

GENE NOTE

Calcium/calmodulin activation of two divergent glutamate decarboxylases from tobacco*

Dmytro P. Yevtushenko, Michael D. McLean, Sriyani Peiris, Owen R. Van Cauwenberghe and Barry J. Shelp[†]

Department of Plant Agriculture, Biotechnology Division, Bovey Building, University of Guelph, Guelph, Ontario, Canada N1G 2W1

Received 30 April 2003; Accepted 7 May 2003

Abstract

Glutamate decarboxylase (GAD, EC 4.1.1.15) catalyses the α -decarboxylation of glutamate to produce γ -aminobutyrate (GABA). The nucleotide sequences of two divergent GADs (designated *GAD1* and *GAD3*) were isolated from a *Nicotiana tabacum* L. cv. Samsun NN leaf cDNA library. Open reading frames indicated that *GAD1* encodes a polypeptide of 496 amino acids and has greater than 99% identity with known tobacco GADs, whereas *GAD3* encodes a polypeptide of 491 amino acids and has about 14% divergence from known tobacco GADs. Genomic DNA analysis suggested that there are at least four tobacco GAD genes, existing in pairs of highly identical genes. An *in vitro* assay at pH 7.3 revealed that activities of the recombinant proteins, after isolation from *Escherichia coli* and partial purification by nickel-affinity chromatography, are 57–133 times the control levels in the presence of 0.5 mM calcium and 0.2 μ M bovine calmodulin.

Key words: cDNA sequences, γ -aminobutyrate, glutamate decarboxylase, recombinant protein, tobacco.

γ -Aminobutyric acid (GABA) is a ubiquitous, non-protein amino acid that accumulates in plants in response to a variety of stress conditions such as temperature shock and hypoxia (see Shelp *et al.*, 1999, for a review). This rapid, stress-induced synthesis of GABA in plants involves the activation of glutamate decarboxylase (GAD) via a signal transduction pathway that involves Ca^{2+} /calmodulin (CaM). Earlier research suggests that all plant GADs, including those from petunia, tobacco and *Arabidopsis*, possess a CaM binding domain. However, Akama *et al.* (2001) recently identified two rice GAD isoforms, *OsGAD1* and *OsGAD2*, which encode polypeptides of 501 and 500 amino acids, respectively, and share 69% amino acid identity to each other. Interestingly, *OsGAD2*, unlike *OsGAD1*, is not activated by Ca^{2+} /CaM *in vitro*.

In order to investigate the possible occurrence in tobacco (*Nicotiana tabacum* L. cv. Samsun NN) of GAD genes, which do not possess a CaM binding domain, two divergent cDNAs from a leaf cDNA library were isolated and then extended according to McLean *et al.* (2003). One of the full-length cDNAs is extremely similar to two tobacco isoforms in the GenBank database (accession

numbers U54774 and AF020425, which are both named *NtGAD1*) and will be referred to here as *NtGAD1*. The *NtGAD1* found here is 1672 nt in length, encoding a predicted polypeptide of 496 amino acids, a molecular mass of 55.9 kDa, and a pI of 5.85. Another isoform of the same length, *NtGAD2* (GenBank accession number AF020424; Yun and Oh, 1998), has about 98% amino acid identity with the three *NtGAD1*s. The second cDNA found in this study, designated as *NtGAD3* cDNA is 1776 nt in length, encoding a predicted polypeptide of 491 amino acids, a molecular mass of 55.9 kDa, and a pI of 5.64. Over the entire length of the polypeptide, *NtGAD3* shares about 86% identity with the four other *NtGAD*s; however, within the 41 C-terminal amino acids there is only about 38% identity and 55% similarity to those sequences. Thus, the *NtGAD3* C-terminus is unique among the tobacco GAD sequences.

High-stringency hybridizations of genomic DNA digested with *EcoRI*, *HindIII* and *BclI* and with *NtGAD3*- and *NtGAD4*-specific probes, reveals different pairs of bands in each lane (Fig. 1), indicating the potential for a minimum of four GAD genes per tobacco genome. The simplest explanation for these results is that tobacco has at least two pairs of divergent GAD genes, which as pairs share high degrees of nucleotide sequence identity. The inbred nature of common tobacco cultivars makes it unlikely that each pair of bands represents sequences of allelic variants; however, each pair may represent alloallelic variants, which exist due to the allotetraploid nature of *Nicotiana tabacum* (Matassi *et al.*, 1991).

When the cDNAs for *NtGAD1* and *NtGAD3* were incorporated into an *E. coli* expression system, using methods modified from (Van Cauwenberghe *et al.* (2002), immunoblot analysis of total crude bacterial protein probed with an Anti-Xpress antibody (specific to the Xpress epitope that was added to the N terminus of the GAD cDNAs) revealed a major recombinant GAD band at approximately the predicted molecular mass of 59.9 kDa (Fig. 2). A second smaller recombinant band was located at approximately 50 kDa. Since this band, like the major band, was not present in the 'no insert' lane, it was probably a proteolytic product of the recombinant GAD. Crude extracts of both GAD lines were partially purified using nickel-affinity chromatography, and then used as a source of enzyme for *in vitro* assay of activity at pH 7.3, a pH known to be optimal for demonstrating Ca^{2+} /CaM activation of GAD activity (Snedden *et al.*, 1995). The addition of Ca^{2+} and bovine CaM dramatically stimulated

* The nucleotide sequences for *NtGAD1* and *NtGAD3* were deposited in the GenBank database under the accession numbers AAK18620 and AAK38667, respectively.

[†] To whom correspondence should be addressed. Fax: +519 767 0755. E-mail: bshelp@uoguelph.ca

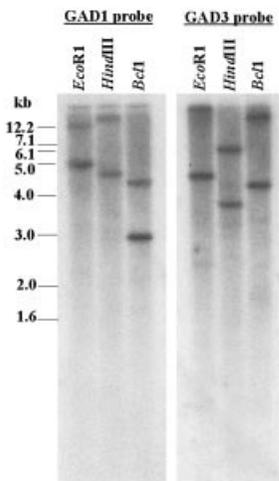


Fig. 1. Organization of GAD genes in the tobacco genome. Autoradiograms of identical Southern blots probed with *NtGAD1* and *NtGAD3* probes, as indicated at the top of the figure, are presented. Lanes with DNA samples digested with *EcoRI*, *HindIII*, and *BclI* are indicated. Molecular weight DNA markers, in kb, are shown on the left.

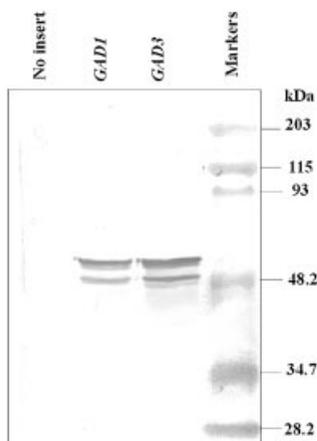


Fig. 2. Immunoblot analysis of total crude protein ($16 \mu\text{g lane}^{-1}$) from *Escherichia coli* cells containing no insert:pTrcHisB, *NtGAD1*:pTrcHisB or *NtGAD3*:pTrcHisB. The first three lanes were probed with an Anti-Xpress antibody specific to the recombinant protein. Molecular mass protein markers, in kDa, are shown in the right lane.

the activities of both recombinant tobacco GAD isoforms (57–133 times the control), despite their relatively high degree of divergence (Table 1). In the present report, no evidence was found for the

Table 1. Responsiveness of recombinant tobacco GAD isoforms to Ca^{2+} /CaM *In vitro*

GAD activity was measured at pH 7.3 in the absence or presence of $0.2 \mu\text{M}$ CaM and 0.5 mM calcium using the radiometric method described previously (Snedden *et al.*, 1995). Data represent the mean \pm SE of three separate experiments.

Treatment	Activity ($\text{nmol min}^{-1} \text{ mg}^{-1} \text{ protein}$)	
	GAD1	GAD3
$-\text{Ca}^{2+}/\text{CaM}$	0.04 ± 0.02	0.44 ± 0.07
$+\text{Ca}^{2+}/\text{CaM}$	5.30 ± 0.45	25.17 ± 1.74

occurrence of tobacco GAD genes, which do not possess a CaM binding domain. Previously, Yun and Oh (1998) showed that the *NtGAD2* isoform also binds CaM. These results are similar to those found with two divergent GAD isoforms from *Arabidopsis* (Turano and Fang, 1998; Zik *et al.*, 1998).

Acknowledgements

Grants from the Natural Sciences and Engineering Research Council (NSERC) of Canada Strategic Grants Program and the Discovery Research Grants Program to BJS supported this research.

References

- Akama K, Akihiro T, Kitagawa M, Takaiwa F. 2001. Rice (*Oryza sativa*) contains a novel isoform of glutamate decarboxylase that lacks an authentic calmodulin-binding domain at the C-terminus. *Biochimica et Biophysica Acta* **1522**, 143–150.
- Matassi G, Melis R, Macaya G, Bernardi G. 1991. Compositional bimodality of the nuclear genome of tobacco. *Nucleic Acids Research* **19**, 5561–5567.
- McLean MD, Yevtushenko DP, Deschene A, Van Cauwenberghe OR, Makhmoudova A, Potter JW, Bown AW, Shelp BJ. 2003. Overexpression of glutamate decarboxylase in transgenic tobacco plants confers resistance to the northern root-knot nematode. *Molecular Breeding* **11**, 277–285.
- Shelp BJ, Bown AW, McLean MD. 1999. Metabolism and functions of γ -aminobutyric acid. *Trends in Plant Science* **4**, 446–452.
- Snedden WA, Arazi T, Fromm H, Shelp BJ. 1995. Calcium/calmodulin activation of soybean glutamate decarboxylase. *Plant Physiology* **108**, 543–549.
- Turano FJ, Fang TK. 1998. Characterization of two glutamate decarboxylase cDNA clones from *Arabidopsis*. *Plant Physiology* **117**, 1411–1421.
- Van Cauwenberghe OR, Makhmoudova A, McLean MD, Clark SM, Shelp BJ. 2002. Plant pyruvate-dependent gamma-aminobutyrate transaminase: identification of an *Arabidopsis* cDNA and its expression in *Escherichia coli*. *Canadian Journal of Botany* **80**, 933–941.
- Yun SJ, Oh SJ. 1998. Cloning and characterization of a tobacco cDNA encoding calcium/calmodulin-dependent glutamate decarboxylase. *Molecules and Cells* **8**, 125–129.
- Zik M, Arazi T, Snedden WA, Fromm H. 1998. Two isoforms of glutamate decarboxylase in *Arabidopsis* are regulated by calcium/calmodulin and differ in organ distribution. *Plant Molecular Biology* **37**, 967–975.