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Short communication

A cDNA clone encoding *Brassica* calmodulin

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Abstract

A 834 bp cDNA encoding calmodulin (CaM) has been isolated from *Brassica juncea*. On Northern analysis this cDNA hybridises this cDNA to mRNAs of about 0.9 kb in leaf, silique and peduncle. Genomic Southern analysis indicates the presence of a CaM multigene family in *Brassica juncea*. Comparison of the predicted amino acid sequence of *Brassica* CaM with that of *Arabidopsis* CaM ACaM-2 and ACaM-3 showed 100% homology, which is not unusual, since both plants belong to the family Cruciferae. *In situ* hybridisation studies on *Brassica* seedlings using a digoxigenin-labelled RNA probe 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. The occurrence of a higher rate of cell division and growth in these regions than its surrounding tissue may possibly be related to higher levels of CaM mRNA.

CaM is a low-molecular weight, heat-stable, acidic calcium-binding protein which is highly conserved in eukaryotes [1]. It is the most ubiquitous of the calcium-binding activator proteins and has been identified in plants [11, 12]. It has been suggested that calcium acts as a second messenger in signal transduction of stimuli such as light, gravity and phytohormones in plants [10, 23]. When the plant cell is activated by external stimuli it has been observed that the intracellular levels of free calcium is raised from 10^{-7} M to up to 10^{-5} M [1]. CaM is one such calcium-binding protein which has affinity for calcium in the micromolar range and can therefore respond to the elevated calcium level and mediate the effects of calcium. Calcium activates CaM by binding to its four calcium-binding domains, the EF hands, 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, NAD^{+} 3-oxidoreductase and protein kinases [1, 17].

In order to study the expression of CaM in the

The nucleotide sequence data reported will appear in the EMBL Nucleotide Sequence Database under the accession number M88307 (*Brassica juncea* calmodulin cDNA).

germinated *Brassica* seedling we have isolated and sequenced a cDNA for *Brassica* CaM and have subsequently used this cDNA to generate an antisense RNA probe for *in situ* hybridisation studies.

A *Petunia* CaM cDNA (gift of Dr Hillel Fromm) was used as a heterologous hybridisation probe to screen a λZAPII *Brassica juncea* cDNA leaf library (gift of Dr Eng-Chong Pua) for *Brassica* CaM clones. *In situ* plaque hybridisation was carried out at 42 °C in a solution containing 30% formamide. Several putative clones were obtained and upon further characterisation by sequence analysis we identified a 834 bp cDNA encoding *Brassica* CaM. This cDNA consists of 47 bp of 5′-untranslated region, 447 bp coding region, 370 bp of 3′-untranslated region and a poly(A) tail. The coding region of 149 amino acids encodes a protein of predicted M_r 16820.

The nucleotide sequence of *Brassica* CaM has 72.6% identify to that of *Petunia*. Comparison of the predicted amino acid sequence of *Brassica* CaM and those of other plant CaMs shows over 90% amino acid similarity (Fig. 1). *Brassica* CaM shows 100% amino acid homology to *Arabidopsis* CaM ACaM-2 [13], ACaM-3 [16] and TCH1 [4], 97.1% to *Arabidopsis* CaM ACaM-1 [13], 98.7% to alfalfa CaM [3] and barley CaM [12] and 91.9% to potato CaM [11]. The high conservation in amino acid homology between *Brassica* and *Arabidopsis* is not surprising since CaM is a highly conserved protein and both these plants belong to the family Cruciferae.

Brassica juncea cv. India Mustard (Czern and Coss) plants were grown in a growth chamber (12 h light/12 h dark cycle at 28/23 °C respectively). Total cell RNA was isolated by the method of Nagy *et al.* [15] from peduncles, siliques and leaves of these plants for northern blot analysis. Peduncles and leaves were obtained from one-month old plants while young and mature siliques were collected from 5–15 days and 15–30 days respectively, after fertilization.

Northern blot analysis showed that the *Brassica* CaM cDNA hybridised to mRNAs of about 0.9 kb in peduncle, silique and leaf (Fig. 2A). The northern blot was stained with methylene blue to

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                                    * * * * *  *
BCaM        MADQLTDDQISEFKEAFSLFDKDGDGCITTKELGTVMR
TCH1                 -------------------------
ACaM-2      -------------------------------------
ACaM-3      -------------------------------------
ACaM-1               -------------------------
Alfalfa     -------E-----------------------------
Barley      ----------A---------------------------
Potato      --E---EE----------------------------

                                  * * * * * *
BCaM        SLGQNPTEAELQDMINEVDADGNGTIDFPEFLNLMAR
TCH1        -------------------------------------
ACaM-2      -------------------------------------
ACaM-3      -------------------------------------
ACaM-1      ------------------------------------K
Alfalfa     -------------------------------------
Barley      -------------------------------------
Potato      --------------S-A---Q----------------

                                  * * * * * *
BCaM        KMKDTDSEEELKEAFRVFDKDQNGFISAAELRHVMTN
TCH1        -------------------------------------
ACaM-2      -------------------------------------
ACaM-3      -------------------------------------
ACaM-1      -------------------------------------
Alfalfa     -------------------------------------
Barley      -------------------------------------
Potato      --------------K----------------------

                                  * * * * * *
BCaM        LGEKLTDEEVDEMIKEADVDGDGQINYEEFVKVMMAK
TCH1        -------------------------------------
ACaM-2      -------------------------------------
ACaM-3      -------------------------------------
ACaM-1      ----------E---R-----------------I----
Alfalfa     --------------R----------------------
Barley      -------------R----------------------
Potato      ------------------I-----V------RM-L--
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Fig. 1. Comparison of the deduced amino acid sequences of *Brassica* CaM and those from other plants including *Arabidopsis* (TCH1 [4], ACaM-1 and ACaM-2 [13], ACaM-3 [16], alfalfa [3], barley [12] and potato [11]. Positions of identity are denoted by dashes. Calcium-binding residues are marked with asterisks.

check the amounts of total mRNA in each lane (Fig. 2B). It was observed that young siliques had higher CaM expression than mature siliques and peduncle had higher expression than leaf. The apparent size differences observed for CaM mRNAs may be due to the presence of different species of CaM mRNAs of about 0.9 kb encoded by a CaM multigene family.

Genomic DNA was obtained from young leaves following the procedure of Dellaporta *et al.* [7] for genomic Southern analysis. When the 0.9 kb *Brassica* CaM cDNA was used to probe a genomic Southern blot of *Brassica* total genomic DNA restricted with *Bam* HI, *Eco* RI, *Eco* RV, *Hind* II, *Hind* III or *Xba* I, multiple bands were seen with each digest (Fig. 3). These results suggest the existence of a CaM multigene family in

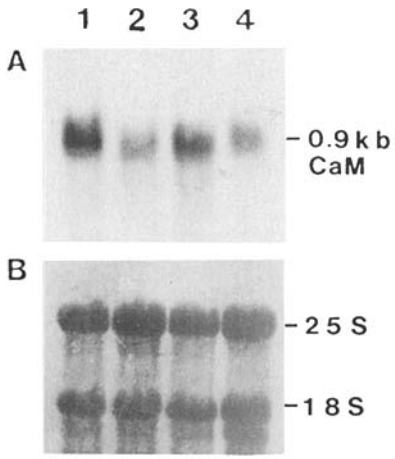

Fig. 2. A. Northern blot analysis. Total RNA (20 μg) from peduncle (lane 1), mature siliques (lane 2), young siliques (lane 3) and leaf (lane 4) were hybridised to the 0.8 kb *Brassica* CaM cDNA. B. The same RNA blot was stained with methylene blue following the procedure of Sambrook *et al.* [21]. 25S and 18S ribosomal RNA bands are marked.

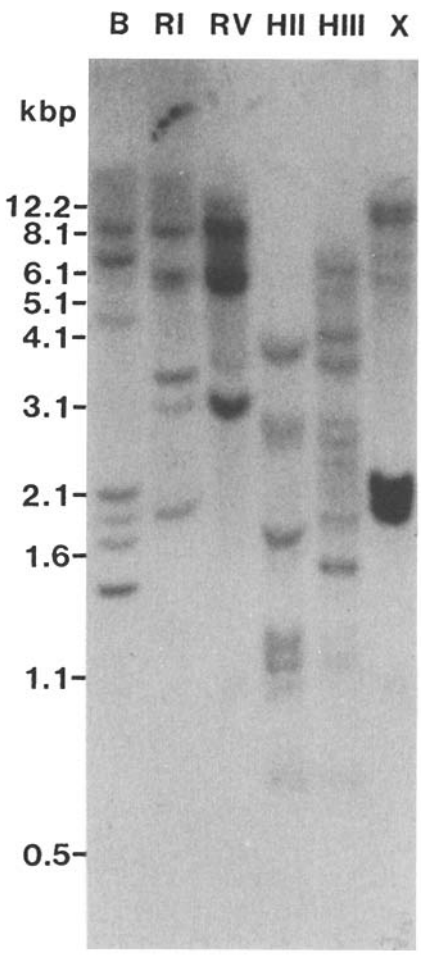

Fig. 3. Genomic Southern analysis. *Brassica* genomic DNA (20 μg) was digested with *Bam* HI (B), Eco RI (RI), *Eco* RV (RV), *Hin*d II (HII), *Hin*d III (HIII) and *Xba* I (X), separated by gel electrophoresis, blotted onto Hybond-N filters and hybridised with the 0.8 kb *Brassica* CaM cDNA probe.

Brassica juncea. In *Arabidopsis*, Ling *et al.* [13] and Perera and Zielinski [16] have identified three CaM cDNAs, ACaM-1, ACaM-2 and ACaM-3. Braam and Davis [4] have also isolated three members of a CaM-related gene family from *Arabidopsis*, TCH1, TCH2 and TCH3. Comparison of the partial amino acid sequence of TCH1 with the amino acid sequences of ACaM-2 and ACaM-3 shows 100% homology. The sequenced regions of TCH2 and TCH3 show 44% and 70% amino acid sequence similarity to TCH1. It has also been reported that in humans [8], CaM is encoded by a family of at least three genes while in the sea urchin there are two CaM isoforms [9].

In situ hybridisation studies were carried out using a digoxigenin-labelled RNA probe to examine the distribution of CaM mRNA in the germinated *Brassica* seedling. These experiments were carried out following conditions described by Cox and Goldberg [5] with a modification in the proteinase K incubation step where the incubation time for the proteinase K (0.5 μg/μl) reaction was reduced from 30 min to 3 min. The 0.8 kb *Eco* RI fragment containing the *Brassica* CaM cDNA was cloned into pBluescript SK – (Stratagene) in opposite orientations. Digoxigenin UTP-labelled antisense and sense RNA probes were synthesised *in vitro* using T7 RNA polymerase from *Xba* I-digested SK – derivatives. The antisense and sense probes were hybridised with 6 μm thick sections overnight at 40 °C. Sections were washed following procedures described [5] and digoxigenin-labelled RNA probes were detected after hybridisation by an alkaline phosphatase-linked immunoassay (Boehringer Mannheim) using a colour reaction with 5-bromo-4-chloro-3-indolyl phosphate (X-phosphate) and nitroblue tetrazolium salt (NBT). Slides were mounted using GeITol aqueous mounting medium (Immunon) and were observed with a Zeiss/Axioskop

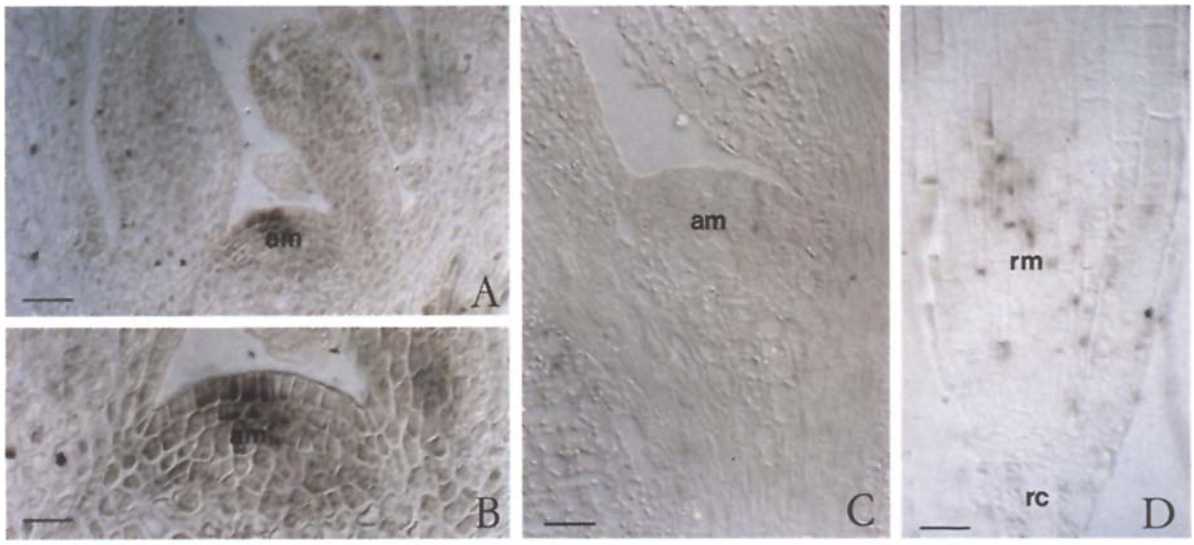

Fig. 4. Localization of CaM mRNA in longitudinal sections of the shoot meristem (A–C) and root meristem (D) of a germinated *Brassica* seedling. Section A was hybridised to the antisense probe and section C to the sense probe. B is an enlargement of A at the apical meristem. am, apical meristem. Bar represents 30 μm in A; 25 μm in B, C and D.

microscope using differential interference contrast (Normarski Optics).

We observed that cells of the leaf primodia and the shoot apical meristem of a germinating seedling had abundant amounts of CaM mRNA which were considerably higher than that of the surrounding tissue (Fig. 4). This difference in mRNA expression is likely to be related to the high number of actively dividing cells in these regions compared to cells of the surrounding tissue which are not in active cell division. We also observed scattered hybridisation at the zone of root elongation of the root tip in the seedling. These cells which are actively growing, had higher levels of CaM mRNA than the adjacent tissue (Fig. 4).

It has been previously shown by NAD-kinase activation assay and radioimmunoassay that plant cells which are in active growth and division contain higher levels of CaM than non-dividing cells [2, 14]. Using northern blot analysis and *in vitro* translation studies Zielinski [24] has shown that CaM mRNA levels are elevated in barley leaf meristematic zones. We have demonstrated by *in situ* hybridisation studies that the leaf primordia, the shoot apical meristem and the zone of elongation of the root tip of a *Brassica* seedling have higher CaM mRNA levels than its surrounding tissues. This study suggests that CaM plays an important role in cell division in these regions of the *Brassica* seedling. The importance of CaM in cell growth and division is further indicated by the studies in mammalian cells [18, 19], *Saccharomyces cerevisiae* [6], *Schizosaccharomyces pombe* [22] and *Aspergillus nidulans* [20] in which deletion of the CaM gene is lethal.

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References

1. Allan E, Helper PK: CaM and calcium-binding proteins. In: Marcus A (ed) The Biochemistry of Plants: A Comprehensive Treatise, vol. 15: Molecular Biology, pp. 455–484. Academic Press, New York (1989).

2. Allan E, Trewavas A: Quantitative changes in CaM and NAD kinase during early cell development in the root apex of *Pisum sativum* L. Planta 165: 493–501 (1985).
3. Barnett MJ, Long SR: Nucleotide sequence of an Alfalfa CaM cDNA. Nucl Acids Res 18: 3395 (1990).
4. Braam J, Davis RW: Rain-, wind- and touch-induced expression of CaM and CaM-related genes in *Arabidopsis*. Cell 60: 357–364 (1990).
5. Cox KH, Goldberg RB: Analysis of plant gene expression. In: Shaw CH (ed) Plant Molecular Biology: A Practical Approach, pp. 1–35. IRL Press, Oxford (1988).
6. Davis TN, Urdea MS, Masiarz FR, Thorner J: Isolation of the yeast CaM gene: CaM is an essential protein. Cell 47: 423–431 (1986).
7. Dellaporta SL, Wood J, Hicks JB: A plant DNA minipreparation: Version II. Plant Mol Biol Rep 1: 19–21 (1983).
8. Fischer R, Koller M, Flura M, Mathews S, Strehler-Page M, Kribs J, Penniston JT, Carofoli E, Strehler E: Multiple divergent mRNAs code for a single human CaM. J Biol Chem 263: 17055–17062 (1988).
9. Hardy DO, Bender PK, Kretsinger RH: Two CaM genes are expressed in *Arbacia punctulata*. J Mol Biol 199: 223–227 (1988).
10. Hepler PK, Wayne RO: Calcium and plant development. Ann Rev Plant Physiol 36: 397–439 (1985).
11. Jena PK, Reddy ASN, Poovaiah BW: Molecular cloning and sequencing of a cDNA for plant CaM: signal-induced changes in the expression of CaM. Proc Natl Acad Sci USA 86: 3644–3648 (1989).
12. Ling V, Zielinski RE: Cloning of cDNA sequences encoding the calcium-binding protein, CaM, from barley (*Hordeum vulgare* L.) Plant Physiol 90: 714–719 (1989).
13. Ling V, Perera I, Zielinski RE: Primary structures of *Arabidopsis* CaM isoforms deduced from the sequences of cDNA clones. Plant Physiol 96: 1196–1202 (1991).
14. Muto S, Miyachi S: Production of antibody against spinach CaM and its application to radioimmunoassay for plant CaM. Z. Pflanzenphysiol 114: 421–431 (1984).
15. Nagy F, Kay Sa, Chua NH: Analysis of gene expression in transgenic plants. In: Gelvin SV, Schilperoort RA (eds) Plant Molecular Biology Manual, pp. B4: 1–29. Kluwer Academic Publishers, Dordrecht (1988).
16. Perera IY, Zielinski RE: Structure and expression of *Arabidopsis* CaM-3 CaM gene. Plant Mol Biol 19: 649–664 (1992).
17. Poovaiah BW, Reddy ASN: Calcium messenger systems in plants. CRC Crit Rev Plant Sci 6: 47–103 (1987).
18. Rasmussen CD, Means AR: CaM is involved in regulation of cell proliferation. EMBO J 6: 3961–3968 (1987).
19. Rasmussen CD, Means AR: CaM is required for cell-cycle progression during G_1 and mitosis. EMBO J 8: 73–82 (1989).
20. Rasmussen CD, Means RL, Lu KP, May GS, Means AR: Characterization and expression of the unique CaM gene of *Aspergillus nidulans*. J Biol Chem 265: 13767–13775 (1990).
21. Sambrook J, Fritsch EF, Maniatis T: Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor NY (1989).
22. Takeda T, Yamamoto M: Analysis and *in vivo* disruption of the gene coding for CaM in *Schizosaccharomyces pombe*. Proc Natl Acad Sci USA 84: 3580–3584 (1987).
23. Trewavas A, Gilroy S: Signal transduction in plant cells. Trends Genet 7: 356–361 (1991).
24. Zielinski RE: CaM mRNA in barley (*Hordeum vulgare* L.). Apparent regulation by cell proliferation and light. Plant Physiol 84: 937–943 (1987).