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MOLECULAR CLONING AND CHARACTERIZATION OF A TOBACCO CALMODULIN BINDING PROTEIN

A DISSERTATION SUBMITTED TO THE GRADUATE DIVISION OF THE UNIVERSITY OF HAWAI'I IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

BOTANICAL SCIENCES

(PLANT PHYSIOLOGY)

MAY 1996

By

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I am indebted to my husband, Kishore, for his patience, care, and understanding.
ABSTRACT

Calmodulin-binding proteins (CaMBP) play important roles in Ca\textsuperscript{2+}/CaM-mediated cellular activities. While many CaMBPs have been isolated and characterized in animals, very little is known about plant calmodulin-binding proteins (CaMBPs). A cDNA clone, pTCB 60, encoding a recombinant tobacco CaMBP was isolated previously by screening a tobacco (Nicotiana tabacum L. cv Wisconsin 38) cDNA library using S\textsuperscript{35}-labeled CaM as a ligand probe. The cDNA clone contained a 1572 bp cDNA insert with an 1184 bp open reading frame encoding 393 amino acid residues. Northern blot analysis of tobacco total RNA with radiolabeled cDNA insert as a probe detected a 2.4 kb mRNA, indicating that pTCB60 is not a full length cDNA clone. The presence of a poly (A) tail at 3' end and the lack of an initiation methionine codon at the beginning of the open reading frame (ORF) of the cDNA sequence, suggest that approximately 830 bases from the 5'end of the message are missing in pTCB60 cDNA clone. The complete sequence of the messenger was determined by two approaches. First, the 800 bp unknown 5'downstream region of the messenger was synthesized and amplified by a slightly modified 5'RACE (rapid amplification of cDNA ends) protocol, cloned and sequenced. Second, a tobacco cDNA library was constructed in λgt11 and rescreened with pTCB60 cDNA probe to isolate a full-length clone. The combined nucleotide sequence of the 5'RACE clone and pTCB60 partial cDNA clone indicated the full length cDNA sequence is 2409 bp containing a 1656 bp ORF encoding 551 amino acid residues. Database searches revealed no similarity with known gene or protein sequences.
The 48.5 kD recombinant tobacco CaMBP encoded by pTCB60 cDNA clone was purified by a two step procedure using CaM-Sepharose chromatography and Protosorb immunoaffinity chromatography. Polyclonal antisera raised against the purified recombinant protein recognized a 60 kD polypeptide in western blots of tobacco cell extracts. Northern blot and immunoblot analyses showed differential expression of TCB60 mRNA and the corresponding protein during tobacco cell culture growth and heat shock response. The western blot of tobacco leaf, stem and root proteins indicated the expression of the protein only in leaf tissues.

Secondary structure analysis of the deduced amino acid sequence of the recombinant CaMBP suggested the presence of a basic amphiphilic α-helix (BAA) motif at the C-terminus of the protein. A synthetic peptide was made corresponding to the putative BAA motif spanning amino acids 520-538 and its interaction with CaM was analyzed by a variety of methods. Calmodulin exhibited a Ca\(^{2+}\)-dependent mobility shift upon binding the synthetic peptide in 4 M urea and native PAGE. The synthetic peptide competitively inhibits CaM-stimulated PDE activity (Ki = 15 nM). Upon binding CaM, the fluorescence emission spectra of the peptide containing two tryptophanyl residues shifted toward blue and increased in intensity. The circular dichroism (CD) spectra show the helicity of CaM and peptide increase upon complex formation. \(^1\)H NMR studies indicate that the peptide interacts with the aromatic residues in the leading helices of domain I and III of CaM. Taken together, these data provide direct evidence that a structurally conserved BAA CaM-binding domain similar to most CaM-binding proteins characterized in animal systems is present in a plant protein.
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LIST OF ABBREVIATIONS

A$_{280}$ absorbance at 280 nm
AMP adenosine monophosphate
BAA basic amphiphilic $\alpha$-helix
BCIP 5-bromo-4-chloro-indolyl-phosphate
bp base pair
BSA bovine serum albumin
$\beta$-gal $\beta$-galactosidase
CaM calmodulin
CaMBP calmodulin-binding protein
cAMP cyclic AMP
CD circular dichroism
CDPK calcium dependent protein kinase
CTAB hexadecyl trimethyl ammonium bromide
cv cultivar
DEPC diethylpyrocarbonate
DMF N,N-dimethyl formamide
DTT dithiothreitol
E. coli Escherichia coli
EDTA ethylenediamine tetraacetic acid
EGTA ethylene glycol bis($\beta$-aminoethyl ether) N, N, N', N'-tetraacetic acid
<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>GCG</td>
<td>genetics computer group</td>
</tr>
<tr>
<td>GES</td>
<td>Goldman-Engelman-Steitz</td>
</tr>
<tr>
<td>GSP</td>
<td>gene specific primer</td>
</tr>
<tr>
<td>HPLC</td>
<td>high-performance liquid chromatography</td>
</tr>
<tr>
<td>HSR</td>
<td>heat shock response</td>
</tr>
<tr>
<td>HSP</td>
<td>heat shock protein</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropyl-β-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>kb</td>
<td>kilobase</td>
</tr>
<tr>
<td>KD</td>
<td>Kyte-Doolittle</td>
</tr>
<tr>
<td>kD</td>
<td>kiloDalton</td>
</tr>
<tr>
<td>Ki</td>
<td>inhibition constant</td>
</tr>
<tr>
<td>MLCK</td>
<td>myosin light chain kinase</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>NBT</td>
<td>nitro blue tetrazolium</td>
</tr>
<tr>
<td>NMR</td>
<td>nuclear magnetic resonance</td>
</tr>
<tr>
<td>NP-40</td>
<td>nonidet P-40</td>
</tr>
<tr>
<td>ORF</td>
<td>open reading frame</td>
</tr>
<tr>
<td>PCR</td>
<td>polymerase chain reaction</td>
</tr>
<tr>
<td>PDE</td>
<td>phosphodiesterase</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>Poly(A)+</td>
<td>polyadenylated</td>
</tr>
<tr>
<td>PVDF</td>
<td>polyvinylidene difluoride</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>PVP</td>
<td>polyvinylpyrrolidone</td>
</tr>
<tr>
<td>RACE</td>
<td>rapid amplification of cDNA ends</td>
</tr>
<tr>
<td>SA-PMP</td>
<td>streptavidin paramagnetic particles</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SSC</td>
<td>sodium chloride and sodium citrate</td>
</tr>
<tr>
<td>SSPE</td>
<td>sodium chloride, sodium phosphate and EDTA</td>
</tr>
<tr>
<td>TCB60</td>
<td>tobacco calmodulin-binding protein 60</td>
</tr>
<tr>
<td>Top Pred II</td>
<td>topology prediction II</td>
</tr>
<tr>
<td>UTR</td>
<td>untranslated region</td>
</tr>
</tbody>
</table>
CHAPTER I

REVIEW OF LITERATURE

Introduction

Calmodulin (CaM) is a 17 kD calcium-binding protein present in all eukaryotes. Upon binding calcium, calmodulin undergoes conformational changes and interacts with a number of enzymes and structural proteins called calmodulin-binding proteins (CaMBPs). In turn, the activated proteins regulate many cellular activities and Ca$^{2+}$-mediated signal transduction pathways. While several calmodulin-binding proteins have been isolated and characterized in animals, very little is known about the structure, function, and distribution of plant CaMBPs. This lack of information on CaMBPs is the major obstacle in elucidating the Ca$^{2+}$/CaM-dependent physiological responses in plants. Thus, identification and characterization of plant CaMBPs would provide some insight into the Ca$^{2+}$/CaM-mediated signal transduction pathways in plants.

Calcium second messenger system in plant cells

Ca$^{2+}$ homeostasis in plant cells

Calcium ions play a major role in various cellular processes and signal transduction pathways (Bush, 1993; Roberts and Harmon, 1992; Poovaiah and Reddy,
1993; Fricker et al., 1990). The "second messenger" functions of calcium ions have been well-documented in animals (Hepler and Wayne, 1985). During past fifteen years there has been considerable progress in the investigation of Ca\textsuperscript{2+}/CaM-mediated processes in plants (Poovaiah and Reddy, 1993). Recently new approaches such as transgenic plants expressing apoaequorin, confocal microscopy and fluorescence imaging have been employed to monitor the changes in cytosolic Ca\textsuperscript{2+} concentration (Read et al., 1992). Under normal physiological conditions the cytosolic Ca\textsuperscript{2+} concentration estimated by all these methods varies between 30 to 200 nM (Bush, 1993). The cytosolic and nuclear calcium concentrations in a plant cell are 3 to 4 times lower than other cellular compartments (Gilroy, 1989). The vacuoles contain high levels (1-2 mM) of Ca\textsuperscript{2+} and help to maintain calcium homeostasis in the plant cell (Randall, 1992). Many proteins involved in calcium transport across the vacuolar membrane have been identified (Bush and Sze, 1986; Joyce et al., 1988; Schumaker and Sze, 1985; Schumaker and Sze, 1987). Under normal growth conditions, Ca\textsuperscript{2+}-transport from external medium and organellar compartments into the cytosol is driven by a steep electrochemical gradient (Hsieh et al., 1991). During signal transduction Ca\textsuperscript{2+} channels on the plasma membrane, vacuolar tonoplast and endoplasmic reticulum membrane open to facilitate Ca\textsuperscript{2+} fluxes into cytosol (Schroeder and Thuleau, 1991). The cytosolic Ca\textsuperscript{2+} level is restored to the submicromolar resting level by active Ca\textsuperscript{2+}-transport systems including Ca\textsuperscript{2+}-pumping ATPases (Briskin, 1990; Kasai and Muto, 1990; Robinson et al., 1988; Rasi-Caldogno et al., 1995). The calcium-binding proteins localized on vacuolar and endoplasmic reticulum membranes may be involved in calcium buffering along with the calcium
transport systems to maintain the low cytosolic Ca\(^{2+}\) levels (Randall, 1992).

**Ca\(^{2+}\)-mediated cellular activities and signal transduction pathways**

Many cellular processes such as polarized growth (Lehtonen, 1984), mitosis (Welsh et al., 1978; Kiehart, 1981), cytoplasmic streaming (Tominaga et al., 1983), circadian leaf movements (Toriyama and Jaffe, 1972), guard cell swelling (McAinsh et al., 1992) are regulated by calcium ions. Shacklock et al., (1992) reported cytosolic free calcium also mediates red light-induced photomorphogenesis. A transient increase of cytosolic Ca\(^{2+}\) concentration has been reported in response to various environmental signals such as wind (Knight et al., 1992), touch and gravity (Knight et al., 1991), light (Chae et al., 1990; Russ et al., 1991), heat shock (Calderwood and Stevenson, 1988), salinity (Lynch et al., 1989), cold shock (Gehring et al., 1990) and hormonal signals such as GA (Bush and Jones, 1987; Gilroy and Jones, 1992), ABA (Gilroy et al., 1990) and cytokinins (Hahm and Saunders, 1991). Three basic patterns are observed in stimuli-induced changes of cytosolic Ca\(^{2+}\)-concentration in plant cells, large and transient increases in response to mechanical stimuli, hypotonic shock and elicitors; steady and modest increases and decreases occur in response to gibberellic acids, cytokinins and light; and oscillatory changes with regular and irregular periods occur in response to auxin and ABA (see Table I) (Bush, 1993). These intracellular Ca\(^{2+}\) concentration changes are sensed by Ca\(^{2+}\)-binding proteins called response elements, such as
calmodulin and calcium-dependent protein kinases, which in turn regulate the cellular metabolism leading to physiological responses (Fig. 1.1).

Fig. 1.1 A model for \( \text{Ca}^{2+} \)-based second messenger system in plant cells
### Table 1.1 Summary of stimulus-induced changes in cytosolic Ca\(^{2+}\) in plant cells

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Ca(^{2+}) Response</th>
<th>Ca(^{2+}) Source</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Mechanical</td>
<td>Transient increase</td>
<td>Extracellular(?)</td>
<td>Knight et al., 1991; Bramm, 1992</td>
</tr>
<tr>
<td>Temperature</td>
<td>Transient increase</td>
<td>?</td>
<td>Knight et al., 1991</td>
</tr>
<tr>
<td>Light</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>red</td>
<td>Increase</td>
<td>Intracellular</td>
<td>Chae et al., 1990</td>
</tr>
<tr>
<td>uv</td>
<td>Increase</td>
<td>Intracellular</td>
<td>Russ et al., 1991</td>
</tr>
<tr>
<td>Hormones</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ABA</td>
<td>Transient increase</td>
<td>Intracellular</td>
<td>Gilroy et al., 1990</td>
</tr>
<tr>
<td></td>
<td>Oscillations</td>
<td>?</td>
<td>Schroeder and Hagiwara, 1990</td>
</tr>
<tr>
<td>Auxins</td>
<td>Oscillations</td>
<td>?</td>
<td>Felle, 1988a</td>
</tr>
<tr>
<td>GA</td>
<td>Steady-state increase</td>
<td>Extracellular</td>
<td>Bush and Jones, 1987; Gilroy and Jones, 1992</td>
</tr>
<tr>
<td>Cytokinin</td>
<td>Steady-state increase</td>
<td>Extracellular</td>
<td>Hahm and Saunders, 1991</td>
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</table>

(Source: Bush, 1993)
Calcium-binding proteins

Over 150 calcium-binding proteins have been identified and characterized in animal systems, suggesting the significance of calcium in regulation of cellular metabolism (Moncrief et al., 1990). Several Ca\(^{2+}\)-binding proteins such as calmodulin, Ca\(^{2+}\)-dependent protein kinases (Harper et al., 1991; Roberts and Harmon, 1992; Blowers and Trewavas, 1989), Ca\(^{2+}\)-stimulated phospholipases (Shorrosh and Dixon, 1991), phosphatases (Kauss and Jeblick, 1991) and Ca\(^{2+}\)-binding annexin-like proteins (Clark et al., 1992) have been isolated and characterized in plants. Recent studies have also indicated the involvement of calcium and Ca\(^{2+}\)-binding proteins in regulation of gene expression in plants and animals (Sheng et al., 1991; Braam, 1992). The regulatory functions of calcium-dependent protein phosphorylation and dephosphorylation of enzymes and structural proteins in biochemical pathways of animal cells is well established (Edelman et al., 1987; Klee, 1991). The wide distribution of calcium and/or calmodulin-dependent protein kinases in flowering plants, algae and protists indicates the importance of these protein in cellular function and growth (Roberts and Harmon, 1992; Ranjeva and Boudet, 1987; Harper et al., 1991; Harmon et al., 1987; Hetherington and Trewavas, 1984).

Recently a soybean cDNA encoding a CDPK (calmodulin-like domain protein kinase) containing a protein kinase catalytic domain and a CaM-like domain with four Ca\(^{2+}\)-binding sites was isolated (Harper et al., 1991). Identification of this new type CDPK in the plant system and several other plant CDPKs (Choi and Suen, 1991; Li et
al., 1991; Polya and Chandra, 1990; Roux and Slocum, 1982; Roberts and Harmon, 1992) suggests the wide occurrence of Ca$^{2+}$-dependent phosphorylation events in physiological processes of plant cells.

Calmodulin

Structure, function and localization

Calmodulin is one of the best characterized intracellular calcium receptors involved in Ca$^{2+}$-mediated cellular processes (Roberts and Harmon, 1992). It was originally discovered by Cheung et al., (1967) as an activator of cyclic nucleotide phosphodiesterase in bovine brain. The presence of CaM in plants was first reported by Muto and Miyachi (1977) and Anderson and Cormier (1978). Calmodulin has been isolated and characterized from a large number of higher plants including spinach (Watterson et al., 1980; Lukas et al., 1984), zucchini (Marme and Dieter, 1983), peanuts (Charbonneau and Cormier, 1979; Anderson et al., 1980), peas, asparagus and mung bean (Anderson, 1983), wheat (Anderson, 1983; Yoshida et al., 1983; Toda et al. 1985), barley (Schleicher et al. 1983), tobacco and Arabidopsis. The crystal structure of Ca$^{2+}$-bound CaM indicated it is a dumbbell-shaped molecule with two globular domains at opposite ends connected by an extended α-helix (Babu et al., 1985). Each globular domain contains two helix-loop-helix calcium binding sites, termed EF hands. The four calcium-binding EF hands (with the exception of Saccharomyces cerevisiae calmodulins)
bind calcium ions with dissociation constants in the range of \(10^4\) to \(10^5\) M under normal physiological conditions (Roberts and Harmon, 1992). Calmodulin is a highly conserved protein in plants and animals (Roberts et al. 1986). Sequences of several plant calmodulins exhibit 92\% or more homology among themselves and with animal calmodulins. The plant calmodulin also undergoes posttranslational trimethylation at lysine 115 like vertebrate CaM (Lukas et al., 1984; Watterson et al., 1980). Calmodulin predominantly occurs in the plant cytosolic soluble fraction. Radioimmunoassays indicate calmodulin is also present in the subcellular fractions of mitochondria, etioplasts, nuclei and the soluble fraction of cell wall and extracellular space of etiolated oat seedlings (Biro et al., 1984) and in the nuclei of pea buds (Matsumoto et al., 1983). Small concentrations of CaM was also reported in the soluble stromal fractions of chloroplast (Roberts et al., 1986).

Several isoforms of CaM (Zielinski et al., 1990), CaM like genes such as TCH 2 and TCH 3 (Bramm et al., 1992) and calcium-binding proteins with a calmodulin-like domain such as CDPK (Harper et al., 1991) have been isolated from plants. Since these proteins are encoded by distinct genes and are differentially expressed in a tissue-specific manner, they may be involved in transducing Ca\(^{2+}\) signals to elicit specific physiological responses. The mRNA encoding a 22 kD CaM-related Ca\(^{2+}\)-binding protein (CaBP-22) is expressed in leaves of both soil-grown and hydroponically-grown Arabidopsis plant but can not be detected in roots and developing siliques. The putative Ca\(^{2+}\)-binding domains of CaBP-22 and CaM are highly conserved with 79\% sequence identity (Ling and Zielinski, 1993). Isolation and characterization of CaM-target proteins would reveal if
these isoforms have differential ability to interact with specific target proteins (Roberts and Harmon, 1992). Calmodulin itself has no enzymatic activity; however, on binding Ca\(^{2+}\) calmodulin undergoes a conformational change making the hydrophobic sites available for interaction with other enzymes and proteins involved in cellular regulation.

**Calmodulin and regulation of cellular processes**

Calmodulin is considered to be a multifunctional protein (Klee, 1991) because of its ability to modulate the activities of many different proteins (figure 1.2). Deletion of the CaM gene is lethal in yeast, indicating a vital role in cellular metabolism (Davis et al., 1986; Takeda and Yamamoto, 1987). Plant studies using CaM antagonists such as chlorpromazine, trifluoperazine and fluphenazine suggest an important role of Ca\(^{2+}\)/CaM complex in polar growth of tips and pollen tubes (Hauber et al., 1984), phytochrome responses (Roux et al., 1986) and hormonal responses (auxin, gibberellic acid, cytokinins) (Elliot et al., 1983) in plants. The physiological role of CaM in plant growth and development has been investigated by over-expression or repression of CaM genes using transgenic CaM cDNA constructs fused to 35SCaMV promoter in sense and antisense orientation (Roberts et al., 1992; Zielinski et al., 1990, and Pooviah et al., 1992). Over-expression of CaM in yeast cells does not show any effect on cell cycle progression and other characteristics (Davis and Thorner, 1989). However, cell proliferation is arrested in transformed mouse cells by antisense CaM mRNA and accelerated by over-production of CaM (Rasmussen and Means, 1989).
Figure 1.2 Calmodulin (CaM) regulated cellular processes

- **Cytoskeleton**
  - MAP-2, Tau, Fodrin, Neuromodulin

- **Cyclic Nucleotide Metabolism**
  - Cyclic nucleotide phosphodiesterase
  - Adenylate cyclase
  - NO synthetase
  - (Guanylate cyclase)

- **Ca²⁺ Transport**
  - Plasma membrane ATPase
  - IP₃ kinase

- **Calcmeurm**
  - Calmodulin-stimulated protein phosphatase
    - (Calcineurin)

- **Protein Phosphorylation**
  - Calmodulin kinase I
  - Calmodulin kinase II
  - Elongation factor-2 kinase
  - Myosin light chain kinase

- **Protein Dephosphorylation**
  - Calmodulin-stimulated protein phosphatase
    - (Calcineurin)
Calmodulin plays a key role in growth and cell division cycle of plants and animals (Rasmussen and Means, 1987; Allan and Trewavas, 1985). In animal systems, elevated levels of CaM are found in regions of high mitotic activity and in cells involved in specialized rapid signalling. The cellular concentration of CaM varies among different tissues and various developmental stages (Roberts and Harmon, 1992; Ling and Assman, 1992). Radioimmunoassays indicate different levels of CaM in leaf, stem, root and cotyledon of pea. The rapidly growing regions contain the highest levels of CaM (Muto and Miyachi, 1984). A 17-fold higher level of CaM is observed in the root apex than the zone of cell elongation (Allan and Trewavas, 1985). In radish embryos a 5-fold increase in calmodulin concentration is observed during germination, that is reversed when germination is inhibited by ABA (Cocucci and Negrini, 1988). Using fluorescent probes with computer-assisted video-imaging, Tirlapur and Cresti (1992) have shown high levels of Ca\(^{2+}\) and CaM near the germinal apertures of hydrated *Nicotiana tabacum* pollen grains from where the pollen tubes emerge. The tip to base Ca\(^{2+}\)/CaM-gradient observed in actively growing pollen tubes, is absent in non-growing tubes. Immunocytochemical localization of CaM on microtubules in mitotic apparatus of plants and animals and in vitro studies on binding of CaM to microtubule associated proteins including MAP-2, tau and tubulin suggest a possible role of CaM in microtubule assembly and disassembly (Lee and Wolff, 1984; Ohta et al., 1990).

Stinemetz et al., (1987) demonstrated that in primary roots of maize CaM gene expression is regulated by gravitropic response. The apical millimeter of root apex containing the root cap shows 4-fold higher CaM concentration than the subtending 3
millimeter segment. No such difference is observed in dark grown gravitropically unresponsive plants. This indicates the Ca\(^{2+}\)/CaM levels in root caps may play a role in the gravitropic responses of plants.

The role of calmodulin in signal transduction processes may be reflected by a change in the expression of the CaM gene in response to various environmental and hormonal signals. In Arabidopsis the levels of TCH1, TCH2, TCH3 mRNA encoding CaM and CaM-related proteins respectively, are induced rapidly in response to touch, wind, rain and wounding (Bramm and Davies, 1992). Expression of these TCH genes is also induced by external Ca\(^{2+}\) and heat shock treatments that elevate the cytosolic Ca\(^{2+}\) level (Braam, 1992). Mechanical wounding also increases TCH1, TCH2 and TCH3 gene expression in apple (Watillon et al., 1992). Plant growth regulators such as auxin, cytokinin and gibberellic acid induces the expression of CaM gene (Elliot et al., 1983). In auxin-responsive strawberry fruit and light-responsive merit corn root the CaM gene expression is induced by auxin and light signals (Jena et al., 1989; Reddy and Poovaiah, 1990). Calmodulin also plays a key role in some of the phytochrome-mediated physiological processes such as chloroplast development and chlorophyll a/b-binding protein gene expression (Neuhaus et al. 1993). The process of leaf senescence may be regulated by CaM. In contrast to the findings of Leshem et al. (1984) in pea leaves, Huang et al. (1990) reported that elevated cytosolic calcium retarded senescence of rice leaves by a CaM-dependent mechanism. In senescing apples, phosphorylation of membrane proteins is promoted by Ca\(^{2+}\) and CaM (Paliyath and Poovaiah, 1985). High CaM concentrations are reported in germinating radish (Cocucci, 1984; Cocucci and
Negrini, 1988) and *Cicer arietinum* (Hernandez-Nistal et al., 1989) embryos. This induction of CaM gene expression is up-regulated by fusicoccin (a stimulator of germination) and down-regulated by ABA (an inhibitor of germination). All these studies indicate CaM not only plays significant role in signal transduction pathways but also in cellular metabolism during growth and development of plants.

**Calmodulin-binding proteins**

The regulatory functions of CaM are mediated through the regulation of a number of enzymes and structural proteins involved in various biochemical activities (Klee, 1991; Roberts and Harmon, 1992; Pooviah and Reddy, 1993). Several enzymes including myosin light chain kinase, calmodulin-dependent protein kinases, phosphorylase kinase, the calcineurin phosphatase, cyclic nucleotide phosphodiesterase, adenylate cyclases, plasma membrane Ca$^{2+}$-ATPases, certain NAD kinases are activated by calmodulin (Roberts and Harmon, 1992). Although many calmodulin-binding proteins have been isolated and characterized in animals, little is known about the numbers, identities and distribution of CaM-binding proteins in plants. The plant enzymes so far known to be Ca$^{2+}$/CaM dependent include Ca$^{2+}$-ATPase (Hetherington and Trewavas, 1982; Dieter, 1984; Rasi-Caldogno et al., 1995), NAD kinase (Allan and Trewavas, 1985; Roberts et al., 1986), nuclear NTPase (Matsumoto et al., 1984, Chen et al. 1987), glutamate decarboxylase (Baum et al., 1993; Ling et al., 1994) and kinases of soluble and
membrane-bound proteins (Polya, 1990) and a slow vacuolar ion channel (Bethke and Jones, 1994).

Recently molecular approaches using $^{35}$S CaM, $^{125}$I CaM and biotinylated CaM have been employed for cloning and characterizing CaMBPs (Flanagan and Yost, 1984; Asselin et al., 1989; Lu and Harrington, 1994). Recently, several plant CaM-binding proteins have been cloned, though their physiological roles are unknown. Due to the combination of genetic and molecular analysis, yeast is considered as a good model system for studying the biological role of the various proteins that interact with calmodulin. Two yeast genes encoding calmodulin-binding proteins homologous to the catalytic subunit of mammalian protein phosphatase and several CaM-binding proteins have been identified and characterized from soluble, plasma membrane and nuclear fractions (Liu et al., 1990; Liu et al., 1991).

Changes in the levels of CaM-binding proteins have been reported during embryogenesis and germination of carrot and radish (Oh et al., 1992; Coccuci and Negrini, 1988). Brawley and Roberts (1989) reported developmental regulation of CaM-binding protein expression in gametes and embryos of fucoid algae. Chen et al. (1994) showed the CaM-binding glutamate decarboxylase is present ubiquitously in *Petunia* organs and the expression of the corresponding mRNA and protein is developmentally regulated. Studies on distribution of CaM-binding proteins indicate tissue-specific expression of these proteins in *Vicia faba* L. (Ling and Assman 1992). Ling et al. (1994) and Snedden et al. (1995) purified glutamate decarboxylase from *Fava* bean roots and various soybean tissues respectively and showed that its activity is stimulated by
Ca\textsuperscript{2+}/CaM. Such identifications and characterizations of CaM-binding proteins and studies on their distribution among various tissues and developmental stages are essential for elucidating the role Ca\textsuperscript{2+}/CaM complex in plant growth.

Ca\textsuperscript{2+}-dependent and Ca\textsuperscript{2+}/CaM dependent protein kinases play a key role in many signal transduction pathways by modulating protein phosphorylation (Trewavas and Gilroy, 1991; Lu and Harrington 1991; Koyasu et al., 1989; Stevenson and Calderwood, 1990). Several animal CaM-dependent protein kinases including CaM kinase I, CaM kinase II, CaM kinase III, phosphorylase kinase and myosin light chain kinase have been well characterized. Although both Ca\textsuperscript{2+}/CaM-dependent and Ca\textsuperscript{2+}-dependent calmodulin independent protein kinases are thought to exist in plants (Poovaiah and Reddy, 1987; Harper et al., 1991; Polya et al., 1983; Blowers et al., 1985), they have not been purified. Recently, Watillon et al. (1993) have cloned and characterized an apple calcium/calmodulin-binding protein kinase homologous to mammalian CaM-kinase II. Purification of CaM-dependent protein kinases and identification of their substrates will shed some light on Ca\textsuperscript{2+}/CaM-mediated protein phosphorylation in signal transduction pathways of plants.

**Calmodulin-binding domains**

The mechanisms involved in the interaction of CaM with such a large number of structurally and functionally diverse target proteins is intriguing. Recent studies on several animal and plant calmodulin binding proteins indicate CaM has a high affinity
and broad specificity for basic amphiphilic α-helices (O'Neil and de Grado, 1990). In basic amphiphilic α-helix CaM-binding domains, the positively charged residues lie on one side of the helix while the other side is predominantly hydrophobic (Lukas et al., 1986). Although the CaM-binding domains are structurally conserved, the absolute amino acid sequences are not conserved (see table 1.2). A tryptophan residue is present in many the CaM-binding domains characterized in animal systems. The plant CaM-binding domains identified so far also contain tryptophan residues (Arazi et al., 1995, Lu and Harrington, 1994; Reddy et al., 1993). The significance of the tryptophan residue in CaM-binding process is well-documented in animal CaMBPs; removing the tryptophan residue by tryptic hydrolysis and by site directed mutagenesis of the cDNA sequences encoding CaMBPs or by substitution of the tryptophan residues in the synthetic binding domain peptides. Recently, Arazi et al., (1995) have shown that the tryptophan residue of the CaM-binding domain of glutamate decarboxylase is indispensable for its interaction with CaM.

Although several basic amphiphilic α-helix CaM-binding domains have been identified in both animal and plant CaMBPs, nothing is known about the tertiary structures of these proteins complexed with CaM in vivo. Thus, information about the conformational changes of the proteins due to complex formation with CaM is derived using synthetic binding domains in vitro (Lukas et al., 1986; O'Neil and de Grado, 1990). The CaM-binding domains of some proteins such as MLCK and the plasma membrane Ca^{2+} pump have dual functions: internal interaction with the enzyme itself for autoinhibition and external binding with CaM for activation of the enzyme (James et al.,
<table>
<thead>
<tr>
<th>Proteins</th>
<th>Amino acid sequences</th>
<th>Residues</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myosin light chain kinase (Lukas et al., 1986)</td>
<td>RRKWQKTGHAVRAIGRLSSS</td>
<td>494-513</td>
</tr>
<tr>
<td>Phosphorylase kinase (Dasgupta et al., 1989)</td>
<td>GKFKVICLTVLASVRIYYQYRRVKP</td>
<td>302-326</td>
</tr>
<tr>
<td>CaM kinase II (Hanley et al., 1987)</td>
<td>NARRKLKGAILTMLATRNFS</td>
<td>163-183</td>
</tr>
<tr>
<td>Calcineurin (Kincaid et al., 1988)</td>
<td>ARKEVINKIRAIGKMARVRSVLR</td>
<td>177-200</td>
</tr>
<tr>
<td>HSP70 family members (Stevenson and Calderwood, 1990)</td>
<td>KRAVRRLRTACERAKRTLSSS</td>
<td>257-277</td>
</tr>
<tr>
<td>Plant glutamate decarboxylase (Baum et al., 1993)</td>
<td>MITAWKKRVEEKKKKTNRVC</td>
<td>481-500</td>
</tr>
<tr>
<td>Tobacco TCB60 (Lu and Harrington, 1994)</td>
<td>GWLKIKAAMRWGRRVRRKA</td>
<td>362-380</td>
</tr>
<tr>
<td>Corn CBP1 &amp; CBP5 (Reddy et al., 1993)</td>
<td>GWLKIKAAMRWGIFVRKK</td>
<td>99-116, 252-269</td>
</tr>
<tr>
<td>Yeast CMP1 (Liu et al., 1991)</td>
<td>RRKALRNKILAIAKVSRMRSVLR</td>
<td>454-476</td>
</tr>
<tr>
<td>Yeast CMP2 (Liu et al., 1991)</td>
<td>RRKALRNKILAVAKVSRMYSVLR</td>
<td>501-523</td>
</tr>
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</table>
1995). Further investigation on the interactions of the binding domains within the enzymes and the effects of CaM phosphorylation on the structure and function of these domains, would provide valuable insight into the roles of CaM as well as CaM-binding domains in Ca\(^{2+}\)-signalling pathways.
CHAPTER II

SIGNIFICANCE AND HYPOTHESES

Calcium and calmodulin play important roles in growth, development and signal transduction pathways of all eukaryotic organisms. Several Ca\textsuperscript{2+}/CaM-regulated enzymes and structural proteins have been isolated and characterized in animal systems. To date, very little is known about the structures, functions and identities of plant CaMBPs. In plants, several enzymes including NAD kinase, Ca\textsuperscript{2+}-ATPases, nuclear triphosphatase, glutamate decarboxylase, an apple protein kinase similar to animal CaM kinase II, and several other protein kinases are known to be activated by Ca\textsuperscript{2+}/CaM. Although several plant CaMBPs of various molecular masses have been characterized recently using molecular cloning techniques, the identities and physiological roles of these proteins still remain unknown.

The broad goal of this study is to provide insight into the understanding of the Ca\textsuperscript{2+}/CaM-mediated signal transduction pathways in plants. Since the regulatory functions of Ca\textsuperscript{2+}/CaM leading to physiological responses are mediated through the CaMBPs, the immediate objective of this research proposal is to clone and characterize cDNAs encoding tobacco (*Nicotiana tabacum* L. cv Wisconsin 38) calmodulin-binding proteins using molecular cloning techniques.
In this project the following hypotheses will be tested:

1. Tobacco cells synthesize a CaMBP corresponding to the calmodulin-binding fusion protein encoded by the cDNA clone, pTCB 60.
2. The putative basic amphiphilic α-helix motif in the C-terminus of TCB 60 fusion protein is the CaM-binding domain.
3. TCB 60 mRNA and the corresponding CaM-binding protein are differentially expressed with respect to cell culture age, tissue type, and heat shock response.

The specific objectives are:

1. To determine the complete coding sequence of the messenger RNA encoding the calmodulin-binding protein to deduce the complete protein sequence and search the data base for similarities with other genes and proteins.
2. To purify the recombinant CaM-binding protein encoded by the cDNA clone to raise polyclonal antisera for immunodetection of the native CaM-binding protein in tobacco cell extracts.
3. To analyze the expression of the gene and the corresponding protein at various developmental phases of tobacco cell culture growth; in different tissues of tobacco plants; and in response to heat shock treatments.
4. To characterize the putative basic amphiphilic α-helix CaM-binding domain of TCB 60 protein.
CHAPTER III

CLONING OF THE 5' END AND CHARACTERIZATION OF THE FULL-LENGTH TCB60 MESSENGER RNA

Introduction

The tobacco cDNA clone, pTCB60, contains a 1572 bp cDNA insert with a 1184 bp ORF encoding a recombinant tobacco CaMBP of 393 amino acid residues. The labeled cDNA probe detected a 2.4 kb mRNA in the northern blot of tobacco total RNA indicating that pTCB60 is not a full length cDNA clone (Lu and Harrington, 1994). The presence of a polyA tail at the 3' end of the cDNA sequence and the lack of an initiation methionine codon at the 5' end of the ORF suggested approximately 800-900 nucleotides are missing from the 5' end of the cDNA clone, pTCB60. Two approaches were employed to isolate cDNA copies of the messenger downstream from the 5' end of the known cDNA sequence. The rapid amplification of cDNA ends (RACE) protocol of Frohman (1990) was used with slight modification to clone the missing 5' end fragment of the messenger. As an alternative approach a cDNA library was constructed in λgt 11 expression vector using tobacco mRNA and screened with pTCB60 cDNA as a probe to isolate a full-length clone. The full-length cDNA was sequenced and the database was searched for homologies with other known sequences. The expression of TCB60 mRNA during growth and heat-shock treatments of cultured tobacco cells was characterized.
Materials and Methods

Plant Material

Tobacco cells (*Nicotiana tabacum* L. cv Wisconsin 38) were grown at 23°C as suspension cultures in Gamborg's B-5 medium (Gamborg, 1970). Cells were isolated from mid log (7 days old) cultures incubated at 38°C for heat shock and 23°C for control treatments (Harrington and Alm, 1988).

Isolation of tobacco total RNA and northern blot hybridization

Cultured tobacco cells from mid-log phase were harvested by vacuum filtration, frozen in liquid N₂ and ground to a fine powder in a precooled mortar and pestle. Eight grams of powdered tissue were placed in a sterile 50 ml Falcon tube and 30 ml of guanidine thiocyanate buffer (McGookin, 1984) containing 5 M Guanidine thiocyanate, 50 mM Tris.Cl (pH 7.5), 10 mM EDTA, 5% β-mercaptoethanol was added. The tissues were lysed completely by several strokes with a tissue homogenizer. Solid N-lauroyl sarcosine to a final concentration of 4% (w/v) and cesium chloride (CsCl) (0.15 g/ml) were added and dissolved by shaking vigorously. The debris was removed by centrifugation at 15,000g at 4°C for 20 min. Ultracentrifuge tubes were filled with 5 ml of 5.7 M CsCl cushion and the supernatant was carefully layered over the cushion. The RNA was centrifuged at 100,000g for 18 hr at 20°C. The homogenate was carefully aspirated with a Pasteur pipet without disturbing the CsCl cushion interface. The walls
of the tube and the surface of the cushion were washed three times with sterile water. The pellet was carefully dissolved in 500 µl sterile water and precipitated with 0.1 vol. of 3M sodium citrate pH 5.2 and 2 vol of ethanol at -20°C overnight. The glasswares were baked at 250°C for 2 hr and all solutions were (with the exception of Tris-HCl) were treated with 0.1% DEPC (diethyl pyrocarbonate). The total RNA sample was separated on a 1.5% denaturing agarose gel containing formaldehyde and transferred to a nylon membrane in 20X SSC (175.3 g sodium chloride and 88.2 g sodium citrate in 1000 ml dH2O, pH 7.2) as described in Sambrook et al. (1989). Plasmid DNA (10 µg) isolated from pTCB60 cDNA clone by alkaline lysis (Sambrook et al., 1989) was digested with XbaI and XhoI restriction enzymes and separated on a 1% agarose gel. A 1.6 kb fragment containing the cDNA insert was purified from the gel using Prep-A-Gene DNA purification kit (Bio Rad). The gel-purified cDNA (100 ng) was boiled for 5 minutes and chilled quickly on ice. Fifteen µl of random primers buffer mixture (0.67 M HEPES, 0.17 M Tris-HCl, 17 mM MgCl2, 33 mM 2-mercaptoethanol, 1.33 mg/ml BSA, 18 OD260 units/ml oligodeoxynucleotide hexamers, pH 6.8), 2 µl of each dNTP (0.5 mM dATP, 0.5 mM dTTP, 0.5 mM dGTP and 0.5 mM α-32P labeled dCTP) and 3 units of Klenow fragment (the large fragment of DNA polymerase I) were added to the heat denatured DNA and incubated at room temperature for 3 hours (Random Primers DNA Labeling System, Gibco-BRL). The unincorporated nucleotides were removed by chromatography on a Sephadex G-50 mini column equilibrated with 10 mM Tris-HCl (pH 8.0), 50 mM NaCl, 0.1 mM EDTA (pH 8.0). The northern blotted RNA was incubated with 4 ml prehybridization solution containing 1 ml 20X SSPE (175.3 g sodium chloride,
27.6 g monosodium phosphate and 7.4 g EDTA in 1000 ml dH₂O, pH 7.4), 2 ml formamide, 40 μl 10% SDS, denatured fragmented salmon sperm DNA to a final concentration of 100 μg/ml, 600 μl 50X Denhardt's solution (5 g Ficoll, 5 g PVP, 5 g BSA in 500 ml dH₂O) and 600 μl dH₂O for 2 hours at 42°C in the hybridization chamber. The probe was boiled for 10 minutes, quickly chilled on ice and added to the pre-hybridization solution and hybridization was done for 20 hours at 42°C. The blots were washed twice (10 min each) at room temperature with 2X SSC and 0.1 % SDS, followed by two-10 min washes at 55°C with 0.1X SSC and 0.1 % SDS solution and exposed to X-ray film at -80°C.

Analysis of TCB60 mRNA expression during growth and heat shock response in cultured tobacco cells

The steady state levels of TCB60 mRNA at different phases of culture growth and at different time intervals of heat shock treatments were analyzed by northern blot hybridization of tobacco total RNA with labeled pTCB60 cDNA probe. To analyze developmental regulation of TCB60 mRNA tobacco cells were harvested from 4, 8, 12 and 16 days old suspension cultures and ground to a fine powder in liquid N₂. For heat-shock treatment, mid-log phase (7 days old) tobacco cell suspension cultures were incubated at 38°C in a shaking water bath for 2, 4 and 8 hours and the harvested cells were ground to a fine powder in liquid N₂. Total RNA was isolated from each sample (8 g tissue) in guanidine thiocyanate buffer as described above. The RNA samples (20
μg) were separated on 1.2% denaturing agarose gels, transferred to nylon membrane in 20X SSC and hybridized with P32-labeled cDNA probe as described previously.

Isolation of Poly(A)+ RNA

Poly (A)+ RNA was isolated from total RNA using a PolyATract mRNA isolation kit (Promega) following the manufacturer's instructions. One mg of tobacco total RNA in a volume of 500 μl of RNase-free water was incubated for 10 min in a 65°C heating block. Biotinylated-oligo(dT) probe (3 μl) and 13 μl of 20X SSC were added to the tube and incubated at room temperature for 10 min. The streptavidin-paramagnetic particles (SA-PMP) were washed three times with 0.3 ml, 0.5X SSC each time capturing them using the magnetic stand and removing the supernatant. The entire annealing reaction was added to the tube containing the washed SA-PMPs and incubated at room temperature for 10 min. The SA-PMPs were captured by the magnetic stand and washed three times with 0.3 ml 0.1X SSC. After the final wash, the aqueous phase was removed as much as possible without disturbing the SA-PMPs. The particles were resuspended in 0.1 ml RNase-free water. The SA-PMPs were magnetically captured and the supernatant containing the poly(A)+ RNA was transferred to a 1.5 ml sterile Eppendorf tube. The elution process was repeated again with 0.15 ml RNase-free water and the final volume was brought to 500 μl for spectrophotometric quantitation of poly(A+) RNA. The mRNA was concentrated in a speed-vac and used for rapid amplification of the 5' end of TCB 60 messenger and construction of a tobacco cDNA library.
Rapid Amplification of cDNA ends (5' RACE)

The RACE protocol of Frohman (1990) was used for the isolation of cDNA copies downstream (toward the 5' end) from the known sequence of the messenger. Poly(A)^+ RNA was isolated from tobacco cells as described above and used with a 5'-AmpliFINDER RACE Kit (CLONTECH Laboratories Inc.) following the manufacturer's directions. The first strand cDNA was synthesized from tobacco mRNA with a gene-specific primer (GSP1) from the 5'-end sequence of the partial cDNA clone and reverse transcriptase. Then the RNA template was hydrolyzed with NaOH and the excess primers were removed using selective binding of cDNA to the glass matrix support. The cDNA was concentrated by ethanol precipitation and the anchor oligonucleotide was ligated to the 3'-end of the cDNA. The anchor-ligated cDNA was used as a template for PCR amplification with a nested 5' gene-specific primer (GSP2) and an anchor primer complementary to the 3' anchor sequence. The protocol can be diagrammatically explained as in figure 3.1.
Figure 3.1 Diagrammatic scheme of 5' RACE Protocol. P1 is gene specific primer for first strand cDNA synthesis and P2 is nested gene specific primer for amplification of the cDNA by PCR (Source: CLONTECH 5'AmpliFINDER RACE Kit protocol)
Construction of a tobacco cDNA library

A tobacco cDNA library was constructed in λgt 11 vector using Pharmacia Time Saver cDNA synthesis kit following the manufacturer’s instructions. PolyA⁺ RNA (1-5 µg) in 20 µl DEPC-treated water was first incubated with methylmercuric hydroxide and then denatured at 65°C for 10 min and chilled on ice. One µl of oligo(dT)₁₂₋₁₈ primer, 1 µl of DTT solution and the heat denatured mRNA were added to the first strand reaction mix of the kit containing reverse transcriptase and incubated at 37°C for 1 hour. The first-strand reaction mix (33µl) was transferred to the tube containing second strand reaction mix containing E.coli RNase H and DNA polymerase, mixed gently and incubated at 12°C for 30 min and then at 22°C for 1 hour. The reaction was then heated to 65°C for 10 min. The mixture was vortexed with 100 µl phenol/chloroform, centrifuged at room temperature and the upper aqueous layer was applied to a Sepharose 4B-CL spin column to purify the cDNA. The EcoRI/NotI adaptors were ligated to the cDNA ends using 30 µl PEG buffer, 1 µl ATP (diluted 1:4) and 1 µl T4 DNA ligase at 16°C for 1 hour. The DNA ligase was inactivated at 65°C and the cDNA ends were phosphorylated with 1.5 µl of 15 mM ATP solution and 1 µl T4 polynucleotide kinase at 37°C for 30 min. The kinase was inactivated at 65°C for 10 min and the reaction mix was vortexed with 140 µl phenol/chloroform. The upper aqueous layer was again purified by Sepharose 4B-CL spin column. The cDNA strands were ligated to λgt 11 arms, precipitated and packaged using Stratagene Giga Pack Gold Packaging Extract. The recombinant phages were titrated using E. coli strain Y1090r- and screened with
P\textsuperscript{32}-labeled TCB 60 cDNA probe (the cDNA was labeled with \(\alpha\)-P\textsuperscript{32}dCTP following the instructions of random priming kit from Gibco-BRL) to isolate full-length clones.

Isolation of tobacco genomic DNA and Southern hybridization

Mid-log phase tobacco suspension culture cells were harvested and ground to a fine powder in liquid N\textsubscript{2}. The powder (0.75 g) was added to 5 ml of preheated (60°C) CTAB DNA isolation buffer (2% w/v CTAB, 1.4 M NaCl, 0.2% v/v 2-mercaptoethanol, 20 mM EDTA, 100 mM Tris-HCl pH 8.0), swirled gently to mix and incubated at 60°C for 30 min (Doyle et al., 1990). The sample was gently extracted once with chloroform/isoamyl alcohol (24:1 v/v), the upper aqueous phase was removed with a wide-bore pipet and precipitated with 2.5 vol. of isopropanol. The precipitated DNA sample was washed with 76% ethanol and 10 mM ammonium acetate, centrifuged at 1600xg for 10 min. The pellet was air dried. The DNA was resuspended in TE buffer and treated with RNase A (10 \(\mu\)g/ml). The genomic DNA samples (20 \(\mu\)g) were digested with HindIII, EcoRI and XbaI and electrophoresed on an 1% agarose gel and transferred onto nylon membrane (Sambrook et al. 1989). A \textsuperscript{32}P-labeled probe was made with pTCB 60 cDNA insert using the Random Primers DNA Labeling System (Gibco-BRL) as described before. The hybridization was done at 60°C and blot was washed twice with 0.1X SSC and 0.1% SDS solution at 65°C and the blot was exposed to X-ray film at -80°C as described before (Sambrook et al. 1989).
Results and discussion

Cloning of the 5‘end and analysis of the full-length sequence of TCB60 messenger

The pTCB60 cDNA probe detected a 2.4 kb mRNA in the northern blot of tobacco total RNA, indicating pTCB60 is a partial cDNA clone (Fig. 3.2). A 900 bp cDNA fragment was obtained by reverse transcription of tobacco poly(A)^+ RNA using a gene specific primer (GSP1) from the known 5‘end sequence of the partial pTCB60 cDNA clone (Fig. 3.3). The PCR amplified 5‘end downstream fragment of TCB 60 messenger was subcloned into PCR II TA cloning vector for sequencing. Purified plasmid DNA isolated from one white colony was sequenced by primer walking using T7, SP6 and gene specific primers. The combined nucleotide sequence of the 5‘end cDNA fragment and the 1572 bp partial cDNA insert from pTCB60 clone is presented in figure 3.4.

The 2409 bp sequence contains a 1653 bp ORF encoding 551 amino acid residues. The ORF starts with the initiation methionine codon at nucleotide position 366 and ends at nucleotide 2019 followed by a TAA stop codon. The full-length mRNA contains a 365 bp 5’ untranslated and a 391 bp 3’ untranslated region. The estimated molecular mass of the protein deduced from the ORF of the full-length cDNA is 61.5 kD.
Figure 3.2 Northern blot hybridization of tobacco total RNA with the

cDNA probe.
Figure 3.3 Analysis of the PCR amplified 5'-end cDNA fragment of TCB60 messenger on 1.5% agarose gel. Lane 1, Mol. wt markers; lane 2, 5'RACE PCR product
Figure 3.4 Nucleotide sequence of the full-length tobacco cDNA clone encoding a calmodulin-binding protein. The deduced amino acid sequence (one-letter code) is indicated below the nucleotide sequence. The polypeptide start and stop codons are underlined. The gene specific primer, GSP1 was used for the synthesis of first strand cDNA by 5'RACE protocol and GSP2, GSP3 are nested gene specific primers for amplification of the 5'RACE product by PCR.

```
1 attgaaagagagagagaaggagaaaaagtggaaccccaagactcaaact
61 aaaccttcaagaattcaagctgcagggtattttggtcatatctgaagactgtatatgag
121 aggccaaaaatctatgaagaggagcttggagggtgatgaagcagcagccagccgtaa
181 aagggctgtctctagctgtatatttggaagcttcagctctcaagttcaaggtct
241 ctgctctagctttggcaccctcttctcgaggtgcttttaaatgagaggtggagcagcat
301 tggctaaattagaccctgtcgcaggttggctgaggttgagctctctcaagagataaggaggaag
361 gcccgatgcagaaaaactttgcaagttcagttcaggtctaggctatctcttttctgtctca

M E E T C S F S S G L G Y L Y P L F
421 tggagccaaagtgagggagcaaggtggtgtgtatctccatgtatgttgtgtgattgatgcag
T G A K V E G E H G A A I H V V L I D A
481 atactggccatctttgtgactactggagctgaatcatgtataaaactggatgttgtgtgc
D T G H L V T T G A E S C I K L D V V V
541 tagaaggtgatatttcaacactgaagagatgagaggtggagcagcaagagagagattgacagtc
L E G D F N T E D D E G W T Q E F D S
601 atggtgttaagagcggctagagaggaagagacatctcttttaactggtgagttgaagttacac
H V V K E R E G K R P L L T G E L Q V T
661 tcaaggaggttggagctcgctctactggagctcagataacatttcaatcagttgatagac
L K G G V G T L G D L T F T D N S S W I
721 gggagggatcaagacttggcagatggaaggttctgtggtatattgtagggactgtacgca
R S R K F R L G M K V A S G Y C E G V R
```
781  tacgagaagcaagactgaggctttcactgtgagatcaccgtggagaattgtacaaga
    IRE A K TE A F T V E D H R G E L Y K
841  aacattatccaccagctctactaatgacgatctgtcggagattggaagaaatttgcagcttg
    GSP3
    K H Y P P A L N D D V W R L E K I G K D
901  gttctttccacaaagagtttaaatagctgcatatattattctgtgagacattcttaggc
    GSP2
    G S F H K R L N K A A I F T V E D F L R
961  ttgtaggtgagagccgcagaattttctttggaagcggatagttacatagaaga
    L V V R D P Q K L R N I L G S M S N K
1021  tggaggtctctactaatgacgatctgatctgttgaggtggaagctttatgtct
    M W D A L I E H A K T C V L S G K L Y V
1081  attatctctgatttttccacaaatatttctgatgagtgaatgtg
    Y Y S D D S R N V G V V F N N I Y E L N
1141  gcttttagctggtgaaacaataactatagcggagcttaatctttcagcagggcaggaagttat
    GSP1
    G L I A G E Q Y Y S A D S L S D S Q K V
1201  atgtagttctcatatgctcaagagggattatggtgaaattgtgaaatgtatg
    Y V D S L V K K A Y D N W N Q V V E Y D
1261  gcaaggcttatagctagcagcaattttgacgctcagtaggtgcggaggtttcagttc
    G K S F L N I K Q N Q K A G S S R N E L
1321  cggaggccaggagttttacacccaacaacatgtagattaatcagctttccacaattcagacttac
    P V G P V D Y P N M N V Q L P Q S R L
1381  cggaggccaggagttttacacccaacaacatgtagattaatcagctttccacaattcagacttac
    P V S V Q S S M H D P N L I G G S
1441  gttataatgacgcataggttctgataagtcttaaaccacaaagtcagatgatgaattctagtgtt
    G Y N D S I V A R M P N Q S Q M N S S
1501  cggaggccaggagttttacacccaacaacatgtagattaatcagctttccacaattcagacttac
    S R S Q F E S T P F A P Q Q Q I T N T H
1561  agcttccaaagtagtaggtggctgctttttcagctgtctgtgctctccgcaaatcat
    Q L Q S T S Y D N N V G L A L G P P Q S
1621 catcattcagacaatgacctcatctcttcacaaaccacatcttaatccttttcaggaact
SSFSQTMTSLSLPQTNLNPFEDE
1681 ggccacacaacaggacacggcctggagtctctgtctctgaggagaatctggatga
WPHNRDKGVDFLSEEEIRM
1741 gaagccagaaatctttgagatgacgagatagcaacacttcgctgactctcagatgg
RSHELNDHQLLRLFSM
1801 gagcggcccatggctctgtcaatgtgtctgaagacggatagctgttttcctgctttcatgc
GGGHSVNVSEDGYGFPSPFM
1861 ccctacatctctactttggtttatgatgagcaccacaaaccttcaggaagaagctgcg
PSPSPFTGYDEDPKPSGKA
1921 tgggttggctgaaattagcctgcatgtgctggagatggagagattctttgtgaggaagaagcgag
VGWLKKIAAMRWFRVRKKA
1981 ctgagagggccacagattgtggaactggtgatgatgaatataatctcagggcttttgcggc
ERRAQIVELDDE
2041 ttacctgtaggcaaacatattcacaaggtctgtgatgatgagccgagtaat
2101 tagctacacagattttattttccccagggattagagtgaacagtctttcttcctttcg
2161 tcaaatagtttcttcatgtacaagacctagtacattttattttatgtctctctattg
2221 ttactcaacctgtctacttgtcttttatctctttcagatgtatagatgtctgag
2281 accaaagggaattataactttcacaagacactctgtgctctcctgtgtgtgtctattgtagg
2341 aactttatgtaatcaacagggatatcttggcttttgcattttgagcgttcaaaaaaa
2401 aaaaaaaaa
A computer-assisted homology search using the NCBI (NIH) nucleotide blast program revealed significant sequence identity with 1 rice, 3 maize and 5 Arabidopsis cDNA clones as summarized in table 3.1.

Table 3.1 Identities of Sag60 nucleotide sequence with other sequences

<table>
<thead>
<tr>
<th>Source organism</th>
<th>Length of the clone (bps)</th>
<th>No. of positives</th>
<th>% identity</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. thaliana</td>
<td>275</td>
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<td>80</td>
</tr>
<tr>
<td>A. thaliana</td>
<td>513</td>
<td>261/341</td>
<td>76</td>
</tr>
<tr>
<td>A. thaliana</td>
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<td>72</td>
</tr>
<tr>
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<td>109/154</td>
<td>70</td>
</tr>
<tr>
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<td>369</td>
<td>198/294</td>
<td>67</td>
</tr>
<tr>
<td>Rice</td>
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<td>78</td>
</tr>
<tr>
<td>Zea mays</td>
<td>583</td>
<td>370/479</td>
<td>77</td>
</tr>
<tr>
<td>Zea mays (CBP1)</td>
<td>671</td>
<td>225/352</td>
<td>69</td>
</tr>
<tr>
<td>Zea mays (CBP5)</td>
<td>991</td>
<td>533/843</td>
<td>62</td>
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</table>

The deduced amino acid sequences of two maize CaMBPs encoded by the partial cDNA clones CBP1 and CBP5, showed 63% and 45% identity with the deduced amino acid residues of full-length TCB60 message, respectively (Fig. 3.6). Significantly, the putative basic amphiphilic α-helix CaM-binding domains of TCB60, CBP1 and CBP5 are identical with the exception of a single amino acid substitution. The putative CaM-binding domains of CBP1 and CBP5 contain an isoleucine residue instead of a phenylalanine residue at position 532 of TCB60 protein (Fig. 3.5).
Figure 3.5 Comparison of the deduced amino acid sequences of tobacco TCB60, maize CBP1 and CBP5 messengers respectively. Asterisks indicate identical amino acids among the sequences. Dashes indicates spaces introduced to maximize alignment. Dots indicate the sequence has not started yet. Underlined sequence indicates the putative basic amphiphilic α-helix CaM-binding domains.

<table>
<thead>
<tr>
<th>Sequence</th>
<th>Sequence</th>
<th>Sequence</th>
<th>Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sag60</td>
<td>Sag60</td>
<td>CBP1</td>
<td>CBP5</td>
</tr>
<tr>
<td>MEETCSFSSG LGYLYPLFTG AKVEGEGAA IHVVLIDADT GHLVTQGAEES</td>
<td>CIKLDVVLTE GDFNEDDEG WTQEEFDHSV VKEREGRPL LTGELQVTLK</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C1</td>
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<td>C1</td>
</tr>
<tr>
<td>Sag60</td>
<td>Sag60</td>
<td>CBP1</td>
<td>CBP5</td>
</tr>
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<td></td>
<td></td>
<td></td>
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</tr>
<tr>
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<td>Sag60</td>
<td>CBP1</td>
<td>CBP5</td>
</tr>
<tr>
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<td></td>
<td></td>
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</tr>
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<tr>
<td>Sag60</td>
<td>Sag60</td>
<td>CBP1</td>
<td>CBP5</td>
</tr>
<tr>
<td>R<em><strong><em>AFC** <strong>S</strong></em>F</strong>SE <strong>D</strong></em>LFA <em>A</em>****** <em>MYI</em>****</td>
<td></td>
<td></td>
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</tr>
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<td>C1</td>
</tr>
<tr>
<td>Sag60</td>
<td>Sag60</td>
<td>CBP1</td>
<td>CBP5</td>
</tr>
<tr>
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<td></td>
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<tr>
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</tr>
<tr>
<td>Sag60</td>
<td>Sag60</td>
<td>CBP1</td>
<td>CBP5</td>
</tr>
<tr>
<td>GL<strong>P</strong>PKK* SA'TGQVETH A<em>R</em><strong>L</strong>S **TMSS<em>AC</em> GHHHQ*ELIL</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C1</td>
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<td>C1</td>
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</tr>
<tr>
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<td>Sag60</td>
<td>CBP1</td>
<td>CBP5</td>
</tr>
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</tr>
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<td>Sag60</td>
<td>Sag60</td>
<td>CBP1</td>
<td>CBP5</td>
</tr>
<tr>
<td><em>GMA</em>TKSQH IHLSCRRPHF MSHRH<em>MTHF RSYHVPC</em>QG LQMM*WVWNN</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C1</td>
<td>C1</td>
<td>C1</td>
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<td>Sag60</td>
<td>CBP1</td>
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<tr>
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<td></td>
<td></td>
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<tr>
<td>Sag60</td>
<td>Sag60</td>
<td>CBP1</td>
<td>CBP5</td>
</tr>
<tr>
<td>A<strong>V</strong>V<strong>I</strong>M<strong>S <em>KTFG</em></strong>*</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>
Computer-assisted structural analysis of TCB60 protein

The hydrophobicity analysis of a protein sequence shows unbroken stretches of hydrophobic and hydrophilic residues and detects the periodicities in the distribution of such residues along the sequence of the protein. Kyte and Doolittle plot (Kyte and Doolittle, 1982) is the most widely method for analyzing the hydrophobicity of proteins. The hydrophobicity or hydropathy profile of the deduced amino acid sequence of the protein encoded by the full-length TCB60 cDNA was analyzed using Kyte-Doolittle scale in GCG (Genetic Computer Group) sequence analysis program. The distribution of the clusters of hydrophobic and hydrophilic residues along TCB60 protein sequence is presented in figure 3.6.
In recent years, hydrophobicity analysis is the most important method for predicting structure of membrane proteins (von Heijne, 1987). The membrane-spanning domains of TCB60 protein were identified using topology prediction II (Top Pred II) computer program based on von Heijne algorithm of distribution of amino acid residues (Claros and von Heijne, 1994). In topology prediction program the 20-residue window length GES scale of Goldman-Engelman-Steitz (Engelman et al., 1986) identified 1 certain and 2 putative transmembrane domains spanning 3-23, 409-429, 489-509 amino acid residues of TCB60 protein respectively (Fig. 3.7 A and B). The Kyte-Doolittle (KD) scale (Kyte and Doolittle, 1982) detected 2 putative membrane-spanning (residues spanning positions 28-48 and 184-204 respectively) segments in TCB60 protein (Fig. 3.8 A and B). Using the 8-residue window length of β-amphipathic scale ten transmembrane segments spanning residues 30-38, 57-65, 103-111, 123-131, 193-201, 232-240, 276-284, 334-342, 349-357, 526-534 amino acid residues respectively were identified. Out of the ten domains, the first one is a hydrophobic region while the remaining nine are amphiphilic segments (Fig. 3.9 A and B).
Figure 3.6 Kyte-Doolittle hydrophobicity plot of TCB60 protein sequence
**Figure 3.7 A** The membrane-spanning hydrophobic domains are predicted based upon the topology prediction II computer program. Using a 20-residue window length, the Goldman-Engelman-Steitz hydrophobicity scale indicates the presence of 1 certain (amino acid residues 3-23) and 2 putative membrane-spanning domains (amino acid residues 409-429 and 489-509 respectively).

**Figure 3.7 B** The topology of the membrane spanning domains identified by GES (Goldman-Engelman-Steitz) hydrophobicity scale of Top Pred II program.
Figure 3.8 A Kyte-Doolittle (KD) hydropathy profile of the deduced amino acid sequence of TCB60 protein based upon the algorithm of von Heijne (Claros and von Heijne, 1994) Top Pred II program. Two transmembrane domains spanning amino acid residues 28-48 and 184-204 were identified.

Figure 3.8 B The topology of the putative membrane-spanning domains identified by Kyte-Doolittle hydrophobicity scale of von Heijne Top Pred II program.
Figure 3.9 A \( \beta \)-amphipathic hydrophobicity profile of TCB60 protein based upon von Heijne topology prediction program. The 8-residue window length identified 10 transmembrane domains spanning amino acid residues 30-38, 57-65, 103-111, 123-131, 193-201, 232-240, 276-284, 334-342, 349-357, 526-534 respectively.

Figure 3.9 B Topology of the membrane-spanning domains based upon the \( \beta \)-amphipathic hydrophobicity scale of von Heijne Top Pred II program
As an alternative approach to determine the complete sequence of the mRNA, a tobacco λgt 11 cDNA library was rescreened using radiolabelled pTCB 60 cDNA as a probe. Six positive clones were isolated, of which one clone contained a much larger insert than TCB 60 cDNA insert. The 3.5 kb new cDNA hybridized strongly with pTCB 60 probe (Fig. 3.10). The 3.5 kb cDNA fragment was amplified by PCR and subcloned into TA cloning vector for sequencing (Fig. 3.11). A 2.4 kb sequence in the orientation of SP6 promoter was identical to the full-length sequence of TCB 60 mRNA obtained with 5' RACE protocol confirming those results. The sequence of the 1.1 fragment from T7 promoter orientation was different. The 1.1 kb fragment downstream from the 2.4 kb fragment was amplified by PCR and a probe was made for northern blot hybridization. The labeled 3.5 kb cDNA insert probe detected two messages (2.4 kb and 1.1 kb) whereas the labeled 1.1 kb fragment probe detected only a 1.1 kb message in northern blots of tobacco total RNA indicating that the cDNA fragment downstream of the 2.4 kb region of the large cDNA may be a cloning artefact due to ligation of another cDNA fragment (fig 3.12).
Figure 3.10 Southern blot hybridization of the 3.5 kb cDNA insert in λgt 11 with pTCB60 cDNA probe.
Figure 3.11  Restriction analysis of the 3.5 kb cDNA subcloned in PCR II TA cloning vector with BsiXI enzyme.
Figure 3.12 Northern blot of tobacco total RNA. Panel A, the 3.5 kb $^{32}$P-labeled cDNA probe hybridized with two bands; Panel B, the 1.1 kb cDNA probe made with PCR product downstream from the 2.4 kb cDNA fragment (the sequence of the 2.4 kb fragment is identical to TCB60 sequence) of the 3.5 kb cDNA clone hybridized with a single 1.1 kb.
Southern detection of the corresponding genomic fragments in tobacco genomic DNA

The $^{32}$P-labeled 1572 bp pTCB60 cDNA insert was hybridized to HindIII, EcoRI and XbaI digested tobacco genomic DNA. Since the full-length TCB60 cDNA sequence contains 1 HindIII, 3 EcoRI and no XbaI sites, Southern hybridization of tobacco genomic DNA digested with HindIII, EcoRI and XbaI enzymes should generate at least 2, 4 and 1 hybridizing bands. The Southern blot autoradiograph revealed the presence of 3 fragments (4.7, 2.6 and 0.7 kb), 4 fragments (5.1, 2.2, 1.7 and 0.9 kb), and 2 fragments (9.2 and 5.3 kb) in HindIII, EcoRI and XbaI digested lanes, respectively (Fig. 3.13). The numbers and sizes of the hybridizing bands in the Southern blot indicate the TCB60 gene in tobacco genomic DNA may contain intervening sequences containing restriction sites for HindIII and XbaI or may be a different copy of the gene since *Nicotiana tabacum* is an aneuploid.

Differential expression of TCB60 mRNA during culture growth and heat shock response of cultured tobacco cells

The steady-state level of every mRNA is regulated both by its rate of synthesis and rate of degradation (Newman et al., 1993). The steady-state levels of TCB 60 mRNA during tobacco cell culture growth and heat shock response (38°C) were analyzed. Analysis of tobacco cell cultures indicates that cell division is most pronounced during the first 2-3 days after transfer (Harrington unpublished results). The northern blot of
Figure 3.13  Southern blot detection of TCB60 corresponding fragments in tobacco genomic DNA. Lanes 1, 2 and 3, 20 μg genomic DNA digested with HindIII, EcoRI and XbaI restriction endonucleases respectively.
tobacco total RNA shows, TCB60 mRNA level is high in rapidly dividing cells and declines sharply in stationary phases of culture growth (Fig. 3.14). Several studies have shown that CaM and CaMBP such MAP-2, tau (Lee and Wolff, 1984; Sobue et al., 1981) and tubulin (Job et al., 1981; Job et al., 1982) are present on the mitotic apparatus of both plant and animal cells and may play a role in the assembly and disassembly of microtubules (Marcum et al., 1978; Job et al., 1981; Lee and Wolff, 1984; Schliwa et al., 1981). High levels of CaM and CaM-dependent enzymes including NAD kinase (Allan and Trewavas, 1985), glutamate decarboxylase (Ling and Assman, 1992; Ling et al., 1994) have been reported in actively dividing meristematic zones of roots (Muto and Miyachi, 1984; Coccuci and Negrini, 1988).

In heat shock response, the synthesis of many mRNAs and proteins are repressed, some mRNAs and proteins are expressed constitutively, while transcription and translation of the mRNA encoding a new set of proteins called heat-shock proteins are initiated to provide thermotolerance to the organisms (Vierling, 1991; Lindquist, 1986, Parsell and Linquist, 1993). The steady-state levels of TCB60 mRNA decreased rapidly (up to 70%) in the initial 2 hours of heat-shock treatment followed by a gradual decrease during the subsequent hours of heat-shock (Fig. 3.15). There may be two possible explanations for this rapid decrease; the rate of TCB60 mRNA transcription is repressed or the mRNA is rapidly degraded during heat-shock treatments. Although previously it was reported that most control mRNA species are maintained in intact polysomal complexes and can be reactivated after recovery from heat-shock (Nover, 1984), a recent study on the inhibition of α-amylase synthesis in barley aleurone layer during heat-shock
Figure 3.14 Northern blot analysis of TCB60 mRNA at different stages of culture growth. Lanes 1, 2, 3, 4, total RNA (20 μg) from 4, 8, 12, 16 days old cultured tobacco cells respectively probed with labeled pTCB60 cDNA.
Figure 3.15 Northern blot showing expression of TCB60 mRNA at different time intervals of heat-shock (38°C) treatments. Lanes 1, 2, 3, 4, total RNA (20 μg) from control, 2h, 4h, and 8h heat-shocked (7 days old) cultured tobacco cells.
treatment supports the hypothesis that rapid mRNA degradation occurs during heat-shock
treatment (Belanger et al., 1986, Sticher et al., 1990).

Recently McClure et al., (1989) identified a 40 bp sequence, designated as DST,
in the 3' untranslated regions of SAUR (small auxin-up RNA) genes of soybean which
control the stability of SAUR transcripts. The DST sequences were also identified in
auxin-inducible cDNAs of mung bean, *Arabidopsis* and tobacco (Newman et al., 1993)
A 13 bp segment of this 40 bp cis-acting element is present in the coding region (bases
876-888) of TCB60 mRNA sequence with the exception of a single base substitution.
Further research is necessary to determine whether this 13 bp segment regulates the
steady-state levels of TCB60 mRNA.
CHAPTER IV

PURIFICATION OF THE RECOMBINANT CALMODULIN BINDING PROTEIN ENCODED BY pTCB60 cDNA CLONE AND IMMUNODETECTION OF THE NATIVE CaM-BINDING PROTEIN IN TOBACCO CELL EXTRACTS

Introduction

The physiological roles of calcium and CaM in cellular metabolism and signal transduction pathways are mediated through the activities of Ca^{2+}/CaM-binding proteins. Although several cDNA clones encoding CaMBPs of various molecular sizes have been isolated from different plants, the identities of these proteins remain unknown. In plants only a few enzymes such as NAD kinase, Ca^{2+}ATPase, nuclear nucleoside triphosphatase and glutamate decarboxylase are known to be stimulated by CaM. Recently, molecular cloning techniques have been used to identify and characterize calmodulin-binding proteins present in low concentrations. In this chapter, a two-step protocol using CaM-sepharose affinity chromatography followed by Protosorb immunoaffinity chromatography to purify the recombinant CaM-binding protein encoded by pTCB60 cDNA clone is presented. The purified recombinant protein was used to raise polyclonal antisera for immunodetection of the native calmodulin-binding protein in tobacco cell extracts. The differential expression of the native CaMBP during growth and heat-shock response of cultured tobacco cells and in various tissues of tobacco plants was analyzed by
immunoblots using the polyclonal antisera raised against the purified recombinant protein.

Materials and methods

Plant Materials

Tobacco (*Nicotiana tabacum* L. cv Xanthi) plants were grown in a growth chamber at 25°C under 16 and 8 hours continuous dark and light cycles. Tobacco (*Nicotiana tabacum* L. cv Wisconsin 38) cell suspension cultures were grown in Gamborg's B-5 medium (Gamborg, 1970). For heat-shock treatment 7-days old cultures were incubated at 38°C shaking water bath (Harrington and Alm, 1988).

Gel electrophoresis of proteins

The protein samples in Laemmli sample buffer were separated in single dimension SDS-PAGE (Laemmli, 1970) and in two-dimensional mini IEF/SDS-PAGE by modified method of O'Farrell et al., 1977. The protein samples were dissolved in IEF sample buffer containing 5% ampholines (pH 3-10 and pH 5-7 in 1:1 volume). The protein samples were applied to the basic end of the prefocussed tube gels containing 5% ampholines. The gels were focused for 10,000 V.h with an additional 2 h focussing at 1,000 V.h (Moisyadi and Harrington, 1989). The tube gels were prepared for separation on SDS-PAGE slab gels with a H₂O wash followed by three washes (10 min. each) in
boiling Laemmli buffer. The tube gels were placed on top 12.5% SDS-PAGE mini slab gels and held in place using 3% agarose in Laemmli sample buffer. Electrophoresis was done at 150 volts until the bromophenol blue reached the running front. The gels were visualized by silver staining (Goederham, 1984) or by 0.1% Coomassie R-250 in aqueous 50% methanol and 10% acetic acid (w:v:v) (Moisyadi and Harrington, 1989).

Purification and analysis of CaM-binding fusion protein

The pTCB 60 cDNA clone was grown in 500 ml LB medium with 5 µg/ml ampicillin, 0.5 mM IPTG at 37°C until the A600 reached 0.6 (Lu and Harrington, 1994). The bacteria were collected by centrifugation at 5000g for 10 min. and resuspended in the extraction buffer (buffer A) containing 50 mM Tris-HCl pH 7.5, 3 mM MgCl2, 5 mM KCl, 0.2 mM EDTA, 1 mM PMSF, 0.5 mM pepstatin, 0.5 mM leupeptin, and 0.5 mM aprotinin. The bacterial cells were lysed by sonication and centrifuged at 5000g for 10 min. at 4°C. The supernatant was adjusted to 1 mM CaCl2 and applied to a 5 ml CaM-Sepharose-4B column. The column was washed with buffer B (25 mM Tris-HCl pH 8.0, 3 mM MgCl2, 2 mM KCl, 0.1 mM CaCl2) and a gradient of 0.15 to 0.3 mM NaCl, at a flow rate of 0.9 ml/min. The putative CaM-binding proteins were eluted as 5 ml fractions in buffer B containing 1 mM EDTA instead of CaCl2. The fractions containing the peak A280 were dialyzed extensively against 20 mM ammonium bicarbonate, lyophilized and dissolved in 200 µl dH2O. The lyophilized sample was resuspended in TEP buffer (100 mM Tris-HCl pH 7.4, 10 mM EDTA and 1 mM PMSF). Three
volumes of saturated ammonium sulfate solution were added to the TEP protein suspension at 4°C. The ammonium sulfate precipitate was centrifuged at 10,000 x g for 20 min, the supernatant was discarded and the pellet was redissolved in a minimum volume of TEP buffer. The protein in TEP buffer was applied to the Protosorb column, equilibrated with 5 ml of 50 mM Tris-HCl buffer pH 7.3. The column was washed with TN buffer (50 mM Tris-HCl pH 7.3, 0.2% NP-40) until the A_{280} reached the baseline. Then the column was eluted with 3 successive 1 ml aliquots of elution buffer (0.1 M NaHCO_3/Na_2CO_3, pH 10.8) followed by 1 ml of TBS (50 mM Tris-HCl pH 7.3, 150 mM NaCl) to re-equilibrate the column. The purified protein was analyzed on 12.5% SDS PAGE. The purified protein (250 µg/injection) was injected into rabbit four times (at two weeks interval each time) to raise polyclonal antisera (Laboratory Animal Science Facility, University of Hawaii at Manoa).

Immunodetection of the native CaMBP in tobacco cell extracts

Total protein was extracted from 7-days old tobacco (Nicotiana tabacum L. cv Wisconsin 38) suspension culture cells in 2X Laemmli buffer (Laemmli, 1970) containing 1 mM PMSF, 0.5 mM pepstatin, 0.5 mM leupeptin and 0.5 mM aprotinin. The protein was precipitated with cold ethanol and resuspended in 1.0 N NaOH. The quantity of protein was estimated by the Lowry et al., (1951) method. The protein extract was separated on a 12.5% SDS PAGE and soaked in transfer buffer (25 mM Tris, 192 mM glycine and 20% methanol) for 15 minutes. The SDS PAGE separated proteins were
electrophoretically transferred to PVDF membrane at 50 volts for 3 hours in cold transfer buffer (Towbin et al., 1979; Pluskal et al., 1986). The blot was equilibrated with phosphate-buffered saline (PBS 150 mM NaCl, 10 mM NaPO₄ pH 7.2) for 15 min. and blocked with blotto (150 mM NaCl, 10 mM Na₂HPO₄/NaHPO₄ pH 7.2, 5% non-fat dry milk, 0.01% antifoam A emulsion) for 1 hour. Then the blot was incubated for 3 hours with the polyclonal antibodies, raised against the purified recombinant protein in rabbit. The blot was washed with Blotto for 20 min followed by blotto/NP-40 for 20 min and Blotto for 20 min. Then the blot was incubated with alkaline phosphatase conjugated goat anti-rabbit second antibody and again washed blotto/NP-40 and TBS (Tris-buffered saline, 10 mM Tris-Cl, 150 mM NaCl pH 8.0). The bands on the blot were visualized by color development with BCIP (50 mg/ml in 70% DMF) and NBT (50 mg/ml in 70% DMF) in alkaline phosphatase buffer (100 mM Tris-Cl, 100 mM NaCl and 5 mM MgCl₂·6H₂O) for 10 min.

Analysis of expression of TCB60 protein during growth and heat shock response of suspension cultured tobacco cells and in various tissues of tobacco plants

The steady state levels of TCB60 protein during tobacco cell culture growth was determined by immunoblot analysis of total protein isolated from 4, 8, 12, 16 and 20 days old cultured tobacco cells. The expression of TCB60 protein during heat shock response was analyzed by immunoblot of total protein isolated from 7-days old (mid-log phase) tobacco suspension culture cells after 0, 2, 4, 6 and 8 hours heat-shock (on water
bath rotary shaker at 38°C) treatments. The protein samples (10 μg from each sample) were separated on 12.5% SDS PAGE, and electrophoretically transferred to PVDF membrane. The steady-state level of TCB60 protein was examined by immunoblot analyses using the polyclonal antisera raised against the purified recombinant CaMBP as described above.

Total protein (20 μg) isolated from tobacco leaves, stems, and roots were separated on a 12.5% SDS PAGE and electrophoretically transferred to PVDF membrane. The tissue-specific expression of TCB60 protein was analyzed by immunodetection with the polyclonal antisera as described above.

Results and discussion

Purification of the CaM-binding fusion protein

The total protein extracts isolated from XL-1 Blue E.coli cells harboring pBluescript phagemid only (control) and the cDNA clone pTCB 60 (pBluescript with a 1572 bp cDNA insert) were analyzed on a 12.5% SDS PAGE (Fig. 4.1). The 1184 bp ORF of pTCB60 encodes 393 amino acid residues with an estimated molecular mass of 48.5 kD. A prominent additional band of molecular mass 48.5 kD was observed in the protein profile of E.coli harboring pTCB60. This 48.5 kD protein disappeared from the protein profile after multiple passes of the crude protein extract through CaM-sepharose-4B column in the presence of 1 mM CaCl$_2$ (Fig. 4.1, lane 3) indicating Ca$^{2+}$-dependent
Figure 4.1 SDS-PAGE separation of 15 μg total protein from lane 1, control cells (XL-1 Blue cells harboring pBluescript phagemid only); lane 2, pTCB60 cDNA clone before loading on CaM-sepharose column; lane 3, pTCB60 cDNA clone after multiple passes through CaM-sepharose column
binding of the protein to CaM-Sepharose-4B affinity column. Analysis of the proteins eluted from CaM-sepharose column with 5 mM EGTA on one and two-dimensional SDS-PAGE revealed the presence of a major 48.5 kD protein along with several minor-nonspecifically bound proteins (Fig. 4.2 and 4.3). The 48.5 kD band was absent from the elution profile of the control cells (Fig. 4.2, lane 2).

Due to the presence of a 4 kD β-gal fragment at the N-terminus of the recombinant protein, the partially purified protein was further purified with Protosorb immuno-affinity chromatography since anti-β-gal is coupled to the Sepharose-4B matrix. Analysis of the proteins eluted in high pH buffer from the Protosorb column on one and two-dimensional SDS-PAGE revealed a single band of molecular weight 48.5 kD (Fig. 4.4 A and 4.4 B).
Figure 4.2 SDS-PAGE analysis of the proteins eluted from CaM-sepharose column chromatography. Lanes 1 and 2 eluted fraction 2 of pTCB60 cDNA clone and control bacterial cells (XL-1 Blue cells containing phagemid only) respectively.
Figure 4.3 Two-dimensional SDS-PAGE separation and silver staining of twenty μg pTCB60 cDNA clone proteins purified by CaM-sepharose affinity chromatography.
Figure 4.4 Purification of CaM-sepharose purified recombinant protein by Protosorb immuno-affinity chromatography and analysis on (A) one-dimensional and (B) two-dimensional SDS-PAGE and visualized by silver staining.
Immunodetection of the native CaM-binding protein

The polyclonal antisera raised against the purified recombinant protein recognized peptides of approximately 50 and 60 kD molecular masses in the western blot of tobacco total protein extracts (Fig. 4.5 A). Upon preadsorbing the polyclonal antisera with the control E.coli (XL-1 Blue bacterial cells containing pBluescript phagemid only) cell lysate a single band of molecular size ~60 kD was observed in the western blot (Fig. 4.5B). Thus the molecular mass of the native tobacco CaMBP detected by the antisera agrees with the predicted size of the protein encoded by the 1653 bp ORF of the full-length coding sequence of the messenger.

Differential expression of TCB60 protein during growth and heat shock response of tobacco suspension cultured cells

Changes in steady-state levels TCB 60 protein at various stages of tobacco cell culture growth were analyzed by immunoblots of tobacco total protein isolated from 4, 8, 12, 16 and 20 days old cultured tobacco cells. The protein level decreased gradually during the mid-log and stationary phases of culture growth following the same pattern expression as TCB60 mRNA during tobacco cell culture growth (Fig. 4.6). This result suggests the steady-state levels of TCB60 protein may be directly correlated to TCB60 mRNA levels during culture growth. Several studies have indicated high levels of CaM
Figure 4.5 Immunodetection of the native tobacco CaM-binding protein by western blot analysis. Panel A antisera without preadsorption and Panel B antisera preadsorbed with control E.coli cell lysate (XL-1 Blue cell with pBluescript phagemid only). Lane 1, five μg of purified recombinant protein; Lane 2, fifteen μg of total tobacco protein.
**Figure 4.6** Immunoblot analysis of tobacco total protein at various stages tobacco cell suspension culture growth. Lanes 1, 2, 3, 4 and 5 total protein (10 µg) from 4, 8, 12, 16 and 20 days old cultured tobacco cells respectively.
and CaMBP in actively dividing cells (Roberts and Harmon, 1992; Ling and Assman, 1992; Coccuci and Negrini, 1988; Muto and Miyachi, 1984).

Immunocytochemical studies have shown the presence of CaM and CaMBP proteins such as MAP-2, tau and tubulin (Lee and Wolff, 1984; Ohta et al., 1990) in the mitotic apparatus of animal cells. Presence of high levels of CaM and CaM-dependent NAD kinase have been reported during early cell development in the root apices of *Pisum sativum* (Allan and Trewavas, 1985). Thus, the high levels of TCB60 protein observed in exponential and mid-log phases of cultured tobacco cells suggest the CaMBP may be involved in some Ca\(^{2+}/\)CaM-dependent processes during cell division and growth.

During heat-shock response (HSR) the protein synthesis patterns change in all organisms. The normal protein synthesis is shut-down or down-regulated and a new set of proteins called heat-shock proteins (HSPs) are synthesized (Nover, 1984; Ho and Sachs, 1989; Vierling, 1991; Lindquist S, 1986; Parsell and Linquist, 1993). In animal cells the cytosolic calcium level increases (Stevenson et al., 1986; Calderwood et al., 1988; Landry et al., 1988) and the expression of many calmodulin-binding heat-shock proteins including hsp70 (Stevenson and Calderwood, 1990), hsp74 (Clark and Brown, 1986), hsp90 (Nishida et al., 1986), hsp100 (Koyasu et al., 1989) are induced in response to heat-shock. To date, nothing is known about the role of the Ca\(^{2+}/\)CaM second messenger system in heat shock-response of plant cells. The steady state level of TCB60 protein decreased approximately 50% in the initial 2 hour of heat-shock (38°C) followed by gradual decrease during prolonged heat-shock treatments (up to 16 hours) (Fig. 4.7). The decrease in TCB60 protein level corresponds to the rapid
Figure 4.7 Western blot of tobacco total protein at various time intervals of heat shock treatment. Lane 1, control; lanes 2, 3, 4, 5 and 6, total protein (20 μg) from 2, 4, 8, 12 and 16 h heat shocked suspension cultured tobacco cells.
decrease observed in the mRNA level during heat-shock treatments of cultured tobacco cells (Fig. 3.11). The decline in TCB60 may be due to low levels of TCB60 mRNA available for translation, due to inhibition of normal protein synthesis, or destabilization of the protein due to abnormal modifications during heat shock. Further research, using mRNA from the heat-shocked cultured tobacco cells in a cell-free translation system would reveal whether TCB60 protein level is a reflection of the decline in TCB60 mRNA level during heat-shock response.

Expression of TCB60 protein in various tissues of tobacco plants

First, to determine whether the 61.5 kD native CaMBP detected in cultured tobacco cells is synthesized in tobacco plants or not; and second, to get some clue about its possible function, the distribution of the protein in various organs of tobacco plants was examined. Immunoblot analysis of tobacco leaf, stem and root proteins indicated that TCB 60 protein was expressed only in tobacco leaf tissues (Fig. 4.8). Many physiological processes occurring in leaf tissues including phytochrome mediated chloroplast development and Cab (chlorophyll a/b-binding protein) gene expression (Neuhaus et al., 1993) and leaf senescence (Huang et al., 1990) are known to be regulated by Ca\(^{2+}\)/CaM. Previous studies also indicate that circadian leaf movements (Toriyama and Jaffe, 1972) and ABA-regulated guard cell swelling leading to stomatal closure (McAinsh et al., 1992; Schroeder and Hagiwara, 1989) are Ca\(^{2+}\)-dependent processes. Thus, the organ-specific expression of TCB60 protein in tobacco leaves may
have some physiological significance in leaf-specific Ca\(^{2+}\)/CaM-regulated pathways.

Several smaller cross-reacting bands were also detected in the root tissues (Fig. 4.8). Since the protease inhibitor cocktail containing PMSF, leupeptin, pepstatin, aprotinin and Na\(_2\)EDTA was used during protein extraction, the smaller cross-reacting bands may not partially degraded fragments of the native CaMBP. Previous studies have reported higher levels CaM are present in root apices of pea (17 fold) (Allan and Trewavas, 1985) and maize (4 fold) than the other parts of the plant (Stinemetz et al., 1987). CaM also plays a role in gravitropic responses (Yang and Evans, 1990; Stinemetz et al. 1987; Muto and Miyachi, 1984). The expression of CaM-binding proteins of various molecular sizes in \(Vicia\ faba\) roots is also regulated tissue-specifically and developmentally (Ling and Assman, 1992). Thus, it may be possible that the cross-reacting bands observed in root tissues are low molecular weight CaMBPs containing similar antibody-binding epitopes as TCB60 protein.
Figure 4.8 Immunoblot analysis of total protein isolated from tobacco leaf, stem and root tissues. Lanes 1, 2, 3, total protein (20 μg) from leaf, stem and root tissues respectively.
CHAPTER V

CHARACTERIZATION OF THE BASIC AMPHIPHILIC &-HELIX
CALMODULIN-BINDING DOMAIN OF THE 61.5 kD TOBACCO PROTEIN

Introduction

Calmodulin (CaM) is one of the best characterized components of the Ca$^{2+}$-signalling pathway. Analysis of the crystal structures show CaM is a small dumb-bell shaped molecule with two globular Ca$^{2+}$-binding domains connected by a long extended &-helix (Babu et al., 1985). Although CaM itself does not have any enzymatic activity, upon binding Ca$^{2+}$ it acts as molecular switch and activates a number of enzymic and non-enzymic proteins. In the absence of Ca$^{2+}$, the hydrophobic sites of CaM are concealed but upon binding Ca$^{2+}$ the two hydrophobic patches become exposed and interact with the target sites of many structurally and functionally diverse proteins (Means, 1988; Meador et al., 1993; Ikura et al., 1992).

Although the sequence of CaM is highly conserved among plants, animals and yeast, the sequences of CaM-binding sites of the target proteins vary considerably (Harrington et al., 1994). In many animal CaMBPs a basic amphiphilic &-helix (BAA) motif has been identified as the CaM binding domain. The BAA is structurally conserved and is characterized by a sequence of 15-20 amino acids that form a helical wheel projection with the predominance of basic residues on one face and hydrophobic
residues on the other. Another common feature of BAA CaM-binding domains is the presence of a tryptophan residue. Using synthetic binding domains and hydrolytic fragments of CaMBPs containing the BAA, it has been shown that CaM interacts with BAA motifs with high affinity and broad specificity.

Helical wheel projection of the deduced amino acid residues encoded by the cDNA clone using computer-assisted protein secondary structure prediction program of Chou and Fasman (1978) and gel overlay assays of cDNA deletion constructs (Lu and Harrington, 1994) indicated the presence of a putative BAA at the C-terminus of the protein (Fig. 5.1). A 19 amino acid residue peptide; Gly-Trp-Leu-Lys-Ile-Lys-Ala-Ala-Met-Arg-Trp-Gly-Phe-Phe-Val-Arg-Lys-Lys-Ala, corresponding to the basic amphiphilic α-helix motif of a recombinant tobacco calmodulin-binding protein (CaMBP) sequence was synthesized and its interaction with CaM was analyzed by gel mobility shift assays, phosphodiesterase competition assays, fluorescence spectroscopy, circular dichroism and nuclear magnetic resonance techniques.

Materials and methods

Peptide synthesis and purification

The synthetic peptide of 19 amino acid residues corresponding to the basic amphiphilic α-helix motif of the fusion protein was made by standard solid-phase synthetic methods using an Applied Biosystems 430 A automated synthesizer (University
Figure 5.1 Chou and Fasman helical wheel projection of the putative CaM-binding domain of TCB60 protein
of Hawaii Biotechnology Facility). The synthetic peptide was purified by reverse-phase high pressure liquid chromatography (HPLC) on a C$_{18}$ column and the most prominent peak eluted with gradients of 0.1% (v/v) aqueous trifluoroacetic acid and acetonitrile was lyophilized and dissolved in nano-pure deionized water. A small fraction of the peptide was hydrolyzed in 5.7 N HCl (containing phenol and β-mercaptoethanol) and heated under vacuum at 110°C and dried. The dried sample was dissolved in sodium citrate buffer pH 2.0 and amino acid analysis was done by the Ninhydrin Detection System of a Beckman Model 6300 Amino Acid Analyzer. The peptide concentration was determined using molar extinction coefficient of 11000 cm$^{-1}$ M$^{-1}$ at 280 nm due to the presence of two tryptophan residues in the peptide (molar extinction coefficient of each tryptophan residue is 5500 cm$^{-1}$ M$^{-1}$).

**Gel mobility shift assays**

The ability of the peptide to bind CaM was confirmed by relative mobility shifts of CaM on 12.5% native, SDS and 4M urea PAGE (Erickson-Viitanen and De Grado, 1987) in presence of the peptide and CaCl$_2$. For native gels 0.5 μM CaM and 0.5 μM synthetic peptide were incubated at room temperature for 30 min in 50% sucrose and 0.1% bromophenol blue sample buffer with 1 mM CaCl$_2$ or 5 mM EGTA (Chrambach et al., 1976) and separated on 12.5% polyacrylamide minigels using 63 mM Tris, 0.05 N HCl (pH 7.47) lower tank buffer and 37.6 mM Tris, 40 mM glycine (pH 8.89) upper tank buffer. For SDS PAGE, 12.5% minigels were made (as described previously by
Harrington and Alm, 1988). CaM (0.5 μM) and the synthetic peptide (0.5 μM) were incubated at room temperature for 30 min in the presence of 1 mM CaCl$_2$ and 5 mM EGTA respectively, then 10 μl of 2X Laemmli buffer were added to the samples and the samples were loaded on the gel. Urea gels contained 12.5% acrylamide, 4 M urea, 0.375 M Tris-HCl pH 8.8 and 0.1 mM CaCl$_2$ or 2 mM EGTA and run at constant voltage of 100 volts in electrode buffer consisting of 25 mM Tris-Cl, 192 mM glycine, pH 8.3 and 0.1 mM CaCl$_2$ or 2 mM EGTA. Calmodulin (100 nM) and increasing concentrations of synthetic peptide (100, 150, 200, 250 and 300 nM) were incubated at RT for 1 h in 100 mM Tris-HCl pH 7.2, 4 M urea and 0.1 mM CaCl$_2$ or 2 mM EGTA and 50% glycerol with tracer bromophenol blue was added before loading the samples on gel.

**Phosphodiesterase competition assay**

In phosphodiesterase competition assays the hydrolysis of cyclic AMP to AMP by phosphodiesterase was measured by HPLC (Watterson et al., 1980) using a Beckman model 110 A pump. For competition assays the reaction mixture contained 40 mM Tris-HCl, pH 8.0, 1 mM CaCl$_2$, 0.4 mM MnCl$_2$, various concentrations of cyclic AMP, enzyme preparations and CaM. For each reaction, 20 μl of sample was injected and eluted isocratically on a (250 x 4.6 mm) of anion exchage resin (Partisil-10 SAX) with 25.0 mM KH$_2$PO$_4$, pH 4.0, at ambient temperature and flow rate of 2.0 ml per min were detected by a Waters 990 Photodiode Array Detector, analyzed by a Waters 990
integrator (installed in a NEC APC IV™ advanced personal computer, Power Mate 2) and recorded by Waters 990 plotter. The basal level of enzyme activity was determined in the absence of CaM and the stimulated activity was determined in the presence of CaM and CaCl₂. Increasing concentrations (20, 40, 60 and 80 nM) of synthetic peptide were used to inhibit stimulation of phosphodiesterase activity in the presence of 10⁻⁸, 10⁻⁷ and 10⁻⁶ M CaM. The column was washed with 50 mM KH₂PO₄, pH 4.0 before starting each experiment.

**Fluorescence spectroscopy**

The presence of a tryptophan residue is a unique feature of most basic amphiphilic α-helix CaM-binding domains identified and characterized so far. Thus, the changes in the microenvironments of the tryptophan residues of the peptide upon binding calmodulin were investigated by fluorescence spectroscopy (Malenick and Anderson, 1984) using a SLM 8000C spectrofluorometer (SLM Aminco, Champaign, Illinois) modified with data acquisition electronics and software from ISS (ISS Inc., Champaign, Illinois). The excitation and emission slit widths were 2 nm with the emission spectra scanning done at 10 nm/min and a 1-cm path length cuvette. The synthetic peptide (5 μM) incubated in 5 mM Tris-HCl pH 7.3 and 0.5 mM CaCl₂ at RT for 30 min was excited at the excitation wavelength for tryptophan residues at 295 nm and the fluorescence emission spectra in the range of 290 nm to 440 nm recorded. Calmodulin (to a final concentration 5 μM) was added to the same cuvette from a highly concentrated stock solution to
minimize dilution effects and the emission maximum shifts between 290 to 440 nm recorded in the presence of 0.5 mM CaCl$_2$. EGTA 2 mM (from a 1 M stock solution) was added to the peptide+CaCl$_2$ sample and peptide+CaCl$_2$+CaM sample and the emission spectra again recorded.

**Circular dichroism**

The circular dichroism spectra was recorded on a Jasco 600 spectropolarimeter equipped with a HP 7475A plotter (Hewlett Packard). Each spectrum was the average of four scans and smoothing of base-line-corrected spectra done by digital filtering. The spectra of 20 µM synthetic peptide and 20 µM CaM in 5 mM Tris-HCl, 0.5 mM CaCl$_2$ (pH 7.3) were recorded independently between 190 and 250 nm using a 1.2 mm cell path length, sensitivity of 10 mdeg/cm, time constant of 16 sec, scan speed of 10 nm/min. The peptide (20 µM) and CaM (20 µM) were mixed in 5 mM Tris-HCl, 0.5 mM CaCl$_2$ pH 7.3 CaM and incubated for 30 min at RT and the spectrum was recorded. To the same sample, EGTA (1 M stock solution) was added to a final concentration of 2 mM and the change in spectra was recorded.

**Nuclear magnetic resonance experiments**

The $^1$H NMR studies were done at the NMR Facility of the Chemistry Department, University of Hawaii. Both CaM and peptide samples were deionized by
passing through neutral Chelex-100. Neutral Chelex was prepared by washing 10 g dry resin with 50 ml 2 N HCl. The slurry was applied to a 5 cm x 0.5 cm column and washed with deionized distilled H₂O until the eluant was neutral. The pH of H₂O from (99.98%, low in paramagnetic impurities from Aldrich Chemical Company) was adjusted to 7.0. The eluant from Chelex-100 column was freeze-dried and dissolved in H₂O. The sample was placed in a 5 mm O.D. NMR tube (William Glass Co.) and freeze-dried. The final sample was prepared by adding 300 µl of H₂O (99.98%) to the NMR tube. All spectra were recorded on GE NMR instruments GN-Omega 500 Fourier Transform NMR spectrometer operating at 500.11 MHz frequency for H NMR. All spectra were recorded at 25°C and referenced to external dioxane in D₂O (δ = 3.53 ppm). One dimensional spectra were recorded using presaturation pulse sequence (t = 1.0 sec irradiation time) and a compensated pulse with a 90° flip angle. The spectra were digitized into 8 K complex data points using quadrature detection and Bessel filtration. Time domain data was exponential weighing function of 0.5 Hz, fourier transformer and phase corrected.

Results and discussion

Gel mobility shift assays

Although an apparent 2 kD increase in CaM Mr was observed in presence of the synthetic peptide and CaCl₂ on SDS PAGE (Fig. 5.2), it was not the best method for
analyzing mobility shift of CaM, because in SDS PAGE CaM shows a characteristic shift in the presence of EGTA (Lu and Harrington, 1994; Roberts et al., 1986). Complex formation between CaM and the peptide was also confirmed by gel electrophoresis in the presence of 4 M urea as low-affinity and nonspecific complexes are dissociated by urea and only tight and specific complexes were observed in the gel. Increasing concentrations of the synthetic peptide (100, 150, 200, 250 and 300 nM) bands with decreased mobilities relative to the CaM band (Fig. 5.3 A, lane 1) indicated the binding of synthetic peptide to CaM (Erickson-Viitanen and DeGrado, 1988). No shift was observed upon addition of 5 mM EGTA to the samples suggesting the binding is Ca$^{2+}$-dependent (Fig. 5.3 B).
Figure 5.2 SDS-PAGE showing the $\text{Ca}^{2+}$-dependent mobility shift of CaM upon binding the 19 amino acid residue (~2 kD) synthetic binding domain. Lane 1, CaM (1 $\mu$M) with 1 mM CaCl$_2$; lane 2, CaM with peptide (250 nM) and 1 mM CaCl$_2$; lane 3, CaM with 5 mM EGTA; lane 4, CaM with peptide (250 nM) and 5 mM EGTA; lane 5, peptide only.
Figure 5.3 Electrophoretic mobility shift of CaM complexed with the synthetic CaM-binding domain in presence of 4 M urea and 0.1 mM CaCl$_2$ (Panel A) or 2 mM EGTA (Panel B). Lane 1, CaM (100 nM) alone; lanes 2, 3, 4, 5 and 6, CaM (100 nM) plus 100, 150, 200, 250 and 300 nM peptide respectively.
Competition assays of the peptide with CaM-dependent phosphodiesterase

Competition assays using a CaM-activated enzyme, cyclic nucleotide phosphodiesterase, were designed to determine if the synthetic binding domain interacts with calmodulin at physiological nanomolar concentrations. As a positive control the basal level of phosphodiesterase activity was determined in the absence of CaM and the stimulation of activity was determined in the presence of CaM (Table 5.1 and Fig. 5.4). Measurements of the conversion of cyclic AMP to AMP by phosphodiesterase in presence of increasing concentrations of synthetic peptide and CaCl₂, indicate that the synthetic peptide competes with phosphodiesterase to bind CaM (Table 5.2 and Fig. 5.5). Based on a Dixon Webb plot of the quantity of AMP produced by CaM-stimulated (10⁻⁸, 10⁻⁷ and 10⁻⁶M CaM) phosphodiesterase in the presence of 20, 40, 60 and 80 nM synthetic peptide, the Ki (inhibition constant) of the synthetic peptide is estimated to be 15 to 20 nM (Fig. 5.6). Previous studies characterizing the CaM-binding domains of various proteins have indicated that CaM has very high affinity for the target sites (O'Neil and DeGrado, 1990). Synthetic peptides corresponding to the binding domains skeletal muscle phosphorylase kinase (Dasgupta et al., 1989; DeGrado et al., 1987) and myosin light chain kinase (Blumenthal et al., 1985) also showed inhibition constants in the nanomolar ranges.
Table 5.1 Measurement of production of AMP (nM) by increasing concentrations of phosphodiesterase (μg) in the presence and absence of CaM

<table>
<thead>
<tr>
<th>PDE concentrations (μg)</th>
<th>No CaM</th>
<th>10⁻⁷ M CaM</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1</td>
<td>4.7</td>
<td>15.22</td>
</tr>
<tr>
<td>2</td>
<td>10.96</td>
<td>27.46</td>
</tr>
<tr>
<td>3</td>
<td>15.17</td>
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<tr>
<td>4</td>
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<td>69.91</td>
</tr>
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<td>5</td>
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<td>70.75</td>
</tr>
</tbody>
</table>

Table 5.2 Inhibition of CaM-stimulated phosphodiesterase activity by TCB60 binding domain. Amount of AMP (nM) formed is measured by HPLC.

<table>
<thead>
<tr>
<th>CaM (M)</th>
<th>No Peptide</th>
<th>20 nM peptide</th>
<th>40 nM peptide</th>
<th>60 nM peptide</th>
<th>80 nM peptide</th>
</tr>
</thead>
<tbody>
<tr>
<td>10⁻⁸</td>
<td>51.00</td>
<td>42.00</td>
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<td>18.79</td>
</tr>
<tr>
<td>10⁻⁷</td>
<td>76.30</td>
<td>57.95</td>
<td>51.37</td>
<td>36.40</td>
<td>30.67</td>
</tr>
<tr>
<td>10⁻⁶</td>
<td>83.66</td>
<td>80.74</td>
<td>71.83</td>
<td>62.09</td>
<td>53.79</td>
</tr>
</tbody>
</table>
Figure 5.4 Stimulation of phosphodiesterase (PDE) activity by CaM. Conversion of cyclic AMP to AMP with increasing concentrations of PDE were assayed in the absence and presence of $10^7$ M CaM.
Figure 5.5 Inhibition of CaM-stimulated PDE activity by TCB60 synthetic binding domain. PDE activity was assayed in presence of varying concentrations of CaM (10⁻⁶, 10⁻⁷ and 10⁻⁸ M) and synthetic peptide (20, 40, 60 and 80 nM). The concentration of PDE was held constant.
Figure 5.6 Dixon-Webb plot of synthetic TCB60 CaM-binding domain inhibition of CaM-dependent phosphodiesterase (PDE). Nanomolar concentrations of peptides were assayed for competition with PDE for calmodulin. The Ki (inhibition constant) for the peptide was determined from the intercept of the Dixon-Webb plot.
Fluorescence emission spectra

Since calmodulin has no tryptophan residues and the synthetic binding domain contains two tryptophan residues, the conformational changes of the binding domain during complex formation with CaM are analyzed from the fluorescence emission spectra of the CaM-bound peptide excited at 295 nm. The emission maximum of the peptide excited at 295 nm depends on the rigidity and polarity of the molecular environment surrounding the indole group of tryptophan (O'Neil and DeGrado, 1990). The emission maximum of the peptide in the buffer only was 356 nm (Fig. 5.7). Upon addition of calmodulin and 0.5 mM CaCl$_2$, the fluorescence emission maximum of the synthetic peptide shifted toward shorter wavelength (from 356 to 335 nm) and increased in intensity (Fig. 5.8), indicating the tryptophan residues were in a hydrophobic environment such as the interior of CaM. Addition of 2 mM EGTA to the same reaction mixture completely reversed the shift in the emission spectrum (Fig. 5.9). The fluorescence spectra of free peptide and CaM-bound peptide were compared as shown in the figure (Fig. 5.10). These results agreed with the previous studies on myosin light chain kinase CaM-binding domain which indicate that the microenvironment of the tryptophan residues changes when calmodulin is bound to the target protein (Malenick et al., 1982; Lukas et al., 1986).
Figure 5.7 Fluorescence emission spectra of the peptide in presence of CaCl$_2$. 

Wavelength (nm)

Peptide + CaCl$_2$
Figure 5.8 Fluorescence emission spectra of CaM and peptide complex in presence of CaCl₂.
Figure 5.9 Fluorescence emission spectra showing the reversal of CaM and peptide interaction in presence of 2 mM EGTA.
Figure 5.10 Comparison of the fluorescence emission spectra of the free peptide and CaM/peptide complex in presence of CaCl₂
IH NMR of the interaction of TCB60 binding domain with Calmodulin

The IH NMR study of the interaction between the binding domains and CaM leads to the identification of the domains in CaM that interact with the peptide (Klevit, 1987; Vorherr et al., 1990). The changes in the aromatic portion of the IH NMR spectra of CaM and CaM/peptide complex were analyzed. The assignments given to the resonances are in general agreement with the previous literature (Seamon, 1980; Krebs and Carafoli, 1982; Ikura et al., 1985; Dalgarno et al., 1984; Vorherr et al., 1990). The aromatic portion of the IH NMR spectra of the free peptide is shown in figure 5.11. A comparison of the NMR spectra of CaM and CaM/synthetic peptide complex suggests that the interaction occurs with aromatic residues in both C-terminal and N-terminal half of calmodulin (Fig. 5.12). The resonances of the phenylalanine F89/F16 residues were more perturbed than the remaining aromatic resonances. The F16 residue was present in domain I and F89 residue was present in domain III at opposite ends of the calmodulin molecule. Thus the peptide interacted with the leading helices of domain I and III. This may lead to the folding of calmodulin molecule in a way to facilitate its interaction with a small specific site of the target protein. From the relative ratios of the signals of the the tryptophan aromatic residues of the peptide and the aromatic residues of CaM, the binding ratio between CaM and peptide was estimated to be 1:2. However, as reported before (Vorherr et al., 1990) it was difficult to make conclusive interpretations of the spectral changes due to interaction between CaM and the peptide based on 1D NMR.
Figure 5.11 The aromatic region of the 500 MHz NMR spectra of the synthetic binding domain.
Figure 5.12 Comparison of the aromatic portions of the $^1$H NMR spectra of (A) calmodulin and (B) calmodulin-peptide complex. The assignments given were in general agreement with those found in the literature as mentioned in the results and discussion. The sharp peaks in the region between 7.2 to 7.5 ppm (B) were due to the free tryptophan resonances of the unbound peptide.
Circular Dichroism

Circular dichroism (CD) is a powerful technique to investigate the changes in secondary structures of proteins (Klevit et al., 1985). The CD spectrum of CaM displayed the minima at 208 and 222 nm and a maximum at 195 nm, typical of most helical proteins. Analysis of the CD spectra of the free peptide, free CaM and CaM/peptide complex in presence CaCl₂ showed the calmodulin peaks at 208nm and 222nm shifted and increased in intensity upon binding CaM (Fig. 5.13). The ellipticity of CaM and peptide complex at 222 nm was more negative than free CaM and peptide, which suggested a significant increase in the α-helix content during complex formation. This result was supported by the previous studies on CaM-binding domains of animal proteins, which showed that the peptides form helices when they bind CaM, thus increasing the intensity of the peaks (Cox et al., 1985; DeGrado et al. 1985; McDowell et al., 1983; Giedroc et al., 1983). The binding was partially reversed by addition of 2 mM EGTA (Fig 5.13) indicating the interaction was Ca²⁺-dependent. Similar changes in ellipticity were observed in case of myosin light chain kinase whole protein as well as synthetic binding domain (Blumenthal and Krebs, 1987).
Figure 5.13 Circular dichroism spectra of calmodulin, peptide, calmodulin-peptide complex in the presence of 0.5 mM CaCl$_2$ and 2 mM EGTA.
CONCLUSION AND FUTURE PROSPECTS

Although calcium and calmodulin are implicated in the regulation of many cellular activities, little is known about the identities and function of calmodulin-binding proteins in plants. Thus, identification and characterization of plant CaMBPs are essential for developing an understanding of Ca\(^{2+}\)/CaM-mediated physiological responses in plants. The missing 5' end of pTCB60 was cloned by 5’ RACE (rapid amplification of cDNA ends) protocol and sequenced. A full-length cDNA clone was isolated by rescreening a tobacco λgt 11 cDNA library with pTCB60 cDNA probe. The full-length sequence the messenger encoding the CaMBP was 2409 bp and contained a 1656 bp ORF encoding 551 amino acid residues. The cDNA sequence showed various degrees of homology with 5 Arabidopsis, 3 maize and 1 rice partial cDNA sequences in the database. Database search of the deduced amino acid sequence of the full-length tobacco cDNA revealed 63\% and 45\% sequence identity with two maize CaMBP encoded by two partial cDNA clones, CBP1 and CBP5, respectively. No significant homology was found with any other known gene or protein sequences.

The recombinant CaMBP (48.5 kD) encoded by the tobacco cDNA clone, pTCB60, was purified from the bacterial expression cells to raise polyclonal antisera. Based on the CaM-binding ability, the protein was first purified by CaM-sepharose chromatography. Taking advantage of the 4 kD β-gal fragment at the N-terminus of the recombinant protein, the partially purified protein was further purified by Prosorb immuno-affinity chromatography. The development of the two-step purification protocol
using both CaM-sepharose and Protosorb immuno-affinity chromatography provides a powerful tool for purifying recombinant CaMBPs. Polyclonal antisera raised against the purified recombinant protein recognized a 61.5 kD polypeptide in western blots tobacco cell extracts that agrees with the predicted size of the polypeptide encoded by the 1653 bp ORF of the full-length cDNA clone.

The TCB60 mRNA and the corresponding protein showed differential expression during the course tobacco cell culture growth and heat shock treatments. The high levels of mRNA in exponential phases of growth and the sharp decline in stationary phases, and the slow and gradual decrease in the protein level during culture growth, may suggest the protein plays some role in plant growth and development. Previously it was shown that the mRNA level decreased 70% during a 2 hour heat-shock (38°C) period (Lu and Harrington, 1994). The mRNA level decreased gradually during prolonged (4 and 8 hours) heat shock treatments. The CaMBP level decreased an estimated 50% in 2 hour heat-shock treatment followed by slight gradual decreases during subsequent hours of heat-shock (4, 8, 12, and 16 hours). The presence of the low levels of protein in the cells even after 16 hours heat-shock implies that the protein may be essential for maintenance of cellular activities during heat-shock response. Immunoblot of tobacco leaf, stem and root proteins exhibited the tissue-specific expression of the protein in leaf tissues.

Calmodulin is known to regulate a large number of enzymes and structural proteins with varying structures and functions. To date little is known about the molecular basis of such interaction of CaM with diverse target enzymes. Many CaM-
dependent enzymes are activated by confirmational changes due to binding of Ca\(^{2+}\)/CaM. Thus it is essential to investigate the structural properties of the CaM-binding domains in the target proteins as well as its interactions with Ca\(^{2+}\)-bound CaM and with the microenvironment of the target protein itself. Secondary structure analysis of amino acid residues encoded by pTCB60 cDNA by helical wheel projection indicated the presence of a putative basic amphiphilic \(\alpha\)-helix CaM-binding domain at the C-terminus of the fusion protein. This study is the first report in plant CaMBPs characterizing the BAA CaM-binding domain using various biochemical and biophysical methods.

A 19 amino acid residue synthetic peptide was made corresponding to the BAA motif and its interaction with CaM in vitro was analyzed by various biochemical methods. The shift in relative mobility of CaM upon binding the synthetic peptide in presence of CaCl\(_2\) and an apparent 2 kD increase in CaM M\(_r\) could be visually observed in 4 M urea gels. The peptide was assayed for competition with CaM-dependent phosphodiesterase for binding calmodulin. Dixon and Webb plot of the competition assay indicate the inhibition was competitive with a Ki of 15-20 nM suggesting a high-affinity (in the nanomolar physiological range) interaction between the synthetic CaM-binding domain and CaM. The increased intensity and shift of the fluorescence maximum of the peptide toward shorter wavelength, upon binding Ca\(^{2+}\)/CaM indicate some change in the microenvironments of the two tryptophanyl residues of the peptide due to complex formation with CaM, as shown in the previous studies. The circular dichroism spectra of CaM/peptide complex relative to the CD spectra of free peptide and CaM, suggested that the helicity of the peptide was altered when it binds CaM. \(^{1}H\) NMR
studies identified that peptide interacts with the aromatic residues of the leading helices of domain I and III of the calmodulin molecule. Taken together, these data confirm that a structurally conserved basic amphiphilic $\alpha$-helix CaM-binding domain, similar to most CaM-binding proteins in animal systems was present in the 61.5 kD tobacco calmodulin-binding protein and its interaction with CaM at nanomolar concentrations, may be physiologically significant.

Future research should focus on identifying the function of the protein. Although the protein is expressed in tobacco leaves, the tissue-specific and subcellular localization of the protein is still unknown. The subcellular localization of the protein may provide help in identifying the function of the protein. It is important to analyze the expression of TCB60 mRNA and the corresponding protein after recovery from heat-shock treatments. Since the amino acid sequence of TCB60 CaM-binding domain is identical to the CaM-binding domains two partial maize CaMBPs with the exception of a single I/F substitution, it would be interesting to make an antibody against the synthetic BAA CaM-binding domain and investigate if this BAA CaM-binding motif was conserved in CaMBPs of various plant species. It was also necessary to find out how CaM interacts with the BAA CaM-binding domain of the protein in vivo and regulates the activity of the protein. These studies may provide some insight to the physiological role of the protein in Ca$^{2+}$/CaM-mediated signal transduction pathways.
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