MOLECULAR CLONING AND CHARACTERIZATION OF A HEAT-SHOCK INDUCED CALMODULIN BINDING PROTEIN GENE AND cDNAs ENCODING GLUTAMATE DECARBOXYLASE FROM TOBACCO

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DEDICATION

TO OUR BROTHER,

SISIRA KEERTHI JAYAWARDANE

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ABSTRACT

Two individual heat shock induced/enhanced tobacco (*Nicotiana tabacum* L. cv Wisconsin-38) CaMBP cDNAs, pTCB48 and pTCB15, were characterized. Both of these clones were partial cDNAs. The 5' end of pTCB48 was obtained using 5' RACE technique. The ORF of this cDNA codes for a protein with a molecular weight of 50 kD. Using several deletions coupled with CaM gel overlay, CaM binding domain of pTCB48 was localized to the very end of the C-terminus of the protein. Structural prediction of this domain revealed a possibility of forming a BAA structure but an alternative β-strand and β-turn structure could not be excluded. Polyclonal antibody was raised against purified TCB48 fusion protein and used in Western analysis.

The gene (TG48) corresponding to the pTCB48 cDNA was isolated and characterized. TG48 contains a total of 6149 bp including 1064 bp of 5' flanking region and 364 bp of 3' flanking sequence. The TG48 gene is consists of 6 exons and 5 introns. The 5' flanking sequence contains common promoter elements such as TATA and CAAT boxes, and 5 putative HSEs, characteristic of heat shock induced genes. These

HSEs may be involved in heat shock induction of this gene. The structure of both TG48 gene and full length cDNA indicate that of two heat shock induced mRNAs, the 1.8 kb transcript is encoded by this gene. Northern analysis indicates this gene is subjected to tissue specific, developmental and environmental regulation.

Sequence analysis of pTCB15 indicated that it encodes a CaM binding glutamate decarboxylase (GAD). Two full length GAD clones were isolated by screening a *N. tabacum* L. cv Xanthi cDNA library. Both clones had similar nucleotide sequences except that GAD9 had a shorter 3' UTR. CaM binding domain of the tobacco GAD may be located at C-terminus and possibly a BAA structure. Results presented here indicate GAD transcript level is subjected to developmental, environmental and tissue specific regulation.

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A	absorbance
ATP	adenosine 5'-triphosphate
BCIP	5-bromo-4-chloro-3-indolyl phosphate
BAA	basic amphiphilic α-helix
CaM	calmodulin
CaMBP	calmodulin binding protein
cDNA	complementary DNA
CDPK	calcium dependant protein kinase
cm	centimeter
cpm	counts per minute
DG	diacylglycerol
DNA	deoxyribonucleic acid
DTT	dithiothreitol
EGTA	ethyleneglycol-bis-(β-aminoethyl ether) N, N, N', N'-tetraacetic acid
EDTA	ethylenediamine tetraacetic acid
ER	endoplasmic reticulum
GABA	γ-aminobutyric acid
GAD	glutamate decarboxylase
g	gram
g	gravity
GRP	glucose regulated protein
GSP	gene specific primer
h	hours
HSC	heat shock cognate
HSE	heat shock element
HSF	heat shock factor
HSP	heat shock protein
HSR	heat shock response
IEF	isoelectric forcusing
IP_3	inositol 1,4,5-triphosphate
IPTG	isopropyl-β-D-thiogalactoside
kb	kilobase
kD	kilodalton
LMW	low molecular weight
M	molar
min	minutes
m	milli-
μ	micro-
mRNA	messenger RNA
NBT	nitroblue tetrazolum
nM	nano meter

PAGE poly acrylamide gel electrophoresis
PCR polymerase chain reaction
PIP phosphatidyl inositol phosphate
PIP_2 phosphatidylinositol bisphosphate
PMSF phenylmethylsulfonyl fluoride
poly(A) poly adenylatetd
PVDF polyvinyl difluoride
RACE rapid amplification of cDNA ends
RNA ribonucleic acid
s seconds
SDS sodium dodecyl sulphate
TBST tris buffer saline, Tween-20
TRIS tris(hydroxymethyl)amminomethane
UTR untranslated region
v volts

Chapter I

LITERATURE REVIEW

INTRODUCTION

Plants are subjected to biotic and abiotic stresses which affect their normal growth and developmental processes. These stresses can act as signals to evoke specific responses in plants. Abiotic factors such as water, light, salinity, and temperature can negatively affect plant developmental processes thus limiting agricultural productivity. Plants have developed special mechanisms to perceive and transduce stress signals in order to respond them successfully. A clear understanding of these signal transduction mechanisms is important in developing agronomically important stress-resistant plants.

Involvement of Ca^{2+}, calmodulin (CaM) and calmodulin binding proteins (CaMBPs) in signal transduction pathways has been studied extensively in animal systems. Although calcium has been established as a messenger in plants, our understanding of signal transduction mechanisms in plants is still in its infancy. The presence of Ca^{2+}

modulated proteins, such as CaM and calcium dependent protein kinase (CDPK), is also well established in plants, but only a few CaMBPs have been characterized.

Plant responses to high temperature have been studied in detail. When plants are exposed to high temperature, they synthesize a set of new proteins called heat shock proteins (HSPs). Several genes which respond to heat shock have been isolated from plants. All of these genes share common nucleotide sequences in their promoter regions. However, to date, neither heat shock nor any other stress-induced calmodulin binding protein genes have been isolated from plants. Research discussed here has focused on isolation and characterization of heat-shock related CaMBPs and their genes or cDNAs from tobacco.

SIGNAL TRANSDUCTION

The role of Ca^{2+} as a second messenger in plant signal transduction pathways has been investigated extensively (for reviews see Bush, 1995; Gilroy *et al*, 1993; Poovaiah and Reddy, 1987, 1993). The cytosolic Ca^{2+} level may range from 0.1 to 1 μM and fluctuates during physiological processes (Bush, 1990; Gehring *et al*, 1990a; Gehring *et al*,

1990b, Gilroy *et al*, 1990; Gilroy and Jones, 1992; Gilroy and Trewavas, 1990; Knight *et al*, 1991; McAnish *et al*, 1990). Environmental stimuli such as touch, cold-shock, fungal elicitors (Kinght *et al*, 1991), wind (Knight *et al*, 1992), salinity (Lynch *et al*, 1989), light, gravity (Gehring *et al*, 1990), and hormones such as auxin, ABA (Gehring, 1990), and GA (Gilroy and Jones, 1992) induce rapid and transient changes in cytosolic Ca^{2+} level. The mechanisms by which signals cause Ca^{2+} fluctuations are poorly understood. However, it is possible that different environmental stimuli may use separate mechanisms to change free cytosolic Ca^{2+} levels. For example, wind induced Ca^{2+} increase is unaffected by plasmamembrane calcium channel blockers but is abolished by organellar calcium channel blockers (Knight *et al*, 1992). Whereas, cold-induced cytosolic Ca^{2+} increase is blocked by plasmamembrane Ca^{2+} channel blockers but is unaffected by organellar Ca^{2+} channel blockers. These observations suggest that the Ca^{2+} source for wind signal is organellar but is extracellular for cold-shock (Knight *et al*, 1992).

It is also likely that the same stimulus may have opposing effects on Ca^{2+} levels depending on the tissue or cell type. For example, ABA

increases cytosolic Ca^{2+} level in maize coleoptiles, parsley hypocotyles, roots (Gehring *et al*, 1990) and stomatal guard cells (Gilroy *et al*, 1991; McAnish *et al*, 1990). Conversely, ABA lowers the cytosolic Ca^{2+} level in barley aleuron protoplasts (Gilroy *et al*, 1992; Wang *et al*, 1991).

As mentioned above how stimuli cause cytosolic Ca^{2+} fluctuations are not clearly understood. However, in animal cells, hydrolysis of phosphatidylinositol-4,5, bisphosphate (PIP_2) plays a crucial role in elevating cytosolic Ca^{2+} (Berridge and Irvine, 1989). Signals, through receptors and G proteins, activate phospholipase C which in turn catalyzes hydrolysis of PIP_2 into inositol-1,4,5 triphosphate (IP_3) and diacylglycerole (DG) (Berridge and Irvine, 1989). IP_3 elevates the cytosolic Ca^{2+} level by releasing Ca^{2+} from internal stores, especially from endoplasmic reticulum (ER). Diacylglycerol and Ca^{2+} activate protein kinase C which regulates the activity of several enzymes and proteins through phosphorylation (Asoka et al, 1992; Nishizuka, 1988). Studies on plants have indicated the presence of many components of the PIP_2 pathway and have suggested possible roles of this pathway in plant signal transduction (Einspahr and Thompson, 1990; Irvine, 1990; Lehle, 1990; Morse *et al*, 1989;). IP_3 signal transduction related functions such

as light and auxin induced hydrolysis of PIP_2 (Lehle, 1990; Morse *et al*, 1989), IP_3 induced Ca^{2+} release from isolated vacuoles (Ranjeva *et al*, 1988; Schumaker and Sze, 1987) and guard cells (Gilroy and Trewavas, 1990), and DG induced ion pumping and stomatal opening (Lee and Assmann, 1991) have been shown in plants. However, there is no conclusive evidence to prove the presence of protein kinase C and DG in plants.

The increase in free cytosolic Ca^{2+} in response to environmental signals is considered as a molecular switch which can turn on/off various cellular processes. In most cases, cytosolic Ca^{2+} changes are sensed by a group of proteins known as calcium binding/modulated proteins.

CALCIUM MODULATED PROTEINS

CaMBPs are believed to be involved in regulation of many cellular activities. Most of these proteins have a structural feature called the 'EF hand' which binds to Ca^{2+} and undergoes a conformational change. This change alters the ability of these proteins to interact with their target proteins (Babu et al, 1988; Heizmann and Hunziker, 1991; Klee and Vanaman, 1982; O'Neil and DeGrado, 1990; Strynadka and James,

1989). In plants, two calcium binding proteins have been well characterized (Poovaiah and Reddy, 1993). Calcium dependant (CaM independent) protein kinase (CDPK), which is unique to plants, has been isolated from several plants (Roberts and Harmon, 1992). Another Ca^{2+} binding protein, calmodulin (CaM) a small protein about 18 kD present in all eukaryotes, is believed to play a key role in the Ca^{2+} signaling pathway (Roberts *et al*, 1992; Roberts and Harmon, 1992).

CDPK is unique among Ca^{2+} regulated plant protein kinases as it binds Ca^{2+} directly. The catalytic domain of the protein is similar to Ca^{2+} / CaM protein kinases while the regulatory domain is similar to the Ca^{2+} binding site of CaM. These two domains of CDPK are separated with a short stretch of amino acids. Experimental evidence suggests that there is a intrasteric inhibition of the catalytic domain by the regulatory domain in the absence of Ca^{2+}. CDPK is localized in the cytosol, plasmamembrane, chromatin, cytoskeleton and symbiosome membrane of nodules. The physiological role of this protein is still not known. However, three possible endogenous substrates, nodulin-26,

plasmamembrane H^+-ATPase, and myosin, suggest CDPK may play a role in regulation of metabolic and ion fluxes in the cell (Roberts and Harmon, 1992).

CaM is a highly conserved protein found in all eukaryotes. So far, cDNA or genomic clones of CaM have been isolated from many plants including spinach (Lukas *et al*, 1985), barley (Ling and Zielinski, 1989), potato (Jena *et al*, 1989), *Arabidopsis* (Braam and Davis, 1990; Ling *et al*. 1991; Perera and Zielinski, 1992) alfalfa (Barnette and Long, 1990), and apple (Wattilon *et al*, 1993).

Recent studies have revealed that CaM genes are highly responsive to both internal and external signals. Jena *et al* (1989) showed that CaM mRNA was enhanced when strawberry fruits were treated with auxin. Studies with other plants have shown that touch, wind, rain (Braam and Davis, 1990), and wounding (Braam and Davis, 1990; Wattilon *et al*, 1992) enhance the CaM mRNA level. In plants, CaM is encoded by a multigene family (Perera and Zielinski, 1992). Expression of CaM genes are differentially regulated probably due to having different regulatory elements (Ling *et al*, 1991; Perera and Zielinski, 1992).

Calmodulin is an essential protein for cell proliferation. In yeast deletion of CaM gene is lethal to cells (Davis *et al*, 1986; Takeda and Yamamoto, 1987). Rusmussen and Means (1989) showed that overproduction of CaM in transformed mouse cells accelerated the proliferation while a decrease in CaM level by antisense RNA resulted in arrest of the cell cycle (Shoemaker *et al*, 1990). Attempts to overexpress CaM in transgenic plants were not successful. In these plants, eventhough the CaM mRNA level was 50 fold higher than controls, the CaM protein level was not significantly affected (Roberts *et al*, 1992; Poovaiah and Reddy, 1993). These results suggest that the amount of CaM protein in plant is regulated either at the translational or post-translational level (Poovaiah and Reddy, 1993).

CaM undergoes post-translation methylation at position 115. This step is catalyzed by a specific CaM-N-methyltransferase (Rowe *et al*, 1986). Methylation of CaM has shown to be important in regulating its activity as well as in protecting it from degradation by ubiquitin (Roberts *et al*, 1992). The expression of methylation mutant CaM in transgenic plants resulted in two fold increase in CaM level. These plants showed

decreased stem internode, reduced seed and pollen viability, and reduced seed production (Roberts *et al*, 1992).

Upon binding to Ca^{2+}, CaM interacts with many other proteins known as calmodulin binding proteins (CaMBPs). Interaction between CaMBP and Ca^{2+}/CaM complex has been shown to be involved in cellular function.

CALMODULIN BINDING PROTEINS

CaMBPs are known to play a key role in cellular regulation (Meader *et al*, 1992). A number of CaMBPs have been isolated, characterized and identified in animals. These include protein kinases, protein phosphatases (calcineurin), nitric oxide synthase, Ca^{2+} ATPase, IP_3 kinase and some structural proteins (Colbran and Soderling, 1990; Klee, 1991). In contrast, very little is known about the number, identity and localization of plant CaMBPs. However, enzymes such as protein kinases (Blowers *et al*, 1985; Blowers and Trewavas, 1987; Lu *et al*, 1993; Parajape *et al*, 1994; Patil *et al*, 1994; Wattilon *et al*, 1992), Ca^{2+} ATPases (Brauer *et al*, 1990; Dieter and Marme, 1980; Dieter and Marme, 1981), NAD kinase and nuclear ATPase (Poovaiah and Reddy,

1987; Roberts and Harmon, 1992) are known to be activated by CaM. The expression of some CaMBPs in plants are regulated developmentally and tissue specifically (Brawley and Roberts, 1989; Ling and Assmann, 1992; Oh and Roberts, 1992).

Sequence analysis of many CaMBPs has shown that there is no reasonable homology among these proteins in their CaM binding domains. However, many of these proteins have a common basic amphiphilic α-helix structure (BAA) as the calmodulin binding domain (Lukas *et al*, 1985; O'Neil and DeGrado, 1990). Recent studies with several plant CaMBPs have also shown the presence of this BAA structure (Baum *et al*, 1993; Lu, 1991; Lu and Harrington, 1994; Lu *et al*, 1995; Reddy *et al*, 1993).

A few CaMBP cDNAs with known functions have been isolated from plants. Wattilon *et al* (1992) and Patil *et al* (1994) isolated plant cDNAs homologous to the mammalian CaM kinase II. Deduced protein sequences of these cDNAs contain a CaM binding domain and the entire conserved domain of protein kinases. Recently, another CaMBP, glutamate decarboxylase, was cloned from a *Petunia* cDNA library. As this enzyme is relevant to the present study, it will be discussed in detail.

GLUTAMATE DECARBOXYLASE

Glutamate decarboxylase (GAD) catalyzes the conversion of glutamic acid to γ-aminbutryic acid (GABA). The presence of GAD activity and GABA in plants has been established for many years (Satyanarayan and Nair, 1990). However, their function in plants is unknown. In animal cells, GABA acts as a major inhibitory neurotransmitter by regulating ion channel conductance (Zhang and Jackson, 1993). In plants, GABA accumulates in response to many stimuli such as mechanical stress, cold-shock and darkness (Wallace *et al*, 1984); hypoxia (Streeter and Thompson, 1972); heat shock (Mayer *et al*, 1990); and water stress (Rhodes *et al*, 1986). Many of these stresses causes cytoplasmic acidification and GABA has been suggested to stabilize cytoplasmic pH by consumption of a proton in the glutamate to GABA conversion (Snedden *et al*, 1992; Crawford *et al*, 1994).

GABA undergoes transamination to yield succinic semialdehyde, that is then converted to succinate (Satyanarayan and Nair, 1990). Transamination of α-ketoglutarate by GABA to produce succinic semialdehyde and glutamate has been suggested as a bypass for direct

conversion of α-ketoglutarate to succinate in the tricarboxylic acid cycle. This direct conversion step may be inhibited under certain physiological conditions (Dixon and Fowden, 1961).

Recently, a GAD clone has been isolated from a *Petunia* cDNA library. The deduced protein sequence of this clone contains a GAD catalytic domain and a CaM binding domain which may be a BAA structure (Baum *et al*, 1993). The enzyme activity of the recombinant GAD expressed in *E. coli* is stimulated by CaM in the presence of Ca^{2+} (Arazi *et al*, 1995). Activity of GAD protein isolated from fava bean roots (Ling *et al*, 1994) and soybean (Snedden *et al*, 1995) is also stimulated 2 - 8 fold by CaM. These results suggest that the GAD activity may be regulated *in vivo* by the Ca^{2+}/CaM messenger system to elevate the GABA level in response to stressful conditions.

HEAT-SHOCK RESPONSE

Every living organism responds to moderate increase in temperature by altering normal cellular functions. This ubiquitous phenomenon known as the 'heat-shock response'(HSR) is characterized by induction of a new or existing set of proteins (heat shock proteins;

HSPs) and acquisition of thermotolerance to otherwise lethal temperatures (Beinz and Pelham, 1987; Lindquist, 1986; Lindquist and Craig, 1988; Marshall *et al*, 1990; Nagao *et al*, 1986; Vierling, 1991). A comparison of the heat shock response in different organism has shown that it is highly conserved in two ways: a) the molecular mechanism of gene induction by heat shows many similarities among diverse eukaryotes; and b) the major HSPs are highly conserved among eukaryotes and in some prokaryotes. The evolutionary conservation of the HSR argues strongly that it is a fundamental and essential process (Vierling, 1991).

In most organisms, the induction of protein synthesis as a result of high temperature coincides with the reduction of the synthesis of many normally synthesized proteins (Kelly and Schlesinger, 1978; Key *et al*, 1981; Lindquist, 1981; Somers et al, 1989). However, in maize (Baszczynski *et al*, 1985; Cooper and Ho, 1987; Cooper and Ho, 1985; Cooper *et al*, 1984) and sugarcane (Moisyadi and Harrington, 1989), heat shock does not affect normal protein synthesis. The reduction in normally synthesized protein may be a direct result of transcriptional inhibition of the respective genes (Findly and Pederson, 1981; Spradling *et al*, 1975) or post-transcriptional inhibition of respective mRNAs

through altered RNA processing (Neumann *et al*, 1984; Yost and Lindquist, 1986), mRNA stability (Lindquist, 1980), or modification of ribosomes (Scharf and Nover, 1982) .

HSPs are induced at very different temperatures in different organisms. A temperature shift from 95°C to 105°C induces HSPs in thermophilic bacteria (Phipps et al, 1993; Trent et al, 1990), but in arctic fishes (Parsell and Lindquist, 1993) and arctic algae (Vayda and Yuan, 1994) shift from 0°C to 5-10°C is sufficient for HSP induction. Plant species adapted to temperate environments, including crop plants such as soybean, pea, maize and wheat begin to synthesis HSPs when tissue temperatures exceed 32-33°C. Generally, temperature of maximum HSP synthesis is about 5-10°C above a particular species' optimum growth temperature (Vierling, 1991).

HSPs can be divided in to high molecular weight (60-110 kD) and low molecular weight (LMW) (15-30 kD) groups depending on their size (Vierling, 1991). Usually, in eukaryotes, HSPs are encoded by multigene families, and contain several isoforms belonging to each size class (Craig *et al*, 1989; Engman *et al*, 1989). High molecular weight HSPs are more prominent in animals, while LMW HSPs are abundant in

plants (Vierling, 1991). Some of these HSPs can also be induced by other stress conditions other than heat shock (Mizzen *et al*, 1989; Munro and Pelham, 1986; Normington *et al*, 1989).

REGULATION OF HEAT-SHOCK GENES

The expression of HSPs are subjected to both transcriptional (Stevenson *et al*, 1986) and post-transcriptional (Klemenz *et al*, 1985; LIndquist, 1987; Matsumoto *et al*, 1984) regulation. Heat shock gene transcription is coordinately regulated in all eukaryotic cells (Somers, *et al* 1989). Moreover, transgenic experiments using chimeric gene constructs from evolutionarily distantly related species have shown that regulatory mechanisms of heat shock gene transcription are highly conserved (Corces *et al*, 1981; Schoffl *et al*, 1989; Spena *et al*, 1985). A short palindromic consensus sequence, CT-GAA--TTC-AG, called the heat shock element (HSE), is essential for heat shock gene induction. This HSE has been found in all eukaryotic heat shock-regulated genes. (Beinz and Pelham, 1987; Bonner *et al*, 1984; Czarnecka *et al*, 1985; Czarnecka *et al*, 1989; Lindquist and Craig, 1988; Nagao *et al*, 1985; Rochester *et al*, 1986). Of these 14 bases, 8 nucleotides are highly

conserved and at least 7 are required to constitute an individual functional HSE (Beinz, 1985). Further studies have shown that HSEs are composed of a contiguous array of the 5 base unit of NGAAN in alternating orientations (Lis *et al*, 1989; Perisic *et al*, 1989; Xiao and Lis, 1988).

Most heat-shock genes contain more than one HSE with the most proximal one being 15-18 bases 5' to the TATA box (Beinz, 1987). Studies so far have shown that multiple HSEs are required for maximal expression of heat shock genes (Czarnecka et al, 1989; Dudler and Trevers, 1984; Gurley *et al*, 1986). The most TATA proximal HSE only accounts for 1% of the total induction (Amin et al, 1985; Dudler and Travers, 1984). Sequences immediately flanking the HSE may also play a role in heat shock induction. These sequences are suggested to be required for optimum binding of the heat shock factor (see below) (Amin *et al*, 1985; Lis *et al*, 1989; Shuey and Parker, 1986).

HEAT-SHOCK FACTOR

A trans-acting DNA-binding protein known as heat-shock factor (HSF), interacts with the HSE and induces transcription of heat-shock

genes (Abravaya *et al*, 1991; Beinz and Pelham, 1987; Cartwright and Elgin, 1986; Dietz *et al*, 1989; Parker and Topol, 1984; Wu. 1984a; Wu 1984b). HSF has been isolated and characterized from yeast (Sorger and Pelham, 1988; Wiederrecht et al, 1988), *Drosophila* (Wiederrecht *et al*, 1987), and tomato (Scharf *et al*, 1990). According to some studies, heat shock genes can be activated without protein synthesis indicating that HSF is present in some form before the heat shock (Ashburner and Bonner, 1979; Zimarino and Wu, 1987).

High affinity binding of HSF to DNA correlates with the conversion of HSF from a monomer to a trimer. This alteration is common to all organisms and may or may not be induced by heat shock (Rabindran *et al*, 1993; Sarge *et al*, 1993). The trimerization of HSF depends on several evolutionary conserved, hydrophobic heptad repeats (zipper motif) located next to the DNA-binding domain (Rabindran *et al*, 1993).

Two mechanisms are known for HSE-HSF regulation of heat shock gene transcription. In yeast, HSF is bound to the HSE under normal conditions both *in vitro* (Sorger *et al*, 1987; Sorger and Pelham, 19880), and *in vivo* (Jakobson and Pelham, 1988). However, the transcriptional

activation of HSP genes only begins after the heat shock (Sorger *et al*, 1987). This may be accomplished through heat induced phosphorylation of the HSF (Sorger *et al*, 1987; Sorger and Pelham, 1988). On the other hand, in human and *Drosophila*, HSF is incapable of binding to the HSE under normal temperatures but binds after heat shock (Kingston *et al*, 1987; Sorger *et al*, 1987; Zimarino *et al*, 1990; Zimarino and Wu, 1987). HSF is interconverted between active and inactive forms in response to temperature change. Interaction of HSF with the HSE is induced by phosphorylation of the HSF (Sarge *et al*, 1993; Sorger and Pelham, 1988). Studies on the *Drosophila* HSP70 gene demonstrate that RNA polymerase II transcribes the gene and forms a nascent mRNA chain about 25 nucleotides at normal temperature but transcription can not be continued without heat shock (Lis *et al*, 1989; Rougvie and Lis, 1988).

FUNCTIONS OF HSPs

The function of HSPs is not well understood. However, several studies including specific HSP mutant experiments indicate that HSPs are involved in the development of thermotolarence (Lindquist, 1986;

Lindquist and Craig, 1988, Vierling, 1991;). The high molecular weight HSPs (HSP90, -70, and -60) are suggested to be involved in transport and maintenance of protein conformation, hence are known as "molecular chaperonins"(Ellis, 1987; Ellis and Van der Vies, 1991; Kiefhaber *et al*, 1991; Kim and Baldwin, 1990; Nilsson and Anderson, 1991; Hendrick and Hartl, 1993; Parsell and Lindquist, 1993).

Heat shock cognate 70 (HSC70), a homolog of HSP70, is known to have a weak ATPase activity (Pelham, 1986; Pelham, 1990), and is associated with ATP dependant disassembly of clathrin-coated vesicles (Chapell *et al*, 1987; Deluca-Flaherty *et al*, 1990; Rothman and Schmid, 1986). Cytoplasmic SSA protein in yeast, a homolog of HSP70, facilitate protein transport to endoplasmic reticulum (ER) and mitochondria (Chirico *et al*, 1988; Deshaies *et al*, 1988). This may be achieved through unfolding of precursor proteins or their maintenance in a suitable conformation for transport (Beckmann *et al*, 1990; Vierling, 1991). In the light of theses findings, HSP70 is thought to be involved in folding of disassociated or denatured protein during heat stress and facilitating protein unfolding and reassembly after the heat stress (Pelham, 1986; Pelham, 1990).

Despite their abundance in plants, a possible role of LMW HSPs in HSR is not still clear. However, these proteins aggregate into 'heat shock granules'(de Sa *et al*, 1989; Nover *et al*, 1989), which may function in preserving normally synthesized mRNAs during heat shock (Nover, 1989). High sequence similarity of some LMW HSPs to α-crystallin (Bossier *et al*, 1989; Ingolia and Craig, 1982) raise the possibility of a structural role in maintaining cellular integrity during heat shock (Lindquist and Craig, 1988).

Although direct evidence is lacking on the function of many HSPs in thermotolerance, several studies suggest at least some HSPs are involved in thermotolerance. For example, mutants of the *Dna*K protein, a bacterial homolog of HSP70, had impaired growth at normal temperature and could not grow at all at high temperature (Georgopolus et al, 1990). Likewise, deletion mutants of yeast HSP104 could not develop thermotolerance (Sanchez and Lindquist, 1990).

Despite extensive studies on heat shock response in plants, how the heat shock signal is transduced to the responsive sites in the cell is still not clear. However, in animal cells the IP_3 pathway may be involved in this process.

SIGNALLING AND THE HSR

Heat shock stimulates phospholipid turnover and increases free cytosolic Ca^{2+} in animal cells (Calderwood et al, 1987; Calderwood, 1988; Jensen et al, 1988; Stevenson et al, 1986). This increase in free Ca^{2+}, IP_3 and diacylglycerol (DG) occurs during the first 30 seconds of stress treatment (Calderwood et al, 1987, Stevenson et al, 1986) suggesting a possible involvement of a second messenger cascade mechanism during the HSR. As discussed in the introduction, the existence of the phosphodylinositol cycle has been established in plants.

Several studies have focused on the role of Ca^{2+} in stress response. Treatment of a variety of animal cells with Ca^{2+} agonists induce the expression of two glucose regulated proteins (GRPs) which are also induced by heat shock (Resendez et al, 1985; Welch and Feramisco, 1985; Welch et al, 1983). Similarly, Ca^{2+} agonists induce the production of GRPs in rat hepatoma cells. These proteins are not produced when cells were subjected to Ca^{2+} depletion by EGTA (Lamarche et al, 1985). These results indicate that Ca^{2+} may be involved in the generation of the signal or transduction of the signal during HSR.

HSR AND CaMBPs

As mentioned earlier, both Ca^{2+} and CaM levels increase in response to many stress conditions. By using CaM-sepharose affinity chromatography, Harrington *et al* (1990) detected a set of newly synthesized CaMBPs ranging from 15 kd to 111 kd in heat shocked cultured tobacco cells. As the synthesis of these CaMBPs are induced or enhanced by heat shock, these proteins can be considered as HSPs. Lu (1991, 1993,) using ^{35}S labeled CaM as a legend probe isolated 25 CaMBP clones from a tobacco heat shock cDNA library. According to northern analysis, four of these cDNA clones represent heat-shock induced mRNAs, and one clone represents an mRNA that is down regulated by heat shock. The remainder of the clones represents CaMBP mRNAs which are not affected by heat shock (Lu, 1991; Lu and Harrington, 1993; Lu *et al*, 1995).

One of the heat-shock induced cDNA clones, pTCB48 (ca 1.6 kb), was studied in detail. In northern analysis, a probe prepared from this cDNA hybridized with two transcripts of 1.9 kb and 2.5 kb. These transcripts were only detected in heat-shocked cells. Lu (1991) suggested that these two transcripts might have resulted from a single gene or from

two different genes with high homology. Sequence comparison of pTCB48 with other known sequences in Genebank did not reveal a significant sequence similarity and the function of this CaMBP remains unknown (Lu, 1991).

Using several deletion constructs from both 5' and 3' ends of pTCB48 cDNA Lu (1991) located the CaM binding domain of the deduced protein to its C-terminus; but the exact domain and location could not be determined. Secondary structure analysis of the deduced amino acid sequence did not reveal the presence of common basic amphiphilic α-helix structure (BAA) found in many CaMBPs. However, the C-terminus showed a possibility of forming a β-strand and β-turn structure. Therefore, Lu (1991) suggested that this CaMBP might contain an alternative CaM binding domain.

Chapter II

SIGNIFICANCE AND HYPOTHESES

The involvement of Ca^{+}/CaM second messenger system in signal transduction in eukaryotic systems has been studied in detail (Roberts *et al*, 1985; Roberts and Harmen, 1992; Poovaiah and Reddy, 1987, 1993). The activities of target enzymes and proteins are modulated through this system in response to various stimuli. Many CaM target proteins (CaMBPs) have been characterized in animals, but only a very few of these have been characterized from plants (Roberts and Harman, 1992). Thus, a study of CaMBPs would help to elucidate how plant cells respond to stress environments.

There is substantial evidence to indicate that a Ca^{+}/CaM messenger system may be involved in the heat shock response of animal systems, however, no similar information is available from plants. Lu (1991) isolated several CaMBP cDNA clones from a tobacco cDNA expression library. One of these, pTCB48, was induced by heat shock. However, this clone was a partial cDNA and the function of the deduced protein was not determined. The CaM binding domain of the predicted protein

was localized to its C-terminus but the precise location was unknown (Lu. 1991). In northern analysis, a probe made from this cDNA recognized two heat shock-induced transcripts, 1.9 kb and 2.5 kb. Lu (1991) suggested that 1.9 kb mRNA may be transcribed from pTCB48 gene, however, the evidence to support this suggestion is not conclusive.

Recently, plant GAD was characterized as a CaMBP (Baum *et al*, 1993). GAD catalyzes the conversion of glutamic acid to GABA which is accumulated in plant tissues in response to various stress conditions including heat shock. This information suggests that GAD expression may be regulated by heat shock. Lu (1991), using ^{35}S-labeled CaM as a ligand probe, screened a heat shock tobacco cDNA library and isolated 25 individual CaMBP clones. One of these clones, pTCB15, was sequenced and found to be homologous to other plant GAD cDNA clones. However, this clone was also a partial cDNA.

The present study was undertaken to investigate the structure and expression of pTCB48 gene and the structure of the CaM binding domain of the corresponding protein. An attempt was also made to isolate and characterize a CaM binding glutamate decarboxylase cDNA clone from

tobacco (*N. tabacum* L. Xanthi). Based on available information on pTCB48 cDNA clone and other known CaMBPs, following hypotheses are proposed.

1) The promoter of the gene (TG48) corresponding to the pTCB48 cDNA clone contains heat shock elements (HSEs).
2) Of two transcripts detected in northern analysis, the 1.9 kb transcript is encoded by this gene (TG48) or corresponds to pTCB48.
3) The deduced protein of pTCB48 contains an alternative CaM binding domain.
4) The expression of tobacco CaM binding GAD is regulated by heat shock.

Specific objectives of this study are to,

(I) isolate 5' end of pTCB48 cDNA by 5' RACE and sequence it.

(II) construct PCR-based nested deletions of pTCB48 cDNA to sequentially delete C-terminus amino acid residues and to use these deletions in CaM gel overlay assay to identify the CaM binding domain.

(III) purify the pTCB48 recombinant protein from *E. coli* and prepare an antibody against this protein.

(IV) analyze the expression of pTCB48 CaMBP by northern and western blot analysis in cultured tobacco cells and tobacco plants.

(V) isolate and characterize the pTCB48 corresponding gene from a tobacco genomic library.

(VI) isolate and characterize a full length glutamate decarboxylase cDNA clone from a tobacco cDNA library and to study its expression under heat shock conditions by northern analysis.

Chapter III

ISOLATION OF THE 5' END OF pTCB48 cDNA AND DENTIFICATION OF THE CaM BINDING DOMAIN

INTRODUCTION

The CaMBP cDNA clone, pTCB48, isolated from a tobacco heat shock cDNA library consists of a 1574 bp insert including a 22 bp poly A tail. This clone was determined to be a partial cDNA based on northern analysis and the lack of an inframe Met initiation codon (Lu, 1991). In northern analysis, a probe from the cDNA insert hybridized to two messages, 1.9 kb and 2.5 kb. These two mRNAs might result from processing of the transcript of a single gene or from two different heat shock induced genes with very high sequence similarity (Lu, 1991). Lu (1991) suggested that if the latter is true, 1.9 kb RNA might be the product of pTCB48 gene. However, there is no direct evidence to support this suggestion.

The CaM binding domain in many known CaMBPs is a basic amphiphilic α-helix (BAA) (O'Neil and DeGrado, 1990). However, an alternative form of CaM binding domain has been reported for

phosphorylase B kinase in which this domain is a β turn and β strand (Dasgupta *et al.*,1985). So far, all known CaM binding domains consist of 17 - 24 amino acid residues (Harrington *et al.*, 1994). Lu (1991), using several deletion constructs from both 5' and 3' ends of the pTCB48 cDNA insert, localized the CaM binding domain to the 40 amino acids in the C-terminus of the predicted protein sequence. The Chou-Fasman secondary structure prediction for this area showed the possibility of forming β turn and β strand structure. Therefore, Lu (1991) suggested that pTCB48 protein might contain an alternative form of CaM binding domain. However, without a precise identification of the CaM binding domain this suggestion is inconclusive. In this chapter, isolation of the 5' end of the pTCB48 cDNA and identification of the CaM binding domain is described.

MATERIALS AND METHODS

Plant material

Tobacco cells (*Nicotiana tabacum* L. Wisconsin 38) were used in all experiments unless otherwise mentioned. Tobacco cells were

maintained as suspension cultures in Gamborg's B-5 medium (Gamborg, 1970). and routinely transferred into new medium at mid log phase (7 day old). Heat shock at 38°C for 2 h was given to 7 day old cells in an orbital shaking water bath.

Isolation of total and Poly(A) RNA

Tobacco cells (50 ml cell suspension in 250 ml Erlenmeyer flasks) were heat shocked at 38°C for 2h, filtered, frozen in liquid N_2 and ground to a fine powder. Total RNA was extracted from 8 g of ground cells using guanidine isothiocyante as described by McGookin (1984). Poly(A) RNA was isolated by two rounds of oligo-dT column chromatography as described by McGookin (1984) or with the PolyAtract system (Promega Co., Madison, WI) according to the manufacturer's instructions.

Northern blot analysis

Total (20 μg/lane) or poly(A) (2 μg/lane) RNA isolated from control or heat shocked tobacco suspension cells were electrophoresed on a 1.5% agarose gel containing formaldehyde and transferred onto Magna

nylon membrane (MSI, Westboro, MA). Membranes were then prehybridized in 5 ml of 50% formamide, 5X Denhardt's solution, 0.1% SDS, 5x SSPE and 100 μg/ml salmon sperm DNA for 2 h. A *Eco*RI/*Xho*I (1 kb) fragment of pTCB48 cDNA and was labelled with ^{32}P using random primer extension (BRL) and added to the prehybridization solution. Hybridization was continued for 16 h at 42^{0}C in a mini-hybridization oven (Labnet, Woodbridge, NJ). Blots were then washed twice with 25 ml of 2X SSC + 0.1% SDS for 10 min each at room temperature, twice with 25 ml of 0.1% SSC + 0.1% SDS for 10 min each at 37^{0}C. Final wash was done with 50 ml of 0.1% SSC +0.1% SDS for 30 - 60 min at 65^{0}C. The filters were then exposed to X-ray film at -80^{0}C.

5' Rapid Amplification of cDNA Ends (5' RACE)

The 5' end of the pTCB48 cDNA was cloned with the 5' RACE technique using the 5' AmpliFINDER RACE kit (Clonetech, Palo Alto, CA). Three gene specific, nested antisense primers (GSP1, GSP2,

GSP3) complementary to the cDNA clone (Fig. 3.4) were synthesized and used with the 5'RACE kit according to the manufacturer's instructions.

Poly(A) RNA (2 μg) isolated from heat shocked tobacco cells was used with GSP1 in the initial reverse transcription. An aliquot (2 μl) of this reaction mixture was used as template DNA for the polymerase chain reaction (PCR). The 5' end of the cDNA was amplified by two rounds of PCR using GSP2 / anchor primer (manufacturer supplied) in the first round and GSP3 / anchor primer in the second round. The PCR product from the 2nd round of amplification was purified with phenol:chloroform, blunt ended with Klenow fragment and then cloned into the *Sma*I site of the pBluskcriptII SK- (Stratagene). The cloned PCR product was sequenced by dideoxy sequencing method (Sequenase Version II, USB).

Preparation of ^{35}S-CaM

Isolation of ^{35}S-CaM was accomplished using the method described by Asselin *et al.* (1989) with minor modifications. An overnight culture (200 μl) of *E. coli* UT481 harboring pVUC-1 was inoculated into 20 ml of PSI medium (Appendix) and incubated at 37°C

until the A_{600} of the culture reached 0.6. Bacteria were collected by centrifugation at 1000 g for 5 min. and washed twice with 20 ml each of 0.1 M Tris-HCl (pH 7.4) , 92 mM NaCl, 40 mM KCl, 19 mM NH_4Cl, 0.26 mM $CaCl_2$, 0.98 mM $MgCl_2$, 0.74 mM $FeCl_{3,}$, 0.639 mM KH_2PO_4, 2.5% glycerol and 25 μg/ml ampicillin. Bacteria were pelleted again at 1000 g and resuspended in 5 ml of the latter medium containing 2.5 mCi of Tran-^{35}S labeled methionine/cysteine (ICN Biomedicals) and IPTG (0.4 mM). Cells were then incubated at 37°C for 3 h and collected by centrifugation as above. The pellet was resuspended in 5 ml of Tris/HCl (pH 7.5), 1 mM $CaCl_2$ and lysed by sonication. The bacterial lysate was centrifuged at 500 g for 5 min. and the supernatant was collected and heated at 90°C for 1 min. The heat stable fraction was separated by centrifugation at 500 g for 5 min. The supernatant was then applied to a 500 ul of phenyl-sepharose column in a 1.5 ml microfuge tube and washed 3 ml of 25 mM Tris/HCl(pH 7.5), 1 mM $CaCl_2$ and 3 ml of 25 mM Tris/HCl (pH 7.5), 1 mM $CaCl_2$, 0.2 M KCl. The labeled CaM was eluted with 500 μl of 25 mM Tris/HCl (pH 7.5) containing 2 mM EDTA.

Construction of pTCB48 deletions

Four nested antisense PCR primers (48D1, 48D2, 48D3, 48D4) complementary to the 3' end of the coding region of the pTCB48 were designed (Fig. 3.1) so that several amino acid residues were sequentially deleted in each construct (Fig. 3.8 D) from the carboxy terminus of the fusion protein by creating a premature stop codon. In each of these primers an *Xho*I restriction site was introduced to facilitate cloning. The sense primer was designed to include a Met initiation codon and a *Bam*HI site (Fig. 3.1). The undeleted construct (48D0) was prepared by using the sense primer and the T7 primer.

The coding region of pTCB48 was amplified by PCR and products were purified by phenol:chloroform extraction. Then PCR products were double digested with BamHI and XhoI and separated on a 0.7% agarose gel. DNA was eluted from the agarose using a gel eluter (Hoffer Scientific) and cloned into the *Bam*HI/*Xho*I site of pBluscriptII SK- (Stratagene). These plasmids were used to transform *E. coli* (XL1- Blue) and colonies containing appropriate size deletions were selected. The

Antisense primer	Deletion
5' 3'	
tgcctcgagctataatcgtaatt	48D1
aatttctcgagttagtctcaattga	48D2
cgtctcgagtgaaaagctactca	48D3
tcactcgagttagttcatgca	48D4

Xho I

Sense primer

gttgagctcacaggatccgatgtcagttgcttgtggag

*Bam*HI

Fig.3.1: Primers used for deletion constructs. *Xho*I site in antisense primers and *Bam*HI site in sense primer are underlined.

accuracy of the coding region was confirmed by sequencing of each deletion by automated sequencing (Applied Biosystems, model 373A).

Expression of recombinant protein

E. coli containing pTCB48 deletions, 48D1, 48D2, 48D3, 48D4 and undeleted plasmid 48D0 were inoculated into LB medium and incubated at 37°C until the A_{600} of the culture reached 0.2. IPTG (10 mM) was then added to the medium and culture growth was continued until the A_{600} was 1.0. Bacterial cells were pelleted in a microfuge for 3 min. and the pellet was resuspended in 500 ul of 2X Lamelli sample buffer (Lamelli, 1970). After 3 min in a boiling water bath the supernatant was subjected to SDS-PAGE (Harrington and Alm, 1988). CaM gel overlay analysis was done according to the method by Burgess *et al.* (1984). After electrophoresis, gels were washed three times with 100 ml of 25% (v/v) isopropanol and 10% (v/v) acetic acid for one hour each and then three times with 100 ml of buffer B (50 mm Tris/HCl pH 7.6 and 0.2 M NaCl) each at room temperature. Gel was then blocked with 30 ml of buffer B containing 0.1% BSA for 2 h and then incubated with 30 ml of buffer B plus 0.1 mM $CaCl_2$ containing 1 x 10^5 cpm ^{35}S-

CaM/ml for 14 h at room temperature. After washing the gel with 100 ml of buffer B plus 0.1 mM $CaCl_2$ three times (1 h each), the gel was stained with Coomassie Blue, destained, dried and exposed to X-ray film at -80°C.

RESULTS AND DISCUSSION

Northern analysis

When a 1 kb cDNA fragment was used as the probe in northern analysis, two messages were recognized (Fig 3.2 A). These two messages were only detected in heat-shocked samples of either total or poly (A) RNA consistent with the earlier report (Lu, 1991). However, according to the present study the sizes of these messages are 1.8 kb and 2.1 kb in contrast to 1.9 kb and 2.5 kb in the earlier report. This discrepancy may be due to an error in size estimation in the earlier study as the size of the full length pTCB48 cDNA is approximately 1.8 kb (Fig. 3.4).

When a ^{32}P-labelled probe prepared from the 3' prime untranslated region of the pTCB48 cDNA was used in northern analysis, only a single

message corresponding to 1.8 kb was detected (Fig. 3.2 B). This indicates that pTCB48 gene codes for the 1.8 kb message while the 2.1 kb message is transcribed from a different gene with sequence similarity to pTCB48.

Cloning of the 5' end of pTCB48

The 5' end of pTCB48 was cloned the by 5' RACE technique. The first round of PCR amplification using the nested GSP2 and anchor primer yielded a PCR product of 396 bp (data not shown). When an aliquot of this reaction mixture was used as the template for the second round of PCR amplification with GSP3 and the anchor primer, a 215 bp band was obtained (Fig. 3.3). Sequence analysis of this product showed that it contained 118 base of the pTCB48 cDNA, 52 bases of the anchor primer, and 35 bases of the 5' end of the cDNA (Fig. 3.4). The putative Met initiation codon was identified by the presence of numerous stop codons in all reading frames upstream to this codon.

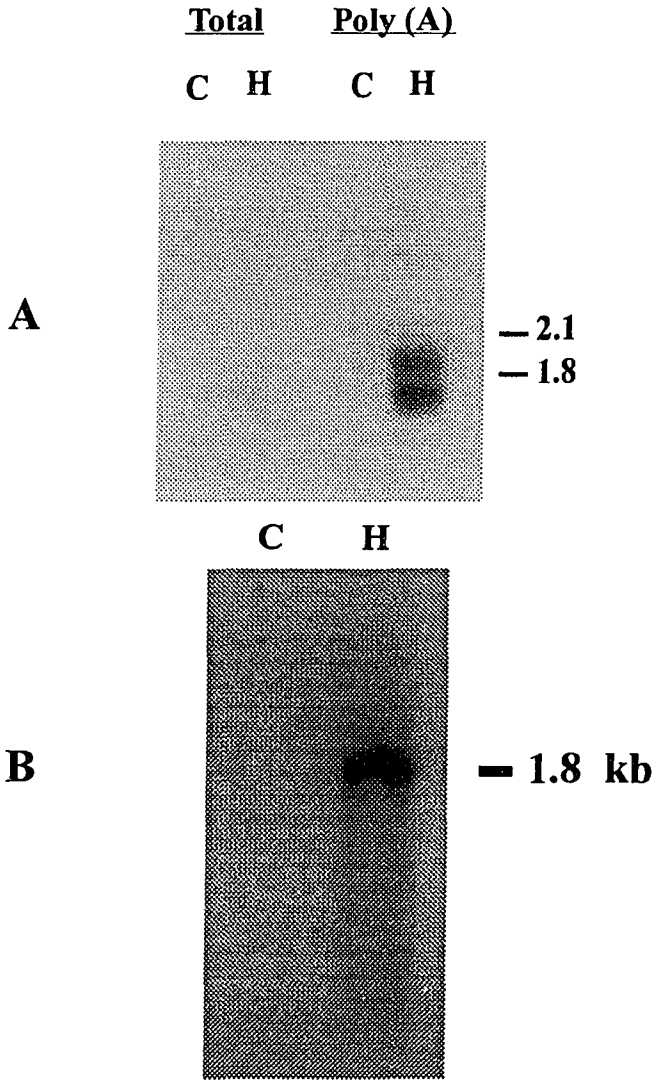

Fig. 3.2 Northern analysis with tobacco (W-38) RNA. A: Total or Poly(A) RNA isolated from cells grown at 23°C (C) or heat shocked at 38°C (HS) was hybridized with 1 kb probe from pTCB48 cDNA. B: Poly(A) RNA probed with 3' UTR of the pTCB48 cDNA.

C: control; HS: heat shocked

In order to confirm the earlier reported sequence (Lu, 1991), the pTCB48 cDNA was sequenced again by automated sequencing (Applied Biosystems). Several errors were noted in the previously reported cDNA sequence (Lu, 1991), including 33 bases missing in the middle of the cDNA (Fig. 3.4). These errors did not introduce a frame shift in the predicted protein sequence except from amino acid residues 68 - 77 (Fig. 3.4).

The full length cDNA contains an open reading frame of 1347 bases with a initiation codon at position 32 and a stop codon at position 1379 (Fig. 3.4). The putative protein sequence consists of 449 amino acids with a calculated molecular weight of 51 kD. However, a slightly bigger protein band was detected in western blot analysis using an antibody raised against the recombinant pTCB48 protein (Chapter IV). Computer searches of data bases using TFASTA or BLAST did not reveal any significant sequence similarity with any known proteins. However, two possible homologs, stress activated mitogen activated protein kinases (MAP kinases) from rat (c-jun)(Kyriakis *et al.*, 1994) and yeast (Hog1)(Brewster *et al.*, 1993), showed 20% identity and 40% similarity to TCB48 protein.

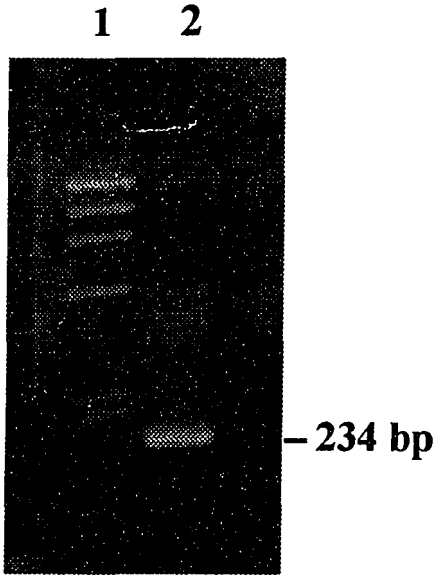

Fig. 3.3: PCR product from the 2nd round of amplification. Lane 1: θX174/*Hae*III DNA molecular weight markers. Lane 2: PCR product.

These two MAP kinases are similar in size to the TCB48 protein and are only 36% identical to each other at amino acid sequence level. However, TCB48 protein does not contain any conserved kinase domains.

Isolation of ^{35}S labelled Calmodulin

As mentioned in materials and methods, calmodulin labeled with ^{35}S was isolated and purified from *E. coli* harboring pVUC-1 . The specific activity of purified CaM was 1 x 10^6 cpm/μg. The purified CaM migrated on SDS-PAGE as a single band and showed the characteristic mobility shift in the presence and absence of Ca^{2+} (Fig. 3.5 A). Chicken gizzard CaM, used as authentic sample also showed the same mobility shift in the presence and absence of Ca^{2+} (Fig 3.5 B).

Identification of the CaM binding Domain

The CaM binding domain of the pTCB48 recombinant protein was localized by using protein derived from different deletion constructs of the pTCB48 cDNA clone (Fig. 3.6 and 3.8 D) in CaM gel overlay assay. Protein isolated from bacteria with only the pBluescript

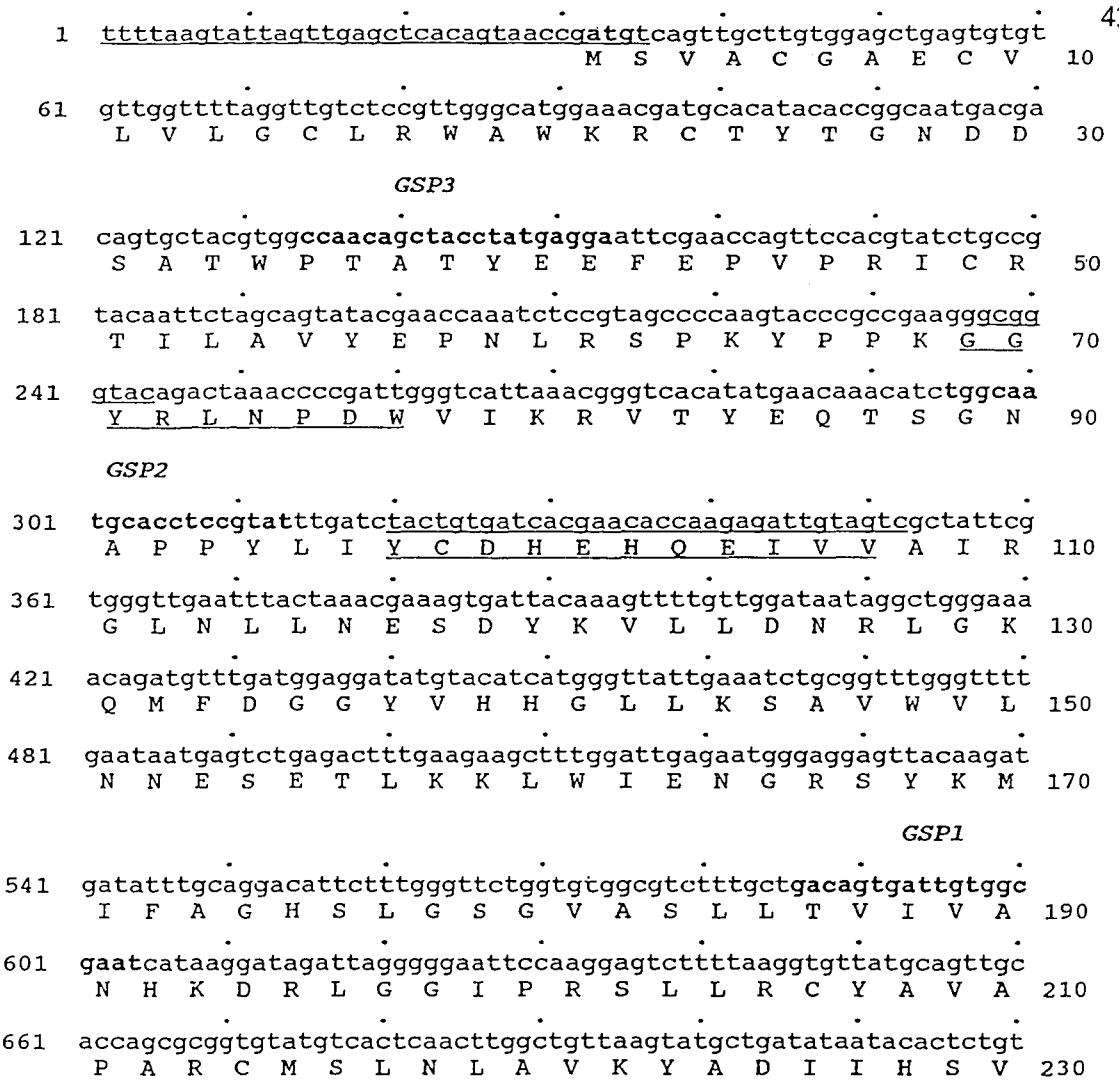

Fig. 3.4: Complete nucleotide sequence of pTCB48 cDNA and its predicted protein sequence. Sequences which were not reported in the earlier study (Lu, 1991) are underlined. Protein sequence used in deletion study is marked with double underlined italic letters. Complementary sequences to GSP1, GSP2, and GSP3 are marked with bold letters.

```
721  ggtattgcaggatgatttcttgccaagaacagccaccccacttgaagatatatttaaatc
      V  L  Q  D  D  F  L  P  R  T  A  T  P  L  E  D  I  F  K  S  250

781  catcttctgtttaccgtgcttgatatttttggtatgcttgagagataccttcattcctga
      I  F  C  L  P  C  L  I  F  L  V  C  L  R  D  T  F  I  P  E  270

841  gggcagaaaactccgagatccaagaagactttatgcaccaggccgtatgtatcacattgt
      G  R  K  L  R  D  P  R  R  L  Y  A  P  G  R  M  Y  H  I  V  290

901  agaaagaagattctgcagatgtgggaggttcactccagatgttagaactgccatcccagt
      E  R  R  F  C  R  C  G  R  F  T  P  D  V  R  T  A  I  P  V  310

961  tgacggaagatttgagcatatcgtgttgtcacgcaatgctacagttgatcatggaatcat
      D  G  R  F  E  H  I  V  L  S  R  N  A  T  V  D  H  G  I  I  330

1021 ttggatagaaagagaatctgaaaaagtattagcaagacttaaggaggctagtgctgagac
      W  I  E  R  E  S  E  K  V  L  A  R  L  K  E  A  S  A  E  T  350

1081 cacaactactccccccaaagtgcagaaaattgaaaggctgaagacattagaaaaagaaca
      T  T  T  P  P  K  V  Q  K  I  E  R  L  K  T  L  E  K  E  H  370

1141 caaggatgcactcgaaagagctgtcagtttaaacataccacacgctgtggacgcagatga
      K  D  A  L  E  R  A  V  S  L  N  I  P  H  A  V  D  A  D  E  390

1201 agaggaatccacggagagtataactgaggagtcatctcagaaacaggaggaagatgcaat
      E  E  S  T  E  S  I  T  E  E  S  S  Q  K  Q  E  E  D  A  M  410

1261 gacaagcaaagcgcagtgcagtgatgcaagaactaactggaatgaagtagttgagaagct
      T  S  K  A  Q  C  S  D  A  R  T  N  W  N  E  V  V  E  K  L  430

1321 tttcaatagagacgaaagtgggaaattacgattaaagagagatgcaactggtcccgagta
      F  N  R  D  E  S  G  K  L  R  L  K  R  D  A  T  G  P  E  *  449

1381 acaactctgttccaaggtgcttttctctcttcttttttatgatggctgtgtatgttaga

1441 tgaaagcattattactcgtgttccaacaactcataattctttattatccatatcgcatag

1501 tacaatatcttctttttgtatatcaacgaccataatgtataatctgaagccccgtcaata

1561 gtacagttggaaattcttttatgcaattattttaggcagttcctcgtggaatgtgatttg

1621 ttcatgaaaaaaaaaaaaaaaaaaaaa
```

Fig. 3.4: continued.

plasmid (Fig 3.7 lane 1) did not bind to CaM. Protein isolated from bacteria carrying the full length ORF (48D0), bound to ^{35}S-CaM when induced by IPTG (Fig 3.7 lane 3). Two bands, approximately 58 kD and 34 kD, were detected in this lane. The predicted molecular weight of the putative TCB48 protein is 51 kD and the molecular weight of the recombinant protein with the portion of the β-galactosidase would be approximately 55 kD. This calculated value corresponds to the observed value of 58 kD. The 34 kD band may be a degradation product of the recombinant protein. These two bands were barely detectable in the absence of IPTG (Fig 3.7 lane 2). Proteins isolated from bacteria containing any of the deletion constructs did not bind to ^{35}S-CaM (Fig 3.7 lanes 4 - 7). The deletion construct 48D1 is missing only the C- terminal 8 amino acid residues of the complete protein sequence. Thus, these 8 C-terminal amino acid residues should resides or overlap with the CaM binding domain of this protein.

Secondary structural prediction of the sequence covered by these deletions (amino acid residues 420 to 449) revealed the possibility of forming a β-turn and β-strand structure (from 424 to 443) by the

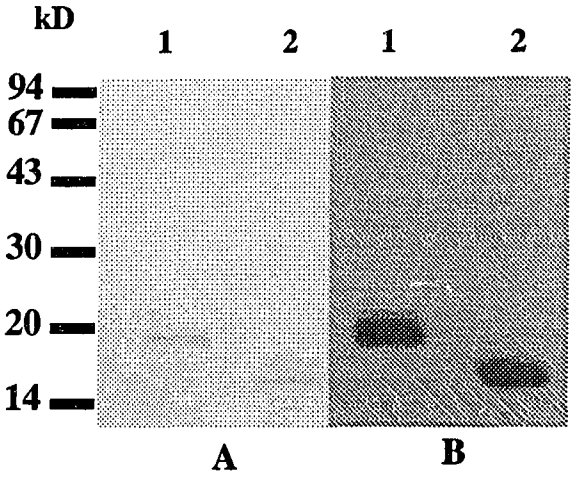

Fig. 3.5: SDS-PAGE of purified ^{35}S-labelled CaM. A: Chicken gizzard calmodulin. B: ^{35}S-labelled CaM isolated from *E.coli*. Lane 1: with 2 mM EDTA. Lane 2: with 1 mM $CaCl_2$. M: molecular weight markers.

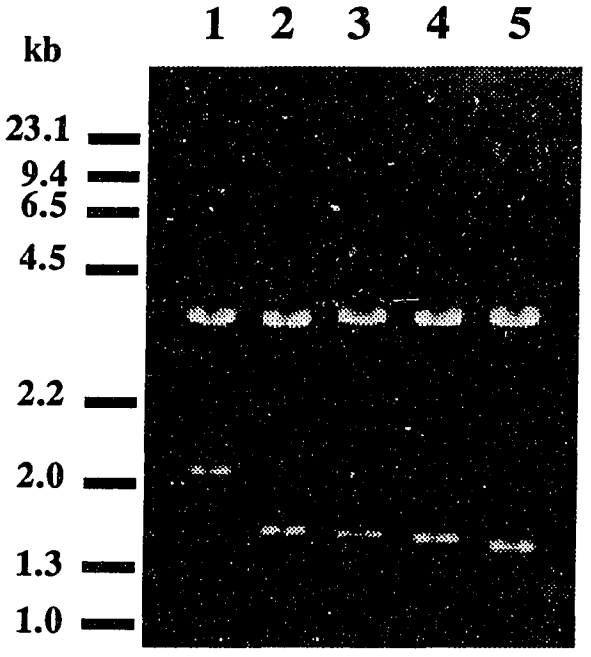

Fig. 3.6: *Bam*HI/*Xho*I digestion of deletion constructs. Lane 1: 48D0 (undeleted clone). Lane 2: 48D1. Lane 3: 48D2. Lane 4: 48D3. Lane 5: 48D4. M: Molecular weight markers.

Chou-Fasman method (Fig 3.8 A) (Chou and Fasman, 1978). In contrast, the Garnier-Osguthorpe-Robson structural prediction for the same area suggest the possibility of forming an α-helix (Fig. 3.8 B). A helical wheel projection of this α-helix revealed a putative BAA structure (Fig. 3.8 C). Interestingly, 48D1 deletion, which lacks 8 C-terminal amino acid residues, does not contain two basic residues found in this BAA structure. This deletion reduces the number of basic residues from 5 to 3 on the hydrophilic face of the BAA. Results of the CaM gel overlay revealed that the recombinant protein from this deletion does not bind to ^{35}S-CaM. Therefore, these results suggest that CaM binding domain of the TCB48 protein may be a BAA structure and resides between the amino acid residues 425 and 443. However, the possibility of this domain being a β-turn and β-strand structure can not be eliminated and require further investigation.

It possible that these deletion mutants were made more susceptible to proteolytic degradation. This would result in little or no observed expression of the protein and in misleading conclusions in overlay

coli produces a major cross-reacting band at approximately 58 to 60 kd; the predicted size of the TCB48 protein. Because of the presence of this band, mutant proteins could not be detected. Furthermore, it was difficult to detect intact TCB48 even though overlay analysis confirmed that this protein was, in fact, present. The nature of this cross-reacting band is presently unknown. However, this band clearly does not bind CaM as indicated by overlay analysis (Fig. 3.7; lane 2). Thus, the results presented here are insufficient to conclusively confirm the precise location of the CaM binding domain.

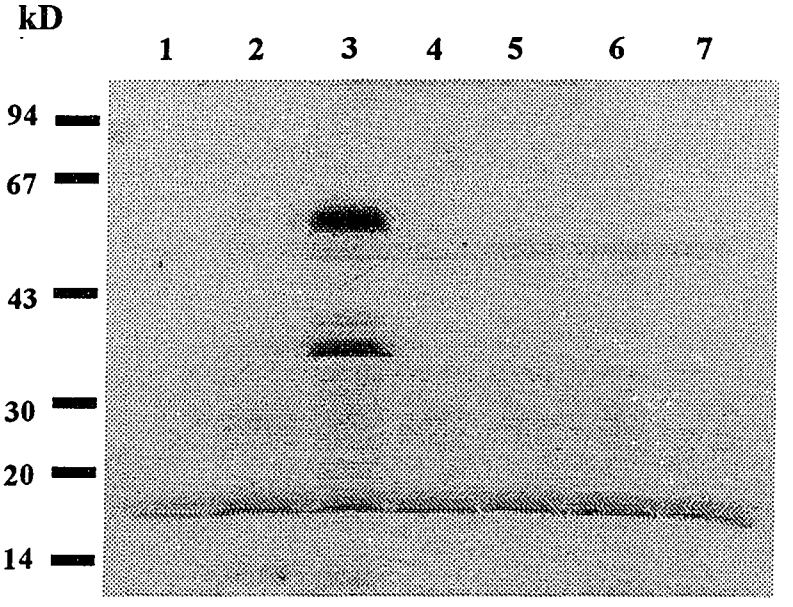

Fig. 3.7: CaM overlay with protein isolated from pTCB48 deletions. Proteins were separated on SDS-PAGE and incubated with ^{35}S-labelled CaM. Lane 1: pBluescript (no insert). Lane 2: 48D0 (undeleted) without IPTG induction. Lane 3: 48D0 induced by IPTG. Lane 4 - 7 48D1, 48D2, 48D3 and 48D4 induced by IPTG.

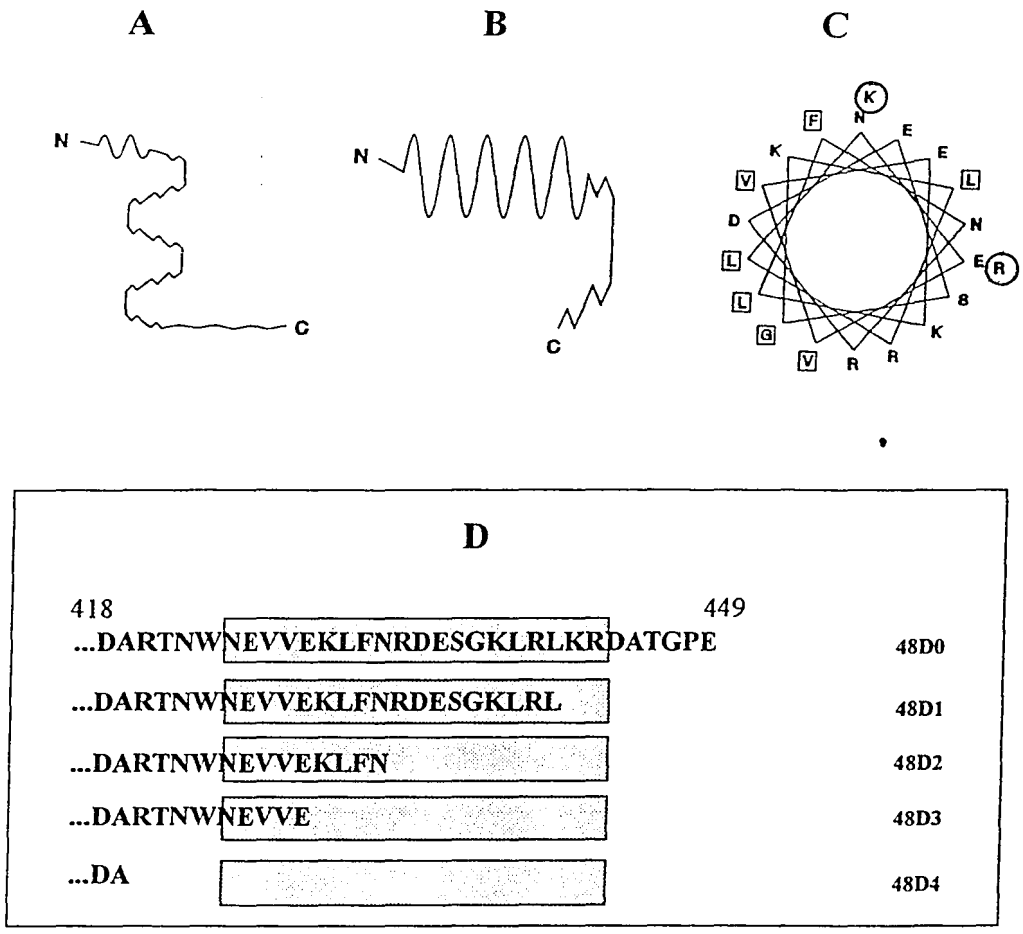

Fig 3.8: Secondary structure prediction for C-terminus of pTCB48. A: Chou-Fasman prediction for amino acid residues 424-443. B: Garnier-Osguthorpe-Robson prediction for residues 424-443. C: Helical wheel projection for the same sequence (NEVVEKLFNRDESGKLRLKR). D: C-terminal sequences of pTCB48 deletion constructs. Sequence shown by the helical wheel is boxed. Circled residues are missing in 48D1.

Chapter IV

STRUCTURE AND EXPRESSION OF TOBACCO TG48 HEAT SHOCK INDUCED CaMBP GENE

INTRODUCTION

In tobacco suspension cells, the gene corresponding to the pTCB48 cDNA clone is induced by heat shock at 38°C. Two transcripts related to this cDNA were detected in heat shocked cells but not in control cells grown at 23°C (Lu, 1991; Lu *et al*, 1995). It was not clear whether these two transcripts resulted from a single gene or from two genes with high sequence similarity.

Induction of certain genes by heat shock is an ubiquitous phenomenum (Lindquist, 1986; Vierling, 1991). Expression of these so called heat shock genes can be regulated at transcriptional and post-transcriptional level (Vierling, 1991). Regulatory mechanisms involved in heat shock gene transcription are highly conserved among distantly related species. Promoters of these genes contain a palindromic consensus nucleotide sequence (heat shock element; HSE), CT_GAA__TTC_AG (Bienz and Pelham, 1987). Heat shock genes

usually contains multiple HSEs in a contiguous array and these multiple HSEs are required for the maximum expression of heat shock genes (Czarnecka *et al*, 1989; Dudler and Travers, 1984; Gurley *et al*, 1986). Eight of 14 nucleotides in the HSE are highly conserved and at least 7 are required to constitute a functional HSE (Beinz and Pelham, 1987).

It is unclear whether the induction of gene corresponding to the pTCB48 cDNA is regulated at transcriptional or post-transcriptional level by heat shock. Isolation of the corresponding gene and its characterization will provide insight into this question. In this chapter isolation of the TG48 gene and its characterization is described. In addition, the expression of TG48 in tobacco suspension cells and plant tissues was studied by northern and western analysis.

MATERIALS AND METHODS

Screening of the genomic library

A tobacco (*N. tabacum* L. W-38) genomic library in EMBL4, prepared from suspension cell cultures was screened with a probe made with the *Eco*RI/*Xho*I 1 kb cDNA fragment from pTCB48. 1 x 10^6 pfu

from the amplified genomic library were used in the primary screening. *E. coli* (K802) host cells were grown at 37°C until the OD_{550} was 0.5 in LB medium containing 0.2% maltose. Bacteria were pelleted by centrifugation and resuspended in 10 mM $MgSO_4$. Aliquots (5×10^4 pfu/plate) of plaques were mixed with *E coli* (K802) cells and incubated for 20 min at 37°C. Bacteria were mixed with 5 ml of melted 0.7% agar and spread on NZY medium in 150 mm Petri dishes. Plates were incubated overnight at 37°C and plaques were transferred on to Magna nylon membranes (MSI) according to manufactures instruction.

Membranes were laid on filter paper pre-wetted with denaturing solution (0.5 N NaOH + 1.5 M NaCl) for 5 min, neutralizing solution (1 M Tris-HCl (pH 8.0), 1.5 M NaCl) for 5 min and then rinsed with 2x SSC. Finally, membranes were baked for 30 min at 80°C. Prehybridization, hybridization and washing of membranes were done according to the manufacturers instructions (MSI) using randomly labeled (Promega) probe prepared from the 1 kb *Eco*RI/*Xho*I fragment from pTCB48. Positive clones were isolated and used in two more rounds of screening.

Subcloning and Sequencing of the gene

Isolation of bacteriophage λ and λ DNA was accomplished according to Sambrook *et al*,(1992). Two genomic clones were isolated from the initial genomic screening. Phage DNA isolated from these two clones was digested with *Sac*I, *Eco*RI and *Sal*I in different combinations to generate a restriction map. The genomic clone 411λ was selected for subcloning and sequencing. 411λ DNA was digested with *Sal*I/*Sac*I/*Eco*RI or *Eco*RI alone. Restricted fragments were separated on a 1% agarose gel and DNA from individual bands were separately recovered either by using the gel eluter (Hoffer Scientific) or using a DNA purification kit (BioRad). Each DNA band was ligated to a appropriate site of pBluscriptII SK- vector (Strategene) and plasmids were amplified using XL1-Blue cells. Appropriate genomic subclones were sequenced either by dideoxy sequencing (Sequinase version 2, USB) or by automated sequencing (Applied Biosystems).

Primer extension

Primer extension using an antisense primer (GSP3 see chapter III) complementary to the 5' end of the pTCB48 cDNA clone, was carried

out to determine the transcription initiation point. The GSP3 primer was end-labeled with ^{32}P using T-4 polynucleotide kinase as described by Zyskind and Bernstein (1992). Poly(A^+) RNA (2 μg) isolated from heat-shocked tobacco cells was mixed with labeled primer (10 μM) in a total of 10 μl reaction volume and incubated at 75°C for 5 min. The reaction mixture was allowed to cool to 52°C and 20 μl of reverse transcription master mix (9.2 μl of DEPC H_2O, 9 μl of 4X reverse transcriptase buffer (Clonetech), 1.6 μl of RNase inhibitor (Clonetech), 3.7 μl of dNTPs (10 mM each) and 25 units of AMV reverse transcriptase (Clonetech)) were then added. This reaction mixture was incubated at 52°C for 30 min and 1 μl of 0.5 M EDTA was added to stop the reaction. DNA was precipitated with EtOH and resuspended in 10 μl of TE. An aliquot (5 μl) of the reaction was run on a 6% denaturing polyacrylamide gel along with end-labeled θX174/HaeIII marker and the rest was run on a sequencing gel along with a sequencing reaction carried out using the same primer with a genomic subclone containing the 5' flanking region of the gene.

Isolation of tobacco genomic DNA

Genomic DNA and total RNA were simultaneously isolated from tobacco cell cultures from the same sample using the guanidine isothiocyanate method. After pelleting RNA, liquid at the interphase of the $CsCl_2$ cushion was collected and diluted with an equal volume of water. Genomic DNA was then precipitated with 2.5 vol. of 95% EtOH. Precipitated DNA was taken up with a sterile tooth pick and washed twice with 1 ml of 70% EtOH and dried in a speed vac (Savant). Genomic DNA was then resuspended in 0.5 ml of 10 mm TE.

Southern blot analysis

Genomic DNA (10 μg) was digested with *Eco*RI, *Xba*I and *Bam*HI for 16 h in a 100 μl reaction. Digested DNA was precipitated with 0.1 volume of 3M NaOAC and 2.5 volume of 95% EtOH, washed twice with 70% EtOH and resuspended in 20 μl of 10 mM TE. DNA was separated on 0.8% agarose gel and transferred onto Magna nylon membrane using a vaccum blotter (BioRad). DNA was then crosslinked to the membrane by baking at 80°C or 30 min. Membrane was prehybridized at 65°C in 5 ml of prehybridization solution (6X SSPE, 3X Denhardt's solution

(appendix), 0.25% SDS and 100 μg/ml Salmon sperm DNA) for 2 h. A 1 kb *Eco*RI/*Xho*I fragment of pTCB48 cDNA was labeled with ^{32}P by random priming (Prime-A-Gene kit, Promega) and added to the prehybridization solution. Hybridization was continued at the same temperature for 16 h. The blot was washed twice in 25 ml each with 5X SSC + 0.1% SDS at room temperature for 10 min, twice in 25 ml each of 1X SSC + 0.1% SDS at 37°C for 10 min and twice in 25 ml each of 0.1X SSC + 0.2% SDS at 65°C for 15 min. The blot was exposed at to X-ray film at -80°C.

Isolation of total and poly(A) RNA

Total RNA was isolated from cultured tobacco cells or tobacco plants as described by McGookin (1984). Poly (A) RNA was isolated using the polyAtract system (Promega) according to the manufacturer's instructions.

Northern blot analysis

Poly(A) RNA (2 μg) from each sample was separated on a 1.5% formaldehyde-agarose gel and transferred to Magna nylon membrane

using a vaccum blotter (BioRad). RNA was crosslnked to the membrane with a UV-crosslinker in autocrosslink mode (Stratagene). Blots were prehybridized, hybridized and washed as described in chapter II. Autoradiography of blots was done at -80°C.

Purification of the recombinant protein

Expression of the recombinant protein :

E. coli(XL1-blue) harboring pTCB48 cDNA was grown in 5 ml LB/ampicillin (50 μg/ml) at 37°C overnight. An aliquot (500 μl) of this culture was used to inoculate 500 ml of LB/ampicillin (50 μg/ml) medium and incubated at 37°C until the absorbance at 500 nm (A_{500}) of the culture reached 0.5. IPTG was then added to the medium to give a final concentration of 5 mM and the incubation was continued for further 2 h. Bacteria were harvested by centrifugation at 5000 g for 10 min and resuspended in 25 ml of buffer A (50 mM Tris-HCl (pH7.5); 3 mM $MgCl_2$; 5 mM KCl; 2 mM EDTA) containing 4 mM DTT and 1 mM PMSF. Cells were lysed by sonication and cell debris was removed by centrifugation at 5000 g for 15 min. $CaCl_2$ was added to the supernatant to give a final concentration of 2 mM.

CaM-Sepharose chromatography :

The recombinant protein was partially purified by chromatography on a CaM-Sepharose column (Pharmacia). The column (10 ml) was preequilbriated with 100 ml of buffer A. Then 100 ml of buffer A containing 1 mM $CaCl_2$ was passed through the column. The bacterial supernatant was then passed through the column at a rate of 0.25 ml/min. Effluent was recycled two more times through the column to ensure maximum protein binding. The column was then washed at a flow rate of 0.9 ml/min with 200 ml of buffer B (25 mM Tris-HCl (pH 8.0); 3 mM $MgCl_2$; 2 mM KCl; 5 mM $CaCl_2$) containing 0.3 M NaCl followed by 100 ml of buffer B. Proteins bound to the column were eluted with buffer C (25 mM Tris-HCl (pH 8.0); 3 mM $MgCl_2$; 2 mM KCl; 2 mM EDTA). Protein elution was monitored with a UV detector (ISCO, UA-5). The fractions containing eluted protein were confirmed by SDS-PAGE. Fractions containing eluted protein were pooled together and dialyzed against 10 mM NH_4HCO_3, lyophilized, and then resuspended in 0.5 ml of Milli-Q water.

Recovery of the recombinant protein from SDS-PAGE and preparation of polyclonal antibody:

200 μg of protein recovered from CaM-Sepharose chromatography was loaded onto a 12.5% polyacrylamide minigel with a single wide well and run at constant voltage (100v). The gel was then rinsed with milli-Q water and negatively stained with 0.3 M $CuCl_2$ for 2 to 4 min. The recombinant protein band was excised and cut into approximately 2 mM pieces. Cu^{2+} ions bound to gel pieces were removed by washing the gel pieces three times (10 min) in 1 ml of 0.25 M EDTA/ 0.25 M Tris-HCl (pH 8.0) for 10 min each. Gel pieces were then finely chopped and protein was eluted in a electrophoretic concentrator (BioRad) for 2 h at 100v. Protein was recovered in a 200 - 400 μl and directly injected in to a rabbit or dialyzed against 10 mM NH_4HCO_3 before injected into a rabbit.

Polyclonal antibody against the recombinant protein was made at the UH Laboratory Animal Service facility.

Isolation of total protein from tobacco

Total protein was isolated from tobacco suspension cells or whole plant tissue using 2x Lamelli buffer (appendix). Tobacco suspension cells at the mid log phase (7 day) were heat shocked at 38°C for 15, 30, 60, 90, or 120 min on a rotary shaker water bath. Cells were frozen in liquid nitrogen, ground to a fine powder and stored at -80°C until use.

Total protein was also isolated in the same manner from leaves, stem and root of tobacco plants that were grown at 23°C (control) or heat shocked at 42°C for 2 h. One g of finely ground cells or tissue was resuspended in 2 ml of 2x Lamelli sample buffer, then heated in a boiling water bath for 5 min. The sample was then incubated at room temperature for 10 min and centrifuged at 9000 xg for 20 min at 23°C. The supernatant was stored at -80°C until further use.

Western blotting

Equal amounts (20 μg/lane) of protein isolated from different samples were separated on 12.5% polyacrylamide-SDS minigels and transferred to PVDF membrane (BioRad) in Towbin buffer (25 mM Tris-HCl, 192 mM glycine and 20% methanol) (Towbin *et al*, 1979) using a

Mini Trans blot transfer unit (BioRad) at 60V for 2 h at room temperate. The membrane was blocked with TBST (10 mM Tris-HCl (pH 8.0), 150 mM NaCl and 0.05% Tween 20) containing 3% BSA for 1 h and then incubated with primary antibody in TBST + 1% BSA for 1 h. The blot was then washed 3 times in 20 ml of TBST for 5 -10 min each and exposed to alkaline phosphatase conjugated goat anti-rabbit IgG (1:1500 dilution) (Sigama) in 15 ml of TBST + 1% BSA for 45 min. Blot was washed twice in 20 ml of TBST, once with 20 ml of TBS and then incubated with BCIP and NBT in alkaline phosphatase buffer (100 mM Tris-HCl, 100 mM NaCl, 5 mM $MgCl_2$ /pH 9.5) until color developed.

RESULTS AND DISCUSSION

Cloning and subcloning of genomic clone

Tobacco genomic library in EMBL4 was screened with a ^{32}P labeled probe prepared from a 1 kb EcoRI/XhoI cDNA fragment from pTCB48. Two genomic clones (411λ, 511λ) were initially isolated by screening 1 X 10^6 pfu. Both clones had similar restriction patterns when digested with individually or with different combinations of *SalI*, *Sac*I

and *Eco*RI. This result suggested that these two clones may be the same. The 411λ clone was selected for further study.

411λ had a genomic insert of approximately 13.0 kb. The restriction map of this clone with *Sal*I, *Sac*I and *Eco*RI is given in figure 4.1A. Digested fragments were cloned into appropriate sites of pBlusckript II SK- in such a way to obtain overlapping subclones (Fig. 4.1B). The orientation of the genomic clone was determined by Southern blots of restricted λDNA probed with 3 different restricted fragments from the pTCB48 cDNA clone (data not shown).

The pTG2.7 genomic subclone (Fig 4.1) was further subcloned using *Hind*III and *Pst*I sites. These different clones were sequenced by dideoxy sequencing method (Sequinase version 2, USB) or by automated sequencing (Applied Biosystems). The sequenced portion (TG48) of the 411λ clone (Fig. 4.1A) contains the complete open reading frame, 5' and 3' untranslated regions along with 1064 bp 5' flanking sequence and 364 bp of 3' flanking sequence. The open reading frame is interrupted with 5 introns (Fig. 4.2 A and 4.2 B). Putative TATA and CAAT sequences are located at -45 and -60 in the 5' flanking sequence. The transcription initiation point was located by primer extension (see below). Five

putative HSEs were identified by the BESTFIT computer program in 5' flanking upstream sequence. These are located at -78, -97, -283, -471, and -908 of the promoter region (Fig. 4.2 A and B). The most proximal HSE is located about 25 bases 5' to the TATA box, a characteristic of many heat shock induced genes (Beinz and Pelham, 1987). These HSEs may be involved in transcriptional activation of this gene under heat shock conditions. However, transgenic expression of a reporter gene under the influence of the TG48 gene promoter is required before this can be concluded.

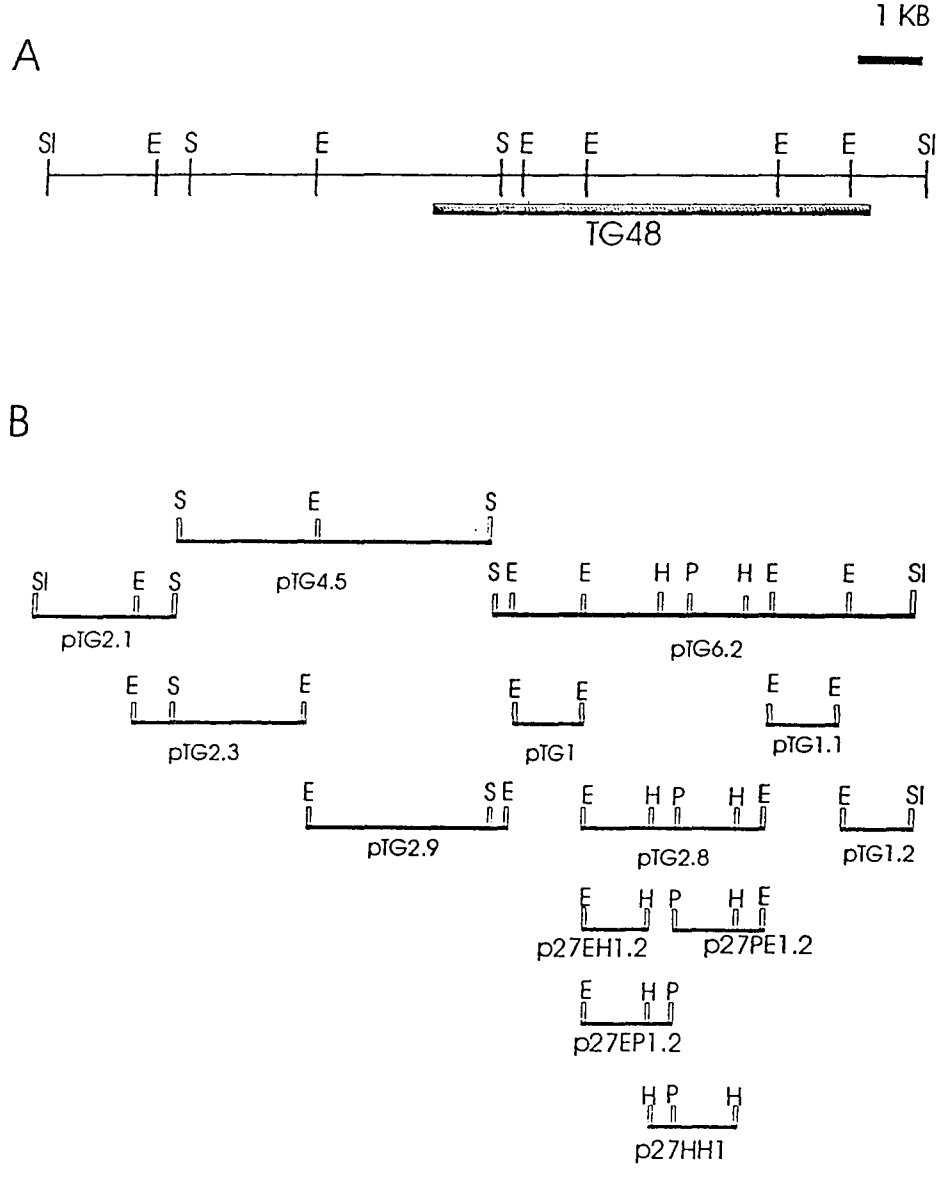

Fig. 4.1: (A) Linear restriction map of 411λ clone. Sl- *Sal*I, S- *Sac*I, E- *Eco*RI. Solid bar represents the region sequenced in this study.

(B). Restriction maps of genomic subclones used in sequencing.

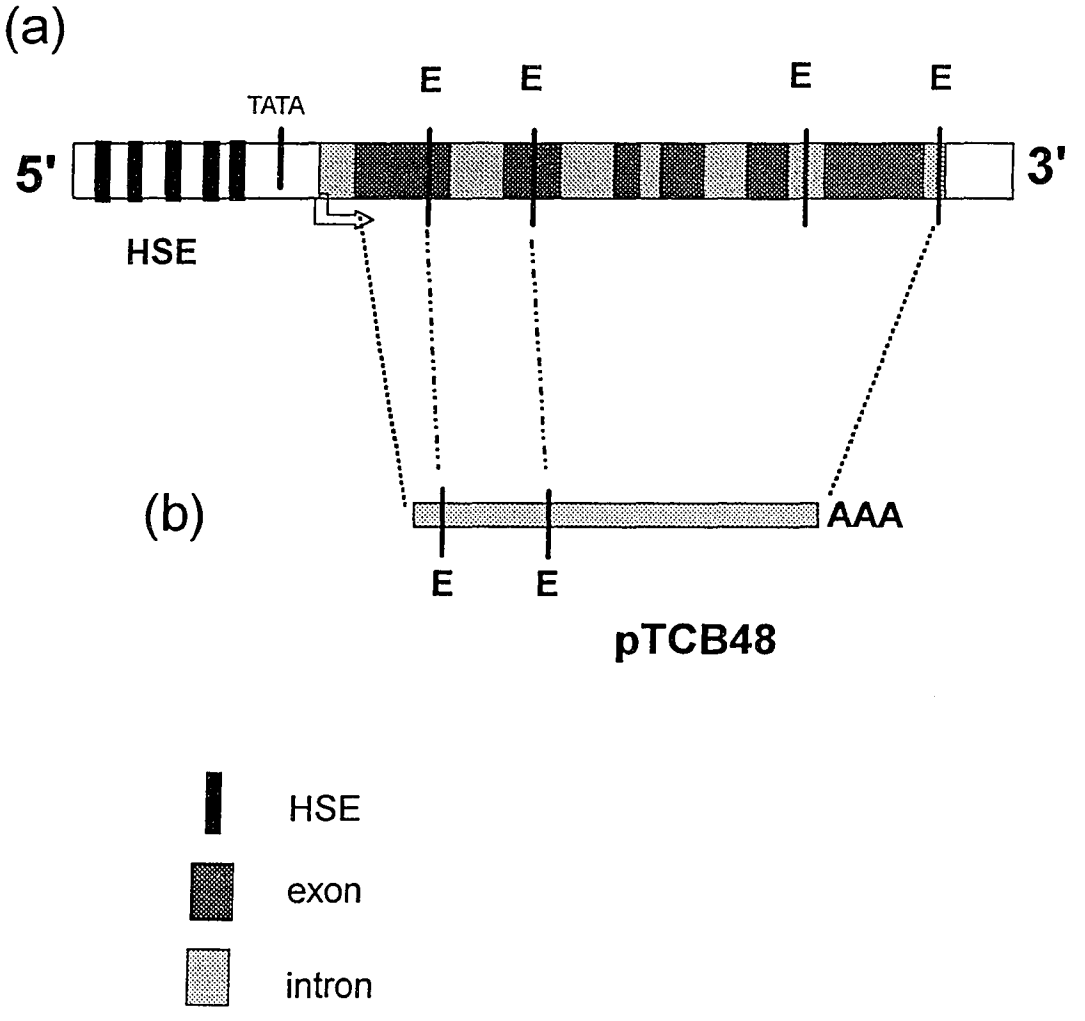

Fig. 4.2 A: Structure of the TG48 gene (a) and pTCB48 cDNA (b). Position of introns, exons, 5' and 3' flanking sequences and HSEs are marked. Transcription initiation point is marked with an arrow. *Eco*RI restriction sites are marked as E.

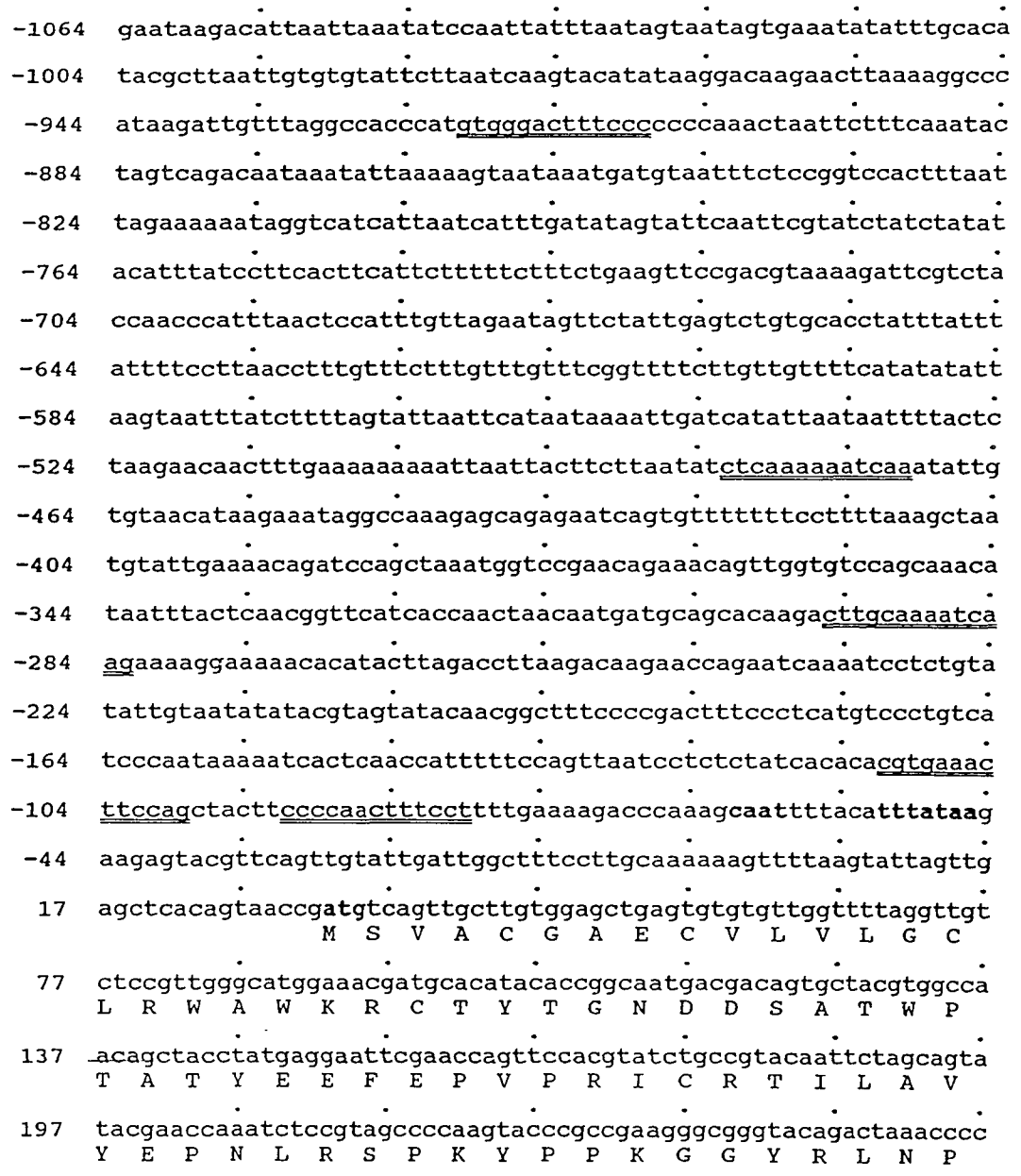

Fig. 4.2 B: Complete nucleotide sequence of the TG48 gene. Putative TATA box, CAAT box, initiation Met cordon, stop cordon are in bold letters. Putative HSEs are double underlined.

```
 257  gattgggtcattaaacgggtcacatatgaacaaacatctggcaatgcacctccgtatttg
      D  W  V  I  K  R  V  T  Y  E  Q  T  S  G  N  A  P  P  Y  L

 317  atctactgtgatcacgaacaccaagagattgtagtcgctattcgtgggttgaatttacta
      I  Y  C  D  H  E  H  Q  E  I  V  V  A  I  R  G  L  N  L  L

 377  aacgaaagtgattacaaagttttgttggataataggctgggaaaacagatgtttgatgga
      N  E  S  D  Y  K  V  L  L  D  N  R  L  G  K  Q  M  F  D  G

 437  ggatatgtacatcatgggttattgaaatctgcggtttgggttttgaataatgagtctgag
      G  Y  V  H  H  G  L  L  K  S  A  V  W  V  L  N  N  E  S  E

 497  actttgaagaagctttggattgagaatgggaggagttacaagatgatatttgcaggtaaa
      T  L  K  K  L  W  I  E  N  G  R  S  Y  K  M  I  F  A  G

 557  tatggcaaataggctggttggattaaattcttagcgaaccttaactgataaaattgttgt

 617  catgtgagcaggaaggtcacgggttcgagccgtggaaacagactcttgcagaaatgcagg

 677  gtaagggacgtacaatagactcatatggtccggccctgaactagggtgctacaatagacc

 737  cttgtggtccggcccttcccggacccgcgcatagcgagaacttggtgcactagactgcct

 797  tttttagcttaagttcttataagtttgtataatttccacctccaaatgtaatttgtattt

 857  ttggagtttcgatgaatttttacctgtctggttacgctatttgacctgatactttgatgg

 917  gtcatttcaagttcaagctggagtaaagtcaacaacaagtcaccctactttgaaccaata

 977  gtttaatgggtcatttcgagtttatcccaccggttgggtggattactaaaaattggttaa

1037  ttttgccacctctattttgcaggacattctttgggttctggtgtggcgtctttgctgaca
                           H  S  L  G  S  G  V  A  S  L  L  T

1097  gtgattgtggcgaatcataaggatagattaggggaattccaaggagtcttttaaggtgt
      V  I  V  A  N  H  K  D  R  L  G  G  I  P  R  S  L  L  R  C

1157  tatgcagttgcaccagcgcggtgtatgtcactcaacttggctgttaagtatgctgatata
      Y  A  V  A  P  A  R  C  M  S  L  N  L  A  V  K  Y  A  D  I

1217  atacactctgtggtattgcaggtaagcgtcggaaactaatccagaattttgagtcagtgg
      I  H  S  V  V  L  Q

1277  gtagattatacacttttcaccggttattcttgtgtggcaaagactgaggacgtgatacaa

1337  atattgaatatttttttcagaaaaagtatcttatatgtaatttgaattgataggaactgct

1397  ttcatatgctgcatctgctcatgctgagaatgctttaaatttgggcaatgtattctggaa

1457  atgttattaatgggttgtgaggtggtcgattcacctgggaaaagagagatggactaggga

1517  ctaattactaacgggttcagagtaatgtagtcagttgatttagctgataaatattgggat

1577  actgtggtgggcagatgctacaaggaaagaacaatttgggtgatcagcagttaatcaccc
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Fig. 4.2 B: continued.

```
1637  ctttgatttacagaaaaatagaatctttactatctgttctctgggggaatttaccgccaa
1697  gagtggaaagttgaagtacattgaaactgtcccaactagaatctcttcccctccttctga
1757  tctaaattaaagcgcctcctcgctcatagatggtatctgggtgttacaggcattatatat
1817  ttatatatttaatattaccacttgaaataccaattggcttgagtttgtacttatcatgtg
1877  ggggggaagacgaatccaaggaatggcgactgagctggtagtttagtcgctaactgataa
1937  cttatttttcagccagttactaagttgaaatttctccaaggtaggggtaaggtttgcct
1997  acacacaccctcccagaccccacagtgtgataatactgggatgttgttgttgttgttacc
2057  tagttgaaatttaaaccattgggcagtgcaattttgctgtgacatttcagtaaaatagtt
2117  gcccacattaacaggaatttccactgtttttctctggtcatatttgcaaatttgagaagt
2177  ttcggtatatgtgttaatttgctccttagcttgccatgtgaaattcatgaaccgtcacat
2237  ccagtgaaataacaatgtaaccatgactctttcctaacaggatgatttcttgccaagaac
                                          D  D  F  L  P  R  T
2297  agccaccccacttggagatatatttaaatccatcttctggttagtttagatccacactct
       A  T  P  L  E  D  I  F  K  S  I  F  C
2357  tgtgcataataaagctttctctctattctctgatttcttgccgttgcatgattgctataa
2417  ttatctcttataccaggcattaatgctttcaacagtttaccgtgcttgatattttggta
                                       L  P  C  L  I  F  L  V
2477  tgcttgagagataccttcattcctgagggcagaaaactccgagatccaagaagactttat
      C  L  R  D  T  F  I  P  E  G  R  K  L  R  D  P  R  R  L  Y
2537  gcaccaggccgtatgtatcacattgtagaaagaagattctgcaggtaaagcacattcaga
      A  P  G  R  M  Y  H  I  V  E  R  R  F  C  R
2597  gagtgttactaggtgtgctatgtactgtcaaagtaatgcaaactagtgatatgatataac
2657  ctagcctctgtcggtgtgactttcacagaaccgccgaccattgcttgtggtcattgtgtg
2717  ctttgtttgacttgttgtagatacgttatatctcccctcagttctgtgcatggcgagagc
2777  tcagtcctttctaatattctgttgtcttgagttccgtgtttatggttcacagcccggctt
2837  atgagtcaatcgaattggtgttgggtgctgaggcttgccactatcaactagcatgagtca
2897  tgccaaacattgtgctcctaagtggtgttcggtcaactatcgatcttgtcaatattcttt
2957  gtcctcatcctgctaaatccactgattaggatatactaaccctgacaaatagaggcattg
3017  tgttccgttgccctgaattagtgaagatattatagggcaagtttcatatgaaatgcatcg
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Fig. 4.2 B: continued.

```
3077  gcatctgttatctcccattaaatagacgagtgcagaataaagttgaactaatctcacata
3137  ctggtgaattattcttacttgttggacgagaagcactttaacctcattgtttttgtttcc
3197  ctagtttggtttaatcttaataaatctgaccctatgtatttattctcttcatgtaactgt
3257  caccgcacgcttaggcatttacgattattgtgcagacgctgctacagcaaaatattttca
3317  gatgagaccaccgttttggatttcaacttaaccattttaagaaaaaaacgttttttcctt
3377  atgttagggcgatttaaagctctcctcttggttatgatattagcactcagtgcttatgat
3437  atttttgcaacagtgaagctttaatactcctgatatgcaatttcctgcttcacagatgtg
                                                              C  G
3497  ggaggttcactccagatgttagaactgccatcccagttgacggaagatttgagcatatcg
        R  F  T  P  D  V  R  T  A  I  P  V  D  G  R  F  E  H  I  V
3557  tgttgtcacgcaatgctacagttgatcatggaatcatttggatagaaagagaatctgaaa
        L  S  R  N  A  T  V  D  H  G  I  I  W  I  E  R  E  S  E  K
3617  aagtattagcagtaagtagcatcaacacacttttccctttgaaaggaaggactccagttg
        V  L  A
3677  gtcatctatgatttcattttggtacaaaacatatgaggagtaatttgccttgctttcgtc
3737  gcgcaaaaatttccaaagacaaaagcataaacctagtcattttgtcattgataagaggag
3797  tggttgtcttcataaaagagaaccaaatatatgtgcatttggacacaaatgatcttttta
3857  ttttatccgtacagtgaaatgatactatctgtatggaaatagattgagagaaggatttca
3917  ctttgcaggaaggtatagatttttgcaacaacagtattgcctttgtgacctataaaacgc
3977  ggcctacttatcccgaattcccaatagaagtttacagtgggtaacatatggtaatttgcg
4037  tccatgaagttatggcttggtcctgtcccatactttagatctcattgttccgttgttta
4097  agtctagaattactgtactccatctgaattacatgctgcatatgatggaactagaatgtc
4157  caccagctcagtttccactgaaatctttcaatgttaagatcctgaaatgcatagtagaaa
4217  aaccagtgtatagtcatccgtgctatatttccgcatgcttgcaagtcattgttgtcttct
4277  ttcagtggttacgtttatcaaacaatcaactatgtctcaatccccaatcagttggggtca
4337  gctatatgaatcctcgttagcataggcagatgtaattttagtggattttacacgtaaatt
4397  tatgctccgcatcaaagttcacataaacccgatactctctatactacacccccccccccc
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Fig. 4.2 B: continued.

```
4457  ccccgggggtgttggacaaatttgggtctagttgagcaaactcttccttgcagagactta
                                                          R  L  K
4517  aggaggctagtgctgagaccacaactactccccccaaagtgcagaaaattggaaggctga
        E  A  S  A  E  T  T  T  T  P  P  K  V  Q  K  I  E  R  L  K
4577  agacattaggaaaagaacacaaggatgcactcgaaagagctgtcagtttaaacataccac
        T  L  E  K  E  H  K  D  A  L  E  R  A  V  S  L  N  I  P  H
4637  acgctgtggacgcagatgaagaggaatccacggagagtataactgaggagtcatctcaga
        A  V  D  A  D  E  E  E  S  T  E  S  I  T  E  E  S  S  Q  K
4697  aacaggaggaagatgcaatgacaagcaaagcgcagtgcagtgatgcaagaactaactgga
        Q  E  E  D  A  M  T  S  K  A  Q  C  S  D  A  R  T  N  W  N
4757  atgaagtagttgagaagcttttcaatagagacgaaagtgggaaattacgattaaagagag
        E  V  V  E  K  L  F  N  R  D  E  S  G  K  L  R  L  K  R  D
4817  atgcaactggtcccgagtaacaactctgttccaaggtgctttttctctcttctttttttat
        A  T  G  P  E
4877  gatggctgtgtatgttagatgaaagcattattactcgtgttccaacaactcataattctt
4937  tattatccatatcgcatagtacaatatcttctttttgtatatcaacgaccataatgtata
4997  atctgaagccccgtcaatagtacagttggaaattcttttatgcaattattttaggcgcag
5057  ttcctcgtggaatgtgatttgttcatgaattcttgtttacaacttgagtgttcatttggt
5117  attctgataccacccgctcaatttgttatacctttacattctaatttttcctgtcaattg
5177  ttcagtgtgcagattagttcattaacttgtctacactctacattaatatcatagcaaaat
5237  ggcatcgacagttcaatactgagagacatttacaactcatgcattctttaaccaagtcat
5297  atatgttatgctttttttatagccagctggtatgtacaaagaccaacccaatagctagttg
5357  caaatatgactgttaggatgctgcttcttccttctttattgtttttaatctttcacataa
5417  gtagagaaggccatctgaacgtagtaataaaaa
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Fig. 4.2 B: cotinued.

Primer extension

The transcription initiation point of the TG48 gene was determined by primer extension technique as described in the materials and methods. A portion of the final reaction products was resolved on 6% denaturing polyacrylamide gel along with end-labeled X174/*Hae*III marker (Fig. 4.3 A). The rest of the reaction products were run on a sequencing gel along with a sequencing ladder (Fig. 4.3B) originated with the same primer used in primer extension. When primer extension reaction product was run on a polyacrylamide gel, a major band about 0.23 kb, and a minor band about 0.45 kb were observed (Fig. 4.3A). When this reaction mixture was run on the sequencing gel, transcription start point lined up with one of the thymine residues of the sense sequence (Fig 4.3B and fig. 4.2). Similarly, the 5' end of the 5'RACE product ended with the same nucleotide (chapter III). Whether the second lighter band represents an alternative transcription start site is not clear. However, in 5' RACE, only a single PCR product corresponding to the 0.23 kb band was observed (Chapter III).

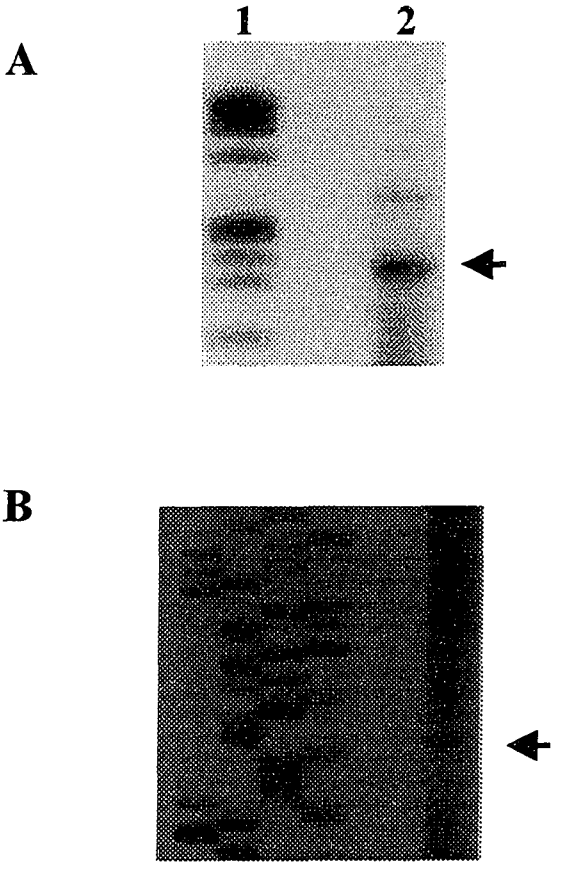

Fig. 4.3: Results of the primer extension experiment. (A) Lane 1: Primer extension product resolved on a polyacrylamide gel. Lane 2: End labeled θX174/*Hae*III marker. (B) Primer extension product run on a sequencing gel. Alignment point is marked with an arrow.

Organization of TG48 genes in tobacco

Genomic DNA isolated from suspension cultured cells was digested with *Eco*RI, *Bam*HI or *Xba*I, separated on a agarose gel, blotted on to nylon membrane and hybridized to a random primed 1 kb *Eco*RI/*Xho*I cDNA fragment. In each enzyme digestion the probe hybridized to multiple bands (Fig. 4.5). In the case of a single gene this probe should recognize only two bands (ca. 1.1 kb and 2.8 kb) when genomic DNA is digested with *Eco*RI. (genomic sequence complementary to the probe sequence has an intron with an *Eco*RI site). Hybridization of the probe to multiple bands in this southern blot indicates TG48 is a member of a small multigene family or an allelic series. As this genomic DNA was isolated from cells cultured over a long period, one might expect a considerable polymorphism at any given locus.

Purification of the recombinant protein

Recombinant protein was isolated form *E coli* harboring the pTCB48 plasmid and partially purified by CaM-Sepharose chromatography. Most of the bacterial proteins were removed by this step; however, SDS-PAGE revealed the presence of several contaminant

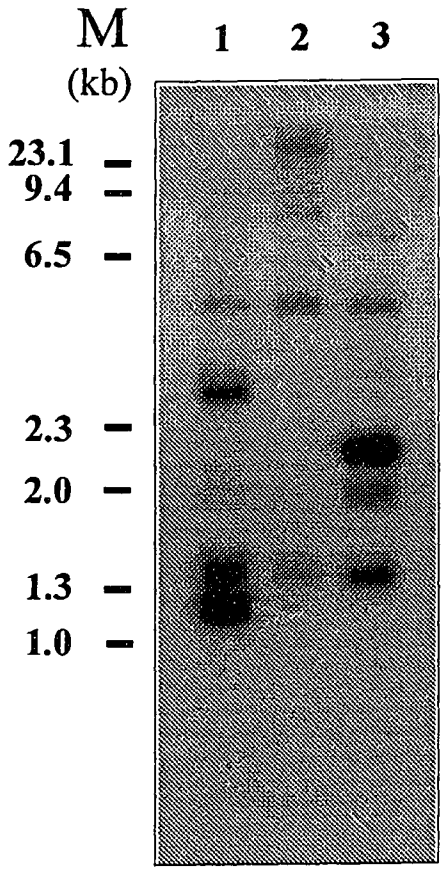

Fig. 4.4: Genomic southern blot probed with 1 kb *Eco*RI/*Xho*I cDNA fragment. Genomic DNA was digested with *Eco*RI (lane 1), *Bam*HI (lane 2) or *Xba*I (lane 3). M- Molecular weight markers.

protein bands along with the recombinant protein (Fig 4.5A). An aliquot of CaM-sepharose purified protein was also separated on a 2-dimensional IEF-PAGE in order to make sure there are no bacterial protein of a similar size co-migrating with the recombinant protein (data not shown). When the recombinant protein band was eluted from SDS-PAGE, purified protein run as a single band on SDS-PAGE (Fig 4.5A, lane 4). Identity of the purified protein was also tested by a CaM gel overlay (Fig. 4.5B) (method described in chapter III).

Northern blot analysis

Poly(A) RNA isolated from tobacco suspension cells or tobacco plants was used in northern analysis. When equal amount (2 μg) of RNA isolated from tobacco cells that were heat shocked at 38°C for different lengths of time ranging from 0 to 120 min, TCB48 RNA was detectable after 15 min of heat treatment (Fig. 4.6). This message accumulated to a maximum at 90 min and then declined. Consistent with earlier reports (Lu, 1991, Lu *et al*, 1995 and chapter III), this transcript was not detected in control cells (Fig. 4.6).

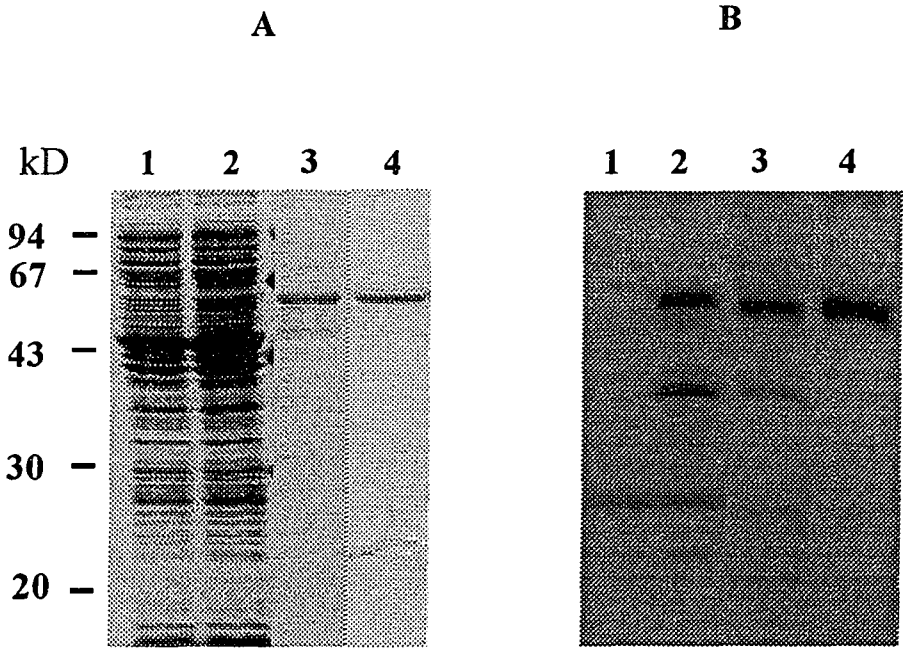

Fig 4.5: (A) Protein isolated from *Ecoli* with pBluescript II SK- (lane 1), with pTCB48 plasmid (lane 2), Protein recovered from CaM-Sepharose column (lane 3), or eluted recombinant protein (lane 4) run on a SDS-PAGE and stained with Coomassie blue. (B) Autoradiograph of CaM gel overlay of the proteins isolated at different purification steps.

To investigate the heat shock inducibility of this gene during the development, northern analysis was carried out using poly(A) RNA isolated from control and heat shocked cells at different culture age ranging from 2 to 15 days. Interestingly, during early stages of the culture (2 and 5 days) this message was detected in control cells at very low level (Fig. 4.7). However, with age, transcript level gradually declined and at 7 day (mid log phase) it was not detectable in control cells. Transcript level of this gene was increased with heat shock in all developmental stages tested (fig. 4.7). However, heat shock inducibility was less prominent in early and late stages of the development but was more pronounced in 7 and 11 day old cultures. This result suggests that this gene is subject to both environmental and developmental regulation.

Western blot analysis

In vivo synthesis of TCB48 protein during heat shock was studied by Western blot analysis using polyclonal antibody raised against TCB48 recombinant protein. Two protein bands of approximately 60 kD were detected in western blots (Fig. 4.6B). These two bands run very closely on the gel. Both bands were detected in control cells, but increased with

time in heat shocked cells. The molecular weight of these two proteins is higher than the calculated value (51 kD) of the deduced protein of pTCB48. This discrepancy may be due to post-translational modification of the protein. In fact, deduced amino acid sequence contains several putative phosphorylation sites (K/RXXT/S). The 1.8 transcript was not detected in mid log phase (7 days) control cells (Fig. 4.6A and fig. 4.7), however, the protein was detected. In fact, northern analysis with culture age (Fig. 4,7) revealed that the transcript was present early stages. Thus it is likely this protein is expressed in young cells and persists throughout the growth cycle.

Expression of TCB48 gene in tobacco plants

Northern analysis was done using poly(A^+) RNA isolated from leaves, stems or roots of 6 week old tobacco plants. Plants were grown in a growth chamber at 23°C and heat shocked at 42°C for 2 h. The TG48 transcript was present in all plant tissue tested in both controls and heat shock treatment (Fig. 4.8). In leaves the steady state level of transcript was higher than that of stem and root tissue. Heat shock induction of the gene was also tissue specific. Leaves had the highest

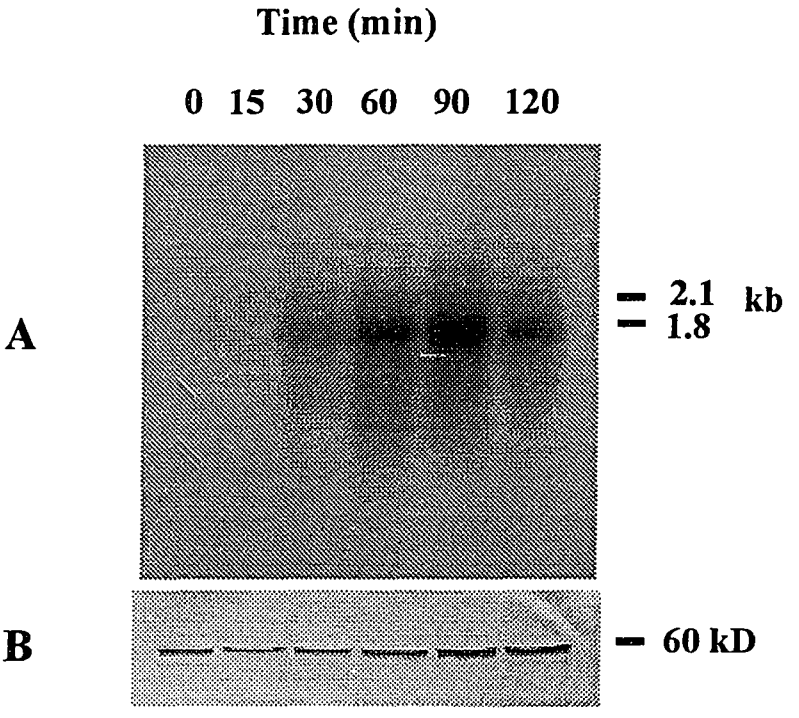

Fig 4.6: (A) Northern bolt analysis of poly(A) RNA isolated from tobacco suspension cells heat shocked at 38°C for different length of time as indicated. (B) Western blot of total protein isolated from the same samples probed with polyclonal antibody against the TCB48 recombinant protein.

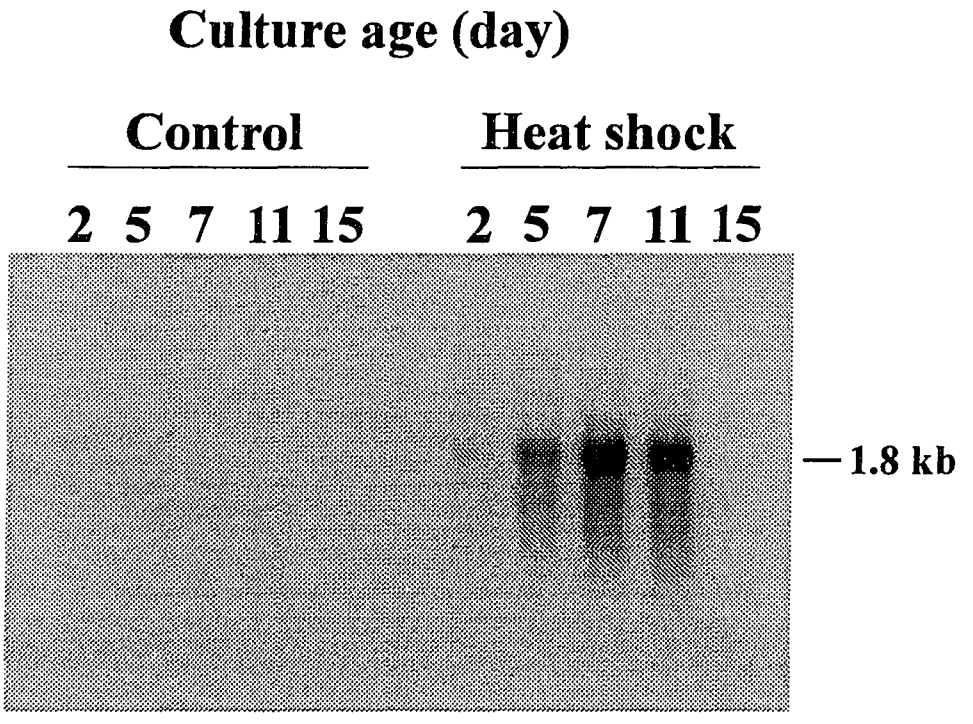

Fig 4.7: Northern blot analysis of poly(A) RNA isolated from tobacco suspension cell at different culture age as indicated. Cells were grown at 23°C and heat shocked for 2 h at 38°C.

level of induction while stem had the lowest. The presence of this transcript in control tissues may represent a fundamental difference between cultured cells and whole plants with respect to gene expression. Taken together, northern analysis results of suspension cells and plant tissues indicates TG48 gene/s is/are subjected organ specific developmental and environmental regulation.

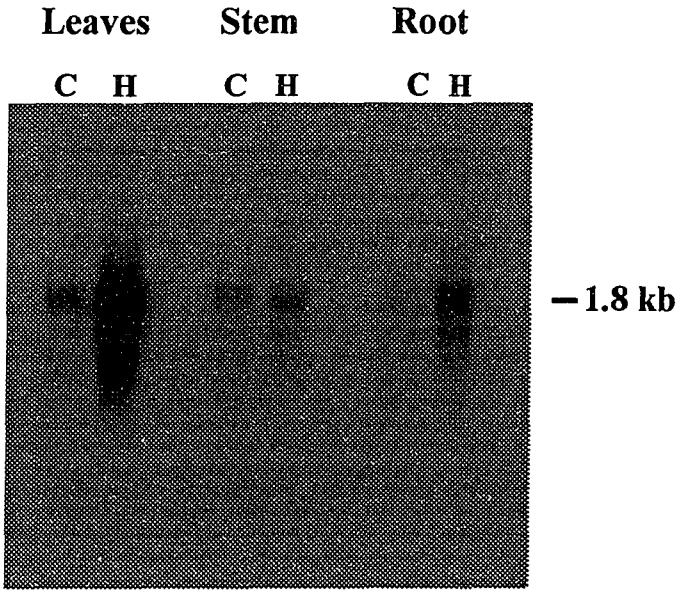

Fig. 4.8: Northern blot analysis of poly(A) RNA isolated from different tissues of tobacco plants heat shocked (H) at 42°C for 2 h. or control plants (C) grown at 23°C.

Chapter V

CLONING AND CHARACTERIZATION OF CALMODULIN BINDING GLUTAMATE DECARBOXYLASE cDNAs FROM TOBACCO

INTRODUCTION

The synthesis and accumulation of γ-aminobutyric acid (GABA), a non-protein amino acid, increases rapidly in response to many environmental conditions including mechanical shock, cold shock, and darkness (Wallace *et al.*, 1984), anaerobiosis (Streeter & Thompson, 1972), heat shock (Mayer *et al*, 1990) and water stress (Rhodes *et al*, 1986). In animal systems GABA functions as a major inhibitory neurotransmitter by modulating ion channels (Erlander & Tobin, 1991). The exact role of GABA in plants is not known, but several functions have been suggested.

Decarboxylation of glutamic acid to GABA in the presence of a proton has been suggested as a mechanism to stabilize the cytosolic pH in plant cells which undergo cytoplasmic acidification during stress (Snedden *et al.*, 1992; Crawford *et al.*, 1994). Consistent with this suggestion, the

activity of GAD, which catalyze this reaction, is enhanced at relatively acidic pH (Snedden *et al.*, 1992; Crawford *et al.*, 1994). Transamination of α-ketoglutarate by GABA to produce succinic semialdehyde and glutamate can regulate the tricarboxylic acid cycle by bypassing the direct conversion of α-ketoglutarate to succinic acid which may be inhibited under certain physiological conditions (Dixon and Fowden, 1961). In addition, GABA may also be involved in nitrogen metabolism and storage in plants (Larher *et al.*, 1983).

GAD activity is stimulated by lower cytosolic pH which may result under stressful conditions. However, reduction of cytosolic pH is not a prerequisite for the stimulation of GAD activity (Crawford *et al*, 1994) suggesting that GAD activity may be regulated through other mechanism(s). Many stress conditions increase both GAD activity and cytosolic Ca^{2+} level in plants. Therefore, Wallace (1984) postulated that GAD activity may be regulated through Ca^{2+} signaling pathways. Recently, several studies have shown that GAD activity in soybean (Snedden *et al.*, 1995), Fava bean (Ling *et al.*, 1994) and *Petunia* (Arazi *et al.*, 1995) is regulated by Ca^{2+}/CaM. The deduced amino acid sequence of a *Petunia* cDNA clone encoding GAD contains a putative

CaM binding domain (Baum *et al.*, 1993) and the GAD activity of the recombinant protein isolated from *E. coli* was stimulated by CaM in the presence of Ca^{2+}.

Lu (1991) showed that pTCB15 encodes a CaMBP. In this chapter sequencing of pTCB15 isolated from a tobacco cDNA library and its identification as GAD is described. Further, expression of GAD gene/s was studied in both tobacco cell culture and plant under normal and heat shock conditions.

MATERIALS AND METHODS

Sequence analysis of pTCB15 cDNA

pTCB15, pBluscriptII SK- plasmid harbouring a 1.2 kb CaMBP cDNA was sequenced by dideoxy sequencing (Sequanase version 2, USB) or automated sequencing (Applied Biosystems).

Screening of the tobacco cDNA library

A tobacco (*Nicotiana tabacum* L. cv. Xanthi) cDNA library in λZAPII was obtained from Dr. Yamamoto's laboratory (Plant Molecular

Physiology Dept., University of Hawaii). *E. coli* (XL1-Blue) cells were infected with recombinant λZAPII phages and grown on NZY (appendix) plates at the density of 50,000 pfu/plate overnight. Plaques were transferred onto Magna nylon membranes for 1 min. Membranes were then denatured for 2 min in 1.5 M NaCl / 0.5 M NaOH , neutralized for 5 min in 1.5 M NaCl / 0.5 M Tris-HCl (pH 8.0) and rinsed for 30 s by submersion in 2X SSC buffer. DNA was crosslinked to the membranes by baking at 80°C for 30 min under vacuum. Membranes were prehybridized at 65°C in 10 ml of prehybridization solution (appendix) in a minihybridization oven (Labnet) for 2 h . A probe prepared from the 1.2 kb cDNA insert of pTCB15 was labeled with ^{32}P by random primer labeling (Prime-A-Gene kit, Promega), and added to the prehybridization solution. Hybridization was continued for 16 h at the same temperature. Membranes were washed twice in 25 ml of 5X SSC + 0.1% SDS for 10 min at room temperature, twice in 25 ml of 1X SSC + 0.1% SDS for 10 min at 37°C and twice in 50 ml of 0.1X SSC + 0.1% SDS for 15 min each at 65°C. Blots were autoradiographed at -80°C overnight.

Positive phages were *in vivo* excised into phagemids using ExAssist/SOLR system according to the manufacturers' instructions

(Stratagene). A positive plaque was cored from the agar plate and transferred to a microfuge tube containing 500 μl of SM buffer and 20 μl of $CHCl_3$, and incubated at room temperature for 1 - 2 h. In a 50 ml flask, 200 μl of XL1-Blue cells were mixed with 100 μl of phage stock and 1 μl of ExAssist helper phage and incubated at 37°C for 15 min. Three ml of 2X YT medium was added to this mixture and incubation was continued for another 2 h in a shaking water bath. The mixture was then heated at 70°C for 20 min and centrifuged at 4000 xg for 15 min. An aliquot (1 μl) of the supernatant was mixed with 200 μl of SOLR cells (OD_{600} = 1.0) and incubated at 37°C for 15 min. A portion of this mixture (50 μL) was plated on LB/ampicillin medium and incubated at 37°C overnight. Plasmid DNA was isolated from colonies appearing on this medium using BioRad plasmid isolation kit and sequenced with automated sequencing (Applied Biosystems).

Isolation of total and poly(A) RNA

Total RNA was isolated from cultured tobacco suspension cells or from tobacco plant tissue by guanidine isothiocyanate method as

described by McGookin (1984). Poly(A) RNA was isolated by using the PolyAtract system (Promega) according to the manufacturers' instructions.

Northern blot analysis

Total (25 μg) or poly(A) RNA (1 μg) isolated from tobacco suspension cell cultures or plant tissue samples were separated on 1.5% agarose-formaldehyde gels and transferred on to Magna nylon membrane (MSI) using a vacuum blotter (BioRad) for 1 h. RNA was crosslinked to the membrane with autocrosslink mode of a UV-crosslinker (Stratagene). Blots were then prehybridized, hybridized and washed as described in chapter III. Blots were exposed to X-ray films at -80°C.

RESULTS AND DISCUSSION

Sequence analysis of pTCB15

The pTCB15 cDNA clone was sequenced by automated sequencing apparatus (Applied Biosystems). The cDNA insert consists of 1175 bp including 28 bp of the polyA tail (Fig. 5.1). It contains an open reading

frame of 1076 bases which codes for 359 amino acids (Fig. 5.1). A putative polyadenylaton signal is located at 1084 bp. This clone was determined to be a partial cDNA based on the absence a Met initiation codon. Moreover, in northern analysis, probe from this cDNA insert recognized a single 2.1 kb transcript (Fig. 5.6).

A Genebank search using the BLAST program showed that pTCB15 nucleotide and deduced amino acid sequences share high sequence similarity with plant (> 85%) and bacterial (47%) glutamate decarboxylase (GAD) (Fig. 5.4 and 5.5). However, as this cDNA was a partial sequence, a full length tobacco GAD cDNA was isolated from another cDNA library.

Screening of the cDNA library

A tobacco (*N. tabaum* Xanthi) cDNA library in λZAPII was screened with a probe prepared from the pTCB15 cDNA insert as described above. Thirty five positive clones were detected in primary screening. Ten individual clones were selected for further analysis. After two more rounds of screening, 10 individual clones were used for *in vivo* excision as described in the methods section.

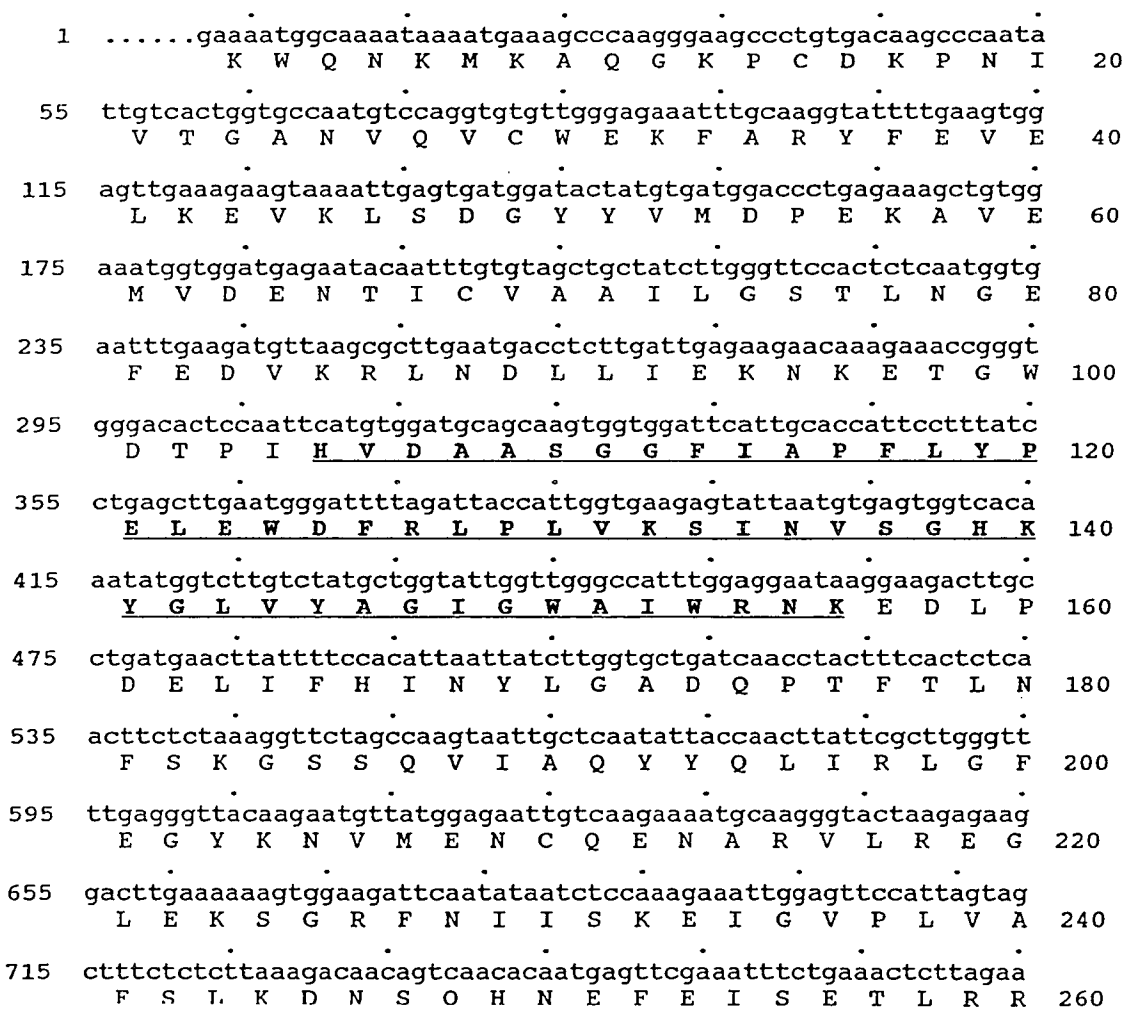

Fig. 5.1: Nucleotide and deduced amino acid sequences of pTCB15 cDNA. Putative stop and polyadinylation sequences are marked with bold lower case letters. Numbers in left and right represents nucleotide and amino acid position respectively. The catalytic domain of the protein is indicated with underlined bold letters.

```
775  gatttggatggattattcctgcatatactatgccaccaaatgctcaacatgtcacagttc
       F  G  W  I  I  P  A  Y  T  M  P  P  N  A  Q  H  V  T  V  L  280
835  tcagagttgttattagagaagatttctcccgtacactcgcggagcgactggtgatagaca
       R  V  V  I  R  E  D  F  S  R  T  L  A  E  R  L  V  I  D  I  300
895  ttgaaaaagtcctccacgagctagacacacttccggcgagggtcaacgctaagctcgccg
       E  K  V  L  H  E  L  D  T  L  P  A  R  V  N  A  K  L  A  V  320
955  tggccgaggcgaatggcagcggcgtgcataagaaaacagatagagaagtgcagctggaga
       A  E  A  N  G  S  G  V  H  K  K  T  D  R  E  V  Q  L  E  I  340
1015 ttactgctgcatggaagaaatttgttgctgataagaagaagaagactaatggagtttgtt
       T  A  A  W  K  K  F  V  A  D  K  K  K  K  T  N  G  V  C  *  359
1075 aatttaatttaaccaaatatgtttataatatgatgatttatgactactagcaatattggt
1135 attgcttgtttttaaaaaaaaaaaaaaaaaaaaaaaaaaa
```

Fig. 5.1: continued.

When the plasmid DNAs isolated from these clones, were digested with *Eco*RI, different insert sizes ranging from 1.6 - 4.1 kb were detected (fig. 5.2A and table 5.1). Southern hybridization of these restricted plasmid DNAs with the same probe used for cDNA screening revealed that all clones except GAD2, are true positive clones (Fig. 5.2B). Of these clones, GAD1 and GAD5 are much larger than expected. However, when plasmid DNA from these two clones were used as template DNA in PCR using internal primers made to the GAD10 sequence an expected size band was observed (data not shown). Thus, GAD1 and GAD5 clones may have resulted from ligating another cDNA to the GAD clones during the cDNA library synthesis. Of these nine positive clones, GAD9 and GAD10 were selected for sequencing.

Table 5.1: Insert sizes of cDNA clones encoding GAD.

Clone	*Size (kb)*	*Clone*	*Size (kb)*
GAD1	4.10	GAD6	1.70
GAD3	1.60	GAD7	1.70
GAD4	1.65	GAD8	1.75
GAD5	2.70	GAD9	1.70
		GAD10	1.90

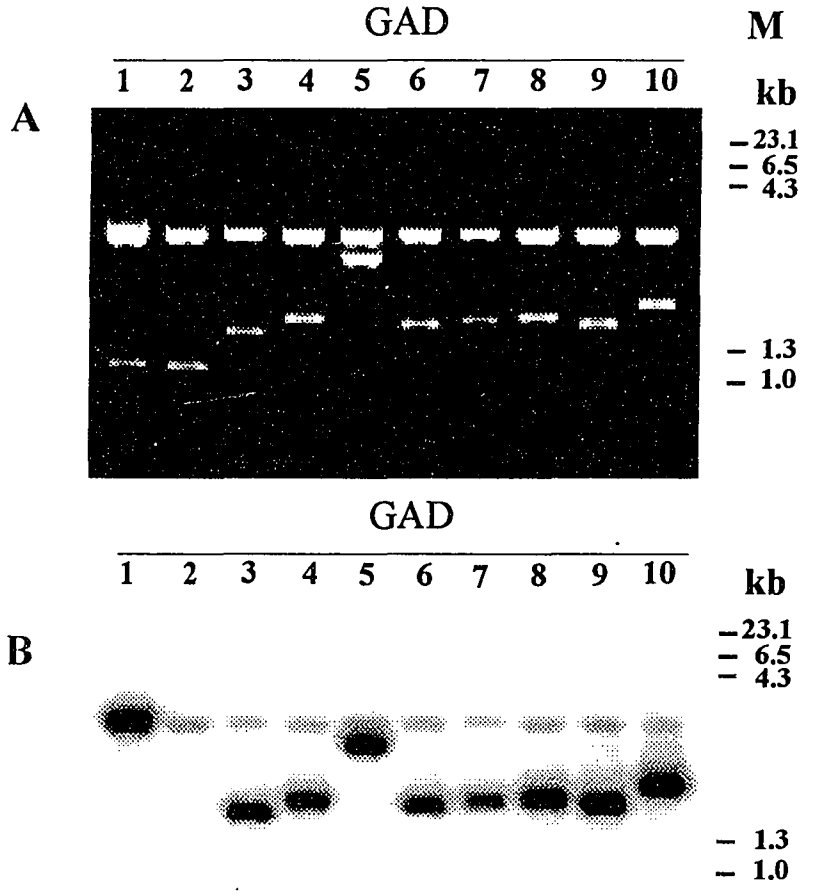

Fig. 5.2: A:- *Eco*RI digested plasmid DNA from different GAD clones were separated in 0.8% agarose gel. B:- Southern hybridization of this plasmid DNA with a probe made from pTCB15 cDNA insert. (M) λ/Hind III molecular weight marker.

Sequence analysis of GAD9 and GAD10

The full length GAD10 cDNA consists of 1929 bp including 12 residues of the poly(A) tail (Fig. 5.3). The longest possible open reading frame (ORF) extends from 66 (Met initiation codon) to the 1556 (stop codon, TAA). A putative polyadinylation signal, ATTAAA, is located at 1887 of the 3' untranslated region. The ORF codes for a protein with a calculated molecular weight of 55 kD. The GAD10 nucleotide sequence was identical to the GAD9 except at the 5' and 3' UTRs. GAD9 contains 5 additional nucleotides at the 5' end of the UTR and has a shorter 3' UTR (Fig. 5.4). These two cDNAs may have arisen from the same mRNA by mispriming the 'oligo dT' primer during cDNA synthesis. Alternatively, it is possible that these two cDNAs may have arisen from two different mRNAs. The 3' RACE technique can be used to resolve this dilemma. These two GAD cDNAs showed 98% sequence identity to pTCB15 clone (Fig. 5.4).

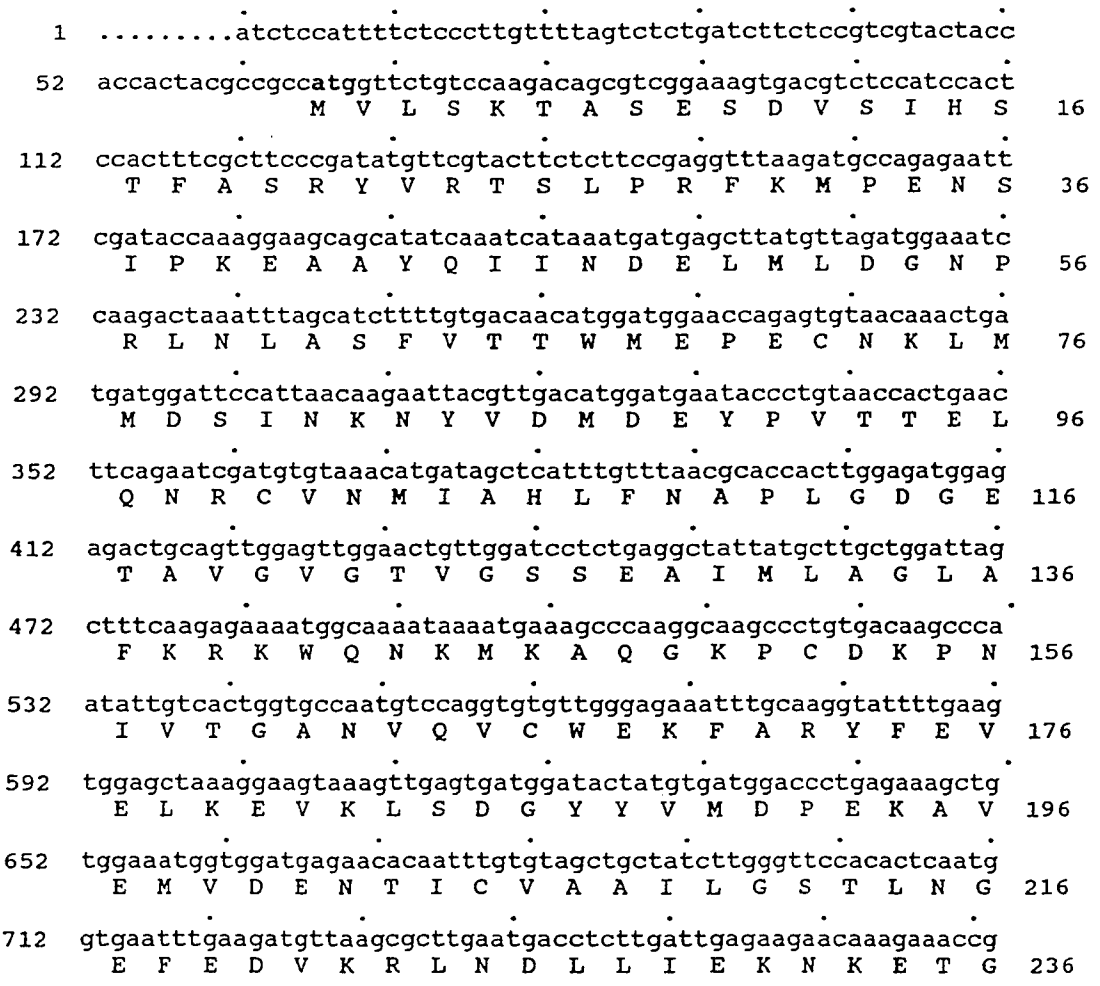

Fig. 5.3: Nucleotide and deduced amino acid sequence of GAD10 clone. Putative Met initiation codon, stop codon and polyadinylation sequences are marked with bold lower case letters. Catalytic domain of the protein is indicated by underlined by bold letters.

```
772  ggtgggacactccaattcatgtggatgcagcaagtggtggatttattgcaccattccttt
       W  D  T  P  I  H  V  D  A  A  S  G  G  F  I  A  P  F  L  Y  256

832  atccagagcttgaatgggactttagattgccattggtgaagagtattaacgtgagtggtc
       P  E  L  E  W  D  F  R  L  P  L  V  K  S  I  N  V  S  G  H  276

892  acaaatatggtcttgtttatgctggtattggctgggccatttggaggaataaggaagact
       K  Y  G  L  V  Y  A  G  I  G  W  A  I  W  R  N  K  E  D  L  296

952  tacctgacgaacttatcttccacattaattatcttggtgctgatcaacctactttcactc
       P  D  E  L  I  F  H  I  N  Y  L  G  A  D  Q  P  T  F  T  L  316

1012 tcaacttctctaaaggttctagccaagtaattgctcaatattaccaacttattcgcttgg
       N  F  S  K  G  S  S  Q  V  I  A  Q  Y  Y  Q  L  I  R  L  G  336

1072 gttttgagggttacaagaatgttatggagaattgtcaagaaaatgcaagggtactaagag
       F  E  G  Y  K  N  V  M  E  N  C  Q  E  N  A  R  V  L  R  E  356

1132 aaggacttgaaaaaagtggaagattcaacataatatccaaagaaattggagttccattag
       G  L  E  K  S  G  R  F  N  I  I  S  K  E  I  G  V  P  L  V  376

1192 tagctttctctcttaaagacaacagtcaacacaatgagttcgaaatttctgaaactctta
       A  F  S  L  K  D  N  S  Q  H  N  E  F  E  I  S  E  T  L  R  396

1252 gaagatttggatggattattcctgcatatactatgccaccaaatgctcaacatgtcacag
       R  F  G  W  I  I  P  A  Y  T  M  P  P  N  A  Q  H  V  T  V  416

1312 ttctcagagttgtcattagagaagatttctcccgtacactcgccgagcgactggtaatag
       L  R  V  V  I  R  E  D  F  S  R  T  L  A  E  R  L  V  I  D  436

1372 acattgaaaaagtcctccacgagctagacacacttccggcgagggtcaacgctaagctag
       I  E  K  V  L  H  E  L  D  T  L  P  A  R  V  N  A  K  L  A  456

1432 ccgtggccgaggcgaatggcagcggcgtgcataagaaaacagatagagaagtgcagcttg
       V  A  E  A  N  G  S  G  V  H  K  K  T  D  R  E  V  Q  L  E  476

1492 agattactactgcatggaagaaatttgttgctgataagaagaagaagactaacggagttt
       I  T  T  A  W  K  K  F  V  A  D  K  K  K  K  T  N  G  V  C  496

1552 gttaatttaatttaacaaaatatgtttataattaatatgatgatttataactactagcag
       *

1612 tggtactgcttgtttttatatttgaattgttgggtttttgagtatgaggagctagctat

1672 ttattgctagtgaaatattggttgattttggactactttgtattattaatgttaattttc

1732 ttaagtacttaatatgaggatatttatcatgcatgtgatatagaaaaaagttgtgagtgc

1792 ctgaggtctagtttaatcctttatattccaatataaaaaacatatacatgggcggaggta

1852 gaaaaaccaatatatttgtattaagaatattattattaaaatttaaattcaattatta

1912 gatcccaaaaaaaaaaaa
```

Fig. 5.3: continued.

```
      1                                                            50
Gad10  .....atctc cattttctcc cttgttttag tctctgatct tctccgtcgt
 Gad9  aaaat***** ********** ********** ********** **********
Ptcb15 .......... .......... .......... .......... ..........

       51                                                          100
Gad10  actaccacca ctacgccgcc atggttctgt ccaagacagc gtcggaaagt
 Gad9  ********** ********** ********** ********** **********
Ptcb15 .......... .......... .......... .......... ..........

       101                                                         150
Gad10  gacgtctcca tccactccac tttcgcttcc cgatatgttc gtacttctct
 Gad9  ********** ********** ********** ********** **********
Ptcb15 .......... .......... .......... .......... ..........

       151                                                         200
Gad10  tccgaggttt aagatgccag agaattcgat accaaaggaa gcagcatatc
 Gad9  ********** ********** ********** ********** **********
Ptcb15 .......... .......... .......... .......... ..........

       201                                                         250
Gad10  aaatcataaa tgatgagctt atgttagatg gaaatccaag actaaattta
 Gad9  ********** ********** ********** ********** **********
Ptcb15 .......... .......... .......... .......... ..........

       251                                                         300
Gad10  gcatcttttg tgacaacatg gatggaacca gagtgtaaca aactgatgat
 Gad9  ********** ********** ********** ********** **********
Ptcb15 .......... .......... .......... .......... ..........

       301                                                         350
Gad10  ggattccatt aacaagaatt acgttgacat ggatgaatac cctgtaacca
 Gad9  ********** ********** ********** ********** **********
Ptcb15 .......... .......... .......... .......... ..........

       351                                                         400
Gad10  ctgaacttca gaatcgatgt gtaaacatga tagctcattt gtttaacgca
 Gad9  ********** ********** ********** ********** **********
Ptcb15 .......... .......... .......... .......... ..........

       401                                                         450
Gad10  ccacttggag atggagagac tgcagttgga gttggaactg ttggatcctc
 Gad9  ********** ********** ********** ********** **********
Ptcb15 .......... .......... .......... .......... ..........

       451                                                         500
Gad10  tgaggctatt atgcttgctg gattagcttt caagagaaaa tggcaaaata
 Gad9  ********** ********** ********** ********** **********
Ptcb15 .......... .......... .......... .....***** **********
```

other tobacco GAD clones, GAD9 and pTCB15. "*" represents the same amino acid residues. "." is used to introduce gaps for optimal alingment.

```
          501                                                       550
Gad10     aaatgaaagc ccaaggcaag ccctgtgaca agcccaatat tgtcactggt
Gad9      ********** ********** ********** ********** **********
Ptcb15    ********** ******g*** ********** ********** **********

          551                                                       600
Gad10     gccaatgtcc aggtgtgttg ggagaaattt gcaaggtatt ttgaagtgga
Gad9      ********** ********** ********** ********** **********
Ptcb15    ********** ********** ********** ********** **********

          601                                                       650
Gad10     gctaaaggaa gtaaagttga gtgatggata ctatgtgatg gaccctgaga
Gad9      ********** ********** ********** ********** **********
Ptcb15    *t*g**a*** *****a**** ********** ********** **********

          651                                                       700
Gad10     aagctgtgga aatggtggat gagaacacaa tttgtgtagc tgctatcttg
Gad9      ********** ********** ********** ********** **********
Ptcb15    ********** ********** *****t**** ********** **********

          701                                                       750
Gad10     ggttccacac tcaatggtga atttgaagat gttaagcgct tgaatgacct
Gad9      ********** ********** ********** ********** **********
Ptcb15    *********t* ********** ********** ********** **********

          751                                                       800
Gad10     cttgattgag aagaacaaag aaaccgggtg ggacactcca attcatgtgg
Gad9      ********** ********** ********** ********** **********
Ptcb15    ********** ********** ********** ********** **********

          801                                                       850
Gad10     atgcagcaag tggtgaattt attgcaccat tcctttatcc agagcttgaa
Gad9      ********** ********** ********** ********** **********
Ptcb15    ********** *********c ********** ********** t*********

          851                                                       900
Gad10     tgggacttta gattgccatt ggtgaagagt attaacgtga gtggtcacaa
Gad9      ********** ********** ********** ********** **********
Ptcb15    *****t**** ****a***** ********** *****t**** **********

          901                                                       950
Gad10     atatggtctt gtttatgctg gtattggttg ggccatttgg aggaataagg
Gad9      ********** ********** ********** ********** **********
Ptcb15    ********** **c******* ********** ********** **********

          951                                                      1000
Gad10     aagacttacc tgacgaactt atcttccaca ttaattatct tggtgctgat
Gad9      ********** ********** ********** ********** **********
Ptcb15    *******g** ***t****** **t******* ********** **********

          1001                                                     1050
Gad10     caacctactt tcactctcaa cttctctaaa ggttctagcc aagtaattgc
Gad9      ********** ********** ********** ********** **********
Ptcb15    ********** ********** ********** ********** **********
```

Fig. 5.4: continued.

```
        1051                                                      1100
Gad10   tcaatattac caacttattc gcttgggttt tgagggttac aagaatgtta
 Gad9   ********** ********** ********** ********** **********
Ptcb15  ********** ********** ********** ********** **********

        1101                                                      1150
Gad10   tggagaattg tcaagaaaat gcaagggtac taagagaagg acttgaaaaa
 Gad9   ********** ********** ********** ********** **********
Ptcb15  ********** ********** ********** ********** **********

        1151                                                      1200
Gad10   agtggaagat tcaacataat atccaaagaa attggagttc cattagtagc
 Gad9   ********** ********** ********** ********** **********
Ptcb15  ********** ********** c********* ********** **********

        1201                                                      1250
Gad10   tttctctctt aaagacaaca gtcaacacaa tgagttcgaa atttctgaaa
 Gad9   ********** ********** ********** ********** **********
Ptcb15  ********** ********** ********** ********** **********

        1251                                                      1300
Gad10   ctcttagaag atttggatgg attattcctg catatactat gccaccaaat
 Gad9   ********** ********** ********** ********** **********
Ptcb15  ********** ********** ********** ********** **********

        1301                                                      1350
Gad10   gctcaacatg tcacagttct cagagttgtc attagagaag atttctcccg
 Gad9   ********** ********** ********** ********** **********
Ptcb15  ********** ********** *********t ********** **********

        1351                                                      1400
Gad10   tacactcgcc gagcgactgg taatagacat tgaaaaagtc ctccacgagc
 Gad9   ********** ********** ********** ********** **********
Ptcb15  ********** ********** *g******** ********** **********

        1401                                                      1450
Gad10   tagacacact tccggcgagg gtcaacgcta agctagccgt ggccgaggcg
 Gad9   ********** ********** ********** ********** **********
Ptcb15  ********** ********** ********** ****c***** **********

        1451                                                      1500
Gad10   aatggcagcg gcgtgcataa gaaaacagat agagaagtgc agcttgagat
 Gad9   ********** ********** ********** ********** **********
Ptcb15  ********** ********** ********** ********** ****g*****

        1501                                                      1550
Gad10   tactactgca tggaagaaat ttgttgctga taagaagaag aagactaacg
 Gad9   ********** ********** ********** ********** **********
Ptcb15  ****g***** ********** ********** ********** *********t*

        1551                                                      1600
Gad10   gagtttgtta atttaattta acaaaatatg tttataatta atatgatgat
 Gad9   ********** ********** ********** ********** **********
Ptcb15  ********** ********** **c******* ****....** **********
```

Fig. 5.4: continued.

```
          1601                                                      1650
  Gad10   ttataactac tagc...agt ggtactgctt gtttttatat ttgaattgtt
   Gad9   ********** ****...*** ********** ********** **********
 Ptcb15   ****g***** ****aat*t* *****t***** ******aaaa aaaaaaaaaa

          1651                                                      1700
  Gad10   gggtttttttg agtatgagga gctagctatt tattgctagt gaaatattgg
   Gad9   ********** ********** ********** ********** **********
 Ptcb15   aaaaaaaaaa aaaa...... .......... .......... ..........

          1701                                                      1750
  Gad10   ttgattttgg actactttgt attattaatg ttaattttct taagtactta
   Gad9   ***aaaaaaa aa........ .......... .......... ..........
 Ptcb15   .......... .......... .......... .......... ..........

          1751                                                      1800
  Gad10   atatgaggat atttatcatg catgtgatat agaaaaaagt tgtgagtgcc
   Gad9   .......... .......... .......... .......... ..........
 Ptcb15   .......... .......... .......... .......... ..........

          1801                                                      1850
  Gad10   tgaggtctag tttaatcctt tatattccaa tataaaaaac atatacatgg
   Gad9   .......... .......... .......... .......... ..........
 Ptcb15   .......... .......... .......... .......... ..........

          1851                                                      1900
  Gad10   gcggaggtag aaaaaccaat atatttgtat taagaatatt attatattaa
   Gad9   .......... .......... .......... .......... ..........
 Ptcb15   .......... .......... .......... .......... ..........

          1901                                  1937
  Gad10   attttaaatt caattattag atcccaaaaa aaaaaaa
   Gad9   .......... .......... .......... .......
 Ptcb15   .......... .......... .......... .......
```

Fig. 5.4: continued.

Comparison of deduced protein sequences of these three tobacco cDNAs to each other as well as to other known plant and bacterial GAD clones showed high homology among these different clones (Fig. 5.5). The GAD10 protein sequence has more than 85% similarity to other plant GAD proteins and more than 44% similarity to the bacterial counterpart. These similarities were more prominent in the catalytic domain (Baum *et al*, 1993) (Fig 5.5 residues 245 to 296) of the protein (Fig. 5.5).

In this study no attempt was made to map the CaM binding domain of the deduced proteins of GAD9 or GAD10. Baum *et al.* (1993) located CaM binding domain of *Petunia* GAD at the very end of the carboxy terminus and concluded that it was a BAA structure. A comparison of amino acid sequences of tobacco GAD clones with *Petunia* GAD clone in this area showed 80% sequence identity (Fig 5.5). The Chou-Fasman secondary structure analysis for this area does not predict the presence of an α-helix for GAD9 or GAD10, however, the Garnier-Osguthorpe-Robson analysis predicts an α-helix. Thus CaM binding domain of tobacco GAD may also be a BAA.

```
            1                                                   50
GAD 10      ...MVLSKTA SESDVSIHST FASRYVRTSL PRFKMPENSI PKEAAYQIIN
GAD 9       ...******* ********** ********** ********** **********
pTCB15      .......... .......... .......... .......... ..........
Pet. GAD    ...******* *Q******** ********** ********** **********
Arab. GAD   ...****HA* ******V*** ********** ********** **********
Ecoli GAD   MDQKL*TDFR **LLD*RFGA K*AISTIAES K**PLHE..M RDDV*F****

            51                                                  100
GAD 10      DELMLDGNPR LNLASFVTTW MEPECNKLMM DSINKNYVDM DEYPVTTELQ
GAD 9       ********** ********** ********** ********** **********
pTCB15      .......... .......... .......... .......... ..........
Pet. GAD    ********** ********** ********** ********** **********
Arab. GAD   ********** ********** ********I* S********* **********
Ecoli GAD   ***Y****A* Q***T*SQ** DDENVR**MD L*****WI*K E***QSAAID

            101                                                 150
GAD 10      NRCVNMIAHL FNAPLGDGET AVGVGTVGSS EAIMLAGLAF KRKWQNKMKA
GAD 9       ********** ********** ********** ********** **********
pTCB15      .......... .......... .......... .......... ..********
Pet. GAD    ********** *****E**** ********** ********** **********
Arab. GAD   ********** *****EEA** ********** ********** *******R**
Ecoli GAD   L*****V*D* WH**APKNGQ ***TN*I*** **C**G*MAM *W**RKRME*

            151                                                 200
GAD 10      QGKPCDKPNI VTGANVQVCW EKFARYFEVE LKEVKLSDGY YVMDPEKAVE
GAD 9       ********** ********** ********** ********** **********
pTCB15      ********** ********** ********** ********** **********
Pet. GAD    ********** ********** ********** *******E** **********
Arab. GAD   E***V***** ********** ********** L******E** *****QQ**D
Ecoli GAD   A***TN***L *CGP.**IS* H*****WD** *R*IPMRPGQ LF***KRMIE

            201                                                 250
GAD 10      MVDENTICVA AILGSTLNGE FEDVKRLNDL LIEKNKETGW DTPIHVDAAS
GAD 9       ********** ********** ********** ********** **********
pTCB15      ********** ********** ********** ********** **********
Pet. GAD    ********** ********** ********** ********** **********
Arab. GAD   ********** D********* *****L**** *V******** **********
Ecoli GAD   AC*****G*V PTF*V*YTGN Y*FPQP*H*A *DKFQAD*GI *IDM*I****

            251                                                 300
GAD 10      GEFIAPFLYP ELEWDFRLPL VKSINVSGHK YGLVYAGIGW AIWRNKEDLP
GAD 9       ********** ********** ********** ********** **********
pTCB15      ********** ********** ********** ********** **********
Pet. GAD    *******I** ********** ********** ********** VV****D***
Arab. GAD   ********** ********** ********** ********** V*********
Ecoli GAD   ***L***VA* DIV******R ****SA**** F**APL*C** V***DE*A**
```

Fig 5.5: Comparison of GAD10 amino acid sequence with other plant and bacterial GAD sequences. "*" represents identical amino acids and "." represents gaps to allow maximal alignment of sequences.

```
          301                                                       350
GAD 10    DELIFHINYL GADQPTFTLN FSKGSSQVIA QYYQLIRLGF.EGYKNVMENC
GAD 9     ********** ********** ********** ********** **********
pTCB15    ********** ********** ********** ********** **********
Pet. GAD  ********** ********** ********** *********Y **********
Arab. GAD E********* ********** ********** *********H **********
Ecoli GAD Q**V*NVD** *GQIG**AI* **RPAG**** ***EFL***R ***TK*QNAS

          351                                                       400
GAD 10    QENARVLREG LEKSGRFNII SK...EIGVP LVAFSLKD.. NSQHNEFEIS
GAD 9     ********** ********** **...***** ********.. **********
pTCB15    ********** ********** **...***** ********.. **********
Pet. GAD  ****S***** ***T****** **...***** ********.. *R********
Arab. GAD R**MI***** ***TE****V **...DE*** ********.. SSC*T*F***
Ecoli GAD YQV*AY*ANE IA*LGPYEF* CTGRPDE*I* A*CFK***GE DPGYTLYDL*

          401                                                       450
GAD 10    ETLRRFGWII PAYTMPPNAQ HVTVLRVVIR EDFSRTLAER LVIDIEKVLH
GAD 9     ********** ********** ********** ********** **********
pTCB15    ********** ********** ********** ********** **********
Pet. GAD  *********V ********** *I******** ********** **R*******
Arab. GAD DM***Y**** ********** *I******** ********** ********MR
Ecoli GAD *R**LR**QV **F*LGGEAT DIV*M*IMC* RG*EMDF*EL *LE*YKASLK

          451                                                       500
GAD 10    ELDTLPARVN AKL.AVAE.. ..ANGSGV.. .HKKTDREVQ LEITTAWKKF
GAD 9     ********** ***.****.. ..******.. .********* **********
pTCB15    ********** ***.****.  ..******.. .********* ****A*****
Pet. GAD  ********** ***.****EQ AA****E*.. .*****S*** **MI******
arab. GAD ***E*****I H*I.SLGQEK SESNSDNLMV TV**S*IDK* RDII*G****
Ecoli GAD Y*SDH*.... .*LQGIAQQN SFKHT..... .......... ..........

          501       513
GAD 10    VADKKKKTNG VC
GAD 9     ********** **
pTCB15    ********** **
Pet. GAD  *EE******R **
Arab. GAD ***.R***SG I*
Ecoli GAD .......... ..
```

Fig. 5.5: continued.

Northern blot analysis

Northern analysis was accomplished using total or poly (A^+) RNA isolated from tobacco suspension cell cultures (W-38) or different tissues of tobacco plants (Xanthi). The pTCB15 cDNA insert was used as the probe in all northern analyses. This probe hybridized to a single 2.1 kb message in all northern blots.

When tobacco cells were subjected to heat shock at 38°C GAD mRNA level increased to a maximum within 15 min and then declined to prestress levels within 2 h (Fig. 5.6). However, whether this increase of mRNA level represents a transcriptional activation or increased mRNA stability during heat shock is not known. When poly(A) RNA isolated from suspension cell cultures at different stages of the culture cycle, GAD mRNA was detected in all stages. The level of mRNA increased with culture age to the mid log phase (7 days) and then decreased sharply (Fig. 5.7). A similar pattern of behavior was observed for GAD mRNA during *Petunia* flower development (Chen *et al*, 1994). GAD mRNA was about 10 times higher in the 2.0 cm long flower than in either the 0.5 cm long stage or fully open stage. Heat shock at 38°C for 2 h

increased the abundance of GAD mRNA in 5 day and, most prominently in 7 day old cell cultures. This heat shock effect on GAD mRNA was not detected in any other stages of the cell culture (Fig. 5.7). These results indicate that GAD mRNA level is both developmentally and environmentally regulated in tobacco suspension cultures.

Northern analysis using total or poly(A^+) RNA isolated from different tissues of tobacco plants indicated that GAD is expressed in leaf, stem, root and flower (Fig. 5.8). However, the abundance of GAD mRNA differed in various tissues. Root tissue of 12 week old plants had the highest level GAD mRNA while leaf tissue had the lowest level in 3 month old plants. However, in 6 weeks old plants leaf tissue had the highest level GAD mRNA while root had the lowest (Fig. 5.9). In *Petunia* the highest level of GAD message was detected in stem while lowest level was found in flowers (Chen *et al*, 1994). These differences in both tobacco and *Petunia* may be related to developmental regulation of GAD gene(s).

A 42°C heat shock for 15 min had a little or no effect on GAD mRNA abundance in 12 week old plants (Fig. 5.8). When 6 week old plants were heat shocked for 2 h at 42°C, the message level declined

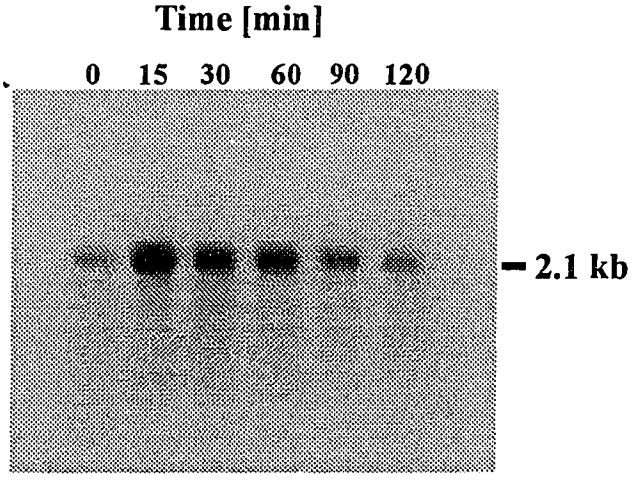

Fig. 5.6: Effect of heat shock at 38°C on GAD mRNA of tobacco cell culture. Equal amount of poly(A) RNA (2 μg) was separated on 1.5% formaldehyde-agarose gel and blot was hybridized to a probe from pTCB15 cDNA.

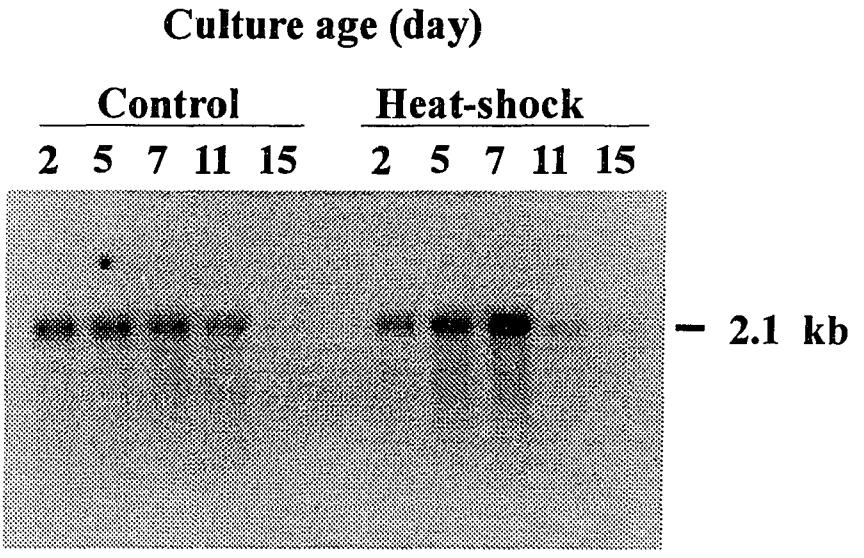

Fig. 5.7: GAD mRNA abundance in tobacco cell culture during different culture age and in response to heat shock at 38°C for 15 min. Equal amounts (1 μg) of poly (A) RNA were separated on 1.5% formaldehyde-agarose gel and probed with pTCB15 cDNA.

sharply and this decline was most apparent in leaf tissue (Fig. 5.9). Many environmental conditions including heat shock are known to increase the level of GABA in plant tissues. This study showed that GAD mRNA level increased during the first 15 minutes of heat shock. This may result in high level of GAD enzyme in plant cells which may ultimately result in higher GABA levels. However, whether GAD protein level and activity are elevated in response to heat shock is not known.

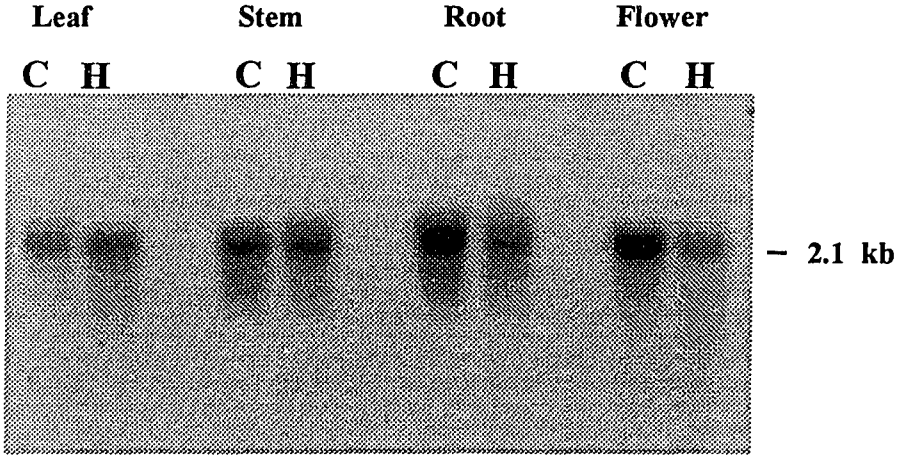

Fig. 5.8: GAD mRNA abundance in different tissues of tobacco plants at 12 weeks of age. Total RNA (25 μg/lane) isolated from control (grown at 23°C) or heat shocked (at 42°C for 15 min) plants were separated on 1.5% formaldehyde-agarose gel and probed with pTCB15 cDNA.

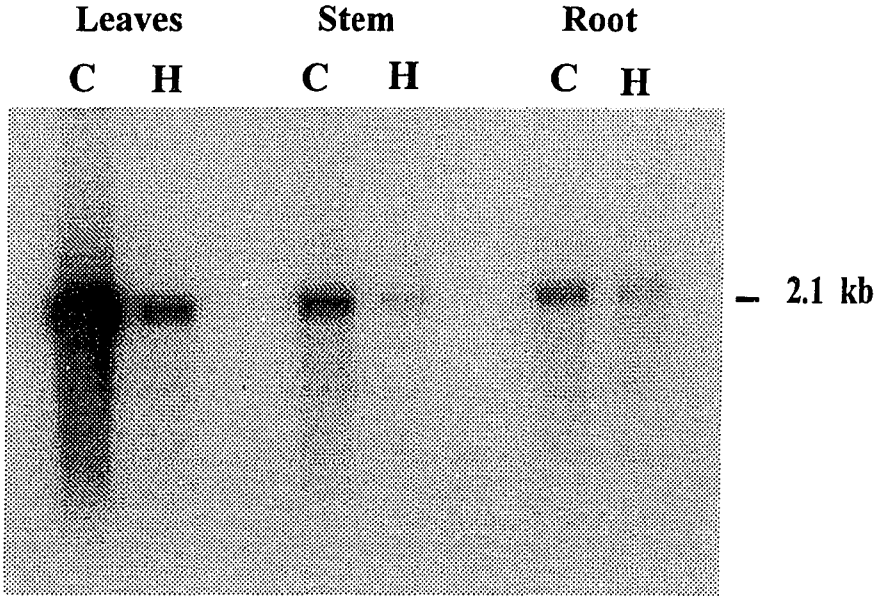

Fig. 5.9: Poly (A) RNA (2 μg/lane) isolated from control (grown at 23°C) or heat shocked (at 42°C for 2 h) tobacco plants at 6 weeks of age was separated on 1.5% formaldehyde-agarose gel and probed with pTCB15 cDNA.

Chapter VI

CONCLUSIONS AND FUTURE PROSPECTS

The study presented here indicates that the two heat shock induced transcripts recognized by the CaMBP cDNA clone pTCB48 are encoded by two different genes. The 1.8 kb transcript is encoded by TG48 gene. The origin of 2.1 kb transcript is currently unknown. The TG48 is either a member of a small multigene family or an allelic series. TG48 contains five introns and six exons. The 5' flanking sequence of this gene consists of putative TATA and CAAT boxes, and five HSEs. These HSEs may be involved in transcriptional regulation of this gene during heat shock induction. However, the possibility of post-transcriptional regulation can not be excluded. This gene exhibits developmental, environmental and tissue specific regulation.

TG48 encodes a CaMBP with a predicted size of 51 kD. Antibody raised against the recombinant protein recognized two proteins approximately 60 kD in Western blots. Heat shock enhances the protein levels, but unlike its transcript, protein is present in both control and heat shocked cells. This study provide the evidence for the presence of a

CaM binding domain at the end of the carboxy terminus of the putative protein sequence. The exact structure of the CaM binding domain is not clear but may be a BAA as in many other known CaMBPs. However, the possibility that it is a β-turn and β-strand structure can not be excluded. The function of this protein still remains unknown.

This study found that pTCB15, another CaM binding cDNA clone isolated from a tobacco cDNA library, encodes a glutamate decarboxylase. This clone was a partial cDNA. Two full length cDNAs of GAD were isolated from another tobacco cDNA library. Both of these cDNAs have an identical nucleotide sequence except that GAD9 contains a shorter 3' UTR. These two cDNAs may have resulted from the same transcript or from two different transcripts. Predicted protein sequences of these clones are highly homologous with other plant GADs and the bacterial counterpart. As expected this homology is more apparent in the catalytic domain of the protein. Comparison of the predicted protein sequence with other plant GAD sequences shows the presence of CaM binding domain similar to BAA structure. This study, along with others, suggests that GAD activity may be regulated *in vivo* by Ca^{+}/CaM messenger system.

Expression of GAD genes also exhibits developmental, environmental and tissue specific regulation. The GAD transcript was found in all developmental stages of suspension cells and all plant tissues tested. However, the relative abundance of the transcript was different in different culture ages and in different tissues. Heat shock at 38°C enhances the transcript level in suspension cells within 15 min of heat shock. However, this enhancement of transcript level depends on the culture age. Heat shock at 42°C for 15 min slightly reduced the transcript level in stem and root tissue. Heat shock at 42°C for longer periods drastically reduced the transcript level in all tissue tested. Transcript level of leaf tissue is more affected than that of stem or root.

Future effort on the work described here should be focused on several areas. Functional identification of the TCB48 protein should be a priority. Subcellular localization of this protein will provide an insight on possible functions. Isolation of homologous genes from other species will help to determine the conserved domains that may be necessary for its function. Antisense techniques to reduce the expression of this gene may also help to determine its function. It is also important to determine how this gene is regulated during heat shock. The presence of heat shock

elements in the promoter region suggests that this gene may be regulated at transcriptional level. However, whether these HSEs are involved in heat shock regulation should be tested by expression of a chimeric gene constructed with a reporter gene fused to the TG48 promoter region. Nuclear run on experiment may also provide additional information.

Future work on GAD should be directed to find the function of GABA in plants, especially during heat shock and other stressful conditions. Moreover, it is important to determine whether the GAD protein level and activity are also enhanced by heat shock. Isolation and sequencing of GAD genes, especially their regulatory sequences, will help to determine how the expression of GAD genes are regulated. Finally, further characterization of CaM binding domains of pTCB48 and GAD clones will elucidate how the functions of these CaMBPs are regulated by Ca^{+}/CaM messenger system.

APPENDIX

PSI medium

NZ amine - 10 g

NaCl - 5 g

Yeast extract - 5 g

Casamino acid - 5 g

$MgSO_4.7\ H_2O$ - 2 g

Adjust the pH to 7.5 with 0.1 N NaOH

2X Lamelli sample buffer

For 100 ml

0.5 M Tris-HCl (pH 6.8) - 50 ml

Milli-Q H^2O - 20 ml

SDS - 4 g

glycerol - 20 ml

bromophenol blue - 3 mg

mercaptoethanol - 10 ml (or 10 ml of 100 mM dithio threitol)

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