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MOLECULAR CLONING, CHARACTERIZATION AND EXPRESSION ANALYSIS OF TWO *LEA* GENES IN CHRYSANTHEMUM

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Abstract

Late embryogenesis abundant protein (LEA) has been demonstrated to play essential roles in plant growth, development, and also in a variety of environmental stress responses. In this study, 2 LEA gene family members were cloned and named CmLEA2-2 and CmLEA6-5, then computationally examined to explore its physical and chemical characteristics, evolutionary links, and expression patterns in different tissues and under low-temperature stress. Phylogenetic analysis showed that *CmLEA2-2* and *TcLEA14* are closely related, while *CmLEA6-5* and *PfLEA* is closely related. The expression of the *CmLEA* gene in distinct tissues of chrysanthemum is tissue specific: the expression of *CmLEA2-2* gene was highest in leaves, second in stems, and lower in roots and flowers; the *CmLEA6-5* gene was highest expressed in stem, but not in other tissues. The expression study of the 2 discovered LEA family members under low-temperature stress revealed that low-temperature stress influenced the transcription of the chrysanthemum LEA gene. The expression of them increased, but there were variances in response time and intensity. They're supposed to help the chrysanthemum cope with stress and protect its cells. This could be owing to the tiny molecular weight and strong hydrophilic properties of the CmLEA protein.

Key words: *Chrysanthemum*; *LEA* gene; Cryogenic stress; Gene expression.

Introduction

Late embryogenesis abundant protein (LEA) was identified in the cotyledons of late stage cotton germ development (Dure *et al*., 1981), and it has since been reported in various plant nutritional tissues, microbes, and invertebrates (Hand *et al*., 2010). The *LEA* gene was shown to be expressed in a variety of plant tissues, including seeds, seedlings, roots, stems, leaves, flowers, and so on (Shao *et al*., 2005). Furthermore, the LEA protein is widely distributed in cells. The LEA protein is found not only in the cytoplasm and plasma membrane but also in the nucleus, endoplasmic reticulum, mitochondria, chloroplasts, peroxysomes, and other organelles associated with its unique mechanism of action (Cantata *et al*., 2014). The LEA protein is abundant in plants and has many members. According to its amino acid sequence, homology, and conserved features, it is classified into 8 catagories: LEA1, LEA2, LEA3, LEA4, LEA5, LEA6, Dehydrin, and seed maturation protein (SMP) (Dure, 1993; El-Gebali *et al*., 2019). Early research on LEA proteins assumed that such proteins remained disordered under climates of sufficient humidity and that when cells lost water under stress, a large number of LEA proteins began to be increased and highly helically folded, thereby retaining cell stability and performing normal functions (Wise & Tunnacliffe, 2004). At the same time, some LEA proteins are associated with the stability of anionic phoshpholipid vesicles, which aids in the stability of the membrane structure in a dry state or at low temperatures (Kosová & Rášil, 2007). Plants have a high tolerance to diverse abiotic stressors (Olvera-Carrillo & Reyes, 2011; Wang *et al*., 2020). These proteins are activated by stress and act as cytoprotective agents. The mode of action of the LEA protein in plant stress response is currently a hot research issue (Wang *et al*., 2003; Gao & Lan, 2016). The LEA gene family has been identified and analysed at the genome-wide level in several plant DNA sequencing species, including *Arabidopsis,* rice (Wang *et al*., 2007), corn (Li & Cao, 2016), potato (Chen *et al*., 2019), tomato (Cao & Li, 2015), rapeseed (Liang *et al*., 2016), cassava (Wu *et al*., 2018), populus (Lan *et al*., 2013), pine oil (Kosová & Rášil, 2007), peanut (Huang *et al*., 2022) and rye (Ding *et al*., 2021) and others. The statistical study of the *LEA* gene in chrysanthemum, as well as the research on the response to cryogenic stress treatment, have not yet been published. Chrysanthemum (*Chrysanthemum morifolium*) is one of China's top 10 traditional renowned florals and one of the world's four major peonies. There is a lot of economic need, but the chrysanthemum is subject to various biological and agronomic stresses during its growth and development, which impairs its aesthetic and economic worth, stifling the industry's healthy and sustainable development (An *et al*., 2014). For the specimens in this investigation, the sliced chrysanthemum typology was chosen. Members of the chrysanthemum LEA gene family were tested from the research group's existing transcriptome database, bioinformatics analysis was performed, and RT-qPCR (Real Time Quantitative Reverse Transcription) technology was used to study the transcriptional activity of the *CmLEA* gene in response to low temperature, laying the groundwork for further physiological identification of the chrysanthemum LEA gene, particularly due to cryogenic temperature stress caused by chrysanthemum growth being hampered and quality degradation.

Material and methods

Plant materials, growing parameters, experimental treatment, and sampling: The experimental material employed here was chrysanthemum cultivar “Jinba”, and the cuttings were obtained from our laboratory. Cuttings with vigorous, regular growth were selected and put them into plastic containers after rooted. The medium is vermiculite, perlite and nutrient soil, mixed in volume ratio of 1:1:1. The plants were placed in a culture room with the growing environment as follows: temperature 22°C, light intensity 100 mol · $m^{-2}·s^{-1}$, with light/dark time of 16 h/8 h. Plants with 6 to 8 leaf ages and regular growth were chosen as test materials. Both the experimental group with the low-temperature treatment and the control group were grown in a light incubator with a photoperiod of 16 h/8 h and a light intensity of 50 mol m^{-2} s^{-1}. The low-temperature experiment group's temperature (LT) was set to 4°C , whereas the control group's (CK) temperature was set at 22°C (Song *et al*., 2014). The sample times are 0, 1, 2, 4, 8, 12, and 24 hours after processing, with three biological replications for each processing and time point. From top to bottom, the sample position is the third completely developed leaf (Xia *et al*., 2014). After sampling, it was immediately frozen in liquid nitrogen before being transferred to a -80°C refrigerator for storage.

RNA extraction from chrysanthemum samples and identification of the whole transcriptosome of the chrysanthemum LEA gene family: The protocols for the fast universal plant RNA extraction Kit 3.0 (Huayueyang biotechnology Co., Ltd, Beijing, China) was used to extract RNA from plant samples. The package includes a DNase for digesting genome. cDNA was generated using RNA as a template and the reverse transcription kit's instructions (Huayueyang biotechnology Co., Ltd, Beijing, China). The sequence of unigenes in the LEA family was evaluated using the research group's pre-transcriptome sequencing results as a database. Download the homologous sequence of CmLEA protein from NCBI and used as query to search for putative LEA protein sequences by using TBtools (Chen *et al*., 2020). After discarding the redundant sequences, the candidate LEA protein sequences were obtained by run blast against SMART, Pfam and NCBI Batch CD-Search. The ProtParam online tool was used for caculating the biochemical properties of two CmLEA protein sequences. The subcellular localization predictions of these proteins were performed using Wolf Psort.

Cloning of *CmLEA* genes: According to manufactures instructions for RNA Extraction Kit, total RNA extraction was successfully performed. The cDNA was subsequently produced according to the SuperScript cDNA Synthesis Kit (WX2050). The above RNA Extraction Kit and cDNA Synthesis Kit were all from Huayueyang Biotech, Beijing, China. The full-length amplification primers were designed by retrieving the sequences of the chrysanthemum *LEA* genes from the previous transcriptome data (Table 1). The reaction system of PCR was set according to manufactures instructions (Yeasen Biotechnology Co., Ltd., Shanghai, China) as follows: 2 μL cDNA, 2.5 μL of each primers (10 $\mu mol·L^{-1}$), 25 μL of 2×Canace® PCR buffer (with Mg^{2+} and dNTPs), 0.5 μL of Hieff Canace® High-Fidelity DNA Polymerase (2U/μL), ddH_2O was used to make up for the final volume as 50 μL. The PCR reaction program was set as follows: first, pre-denaturation at 98°C for 3 min; second, a 35 cycles of denaturation at 98°C for the 10 sec, annealing at 60°C for 20 s, and extension at 72°C for 30 sec; last, a final extension at 72°C for 5 min. After that, DNA amplicon were collected using a kit from Tiangen Biotech Co., Ltd. (Beijing, China) and then a kit from the same company was used to add A tail to the target segment. Then the product was connected with the vector pMD19-T (Takara Co, Ltd. Japan) and picked for sequencing.

Bioinformatic analysis of the two *CmLEA* genes: Online software ProtScale (https://web.expasy.org/protscale/) was utilized to analysis the physicochemical and hydrophilic properties of CmLEAs. Protein 3D structures were predicted through the ColabFold notebook, which uses AlphaFold2 to predict the structure and MMseqs2 to quickly create an alignment of multiple sequences, while the optimal protein 3D structure model in PDB format was submitted to PyMOL to generate the final image(Martinez *et al*., 2019). The CLUSALX was used to perform multi-sequence alignment of two CmLEAs and other LEAs, and the Neighbor-joining technique was utilised in MEGA7.0 software to construct an evolutionary history with a Bootstrap detection value of 1000.

Detection and analysis of the two *CmLEA* genes using qRT-PCR: The CmEF1α-F and CmEF1α-R primers were developed using the registered chrysanthemum gene CmEF1α as a reference gene. Prime Premier 5.0 was used to build primers based on the *CmLEA* gene sequence.The sequences of all primers were presented in Table 1. The CFX96 real-time fluorescence quantitative PCR apparatus (BIO-RAD) was used here. A 2×SYBR Green qPCR Mix (Sparkjade, Nanjing, China) was used for the reaction and the procedure as follow: 94°C 3min; 94°C 20s; 55°C 20s, 72°C 30s, 40 cycles. Three replicates for each sample were made to minimize the error. The relative expression of genes was determined according to the $2^{-\Delta\Delta CT}$ formula (Livak & Schmittgen, 2001).

Table 1. Sequences of primers used in this investigation.

Gene	Primer F (5’-3’)	Primer R (5’-3’)	Usage
CmLEA2-2	ATGGCTGGAATGCTTGAC	TTTTCACTTCCACACGTCGGAGAGT	Amplification of full- length primer
CmLEA6-5	ATGGCAGATCACCAAAGAATCCACC	TAACTTAAACTTGAAACTACAATCA	Amplification of full- length primer
CmLEA2-2	GGTTAAGGACATTGCTCG	TTGATCTCACCTTTGCTGT	qRT-PCR primer
CmLEA6-5	AGTACTTAGAGCTAAAGTTCCTGT	ATTCTAAGCGAACCAGCA	qRT-PCR primer
CmEF1α	TTTTGGTATCTGGTCCTGGAG	CCATTCAAGCGACAGACTCA	reference gene primer

Results and analysis

Identification and evolutionary study of the *CmLEA* genes: In this study, we successfully cloned two *CmLEA* genes, *CmLEA2-2* and *CmLEA6-5*. The PCR products of *CmLEA* genes were presented in Fig. 1. The *CmLEA2-2* gene encodes a protein with 153 amino acids. Its theoretical isoelectric point (PI) is 4.83, and its molecular weight is 16328.87 Da. It has an average hydrophilic coefficient of 0.042, implying a hydrophilic protein (Fig. 2A). *CmLEA6-5* gene encodes 251 amino acids, with a molecular weight of 27760.26 Da and an average hydrophilic coefficient of 0.274 which indicating a hydrophilic protein (Fig. 2B). Protein 3D structures of the two genes were predicted through the ColabFold notebook, and the results are present in Fig. 3. The yellow parts in the figure represent the typical domain of the group 2 of LEA protein. For CmLEA2-2, the domain harbours 96 amino acids from the 44th to 140th; the domain of CmLEA6-5 consists of 105 amino acids from 124th to 229th. The sequence analysis of two CmLEAs shows the evolutionary relationship between CmLEA family members and homologous proteins of other species (Fig. 4.). According to the graphical evolutionary relationship, CmLEA2-2 and TcLEA14 are closely related, while CmLEA6-5 and PfLEA are closely related, as shown in Fig. 4. Subcellular localization prediction showed CmLEA2-2 and CmLEA6-5 proteins were localized in the cytoplasm.

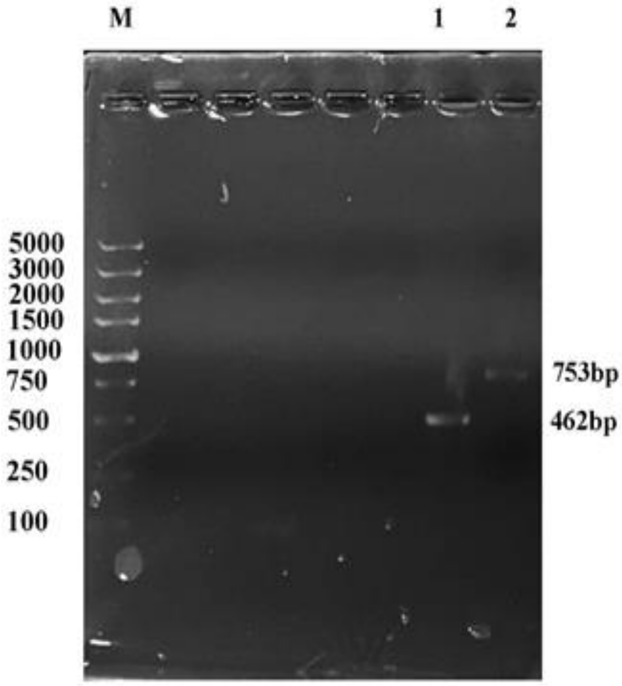

Fig. 1. The PCR amplicon of *CmLEA*.
(M: Marker 5000; 1: the PCR product of CmLEA2-2; 2: the PCR product of CmLEA6-5)

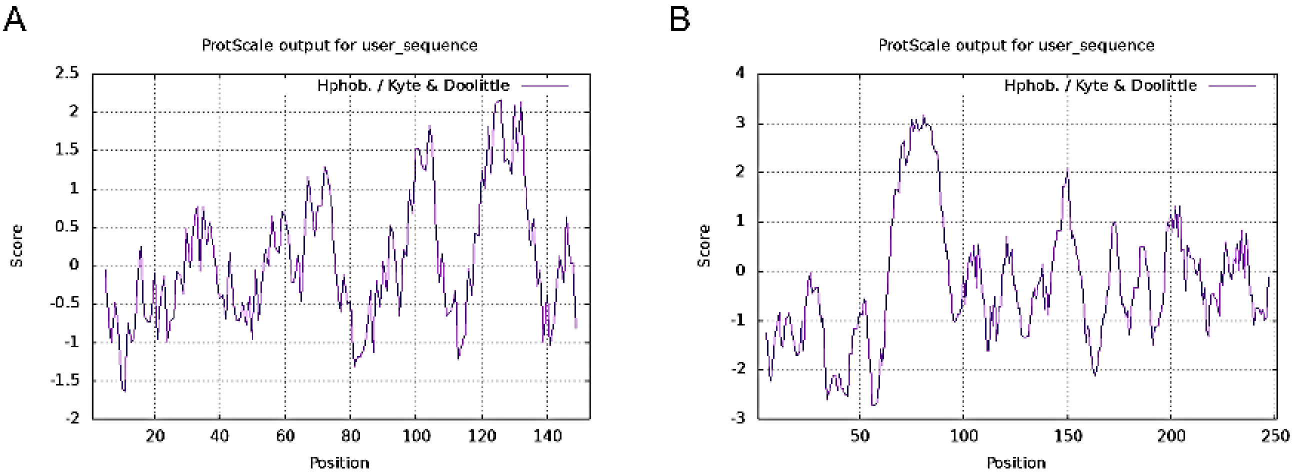

Fig. 2. The hydrophilicity features of CmLEA2-2 (A) and CmLEA6-5 (B).

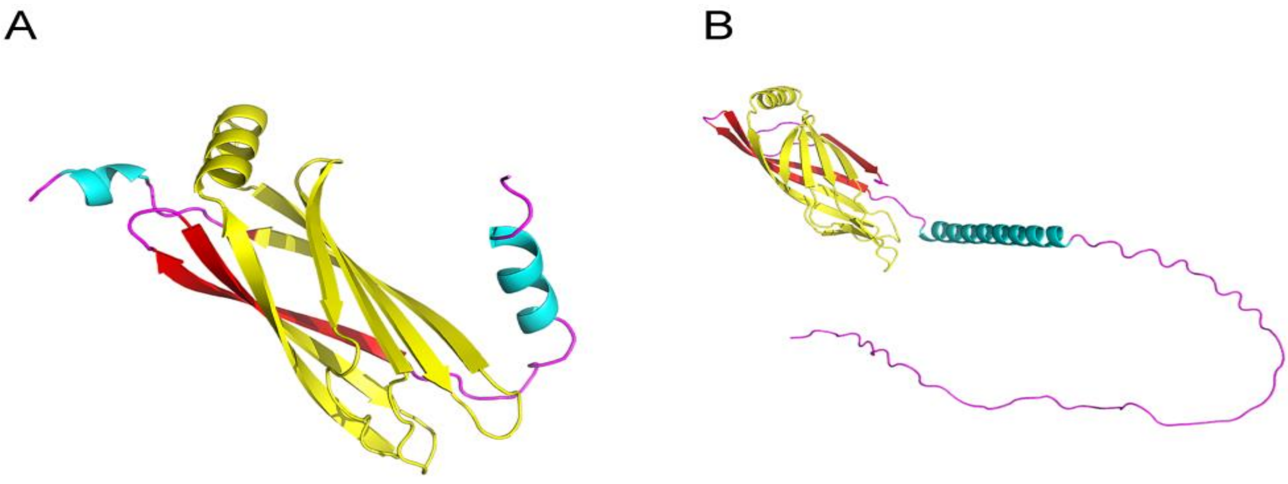

Fig. 3. The tertiary structure prediction of CmLEA2-2 (A) and CmLEA6-5 (B) proteins.

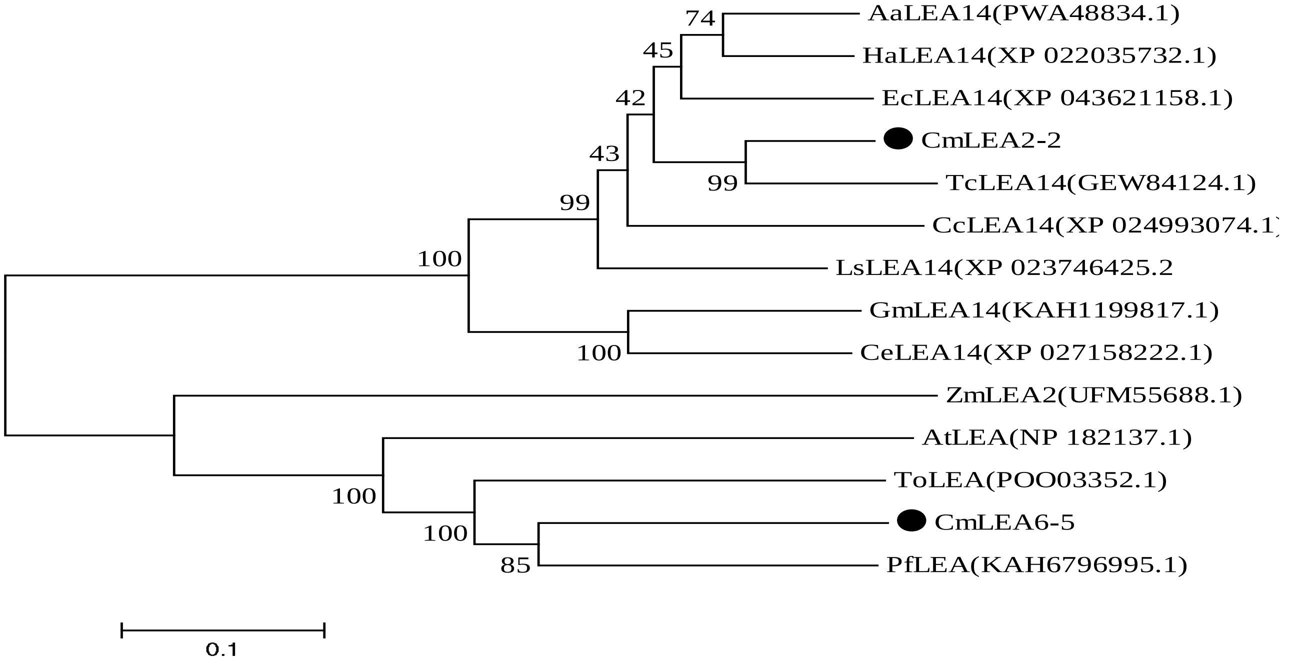

Fig. 4. Phylogenetic analysis of the CmLEA2-2, CmLEA6-5 and other LEAs.
(Aa: *Artemisia annua*; At: *Arabidopsis thaliana*; Cc: Cynara cardunculus var. scolymus; Ce: Coffea eugenioides; Cm: *Chrysanthemum morifolium*; Ec: Erigeron canadensis;
Gm: Glycine max; Ha: Helianthus annuus; Pf: Perilla frutescens var. hirtella; Tc: Tanacetum cinerariifolium; To: Trema orientale; Zm: Zea mays.)

Expression of the *CmLEA* genes in various tissues: The expression of the *CmLEA* gene in roots, stems, leaves, and flowers was examined (Fig. 5). The expression of the *CmLEA* gene in distinct tissues of chrysanthemum is tissue specific: the expression of *CmLEA2-2* gene was highest in leaves, second in stems, and lower in roots and flowers; the *CmLEA6-5* gene was highest expressed in stem, but not in other tissues. It can be seen that the *CmLEA* gene is engaged in the growth and development of numerous chrysanthemum tissues.

Expression analysis of the *CmLEA* genes under low temperature stress: Fig. 6 shows the change of expression of two *CmLEA* genes under 4℃ treatment. It can be seen from the figure that both genes respond to low temperature stress treatment, but the response trend is slightly different: the expression trend of the two genes is first increased, then decreased, and then increased. The turning point of *CmLEA2-2* gene expression trend was 12h, that is, the expression level showed an upward trend before 12h, the expression level fell below 4h at 12h, and the expression level increased significantly in 24h; The expression of *CmLEA6-5* gene began at 2h after low temperature treatment, decreased at 4h, and gradually increased at 8h. It can be seen from the figure that *CmLEA2-2* gene ratio *CmLEA6-5* gene responds to low temperature stress earlier, and the overall relative expression was higher.

Discussion

Plant LEA protein has protective activities. It is essential for plant embryonic development and responds to biotic stress (Zhang & Zhang, 2017). With the availability of the entire genome sequences of *Arabidopsis* (Initiative & Copenhaver, 2000) and wheat (Walkowaik *et al*., 2020), corn (Hufford *et al*., 2021), rice (Goff, 2005), and other crops, the *LEA* gene has been found in an increasing number of species. It was not, however, reported in chrysanthemum, nor has it been reported on chrysanthemum cold resistance. Here, 2 *CmLEA* family members were identified by using bioinformatic methods and the expression in tissues and exposing to low temperature were analyzed. It’s found that the members of the *LEA* gene family are widely distributed in multiple organelles of plants through the study on the subcellular localization of each member of the *Arabidopsis*, which may highlight the functional mechanism of LEA protein to protect each cell partition against drought or cold stress (Adrien *et al*., 2014). Through subcellular localization prediction, the two cloned CmLEA proteins were localized in cytoplasm, which is comparable to the distribution of the LEA gene family in *Arabidopsis*.

According to the reported research, the accumulation of LEA3 protein in wheat and rye chloroplasts can greatly increase plant cold tolerance (Ndong, 2002). Over-expression of ectopic wheat *TaLEA3* in yeast could increase its capacity to osmotic stress, salty and cold stress (Yu *et al*., 2010). The tea tree *CsLEA1* gene was overexpressed in *E. coli* and yeast, and both showed increased cold tolerance (Gao *et al*., 2021). *PcLEA14*, a unique 5C late embryogenesis abundant (LEA) protein group gene in *Pyrus communis*, was proved to exert its role in resisting cold stress because of its induced expression under low temperature, expression pattern with seasonal fluctuation, and clarification of low temperature tolerance in transgenic *Arabidopsis* with over-expressed *PcLEA14* gene (Shibuya *et al*., 2020). In this study, the expression profile of the two cloned *CmLEA* genes have shown that they all respond to stress treatment at low temperatures. However, there are variances in responsive time and intensity. *CmLEA2-2* and *CmLEA6-5* show high expression during low-temperature stress. It is hypothesised that the two *CmLEA* genes are both involved and play a function in the process of chrysanthemum reacting to low-temperature stress.

High hydrophilicity of most LEA proteins might be due to their varied amino acid composition (Eriksson *et al*., 2011); this feature contributes to the protection against from the detrimental effects of many harsh conditions, including drought and cold (Paul *et al*., 2014). Furthermore, earlier research has demonstrated that molecular weight (MW, 10-30 kD) constitutes an essential feature of LEA protein in higher plant in cell protection (Wang *et al*., 2019). Our findings suggest that both CmLEA2-2 and CmLEA6-5 have higher MW and are hydrophilic. As a result, it is fair to propose that CmLEA has a cell-protective function. Under low-temperature stress, it may be associated to lower MW and higher hydrophilic features. In this study, the *LEA* genes linked to low-temperature resistance in *Chrysanthemum* were screened, which provided a basis for further studying their functions and using them to improve the resistance and quality of *Chrysanthemum*.

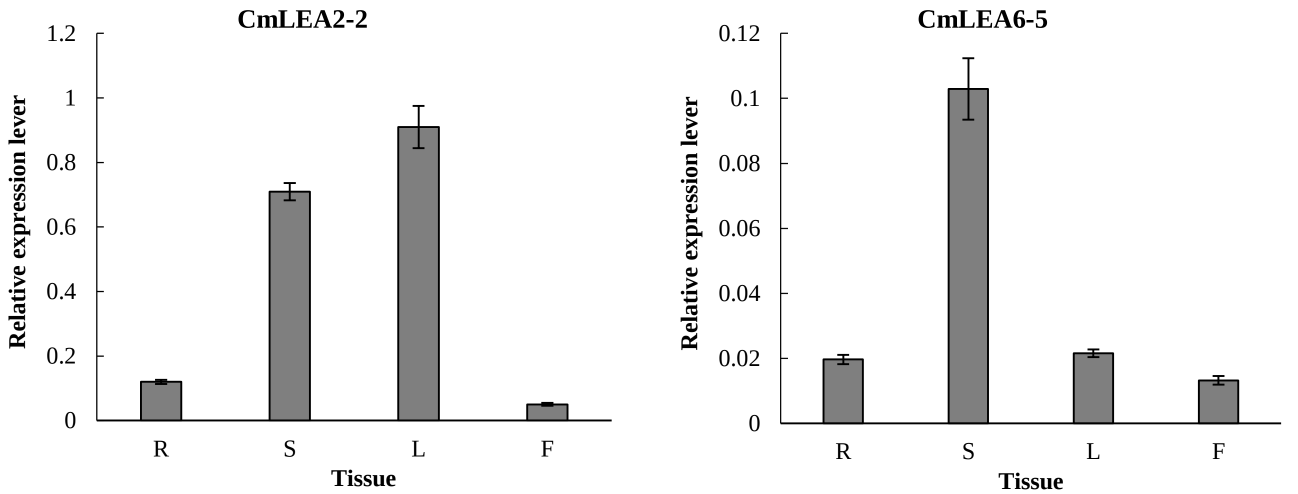

Fig. 5. The expression level of *CmLEA* in different tissues of chrysanthemum.

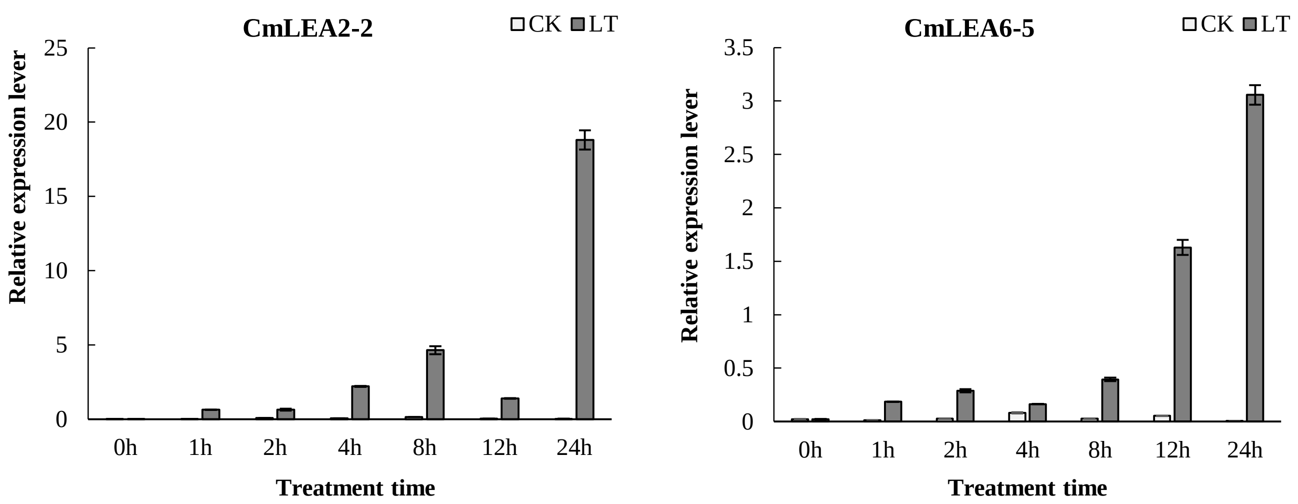

Fig. 6. Changes in the relative expression level of *CmLEA* genes under low-temperature treatment.

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