Cloning and Characterization of a Rice cDNA Encoding Glutamate Decarboxylase

Suk-Heung Oh*, Won-Gyu Choi, In-Tae Lee and Song Joong Yun†
Department of Biotechnology, Woosuk University, Jeonju 565-701, Korea
†Division of Biological Resources Science, Chonbuk National University, Jeonju 561-756, Korea

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In this study, we have isolated a rice (Oryza sativa L.) glutamate decarboxylase (RicGAD) clone from a root cDNA library, using a partial Arabidopsis thaliana GAD gene as a probe. The rice root cDNA library was constructed with mRNA, which had been derived from the roots of rice seedlings subjected to phosphorus deprivation. Nucleotide sequence analysis indicated that the RicGAD clone was 1,712 bp long, and harbors a complete open reading frame of 505 amino acids. The 505 amino acid sequence deduced from this RicGAD clone exhibited 67.7% and 61.9% identity with OsGAD1 (AB056060) and OsGAD2 (AB056061) in the database, respectively. The 505 amino acid sequence also exhibited 62.9%, 64.1%, and 64.2% identity with Arabidopsis GAD (U9937), Nicotiana tabacum GAD (AF020425), and Petunia hybrida GAD (L16797), respectively. The RicGAD was found to possess a highly conserved tryptophan residue, but lacks the lysine cluster at the C-proximal position, as well as other stretches of positively charged residues. The GAD sequence was expressed heterologously using the high copy number plasmid, pVUCH. Our activation analysis revealed that the maximal activation of the RicGAD occurred in the presence of both Ca²⁺ and calmodulin. The GAD-encoded 56-58 kDa protein was identified via Western blot analysis, using an anti-GAD monoclonal antibody. The results of our RT-PCR analyses revealed that RicGAD is expressed predominantly in rice roots obtained from rice seedlings grown under phosphorus deprivation conditions, and in non-germinated brown rice, which is known to have a limited phosphorus bioavailability. These results indicate that RicGAD is a Ca²⁺/calmodulin-dependent enzyme, and that RicGAD is expressed primarily under phosphate deprivation conditions.

Keywords: Activation, Calmodulin, Expression, Glutamate decarboxylase, Rice

Introduction

Glutamate decarboxylase (GAD) catalyzes the conversion of L-glutamate to γ-aminobutyric acid (GABA). The presence of GAD activity and GABA in plants was first detected at least half a century ago (Satyanarayan and Nair, 1990; Bouche and Fromm, 2004). The role played by GABA in plants, however, remains to be precisely delineated, whereas its function as an inhibitory neurotransmitter in animals is fairly well understood (Erlander and Tobin, 1991; Mody et al., 1994; Lee et al., 2001).

There is ample evidence in the relevant literature demonstrating that GABA is rapidly and abundantly accumulated in a variety of plant tissues, under several environmental stress conditions, including mechanical stimulation, damage, cold shock, heat shock, hypoxia, cytosolic acidification, darkness, water stresses, and hormonal changes (Shelp et al., 1999; Bouche and Fromm, 2004). Many of the stresses known to induce GABA generation in plants also induce increases in the levels of cytosolic Ca²⁺ (Knight et al., 1991).

Transient increases in cytosolic Ca²⁺ levels are transmitted via Ca²⁺-modulated proteins, most notably calmodulin. Calmodulin activates a number of target enzymes, including calcium/calmodulin-dependent protein kinases and phosphatases (Roberts and Humon, 1992; Bandyopadhyay et al., 2004). Ca²⁺-stimulated GAD activity has been previously observed via the screening of a petunia cDNA expression library with 35S-labeled calmodulin (Baum et al., 1993). Recently, a series of cDNA clones encoding Ca²⁺/calmodulin-dependent GADs have been isolated and characterized from a variety of dicotyledonous species, including petunia, tomato, tobacco, and the fava bean (Shelp et al., 1999; Bouche and Fromm, 2004). Transgenic studies of petunia GAD revealed that the calmodulin-mediated modulation of GAD activity is crucial for both the growth and development of transgenic tobacco plants (Baum et al., 1996).

More recently, Akama et al. (2001) demonstrated that monocotyledons, such as rice, harbor at least two divergent
GAD isoforms, namely, OsGAD1 and OsGAD2. The OsGAD1 protein harbors a calmodulin-binding domain, as do all dicot GADs thus far analyzed. However, the OsGAD2 protein is quite likely to lack an authentic calmodulin-binding domain at its C-terminal domain (Akama et al., 2001). In order to accurately determine the mechanisms underlying GAD regulation at the molecular level, as well as the physiological role played by GABA in monocot plants, we isolated a novel GAD clone from a rice root cDNA library constructed with mRNA derived from the roots of rice seedlings which had been subjected to phosphorus deprivation. In this study, we report the sequence and expression patterns of the RicGAD clone, along with the functional characteristics of the expressed protein.

Materials and Methods

Materials  The side arm flasks used in our GAD assay were obtained from Korres (New Jersey, USA) and the L-[U-14C] glutamic acid (55 mCi/mmol) was purchased from Amersham (Buckinghamshire, England). The chitosan was supplied by EL-Chitosan Korea Co., Ltd., located in Jeonju, Korea. The brown rice was obtained from a marketplace in Jeonju, Korea. All of the other reagents used in this study were purchased from commercial sources, and were of the highest available grade.

cDNA clone isolation and sequence analysis A partial Arabidopsis thaliana GAD (AIGAD) gene (Oh et al., 2001) was employed as a probe for the screening of a rice root cDNA library. This rice root cDNA library was constructed using a Lambda ZAP-cDNA library construction kit ( Stratagene, La Jolla, USA), in accordance with the manufacturer’s instructions. The mRNA was prepared from the roots of 4-week-old rice seedlings (Oryza sativa L. cv. Dongjin) which had been subjected to phosphorus deprivation for the prior one week. AIGAD was selected for study, due to the high homology of its sequence as compared with those of other plant GADs (Baum et al., 1993; Oh et al., 2001). A hybridization probe was prepared via the random-oigomeric acid priming of the AIGAD gene, using a DIG DNA labeling kit (Boehringer Mannheim, Germany). A putative rice GAD clone was then isolated from 5 x 10⁴ pfu of the rice root cDNA library, and was designated as RicGAD (GenBank Accession number: AF428025). Both strands of the clone were sequenced via dideoxynucleotide termination (Sanger et al., 1977), conducted by a commercial sequencing service program. Restriction enzyme sites, as well as the nucleotide and deduced amino acid sequences, were analyzed using DNASIS software (Hitachi Software Engineering Co., USA) and NCBI database search programs.

Protein expression and cell extract preparation The complete open reading frame (ORF) sequence of RicGAD was then cloned into the pVUCH expression vector ( Lukas et al., 1987) using the EcoRI site, which contains an ATG start codon and a 3'-end KpnI site. The recombinant expression vector was then transformed into the UT481 E. coli strain, and was expressed via the addition of isopropyl-beta-D-thiogalactopyranoside ( IPTG, final concentration of 0.5 mM) to the culture. E. coli cell cultures, grown at 37°C with IPTG induction, were then harvested, and the pellets were resuspended in 50 mM Tris-HCl, pH 7.5, 0.2 mM ethylenediaminetetraacetic acid (EDTA), 1 mM dithiothreitol (DTT), 10% glycerol (v/v), and 1 mM phenylmethylsulphonyl fluoride (PMSF). Lysozymes were added to a concentration of 200 µg/ml, and the resultant lysates were incubated for 10 minutes on ice. The samples were then sonicated for 30 seconds, followed by 1 minute of rest (repeated 3 times), and then centrifuged for 1.5 minutes at 4°C at full speed in a microcentrifuge. The resulting supernatants were harvested and used in the GAD assay.

GAD assay The GAD assay was performed via a radiometric technique, which is predicated on L-[1-14C]Glut-dependent CO₂ production ( Snedden et al., 1995; Oh, 2003) with some modifications. The samples were incubated with agitation in a water bath at 30°C for 10 minutes in 25 ml sealed flasks, with a side arm containing a CO₂ trap, consisting of 0.4 ml of 0.1 N NaOH and 2 ml of reaction medium. The reaction medium was composed of a 20–100 µl sample, 100 mM biss-Tris-HCl buffer (pH 7.0), 1 mM DTT, 0.5 mM pyridoxal 5'-phosphate (PLP), and 10% glycerol (v/v) with 2.5 mM L-glutamate (0.1 µCi/reaction; Amersham). Calcium (as CaCl₂) and calmodulin were both included in the reaction mixture at the concentrations listed in the figure legends. All reactions were initiated by the addition of the GAD sample into the reaction medium, via injection through the rubber stopper with a microsyringe. The samples were then incubated at 30°C for 40 minutes, and the reactions were terminated via the addition of 0.1 ml of 0.9 N H₂SO₄. The reaction flasks were then allowed to stand at 30°C overnight, in order to ensure the complete evolution of the CO₂ and absorption by the sodium hydroxide trap, prior to the determination of the 14C content of the CO₂ trap, which was accomplished by liquid scintillation counting (LS3801, Beckman, USA).

Western blot analysis The protein samples were separated on 12.5% SDS-PAGE gel, and transferred to nitrocellulose membranes (0.2 µm, Bio-Rad, USA), and the presence of GAD was detected using anti-GAD monoclonal antibody (mAb-107.1, Snedden et al., 1996) using a chemiluminescence protocol with an ECL kit (Amersham, UK), as has been previously described (Ahn et al., 2004).

Expression patterns of RicGAD in rice roots and brown rice The patterns of RicGAD expression in the rice roots and the brown rice (Oryza sativa L. cv. Dongjin) were evaluated via RT-PCR analysis. The rice roots were acquired from 4-week-old rice seedlings which had been grown in a standard nutrient solution, or had been subjected to phosphorus deprivation conditions, as previously described ( Lim et al., 2003). In order to obtain the brown rice samples, we removed the hulls of the rice seed's. The dehulled rice seed's are referred to as non-germinated brown rice. In order to obtain germinated brown rice, non-germinated brown rice (30 g) was soaked in distilled water at 25~26°C in the dark, for 72 hours. The germinated brown rice which exhibited roots of 3-4 mm in length was then harvested. The samples were air-dried and frozen in liquid nitrogen, then ground with a mortar and pestle, as previously described (Oh, 2003). The total RNA was isolated from the samples using an RNA extraction kit (Sigma, St. Louis, USA). The sequences of the primers for RT-PCR were identical to the
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Results and Discussion

In the present study, we report the structural and functional characteristics of a novel GAD clone, **RicGAD**, derived from rice. The 1,712 bp nucleotide sequence of **RicGAD** harbors an ORF consisting of 505 amino acids, and nontranslated 5' and 3' flanking sequences, including an 18 bp poly(A) tail. The entire RicGAD cDNA nucleotide sequence exhibited 84.5% and 54.7% identity to the rice GAD sequences deposited in the GenBank database, **OsGAD1** (AB056060) and **OsGAD2** (AB056061), respectively (Fig. 1A). The deduced amino acid sequence from the ORF of **RicGAD** exhibited 67.7% and 61.9% identity to the **OsGAD1** and **OsGAD2** sequences deduced from **OsGAD1** and **OsGAD2**, respectively. **RicGAD** and **OsGADs** share nearly complete identity within their putative active site domains. These domains harbor a conserved S-X-X-K motif, in which a lysine (K) is the site responsible for binding to a cofactor pyridoxal 5'-phosphate and is conserved in all currently analyzed plant GAD sequences (Fig. 1B) (Akama et al., 2001).

Plant GADs, including **RicGAD**, **OsGAD1**, and **OsGAD2**, exhibit an approximately 30 amino acid extension in their C-terminal regions, which has never been detected in bacterial or animal GADs (Ueno, 2000). The multifunctional role of this C-terminal extension has been amply demonstrated by the results of studies using petunia GAD. This region appears to be required for the binding of GAD to calmodulin, and the tryptophan and C-proximal lysine cluster in this region constitute the most important residues in the process of calmodulin binding. GAD activity is stimulated by Ca\(^{2+}\)/calmodulin (Snedden et al., 1996). It has been previously demonstrated that **OsGAD1** possesses this highly conserved tryptophan residue and C-proximal lysine cluster, but that **OsGAD2** does not (Akama et al., 2001). Fig. 2A shows a
comparison of the deduced C-terminal amino acid sequences of rice GADs (RiGAD) and tobacco GAD (NtGAD2) as well as rice GADs (OsGAD1 and OsGAD2). Identical and similar amino acids are marked by black boxes. (B) Hydrophobic analysis of the C-terminal regions. As is shown in Fig. 2B, hydrophobic plots from the C-terminal regions of RiGAD, OsGAD1, and OsGAD2 display differing features. These observed differences between the C-terminal regions of RiGAD, OsGAD1, and OsGAD2 suggest possible differences in the calmodulin-binding ability of the three.

In order to verify that the RiGAD gene encodes a rice GAD, RiGAD was expressed in E. coli, utilizing the high
The GAD assay was performed using a radiometric method based on L-[1-14C]glutamate-dependent CO₂ production (Snedden et al., 1996) with modifications. Without the addition of Ca²⁺ and calmodulin (CaM) (-Ca²⁺/-CaM), with the addition of 2.5 mM CaCl₂ (+Ca²⁺), with the addition of 200 nM spinach calmodulin (+SPCaM), and with the addition of 2.5 mM CaCl₂ and 200 nM spinach calmodulin (+Ca²⁺/+SPCaM). Spinach calmodulin was purified by hydrophobic phenyl-Sepharose column chromatography of Gopalakrishna and Anderson (1982) as described (Oh and Yun, 1999).

Values represent the means of three independent determinations with standard errors shown in parenthesis.
of rice seeds appears to have induced activities (Seshu and Dadlani, 1991). Thus, the hull removal substantially, but augmented amylase and dehydrogenase activities. Hull removal reduced peroxidase activity seeds to moist heat treatment, as well as hull removal (Seshu and Dadlani, 1991). Hull removal reduced peroxidase activity. Dormancy in rice seeds is imposed by the physical and chemical factors associated with its covering structures, rice. Dormancy in rice seeds is imposed by the physical and chemical factors associated with its covering structures, including the hull and the pericarp. However, dormancy in all rice cultivars was completely released via the exposure of the seeds to moist heat treatment, as well as hull removal (Seshu and Dadlani, 1991). Hull removal reduced peroxidase activity substantially, but augmented amylase and dehydrogenase activities (Seshu and Dadlani, 1991). Thus, the hull removal of rice seeds appears to have induced RicGAD expression, and 72 hours of germination of the dehulled rice seeds (brown rice) significantly attenuated the expression of RicGAD (Fig. 4). It has been previously demonstrated that GAD mRNA is undetectable in dry petunia seeds, but that GAD protein is clearly apparent (Chen et al., 1994). GAD activity was detected at very early germination stages, and GAD activity increased with the onset of growth (Trinomi and Slaugher, 1971; Vandewalle and Olsson, 1983). The results of the expression analysis conducted in this study suggest that RicGAD transcription is regulated in a stage-specific manner, to some extent (Fig. 4). All plant seeds and cereals contain phytate, which is the principal form in which phosphorus is stored, and accounts for more than 70-80% of the total phosphorus in seeds (Novak and Haslberger, 2000; Kim et al., 2002). The levels of plant phytases, phosphatase enzymes that hydrolyze phytate to myo-inositol and phosphate, have been shown to be elevated during the germination of plant seeds (Barnick and Szafiranska, 1987; Laboure et al., 1993). Therefore, the possible increase of phosphate levels in the brown rice which was germinated for 72 hours may have been the cause of the observed suppression of RicGAD expression. Further studies involving expression analyses of RicGAD and other GADs, such as OsGAD1 and OsGAD2, at different stages of development, may provide further insights into the temporal and tissue-specific aspects of GAD expression in rice plants. In summary, we have cloned a novel glutamate decarboxylase (RicGAD) clone from a rice root cDNA library. The RicGAD is a Ca<sup>2+</sup>/calmodulin-dependent enzyme. The RicGAD is expressed primarily under phosphate deprivation conditions, and its expression appears to be regulated in a stage-specific manner, to some extent, by transcriptional processes. The role of RicGAD expression in rice development, however, remains to be elucidated. Further research with the RicGAD gene obtained in this study may facilitate future study of the molecular mechanisms underlying GABA metabolism in rice plants.

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References


