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Isolation and characterization of calmodulin-binding heat shock proteins and cDNAs encoding calmodulin-binding proteins in cultured tobacco cells

Lu, Yingtang, Ph.D.

University of Hawaii, 1991
ISOLATION AND CHARACTERIZATION
OF CALMODULIN-BINDING HEAT SHOCK PROTEINS
AND cDNAs ENCODING CALMODULIN-BINDING PROTEINS
IN CULTURED TOBACCO CELLS

A DISSERTATION SUBMITTED TO THE GRADUATE DIVISION OF THE UNIVERSITY OF HAWAII IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY IN BOTANICAL SCIENCES (PLANT PHYSIOLOGY) AUGUST 1991

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Special thanks to Dr. John I. Stiles in whose lab I was well trained in molecular biology.

My wife, Shuping Liang's support and understanding helped to finish this study.
ABSTRACT

Calmodulin-binding heat shock proteins were characterized in cultured tobacco cells (Nicotinan tabacum L. cv Wisconsin-38). Analyses of $^{35}$S-labeled calmodulin-binding proteins (CaMBPs) purified by affinity chromatography and SDS-PAGE indicated that heat shock (38°C) enhanced/induced the synthesis of some CaMBPs while the synthesis of others was repressed during the heat shock response (HSR). The synthesis of CaMBPs with apparent molecular weights of 82, 78, 71, 68, 22, 20, 19.5 and 17 Kd was stimulated by heat shock.

A procedure for the isolation of cDNA clones encoding CaMBPs was refined. Twenty five positive cDNA clones were isolated by screening a tobacco heat shock cDNA expression library with $^{35}$S-CaM as a ligand probe. These clones produced peptides exhibiting Ca$^{2+}$-dependent, CaM-binding activity when assayed by gel overlay analysis. While most cloned mRNAs such as pTCB40 were unaffected by heat shock, two clones, pTCB60 and pTCB48 were heat shock-related. Analysis of Northern blot demonstrated that a 2.1 kb mRNA recognized by pTCB60 decreased by at least 70% in a 2 hour heat shock treatment. The expression of pTCB48 mRNA was induced by heat shock. This translationally active mRNA was detected after 15 minutes of heat shock and accumulated to maximum amounts after 1.5 hours. These results suggest that
The Ca\textsuperscript{2+}/CaM second messenger system plays a role in tobacco heat shock response.

The natures of CaM-binding domains were determined for pTCB48 and pTCB60. DNA sequences of these clones were determined and several deletion constructs from both the 5' and 3' ends of the inserts were constructed. The CaM-binding activities of the proteins generated from these deletion constructs were assayed by gel overlay analysis. These data combined with secondary structure analyses of deduced proteins localized the CaM-binding domains in the C-terminus of these proteins. The CaM-binding domain of TCB60 was a basic amphiphilic α-helix similar to that of several animal and human CaMBPs. No similar structure was found in the C-terminal region of TCB48 suggesting that an alternative structure is responsible for the CaM-binding.
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<tr>
<td>$A_{260}$</td>
<td>absorbance at 260 nm</td>
</tr>
<tr>
<td>$A_{600}$</td>
<td>absorbance at 600 nm</td>
</tr>
<tr>
<td>BSA</td>
<td>bovine serum albumin</td>
</tr>
<tr>
<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>CaM</td>
<td>calmodulin</td>
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<tr>
<td>CaMBP</td>
<td>calmodulin-binding protein</td>
</tr>
<tr>
<td>cAMP</td>
<td>cyclic AMP</td>
</tr>
<tr>
<td>cpm</td>
<td>counts per minute</td>
</tr>
<tr>
<td>cv</td>
<td>cultivar</td>
</tr>
<tr>
<td>DAG</td>
<td>diacylglycerol</td>
</tr>
<tr>
<td>DEPC</td>
<td>diethylpyrocarbonate</td>
</tr>
<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
</tr>
<tr>
<td>DNAse</td>
<td>deoxyribonuclease</td>
</tr>
<tr>
<td>DTT</td>
<td>dithiothreitol</td>
</tr>
<tr>
<td>E.coli</td>
<td>Escherichia coli</td>
</tr>
<tr>
<td>EDTA</td>
<td>ethylenediamine tetraacetic acid</td>
</tr>
<tr>
<td>EGTA</td>
<td>ethyleneglycol-bis-(β-amoetaethyl ether) N,N,N',N'-tetraacetic acid</td>
</tr>
<tr>
<td>g</td>
<td>gram</td>
</tr>
<tr>
<td>g</td>
<td>gravity</td>
</tr>
<tr>
<td>HS</td>
<td>heat shock</td>
</tr>
<tr>
<td>HSE</td>
<td>heat shock element</td>
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<tr>
<td>HSF</td>
<td>heat shock factor</td>
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<td>HSP</td>
<td>heat shock protein</td>
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<tr>
<td>HSR</td>
<td>heat shock response</td>
</tr>
<tr>
<td>IEF</td>
<td>isoelectric focus</td>
</tr>
<tr>
<td>IPTG</td>
<td>isopropylthiogalactoside</td>
</tr>
<tr>
<td>IP$_3$</td>
<td>inositol 1,4,5-triphosphate</td>
</tr>
<tr>
<td>Kb</td>
<td>kilobase</td>
</tr>
<tr>
<td>Kd</td>
<td>kilodalton</td>
</tr>
<tr>
<td>MLCK</td>
<td>myosin light chain kinase</td>
</tr>
<tr>
<td>MOPS</td>
<td>3-(N-morpholino)propanesulfonic acid</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger RNA</td>
</tr>
<tr>
<td>NP-40</td>
<td>nonidet P-40</td>
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<tr>
<td>oligo(dT)</td>
<td>oligothymidylic acid</td>
</tr>
<tr>
<td>PEG</td>
<td>polyethylene glycerol</td>
</tr>
<tr>
<td>pfu</td>
<td>plaque forming units</td>
</tr>
<tr>
<td>PIP$_2$</td>
<td>phosphatidylinositol-4,5-bisphosphate</td>
</tr>
<tr>
<td>PKC</td>
<td>protein kinase C</td>
</tr>
<tr>
<td>PLC</td>
<td>phospholipase C</td>
</tr>
<tr>
<td>PMSF</td>
<td>phenylmethylsulfonyl fluoride</td>
</tr>
<tr>
<td>poly(A)</td>
<td>poly(adenylated)</td>
</tr>
<tr>
<td>PVP</td>
<td>polyvinylpyrrolidone</td>
</tr>
<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
</tr>
<tr>
<td>RNP</td>
<td>ribonucleoprotein</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulfate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>SDS-polyacrilamid gel electrophoresis</td>
</tr>
<tr>
<td>Tris</td>
<td>Tris[hydroxymethyl]aminomethane</td>
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CHAPTER I
LITERATURE REVIEW

INTRODUCTION

All organisms are subjected to a large number of biotic and abiotic stresses. Environmental perturbations such as light, heat, anaerobiosis influence gene expression. Recent studies on the responses of organisms to stress have focused on the analysis of gene expression. The heat shock response (HSR) provides a convenient system for investigating mechanisms of gene expression in a wide variety of organisms and is the subject of intense investigation.

The HSR was first investigated in the fruit fly Drosophila melanogaster by Ritossa (227). When Drosophila cells are shifted from normal growing temperature (25°C) to an increased temperature (37°C), a novel set of heat shock proteins (HSPs) is synthesized with the rapid shutdown of most normal protein synthesis. The mRNAs encoding the HSPs result from de novo transcription and are selectively translated during heat shock. Thus, the HSR involves both transcriptional and translational control of gene expression. A number of lines of evidence support the contention that the presence of HSPs in cells confers
tolerance to subsequent, more intense heat stress. However, little information is available on the possible functions of HSPs in thermotolerance. Moreover, the mechanisms by which heat-shock induces/represses genes and how the cell senses the heat-shock signal and converts the signal into responses are unclear. Several lines of evidence suggest that Ca\(^{2+}\)/calmodulin mediated processes are involved in the HSR (56, 141, 151, 192, 271, 299).

**HEAT SHOCK RESPONSE**

All organisms respond to higher than normal growing temperatures through profound alterations in gene expression. The major features of the HSR are the shutdown of most normal protein synthesis; de novo synthesis of heat shock mRNAs and heat shock proteins (HSPs), and the acquisition of thermotolerance to otherwise non-permissive heat stress (66, 132, 133, 163, 164, 189). In general, the initiation of HSP synthesis occurs within minutes after the start of heat shock treatment. The maximum induction of HSP synthesis requires about 10°C increase above normal growth temperature for a variety of different organisms. For example, *Drosophila* cells are normally grown at 25°C and HSPs are initially induced when the temperature is raised to 29°C. Optimum HSP synthesis occurs at 36-37°C (161). At the
optimum temperature, heat shock mRNAs are produced within four minutes and within an hour several thousand heat shock-specific transcripts are present in each cell (160). The heat shock specific mRNAs are translated with very high efficiency (160). At the same time, both the transcription of previously active genes (20, 95, 124, 269) and the translation of pre-existing control mRNAs are suppressed (162). Normal protein synthesis gradually resumes when cells are returned to normal temperature (163).

The HSR of most plants such as tobacco (111), spinach (263), wheat (113, 143), soybean (135), barley (57) and sorghum (57) is generally similar to that of Drosophila and other systems (163). In soybean seedlings the synthesis of normal proteins is greatly decreased and HSPs are induced when subjected to heat shock treatment at 40°C for 4 hours (131). Similar results are also obtained in cultured tobacco and tomato cells in which heat shock proteins are induced by heat shock treatment with little constitutive proteins synthesis (111, 195). Some exceptions are observed in the tropical crops such as maize (61, 62) and sugarcane cells (185) where the HSR does not result in complete shutdown of normal protein synthesis. This may mean that different regulation mechanisms for the HSR exist in these tropical plants.
The relationship of the HSPs and development of thermotolerance has been subjected to great attention. Many lines of evidence indicate that the synthesis of heat shock proteins is necessary for the development of thermotolerance (18, 100, 111, 150, 158, 168, 177, 185, 235, 246, 275) although a few contradictory reports also exist (33, 109, 220). In brief, data show increased survival of cells exposed to a normally lethal temperature treatment if the cells are first subjected to a less extreme heat treatment (heat shock). The development and decay of thermotolerance closely parallels the rate of HSP accumulation and decay when cells are returned to normal temperature. Similar results are obtained in plant systems. In cultured tobacco cells, treatment of heat shock (38°C for two hours) confers thermotolerance to otherwise lethal temperature (8 minutes at 54°C) (111). Tomato and sugarcane cells require the synthesis of the small heat shock proteins to achieve thermotolerance (185, 194).

Several lines of direct evidence for the essential role of HSPs has been reported (152, 226, 235). HSP 70 has been demonstrated to be necessary for thermotolerance in animal systems (226). Injection of antibodies against HSP 70 into rat cells results in the inability to develop thermotolerance (226). A role of small HSPs in thermotolerance development is suggested since a
Dictyostelium mutant which is deficient for the synthesis of several of the small heat shock proteins lacks the ability to survive extreme temperature (235). Recently, genetic transfection experiments indicate that HSP 27 plays a major role in thermoresistance. The thermotolerant phenotype can be conferred to Chinese hamster and mouse cells by transfection with the human HSP 27 gene (152).

Heat shock proteins are induced not only by heat shock, but also by a wide variety of agents such as amino acid analogs (129), arsenite (134), ABA and water-stress (112), salt stress (111), methomyl (224), metals (69, 283), light (103) and many other factors (43). Some stresses induce a complete set of HSPs while others do not, suggesting that there is a set of stress proteins common to several forms of stress. Further evidence demonstrates that the expression of certain heat shock genes including HSP 90 and small HSPs are developmentally regulated (32, 51, 69, 74, 107), implying a role of the HSPs in normal cellular growth.

HEAT SHOCK PROTEINS

Heat shock proteins are generally defined as whose synthesis is sharply and dramatically induced/enhanced at high temperature. The genes for such proteins have heat
shock elements (HSE, see below) which account for strong
induction upon exposure to elevated temperature (163, 276).
The major HSPs can be grouped into two size classes based on
SDS-PAGE. The large HSPs range from 68 to 110 Kd while
small HSPs range from 15 to 30 Kd (35). When proteins
extracted from heat-shocked soybean seedlings are separated
by SDS-polyacrylamide gel electrophoresis, ten HSP bands are
distinguished on one-dimensional gels. A more complex
pattern appears on two-dimensional gels with more than 60
labeled proteins (131). These phenomena are ubiquitous in a
variety of experimental systems. Using cultured tobacco
cells, Harrington and Alm (1988) indicate that many proteins
are induced during heat shock. Apparent molecular weights
of 94, 80, 71, 50, 48, 44, 41, 40, 36, 30, 28, 26, 25, 23,
22 and 20 to 15 (kd) have been reported (111). Two-
dimensional gels reveal that there may be as many as 100
polypeptides synthesized during heat shock in tobacco cells
(S. Dharmasiri, personal communication). Of these HSP 94,
80, 71 and small HSPs (20 to 15 Kd) are most prominent.

The HSPs show a remarkable conservation throughout
evolution. The larger HSPs appear to be more highly
conserved than the smaller HSPs. In fact, HSP 70 has been
suggested to be the most conserved protein in nature. A
polyclonal antibody against chicken HSP 70 cross-reacts with
HSP 70 from yeast, dinoflagellates, slime molds, maize,
worm, frogs, Drosophila, mice, rats and humans (130). Analyses of HSP 70 genes from different species indicate that eukaryotic families of related genes for HSP 70 evolved from a single bacterial gene (66, 115, 163). Yeast has eight genes for the HSP 70 family and these are grouped into four subfamilies (SSA, SSB, SSC and SSD) with homologies ranging from 96% to 50% with each other (165). Comparison of the deduced amino acid sequences of cloned HSP 70 genes from different eukaryotic species reveals a high degree of homology, ranging between 60% and 70%. (66, 163). Furthermore, E. coli HSP 70, DnaK gene product, is 48% identical to the HSP 70 of yeast and Drosophila (12). Plant HSP 70 genes have been isolated from maize (232), Arabidopsis (303), Petunia (305) and soybean (189). All these genes have homologies with each other ranging from 72% to 86%. With the exception of the soybean gene further similarities exist in the presence of an intron located in all of these plant genes (189).

Other major HSP families are HSP 110 and HSP 90. Mammalian cells produce proteins of 110 Kd and 100 Kd which do not appear to have counterparts in Drosophila (165). For the HSP 90 family, the genes have been cloned from several evolutionarily diverse organisms, including Drosophila, yeast, chickens, mammals and bacteria. Sequence analysis reveals that HSP 90 is the second-most highly conserved HSP
examined to date. The proteins from eukaryotic species have 50% identity and all have greater than 40% identity with the *E. coli* protein (13, 94, 165). Using a *Drosophila* HSP 83 gene fragment as probe, HSP 90 genes have been isolated from soybean (229), maize (262) and *Arabidopsis* (60).

Small HSPs have also been the subject of great interest, especially in plants where they are abundantly expressed during the HSR. In animal systems, the greatest proportion of HSP synthesis is represented by the high molecular mass HSPs of 68 to 110 kd with HSP 70 being the most abundant species (198). In *Drosophila*, several low molecular weight HSPs have been reported (19). In contrast, as many as 27 small HSPs have been detected in soybean seedlings (173). Varying numbers of small HSPs have been also reported in other plants: pea, sunflower, wheat, rice, maize, millet (173), cotton (42), tomato (195), tobacco (111) and sugarcane (185). Many genes for these small HSPs have been isolated from different organisms and sequence analyses indicate that these small HSPs of different organisms are clearly related (165, 274). However, small HSPs show much greater homology within organisms than between organisms. For example, members of a subgroup of the soybean small HSP family have 90% amino acid identity with each other but only 20% amino acid identity with the proteins of *Drosophila*, *Xenopus* and *Caenorhabditis elegans*
Yeast HSP 26 exhibits 30-50% C-terminal homology with small HSPs from *Drosophila*, *Xenopus*, Human and *C. elegant* (35).

HSPs may be modified by phosphorylation, methylation, ADP-ribosylation and/or glycosylation (89, 195, 196). HSP 89 is modified by phosphorylation (99) and methylation (290, 291). This is also true in tomato where HSP 80 and 70 are phosphorylated and methylated (195). Moreover, that many pre-existing proteins are modified during heat shock is evidenced in tomato (243). Ribosomal protein S6 is rapidly dephosphorylated when cells are subjected to heat shock and the protein is rephosphorylated after cells return to normal temperature (243). Further experiments also demonstrated that several other ribosomal proteins become phosphorylated during heat shock (244). Nuclear proteins are also modified during heat shock (45, 46). In light of these discoveries, it is possible that heat shock may result in induction or inhibition of specific phosphatases and/or protein kinases.

FUNCTIONS OF HEAT SHOCK PROTEINS

All available evidence indicates that the HSPs protect cells from damage of heat and other stresses (163, 165). The determination of the cellular localization of HSPs is of
great importance because it is reasonable to suppose that there is a logical connection between the HSP function and localization (184). Many methods including cell fractionation (287, 294), direct autoradiographic analysis (5, 284) and immunofluorescence (6, 286) have been employed for HSP localization.

Localization of Drosophila HSP 70 reveals that the protein is concentrated mainly within the nucleus and secondarily at the cell membrane after heat shock. During recovery from heat shock, the protein delocalizes from the nucleus and is found mainly in the cytoplasm (286). Furthermore, when the Drosophila HSP 70 gene is introduced into mammalian cells, the protein shows a very similar pattern of localization (213). This is consistent with the hypothesis that macromolecular complexes can be prevented from unfolding and denaturation by association with HSPs (247).

Heat shock causes precipitation of numerous nuclear proteins, damages the structure of partially assembled ribosomes and completely blocks nucleolar function (24). HSP 70 concentrates in nucleoli, binds to the nuclear matrix, associates with cytoskeleton and pre-ribosomes, and may protect pre-ribosomes from heat damage (56). Direct evidence for this comes from the studies on genetic
transfected animal cells (214). The *Drosophila* HSP 70 gene was placed under the control of adenovirus major late promotor and introduced into mouse L cells and monkey COS cells. HSP 70 was mostly found in the nucleus of unstressed cells but strongly concentrated in nucleoli after heat shock. When cells containing this chimeric gene were treated with actinomycin, followed by heat shock and recovery, nucleolar morphology and ribosome export resumed much more rapidly than in cells carrying no chimeric gene. This recovery requires neither RNA or protein synthesis. Taken together with observations that heat shock disrupts pre-ribosomal RNPs, these imply that HSP 70 may bind to damaged RNPs and catalyze their ordered reassembly (214).

HSP 70 and related HSPs are involved in a variety of cellular processes including DNA replication (165, 190), post-translational translocation of proteins across membranes (17, 53, 80), protection of RNA splicing (310), association with hnRNA (138) and organization of cytoskeleton (56), and uncoating coated vesicles (52, 280). These diverse functions have been suggested to be due to the ability of HSP 70 to prevent or disrupt inappropriate protein-protein interactions by binding to hydrophobic regions of proteins. This binding can be reversed with the aid of ATP hydrolysis (145). The fact that HSP 70 family can bind and hydrolyze ATP favors this idea (52, 295, 316).
Using ATP-derived energy, HSP 70 breaks protein-protein interactions and allows the denatured protein to refold or reassemble into the normal state. Recent evidence from *in vitro* experiments demonstrates that HSP 70 from soybean seedlings, together with other HSPs, has the ability to protect the control proteins from heat denaturation (126).

HSP 90 associates with membrane ATPase (39, 68), steroid hormone receptors (50, 237, 252) and tyrosine kinase (37, 311). The transforming protein of Rous sarcoma virus, pp60^src^ a tyrosine kinase, associates with HSP 90 and a 50 Kd phosphoprotein immediately after synthesis. When the kinase is disassociated from HSP 90, the kinase inserts into the membrane as a phosphoprotein and acts as a kinase (37, 38, 65, 204). These results suggest that HSP 90 may function to keep its targets in an inactive state. This suggestion has been supported by the observation that HSP 90 associates with glucocorticoid receptors preventing binding to DNA. The inactive complex is maintained until hormone disrupts the association of HSP 90 to the receptor (16, 44, 165, 239). Recent results have demonstrated that HSP 90 stimulates eIF-2α kinase activity by association with it (144, 233, 234). While HSP 90 is abundant in most cells, only a small portion is found to be associated with these cellular proteins and the bulk of HSP 90 is present as a
monomer (146, 153, 163). The significance of the excess amount of HSP 90 is presently unclear.

Structural characterization of small HSPs demonstrates that these HSPs have structural homology to the α-crystallin (35, 79, 120). In line with these characteristics, small HSPs may mediate effects in stressed cells via molecular aggregation either with themselves or with other related proteins. Recent evidence has shown that the major portion of small HSPs is present in large aggregates called heat shock granules. These are found mainly in the perinuclear region of heat shocked cells of tomato (194, 200), Drosophila (7) and vertebrates (8, 59). Untranslated constitutive or normal mRNAs are detected to associate with this cytoplasmic heat shock granule fraction (200), suggesting that small HSPs may play some role in conservation of untranslated control mRNAs during heat shock.

Ubiquitin, a highly conserved protein with a mass of approximately 8 KD, is found in all eukaryotic cells (30, 31, 97, 248, 256). It is synthesized as polyubiquitin and found in cells either free or linked via its terminal glycine residue to a variety of cellular proteins. Ubiquitin forms conjugated complexes with aberrant or unstable proteins in an ATP-dependent manner, conferring
selective degradation of these proteins (166, 207, 248). It has been suggested that ubiquitin and other HSPs provide complementary methods of dealing with the production of denatured protein aggregates in heat shocked cells (96).

Many other HSPs have also been characterized for their functions. For example, yeast hsp 48 is enolase (118) and another HSP is glyceraldehyde-3-phosphate dehydrogenase (G3PDH) (165). Recently, Ostermann and co-workers discovered that proteins imported from the cytosol into mitochondria do not refold spontaneously once translocation across the mitochondrial membrane is completed (205). A nuclear-coded, mitochondrial HSP 60 is involved in the folding of imported proteins in conjunction with ATP (205).

**REGULATION OF HEAT SHOCK GENE EXPRESSION**

The heat shock response is an ideal system for investigating molecular mechanisms of gene expression because of the speed of induction, the magnitude of the response and ubiquity in a wide variety of organisms. Many lines of evidence indicate that heat shock gene expression is under the control at either transcriptional or translational levels or both, depending on the organism. In *E.coli* (190, 307) and in yeast (162, 176), the response is
controlled primarily at the level of transcription. In contrast, the response of *Xenopus* oocytes is controlled at the translational level (22). In other systems including animals, plants and *Drosophila*, both transcriptional and translational controls are active. While the translational repression of most pre-existing normal mRNA during the HSR is common in many systems, translation of normal mRNA in sugarcane seems not to be repressed by heat (185). These differences provide impetus for investigation of the mechanisms of gene regulation.

**TRANSCRIPTIONAL REGULATION**

**Chromatin structure.** Many heat shock genes are quickly activated in a manner that results in the rapid accumulation of heat shock mRNAs under inductive conditions. The products of most heat shock genes are barely detectable or undetectable under normal conditions. For example, transcription of the heat shock genes in *Drosophila* can be fully induced within minutes of temperature elevation (161) and message levels increase 1000-fold within an hour after heat shock induction (285). It has been suggested that heat shock genes are preassembled into an open chromatin configuration at normal temperature, thus facilitating immediate activation under heat shock. This is based on the
results from the studies of hypersensitive sites of heat shock genes in chromatin (49, 64, 83, 279, 300). The genes for HSP 70, 83, 22, 23, 26, and 28 contain two to five DNase I hypersensitive sites at their 5' ends at normal temperature. However, heat shock treatment results in the changes in the position and number of hypersensitive sites (64, 279, 300). Evidence obtained from Drosophila HSP 70 and 83 genes indicates that the promoter regions containing heat shock elements (HSEs) are protected only after temperature upshift while the TATA-box region is protected in both heat-shocked cells and control cells (301). Moreover, the HSEs can be protected against DNase I digestion by applying an extract from heat shocked nuclei to unshocked nuclei (302). Similar results are also obtained for small Drosophila HSP gene promoters (49). Several regions containing HSEs are hypersensitive in non-heat shocked cells but protected after heat shock. These results are interpreted as evidence for binding protection by transacting protein factors. What prevents nucleosomes from covering the HSE region of HSP gene promoters is unclear although the results above suggest it may be the case. HSP gene transformation experiments done by Costlow and Lis (64) may provide a clue for this. After the Drosophila HSP 70 and 83 genes were introduced and integrated into yeast genome, the DNase I hypersensitivity of the promoter sequence was preserved. This implies that heat shock gene
sequences carry information necessary for specific hypersensitive structure in chromatin.

**Promotor structure.** Heat shock gene transcription is coordinately regulated and highly conserved in all eukaryotic species examined thus far. In spinach, *in vitro* translation of heat shock mRNAs results in the synthesis of all 35 HSPs. The mRNAs for all 35 HSPs are induced by heat shock at 32°C, indicating coordinate transcriptional regulation of all heat shock genes with respect to temperature while non-coordinate synthesis of HSPs is recorded (263). Similarly, coordinate heat shock mRNA expression has been also documented in maize (15). Additionally, transgenic expression experiments indicate that regulatory mechanisms of the heat shock gene transcription are highly conserved in different organisms (63, 251, 267). A chimeric gene construct which contained *Drosophila* HSP 70 promotor and the reporter gene, neomycin phosphotransferase II (NPT II), was introduced into tobacco cells. The NPT II gene was expressed in a heat-induced fashion in tobacco (267), suggesting a similar mechanism regulating heat shock gene expression across widely divergent species. This view is further strengthened by the observation that the cloned *Drosophila* HSP 70 gene is under heat shock control in mouse fibroblasts (63). The conservation of heat shock promotor function has been
indicated by the expression of the Drosophila HSP 70 promoter in mammalian (41, 63, 182, 212), amphibian cells (288) and in regenerated tobacco plants (268). These results suggest that heat shock gene promoter sequence *per se* carries the information required for heat shock activation and that the transacting factor(s) are conserved.

Sequence analyses of heat shock genes have identified a short sequence upstream of the TATA-box of Drosophila HSP 70 gene promoter that is essential for heat inducibility (182, 211). This palindromic consensus sequence (CT-GAA--TTC-AG), called the heat shock element (HSE), has been found within the first 400 base pairs upstream of every eukaryotic heat shock gene sequenced to date (24, 70, 71, 165, 187, 232, 249). Within the HSE, eight nucleotides, C--GAA--TTC--G, are highly conserved and seven of these are required in order to constitute an individual, functional HSE (23). Many lines of evidence show that it is HSE that confers heat-inducibility of heat shock genes (for review, see 24). This conclusion is strengthened by the findings that one synthetic HSE is sufficient for heat inducible transcription of the Herpes simplex virus thymidine kinase gene (212).

Many heat shock gene promoters contain multiple HSEs and the most proximal one is usually found 15-18 bp immediately 5' to TATA box (24). As many as seven HSE
copies have been reported in *Drosophila* HSP 26 gene promotor (83). Analyses of the 5' deletion mutants of the *Drosophila* HSP 70 gene in *Drosophila* cells (3) or germline transformants (87) indicated that deletion mutants which retained only the TATA-proximal HSE showed only 1% of the normal heat-induced expression. Thus, at least two copies of the HSE are needed for high levels of heat shock induced expression for *Drosophila* heat shock genes (87). This is consistent with the observation that multiple HSEs are required for maximal expression of heat shock genes in plant cells (71, 105). These results suggest the cooperative binding of multiple heat shock factors (HSFs, see below) to separate HSEs because the binding of HSF to HSE is necessary for the activation of heat shock genes (278).

The sequences immediately flanking the 14 base pair HSE have been demonstrated to play a role in the heat shock gene expression by using HSP 70-LacZ fusion genes containing variant synthetic regulatory regions in *Drosophila* (167). The importance of sequences flanking HSEs has been reinforced by mutational analyses of the *Drosophila* HSP 70 gene (4). The employment of high resolution methylation interference mapping also supports the importance of three to four bases flanking HSE for optimum binding of HSF (258). Based on the observations of *in vivo* assays of expression of HSP 70 gene containing variant synthetic regulatory regions,
it has been suggested that heat shock regulatory elements in gene promoters are composed of contiguous arrays of a 5 bp unit sequence, -GAA-, in alternating orientations (167). When normal upstream regulatory region of HSP 70 gene is replaced with two copies of -TTC--GAA--TTC--GAA- which are separated by 11 bp, This gene is expressed at a 6-fold higher level than HSP 70 gene containing the pair of perfect 14 bp consensus sequences and at a 5-fold higher level than HSP 70 gene containing two native HSEs separated by 11 bp (167, 306). This is supported by the evidence that heat shock factor can form a stable complex in vitro with an inverted repeat of 5 bp recognition unit, -GAA- (218).

**Heat shock factor.** Studies of heat shock gene regulation indicate that a transacting protein, heat shock factor (HSF), is involved in the activation of heat shock genes (1, 24, 49, 83, 208, 301, 302) even an HSF-independent mechanism for heat shock induction of transcription has been recently described (139). The HSF is present in some form prior to heat shock because heat shock genes can be activated without protein synthesis (9, 312). The binding of HSF to HSE-containing regions of the promoter upon heat shock is supported by studies of DNase I hypersensitive sites (301, 302). This is reinforced by similar recent observations in mouse embryonal carcinoma (EC) cells (181). Several correlations suggest the importance of HSF in the
induction of HSP genes by using heat shock inducible and non-inducible EC cell lines. These include the high spontaneous expression of some HSPs and the constitutive level of HSF activity at normal temperature, the heat-induction of HSP gene transcription and marked increase in HSF activity in inducible cells, and the deficiency in heat-activation of HSP genes and the loss of HSF activity in non-inducible cells upon temperature upshift. Moreover, yeast HSF and the Drosophila HSF can bind to each other's HSEs with approximately equal affinities (297).

The interaction between HSF and HSE for heat shock gene activation and the heat activation of chimeric genes with same heat shock upstream region in transformed cells of different organisms suggest that the HSF is also functionally conserved. This is supported by the isolation and characterization of HSFs in yeast (265, 298) and tomato (245). Sequence analysis indicates that yeast HSF is composed of 833 amino acids with a mass of about 93 Kd. A 118 amino acid region between positions 166 and 285 has been defined as the DNA-binding domain. However, analysis of this domain did not reveal resemblance to any currently known secondary structural motifs implicated in DNA recognition and binding. The HSF may have a novel secondary structural motif involved in a DNA binding (298).
The HSF exists in an inactive form in certain non-heat shocked cells (9, 191, 312). In human and Drosophila cells, pre-heat shock HSF is not able to efficiently bind HSE but acquires strong HSE-binding activity upon temperature upshift (136, 264, 312, 313). The HSF is interconverted between active and inactive forms in dynamic response to heat shock and recovery in the presence of protein synthesis inhibitor, cycloheximide. It is highly likely that some posttranslational modification is involved in this interconversion but the exact nature of this change has not yet been identified (312). Recent data indicate that human HSF can be activated to bind to HSE by treatment of pH, calcium and non-ionic detergent in vitro (186). A role of possible interaction between HSP 70 and HSF in the expression of all HSPs has been recently suggested by Craig and Gross (67).

Heat shock does not modify the HSE-binding activity of yeast HSF, in contrast. The yeast HSF binds HSE at all temperatures both in vitro (264, 265) and in vivo (122) and HSP genes are activated only after heat shock (264). Thus, the regulation of HSF-mediated activation of HSP genes does not occur at the DNA-binding step but involves the subsequent ability to activate transcription. Posttranslational activation of yeast HSF may be due to heat-induced phosphorylation. The mobilities of protein-HSE
complexes from heat shock and control are different and this
difference can be significantly reduced by the treatment of
crude heat shock extracts with phosphatase (264, 265).

RNA polymerase II must be involved in heat shock gene
transcription because it is responsible for mRNA synthesis.
Studies on the Drosophila HSP 70 gene demonstrate that this enzyme is associated with promotor region (between nucleotides -12 to +65 relative to the transcription start site at +1) in the absence of heat induction (101). RNA polymerase II partially transcribes Drosophila HSP 70 gene and forms a nascent RNA chain of about 25 nucleotides at normal temperature. Additional transcription can not continue without heat induction (236). Thus, heat shock promotor appears to be ready for transcription with the TATA factors constitutively bound and RNA polymerase engaged in transcription, but complete transcription is impossible until HSF is activated. However, how the heat shock signal is perceived and transduced and finally triggers the activation of HSF is still unknown.

TRANSLATIONAL REGULATION

Structure of heat shock mRNA. Inspection of heat shock mRNA structure reveals an unusually long 5' untranslated
leader sequence (longer than 200 base pairs). This region appears to contain two conserved sequences located in the middle and at the 5' end. Both are rich in adenosine residues with little secondary structure (108, 116, 119, 163, 164, 266). The translational preference of Drosophila HSP mRNAs during heat shock is suggested to be due to this relatively long 5' untranslated leader sequence (116, 137). This is reinforced by the observations that chimeric messages from fused genes which contain HSP 70 gene promoter and untranslated leader sequences are translated at high temperature (34, 84). A complementary result was obtained from the analyses of deletion mutants in the 5' untranslated leader of HSP 70 gene (178). The gene carrying a large deletion of 204 nucleotides from the total 242 nucleotide leader was transcribed very efficiently during heat shock, but the mRNA was not translated. These results clearly demonstrate that the 5' untranslated leader sequence is an important factor in determining selective translation during heat shock.

The identification of the precise sequence required for selective translation in the 5' leader sequence has proven difficult. The length of the 5' leader sequence is not a determining factor for selective translation (164, 178). A message from a reconstructed HSP 70 gene carrying inverse orientation of DNA fragment of nucleotides +2 to +205 in
leader sequence is not translated during heat shock (164). In contrast, messages from mutated HSP 70 and HSP 22 genes, in which nucleotides from +37 to +205 and from +27 to +242 were deleted respectively, were translated during heat shock (117). These results imply the importance of a conserved sequence at the 5' end because it was not deleted in these deletion mutants. However, deletion of the 5' conserved sequence or conserved middle sequence, even both conserved sequences does not affect the translation of messages from these mutants (164, 178).

A possible explanation for these results is that more than one specific sequence in leader including the 5' conserved sequence is required for selective translation. This view is reinforced by experiments with two mutants containing large deletions, one including the 5' conserved sequence and another not. Results indicated that the message lacking this conserved sequence was not translated during heat shock but the message containing this conserved sequence was translated (164, 178). However, comparison shows that sequences homologous to the conserved motifs in Drosophila heat shock messages are not found in soybean (250). Using deletion mutants of soybean heat shock gene Gmhsp 17.3-B promotor including the leader sequence and a reporter gene (CAT) encoding sequence, Schoffl et al (1989) demonstrated that effective translation during heat shock
requires sequences in heat shock mRNA leader region. But, these sequences can be functionally replaced by the 5' leader sequence of the CaMV 35S promotor (251). These results suggest that secondary structure, not certain sequences in the leader region may play an important role in translational efficiency of heat shock messengers (164, 250). Kozak (1988) demonstrated a dramatic reduction of the translational efficiency under hypertonic stress if the leader sequence of the reporter gene (preproinsulin II gene) mRNA is modified by incorporation of secondary structure elements (142). It is conceivable that a low potential for secondary structure formation, as indicated in heat shock leader sequence, is a prerequisite for Cap-independent unwinding and initiation of mRNA translation during heat shock (206).

Certain viral RNAs are translated efficiently in the absence of active Cap binding factors (216, 217) or during heat shock (77). These data favor the importance of having little secondary structure in the leader region for selective translation. This is reinforced by the recent evidence that heat shock impairs the interaction of the cap-binding protein complex with mRNA 5' cap (148, 149). The cap-binding protein complex binding to mRNA 5' cap with several other initiation factors mediates binding of the small ribosomal subunit to mRNA. Part of the function of
these factors appears to be the unwinding of mRNA secondary structure facilitating ribosome binding. As complex binding to cap is impaired during heat shock, unwinding must also be impaired. This will result in most mRNA being translated poorly. However, mRNAs with little secondary structure, such as HSP mRNAs, will have a selective advantage.

Cellular Components. One important change that occurs in cells during heat shock is the rapid disaggregation of non-heat shock polysomes and formation of heat shock polysomes (131, 160, 180). This rapid decay of control polysomes is evidently a typical stress phenomenon. The cytoskeleton, which is thought to be necessary for efficient translation under non-stress conditions (156), is disrupted during temperature upshift (25, 93). However, translation under heat shock conditions proceeds largely independent of cytoskeleton on free polysomes (93, 131). This difference may be important for translational regulation.

One aspect of the characterization of the mechanisms of translation is the identification of cellular factors involved in selective translational regulation. Lysates from control and heat-shocked cells were used for cell-free translations and the results indicate that heat shock lysates do not contain factors that inhibit the translation of control mRNAs in control lysates. Control lysates do
contain a factor that stimulates the translation of control mRNAs in heat shock lysates (240, 254, 255). In some cases this stimulatory activity was found in the ribosomal pellet (254, 255), or in the supernatants in others (240). This discrepancy may be due to different methods of fraction preparation. These results suggest that selective translation during heat shock may be controlled, in part, by heat-induced change of a factor that is required for the translation of control mRNAs.

Analyses of ribosomal proteins indicate that some proteins are phosphorylated while other are dephosphorylated during heat shock (243, 244). Of these, a ribosomal protein, S6, may be a good candidate as a cellular determining factor in translational control. This protein is rapidly dephosphorylated when plant cells are subjected to heat shock and rephosphorylated after cells are returned to normal temperature (243, 244). Considering its localization in the neck region of the small ribosomal subunit, which is part of the initiator tRNA binding site (28), S6 could be involved in selectively translational control during heat shock. However, prolonged incubation of tomato cells under heat shock (12 hr at 37°C) results in restoration of normal protein synthesis while S6 remains unphosphorylated (243).
The possible role of protein synthesis initiation factors in selectively translational control during heat shock has also been studied. Heat shock results in the activation of a protein kinase which phosphorylates the α-subunit of the eukaryotic initiation factor of eIF-2 (eIF-2α) and phosphorylated eIF-2α is dephosphorylated by a specific phosphatase (29, 78, 88, 91). Further analyses indicate that HSP 90 may be involved in the phosphorylation of eIF-2α because it is contained in a highly purified preparation of the heme-controlled eIF-2α kinase of rabbit reticulocytes (144, 233). HSP 90 does not phosphorylate eIF-2α or inhibit the eIF-2-mediated binding of Met-tRNA to 40S ribosomal subunits. HSP 90 has been suggested to increase the kinase activity based on the observation that enzymatic activity of the eIF-2α kinase is markedly increased by addition of the purified HSP 90 (144, 233, 234). The phosphorylation of eIF-2α leads to a failure of guanine nucleotide exchange on the initiation factor and subsequent inhibition of protein synthesis (78, 234). These results appear to implicate eIF-2α phosphorylation as a contributory mechanism in the inhibition of translational activity immediately following heat shock treatment and indicates a possible role of HSP 90 in the regulation of eIF-2α kinase activity (233, 234). The role of eIF-2α phosphorylation in regulating heat-shock translation is still controversial because others have not found this
phosphorylation (174). Recently, eIF-2α has been indicated to be a heat shock protein because its synthesis is stimulated by heat shock (58). This stimulation is even greater during the recovery period, suggesting that the induction of eIF-2α in the HSR may be important in restoring the ability to initiate normal protein synthesis (58).

Many lines of evidence suggest that a specific quantity of functional HSPs in a given heat treatment is needed for the recovery of normal protein synthesis during recovery period (81, 164). Drosophila HSP 70 is always the first protein to be repressed and the time at which HSP 70 reaches approximately 50% repression always coincides with the time at which normal protein synthesis reaches 50% recovery during recovery period (164). Furthermore, the time when normal protein synthesis is restored and HSP 70 synthesis is repressed correlates with the time when HSP 70 moves from the nucleus to cytoplasm (286). Therefore, HSP 70 may play a role in recovery of control message translation and in suppression of heat shock message translation (164, 183). Results from the distribution of small HSPs suggest a role in conservation of untranslated control mRNAs during heat shock. A major portion of these small HSPs is contained in heat shock granules carrying sequestered control messages (200). HSPs may also act to increase the instability of
heat shock messages during recovery because heat shock messages are stable in the absence of HSPs, even when cells are returned to normal temperature (81, 82).

CALCIUM, CaM AND CaM-BINDING PROTEINS IN THE HSR

Calcium and cAMP are two major second messengers for signal perception and transmission in animal cells (21, 222, 223). However, cAMP is apparently not a second messenger in plants (175, 221, 222) although there is convincing evidence for the existence of cAMP in plants (36). Calcium has been shown to mediate various plant physiological processes elicited by extracellular signals such as light, hormones and gravity (222). In plant and animal cells, the free calcium concentration is submicromolar in the resting state, rising up to as high as micromolar during excitation (2). Cells may regulate cytoplasmic calcium in a number of ways, including membrane permeability, calcium channels, inositol 1,4,5-triphosphate (IP₃), calcium-induced calcium release, Ca²⁺/H⁺ and Na⁺/Ca²⁺ exchange, and Ca²⁺-ATPase (2). Of these IP₃, the product of phospholipase C-catalyzed breakdown of phosphatidylinositol-4,5-bisphosphate (PIP₂), has been suggested to be involved in heat-induced increase of cytosolic Ca²⁺ (48, 270). Another product of PIP₂ breakdown, diacylglycerol (DAG), stimulates the activity of protein
A number of studies have shown that rapid increase of cytosolic calcium ([Ca\textsuperscript{2+}]\textsubscript{i}) is a common feature in a variety of organisms during heat shock (47, 48, 86, 147, 151, 270). The resting level of [Ca\textsuperscript{2+}]\textsubscript{i} in Drosophila melanogaster larval salivary gland cells is about 200 nM and increases approximately 10-fold, to 2 \textmu M during heat treatment (85, 86). This increase of [Ca\textsuperscript{2+}]\textsubscript{i} is very rapid with the concentration doubling by two minutes and increasing up to five-fold by five minutes after initiation of heat shock (270). The close correlation of this change with heat-induced generation of IP\textsubscript{3} suggests that the phosphoinositide pathway may be involved in the modulation of cytosolic calcium concentration during heat shock (48, 270). Several groups suggest that heat shock alters cellular Ca\textsuperscript{2+} through Ca\textsuperscript{2+} influx into cytoplasm from both internal stores and external medium (47, 48, 270). The rapidity and ubiquity of heat shock-induced increase in cytosolic calcium concentration underscore the possible importance of calcium in the HSR.

Cells treated with the ionophores valinomycin, dinactin, A23187 or ionomycin synthesize HSPs (9, 225, 293, 304). The long-term depletion of cellular calcium with EGTA
in rat hepatoma and Chinese Hamster Ovary cells inhibits the HSR (147). However, some contradictory results have been recorded in experiments in which the Ca\(^{2+}\) was maintained at a low level during heat shock. Calcium-depleted Drosophila salivary glands and BAPTA-leaked Kc cells maintain a low [Ca\(^{2+}\)]\(_i\) level during heat shock but are competent to synthesize a complete set of HSPs (85, 86). This suggests that Ca\(^{2+}\) is not essential or extremely small amounts of Ca\(^{2+}\) are capable of inducing HSP synthesis.

One type of intracellular calcium target which may be involved in signal transduction in eukaryotic cells is a class of calcium-binding proteins represented by calmodulin (CaM) (for review, see 175, 222). Calmodulin has been isolated and characterized and CaM genes have been cloned from many different organisms (14, 125, 166, 193, 314, for reviews, see 221, 222, 230). Calmodulin is a highly conserved, heat-stable, acidic protein with four Ca\(^{2+}\)-binding domains and is ubiquitous among eukaryotes. The Ca\(^{2+}\)/CaM complex may, directly or indirectly, regulate activities of many enzymes such as ATPase, NAD kinase, H\(^+\)-ATPase, quinate:NAD\(^+\) oxidoreductase, phospholipase, protein phosphatases and protein kinases. Many lines of evidence demonstrate that levels of CaM differ with respect to tissue type as well as on the physiological and developmental state of cells (26, 125, 170, 221, 230). The level of CaM
apparently increases two to three-fold during heat shock
(Harrington HM, personal communication). A sequence similar
to HSE in the upstream region of Chlamydomonas CaM gene
promotor may provide the molecular basis for such heat
regulation (314).

Using CaM inhibitors, Wiegant et al (299) concluded
that CaM antagonists sensitize cells to heat by inhibiting
cytoskeleton rearrangements mediated by Ca\(^{2+}\)/CaM. The CaM
antagonistic drug W13 potentiates hyperthermic cell killing
but the nonfunctional analog W12 has little influence,
supporting the idea that Ca\(^{2+}\)/CaM mediated processes are
involved in the HSR (151). This view has been reinforced by
the findings that some HSPs, such as HSP 70 (56, 271), 90
(192) and 100 (141) in animal systems are cytoskeleton­
associated, Ca\(^{2+}\)/CaM binding proteins. Taken together,
these data underscore the potential importance of Ca\(^{2+}\)/CaM
mediated processes in the HSR.

Recently, Landry and co-workers demonstrated that
transcriptional activation of the HSP 68 gene by heat and
cytoplasmic accumulation of mRNA are considerably reduced in
cells incubated prior to heat in EGTA-containing medium and
suggested that the block occurs very early at a
pretranscriptional site (151). These data, together with
several other important observations including a.) that
Ca\textsuperscript{2+}/CaM stimulates activities of many protein kinases (253); b.) that phosphorylation may be involved in the activation of HSF (264); and c.) that many proteins are modified with phosphorylation/dephosphorylation during heat shock (45, 46, 233, 234, 243, 244) provide the rationale for the characterization and identification of Calmodulin-binding proteins (CaMBPs) in the HSR.
CHAPTER II
SIGNIFICANCE AND HYPOTHESIS

As discussed in Chapter I, Ca\(^{2+}\)/CaM mediated processes may be involved in the HSR of animal systems but no similar information is available in plants. This lack of understanding of the Ca\(^{2+}\)/CaM system in plants provides the rationale for the present studies. The characterization of heat-induced genes for CaMBPs and the subsequent identification of functions for these proteins are both theoretically and practically important. In theory, identification and characterization of CaMBP genes will enhance our understanding of how cells perceive environmental signals and how such stresses affect physiological and biochemical processes. This research will also enhance our understanding of molecular mechanisms of gene regulation in plants. Finally, the elucidation of molecular mechanisms of the HSR will provide clues which will be ultimately useful in the development of stress-resistant crops.

The hypothesis to be tested in this research is:

The expression of some genes for CaMBPs is regulated by heat shock in cultured tobacco cells.
This hypothesis will be tested by following specific objectives:

I. To characterize CaMBPs in cultured tobacco cells during heat shock;

II. To isolate cDNA clones for CaMBPs;

III. To characterize a cDNA clone encoding a heat shock-induced CaMBP;

IV. To analyze the transcriptional expression of the cloned CaMBP genes;

V. To characterize calmodulin-binding domains by deletion analysis.
CHAPTER III
CHARACTERIZATION OF HEAT SHOCK INDUCED CALMODULIN-BINDING PROTEINS IN CULTURED TOBACCO CELLS

INTRODUCTION

Calcium is an important second messenger which mediates various plant physiological processes elicited by extracellular signals such as light, hormones and gravity (21, 222, 223). One target involved in signal transduction in eukaryotic cells is a class of Ca$^{2+}$-binding proteins exemplified by calmodulin (CaM). This 17 Kd, heat-stable, acidic protein contains four EF hand Ca$^{2+}$-binding domains (221, 230). Calmodulin regulates activities of many enzymes including ATPase, NAD kinase, phospholipase, quinate:NAD$^+$ oxidoreductase, protein phosphatases and kinases in a Ca$^{2+}$-dependent manner (221, 222, 230).

A number of studies have shown that rapid increase of cytosolic calcium is a feature common to variety of organisms during heat shock (47, 48, 86, 147, 151, 270). The control cells (not heat shocked) treated with the ionophores valinomycin, dinactin, A23187 or ionomycin synthesize HSPs (9, 225, 293, 304). Alternatively, long-term Ca$^{2+}$-depletion with EGTA inhibits the HSR (147).
Recent evidence demonstrates that calcium activates HSF binding activity to HSE in vitro (186). Taken together, these findings imply that calcium may be involved in the HSR. Other studies indicate that calcium is not necessary for HSP synthesis per se (85, 86). A role of CaM in the HSR may be implied since the anti-CaM drug, W13, potentiates hyperthermic cell killing while the non-functional analog, W12, has little influence (151). This view is reinforced by the findings that some proteins, such as HSP 70 (56, 271), 90 (192) and 100 (141) from animal cells are CaM-binding proteins (CaMBPs). These results serve to underscore the potential importance of the Ca²⁺/CaM second messenger system in the HSR. However, similar information is unavailable for the plant HSR. Few, if any, studies have systematically analyzed CaMBPs during the HSR and there are limited data on CaMBPs in plants. This report details the characterization of CaM-binding HSPs in cultured tobacco cells.

**MATERIALS AND METHODS**

**Materials.** Tobacco cells (*Nicotiana tabacum* L. cv Wisconsin-38) were grown at 23°C in the dark as suspension cultures in Gamborg's B-5 medium (98). Cell cultures were maintained by transferring 6 ml of mid log phase (7 days old) culture into 70 ml fresh B-5 medium contained in a 250
ml erlenmeyer flask. Cells from mid log phase cultures were used in all experiments.

Isolation of CaM-binding proteins. Tobacco cells (5g/15 ml culture) were incubated in shaking water baths for 15 minutes at 23°C for control and 38°C for heat shock treatment and then 250 μCi ^35S-Trans Label (ICN) was added. The cultures were continued for 4 hours at the same temperatures. The labeled cells (20g) were ground into fine powder under liquid nitrogen and extracted with buffer A (3 ml/g cell) containing 50 mM Tris/HCl pH 7.5, 3 mM MgCl₂, 5 mM KCl and 0.2 mM EDTA at 1°C. This extract was centrifuged at 20,000g for 20 minutes. The supernatant was adjusted to 1 mM CaCl₂ final concentration and applied to a CaM-sepharose-4B column (5 ml) (292). The column was washed at flow rate of 0.9 ml/minute at 1°C and 4.5 ml fractions were collected. The column was step eluted with 25 column volumes each of buffer B (25 mM Tris/HCl pH 8.0, 3 mM MgCl₂, 2 mM KCl and 0.1 mM CaCl₂), buffer B plus 0.15 M NaCl, buffer B plus 0.3 M NaCl. The putative CaMBPs were eluted in buffer B lacking CaCl₂ with the addition of 1 mM EGTA. The fractions containing peaks of radioactive materials were exhaustively dialyzed against 20 mM ammonium bicarbonate, lyophilized and resuspended in 2X Lamelli sample buffer (145). This procedure did not result in obvious protein degradation. Experiments in which crude extracts and other
fractions from the CaM-sepharose-4B columns were allowed to stand for up to 10 hours at 1°C revealed little proteolysis as evidenced by SDS-polyacrylamide gel electrophoresis (SDS-PAGE).

**Analysis of labeled proteins.** Labeled proteins were detected on SDS-mini gels as previously described by Harrington and Alm (111). Samples containing equal amount of radioactive proteins were loaded on 12.5% SDS-polyacrylamide gels. After electrophoresis, gels were stained with Coomassie Blue, destained, dried and autoradiographed at -80°C to locate labeled proteins. Incorporation of radioactive amino acids into proteins was estimated by the method of Mans and Novelli (172). All radioactivities were determined by liquid scintillation spectrometry.

**RESULTS AND DISCUSSION**

**Calmodulin-sepharose chromatography of tobacco proteins.** Initially, $8.5 \times 10^8$ and $5.8 \times 10^8$ cpm of radioactive labeled materials for control and heat shock treatments respectively were loaded onto CaM-sepharose-4B columns (5 ml) and eluted with over 25 column volumes (130 ml) of each wash buffer. At the end of each wash the
radioactive counts (cpm) stabilized at a low level (95-450 cpm/50 μl). Results in Figure 1 indicated similar elution profiles for control and heat shock samples. The wash of the columns with buffer B plus 0.15 M NaCl resulted in the elution of a radioactive peak containing 3412 cpm/50 μl for control and 1322 cpm/50 μl for heat shock treatments. A similar smaller peak was observed when the columns were washed with buffer B plus 0.3 M NaCl (1249 cpm/50 μl for control and 641 cpm/50 μl for heat shock treatments). The possibility that CaMBPs were eluted in salt washes (0.15 and 0.3 M NaCl) can not be excluded because the binding of a known CaM-target, myosin light chain kinase is easily disrupted by relatively low levels of salt (0.1 M). Elution of the columns with buffer B plus EGTA produced a large radioactive peak (9720 cpm/50 μl for control and 4527 cpm/50 μl for heat shock treatment). The fact that EGTA washes released labeled materials after high ionic strength salt wash suggests that these peptides exhibit highly specific, Ca²⁺-dependent binding activity. These fractions and other column effluents were analyzed by SDS-PAGE.

To confirm the binding specificity of these putative CaMBPs, parallel experiments were run using sepharose-4B columns. Equal amounts of radioactive proteins (5.8 x 10⁸ cpm) of heat shock extracts were loaded into CaM-sepharose-4B and sepharose-4B columns and eluted as described above.
Figure 1. CaM-sepharose chromatography elution profile
Results summarized in Table 1 indicate that few radioactive proteins bound to sepharose-4B. If the data are expressed as the ratio of sepharose-4B/CaM-sepharose-4B, 2.9% of the counts were released from sepharose-4B by the 0.3 M salt wash. Only 1.6% of the counts were eluted by EGTA wash. These results suggest that the peptides released from CaM-sepharose-4B with EGTA washes specifically bind to CaM and not to the column supports.

Analysis of labeled proteins by SDS-PAGE. Equal amounts of labeled proteins (30,000 cpm/lane) were separated on 12.5% SDS-gels. The results in Figure 2 show a comparison of total labeled proteins extracted with 2X Lamelli buffer from control (lane A1) and heat shocked cells (lane B1). These data indicate strong induction of HSPs, especially HSP 96, 82 and 71 and abundant small HSPs in agreement with Harrington and Alm (111). Few constitutive proteins were synthesized during heat shock. The extraction of cells with Buffer A resulted in the detection of significantly fewer labeled bands for both treatments (Fig. 2, lanes A2 and B2). For example, HSP 96 was not extracted by this procedure (Fig. 2, lane B2). Chromatography of these Buffer A extracts on CaM-sepharose-4B as above resulted in the elution of specific peptides.
Table 1. Comparison of radioactive proteins recovered from CaM-sepharose-4B and sepharose-4B columns

<table>
<thead>
<tr>
<th>Column material</th>
<th>0.3 M NaCl (cpm)</th>
<th>EGTA (cpm)</th>
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Figure 2. SDS-PAGE of CaM-sepharose chromatography fractions.

Equal amounts of labeled proteins (30,000cpm/lane) were separated on 12.5% SDS-polyacrylamide gels. Labeled proteins were visualized by autoradiography.

A, Proteins were isolated from cells treated at 23°C for control. B, Proteins were extracted from cells treated at 38°C for heat shock treatment.

Lane 1, SDS extraction buffer; lane 2, CaM-sepharose extraction buffer A; lane 3, Initial non-bound fraction; lane 4, 0.1 mM Ca²⁺ wash; lane 5, 0.15 M NaCl wash; lane 6, 0.3 M NaCl wash; lane 7, 1 mM EGTA wash after 0.15 M NaCl wash and Lane 8, 1 mM EGTA wash after 0.3 M NaCl wash.
When the CaM-sepharose-4B wash buffer Ca\(^{2+}\) concentration was decreased to 0.1 mM, a unique HSP 25 (25 Kd) was eluted (Fig. 2, lane B4). There were also other major bands corresponding to 85, 82 and 71 Kd of HSPs in this elution. A 41 Kd peptide was present both in control and heat shock samples (Fig. 2, lanes A4 and B4). Column elution with salt-containing buffers released additional labeled peptides with major HSP bands at molecular weights of 90, 85, 82, 71, 68, 34, 32, 22, 21 Kd (Fig. 2, lane B5 and B6).

The EGTA washes were used to release peptides exhibiting Ca\(^{2+}\)-dependent binding to CaM-sepharose-4B. These fractions contained the putative CaMBPs. With the exception of a HSP 17 (17 Kd), the profiles for heat shock samples were similar to those obtained with salt wash samples (Fig. 2, lanes B5, B6, B7 and B8). Our recent data with gel overlay analyses indicated that the proteins released by EGTA washes bound to \(^{35}\)S-labeled calmodulin while the proteins obtained with salt washes did not, suggesting specific CaM-binding of the proteins in EGTA wash samples (Data not shown). These EGTA wash fractions contained heavily labeled heat shock peptides with molecular weights of 90, 85, 82, 78, 71, 68, 22, 20, 19.5 and 17 Kd. By comparison, control EGTA fractions contained few distinctive
labeled bands including peptides with molecular weights of 100, 50 and 35 Kd (Fig. 2, lane A7 and A8).

The above results indicate that heat shock induced the synthesis of CaMBPs/HSPs in cultured tobacco cells. These findings are consistent with recent discoveries in animal system that HSP 100, 90 and 70 are CaMBPs (141, 192, 271). The synthesis of CaMBPs during heat shock suggests that the Ca$^{2+}$/CaM second messenger system may play a role in the plant HSR. However, the identities and functions of the CaMBPs/HSPs in tobacco are currently unknown. Possible roles could include CaM-dependent protein kinases and other enzymes that regulate existing metabolic processes, repair heat damage or protect cellular structures during heat shock. This first indication that CaMBPs/HSPs are involved in tobacco HSR provides the rationale for future characterizations of Ca$^{2+}$/CaM-mediated processes during heat shock in plant cells.

Since most CaMBPs are present in extremely small amount, it would be difficult to purify sufficient amounts of the protein for biochemical identification and molecular analysis. An alternative approach to the identification of CaMBPs is to isolate cDNA clones for CaMBPs by screening an expression cDNA library with radioactive labeled CaM. The nucleotide sequences from such clones may provide clues as
to identities of CaMBP/HSPs and may suggest biochemical
assays to confirm functions.
CHAPTER IV
ISOLATION AND CHARACTERIZATION OF cDNA CLONES
ENCODING TOBACCO CALMODULIN-BINDING PROTEINS

INTRODUCTION

All organisms are subjected to a large number of environmental and biological stresses. One of the important environmental factors for plants in agriculture is heat shock. Plants, when subjected to a moderate upshift temperature, undergo a phenomena called heat shock response (HSR). The HSR is characterized by the rapid shutdown of most normal mRNA and protein synthesis; induction of de novo synthesis of heat shock mRNAs and proteins and the acquisition of thermotolerance to otherwise lethal high temperature (66, 132, 163, 189). Much progress has been made in the understanding of the structure and regulation of heat shock genes. However, little is known about the biochemical function of individual HSPs.

Calcium, acting as a second messenger, plays a role in various plant physiological processes elicited by extracellular signals such as light, hormones and gravity (175, 222). As in animal cells, many of the effects of calcium ions in plant cells are mediated through the calcium
responsive element, calmodulin (CaM) (114, 175, 221, 230, 281). Calmodulin is a small, heat-stable, acidic protein with four EF hand Ca^{2+}-binding domains that has been identified in all eukaryotic species examined (230). It is highly conserved in structure and is functionally interchangeable among different species (76, 230), suggesting that CaM is an essential protein for normal growth and development performing similar functions in all eukaryotes (75, 230). The Ca^{2+}/CaM complex regulates activities of many enzymes including Ca-ATPase, NAD kinase, phospholipases, protein phosphatase and a variety of kinases (221). To date, few CaM-dependent processes have been well-characterized in plants (222, 230). This lack of information on the role of CaMBPs has hindered the development of an integrated view of signal transduction in plant cells. Furthermore, a number of studies suggests that Ca^{2+}/CaM mediated processes may be involved in the HSR (47, 48, 147, 151, 186, 271, 293) even though some data suggest that calcium is not necessary for HSP synthesis (85, 86). Therefore, the characterization of CaMBPs will enhance our understanding of mechanisms of how CaM acts to regulate physiological, metabolic and molecular processes including the HSR in plants.

Calmodulin-binding proteins have been extensively studied in animal systems (175, 203). Characterization of
CaM-binding sites of several animal and human CaMBPs have defined a basic amphiphilic α-helix as the common feature of CaM-binding domain (27, 73, 157, 169, 289). This structure is characterized by the presence of basic amino acid residues on one side of the helix while hydrophobic residues on the opposite side. Short synthetic peptides containing this structure can efficiently bind to CaM (73, 157, 169, 289). Moreover, two groups have reported successful screening of expression cDNA libraries in animal systems with $^{125}$I- or $^{35}$S-CaM (259, 296). Such results provide the rationale for the isolation of plant CaMBP cDNA clones from an expression library using CaM as a ligand probe. Here we report the results of isolation and identification of cDNA clones for heat shock-induced and constitutive CaMBPs in tobacco and tentative characterization of the CaM-binding domain of the deduced protein.

**MATERIALS AND METHODS**

Tobacco cells (*Nicotiana tabacum* L. cv Wisconsin-38) were grown at 23°C as suspension culture in Gamborg's B-5 medium (98). Cells from mid log phases culture (7-day old) were incubated for 2 hours at 38°C for heat shock and at 23°C for control treatments. These cells were used in all experiments. *Escherichia coli* UT481 harboring pVUC-1 for
preparation of $^{35}$S-CaM was a gift from D. Martin Watterson (Vanderbilt University) (231).

**Preparation of $^{35}$S-CaM.** The isolation of $^{35}$S-CaM was done essentially as described by Asselin et al (10). An overnight culture (200 µl) of *E. coli* UT481 harboring pVUC-1 was inoculated into 20 ml of 1% NZ amine, 0.5% yeast extract, 0.5% NaCl, 0.1% casamino acids, 0.2% MgSO$_4$, pH 7.5, 2.5% glycerol and 25 µg/ml ampicillin and incubated at 37°C until the $A_{600}$ of the culture reached 0.6. The bacteria were collected by centrifugation (1000g, 5 minutes) and resuspended in 50 ml of 0.1 M Tris/HCl pH 7.4, 92 mM NaCl, 40 mM KCl, 19 mM NH$_4$Cl, 0.26 mM CaCl$_2$, 0.98 mM MgCl$_2$, 0.74 µM FeCl$_3$, 0.639 mM KH$_2$PO$_4$, 2.5% glycerol and 25 µg/ml ampicillin. The cells were centrifuged as above and resuspended in 20 ml of the latter medium containing 5 mCi of $^{35}$S-sodium sulfate (1200-1400 Ci/mM, NEN) and incubated for 3 hours at 37°C. The incubation was continued for an additional 3 hours after addition of 16 µl of 0.5 M IPTG. The bacteria were collected and resuspended in 5 ml of 25 mM Tris/HCl pH 7.5, 1 mM CaCl$_2$ and lysed by sonication (10). The mixture was heated at 90°C for 1 minute and centrifuged at 1000g for 5 minutes. The supernatant was then applied to a 500 µl column of phenyl-sepharose in a 1.5 ml microfuge tube and washed with 1 ml of 25 mM Tris/HCl, 1mM CaCl$_2$, pH 7.5 and 3 ml of 25 mM Tris/HCl, 1mM CaCl$_2$, 0.2 M KCl, pH 7.5.
The CaM was eluted from the column with 500 µl of 25 mM Tris/HCl, 1 mM EDTA pH 7.5. This \(^{35}\)S-CaM was analyzed by SDS-PAGE and autoradiography. The mobility of \(^{35}\)S-labeled CaM shifted on the SDS gels in the presence or absence of CaCl\(_2\) as expected (Fig. 3, lanes A2, A3, B2 and B3). The behavior was identical to authentic CaM from chicken gizzard (Fig. 3). The specific activity of labeled CaM was approximately \(1.5 \times 10^7\) cpm/µg.

**Isolation of total and polysome RNAs.** Isolation of total RNAs was done as described by McGookin (179). Filtered tobacco cells (5g) were frozen with liquid nitrogen and ground into a fine powder in a prechilled mortar and pestle. Four volumes of 5 M guanidine thiocyanate, 50 mM Tris/HCl pH 7.5, 10 mM EDTA and 5% β-mercaptoethanol were added to the powder and the mixture was homogenized with a glass homogenizer. Sarkosyl and CsCl were then added to final concentrations of 4% (W/V) and 15% (W/V) respectively. After centrifugation at 15,000g for 20 minutes at 4°C, the supernatant was layered on a 4.5 ml cushion of 5.7 M CsCl, 100 mM EDTA, pH 7.5 and centrifuged at 100,000g for 18 hours at 20°C. The pellet was resuspended in 200 µl of TE (10 mM Tris/HCl, 1 mM EDTA pH 7.5) and precipitated by the addition of 4% (V:V) 6 M ammonium acetate and 2 volumes of ethanol at -20°C. The resulting RNA pellet was collected by
Figure 3. SDS-polyacrylamide gel electrophoresis of $^{35}$S-labeled calmodulin.

12.5% SDS-polyacrylamide gels containing 5 mM EDTA (A) or 1 mM CaCl$_2$ (B) were used to separate the calmodulin. Lanes 1 and 2, Coomassie blue stain; lane 3, autoradiography.

Lane 1, Chicken gizzard calmodulin; lanes 2 and 3, $^{35}$S-labeled calmodulin (1 x 10$^6$ cpm/lane).
centrifugation, washed with 70% ethanol, resuspended in H$_2$O and stored at -80°C until use.

Polysomal RNA was isolated as described by Laroche and Hopkins (154). Filtered tobacco cells were frozen with liquid nitrogen and homogenized into fine powder with an Omni Mixer. Ice-cold isolation buffer (3 ml/g cells) (200 mM Tris/HCl pH 8.95, 200 mM KCl, 35 mM MgCl$_2$, 250 mM sucrose, 12.5 mM EGTA and 15 mM DTT) was added and the mixture was homogenized in a Polytron. The mixture was filtered through miracloth and NP-40 was added to a final concentration of 1% (V:V). After 10 minutes on ice, the mixture was centrifuged at 15,500g for 15 minutes. The supernatant was loaded on a 5 ml sucrose cushion (40 mM Tris/HCl pH 8.95, 40 mM KCl, 7 mM MgCl$_2$, 1.5 M sucrose, 5 mM EGTA and 5 mM DTT) and centrifuged at 180,000g for 1.5 hours at 4°C. The pellet was rinsed 3 times with 40 mM Tris/acetate pH 8.5, 20 mM KCl and 1 mM MgCl$_2$ and resuspended in the same buffer. The resuspension was then extracted twice with phenol, three times with phenol/chloroform (1:1) and twice with chloroform. The RNAs were precipitated with 0.1 volume of 3 M sodium acetate pH 5.2 and 2.5 volume of ethanol at -20°C and centrifuged at 11,000g for 10 minutes at 4°C. The pellet was washed with 70% ethanol, resuspended in H$_2$O and stored at -80°C until use.

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Construction of expression CDNA library. Poly(A) RNA was purified from heat shock polysome RNAs by oligo(dT)-cellulose chromatography (11). The RNA sample was heated for 2 minutes and loaded on a column containing 1 g oligo(dT)-cellulose. The column was washed with 10 mM Tris/acetate pH 7.5, 0.4 M NaCl to remove unbound RNA until the A_{260} was at the baseline. Then, the poly(A) RNA was collected in a wash of 10 mM Tris/acetate pH 7.5. The RNA was subjected to the same procedure two more times. Finally, the poly(A) RNA was precipitated with 0.1 volume of 20% potassium acetate pH 5.5 and 2.5 volumes of ethanol at -20°C. This purified poly(A) RNA was dissolved in H_{2}O and used (5 μg) in the construction of a cDNA expression library with a λZAPII-cDNA synthesis kit (Strategene). The library was constructed following the manufacturer's instructions. The synthetic cDNAs with 13 bp EcoRI adaptor (AATTCGGCACGAG) at the 5' end and 6 bp XhoI site (CTCGAG) at the 3' end were inserted in the EcoRI/XhoI site of the vector λZAPII which can be in vivo excised into phagemid pBluescript SK- (Fig. 4) (257). A total of 1.1 x 10^6 recombinants were obtained.

Screening of cDNA library with ^35S-CaM. Escherichia coli BB4 was infected by recombinant λ-ZAP II phages and grown on NZY (0.5% NaCl, 0.2% MgSO_4, 0.5% yeast extract, 1% NZ amine pH 7.5) plates at the density of 50,000 pfu/plate for 3 hours at 42°C. Nitrocellulose filters saturated with
Figure 4. pBluescript SK- vector.
10 mM IPTG were layered on the plates and the incubation continued for an additional 6 hours. The filters were washed in 100 ml of buffer A (50 mM Tris/HCl pH 7.5, 150 mM NaCl, 1 mM MgCl₂) plus 0.1% BSA for one hour at 37°C. The filters were then incubated in 50 ml of buffer A plus 1 mM CaCl₂ containing 1 x 10⁶ cpm/ml of ³⁵S-CaM for 3 hours at room temperature and washed three times with 100 ml of buffer A plus 1 mM CaCl₂ for 10 minutes each. Dried filters were autoradiographed at -80°C to locate positive signals. The positive plaques were further purified by 3 more rounds of screening as described above.

In vivo excision of positive phages into phagemids.

Positive phages were in vivo excised into phagemids as described by Short et al (257). A mixture of 200 μl of E.coli XL1-Blue (A₆₀₀=1.0), 200 μl of phage (>1 x 10⁵ pfu) and 10 μl of R408 helper phage was incubated for 15 minutes at 37°C. Five ml of 2xYT medium (1% NaCl, 1% yeast extract and 1.6% tryptone pH 7.4) were added and the incubation was continued for 3 hours at 37°C. After 20 minutes at 70°C, the cultures were centrifuged at 4,000g for 5 minutes. An aliquot of the supernatant (200 μl) and 200 μl of E.coli XL1-Blue were mixed and incubated for 15 minutes at 37°C. The culture was then distributed on LB plates (1% tryptone, 0.5% yeast extract and 0.5% NaCl pH 7.5) containing 50 μg/ml
ampicillin and incubated overnight at 37°C. Colonies obtained were used for further analyses.

**Gel overlay analysis of positive colonies.** A positive colony was inoculated into LB medium and incubated at 37°C until the A₆₀₀ of the culture reached 0.2. IPTG was then added to 10 mM and the culture growth was continued to A₆₀₀ of 1.0. The culture was microfuged for 3 minutes and the pellet was resuspended in 2X Lamelli sample buffer (145). After 3 minutes in a boiling water bath, the sample was microfuged for 2 minutes and the supernatant was subjected to SDS-PAGE as described by Harrington and Alm (111). Gel overlay analysis was done by the methods of Burgess *et al.* (40). After electrophoresis the gels were washed three times in 100 ml of 25% (V:V) isopropanol and 10% (V:V) acetic acid for one hour each and three times with 100 ml of buffer B (50 mM Tris/HCl pH 7.6 and 0.2 M NaCl) for one hour each at room temperature. The gels were then blocked in buffer B plus 0.1% BSA for 2 hours and then incubated in 30 ml of buffer B plus 0.1 mM CaCl₂ or 5 mM EDTA containing 1 x 10⁵ cpm ³⁵S-CaM/ml for 14 hours at room temperature. After three washes with 100 ml of buffer B plus 0.1 mM CaCl₂ or 5 mM EDTA for one hour each at room temperature, the gels were stained with Coomassie Blue, destained, dried and autoradiographed at -80°C to detect CaMBPs.
DNA deletion and sequencing. Phagemid DNAs were purified by CsCl procedure as described by Maniatis et al (171). Phagemid DNA from clone pTCB40 was double-digested with SacI/EcoRI for deletion from 5' end of the insert. The deletion was accomplished with an ExoIII/mung bean deletion kit (Strategene) essentially as described in the manufacturer's instructions. Colonies containing appropriately-sized deletions were selected based on restriction enzyme digestion and subjected to double-stranded DNA sequencing analysis with a Sequenase version 2.0 DNA sequencing kit (United States Biochemicals). Sequencing electrophoresis was done on 7 M urea/6% polyacrylamide gels with constant power (38W) at 50°C.

Northern blot/hybridization analysis. Total tobacco RNAs (20 μg) were loaded on a 1.5% formaldehyde agarose gel and electrophoresed in 20 mM MOPS, 5 mM sodium acetate and 1 mM EDTA pH 7.0. The RNAs were transferred by blotting to nitrocellulose filters in 10X SSC (1.5 M NaCl, 150 mM sodium citrate pH 7.0) for overnight at room temperature (277). The filters were incubated in 50% formamide, 5X Denhardt's (0.1% Ficoll, 0.1% PVP and 0.1% BSA), 0.1% SDS, 5X SSPE (750 mM NaCl, 50 mM NaH₂PO₄, 5 mM EDTA pH 7.4) and 100 μg/ml salmon sperm DNA for 2 hours at 42°C. The filters were transferred to 50% formamide, 2X Denhardt's (0.04% Ficoll, 0.04% PVP and 0.04% BSA), 0.1% SDS, 5X SSPE, 100 μg/ml
salmon sperm DNA containing $3 \times 10^6$ cpm/ml of $^{32}$P-labelled DNA made from pTCB40 by random primer extension (Random Primers DNA Labeling System, Bethesda Research Laboratories Life Technologies, Inc.) for 16 hours at 42°C. After hybridization the filters were washed twice in 100 ml of 2X SSC (300 mM NaCl, 30 mM sodium citrate pH 7.0) containing 0.1% SDS for 10 minutes each and twice in 100 ml of 0.1X SSC containing 0.1% SDS for 10 minutes each at room temperature. A final wash was done in 100 ml of 0.1 X SSC containing 0.1% SDS for one hour at 55°C. The filters were autoradiographed at -80°C.

**RESULTS AND DISCUSSION**

A. Isolation and Confirmation of cDNA Clones Encoding Calmodulin-Binding Proteins

Synthesized cDNA from tobacco polysomal/poly(A) RNA was inserted into the bacteriophage expression vector λ-ZAP II and the resultant library was screened for the presence of plaques containing fusion proteins that bind to radioactive CaM. Initial screens used $^{125}$I-labeled CaM (lactoperoxidase method); however, the results were negative and the high background was high. These negative results may be due to structural damage to CaM caused by the
iodination procedure. To overcome these potential problems, in vivo labeled $^{35}$S-labeled CaM encoded by pVUC-1 was used to screen the library. The results showed distinct positive spots with very low background (Fig. 5). This method was very efficient because 25 independent positive clones were obtained from $1 \times 10^6$ screened clones. All these positive phages were converted into phagemids by in vivo excision as described in the methods section. The recombinant DNAs from these clones were isolated and double-digested with EcoRI and XhoI. These two enzymes were used during construction of the library and should excise the insert from the vector. These digested DNAs were analyzed on agarose gel (Fig. 6) and the sizes of the inserts from these clones are summarized in Table 2.

In order to confirm that positive clones obtained in the screening procedure encoded CaMBPs, gel overlay analysis was carried out on extracted proteins. A polypeptide from the first clone tested (pTBC01) bound to CaM in the presence of 0.2 M NaCl and 0.1 mM CaCl$_2$ (Fig. 7, B4). This binding was readily inhibited by the omission of CaCl$_2$ and addition of EDTA into the overlay incubation buffer (Fig. 7, lane C4), suggesting that CaM binding was Ca$^{2+}$-dependent. Subsequent experiments demonstrated that all 25 positive clones synthesized proteins that bound to CaM in a Ca$^{2+}$-dependent manner (Fig. 8). Some fusion proteins were not
Figure 5. Autoradiograms of blotted phages screened with $^{35}$S-labeled calmodulin.

A, Primary screen in which a positive signal (arrow) was evident. B, Screen of purified phages in which all plaques bound calmodulin.
Figure 6. DNAs from pBluescript recombinants encoding calmodulin-binding proteins.

DNAs were double-digested with EcoRI and XhoI and separated on 1% agarose gel.

Samples for lanes from left to right are pBluescript, pTCB01, 03, 04, 05, 06, 10, 14, 15, 18, 22, 23, 25, 28, 29, 31, 38, 40, 46, 48, 51, 55, 56, 58, 59, 60 and molecular weight marker.
Table 2. Sizes of the inserts of cDNA clones encoding CaMBPs

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Figure 7. Calmodulin gel overlay analysis of the protein generated from clone pTCB01.

Equal proteins (20μg/lane) were separated on 12.5% SDS-polyacrylamide gels and gel overlay analysis was done in the presence of 0.1 mM CaCl$_2$ (B) or 5 mM EDTA (C) as described in Materials and Methods.

A, Coomassie blue stain; B and C, Autoradiography.

Lane 1, XL1-Blue; lane 2, XL1-Blue harboring pBluescript; lane 3, XL1-Blue harboring pBluescript with IPTG as inducer and lane 4, XL1-Blue harboring pTCB01 with IPTG as inducer.
Figure 8. Calmodulin gel overlay analysis of extracts from 25 independent recombinants.

Equal proteins extracted from cultures of *E. coli* XL1-Blue harboring different recombinant phagemids without (lanes 1 and 2) or with (remaining lanes) IPTG as inducer were separated on 12.5% SDS-polyacrylamide gels and gel overlay analysis was done in the presence of 0.1 mM CaCl$_2$ as described in Materials and Methods.

Lane 1, No phagemid; lanes 2 and 3, pBluescript; Remaining lanes from lane 4 to 28, pTCB01, 03, 04, 05, 06, 10, 14, 15, 18, 22, 28, 23, 25, 28, 31, 38, 40, 46, 48, 51, 55, 56, 58, 59 and 60 in order.

When gel overlay analysis was done in the presence of 5 mM EDTA, no CaM-binding activity was detected (Data not shown).
stable in *E.coli* (Fig. 8); however, partially degraded peptides still retained the ability to bind CaM. Several clones such as pTCB 25, 31, 38 and 48 showed the same patterns for the DNA restriction maps and gel overlay analysis (Fig. 6, lanes 13, 16, 17, 20 and Fig. 8, lanes 16, 18, 19, 22). These results suggest that the CaM-binding domain is functional even when they do not reside in an intact native protein. Similar results have been demonstrated by using short synthetic peptides in the studies of CaM-binding domains (203). This feature provides the rationale for the characterization of CaM-binding domains using expression vector and DNA deletion analysis. The data in Figure 7 also indicated that *E.coli* contained two minor peptides which bound to CaM. However, these did not interfere with the screening procedure used here as positive clones produced fusion proteins with strong \(^{35}\text{S}-\text{CaM}\) signals. While *E.coli* lacks CaM (55, 259), there is protein with EF hand Ca\(^{2+}\)-binding domains present in this organism (273). Since the EF hand structure is highly conserved, this finding may be the result of CaM-binding to protein targets for the *E.coli* EF hand protein.

**B. Characterization of Calmodulin-Binding Protein Clone pTCB40**

Of 25 clones obtained by the screening procedure, the mRNA levels of 20 clones were unaffected by heat shock
treatment. Clone pTCB40 was selected as representative of this class of the clones (Fig. 9) and subjected to further analysis.

A polypeptide (TCB40) generated from pTCB40 was detected by $^{35}$S-CaM gel overlay analysis (Fig 12, lane A4). A 4.3 kd N-terminal fragment of $\beta$-galactosidase should fuse with the peptide encoded by the insert provided that the translation of TCB40 begins from $\beta$-galactosidase initiation codon. The size (27 Kd) of TBC40 suggested that the cDNA insert contained an open reading frame (ORF) sufficient to encode a sequence of approximately 210 amino acids.

To gain more precise information on the ORF and possible clues as to the identity of the cloned TCB40, the sequence of the cDNA insert of pTCB40 was determined (Fig. 10). The 928 bp insert contains an ORF encoding 233 amino acids sufficient to code for a peptide with a calculated molecular weight of about 27 Kd. Following the stop codon TAG at positions 814-816 is an untranslated sequence of 112 bp including an 18 bp poly(A) sequence. The polyadenylylation signal TAATAT is located at the positions 888-893, 17 bp upstream from poly(A) sequence. Numerous stop codons are present in the 114 bp 5' untranslated leader
Figure 9. Northern blot analysis of pTCB40.

Equal amounts (20 μg/lane) of total RNAs isolated from control (C) and heat shocked (HS) tobacco cells were separated on 1.5% formaldehyde agarose gel and transferred by blotting to nitrocellulose. The mRNA was probed with $^{32}$P-labeled DNA made from pTCB40 by random primer extension.
Figure 10. DNA sequence and deduced amino acid sequence of pTCB40.
for all three reading frames, suggesting that TCB40 is not a fusion protein translated from the ATG initiation codon of β-galactosidase. The predicted molecular weight based on the DNA sequence is similar to what detected by gel overlay analysis (Fig. 12, lane A4). These two factors suggest that translation of the cDNA insert of pTCB40 may use the ATG initiation codon located at the positions 115-117 even though no conserved sequence for eukaryotic translation initiation was found bordering this ATG. The high copy number of mRNAs transcribed from the insert and the presence of a Shine-Dalgarno like sequence, GAAGGT, (Shine-Dalgarno consensus: GGAGGT) at positions 105-110, suggest efficient synthesis of TCB40 even though the initiation codon (ATG at the positions 114-117) is remote from terminal codon TAA (position 59-61) for the β-galactosidase fragment.

In order to confirm this hypothesis, the insert of pTCB40 was subjected to deletion and several clones containing different deletion constructs (Fig. 11) were used for 35S-CaM gel overlay analysis. The first construct, D-6, removed 62 bp from the vector resulting in an out-of-frame mutation for the fusion protein. The second deletion construct, D64, deleted 63 bp from putative 5' untranslated leader region in addition to 67 bp from the vector. Both deletions would not interrupt TCB40 provided that translation begins from ATG (position 115-117) of the ORF of
Figure 11. Deletion constructs of pTCB40. First nucleotide from the 5' end of the insert is referred as number 1.
Figure 12. Calmodulin gel overlay analysis of extracts from *E. coli* XL1-Blue cells harboring pTCB40 or different deletion constructs.

Equal proteins extracted from cultures of XL1-Blue cells harboring different phagemids with (lanes 3, 4, 5, 6 and 7) or without (lanes 1 and 2) IPTG as inducer were separated on 12.5% SDS-polyacrylamide gels and gel overlay analysis was done in the presence of 0.1 mM CaCl$_2$ (A) or 5 mM EDTA (B) as described in Materials and Methods.

Lane 1, No phagemid; lanes 2 and 3, pBluescript; lane 4, pTCB40; lane 5, D-6; lane 6, D64; lane 7, D115 and lane 8, D337.
pTCB40 insert. The CaMBPs generated from both deletions were the same size, 27 Kd, as TCB40 (Fig. 12, lanes A4, A5 and A6), demonstrating that the deleted sequences were not required for the synthesis of TCB40. The third deletion, D115, in which the entire 5' leader sequence was exactly removed, produced a 29 Kd CaMBP, 2 kd larger than TCB40 (Fig. 12, lane A7). This was predicted since this in-frame deletion fused the ORF of pTCB40 with the 19 amino acids of the N-terminus of β-galactosidase. Taken together, these results indicate that TCB40 is translated from the ORF of the insert.

The size of mRNA for pTCB40 detected by Northern blot was 2.5 Kb (Fig. 9). This estimated size is much larger than expected based on DNA sequence data (Fig. 10). These conflicting data may arise from errors in cDNA synthesis during library construction if some sequence of the mRNA was not copied into the cDNA due to secondary structure. It is highly unlikely that such rare events would create CaM-binding domains for the following reasons. First, frame shifts may introduce numerous stop codons and abort translation. Second, the special domains such as a basic amphiphilic α-helix are required for CaM-binding. The probability of creating such a structure as an artifact is extremely low.
Figure 13. Predicted secondary structure of TCB40 protein with Chou-Fasman method.
A fourth deletion construct D377 lacking 86 amino acids of the N-terminus of TCB40 retained CaM-binding activity (Fig. 12, lane A8). This result suggests that CaM-binding site resides in C-terminal 147 amino acids. Since a basic amphiphilic α-helix is the CaM-binding domain of several animal CaMBPs (203), a secondary structural analysis of TCB40 was done with the Chou-Fasman method using the University of Wisconsin sequence analysis software package (54). The results indicate that three regions around the positions 100, 155 and 195 have high probabilities for forming α-helical structure (Fig. 13). However, there is insufficient evidence to assign the CaM-binding domain to a specific region. Furthermore, DNA and protein sequence database searches did not reveal any significant homologies between pTCB40 and other reported genes or proteins.

C. Characterization of Calmodulin-Binding Protein Clone pTCB60 and Identification of Calmodulin-Binding Domain.

Analysis of Northern blots demonstrated that a 2.1 Kb mRNA was recognized by a probe made from pTCB60. This mRNA decreased by at least 70% over a 2 hour heat shock treatment (Fig. 14). Since no degradation of mRNA during heat shock has been suggested (162, 200, 269) and our previous results also supported this idea, the apparent specific degradation of the mRNA for TCB60 may play a role in the HSR. This
Figure 14. Northern blot analysis of pTCB60.

Equal amounts (20 μg/lane) of total RNAs isolated from control and heat shocked tobacco cells were separated on 1.5% formaldehyde agarose gel and transferred by blotting to nitrocellulose. The mRNA was probed with \(^{32}\text{P}\)-labeled DNA made from pTCB60 by random primer extension.
degradation of mRNA would presumably affect a metabolic activity or affect distribution of free CaM.

**Analysis of DNA sequence.** The phagemid of pTCB60 was purified and sequenced. A 1572 bp insert containing an 1184 bp ORF encoding 393 amino acids was found (Fig. 15). Following the TAA stop codon (positions 1182-1184) is an untranslated sequence of 388 bp including a poly(A) terminus of 19 bp. A sequence, ATAAA, at the positions 1514-1518, 35 bp upstream from poly(A) is probably the polyadenylylation signal. This recombinant phagemid produced a CaM-binding fusion protein of 435 amino acids with a calculated molecular weight of 48.5 Kd. This size is somewhat larger than that detected by $^{35}$S-CaM gel overlay analysis of clone pTCB60 (38 Kd) (Fig. 17, lane A4). This may be due to unstability of the fusion proteins in *E.coli* cells mentioned earlier. In order to obtain insight as to identities of this clone, DNA and protein sequence database searches were done; however, no significant homology between pTCB60 and other reported genes and proteins was revealed.

**Preliminary identification and characterization of the CaM-binding domain.** Recent studies on several animal and human CaMBPs, including non-erythroid spectrin (157), smooth muscle MLCK (169), skeletal muscle MLCK (27), the Ca$^{2+}$ pump (289), calmodulin kinase II (110) and the γ-subunit of
Figure 15. DNA sequence and deduced amino acid sequence of pTCB60.
phosphorylase b-kinase (73), indicate that one class of CaM-binding domain is a basic amphiphilic α-helix. To determine the CaM-binding site of TCB60, several deletion mutants from both the 5' and 3' ends of the insert of pTBC60 were constructed (Fig. 16) and assayed for the CaM-binding activity by gel overlay analysis. The protein generated from mutant D684, in which N-terminal 227 amino acids encoded by the insert were deleted, bound to CaM, suggesting that the C-terminal 166 amino acids contained the CaM-binding domain (Fig. 17, lane A12). Similarly, the protein produced from deletion R1489 in which 64 bp of the 3' untranslated region were removed retained the ability to bind to CaM (Fig. 17, lane A5). Alternatively, R1129 in which all of the 3' untranslated region and 17 amino acids from the C-terminus of TCB60 were deleted, including three positively charged amino acids at the positions 377-379, lacked CaM-binding activity (Fig. 17, lane A6). These results indicate that the CaM-binding domain is located in the C-terminus of the protein.

Secondary structural analysis of TCB60 protein using Chou-Fasman method in the University of Wisconsin sequence analysis software package indicates that the C-terminus of TCB60 has a high probability for forming an α-helix (Fig. 18) (54). The sequence of amino acids from 362 to 380 is depicted as the helical wheel in Figure 19. In this
Figure 16. Deletion constructs of pTCB60.  
First nucleotide from the 5' end of the insert is referred as number 1.
Figure 17. Calmodulin gel overlay analysis of extracts from E.coli XL1-Blue cells harboring pTCB60 or different deletion constructs.

Equal proteins extracted from cultures of XL1-Blue cells harboring different phagemids without (lanes 1 and 2) or with IPTG (remaining lanes) as inducer were separated on 12.5% SDS-polyacrylamide gels and gel overlay analysis was done in the presence of 0.1 mM CaCl$_2$ (A) or 5 mM EDTA (B) as described in Materials and Methods.

Lane 1, No phagemid; lanes 2 and 3, pBluescript; lane 4, pTCB60; lane 5, R1489; lane 6, R1129; lane 7, R953; lane 8, D132; lane 9, D456; lane 10, D636; lane 11, D681 and lane 12, D684.
Figure 18. Predicted secondary structure of TCB60 protein with Chou-Fasman method.
Figure 19. Helical wheel projection for tentative calmodulin-binding domain of TCB60 protein.
Amino acid sequence is GWLKIKAAMRWGFFVRKKA at positions 362-380 of TCB60 protein. The underlined amino acid residues are deleted from the protein generated from R1129.
projection, six positively charged residues lie on one side of the helix while the opposite side is predominantly hydrophobic. This structure is striking in its similarity to the basic amphiphilic α-helices identified as CaM-binding domain from a variety of animal and human CaMBPs (169, 203, 271). The helical wheel of the CaM-binding domain from chicken gizzard myosin light chain kinase (MLCK) depicted by Lukas et al (169) is presented here for comparison (Fig. 20) (169).

Both hydrophobic and electrostatic interactions between CaM and the CaM-binding domain have been identified by using the replacement of certain amino acids in CaM-binding domain (203). The deletion of the sequence RKK (positions 377-379) in pTCB60 deletion mutant R1129 would be predicted to reduce the electrostatic interaction which may result in the loss of CaM-binding activity. Indeed the R1129 mutant protein failed to bind to CaM (Fig. 17, lane A6). The presence of tryptophan residues (positions 363 and 372) is also a feature of the known CaM-binding domains. These residues appear to be essential for high affinity CaM-binding as evidenced by amino acid replacement experiments (289). The bulky tryptophanyl residue hinders the formation of a stable secondary structure. This provides flexibility of the CaM-binding domain which may be required for optimal packing and high-affinity binding (289). The results presented here
Figure 20. Helical wheel projection for calmodulin-binding domain from chicken myosin light chain kinase (ref. 169).
demonstrated that TCB60 contains a basic amphiphilic α-helix CaM-binding domain as described in animal CaMBPs. This is the first such evidence for this structure in plant CaMBPs.

D. Characterization of a cDNA Clone Encoding a Heat Shock-Induced Calmodulin-Binding Protein.

Clone pTCB48 was further confirmed to code for a CaM-binding protein by gel overlay analysis. Total proteins isolated from the culture of E.coli XL1-Blue containing this phagemid were used for gel overlay analysis under more stringent conditions. Several peptides with molecular weights of 55, 38, 34 and 24 Kd generated from pTCB48 bound to CaM in the presence of 0.2 M NaCl and 0.1 mM CaCl₂ (Fig. 22, lane A4). CaM-binding activity was not detected in the presence of EDTA (Fig. 22, lane B4), suggesting that the interaction of CaM with these peptides is Ca²⁺-dependent. These CaM-binding peptides were probably expressed from one ORF of the insert of pTCB48 and resulted due to instability of fusion proteins in E.coli cells. This was evidenced by deletion and sequencing analysis (see below).

Analysis of gene expression. Equal RNAs from control and heat shock treatments were electrophoresed on formaldehyde agarose gels and probed with a ³²P-labeled DNA probe made from pTCB48. Two mRNA bands with molecular
Figure 21. Deletion constructs of pTCB48. First nucleotide from the 5' end of the insert is referred as number 1.
Figure 22. Calmodulin gel overlay analysis of extracts from *E. coli* XL1-Blue cells harboring pTCB48 or different deletion constructs.

Equal amounts of proteins extracted from cultures of XL1-Blue cells harboring different phagemids without (lanes 1 and 2) or with IPTG (remaining lanes) as inducer were separated on 12.5% SDS-polyacrylamide gels and gel overlay analysis was done in the presence of 0.1 mM CaCl₂ (A) or 5 mM EDTA (B) as described in Materials and Methods.

Lane 1, No phagemid; lanes 2 and 3, pBluescript; lane 4, pTCB48; lane 5, D180; lane 6, D747; lane 7, D835; lane 8, D867; lane 9, D982; lane 10, R1516; lane 11, R1480; lane 12, R1184; lane 13, R1162 and lane 14, R1087.
weights of 1.9 and 2.5 Kb were detected in the RNA sample isolated from heat-shocked cells but not in control extracts (Fig. 23, lanes 1 and 2). This indicates that these two RNA species were induced by heat shock. In addition, it is likely that both RNAs were active in the translation during heat shock since both were present in heat-shocked polysomal RNA fraction (Fig. 23, lanes 4 and 5). A time course analysis of expression indicated that these two mRNAs were barely detected after a 15 minute heat shock treatment at 38°C (Fig. 24). These RNAs accumulated to maximum amounts after 1.5 hours of heat shock (Fig. 24).

These two RNAs may result from processing of the transcript of a single gene. Alternatively, they may result from two different heat shock-induced genes of very high homology. If the latter case is true, we suggest that 1.9 kb RNA is transcribed from pTCB48 gene for the following reasons. First, the much stronger hybridization signal for the 1.9 Kb RNA on Northern blots suggests a high abundance of this RNA in the polysomal RNA population as compared with the 2.5 Kb RNA. The latter band was just above the level of detection under our conditions (Fig. 23, lanes 4 and 5).

Secondly, our cDNA library was constructed with the poly(A) RNAs purified from polysomal RNAs. Finally, three (pTCB25, 31 and 38) of 25 putative CaMBP clones obtained
Figure 23. Northern blot analysis of pTCB48.

Equal amounts (20 µg/lane) of total (lanes 1 and 2) and polysomal (lanes 3, 4 and 5) RNAs isolated from control (lanes 1 and 3) and heat shocked (lane 2, 4 and 5) tobacco cells were separated on 1.5% formaldehyde agarose gel and transferred by blotting to nitrocellulose. The RNAs were probed with 32P-labeled DNA made from pTCB48 by random primer extension. Note: Lane 5 was the same as lane 4 but exposed for longer time.
Figure 24. Time course of expression of pTCB48 mRNA for different lengths of heat treatment.

Equal amounts (20 µg/lane) of total RNAs isolated from tobacco cells which have been treated at 38°C for different lengths were separated on 1.5% formaldehyde agarose gel and transferred by blotting to nitrocellulose. The RNAs were probed with 32P-labeled DNA made from pTCB48 by random primer extension.
from screened recombinant phages are identical to pTCB48, further indicating the abundance of this RNA.

**Analysis of DNA sequence.** The sequence of pTCB48 was determined (Fig. 25). A 1574 bp insert contains an 1307 bp ORF which encodes for 434 amino acids (Fig. 25). Following the TAA stop codon (position 1305-1307) is an untranslated sequence of 267 bp including a poly(A) terminus of 22 bp. A polyadenylation signal is the sequence, AATTATTTTT, located at the positions 1511-1519, 33 bp upstream from poly(A) sequence. The predicted size of the fusion protein produced from clone pTCB48 based on the sequence data is 53.5 Kd which is in agreement with the size of the CaMBP (55 Kd) identified by overlay analysis (Fig. 22, lane A4). No significant homology between pTCB48 and other reported genes from DNA and protein database was found.

**Tentative identification and features of the CaM-binding domain.** Recent studies on animal and human CaMBPs, including non-erythroid spectrin (157), smooth muscle MLCK (169), skeletal muscle MLCK (27), the Ca$^{2+}$ pump (123, 289), calmodulin kinase II (110) and the γ-subunit of phosphorylase b-kinase (73), indicate that one type of CaM-binding domain is a basic amphiphilic α-helix (Fig. 20). To determine the CaM-binding site of TCB48, several deletion mutants from both the 5' and the 3' ends of pTCB48 insert
Figure 25. DNA sequence and deduced amino acid sequence of pTCB48.
were constructed (Fig. 21). The CaM-binding activity of the proteins from these various deletions was assayed by gel overlay analysis with $^{35}$S-labeled CaM. The proteins generated from all 5' deletion constructs, including D982 which lacked 327 amino acids from the N-terminus encoded by pTCB48 insert, retained the ability to bind to CaM (Fig. 22, A5, A6, A7, A8 and A9). These results localized the CaM-binding domain to a sequence of 107 C-terminal amino acids (from 328-434) of TCB48 (Fig. 25). The fact that D982 synthesized only one peptide (16 Kd) that bound to CaM indicated that the ORF of pTCB48 was responsible for all four CaM-binding peptides produced from clone pTCB48 (Fig. 22, A9). Analysis of several 3' deletion constructs further define the binding domain. The protein produced from deletion construct R1184, in which 40 C-terminal amino acids were removed, lacked CaM-binding activity (Fig. 22, lane A12). Thus, the CaM-binding domain resides either in the C-terminal 40 amino acids or overlaps the deletion point.

A secondary structure analysis of TCB48 protein was done with Chou-Fasman method in the University of Wisconsin sequence analysis software package (54). This analysis suggests that the region from amino acid residue 400 to 434 has a high probability of forming $\beta$-sheets and $\beta$-turns (Fig. 26). The region from amino acid residue 330 to 400 has a
Figure 26. Predicted secondary structure of TCB48 protein with Chou-Fasman method.
propensity for forming α-helices. However, neither region apparently contains the classic CaM-binding basic amphiphilic α-helix domain. Even though α-helices are predicted in the region from amino acid 330 to 400, a total of 18 negatively charged, acidic residues is dispersed throughout the sequence. This would prevent the formation of a strict basic amphiphilic α-helix. Recent experiments using short synthetic peptides of 25 amino acids based on the γ-subunit of phosphorylase kinase indicate an alternative CaM-binding domain structure (73). A peptide of 25 amino acids which binds to CaM forms a β-turn followed by β-strand structure (73). The data presented in this study suggest that some CaM-binding domain other than a basic amphiphilic α-helix is also present in plant CaMBPs.
CHAPTER V
CONCLUSION

The study presented here provides first indication of the synthesis of CaMBPs as HSPs during plant HSR. Beside HSP 70, the synthesis of several other CaMBPs with molecular weights ranging from 17 to 82 Kd was enhanced/induced by heat shock. These results suggest a role of CaM-mediated processes in plant HSR.

A procedure for the isolation of CaMBP clones by screening a cDNA expression library with radioactively-labeled CaM as a ligand probe was highly refined. Twenty-five independent tobacco cDNA clones were isolated using this screening method and confirmed to encode CaMBPs by gel overlay analyses. The expression of all these cloned mRNAs was analyzed with Northern blots. These clones can be divided into three classes based on the differential expression during heat shock. The expression of pTCB48 mRNA was induced while the level of pTCB60 mRNA was reduced by heat shock. However, the levels of most cloned mRNAs as represented by pTCB40 were unaffected by heat shock. These three clones were sequenced and database searches of DNA and protein sequences did not reveal any significant homology between these clones and other reported genes or proteins.

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Thus, these cDNAs apparently represent new sequences encoding CaMBPs of unknown functions. Based on the present study and data from literature, a model for the possible functions of CaMBPs in the HSR is suggested in Figure 27.

This study is the first report for the isolation and characterization of cDNA clones for CaMBPs in plants. Furthermore, this research provides the first evidence for the presence of a basic amphiphilic α-helix as CaM-binding domain in a plant CaMBP. An alternative structure other than a basic amphiphilic α-helix is also suggested to be a CaM-binding domain in a plant CaMBP. This structure may be a β-sheet following a β-turn.

Future efforts should focus on the functional identification of these cloned CaMBPs. The sequence analyses of the whole coding regions and promoters may provide insight into the regulation of these proteins during heat shock. Finally, further characterization and identification of the nature of the CaM-binding domains as well as the interactions with CaM will provide a better understanding of the mechanisms by which CaM regulates a variety of protein targets.
Figure 27. A model for the possible functions of CaMBPs in the HSR.
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