Effects of Various Calmodulins on the Activation of Glutamate Decarboxylase and Nicotinamide Adenine Dinucleotide Kinase Isolated from Tobacco Plants

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Plants have been shown to contains Ca^{2+}/calmodulin-stimulated GAD and NAD kinase. To test how calmodulin and calmodulin methylation affect the activation of GAD and NAD kinase, GAD and NAD kinase were partially purified from tobacco plants. GAD was also partially purified from *E. coli* transformed with a plasmid carrying a cloned tobacco GAD gene. We find that GAD from the transformed *E. coli* showed 60-fold Ca^{2+}/calmodulin-dependent activation. However, GAD from tobacco plants was stimulated only about 3.8-fold by the addition of calmodulin in the presence of calcium, suggesting high background activity of the enzyme was possibly due to bound endogenous tobacco calmodulin. There were no significant differences in the tobacco GAD activator properties between calmodulins. A monoclonal antibody against petunia GAD interacted strongly with both GAD from tobacco plants and GAD from cloned gene. NAD kinase from tobacco plants showed a complete Ca^{2+}/calmodulin dependency for activity. Unmethylated calmodulins activated GAD in a manner similar to methylated calmodulin. However, the maximum level of NAD kinase activation obtained with unmethylated calmodulins is approximately 4-fold higher than methylated calmodulins. These data suggested that endogenous tobacco calmodulin may interact more tightly with GAD than NAD kinase and that calmodulin methylation affects the activator properties of calmodulins for tobacco NAD kinase but not for GAD.

Key words: *tobacco glutamate decarboxylase, NAD kinase, calcium, calmodulin*

Calcium plays a key role as a signal molecule in the regulation of intracellular events in eukaryotes. Calcium serves as an intracellular signal transducer by reversibly interacting with specialized calcium-binding proteins known as calcium-modulated proteins.[1)] Calmodulin is a calcium-modulated protein found in all eukaryotic cells examined and interacts with and regulates a variety of enzymes.[1,2)]

Plants have been shown to contains Ca^{2+}/calmodulin-stimulated GAD and NAD kinase.[3-5)] GAD catalyzes the decarboxylation of glutamate to CO_2 and GABA. In animals, GABA is an inhibitory neurotransmitter.[6)] The role of GABA in plants is unclear. GABA is known to be rapidly accumulated by a variety of environmental stress conditions including stimulation of the mechanical damage resulting from phytophagous activity of insects.[7)]

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Abbreviations: AOS, active oxygen species; DTT, dithiothreitol; GABA, gamma-aminobutyrate; GAD, glutamate decarboxylase; NAD, nicotinamide adenine dinucleotide; NADP, nicotinamide adenine dinucleotide phosphate; PEG, polyethylene glycol; PLP, pyridoxal 5'-phosphate; PMSF, phenylmethylsulfonylfluoride; PVPP, polyvinylpolypyrrolidone.

NAD kinase catalyzes the phosphorylation of NAD to NADP using ATP as a co-substrate. In most animal tissues, NAD kinase is not a calmodulin-stimulated enzyme. However, an exception to this is the Ca^{2+}/calmodulin-stimulated NAD kinase from human neutrophils.[8)] NADPH is utilized by NADPH oxidase in the generation of AOS for bactericidal activity in the neutrophils.[9)] Therefore, the elevation of NADP by Ca^{2+}/calmodulin-stimulated NAD kinase could be necessary to provide a rapid increase in substrate NADPH for the reductive processes. From these, it can be proposed that Ca^{2+}/calmodulin modulation of NAD kinase may serve *in vivo* as a regulatory mechanism for the oxidative burst response of plants to pathogens.[10)]

Previously it has been shown that pea NAD kinase is differentially activated by calmodulins from different sources.[11,12)] Further, the enzyme is sensitive to calmodulin methylation. For example, the level of enzyme activation by unmethylated calmodulins is at least 3-fold greater than the activation by methylated calmodulin.[12)] Although most calmodulin isolated from plant tissues is largely methylated, calmodulin methylation can vary depending on the developmental state of the plant roots.[13)] Based on the recent data from Ling *et al.*,[14)] the distribution of GAD in fava

bean roots parallels that of methylated calmodulin, which is highest in differentiated roots and lowest in the first centimeter section of root tips.[13] From these, it could be suggested that the degree of activation of tobacco NAD kinase and GAD by calmodulins is different, and calmodulin methylation may play a role in the activation of the tobacco enzymes.

In order to test how calmodulin and calmodulin methylation affect the activation of GAD and NAD kinase, GADs and NAD kinase were partially purified from tobacco plants and from *E. coli* carrying a plasmid with cloned tobacco GAD gene. The ability of calmodulins from various sources to activate the tobacco enzymes was tested. The present study shows: i) complete Ca^{2+}/calmodulin-dependent activation of tobacco GAD and NAD kinase; ii) different activation of tobacco NAD kinase by calmodulins and calmodulin methylation mutants.

Materials and Methods

Materials. Flasks with side arm for GAD assay were purchased from Kontes (New Jersey, USA), and L-[1-^{14}C]glutamic acid (55 mCi/mmol) were from Amersham (Buckinghamshire, England). Other chemicals with the highest grade were purchased from Sigma (St. Louis, USA). Tobacco plants (*Nicotiana tabacum* L. cultivar Wisconsin 38) were grown under standard conditions as described previously.[14,15]

Calmodulin purification. Calmodulins used in this study were purified from tobacco plants, bovine brain, chicken gizzard and *E. coli* carrying the calmodulin expression vector[16] by hydrophobic phenyl-Sepharose column chromatography of Gopalakrishna and Anderson.[17] SDS-PAGE of the fractions of the phenyl-Sepharose columns was done by using the method of Laemmli.[18] EGTA (1 mM) was added to the gel for the analysis to prevent the anomalous migration of calmodulin.[16] The fractions containing calmodulin were pooled and dialyzed against 20 mM NH_4HCO_3 and then extensively against deionized water. The dialized sample was frozen at -70°C and then lyophilized in an acid washed container. The lyophilized calmodulin was resuspended in 10 mM NH_4HCO_3 and was stored frozen at -70°C.

Partial purification of GAD from tobacco leaves. GAD from tobacco leaves was partially purified by a combination of ammonium sulfate precipitation and anion-exchange chromatography as described[4,19] with modifications. Fully expanded upper leaves from 3-month-old tobacco plants were harvested and immediately frozen in liquid nitrogen. The samples were ground to a fine powder with a mortar and pestle and transferred to 50 mM bis-Tris-HCl (pH 7.0), 1 mM EDTA, 1 mM DTT, 0.1 mM PLP, 1 mM PMSF, 10% (w/v) PVPP, 10% (v/v) glycerol. The homogenate was centrifuged at 23,000×g for 20 min, and the supernatant was used to prepare a 20 to 60% (w/v) ammonium sulfate pellet, which was solubilized in extraction buffer. The sample was then dialyzed overnight at 4°C against 50 mM bis-Tris-HCl (pH 7.0) buffer containing 1 mM EDTA, 1 mM DTT, 1 mM PMSF, and 10% (v/v) glycerol with several changes. The sample was applied to a 2.5×10 cm column of DEAE-cellulose pre-equilibrated with the dialysis buffer and washed with the same buffer. After washing, the column was eluted with a linear NaCl gradient (0~1.0 M) in equilibration buffer. The enzyme peak was pooled and concentrated by ultrafiltration on a Amicon YM-10 membrane.

Partial purification of GAD from *E. coli* expressing a cloned tobacco GAD gene. Tobacco GAD expressed in *E. coli*[20] was purified by the method of calmodulin-agarose chromatography as described previously[19] with modifications. *E. coli* cell cultures grown at 37°C with IPTG induction were harvested, and the pellets were resuspended in 50 mM Tris-HCl, pH 7.5, 0.2 mM EDTA, 1 mM DTT, 10% glycerol and 1 mM PMSF. Lysozyme was added to a concentration of 200 μg/ml, and the lysates were incubated on ice for 10 min. The samples were sonicated for 30 sec followed by 1 min rest (repeated 3 times) and centrifuged for 15 min at 4°C at full speed on a microcentrifuge, and supernatants were collected. $CaCl_2$ was added to the extract to final concentration of 10 mM, and the sample was passed through a 0.22-μm filter. The sample was loaded onto calmodulin-agarose column pre-equilibrated with 50 mM Tris-HCl, 1 mM $CaCl_2$, 150 mM NaCl, 10% glycerol, and 1 mM PMSF, pH 7.5. The column was washed with the equilibration buffer (20 column volumes). Then, the column was eluted with 25 mM Tris-HCl, 2 mM EDTA, 150 mM NaCl, 10% glycerol, and 1 mM PMSF, pH 7.5. The eluents were collected in Eppendorf tubes (0.5 ml each) and assayed for GAD activity and stored at -70°C.

Partial purification of NAD kinase from tobacco leaves. NAD kinase was extracted and partially purified using modifications of previously described procedures.[11,21] Frozen tobacco tissue powders were homogenized in 50 mM Tricine-HCl, pH 7.8, 250 mM sucrose, 5 mM $MgCl_2$, 0.5 mM EGTA, 2 μg/ml leupeptin, 0.3 μg/ml pepstatin, 1 mM PMSF, and 10% (v/v) PVPP. The homogenate was centrifuged at 18,000×g for 20 min at 4°C. NAD kinase in the supernatant was purified through the protimine sulfate precipitation (0.5 g/L) and PEG precipitation (425% fractionation) steps. The precipitate obtained from the PEG step was dissolved in 50 mM Tris-HCl, pH 7.5, 100 mM KCl, 5 mM $MgCl_2$, 0.2 mM EGTA, 0.1 mM DTT, 0.5 μg/ml leupeptin and was passed through DEAE-Sephadex A 25 column (2.5×5 cm) pre-equilibrated with the resuspension buffer. The effluent was used for NAD kinase assays without further purification since the the enzyme solution showed a complete Ca^{2+}/calmodulin dependency for activity. The enzyme partially purified was stored at -70°C until use.

Enzyme assays. GAD assays were performed using a radiometric method based upon L-[1-^{14}C]Glu-dependent $^{14}CO_2$ production.[5,19] Samples were incubated in a shaking water bath at 30°C for 30 min in 25 ml sealed flasks with side arm containing a CO_2 trap of 0.4 ml of 0.1 N NaOH and 2 ml of reaction medium. The reaction medium consisted of a 20~100 µl sample, 100 mM bis-Tris-HCl buffer (pH 7.0), 1 mM DTT, 0.5 mM PLP, 10% glycerol with 2.5 mM L-glutamate (0.1 µCi/reaction, Amersham). Calcium (as $CaCl_2$) and calmodulins were included in the reaction mixture at concentrations indicated in the figure legends. All reactions were initiated by the addition of purified GAD using microsyringe through rubber stopper on side arm into the reaction medium and incubated. Reactions were terminated by injection of 0.1 ml of 9 N H_2SO_4. Reaction flasks were left at 4°C overnight to ensure complete evolution of CO_2 and absorption by the sodium hydroxide trap before the ^{14}C content of the CO_2 trap was determined using liquid scintillation counter (Beckman, USA).

NAD kinase activity was assayed by the method of Harmon *et al.*[22] with slight modifications. The assay mixture contained 50 mM Tricine, pH 8.0, 3 mM $MgCl_2$, 2 mM NAD, 3 mM ATP, 2.5 mM $CaCl_2$ and 200 nM calmodulin. The assay was initiated by the addition of NAD kinase, and the assay tubes were incubated for 30 min at 30°C. The assay was terminated by placing the tubes in a boiling waterbath for 3 minutes and centrifuged in microcentrifuge for 10 min to remove debris. The supernatant (400 µl) was mixed with 0.5 ml of 500 mM Tris-HCl, pH 8.0, 30 µl of dichlorophenolindophenol (1 mg/ml), 20 µl of phenazine-methosulfate (1 mg/ml), 20 µl of glucose-6-phosphate (2.8 mg/ml). The mixture was transferred to a cuvette, and 30 µl of 10 units/ml glucose-6-phosphate dehydrogenase was added. The changes in absorbance at 600 nm per min was measured with a spectrophotometer (Shimazu, Japan).

Immunodetection of GAD on western blots. Protein samples were separated by SDS-PAGE on 12.5% (w/v) polyacrylamide gels and transferred to nitrocellulose membrane (0.2 µm, BioRad, USA). The presence of GAD was detected with an anti-GAD monoclonal antibody (mAb-107.1)[19] by using a chemiluminescence protocol with the ECL kit (Amersham, UK).

Results and Discussion

The ability of various calmodulin preparations to activate GAD and NAD kinase was examined. For these assays, calmodulins from various sources including tobacco, bovine brain, chicken gizzard and *E. coli* transformed with a plasmid carrying a recombinant DNA-derived calmodulin gene or mutant calmodulin gene were purified by a Ca^{2+}-dependent hydrophobic chromatography on phenyl-Sepharose column.[17] SDS-PAGE analysis of the fractions from the column shows that the only detectable proteins

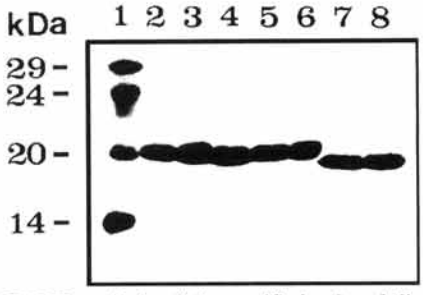

Fig. 1. SDS-PAGE analysis of the purified calmodulins from various sources. 10 µl of sample mixtures were loaded to the wells of 15% (w/v) SDS-polyacrylamide gel containing 1 mM EGTA. After electrophoresis, the proteins were stained with Coomassie Brilliant Blue. Lane 1, molecular weight markers; lane 2, chicken gizzard calmodulin; lane 3, recombinant DNA-derived calmodulin (lys 115, VU-1 calmodulin); lane 4, bovine brain calmodulin; lane 5 and 6, recombinant DNA-derived calmodulin mutant (ile 115, VU-4 calmodulin); lane 7 and 8, tobacco calmodulin. Animal calmodulins, recombinant DNA-derived calmodulin and calmodulin mutants show slower mobility than plant calmodulins as shown previously.[29]

were calmodulins (Fig. 1). GAD and NAD kinase were partially purified from tobacco plants and GAD was also partially purified from *E. coli* transformed with a plasmid carrying a cloned tobacco GAD gene. Immunodetection of GADs from tobacco leaf and the transformed *E. coli* with an anti-petunia GAD monoclonal antibody, which detects tobacco GAD,[23] demonstrated that 56~58 kDa GADs were obtained from the GAD preparations (Fig. 2).

To determine whether the tobacco leaf GAD is activated by calmodulin and whether activation is Ca^{2+}-dependent, GAD activity was measured in the presence or absence of Ca^{2+} and calmodulin. Fig. 3(A) shows that maximal activation of the GAD occurred in the presence of both Ca^{2+} and calmodulin. The activation of tobacco GAD by Ca^{2+}/calmodulin (376%) was higher than the activation of petunia GAD (276%)[3] and the activation of *Vicia fava* root GAD (212%).[4] Stimulation of the GAD even in the absence of added calmodulin (Fig. 3A, control and +Ca^{2+})

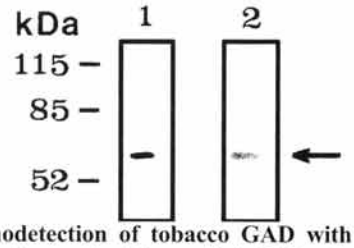

Fig. 2. Immunodetection of tobacco GAD with an anti-GAD monoclonal antibody. Partially purified GADs from tobacco leaf and *E. coli* transformed with a plasmid carrying cloned tobacco GAD gene were applied on 12.5% (w/v) SDS-polyacrylamide gels and transferred to nitrocellulose membranes, and the presence of GADs was detected with an anti-GAD monoclonal antibody (mAb-107.1)[19] by using a chemiluminescence protocol with ECL kit (Amersham). The antibody was provided by Dr. Hillel Fromm of Weizmann Institute of Science, Rehovot, Israel. The arrow indicates the position of the tobacco GAD. Lane 1, GAD from tobacco leaf; lane 2, GAD from the transformed *E. coli*.

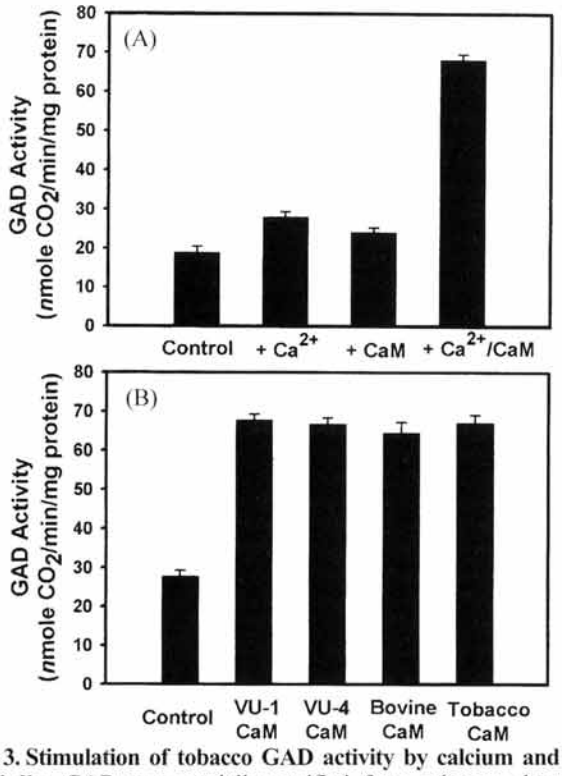

Fig. 3. Stimulation of tobacco GAD activity by calcium and calmodulin. GAD was partially purified from tobacco plants as described in Materials and Methods. GAD activity was determined by assaying L-[1-^{14}C]Glu-dependent $^{14}CO_2$ production.[5] (A) Response of GAD activity to calcium and calmodulin. Without the addition of Ca^{2+} and calmodulin (CaM) (Control). With the addition of 2.5 mM $CaCl_2$ (+Ca^{2+}), 200 nM VU-1 calmodulin (+CaM), or 2.5 mM $CaCl_2$ and 200 nM VU-1 calmodulin (+Ca^{2+}/CaM). (B) Activation of GAD by various calmodulins. Without the addition of calmodulin (Control) or with the addition of 200 nM calmodulin each. 2.5 mM $CaCl_2$ was added to the assay mixtures. Values are the means of three independent determinations with the standard error of the mean.

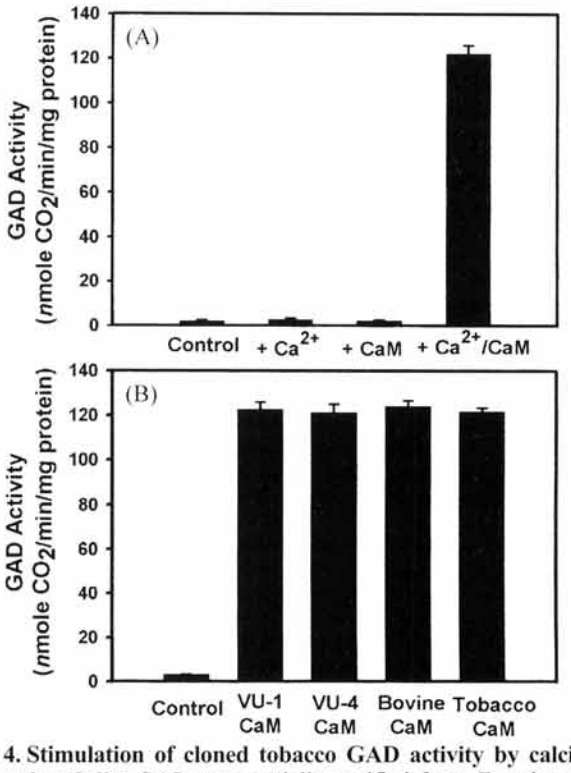

Fig. 4. Stimulation of cloned tobacco GAD activity by calcium and calmodulin. GAD was partially purified from *E. coli* transformed with a plasmid carrying a cloned tobacco tobacco GAD gene as described in Materials and Methods. GAD activity was determined with same conditions by assaying L-[1-^{14}C]Glu-dependent $^{14}CO_2$ production as described in the legend in Fig. 3. (A) Response of GAD activity to calcium and calmodulin. (B) Activation of GAD by various calmodulins. Without the addition of calmodulin (Control) or with the addition of 200 nM calmodulin each. 2.5 mM $CaCl_2$ was added to the assay mixtures. Values are the means of three independent determinations with the standard error of the mean.

may be interpreted as evidence for bound calmodulin. It has been observed that bound calmodulin in final protein preparation results in high background activity of plant Ca^{2+}/calmodulin-dependent enzymes.[24,25] A small increase in GAD activity with calmodulin in the absence of Ca^{2+} may be explained by calmodulin/GAD interactions in the absence of Ca^{2+} as observed.[3] To exclude the possible inability of removal of the bound calmodulin during partial purification of GAD, we utilized *E. coli* expression system to obtain calmodulin free GAD enzyme. GAD purified from *E. coli* carrying a plasmid containig tobacco GAD gene shows about 60-fold Ca^{2+}/calmodulin-dependent activation (Fig. 4A). The activity was not stimulated by the addition of Ca^{2+} or calmodulin alone. To test whether the tobacco GAD activation is affected by the state of calmodulin methylation, GAD activity was measured with various calmodulins. Figs. 3B and 4B show that there were no significant differences in the tobacco GAD activator properties between calmodulins. VU-1 and VU-4 calmodulin possess an unmethylated lysine or an unmethylatable isoleucine at position 115, respectively, rather than the methylated lysine residue characteristic of animal and higher plant calmodulins.[12,14,16,26]

In order to determine whether the tobacco NAD kinase is activated by Ca^{2+} and calmodulin, NAD kinase activity was measured in the presence or absence of Ca^{2+} and calmodulin. Fig. 5A shows that maximal activation of the NAD kinase occurred in the presence of both Ca^{2+} and calmodulin. In addition, NAD kinase purified from tobacco plants has a specific activity of 852 to 885 nmole NADP/min/mg protein with saturating VU-1 or VU-4 calmodulin in the presence of calcium. However, the specific activity of the NAD kinase measured with bovine brain or tobacco calmodulin was 231 to 242 nmole NADP/min/mg protein under the same conditions (Fig. 5B). Previous results have shown that pea NAD kinases are differently activated by calmodulins[12] and calmodulin isoforms.[27] As discussed above, VU-1 and VU-4 calmodulin possess an unmethylated lysine or an unmethylatable isoleucine at position 115, respectively, rather than the methylated residue characteristic of animal and higher plant calmodulins. Thus, it can be

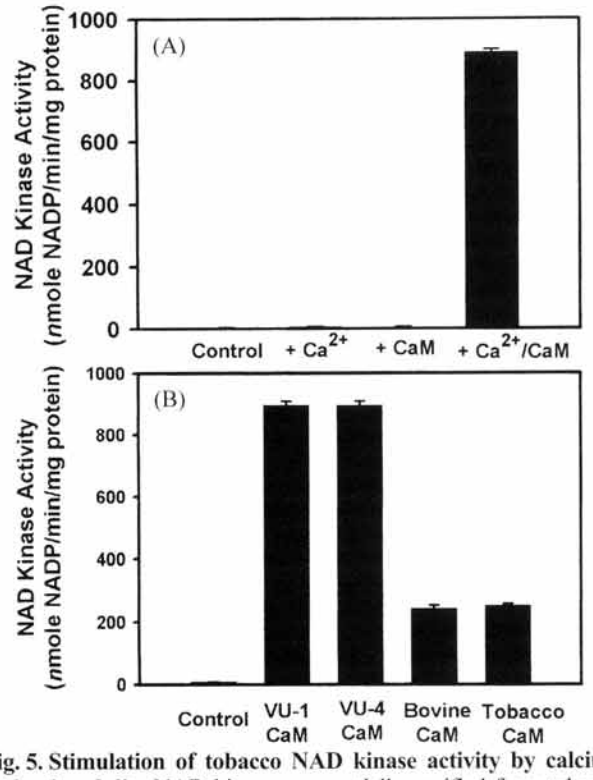

Fig. 5. Stimulation of tobacco NAD kinase activity by calcium and calmodulin. NAD kinase was partially purified from tobacco plants and assayed as described in Materials and Methods. (A) Response of NAD kinase activity to calcium and calmodulin. Without the addition of Ca^{2+} and calmodulin (CaM) (Control). With the addition of 2.5 mM $CaCl_2$ ($+Ca^{2+}$), 200 nM VU-1 calmodulin (+CaM), or 2.5 mM $CaCl_2$ and 200 nM VU-1 calmodulin ($+Ca^{2+}$/CaM). (B) Activation of NAD kinase by various calmodulins. Without the addition of calmodulin (Control) or with the addition of 200 nM calmodulin each. 2.5 mM $CaCl_2$ was added to the assay mixtures. Values are the means of three independent determinations with the standard error of the mean.

suggested that the reduction of the tobacco NAD kinase activation is the direct result of calmodulin methylation at position 115. These results confirm that the activation of Ca^{2+}/calmodulin-dependent plant NAD kinase is attenuated by posttranslational methylation.[12]

In this study, calmodulins isolated from various sources were used in activity assays of tobacco GADs and NAD kinase, but it remains to be determined to what extent calmodulin isoforms are involved in stimulating GADs and NAD kinase activities in tobacco. It has been shown that plants can have more than one type of calmodulin[1,27] and NAD kinase from pea can be activated differently by calmodulin isoforms.[27,28] Future work involving the purification of the calmodulin isoforms, analysis of tissue distribution, and identification of the potential targets is important to understand the roles of calmodulin isoforms existing in the same organism.

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