To the Graduate Council:

I am submitting herewith a dissertation written by Suk-Heung Oh entitled "In vitro and In vivo Studies on Posttranslational Calmodulin Methylation in Plants." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Biochemistry.

Daniel M. Roberts, Major Professor

We have read this dissertation
and recommend its acceptance:

Accepted for the Council:

Associate Vice Chancellor
and Dean of The Graduate School

IN VITRO AND IN VIVO STUDIES ON POSTTRANSLATIONAL CALMODULIN METHYLATION IN PLANTS

A Dissertation

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Degree

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ABSTRACT

A recombinant DNA-derived calmodulin (VU-1, lys 115) was used as a substrate to purify a specific calmodulin-lysine N-methyltransferase to homogeneity from sheep brain. The enzyme is a monomer with an apparent molecular weight of 38,000. The enzyme is highly specific for lysine 115 of calmodulin since the site-directed mutants of calmodulin with substitutions at this position (VU-3, lys to arg, 115; VU-4, lys to ile, 115) were unable to be methylated. These calmodulins were also strong competitive inhibitors of methylation. Substrates of different protein methyltransferases also were not methylated by the enzyme. The enzyme requires μM calcium for activity, suggesting that the calcium bound form of calmodulin is preferred as a substrate.

A radiometric assay based on the purified calmodulin-lysine N-methyltransferase has been developed to analyze the degree of methylation of calmodulins from developing plant tissues. By this approach, the presence of undermethylated calmodulin from plant tissues was detected. Further, there are developmental differences in calmodulin methylation in plants. Calmodulin methylation levels were lower in apical root segments and in the young lateral roots compared with the mature, differentiated root tissues. The endogenous methylation of calmodulin from carrot culture cells in the early exponential stage of growth is substantially lower than

that from non-proliferating cells in the stationary stage. The level of endogenous calmodulin methylation rapidly decreases upon inoculation of these cells into fresh media. Calmodulin protein levels were similar in cells from exponential and stationary stages. These findings suggest that calmodulin methyltransferase activity is regulated depending upon the growth or developmental stage, and/or that other factors, such as the availability of co-substrate, S-adenosyl methionine and calcium ion fluxes, are involved in controlling calmodulin methylation during plant cell growth and development. The data also suggest that proliferating cells have lower endogenous methylation of lysine 115 of calmodulin.

We utilized carrot embryogenesis to study the calmodulin system in undifferentiated tissues vs. differentiated tissues. Calmodulin, calmodulin methylation, and calmodulin-binding proteins were analyzed during the formation and germination of embryos. During the germination of carrot embryos, the level of calmodulin was doubled and calmodulin binding to a 54,000 M_r protein increased substantially. The changes in calmodulin methylation were not as drastic as those seen in the proliferating cells. The 54,000 M_r calmodulin-binding protein was purified by using chromatography on calmodulin-Sepharose. These data suggest that changes in the calmodulin regulatory pathway occur during the germination of carrot embryos. These changes may reflect

an altered role of calcium signal transduction during early growth and development of germinating embryos.

In order to address questions of the biological significance of calmodulin methylation in plants, transgenic tobacco plants expressing VU-1, VU-3 or VU-4 calmodulin have been generated. The VU-calmodulins were stably expressed in transgenic tobacco plants. The expression levels in transgenic tobacco tissues were maximally twice that of endogenous calmodulin. As expected, the transgenic VU-1 calmodulin is methylated at position 115, similar to the endogenous tobacco calmodulin. However, VU-3 calmodulin is incapable of methylation. These differences in posttranslational modification result in altered plant NAD kinase activation, with VU-3 calmodulin activating the enzyme to a level three-fold higher than transgenic VU-1 and endogenous tobacco calmodulins. As previously shown by Besl (1991), severe defects in growth and development (i.e., dwarfism and low seed yield) were observed in tobacco plants that express VU-3 calmodulin. The same abnormalities in growth and development were observed with 20 % of the F_0 VU-4 transgenic plants.

Overall, the data show unmethylated calmodulins exist <u>in vivo</u>, and that the methylation process is controlled developmentally. Combined with the transgenic plant and NAD kinase activator data, the results suggest that posttranslational methylation of calmodulin could be a

mechanism for regulating calmodulin activities *in vivo*.

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LIST OF ABBREVIATIONS

ABA, abscisic acid; **AdoMet**, S-adenosyl-L-methionine; **^{3}H-AdoMet**, S-adenosyl-L-[methyl-^{3}H]methionine; **βME**, β-mercapto-ethanol; **BSA**, bovine serum albumin; **2,4-D**, 2,4-dichloro-phenoxy acetic acid; **DTT**, dithiothreitol; **EGTA**, ethyleneglycol -bis-(β-aminoethyl ether, N, N, N', N'-tetraacetic acid; **Hepes**, 4-(2-hydroxyethyl)-1-piperazineethane-sulfonic acid; **IPTG**, isopropyl-β-D-thiogalacto-pyranoside; **MLCK**, myosin light chain kinase; **MOPS**, 3-(N-Morpholino) propanesulfonic acid; **PEG**, polyethylene glycol; **PMSF**, phenylmethyl sulfonyl fluoride ; **PVPP**, polyvinyl-polypyrrolidone; **RIA**, radioimmunoassay; **RUBISCO**, ribulose bisphosphate carboxylase/oxygenase; **SDS-PAGE**, SDS-polyacrylamide gel electrophoresis; **VU-1 calmodulin**, calmodulin derived from a synthetic gene; **VU-3 calmodulin**, site-directed mutant of VU-1 calmodulin with a lysine to arginine substitution at position 115; **VU-4 calmodulin**, site-directed mutant of VU-1 calmodulin with lysine to isoleucine substitution at position 115; **VU-8 calmodulin**, VU-1 calmodulin with lysine substitutions at position 82-84; **VU-12 calmodulin**, VU-1 calmodulin with lysine substitutions at position 118-120.

CHAPTER I

INTRODUCTION

Protein methylation : An overview

The enzymatic methylation of proteins is an ubiquitous phenomenon (for a recent review see Paik and Kim, 1990). Protein methyltransferases are selective and catalyze the methylation of specific residues within specific recognition sites (Paik and Kim, 1985; Frost et al., 1990). Among the group of methylation enzymes are protein-lysine N-methyltransferases, protein-arginine N-methyltransferases, protein-carboxyl O-methyltransferases, and protein-histidine N-methyltransferases (Paik and Kim, 1985, 1990; Frost et al., 1990). Although the protein substrate specificity is diverse, S-adenosyl-L-methionine (AdoMet) is the only known methyl group donor in all enzymatic protein methylation reactions (for reviews see Tabor and Tabor, 1984; Frost et al., 1990).

Since the involvement of protein-bound N-trimethyllysine as a metabolic precursor for carnitine biosynthesis was reported (LaBadie et al., 1976), the biological significance of protein methylation has been continuously explored (for reviews see Waarde, 1987; Paik and Kim, 1985, 1990; Frost et al., 1990). In some cases the biological roles of protein methylation are well established. For example, methylation of D-aspartyl or L-isoaspartyl residues is involved in a

repair system of damaged proteins, or degradation of aged proteins (Waarde, 1987; Fu et al., 1991). The combination of isoprenylation and methylation of several membrane-associated proteins at a C-terminal cysteine residue helps insert the proteins into their functional locations in the membrane (Clarke et al., 1988; Casey et al., 1989; Stephenson and Clarke, 1990; for a recent discussion see Hoffman, 1991). Methylation of chemotactic receptors at glutamyl residues is a key biochemical signal for sensory input in bacterial chemotaxis (for reviews see Frost et al., 1990 and Waarde 1987). However, the biological role of lysine methylation has been less clear. Histone H3 and H4 from animals and Drosophila contain ϵ-N-methyllysines (Paik and Kim, 1980; Desrosiers and Tanguay, 1988) and a protein methyltransferase specific for lysine residues in histones has been identified (Wallwork et al., 1977). Interestingly, the methylation state of histone H3 from heat-shocked Drosophila cells at 37 ^{o}C was decreased more than 3-fold compared with that from cells maintained at 23 ^{o}C (Desrosier and Tsanguay, 1988). Although a potential role of this change in methylation in connection with changes in chromatin structure has been suggested (Desrosiers and Tanguay, 1988), the precise function(s) of this modification is unclear. Based on in vitro studies with methylated and unmethylated apocytochrome c, Park et al., (1987) observed that methylation of apocytochrome c increased its import 2 to 4-fold into yeast mitochondria. However, the

methylation did not affect its import into other mitochondria and it is unknown whether the methylation of apocytochrome c in yeast affects its import into mitochondria in vivo.

Calmodulin-lysine N-methylation was identified from the amino acid sequence and composition analysis of bovine brain calmodulin (Watterson et al., 1980a). This analysis showed that bovine brain calmodulin contains ε-N-trimethyllysine at position 115. Since then, many attempts including this study have been made to understand the nature of the enzyme (Oh and Roberts, 1990) responsible for the specific methylation (Rowe et al., 1986; Morino et al., 1987) and to gain insight into the biological relevance of the modification (Roberts et al, 1986b). In the following section, I present an overview of calmodulin structure and function, and then consider the potential biological significance of lysine-115 methylation.

Calmodulin structure and function

Calcium has been recognized as an important signal molecule that is involved in the regulation of many key cellular activities in eukaryotes. (for reviews see Hepler and Wayne, 1985: Rasmussen, 1989, 1990; Berridge and Irvine, 1989; Berridge, 1990). In animals, calcium is believed to be involved in the regulation of muscle contraction and relaxation (van Breemen and Saida, 1989; Bers, 1990), mitotic events (Hepler, 1989; Hepler et al., 1990), egg fertilization (Epel, 1978), neurotransmitter release (DeLorenzo, 1982), and

many others (for reviews see Rasmussen, 1990; Berridge, 1990). In plants calcium also has been implicated in the regulation of a variety of growth and developmental processes such as cell motility, chromosome motion, cell proliferation, and germination, among others (for reviews see Hepler and Wayne, 1985; Hepler, 1990). The biochemical targets of intracellular calcium signals are a set of structurally related calcium-modulated proteins (Kretsinger, 1980: Van Eldik et al., 1982; Strynadka and James, 1989; Moncrief et al., 1990). All of these calcium-modulated proteins have a common helix-loop-helix motif known as the EF-hand (for reviews see Kretsinger, 1980; Strynadka and James, 1989). These structures allow high affinity calcium binding and undergo a change in activity that is related to a calcium-induced change in conformation (Kretsinger, 1980; Strynadka and James, 1989).

Calmodulin is a highly conserved, ubiquitous, multifunctional calcium-modulated protein that interacts with a variety of enzymes and proteins (Roberts et al., 1986a; Haiech and Watterson, 1988; Cohen and Klee, 1988; Roberts and Harmon, 1992). It is an acidic protein (pI≈4.0) consisting of a single polypeptide chain that binds four calcium ions reversibly with dissociation constants in the micromolar range (for reviews see Klee and Vanaman, 1982; Manalan and Klee, 1984; Roberts et al., 1986a). The amino acid sequence of calmodulin can be divided into four structural domains that are homologous to each other (Watterson et al., 1980a). Each

domain contains one EF hand. The crystal structure of calmodulin (2.2 Å resolution, Babu et al., 1988) has seven α-helices which comprise 66 % of the total amino acids in the molecule. Calmodulin has two lobes that are connected by an unusual central helix (a seven-turn α-helix). Each of the lobes, which have no contact with each other, contain two EF hand calcium binding domains that interact with each other to allow cooperative Ca^{2+}-binding (Babu et al., 1988; Haiech et al., 1991). Each calcium binding domain consists of a 12 amino acid loop that contributes seven oxygen ligands to calcium coordination, and two flanking amphipathic α-helical regions. The first three oxygen ligands are furnished by Asp or Asn side chains. The last two ligands are furnished by a Glu side chain that always contributes two carboxyl group oxygen atoms to the co-ordination (Strynadka and James, 1989). Other oxygen ligands are furnished by a water molecule and a peptide carbonyl oxygen.

Upon binding calcium, conformational changes in calmodulin are observed by a number of analyses. For example, small-angle X-ray scattering studies show that calcium-saturated calmodulin has an increased radius of gyration (R_g) compared with calcium-free calmodulin (Seaton et al., 1985; Heidorn and Trewhella, 1988). A number of studies with ^1H NMR spectroscopy indicate that there are changes in the conformation of calmodulin upon binding calcium (Seamon, 1980; Krebs and Carafoli, 1982; Ikura et al., 1983; Klevit et al.,

1984). Circular dichroism studies show that binding of calcium to calmodulin causes an increase in α-helix content (Kuo and Coffee, 1976; Wolff et al., 1977; Crouch and Klee, 1980; Dieter et al., 1985; Hennessey et al., 1987). Fluorescence spectroscopic studies using naturally occurring calmodulin with intrinsic tyrosine residues or by using an engineered calmodulin with a tryptophan residue in the calcium binding domain(s) show that calcium-binding to calmodulin induces marked conformational changes in the protein (Kilhoffer et al., 1981, 1989). Since the crystallographic data of calcium-free calmodulin is not available, the structural differences between the calcium-bound and calcium-depleted molecules have been proposed based on a comparison of the crystal structure of calmodulin and troponin C (Strynadka and James, 1989). Troponin C has four EF-hand calcium-binding motifs. Troponin C and calmodulin show 78 % sequence homology (Watterson et al., 1980a) and possess similar tertiary structure (reviewed by Strynadka and James, 1989). In addition, the crystal structure of troponin C is available with only the C-terminal calcium binding sites saturated (Herzberg and James, 1985a, 1985b). Thus, it was possible to compare the calcium underloaded troponin C molecule with the calcium-saturated calmodulin molecule. By this comparison, Babu et al., (1988) observed that there is a great superimposition in the C-terminal domains of the crystal structures of the two molecules. However, there are

significant differences in the imposition of N-terminal domains of the crystals suggesting calcium-induced changes in the crystal structure of calmodulin. From the crystal structure of the Ca^{2+}-underloaded troponin C, it has been observed that the Ca^{2+}-free N-terminal domain has a closed conformation in which the hydrophobic surfaces of the E and F helices are not accessible to solvent. Upon binding calcium, the N-terminal domain has a more open conformation that leads to the exposure of hydrophobic surfaces (Herzberg et al., 1986; Strynadka and James, 1989). The X-ray crystallographic study also shows that calmodulin contains a hydrophobic patch in each half of the molecule. These patches seem to be exposed on surfaces only in the calcium-saturated form, and may be the key structural features that allow many calcium-dependent calmodulin actions including drug-binding interactions and binding to calmodulin-binding peptides (O'Neil and Degrado, 1990).

Since the first detection of calmodulin from bovine brain as an activator of phosphodiesterase (Kakiuchi et al., 1970: Cheung, 1970), calmodulin has been found widely distributed in eukaryotes ranging from fungi to humans, and a number of calmodulin target proteins have been identified (for reviews see Klee and Vanaman, 1982; Manalan and Klee, 1984; Roberts et al., 1986a; Cohen and Klee, 1988; Roberts and Harmon, 1992). Therefore, it is believed that calmodulin, which itself has no intrinsic enzymatic activity, mediates its

cellular regulation through interaction with target enzymes or other nonenzymic proteins. Thus far, over 20 calmodulin-dependent enzymes and calmodulin-binding proteins in animals have been identified and characterized (Cohen and Klee, 1988). Among the groups of calmodulin-dependent enzymes are phosphorylase kinase (Cohen et al, 1978), myosin light chain kinase (Yagi et al., 1978), calmodulin-dependent kinase I (Greengard, 1987), II (Schulman, 1988), III (Nairn and Palfrey, 1987), cyclic nucleotide phosphodiesterase (Cheung, 1970; Kakiuchi et al.,1970), several adenylate cyclases (Brostrom et al., 1975; Minocherhomjee et al., 1988), Ca^{2+}-ATPases (Jarrett and Penniston, 1977), NAD kinases (Anderson and Cormier, 1878; Epel et al., 1981), and protein phosphatases (Stewart et al., 1983). Examples of identified calmodulin-binding proteins with no known enzymatic activities are microtubule-associated proteins (MAPs) (Lee and Wolf, 1984), tau factor (Kakiuchi and Sobue, 1983), stable tubule-only proteins (STOPs) (Job et al., 1981, 1982), fodrin (Kakiuchi and Sobue, 1983), caldesmon (Ngai and Walsh, 1985), and synapsin I (Okabe and Sobue, 1987). This diversity reflects the multiple roles of calmodulin in regulating cell processes.

In order to interact with the majority of target enzymes and proteins, calmodulin must bind calcium. However, the possibility of Ca^{2+}-independent action of calmodulin has been suggested from the recent observations of calcium-independent

interaction with some calmodulin-stimulated enzymes and target proteins, such as the Bordetella pertussis adenylate cyclase (Greenlee et al., 1982) and neuromodulin (Alexander et al., 1988). In addition, Geiser et al., (1991) observed that yeast strains deleted for calmodulin were not viable but reintroduction of any of several mutant calmodulins with defects in Ca^{2+}-binding can restore viability. Currently, it is unknown what essential Ca^{2+}-independent roles calmodulin plays in eukaryotes.

From a number of analyses, it also becomes clear that the levels of calmodulin can significantly vary from tissue to tissue and from organism to organism. For example, brain and testis are rich sources of calmodulin in animals (Klee and Vanaman, 1982), whereas calmodulin levels are low in other tissues, such as skeletal and cardiac muscles. In addition, among brain tissues, the levels of calmodulin are higher in the cadudate nucleus and cerebral cortex than in the posterior and anterior pituitary gland (Klee and Vanaman, 1982). It is also interesting to note that calmodulin is 2 to 5 % of the total protein in extracts of the electroplax of eel, which is the highest concentration of calmodulin among any vertebrate tissues (Childers and Siegel, 1975; Chafouleas et al., 1979; Munjaal et al., 1986). Evidence showing that the levels of calmodulin can vary depending on the state of cell growth and development comes from the analyses of transformed and normal cell cultures (Watterson et al., 1976; Connor et al., 1983;

Veigl et al., 1982). Cells transformed by either a oncogenic virus or chemical carcinogens have enhanced calmodulin levels (2- to 4-fold) compared with normal cells.

From the above observations, it has been suggested that calmodulin may be involved in regulating cell growth. In this regard, the following exciting findings are supportive of the possible involvement of calmodulin in cell growth and development. First, in synchronized cells, the concentrations of calmodulin are doubled at the G1/S boundary (Chafouleas et al., 1982; Feinberg et al., 1987). Second, the transiently increased calmodulin levels cause acceleration of cell proliferation by shortening the length of G1 phase (Rasmussen and Means, 1987, 1989). Third, a slight reduction (30 %) in calmodulin levels by utilizing a expression vector for anti-sense calmodulin RNA causes a temporary cell cycle arrest at the G1 phase or metaphase (Rasmussen and Means, 1989). Fourth, <u>in vivo</u> disruption of the gene encoding for calmodulin causes an inability of the fungi to grow from spores (Davis et al., 1986; Takeda and Yamamoto, 1987; Rasmussen et al., 1990). Further, the growth of a mutant yeast in which the expression of calmodulin is galactose-dependent is arrested in galactose free-medium but it grows normally in medium containing galactose (Ohya and Anraku, 1989).

As discussed thus far, calmodulin is a highly conserved high-affinity calcium-binding protein that is ubiquitous in all eukaryotic cells, has multiple regulatory activities and

is essential for cell growth. In the following section, the focus will be on the structure, function and homeostasis of plant calmodulin, calmodulin-dependent NAD kinase, and calmodulin-lysine N-methylation, with an emphasis on their potential significance in nicotinamide co-enzyme regulation.

Plant calmodulin structure, function and homeostasis

Comparison of the amino acid sequences between vertebrate calmodulins and plant calmodulins shows over 90 % sequence identity between the proteins (reviewed by Roberts et al., 1986a and Roberts and Harmon, 1992) (Figure 1). Like vertebrate calmodulins, plant calmodulins bind four calcium ions with high affinity (Yoshida et al., 1983) and activate a number of calmodulin-dependent enzymes with similar dependency on the free calcium concentration (Watterson et al., 1980b; Marme and Dieter, 1983; Roberts et al., 1984). In addition to the sequence homology and functional properties discussed above, another common characteristic shared by both animal and higher plant calmodulin is the posttranslational trimethylation of lysine 115 (Watterson et al., 1980a, 1980b; Anderson et al., 1980; Schleicher et al., 1983; Lukas et al., 1984).

Plant calmodulin possesses some unique and unusual substitutions (i.e., threonine to cysteine-26, in the first the calcium-binding site and glycine to glutamine-96 in the third calcium binding site) that are not necessary for function but which confer some unique physicochemical

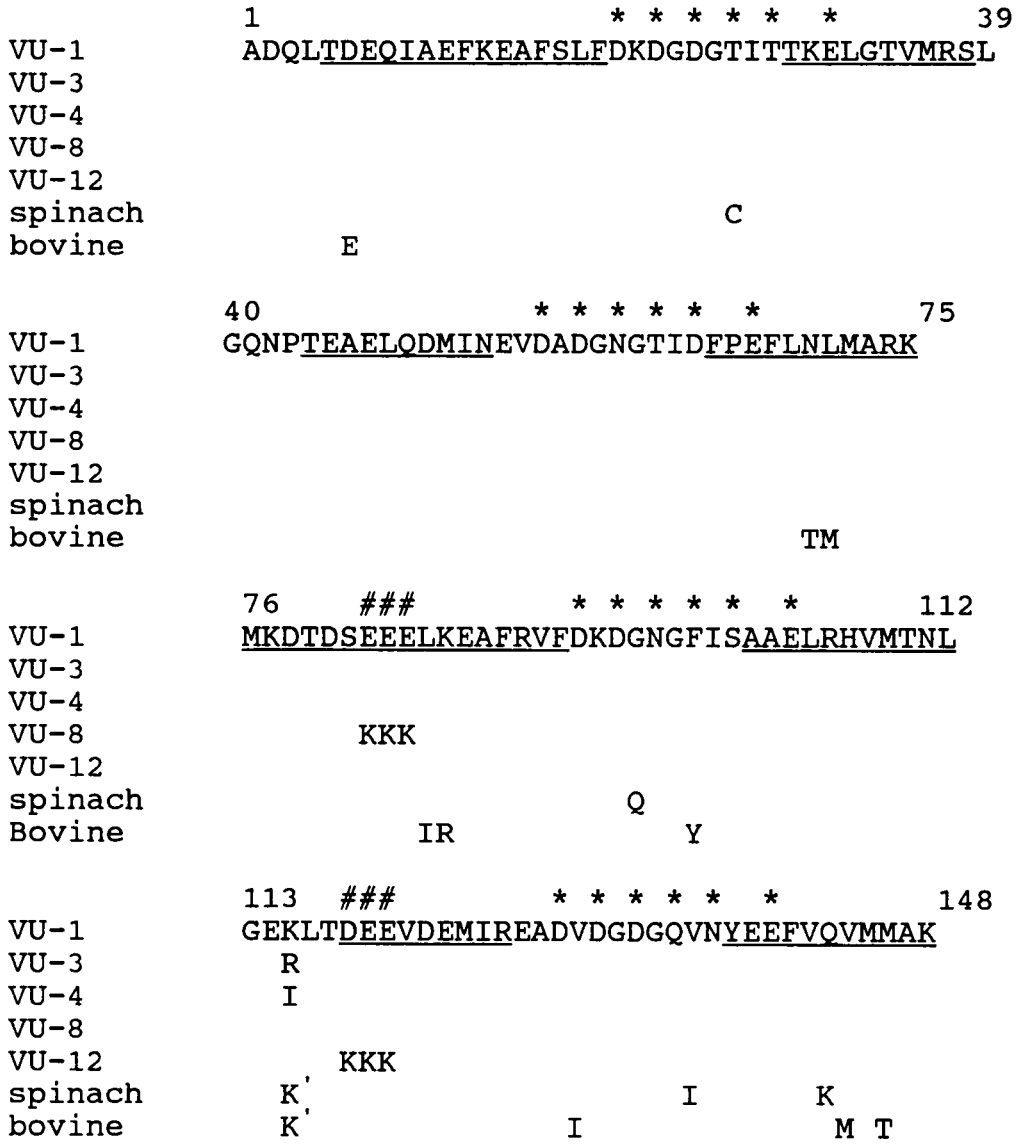


Figure 1. Comparision of calmodulin amino acid sequences. The complete sequence of VU-1 calmodulin (Roberts et al., 1985) is shown and only the differences from the VU-1 calmodulin sequence are shown in VU-3, VU-4 (Roberts et al., 1986b), VU-8 (Craig et al., 1987), VU-12 (Weber et al., 1989), spinach (Lukas et al., 1984), and bovine brain (Watterson et al., 1980a). The four calcium binding domains are shown with calcium binding residues (*) indicated and α-helical residues underlined. The # symbol represents amino acids that composing acidic clusters, and K' at position 115 of spinach and bovine brain calmodulin refers to trimethyllysine.

properties (Roberts et al., 1986a). For example, Watterson et al., (1980b) found a clear mobility difference between plant calmodulin and vertebrate calmodulin on SDS-PAGE in the presence of EGTA. The estimated molecular weights of the bovine brain calmodulin and spinach leaf calmodulin based on the SDS-PAGE analyses were 18,000-20,000 and 17,000-19,000, respectively, showing a difference of at least 1,000 in the apparent molecular weight. However, the numbers of total amino acid residues composing the calmodulins turned out to be identical (Lukas et al., 1984). Although the possibility was raised that one or two amino acid substitutions could result in altered SDS-binding (Watterson et al., 1980b), a clear explanation for the apparent difference in electrophoretic mobility is not available. A recombinant DNA-derived VU-1 calmodulin, which differs from plant calmodulin at only 4 residues (Figure 1), has an electrophoretic mobility on SDS-PAGE similar to that of vertebrate calmodulin (Roberts et al., 1985). As discussed later, we utilized this mobility difference on SDS-PAGE between VU-1 calmodulin and plant calmodulin in the analysis of transgenic plants to estimate the relative expression levels of foreign VU-1 calmodulin.

Another interesting finding is that one organism can have more than one type of calmodulin (Reviewed by Roberts et al., 1986a; Roberts and Harmon, 1992). For example, calmodulin genes encoding calmodulin isoforms and calmodulin-like proteins have been detected and sequenced from

Arabidopsis and barley (Braam and Davis, 1990; Zielinski et al., 1990; Ling et al., 1991; Perera et al., 1991). The deduced amino acid sequences of two cDNAs of the two calmodulin isoforms from Arabidopsis leaf showed 97 % sequence homology (Ling et al., 1991). These two calmodulin isoforms differ by four amino acid substitutions which do not occur within the four calcium-binding domains. Genomic Southern blot assays revealed that they are derived from two different genes. The reason for multiple isotypes is unknown, but the expression of the genes may be differently controlled to meet cell requirements.

Braam and Davis (1990) detected and sequenced three touch-induced (TCH) cDNAs that encode calcium modulated proteins with homology to calmodulin in Arabidopsis. TCH 1 cDNA encodes Arabidopsis calmodulin 2 (Ling et al., 1991). TCH 2 and TCH 3 also encode proteins with 44 % and 70 % amino acid sequence identities to calmodulin, respectively. Currently, the functional roles of the different calmodulin forms is unclear. However, the finding showing the existence of calmodulin isoforms and calmodulin-like proteins within the same organism may suggest that they have different roles in the regulation of cellular processes. Further work involving the purification of the calmodulin isotype proteins, analysis of tissue or cell distribution, and identification of the potential targets is important to understand the roles of calmodulin isoforms existing in the same organism.

In plants, it has been shown that the levels of calmodulin also vary from tissue to tissue depending on the developmental stages (Muto and Miyachi, 1984; Allan and Trewavas, 1985; Zielinski, 1987). In general, based on radioimmunoassay or enzyme activator analyses, higher amounts of calmodulin have been found from young meristematic tissues compared with mature tissues. In addition, immunohistochemical and immunocytochemical analyses of apical regions (Lin et al., 1986; Dauwalder et al., 1986) show that the root cap and the meristem have the highest concentrations of calmodulin. Analyses of the germinated seed embryos (Cocucci, 1984; Cocucci and Negrini, 1988; Hernandez-Nistal, 1989) show that the levels of calmodulin can vary depending on the state of plant development. The germinated embryos have significantly increased calmodulin levels compared with undifferentiated cells or non-germinated embryos. The developmentally different distribution between tissues may reflect a close relationship between calmodulin level and plant cell growth and development. In this regard, it is important to note that even minor changes in calmodulin levels can drastically influence growth (Rasmussen and Means, 1987, 1989).

Since calmodulin mediates its effects as a cell regulator through its interaction with calmodulin-dependent target proteins, it is important to consider the types and distribution of these proteins. Several calmodulin-dependent

enzyme activities and calmodulin-binding proteins have been detected in plant systems (Roberts et al., 1986a; Roberts and Harmon, 1992). Among the groups of calmodulin-dependent enzyme activities detected in plants are NAD kinase (Anderson and Cormier, 1978), Ca^{2+}-ATPase (Dieter and Marme, 1980, 1981; Briars et al., 1988), and a nuclear nucleoside triphosphatase (Matsumoto et al., 1984; Chen et al., 1987). However, except for NAD kinase (Roberts et al., 1985; 1990; Harmon et al., 1984), information on the purification and characterization of these calmodulin-stimulated enzymes is lacking and in some cases it is unclear whether calmodulin is the in vivo regulator of their activities. Among other groups of calmodulin-binding proteins identified in plants are 30,000 to 33,000 M_r protein from from pea and spinach (Roberts et al., 1983), and 13,000 M_r protein from germinating radish seed embryos (Cocucci and Negrini, 1988). Additionally, Brawley and Roberts (1989) identified 45,000 M_r and 72,000 M_r calmodulin-binding proteins that are found predominantly in sperm and zygotes respectively of the marine brown algae Fucus vesiculosus. Interestingly, the 72,000 M_r protein was not detectable in the unfertilized egg and started to appear 1 hr postfertilization and maintained during early embryogenesis. Thus, there is precedent for developmental regulation of calmodulin target proteins. However, precise roles for calmodulin and the calmodulin-binding proteins in the system are unknown. To understand the mechanism by which calmodulin

and calmodulin target proteins exert their roles in plant systems, it is necessary to identify the activities of these calmodulin receptor proteins.

Calmodulin-dependent NAD kinase

Since the first demonstration of plant NAD kinase activation by Ca^{2+}/calmodulin (Muto and Miyachi, 1977; Anderson and Cormier, 1978), Ca^{2+}/calmodulin-dependent NAD kinases have been found in many plant tissues (Roberts et al., 1986a; Roberts and Harmon, 1992), in sea urchin eggs (Epel et al., 1981), and in human neutrophils (Williams and Jones, 1985). Among these enzymes, pea NAD kinase is the best characterized activity. Roberts et al., (1990) obtained a purified preparation of the enzyme from pea seedlings with apparent molecular weight ranging from 50,000 to 55,000 based on SDS-PAGE analysis and molecular exclusion chromatography. The purified enzyme is completely dependent upon the presence of added calcium and calmodulin. The enzyme is particularly sensitive to the state of posttranslational calmodulin methylation (see below for discussion).

NAD kinase catalyzes the phosphorylation of NAD to NADP using ATP as a co-substrate (for a review see McGuinness and Butler, 1985). NAD and NADP are utilized in different metabolic pathways (for reviews see Lowenstein, 1961; Roberts et al.,1990). NAD(H) is mainly used in catabolic reactions for energy production. NADP(H) is mainly used in biosynthetic

reactions and in certain catabolic pathways such as the pentose phosphate pathway. Availability of NADP is the rate limiting step for certain pathways including the pentose phosphate pathway (Yamamoto, 1963). Therefore, calcium fluxes and the Ca^{2+}/calmodulin-dependent NAD kinase could be important in triggering a rapid shift of metabolism in response to external signals by modulating NAD/NADP ratios.

Epel (1964, 1978) observed that there are increases of free Ca^{2+} and a burst of NADPH after fertilization of sea urchin eggs. Ca^{2+}/calmodulin-dependent NAD kinase was proposed to be responsible for this increase (Epel et al., 1981) and was proposed to be necessary to provide cellular NADP(H) for reductive biosynthetic processes or for protection of the egg from oxidative stress (Epel, 1964; Epel et al., 1981; Shapiro, 1991). Williams and Jones (1985) found another Ca^{2+}/calmodulin-dependent NAD kinase from human neutrophils. NADPH is utilized by NADPH oxidase in the production of superoxide for bactericidal activity in the neutrophils (Baggiolini and Wymann, 1990). Therefore, the elevation of NADP by calmodulin-dependent NAD kinase could be necessary to provide a rapid increase in substrate NADPH for reductive processes in these cells.

Presently, the role of calmodulin-dependent NAD kinase in plants is unknown. Previously, it was proposed that calmodulin-dependent NAD kinase may be involved in the light - dependent increase in the NADP/NAD ratio (Oh-hama and Miyachi,

1960; Ogren and Krogmann, 1965). Additionally, Muto et al., (1981) observed that light increases the NADP/NAD ratio through stimulation of NAD kinase activity in the chloroplast stroma. From this, it was speculated that calmodulin-dependent NAD kinase plays a role in photosynthesis by providing an electron acceptor, NADP (Muto et al., 1981; Jarrett et al., 1982). However, it has been shown that the light-activated stromal NAD kinase activity is not the calmodulin-stimulated enzyme (Simon et al., 1982; Muto and Miyachi, 1986). Further, it has been found that the chloroplast has at least two forms of NAD kinase activity, an envelope calmodulin-dependent activity and a stromal calmodulin-independent activity (Simon et al., 1984; Muto and Miyachi, 1986). However, other studies have shown that most of the NAD kinase activity in plants is calcium and calmodulin dependent (Dieter and Marme, 1984, 1986; Kansara et al., 1989; Roberts et al., 1990). Therefore, the widespread nature of this enzyme in higher plants argues for a fundamental function of calcium in controlling redox metabolism.

From the physiological perspective, it is important to consider how NAD kinase is regulated during plant growth and development. From the preliminary studies done by Allan and Trewavas (1985), it was found that the extractable activity of calcium-stimulated NAD kinase increases substantially during the transition of pea root cells from the proliferative, meristematic stage to the elongation and maturation stages.

Further, the distribution of calcium-dependent activity in the root paralleled the increase in the activity of the pentose phosphate pathway. An interesting proposal is that NAD kinase might influence the flux of carbon through glycolytic and pentose phosphate pathways by controlling the availability of NAD and NADP. In this regard it is important to note that the levels of nicotinamide co-enzymes are low in many plant tissues and may be the limiting factor that controls the flux of certain metabolites through different pathways (Yamamoto, 1963).

Calmodulin-lysine N-methylation: Significance to NAD kinase regulation

ϵ-N-trimethyllysine is a posttranslational modification that is found at position 115 of many calmodulins (Watterson et al., 1980a; Marshak et al., 1984; Lukas et al., 1984, 1985). Based on x-ray crystallographic data (Babu et al., 1988), it is clear that lysine-115 is located in between the third and the fourth calcium binding domain, is the most solvent exposed lysine residue, and has no interaction with any other residues in the structure. This site is methylated by a calmodulin:lysine N-methyltransferase (Rowe et al., 1986; Morino et al., 1987; Oh and Roberts, 1990). Additionally, although much less common, other sites of methylation also have been found in certain calmodulins. For example, the calmodulin from Paramecium tetraurelia contains ϵ-

N-dimethyllysine at residue 13 (Schaefer et al., 1987).

The biological role of calmodulin methylation is unclear. Although trimethyllysine-115 is commonly found in calmodulin, naturally occurring calmodulins that lack trimethyllysine have been purified and characterized from some organisms including Chlamydomonas reinhardtii (Van Eldik et al., 1980a; Schleicher et al., 1984; Lukas et al., 1985), Dictyostelium discoideum (Bazari, 1981; Marshak et al., 1984), trypanosomes (Ruben et al., 1984), crayfish (Hergenhan et al, 1984), and Neurospora crassa (Cox et al., 1982). Additionally, active recombinant DNA-derived calmodulins that do not possess trimethyllysine-115 have been expressed in E. coli from cloned calmodulin genes (Roberts et al., 1985) or cDNAs (Putkey, 1985, 1986; Persechini et al., 1988).

Based on in vitro studies with unmethylated and methylated calmodulins, Gregori et al. (1985, 1987) have suggested that lysine 115 of calmodulin is a site for ubiquitin conjugation. They also have proposed that the posttranslational methylation of lysine 115 and acetylation of the amino terminus of calmodulin may serve the function of protecting calmodulin from ubiquitination, and from degradation by the cytoplasmic ubiquitin-dependent proteolytic pathway. However, it has yet to be determined whether unmethylated calmodulins are conjugated to ubiquitin in vivo and are turned over more rapidly. Also, Ziegenhagen and Jennissen (1988) showed that bovine brain calmodulin, which

contains trimethyllysine at position 115, can be coupled to ubiquitin *in vitro* in the presence of calcium. Ziegenhagen et al. (1988) observed that the conjugating activity depends on the presence of micromolar calcium concentrations. From these findings, it has been suggested that the Ca^{2+}-bound form of calmodulin is preferred by the ubiquitination system. Jennissen and Laub (1988) demonstrated that the activity that conjugates ubiquitin to calmodulin is also present in extracts from other tissues, such as cardiac muscle, suggesting a wide distribution of this activity. More recently, Ziegenhagen et al. (1990) demonstrated that the ubiquitination product of calmodulin still contains one mole of trimethyllysine per mole of calmodulin, and proposed that trimethylation does not prevent ubiquitination. In addition, evidence from animal cells shows that the methylation state of calmodulin varies from tissue to tissue (Molla et al., 1981; Rowe et al., 1986). Thus, unmethylated calmodulin can exist in an undegraded state *in vivo*. Presently, the reason for the discrepancies between the observations of Ziegenhagen and co-workers and Gregori and co-workers is not clear. One possibility is that lysine 115 of calmodulin may be an additional site of ubiquitination under some conditions. Further work involving an *in vivo* analysis of calmodulin ubiquitination and turnover will help reveal the significance of these processes in calmodulin homeostasis.

With respect to the regulatory activities of

calmodulin, unmethylated calmodulins are not significantly different from methylated calmodulins in their ability to bind calcium and activate a number of enzymes (Putkey et al., 1985, 1986; Rowe et al., 1986; Roberts et al., 1986b). However, one calmodulin-dependent enzyme, plant NAD kinase, is sensitive to calmodulin methylation. For example, NAD kinase purified from pea plants has a specific activity of 18 μmole NADP/min/mg protein with saturating VU-1 calmodulin in the presence of calcium and saturating levels of NAD and ATP (Roberts et al., 1990). However, the specific activity of the enzyme measured with spinach calmodulin was 2 to 4 μmole NADP/min/mg protein under the same conditions. VU-1 calmodulin is a recombinant DNA-derived calmodulin which has unmethylated lysine at position 115 (Roberts et al., 1985, 1986b). On the other hand, spinach calmodulin has trimethyllysine at position 115 (Lukas et al., 1984). Upon in vitro enzymatic trimethylation, the NAD kinase activator properties of VU-1 calmodulin are reduced to the level of that seen with spinach calmodulin (Roberts et al., 1986b). Thus, the reduction of the level of activation of NAD kinase is the direct result of trimethylation of calmodulin at position 115. These results raise the possibility that selective calmodulin activator activities could be attenuated by posttranslational methylation of lysine-115. For example, the methylation of calmodulin could result in an overall reduction in NAD kinase activation whereas other regulatory targets may not be

similarly affected.

In order to address the potential regulatory significance of calmodulin methylation, it is necessary to examine the factors that control methylation in vivo and to determine the distribution of unmethylated or undermethylated calmodulin in vivo. Based on the data from amino acid composition analysis (Anderson et al., 1980; Watterson et al., 1980b; Marme and Dieter, 1983; Biro et al., 1984) and amino acid sequence determination (Schleicher et al., 1983; Lukas et al., 1984), higher plant calmodulins appear to possess one mole of trimethyllysine per mole calmodulin, suggesting stoichiometric methylation. However, it has to be pointed out that the calmodulins were purified from whole seedlings, and small or transient amounts of unmethylated calmodulins that are specifically localized in certain tissues may have evaded detection by the insensitive protein chemistry methods. In this regard, it is interesting to note that the methylation state of calmodulin and the levels of calmodulin methyltransferase vary in different tissues within the same organism (Seamon and Moore, 1980; Molla et al., 1981; Rowe et al., 1986). In order to investigate the functional significance of lysine methylation in the control of nicotinamide metabolism it would be beneficial to have a system to perturb the calmodulin/NAD kinase regulatory system in vivo. In this regard the site-directed mutants of calmodulin with substitution at position 115 may provide

interesting information on the functional effects of calmodulin methylation by using transgenic plant technology. For example, Roberts et al., (1986b, 1990) showed that the calmodulin derivatives, VU-3 calmodulin (lys to arg 115) and VU-4 calmodulin (lys to ile 115), retain calmodulin activities, and activate NAD kinase to an activity that is 3-fold higher than trimethylated calmodulin. Therefore, introduction of these calmodulin mutants into higher plants may perturb the control of nicotinamide co-enzyme metabolism.

The present study was undertaken to address the following questions: 1. What are the characteristics of the calmodulin methylating enzyme ?; 2. How is calmodulin methylation controlled in vivo and do pools of methylated and unmethylated calmodulin exist ?; 3. What is the biological significance of calmodulin methylation and how can the system be manipulated ? To address these questions, the calmodulin methyltransferase from sheep brain was purified and characterized, and was used to design a specific assay for the analysis of calmodulin methylation states. I have analyzed the methylation states of calmodulin, calmodulin protein levels, and calmodulin-target proteins from several plant tissues and tissue cultures. Finally, transgenic tobacco plants expressing calmodulin derivatives that are defective in their ability to be methylated were generated and characterized to analyze the role of calmodulin methylation in calmodulin cell function.

CHAPTER II

MATERIALS AND METHODS

Materials

^{3}H-AdoMet, 76.5 Ci/mmol was purchased from Du Pont. Nonradioactive S-Adenosyl-L-methionine and Bis-Tris were obtained from Sigma. Sheep brains were obtained from the Large Animal Clinic, School of Veterinary Medicine, University of Tennessee. Phenyl-Sepharose CL-4B and CNBr activated Sepharose 4B were purchased from Pharmacia Fine Chemicals. Mono Q HR 5/5 column was from Pharmacia LKB. Phosphocellulose (P11) was purchased from Whatman. Chicken gizzard calmodulin-Sepharose 4B was prepared by the method of Klee and Krinks (1978). Chemicals for the generation of transgenic tobacco plants were purchased from Sigma except hygromycin (Boehringer Mannheim). All other reagents were purchased from commercial sources and were of the highest grade.

Calmodulin mutagenesis, expression cloning and purification

VU-1, VU-3 (lys to arg 115) , VU-4 (lys to ile), VU-8 (glu-glu-glu to lys-lys-lys 82-84), and VU-12 (asp-glu-glu to lys-lys-lys 118-120) calmodulins were produced as previously described (Roberts et al., 1985, 1986b, 1987; Craig et al., 1987; Weber et al., 1989). In order to obtain high levels of expression of the calmodulins we cloned them into a higher

copy number plasmid, pVUCH-1. pVUCH-1 plasmid (6 μg) containing the synthetic VU-1 calmodulin gene (Roberts et al., 1985) was digested at 37 °C overnight in a 50 μl reaction mixture containing EcoRI (40 units), NruI (20 units), 10 mM $MgCl_2$, 50 mM NaCl, 50 mM Tris-HCl, pH 8.0. 1 unit of calf intestine alkaline phosphatase was then added to the digest and the mixture was incubated at 37 °C for 1 hr, and then at 60 °C for 15 min. pVUC-3 (75 μg) containing the VU-3 calmodulin gene (Roberts et al., 1986b) was digested at 37 °C overnight in a 200 μl reaction mixture containing EcoRI (150 units), NruI (75 units), 10 mM $MgCl_2$, 50 mM NaCl, 50 mM Tris-HCl, pH 8.0. Digested DNA was extracted with phenol/ chloroform/isoamyl alcohol (25 : 24 : 1) and chloroform/ isoamyl alcohol (24 : 1), and was precipitated with ethanol and resuspended in 1 mM Tris-HCl, pH 7.5, 0.1 mM EDTA. The DNA fragments of pVUC-3 were resolved by electrophoresis on a 8 % (w/v) polyacrylamide gel, and the small fragment was sliced from the gel. The DNA was extracted from the sliced gel by using a modified protocol (Craig et al., 1987) from the method developed by Maxam and Gilbert (1980). The purified product was resuspended in 1 mM Tris-HCl, pH 7.5, 0.1 mM EDTA, and was stored at 4 °C. The VU-3 calmodulin gene fragment (90 ng) and the EcoRI/NruI-cleaved vector (400 ng) were combined and treated with 1 unit of T4 DNA ligase at 15 °C overnight in a 40 μl reaction mixture containing 1 mM ATP, 50 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, 10 % (w/v) PEG, 10 mM

DTT. E. coli UT481 cells were transformed with the ligation mixture as previously described by Roberts et al.(1985). The pVUCH-3 plasmid containing the VU-3 calmodulin gene was prepared by the alkaline lysis method of Birnboim and Doly (1979) as described previously (Roberts et al., 1985). Plasmids that contain VU-3 calmodulin gene were shown to lack the HindIII site within the coding sequence but yielded the expected restriction endonuclease maps with BamHI and EcoRI (Roberts et al., 1986b). The loss of the HindIII site is due to the replacement of lysine-115 codon (AAG) by the arginine codon (CGT) in the case of VU-3. DdeI restriction maps were used to confirm the VU-8 and VU-12 insertions. pVUCH-3 and pVUCH-4 plasmids were screened for the presence of the mutation by restriction mapping with BamHI/HindIII. Recombinant calmodulins and calmodulin mutants were expressed in E. coli (UT481) and were purified as previously described (Roberts et al., 1985, 1987). Chicken gizzard calmodulin and spinach calmodulin were purified by using previously described procedures (Van Eldik and Watterson, 1979; Lukas et al., 1984). The final purification of the calmodulins was achieved by Ca^{2+}-dependent hydrophobic chromatography on a phenyl-Sepharose column (Gopalakrishna and Anderson, 1982).

Purification of calmodulin-lysine N-methyltransferase

One sheep brain (90 g) was homogenized in 4 volumes of 25 mM Hepes-NaOH, pH 7.4, 4 mM βME, 0.25 M sucrose, 5 mM EGTA,

0.1 mM PMSF, and was centrifuged for 30 min. at 10,000 x g. Supernatants were brought to 35 % saturation with solid ammonium sulfate and were stirred for 30 min. The sample was centrifuged for 30 min. at 12,000 x g. Ammonium sulfate was added to the supernatant to a final concentration of 70 % saturation and was stirred for 30 min. The sample was centrifuged for 30 min. at 12,000 x g. The pellet was resuspended in 30 ml of homogenization buffer (pH 6.8) without sucrose and then dialyzed overnight at 4 ^{o}C against 4 liters of this buffer with 4 changes. An equal volume of phosphocellulose equilibration buffer (25 mM Hepes-NaOH, pH 6.8, 4 mM β-ME, 0.1 mM PMSF, 0.01 % (w/v) Triton X-100) was added to the dialyzed sample. The sample was applied to a 2.5 x 10 cm column of phosphocellulose and the column was washed with 400 ml of the equilibration buffer. After washing, the absorbance of the effluent at 280 nm was less than 0.025. The column was eluted with a linear NaCl gradient (0-0.5 M) in equilibration buffer. The enzyme peak was pooled and concentrated by ultrafiltration on a Amicon YM-10 membrane. The concentrated enzyme was dialyzed against calmodulin-Sepharose buffer, 5 mM Tris-HCl, pH 7.4, 2 mM DTT, 0.1M NaCl, 0.1 mM PMSF, 0.01 % (w/v) Triton X-100, 0.1 mM $CaCl_2$. The solution was applied to a 1.0 x 4 cm column of chicken gizzard calmodulin-Sepharose equilibrated in calmodulin-Sepharose buffer. The column was washed with 100 ml of the calmodulin-Sepharose equilibration buffer and then with 125 ml of the

same buffer containing 0.5 M NaCl. The enzyme was eluted with calmodulin-Sepharose elution buffer, 5 mM Tris-HCl, pH 7.4, 2 mM DTT, containing 0.01 % (w/v) Triton X-100, 1.0 M NaCl, and 1 mM EGTA. The active fractions were pooled and concentrated by ultrafiltration on a YM-10 membrane. The concentrated enzyme (0.5 ml) was applied to a 1.5 x 84 cm column of Biogel A-1.5 equilibrated in 25 mM Hepes-NaOH, pH 7.4, 2 mM DTT, 0.15 M NaCl, 0.01 % (w/v) Triton X-100. The column was eluted at 4 ml/h with this buffer. Fractions of 1.4 ml were collected. The active fractions were pooled and applied to a 1.5 x 12 cm column of Sephadex G-10, previously equilibrated in 25 mM Hepes-NaOH, pH 7.5, 2 mM DTT, 0.01 % (w/v) Triton X-100. Active samples were pooled and were stored frozen at -80 °C. FPLC chromatography on a Superose 12 (1.0 x 30 cm) column has also been used as an alternative to chromatography on Biogel A-1.5. For the chromatography on Superose 12, the column was equilibrated with 25 mM Hepes-NaOH, pH 7.5, 2 mM DTT, 0.2 M NaCl, 0.05 % (w/v) CHAPS.

Calmodulin-lysine N-methyltransferase assay

Radiometric calmodulin N-methyltransferase assay procedures were done as previously described (Rowe et al., 1983). Assays were done under conditions where enzyme activity is constant with time and proportional to enzyme concentration. Typical reaction mixtures (total volume of 100 μl) contained VU-1 calmodulin (3 μg), 12 μM ^{3}H-AdoMet (0.5

μCi), 0.1 M NaCl, 2 mM $MgCl_2$, 5 mM DTT, 0.01 % (w/v) Triton X-100, 0.1 mM $CaCl_2$, 0.1 M glycylglycine-NaOH, pH 8.0. Reactions were initiated by the addition of enzyme. The mixture was incubated for 20 min at 37 °C and the reactions were terminated by heating at 90 °C for 3 min. The samples were centrifuged in an Eppendorf model 3514 microfuge for 6 min. [^{3}H]calmodulin was separated from the other reaction components by calcium-dependent adsorption to phenyl-Sepharose. The supernatants were combined with 200 μl of phenyl-Sepharose equilibrated with 50 mM Tris-HCl (pH 8.0), 0.3 M NaCl, 0.1 M $CaCl_2$. The resin was mixed and collected by centrifugation. The resin was washed with 4 ml of 50 mM Tris-HCl (pH 8.0), 0.3 M NaCl, 0.1 M $CaCl_2$. [^{3}H]calmodulin was eluted with 0.1 M NH_4HCO_3 (pH 8.0), 2 mM EDTA and counted in a Beckman model 3081 liquid scintillation counter. The recorded cpm (counts per minute) values were converted into dpm (disintegrations per minute) values using counting efficiency which is calculated by using standard sets with known dpm values and the cpm recorded by the instrument. Under the conditions of the assay, the incorporation of as little as 0.015 mol of [^{3}H]methyl groups per mole calmodulin could be detected. In assays of calcium dependence of the enzyme, the incubation buffer was modified to contain 25 mM Hepes-NaOH, pH 7.5, 2 mM DTT, containing 0.01 % (w/v) Triton X-100. The reaction mixture contained the buffer and various amounts of $MgCl_2$, $CaCl_2$, and EGTA, and constant amounts of

VU-1 calmodulin and enzyme. A computer program (Perrin and Sayce, 1967) and the stability constants of Martel and Smith (1974) were used to determine the amounts of $MgCl_2$, $CaCl_2$, and EGTA required to yield a Mg^{2+} concentration of 5 mM and a free Ca^{2+} concentration that varied from 10^{-2} to 10^{-9} M. All buffers were treated with Chelex resin to remove metal ions. Metal ion content of stock solutions was determined by atomic absorption spectroscopy as previously described (Roberts, 1989).

Immunochemistry

Antibodies against VU-1 calmodulin were produced in New Zealand White rabbits by subcutaneous injection of VU-1 calmodulin (1 mg) emulsified 1:1 with Freund's complete adjuvant. After one week the injection was repeated with VU-1 calmodulin (1 mg) in Freund's incomplete adjuvant. After two additional weeks the injection was repeated again. The rabbit was bled at weekly intervals and the antisera was stored at -80 ^{o}C.

The calmodulin concentrations were determined by radioimmunoassay. VU-1 calmodulin was iodinated by the chloramine T method as previously described (Van Eldik and Lukas, 1987). ^{125}I-VU-1 calmodulin (80,000 cpm) was incubated with anti-VU-1 calmodulin serum in a 200 μl reaction mixture containing 1.3 % (v/v) normal rabbit sera, 2 % (w/v) bovine serum albumin, 50 mM Tris-HCl, 100 mM NaCl, pH 7.5, and

various amounts of unlabeled calmodulin standards or samples. After 8 hr at 4° C, 10 μl of goat-anti-rabbit IgG sera (Cappel Laboratories) was added and the reaction was continued at 30°C for 1 hr. An equal volume of 6 % (w/v) polyethylene glycol 8000 was added and the samples were placed on ice for 30 min. The precipitated antigen and antibody complexes were collected by centrifugation in an Eppendorf model 3514 microcentrifuge for 10 min. The pellets were washed once with 3 % (w/v) polyethylene glycol 8000 in 50 mM Tris-HCl, 100 mM NaCl, pH 7.5, and were counted in a Beckman 5500B gamma counter. The amount of calmodulin in unknown samples was determined by constructing standard curves based on the ability of VU-1 calmodulin to compete for ^{125}I-calmodulin.

Analysis of kinetic data

Substrate interaction data was derived from:

$$V = \frac{Vmax[A][B]}{K(ia)Km(B) + [A]Km(B) + [B]Km(A) + [A][B]} \quad [1]$$

or

$$V = \frac{Vmax\ [A][B]}{[A]Km(B) + [B]Km(A) + [A][B]} \quad [2]$$

Where A and B are the varied substrates, Km(A) and Km(B) are the Michaelis-Menten constant for substrates A and B, respectively. Vmax is the apparent maximum velocity, and

K(ia) is the dissociation constant for enzyme and substrate A complex. Inhibition study data were derived from equation [3] for competitive inhibition as determined by inspection of the double-reciprocal plots.

$$V = \frac{Vmax}{1 + Km/[A] + Km[I]/Ki[A]} \qquad [3]$$

Where A is the variable substrate, I is the inhibitor, Km and Vmax are the Michaelis constant and maximum velocity, respectively, in the presence of inhibitor and Ki is the inhibition constant. Km_{app} replots versus fixed inhibitor concentrations were used to derive inhibitor constants.

Investigation of calmodulin levels and calmodulin methylation in plant tissues and plant tissue cultures

Pea seeds (Pisum sativum L. var Early Alaskan) were surface-sterilized with 5 % (v/v) Clorox for 5 min and were washed extensively with water to remove Clorox residual. The seeds were aerated in water for 8 h and then germinated in trays of vermiculite in the dark at 22 - 24 °C. Etiolated plants were obtained by growing under these conditions for 72 h. Plants with roots 3.5 - 4.0 cm long were harvested, dissected into 0.6 cm root segments and epicotyls, and immediately frozen in liquid nitrogen and stored at -80 °C until analyzed. Green seedlings were germinated as described above, and were grown in a growth chamber at 22 - 24 °C for 6

days with 12 h light/dark cycles. Plants with primary roots of 9.0 cm to 9.5 cm were harvested, and were dissected into 1 cm root segments. Lateral roots, stems, and the young primary leaves were also dissected and frozen in liquid nitrogen immediately and stored at -80 °C until analyzed.

Soybean seeds (Glycine max c.v. Essex) were surface-sterilized with 20 % (v/v) Clorox for 10 min and washed 3 times with sterile deionized water. The seeds were then soaked in 0.01 N HCl for 10 min and washed 6 times with sterile deionized water. The seeds were transferred to a sterile petri dish plate with 10 ml of sterile water. The plates were placed in the dark for 3 days. Germinated soybeans with 3.0 - 4.0 cm long roots were selected, were dissected into 1.0 cm segments, and were frozen in liquid nitrogen, and were stored at -80 °C until analyzed. To obtain young nodules, the germinated soybeans were transferred to sterile vermiculite and infected with Bradyrhizobium japonicum strains 61A101C or USDA 110 (So et al., 1986). Nodules were harvested from 14 day-old soybean plants, were frozen in liquid nitrogen, and were stored at -80 °C as described above.

Carrot cell cultures and carrot embryos were obtained in collaboration with Dr. Don Dougall and Henry Steiner in the Botany Department at the University of Tennessee. Carrot cell (Daucus carota L.) line 13-1 was grown in WCM-4 media (Dougall and Weyrauch, 1980) containing 2.2 mg/l 2,4-dichlorophenoxy acetic acid (2,4-D). 7-day old cells

maintained on WCM-1 media (Wetherell, 1969) were inoculated into the WCM-4 media with an inoculum density of 1:10. Cultures were grown at 25 °C in a gyrotory shaker at 120 rpm. Samples were harvested from various stages of the cell growth curve (i.e., 2, 4, 5, 6, 8, 11, 14-days) for the first growth cycle. For the second growth cycle, 14-day old stationary phase cells were inoculated into fresh WCM-4 media and were grown under conditions identical to the first cycle. Samples were taken at 0.5, 1, 2, 3, 4, 8-day intervals. The harvested tissues were collected by filtration on 70 mm Whatman #2 filter paper, were weighed, and were immediately frozen in liquid nitrogen and stored at -80 °C.

Embryogenic carrot cultures were maintained by weekly serial passages using a 10 % (v/v) inoculum. Media containing MS salts and vitamins supplemented with 30 g/l sucrose, 146 mg/l glutamine, 0.1 mg/l γγ dimethylallylamino purine and 0.5 mg/l 2,4-dichlorophenoxy acetic acid (2,4-D) were used for culture growth. Cultures were grown at 25 °C in the dark in a gyrotory shaker at 150 rpm. For initiation of embryogenesis, one week old cultures were passed through a 105 μm screen and retained on a 44 μm screen. The tissue was washed twice on the 44 μm screen with the above medium minus 2,4-D (embryogenesis media). The tissue was further washed twice on the screen with embryogenesis media. The resulting tissue was incubated in embryogenesis media at a density of 16-20 μl packed tissue volume (PTV)/ml of medium for 3 hr.

The cells were centrifuged and resuspended in the same medium at 4-5 μl PTV/ml. After 4 days the cultures were again diluted 1:5 with fresh embryogenesis media. At this stage the formation of globular embryos had begun. After 6 days, the cultures were diluted 1:10 with embryogenesis media, at which time a variety of early stage embryos were present. Seven days later, the cultures were again diluted 1:10, and advanced stages of embryos were now also present. At this stage tissue which passed through 310 μm screen was globular and early heart shaped embryos. Material which was smaller than 570 μm but bigger than 310 μm was principally comprised of torpedo shaped embryos. At the end of this seven days of incubation the cultures consisted of many large, germinated embryos, smaller well developed embryos and some debris. All samples were frozen in liquid nitrogen immediately after harvest and stored at - 80 ^{o}C until analyzed.

Pea and soybean calmodulin was extracted and partially purified from the tissues by using a modification of the protocol described by Zendegui et al., (1984). This protocol takes advantage of the differential solubility of calmodulin in ethanol compared to most other cellular proteins. The procedure is rapid and yields calmodulin that is undegraded. Samples were frozen in liquid nitrogen and ground by using a mortar and pestle. The ground samples were thawed in a volume of homogenization buffer (75 mM Tris-HCl, pH 7.5, containing 10 mM EDTA, 1% (w/v) PVPP, 2 mM thioglycolate, and 10 mM

isoascorbate) that was three times the frozen weight of the tissue. The mixture was stirred and centrifuged at 27,000 x g at 4 °C for 20 min. The supernatant was brought to 50 % (v/v) ethanol and stirred for 20 min. The sample was centrifuged for 20 min. at 27,000 x g. Ethanol was added to the supernatant to a final concentration of 80 % (v/v) and stirred for 40 min. The sample was centrifuged for 30 min. at 27,000 x g at 4 °C. The pellet was lyophilized and resuspended with 50 mM Tris-HCl, pH 7.5, 1 mM βME. The amount of calmodulin was quantitated by RIA.

Calmodulin was extracted from carrot cell cultures and embryos by using the procedures described above with modified buffer conditions. Carrot samples were frozen in liquid nitrogen and ground with a pestle in a mortar. The ground samples were thawed in a volume of extraction buffer that was three times the frozen weight of the samples. The extraction buffer consisted of 100 mM potassium phosphate, 5 mM EDTA, 20 mM βME, 1 μg/ml leupeptin, 1 mM PMSF, 10 mM isoascorbate, 10 % (w/v) PVPP, pH 7.4. The mixture was stirred and then centrifuged at 30,000 x g at 4 °C for 15 min. The supernatant was removed and the pellets were reextracted in 3 volumes of the fresh extraction buffer. The supernatants were combined. Calmodulin in the extract was measured by RIA. Since further extraction with fresh buffer containing 8 M urea showed extremely low amounts of calmodulin (Table I), it is believed that the first two extractions gave almost complete extraction

TABLE I

Extraction of calmodulin from carrot cell cultures[a]

Extraction[b]	calmodulin (pmol)[c]	percentage[d]
Step I	511	63
Step II	231	29
Step III	66	8

[a]2-day old carrot cells (1.5 g) of the first cycle of the growth curve were used for the experiments.

[b]Step I, the first supernatant using the extraction buffer (see Materials and Methods). Step II, re-extracted supernatant from the pellets of the first extraction using extraction buffer. Step III, the supernatant from the pellets of the second extraction obtained by re-extraction with extraction buffer containing 8 M urea.

[c]Calmodulin in the extracts was measured by RIA.

[d]Values represent the percentage of total amounts of calmodulin measured by RIA.

of calmodulin from carrot samples. It is possible that the detection of calmodulin from the urea extracted supernatant (Table I) is due to the residue in pellets after removing supernatant from the reextracted sample.

In order to determine the degree of methylation of the purified calmodulin samples, they were tested as substrates for the purified calmodulin N-methyltransferase from sheep brain. Typically, calmodulin (0.5 μg) was incubated with 0.25 μg of purified enzyme and 12 μM ^{3}H-AdoMet (0.5 μCi) at 37 ^{o}C for 4 h, in 100 μl of calmodulin N-methyltransferase assay mixture. For a positive control, VU-1 calmodulin was treated under the same conditions. As negative controls, VU-1 calmodulin was incubated under the same conditions with no added enzyme and VU-4 calmodulin and chicken gizzard calmodulin were incubated with enzyme. After 4 h, the reaction mixtures were heated at 90 ^{o}C for 3 min., then centrifuged for 6 min in a microfuge to remove insoluble debris. ^{3}H-Calmodulin was separated from other reaction components in the supernatant by phenyl-Sepharose chromatography and was counted by liquid scintillation spectrometry as described above. Fluorography was done on labeled samples after SDS-PAGE. Gels were fixed with 10 % (v/v) acetic acid, 30 % (v/v) methanol, and then incubated with EN^{3}HANCE (Du Pont). The fluors were precipitated with cold water and the gel was dried under heat (60 - 70 ^{o}C) and vacuum. The dried gel was exposed to Wicor-X RP x-ray film

at - 80 °C.

Identification and purification of calmodulin-binding proteins

Calmodulin-binding proteins were detected on SDS-PAGE gels by an ^{125}I-calmodulin overlay protocol (Burgess et al., 1984) with the modifications described below. Bovine brain calmodulin was radioiodinated by the gentle lactoperoxidase method (Van Eldik and Burgess, 1983). SDS-PAGE (12.5 %) was performed in duplicate to separate carrot proteins in extracts. Gels were washed with a solution containing 25 % (v/v) isopropanol and 10 % (v/v) acetic acid to remove SDS, were washed with 50 mM Tris-HCl, 0.2 M NaCl, pH 7.6 to allow in situ protein renaturation. After the washing and renaturation steps, the gels were incubated overnight with ^{125}I-calmodulin (10^7 cpm/gel) in 50 mM Tris-HCl, 0.2 M NaCl, 1 mg/ml BSA, pH 7.6 containing either 1 mM $CaCl_2$ or 5 mM EDTA. After incubation overnight, the gels were washed with 100 ml of 50 mM Tris-HCl, 0.2 M NaCl, pH 7.6, containing either 0.5 mM $CaCl_2$ or 2 mM EDTA, for three hours with three changes. All buffers were filtered through millipore filters (0.2 μm) into acid washed glassware. These modifications reduce the non-specific background staining of the gel matrix (C.D. Weaver and D.M. Roberts, unpublished). The washed gels were stained in Coomassie-Blue and then destained. The gels were dried and then exposed to x-ray film at -80 °C for 2 to 7 days with a Cronex intensifying screen.

In order to purify calmodulin-binding proteins from carrot embryos, 24-day old germinated samples (8 g) were frozen in liquid nitrogen and ground with a pestle in a mortar. The ground samples were thawed in 25 ml of homogenization buffer (50 mM Tris-HCl [pH 7.5], containing 5 mM EDTA, 10 % [w/v] PVPP, 20 mM βME, 1 μg/ml leupeptin, 1 mM PMSF, 10 mM isoascorbate, and 0.2 M NaCl). The mixtures were stirred and then centrifuged at 30,500 x g at 4 °C for 15 min. The supernatants were applied to a DEAE-cellulose column (1.5 x 6 cm) equilibrated with 50 mM Tris-HCl, 0.2 M NaCl, 3 mM $MgCl_2$, 1 mM EGTA, pH 7.5, and the eluent was collected. $CaCl_2$ was added to a final concentration of 1 mM and the samples were applied to bovine brain calmodulin-Sepharose column (1.0 x 1.0 cm) equilibrated with 25 mM Tris-HCl, 0.25 mM $CaCl_2$, 3 mM $MgCl_2$, 0.15 M NaCl, 1 mM DTT, pH 7.5. The column was washed with 50 ml of equilibration buffer and eluted with 25 mM Tris-HCl, 1.5 mM EGTA, 3 mM $MgCl_2$, 0.15 M NaCl, 1 mM DTT, pH 7.5. The eluant was concentrated and stored at -80 °C until ^{125}I-calmodulin overlay analysis.

Generation of transgenic tobacco plants

Plant expression vector construction: Plasmids pVUC-4 (Roberts et al., 1986b), pRT101 (Topfer et al., 1987), and pGDW31 (D. Wing, unpublished) were prepared by using the pZ523 protocol (5 prime -> 3 prime, Inc.). The pVUC-4 plasmid (150 μg) containing the synthetic VU-4 calmodulin gene was digested

at 37 °C for 1 hr in a 50 μl reaction mixture containing EcoRI (150 units), BamHI (150 units), 50 mM Tris-HCl, 10 mM $MgCl_2$, 100 mM NaCl, 1 mM DTT, pH 7.9. The DNA fragments were resolved by electrophoresis on 5 % (w/v) polyacrylamide gel, and the 455 bp VU-4 calmodulin gene fragment was sliced from the gel. The DNA was extracted from the sliced gel as described above. The purified product was resuspended in 0.1 x TE buffer (1 mM Tris-HCl, pH 7.5, 0.1 mM EDTA), and was stored at 4 °C. Plasmid pRT101 (50 μg) carrying the cauliflower mosaic 35S promoter and the polyadenylation signal (Topfer et al., 1987) was digested at 37 °C for 2 hr in a 100 μl reaction mixture containing EcoRI (250 units), BamHI (250 units), 50 mM Tris-HCl, 10 mM $MgCl_2$, 100 mM NaCl, 1 mM DTT, pH 7.9. 0.5 unit of alkaline phosphatase from calf intestine was then added to the digest and the mixture was incubated at 37 °C for 30 min. At the end of incubation, 10 μl of 500 mM EDTA (pH 8.0) was added to the digest and the mixture was incubated at 65 °C for 45 min to inactivate the phosphatase. The digested DNA was extracted as described above and precipitated with ethanol and resuspended in 1 mM Tris-HCl, pH 7.5, 0.1 mM EDTA. The VU-4 calmodulin gene fragment (200 ng) and EcoRI/BamHI-cleaved pRT101 vector (200 ng) were combined and incubated with 2 units of T_4 DNA ligase at room temperature for 1 hr in a 40 μl reaction mixture containing 1 mM ATP, 70 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, 5 mM DTT. E. coli UT481 cells were transformed with the ligation mixture as described

(Roberts et al., 1985). The resulting plasmid, pRT/VU-4 (100 μg) was digested at 37 °C for 3 hr in 100 μl reaction mixture containing HindIII (200 units), 100 mM Tris-HCl, 100 mM $MgCl_2$, 50 mM NaCl, 1 mM DTT, pH 7.9. The 1.1 kbp fragment containing the 35 S promoter/VU-4 calmodulin/polyadenylation signal cassette was separated by electrophoresis on a 0.8 % (w/v) low-melting point agarose gel, and the cassette gene fragment (1.1 kbp) was sliced from the gel, and purified. The 1.1 kbp cassette fragment (420 ng) was combined with HindIII-cleaved and alkaline phosphatase-treated 9.1 kbp of pGDW31 vector (D. Wing, unpublished), and was treated with 10 units of T_4 ligase at 15 °C overnight in 20 μl reaction mixture containing 1 mM ATP, 70 mM Tris-HCl, pH 7.8, 10 mM $MgCl_2$, 5 mM DTT. E.coli S17-1 cells (Simon, 1983) which can produce the transfer (tra) and mobilization (mob) gene products were transformed with the ligation mixture as described above. Small amounts of plasmids were prepared from transformants and the presence of 10.2 kbp binary vector (pGDWVU-4) containing the 1.1 kbp cassette gene (35S promoter/VU-4 calmodulin/polyadenylation signal) was confirmed by HindIII digestion. The binary vector pGDWVU-4 in the cells of E.coli S17-1 strain was transferred into Agrobacterium tumefaciens PMP90RK (Koncz and Schell, 1986) by using a modified Agrobacterium conjugation procedure (Besl, 1991) from the method of Ditta et al., (1981). Once conjugated with the S17-1 strains, the Agrobacterium tumafaciens strain PMP90RK showed resistance to rifampicillin

and kanamycin, as well as carbenicillin, indicating that the pGDWVU-4 that contains the β-lactamase gene was transferred into the Agrobacterium tumafaciens clones. The identity of the transferred binary vector was further confirmed by backmating the Agrobacterium tumafaciens PMP90RK into E.coli S17-1. Plasmids were prepared from the backmated S17-1 strain and the presence of pGDWVU-4 vector containing 1.1 kbp cassette gene was confirmed by HindIII digestion.

Plant transformation: To obtain expression of VU-4 calmodulin in transgenic tobacco plants, the general transformation procedures originally developed by Horsch et al. (1985) were used with modifications. For transformation, leaves from tobacco plants (Nicotina tabacum cv. Wisconsin 38), grown in a sterile Magenta vessel (Sigma) on MS agar (4.6 g/l MS salts, 30 g/l sucrose, 9 g/l agar, 0.1 g/l myo-inositol, 2 mg/l glycine, 0.5 mg/l nicotinic acid, 0.5 mg/l pyridoxine, 0.1 g/l thiamine, 1 mg/l biotin, pH 5.8) were wounded and the leaf pieces were incubated for 10 min with Agrobacterium suspension prepared as described (Besl, 1991). The leaf pieces then were removed and blotted onto sterile Whatman filter paper, and were placed onto MS agar containing 0.5 mg/l 6-benzylaminopurine (BAP) and 0.1 mg/l α-naphthalene acetic acid (NAA). The agar plates were sealed with parafilm and placed in a growth chamber with fluorescent lights (28 °C) for 3 days. Three days later, the leaf pieces were washed 3 times with sterilized MS liquid media and were blotted on

sterile Whatman paper. The leaf pieces were placed on MS agar plus 0.1 mg/l NAA, 0.5 mg/l BAP, 20 mg/l hygromycin, 100 mg/l cefotaxime. As positive control for growth, untransformed leaf pieces were maintained on MS agar containing 0.5 mg/l BAP and 0.1 mg/l NAA. As a negative control for hygromycin selection, untreated leaf pieces were placed on MS agar containing 0.5 mg/l BAP, 0.1 mg/l NAA, 20 mg/l hygromycin, 100 mg/l cefotaxime.

After 2 to 3 weeks, the leaf pieces had produced small calli. These calli were removed from the leaf pieces and were transferred to shooting medium (MS agar plus 50 mg/l isopentyladenine, 0.1 mg/l chlorophenoxy acetic acid, 20 mg/l hygromycin, and 100 mg/l cefotaxime). 10 days later, the calli had produced shoots. These shoots (0.5 cm to 1.0 cm) were removed from the calli and transferred to MS agar containing 0.1 mg/l NAA, 0.5 mg/l BAP, 20 mg/l hygromycin, and 100 mg/l cefotaxime. After approximately 10 days, the shoots had produced roots. After several healthy roots were produced, the tobacco plants were transferred into soil. The tobacco plants were then grown under standard green house conditions.

Seeds of transgenic plants generated by self fertilization of F_0 plants (F_1 generation) were surface sterilized with 2 % (v/v) of sodium hypochlorite and were germinated on MS agar (Bachmair et al., 1990) containing 50 µg/ml hygromycin. The seeds were germinated in the dark until

the radical was 5 mm in length, and were then transferred to the light. The plants were grown under continuous light for 5 days and then were transferred to soil and were grown for 37 days under standard greenhouse conditions. Plants were harvested and frozen in liquid nitrogen and stored at -80° C until analysis.

Characterization of calmodulin from VU-1 and VU-3 transgenic tobacco plants

VU-1 and VU-3 transgenic tobacco plants were produced by Besl (1991) by the general protocol described above. To estimate the VU-calmodulin expression levels in transgenic tobacco plants, plant tissues were ground in liquid nitrogen and were thawed in 100 mM KPO_4, 5 mM EDTA, 20 mM 2ME, 1 μg/ml leupeptin, 1 mM PMSF, 10 mM sodium isoascorbate, 10 % (w/v) polyvinylpolypyrrolidone, pH 7.5 (3 ml of buffer per g tissue). The extract was filtered through cheesecloth and was centrifuged at 8,000 rpm at 4° C for 30 min in a GS-3 rotor. The contents of calmodulin in crude extracts were measured by using a competitive RIA as described before. To purify calmodulin, the extract was adjusted to 55 % saturation by the addition of solid ammonium sulfate. After removal of precipitated proteins by centrifugation, calmodulin was precipitated from the supernatant by lowering the pH to 4.0 with dilute H_2SO_4. The precipitated calmodulin was collected by centrifugation as described above. The sample was

resuspended in 50 mM Tris HCl, 1 mM EDTA, 1 mM βME, 1 mM sodium isoascorbate, pH 7.5 and was dialyzed against the same buffer (20 to 40 times the sample volume) overnight at 4° C. The sample was applied to a phenyl-Sepharose column equilibrated with 50 mM Tris HCl, 1 mM EDTA, 1 mM βME, pH 7.5, and the effluent was collected. $CaCl_2$ was added to a final concentration of 2 mM and the sample was reapplied to a second phenyl-Sepharose column (1.5 x 4 cm) equilibrated with 50 mM Tris-HCl, 0.1 mM $CaCl_2$, 1 mM 2ME, pH 7.5. The column was washed and eluted according to the protocol of Gopalakrishna and Anderson (1982). The calmodulin fractions were pooled and dialyzed against 20 mM ammonium bicarbonate, and then extensively against deionized water, and were lyophilized.

Separation of VU-1 or VU-3 calmodulin from endogenous tobacco calmodulin was accomplished by FPLC chromatography on a mono Q column. The lyophilized calmodulin mixture from phenyl-Sepharose chromatography was dissolved in mono Q buffer (20 mM bis-Tris, pH 6.1 containing 0.2 M NaCl, 0.1 mM EDTA, and 0.1 mM dithiothreitol), and was applied to a mono Q column (0.5 x 5 cm) equilibrated in the same buffer. The column was eluted with a linear NaCl gradient (0.2 to 0.4 M). Fractions were analyzed for calmodulin by SDS-PAGE and calmodulin RIA. Fractions containing calmodulin were pooled, concentrated, and dialyzed against 5 mM ammonium bicarbonate. The buffers were freshly prepared with nanopure deionized water and filtered through membrane filters with pore size 0.2 μm and degassed

before use.

General methods

The apparent molecular weight of purified calmodulin N-methyltransferase was determined by gel filtration on the Biogel A-1.5 column or Superose 12 column in the presence of the following internal standards: thyroglobulin (670,000), gamma globulin (158,000), ovalbumin (44,000), myoglobin (17,000), and vitamin B-12 (1,350). The buffer conditions used were 25 mM Hepes-NaOH, pH 7.4, 2 mM DTT, 0.15 M NaCl.

SDS-PAGE of calmodulin and calmodulin N-methyltransferase was done by using the method of Laemmli (1970). EGTA (1 mM) was routinely added to gels for calmodulin analysis to prevent anomalous migration (Burgess et al., 1980). Molecular weight marker proteins were phosphorylase b (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,100), and α-lactalbumin (14,400). Calmodulin concentrations for the analysis of kinetic data were determined by amino acid composition analysis on a Waters Picotag system by using the general method of Bidlingmeyer et al. (1984). The concentration of other proteins was determined according to the method of Bradford (1976) by using the Bio-Rad reagent and gamma globulin as a standard protein. Calmodulin-dependent NAD kinase activator analysis was done as described by Roberts et al. (1985).

The calculation of net charge as a function of pH for calmodulin sequences was done by using the sequence analysis software package of the Genetic Computer Group (GCG) (University of Wisconsin Biotechnology Center).

CHAPTER III

RESULTS

Purification and characterization of calmodulin-lysine N-methyltransferase

The purification of calmodulin-lysine N-methyltransferase from sheep brain was performed to develop an assay for the analysis of the endogenous calmodulin methylation state and to obtain large amounts of the enzyme for future studies which may involve purification of the plant calmodulin methyltransferase. Since it has been demonstrated that: 1. animal calmodulin and plant calmodulin share over 90 % sequence homology (Roberts et al., 1986a; Figure 1); 2. both animal and plant calmodulins undergo posttranslational trimethylation of lysine at position 115 (Fig. 2); and 3. brain tissue has more calmodulin than other sources (Manalan and Klee, 1984), we chose the calmodulin-rich sheep brain as the calmodulin methyltransferase source for the present study.

The enzyme was initially purified by differential ammonium sulfate precipitation and ion-exchange chromatography on phosphocellulose. The sample was then chromatographed on calmodulin-Sepharose as described in the Materials and Methods. After applying enzyme, the calmodulin Sepharose resin was washed with a step-wise NaCl gradient containing calmodulin-Sepharose equilibration buffer (0.1 M to

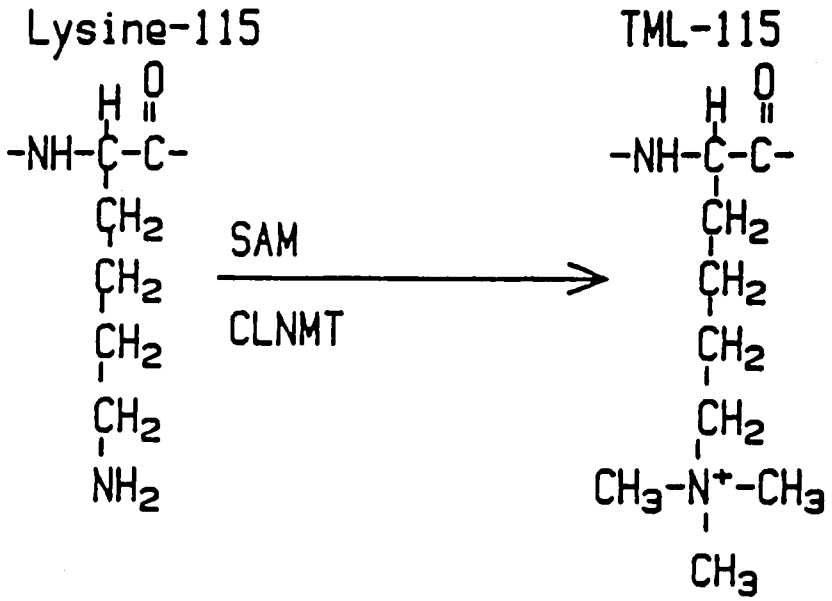


Figure 2. Calmodulin methylation reaction. SAM, S-adenosyl-L-methionine; CLNMT, calmodulin-lysine N-methyltransferase; TML, trimethyllysine.

0.5 M NaCl). Finally, the enzyme was eluted in column buffer in the presence of EGTA (Fig. 3). Affinity chromatography on chicken gizzard calmodulin-Sepharose gave a better yield of the enzyme than chromatography on immobilized VU-1 and VU-3 calmodulins. Therefore, we chose to use this resin in the calmodulin methyltransferase purification procedures. The above data indicate that it is not necessary to use unmethylated calmodulin to purify the enzyme by using calmodulin-Sepharose. Also the data suggests that chicken gizzard calmodulin and VU-3 calmodulin can bind the enzyme. Further, the chromatography results indicate that the interaction of the enzyme with calmodulin is calcium-dependent. These properties were investigated further below.

Overall, the purification protocol resulted in 28,000-fold purification and a specific activity of 12,000 (pmol/min/mg) (Table II). SDS-PAGE of the purified enzyme preparation revealed a major band with an apparent M_r of 38,000 (Fig. 4). The enzyme also was eluted from the Biogel A-1.5 with an apparent molecular weight of 38,000 (Fig. 5). Thus, the data suggest that calmodulin-lysine N-methyl-transferase from sheep brain is a monomeric enzyme with molecular weight 38,000.

The observation that affinity chromatography of calmodulin methyltransferase on calmodulin-Sepharose results in a calcium-dependent interaction raised the interesting possibility that calcium could regulate the interaction of

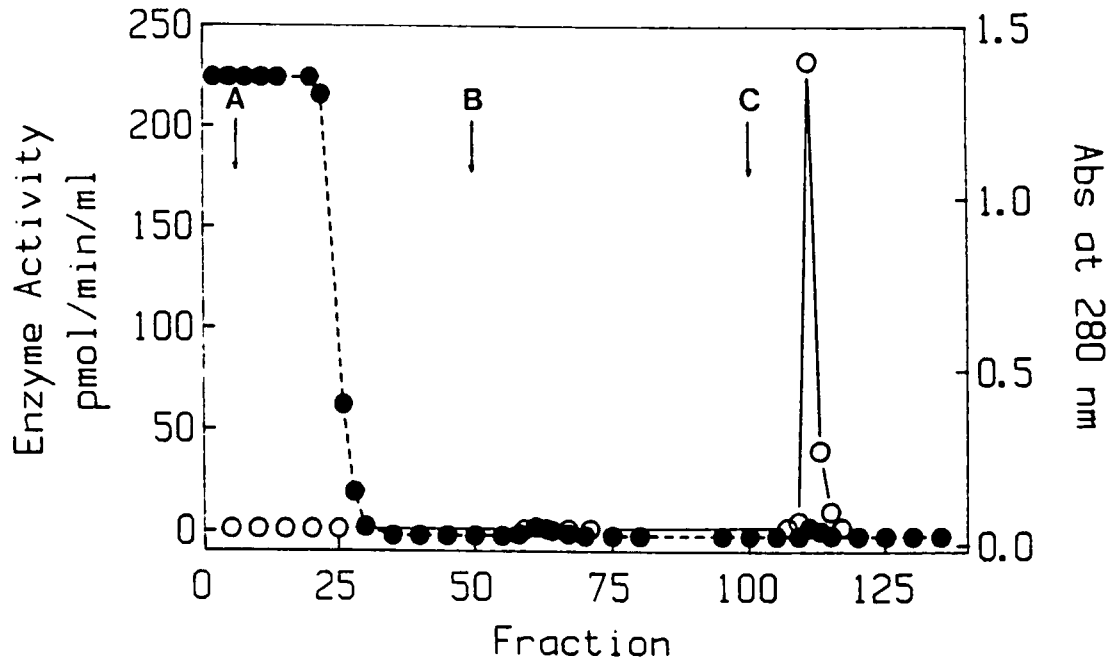


Figure 3. Affinity chromatography on chicken gizzard calmodulin-Sepharose. The pooled fractions from the phosphocellulose column were dialyzed against the calmodulin-Sepharose equilibration buffer and applied to a column of chicken gizzard calmodulin-Sepharose (1 x 4 cm). The column was washed sequentially with 100 ml of calmoduin-Sepharose equilibration buffer containing 0.1 M NaCl and 0.1 mM $CaCl_2$ (A), and with 125 ml of the same buffer containing 0.5 M NaCl and 0.1 mM $CaCl_2$ (B). The enzyme was then eluted with 80 ml of the buffer containing 1.0 M NaCl and 1 mM EGTA (C). Fractions of 2.5 ml were collected at a flow rate of 25 ml/h. Absorbance at 280 mm (closed circles), calmodulin N-methyltransferase activity (open circles).

TABLE II

Purification of calmodulin-lysine N-methyltransferase from sheep brain

Step	Total protein (mg)	Total activity[a]	Specific activity[b]	Purification (fold)	Recovery (%)
Soluble extract	7,800	3,360	0.43	1	100
Ammonium sulfate	2,200	2,330	1.06	2.5	69.5
Phospho-cellulose	201.0	2,470	12.30	28.5	73.5
Calmodulin-Sepharose	0.156	1,340	8,600	20,000	40.0
Biogel A-1.5	0.037	477	12,100	28,100	13.3

[a]Total activity is expressed as pmol of [^{3}H]-methyl groups incorporated into VU-1 calmodulin per min.

[b]Specific activity is standardized to mg of protein in each sample.

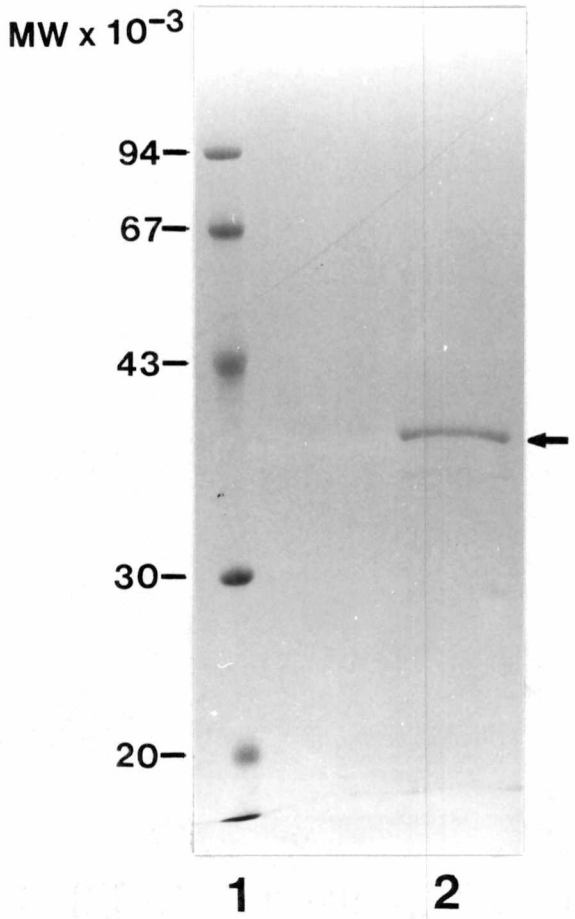


Figure 4. SDS-PAGE of the purified enzyme. Electrophoresis in 12.5 % (w/v) polyacrylamide gel in the presence of 0.1 % (w/v) SDS was performed as described in Materials and Methods. Lane 1, molecular weight standards; lane 2, the purified calmodulin methyltransferase from sheep brain.

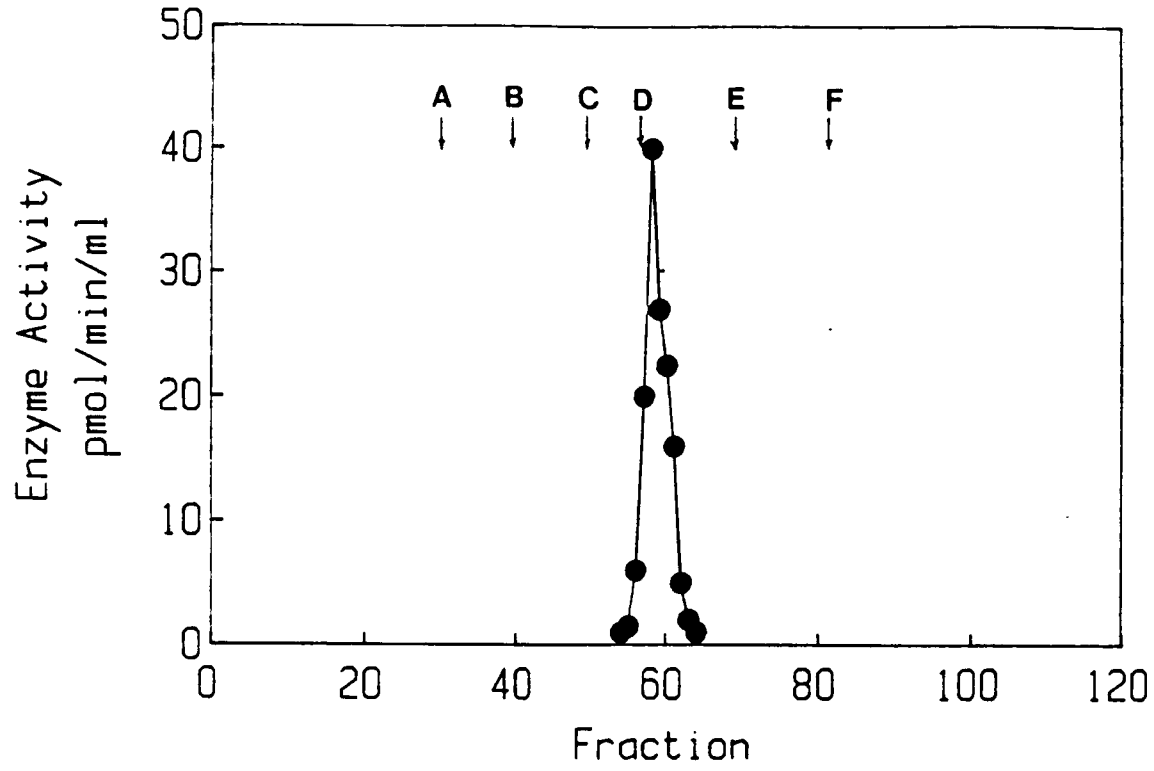


Figure 5. Gel filtration on Biogel A-1.5 of calmodulin-lysine N-methyltransferase. The standard mixture was dissolved in 0.5 ml of the calmodulin-Sepharose purified enzyme and was applied to a column of Biogel A-1.5 (1.5 x 84 cm). The column was eluted with calmodulin-Sepharose elution buffer with no Triton X-100 at 5 ml/h. Fractions of 1.4 ml were collected, and aliquots of 30 μl were assayed for the enzyme activity. A, protein aggregates (void peak); B, thyroglobulin (670,000); C, gamma globulin (158,000); D, ovalbumin (44,000); E, myoglobin (17,000); F, vitamin B-12 (1,350).

the enzyme with calmodulin. In order to investigate this further, the ability of calcium to stimulate the enzyme in the presence of physiological levels of Mg^{2+} was tested. The enzyme was stimulated in the presence of low concentrations of free calcium (Fig. 6) and the concentration of calcium required for half-maximal stimulation ($K_{0.5}$) was 3.0×10^{-6}M. At higher, non-physiological concentrations of calcium, the enzyme was inhibited. Calmodulin binds four calcium ions per molecule with dissociation constants within the physiological range of intracellular calcium concentrations (10^{-5} to 10^{-7}M). Upon binding calcium, conformational changes in calmodulin are observed (Klevit, 1983: Seaton et al., 1985; Kupke and Dorrier, 1986; Kilhoffer et al., 1988, 1989). Therefore, the above data, along with the calcium-dependent nature of the interaction of the enzyme with calmodulin-Sepharose, suggest that the stimulation of calmodulin N-methyltransferase from sheep brain by calcium is due to a preference for the calcium-bound form of calmodulin.

Kinetic properties of the enzyme

VU-1 calmodulin is encoded by a synthetic gene and possesses a high degree of homology to vertebrate and higher plant calmodulins (Roberts et al., 1985). However, whereas higher plant and vertebrate calmodulins generally possess trimethyllysine-115 (Watterson et al., 1980a; Lukas et al., 1984), VU-1 calmodulin possesses an unmethylated lysine at

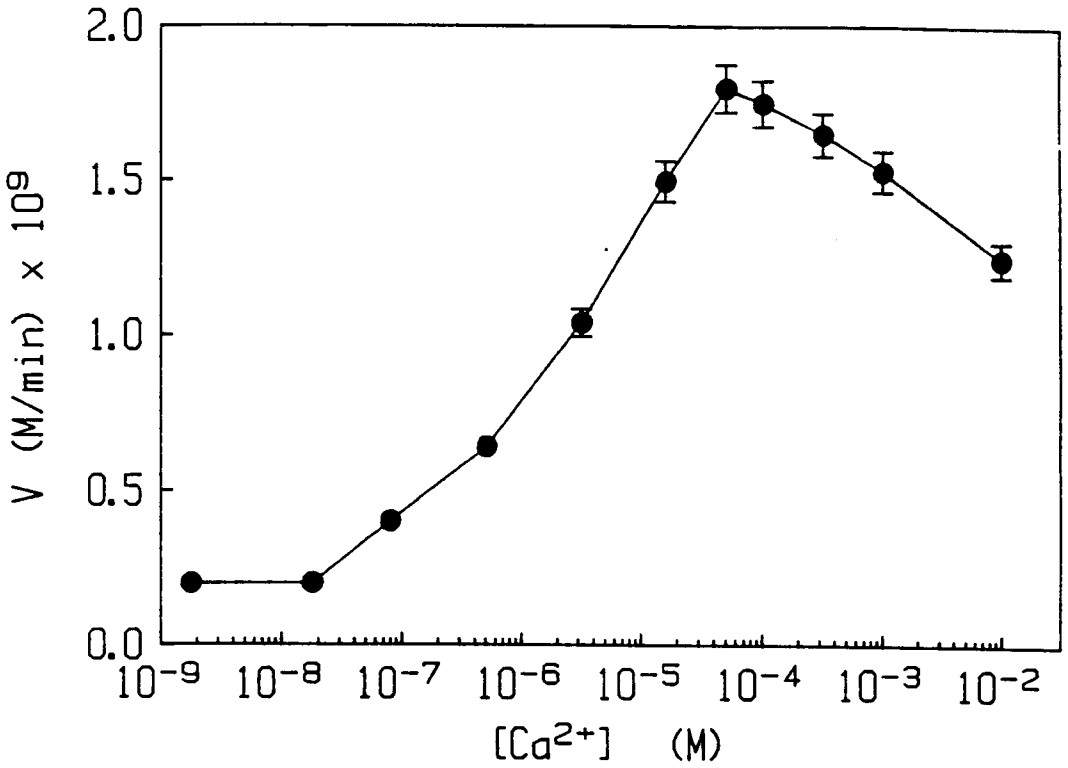


Figure 6. Calcium requirement of calmodulin-lysine N-methyltransferase. The activity was determined at pH 7.5 in 25 mM Hepes-NaOH, 2 mM DTT and 0.01 % (w/v) Triton X-100. A calcium buffer system consisting of $MgCl_2$, $CaCl_2$ and EGTA was used to give a final free Mg^{2+} concentration of 5 mM and the free Ca^{2+} concentrations shown. The points shown are the average of three determinations and the error bars show standard error for the data set.

residue 115 (Roberts et al., 1985) (Fig. 1). This probably is the result of the fact that E. coli does not possess calmodulin (Burgess et al., 1980) and thus does not have a calmodulin methylating enzyme. Hence, VU-1 calmodulin is a suitable substrate with which to study the kinetic properties of calmodulin methyltransferase. Double-reciprocal plots of kinetic data (Fig. 7) were obtained by using VU-1 calmodulin as a variable substrate at several fixed concentrations of AdoMet. The data showed intersecting lines. Slope and intercept replots versus the reciprocal AdoMet concentrations (inset in Fig. 7) produced straight lines. The initial velocity data obtained were consistent with a sequential binding mechanism. Therefore, the experimental data are represented by Equation [1], the equation for a sequential bireactant mechanism (Cleland, 1967). The Km value for AdoMet, which was calculated from the intercept replot, was 1.11×10^{-6}M.

Properties of calmodulin derivatives produced by site-directed mutagenesis

The enzyme also was assayed with several site-directed mutants of calmodulin: VU-3 calmodulin, lys to arg 115; VU-4 calmodulin, lys to ile 115; VU-8 calmodulin, glu-glu-glu to lys-lys-lys, 82 - 84; and VU-12 calmodulin, asp-glu-glu to lys-lys-lys, 118 - 120 (Fig. 1). Because of substitutions at position 115, VU-3 and VU-4 calmodulins are not expected to be

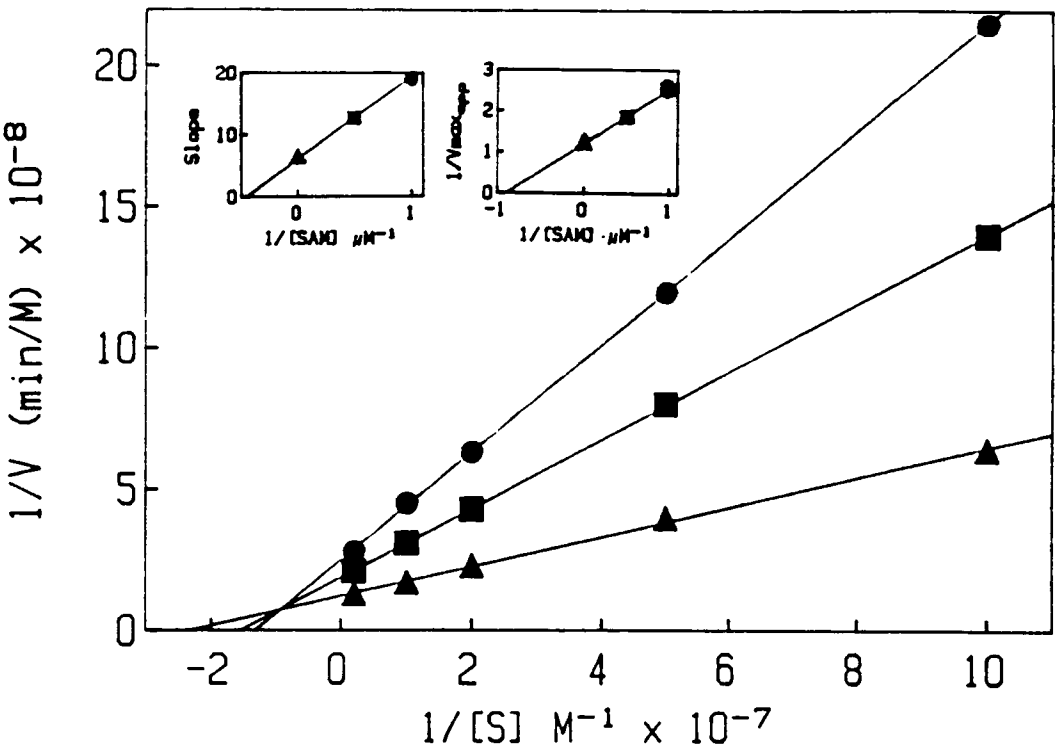


Figure 7. Double-reciprocal plots of initial velocities of VU-1 calmodulin methylation. Calmodulin methylation was assayed at 1 μM (circles), 2 μM (squares), and 12 μM (triangles) AdoMet with VU-1 calmodulin as the variable substrate. The inset shows slope and intercept replots.

methylation substrates. However, the affinity chromatography data above suggests VU-3 and VU-4 calmodulins may retain the ability to interact with the enzyme and may serve as good competitive inhibitors of calmodulin methylation. VU-8 and VU-12 calmodulins possess mutations in non-calcium binding acidic amino acid clusters in the central helix and between Ca^{2+} binding sites III and IV, respectively. These clusters are found in all calmodulins characterized from yeast to human (Roberts et al., 1986a). Since calmodulin-binding structures are basic, amphipathic α-helices, it has been postulated that these negative charge clusters could serve as a part of the recognition sites for calmodulin-dependent enzymes (Craig et al., 1987). This hypothesis has been supported by model peptide studies that show that alterations or substitutions of basic residues on calmodulin-binding peptides result in a lower affinity of the peptide for calmodulin (Lukas et al., 1986, 1987). Calmodulin mutants with substitutions within these negative charge clusters have been found to have selective defects in their abilities to activate several calmodulin-dependent enzymes. For example, VU-8 calmodulin activated phosphodiesterase to the same maximal extent as VU-1 calmodulin. In contrast, MLCK was activated to only 30 % of maximal activity and NAD kinase was not activated (Craig et al., 1987). VU-12 calmodulin has been found to have a reduced ability to activate some enzymes, such as calmodulin-dependent kinase II and myosin light chain kinase, whereas other

calmodulin-dependent enzymes are not affected (Weber et al., 1989).

VU-1 calmodulin and VU-8 calmodulin were found to be good substrates, as demonstrated by their relatively low apparent Km values; 50 nM for VU-1, 51.3 nM for VU-8, and comparable kcat/Km ratios; 7.3 x 10^4 ($s^{-1}M^{-1}$) for VU-1, 6.4 x 10^4 ($s^{-1}M^{-1}$) for VU-8. On the other hand the VU-12 CaM showed a 4-fold higher Km value than that of VU-1 calmodulin and a lower kcat. Overall, the kcat/Km ratio of VU-12 calmodulin was over 11-fold lower than the ratios for VU-1 and VU-8 calmodulins (Table III). VU-3 calmodulin and VU-4 calmodulin derivatives showed no ^{3}H-methyl incorporation in the assays (Fig. 8). Rather, VU-3 and VU-4 calmodulins were competitive inhibitiors of the enzyme with inhibition constants (Ki) of 40 nM and 2.2 nM, respectively (Fig. 9, 10). Interestingly, VU-4 calmodulin, which contains the uncharged and hydrophobic isoleucine 115 instead of lysine 115, showed better inhibitor activity than VU-3 calmodulin which contains arginine 115. The enzyme was also competitively inhibited by trimethylated chicken gizzard calmodulin and spinach calmodulin, but the degree of inhibition (Ki values: 88 nM for chicken gizzard calmodulin and 98 nM for spinach calmodulin) was less than that of VU-3 and VU-4 calmodulins. Thus, the enzyme is subject to product inhibition.

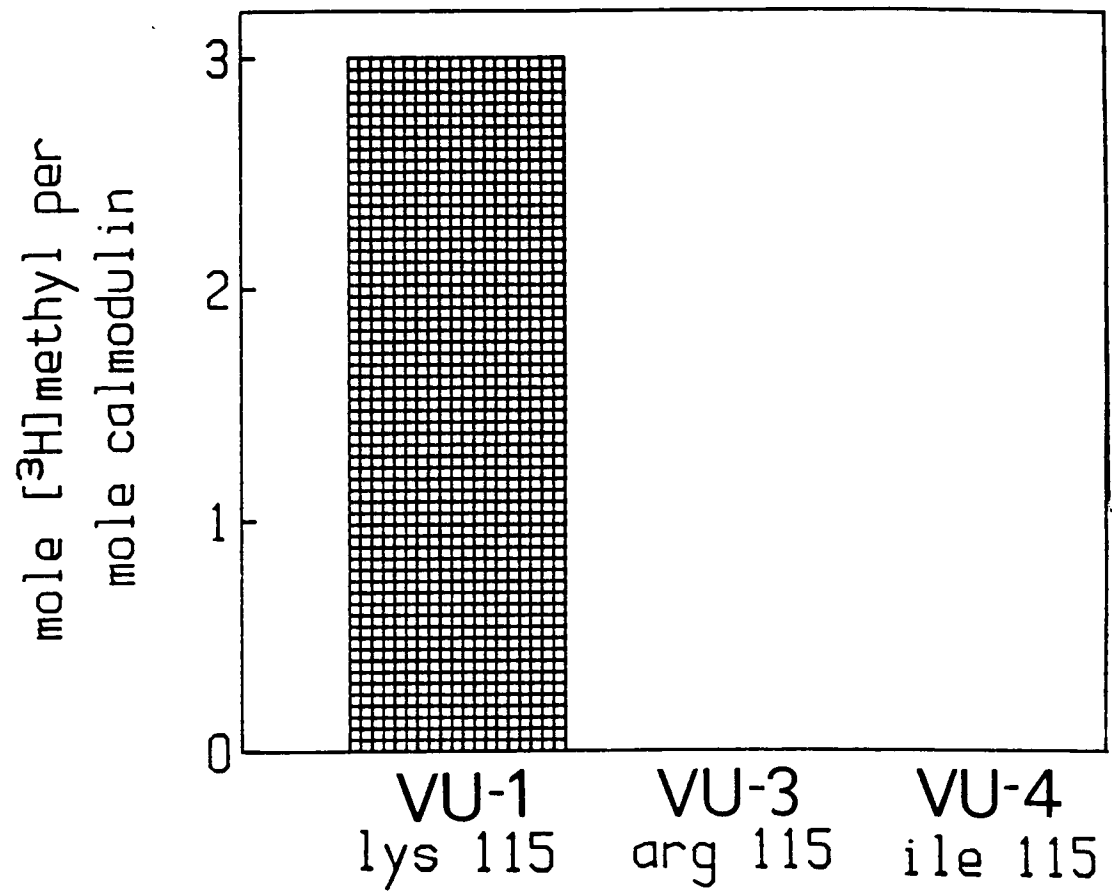


Figure 8. The comparison of methylation of the VU-1, VU-3, and VU-4 calmodulins by the calmodulin-lysine N-methyltransferase. Calmodulins (0.5 μg) were incubated with 0.5 μg of purified enzyme and 12 μM ^{3}H-AdoMet (0.5 μCi) at 37 °C for 3 hr, in 100 μl of the standard calmodulin N-methyltransferase reaction mixture.

TABLE III

Catalytic parameters of sheep brain calmodulin-lysine N-methyltransferase

CaM or CaM derivatives[a]	Km[b] (nM)	kcat[c] (sec^{-1})	kcat/Km ($sec^{-1}M^{-1}$)
VU-1	50.0	3.65×10^{-3}	7.3×10^{4}
VU-8	51.3	3.30×10^{-3}	6.4×10^{4}
VU-12	222.0	1.25×10^{-3}	5.6×10^{3}
	Ki (nM)		
VU-3	40.0		
VU-4	2.2		
Chicken gizzard	88.0		
Spinach	98.0		

[a]CaM, calmodulin.

[b]Km values were calculated from intercept and slope replots versus the reciprocal of AdoMet concentrations. Ki values were derived from Km_{app} replots versus fixed inhibitor concentrations.

[c]Kcat values derived from $Vmax/[E]_t$, where $[E]_t$ is the total enzyme concentration. The molecular weight of the enzyme is 38,000.

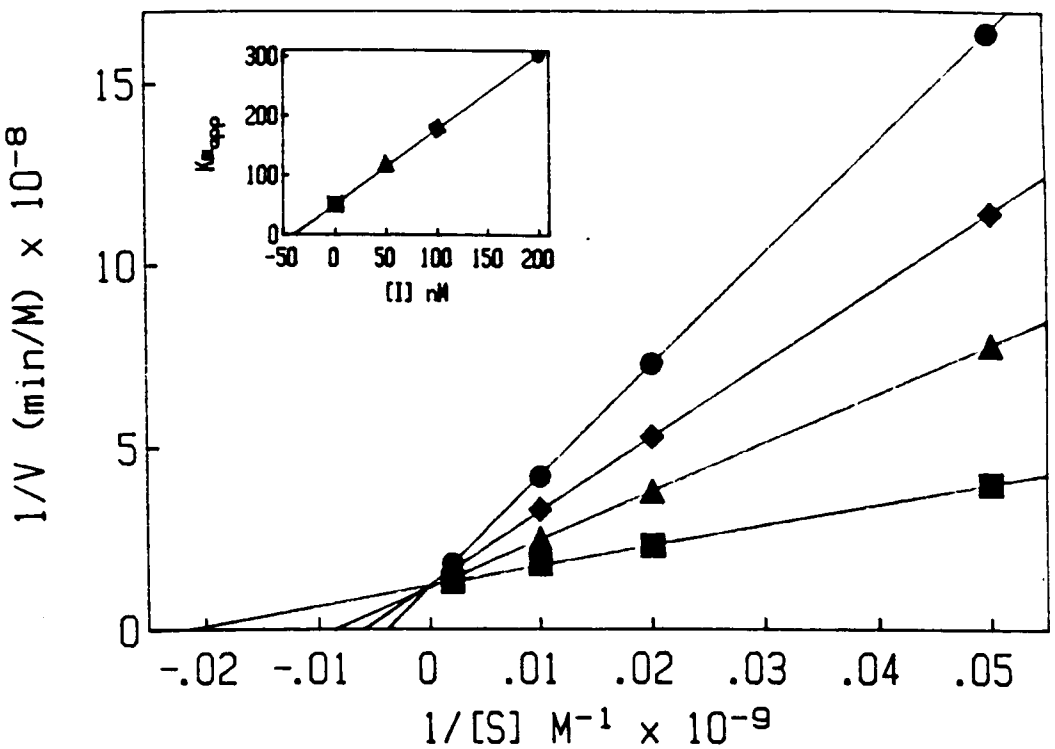


Figure 9. VU-3 calmodulin inhibition kinetics of the calmodulin-lysine N-methyltransferase. Inhibition by VU-3 calmodulin with VU-1 calmodulin as the variable substrate at a constant concentration of 12 μM AdoMet. The concentration of VU-3 calmodulin was varied at 200 nM (circles), 100 nM (diamonds), 50 nM (triangles), 0 nM (squares).

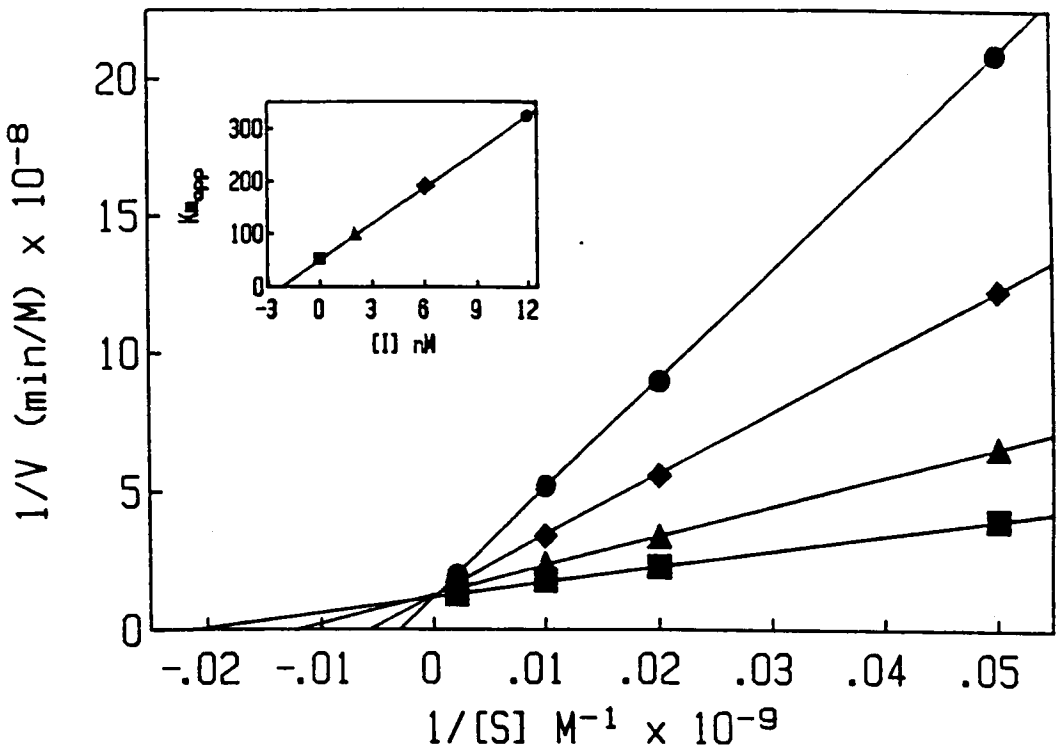


Figure 10. VU-4 calmodulin inhibition kinetics of the calmodulin-lysine N-methyltransferase. Inhibition by VU-4 calmodulin with VU-1 calmodulin as the variable substrate at a constant concentration of 12 μM AdoMet. The concentration of VU-4 calmodulin was varied at 12 nM (circles), 6 nM (diamonds), 2nM (triangles), 0 nM (squares).

Design of a specific assay for the degree of methylation of plant calmodulins

In order to quantitate the level of methylation in plant calmodulins, we designed a specific and sensitive radiometric assay using the purified calmodulin methyltransferase. Conditions for stoichiometric methylation were established with VU-1 calmodulin. Time course experiments of VU-1 calmodulin showed that stoichiometric methylation (3 mole of methyl groups/1 mole of calmodulin) was attained after 3 hours (Fig. 11). The final product was heat stable, and SDS-gel electrophoresis and fluorography showed that the only detectable methylated product was calmodulin (Fig. 12). As discussed above, the methylation is specific for position 115, since neither VU-3 nor VU-4 methylation is detected by scintillation counting nor by fluorographic analysis (Fig. 8, 12). In addition, substrates (RUBISCO large subunit, histone, and myosin light chain) of other protein methyltransferases were not methylated by the assay system (data not shown). Overall, the results show that the purified calmodulin methyltransferase is highly specific for the 115 site and can stoichiometrically incorporate 3 mole of [^{3}H]methyl groups into this site.

Detection of undermethylated calmodulin from higher plant tissues

To test for the presence of unmethylated calmodulins,

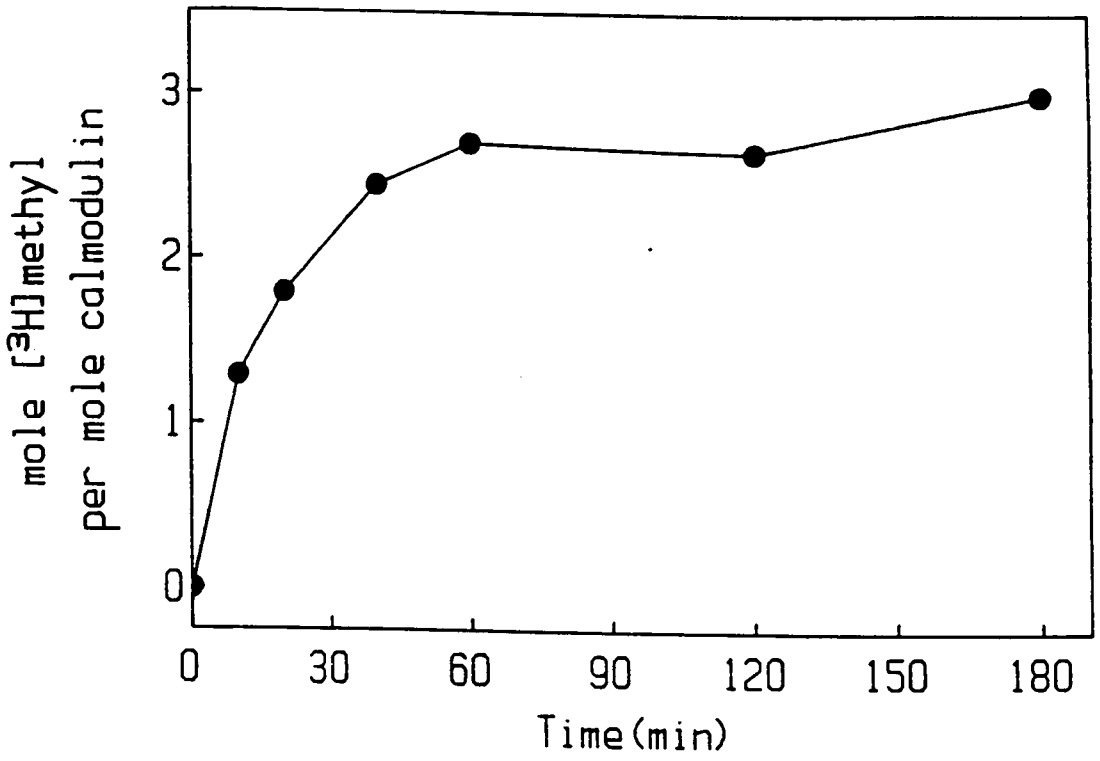


Figure 11. Stoichiometric methylation of VU-1 calmodulin. VU-1 calmodulin (1.0 μg) was incubated with 0.7 μg of purified calmodulin-lysine N-methyltransferase and 12 μM ^{3}H-AdoMet (0.5 μCi). The degree of methyl groups incorporation into VU-1 calmodulin was analyzed at various time as described in the Material and Methods.



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Figure 12. Identification of methylated VU-1 calmodulin. Fluorography of methylation assay products. VU-1 calmodulin and negative controls (chicken gizzard calmodulin and VU-4 calmodulin) were incubated with calmodulin-lysine N-methyltransferase as described in the legend in Figure 11 and then analyzed by SDS-PAGE and fluorography. Lane 1, VU-1 calmodulin + calmodulin methyltransferase; lane 2, VU-1 calmodulin with no added enzyme; lane 3, VU-4 calmodulin + calmodulin methyltransferase; lane 4, chicken gizzard calmodulin + calmodulin methyltransferase. The arrow indicates the position of calmodulin.

the calmodulin pools isolated from plant tissues were quantitated by a calmodulin specific RIA, and were tested for their ability to serve as substrates for the calmodulin methyltransferase by using the conditions for stoichiometric methylation described above. Thus, if calmodulin is not completely methylated, they will be able to incorporate [^{3}H]methyl groups from ^{3}H-AdoMet.

As a starting point we used the root as a developmental model. The distribution of undermethylated calmodulins in 3-day old etiolated pea plants is shown in Figure 13. In order to test different tissues at various states of development, roots were dissected into 0.6 cm segments and were analyzed. Undermethylated calmodulins were detected in all tissues tested, but the highest incorporation of [^{3}H]methyl groups occurred with calmodulin isolated from 0.6 cm tip portion of the root, and corresponds to a value of 0.5 mol methyl groups incorporated per mol calmodulin. The level declined greatly as calmodulin from successive segments were tested, and the level of [^{3}H]methyl group incorporation into calmodulin isolated from the mature tissues at the base of the root was 12-fold lower than calmodulin from the root apex (Fig. 13). SDS-PAGE and fluorography (Fig. 14) verified the pattern of incorporation, and showed that methylated calmodulin was the only product.

A similar pattern was observed in 6-day old green pea seedlings (Fig. 15). At this age, the tap root is much more

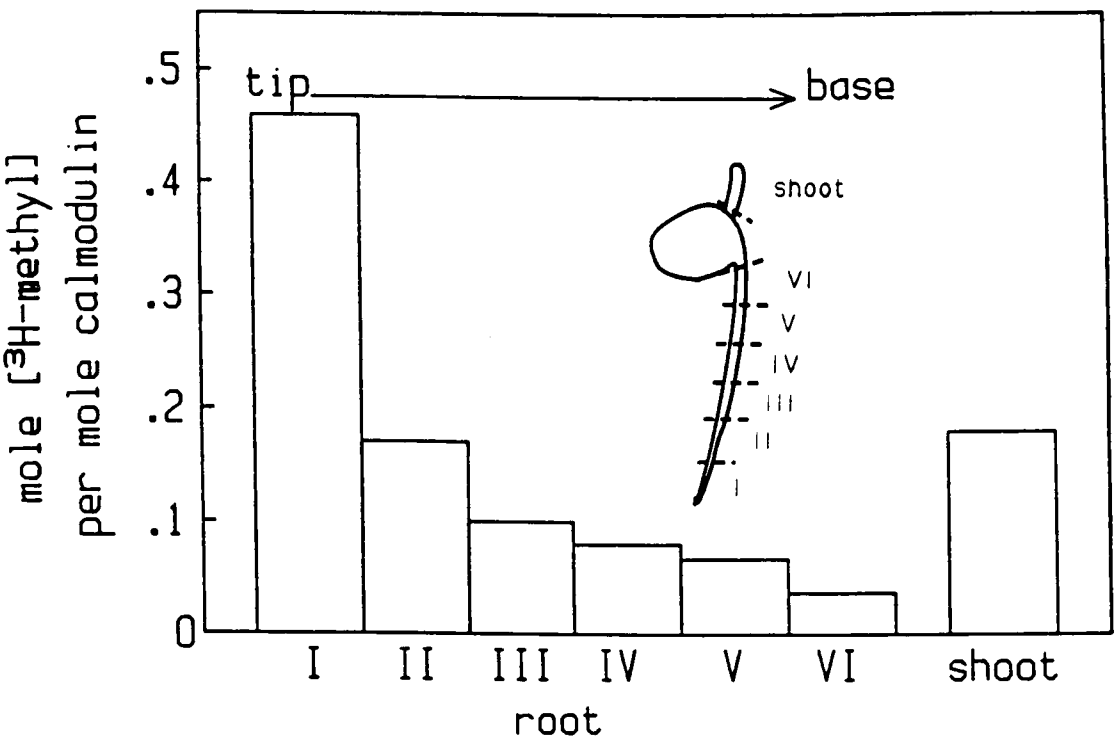


Figure 13. Analysis of the state of calmodulin methylation in developing, etiolated pea roots. Three-day old etiolated pea plants were dissected into shoots and root segments. The roots were dissected further into 0.6 cm segments: I, tip to 0.6 cm; II, 0.6 - 1.2 cm; III, 1.2 - 1.8 cm; IV, 1.8 - 2.4 cm; V, 2.4 - 3.0 cm; VI, 3.0 cm to cotyledon node. The state of methylation was determined by incubation with ^{3}H-AdoMet and calmodulin methyltransferase and by liquid scintillation counting as described in the legend in Figure 11. Inset, diagram of 3-day old etiolated pea with dissection marks.



Figure 14. Verification of the pattern of methyl group incorporation into etiolated pea calmodulin. SDS-PAGE on 15 % (w/v) polyacrylamide gels and fluorography analyses of the assay products from the analysis of Figure 13.

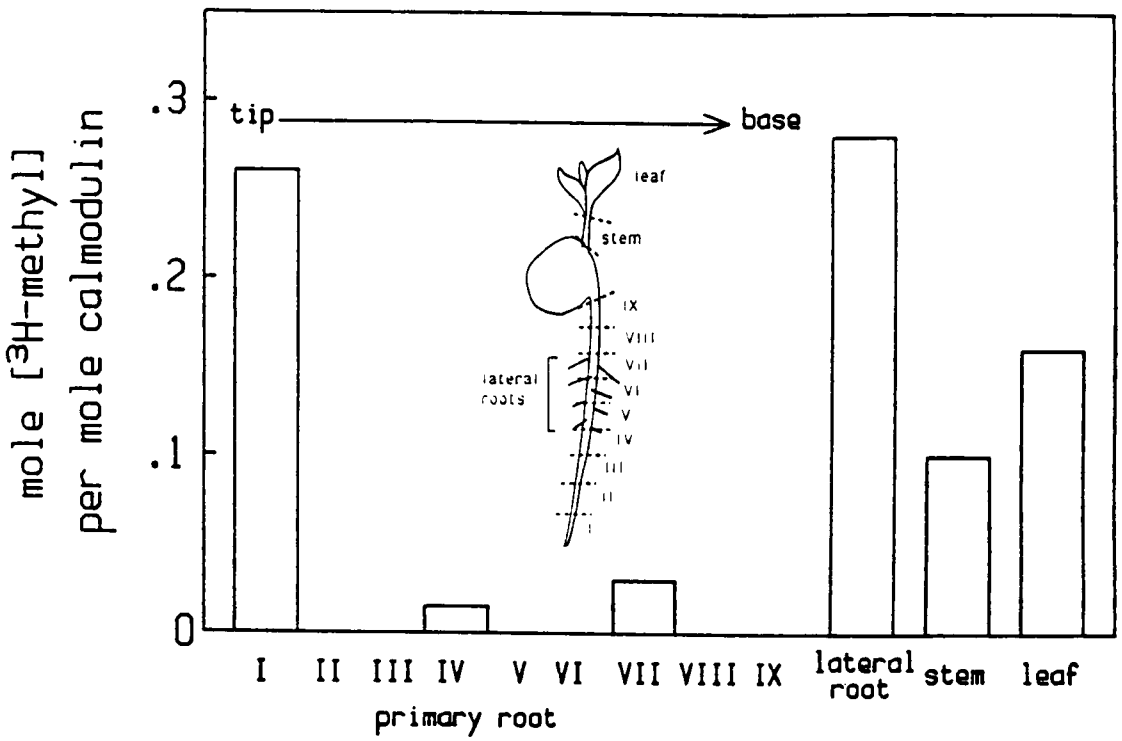


Figure 15. Analysis of the state of calmodulin methylation in 6-day old green pea seedlings. Plants were dissected into lateral and primary roots, stems, and leaves. The primary roots were dissected further into 1.0 cm segments: I, tip to 1.0 cm; II, 1.0 - 2.0 cm; III, 2.0 - 3.0 cm; IV, 3.0 - 4.0 cm; V, 4.0 - 5.0 cm; VI, 5.0 - 6.0 cm; VII, 6.0 - 7.0 cm; VIII, 7.0 - 8.0 cm; IX, 8.0 cm to cotyledon node. The state of methylation was determined as described in the legend in Figure 13. Inset, diagram of 6-day old green pea with dissection marks.

developed, and small, young, 1.0 to 1.5 cm lateral roots are apparent at the base. The epicotyl consists of a very small stem and primary leaves. The plants were dissected as shown in Figure 15. The highest levels of methyl group incorporation were associated with calmodulin from the tip of the primary roots and from the young lateral roots (Fig. 15). The values are somewhat lower than those found in etiolated root tips and correspond to about 0.26 to 0.28 mol per mol calmodulin. Calmodulins from most of the other root tissues appear to be completly methylated since they were poor substrates for the enzyme. The detection of low amounts of undermethylated calmodulin in sections IV and VII of the primary root may be due to contamination with calmodulin from lateral root tissue that may remain after dissection. Undermethylated calmodulins were also readily observed in the young stem and primary leaves. Again, the liquid scintillation counting data was supported by fluorographic analysis (Fig. 16). As an additional test of whether the methylation of pea calmodulins in Figures 13 and 15 was the result of specific incorporation into site 115, we tested the ability of added VU-4 calmodulin to inhibit pea calmodulin methylation (Fig. 17). Added VU-4 calmodulin inhibited the methylation of pea calmodulin in a dose-dependent manner.

A corresponding pattern was also obtained from the analysis of soybean tissues. Calmodulins isolated from the root apex and 14-d-old young nodules have much higher ability

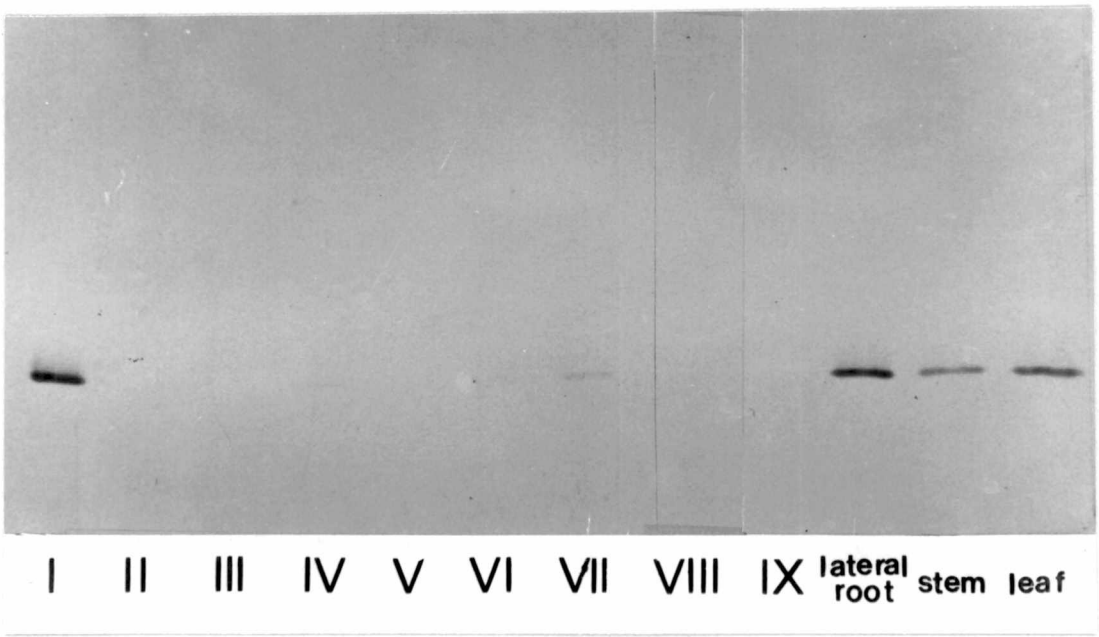


Figure 16. Verification of the pattern of methyl group incorporation into green pea calmodulin. SDS-PAGE on 15 % (w/v) polyacrylamide gels and fluorography analyses of the assay products from the analysis of Figure 15.

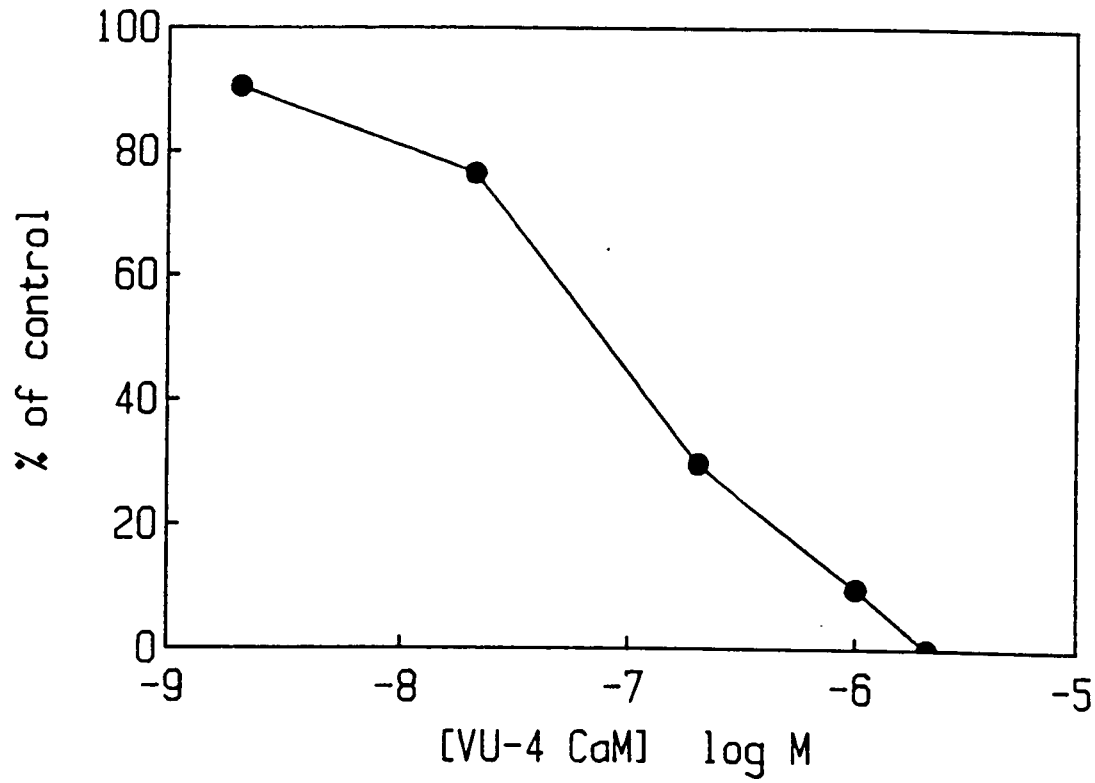


Figure 17. Inhibition of methylation of pea calmodulin by VU-4 calmodulin. Calmodulin (1.25 μg) from 6-day old green pea seedlings were treated with calmodulin-lysine N-methyltransferase and 6.0 μM ^{3}H-AdoMet (1.5 μCi) in the absence or in the presence of VU-4 calmodulin. The results are expressed as % of control (no added VU-4 calmodulin) vs. the amount of VU-4 calmodulin added.

to accept [^{3}H]methyl group, than that of calmodulin from the root base (Fig. 18). Overall, the data clearly show that small pools of undermethylated calmodulins exist in plant tissues, and that in developing roots the levels are highest in the youngest tissues.

Calmodulin and calmodulin methylation during carrot cell growth

The data showing that undermethylated calmodulins are preferentially localized in zones of cell proliferation, such as the root apex, led us to set up a cell culture model system to analyze the level of calmodulin and calmodulin methylation in proliferating vs. nonproliferating tissues. We set up such a system with carrot cultures (Dougall and Weyrauch, 1980). With this approach, we are able to get large amounts of samples from various stages of the growth curve without the difficulties in sampling that is characteristic of plant apicies. As shown in Figure 19, carrot cells have clear growth stages (i.e., lag phase, exponential phase, stationary phase). Upon reinoculation into fresh media, cells enter exponential phase within 4 days (Fig. 19). Calmodulin was exhaustively extracted from the carrot cell samples collected during the various stages of growth as described in the Materials and Methods, and the levels of calmodulin were determined by RIA. Although slight fluctuations were observed from sample to sample, there was no clear trend of calmodulin

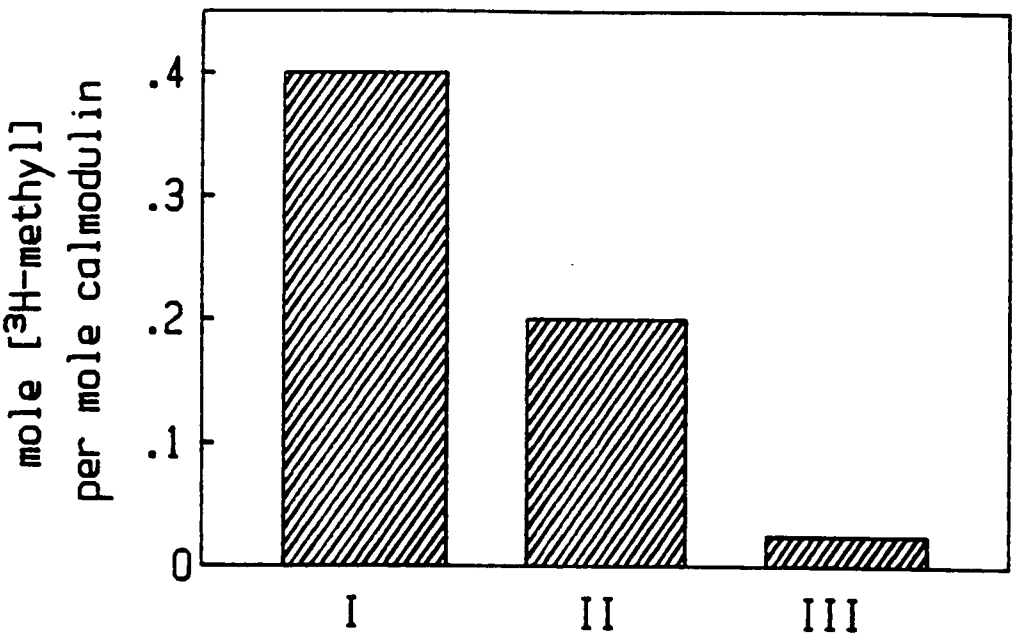


Figure 18. The state of calmodulin methylation in soybean root nodules and root tissues. The level of methylation of calmodulin from tissues was determined as described in the legend in Figure 15. Samples are : I, 14-day old root nodules from soybean infected with Bradyrhizobium japonicum; II, root apex from 3-day old soybean plants; III, root base from 3-day old soybean plants.

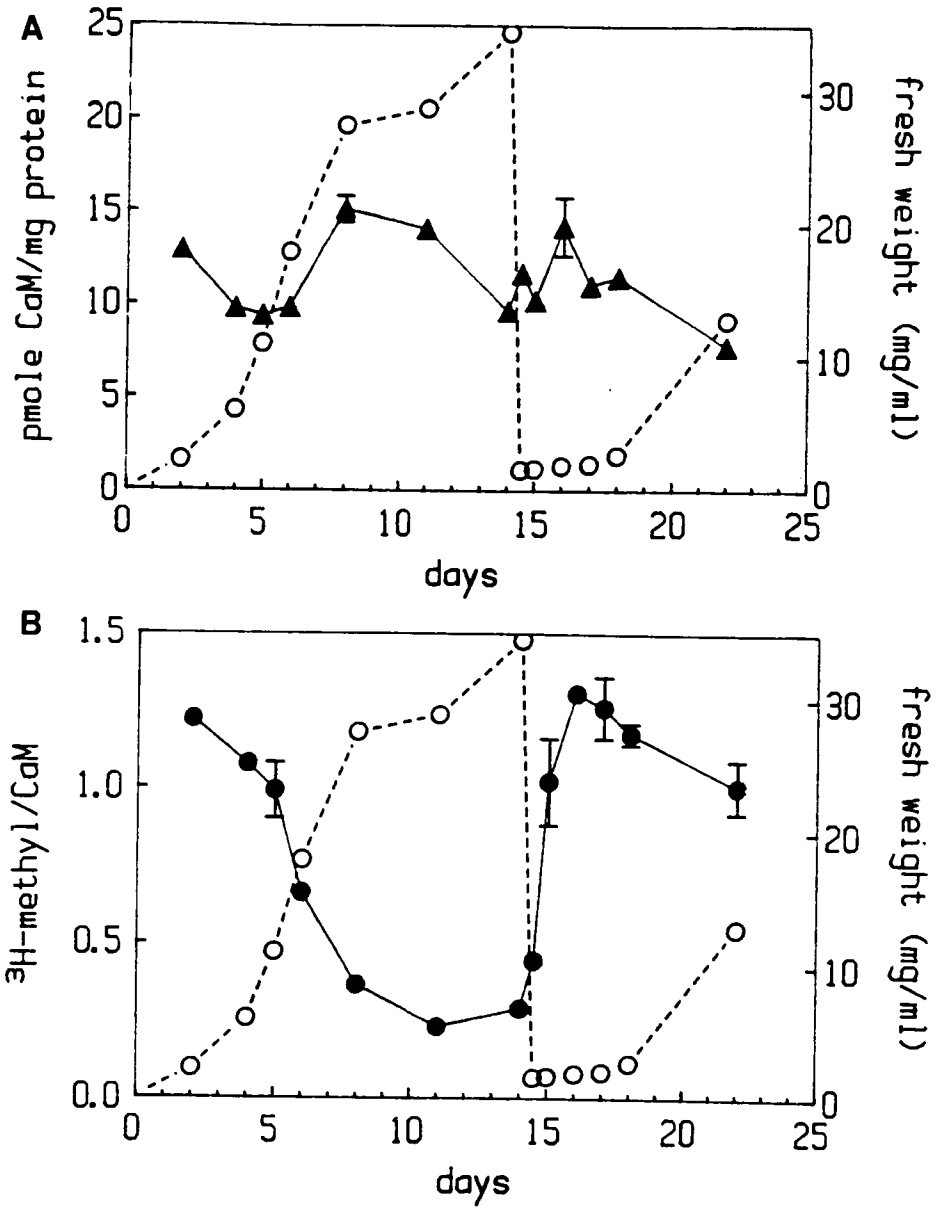


Figure 19. Analysis of the levels of calmodulin protein and calmodulin methylation in carrot cell cultures. Carrot cells from various parts of growth curve (2-day, 4-day, 5-day, 8- day, 11-day, and 14-day of the 1st cycle; 12 hr, 1-day, 2-day, 3- day, 4-day, and 8-day of the 2nd cycle) were harvested. **(A)** The levels of calmodulin protein during carrot growth. The triangles represent pmol calmodulin per mg protein. **(B)** Calmodulin methylation levels during carrot growth. The closed circles show moles of ^{3}H-methyl incorporated per mole of calmodulin. The open circles show carrot cell growth curves. Values average of two determiation, with error bars showing standard error of mean. The absence of error bars indicates that the range is smaller than the size of the symbol. CaM, calmodulin.

increase, and the overall levels of calmodulin were similar in cells from exponential and stationary phases (Fig. 19a).

To analyze the degree of carrot calmodulin methylation, the calmodulin pool was isolated from the carrot extracts and the degree of methyl group incorporation was measured by using the calmodulin methylation assay. As shown in Figure 19b, extreme differences in the state of calmodulin methylation during carrot cell growth were observed. For example, calmodulin isolated from the early exponential stages of the growth curves (i.e., 2-day and 4-day of the first cycle, 2-day, 3-day and 4-day of the second cycle) has ^{3}H-methyl group accepting ability ranging from 1.05 to 1.36 mole per mole calmodulin. On the other hand, calmodulin isolated from the stationary stage of the growth curve (i.e., 11-day and 14-day of the first cycle) has a lower ability to accept methyl groups ranging from 0.22 to 0.30 mole per mole calmodulin. These observations are consistent with our previous findings with developing plant tissues, and show that unmethylated calmodulin is preferentially associated with proliferating cells. Further, the methylation state of calmodulin found in carrot cells is the lowest of any plant calmodulin detected thus far. Another interesting observation from the analyses is that the degree of methyl group incorporation into calmodulin isolated from the early lag phase of the carrot growth (i.e., 12 hrs or 1-day of the second cycle) is low. However, during the later part of the lag phase and the very

early exponential stage, undermethylated calmodulin quickly accummulates independently of calmodulin protein concentration and then starts to decrease as cells grow further. SDS-PAGE and fluorography verified the pattern of incorporation, and showed that methylated calmodulin was the only product (Fig. 20). Overall, the data clearly demonstrate that calmodulin is highly unmethylated at position 115 in the early exponential stage of carrot cell growth and that the level of endogenous calmodulin methylation increases as cells enter the stationary phase. Further, unmethylated calmodulin reappears quickly as the second growth cycle starts.

Calmodulin, calmodulin methylation and calmodulin-binding proteins during carrot embryogenesis

In order to further investigate the calmodulin system during plant development, we have utilized an embryogenic carrot culture. Embryogenic carrot cell cultures grown in the medium devoid of 2,4-D develop into embryos within 17 days. By the end of the 24th day the embryos germinate. Carrot samples from the 0-day, 10-day, 17-day, and 24-day of embryogenesis were harvested and analyzed by using the same protocols described above. As shown in Figure 21a, no significant differences were observed in the levels of calmodulin and calmodulin methylation between the samples from 0-day, 10-day, and 17-day. However, after passing the 17-day point, the level of calmodulin started to increase and doubled



1 2 3 4

Figure 20. Identification of [^{3}H]methylated carrot calmodulin. Calmodulin from 2-day (lane 1), 4-day (lane 2), 8-day (lane 3), 11-day (lane 4) cultures of the first cycle in the Figure 19. Fluorogram after SDS-PAGE on 15 % polyacrylamide gel. Each lane contains 0.15 μg of the calmodulin indicated. Other conditions were same as in the legend in Figure 19b.

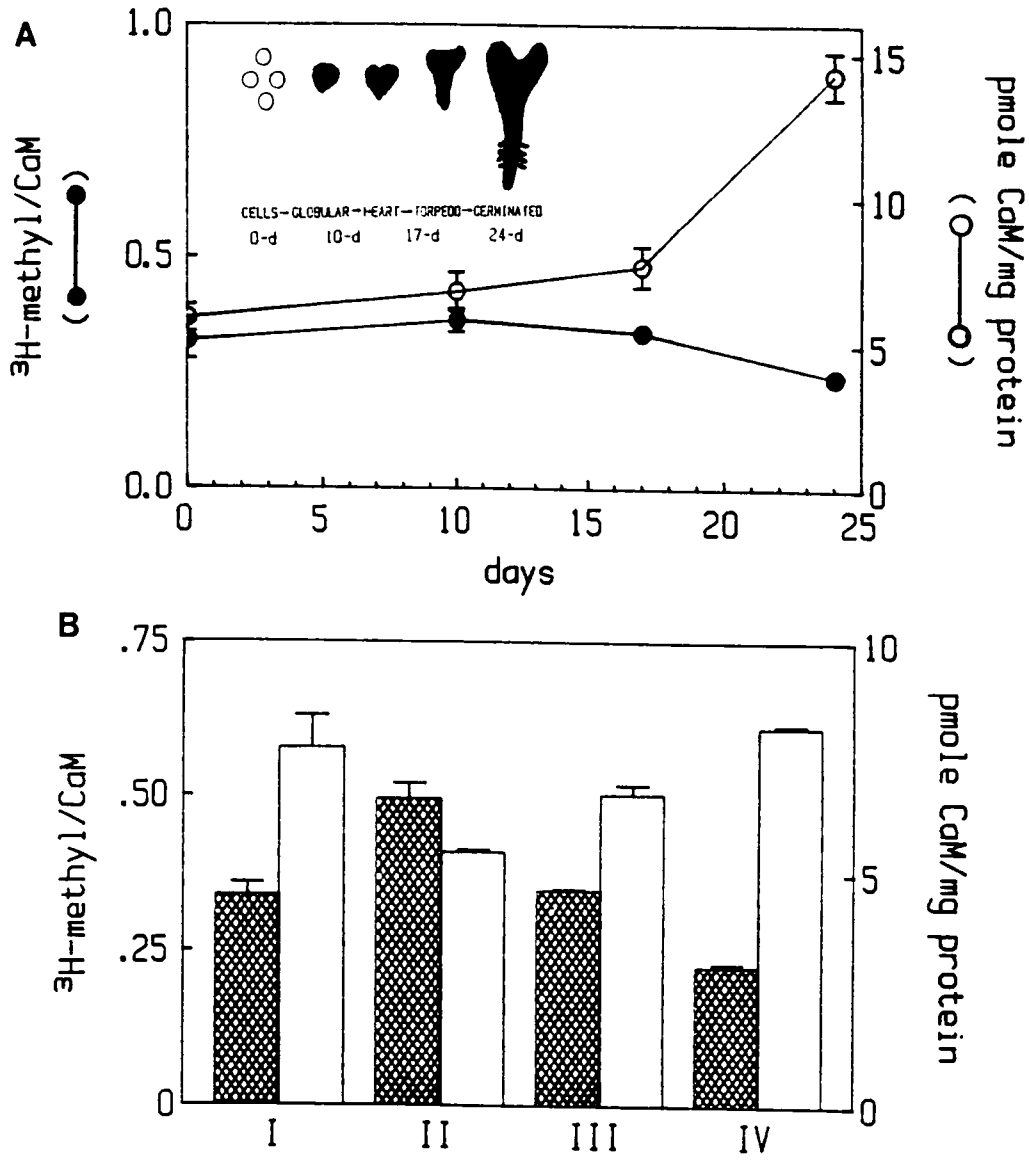


Figure 21. Analysis of the levels of calmodulin protein and calmodulin methylation during carrot embryogenesis. **(A)** The levels of calmodulin and the state of calmodulin methylation during carrot embryogenesis. ^{3}H-methyl/CaM represents moles of ^{3}H-methyl incorporated per mole of calmodulin. Inset, stages of carrot embryo development. **(B)** The levels of calmodulin and the state of calmodulin methylation in different stages of 17-day-old carrot embryos: I, tissue not screened; II, tissue screened with pore size 0 to 310 μm; III, tissue screened with pore size 310 to 570 μm; IV, tissue screened with pore size above 570 μm. The crossed bars show mole [^{3}H-methyl] incorporated per mole calmodulin and the open bars show the concentration of calmodulin based on total cellular protein. Values average of two determination, with error bars showing standard error of mean. CaM, calmodulin.

by the end of the 24th day and the degree of methyl group incorporation decreases slightly. To investigate further the distribution of calmodulin and calmodulin methylation among various embryonic stages, 17-day old carrot embryos were screened based on their sizes and analyzed. Tissue which passed through 310 μm screen is globular and early heart shaped embryos, while tissue which is bigger than 310 μm but smaller than 570 μm has torpedo shaped embryos. Again, the level of calmodulin starts to increase as carrot embryos become further developed (Fig. 21b). However, the changes in calmodulin methylation were not as significant as those seen during carrot cell proliferation (Fig. 19).

Calmodulin overlay analysis with crude extracts and calmodulin-Sepharose purified samples was done to identify target proteins of calmodulin action and to determine whether any differences in calmodulin target proteins occur during the formation and germination of embryos. Previous studies with a germinating seed system (Cocucci and Negrini, 1988) and a developing algal system (Brawley and Roberts, 1989) have shown that there are developmental differences in calmodulin target proteins. As shown in Figure 22, there are several calmodulin-binding proteins in the embryogenic extracts with apparent molecular weights of 81,000, 54,000, 50,000, 32,000, 26,000, and 20,000. Several high molecular weight (>100,000) calmodulin-binding proteins are also present in the extracts. The levels of some of these proteins appear to change during

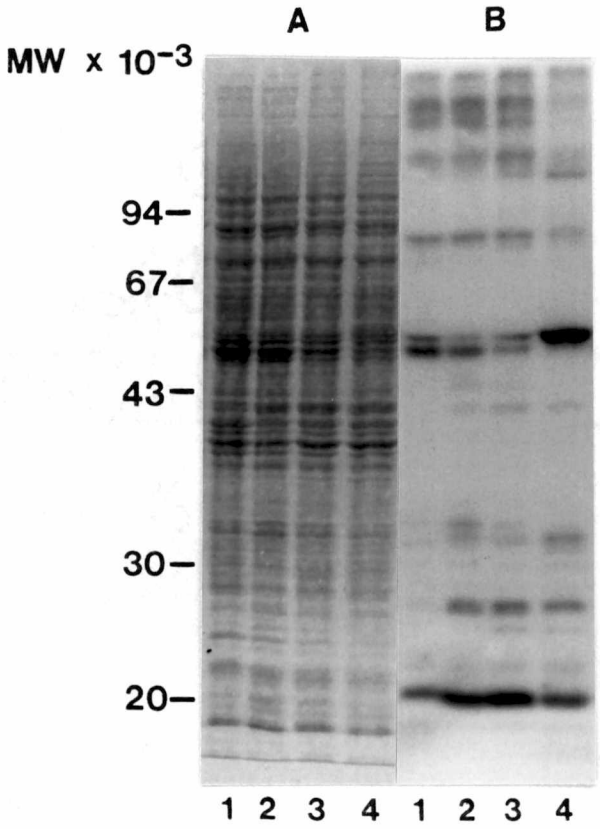


Figure 22. ^{125}I-calmodulin overlay on carrot cell extracts. 17 μg of protein from 0-day (lane 1), 10-day (lane 2), 17-day (lane 3), and 24-day (lane 4) embryogenic carrot samples was applied on 12.5 % SDS-polyacrylamide gel to separate proteins. **(A)** Coomassie-Blue stained gel **(B)** Autoradiogram of the gel exposed to ^{125}I-calmodulin in the presence of 1 mM $CaCl_2$. Since no bands were observed on the autoradiogram of a duplicate gel exposed to ^{125}I-calmodulin in the presence of 5 mM EDTA (data not shown), the bands appeared on the above autoradiogram indicate calcium-dependent calmodulin-binding proteins.

carrot embryogenesis. For example, while calmodulin binding to the 81,000 M_r and 50,000 M_r proteins decreased slightly in the 24-day germinated cell extract, calmodulin binding to the 54,000 M_r protein increased substantially in the 24-day extract.

In order to verify that these proteins are calmodulin-binding proteins and as an initial step towards their purification, we performed affinity chromatography on calmodulin-Sepharose. Since a significant increase in the apparent level of 54,000 M_r calmodulin-binding protein during the germination of embryos was observed from the overlay experiments, we chose 24-day old germinated embryos for further study. Before applying to the calmodulin-Sepharose column, endogenous calmodulin in the extract of germinated embryos was removed by anion-exchange chromatography as described (Roberts et al., 1990). The calmodulin depleted samples were then applied to Ca^{2+}-dependent calmodulin-Sepharose column and washed as described in Materials and Methods. Calmodulin-binding proteins were finally eluted by adding calcium chelator EGTA in buffer (see Materials and Methods). SDS-PAGE analysis (Fig. 23a) showed that a 54,000 M_r protein was the only protein detected. Again, ^{125}I-calmodulin overlay analysis (Fig. 23b) of the calmodulin-Sepharose purified sample preparations verified that the 54,000 M_r protein is the major calmodulin-binding protein. All calmodulin binding was calcium-dependent because no bands

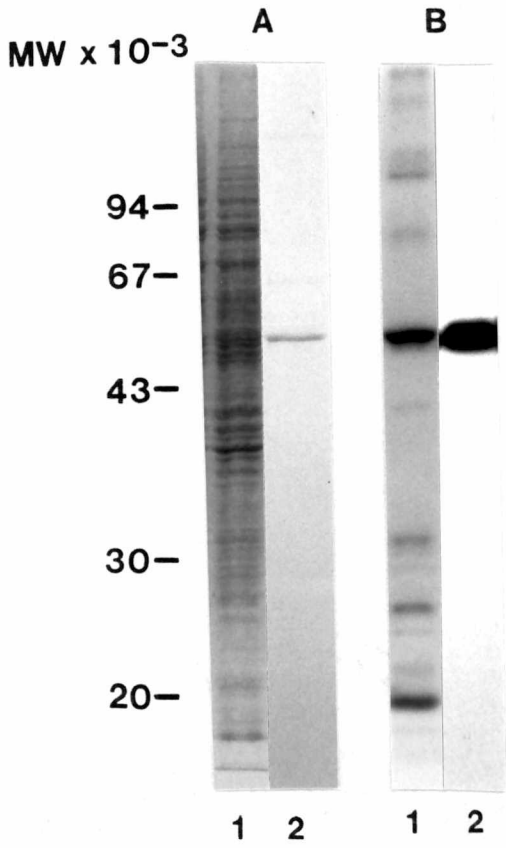


Figure 23. Purification of calmodulin-binding protein from 24-day old germinated carrot samples. Calmodulin-binding proteins were purified by DEAE-cellulose and calmodulin-Sepharose chromatography. **(A)** Coomassie-Blue stained 12.5 % SDS-polyacrylamide gel. **(B)** Autoradiogram of the gel exposed ^{125}I-calmodulin in the presence of 1 mM $CaCl_2$. Lane 1, crude extract; lane 2, calmodulin-Sepharose purified protein.

were observed on the autoradiogram of a duplicate gel exposed to ^{125}I-calmodulin in the presence of 5 mM EDTA (data not shown).

Analysis of the expression of VU-calmodulins in transgenic tobacco plants

In order to understand the role of plant calmodulin and calmodulin methylation *in vivo*, transgenic tobacco plants expressing a recombinant DNA-derived calmodulin (VU-1) or a methylation site mutant (VU-3) have been generated using transgenic plant technology in collaboration with Dr. Gary Stacey and Linda Besl in the Plant Physiology and Genetics Program at the University of Tennessee. To generate the transgenic plants, the binary vectors containing either VU-1 calmodulin or VU-3 calmodulin gene were constructed (Besl, 1991). The foreign calmodulin genes were inserted into the pRT101 plasmid (Topfer et al., 1987), which has the cauliflower mosaic virus 35 S promoter, and then subcloned between the T-DNA borders of a binary vector (pGDW31) which has a hygromycin resistance marker (Fig. 24). These constructs were then transferred into tobacco cells by using *Agrobacterium tumefaciens*. VU-3 was chosen since under *in vitro* conditions it has at least 3-fold higher NAD kinase activator activity than plant calmodulin and it has no methyl accepting ability but competitively inhibits the methylation reaction.

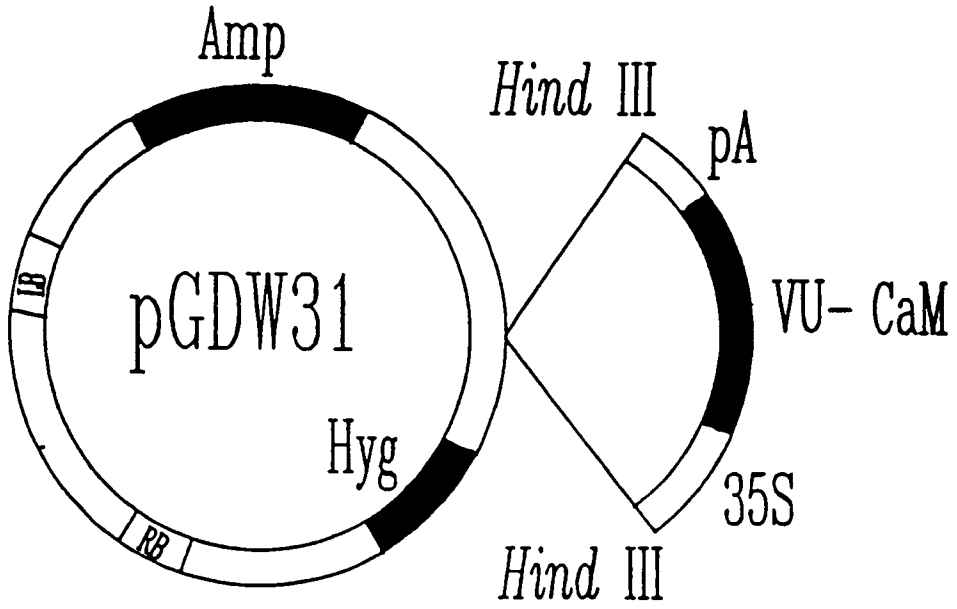


Figure 24. Binary vector construction for the generation of transgenic plants with VU-calmodulins. VU calmodulin genes were cloned into the binary vector, pGDW31 as shown. The calmodulin genes (VU-CaM) are inserted behind the cauliflower mosaic virus 35S promoter and in front of a polyadenylation sequence (pA), (Topfer et al., 1987). The entire construct is inserted between the left and right T-DNA borders to facilitate integration into the plant genome. The plasmid has a β-lactamase gene (Amp) for selection in bacteria and a hygromycin (Hyg) resistance gene for selection in plants.

VU-calmodulin expression in the transgenic tobacco plants has been confirmed by SDS-PAGE analysis. Since VU-calmodulins have subtle differences from plant calmodulin (Fig. 1), we were able to selectively analyze the relative levels of foreign calmodulin protein. SDS-PAGE analysis of purified calmodulin from transgenic plants revealed two bands with electrophoretic mobilities identical to standard VU-calmodulin and plant calmodulin (Fig. 25). On the other hand, normal untransformed tobacco plants showed only one band co-migrating with standard plant calmodulins. To establish that the identity of the upper band as the expressed product of the foreign VU gene, we fractionated the VU calmodulins from endogenous tobacco calmodulin by anion exchange chromatography. This method was selected because of the slight differences in the net charge of VU and plant calmodulins due to amino acid sequence differences (Fig. 26). By examining the isoelectric plots, we were able to choose a suitable pH for the fractionation using anion-exchange chromatography on a Mono Q column. At the buffer pH we used (pH 6.1), the Mono Q chromatography resolves the calmodulin isolated from the VU transgenic plants into two major peaks, I and II (Fig. 27), that correspond to the lower and upper calmodulin bands on an SDS-PAGE gel (Fig. 28). Further evidence that the upper calmodulin band from VU-3 transgenic plants is VU-3 calmodulin was established by amino acid composition analysis that showed that this calmodulin is

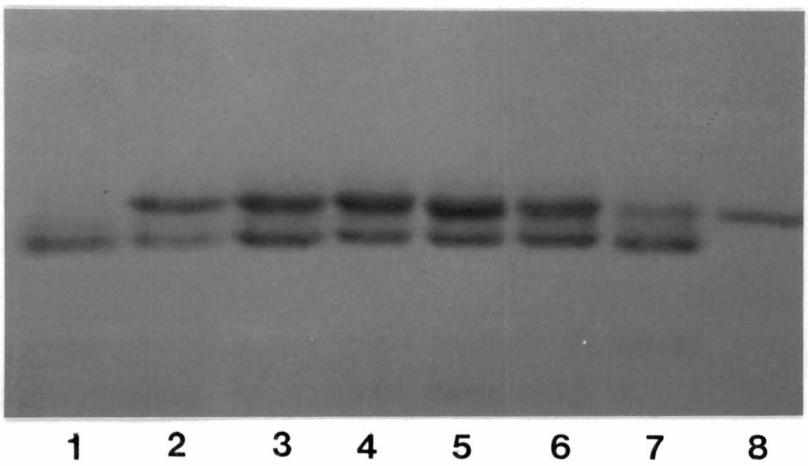


Figure 25. SDS-PAGE analysis of calmodulin purified from control tobacco and VU-transgenic tobacco plants.
Calmodulin was purified from 42-day old tobacco plants (F_1 generation) and was analyzed by SDS-PAGE on 15 % (w/v) polyacrylamide gels in the presence of 1 mM EGTA. The gel was stained with Coomassie blue. Each lane contains 10 pmoles of calmodulin. Lane 1, calmodulin from untransformed W38 tobacco plants; lanes 2 to 4, calmodulins from VU-1 transgenic plants (lines 1-1, 1-4, and 1-13, respectively); lanes 5 to 7, calmodulins from VU-3 transgenic tobacco plants (lines 3-2, 3-3, 3-13, respectively); lane 8, VU-1 calmodulin derived from expression in *E. coli*.

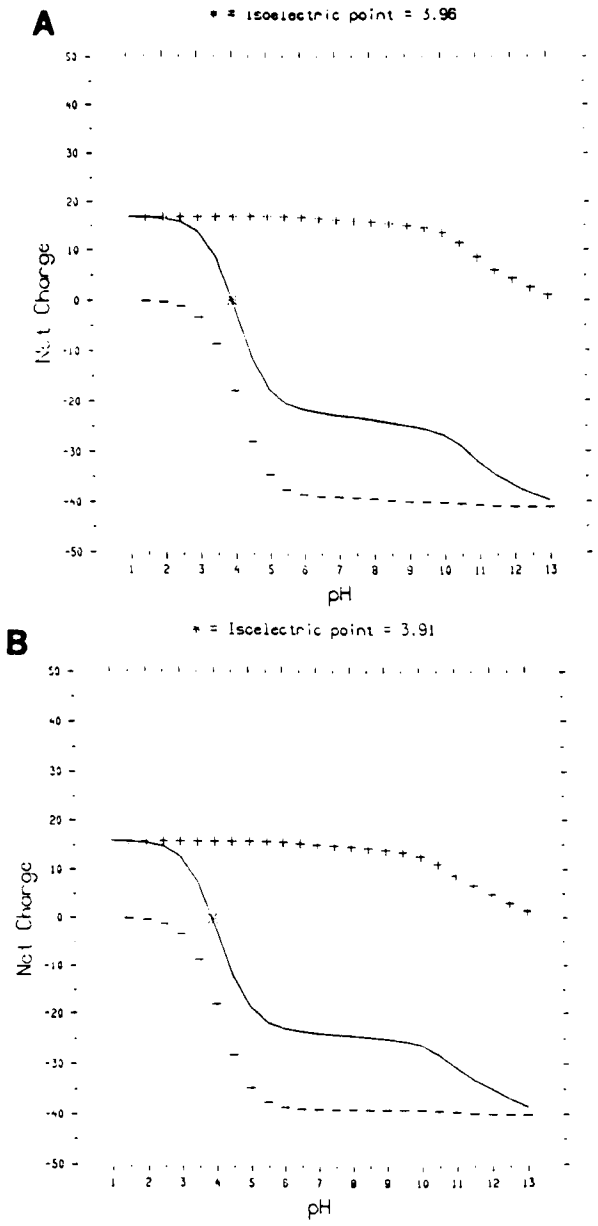


Figure 26. Comparison of isoelectric plots of plant calmodulin and VU-3 calmodulin. Net charges as a function of pH for the plant calmodulin sequence (Lukas et al., 1984) **(A)** and VU-3 calmodulin sequence (Roberts et al., 1986) **(B)** were plotted by using the sequence analysis software package of the Genetic Computer Group (GCG) (University of Wisconsin Biotechnology Center). Plus and minus marks indicate the total positive and negative charges of the calmodulins as a function of pH, respectively. The solid lines indicate the net charges of the calmodulins as a function of pH. The asterisks indicate the isoelectric points of the calmodulins.

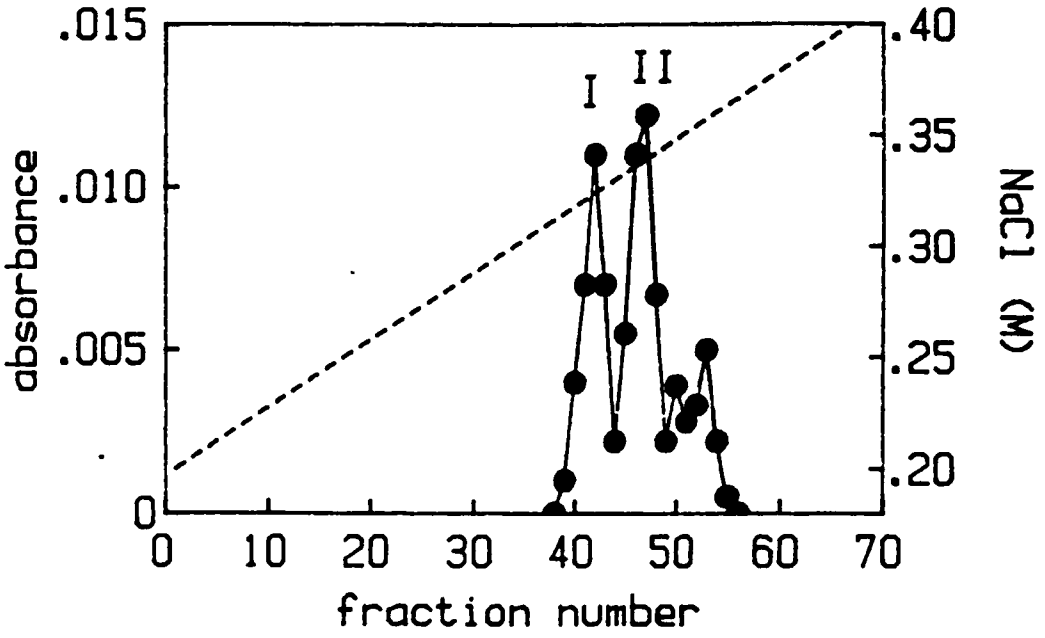


Figure 27. Fractionation of VU-transgenic calmodulin from endogenous tobacco calmodulin. The elution profile of calmodulins from VU-3 transgenic tobacco on a Mono Q HR 5/5 column is shown. Fractions of 0.3 ml were collected. The closed circles show the absorbance of the column effluent, and dashed line shows the NaCl gradient. The two major peaks (I and II) are indicated.

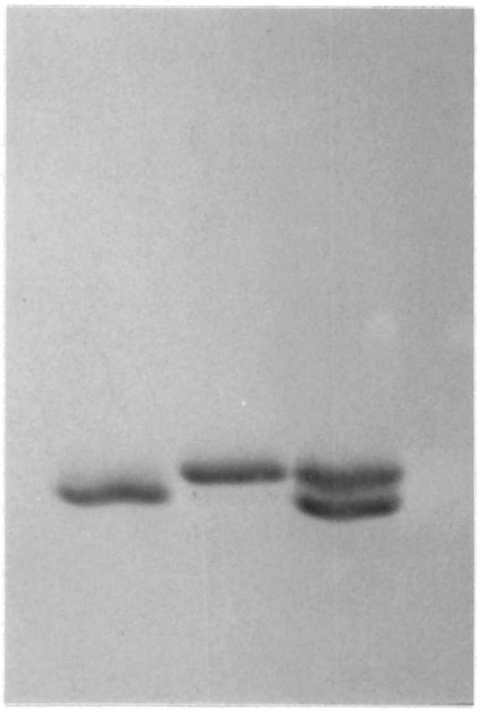


Figure 28. SDS-PAGE analysis of transgenic calmodulins fractionated by Mono Q chromatography. Calmodulin was analyzed by SDS-PAGE on 15 % (w/v) polyacrylamide gels in the presence of 1 mM EGTA, and the gel was stained with Coomassie blue. Lane A is 0.33 μg of endogenous tobacco calmodulin (peak I from Figure 27), lane B is 0.33 μg of transgenic VU-3 calmodulin (peak II from Figure 27), lane C is 0.66 μg of calmodulin mixture from VU-3 transgenic tobacco plants (before Mono Q chromatography).

indistinguishable from recombinant DNA derived VU-3 calmodulin expressed in E. coli (Table IV).

NAD kinase activator analysis shows that VU-3 calmodulin isolated from transgenic plants has the higher activator activity characteristic of E. coli expressed VU-1 and VU-3 calmodulins (Fig. 29a). On the other hand, VU-1 calmodulin from transgenic plants differed from E. coli expressed VU-1 and VU-3 calmodulins in that it activated NAD kinase to a much lower level, characteristic of normal plant calmodulin (Fig. 29b). This suggested that VU-1 calmodulin expressed in transgenic plants was trimethylated by the host calmodulin methyltransferase in vivo. As shown in Table V, transgenic VU-1 calmodulin appears to be methylated to near stoichiometric levels, explaining the activator data. In order to test the effect of the VU-3 calmodulin introduction into tobacco cells on plant calmodulin methylation system, the Mono Q fractionated endogenous calmodulins from VU-3 transgenic plants were tested for methyl group accepting ability using the radiometric assay. Since VU-3 calmodulin is a competitive inhibitor for the calmodulin methyltransferase, the levels of methylation of endogenous calmodulin might be reduced in the VU-3 transgenic plants. However, the results show that there were no significant differences in the endogenous methylation compared with that of the calmodulin from normal untransformed plants (Table VI). One possible explanation is that the VU-3 calmodulin level is not high

TABLE IV

Amino acid composition analysis of VU-3 calmodulin[a]

Amino acid	E. coli VU-3[b]	transgenic VU-3[c]	plant calmodulin[d]
arginine	6.2	6.2	5.0
lysine	8.4	8.2	9.0
glycine	11.1	11.0	10.0
threonine	10.6	10.2	9.0
TML	-	-	1.0

[a]Only key amino acid residues are compared. Amino acid composition analysis was done by using the general method of Bidlingmeyer et al., (1984) (see Materials and Methods). TML, trimethyllysine.

[b]Recombinant DNA-derived VU-3 calmodulin expressed in E. coli.

[c]Transgenic VU-3 tobacco calmodulin fractionated from endogenous tobacco calmodulin by Mono Q chromatography.

[d]Composition of plant calmodulin calculated from amino acid sequence (Lukas et al., 1984).

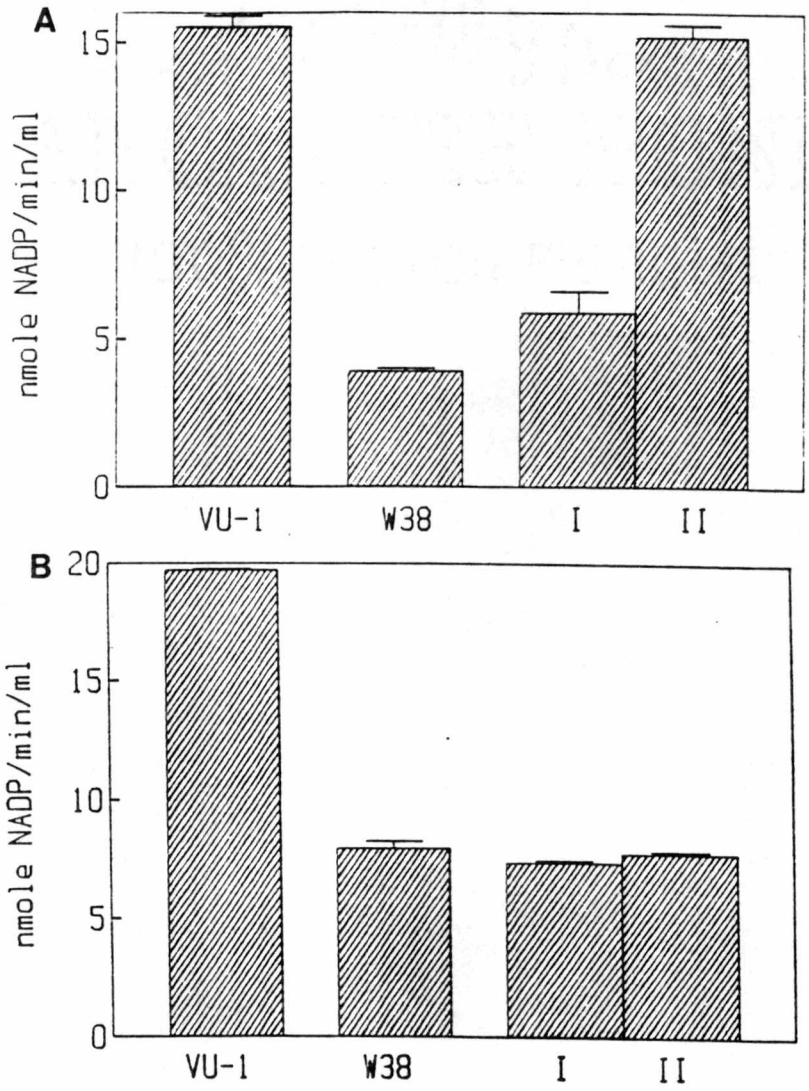


Figure 29. Analysis of the NAD kinase activator properties of calmodulins from transgenic plants. The histograms show the level of activation of purified calmodulin-dependent NAD kinase by a saturating dose (100 nM) of each calmodulin. **(A) Activation by calmodulins from VU-3 transgenic plants. VU-1**, control, unmethylated VU-1 calmodulin expressed in E. coli; **W38**, calmodulin purified from control, untransformed W38 tobacco plants; **I**, endogenous tobacco calmodulin purified by mono Q chromatography (peak I from Figure 27); **II**, VU-3 calmodulin purified by mono Q chromatography (peak II from Figure 27); **(B) Activation by calmodulins from VU-1 transgenic plants. VU-1**, control, unmethylated calmodulin expressed in E. coli; **W38**, calmodulin from control, untransformed W38 plants; **I**, endogenous tobacco calmodulin purified from VU-1 transgenic plants by mono Q chromtography; **II**, VU-1 calmodulin purified from VU-1 transgenic plants by mono Q chromatography.

TABLE V

Methylation state of transgenic VU-1 calmodulin and control VU-1 calmodulin expressed in E. coli[a]

Calmodulins	dpm[b]	moles ^{3}H-methyl groups	moles TML[c]
Transgenic VU-1	1,010	0.18(0.01)	2.82
Control VU-1	16,520	3.02(0.07)	0

[a]0.10 μg of each calmodulin was incubated with 12 μM ^{3}H-AdoMet (0.5 μCi) and 0.5 μg of purified calmodulin-lysine N-methyltransferase from sheep brain.

[b]The values represent dpm of ^{3}H-methyl groups incorporated into calmodulin. The moles ^{3}H-methyl groups per mole of calmodulin is calculated from this. The values in parenthesis represent the standard error of the mean.

[c]This value represents the endogenous level of trimethylation of the two calmodulins. Control VU-1 calmodulin was previously shown to be unmethylated at position 115 (Roberts et al., 1985).
TML, trimethyllysine.

TABLE VI

Analysis of the methylation state of endogenous calmodulins from untransformed tobacco and VU-3 transgenic tobacco plants[a]

Calmodulins[b]	dpm	moles ^{3}H-methyl groups	moles of TML[c]
Control W38	1,258	0.23(0.01)	2.77
Endo. VU-3 (line 3-2)	1,586	0.29(0.01)	2.71
Endo. VU-3 (line 3-3)	1,313	0.24(0.01)	2.76
Control VU-1	16,520	3.02(0.07)	0

[a]The state of methylation was determined as described in the legend in Table 3.

[b]Calmodulin was purified from 42-day old untransformed (control W38) and VU-3 transgenic tobacco plants. Endogenous VU-3 calmodulin (Endo. VU-3) was obtained from the Mono Q chromatography of the purified calmodulin. Control VU-1, VU-1 calmodulin expressed in E. coli.

[b]TML, trimethyllysine.

enough to exert the inhibitory activity that is seen in vitro.

Since the upper bands on SDS-gels (Fig. 25, 28) are identified as foreign VU-calmodulins, the expression levels can be roughly estimated by the relative intensities of Coomassie blue staining of the two bands. Based on the intensities, the relative levels of the foreign and endogenous calmodulins are equivalent. This estimation is supported by calmodulin specific RIA analyses showing that calmodulin levels in whole transgenic tobacco tissues (Table VII) were maximally twice that of endogenous calmodulin. Although the overall calmodulin levels in the VU-transgenic plants are not significantly changed, some clear phenotypic differences between VU-3 transformed, VU-1 transformed or untransformed plants have been observed by Besl (1991). For example, VU-3 plants have much shorter internode length than control plants or VU-1 plants. Further the VU-3 plants have decreased seed number or show lack of ability to form seed in the F_2 generation. Thus, the data suggest that the abnormal phenotypes observed only in VU-3 plants result from the expression of the calmodulin mutant and not from a change in calmodulin levels.

As a continued project concerning the effect of calmodulin methylation on calmodulin function in vivo, we have generated new transgenic tobacco plants expressing VU-4 calmodulin. Like VU-3 calmodulin, VU-4 calmodulin has no methyl accepting abilty, but it is a better inhibitor for the

TABLE VII

Comparison of calmodulin protein levels in normal and transgenic plants

Plant	line[a]	pmol CaM/mg protein[b]
Control	-	15.8 (0.2)
VU-1	1-1	28.9 (0.4)
	1-2	19.7 (0.3)
	1-3	24.0 (0.2)
	1-9	18.8 (0.2)
	1-13	23.2 (0.5)
VU-3	3-2	31.1 (0.6)
	3-3	29.0 (0.3)
	3-4	24.8 (0.3)
	3-5	24.0 (0.2)
	3-6	33.3 (0.4)
	3-13	24.6 (0.2)

[a]Each represents VU-1 or VU-3 transgenic plants (42 days postgermination) derived from a separate transformed line. The plants analyzed are from the F_1 generation.

[b]The values represent the pmoles of calmodulin (by RIA) per mg of soluble protein. The values in parenthesis represents the standard error of the mean. CaM, calmodulin.

calmodulin methyltransferase than VU-3 calmodulin as mentioned above. In contrast to VU-3 transgenic plants, only 20 % of the VU-4 transgenic tobacco plants (F_0) showed dwarf phenotypes and other defects in development such as decreased seed yield. In contrast, all VU-3 plants that showed hygromycin resistance also showed the dwarf phenotypes and other developmental defects (Besl, 1991). Representative normal and dwarf VU-4 plants are compared with control W38 plants in Figure 30.

To confirm that VU-4 calmodulin was expressed in the plants, SDS-PAGE analysis was used. As shown in Figure 31, SDS-PAGE analysis of protein from the selected VU-4 transgenic plants revealed two bands which is characteristic of the VU-calmodulin expression. To estimate the levels of VU-4 calmodulin expression and to find a relationship between the expression levels and the phenotypes, several VU-4 transgenic tobacco plants showing normal or dwarf phenotype were selected and analyzed by RIA. Although there are some variations in the RIA values between the lines tested, the levels of calmodulin of the VU-4 transgenic plants were clearly higher than those of the untransformed control tobacco plants (Table VIII). However, there was no significant correlation between the phenotypes and calmodulin levels.



Figure 30. Comparison of growth phenotype between control tobacco and dwarf VU-4 and normal VU-4 transgenic tobacco plants. Seventy-day old plants (F_0) of: A, control W38; B, VU-4 plant line 4; C, VU-4 plant line 7.

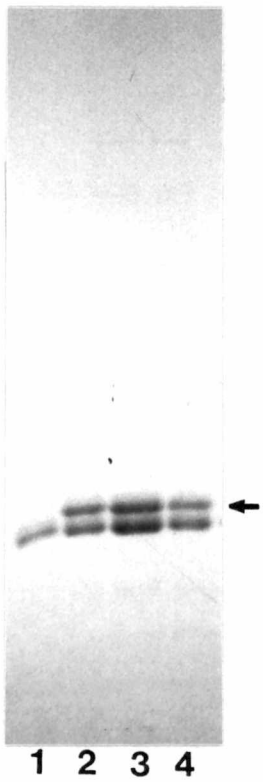


Figure 31. SDS-PAGE analysis of calmodulin purified from control and VU-4 transgenic tobacco plants. Calmodulin was purified from 60-day old F_0 tobacco plants and analyzed by SDS-PAGE on 15 % (w/v) polyacrylamide gel in the presence of 1 mM EGTA, and the gel was stained with Coomassie blue. Lane 1, calmodulin from control untransformed tobacco plants; lane 2 to 4, calmodulins from VU-4 transgenic tobacco plants (lines 4, 7, 10, respectively). The arrow indicates the electrophoretic position of VU-calmodulin.

TABLE VIII

Comparison of calmodulin protein levels, growth phenotype and seed number of untransformed (W38) and VU-4 transgenic plant lines

Plant[a]	pmole CaM/ mg protein[b]	growth phenotype[c]	mean seed number per pod[d]
W38 # 5	7.2 (1.4)	normal	1540 (530)
10	11.9 (1.8)	normal	1574 (482)
VU-4 # 7	43.1 (1.2)	normal	1621 (626)
8	22.0 (0.8)	normal	1307 (503)
11	20.4 (2.3)	normal	1531 (658)
20	10.4 (3.8)	normal	1882 (755)
26	32.3 (1.7)	normal	1149 (392)
4	38.7 (1.3)	dwarf	162 (72)
10	43.9 (1.1)	dwarf	132 (66)
15	15.9 (2.5)	dwarf	84 (41)
23	30.7 (2.0)	dwarf	116 (63)

[a]Each control or VU-4 plant was derived from a separate normal or transformed callus. The plants analyzed were from F_0 generation.

[b]The values represent the pmoles of calmodulin (by RIA) per mg of soluble protein from tobacco leaves. The values in parenthesis represent the standard error of the mean.

[c]The growth phenotypes were determined by measuring internode length based on height and leaf number.

[d]Seed number derived from dividing total seed weight by mean average weight per seed. The values in parenthesis represent the standard deviation from an average of 10 pods.

CHAPTER IV

DISCUSSION

Calmodulin methyltransferase

To investigate the properties of calmodulin-lysine N-methyltransferase, we purified the enzyme from sheep brain 28,000-fold (12,000 pmol/min/mg) to apparent homogeneity. This is the first homogeneous preparation of this enzyme. The purified enzyme is a monomer of 38,000 M_r. The interaction of calmodulin N-methyltransferase with recombinant DNA-derived calmodulins was studied. VU-1 calmodulin and VU-8 calmodulin were found to be equally suitable as substrates for the enzyme, but VU-12 calmodulin had reduced activity. VU-3, VU-4, spinach, and chicken gizzard calmodulins were unable to serve as substrates, but were competitive inhibitors of the enzyme. The enzyme was stimulated by calcium ($K_{0.5}$, 3.0×10^{-6} M) under physiological conditions of ions and pH.

Investigation of the cluster of negatively charged amino acid side chains on the calmodulin molecule revealed the importance of an acidic glutamic acid cluster at residues 82 - 84 in the central helix for the activation of some calmodulin-dependent enzymes (Craig et al., 1987). This cluster is highly conserved among all calmodulins (Roberts et al., 1986a) and numerous troponin C (Kretsinger, 1980) molecules and has been proposed as a possible site of

interaction between calmodulin and calmodulin-dependent enzymes (Craig et al., 1987; Weber et al., 1989). Notably, structures that bind calmodulin with high affinity generally form an amphipathic, basic α-helix (Blumental et al., 1985; Lukas et al., 1986) and electrostatic interactions have been proposed to be involved in the interaction between calmodulin and these helical structures (O'Neil and DeGrado, 1985; Craig et al., 1987). VU-8 calmodulin, which possesses lysine substitutions at residues 82-84, was found to be a poorer activator of several calmodulin dependent enzymes such as myosin light chain kinase and NAD kinase, whereas other enzymes such as phosphodiesterase are not drastically affected (Craig et al., 1987). Our results show that the integrity of this cluster in the central helix is not necessary for the recognition of VU-8 calmodulin by calmodulin methyltransferase.

However, the alteration of a homologous cluster of residues at position 118-120 between calcium binding sites III and IV results in a reduction in the substrate activity of calmodulin for the methyltransferase. In addition, this mutant (VU-12 calmodulin) has been found to have a reduced ability to activate some enzymes, such as calmodulin-dependent kinase II and myosin light chain kinase, whereas other calmodulin-dependent enzymes such as phosphodiesterase and NAD kinase are not affected (Weber et al., 1989). Overall, these data suggest that calmodulin-dependent enzymes interact

differently with the calmodulin structure. In the case of calmodulin methyltransferase, it is not known whether these residues are part of the recognition site for the calmodulin methyltransferase or influence activity indirectly. Further studies will be necessary to obtain a clearer definition of the role of these residues in substrate recognition, and whether the calmodulin methyltransferase resembles other calmodulin-dependent enzymes in its mode of interaction with calmodulin.

The results that show that VU-3 and VU-4 calmodulins are good competitive inhibitors of the enzyme suggest that the enzyme has a high degree of specificity for lysine 115 of calmodulin, and that the recognition site for the enzyme is still intact in these derivatives. Interestingly, VU-4 calmodulin is a 20-fold better inhibitor of methylation suggesting that the substitution of a neutral, hydrophobic isoleucine for a lysine generates a calmodulin mutant that may have a higher affinity for the enzyme. The reason for this is presently unclear. However other mutations in this region and adjacent sites may generate even stronger inhibitors of the methylation reaction that may be useful in <u>in vivo</u> studies. These catalytic data are potentially interesting, because the introduction of foreign calmodulin derivatives with substitutions at position 115 may be used to perturb calmodulin methylation. This approach will be discussed further below.

Of particular interest here is the observation that calcium stimulates methylation in the physiological range. Calcium is likely to be acting by binding to calmodulin since $^{45}Ca^{2+}$-binding analysis showed that the Ca^{2+} does not bind to purified calmodulin methyltransferase from sheep brain (Richardson and Roberts, unpublished). Thus, my interpretation is that the conformational change that results from calcium binding to calmodulin may make it a preferred substrate for the enzyme. X-ray crystallographic (Babu et al., 1985; Kretsinger et al., 1986; Babu et al., 1988) and NMR data (Seamon, 1980; Klevit et al., 1984) show that trimethyllysine is exposed to the solvent in the calcium-bound as well as the calcium-depleted forms of calmodulin. Less is known regarding the conformation of the unmethylated lysine at this position and the adjacent residues that comprise the recognition site. It will be of interest to test other calmodulin derivatives and calmodulin peptides with altered calcium-binding properties to gain insight into the role of calcium in stimulating the methyltransferase activity. Lukas et al., (1989) observed that calmodulin from a mutant Paramecium has a substitution of a threonine for a valine residue at position 136 in the fourth calcium-binding site, and has substantially reduced methylation of lysine-115 (i.e., 0.2 mol of ϵ-N-dimethyllysine, 0.3 mol of ϵ-N-trimethyllysine, and an undetermined amount of ϵ-N-monomethyllysine). The data raise the possibility that calmodulin intermediates with

monomethylated or dimethylated lysine at position 115 can occur under certain conditions and that calmodulin might dissociate from the calmodulin methyltransferase between the transfer of the first and second or second and third methyl groups. If calmodulin intermediates occur during the methylation reaction, the Km and kcat values for VU-1, VU-8, and VU-12 calmodulins could be combinations of various rate and equilibrium constants for each methylation event. Therefore, further work involving the detection of calmodulin intermediates between successive methylation reactions will help us to understand the kinetic mechanism by which calmodulin methyltransferase catalyzes the methylation reaction of calmodulin. In addition, further studies involving identification of the recognition sites responsible for the high degree of specificity will help us understand the mechanism by which calmodulin methyltransferase interacts with calmodulin.

The state of calmodulin methylation <u>in vivo</u>

The purified enzyme from sheep brain was used in a sensitive radiometric assay to evaluate the methylation state of calmodulins from selected higher plant tissues. The assay measures the incorporation of ^{3}H-methyl groups into calmodulin isolated from various tissues. Several lines of evidence indicate that the ^{3}H-product of this assay results from the methylation of undermethylated plant calmodulin at lysine

115:(a) the reaction product is stable to heat and electrophoresis under alkali conditions and thus is characteristic of methylated lysine derivatives (Rowe et al., 1986; Paik and Kim, 1990); (b) all methylated products bind to phenyl-Sepharose in a calcium-dependent manner and comigrate with plant calmodulin on SDS-gels; (c) the enzyme used shows high specificity for calmodulins with lysine-115; and (d) methylation of pea calmodulins is inhibited in a competitive manner by site-specific mutants with substitutions at position 115. Whether these undermethylated calmodulins consist of unmethylated lysine or a mixture of unmethylated and partially methylated residues (i.e., monomethylated and dimethylated lysines) will require more detailed analyses, including amino acid analysis for the various methylated forms of lysine (Lukas et al., 1989).

By using this assay, we have shown that calmodulin isolated from several tissues can serve as substrates for the methyltransferase, and that the level of [^{3}H]methyl group incorporation varied with the developmental and growth state of the plant tissues. For example, calmodulin methylation levels were lower in apical root segments of both etiolated and green seedlings, and in young lateral roots compared with the mature tissues. The results of a survey of different tissues including soybean root nodules reveal that the apical sections of roots are not the only tissues that possess high levels of unmethylated calmodulins, supporting the idea that

posttranslational methylation of lysine 115 of calmodulin varies with developmental state and tissue type. Although calmodulin methylation levels were lower in the apical region of developing roots, it is still apparent that calmodulin isolated from these tissues possess a substantial degree of endogenous methylation. It is important to note that this region consists of several different tissues including the root cap, the apical meristemic region, and cells undergoing rapid cell expansion and differentiation. In this regard, it is interesting to note that immunohistochemical analyses have demonstrated that calmodulin is highly concentrated in selected cells, such as the root cap and in the apical meristem (Lin et al., 1986; Dauwalder et al., 1986). These observations suggest that higher concentrations of calmodulin are associated with certain cells in the root apex. Further studies with dissected apical tissues such as the root cap, the root meristem, the cortex and the stele will be of interest to determine whether unmethylated calmodulins are associated with a particular tissue in the apical portion of roots.

However, to avoid the potential complexity of these different cell types in the root apex and to examine calmodulin methylation in proliferating vs. non-proliferating tissues, we have used a carrot cell culture system (Dougall et al., 1980). By this approach, we were able to obtain large amounts of plant cells from the various stages of the growth

curve without the complexity and difficulties in sampling that is characteristic of plant apicies. My analysis showed that the endogenous methylation of calmodulin from the early exponential stage of carrot cell growth is extremely low and starts to increase as the cells grow further. In addition, upon re-inoculation into fresh media, unmethylated calmodulin quickly accumulates independently of calmodulin protein concentration. This observation confirms that developmental differences in lysine 115-methylation of calmodulin do exist in vivo and that unmethylated calmodulins are preferentially associated with proliferating cells.

The data show that unmethylated calmodulin levels fluctuate regardless of calmodulin protein. Therefore, it is not likely that the amounts of unmethylated calmodulin parallel the levels of newly synthesized calmodulin protein. Possible factors for the control of calmodulin methylation are: 1. the levels of calmodulin methyltransferase present in the cells; 2. the availability of co-substrate, AdoMet; and 3. calcium fluxes. At the present time, little is known regarding the properties of plant calmodulin methyltransferases. However, since plant and vertebrate calmodulins are highly homologous (92 % identity) (Lukas et al., 1984; reviewed by Roberts et al., 1986a), it is logical that the properties of the enzyme are also highly conserved. Future studies with this enzyme in plants may provide insight into whether its levels are controlled.

The degree of calmodulin methylation also could be controlled by the availability of AdoMet in the cells. AdoMet is a co-substrate for the calmodulin methyltransferase (Sitaramayya et al., 1980; Murtaugh et al., 1983; Rowe et al., 1986; Morino et al., 1987; Oh and Roberts, 1990). AdoMet synthetase is an enzyme that catalyzes the biosynthesis of AdoMet from methionine and ATP (see Tabor and Tabor, 1984 for a review). Recently, Minocha et al., (1991) observed that the specific activity of AdoMet synthetase can vary significantly during the carrot cell growth. For example, the specific activity of the enzyme in the extract of 5-day old carrot cells grown in the presence of 2,4-D is more than 25-fold higher than that in the extract of 1-day old carrot cells. These observations in connection with our findings showing the existence of highly unmethylated calmodulin in the early exponential stage of carrot growth suggest that the availability of AdoMet also could be a factor in controlling the degree of calmodulin methylation.

Calcium ion fluxes could also be a factor for the control of calmodulin methylation. Calmodulin methylation is stimulated by micromolar concentrations of calcium. As discussed above, this finding suggests that calcium stimulation of calmodulin methylation is due to a preference for the calcium-bound form of calmodulin by the calmodulin methyltransferase. As discussed above, Lukas et al. (1989) found that a Paramecium calmodulin mutant that has a

substitution in the fourth calcium binding site has substantially reduced lysine 115-methylation. Thus, mutations that result in reduced calcium binding and an altered phenotype can lead to reduced in vivo calmodulin methylation.

From the physiological perspective the present results raise some interesting possibilities on the biological role of methylation in calmodulin function. As pointed out earlier, enzymatically methylated calmodulins activate pea NAD kinase to a maximal level that was at least 3-fold lower than the level of activation obtained with the corresponding unmethylated calmodulins (Roberts et al., 1986b). This raises the possibility that the regulation of posttranslational methylation of calmodulin in vivo could be a mechanism for attenuation of cellular NAD kinase activity. Currently, it is unclear whether plant NAD kinase activity and NAD/NADP ratios are affected by calmodulin methylation in vivo. Such analyses are complicated because one needs to take into account: 1. cytosolic vs. organellar pools of oxidized and reduced NAD and NADP; 2. the likelihood of calmodulin-independent isozymes of NAD kinase (Muto and Miyachi, 1986); 3. the overriding control by calcium fluxes; and 4. the prospect for control of NAD/NADP interconversions by an NADP(H) phosphatase (Viegel and Vanaman, 1980). However, further analyses of the distribution of calmodulin, calmodulin methyltransferase, NAD kinase protein levels, and cytosolic NAD/NADP ratios will be initial steps towards defining how calmodulin controls nicotinamide

coenzyme metabolism.

Calmodulin protein levels were similar in cells from exponential and stationary phases. These results contrast with those of previous reports. In studies with animal tissues, it has been shown that calmodulin levels are significantly enhanced in proliferating cells (Watterson et al., 1976; Chafouleas et al., 1981; Van Eldik et al., 1984). In addition, it has been suggested that calmodulin levels are elevated in tissues that contain proliferating plant cells. For example, by NAD kinase activation assays and two-dimentional electrophoresis, Allan and Trewavas (1985) showed that significantly higher levels of calmodulin activity are localized within the meristematic region of the pea root compared with the mature root tissues. Using RIA, Muto and Miyachi (1984) found that higher concentrations of calmodulin were detected in the pea shoot apex containing young and actively dividing cells compared with those in mature tissues. Zielinski (1987) showed the detection of 3- to 4-fold higher levels of calmodulin mRNA in the meristematic region of the barley leaf compared with the mature leaf tissues. As discussed before, high concentrations of calmodulin among certain cells in the apical regions such as the meristem and root cap, have also been detected by immunocytochemical analysis (Lin et al., 1986; Dauwalder et al., 1986). Several possible explanations for these apparent discrepancies exist. With respect to the mRNA data, a possible explanation based on

studies by Zielinski and co-workers (Zielinski, 1987; Zielinski et al., 1990) is that calmodulin protein regulation is distinct from the regulation of calmodulin mRNA levels. As discussed below in the section on transgenic plants, there may be translational or posttranslational controls of calmodulin expression in plants that regulate protein levels independent of mRNA. However, this explanation cannot explain the apparent discrepancies between our data and those of Allan and Trewavas (1985) and Muto and Miyachi (1984). In this regard, it is important to point out the fundamental differences between the two systems of study. In our case we are studying cells in culture that can exist in an undifferentiated proliferating or non-proliferating state. In contrast, the studies of Allan and Trewavas (1985) and Muto and Miyachi (1984) deal with changes in heterogenous populations of cells that are differentiating into various specialized tissue and cell types, and thus are not simply a comparison of proliferating vs. non-proliferating cells.

One of the possible future attempts to address the regulatory significance of calmodulin and calmodulin methylation in plant cell growth and development is the analysis of fluctuations in the calmodulin system in synchronized cells. It is interesting to note that studies with CHO (Chinese hamster ovary) cells and mouse cells show calmodulin levels double at the G1/S boundary, and that this increase is related to the rate of cell cycle progression

(Chafouleas et al., 1982; Rasmussen and Means, 1989). Since the carrot cell culture system contains a heterogeneous mixture of cells from various cell cycle stages, there may be difficulty in the detection of rapid fluctuations of calmodulin protein which may occur during cell cycle progression in plants. If such a fluctuation is required for plant cell cycle progression, not only rapid increases in synthesis but fast turnover of calmodulin may be necessary. The possible involvement of calmodulin methylation in the ubiquitin-dependent calmodulin turnover has been suggested (Gregori et al., 1985, 1987). In this regard, it may be possible to test whether calmodulin methylation has any relationship to the rate of calmodulin turnover.

Calmodulin and calmodulin-binding proteins during carrot embryogenesis

To investigate the calmodulin system in undifferentiated vs. differentiated tissues, we have utilized an embryogenic carrot cultures (Zimmerman et al., 1989). By analyzing carrot embryos, we have shown that there are little differences in the state of calmodulin methylation during carrot embryogenesis. These insignificant changes in calmodulin methylation during carrot embryogenesis as compared with the significant changes during carrot cell proliferation are not surprising because we have used the culture grown at the stationary phase to induce embryogenesis. However, we

found a clear change in the levels of calmodulin protein during the germination of the embryos. Germinated embryos have more than 2-fold higher calmodulin protein than undifferentiated cells or ungerminated embryos. Cocucci (1984) and Cocucci and Negrini (1988) observed significant enhancements in the levels of calmodulin during the early germination of radish seed embryos. In addition, Hernandez-Nistal (1989) reported a similar result in embryonic axes of germinated chick-pea seeds. Interestingly, in the work with seed embryos, the calmodulin levels were enhanced further by applying a stimulator of germination, fusicoccin (Cocucci and Negrini, 1988). The increase was suppressed by adding a germination inhibitor, abscisic acid, to the embryos (Cocucci and Negrini, 1988; Hernandez-Nistal, 1989). Currently, the question of the relationship between the enhanced calmodulin levels and embryo germination is unclear. The elevation of calmodulin levels may reflect an increased requirement of calmodulin by cells for the activation of germination processes.

Since calmodulin mediates its cellular regulation through interaction with target proteins (see Roberts et al., 1986a for a review), I have investigated the calmodulin-binding protein content of the various embryonic samples by using a calmodulin overlay method. By this approach, we have identified several calcium-dependent, calmodulin-binding proteins in embryo extracts with apparent molecular weights of

81,000, 54,000, 50,000, 32,000, 26,000, and 20,000. In addition to the calmodulin-binding proteins mentioned above, all samples of carrot embryogenesis contain several high molecular weight calmodulin-binding proteins. Some high molecular weight proteins in animals that have been known to have calmodulin-binding properties are microtubule-associated proteins (MAPS) (Lee and Wolf, 1984), Ca^{2+}/calmodulin-regulated ATPase (Jarrett and Penniston, 1977), and fodrin (spectrin) (Kakiuchi and Sobue, 1983; Larson et al., 1990). In plants, a Ca^{2+}-ATPase (Briars et al., 1988) with a relative molecular mass (M_r) of 140,000 has been shown to have calmodulin binding properties and is stimulated by calmodulin. Currently, it is unknown whether the high molecular weight calmodulin-binding proteins from the carrot embryo samples have similar properties to these proteins.

Cocucci and Negrini (1988) detected a low molecular weight basic polypeptide (13,000 M_r) from radish seed embryos which inhibits calmodulin action in the calmodulin-dependent enzyme assay system. Interestingly, the levels of this inhibitor were higher in ungerminated embryos and lower in germinated embryos. However, calmodulin-binding proteins smaller than 20,000 M_r were not detected in our samples of carrot embryos. Also, there was no such a trend in the apparent levels of 20,000 M_r calmodulin-binding protein.

To verify that these proteins are calmodulin-binding proteins by a separate criterion, and as an initial step

towards their purification, affinity chromatography on calmodulin-Sepharose was performed. From the SDS-PAGE and ^{125}I-calmodulin overlay analyses of the calmodulin-Sepharose purified sample preparations, it has been shown that the 54,000 M_r protein is the major protein purified from germinated embryos. This is a major protein of interest since this band undergoes substantial enhancement during germination. Other protein bands were not visible possibly due to the relatively lower amounts of protein levels in the purified sample preparations or possibly due to fractionation during the DEAE cellulose step. There is evidence that calmodulin-binding proteins are developmentally regulated in plant systems (Cocucci and Negrini, 1988; Brawley and Roberts, 1989). There is also evidence that calmodulin-dependent NAD kinase plays an important role during early embryogenic development in the sea urchin eggs (Epel et al., 1981). In this regard, it is interesting to note that plant NAD kinases have an apparent molecular weight ranging from 50,000 to 55,000 (Roberts et al., 1990). Our preliminary data suggest that carrot NAD kinase activity in the germinated embryos is 3 to 4 times higher than that in the non-germinated embryos. Although, these observations may suggest an intriguing possibility that the 54,000 M_r calmodulin-binding protein is carrot NAD kinase, the identity of the 54,000 M_r protein is unknown at the present time. Detection of NAD kinase activity from the extracts and the purified sample preparations has

been difficult, and may be due to the high instability of the enzyme (for a recent review see Roberts and Harmon, 1992). Future work involving characterization of the calmodulin-stimulated activity associated with the 54,000 M_r protein is necessary to understand what roles it plays during the germination of embryos.

Transgenic plants expressing VU-calmodulins as an approach to perturb the calmodulin/NAD kinase system <u>in vivo</u>

From a technical perspective, the use of the synthetic gene system offers certain advantages for studies of calmodulin in transgenic plant organisms. For example, the nature of the synthetic gene allows the facile construction of multiple recombinant vectors for plant transformation. In addition, due to slight differences in biochemical properties, it is easy to distinguish the foreign VU calmodulin from endogenous plant calmodulin. Thus, it is easy to discern the level of expression and to purify and characterize the expressed product.

Transgenic plants expressing a synthetic gene-encoded calmodulin (VU-1 calmodulin) and calmodulin derivatives with arginine (VU-3 calmodulin) or isoleucine (VU-4 calmodulin) at position 115 have been generated using transgenic plant technology. VU-3 and VU-4 calmodulins are not drastically different from endogenous calmodulins with respect to most activities. In fact, arginine substitutions at position 115

are observed with naturally occurring calmodulins such as that found in Schizosaccharomyces pombe (Takeda and Yamamoto, 1987). However, VU-3 and VU-4 calmodulin differ from wild type plant calmodulin in two major respects: 1. they activate NAD kinase to a higher maximal activity than endogenous plant calmodulin (Roberts et al., 1986b, 1990); and 2. they are not susceptible to lysine methylation (Roberts et al., 1986b). From the perspective of plants, the introduction of calmodulin derivatives into plants may allow the selective disruption of the NAD kinase regulatory system.

To obtain a high expression of synthetic VU calmodulins in transgenic tobacco plants, the calmodulin genes are cloned behind the 35S cauliflower mosaic virus promoter which is known as a promoter that confers constitutive gene expression (Benfey and Chua, 1989). Although, this is a strong promoter, the expression levels of calmodulins in the transgenic plants were only about two-fold greater than those in untransformed contol plants based on RIA and SDS-PAGE analysis. Recently, Zielinski et al. (1990) have transformed tobacco plants with a cauliflower mosaic virus 35S promoter and barley calmodulin cDNA construct and observed highly enhanced calmodulin mRNA levels in the transgenic plants. However, the change in overall calmodulin levels appeared to be minor. Therefore, these data suggest that translational or posttranslational control of calmodulin levels could be important in the regulation of calmodulin expression in plant tissues.

The VU-1 and VU-3 calmodulins expressed in transgenic plants were separated from endogenous tobacco calmodulin using anion-exchange Mono Q chromatography. The analysis of the isolated calmodulins showed that the VU-1 calmodulin is trimethylated at lysine 115 and has a reduced NAD kinase activator activity. However, VU-3 calmodulin, which is incapable of posttranslational methylation, retains its higher NAD kinase activator activity, a characteristic of unmethylated calmodulins. The observation that VU-3 calmodulin expressed in transgenic plants retains its elevated NAD kinase activation properties argues that this protein should exhibit this property in transgenic tobacco in vivo. In this regard, it is of interest to note that VU-3 transgenic tobacco plants have altered properties of growth and development that are not apparent in VU-1 transgenic nor control tobacco plants (Besl, 1991). Currently, it is unclear whether the phenotypic results are due to the perturbation of calmodulin/NAD kinase regulatory system or other defects of the calmodulin-dependent pathway in the plants. Future work involving comparison of NAD/NADP fluxes in transgenic and normal plants will us help to understand whether NAD kinase is affected, and may provide insight into which metabolic processes are involved.

Like VU-3 calmodulin, VU-4 calmodulin has the high NAD kinase activator properties of the unmethylated calmodulins (Roberts et al., 1986b, 1990) and is resistant to the

methylation of calmodulin at position 115. Further, it showed 20-fold higher inhibitor activity than VU-3 calmodulin for the calmodulin-lysine N-methyltransferase. 20 % of the F_0 VU-4 plants showed the same abnormalities in growth and development (i.e., dwarfism and low seed yield) that are observed in VU-3 plants. Based on preliminary analysis, there is no difference in expression levels of calmodulin in normal and abnormal VU-4 plants. Presently, the reason for the discrepancies between the VU-1 (no aberration), VU-4 (20 % aberration) and VU-3 plants (100 % aberration) is not known. Among the possible explanations are: 1. mutations or modifications in the VU-4 gene in the normal VU-4 plants; 2. more severe effects of VU-3 calmodulin than VU-4 calmodulin on plant signal transduction processes; 3. position of T-DNA insertion; and 4. somaclonal variation. With respect to the first possibility, future work including the isolation and characterization of the VU calmodulins from the representative normal and dwarf VU-4 plants should help address this possibility. The second possibility seems unlikely considering the known <u>in vitro</u> properties of the two calmodulins. The third possibility, position of T-DNA insertion, could be a factor for the phenotypes found in VU-4 transgenic plants. Presently, the molecular mechanism by which the T-DNA integrates into the plant genome is unclear. From the studies done by Gheysen et al., (1987), it has been found that the T-DNA integrates into the plant genome in a quite random manner. However, using T-

DNA to tag promoter sequence, Koncz et al. (1989) showed that the T-DNA frequently integrates into sequences that potentially can be transcribed. If the T-DNA is inserted into regions of tobacco genome that lead to inactivation of existing genes that are responsible for the growth and development, it could be a factor for the different phenotypes found in VU-4 transgenic tobacco plants. There is evidence that phenotypic mutants of plants are generated by T-DNA insertion mutagenesis (Feldmann et al., 1989; reviewed by Walden and Schell, 1990). In addition, if multiple insertions of the T-DNA containing calmodulin expression cassette take place, it could cause more substantial perturbation of the plant genome and of possibly calmodulin system compared with single copy insert of the T-DNA. The multiple insertions of T-DNA might be probable when the Agrobacterium transfers more than one copy of the T-DNA or when the T-DNA is replicated prior to its integration (Depicker et al., 1985; reviewed by Walden and Schell, 1990). Therefore, copy number effect could be factor for the different phenotypes found in VU-4 transgenic plants.

However, insertion effect (i.e., position effect, copy number effect) is an unlikely explanation for the VU-1 or VU-3 transgenic plants since all lines of the VU-1 or VU-3 plants showed the same phenotype. Further, Besl (1991) observed that the T-DNA insertion occurs at random in the VU-transgenic tobacco plants and that the phenotype of the plants appears to

be unrelated to the site of insertion. Future work including the determination of the target sites, the copy number, and the structure, of the T-DNA, in the genome of normal vs. dwarf VU-4 transgenic plants should help address this possibility.

The fourth possibility, somaclonal variation, could also be a factor for the abnormal phenotypes found in the VU-3 and VU-4 transgenic tobacco plants. It has become clear that somaclonal variation can occur mainly during tissue culture processes due to chromosomal modifications and rearrangements such as deletions, translocations, chromosome fusion and interchanges (reviewed by Scowcroft and Larkin, 1988). To regenerate and to select transformed plants, a series of tissue culture procedures are inevitable. In fact, somaclonal variation has been found in many cases from the regeneration of plants through tissue culture (Scowcroft, 1985). It is interesting to note that many somaclonal variants showed abnormalities in growth and development (i.e., height, leaf color, leaf shapes, seed number, seed size, etc) (for a review see Scowcroft, 1985). One possible interpretation is that the transformation of tobacco with VU-3 or VU-4 calmodulin results in increased somaclonal variation compared to that of VU-1 calmodulin, and that the mutant calmodulins cause an increase in the frequency of this event. However, it is important to note that Besl (1991) found no evidence of chromosomal aberrations in transformed VU-3 plants, which would argue against somaclonal variation. Further work including the

demonstration that antisense VU-3 or VU-4 constructs restore a normal phenotype, and southern blot analysis to probe for the rearrangement of the inserted DNA may help address this issue further.

Other evidence suggests that the perturbation of calmodulin system can cause decreases in growth characteristic of dwarfism. For example, Braam and Davis (1990) demonstrated that mechanical stimulation of Arabidopsis results in decreased stem growth and stem thickening that is accompanied by as high as a 100-fold change in calmodulin mRNA levels. In the case of VU-transgenic plants, the overall expression levels of calmodulin are about 2-fold increased as compared with untransformed normal plants. Further, the VU-1 transgenic plants which have trimethylated calmodulin at position 115 showed no abnormalities in the growth and reproduction, while retarded growth phenotypes were only associated with plants expressing the mutant calmodulins. Therefore, it is more likely that the observed abnormal phenotypes in VU plants are due to the expression of the calmodulin mutants rather than an alteration of calmodulin levels. However, the results with Arabidopsis and VU transgenic plants argue that altered stem growth is related to the perturbation of calmodulin system. Future studies with VU-3 and VU-4 transgenic plants will help us to understand what calmodulin-dependent pathways are affected by the expression of the methylation site calmodulin mutants and what

roles they play for plant growth and development.

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VITA

Suk-Heung Oh was born in Chonbuk province in Korea on November 24, 1960. He graduated from Namsung High School at Iri, Korea, in February, 1978. He entered Chonbuk National University, Chonju, Korea, in March, 1978 and graduated with a Bachelor of Science degree in Agricultural Chemistry in February, 1982. He continued his graduate study and received the degree of Master of Science in Agricultrual Chemistry from the Chonbuk National University in February, 1984. He served in the Army as a Reserve Officer from September 1984 to February 1985. He was employed by the Chonbuk National University as a teaching assistant from May 1983 to August 1987 except for the military service period.

He entered the University of Tennessee, Knoxville, in September, 1987 and received a Ph.D. in Biochemistry in May, 1992. He was employed by the University of Tennessee as a GA (graduate assistant) or GRA (graduate research assistant) from April 1988 to December 1991 and as a post-doctoral associate from January 1992. During the period of graduate work at the University of Tennessee, he received a Science Alliance Upgrade award three times (1989, 1990, and 1991) and a Science Alliance Half-time teaching Upgrade award two times (1990 and 1991) from the University. He presented his research data through oral or poster presentation at several scientific meetings including 1990 American Society of Plant

Physiologists Annual Meeting and the 75th FASEB (Federation of American Societies for Experimental Biology) Meeting. He also presented his data at several seminars and published several papers (see below for details).

He was married to Youn-Soo Cha in 1985 and they have a son, Jun-Ho.

Published Papers and Abstracts

(1) **Suk-Heung Oh** and Daniel M. Roberts (1990)
Analysis of the state of posttranslational calmodulin methylation in developing pea plants.
Plant Physiol. 93: 880 - 887.

(2) **Suk-Heung Oh** and Daniel M. Roberts (1990)
Developmental differences in posttranslational calmodulin methylation in pea plants (abstract).
Plant Physiol. Suppl. 93: 19.

(3) Daniel M Roberts, **Suk-Heung Oh**, Linda Besl, C. David Weaver and Gary Stacey (1990)
Attenuation of calmodulin-dependent NAD kinase activation by posttranslational methylation.
Current Topics in Plant Biochemistry and Physiology 9: 67-84.

(4) Linda Besl, **S.-H. Oh**, R. Masterson, D.M. Roberts, and G. Stacey (1990)
Investigations of calmodulin function using transgenic plants expressing mutant calmodulin genes (abstract).
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(5) **Suk-Heung Oh**, Linda Besl, Gary Stacey, and Daniel M. Roberts (1991)
Analysis of a calmodulin mutant defective in methylation in transgenic plants (abstract).
The FASEB Journal, 5: A449.

Work in preparation

(1) Daniel M. Roberts, Linda Besl, **Suk-Heung Oh**, Robert V. Masterson, and Gary Stacey
Effects of the expression of a calmodulin methylation mutant on the growth and development of transgenic tobacco plants, in preparation.

(2) **Suk-Heung Oh** et al.,
Isolation and characterization of transgenic tobacco calmodulin and calmodulin-mutants derived from a synthetic gene, in preparation.

(3) **Suk-Heung Oh**, Henry Stenier, Donald Dougall, and Daniel M. Roberts
Modulation of calmodulin methylation and calmodulin binding proteins during carrot cell growth and embryogenesis, in preparation.

Presentations at scientific meetings

(1) Jayant G. Joshi, **Suk-Heung Oh** and Sungwoo Cho
2nd International Congress of Plant Molecular Biology, Nov. 13 - 18 (1988), Jerusalem, Israel.

(2) **Suk-Heung Oh**
1st Annual Gatlinburg Symposium of Legume-Microbe Interactions, Jan. 8 (1990), Gatlinburg, TN

(3) **Suk-Heung Oh** and Daniel M. Roberts
9th Annual Symposium of Current Topics in Plant Biochemistry and Physiology, April 4 - 6 (1990), Columbia, MO

(4) Linda Besl, **S.-H. Oh**, R. Masterson, D.M. Roberts, and G. Stacey
9th Annual Symposium of Current Topics in Plant Biochemistry and Physiology, April 4 - 6 (1990), Columbia, MO

(5) **Suk-Heung Oh** and Daniel M. Roberts
American Society of Plant Physiologists Annual Meeting, July 29 - August 2 (1990), Indianapolis, Indiana

(6) Linda J. Besl, **S.-H. Oh**, R. Masterson, D.M. Roberts, and G. Stacey
2nd Annual Gatlinburg Symposium of Plant Biotechnology, Jan. 6 - 9 (1991), Gatlinburg, TN

(7) **Suk-Heung Oh**, Linda Besl, Gary Stacey, and Daniel M. Roberts
Federation of American Societies for Experimental Biology Annual Meeting, April 21 - 25 (1991), Atlanta, GA

(8) D.M. Roberts, **S.-H. Oh**, L. Besl, R. Masterson, and G. Stacey
3rd International Congress of Plant Molecular Biology, October 6 - 12 (1991), Tucson, Arizona

Seminars

(1) Invited speaker, Korean Scientists and Engineers Association (KSEA) in America, Tennessee Chapter Meeting, September 8, (1990), Knoxville, TN

(2) Seminar, Department of Agricultural Chemistry, Chonbuk National University, Chonju, Korea. December 21, 1990

(3) Seminar, Department of Biochemistry, University of Tennessee, Knoxville, November 27, 1991