Evolution and expression analysis of the soybean glutamate decarboxylase gene family

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Glutamate decarboxylase (GAD) is an enzyme that catalyses the conversion of L-glutamate into γ-aminobutyric acid (GABA), which is a four-carbon non-protein amino acid present in all organisms. Although plant GAD plays important roles in GABA biosynthesis, our knowledge concerning *GAD* gene family members and their evolutionary relationship remains limited. Therefore, in this study, we have analysed the evolutionary mechanisms of soybean *GAD* genes and suggested that these genes expanded in the soybean genome partly due to segmental duplication events. The approximate dates of duplication events were calculated using the synonymous substitution rate, and we suggested that the segmental duplication of *GAD* genes in soybean originated 9.47 to 11.84 million years ago (Mya). In addition, all segmental duplication pairs (*GmGAD1/3* and *GmGAD2/4*) are subject to purifying selection. Furthermore, *GmGAD* genes displayed differential expression either in their transcript abundance or in their expression patterns under abiotic stress conditions like salt, drought, and cold. The expression pattern of paralogous pairs suggested that they might have undergone neofunctionalization during the subsequent evolution process. Taken together, our results provide valuable information for the evolution of the *GAD* gene family and represent the basis for future research on the functional characterization of *GAD* genes in higher plants.

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1. Introduction

GABA (γ-aminobutyric acid) is a four-carbon non-protein amino acid found in prokaryotes and eukaryotes (Bouché *et al*. 2003). It is synthesized primarily by decarboxylaytion of L-glutamic acid, catalysed by glutamate decarboxylase (GAD; EC 4.1.1.15), transported into mitochondria, and then converted to succinic semialdehyde by GABA transaminase (GABA-T; EC 2.6.1.19) (Shelp *et al*. 1999). This indicates that the metabolism of GABA takes place in two cellular compartments, cytosol (GABA synthesis) and mitochondrion (GABA degradation). In animals, GABA is known as the

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major inhibitory and the major excitatory neurotransmitter in the mammalian central nervous system (Ben-Ari *et al*. 2012); it is involved in most aspects of normal brain function like memory, learning, and cognition (Zhou and Danbolt 2013). In addition, GABA promotes parasympathetic activity to provide beneficial effects including anti-stress, insomnia, and relaxation and has several beneficial effects on human health by preventing chronic alcohol-related diseases, inhibiting the proliferation of cancer cells, and decreasing blood pressure (Oh *et al*. 2003; Oh and Oh 2004; Yoshimura *et al*. 2010; Takeshima *et al*. 2014). Since GABA in animals has received increased attention due to its effects on human health, it has been demonstrated that the accumulation of GABA is associated with stress factors in plants (Bouche and Fromm 2004; Nogata and Nagamine 2009; Molina-Rueda *et al*. 2010; Renault *et al*. 2010; Yang *et al*. 2013). In plants, GABA plays a role in buffering mechanisms in C and N metabolism, cytosolic pH regulation, and protection against oxidative stress (Roberts 2007). GABA has been shown to act as a signaling molecule, which can modulate nitrate uptake in roots (Beuve *et al*. 2004) and the expression of *Arabidopsis* 14-3-3 gene-family members (Lancien and Roberts 2006). Recently, it has been suggested that calcium-dependent GABA homeostasis regulates pollen germination and polarized tube growth by affecting actin filament pattern, vesicle trafficking, and the deposition of cell wall components (Ling *et al*. 2013). These findings suggest that GABA might act as a signaling molecule, which can modulate gene expressions and plant development.

The soybean (*Glycine max* L.) is one of the most important agricultural crops and has become more popular in Western diets due to its nutritional and pharmacological importance (Wang *et al*. 2013). During the germination process of soybean, the level of an antinutritional factor, trypsin inhibitor, decreases (Kumar *et al*. 2006), whereas the amount of some nutrients and phytochemicals such as isoflavone, tocopherols, and amino acid increase (Shi *et al*. 2010a). The level of GABA is also altered in soybean during seed germination and is mediated by *GAD* gene expression and GAD activities (Matsuyama *et al*. 2009; Xu and Hu 2014). GABA synthesis under environmental stress conditions is mediated by the changing catalytic properties of GADs on cytosolic pH and the activity of Ca^{2+}/calmodulin complex (Baum *et al*. 1993; Snedden *et al*. 1995; Renault *et al*. 2010). In a salt stress condition, a significantly increased level of GABA has been analysed in soybean, suggesting that the salt-induced accumulation of GABA in soybean roots resulted from the activation of GAD, together with enzymes involved in the polyamine degradation pathway (Xing *et al*. 2007; Queiroz *et al*. 2012). Based on genome-wide analysis, five *GAD* genes (*GmGAD1* to *5*) that contain conserved residues and calmodulin (CaM)-binding domain in the C-terminal region have been identified in the soybean genome and an increasing level of these genes' expression has been determined during the germination process (Hyun *et al*. 2013). Although this indicates a positive correlation between the expression of soybean G*AD* (*GmGAD*) genes and GABA accumulation during the germination process, systematic investigations including evolution mechanisms and expression divergence on *GmGAD* genes are still needed to fully understand this relationship.

In this study, to investigate the evolutionary relationships of *GmGAD* genes, we evaluated their expansion and evolutionary mechanisms by their duplication, distribution on chromosomes, and relative-rate tests. In addition, *cis*-elements in promoters and the expression profiles of all *GmGADs* during response to abiotic stresses were determined to analyse the expression divergence between *GmGADs*. Taken together, our results will facilitate our understanding of the evolution of dynamic functions for *GmGADs*, which provides insights into the functional characterization of GABA in higher plants.

2. Materials and methods

2.1 *Analysis of soybean GAD genes' duplication*

To infer their evolutionary history, phylogenetic analysis based on the protein sequences of the GADs was performed using the Phylogeny.fr server (*http://www.phylogeny.fr*) in 'one click' mode. The reliability of the trees obtained was tested using bootstrapping with 1000 replicates.

To categorize the expansion of the *GmGAD* genes, we investigated the gene expansion pattern mediated by segmental duplication (chromosomal segments). Segmental duplication genes were identified on the basis of their physical location on individual chromosomes, with not more than one intervening gene. Then, the locus identifier of *GmGADs* was submitted to the plant genome duplication database (*http://chibba.agtec.uga.edu/duplication/*).

2.2 *Estimation of gene evolution rates*

To analyse the evolutionary dates of the segmental duplication, the coding sequences of *GmGAD* genes were aligned on the basis of the corresponding aligned protein sequences using the PAL2NAL Web server (*http://www.bork.embl.de/pal2nal/*). The ratio of nonsynonymous substitutions per nonsynonymous site (Ka) to synonymous substitutions per synonymous site (Ks) in homologous gene pairs was calculated by using the yn00 procedure of the PAML package (Yang 2007). Then, the approximate date of the duplication event was calculated using the mean Ks values ($T=Ks/2\lambda$), assuming clock-like rates (λ) of synonymous substitution of 6.1×10^{-9} substitutions/synonymous site/year for soybean (Lynch and Conery 2000). Tajima relative rate tests (Tajima 1993), as implemented in MEGA version 4.0 (Tamura *et al*. 2007), were performed with amino acid sequences for the two soybean GAD duplicate pairs.

2.3 *Analysis of cis-elements in soybean GAD promoter regions*

The 1.5 kb upstream sequences of the ATG initial codon for each soybean *GAD* gene were obtained from Phytozome v9.1 (*http://www.phytozome.net/*). Promoter elements for all the sequences were analysed using PLACE (*http://www.dna.affrc.go.jp/PLACE/*).

2.4 *Plant growth and the treatment of abiotic stresses*

Soybean (cultivar Baktae) seeds were germinated and grown in a greenhouse with day and night temperatures of 25°C and 20°C, respectively. To determine the expression pattern of *GmGADs* under abiotic stress conditions, 20 day-old plants were watered with 250 mM NaCl or 10% (w/v) PEG8000. For cold treatment, plants were watered with cold water (4°C) and maintained in a cold room at the same temperature. Samples for RNA analysis were collected at the times indicated in figure 3, quickly frozen in liquid nitrogen, and stored at −80°C until analysis.

2.5 *Quantitative real-time PCR analysis*

Total RNA was extracted using the RNeasy Plant Mini kit according to the manufacturer's instructions. Then, a 1 μg aliquot of total RNA was reverse-transcribed into cDNA using the QuantiTect® Reverse Transcription kit. Quantitative real-time PCR reactions were carried out in 20 μL volumes containing 0.4 μM of each primer and 10 μL of the SYBR Green Real-time PCR Master Mix kit. All reactions took place in the ECO™ Real-time PCR system with the following program: one cycle of 95°C for 10 min and 40 cycles of 95°C for 15 s, 60°C for 15 s, and 72°C for 15 s. The expression levels of different genes were normalized to the constitutive expression level of soybean ubiquitin-3 (Schneider *et al*. 2011). For all qRT-PCR analyses, the means of the three biological experiments were calculated to estimate gene expression. The primers used for qRT-PCR are listed in table 1.

Table 1. List of primers used for quantitative real-time PCR analysis

Primer name	Nucleotides
GmGAD1-F	5′-AGCAGGTGAAGAAAATGACGA-3′
GmGAD1-R	5′-TCTTCTTCCTGTCCATCACAAA-3′
GmGAD2-F	5′-CCACTCACCCAGATGAAAAAG-3′
GmGAD2-R	5′-AGCTGCATCCTTTGGTATTGA-3′
GmGAD3-F	5′-TTCCTGTGTGAGTACGTGTGC-3′
GmGAD3-R	5′-CATCATTGCGCTCATAATCCT-3′
GmGAD4-F	5′-GGTGAGAAGATTAAGAAAGCTGC-3′
GmGAD4-R	5′-GGTAAGCCTAGCATGCTCCA-3′
GmGAD5-F	5′-CTCAGTGCAGAAGAAAATGGC-3′
GmGAD5-R	5′-ACACCCCCTTGAAGCTAACAC-3′
UBI-F	5′-GTGTAATGTTGGATGTGTTCCC-3′
UBI-R	5′-ACACAATTGAGTTCAACACAAACCG-3′

3. Results and discussion

3.1 *Evolutionary patterns and exon structure of GmGADs*

Gene duplication is a key process for the generation of new genes, which in turn facilitates the generation of new functions (Flagel and Wendel 2009). Following whole genome duplication, small-scale duplications including segmental and tandem duplications arise during DNA replication and recombination (Flagel and Wendel 2009; Lestari *et al*. 2013). A tandem duplication event is characterized as the genes of one family occurring within the same intergenic region or neighboring intergenic regions, whereas gene duplication on different chromosomes is characterized as a segmental duplication event (Flagel and Wendel 2009; Jiang *et al*. 2013). In addition, segmental duplication is a frequent event in higher plants because most plants are diploidized polyploids and retain numerous duplicated chromosomal blocks within their genomes (Yin *et al*. 2013). In the soybean genome, five *GAD* genes are located on five different chromosomes (Hyun *et al*. 2013), suggesting that tandem duplication might not contribute to the expansion of the *GAD* genes in soybean. To identify the chromosomal homologous segments within genomes (known as paraplogous), we downloaded the within-genome duplication information of soybean from the Plant Genome Duplication Database (PGDD; *http://chibba.agtec.uga.edu/duplication/*). A search for duplicated genes revealed the existence of two gene pairs (*GmGAD1/3* and *GmGAD2/4*) in soybean (figure 1A), indicating that segmental duplication is a major contributory event leading to the expansion of *GAD* genes in soybean.

In order to trace the dates of the segmental duplication events, the PAML software was used to estimate the Ks (the number of synonymous substitutions per synonymous site) and Ka (the number of nonsynonymous substitutions per nonsynonymous site) distances, as well as the Ka/Ks ratios, and the approximate dates of duplication events were calculated using Ks. Ks in segmental duplication in *GmGAD* genes ranged from 0.1155 to 0.1444, dating the duplication event to have occurred between 9.47 million years ago (Mya) to 11.84 Mya (table 2). Since soybean has undergone two separate polyploidy events 13 and 59 Mya (Schmutz *et al.* 2010; Severin *et al*. 2011), the segmental duplications

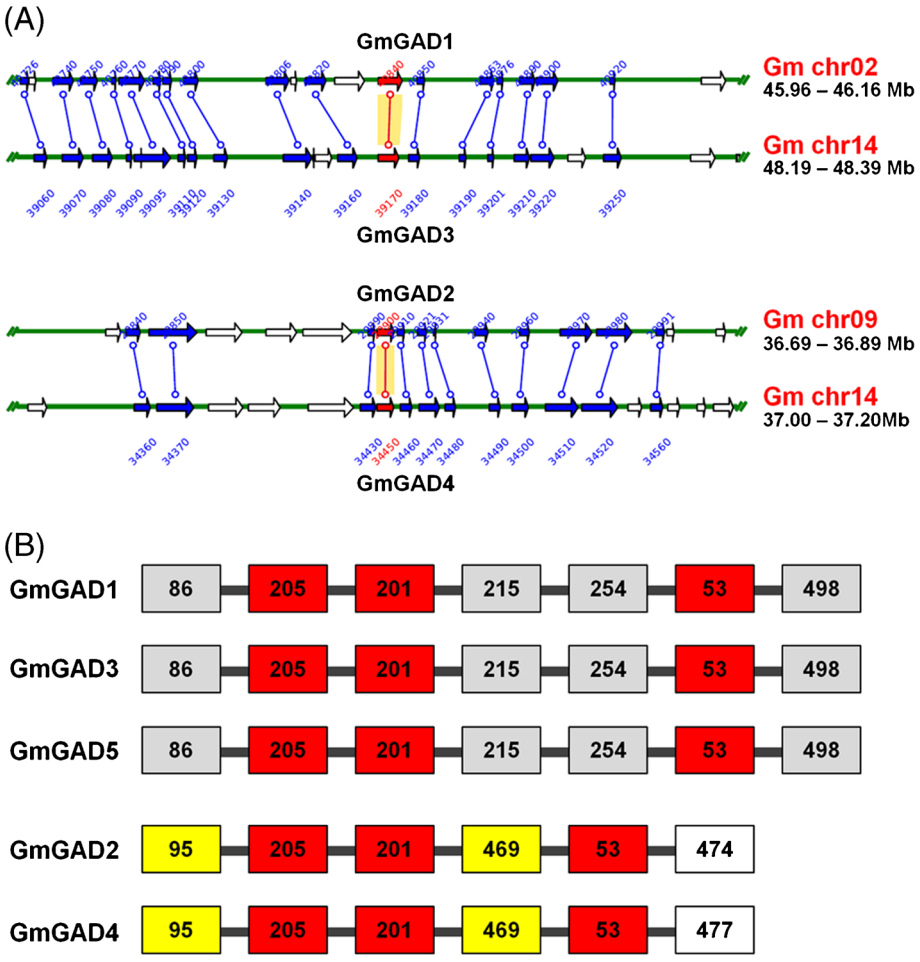

Figure 1. Genome duplication and exon structure analysis of segmental duplicated soybean *GAD* pairs. (**A**) The segmental duplications in regions of the soybean genome encompassing *GmGAD*. Paralogous gene pairs were generated by gene duplications (*http://chibba.agtec.uga.edu/duplication/*). All intra/cross-species blocks for each query gene display regions of ~200 kb. Blue arrows indicate the other anchor gene in the region, whereas red arrows indicate the query locus. Green lines connect gene pairs. (**B**) Exon structure of *GmGAD* genes. The numbers in the boxes are the nucleotide length of each exon. Exons colored red is are conserved in length among all soybean *GAD* genes. Exons with different color are conserved in length between each duplicated pair.

of the *GmGAD* genes occurred around 13 Mya. This estimate indicates that the segmental duplications of the *GmGAD* genes are produced from recent Glycine-specific genome duplication (~13 Mya). To characterize the evolution rate between two sequences, the Ka/Ks ratio has been used. In general, when positive selection dominates, the Ka/Ks ratio is greater than 1. In addition, Ka/Ks<1 indicates genes subject to negative selection, whereas Ka/Ks ratio of 1 indicates that the positive and negative selection forces balance each other (Yang and Nielsen 2000). The nonsynonymous substitution rates of 2 segmental duplication pairs are markedly lower than their synonymous substitution rates, and their Ka/Ks values are <1 (table 2), suggesting that these segmental duplication pairs were subjected to negative selection. Furthermore, the Tajima relative rate tests (Tajima 1993) were conducted to investigate whether duplicated genes evolve at the same rate, and the orthologous sequences from *Arabidopsis GAD* genes (supplementary figure 1) were used as an outgroup. The null hypothesis of equal rates of evolution between these lineages was supported because the P-value is greater than 0.05 (table 3). This indicates that all segmental duplicated pairs in *GmGADs* evolved at a similar rate.

Conserved exonic structures, including the conserved gene structure with the same number of nucleotides in the exons and the conserved intron phases, indicate the similarities of the studied genes (von Schantz *et al*. 2006). The intron phage analysis of *GmGADs* based on the splicing sites

Table 2. Divergence between paralogous GAD gene pairs in soybean

Gene 1	Gene 2	S	N	Ks	Ka	Ka/Ks	Mya
GmGAD1	GmGAD3	373.1	1135.9	0.1155	0.0170	0.1467	9.47
GmGAD2	GmGAD4	373.5	1120.5	0.1444	0.0153	0.1062	11.84

These gene pairs were identified at the terminal nodes of the gene tree shown in supplementary figure 1. The number of synonymous sites (S), number of non-synonymous sites (N), synonymous substitution rate (Ks), and non-synonymous substitution rate (Ka) are presented for each pair.

The data of the duplication event were estimated according to $T = Ks/2\lambda$. Mya, million years ago.

between the adjacent intron's average length suggested that *GmGADs* share exons with almost the same intron phase (Hyun *et al*. 2013). As shown in figure 1B, *GmGAD1*, *3* and *5* shared 7 exons with the same number of nucleotides and the same intron phase (Hyun *et al*. 2013), whereas *GmGAD2* and *4* shared 5 exons with the same number of nucleotides. In addition to the commonly shared exons with the same numbers of nucleotides mentioned above, there are equal-length-exons (colored red) shared by all *GmGAD* genes (figure 1B), supporting a common ancestral relationship between each duplicated pair.

3.2 *Cis-regulatory elements in the promoter region of GmGADs*

To characterize the general features of the promoter regions of *GmGAD* genes, 1.5-kb sequences upstream of the ATG start codon were used to search against known *cis*-regulatory elements in the PLACE database using the Signal Scan Search (*http://www.dna.affrc.go.jp/PLACE/*). The elements presented in the 5′-UTR regions of the coding strands are represented in figure 2. Several phytohormone-responsive elements were found in the *GmGAD* gene promoters, including ABRE (ABRELATERD1) for abscisic acid (ABA) responses, GARE (GAREAT) for gibberellins (GA) responses, Auxin response factor binding site (ARFAT), Cytokinin-responsive element (ARR1AT), and Ethylene-responsive element (ERELEE4). Among the phytohormone-responsive elements identified here, the most abundant phytohormone-responsive element is ARR1AT with 4 to 11 duplications in each *GmGAD* gene (figure 2). This indicates that *GmGAD* expression might be the strongly induced by cytokinin. In addition, T/GBOXATPIN2 for jasmonate signaling was identified in *GmGAD1*, *3*, and *5* promoters, whereas MYB1LEPR, which regulates defense-related gene expression in tomato (Reddy *et al*. 2014), was only found in *GmGAD4*. The MYC recognition site found in the promoters of the dehydration-responsive gene (S000407) was identified in all *GmGAD* genes with 3 to 7 copies in each *GmGAD* gene. Other stress-response elements, including S000453 (salt-responsive element; Park *et al*. 2004), BIHD1OS (disease resistance responses; Luo *et al*. 2005) and WBOXATNPR1 (response to environmental stresses; Chen *et al*. 2002), were present in the *GmGAD* promoters. All of the aforementioned putative *cis*-regulatory elements suggested that *GmGADs* might respond to environmental stresses via a complex mechanism mediated by cross-talk between phytohormones.

3.3 *Expression patterns of soybean GAD genes under different abiotic stress conditions*

The accumulation of GABA in response to many diverse stimuli, such as drought, salt, mechanical stimulation, heat

Table 3. Tajima relative rate tests of soybean GAD plicate genes[a]

Testing group	Mi	Md	M1	M2	M3	$\chi 2$	*P-value*
GmGAD1/3 with AtGAD1	407	10	3	4	78	0.14	0.70546
GmGAD2/4 with AtGAD5	42	27	3	4	418	0.14	0.70546

[a] The Tajima relative rate test was used to examine the equality of evolutionary rates between soybean duplicate pairs.

Mi is the identical sites in all three sequences.

Md is the divergent sites in all three sequences.

M1 is the number of unique differences in the first paralog.

M2 is the number of unique differences in the second paralog.

M3 is the number of unique differences in the three paralog.

P-value less than 0.05 is often used to reject the null hypothesis of equal rates between lineages.

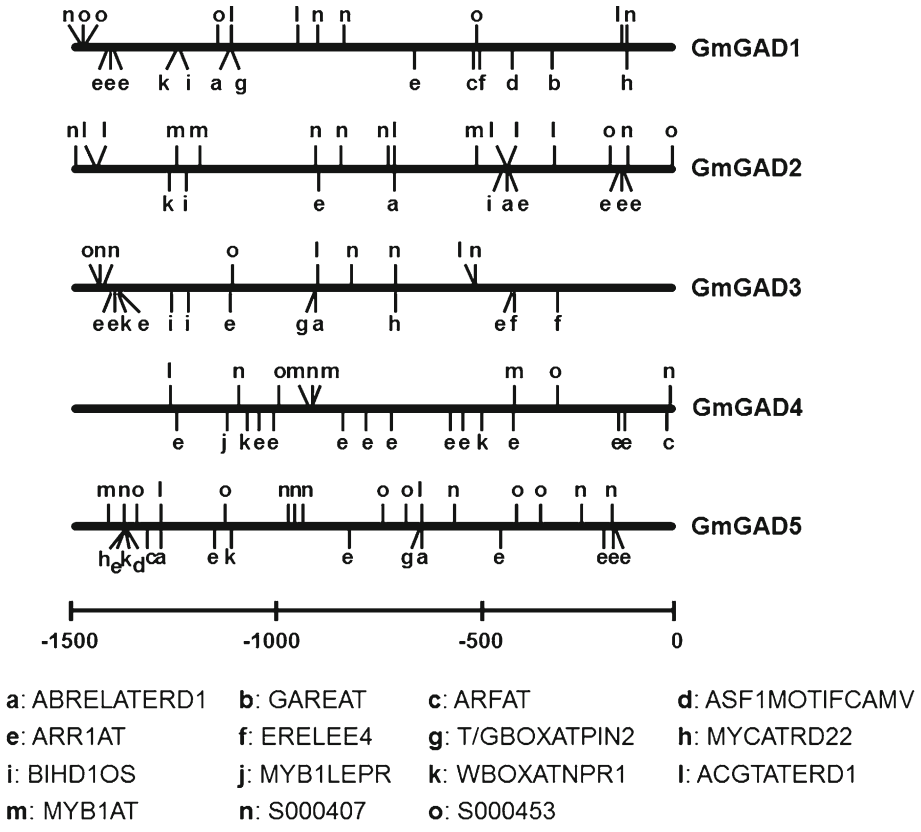

Figure 2. Putative *cis*-acting regulatory elements related to stress and hormone responses in promoter regions of the *GmGAD* gene family.

shock, and plant hormone (Shi *et al.* 2010b) suggests that GABA might be involved in the stress signaling pathway. In fact, GABA plays a role as a signaling molecule, which can modulate gene expression in higher plants (Shelp *et al.* 2012). Under stress conditions, GABA synthesis is mediated by the changing catalytic properties of GAD depending on the activity of the Ca^{2+}/CaM complex and the cytosolic pH (Baum *et al.* 1993; Snedden *et al.* 1995; Hyun *et al.* 2013). Therefore, it is generally supposed that GABA accumulation induced by stress results from the activation of GAD (Bown *et al.* 2006). To gain insight into the potential functions of soybean *GAD* genes during their response to environmental stresses, we investigated the expression profiles of *GmGADs* in various tissues exposed to high salinity, cold stress, and PEG-induced drought stress. As shown in figure 3, short-term exposure of soybean plants to these stresses induced or repressed the expression of *GmGAD* genes, and the effect varied with tissues, i.e. leaves, stems, and roots. In leaves and stems, *GmGAD2* and *3* were induced by salt treatment (figure 3B and C), whereas other *GmGADs* were down-regulated. During response to PEG-induced drought stress, *GmGAD2* (figure 3B) and *GmGAD5* (figure 3E) exhibited similar expression patterns in leaves and roots. In addition, all *GmGAD* genes were transiently down-regulated in response to drought stress, and then increased (figure 3). In addition, the transcript level of most *GmGAD* genes was down-regulated under cold stress (figure 3F), whereas an increasing level of *GmGAD3* (figure 3C), *GmGAD4* (figure 3D) and *GmGAD5* (figure 3E) transcripts was observed in roots when plants were treated with cold stress. According to the above results, *GmGAD2* might be important GAD family for salt and drought responses in soybean, since it was up-regulated by these stresses. Although several *cis*-elements including environmental stress and hormone response related *cis*-acting regulatory elements were found in the *GmGAD* gene promoters, transcript levels for *GmGAD 1, 4* and *5* were hardly changed under drought, salt and cold stresses. One explanation for this might be that some unidentified *cis*-regulated elements play an important role in regulating the expression of those *GmGADs* in stress response in soybean. The expression pattern of two paralogous pairs (*GmGAD1/3* and *GmGAD2/4*) diverged dramatically (figure 3A to D), indicating substantial neofunctionalization during subsequent evolution processes. This suggests that the expression pattern of *GmGAD* genes had diversified substantially, which might

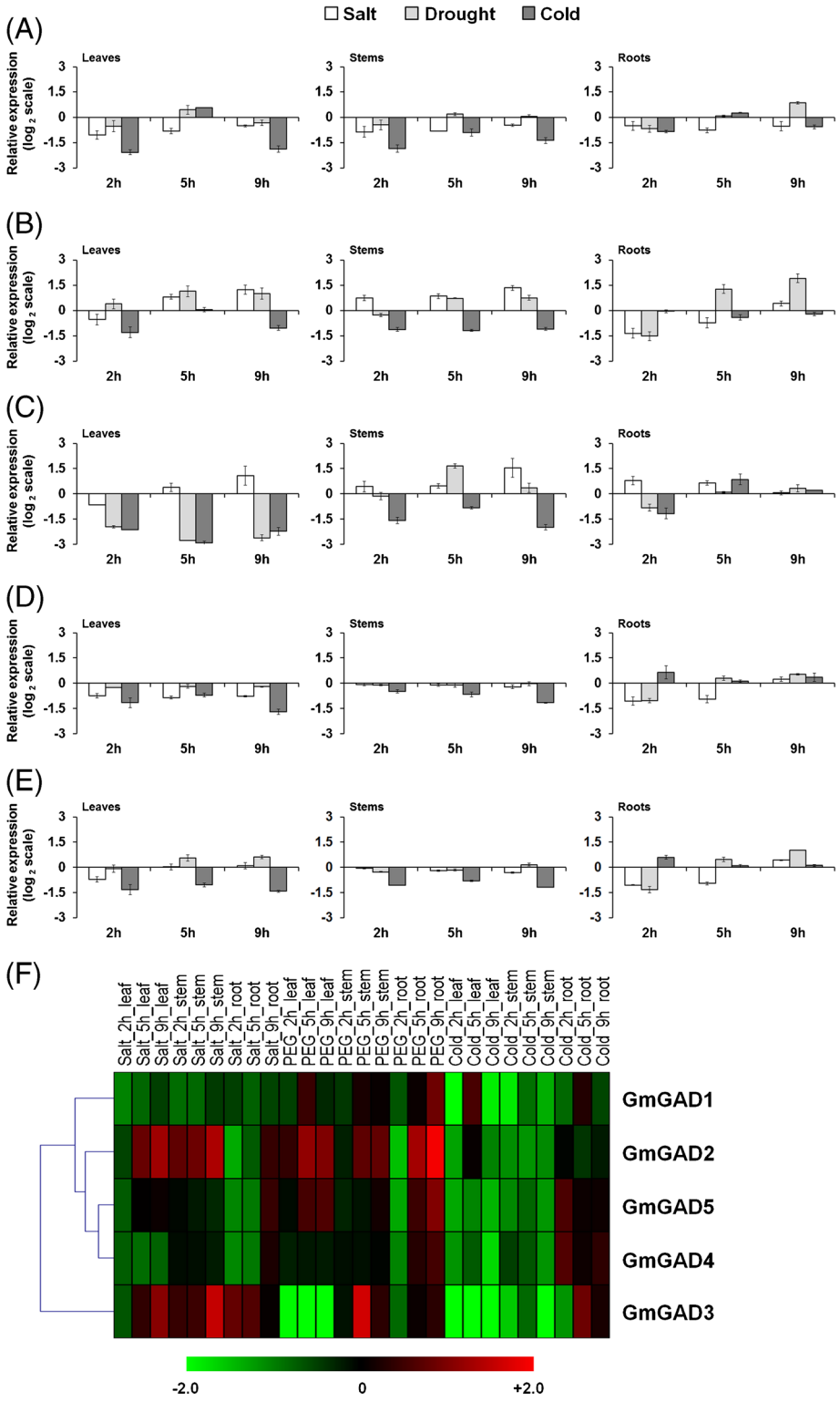

Figure 3. Expression pattern of *GmGAD* genes in various tissues exposed to high salinity, cold stress, and PEG-induced drought stress. Transcript levels *GmGAD1* to *5* (**A** to **E**) were normalized to the constitutive expression level of soybean ubiquitin-3 and were expressed relative to the values at 0 h (control). The Y-axis represents the normalized relative expression values (Log_2). The means and standard errors were calculated from three independent experiments. (**F**) Heatmap of *GmGAD* expression profiles. A heatmap displaying the transcript abundance is produced by quantitative real-time PCR analysis. Log_2-based fold-changes were used to create the heatmap. Blocks with colors indicate decreased (green) or increased (red) transcript accumulation relative to the control.

be due to the different divergent fates after segmental duplications.

4. Conclusion

In this study, we provide new insight into the genic evidence of GADs and how these genes evolved in soybean from the perspective of bioinformatics. The majority of *GAD* gene duplications in soybean appeared to have been caused by segmental duplication, which occurred within the last 9.47 to 11.84 million years. In addition, adaptive evolution analysis showed that purifying selection (negative selection) has driven the evolution of segmental duplication pairs (*GmGAD1/3* and *GmGAD2/4*). Furthermore, an in-depth analysis of *GmGAD* gene expression patterns under different stress conditions suggested clear demarcation in the roles assigned to each gene. Taken together, these data provide a solid foundation for further understanding of the underlying evolution mechanisms in *GAD* genes in higher plants and new fundamental information that should permit future researchers to characterize the function of *GAD* genes in soybean.

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