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Cloning and Characterization of a Rice cDNA Encoding Glutamate Decarboxylase

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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 to *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 Ca^{2+} 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 Ca^{2+}/calmodulin-dependent enzyme, and that *RicGAD* is expressed primarily under phosphate deprivation conditions.

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

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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 Ca^{2+} (Knight *et al*., 1991).

Transient increases in cytosolic Ca^{2+} levels are transmitted via Ca^{2+}-modulated proteins, most notably calmodulin. Calmodulin activates a number of target enzymes, including calcium/calmodulin-dependent protein kinases and phosphatases (Roberts and Harmon, 1992; Bandyopadhyay *et al*., 2004). Ca^{2+}-stimulated GAD activity has been previously observed via the screening of a petunia cDNA expression library with ^{35}S-labeled calmodulin (Baum *et al*., 1993). Recently, a series of cDNA clones encoding Ca^{2+}/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 Kontes (New Jersey, USA) and the L-[U-^{14}C] 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 (AtGAD) 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. AtGAD 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-oligonucleotide priming of the AtGAD gene, using a DIG DNA labeling kit (Boehringer Mannheim, Germany). A putative rice GAD clone was then isolated from 5×10^4 *pfu* of the rice root cDNA library, and was designated as RicGAD (GenBank Accession number: AY428025). 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 *Eco*RI site, which contains an ATG start codon and a 3'-end *Kpn*I 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 15 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-^{14}C]Glu-dependent $^{14}CO_2$ 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 CO_2 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 bis-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 $CaCl_2$) 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 9 N H_2SO_4. The reaction flasks were then allowed to stand at 4°C overnight, in order to ensure the complete evolution of the CO_2 and absorption by the sodium hydroxide trap, prior to the determination of the ^{14}C content of the CO_2 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 seeds. The dehulled rice seeds are referred to as non-germinated brown rice. In order to obtain germinated brown rice, non-germinated brown rice (50 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

sense (from bp number 47 to 72, 5'-GCACGCGAGCTCCGGCCG GGACGACG-3') or antisense (from bp number 1,476 to 1,497, 3'-CACACCGAGACACTCTTTACGA-5') sequences of RicGAD cDNA. RT-PCR was conducted with the One Step RNA PCR kit, from Takara (Japan), with the primers and 500 ng of the total RNA. The RT-PCR products were identified via analysis on 1% (w/v) agarose gel.

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

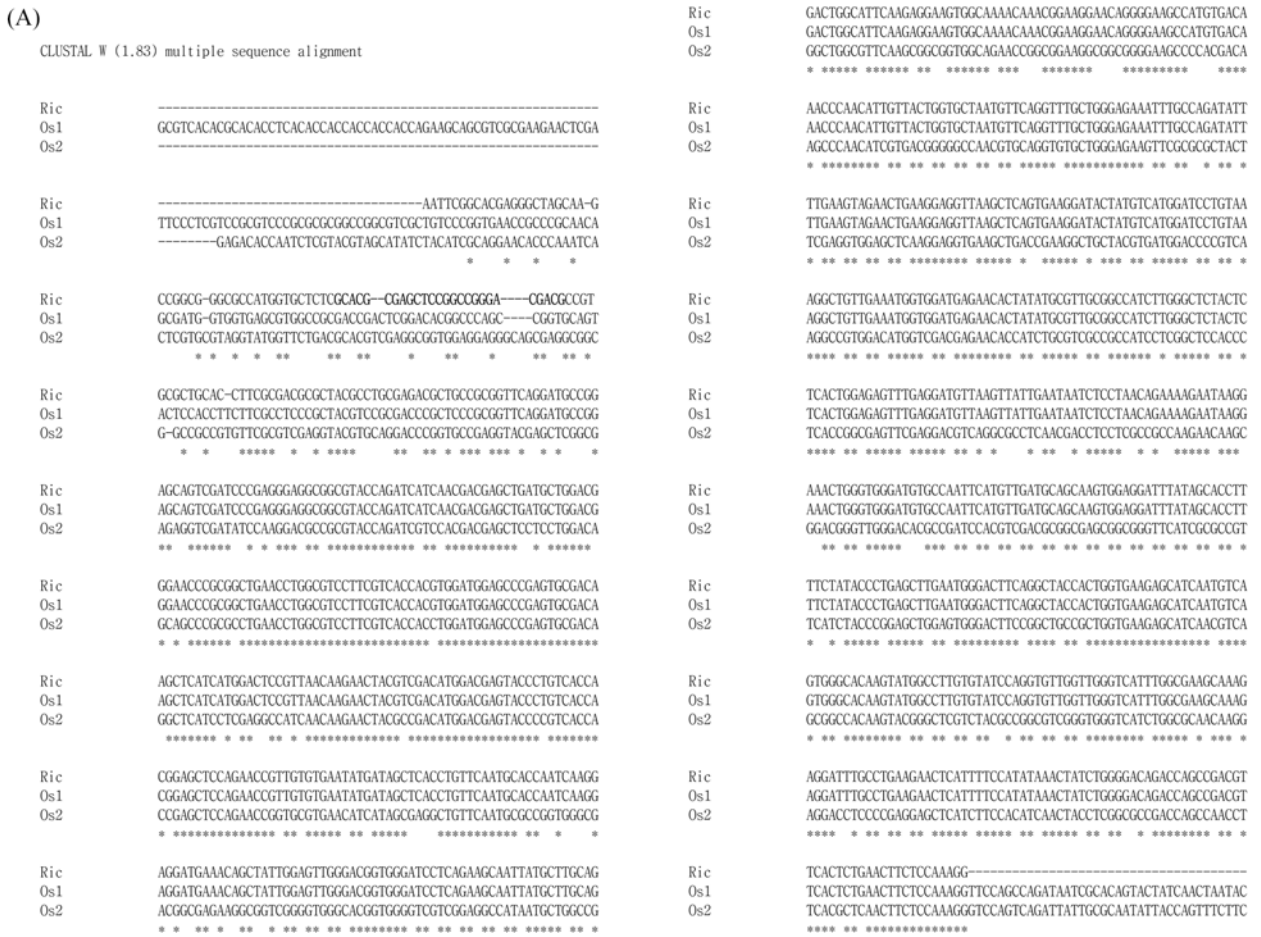

Fig. 1. Alignment of the entire nucleotide sequence (A) and the active site amino acid sequence (B). Ric, Os1 and Os2, *Oryza sativa* (Ric, this study AY428025; Os1, AB56060 and Os2, AB56061); Nt, *Nicotina tabacum* (AF020424); Ph, *Petunia hybrida* (L16797). Gaps are marked by hyphens. Bold letters indicate the sequences of the RT-PCR primers (A) and the consensus motif for the binding of pyridoxal 5'-phosphate (B).

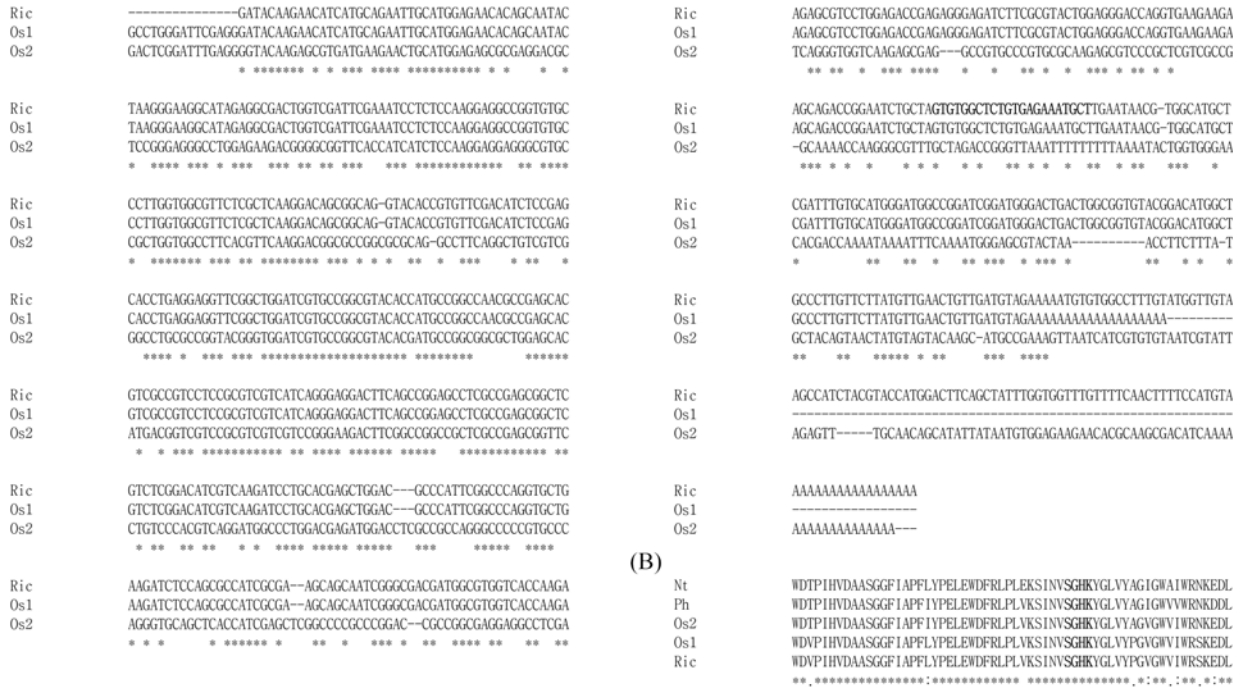

Fig. 1. Continued

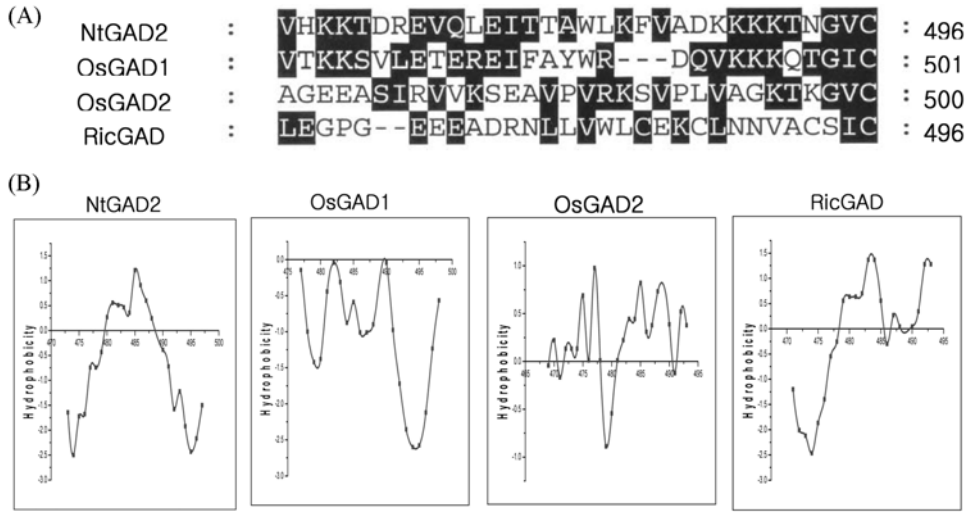

Fig. 2. (A) Comparison of the C-terminal amino acid sequences of rice GAD (RicGAD) to those of tobacco GAD (NtGAD2) and rice GADs (OsGAD1 and OsGAD2). Identical and similar amino acids are marked by black boxes. (B) Hydropathic analysis of the C-terminal regions. Each plot was predicted with the hydrophobicity values described by Kyte and Doolittle (1982).

comparison of the deduced C-terminal amino acid sequences of rice GADs and a tobacco GAD. RicGAD has also been determined to harbor a highly conserved tryptophan residue in this position, but lacks the characteristic lysine cluster at the C-proximal position, as well as certain other stretches of positively charged residues (Fig. 2A). The C-terminus of the RicGAD protein exhibits 20%, 17%, and 13% identity with the C-terminal regions of tobacco GAD (NtGAD2), OsGAD1, and OsGAD2, respectively. A 34% identity has been determined to exist between NtGAD2 and OsGAD1, representing the highest degree of identity currently known among GAD C-termini (Akama *et al*., 2001). Fig. 2B shows a hydropathic analysis of the C-terminal regions. As is shown in Fig. 2B, hydropathic plots from the C-termini of both the RicGAD and NtGAD2 proteins display similar features only in the first half of the region. Plots of the RicGAD, OsGAD1, and OsGAD2 proteins reveal differing features. These observed differences between the C-terminal regions of RicGAD, OsGAD1, and OsGAD2 suggest possible differences in the calmodulin-binding ability of the three.

In order to verify that the RicGAD gene encodes a rice GAD, *RicGAD* was expressed in *E. coli,* utilizing the high

Table 1. Response of GAD activity to calcium and calmodulin

Treatment	Activity	
	nmol CO_2/min/mg protein	% of control
-Ca^{2+}/-CaM	25.2 (1.5)[a]	100
+ Ca^{2+}	37.2 (2.1)	148
+ SPCaM	35.8 (2.5)	142
+ Ca^{2+}/+SPCaM	75.6 (3.4)	300

The GAD assay was performed using a radiometric method based on L-[1-^{14}C]Glu-dependent $^{14}CO_2$ production (Snedden *et al*., 1995) with modifications. Without the addition of Ca^{2+} and calmodulin (CaM) (-Ca^{2+}/-CaM), with the addition of 2.5 mM $CaCl_2$ (+Ca^{2+}), with the addition of 200 nM spinach calmodulin (+SPCaM), and with the addition of 2.5 mM $CaCl_2$ and 200 nM spinach calmodulin (+Ca^{2+}/+SPCaM). Spinach calmodulin was purified by hydrophobic phenyl-Sepharose column chromatography of Gopalakrishna and Anderson (1982) as described (Oh and Yun, 1999).

[a]Values represent the means of three independent determinations with standard errors shown in parenthesis.

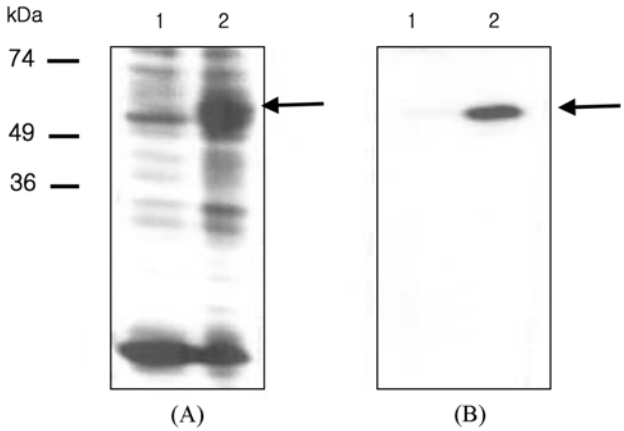

Fig. 3. SDS-PAGE and Western-blot analyses of *RicGAD*-encoded protein expression. Lanes 1 and 2, protein extracts of *E. coli* transformed with pVUCH-*RicGAD* before and after induction by IPTG. (A) Coomassie blue-stained gel. (B) Western-blot, detected with an anti-GAD monoclonal antibody. The arrows indicate the positions of the RicGAD protein expressed in *E. coli*.

copy number plasmid pVUCH with the tac promoter and the ampicillin resistance gene (Lukas *et al*., 1987). An extract of the transformed *E. coli* cells was then used to determine the GAD activity in the presence or absence of calcium and calmodulin. Activation analysis revealed that the maximal activation of the GAD occurred in the presence of both Ca^{2+} and calmodulin (Table 1). We detected no significant differences in the GAD activator properties between calmodulins, such as bovine brain calmodulin and spinach calmodulin (data not shown). These data indicate that calcium and calmodulin exert a synergistic effect on the activation of RicGAD derived from a cloned gene. In a previous *in vitro* assay, Akama *et al*. (2001) demonstrated that OsGAD1 is able to bind specifically to bovine calmodulin, but the activation of OsGAD1 by calmodulin was not detected in that study. The stimulation of RicGAD with calmodulin in the absence of added Ca^{2+} (+SPCaM) can be explained by calmodulin/GAD interactions in the absence of Ca^{2+}, as has been previously observed (Arazi *et al*., 1995). The increase in RicGAD activity with calcium in the absence of calmodulin (+Ca^{2+}) has yet to be explained. Although RicGAD is clearly activated by calcium and calmodulin, the extent to which purified RicGAD is stimulated by calcium and calmodulin isoforms has yet to be precisely determined. In this regard, it should be noted that rice harbors several isoforms of calmodulin (OsCaMs) (Lee *et al*., 2000). It has been previously shown that other types of calmodulin binding proteins (plant GADs and NAD kinases) are differentially activated by different calmodulin isoforms (Oh and Yun, 1999). The differential abilities of GADs in the binding of calmodulin and calmodulin isoforms suggest that GAD isoforms may have their own target calmodulins. In future studies, isoform-specific calmodulin-binding assays for the RicGAD protein may allow us to more clearly understand the mechanism underlying the interaction of RicGAD with calmodulin.

E. coli cells, when transformed with pVUCH-*RicGAD* and induced with IPTG, generated an abundance of a 56~58 kDa protein (Fig. 3A). An anti-GAD monoclonal antibody against the petunia GAD which lacks the calmodulin-binding domain (Snedden *et al*., 1996) recognized this 56~58 kDa RicGAD protein in the *E. coli* cell extracts (Fig. 3B). The petunia GAD region used to raise the antibody shares 90% amino acid sequence identity with the corresponding region of RicGAD, and exhibits 48% identity with *E. coli* GAD. The molecular weight of the protein recognized by the antibody was approximately 56~58 kDa (Fig. 3), larger than that of the *E. coli* GAD, which is 53 kDa (Yun and Oh, 1998; Ueno, 2000). These data clearly suggest that *RicGAD* encodes for a GAD protein.

We also assessed *RicGAD* expression in rice roots and brown rice, via RT-PCR analysis. *RicGAD*-specific primers were used for the PCR amplification of the cDNA products synthesized from the RNA samples. Fig. 4 shows that *RicGAD* was expressed predominantly in the rice roots grown under phosphorus deprivation conditions, as well as in the non-germinated brown rice. The enhanced expression of *RicGAD* in the phosphorus-deficient roots, as is shown in Fig. 4, can be attributed to the growth condition of the seedlings for mRNA preparation. The mRNA was prepared from roots which had been subjected to phosphorus deprivation conditions. Under stressed conditions such as those associated with phosphorus deprivation, plants exhibit a variety of biochemical adaptive responses, including the induction of acid phosphatase activity (Yun and Kaeppler, 2001). The levels of GABA in plants also tend to be elevated during high stress conditions, including hypoxia, darkness, and drought (Shelp *et al*., 1999; Bouche and Fromm, 2004). It is tempting to speculate that GAD activity increases, and GABA accumulates, in plants starved of phosphorus. Future studies

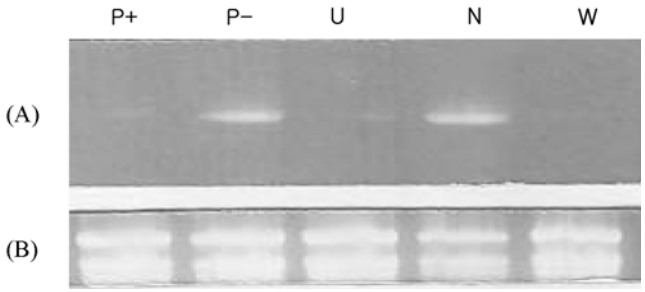

Fig. 4. Patterns of *RicGAD* expression in rice roots, seeds, and brown rice. (A) RT-PCR analysis of rice RNAs. RT-PCR was performed with the primers and total RNA, as was described in the Materials and Methods section; (B) ethidium bromide stain of total RNAs (1.0 μg/lane) from rice roots and brown rice. P+, rice roots grown normally in a standard nutrient solution; P-, rice roots grown under phosphorus deprivation conditions; U, undehulled rice; N, dehulled, but non-germinated brown rice; W, germinated brown rice in double-distilled water. The RT-PCR products and total RNAs were analyzed via 1% agarose gel electrophoresis.

with this rice system (including analyses of the changes in GAD enzyme and GABA levels in response to stresses such as phosphorus deprivation) may provide further insights into the roles played by *RicGAD* in monocotyledonous species.

The fact that *RicGAD* was detected predominantly in the non-germinated brown rice may be attributed to dormancy release, and to the limited bioavailability of phosphorus in the 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 (Inatomi and Slaughter, 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 (Bartnick and Szafranska, 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^{2+}/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* expressional regulation 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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