GENE NOTE

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

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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 μM 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 Ca2+/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 Ca2+/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 NtGAD1s. 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 NtGADs; 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 Cauwenbergh 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 Ca2+/CaM activation of GAD activity (Snedden et al., 1995). The addition of Ca2+ 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.

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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 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).

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

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Activity (nmol min(^{-1}) mg(^{-1}) protein)</th>
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<tbody>
<tr>
<td></td>
<td>GAD1</td>
</tr>
<tr>
<td>-Ca(^{2+})/CaM</td>
<td>0.04±0.02</td>
</tr>
<tr>
<td>+Ca(^{2+})/CaM</td>
<td>5.30±0.45</td>
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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 \(\mu\)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.

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References


