荧光定量PCR检测干旱胁迫下长春花*Crlea*基因的相对表达

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摘 要 首次从长春花中克隆了*Crlea*(*Crlea* for *Catharanthus roseus* late embryogenesis abundant)的全长基因,采用荧光定量PCR方法对干旱胁迫下长春花叶片和根部*Crlea*基因的表达模式进行监测,结果表明,在0.5~8 h的胁迫时间中,叶片和根部的*Crlea*基因表现出相似的积累模式。长春花*Crlea*基因的表达随着胁迫时间的延长而表达增强。在叶片中,在6 h和8 h的干旱处理后,*Crlea*基因表达显著提高,分别是未处理材料的9.984和20.431倍。在根部,在8 h的处理后,*Crlea*基因的表达量显著提高(2.831倍于对照)。初步结果表明*Crlea*基因的表达没有组织特异性,并且为干旱胁迫正调控。

关键词 长春花;干旱胁迫;晚期胚胎丰富蛋白;荧光定量;相对表达

Comparative expression analysis of *Crlea* gene in *Catharanthus roseus* under drought stress by real-time quantitative PCR

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Abstract A full-length *Crlea*(*Crlea* for *Catharanthus roseus* late embryogenesis abundant) gene was first isolated from *Catharanthus roseus*. Gene expression profiles of *Crlea* gene in leaves and roots under drought stress were monitored by real-time quantitative PCR. The results showed that a similar accumulation pattern of *Crlea* gene in leaves and roots over the observation period of 0.5 to 8 hours. The expression of *Crlea* mRNA was strengthened with the prolongation of stress time. In leaves, expression amounts of *Crlea* gene were 9.984 and 20.431 times higher than that of control respectively at 6 and 8 h. Similarly, the expression amounts of *Crlea* gene in root obviously increased (2.831 times higher than that of control) at 8 h. Primary results show the expression of *Crlea* gene is non-tissue-specific and up-regulated under drought stress.

Key words *Catharanthus roseus*;drought stress;*lea* gene;real time quantitative PCR;comparative expression

Drought stress is one of the most serious menaces in abiotic stresses, which inhibits the growth of plant, makes the ecology harmfulness and reduces the yield of crops[1,2]. Plant undergoes various physiological and biochemical changes under drought stress, including alterations in gene expression[3]. Functional studies of drought-responded genes and mechanisms using molecular biological techniques will be helpful for people to breed stress tolerance plants to resist dehydration stress by the means of gene manipulation.

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LEA proteins are one of the important proteins in stress response which have been described as having a potential role in drought tolerance[4]. LEA proteins were first found to accumulate to high levels in cotton embryos during the maturation phase of seed development. For their unusual structural characteristics, such as extremely hydrophilicity, LEA proteins were predicted to protect cells from drought stress[5].

Until now, more and more LEA proteins have been isolated from different species, not only in plants such as *Arabidopsis thaliana*[6], wheat[7], rice[8], tomato[9], soybean[10], etc, but also in *E. coli*[11], yeast[12] and anhydrobiotic nematode[13]. These LEA proteins have the general features: high hydrophilicity and heat-stability, advanced structures composed by amphiphilic α- helices or coiled coils and so on. Further studies show that lea mRNAs or proteins could be induced by drought, osmotic stress, cold or salt stress[3,7], it can also express in vegetative tissues of plants exposed to stress. Yeast function assay[5] and transgenic plants[14] with *lea* genes exhibited an improved tolerance to water stress and other stresses. However, the evidence of physiological function of LEA proteins in plant is scarce. Grelet J identified a LEA protein of group 3 (PsLEAm) which was localized within the matrix space of pea (*Pisum sativum*) seed mitochondria. The recombinant PsLEAm was shown to protect two mitochondrial matrix enzymes, fumarase and rhodanese, during drying in vitro assay. This is the first experimental evidence for a beneficial role of a LEA protein with protection to proteins during desiccation[15]. Nevertheless, more functional studies on LEA protein should be further carried out.

We have established a cDNA library of leaves in *Catharanthus roseus* under drought stress. Through random sequencing and screening, a full-length *Crlea* (*C. roseus* late embryogenesis abundant) gene in *C. roseus* has been first cloned. To further investigate the characteristics and the expression pattern of *Crlea* gene responding to drought stress, real-time quantified PCR with SYBR Green Ⅰ was performed to analyze the expression changes in leaves and roots of *C. roseus* at different stress levels. The expression patterns of *Crlea* gene in different tissues are elucidated.

1 Materials and methods

1.1 Plant materials

Seeds of *C. roseus* were sown and cultivated in growth chamber with a substrate of perlite with a daytime temperature of 25℃, 70% humidity and 16 h photoperiod (light intensity of 12 870 lx), watering everyday to provide healthy growth. One-month-old seedlings were treated by drying on paper towel for 0.5, 1, 2, 3, 4, 6, 8 h, respectively, and then stressed tissues (leaves and roots) were harvested, immediately frozen in liquid nitrogen, and stored at −80℃.

1.2 RNA extraction, quantification and reverse transcription

Total RNA was extracted from 200 mg samples (both leaves and roots) by TRIZOL reagent (Invitrogen), and the concentration of RNAs was assessed by GeneQuantII (Amersham). The structural integrity of the RNAs (1 μg of each sample) was detected with non-denaturing agarose gel containing ethidium bromide. RNAs (1 μg) were reverse-transcribed using random primers and the reaction mixture containing 4 μL 5 × buffer, 2 μL 10 mmol · L^{-1} NTP, 50 μmol · L^{-1} random primer, 20 U RNase inhibitor and 10 U AMV was incubated for 10 minutes at room temperature, and then incubated at 42℃ for 1 hour. cDNA was cooled in ice-water for 2 minutes then stored at −20℃ until further used.

1.3 Real-time quantitative PCR

Real-time quantitative PCR was performed by chromo 4 (MJ Research Company), the results were analyzed using an Opticon version 2.03.5 (MJ) software. 18 S rRNA of tobacco used as inner control was amplified in parallel with the target gene allowing gene normalization and providing quantification. Detections of real time PCR products were carried out by the SYBR Green I fluorescence dye. A 20 μL PCR amplification mixture contained 2 μL 10 × buffer, 0.5 μL of 25 mmol · L^{-1} $MgCl_2$, 2 μL dNTP, 0.5 μL SYBR Green I (10,000-fold dilution), 0.2 μL rTaq, 0.5 μL of each 10 μmol · L^{-1} PF and PR primers, 0.5 μL cDNA (the equivalent of 25 ng total RNA) as template and DEPC-treated water. PCR was per-

formed at 94℃ for 5 min, then at 94℃ for 10 s, 55℃ for 30 s and 72 for 40 s, 80℃ reading plate for 1 s for 40 cycles, and remained at 72℃ for 2 min. For each sample, reactions were repeated three times to ensure the reproducibility of the results. After PCR reaction, a melting curve was obtained by opticon version 2.03.5 and parameters were set as reading plate for 1 s every increasing 0.5 ℃ in the range from 70℃ to 95℃.

1.4 Primers design and data treatments

Primers were designed based on the sequence of cloned full-length cDNA of *Crlea* gene using primer version 5.0, and the primer sequences were listed in Table 1.

Table 1　Primer sequences

Gene name	Primer pairs	Primer sequences (5′−3′)
Crlea	*Crlea*F	TCCAAAATGGCATCCCACG
Crlea	*Crlea*R	AGTTATACGCTTCCTCCCCG

The comparative C_T ($\triangle\triangle C_T$) method was used to analyze the relative transcript levels of *Crlea* gene for different drought-stress time. Firstly, optimal C_T was determined by subtracting the background fluorescence of SYBR Green I, and the threshold of the triplicate PCRs including *Crlea* gene and inner control 18S rRNA were averaged and used for quantification of transcripts. Secondly, $\triangle C_T$ value was determined by subtracting the corresponding average 18S rRNA C_T value from the average *Crlea* gene C_T value. Lastly, $\triangle\triangle C_T$ value was determined by subtracting $\triangle C_T$ control (untreated samples) from $\triangle C_T$ of each treated sample. The $2^{-\triangle\triangle CT}$ value was given to estimate the relative expression of drought stress materials according to the control [1,16].

2 Results and analysis

2.1 Optimization of PCR condition and amplification of special products

SYBR Green I can be used to quantify the copies of template in the reaction system with greatly enhancing fluorescence once incorporating into dsDNA. However, it binds to any dsDNA, and can not distinguish specific and nonspecific dsDNA PCR products and primer dimers. To avoid these factors, primer concentration was decreased to 0.25 μmol · L^{-1} and plate-reading temperature was increased to 80℃, which can eliminate the possibility of dimers forming and acquire correct fluorescence value corresponding to SYBR Green I. Melting curves showed Tm (melting temperature) values of *Crlea* gene and 18 S rRNA were 87 and 82.5℃, respectively (Fig. 1), which also exhibited a single peak, representing a specific product. Dissociation curves also indicated the Tm value of non-specific products was 76℃ (Fig. 2). Setting plate-reading temperature at 80℃ can eliminate the fluorescence of non-specific products effectively through denaturing the dsDNA to ssDNA products.

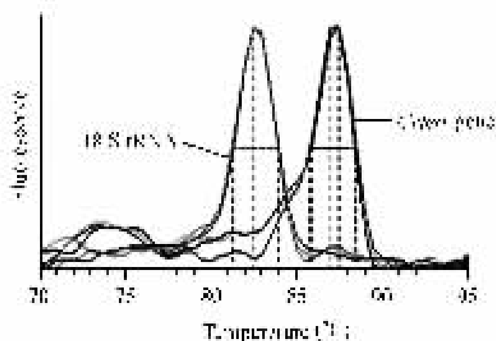

Fig. 1　Dissociation curves of *Crlea* gene and 18S rRNA

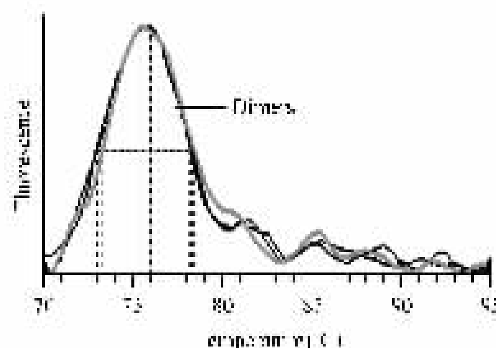

Fig. 2　Dissociation curve of nonspecific products

2.2 Relative expression analysis of sample at different time of drought stress

From the amplification curves of *Crlea* gene, we

can see that C_T value of *Crlea* gene change obviously in the same threshold value compared with 18 S rRNA (Fig. 3), which represents the different initial amount of templates. The number of copies of *Crlea* gene, $\triangle C_T$ and $\triangle\triangle C_T$, and the relative number of copies for *Crlea* gene compared to the untreated mRNA of that gene were determined by average C_T value (Table 2,3). The ranges of copy numbers were obtained from $\triangle\triangle C_T$ and its standard deviations. From Fig. 4 and Fig. 5, we can see the expression pattern of *Crlea* gene from leaves and roots under drought stress is similar. With the increase of stress time, all mRNA levels of *Crlea* gene presented an increasing trend. In leaves, expression of *Crlea* gene showed an obviously increase at the treatments of 6 and 8 hour, which were separate 9.984 and 20.431 folds higher than those of the control. In roots, the expression exhibited an obviously increase after a treatment of 8 hour, which was 2.831 fold higher than that of the control. These results indicate the *Crlea* gene play a very important role in the molecular response to drought stress in *C. roseus*.

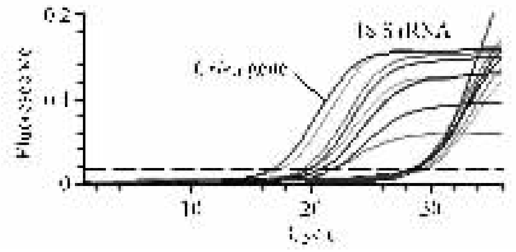

Fig. 3 The amplification curves for quantifying *Crlea* gene and 18 S rRNA gene

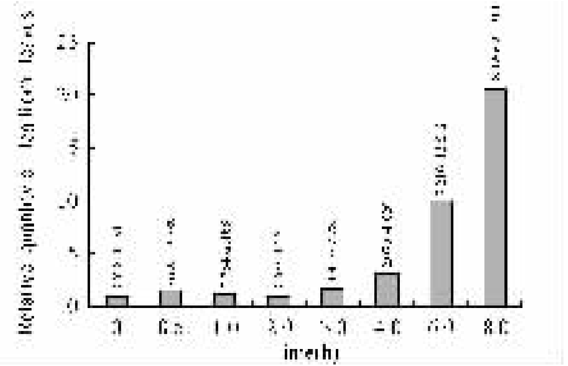

Fig. 4 Bar graphs show the relative gene expression of *Crlea* gene to the corresponding mRNA level of control *C. roseus* Leaves with range, after normalization with 18 S rRNA internal standard

Table 2 Comparative quantification of *Crlea* gene from *C. roseus* leaves using comparative C_T method

Time	*Crlea* gene Average C_T	18 S Average C_T	$\triangle C_T$ *Crlea*-18 S	$\triangle\triangle C_T$ $\triangle C_T - \triangle C_{T\ control}$	*Crlea* gene (Relation to control with range)
0	19.82 +/ −0.21	28.63 +/ −0.46	−8.80 +/ −0.67	0 +/ −0.67	1.000 (0.628 ~ 1.591)
0.5 h	19.05 +/ −0.31	28.56 +/ −0.49	−9.50 +/ −0.78	−0.70 +/ −0.78	1.630 (0.946 ~ 2.789)
1.0 h	19.70 +/ −0.33	28.90 +/ −0.42	−9.20 +/ −0.74	−0.39 +/ −0.74	1.319 (0.784 ~ 2.188)
2.0 h	20.00 +/ −0.49	28.71 +/ −0.30	−8.71 +/ −0.42	0.08 +/ −0.42	0.941 (0.707 ~ 1.265)
3.0 h	18.46 +/ −0.45	28.13 +/ −0.17	−9.67 +/ −0.33	−0.86 +/ −0.33	1.824 (1.443 ~ 2.281)
4.0 h	18.15 +/ −0.13	28.58 +/ −0.45	−10.43 +/ −0.58	−1.63 +/ −0.58	3.097 (2.070 ~ 4.626)
6.0 h	16.40 +/ −0.15	28.53 +/ −0.31	−12.12 +/ −0.46	−3.31 +/ −0.46	9.984 (7.210 ~ 13.642)
8.0 h	15.61 +/ −0.11	28.76 +/ −0.28	−13.15 +/ −0.17	−4.35 +/ −0.17	20.431 (18.126 ~ 22.940)

Table 3 Comparative quantification of *Crlea* gene from *C. roseus* roots using comparative C_T method

Time	*Crlea* gene Average C_T	18 S Average C_T	$\triangle C_T$ *Crlea*-18 S	$\triangle\triangle C_T$ $\triangle C_T - \triangle C_{T\ control}$	*Crlea* gene (Relation to control with range)
0	20.13 +/ −0.09	29.43 +/ −0.30	−9.30 +/ −0.20	0 +/ −0.20	1 (0.870 ~ 1.148)
0.5 h	19.38 +/ −0.16	28.67 +/ −0.10	−9.29 +/ −0.20	0.009 +/ −0.20	0.993 (0.865 ~ 1.141)
1 h	19.27 +/ −0.68	27.36 +/ −0.66	−8.09 +/ −0.61	1.21 +/ −0.61	0.432 (0.283 ~ 0.659)
2 h	19.29 +/ −0.09	27.43 +/ −0.41	−8.14 +/ −0.43	1.16 +/ −0.43	0.447 (0.332 ~ 0.602)
3 h	19.37 +/ −0.29	27.69 +/ −0.36	−8.32 +/ −0.66	0.97 +/ −0.66	0.507 (0.323 ~ 0.806)
4 h	18.99 +/ −0.007	28.21 +/ −0.67	−9.21 +/ −0.66	0.09 +/ −0.66	0.939 (0.594 ~ 1.484)
6 h	18.72 +/ −0.29	28.41 +/ −0.05	−9.68 +/ −0.34	−0.37 +/ −0.34	1.298 (1.021 ~ 1.635)
8 h	17.74 +/ −0.25	28.55 +/ −0.44	−10.80 +/ −0.69	−1.50 +/ −0.69	2.831 (1.753 ~ 4.563)

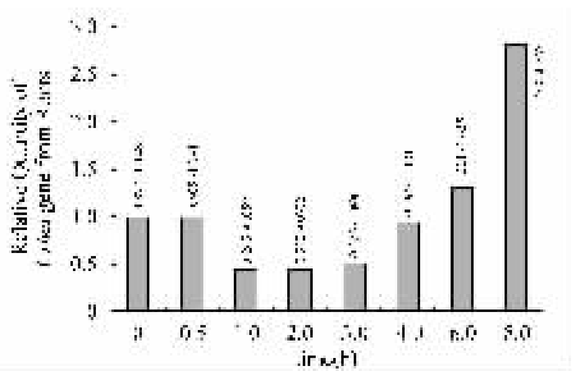

Fig. 5 Bar graphs show the relative gene expression of *Crlea* gene to the corresponding mRNA level of control *C. roseus* roots with range, after normalization with 18 S rRNA internal standard

3 Discussion

In real-time quantitative PCR technique, fluorescence molecules are added into PCR system, and the whole reaction process can be monitored through the accumulation of fluorescence signal. The unknown numbers of templates can be analyzed quantitatively based on the standard curves or internal standard control. Real-time quantitative PCR technique can detect PCR amplification and measure the kinetics of the reaction in the early phase of PCR, which has a distinct advantage over the detection of PCR amplification at the final phase or end-point of the PCR reaction in traditional PCR system. Meantime, it has many advantages such as wide inspection range (-10^8), high sensitivity (<5 copies), high precision (CV <2%), and high throughput etc [17].

The use of SYBR Green I in real-time quantitative PCR is simple and economical and only requires specific primers synthesized according to interest gene. However, it has the disadvantages of interfering by primer dimers and non-specific products. As for the false positive of dimers, second structure of single-strand DNA and wrong amplifying products, the reaction can be optimized through changing PCR conditions, inspecting the fluorescence after increasing the temperature of reading plate and selecting the optimal fluorescence catch-point to decrease the effect of non-specific products. At the same time, dissociation curves and electrophoresis also can be used to analyze the specificity and homogeneity of products [18].

Crlea gene has been first isolated from subtropical plant *C. roseus*. We have performed northern blotting to analyze the expression profile of *Crlea* gene in leaves under different levels of water stress before, results indicated *Crlea* gene was expressed at the time point of 6 h, but compared with 8 and 12h, expression intensity at different time points showed almost no difference (results not showed). Another study on the same templates with real-time PCR showed the specific products of *Crlea* gene could be detected beginning with 3 h in leaves. Compared with all samples, a obviously increase with 9.984 folds and 20.431 folds respectively at treatment of 6 and 8 h can be monitored and calculated. These results indicate real-time PCR technique is more sensitive in expression detection and can be widely used in relative quantification.

The tissue-specific expression of many *lea* genes in other plants has been analyzed. Bray found that exogenous ABA and cold stress can induce the expression of lea mRNA and proteins in almost all tissues of root, stem, leaves, this showed the expression of *lea* gene and LEA protein is non-tissue specific [19]. Other cellular localization analysis of LEA proteins show it mainly locates in cytosol [20] and nuclear [21], which indicates that the expression of *lea* gene is non-tissue-specific. The successfully induced expression of *Crlea* gene in leaves and roots under dehydration stress in our results also supports this viewpoint. In conclusion, we have studied the relative expression profiles of *Crlea* gene of leaves and roots in *C. roseus* under drought stress. Our results showed that the expression of *Crlea* gene in leaves and roots were both up-regulated by drought. The induced expression amounts of *Crlea* mRNA in leaves was obviously higher than in roots, especially with a drastic increase at 6 and 8 h. But in roots, there was a slightly increase after treatment of the first several hours. Until 8 h, an obvious increase was detected, which was 2.831 folds higher than that of control. Primary results show *Crlea* gene has a potential protecting function to plant in drought stress. However further studies of CrLEA protein should be performed to identify the function, with methods such as prokaryotic and eukaryotic expression and plant transgenic techniques and so on.

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