA* genes. The corresponding genes in *R. beggeriana* were identified by sequence alignment using the *R. chinensis* 'Old Blush' genome as a reference, following a one-to-one correspondence screening criterion. The tissue-specific expression heatmaps for both species were generated using TBtools based on fragments per kilobase of transcript per million mapped reads (FPKM) values. Total RNA from the cold-treated samples described in Section 2.5 was used for transcriptome sequencing. The raw data for *R. beggeriana* are provided in Tables S7 and S8. The expression profiles of *RbLEA* genes under cold stress were visualized as heatmaps using TBtools based on FPKM values.

2.7. Experimental Validation by Quantitative Real-Time PCR (qRT-PCR)

To validate the RNA-Seq results, qRT-PCR was performed on the cold-treated samples described in Section 2.5. Total RNA was extracted using the E.Z.N.A.® Plant RNA Kit (Omega Bio-Tek, Norcross, GA, USA). First-strand cDNA was synthesized using the PrimeScript™ RT reagent Kit (Takara, Kusatsu, Japan). qRT-PCR reactions were conducted using SupRealQ Ultra Hunter SYBR qPCR Master Mix (Vazyme, Nanjing, China) on a CFX96 Real-Time PCR System (Bio-Rad, Hercules, CA, USA). Gene-specific primers for LEA genes were designed using NCBI Primer-BLAST (primer sequences are listed in Table S9). Rba-Tubulin was used as the reference gene for normalization. Relative expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method, and statistical significance was assessed as described in Section 2.8.

2.8. Statistical Analysis

All experiments were conducted with three independent biological replicates. Data are presented as mean ± standard error (SE). Statistical analyses were performed using GraphPad Prism v10.1.2. For qRT-PCR data, differences between treatments were evaluated by one-way ANOVA followed by Fisher's Least Significant Difference (LSD) post hoc test. A p-value < 0.05 was considered statistically significant. Fold-change thresholds (>2-fold) were used to define biologically relevant differential expression.

3. Results

3.1. Identification and Characterization of LEAs in R. chinensis 'Old Blush'

R. chinensis 'Old Blush' had 23 *LEA* genes (Table S1). A neighbor joining (NJ) tree was constructed for the LEA members of *A. thaliana* and *R. chinensis* 'Old Blush' (Tables S2 and S3). In total, 74 *LEA* genes were grouped into eight categories: LEA1, LEA2, LEA3, LEA4, LEA5, LEA6, SMP, and DHN (Figure 1). After analysis, the *DHN* gene family was the maximum subgroup including 6 of 23 *LEA* genes from *R. chinensis* 'Old Blush'. The subfamily LEA2 was the next largest subgroup, comprising four LEA from *R. chinensis* 'Old Blush'. Gene numbers differ among groups, with substantial variation compared to Arabidopsis. The species-specific group (AtM) of *A. thaliana* was not present in *R. chinensis* 'Old Blush'. Compared with *A. thaliana*, the number of genes in LEA4 of *R. chinensis* 'Old Blush' is significantly reduced.

3.2. Motif Analysis, Conserved Domain Analysis, and Gene Structure Analysis of LEAs in R. chinensis 'Old Blush'

To analyze the structural features of RcLEA proteins, an unrooted phylogenetic tree incorporating motif, CDS, UTR, and gene structure information was constructed based

on the full-length sequences of the 23 *RcLEA* genes (Figures 2, S1 and S2). The location of motif overlaps with that of the conserved domain (Figure 2b,c). The *DHN* subfamily was predicted to have the largest number of motifs, with three types. The *SMP* subfamily had two types, while each of the other subfamilies had only one type. Meanwhile, the exon-intron structure of *RcLEA* genes was analyzed (Figure 2d). Notably, the DHN subfamily exhibits the widest range of structural diversity among all *RcLEA* groups, containing the most genes and displaying the most pronounced differences in exon–intron architecture. *RcSMP1* has four exons. *RcDHN2*, *RcDHN3*, and *RcLEA2-4* have three exons. *RcLEA6* and *RcLEA3-1* have one exon. The remaining genes all contain two exons. In general, *RcLEA* genes categorized under the same phylogenetic group were characterized by conserved exon–intron structures, a finding consistent with and supportive of the established evolutionary relationships and group classification.

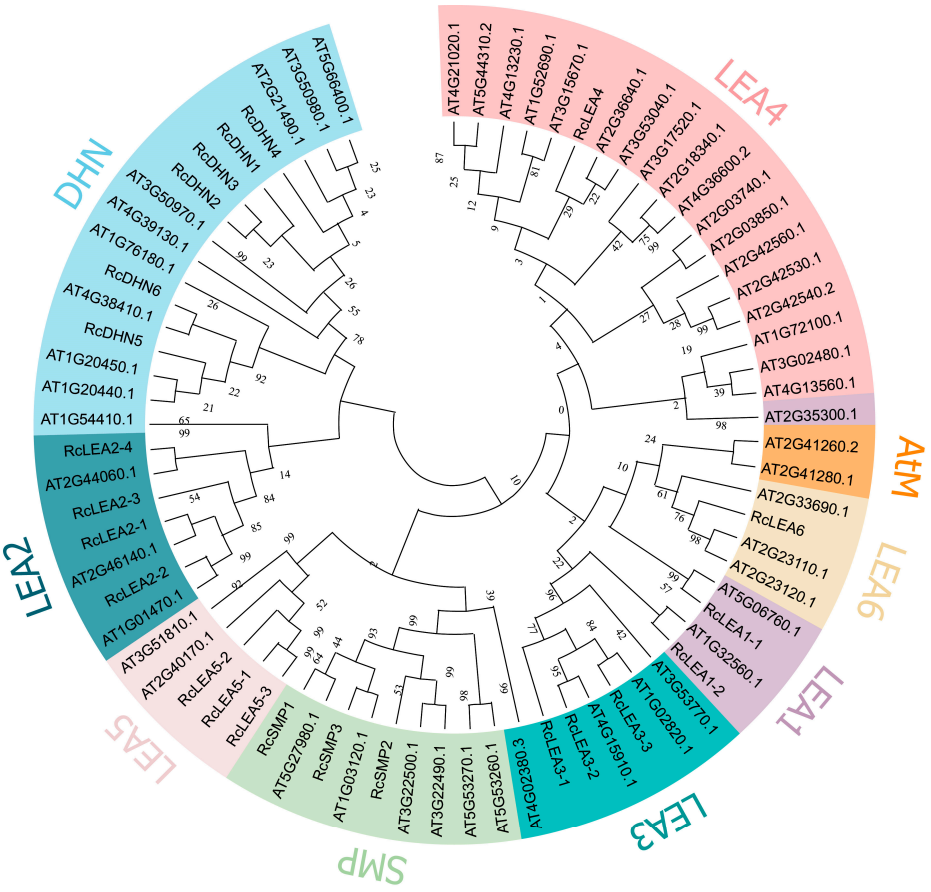


Figure 1. Phylogenetic relationships of LEA proteins of *A. thaliana* and *R. chinensis* 'Old Blush'. Phylogenetic analysis was performed in MEGA 11 employing the neighbor-joining algorithm. The 8 subfamilies are distinguished by different colors.

3.3. Synteny and Chromosomal Location Analysis of LEAs in R. chinensis 'Old Blush'

Twenty-three *RcLEAs* are distributed on the six chromosomes (Figure 2a). The *LEA* genes were unevenly distributed along the chromosome of *R. chinensis* 'Old Blush'. There was no *LEA* gene distribution on Chr 5. The largest quantity of *RcLEAs* was found on Chr 2 and Chr 3, followed by Chr 6. Three genes located on Chr 1 and two genes on Chr 4. Chr 7 carried only one gene. Genes are non-uniformly distributed across chromosomes, with certain gene clusters exhibiting aggregation.

Gene family expansion and evolution are primarily driven by tandem and whole-genome duplication events. For *RcDHN1* and *RcDHN2*, these two tandemly duplicated genes positioned on Chr 2. There are also two clusters of tandem duplications, one on Chr

1 consisting of *RcLEA2-1*, *RcLEA2-2*, and *RcLRA2-3*. The other group is located on Chr 3, which consisted of *RcLEA5-1*, *RcLEA5-2*, and *RcLEA5-3*. Furthermore, two whole-genome duplication events, including *RcDHN5*, *RcDHN6*, *RcSMP2*, and *RcSMP3*, were identified on three separate chromosomes (Figure 3b).

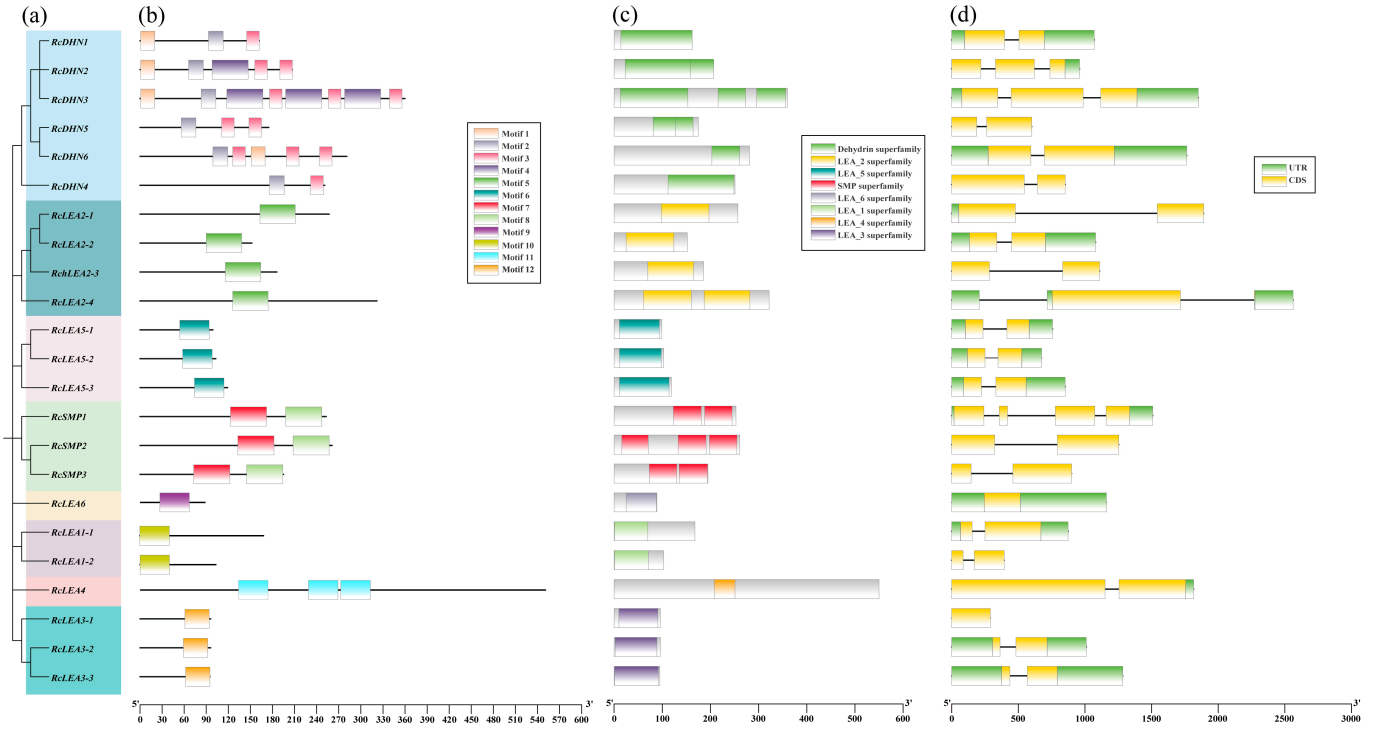


Figure 2. Phylogenetic relationships, motif distribution, conserved protein motifs, and gene structure analysis of *LEA*s in *Rosa chinensis* 'Old Blush'. (**a**) Phylogenetic relationships among RcLEA proteins. MEGA 11 was used to construct phylogenetic tree by NJ method. Using the sequences of 23 RcLEA proteins, the bootstrap analysis was performed with 1000 replicates. (**b**) Distributions of motifs in *RcLEA* genes. Twelve types of predicted motifs are marked with distinct colored boxes. (**c**) Analysis of conserved domain of 23 RcLEA proteins. (**d**) Gene structure of *RcLEA* genes. CDS are represented by orange boxes; introns are represented by black lines.

The selective pressure on homologous gene pairs in *R. chinensis* 'Old Blush' were further investigated. We analyzed their Ka/Ks values (Table S4). All *LEA* homologous gene pairs exhibited Ka/Ks ratios under 1, reflecting the action of purifying selection throughout evolution. We also predicted the Ka/Ks ratios of *AtLEAs* (Table S4). Both ratios of these two species are less than 1, indicating that the family subjected to strong purifying selection and has highly conserved functions.

3.4. Promoters Analysis of the RcLEA Genes

For the analysis of cis-regulatory elements in *RcLEAs*, sequences spanning 2 kb upstream of the translation start sites of all *LEA* genes were retrieved from the *Rosa chinensis* 'Old Blush' genome (Figure 4). In total, 14 types of cis-regulatory elements linked to stress tolerance were found, among them ABRE, ARE, DRE core, F-box, GARE-motif, GATA-motif, LTR, MBS, MYB, MYC, STRE, TGA-element, W-box, and WRE3 (Figure 4a). MYB transcription factors accounted for the largest proportion, whereas GARE transcription factors were the least represented, indicating the diversity of transcription factors associated with *RcLEAs* (Figure 4b). All *RcLEA* members harbored at least two cis-elements associated with stress adaptation. Among these, ABRE, MYB, and WRE3 motifs were particularly enriched, indicating their dominant role in the regulation of the *RcLEA* gene

family (Figure 4). Table S5 summarizes the detailed profiles of cis-acting elements identified in each *RcLEA* gene.

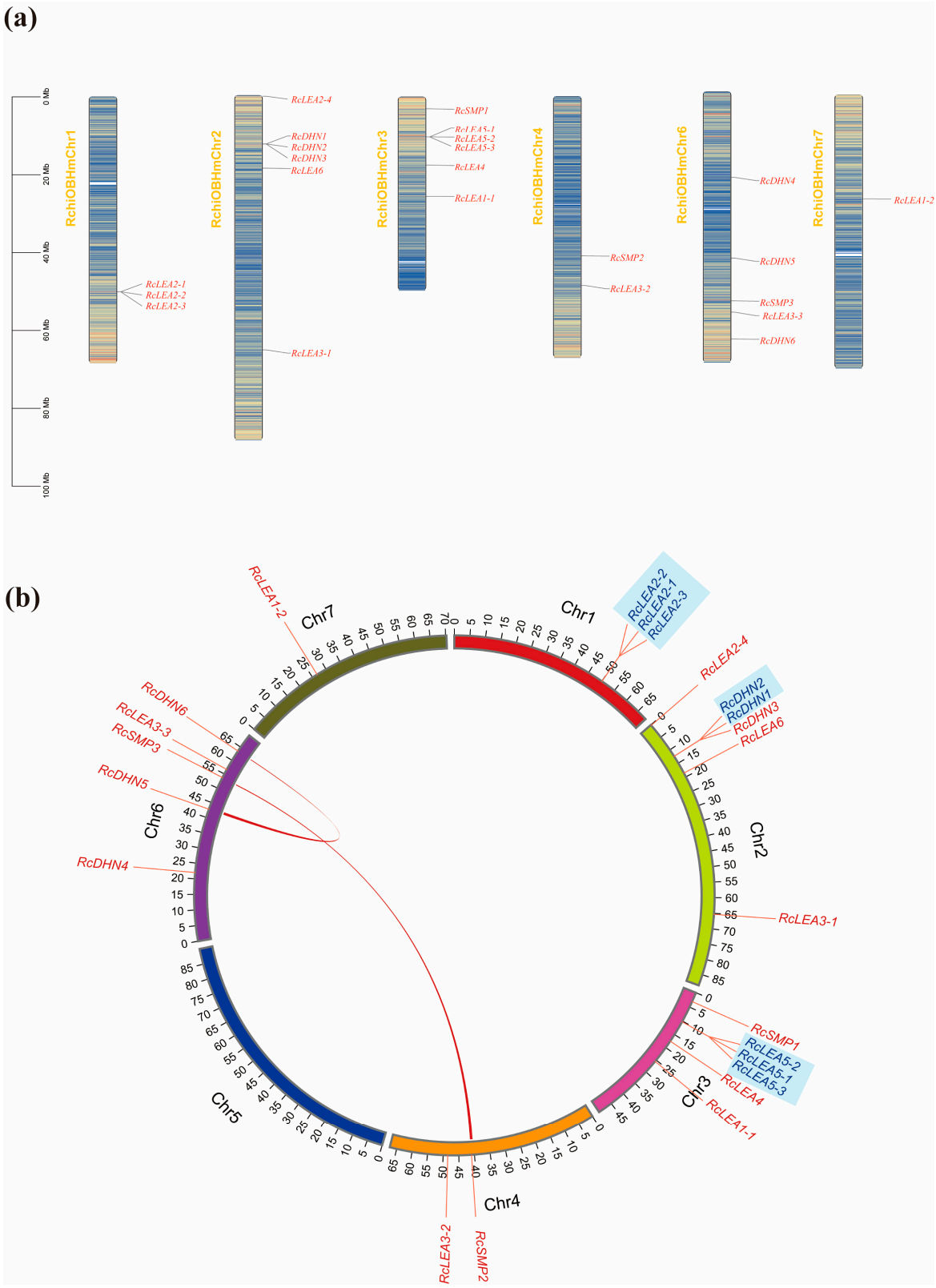


Figure 3. Gene duplication and chromosomal location of the *RcLEA* genes. (**a**) The position of *RcLEAs* on chromosomes. (**b**) Whole-genome duplication and tandem duplication of the *RcLEA* genes. Whole-genome duplicated gene pairs are connected by red lines, while tandemly duplicated genes are highlighted in light blue.

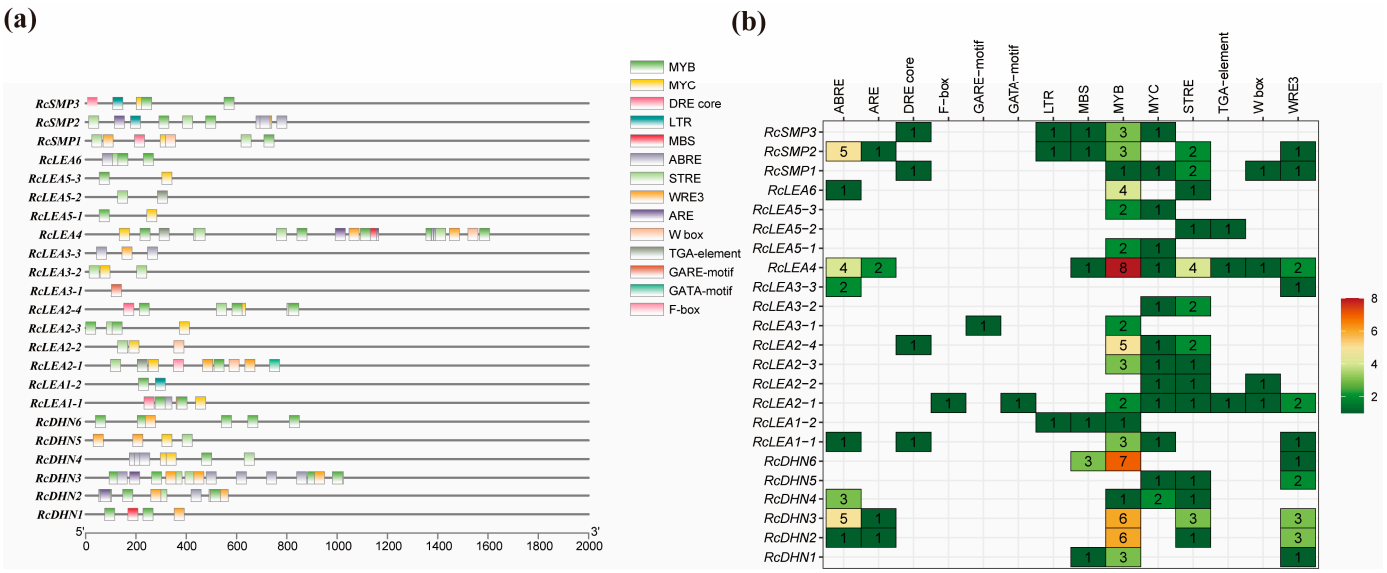


Figure 4. Predicted cis-acting elements in the promoters of *RcLEA* genes. (**a**) Promoter regions of *RcLEA* genes containing cis-elements. Different element classes are indicated by distinct colors and shapes. (**b**) Quantitative heatmap of cis-acting elements across the *RcLEA* gene promoters. The color gradient represents the predicted number of cis-acting elements, with green indicating a lower count and red indicating a higher count.

3.5. Expression Profiling of LEA Genes Under Cold Stress in Rosa chinensis 'Old Blush', with Comparative Patterns Assessed in Rosa beggeriana

RNA-Seq analysis revealed that *RcLEA* genes in *Rosa chinensis* displayed tissue-biased expression across stamen, root, stem, prickle, leaf, and pistil (Figure 5). *RcDHN6* and *RcLEA3-3* exhibited very high transcript abundance across multiple tissues, with peaks in prickle and stamen, whereas *RcLEA1-1* and *RcSMP1* were specifically enriched in stamen. *RcLEA2-4*, *RcLEA2-2*, and *RcLEA6* exhibited high transcript abundance across multiple tissues at the same time. In *Rosa beggeriana*, *RbDHN6* showed higher expression across multiple tissues, with peaks in leaf. In stem, expression was the most pronounced, with several genes reaching extremely high transcript levels, making this tissue the dominant site of *RbLEA* activity. In root, expression was moderate overall, with a few genes contributing prominently. In conclusion, the strongest *RbLEA* signals were concentrated in stem, leaf, and flower, while root and fruit showed more selective expression patterns.

Within the *LEA1* group, *RcLEA1-1* was highly expressed in reproductive tissues, whereas its homolog *RbLEA1-1* showed extremely high expression in flower, indicating a conserved reproductive bias. In the *LEA3* group, *RcLEA3-3* exhibited very strong expression in reproductive tissues and root, while its homolog *RbLEA3-3* was predominantly expressed in flower, reflecting redistribution of expression within reproductive organs. *RcLEA2-1* was highly expressed in stamens, whereas its homolog *RbLEA2-1* showed strong expression in stems and leaves, reflecting a marked shift in tissue specificity. In contrast, *RcLEA6* was moderately expressed across multiple tissues, while its homolog *RbLEA6* showed no detectable expression, suggesting loss of activity in *R. beggeriana*. Among the *DHN* genes, *RcDHN3* was strongly expressed in root and reproductive tissues, whereas its homologs, *RbDHN3-1* and *RbDHN3-2*, were highly expressed in stem and leaf, indicating a major tissue shift. *RcDHN1* and *RcDHN2* showed weak expression in *R. chinensis* 'Old Blush', but their homologs, *RbDHN1* and *RbDHN2*, were strongly expressed in fruit and stem, representing a gain of expression in *R. beggeriana*. By contrast, *RcDHN6* and *RbDHN6* both displayed very high expression across multiple tissues, reflecting a conserved high-expression pattern. Overall, these results demonstrate that, while some homologous pairs

retained conserved expression, such as *DHN6*, many exhibited pronounced changes in both tissue specificity and transcript abundance, with *R. chinensis* 'Old Blush' genes enriched in reproductive tissues and root and *R. beggeriana* homologs more strongly expressed in stem, leaf, and fruit.

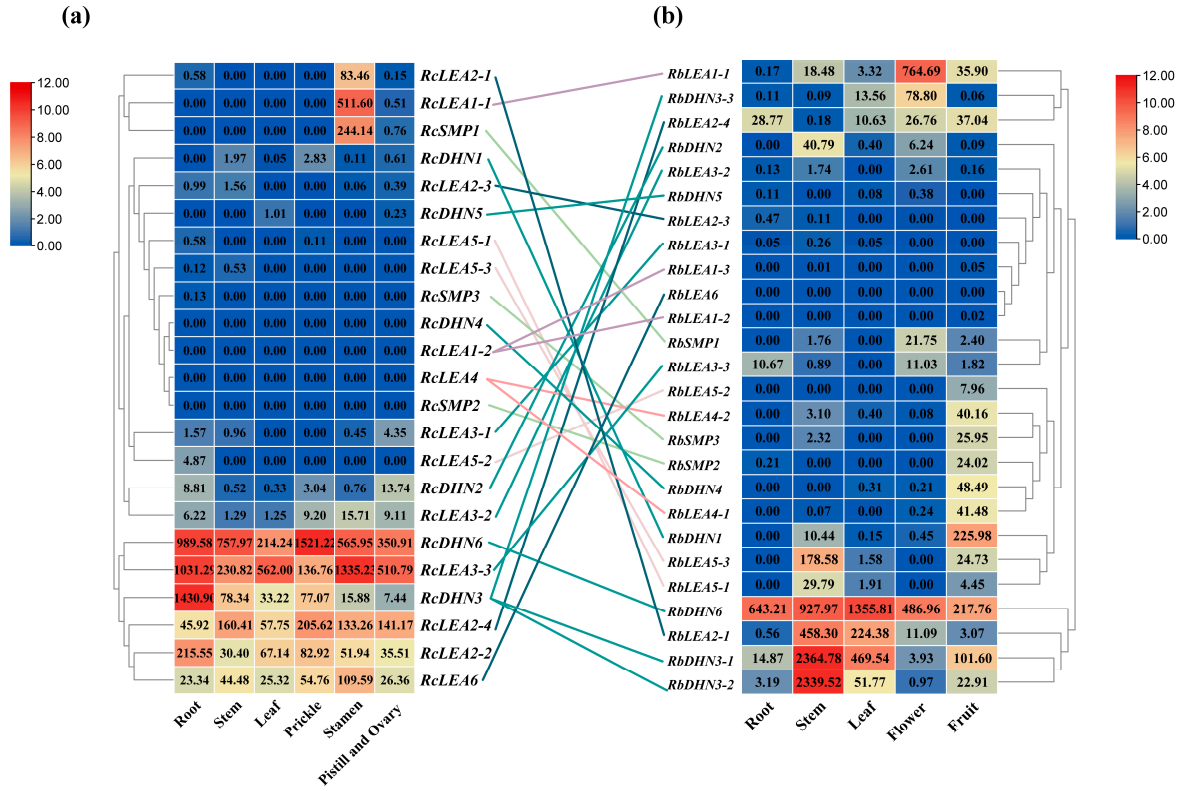


Figure 5. Expression profiles of LEA genes in different tissues of two rose species. (**a**) *Rosa chinensis* 'Old Blush' (*RcLEA*). (**b**) *Rosa beggeriana* (*RbLEA*). The number in each square (heatmap cell) represents the FPKM. The color gradient from red to blue represents $\log_2$-transformed FPKM values, indicating high to low expression levels, respectively. Homologous genes between the two species are connected by colored lines.

Twenty-six *RbLEA* genes were found in *R. beggerian* through transcriptome analysis of leaves and stems under chilling treatment (Figure 6 and Table S7). Over 10 genes were revealed with significance under the 23/18 °C treatment, including *RbLEA6*, *RbLEA3-2*, *RbLEA3-3*, *RbLEA3-1*, *RbDHN6*, *RbLEA2-1*, *RbLEA5-3*, *RbSMP1*, *RbLEA2-3*, *RbLEA1-1*, *RbDHN2*, and *RbLEA2-4* in both tissue. Differentially expressed genes (DEGs) in *R. beggeriana* were identified from transcriptomes of leaves and stems following chilling treatment at 4 °C. In leaves, the expressions of *RbLEA6*, *RbLEA3-2*, *RbLEA3-3*, *RbLEA3-1*, *RbDHN6*, *RbLEA2-1*, and *RbDHN1* were up-regulated under cold stress. The expression levels of *RbLEA5-3* were up-regulated at early time points and then down-regulated. In stems, the expressions of *RbLEA6*, *RbLEA3-2*, *RbLEA3-3*, *RbLEA3-1*, *RbDHN6*, *RbLEA2-1*, *RbLEA5-3*, *RbSMP1*, *RbDHN1*, and *RbDHN4* were up-regulated under cold stress. The expression levels of *RbDHN1*, *RbDHN3*, *RbLEA5-3*, and *RbLEA6* were much higher in stems than in leaves. The expression levels of *RbLEA3-1*, *RbLEA3-2*, *RbLEA5-1*, and *RbSMP1* fluctuated under cold stress. In addition, *RbLEA1-2*, *RbLEA2-1*, *RbSMP1*, and *RbSMP2* showed comparable effects on growth temperature.

In *R. beggeriana*, qRT-PCR analysis revealed that the majority of *RbLEA* genes exhibited expression profiles in both leaves and stems that were consistent with transcriptome data (Figure 7). In leaves, *R. beggeriana* showed much stronger and more sustained induction of *LEA6*, *DHN3*, and *DHN6* than *R. chinensis*. In stems, the same trend was observed,

with genes such as *LEA5-3* and *DHN6* up-regulated more strongly in *R. beggeriana*. Within species, *R. beggeriana* generally displayed higher expression in leaves than stems, while *R. chinensis* showed weaker and less tissue-differentiated responses. These results indicate that *R. beggeriana* activates *LEA* and *DHN* genes more robustly under cold stress, consistent with its greater cold tolerance.

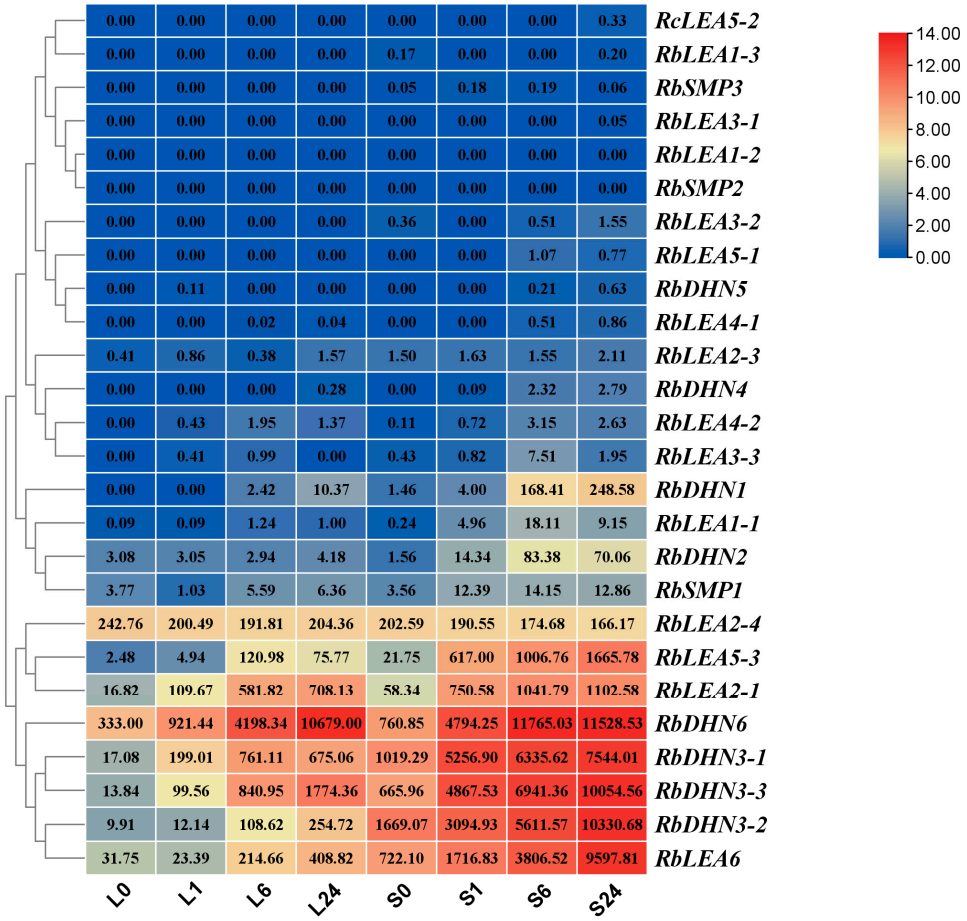


Figure 6. Expression of *RbLEA* genes under cold stress. Heatmap depicting the expression dynamics of *RbLEA* genes in leaves (L) and stems (S) of *R. beggeriana* after chilling treatment at 4 °C for 0, 1, 6, and 24 h. The number in each square (heatmap cell) represents the FPKM. The color gradient from red to blue represents $\log_2$-transformed FPKM values, indicating high to low expression levels, respectively.

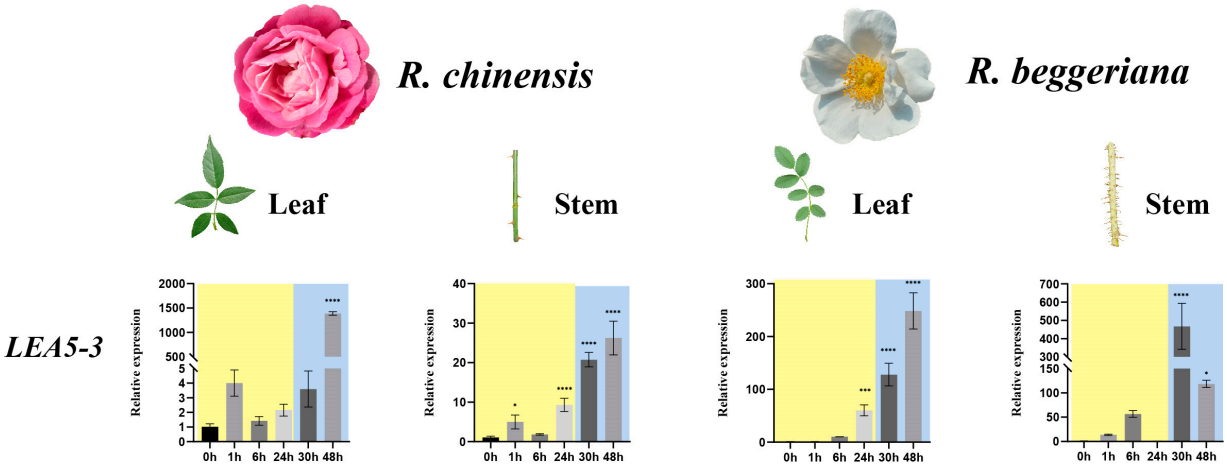


Figure 7. *Cont.*

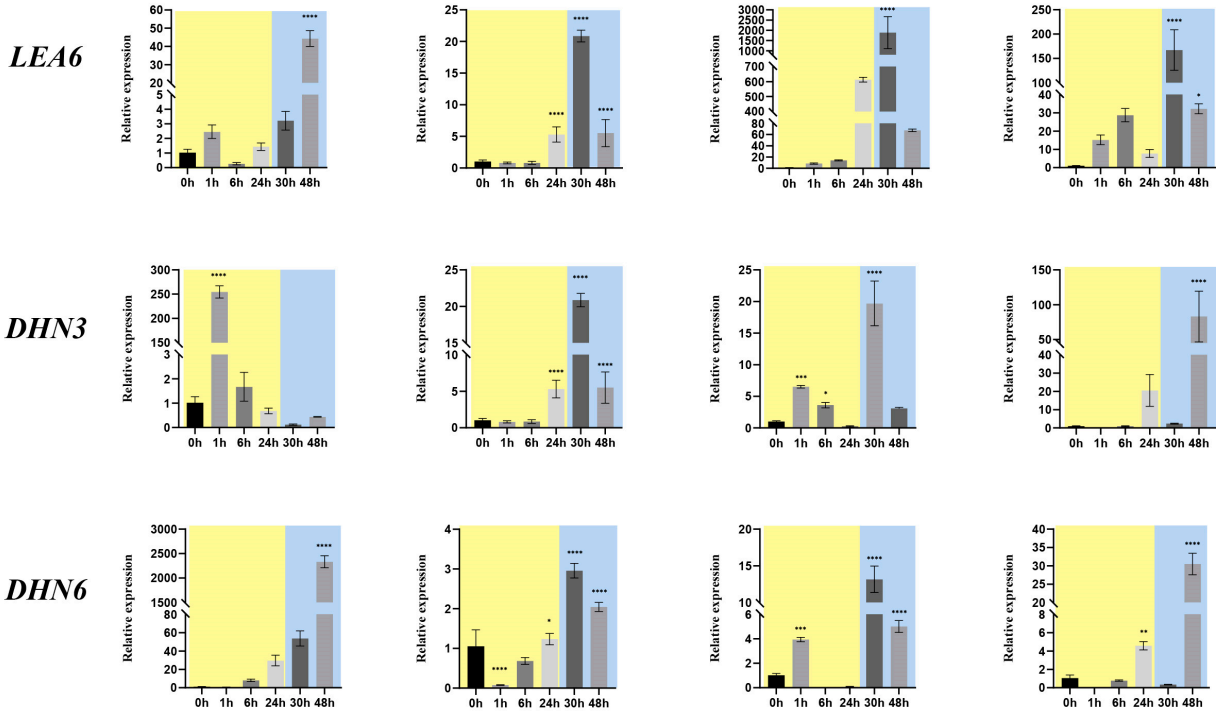


Figure 7. qRT-PCR profiling of *LEA* genes with elevated expression in *Rosa chinensis* 'Old Blush' and *Rosa beggeriana*. Standard deviations are shown as error bars, and statistical significance is indicated by asterisks when comparing the untreated control (0 h) with samples exposed to cold at 4 °C for 1, 6, and 24 h or −4 °C for 6 and 24 h (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$). Stages of 4 °C treatment are highlighted with a yellow background, whereas −4 °C treatment stages are marked with a blue background.

4. Discussion

4.1. Structural and Evolutionary Features of LEA Genes and Their Significance in Rose

The late embryogenesis abundant (LEA) gene family plays a central role in adaptation to abiotic stresses, especially in plants. LEA proteins are typically highly hydrophilic, glycine-rich, and contain intrinsically disordered regions, which enable them to stabilize proteins and membranes under dehydration and freezing conditions [47]. In *Arabidopsis thaliana*, genome-wide surveys revealed that *LEA* genes can be classified into multiple subfamilies, with LEA2 being the largest, and many members are strongly induced by abiotic stresses [48]. These comparative studies suggest that the expansion or contraction of specific LEA subfamilies reflects their central roles in stress adaptation.

In our study, we identified 23 *LEA* genes in *Rosa chinensis*, distributed across eight subfamilies, with DHNs forming the largest group. Rosa exhibits lineage-specific evolutionary features. Tandem duplications were detected in the *LEA2* and *LEA5* clusters, while whole-genome duplications contributed to the expansion of *SMP* and *DHN* genes. Such duplication events are likely the main drivers of functional redundancy and divergence. Although most *LEA* genes maintain conserved gene structures within subfamilies, *DHNs* display substantial structural variability, which may underlie their functional diversification. Compared with other Rosaceae species, *R. chinensis* 'Old Blush' has been domesticated as an ornamental plant in temperate climates. These contrasting evolutionary trajectories likely shaped the structural diversification and regulatory potential of their *LEA* gene families. In this study, all the *LEA* genes showed Ka/Ks < 1, indicating that this family has undergone strong purifying selection and maintains high functional conservation. Compared to *A. thaliana*, the number of *LEA* genes in *R. chinensis* 'Old Blush' is lower,

which may reflect the different whole-genome duplication histories among species and the differences in adaptation strategies for specific habitats.

In summary, the structural conservation of LEA proteins provides the biochemical foundation for stress protection, while lineage-specific duplication and divergence explain the unique features of Rosa. The predominance of *DHNs,* together with Rosa-specific duplication patterns, highlights the evolutionary innovations that may underlie the genus's adaptation to diverse environments.

4.2. Upstream Transcription Factors, Tissue-Specific Expression, and Divergent Cold Responses

The transcriptional regulation of *LEA* genes is tightly controlled by upstream transcription factors. Promoter analyses revealed abundant ABRE, MYB, MYC, and DRE/CRT elements, indicating regulation via both ABA-dependent and ABA-independent signaling pathways [49]. Transcription factors from the MYB, NAC, WRKY, and AP2/ERF families are key regulators orchestrating cold stress responses [50].

Promoter analysis of *RcLEA* genes revealed abundant cis-acting elements correlated with abiotic stress and hormone signaling. Elements such as ABRE, MYB, DRE core, and LTR were frequently detected, suggesting responsiveness to abscisic acid, drought, and cold [51,52]. In our study, 14 types of cis-acting elements implicated in stress were identified, including ABRE, DRE core, ARE, F-box, MBS, GARE-motif, GATA-motif, LTR, MYB, MYC, STRE, TGA-element, W-box, and WRE3. Notably, *RcLEA2-4*, *RcLEA3-3*, and *RcDHN6* harbored multiple ABRE and MYB motifs, consistent with their strong cold-induced expression [13]. The diversity of cis-acting elements in *DHN* promoters may contribute to their transcriptional flexibility, as also observed in other stress-inducible *LEA* genes [53]. Tandemly duplicated genes like *RcLEA2-1* to *RcLEA2-3* shared similar regulatory motifs, indicating conserved stress-responsive regulation. The cis-acting elements landscape supports the hypothesis that *RcLEA* genes are tightly regulated under cold stress, providing a molecular basis for their differential expression and potential roles in cold acclimation [54]. Some transcriptions have been proven to bind to the promotor of LEAs and enhance plants' abiotic tolerance in other species. *AmWRKY45* can combine with the promotor of *AmDHN4* and enhance the low-temperature and drought tolerance in *Ammopiptanthus mongolicus* [30]. The interaction between *ClWRKY61* and *ClLEA55* enhances salt tolerance in watermelon [55]. In *Anoectochilus roxburghii*, a plasma membrane complex formed by *ArWRKY57* and *ArWRKY70* directly regulates the transcription of *ArLEA5,* enhancing drought tolerance in this plant [56]. The *SAG21* promoter was targeted by 33 transcription factors belonging to nine families, notably *ERF*, *WRKY*, and *NAC* [57], the transcriptions we predicted may be involved in low-temperature tolerance in *Rosa chinensis* 'Old blush'. We also predicted the ABRE and DRE; there is early research about these two transcription factors. Analyses of the mutant aba1, which lacks sufficient ABA, and abi1, which is insensitive to ABA, revealed that the *RD29A* promoter, containing multiple DREs and one ABR, is primarily induced via the ABA-independent pathway, whereas the *RD29B* promoter, harboring several ABREs, is predominantly controlled by ABA [58–61]. These two types of transcriptions make great impact on plants' response to abiotic treatment. CRT/DRE binding factor is the CBFs binding site. Overexpression of *CBF1*, *CBF2*, and *CBF3* activated expression of the *COR6.6*, *COR15a*, *COR47*, and *COR78* genes [62–64].

Striking differences in tissue-specific expression were revealed by transcriptome data when comparing *Rosa chinensis* 'Old Blush' with *Rosa beggeriana*. For example, *RcLEA2-1* was highly expressed in stamens, whereas its homolog *RbLEA2-1* showed strong expression in stems and leaves, reflecting a marked shift in tissue specificity. *RcLEA3-3* was abundant in reproductive tissues and roots, while its homolog *RbLEA3-3* was enriched in flowers, indicating intra-organ divergence. *RcDHN3* was strongly expressed in roots

and reproductive tissues, but its homologs *RbDHN3-1* and *RbDHN3-2* were markedly expressed in stems and leaves, suggesting major tissue reallocation. In contrast, *RcDHN6* and its homolog *RbDHN6* both displayed very high expression across multiple tissues, representing a conserved high-expression pattern. qRT-PCR validation further confirmed that *R. beggeriana* activates *LEA* and *DHN* genes more strongly and persistently under cold stress. For example, *RbDHN6* expression increased more than 10-fold within six hours of cold treatment and remained elevated at 24 h, whereas *RcDHN6* induction was weaker and transient. *RbLEA3-3* and *RbLEA5-3* were robustly up-regulated in both leaves and stems, while *RcLEA3-3* and *RcLEA5-3* showed only moderate induction. These results indicate that *R. beggeriana* reallocates *LEA* and *DHN* expression to vegetative tissues under cold stress, which likely contributes to its superior freezing tolerance.

In other species, homologous genes of *RcLEAs* exhibit similar functions. *MsLEA60*, as the homolog of *RcLEA1-1*, responds significantly to drought, low temperature, and salt stress [65]. Under drought and 4 °C chilling treatment, its expression continuously increases, while, under salt stress, its expression first rises and then declines. In *Malus domestica*, the *RcLEA1-1* homolog *MdLEA60* enhances the resistance of transgenic tobacco to cold, drought, and saline-alkaline stress [66]. The *RcLEA6* homolog *BnaA.LEA6.a* is cold-responsive at low temperatures, and studies have shown that its expression in cold-acclimated *Brassica napus* is higher than in non-acclimated controls, with the difference being more pronounced under −4 °C treatment [67]. Meanwhile, *DHNs* have also been shown in other species to enhance plant tolerance to abiotic stresses. In *Hevea brasiliensis*, overexpression of *HbDHNs* in *Arabidopsis thaliana* significantly improved tolerance to salt, drought, and osmotic stress. The function of DHNs is not limited to cold, drought, and salinity [68]. For example, *OsDHN2* has been identified as a cadmium-responsive gene with the potential to enhance cadmium resistance in rice. This inspires further exploration of the diverse functions of *LEA* gene family members [69]. However, studies have also shown that homologous *RcLEA* genes may exhibit functional divergence in other species. In *Caragana korshinskii*, *CkLEA3-3* shows low expression under 4 °C treatment, and *CkDHN3* shows no significant expression [70]. Similarly, the *RcLEA3-3* homologs *CsLEA10* and *CsLEA7* in *Camellia sinensis* do not exhibit significant expression under cold stress [71]. These findings suggest that the functions of *LEA* gene family members are species-specific. Research on abiotic stress in Rosa extends beyond low-temperature stress. In molecular breeding for thermotolerance in *R. chinensis*, *RcTPS7b* functions as a signaling regulator that enhances heat adaptation by activating the heat shock signaling pathway (HSF/HSP) and maintaining redox homeostasis [72]. In *Rosa rugosa*, saline-alkali stress modulates the transcription factor *MYB5*, which affects the expression of downstream genes *ANR* and *TPS31*, thereby dynamically regulating the accumulation of proanthocyanidins and sesquiterpenes in rose petals and improving plant adaptation to salt stress. Therefore, *RcLEAs* may also play crucial roles in other types of abiotic stress [73].

In summary, these findings suggest that Rosa *LEA* regulation involves both conserved and divergent elements. These differences are likely mediated by variation in promoter architecture and transcription factor binding, particularly involving MYB, NAC, WRKY, and CBF families. Understanding these regulatory networks provides a foundation for breeding cold-tolerant roses by introgressing *R. beggeriana* alleles into cultivated backgrounds.

5. Conclusions

In *Rosa chinensis* 'Old Blush', 23 *LEA* genes were identified and classified into eight subfamilies, with chromosomal mapping revealing their distribution across six chromosomes and partial gene clustering. All RcLEA proteins contained conserved LEA motifs and exhibited simple gene structures with few introns. Promoter analysis indicated that

RcLEA genes harbor abundant cis-elements associated with abiotic stress and hormone responses. Comparative expression analysis and qRT-PCR validation showed that several *LEA* homologs, including *DHN3*, displayed strong cold-responsive expression patterns, suggesting their potential as candidate genes for future functional studies. This study also provides a genome-wide framework for understanding *LEA* gene organization and cold-responsive expression in roses. Future work involving transgenic or gene-editing approaches will be necessary to clarify the specific contributions of LEA genes to cold tolerance and to evaluate their potential utility in breeding cold-resilient rose cultivars.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/horticulturae12020136/s1, Table S1: Identification and characterization of *LEAs* in *R. chinensis* 'Old Blush'; Table S2: DNA sequences of 23 *RcLEA* genes; Table S3: Protein sequences of 74 LEA proteins from two species; Figure S1: Motif analysis of *RcLEAs*; Figure S2: Conserved analysis of *RcLEAs*; Table S4: Ka/Ks ratios of *RcLEA* genes; Table S5: Cis-acting elements in the promoters of 23 *RcLEA* genes; Table S6: RNA-seq data of *RcLEA* genes in different tissues (PRJNA546486); Table S7: RNA-seq data of *RbLEA* genes in different tissue; Table S8: RNA-seq data of *RbLEA* genes in leaves and stems under cold treatment; Table S9: Primers used in this study.

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Abbreviations

The following abbreviations are used in this manuscript:

LEA	Late Embryogenesis Abundant proteins
ABA	Abscisic Acid
DHN	Dehydrin
SMP	Seed maturation proteins
NJ	Neighbor-Joining
FPKM	Fragments Per Kilobase of transcript per Million mapped reads
CDS	Coding Sequence
UTR	Untranslated Region
ABRE	ABA-Responsive Element
ARE	AU-Rich Element
DRE	Dehydration-Responsive Element/Drought-Responsive Element
LTR	Low Temperature Responsive element

MBS	MYB Binding Site
MYB	Myeloblastosis oncogene
MYC	Myelocytomatosis oncogene
STRE	Stress-Responsive Element
WRE3	Wound-Responsive Element 3
RNA-seq	RNA Sequencing
qRT-PCR	Quantitative Real-Time Polymerase Chain Reaction
DEGs	Differentially Expressed Genes

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