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Characterization of an Easter Lily Calmodulin cDNA Clone

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A clone, LCM1, which encodes calmodulin (CaM) was isolated and characterized from monocot lily (*Lilium longiflorum* Thunb.) plants. The clone is 681 bps and contains the 447 bp coding region, 8 bp leader sequence, 210 bp 3'-untranslated region, and a poly(A) tail. The coding region of 149 amino acids encodes a protein of predicted *Mr* 17 kD. Comparison of the LCM1 amino acid sequence with other CaMs revealed that the protein is highly conserved among various living organisms. The expression level of calmodulin gene in lily was studied by RNA blot analysis. The LCM1 mRNA was present in all tissues tested. However, a higher level of calmodulin was observed in anther and floral bud. The level of calmodulin mRNA in anther was about 10 times higher than that in vegetative tissues. The anther preferential expression of CaM in lily is currently investigated in dicot plants.

Keywords: calmodulin, cDNA, flower, Easter lily, anther-preferential

It has been indicated that calcium acts as an intracellular messenger in signal transduction in eukaryotes. The changes in cytosolic calcium are transmitted to the metabolic machinery through calcium binding proteins (reviewed by Poovaiah and Reddy, 1987). Calmodulin (CaM), one of the calcium binding proteins, is found in all eukaryotes and is known to be a primary transducer of intracellular calcium signal. It mediates a number of calcium regulated events in eukaryotic cells and is required for cell cycle progression. It has been observed that when the plant cell is activated by external stimuli the intracellular levels of free calcium is increased up to 100-fold (Allan and Helper, 1989). Calcium activates CaM by binding to its four calcium-binding domains, thus causing a conformational change. The calcium-CaM complex can then regulate the activity of many plant enzymes including NAD kinase, Ca^{2+}-ATPase, H^{+}-ATPase and protein kinases (Allan and Helper, 1989).

CaM cDNA clones were isolated and characterized from several plant species including potato (Jena *et al.*, 1989), barley (Ling and Zielinski 1989), alfalfa (Barnett and Long 1990), *Arabidopsis* (Braam and Davis 1990), *Brassica* (Chye *et al.*, 1995), and rice (Choi *et al.*, 1993). All of these cDNA clones code for 149 amino acid peptides which are highly homologous to other eukaryotes. These results suggest that the biological function of CaM is conserved throughout living organisms. It has been shown that CaM mRNA is present in different tissues of potato plants in different amounts, suggesting that it is involved in developmental regulation (Jena *et al.*, 1989). *In situ* studies on *Brassica* seedlings showed that high levels of CaM mRNA were detected in the leaf primordia and the shoot apical meristem, and to a lesser degree, in the zone of root elongation of the root tip (Chye *et al.*, 1995). It was also indicated that CaM activity is regulated by a variety of environmental signals such as hormone, light, touch and wounding (Jena *et al.*, 1989, Braam and Davis 1990). Recently it has been reported that the 5'-flanking region of PCM1, a potato CaM gene, is required for the developmental and touch-induced expression (Takezawa *et al.*, 1995). The fact that CaM gene expression is regulated by a number of signals and developmental factors suggests that there might be multiple regulatory elements in the CaM promoters. However, the molecular mechanisms underlying the pattern of these expression of calmodulin genes are largely unknown.

In the process of obtaining anther-preferential

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cDNA clones by differential screening from lily plants, a clone, LCM1 which codes for CaM was isolated and characterized. The CaM gene of lily is actively transcribed in mature anther and immature floral bud.

MATERIALS AND METHODS

Plant samples and bacterial strains

Pollen from *Lilium longiflorum* Thunb., cv. Nellie White harvested from commercial bulb fields during the 1989 season, was kindly provided by Dr. Frank Loewus (Washington State University). The anthers gathered during the final bud stage preceding anthesis were dried at room temperature and the pollen recovered by passage through a No. 10 brass screen. Pollen was stored in plastic containers at −20°C until used. For the northern blot analysis, lily bulbs, 7~10 cm in diameter, were planted in sterilized soil and grown under greenhouse conditions to the flowering stage. *E. coli* strains, MC1000 [F-, araD139, (araABC-leu)7679, galU, galK, (lac)X74, thi, rpsL (Str^r)] and XL-1 Blue [F'::Tn10 proA+B+, lacIq, (lacZ)M15/ recA1, endA1, gyrA96(Nalr), thi, hsdR 17(rk-mk+), supE44, relA1, lac] were used as hosts for molecular cloning. The f1 helper phage, R408, was used for *in vivo* excision of pBluscript plasmid vector from the λ ZapII phage.

Isolation of RNA and construction of cDNA library

Total RNA was isolated using the method developed by Davis *et al.* (1986). Plant samples were ground to a fine powder in the presence of liquid nitrogen with mortar and pestle. After guanidine isothiocyanate extraction, insoluble material was removed either by centrifugation at 8,000×g for 10 min or by filtration through a Miracloth (Calbiochem). The aqueous solution was centrifuged on 5.7 M CsCl cushion and the RNA pellet was collected and stored at −70°C until used. The total RNA was used either for northern blot analysis or for isolating poly(A) RNA using oligo(dT)-cellulose (Pharmacia) column chromatography (Theologis *et al.*, 1985). Using the poly(A) RNA as template and oligo(dT) as primer, the cDNA was synthesized, ligated into λ ZAPII vector, then in vitro packaged as described (Kim *et al.*, 1993).

LCM1 cloning from lily cDNA libraries

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1   GCGGCCCGATGGCGGATCAGCTCACTGACGATCAGATCTCCGAGTTTAAGGAGGCGTTCA
          A  D  Q  L  T  D  D  Q  I  S  E  F  K  E  A  F  S   17
61  GCCTGTTCGATAAGGACGGCGATGGTTGCATCACAACCAAAGAGCTCGGGACCGTTATGC
    L  F  D  K  D  G  D  G  C  I  T  T  K  E  L  G  T  V  M  R   37
121 GCTCCCTGGGCCAGAACCCAACAGAGGCAGAGCTCCAGGACATGATCAATGAGGTAGATG
    S  L  G  Q  N  P  T  E  A  E  L  Q  D  M  I  N  E  V  D  A   57
181 CTGACGGCAATGGGACCATTGATTTCCCAGAATTTCTCAACTTGATGGCCCGCAAAATGA
    D  G  N  G  T  I  D  F  P  E  F  L  N  L  M  A  R  K  M  K   77
241 AAGACACCGACTCAGAGGAGGAGCTCAAGGAGGCCTTCAGAGTGTTTGACAAGGACCAGA
    D  T  D  S  E  E  E  L  K  E  A  F  R  V  F  D  K  D  Q  N   97
301 ATGGCTTCATCTCTGCTGCTGAGCTCCGCCATGTTATGACCAACCTTGGCGAGAAACTGA
    G  F  I  S  A  A  E  L  R  H  V  M  T  N  L  G  E  K  L  T  117
361 CAGACGAGGAGGTTGATGAGATGATCCGCGAGGCTGACGTCGATGGCGATGGCCAGATCA
    D  E  E  V  D  E  M  I  R  E  A  D  V  D  G  D  G  Q  I  N  137
421 ACTACGAGGAGTTTGTCAAAGTCATGATGGCCAAGTAAAATTTGACTGGTTGATCACTAG
    Y  E  E  F  V  K  V  M  M  A  K                            148
481 TATGATGCGATTCTCCTTTCTCTATTACCTGGTAGTAATCTGGAATTAAGATGTAGTTGT
541 GAAATCAGAAAAGAGGATGATAGTATCATGTACTCTCTTACTCTCTCTGTTGGTTGTGTT
601 TGATCTCTGTTTATCGGGGATTGTTGCCTGTTATCCTTTTGGTGTGATTGGGATCTAAGT
661 TCTATAAAAAAAAAAAAAAAA
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Fig. 1. Nucleotide and deduced amino acid sequences of lily CaM clone, LCM1. The numbers indicate position of nucleotide and amino acid sequences.

During the process of isolating pollen-preferential cDNA clones by differential screening from lily plants, a clone, LCM1 which codes for CaM was isolated and characterized. The plasmid pBluscript containing LCM1 cDNA was *in vivo* rescued from lily pollen cDNA library using f1 helper phage, R 408, following maunfacturer's mannual (Stratagene). Ampicillin resistant colonies were selected and plasmid DNA was prepared by the alkaline lysis method (Birnboim and Doly, 1979). The *in vivo* excised plasmids was cut with *Eco*RI and the cDNA inserts were separated on 0.7 % agarose gel.

Northern blot analysis

Northern blot analysis was conducted using total RNA prepared from various organs. Ten μg of total RNA was run on 1.2% formaldehyde agarose gel, blotted onto a nylon membrane (Amersham), and hybridized with a radioactively labelled probe (Davis *et al.*, 1986). The homologous hybridization was performed for 22 h at 42°C in a solution containing 5X SSPE, 50% formamide, 2X Denhardt's, 0.1% SDS,

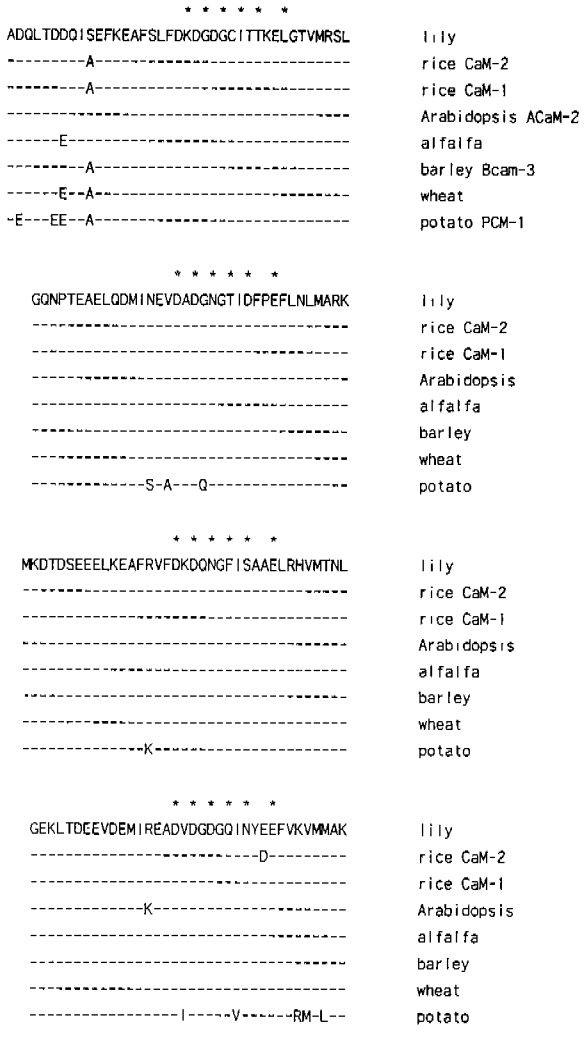

Fig. 2. Amino acid comparison of calmodulins. Amino acid sequence from rice (Choi *et al.*, 1993), *Arabidopsis* (Ling *et al.*, 1991), alfalfa (Barnett and Long, 1990), barley (Ling and Zielinski, 1989), wheat (Toda *et al.*, 1985), and potato (Jena *et al.*, 1989) are compared. Four calcium binding regions are underlined and the amino acids involved in calcium binding are shown with asterisks.

and 50 μg/mL denatured salmon sperm DNA (Sambrook *et al.*, 1989). The heterologous hybridization was performed for 22 h at 40°C in a solution containing 6X SSPE, 20% formamide, 5X Denhardt's, 0.1% SDS, and 100 μg/mL denatured salmon sperm DNA. Washing condition was at 2X SSC, 0.1% SDS. If necessary, the membrane was further washed in 0.1X SSC, 0.1% SDS at 65°C until the background radioactivity became low.

Preparation of labeled probes

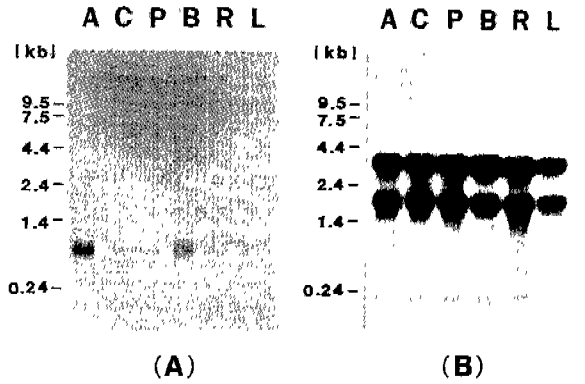

Fig. 3. (A) Northern analysis of the LCM1 gene. Ten μg of total RNA of lily was loaded on each lane and hybridized with the LCM1 3'- probe. The numbers indicate the size of RNA markers (BRL, USA) in kb. (B) The northern blot was deprobed in 0.1X SSC, 0.5% SDS for 20 min twice then reprobed with rice ribosomal DNA pRR217 (Taniai *et al.*, 1988). A, anther; C, carpel; P, petal; B, flower bud; R, root; L, leaf.

After digestion of DNA with appropriate restriction enzymes the probes were purified either by low melting agarose gel electrophoresis by electroelution. DNA was radioactively labelled using [α-^{32}P]dCTP (3000 Ci/mmol) by the random priming method (Feinberg and Vogelstein, 1983). In this method, heat denatured probe was mixed with random hexamer primers and deoxynucleotides. Radioactively labelled probe was generated by T7 DNA polymerase (Pharmacia). The specific activity of probe was greater than 5×10^7 dpm/μg. Unincorporated nucleotides were removed by G-50 Sephadex column chromatography. The radioactively labelled probe was denatured at 95°C for 3 min before use for hybridization.

DNA sequencing and analysis

Both strands of the cDNA inserts were sequenced according to the dideoxynucleotide chain termination method using double stranded DNA as a template (Sanger *et al.*, 1977). Computer softwares, GenePro (Reverside Scientific Enterprises), DNAsis (Hitachi) and GCG programs (Genetics Computer Group, Inc.), were used for sequence analysis. GenBank, EMBL and Swissprot databases were searched for sequence homology. Protein sequence similarity comparisons were based on Lipman and Pearson (1985)

RESULTS AND DISCUSSION

Lily CaM is highly conserved among other living organisms

During the characterization of the lily pollen cDNA clones, we obtained a clone which codes for CaM (Fig. 1). The clone has 681 bps and contains the 447 bp coding region, 8 bp leader sequence, 210 bp 3'-untranslated region, and a poly(A) tail. The coding region of 149 amino acids encodes a protein of predicted 17 kD. Comparison of the LCM1 amino acid sequence with other CaMs shows over 90% amino acid similarity revealing that the protein is highly conserved among various living organisms.

The amino acids involved in calcium binding are perfectly matched to other CaMs. A few differences exist as neutral substitutions at either the N- or C-terminal end (Fig. 2). Whether alanine to serine substitution at the terminal end plays important role is unknown.

A higher level of calmodulin expression observed in anther tissue of lily plant

The expression level of calmodulin gene in lily was studied by RNA blot analysis. Total RNA was isolated by the method of Davis *et al.* (1986). LCM1 cDNA hybridized to mRNAs of about 0.8 kb in anther, carpel, petal, floral bud, root and leaf (Fig. 3). Using either the entire cDNA coding region (data not shown) or a 3'-untranslated region as probes similar result was obtained that the CaM mRNA was present in all tissues tested (Fig. 3A). However, a higher level of calmodulin was observed in anther and flower bud than in other tissues. The level of calmodulin mRNA in anther was about 10 times higher than in vegetative tissues. Although it has been known that there is a multigene family for CaM in other organisms (Chye *et al.*, 1995, Fisher *et al.*, 1988), only a single band of 0.8 kb mRNA was hybridized suggesting that there is nucleotide sequence variation among the LCMs enough not to hybridize to LCM1 in our hybridization condition. The northern blot was deprobed in 0.1X SSC, 0.5% SDS for 20 min twice then reprobed with rice ribosomal DNA pRR217 (Taniai *et al.*, 1988) to evaluate the amounts of total RNA loaded (Fig. 3B). In Southern blot analysis for characterising the genomic organization of lily CaM we didnot detect any hybridization signal. It is thought because the genomic size of lily is so huge ($> 10^{10}$ bp), the hybridization was not detected in our experimental condition.

We showed that the high level expression of a CaM gene in anther tissue of lily plant. It has been observed by using *in situ* hybridization and northern analysis that cells of the leaf primordia, leaf meristematic zones, and the shoot apical meristem have abundant amounts of CaM mRNA which were considerably higher than that of the surrounding tissue (Zielinski, 1987; Chye *et al.*, 1995). These results agree with that plant cells which are actively dividing contain higher levels of CaM than resting cells (Allan and Trewavas, 1985). Nuclear run-on assay of *Arabidopsis* calmodulin genes suggested that calmodulin gene expression is controlled at the transcription level (Perera and Zielinski, 1992). It is hypothesized that the CaM is highly expressed in male reproductive organ. To analyze which cell types in male organ have high amount of CaM *in situ* hybridization experiment will be necessary.

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백합식물에서 하나의 Calmodulin cDNA 클론 연구

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적 요

단자엽 식물인 백합으로부터 calmodulin을 코딩하는 cDNA 클론, LCM1을 분리하여 연구하였다. 681 bp의 이 클론은 447 bp의 open reading frame (ORF)과 각각 8 bp 및 210 bp의 5', 3' 비해독부위 그리고 poly(A) tail로 이루어졌다. ORF로부터 추론된 단백질은 149개의 아미노산으로 구성되며 예상되는 분자량은 17 kD이다. LCM1의 아미노산 배열과 다른 CaM들의 배열을 비교한 결과 매우 높은 정도로 유사함을 알 수 있었다. Northern 분석에서 LCM1 mRNA는 조사된 모든 조직에 분포하였다. 특히 약조직에서는 조사된 영양조직에서 보다 10배 가량 더 풍부하게 존재함으로 미루어 웅성 조직에서 CaM 발현이 웅성기관의 발달과 생식과정에 중요한 영향을 미치는 것으로 생각된다.

주요어: calmodulin, cDNA, 꽃, 백합, 약-우세적

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