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Cloning and Characterization of a Maize cDNA Encoding Glutamate Decarboxylase

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Abstract A putative glutamate decarboxylase (GAD) gene, designated *ZmGAD1*, was cloned from *Zea mays* with a combination of reverse-transcriptase polymerase chain reaction (RT-PCR) and bioinformatic approaches. The *ZmGAD1* cDNA sequence contained a complete open reading frame encoding a putative protein of 496 amino acids, which contained a pyridoxal-5′-phosphate binding domain and a calmodulin (CaM)-binding domain found in nearly all GADs from plants. Sequence analysis showed that it had highest similarity with rice GAD1. Recombinant ZmGAD1 protein was expressed in *Escherichia coli*, purified and used to measure enzyme activity, which confirmed *ZmGAD1* was really a glutamate decarboxylase gene. Southern blotting analysis suggested that *ZmGAD1* was present as a single copy gene in the maize genome. RT-PCR analysis revealed that *ZmGAD1* was expressed in all examined tissues including the roots, stems, leaves, ears, and tassels. The expression of the *ZmGAD1* gene was upregulated and GAD activity was increased in the leaves and roots after treatment with ABA, MeJA, NaCl, PEG, or cold stress. Several stress-related *cis*-elements were present in the *ZmGAD1* promoter cloned from maize genomic DNA. These results suggested that *ZmGAD1* might play an important role in responses to abiotic factors and hormone treatments.

Keywords Activity · Expression · Glutamate decarboxylase · Stress · *Zea mays*

Introduction

Glutamate decarboxylase (GAD; EC 4.1.1.15) is a pyridoxal-phosphate (PLP)-dependent enzyme that catalyses the irreversible α-decarboxylation of L-glutamate to γ-aminobutyrate (GABA). 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 (Lee et al. 2001). Genes encoding GAD proteins have been identified in a number of different plants such as *Arabidopsis thaliana* (Bouché et al. 2004; Turano and Fang 1998), rice (Akama et al. 2001; Ohs et al. 2005), cowpea (Johanson et al. 1997), tomato (Gallego et al. 1995), and tobacco (Yuns and Ohs 1998). Sequence analysis showed that this protein has two conserved domains of glutamate decarboxylase, a pyridoxal-5′-phosphate binding domain in the middle region and a calmodulin (CaM)-binding domain at the carboxyl terminus, which is a unique feature in plants (Arazi et al. 1995; Baum et al. 1993; Gallego et al. 1995). The activity of the plant enzyme in vitro was maximal at pH 5.8, but at neutral pH, the enzyme was barely active without Ca^{2+}/CaM, and was substantially activated by Ca^{2+}/CaM (Snedden et al. 1995; Snedden et al. 1996; Turano and Fang 1998; Zik et al. 1998). Therefore, the possibility of a

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correlation between GABA production and Ca^{2+} signaling has been suggested (Wallace et al. 1984). There is ample evidence in the relevant literature demonstrating that abundant amounts of GABA rapidly accumulates in a variety of plant organs under several environmental stress conditions (Bouché and Fromm 2004; Shelp et al. 1999). GAD activity and GABA have been associated with various physiological responses including regulation of cytosolic pH, carbon effluxes into the tricarboxylic acid cycle, nitrogen metabolism, transport and storage, and deterrence of insects (Bouché and Fromm 2004).

In this study, we isolated a GAD gene from *Zea mays*, designated *ZmGAD1*. ZmGAD1 was expressed in *Escherichia coli* and the activity of the protein was determined. Expression analyses showed that this gene was expressed in different tissues and upregulated in the leaves and roots when plants were subjected to ABA, MeJA, NaCl, polyethylene glycol (PEG), or cold stress. Also, the activity of ZmGAD1 was increased in response to these treatments.

Materials and Methods

Plant Material and Stress Conditions

Sterilized germinated seeds from maize (inbred line DH4866) were grown hydroponically in MS medium (Murashige and Skoog 1962) and transferred to a 25°C growth chamber with 70% relative humidity. All plants used for treatments described below were grown under normal conditions (16 h light/28°C, 8 h dark/19°C). For cold treatments, the 7-week-old seedlings grown at 28°C were shifted to 4°C. For osmotic or salt stress and phytohormone treatment experiments, the plants were grown for 7 weeks in MS medium, and transferred to MS medium supplemented with 20% (*w*/*v*) polyethylene glycol (MW 6000; osmotic stress), 0.8% (*w*/*v*) NaCl, 100 μM ABA, or 100 μM MeJA. The roots and leaves of the plants were harvested carefully and immediately frozen in liquid nitrogen at the indicated times. The entire experiments were repeated at least three times.

Cloning the *ZmGAD* Gene and Analyzing the Sequence and Promoter

The cDNA was synthesized with a SMART Kit from Clontech following the manufacturer's instructions with mRNA from maize seedlings that were treated with 0.8% (*w*/*v*) NaCl for 24 h. From our microarray experiments (Zhuang et al. 2007), a putative glutamate decarboxylase gene was induced under stressed conditions. The sequence (MZ00027324) of the putative GAD gene in the Maize Microarray Database (http://www.maizearray.org) was used for analysis. The open reading frame (ORF) of the sequence was determined with the program NCBI ORF finder. According to the predicted sequence including the complete ORF, designated *ZmGAD1*, a pair of primers 5′-CACCA CCGAAAACGAATC-3′ and 5′-AACATACGGCACCA AACC-3′ were designed to amplify the *ZmGAD1* cDNA with Prime STAR® HS DNA Polymerase (TAKARA, China). The PCR program was: 95°C for 5 min, 30 cycles of 95°C for 1 min, 56°C for 1 min, and 72°C for 2 min with a final extension at 72°C for 10 min. The amplified 1.7-kb cDNA fragment was modified using the A-tailing procedure and inserted into the pGEM® T-easy vector (PROMEGA, USA) according to the manual. The cDNA was sequenced and the sequence was then used as a query to search the MaizeGDB genomic database. A BAC clone (AC203363) containing upstream sequence of *ZmGAD1* was obtained. A DNA fragment of about 1.5-kb before the initiation codon was amplified using a pair of primers 5′-TGTGCCTGAA GTTGGTGAGTC-3′ and 5′-TGGGATTCGTTTTCGGTG-3′. DNA sequencing was performed by the BioAsia Company (Shanghai, China). Protein sequences of GADs from other plants were obtained from the GenBank database and alignments were performed using the Clustal W program. The promoter sequence was analyzed with the PlantCARE program (Lescot et al. 2002).

Expression of the Recombinant ZmGAD1 in *E. coli* and Determination of its Activity

The complete coding sequence for *ZmGAD1* was subcloned into the *Nco*I site of expression vector pET-30a (NOVAGEN, Germany). All constructs were confirmed by restriction enzyme analysis and DNA sequencing. Successful constructs were transformed into *E. coli* BL21 (DE3) and expected to express a ZmGAD1 fusion protein with a hexahistidine tag at the N terminus. Recombinant ZmGAD1 protein was purified with His binding resin chromatography (NOVAGEN, Germany) according to the manufacturer's protocol. The GAD activity of the recombinant protein was determined in vitro with the method of Johanson et al. (1997). The ZmGAD1 fusion protein was separated on 8% SDS-PAGE gels.

DNA Gel Blot Analysis

Twenty-microgram aliquots of DNA from maize plants were digested with *Xba*I, *EcoR*V, *EcoR*I, and *Hind*III restriction enzymes, and separated by 0.8% agarose gel electrophoresis. The samples were transferred to a nylon membrane. The 3′-end cDNA fragment (1.2-kb) of *ZmGAD1* was used as a template for random primed labeling. Probe labeling (using dUTP-DIG), hybridization, and detection were performed as description in the DIG System Manual (ROCHE, Switzerland).

Expression and Activity Analysis of *ZmGAD1*

Tissue-specific expression analysis was performed with RT-PCR, and expression analysis in the leaves and roots under stressed conditions with real-time PCR. Total RNA was extracted with Trizol reagent (SANGON, China) from samples and treated with RNase-free DNase I. cDNA synthesis was performed with the RT reagent kit (TAKARA, China) according to the manufacturer's protocol. Real-time quantitative RT-PCRs were performed on a Chromo 4^{TM} continuous fluorescence detector (MJ RESEARCH, USA) with the SYBR® RT–PCR Kit (TAKARA, China). A pair of specific primers (5′-GCTCCGAGTCGTCGTCAG-3′ and 5′-GGCGAAACCTCCCAGTCT-3′) were designed based on the *ZmGAD1* sequence to amplify a fragment of 274 bp. Amplification conditions were: 2 min at 95°C; 40 cycles of 15 s at 95°C, 30 s at 58°C and 30 s at 72°C, with a final extension of 5 min at 72°C. Fold changes in the amounts of RNA transcripts were calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001) with maize *α-tubulin* (X73980; primers: 5′-CACTGATGTTGCTGTCCTGC-3′ and 5′-CGCTGTTGGTGATTTCGG-3′) as an internal control. The entire experiments were repeated three times. Simultaneously, the GAD activity of these samples from stress treatments was determined in vitro by the method of Johanson et al. (1997) with the modifications of adding CaM and sodium phosphate (pH7.0) to the assays.

Results and Discussion

Cloning and Analysis of a cDNA Encoding GAD from Maize

A 1,736-bp cDNA fragment was amplified by RT-PCR based on the sequence (1,739 bp) from the Maize Microarray Database. Sequence analysis showed that the fragment contained a complete ORF of 1,491 nucleotides, and had 99% similarity with the sequence (MZ00027324) in the Maize Microarray Database. The 1,491 nucleotides encoded a protein of 496 amino acids with a calculated molecular weight of 56.2 kDa and a pI of 5.43. The protein had the highest similarity of 86% to OsGAD1 (BAB32870) from rice. Therefore, it was designated *ZmGAD1*. The full-length cDNA sequence of *ZmGAD1* was deposited in GenBank (accession no. EU302127).

Alignment of the deduced protein sequence of ZmGAD1 and GADs from other species was performed using the Clustal W program (Fig. 1). The predicted protein sequence was highly similar to the previously determined GAD sequences, showing 86% identity to the GAD from rice, 80% to barley, 77% to soybean, and 77% to tobacco, 76% to *Arabidopsis*, 76% to petunia and 75% identity to the GAD in tomato. The amino acid sequence of the predicted protein contained a PLP binding site and an SGHK motif that were reportedly conserved among the various GADs from plants. An approximately 30-amino acid extension in their C-terminal regions appeared to be required for the binding of GAD to CaM, in which tryptophan and the C-proximal lysine cluster constituted the most important residues in the process of CaM binding. These analyses showed that the primary structure of ZmGAD1 had the typical features of a GAD enzyme.

Expression of Recombinant ZmGAD1 Protein and Determination of its Activity

To determine whether the ZmGAD1 protein can function to catalyze α-decarboxylation of L-glutamate to GABA, the complete coding sequence of the *ZmGAD1* cDNA was subcloned into pET-30a. After induction with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 28°C for 6 h, a specific protein band was found in the *E. coli* extracts in SDS-PAGE analysis (Fig. 2a). The molecular weight of the induced protein band was about 61 kDa, which was in accordance with the predicted amino acid sequences (ZmGAD1 496 aa + His and S tag 36 aa). The recombinant protein was purified by Ni^{2+} resin, and used as a source of enzyme for in vitro activity assays at pH5.8, the pH known to be optimal for determining GAD activity (Johanson et al. 1997). Based on measurements of GAD activity (208.2± 14.9 nmol $min^{-1} mg^{-1}$ protein), we concluded that the *ZmGAD1* gene really encodes a glutamate decarboxylase.

DNA Gel Blot Analysis

Southern blot analysis was used to determine the number of copies of the *ZmGAD1* gene in maize (Fig. 2b). A probe from a 1.2-kb fragment of the region proximal to the 3′ end were hybridized under highly stringent conditions to a Southern blot of maize genomic DNA digested with *Xba*I, *Eco*RV, *Eco*RI, and *Hind*III. It was evident that under the hybridization conditions used in this experiment, one strong band was apparent in each line. This indicated that *ZmGAD1* was a single copy gene in the genome of *Z. mays*. However, another slight band was also detected in each digest. To discover the identity of this band, the 3-end sequence (1.2-kb) of the *ZmGAD1* gene was used as a query to search the MaizeGDB database, and an mRNA sequence (AY104454; 1,154 bp) was obtained that had high similarity of 80%. The sequence (AY104454) was used as a seed to obtain a 1,738 bp cDNA sequence that contained the full ORF by in silico cloning. The 1,738 nucleotides encoded a protein of 516 amino acids with 83% similarity to GAD2 (BAB32869.1) from rice. Thus, it is possible that the faint band in Fig. 2b might represent another GAD gene

Fig. 1 Sequence alignment of GADs from various plants. Identical and similar amino acid residues are indicated by *asterisks* and *double dots*, respectively. Gaps are shown by *hyphens*. The active site domain of the GADs is indicated by a *box*, and the C-terminal peptide extension that is unique to plant GADs is shown by a *line*. *Gray* indicates the consensus motif for the binding of pyridoxal 5′-phosphate. Italic in the C terminus indicate the most important residues in calmodulin binding. Plant species and accession nos. are as follows: AtGAD1 (*Arabidopsis thaliana*, AAA93132); GmGAD (*Glycine max*, BAF80895); PetGAD (*Petunia hybrida*, AAA33709); NtGAD1 (*Nicotiana tobacum*, AAK18620), OsGAD1 (*Oryza sativa*, BAB32870), HvGAD (*Hordeum vulgare*, AAP46640); and TomGAD (*Solanum lycopersicum*, P54767)

(designated *ZmGAD2*) in the maize genome. However, this needs to be confirmed. Interestingly, sequence analysis of *ZmGAD2* showed that the C-terminal regions of the predicted protein could not combine with CaM, which was different from ZmGAD1.

Expression Analysis of *ZmGAD1* and Cloning of the *ZmGAD1* Promoter

To determine the tissue-specific expression patterns of *ZmGAD1*, RT-PCR was performed in which 0.5 µg of total RNA from various tissues was used as the template. RT-PCR analysis indicated that the *ZmGAD1* gene was expressed in the ears, tassels, stems, leaves, and roots under normal growth conditions (Fig. 2c). However, the transcripts appeared to be present the most abundantly in the ears and to a lesser extent in the leaves. This was similar to a report that the rice GAD genes had different expression patterns in different organs (Akama et al. 2001). These results indicated that GAD might play different roles in mediating the carbon–nitrogen balance and the metabolism of nitrogen in different organs (Bouché and Fromm 2004).

In addition, we analyzed expression of the *ZmGAD1* gene under different environmental stress conditions by quantitative real-time PCR. There were different patterns of expression in the roots and leaves when the plants were subjected to different stress treatments as detected by real-time RT-PCR. In the leaves the expression of *ZmGAD1* was upregulated more than twofold during treatment with ABA, MeJA, NaCl, PEG, or cold (Fig. 3a), though in different patterns and at different times. Changes in the relative expressions levels in the roots were less obvious than in the leaves, though there were similar patterns with MeJA, NaCl, or PEG treatments (Fig. 3b). The possible reason was

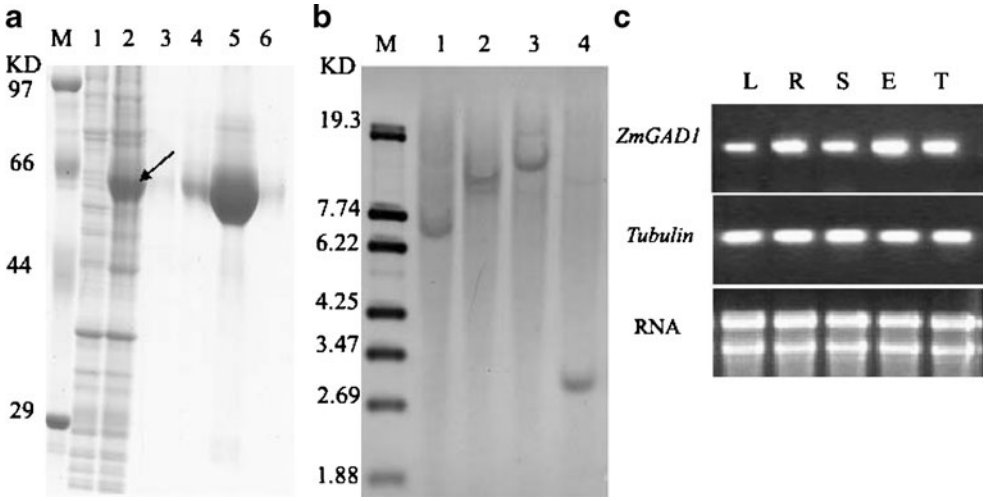

Fig. 2 **a** Expression and purification of recombinant ZmGAD1 in *E. coli* BL21 (DE3). *Lane M* low-weight protein marker. *Lane 1* bacterial proteins from BL21 (DE3) transformed with pET30a-*ZmGAD1* uninduced; *lane 2*, bacterial proteins from BL21 (DE3) transformed with pET30a-*ZmGAD1* and induced with 1 mM IPTG at 28°C for 6 h; *lanes 3–6* different tubes of ZmGAD1 fusion protein sequentially eluted with His binding resin chromatography. The expressed ZmGAD1 fusion protein is indicated with an *arrow*. **b** DNA gel blot analysis of the *ZmGAD1* gene. Twenty-microgram aliquots of DNA were digested with 50 U of *Xba*I, *EcoR*V, *EcoR*I, and *Hind*III and then transferred to a membrane. Gene-specific probes for *ZmGAD1* were labeled in the presence of DIG-dUTP, and hybridized with the membrane. The blot was washed under high stringency conditions (twice at 68°C for 15 min in 0.2×SSC, 0.1% SDS). *M* represents the λDNA/EcoT14 I molecular weight marker; *lanes 1–4* DNA digested with *Xba*I, *EcoR*V, *EcoR*I, and *Hind*III. **c** Expression patterns of the *ZmGAD1* gene in the different organs of maize by RT-PCR detection. *L* leaf, *R* root, *S* stem, *E* ear, *T* tassel. The transcripts of tubulin (X73980) in the samples were used as a reference

that there were more *ZmGAD1* transcripts in the roots than in the leaves (Fig. 2c).

To determine whether some stress-related *cis*-elements were present in the *ZmGAD1* promoter, a 1.5-kb DNA fragment upstream from the initiation codon was obtained by PCR amplification based on a BAC clone (AC203363) sequence. This was obtained by similarity searching in the maize genomic database using the *ZmGAD1* cDNA sequence as a query. PlantCARE program analysis showed that there were several stress-related *cis*-elements present in the *ZmGAD1* promoter, such as ABRE and C-repeat/DRE (Fig. 4; Table 1 in the supplement). These *cis*-elements may have contributed to induced expression of *ZmGAD1* under ABA, MeJA, PEG, NaCl, or cold treatment conditions. These results also suggested that expression of *ZmGAD1* might be regulated at the level of transcription.

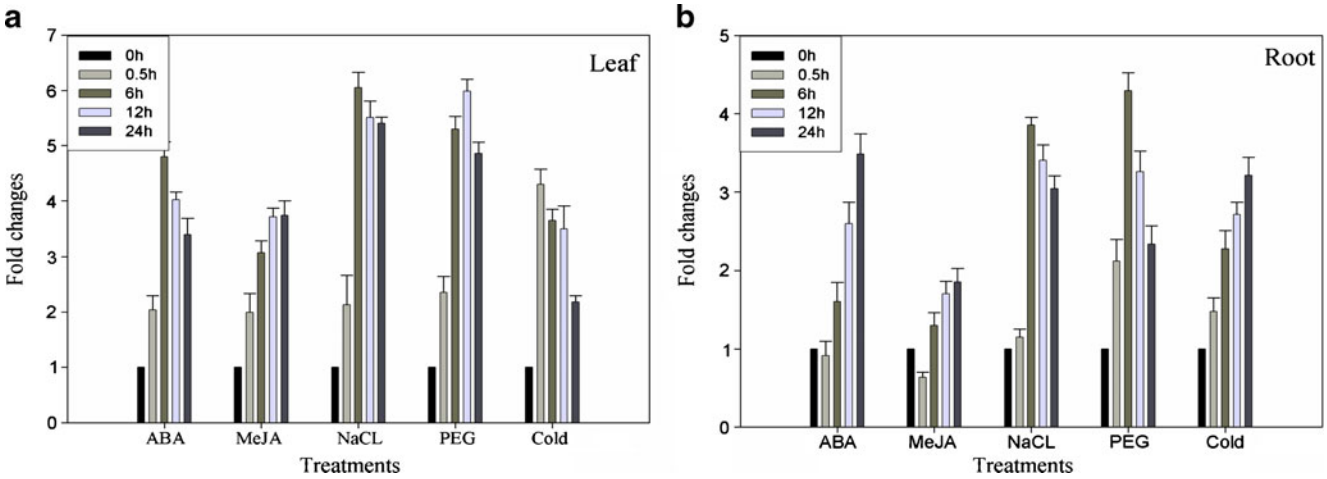

Fig. 3 Expression patterns of the *ZmGAD1* gene in maize leaves (**a**) and roots (**b**) during different treatments by real-time RT-PCR detection. Total RNA was isolated from the leaves and roots of plants treated with 100 μM ABA, 100 μM MeJA, 0.8% NaCl, 20% PEG, or 4°C (cold) at five different time points (0, 0.5, 6, 12, and 24 h). Fold changes of RNA transcripts were calculated by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001) with *α-tubulin* as an internal control

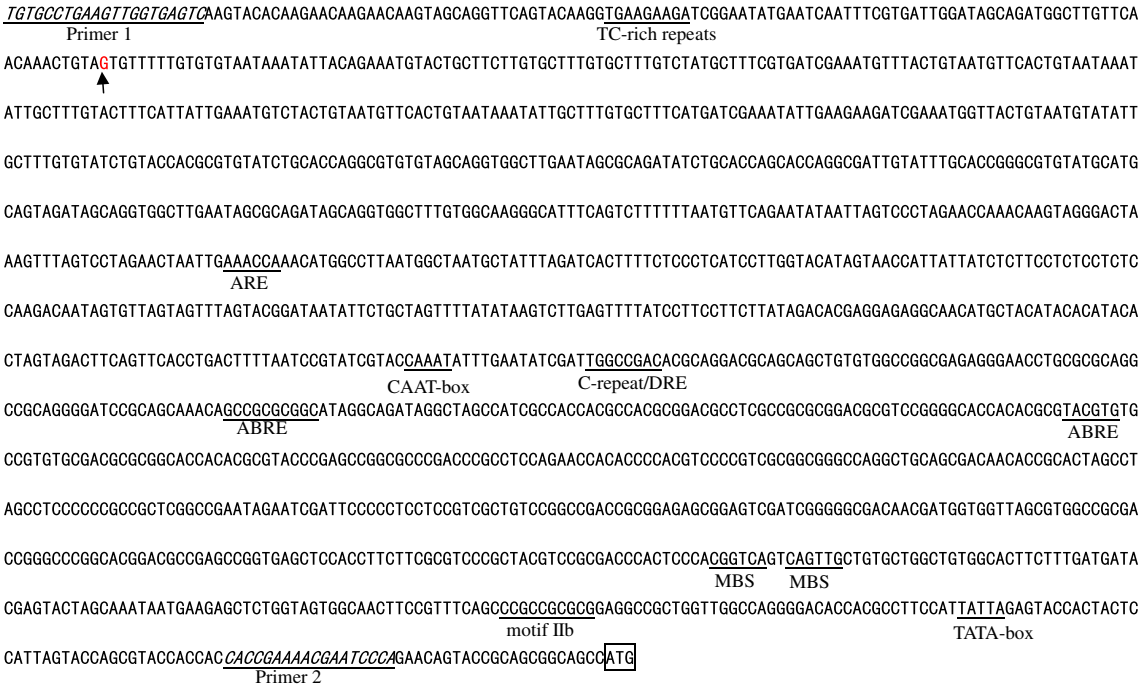

Fig. 4 The upstream sequence of the *ZmGAD1* gene. The sequence was amplified with Primer1 and primer2, which are *underlined*. Other *underlined* sequences indicate *cis*-elements. The start codon is indicated by a *box*. A substituted base compared to the BAC clone (AC203363) is indicated by an *arrow*

Analysis of ZmGAD1 Activity in Response to ABA, MeJA, PEG, NaCl, and Cold

To separate the activity of ZmGAD2 from ZmGAD1, the method described by Johanson et al. (1997) was modified by the addition of CaM and sodium phosphate (pH7.0) to the assays. The activities of ZmGAD1 in the leaves and roots from plants under different treatment conditions were determined in vitro (Fig. 5). The results showed that GAD activity increased gradually during the treatments extending for 24 h. These results support the possibility that a correlation exists between stress responses and GAD activity and the consequent synthesis of GABA, and that the GABA shunt may play a role in the plant response to different stresses. As hypothesized previously (Bouché and Fromm 2004; Shelp et al. 1999), the protective effect of the GABA shunt may

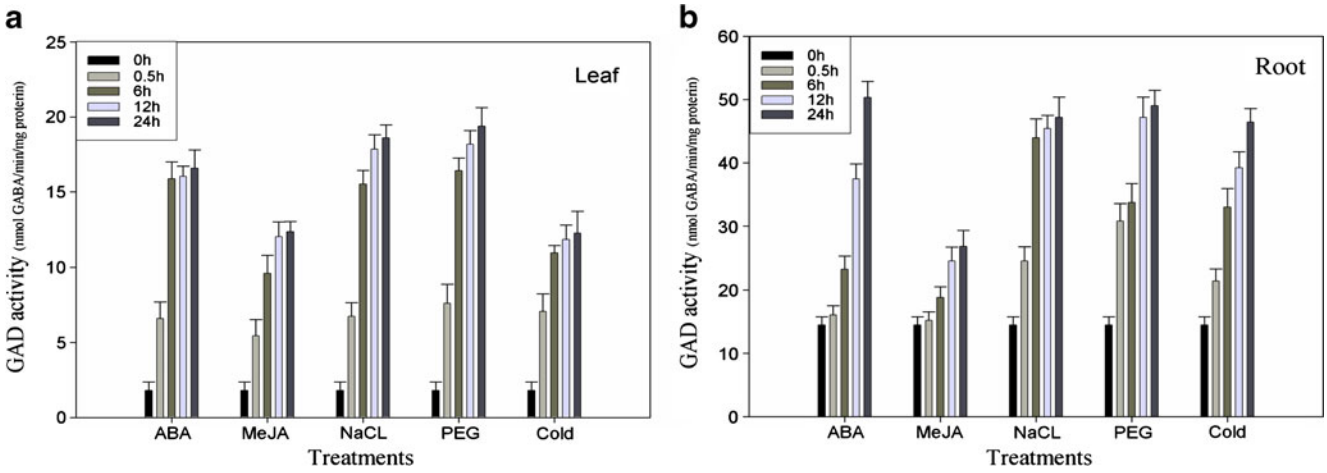

Fig. 5 ZmGAD1 activity changes in the leaves (**a**) and roots (**b**) of plants subjected to the treatment of ABA, MeJA, NaCl, PEG, or cold. Crude protein extracts were isolated from the leaves and roots of *Z. mays* treated with 100 μM ABA, 100 μM MeJA, 0.8% NaCl, 20% PEG, or 4°C (cold) at 0, 0.5, 6, 12, and 24 h. GAD activity was determined by the modified method described by Johanson et al. (1997)

depend on the reaction catalyzed by GAD. The levels of GABA in plants tends to be elevated during high stress conditions, including hypoxia, darkness, and drought (Bouché and Fromm 2004; Shelp et al. 1999). It was reported that GABA stabilizes and protects isolated thylakoids against freezing damage in the presence of salt, and exceeds the cryoprotective properties of proline (Shelp et al. 1999). ZmGAD1 activity was much higher in the roots than in the leaves at the same time under different conditions. It is possible that the roots experienced more severe stress conditions than the leaves, and that the activity of ZmGAD1 may be regulated post-translationally. GAD activity may be regulated at both the transcriptional level and the post-translational level. It was reported that the activity of GAD was developmentally controlled at both the transcriptional and translational levels in petunias (Chen et al. 1994). Our study provides further insight into the roles of ZmGAD1 and facilitates future study of the molecular mechanisms underlying GABA metabolism in plants.

In summary, a full-length GAD gene from *Z. mays* was cloned and characterized. This was the first *ZmGAD* gene isolated from *Z. mays*. Multiple alignments showed that the deduced ZmGAD1 protein was highly homologous with other GAD proteins, especially with OsGAD1 (BAB32870), and contained all the conserved residues present in the plant GAD protein family. Abundant soluble ZmGAD1 protein was expressed in *E. coli*, purified by His binding resin chromatography, and the activity of ZmGAD1 was determined. *ZmGAD1* was ubiquitously expressed in all tested tissues, and the transcript level was much higher in the ears than in other tissues. Moreover, the expression of the *ZmGAD1* gene was upregulated and GAD activity was increased in the leaves and roots during treatment with ABA, MeJA, NaCl, PEG, or cold. Several stress related *cis*-elements were present in the *ZmGAD1* promoter cloned from maize genomic DNA. The cloning and characterization of *ZmGAD1* will be helpful for understanding the detailed functions or roles of GAD family members in *Z. mays*.

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