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Regulation of γ-Aminobutyric Acid Production in Tobacco Plants by Expressing a Mutant Calmodulin Gene

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In order to understand the biological role of calmodulin in plants, transgenic plants expressing a mutant calmodulin (VU-4, lys to ile-115) have been analyzed. We found that tobacco plants expressing VU-4 calmodulin have approximately twofold higher γ-aminobutyric acid (GABA) levels than the control plants. Cell suspension cultures established from the stem explants of the transgenic tobacco seedlings also have higher levels of GABA than the control cell cultures. Specific activity of glutamate decarboxylase (GAD), which catalyzes the decarboxylation of glutamate to CO_2 and GABA, of the transgenic tobacco cell extracts was about twofold higher than the activity of the control cell extracts. Western-blot analysis showed that the GAD is highly expressed in the transgenic tobacco plants. GAD partially purified from tobacco cell extracts showed approximately threefold Ca^{2+}/calmodulin-dependent activation. These data suggest that GABA synthesis in the transgenic tobacco plants is elevated, possibly due to higher levels of the calmodulin-dependent GAD enzyme and/or as a result of enhanced activation due to increased levels of the foreign calmodulin.

Key words : *γ-aminobutyric acid, glutamate decarboxylase, calmodulin, transgenic tobacco plant.*

Calcium plays a key role as a second messenger in plants and is involved in the physiological responses to many environmental stimuli.[1)] The biochemical targets of calcium signals are structurally related calcium-modulated proteins. Calmodulin is such a calcium-modulated protein found in all eukaryotic cells examined and interacts with and regulates many enzymes and proteins.[1,2)]

Plants have been shown to contain Ca^{2+}/calmodulin-stimulated GAD which catalyzes the decarboxylation of glutamate to CO_2 and GABA.[3,4)] In animals GABA is an inhibitory neurotransmitter,[5)] but the role of GABA in plants is unclear. GABA is known to be rapidly accumulated through a variety of environmental stress conditions including hypoxia, temperature shock, and mechanical manipulation.[6)] Interestingly, many of the same stresses that stimulate GABA accumulation in plants also cause fluxes in cytosolic Ca^{2+}.[3)] From these, it can be suggested that Ca^{2+}/calmodulin modulation of GAD may serve *in vivo* as a regulatory mechanism for environmental induction of GABA synthesis. In addition, the transgenic tobacco plants expressing calmodulin or calmodulin-dependent enzyme raise the possibility of generating plants more resistant to the environmental stresses.

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Abbreviations: DTT, dithiothreitol; GABA, γ-aminobutyric acid; GAD, glutamate decarboxylase; PLP, pyridoxal 5'-phosphate; PMSF, phenylmethylsulfonylfluoride; PVPP, polyvinylpolypyrrolidone.

Previously, transgenic tobacco plants that express a wild-type methylatable calmodulin (VU-1 calmodulin with lys-115) were generated and characterized.[7)] A second set of transgenic tobacco plants expressing a calmodulin mutant (VU-4 calmodulin) with a substitution of an isoleucine for lysine-115 were also generated and characterized.[8)] Such substitution renders this calmodulin incapable of posttranslational methylation. VU-1 and VU-4 calmodulins are stably expressed in the transgenic plants and elevate the overall calmodulin protein expression levels to twice that of the nontransformed plants. VU-4 transgenic tobacco plants have enhanced production of active oxygen species,[8)] possibly due to the expression of unmethylatable VU-4 calmodulin which activates tobacco NAD kinase fourfold higher than tobacco calmodulin.[9)] However, calmodulin methylation does not affect Ca^{2+}/calmodulin dependency for the activation of tobacco GADs, and VU-4 calmodulin activates tobacco GADs in a manner similar to tobacco calmodulin.[9)]

The present study aims to ascertain whether the expression of foreign calmodulin in tobacco plants changes GABA production system. We determined GABA content, GAD activity, and GAD protein levels on Western blots in the transgenic tobacco plants and cultured cells expressing VU-4 calmodulin.

Materials and Methods

Materials. Flasks with side arm for GAD assay were purchased from Kontes (Vineland, New Jersey, USA). L-[1-

^{14}C]Glutamic acid (55 mCi/mmol) and Western blotting detection reagents were from Amersham (Buckinghamshire, England). Other chemicals of the highest grade were purchased from Sigma (St. Louis, Missouri, USA). F_1 seeds from the transgenic tobacco plants were imbibed and germinated on MS agar[10] containing 50 μg/ml hygromycin. Tobacco plants were transferred and grown under standard conditions as described previously.[8] Stable suspension cell cultures were established from stem explants of 4-week-old control and VU-4 transgenic tobacco seedlings using the protocol of Harding *et al.*[7]

Determination of GABA contents in tobacco plants and cells. Free amino acids including GABA were extracted from the tobacco plants and cells using a modified procedure of Baum *et al.*[4] Fully expanded upper leaves of 3-month-old tobacco plants and 3-day-old cultured suspension cells were harvested and immediately frozen in liquid nitrogen. The samples were ground to a fine powder with a mortar and pestle. The tobacco powder (~200 mg) was transferred to 800 μl mixture of water:chloroform:methanol (3 : 5 : 12, v/v/v). The homogenate was centrifuged at 12,000 g for 15 min, and the aquous fraction of supernatant was collected. The pellet was dissolved by adding 200 μl chloroform and 400 μl water and centrifuged at the same conditions as mentioned above, and the aquous phase was collected. The collected fractions were combined, dried in a freeze dryer, and dissolved in water. Samples were passed through 0.45 μm PVDF filters (Millipore) and analyzed by using an AccQ · Tag amino acid analysis system (Waters).

Extraction of GAD from tobacco plants and cells. Extraction of tobacco plants and cells for GAD actvity measurement was done as described previously.[3,11] The tobacco 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, and 10% (v/v) glycerol. The homogenates were centrifuged at 23,000 g for 20 min, and the supernatants were collected. The samples were assayed for GAD activity.

Partial purification of GAD from suspension tobacco cells. To purifiy GAD from the extracts, the combined method of ammonium sulfate precipitation and anion-exchange chromatography[3] was used with modifications. Ammonium sulfate pellets, 20 to 60% (w/v), were obtained from the control suspension cell extract and then solubilized in the 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 through ultrafiltration on an Amicon YM-10 membrane.

GAD assay. GAD assays were performed using a radiometric method based upon L-[1-^{14}C]Glu-dependent $^{14}CO_2$ production.[3,11] Samples (20~100 μl) were pre-incubated in a shaking water bath at 30°C for 15 min in 25-ml sealed flasks with a side arm containing 100 mM bis-Tris-HCl buffer (pH 7.0), 1 mM DTT, 0.5 mM PLP, and 10% glycerol in a final volume of 1.9 ml. Purified calmodulin and calcium (as $CaCl_2$) were added during preincubation as required to final concentrations of 200 nM and 2.5 mM, respectively. After 15 min, a CO_2 trap containing 0.4 ml of 0.1 N NaOH (prepared fresh) was added to the flask, and the reaction was initiated by the addition of L-glutamate to a final concentration of 2.5 mM (0.1 μCi/reaction, Amersham) through rubber stopper on the side arm into the reaction medium and incubated for an additional 30 min. Reactions were terminated by injecting 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, Fullerton, California, USA).

Immunodetection of GAD on Western blots. Plant proteins were extracted using 100 mM KH_2PO_4-NaOH buffer (pH 7.4) containing 1 mM EDTA, 1 mM DTT, 0.1 mM PLP, 1 mM PMSF, 10% (w/v) PVPP, and 10% (v/v) glycerol. Protein samples were separated through 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) through a chemiluminescence protocol using an ECL kit (Amersham, UK).[8]

Calmodulin purification. Calmodulins used in this study were purified from *E. coli* carrying the calmodulin expression vectors[12] through hydrophobic phenyl-Sepharose column chromatography.[9] The fractions containing calmodulin estimated through SDS-polyacrylamide gel electrophoresis analysis were pooled and dialyzed against 20 mM NH_4HCO_3 and then extensively against deionized water. The dialized sample was frozen at -80°C and then lyophilized in an acid washed container. The lyophilized calmodulin was resuspended in 10 mM NH_4HCO_3 and stored frozen at -80°C.

General methods. SDS-PAGE was done using the method of Laemmli.[13] Protein concentrations were determined through the method of Bradford.[14]

Results and Discussion

GAD catalyzes the conversion of glutamate to GABA. Although several forms of GAD from a variety of sources have been described,[15,16] only plant GAD showed Ca^{2+}/calmodulin-dependent activation.[3,4]

To investigate whether the expression of a foreign calmodulin in tobacco plants changes GABA production system, we have analyzed transgenic tobacco plants and

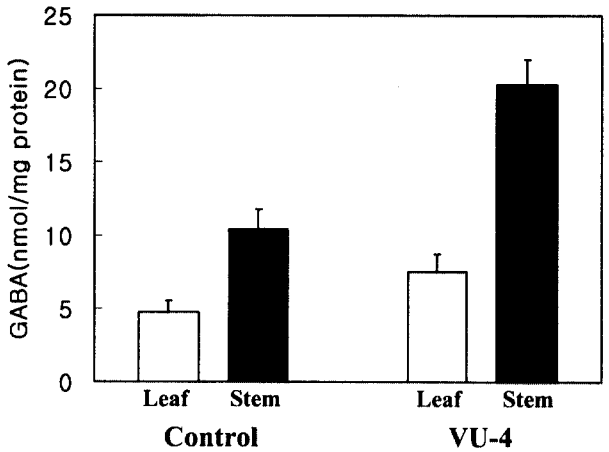

Fig. 1. GABA levels in control and VU-4 transgenic tobacco plants. Total free amino acids were extracted from leaves and stems of the control and VU-4 calmodulin plants as described in Materials and Methods. Levels of GABA were determined through AccQ · Tag amino acid analysis system (Waters). Histograms represent the mean of three independent determinations with error bars showing the standard error of the mean.

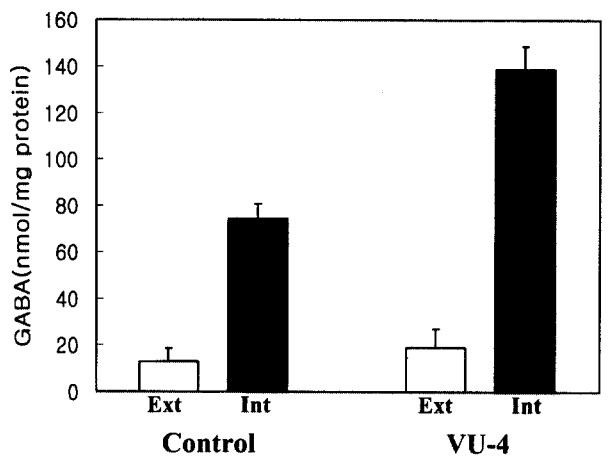

Fig. 2. GABA levels in control and VU-4 transgenic tobacco cells. Stable suspension cell cultures were generated from the control and VU-4 tobacco plants as described in Materials and Methods. Three-day-old cultured suspension cells were centrifuged at 1,500 g for 5 min and total free amino acids were extracted from the extracellular media and pellets as described in Materials and Methods. Levels of GABA were determined through AccQ · Tag amino acid analysis system (Waters). Histograms represent the mean of three independent determinations with error bars showing the standard error of the mean. Ext, extracellular; Int, intracellular.

cells expressing a mutant VU-4 calmodulin. Previously, it has been shown that the mutant calmodulin is stably and highly expressed in the transgenic plants.[8] Soluble free amino acids were extracted from tobacco leaves, stems and cells and analyzed through the AccQ · Tag amino acid analysis system. GABA levels were approximately twofold higher in VU-4 transgenic tobacco plants and cells compared to the control tobacco (Figs. 1 and 2). Higher GABA levels were detected in tobacco stems as compared

Table 1. GAD activities in control and VU-4 transgenic tobacco plant and cell extracts[a].

Tobacco sample		Activity	
		nmol CO_2/min/ mg protein	% of control
Leaf	Control	25.1 (1.1)[b]	100
	VU-4	34.4 (1.3)	137
Stem	Control	44.7 (0.7)	100
	VU-4	69.3 (1.2)	155
Cell	Control	67.1 (1.6)	100
	VU-4	132.9 (2.2)	198

[a]Extracts of fully expanded upper leaves (leaf), expanding upper stems (stem) from 3-month-old tobacco plants, and 3-day-old cell suspension cultures (cell) were prepared and assayed for GAD activity in the presence of 200 nM VU-4 calmodulin and 2.5 mM $CaCl_2$ as described in Materials and Methods. The GAD activities were standardized to total soluble protein in the extracts.

[b]Values show the means of three independent determinations with the standard errors in parenthesis.

with tobacco leaves (Fig. 1). In addition, GABA levels were significantly higher in the inside cells than in the extracellular media (Fig. 2).

The presence of GABA in plants has been known for at least half a century.[17] The role of GABA in plants mostly remains unknown, whereas its roles in animals is well-understood.[5] In animals GABA functions as a major inhibitory neurotransmitter by modulating the conductance of ion channels. Initially, Wallace *et al.*[18] proposed that GABA accumulation in plants may be a defence mechanism against phytophagous insects. The hypothesis that the phytophagous insect activity disrupts the normal cellular compartmentation, thus increasing the H^+ and Ca^{2+} levels in the cytosol, which stimulates the GABA synthesis, which in turn inhibits the growth and development of oblique-banded leaf-roller larvae, was investigated.[19] Stimulation of the mechanical damage resulting from phytophagous activity increased the soybean leaf GABA 10- to 25-fold within 1 to 4 min to 2.15 μmol GABA g^{-1} fresh weight. Introducing GABA of this level into a synthetic diet reduced the growth, developmental, and survival rates of cultured phytophagous larvae of the oblique-banded leaf roller.[19] In addition, more than 90% of oblique-banded leaf-roller larvae were found on light-green expanding leaves of apple trees, which produced lower GABA levels than dark-green mature leaves when mechanically damaged. Thus, the present findings raise the possibility of generating plants more resistant to the environmental stresses such as phytophagous insect attack.

To ascertain whether the expression of VU-4 calmodulin in tobacco plants changes GAD levels, GAD activity and GAD protein levels in tobacco plants and cells were examined. The specific activities of GAD were higher in VU-4 transgenic tobacco plant tissues and cells than those of

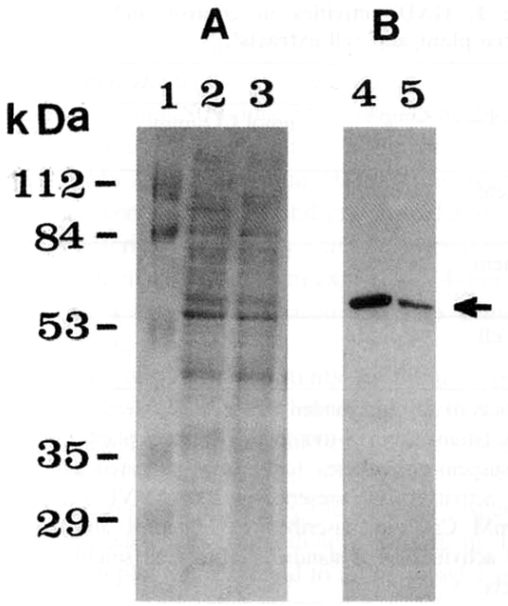

Fig. 3. Immunodetection analysis of GAD levels in control and transgenic tobacco cell cultures. Proteins (5.0 μg) from control and VU-4 tobacco cell cultures were resolved through SDS-PAGE on 12.5% polyacrylamide gels. (A) Coomassie blue-stained gel. (B) Western blot showing the presence of 56~58-kDa GAD, detected with an anti-GAD monoclonal antibody with a chemiluminescence protocol using an ECL kit (Amersham).[8] The arrow indicates the position of the tobacco GAD. Lane 1, Prestained protein standards; lane 2, proteins from VU-4 tobacco cell cultures; lane 3, proteins from control tobacco cell cultures; lane 4, GAD from VU-4 tobacco cell cultures; lane 5, GAD from control tobacco cell cultures.

control plants and cells (Table 1). GAD activity in stem extracts was higher than in leaves, and the highest specific activity was observed in the extracts of VU-4 cells which were established from stem explants of 4-week-old VU-4 transgenic tobacco seedlings. Immunodetection of GAD with an anti-petunia GAD monoclonal antibody, which detects tobacco GAD,[4] showed that 56~58 kDa GAD was present in tobacco cell extracts (Fig. 3), and changes in GAD protein level in the cell extracts coincided with changes in GAD activity (Fig. 3; Table 1), both showing approximately twofold increases in VU-4 tobacco cell cultures compared with the control cell cultures. The estimated molecular weight was similar to those of a cloned tobacco GAD and other plant GADs.[4,20] Previously, it has been suggested that Ca^{2+} and calmodulin might regulate the expression of genes by complex signaling cascades or by direct binding to transcription factors.[1] Genes involved in the development of photosynthetic complexes can be induced in a phytochrome-deficient *aurea* mutant of tomato through microinjection of Ca^{2+}-calmodulin.[21] In addition, UV-B light appears to modulate chalcone synthase gene expression in *Arabidopsis* cells through a calmodulin-mediated pathway.[22] Thus, the expression of foreign calmodulin in tobacco cells could cause the enhanced expression of GAD.

With calmodulin and GAD interaction mentioned above taken into consideration, we attempted to determine whether tobacco GAD is activated by calmodulin and whether the activation is Ca^{2+} dependent. GAD was partially purified from tobacco cell extracts, and the activity was measured in the presence or abscence of Ca^{2+} and calmodulin. Table 2 shows that maximal activation of the GAD occurred in the presence of both Ca^{2+} and calmodulin. The activation of GAD from tobacco cells by Ca^{2+}/calmodulin (289%) was higher than those of petunia GAD (269%) and *Vicia fava* root GAD (212%).[11,23] Stimulation of the GAD even in the absence of added calmodulin may be interpreted as an 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] 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.[23] The possible inability to remove the bound calmodulin during partial purification of tobacco GAD was excluded in utilizing *E. coli* expression system of cloned tobacco GAD gene[20] due to the fact that *E. coli* does not possess calmodulin and thus does not have a calmodulin-binding GAD.[20,25] GAD from the transformed *E. coli* showed a complete Ca^{2+}/calmodulin-dependency for activity.[20] The activity was not stimulated by the addition of Ca^{2+} or calmodulin alone.

Table 2. Calcium/calmodulin dependent activation of partially purified tobacco GAD[a].

Treatment	Activity	
	nmol CO_2/min/	% of control
Control (-Ca^{2+},-CaM)	0.56 (0.05)[b]	100
+Ca^{2+}	0.87 (0.09)	155
+CaM	0.77 (0.07)	138
+Ca^{2+}/CaM	1.62 (0.10)	289

[a]GAD was partially purified from the control tobacco cell extract through a combination of ammonium sulfate precipitation (20~60% saturation) and a DEAE-cellulose column chromatography. GAD assay was performed as described in Materials and Methods without the addition of Ca^{2+} and calmodulin (CaM) (control), and with the addition of 2.5 mM $CaCl_2$ (+Ca^{2+}), 200 nM VU-4 calmodulin (+CaM), and 2.5 mM $CaCl_2$ and 200 nM VU-4 calmodulin (+Ca^{2+}/CaM).
[b]Values represent the means of three independent determinations with standard errors shown in parenthesis.

Overall, the data suggest that GABA synthesis in transgenic plants expressing a calmodulin mutant is elevated, possibly due to higher levels of the calmodulin-dependent GAD enzyme and/or as a result of enhanced activation due to the increased levels of the foreign calmodulin. GAD is a well-characterized enzyme among the Ca^{2+}/calmodulin-dependent enzymes detected in plants.[1,6] Evidences show that many environmental stresses cause fluxes in cytosolic Ca^{2+} and the increased Ca^{2+} stimulates Ca^{2+}/calmodulin-dependent GAD.[6] GABA has been

postulated to have a role in nitrogen metabolism and storage[11] and in the plant's defence against phytophagous insects.[19] The regulation of GAD activity by Ca^{2+} and calmodulin is necessary for normal plant growth and development.[4] Integrated studies in model systems, such as genetically engineered plants expressing calmodulin and/or GAD, may provide further insight into the defence mechanism of plants during pathogen infection, phytophagous attack, and other environmental stresses.

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