Identification of two *CiGAD*s from *Caragana intermedia* and their transcriptional responses to abiotic stresses and exogenous abscisic acid

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Background. Glutamate decarboxylase (GAD), as a key enzyme in the γ-aminobutyric acid (GABA) shunt, catalyzes the decarboxylation of L-glutamate to form GABA. This pathway has attracted much interest because of its roles in carbon and nitrogen metabolism, stress responses, and signaling in higher plants. The aim of this study was to isolate and characterize genes encoding GADs from *Caragana intermedia*, an important nitrogen-fixing leguminous shrub.

Methods. Two full-length cDNAs encoding GADs (designated as *CiGAD1* and *CiGAD2*) were isolated and characterized. Multiple alignment and phylogenetic analyses were conducted to evaluate their structures and identities to each other and to homologs in other plants. Tissue expression analyses were conducted to evaluate their transcriptional responses to stress (NaCl, $ZnSO_4$, $CdCl_2$, high/low temperature, and dehydration) and exogenous abscisic acid.

Results. The *CiGAD*s contained the conserved PLP domain and calmodulin (CaM)-binding domain in the C-terminal region. The phylogenetic analysis showed that they were more closely related to the *GAD*s of soybean, another legume, than to *GAD*s of other model plants. According to Southern blotting analysis, *CiGAD1* had one copy and *CiGAD2*-related genes were present as two copies in *C. intermedia*. In the tissue expression analyses, there were much higher transcript levels of *CiGAD2* than *CiGAD1* in bark, suggesting that *CiGAD2* might play a role in secondary growth of woody plants. Several stress treatments (NaCl, $ZnSO_4$, $CdCl_2$, high/low temperature, and dehydration) significantly increased the transcript levels of both *CiGAD*s, except for *CiGAD2* under Cd stress. The *CiGAD1* transcript levels strongly increased in response to Zn stress (74.3-fold increase in roots) and heat stress (218.1-fold increase in leaves). The transcript levels of both *CiGAD*s significantly increased as GABA accumulated during a 24-h salt treatment. Abscisic acid was involved in regulating the expression of these two *CiGAD*s under salt stress.

Discussion. This study showed that two *CiGAD*s cloned from *C. intermedia* are closely related to homologs in another legume, soybean. *CiGAD2* expression was much higher than that of *CiGAD1* in bark, indicating that *CiGAD2* might participate in the process of secondary growth in woody plants. Multiple stresses, interestingly, showed that Zn and heat stresses had the strongest effects on *CiGAD1* expression, suggesting that *CiGAD1* plays important roles in the responses to Zn and heat stresses. Additionally, these two genes might be involved in ABA dependent pathway during stress. This result provides important information about the role of *GAD*s in woody plants′ responses to environmental stresses.

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18   **ABSTRACT**

19   **Background.** Glutamate decarboxylase (GAD), as a key enzyme in the γ-aminobutyric acid
20   (GABA) shunt, catalyzes the decarboxylation of L-glutamate to form GABA. This pathway has
21   attracted much interest because of its roles in carbon and nitrogen metabolism, stress
22   responses, and signaling in higher plants. The aim of this study was to isolate and characterize
23   genes encoding GADs from *Caragana intermedia*, an important nitrogen-fixing leguminous
24   shrub.

25   **Methods.** Two full-length cDNAs encoding GADs (designated as *CiGAD1* and *CiGAD2*) were
26   isolated and characterized. Multiple alignment and phylogenetic analyses were conducted to
27   evaluate their structures and identities to each other and to homologs in other plants. Tissue
28   expression analyses were conducted to evaluate their transcriptional responses to stress (NaCl,
29    $ZnSO_4$, $CdCl_2$, high/low temperature, and dehydration) and exogenous abscisic acid.

30   **Results.** The *CiGAD*s contained the conserved PLP domain and calmodulin (CaM)-binding
31   domain in the C-terminal region. The phylogenetic analysis showed that they were more closely
32   related to the *GAD*s of soybean, another legume, than to *GAD*s of other model plants.
33   According to Southern blotting analysis, *CiGAD1* had one copy and *CiGAD2*-related genes were
34   present as two copies in *C. intermedia*. In the tissue expression analyses, there were much
35   higher transcript levels of *CiGAD2* than *CiGAD1* in bark, suggesting that *CiGAD2* might play a
36   role in secondary growth of woody plants. Several stress treatments (NaCl, $ZnSO_4$, $CdCl_2$,
37   high/low temperature, and dehydration) significantly increased the transcript levels of both
38   *CiGAD*s, except for *CiGAD2* under Cd stress. The *CiGAD1* transcript levels strongly increased in
39   response to Zn stress (74.3-fold increase in roots) and heat stress (218.1-fold increase in leaves).
40   The transcript levels of both *CiGAD*s significantly increased as GABA accumulated during a 24-h
41   salt treatment. Abscisic acid was involved in regulating the expression of these two *CiGAD*s
42   under salt stress.

43   **Discussion.** This study showed that two *CiGAD*s cloned from *C. intermedia* are closely related to
44   homologs in another legume, soybean. *CiGAD2* expression was much higher than that of
45   *CiGAD1* in bark, indicating that *CiGAD2* might participate in the process of secondary growth in
46   woody plants. Multiple stresses, interestingly, showed that Zn and heat stresses had the
47   strongest effects on *CiGAD1* expression, suggesting that *CiGAD1* plays important roles in the
48   responses to Zn and heat stresses. Additionally, these two genes might be involved in ABA
49   dependent pathway during stress. This result provides important information about the role of
50   *GAD*s in woody plants' responses to environmental stresses.

51   **1. Introduction**

52   γ-Aminobutyric acid (GABA) is a non-protein amino acid present in animals, plants, and other
53   organisms (Batushansky et al., 2014; Gilliham & Tyerman, 2016; Michaeli & Fromm., 2015;
54   Shelp et al., 2012a,b). It functions as a major inhibitory neurotransmitter in the brain tissues of
55   mammals (Granger et al., 2016; Robel & Sontheimer, 2015). In plants, GABA plays roles in

56 diverse processes including the carbon:nitrogen balance, signaling, regulation of redox status,
57 development, stress responses (Batushansky et al., 2014, 2015; Michaeli & Fromm, 2015,
58 Molina-Rueda et al., 2015; Shi et al., 2010; Yu et al., 2014). Recent studies have indicated that
59 GABA is required for proper growth during exposure to abiotic stresses such as salt (Renault et
60 al., 2013), drought (Mekonnen et al., 2016), low temperature (Aghdam et al., 2016), Zn^{2+} (Daş et
61 al., 2016), and cadmium (Sun et al., 2010). Additionally, exogenous GABA has been shown to
62 regulate the expression of genes such as *BnNrt2* in *Brassica napus* (Beuve et al., 2004), *14-3-3* in
63 *Arabidopsis thaliana* (Lancien & Roberts, 2006), *SAMDC* (S-adenosylmethionine decarboxylase)
64 in *Cucumis melo* (Wang et al., 2014), and to control gene transcription in *Caragana intermedia*
65 (Shi et al., 2010) and *A. thaliana* (Batushansky et al., 2014). The metabolite GABA has been
66 shown to play roles in a diverse range of cellular processes ranging from neuronal inhibition in
67 animals to pollen-tube development in plants (Michaeli & Fromm, 2015). Consequently, many
68 researchers have focused on the production of GABA, and in particular, the role of glutamate
69 decarboxylase (GAD), which is the unique pyridoxal enzyme catalyzing the α-decarboxylation of
70 L-glutamate to form GABA (Bouché & Fromm, 2004; Shelp et al., 2012a,b).

71 The pathway of glutamate decarboxylation catalyzed by GAD is widely distributed in organisms
72 (Liu et al., 2014), and is considered to be the main pathway of GABA production in plants (Shelp
73 et al., 2012a). Genes encoding GAD enzymes have been successfully identified in many
74 herbaceous plants such as rice (Akama et al., 2001), *Arabidopsis* (Bouché et al., 2004), maize
75 (Zhuang et al., 2010), and *Panax ginseng* (Lee et al., 2010) and in some woody plants, such as
76 pine (Molina-Rueda et al., 2010), apple (Trobacher et al., 2013), citrus (Liu et al, 2014), and Tea
77 (Mei et al., 2016). Bioinformatics analyses and experimental characterizations have shown that
78 plant GADs contain a calmodulin (CaM)-binding domain that is generally responsible for the
79 cytosolic decarboxylation of glutamate to GABA (Shelp et al., 2012a), although one functional
80 *OsGAD*2 without a Ca^{2+}/calmodulin domain has been isolated from rice (Akama et al., 2001).
81 Several studies have demonstrated spatiotemporal differences in the expressions of *GAD* gene
82 family member, although most *GAD* families studied to date have fewer than nine members
83 (Shelp et al., 2012a). For example, *AtGAD1* is mainly expressed in *Arabidopsis* roots (Miyashita
84 & Good, 2008), while *ZmGAD1* is expressed in the leaves, stems, and roots of maize (Zhuang et
85 al., 2010). In rice, *OsGAD1* is mainly expressed in seeds but *OsGAD2* is mainly expressed in roots
86 (Akama et al., 2001). In citrus, *CsGAD1* is predominantly expressed flowers but *CsGAD2* is
87 predominantly expressed in fruit (Liu et al., 2014). Interestingly *PpGAD* expression was shown
88 to be correlated with vascular differentiation in pine seedlings (Molina-Rueda et al., 2010).
89 Additionally, environmental stimuli were shown to induce the expression of *ZmGAD1* in maize
90 (Zhuang et al., 2010) and *PgGAD* in *P. ginseng* (Lee et al., 2010). These studies indicate that
91 plant *GAD*s might play important roles in plant development and stress responses. However,
92 few studies have focused on the expression of *GAD*s in woody plants under environmental
93 stresses.

94 Our previous studies showed that salt stress induced significant expression of EST sequences
95 (Supplementary T1) homologous to plant *GAD* genes in the desert legume shrub, *C. intermedia.*

96   We also showed that exogenous GABA can enhance the expression of genes, including those
97   related to hormones and reactive oxygen species (ROS), which involved the stress responses of
98   this shrub (Shi et al., 2010). Therefore, in the present study, we cloned two full-length cDNAs,
99   *CiGAD1* and *CiGAD2*, from *C. intermedia* based on known EST sequences using the rapid
100   amplification of cDNA ends (RACE) technique. We analyzed changes in their transcript levels in
101   response to different stress factors and exogenous abscisic acid (ABA). These results allowed us
102   to explore the relationship between *GAD* expression and GABA accumulation, which will
103   increase our understanding of the roles of GABA in stress adaptation in plants.

104   **2. Materials and Methods**

105   **2.1 Plant materials and treatments**

106   Seeds of *C. intermedia* were collected from a desert habitat in Inner Mongolia, China. Seedlings
107   were cultivated as described by Shi et al. (2010). Briefly, seeds were washed and then
108   germinated in sand. Three-day-old seedlings (radical length, 30–40 mm) were selected and
109   cultivated with water in pots in a growth chamber under a 14-h light/10-h dark photoperiod
110   and a day/night temperature of 23/18 °C. The seedlings were grown for 2–3 weeks, and the
111   water was refreshed every 2 days. Subsequently, the seedlings were subjected to the following
112   stress treatments: (1) NaCl (200 mM), $ZnSO_4$ (100 µM), $CdCl_2$ (500 µM), high temperature (40
113   °C), low temperature (4 °C), or dehydration (for 3 h); (2) NaCl (200 mM) for different durations
114   (0, 3, 6, 12, and 24 h); and (3) exogenous ABA (0, 0.5, 1, 10, 100, and 200 µM) combined with
115   the 200 mM NaCl treatment for 0, 3, and 24 h. Roots and leaves were harvested, frozen
116   immediately in liquid nitrogen, and then stored at −80 °C. Also, different tissues including roots,
117   stems, leaves, seeds, testae, and bark were collected from mature individuals growing at the
118   Chinese Academy of Forestry, Beijing, frozen immediately in liquid nitrogen, and then stored at
119   −80 °C. Every experimental treatment had three biological replicates.

120   **2.2 Cloning, sequencing, and bioinformatic analysis**

121   Total RNA was extracted from pooled roots and leaves with Trizol reagent (Invitrogen, Carlsbad,
122   CA, USA), and complementary DNA (cDNA) was generated with the SMARTer™ RACE cDNA
123   amplification kit (Clontech, Palo Alto, CA, USA) according to the manufacturer's instructions.
124   Then, 5' and 3' RACE were performed using nested gene-specific primers (Supplementary table
125   S1). The obtained amplification products were cloned into the pMD19-D vector (Takara, Dalian,
126   China) and sequenced. Then, the two full-length sequences were assembled by DNAMAN 6.0.
127   *CiGAD1* and *CiGAD2* have been deposited in GenBank under the accession numbers KU586714
128   and KU586715, respectively.

129   The two full-length cDNA sequences of *CiGAD1* and *CiGAD2* were analyzed with ORF Finder
130   (https://www.ncbi.nlm.nih.gov/orffinder/), and the isoelectric point (pI) and molecular weight
131   (MW) of the predicted protein were estimated using ExPASy
132   (http://web.expasy.org/compute_pi/). Sequences for the protein homology analysis were
133   obtained from phytozome (https://phytozome.jgi.doe.gov/pz/portal.html), and aligned using

134 DNAMAN 8.0. The phylogenetic tree was constructed using MEGA 6.06 software by the
135 maximum-likelihood algorithm with 1000 bootstrap replicates.

2.3 Southern blot analysis

137 Genomic DNA was isolated from leaves of *C. intermedia* using CTAB method (Porebski et al.,
138 1991), and 20 μg DNAs were digested with BamH I and EcoR V, respectively. The obtaining
139 fragments were separated by electrophoresis through a 1.0% agarose, then DIG-labeled DNA
140 probes of *CiGAD1* and *CiGAD2* were hybridized followed the protocol of DIG-High Prime DNA
141 Labeling and Detection Starter Kit I (Roche Applied Science, Mannheim, Germany). Briefly, DIG-
142 labeled DNA probe was prehybridized at 60 °C for 2 h and then hybridized 37 °C for 12 h.
143 Following hybridization, the blot was washed twice in 2×SSC containing 0.1% SDS at 25 °C for 5
144 min and was washed twice in 0.1×SSC containing 0.1% SDS at 50 °C for 15 min. The
145 immunodetection of the DIG-labeled probe was performed with 1:10000 anti DIG-AP. The blot
146 was exposed to X-ray film (AGFA, Germany). DNA probes were amplified from genomic DNA by
147 using the primers designed in the sequenced DNA fragments based on the cDNA sequences of
148 *CiGAD1* and *CiGAD2*, while the probe for *CiGAD1* was mainly in the region from 1076-1370 bp in
149 the full-text cDNA, and the probe for *CiGAD2* was mainly in the region from 639-1031 bp in the
150 full-text cDNA. Primers listed in Supplementary table S1 and probe sequences listed in
151 Supplementary T3.

2.4 Assay of GAD activity

153 About 0.1 g sample (root, stem, leaf, seed, or bark) was ground into a powder in liquid nitrogen
154 and transferred to a 2-mL centrifuge tube. Then, 1 mL pre-cooled extraction buffer [150 mM
155 potassium phosphate, 5 mM EDTA, 1 mM magnesium chloride, 0.5% (w/v) polyvinylpyrrolidone,
156 3 mM 2-mercaptoethanol, 0.2 mM PLP (pyridoxal 5'-phosphate), 10% (v/v) glycerol, 1 mM
157 phenylmethylsulfonyl fluoride, pH 5.8] was added, and the homogenate was mixed well. After
158 15 min incubation on ice, the homogenate was centrifuged at 10,000 g for 15 min at 4 °C. The
159 supernatant was collected into a 2-mL volumetric flask and the volume was adjusted to 1 mL.
160 This was used as the crude enzyme solution (CES; Liu et al., 2014).

161 To measure GAD activity, the assay mixture (1 mL) contained 0.4 mL 0.1% sodium glutamate,
162 0.2 mL 0.25 mM PLP, and 0.4 mL CES. After mixing well, the reaction mixture was incubated at
163 40 °C for 5 h and then boiled for 5 min to end the reaction. Sodium glutamate was replaced by
164 0.4 mL distilled water in the blank control. After cooling, the absorbance of the mixture was
165 measured at 640 nm using a Multi-Mode Detection Platform (SpectraMax Paradigm, Molecular
166 Devices Co., Sunnyvale, CA, USA). A GABA standard curve was constructed and used to calculate
167 the GABA content in the samples based on absorption value, as described by Yang et al. (2006).
168 And protein contents were tested by Bradford method (Kruger, 1994) with bovine serum
169 albumin as standard. One unit (U) was defined as the amount (μmol) of GABA produced by the
170 action of one mg protein per hour. All determinations were representative of three biological
171 experiments and four technical replicates.

172 **2.5 Determination of GABA**

173 The plant tissues were ground to a fine powder in liquid nitrogen, and about 0.1 g of the frozen
174 homogenate was extracted using methanol and lanthanum chloride, and then centrifuged at
175 13000g for 5 min, and 0.8 ml of the supernatant was transferred to a new Eppendorf tube. The
176 160 µl of 1 M KOH was added, and centrifugation as before. The 100 µl resulting supernatant
177 was used in the spectrophotometric GABA determination (Zhang & Bown 1997). The assay
178 mixture (300 µl) also contained 75 mM potassium pyrophosphate (pH 8.6), 3.3 mM 2-
179 mercaptoethanol, 1.25 mM β-NADP$^+$, 5 mM 2-ketoglutarate and 0.02 units of GABase (Sigma).
180 The increase of OD340 nm was recorded using 96-well microplate reader. The amount of GABA
181 was calculated according to external calibration curve of GABA (Renault et al., 2010). Values
182 shown were representative of three biological experiments and four technical replicates.

183 **2.6 qRT-PCR**

184 Total RNAs were extracted from a pool of roots and leaves with Trizol reagent (Invitrogen), and
185 then cDNAs were synthesized with 0.5 µg RNAs using the Prime Script^{TM} RT reagent kit (Perfect
186 Real Time; Takara) according to the manufacturer's instructions. Specific RT-PCR primers
187 (Supplementary table S2) were designed to have melting temperatures of 60 °C and amplicon
188 lengths of 150–200 bp using Primer3 software (http://primer3.ut.ee/). Real-time qRT-PCR was
189 performed in quadruplicate using the SYBR Premix Ex Taq™ II kit (Takara) on a Roche lightCycler
190 480 (Roche Applied Sciences, Penzberg, Germany) according to the manufacturer's instructions.

191 The $2^{-\triangle Ct}$ method was adopted to analyze the qRT-PCR result, and $\triangle Ct = Ct_{target} - Ct_{EF1\alpha}$. Values

192 shown were representative of three biological experiments.

193 **2.7 Statistical analysis**
194 Data were evaluated by Duncan's multiple test in SPSS v.19. Differences were considered
195 significant at P<0.05.

196 **3. Results**

197 **3.1 Isolation of *CiGAD*s and bioinformatics analyses**

198 Two full-length cDNAs of glutamate decarboxylase (*GAD*) genes were isolated from *C.*
199 *intermedia* by 5'- and 3'-RACE, as shown in Supplementary fig. S1, and designated as *CiGAD1*
200 (GenBank ID: KU586714) and *CiGAD2* (GenBank ID: KU586715). Bioinformatics analyses showed
201 that *CiGAD1* had a 1922-bp full-length mRNA sequence containing a 1521-bp open reading
202 frame (ORF), a 107-bp 5'puntranslated region (UTR), and a 294-bp 3'-UTR; and that *CiGAD2* had
203 a 1797-bp full-length mRNA sequence containing a 1797-bp ORF, a 60-bp 5'-UTR, and a 237-bp
204 3'-UTR. The alignment showed that the nucleotide identity between *CiGAD1* and *CiGAD2* was
205 69.0%. Theoretically, *CiGAD1* encodes a 57.3-kDa peptide consisting of 506 amino acids with
206 the theoretical isoelectric point (pI) of 5.5, and *CiGAD2* encodes a 56.1-kDa peptide consisting
207 of 499 amino acids with the theoretical isoelectric point (pI) of 5.6.

208   The deduced amino acid sequences of the two CiGADs were aligned with GAD family members
209   from three typical model plants; *Arabidopsis*, *Glycine*, and *Populus* (Supplementary fig. S2). The
210   amino acid identity between CiGAD1 and CiGAD2 was 71.4%. CiGAD1 shared 88.5%, 79.9% and
211   79.4% amino acid identity with *Glycine* GAD5, *Arabidopsis* GAD1, and *Populus* GAD1,
212   respectively. CsGAD2 shared 83.1%, 75.0%, and 72.1% amino acid identity with *Glycine* GAD2,
213   *Populus* GAD5, and *Arabidopsis* GAD5, respectively. Both CiGAD proteins contained two
214   conserved domains: a PLP-binding domain in the middle region and a CaM-binding domain at
215   the carboxyl terminus (Fig. 1). The ML tree showed that CiGAD1 was located close to GmGAD5,
216   GmGAD1, and GmGAD3; while CiGAD2 was in a completely different clade, close to GmGAD2,
217   GmGAD4, PtGAD5, and AtGAD5 (Fig. 2). These findings indicated that CiGAD proteins from *C.*
218   *intermedia*, a salt- and drought-resistant desert legume shrub, had higher homologies with GAD
219   homologs in soybean, another well-known legume.

220   We also performed a southern blot analysis to estimate the copy number of two *GAD* genes in
221   *C. intermedia*. The result showed one band in BamH I or EcoR I restriction digest lane of *CiGAD1*
222   (Fig. 3A) and two bands in the BamH I or EcoRI restriction digest lane of *CiGAD2* (Fig. 3B),
223   respectively. This confirmed that *CiGAD1* had one copy, and *CiGAD2* -related genes were
224   present as two copies in the genome of *C. intermedia*.

225   **3.2 Tissue-specific expression of two *CiGAD*s**

226   The expression of two *CiGAD*s showed different patterns in various issues including roots, stems,
227   leaves, seeds, testae, and bark from mature individuals (Fig. 4A). The transcript levels of *CiGAD1*
228   were lower than those of *CiGAD2* in roots, seeds, testae, and bark, but the opposite pattern
229   was observed in other tissues. The two genes showed contrasting trends in mRNA abundance in
230   most tissues. The highest transcript level of *CiGAD1* was in testae, followed by leaves and stems,
231   while it was almost undetectable in roots and bark. In contrast, *CiGAD2* showed the highest
232   transcript level in the bark (37.9-fold that of *CiGAD1* in the bark), followed by testae and roots
233   (1.9 and 21.3 fold higher, respectively, than that of *CiGAD1*). The GAD activity was highest in
234   testaes (9.9 U), followed by seeds (3.2 U). But its activity was lower, and similar, among the
235   other tested tissues (1.1–1.9 U) (Fig. 4B).

236   **3.3 Stress-specific expression of two *CiGAD*s**

237   To investigate whether the two *CiGAD*s were expressed differently in response to various stress
238   conditions, total RNA was extracted from roots and leaves of young seedlings under several
239   different stress treatments. Both *CiGAD*s showed dramatic increases in their transcript levels in
240   roots and leaves in response to stress (Fig. 5A, B). Under Zn stress, the transcript level of
241   *CiGAD1* increased by 74.3-fold and that of *CiGAD2* increased by 6.3-fold, compared with their
242   respective levels in control roots. *CiGAD1* showed 3.4–11.3 fold increases in its transcript levels
243   in roots under dehydration, Cd, Na, and low/high temperature stresses. *CiGAD2* transcript
244   levels showed 1.0–5.5 fold increases in roots under all of the stresses except Cd stress (Fig. 5A).
245   In leaves, the transcript levels of *CiGAD1* and *CiGAD2* strongly increased in response to high
246   temperature, by 218.1-fold and 114.2-fold, respectively, compared with their respective levels

247 in the control leaves (Fig. 5B). The transcript levels of *CiGAD1* and *CiGAD2* increased by 20.6-
248 fold and 36.0-fold, respectively, under NaCl stress, and by 19.7-fold and 21.8-fold, respectively,
249 under low temperature stress. Under Zn, Cd, and dehydration stress, *CiGAD1* expression
250 increased by 4.4–8.5 fold, and *CiGAD2* expression increased by 1.5–5.2 fold. These results
251 indicated that the transcript levels of *CiGAD1* and *CiGAD2* increased to varying degrees in
252 response to various stresses.

253 To analyze the expression of the two *CiGAD*s under stress in more detail, seedlings were
254 treated with NaCl for different times, as shown in Fig. 6. The transcript levels of *CiGAD1* and
255 *CiGAD2* significantly increased as the duration of the NaCl treatment extended, although there
256 were some fluctuations in their transcript levels in leaves at 12 h and in roots at 6 h (Fig. 6A, B).
257 In roots, the transcript levels of *CiGAD1* and *CiGAD2* had increased by 20.8-fold and 16.4-fold,
258 respectively, after 6 h of salt stress, and by 41.2-fold and 12.0-fold, respectively, after 24 h of
259 salt stress, compared with their respective levels at 0 h (Fig. 6A). However, in the leaves,
260 *CiGAD1* and *CiGAD2* transcript levels remained relatively stable except at 6 h of salt stress,
261 when they were increased by 20.6–23.7 fold, and 11.5–13.8 fold, respectively, compared with
262 their respective levels at 0 h (Fig. 6B). Further analyses showed that *CiGAD1* was more strongly
263 induced than was *CiGAD2* as the NaCl treatment extended. Corresponding to the expression of
264 *CiGAD*s, there was a considerable increase in endogenous GABA with increasing duration of the
265 NaCl treatment (Fig. 6C). The GABA content in roots increased rapidly, to 5.9-fold its initial level
266 after 3 h of NaCl stress, and to more than 10-fold its initial level at 12 and 24 h of NaCl stress. In
267 the leaves, the GABA content slightly increased after 3 h of NaCl stress, and markedly increased
268 to 4.7–5.6 fold its initial level by 6 h of NaCl stress. These findings suggested that there was a
269 positive correlation between the expression of *CiGAD* genes and GABA accumulation during
270 NaCl stress.

271 **3.4 ABA regulated *CiGAD*s expression under NaCl stress**

272 Treatment of NaCl-stressed seedlings with exogenous ABA significantly affected the expression
273 of both *CiGAD*s, and the changes in expression differed between the two genes (Fig. 7). In the
274 roots, both *CiGAD*s showed gradually increasing transcript levels with increasing concentrations
275 of exogenous ABA at 3 h. However, *CiGAD1* showed a 3.8-fold increase in its transcript levels in
276 response to 0.5 μM ABA, similar to the increase in response to 200 μM ABA. The two genes
277 showed almost opposite trends in expression in the roots at 24 h of treatment with ABA; the
278 transcript levels of *CiGAD1* decreased with increasing ABA concentrations, but were still much
279 higher than the transcript levels of *CiGAD2* (Fig. 7A). In the leaves, however, after 3 h of ABA
280 treatment, low concentrations of exogenous ABA (0.5 and 1.0 μM) inhibited the expression of
281 both *CiGAD*s, but higher concentrations of ABA induced the expression of both genes. After 24
282 h of treatment with ABA, 0.5 μM ABA considerably increased the expression levels of both
283 genes to more than 5.6-fold that in the control (0 μM ABA), but ABA at other concentrations
284 drastically reduced the transcript levels of both genes, except for *CiGAD1* at 10 μM ABA (Fig.
285 7B). These results demonstrated that, even at micromolar concentrations, ABA participates in
286 regulating *CiGAD1* and *CiGAD2* transcription during salt stress.

287 **4. Discussion**

288 Much attention has been paid to the roles of GABA in the development and stress responses of
289 plants (Batushansky et al., 2014, 2015; Michaeli and Fromm, 2015, Molina-Rueda et al., 2015;
290 Shi et al., 2010). Because GAD is a key enzyme regulating GABA production and the GABA shunt
291 pathway (Michaeli and Fromm, 2015; Shimajiri et al., 2013). In this study, two *GAD* genes
292 (*CiGAD1* and *CiGAD2*) were cloned based on specific EST sequences (Supplementary T1)
293 identified in our previous work on the molecular response of *C. intermedia* to salt stress. These
294 two genes had ORFs encoding 498- and 494-amino acid residues. Homology analyses showed
295 that these two CiGADs exhibited relatively high similarity (72.1%–88.5%) to GADs from three
296 model plants: soybean, Arabidopsis*,* and poplar, so *CiGADs* were classified into the GAD family
297 (Supplementary fig. S2). The CaM/Ca^{2+}-dependent GADs conserved the putative CaM-binding
298 domain in plants, such as Arabidopsis (Turano et al., 1998), tomato (Gallego et al., 1995), and *P.*
299 *ginseng* (Lee at al., 2010). But some GADs did not include CaM-binding domain, such as rice
300 GAD2 (Akama et al., 2001). Sequence analyses confirmed that both of the CiGADs included a
301 conserved PLP domain and a CaM-binding domain (Fig. 1; Trobacher et al., 2013), but whether
302 both CiGADs were indeed activated via CaM or not need a further research (Mei et al., 2016). A
303 phylogenetic analysis indicated that *CiGAD1* and *CiGAD2* were much more closely related to
304 *GAD* genes in soybean than to *GAD* genes in the woody plant poplar (Fig. 2). Southern blot
305 analysis confirmed that *CiGAD1* had one copy and *CiGAD2* -related genes were present as two
306 copies in *C. intermedia* (Fig. 3). However, both *AtGAD1* and *AtGAD2* are single copy genes in
307 Arabidopsis (Turano et al., 1998), and single copy of *GmGAD1* is also observed in soybean
308 (Matsuyama et al., 2009). But there are at least two copy of the *PgGAD* in *P. ginseng* (Lee at al.,
309 2010).These results indicate that the copy number of *GAD* genes depends on each plant.

310 The cloning of *CiGAD*s genes will be useful for further research on their functions during plant
311 development and stress responses. As mentioned in the Introduction, *GAD*s are expressed in
312 most tissues, but different *GAD*s show different spatial and temporal expression patterns
313 (Miyashita & Good, 2008; Akama et al., 2001; Liu et al., 2014). Our results showed that *CiGAD1*
314 was mainly expressed in the stems, leaves, and testae; whereas *CiGAD2* was expressed at high
315 levels in bark and was also expressed in the roots and testae (Fig. 4A). The expression patterns
316 indicated that *CiGAD2* might participate in vascular differentiation, like *PpGAD* in maritime pine
317 (Molina-Rueda et al., 2010). Molina-Rueda et al. (2015) also reported that vascular
318 development was closely linked to GABA production corresponding to *PpGAD* expression.
319 Because the GAD activities were higher in testae and seeds, *CiGAD1* and *CiGAD2* may also play
320 a role in plant reproduction like *GADs* in citrus (Liu et al., 2014) and apple (Trobacher et al.
321 2013).

322 Several studies have shown that plant *GAD*s expression responds differently to various abiotic
323 stresses in herbaceous plants (Zhuang et al., 2010; Lee et al., 2010). Our previous findings
324 showed that exogenous GABA can regulate the molecular responses of *C. intermedia* during salt
325 stress via its roles in regulating signaling (reactive oxygen species, ethylene, and ABA-producing
326 enzymes RBOH/ACO/ABA2) and nitrogen metabolism (nitrate transporter, arginase, nodulin)

327   (Shi et al., 2010). These results prompted us to analyze the expression patterns of the two
328   identified *CiGAD*s in response to various stresses and exogenous ABA, which orchestrates the
329   interaction between biotic and abiotic stresses via signaling pathways (Atkinson & Urwin, 2012).
330   When *Caragana* seedlings were subjected to short-term (3 h) stress treatments including
331   salinity (Na), heavy metals (Zn and Cd), heat (40°C), cold (4°C), and drought (3 h dehydration),
332   the transcript levels of *CiGAD1* and *CiGAD2* increased in the roots and leaves (Fig. 3), possibly as
333   a result of an increase in Ca^{2+} induced by each stress treatment (Akihiro et al., 2008). The only
334   exception was that *CiGAD2* transcript levels did not increase in response to Cd^{2+} (Fig. 4A, B),
335   which is hypothesized to displace metal cofactors (such as Zn^{2+}) from proteins or compete with
336   Ca^{2+} to bind to Ca^{2+}-binding proteins (Stohs & Bagchi, 1995). The salt stress treatment induced
337   *CiGAD1* and *CiGAD2* expression more strongly in leaves (20.6–36.0 fold) than in roots (4.4–5.5
338   fold) (Fig. 5A, B). For comparison, *PgGAD* expression in *P. ginseng* increased 3-fold after 8 h of
339   salt treatment (Lee et al., 2010). This result indicated that the two *CiGAD*s might play a crucial
340   role in the salt stress response in leaves.

341   In terms of heavy metal stress responses, the Zn treatment significantly increased expression of
342   both *CiGAD*s in leaves and roots, and induced a substantial increase in *CiGAD1* expression (a
343   74.0-fold increase) in roots (Fig. 5A), whereas Cd had opposite effects on the two genes,
344   inducing an increase in *CiGAD1* expression (4.4–6.0 fold), but no change or a decrease in
345   *CiGAD2* expression (Fig. 5A, B). An analysis of the *Arabidopsis* transcriptome showed that
346   *AtGAD4* transcript levels increased 8.4-fold under a 50 μM Cd^{2+} treatment (Weber et al., 2006).
347   A surprising result in this study was that heat and cold stresses strongly induced expression of
348   *CiGAD1* and *CiGAD2*, especially in leaves; the transcript levels of these genes increased by
349   218.1-fold and 114.2-fold, respectively, in response to heat, and by 19.7-fold and 21.8-fold,
350   respectively, in response to cold (Fig. 5B). For comparison, *PgGAD* expression in *P. ginseng*
351   increased by 3–5 fold after 8-h heat and cold treatments (Lee et al., 2010). Next, we examined
352   changes in the transcript levels of two *CiGAD*s during a 24-h salt treatment, and observed
353   constant increases in their expression (Fig. 6A, B) consistent with GABA accumulation (Fig. 6C).
354   The present result suggested that these two *CiGAD*s in *Caragana* were involved in multiple
355   stress responses, especially the responses to Zn and heat stress.

356   In plants, the phytohormone ABA is a key endogenous signaling molecule in the responses to
357   various stresses. Understanding ABA-signaling is essential for improving plant performance in
358   the future (Atkinson & Urwin, 2012; Raghavendra et al., 2010). In our previous study, we found
359   that exogenous GABA induced the expression of the ABA-biosynthetic gene *ABA2* in *Caragana*
360   under salt stress (Shi et al., 2010). Exogenous ABA has also been shown to enhance GAD activity
361   and increase the GABA content in fava bean (Yang et al., 2016), and to induce the expression of
362   *ZmGAD1*, which harbors ABA-related *cis*-elements in its promoter (Zhuang et al., 2010). In the
363   present study, we found that exogenous ABA increased the transcript levels of *CiGAD1* and
364   *CiGAD2* at an early stage (3 h) of the salt treatment (Fig.7A, B); while after 24 h of salt
365   treatment, the transcript levels of both genes were reduced in the roots of ABA-treated plants,
366   but strongly increased in the leaves of plants treated with 0.5 μM ABA (Fig. 7A, B). These

367 different transcriptional responses of *CiGAD1* and *CiGAD2* to exogenous ABA may be because of
368 differences in ABREs in their promoter regions (Liu et al., 2014).

369 In conclusion, the results of this study showed that two *CiGAD*s cloned from *Caragana* are
370 closely related to homologs in another legume, soybean. The transcript levels of *CiGAD2* were
371 much higher than those of *CiGAD1* in bark, suggesting that *CiGAD2* might play a role in
372 secondary growth of woody plants. The GAD activities were higher in testae and seeds than
373 other tissues, *CiGADs* may also play a role in plant reproduction. Multiple stresses significantly
374 affected the transcript levels of both *CiGAD*s. Interestingly, Zn and heat stresses had the
375 strongest effects on *CiGAD1* expression, indicating that *CiGAD1* plays important roles in the
376 responses to Zn and heat stresses. Our results also suggested that ABA is involved in regulating
377 these two *CiGAD*s during stress in *Caragana*, an important nitrogen-fixing legume shrub. These
378 results will be useful for further research on the role of *GADs* in carbon and nitrogen
379 metabolism and in signal transduction during the responses to environmental stresses.

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Figure 1

Alignment of the deduced amino acid sequence of two CiGADs in *Caragana intermedia* with the deduced amino acid residues of GADs in *Arabidopsis thaliana*, *Populus trichocarpa*, *Glycine max*.

Accession number: AtGAD1 (AT5G17330), AtGAD2 (AT1G65960), AtGAD3 (AT2G02000), AtGAD4 (AT2G02010), AtGAD5 (AT3G17760), GmGAD1 (Glyma02g40840), GmGAD2 (Glyma09g29900), GmGAD3 (Glyma14g39170), GmGAD4 (Glyma16g34450), GmGAD5 (Glyma18g04940), PtGAD1 (Potri.T059200), PtGAD2 (Potri.004G075200), PtGAD3 (Potri.004G075300), PtGAD4 (Potri.010G100500), PtGAD4.1 (Potri.008G141100), and PtGAD5 (Potri.012G039000). The abbreviation of gene names are as follows: At, *Arabidopsis thaliana*; Pt, *Populus trichocarpa*; Gm, *Glycine max*. Identical and similar amino acid residues were shown in black or pink, respectively. The pyridoxal-5-phosphate binding domain of the GADs was underlined with a thin line, and the CaM binding domain was underlined with a thick line; Trp (W), the important site for in vitro binding to CaM, was indicated by an asterisk.

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CiGAD1   TGWBTPIHVDAASGGFIAPFLYPELEWDFRLPLVKSINVSGHKYGLVYAGIGWVIWRSKVDLEDBIIFHINYLGADQPTF   314
CiGAD2   TGWDTPIHVDAASGGFVAPFLYPELEWDFRLPLVKSINVSGHKYGLVYAGVGWVVWRSKADLEDDIVFHINYLGSDQPTF   317
AtGAD1   TGWDTPIHVDAASGGFIAPFLYPELEWDFRLPLVKSINVSGHKYGLVYAGIGWVVWRNKBDLEEDIIFHINYLGADQPTF   314
AtGAD2   TGWNTPIHVDAASGGFIAPFIYPELEWDFRLPLVKSINVSGHKYGLVYAGIGWVVWRAABDLEEDIIFHINYLGADQPTF   313
AtGAD3   TGWDTPIHVDAASGGFIAPFLYPDLEWDFRLPLVKSINVSGHKYGLVYAGIGWVVWRTKTDLEDBIIFHINYLGADQPTF   314
AtGAD4   TGWDTPIHVDAASGGFIAPFLYPELEWDFRLPLVKSINVSGHKYGLVYAGIGWVVWRTKTDLEDDIIFHINYLGADQPTF   314
AtGAD5   TGWBTPIHVDAASGGFIAPFLYPDLEWDFRLPWVKSINVSGHKYGLVYAGVGWVVWRTKDDLEEDLVFHINYLGADQPTF   313
PtGAD1   TGWDTPIHVDAASGGFIAPFIYPELEWDFRLPLVKSINVSGHKYGLVYAGVGWVVWRNKBDLEEDIIFHINYLGADQPTF   314
PtGAD2   TGWDTPIHVDAASGGFIAPFIYPELEWDFRLPLVKSINVSGHKYGLVYAGVGWVIWRNKEDLEEDIIFHINYLGADQPTF   314
PtGAD3   TGWDTPIHVDAASGGFIAPFIYPDLEWDFRLPLVKSINVSGHKYGLVYAGIGWVIWRSKBDLEEDIIFHINYLGADQPTF   314
PtGAD4   TGWDTPIHVDAASGGFIAPFLWPDLEWDFRLPLVKSINVSGHKYGLVYAGVGWVIWRSKBDLEEDIIFHINYLGTDQPTF   314
PtGAD5   TGWNTPIHVDAASGGFIAPFIWPDLEWDFRLPLVKSINVSGHKYGLVYAGVGWVVWRTKBDLEDBIIFHINYLGSDQPTF   317
GmGAD1   TGWDTPIHVDAASGGFIAPFLYPELEWDFRLPLVKSINVSGHKYGLVYAGIGWVIWRSKBDLEEDIIFHINYLGADQPTF   314
GmGAD2   TGWDTPIHVDAASGGFIAPFLYPDLEWDFRLPLVKSINVSGHKYGLVYAGVGWVVWRSKDDLEDBIVFHINYLGSDQPTF   317
GmGAD3   TGWDTPIHVDAASGGFIAPFLYPELEWDFRLPLVKSINVSGHKYGLVYAGIGWVIWRSKBDLEEDIIFHINYLGADQPTF   314
GmGAD4   TGWDTPIHVDAASGGFIAPFLYPDLEWDFRLPLVKSINVSGHKYGLVYAGVGWVVWRSKDDLEDBIVFHINYLGSDQPTF   317
GmGAD5   TGWDTPIHVDAASGGFIAPFIYPELEWDFRLQLVKSINVSGHKYGLVYAGIGWVIWRSKQDLEEDIIFHINYLGADQPTF   314
         tgw tpihvdaasggf apf  p lewdfrl  vksinvsghkyglvy g gwv wr   dlp  l fhinylg dqptf
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                   _____________________________________
                                     *
CiGAD1   NNDGKVVAKKSALETQREITAVWKKFVLERKKLNDKMSGVC.........   506
CiGAD2   .....EKVEKSITETQAEIAKYWKRLVEGK......RVGTC.........   499
AtGAD1   SDNLMVTVKKSDIDKQRDIITGWKKFVADRKK....TSGIC.........   502
AtGAD2   .IAENVKEKKMEKEILMEVIVGWRKFVKERKK....MNGVC.........   494
AtGAD3   .......VKKTPEETQREVTAYWKKFVDTKTD....KNGVPLVASITNQ.   500
AtGAD4   .......VKKTPEETQREVTAYWKKLLETKKT...NKNTIC.........   493
AtGAD5   ......VKVKTAKMSLEDITKYWKRLVEHK......RNIVC.........   494
PtGAD1   KEAAANKEKRDLEKT.REITTVWRKFVMQR.K.....MNGVC.........   496
PtGAD2   KDAVADKDKKDVQKETREIITAWRKLVIQRKK....MNGVC.........   498
PtGAD3   EQHGAVVKKTALETQREITTIWRKFVMEKKK....MNGVC.........   500
PtGAD4   GKNGTTVVKKTAAETQREITTYWKNFVTAKKS...NRNKIC.........   503
PtGAD5   .MDGKKTVKKSSREIQEEVTLYWRRLASEK......RTGAC.........   504
GmGAD1   NDEKVLFAKKSEIETQREITKAWKKFVMDRKK....MNGVC.........   503
GmGAD2   .....EKVK.SAIETQKEIALYWKRLVDGK......RLGAC.........   498
GmGAD3   NEEKVLFAKKSEIETQREITSVWKKFVMERKK....MNGVC.........   503
GmGAD4   .....EKIKKAAIETQKEIAFYWKRLVDGK......RLGAC.........   499
GmGAD5   ENGKVVVAKKNPMETQREITAIWKKFVLERKKNNDKMNGVC.........   503
                                     w
```

Figure 2

Phylogenetic analysis of GAD sequences from Caragana intermedia and other three model plants *Arabidopsis thaliana*, *Populus trichocarpa*, *Glycine max*.

The consensus tree was obtained by the Maximum-Likelihood method in MEGA 6.06. A bootstrap analysis of 1,000 replicates was performed. Accession numbers were as shown in Legend of Figure 1. The candidate GADs were marked with black triangle.
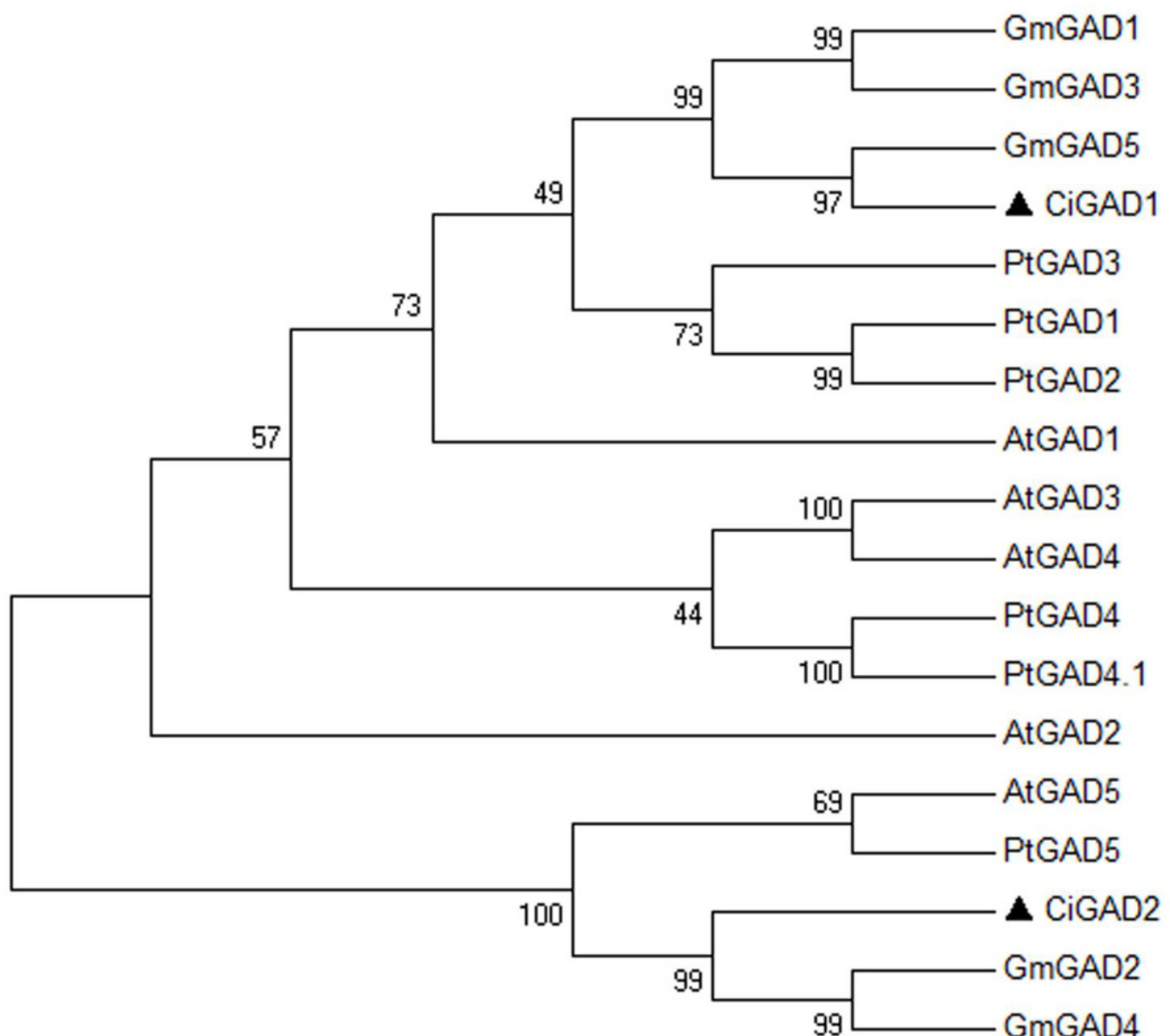
Figure 3

Southern blotting analysis of *CiGAD1* (A) and *CiGAD2* (B)

Lane 1: BamH I; Lane 2: EcoR V. Red arrows were targeted bands; M: 10,000 bp DNA marker; blank: DNA probe.
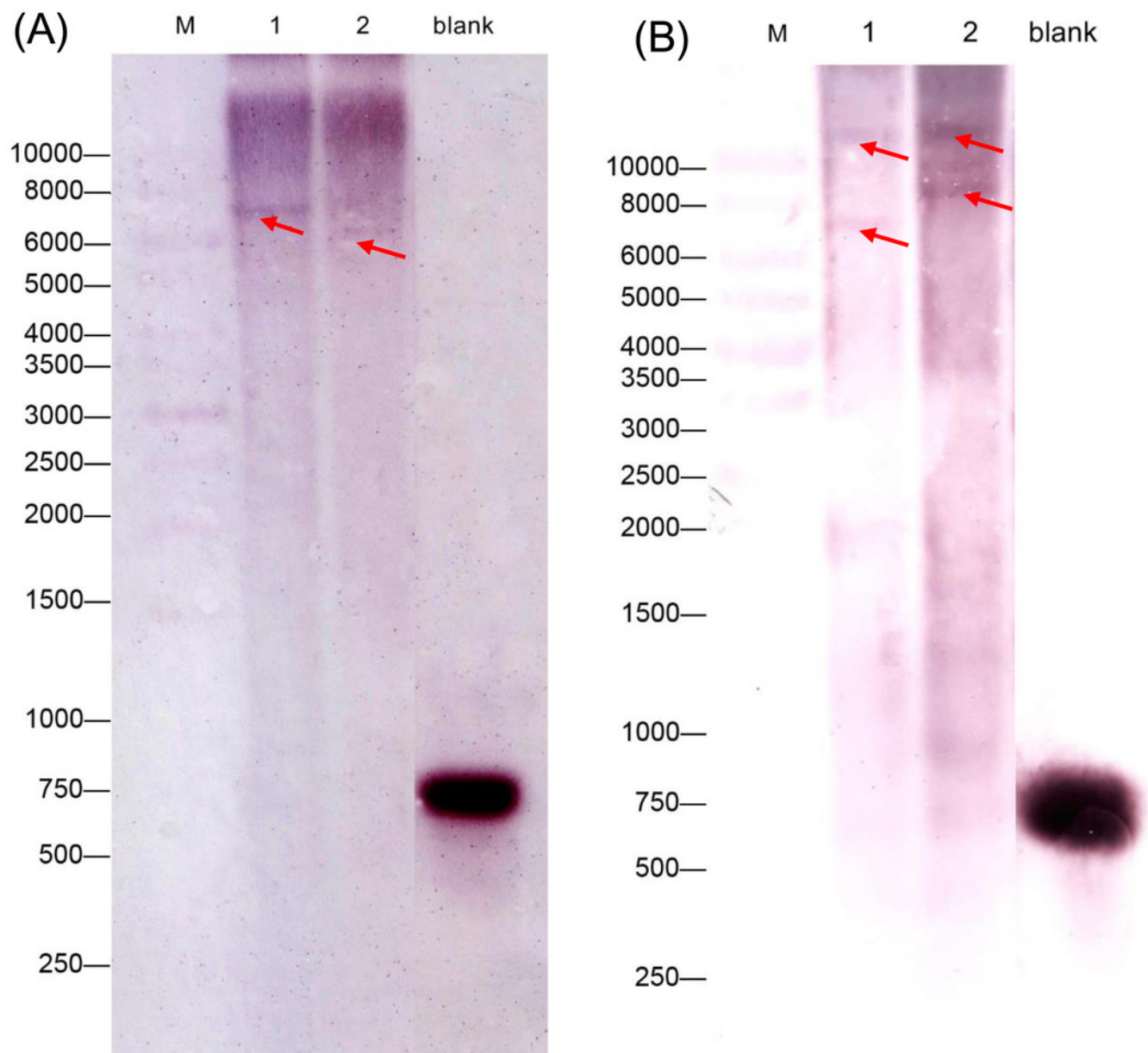
Figure 4

Expression of two *CiGADs* (A) and total GAD activity (B) in different tissues of the mature *Caragana intermedia*, including roots, stems, leaves, seeds, testae and bark.

The means and standard errors were calculated from three biological replicates. Different lower-case letters between any two sampling points indicate signification difference at $P < 0.05$ by Duncan's test.
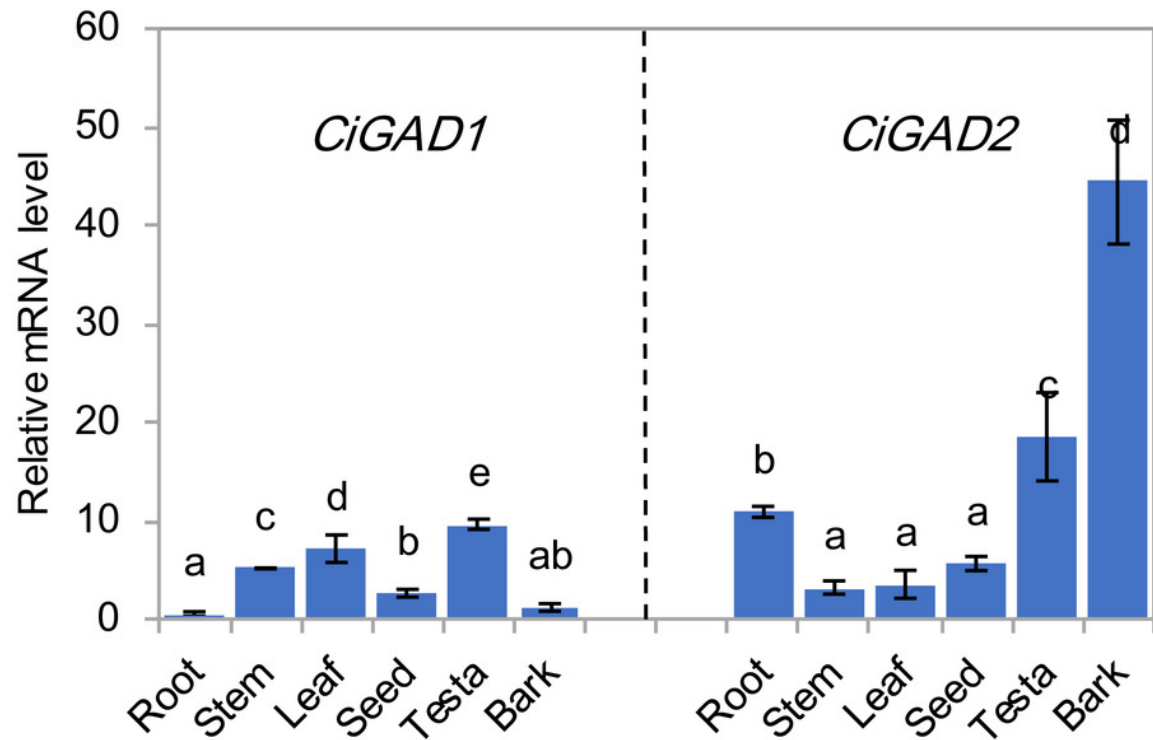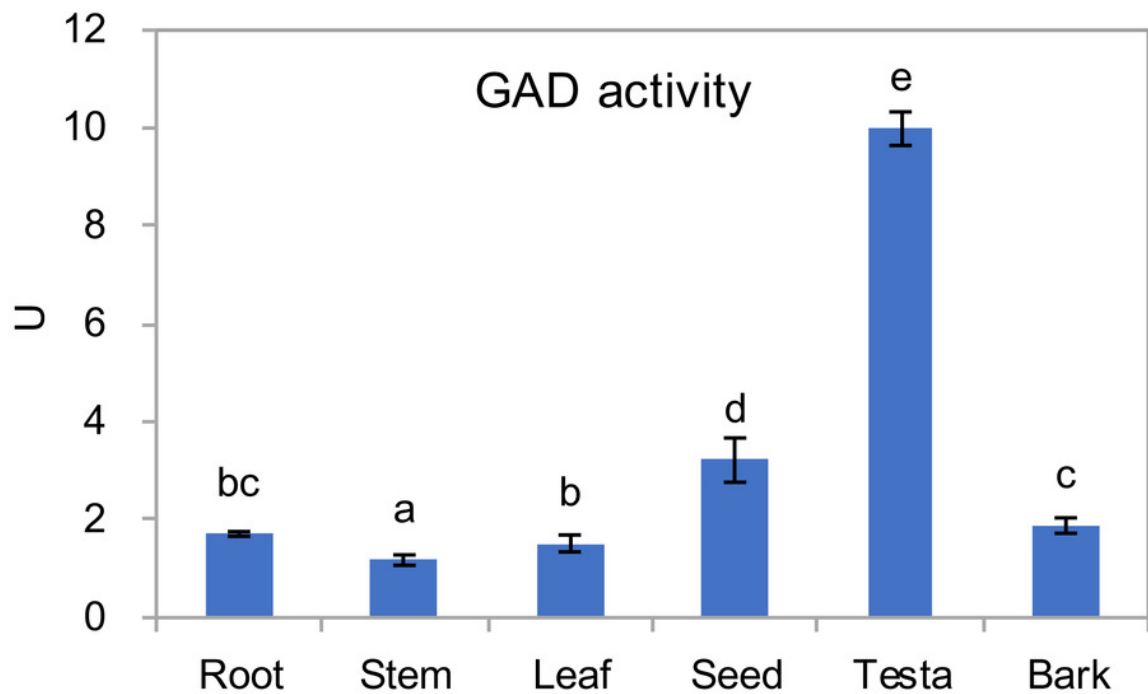
Figure 5

Stress-specific expression of two *CiGADs* in roots and leaves of young *Caragana intermedia* seedlings under different stress treatments for 3 h.

CK: Water; Na: 200 mM NaCl; Zn: 100 μM $ZnSO_4$; Cd: 500 μM $CdCl_2$; HT: High temperature 40 °C; LT: Low temperature 4 °C; DE: Dehydration. The means and standard errors were calculated from three biological replicates. Different lower-case letters between any two sampling points indicate signification difference at $P < 0.05$ by Duncan's test.
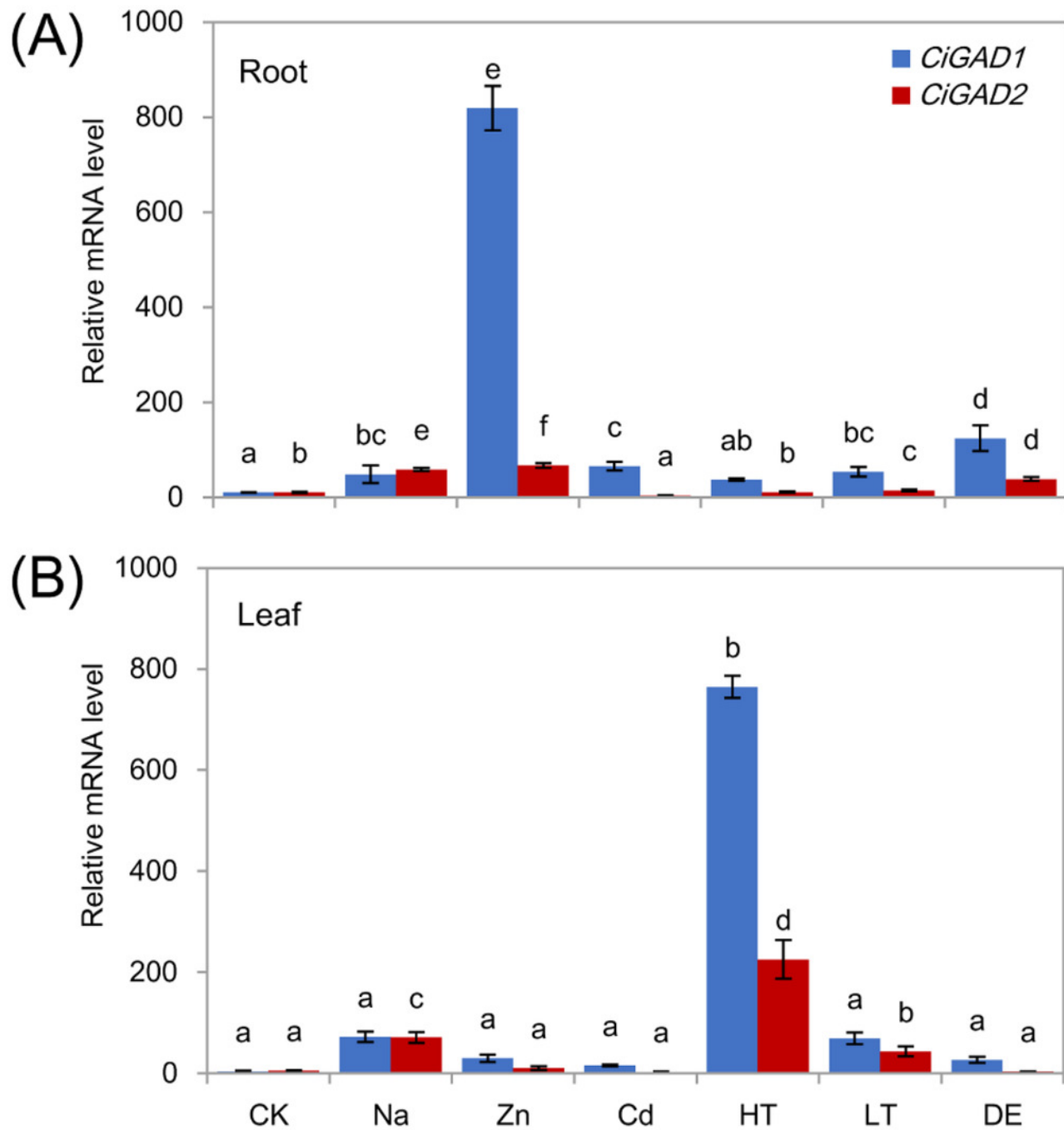
Figure 6

Expression of *CiGADs* and production of GABA in roots and leaves of young Caragana intermedia seedlings under 200 mM NaCl treatment for 0, 3, 6, 12 and 24 h.

(A) and (B) *CiGADs*' expression in root and leaves, respectively; (C) GABA production. The means and standard errors were calculated from three biological replicates. Different lower-case letters between any two sampling points indicate signification difference at $P < 0.05$ by Duncan's test.
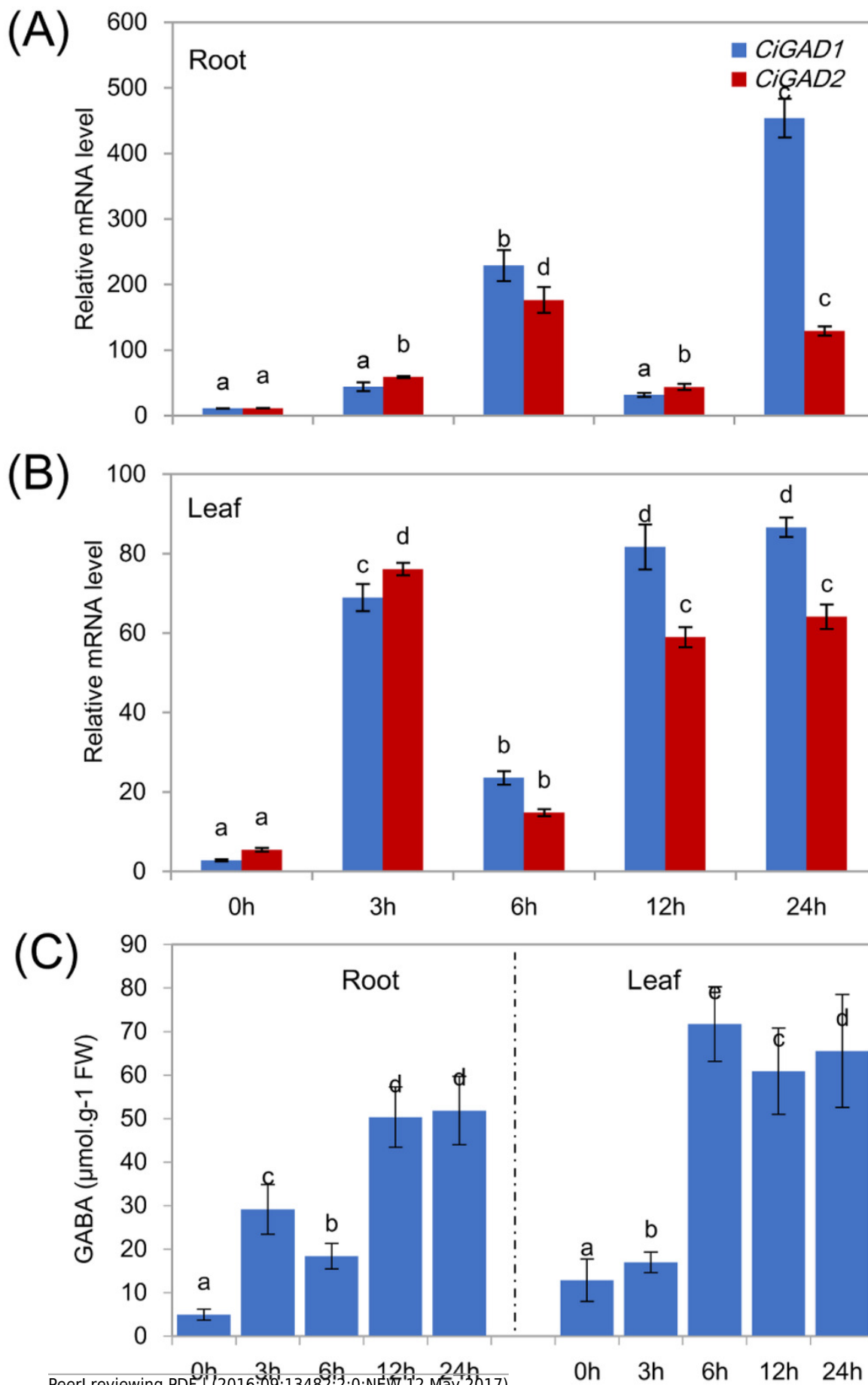
Figure 7

Effects of ABA on *CiGADs*' expression in roots (A) and leaves (B) of young *Caragana intermedia* seedlings under 200 mM NaCl treatment for 3 and 24 h.

The concentration of exogenous ABA was 0, 0.5, 1, 10, 100 and 200 μM. The means and standard errors were calculated from three biological replicates. Different lower-case letters between any two sampling points indicate signification difference at $P < 0.05$ by Duncan's test.