Chimeric plant calcium/calmodulin-dependent protein kinase gene with a neural visinin-like calcium-binding domain

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ABSTRACT Calcium, a universal second messenger, regulates diverse cellular processes in eukaryotes. Ca2+ and Ca2+/calmodulin-regulated protein phosphorylation play a pivotal role in amplifying and diversifying the action of Ca2+-mediated signals. A chimeric Ca2+/calmodulin-dependent protein kinase (CCaMK) gene with a visinin-like Ca2+-binding domain was cloned and characterized from lily. The cDNA clone contains an open reading frame coding for a protein of 520 amino acids. The predicted structure of the CCaMK gene with a neural visinin-like calcium-binding domain was cloned and characterized from lily. The amino-terminal region of CCaMK contains all 11 conserved subdomains characteristic of serine/threonine protein kinases. The calmodulin-binding region of CCaMK has high homology (79%) to α subunit of mammalian Ca2+/calmodulin-dependent protein kinase. The calmodulin-binding region is fused to a neural visinin-like domain that contains three Ca2+-binding EF-hand motifs and a biotin-binding site. The Escherichia coli-expressed protein (~56 kDa) binds calmodulin in a Ca2+-dependent manner. Furthermore, "Ca-binding assays revealed that CCaMK directly binds Ca2+. The CCaMK gene is preferentially expressed in developing anthers. Southern blot analysis revealed that CCaMK is encoded by a single gene. The structural features of the gene suggest that it has multiple regulatory controls and could play a unique role in Ca2+ signaling in plants.

Calcium plays a pivotal role as a second messenger by regulating many aspects of cellular signaling in plants and animals. The signal-induced changes in free Ca2+ concentration in a cell have been portrayed as a switch turning on various cellular processes (1–5). Protein phosphorylation is one of the major mechanisms by which eukaryotic cells transduce extracellular signals to intracellular responses (6, 7). Ca2+ and Ca2+/calmodulin-dependent protein kinases are involved in amplifying and diversifying the action of Ca2+-mediated signals (5, 6, 8–12). In animals, multifunctional Ca2+/calmodulin-dependent protein kinase (CaMKII) is known to play a pivotal role in cellular regulation because of its ability to phosphorylate various proteins upon binding to Ca2+/calmodulin (9, 13). Although not much is known about Ca2+/calmodulin-dependent protein kinases in plants (5, 14, 15), Ca2+-dependent calmodulin-independent protein kinases have been well documented (10, 11). These kinases are characterized by the presence of a calmodulin-like Ca2+-binding domain. This report describes a chimeric Ca2+/calmodulin-dependent protein kinase (CCaMK) gene† in plants with some of the structural features resembling both mammalian Ca2+/calmodulin-dependent protein kinases and plant Ca2+-dependent protein kinases.

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MATERIALS AND METHODS

Plant Material. Lily (Lilium longiflorum Thunb cv. Nellie White) plants were grown under greenhouse conditions and various parts were excised and frozen in liquid nitrogen.

PCR and cDNA Library Screening. Three lily cDNA libraries made from developing anthers and mature and germinating pollen were used for PCR. Degenerate oligonucleotides corresponding to two highly conserved regions, DLKPEN and FNARRKL, of mammalian Ca2+/calmodulin-dependent protein kinases were used as primers for PCR (16). The amplification reaction mixture contained 1× PCR buffer (Cetus), all four dNTPs (each at 200 μM), 50 pmol of each primer, 1.5 mM MgCl2, 2 μl of cDNA library (105 plaque-forming units/ml), and 2.5 units of Taq DNA polymerase in a 100-μl total reaction volume. The cycling profile was 30 cycles of 94°C for 1 min, 48°C for 1 min, and 72°C for 1 min. The specific PCR product of the expected size (471 bp) was subcloned into pBluescriptII KS(+) (Stratagene) and sequenced. This fragment was used to screen the cDNA library (17) from developing anthers (18) to obtain the cDNA clone.

Sequence Analysis. The sequencing of the cDNA was carried out by using the Sanger dyeoxynucleotide chain-termination method (19). A search of the GenBank database (March 1994) was done by using Genetics Computer Group version 7.0 software (20).

The Expression of the CCaMK Gene. The RNAse protection assay (17) was performed by using total RNA (20 μg) from various parts of the lily. Total RNA was isolated from leaf, stem, and various organs from immature flower (21). A 612-bp fragment of the CCaMK coding region (nt 1010–1621) was subcloned into pBluescriptII KS(+) (Stratagene) and used as a template for making the 32P-labeled RNA probe.

Southern Blot Analysis. Lily genomic DNA (5 μg) was digested with various restriction enzymes and transferred to nylon membrane, and Southern blot analysis was carried out by using standard protocols (17).

Expression of CCaMK in Escherichia coli. The CCaMK protein was expressed in E. coli from the pET3b vector (22). E. coli BL21 (DE3)-pLysS was transformed with the pET3b expression vector containing CCaMK cDNA. Bacteria were grown at 35°C in M9 minimal medium supplemented with Casamino acids (2 g/liter), ampicillin (100 mg/liter), and chloramphenicol (25 mg/liter). The protein was induced by adding 0.5 mM isopropyl β-D-thiogalactoside when the OD600 reached 0.5–0.7 unit. Three hours after induction, cells were collected by centrifugation, and the protein was then extracted and purified by using a calmodulin-Sepharose 4B affinity column as described by Hagiwara et al. (23). The quality of the purified protein was checked by SDS/PAGE.

Abbreviations: CCaMK, chimeric Ca2+/calmodulin-dependent protein kinase; CaMKII, multifunctional Ca2+/calmodulin-dependent protein kinase.

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†The sequence reported in this paper has been deposited in the GenBank database (accession no. U24188).
Preparation of 35S-labeled Calmodulin and Calmodulin-Binding Assay. 35S-labeled calmodulin was prepared as described by Fromm and Chua (24) by using a calmodulin cDNA (25) cloned into the pET3b expression vector. The CCaMK protein (250 ng) was electrophoretically transferred onto a nitrocellulose filter and incubated in a solution containing 50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1% nonfat dry milk, 50 mM 35S-labeled calmodulin (0.5 × 106 cpm/μg), and either 1 mM CaCl2 or 5 mM EGTA (26). An excess amount (50×) of unlabeled calmodulin was used as a competitor to show specific binding of calmodulin to CCaMK. The calmodulin binding to CCaMK was quantified by measuring radioactivity in each slot with a liquid scintillation counter.

RESULTS AND DISCUSSION

A partial clone of CCaMK (471 bp) was obtained from developing anthers of lily by using PCR with degenerate primers (DLKPEN and FNARRK) as indicated by asterisks, and the hatched region indicates the putative biotin-binding site (LKAMKMNSLI).

**FIG. 1.** Nucleotide and deduced amino acid sequences of CCaMK. The diagnostic sequences (GKGGFS, DLKPEN, and SIDYVSP) for serine/threonine kinases are underlined. Sequences corresponding to serine/threonine kinases are underlined. Sequences corresponding to the two PCR primers (DLKPEN and FNARRK) are indicated by boxes, putative auto-phosphorylation sites (RXXS/T) are indicated by asterisks, and the hatched region indicates the putative biotin-binding site (LKAMKMNSLI).
oligonucleotide primers corresponding to two highly conserved regions of mammalian Ca\(^{2+}\)/calmodulin-dependent protein kinases. This fragment was not amplified when the cDNA libraries made from mature and germinating pollens were used. The nucleotide sequence of the PCR-amplified fragment contained conserved sequences corresponding to catalytic subdomains VI-XI and part of the calmodulin-binding domain of mammalian CaMKII (28).

A cDNA clone of CCaMK (2514 bp) was obtained by screening the cDNA library by using the PCR-amplified fragment as a probe and its nucleotide sequence was determined (Fig. 1). The cDNA codes for a polypeptide of 520 amino acids flanked by a 634-bp untranslated region at the 5' end and a 317-bp untranslated region at the 3' end. This polypeptide contains all 11 major conserved subdomains of the catalytic domain of serine/threonine kinases (28). Sequence comparisons revealed that CCaMK has high homology to Ca\(^{2+}\)/calmodulin-dependent protein kinases, especially in the kinase and the calmodulin-binding domains (amino acids 1-338). This region of CCaMK has highest homology to kinases from apple (Gen3: Mdstpkn), rat (Gen2: Ratp2g), human (Gen1: Humccdpkb), and fruit fly (Gen2: Drocdpkb, Gen2: Drocdpdl), where data in parentheses are names for corresponding genes in GenBank.

The calmodulin-binding region of CCaMK (ARRKLRAAA-LASVL, residues 325-338) has 79% similarity to the calmodulin-binding domain (ARRKLGAAILTTML, residues 296-309) of a subunit of mammalian CaMKII, a well-characterized Ca\(^{2+}\)/calmodulin-dependent protein kinase (29). However, the calmodulin-binding domain of CCaMK has 43% and 50% similarity to the calmodulin-binding domains of CaMKII homologs of yeast and Aspergillus, respectively (30, 31). The helical wheel projection of the calmodulin-binding domain (amino acids 325-338) of CCaMK formed a basic amphipathic α-helix (32), a characteristic feature of calmodulin-binding sites (data not shown).

The sequence downstream of the calmodulin-binding region of CCaMK (amino acids 339-520) does not have significant homology to known Ca\(^{2+}\)/calmodulin-dependent protein kinases. Further analysis of this region revealed the presence of three Ca\(^{2+}\)-binding EF-hand motifs that had the highest homology (52-54% similarity; 32-35% identity) to a family of genes belonging to visinin-like Ca\(^{2+}\)-binding proteins (Fig. 2), found mainly in neural tissue (33-37). Even though four EF-hand motifs are present in the calmodulin-like domain of Ca\(^{2+}\)-dependent calmodulin-independent protein kinases, this domain shared only 25% identity with the visinin-like domain of CCaMK. Out of the six residues of the EF-hand [positions 1(X), 3(Y), 5(Z), 7(−Y), 9(−X), and 12(−Z), where X, Y, and Z refer to Ca\(^{2+}\)-ligating residues] involved in Ca\(^{2+}\) binding, position 7(−Y) is not conserved in CCaMK. A similar deviation is also observed in visinin-like proteins, wherein the residue at position 9(−X) of the EF-hand motifs of visinin-like proteins (Fig. 2) is not conserved. These differences between the EF-hands of the visinin-like domain of CCaMK and other Ca\(^{2+}\)-binding proteins may affect Ca\(^{2+}\)-binding and protein-protein interactions.

Frequenin, neurocalcin, hippocalcin, and visinin-like neural Ca\(^{2+}\)-binding proteins are members of a family of Ca\(^{2+}\)-sensitive regulators, each with three Ca\(^{2+}\)-binding EF-hand motifs. The presence of such proteins has not been reported in plants. These proteins are activated at nanomolar concentrations of Ca\(^{2+}\). At such low levels, calmodulin-dependent pathways are not activated. Frequenin acts as a Ca\(^{2+}\)-sensitive activator of a photoreceptor particulate guanylyl cyclase (37). It has also been suggested that frequenin might be involved in activating protein kinases and phosphatases in response to changes in intracellular Ca\(^{2+}\), similar to the action of calmodulin (37).
An unusual feature of CCaMK is the presence of a putative biotin-binding site (LKAMKMNSLI) within the visinin-like domain (Fig. 2). Such a biotin-binding site has not been observed in neural visinin-like proteins. Although, biotin is known to play a catalytic role in several essential metabolic carboxylation and decarboxylation reactions (38), its role in the regulation of CCaMK is not known. CCaMK also contains two consensus motifs, RXXT/S (Figs. 1 and 2), analogous to the autophosphorylation site of mammalian CaMKII and its homologs (9).

The structural features of the CCaMK gene indicate that it is a chimeric Ca\(^{2+}\)- and Ca\(^{2+}/\)calmodulin-dependent protein kinase with two discrete regulatory domains, a calmodulin-binding domain and a visinin-like Ca\(^{2+}\)-binding domain (Fig. 3). The presence of these distinct domains suggests dual modes of regulation. Furthermore, the presence of a putative biotin-binding site suggests yet another mode of regulation, adding to the functional diversity of CCaMK. The chimeric feature of the CCaMK gene suggests that it has evolved from a fusion of two genes that are functionally different and phylogenetically diverse in origin.

To study the functional role of the predicted structural motifs of CCaMK, the E. coli-expressed protein was used for Ca\(^{2+}\)- and calmodulin-binding assays. The protein was expressed in E. coli (Fig. 4A) and purified by calmodulin affinity chromatography to near homogeneity as judged by SDS/PAGE. The calmodulin-binding assay confirmed that calmodulin binds to CCaMK only in the presence of Ca\(^{2+}\) (Fig. 4B). Furthermore, when incubated with excess amounts (50-fold) of unlabeled calmodulin, the binding of \(^{35}\)S-labeled calmodulin to CCaMK was effectively reduced, suggesting that calmodulin binding to CCaMK was specific. To determine the functional role of the EF-hand motifs within the visinin-like domain, Ca\(^{2+}\)-binding assays were carried out. The results revealed that Ca\(^{2+}\) directly binds to CCaMK (Fig. 4C). Moreover, CCaMK also showed a Ca\(^{2+}\)-dependent shift in mobility by SDS/PAGE (data not shown). Although, these results suggest that CCaMK has some of the structural properties of both Ca\(^{2+}\)-dependent and Ca\(^{2+}/\)calmodulin-dependent protein kinases, an in-depth study is required to conclusively demonstrate a functional link across the three enzymes (Fig. 5).

The CCaMK gene was preferentially expressed during phase III (40) of anther development as revealed by the ribonuclease protection assay (Fig. 5). The expression of CCaMK during phase III suggests that it may be involved in microsporogenesis. Some of the EF-hand proteins like calmodulin (41) are ubiquitous and are active in diverse tissues. However, visinin-like proteins are restricted to specialized tissues such as neurons. Interestingly, CCaMK, which has a visinin-like domain, is also expressed in an organ-specific manner. Genomic Southern blot analysis revealed that CCaMK is encoded by a single gene (Fig. 6). Hybridization at low stringency using the CCaMK probe indicated the presence of a CCaMK homolog in other plants, such as Arabidopsis, apple, and tobacco (data not shown). We have also cloned a CCaMK homolog from tobacco with structural components similar to lily, including calmodulin-binding and visinin-like domains. These results suggest that the CCaMK-like gene is present in both monocotyledonous and dicotyledonous plants.

The Ca\(^{2+}\)-signaling pathway mediated through Ca\(^{2+}/\)calmodulin-dependent protein phosphorylation is well established in animals. This report confirms the presence of a Ca\(^{2+}/\)calmodulin-dependent protein kinase in plants. However, the presence of a visinin-like Ca\(^{2+}\)-binding domain in CCaMK adds an additional Ca\(^{2+}\)-sensing mechanism not previously known in these kinases. This feature distinguishes CCaMK from all known Ca\(^{2+}/\)calmodulin-dependent protein kinases. The discovery of the CCaMK gene increases our understanding of Ca\(^{2+}\)-mediated signal transduction in plants.

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