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A 14-3-3 Family Protein from Wild Soybean (*Glycine Soja*) Regulates ABA Sensitivity in *Arabidopsis*

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Abstract

It is widely accepted that the 14-3-3 family proteins are key regulators of multiple stress signal transduction cascades. By conducting genome-wide analysis, researchers have identified the soybean 14-3-3 family proteins; however, until now, there is still no direct genetic evidence showing the involvement of soybean 14-3-3s in ABA responses. Hence, in this study, based on the latest *Glycine max* genome on Phytozome v10.3, we initially analyzed the evolutionary relationship, genome organization, gene structure and duplication, and three-dimensional structure of soybean 14-3-3 family proteins systematically. Our results suggested that soybean 14-3-3 family was highly evolutionary conserved and possessed segmental duplication in evolution. Then, based on our previous functional characterization of a *Glycine soja* 14-3-3 protein GsGF14o in drought stress responses, we further investigated the expression characteristics of *GsGF14o* in detail, and demonstrated its positive roles in ABA sensitivity. Quantitative real-time PCR analyses in *Glycine soja* seedlings and GUS activity assays in $P_{GsGF14O}$:GUS transgenic *Arabidopsis* showed that *GsGF14o* expression was moderately and rapidly induced by ABA treatment. As expected, *GsGF14o* overexpression in *Arabidopsis* augmented the ABA inhibition of seed germination and seedling growth, promoted the ABA induced stomata closure, and up-regulated the expression levels of ABA induced genes. Moreover, through yeast two hybrid analyses, we further demonstrated that GsGF14o physically interacted with the AREB/ABF transcription factors in yeast cells. Taken together, results presented in this study strongly suggested that *GsGF14o* played an important role in regulation of ABA sensitivity in *Arabidopsis*.

Introduction

Abscisic acid (ABA) is a key plant hormone that regulates a wide variety of developmental and physiological processes, including maintenance of seed dormancy [1–3], inhibition of seed germination and seedling growth [4,5], accumulation of free proline [6], control of stomata

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movement [7,8], regulation of gene expression [9] and plant responses to environmental stress [10]. It has been well demonstrated that ABA participates in plant stress responses through several pathways. Firstly, environmental stress promotes the accumulation of endogenous ABA in leaves [11], and the increased ABA content induces stomata closure in guard cells [12]. Consequently, stomata closure reduces the leaf water loss and protects plant from damages caused by water deficit [13]; at the same time, stomata closure also might depress the gas exchange of plant leaves, decrease the photosynthetic activity and thus lead to growth penalty [12]. Furthermore, ABA also induces the expression of a considerable number of genes, which are always involved in stress responses [14], and promotes the accumulation of free proline [15], which is helpful for osmotic regulation under environmental challenges. Considering the key regulatory roles of ABA in plant development and stress responses, a considerable amount of researches during the past decades have focused on elucidating the ABA signaling transduction pathway.

After years of studies, scientists have established the central ABA signaling transduction pathway, namely the PYR/PYL/RCAR-PP2C-SnRK2 complex mediated signaling cascade [12,16,17]. Under normal conditions, PP2C phosphatases negatively regulate SnRK2 protein kinases by direct interaction and de-phosphorylation of multiple residues within SnRK2s, so the ABA signaling cascade is blocked [18–20]. Upon environmental stress, endogenous ABA accumulates rapidly, and PYR/PYL/RCAR receptors bind ABA, which results in structure change of themselves [21,22]. The ABA-bound PYR/PYL/RCARs then interact with PP2Cs and inhibit PP2C phosphatase activity, and thereby SnRK2s are released and activated [21]. Activated SnRK2s could phosphorylate downstream factors to trigger a series of consequences, such as the membrane ion channels to induce stomata closure [23], or the AREB/ABF transcription factors to regulate ABA induced gene expression [24,25].

The AREB/ABF genes encode the group A subfamily bZIP transcription factors, and nine AREB/ABF homologs have been identified in *Arabidopsis* [9,26,27]. Among them, five AREB/ABFs are well known to be involved in ABA responses, including AREB1/ABF2, AREB2/ABF4, AREB3/ABF1, ABF3, and ABI5. They directly recognize and bind the ABRE cis-elements in the promoter regions of the ABA-responsive genes [26,28]. In addition to SnRK2s, recent studies uncovered that 14-3-3 proteins could also interact with AREB/ABFs and participate in ABA stress responses in plant cells [29–32].

The 14-3-3 family proteins are phosphor-serine/threonine-binding proteins that regulate a wide array of targets via direct protein-protein interaction [33,34]. Up to now, researches have demonstrated the crucial regulatory roles of 14-3-3s in ABA stress responses [35,36]. The 14-3-3s and ABFs interaction has been identified in *Arabidopsis* [36], *Thellungiella* [32] and barley [29]. By conducting genome-wide analysis, previous studies have identified the 14-3-3 family proteins in soybean [37,38]. Among them, SGF14c and SGF14l are found to play critical roles during the early developmental stages of soybean nodules [39]. In addition, SGF14l also could affect isoflavonoid synthesis by regulating the intracellular localization of the GmMYB176 transcription factor [40,41]. In a previous study, we functionally characterized a 14-3-3 family gene from wild soybean (*Glycine soja*), *GsGF14o*, which participated in stomata and root hair development, and negatively regulated plant drought tolerance [37]. However, until now, there is no direct genetic evidence showing the involvement of soybean 14-3-3 proteins in ABA responses.

Hence, in this study, based on the latest *Glycine max* genome on Phytozome v10.3, we initially analyzed the evolutionary relationship, genome organization, gene structure and duplication, and three-dimensional structure of soybean 14-3-3 family members systematically. Then, based on our previous studies [37], we further investigated the expression characteristics of *GsGF14o* in detail, and demonstrated its positive roles in ABA sensitivity. *GsGF14o* was found to be moderately and rapidly induced by ABA stress, and have obvious effect on plant ABA

sensitivity, including ABA inhibition on seed germination and seedling growth, as well as ABA induction on stomata closure and gene expression. Finally, according to the protein interaction of GsGF14o with AREB/ABFs in yeasts, we proposed that *GsGF14o* positively regulated plant ABA sensitivity, maybe by directly interacting with AREB/ABF transcription factors.

Results

Phylogenetic and Gene Structure Analysis of Soybean 14-3-3 Gene Family

A keyword (14-3-3) search against the soybean (*Glycine max* Wm82.a2.v1) genome at Phytozome v10.3 (http://phytozome.jgi.doe.gov/pz/portal.html) identified a total of twenty sequences containing the 14-3-3 domain (PFAM: PF00244). According to the expression data on Phytozome, two of them (Glyma.20g043700 and Glyma.17G208100) did not express in all detected tissues (S1A Fig). Our previous RNA-seq data of *Glycine soja* roots in response to alkaline stress [42] also showed no expression values for these two genes (S1B Fig). And further check revealed that both of them encoded much fewer amino acids than other soybean 14-3-3 genes, and lacked at least one or more α-helices (nine α-helices for other 14-3-3s). Hence, they are excluded in this study, and detailed information of the remaining eighteen 14-3-3 genes was showed in Table 1.

Previous studies have revealed the conserved structure of 14-3-3 proteins. In this study, we further investigated the protein sequence characteristics and conformational features of soybean 14-3-3 proteins in detail (Fig 1). Protein sequence alignment revealed that similar to 14-3-3s in other species, soybean 14-3-3 proteins were highly conserved in amino acid architecture, and consisted of nine α-helices (α1 to α9). Among them, five α-helices (α1, α3, α5, α7, and α9) were relatively conserved in amino acid sequence than the other four helices, indicating that these five helices might play conserved and important functions during evolution. Notably, the sequences in the C-terminus of soybean 14-3-3s were divergent, and SGF14k possessed an N-terminal extension.

Furthermore, the phylogenetic comparison of the soybean, rice and Arabidopsis 14-3-3s revealed that these 14-3-3 proteins could be clustered into two subfamilies, the ε subfamily and the non-ε subfamily (Fig 2), the same as described previously [38]. Subsequently, the non-ε subfamily was further divided into two groups (Group I-II) and the ε subfamily was divided into three groups (Group III-V). Notably, group I, III and IV only contained soybean and *Arabidopsis* 14-3-3 proteins, while group V only included rice and *Arabidopsis* 14-3-3s. As expected, soybean 14-3-3 family was only consisted of four groups (Group I-IV, Fig 3A), without the fifth group in Fig 2.

Generally speaking, the divergence of exon-intron structure within families always contributes to the evolution of multiple gene families [43]. Paralogous genes within families usually show highly conserved exon-intron organization [44]. Hence, to gain further insights into the structural diversity of soybean 14-3-3 family genes, we then investigated and compared the exon-intron organization in their coding sequences (Fig 3B). As shown in Fig 3B, members from the ε subfamily (group III and IV) averagely shared more introns than the non-ε subfamily (group I and II) members. In details, all of the ε subfamily genes had five introns, while members from the non-ε subfamily averagely possessed three introns (Fig 3B). Furthermore, members within each individual group exhibited similar exon-intron organization pattern, with similar exon numbers and nearly identical exon lengths (Fig 3B). These findings suggested that the exon-intron organization of soybean 14-3-3s was highly conserved within the same group.

Table 1. Detailed information of soybean 14-3-3 family genes.

Subfamily	Gene Name	locus ID	Location	Alternative Splices	Sequence Length				Position of 14-3-3 Domain
					DNA (bp)	mRNA (bp)	CDS (bp)	Protein (aa)	
non-ε	SGF14g	Glyma.02G208700	Chr02:39388574..39391014 forward	2	2441	1393	789	262	9–245
subfamily	SGF14k	Glyma.14G176900	Chr14:43637893..43642553 forward	2	4661	1377	945	314	61–297
	SGF14i	Glyma.06G101500	Chr06:8052625..8054939 reverse	4	2315	1266	840	279	8–242
	SGF14h	Glyma.04G099900	Chr04:9132954..9135203 reverse	3	2250	1329	867	288	8–242
	SGF14j	Glyma.06G094400	Chr06:7432085..7434388 forward	1	2304	1071	753	250	9–246
	SGF14b	Glyma.04G092600	Chr04:8158031..8160711 forward	1	2681	1344	753	250	9–246
	SGF14a	Glyma.18G298300	Chr18:57587135..57590454 forward	1	3320	1612	774	257	6–242
	SGF14m	Glyma.08G363800	Chr08:47528826..47532060 reverse	3	3235	2054	783	260	6–239
ε subfamily	SGF14r	Glyma.20G025900	Chr20:2845106..2852380 reverse	1	7275	1201	783	260	7–241
	SGF14q	Glyma.07G226000	Chr07:40298318..40302692 reverse	1	4375	1143	780	259	7–241
	SGF14f	Glyma.02G115900	Chr02:11280858..11285331 forward	2	4474	1867	780	259	7–241
	SGF14e	Glyma.01G058000	Chr01:7642485..7646277 forward	1	3793	1358	780	259	7–241
	SGF14p	Glyma.13G270600	Chr13:37265741..37269626 forward	2	3886	1276	792	263	8–242
	SGF14n	Glyma.12G229200	Chr12:38919217..38923409 reverse	2	4193	1227	798	265	13–247
	SGF14d	Glyma.13G290900	Chr13:39120795..39124124 forward	2	3330	1293	786	261	7–240
	SGF14o	Glyma.12G210400	Chr12:36943077..36946491 reverse	2	3415	1370	786	261	7–240
	SGF14l	Glyma.08G115800	Chr08:8877809..8881104 forward	6	3296	1408	780	259	7–241
	SGF14c	Glyma.05G158100	Chr05:35025422..35029392 forward	8	3971	1497	780	259	7–240

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Chromosomal Localization and Segmental Duplication of Soybean 14-3-3 Genes

From the above phylogenetic tree (Fig 2 and Fig 3A), we noticed that all soybean 14-3-3 genes appeared in pairs, indicating possible gene duplication during evolution of the 14-3-3 family. Therefore, we operated chromosome localization and synteny analyses to determine the potential gene duplication within the soybean 14-3-3 family. The chromosomal localization analysis revealed that 18 soybean 14-3-3 genes were distributed among 12 chromosomes (Fig 4). In line with results from phylogenetic tree, synteny analyses further confirmed that the soybean 14-3-3 family did possess gene duplication (Fig 4). Members from group I (linked by light blue

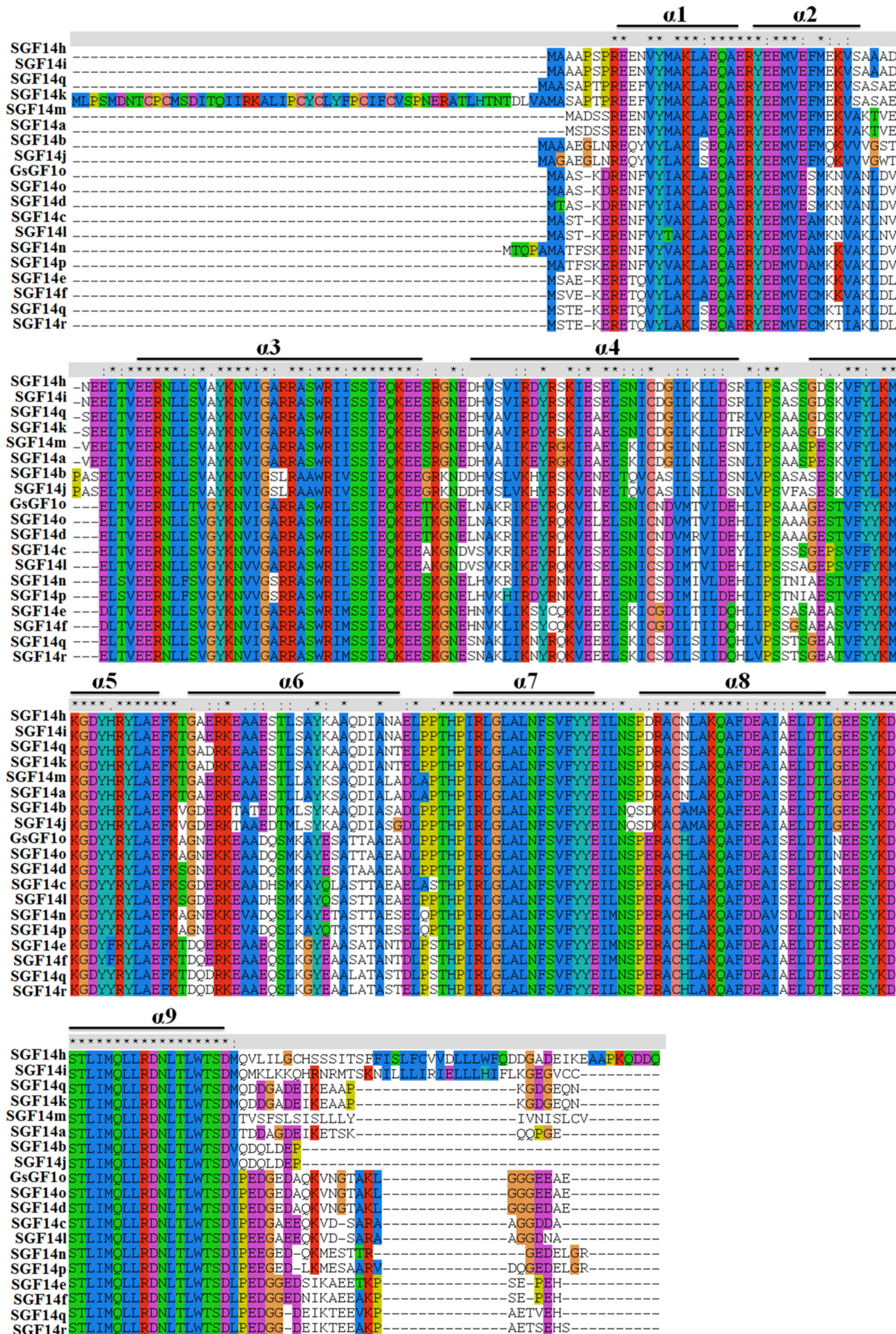

Fig 1. Protein sequence alignment of soybean 14-3-3 family members. Multiple sequences alignment of 14-3-3 proteins was performed by using ClustalX program, and nine α-helices (α1 to α9) were marked with black solid lines.

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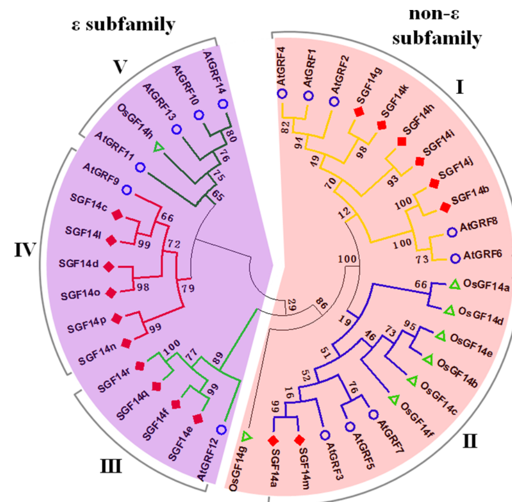

Fig 2. Phylogenetic analysis of the 14-3-3 family proteins from soybean, Arabidopsis and rice. The maximum likelihood phylogenetic tree was constructed by using MEGA5.0 based on the full-length amino acid method with 1000 bootstrap replicates.

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lines), group II (linked by dark blue lines), group III (linked by green lines), and group IV (linked by red lines) were found to be located in duplicated blocks, respectively (Fig 4).

It is reported that soybean underwent at least two rounds of genome wide duplications approximately 13 and 59 million years ago [45]. Based on the locus search at PGDD website (Plant Genome Duplication Database, http://chibba.agtec.uga.edu/duplication/)[46], we found that all of the nine 14-3-3 paralogous gene pairs in soybean genome were generated by segmental duplication (Fig 5). Remarkably, four genes in group IV (SGF14o, SGF14p, SGF14n, and SGF14d) were supposed to be within the same duplication blocks (Fig 5D). Moreover, the four 14-3-3 proteins displayed over 80% sequence identity (S2 Fig) and showed closely related location in the phylogenetic tree (Fig 2 and Fig 3A). These findings suggested that these two paralogous gene pairs might originate from a common ancestor, which firstly underwent tandem duplication prior to segmental duplication.

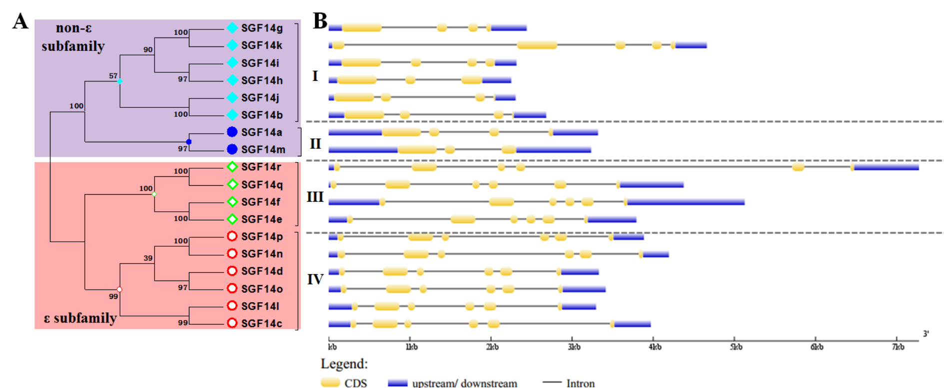

Fig 3. Phylogenetic analysis and exon-intron structures of soybean 14-3-3 family members. (A) The phylogenetic tree of soybean 14-3-3 family proteins. **(**B) The exon-intron structures of soybean 14-3-3 family genes. Yellow and blue boxes represented exons and lines represented introns.

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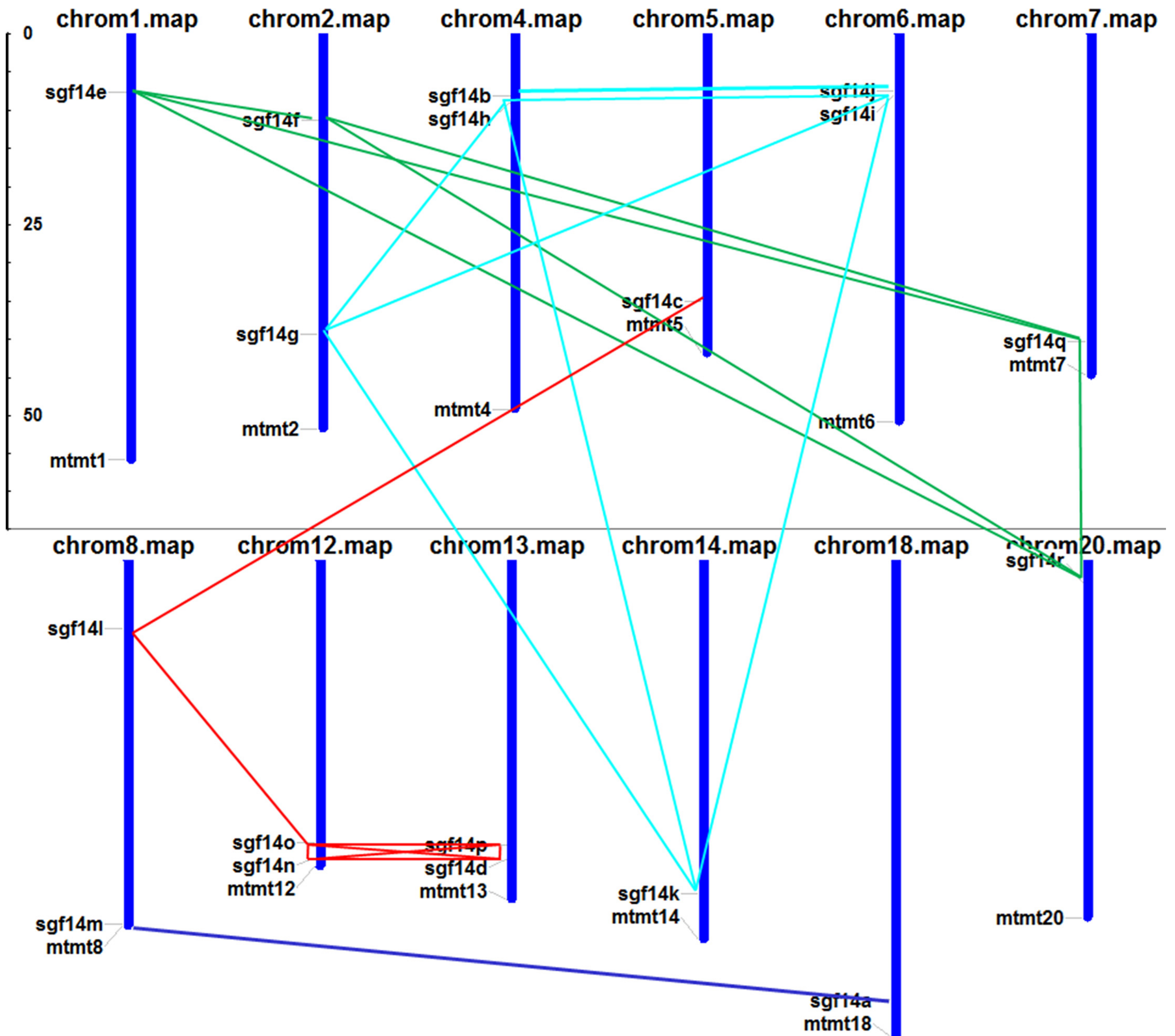

Fig 4. Chromosomal localization and synteny analysis of soybean 14-3-3 family genes. The chromosomal localization of the 14-3-3 family genes was determined by using the MapInspect software. For synteny analysis, the synteny blocks of the soybean genome were downloaded from the Plant Genome Duplication Database (PGDD, http://chibba.agtec.uga.edu/duplication/). Duplicated gene pairs were connected by light blue lines for group I, dark blue lines for group II, green lines for group III, and red lines for group IV.

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Structure Analysis of *Glycine soja* GsGF14o Protein

In previous studies, we have isolated a *Glycine soja* 14-3-3 family gene *GsGF14o* [37], which displayed the highest sequence identity to *Glycine max* SGF14o [38], and demonstrated that it negatively regulated plant responses to drought stress. In that research, we showed that GsGF14o shared the highly conserved sequence features with other identified 14-3-3s, including five highly conserved blocks (I-V), two signature motifs (RNLLSVAYKNV and

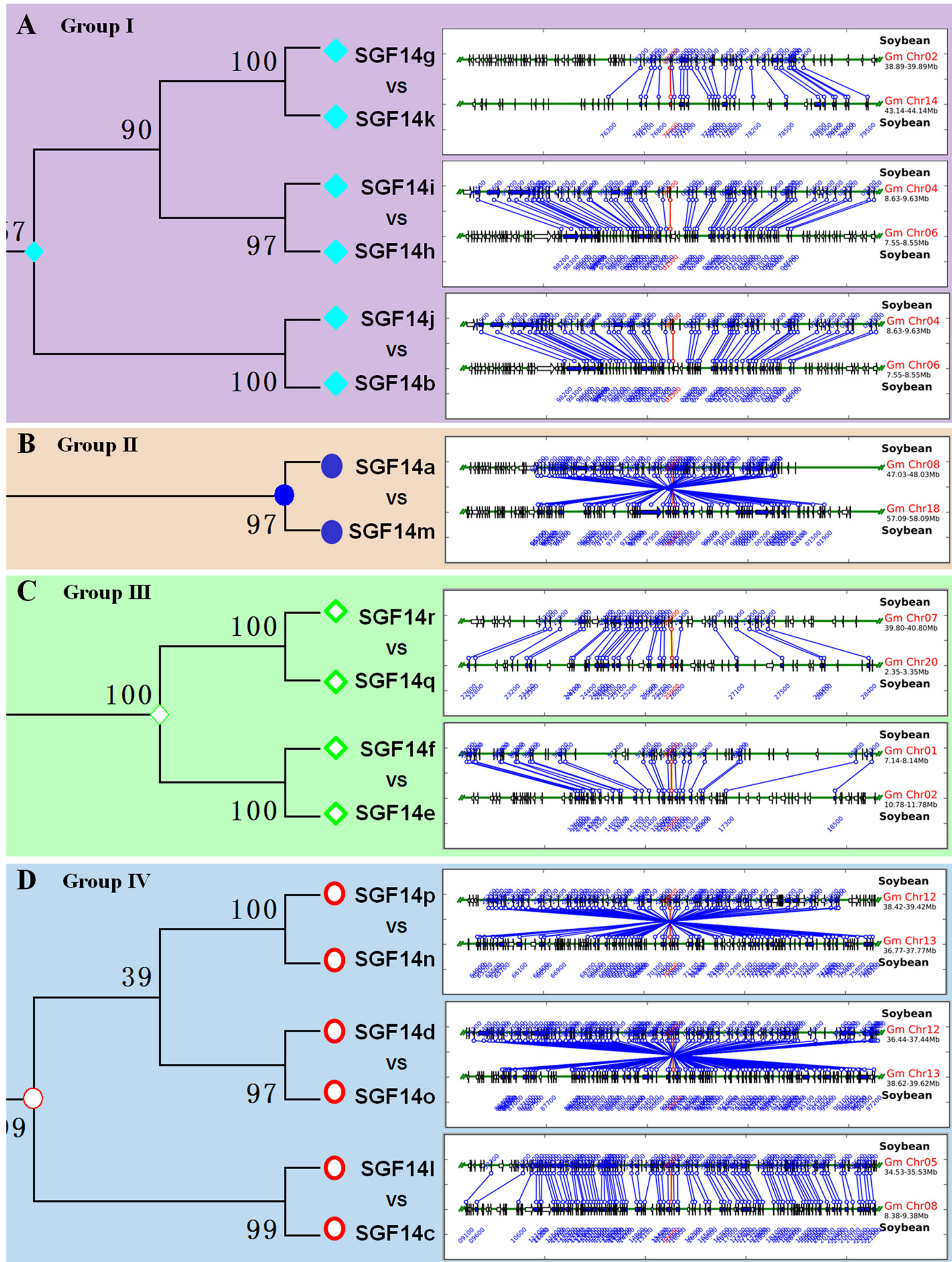

Fig 5. Segmental duplication analyses of soybean 14-3-3 family genes in Group I (A), Group II (B), Group III (C), and Group IV (D). The segmental duplication of soybean 14-3-3 genes was obtained based on the locus search at PGDD website.

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SYKDSTLIMQLLRDNLTLWT), one pseudosubstrate domain for protein kinase C (GARR), one proposed EF-hand domain (SELDTLGEESYKD) and one nuclear exclusion sequence (LIMQLLRDNLTLWT). As shown in Fig 1, GsGF14o also consisted of nine α-helices (α1 to α9).

To get better understanding of the conformational features of GsGF14o protein, we further predicted the three-dimensional structure of GsGF14o by using the I-TASSER one-line software. As shown in Fig 6A, GsGF14o showed the highest similarity in terms of conformational structure to Nt14-3-3 (PDB: 2o98B) in the Protein Data Bank database (http://www.rcsb.org/pdb/home/home.do). Results of the three-dimensional structure analysis also confirmed that GsGF14o consisted of a bundle of nine α-helices (α1 to α9), which were organized into groups of two (α1 and α2), two (α3 and α4), two (α5 and α6), and three (α7, α8 and α9) helices (Fig 6B). Out of them, the first four helices were reported to be essential for dimer formation [47], and helices α3, α5, α7 and α9 could form a conserved peptide-binding groove (Fig 6C).

GsGF14o Expression Was Induced by ABA Stress

In our previous study, we illustrated that the expression levels of *GsGF14o* were greatly and specifically induced by drought stress, but slightly affected by salt and cold stresses [37]. Considering the involvement of 14-3-3s in ABA responses [29,30], we then searched the ABA responsive cis-elements in *GsGF14o* promoter by using PLACE on-line software to check whether *GsGF14o* expression responds to ABA stress. As shown in Fig 7A, we observed several cis-elements related to ABA responses, including three ABRELATERD1 (ACGTG) [48], seven ABRERATCAL (MACGYGB) [49] and one ACGTABREMOTIFA20SEM (ACGTGKC) [50,51] (Fig 7A). The existence of these cis-elements indicated the possible involvement of *GsGF14o* in plant ABA responses.

In this condition, we then investigated the expression profile of *GsGF14o* under ABA stress through quantitative real-time PCR analysis by using RNA extracted from the leaves of 3-week-old *Glycine soja* seedlings treated with 100μM ABA. Our results revealed that without ABA stress, *GsGF14o* displayed stable expression levels (Fig 7B, left), while ABA treatment rapidly increased the transcript accumulation of *GsGF14o* (Fig 7B, right). In details, after ABA treatment, the transcript level of *GsGF14o* started to increase and reached a maximum level at 6 h (about 5 folds), indicating that *GsGF14o* expression responded to ABA stress at the very early stage.

In order to further verify the ABA induction of *GsGF14o* expression, we determined and analyzed the changes in GUS activity in response to ABA stress, by using the $P_{GsGF14o}$:GUS transgenic *Arabidopsis*. To this end, the T_2 transgenic seedlings grown on normal 1/2MS medium were firstly moved to 1/2MS liquid medium lacking sucrose for hydroponics for 12 h, and then were transferred to 1/2MS medium supplemented with 100μM ABA for ABA stress treatment for 6 h. The GUS staining results showed that the ABA treated seedlings (Fig 7C, ABA) displayed much higher GUS activity than non-treated seedlings (Fig 7C, Control). Specifically speaking, under control condition, the 2-day-old seedlings displayed relatively higher GUS activity, and GUS expression decreased along with the seedling growth (Fig 7C, Control), which is in line with our previous results [37]. However, robust GUS expression was detected in the whole seedlings (2-, 4-, 6-, 8-day-old) treated with ABA (Fig 7C, ABA). Notably, the root tips and hypocotyls of ABA-treated seedlings exhibited much higher GUS activity than that of non-treated plants (Fig 7D). Taken together, these findings strongly suggested that *GsGF14o* expression was indeed induced by ABA stress, and implied potential role of *GsGF14o* in regulating ABA sensitivity.

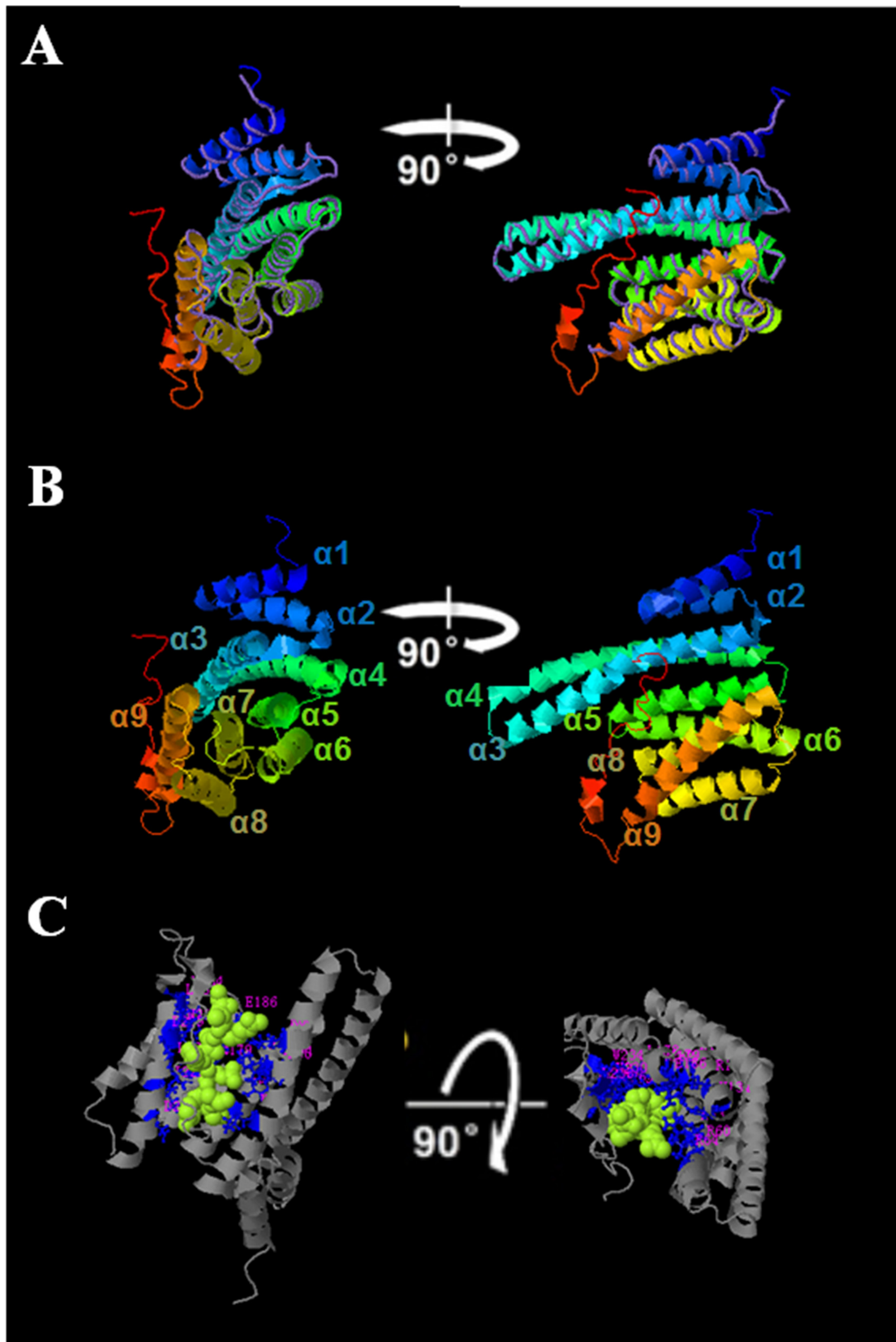

Fig 6. Structural analysis of the *Glycine soja* 14-3-3 protein GsGF14o. (A) Structural comparison of GsGF14o and Nt14-3-3 (PDB: 2098B). GsGF14o structure was shown in cartoon, while Nt14-3-3 structure was shown by using backbone trace. (B) Monomeric structure of the GsGF14o protein. (C) Predicted binding model of GsGF14o. Each structure is rotated 90° to show different view sides of the protein.

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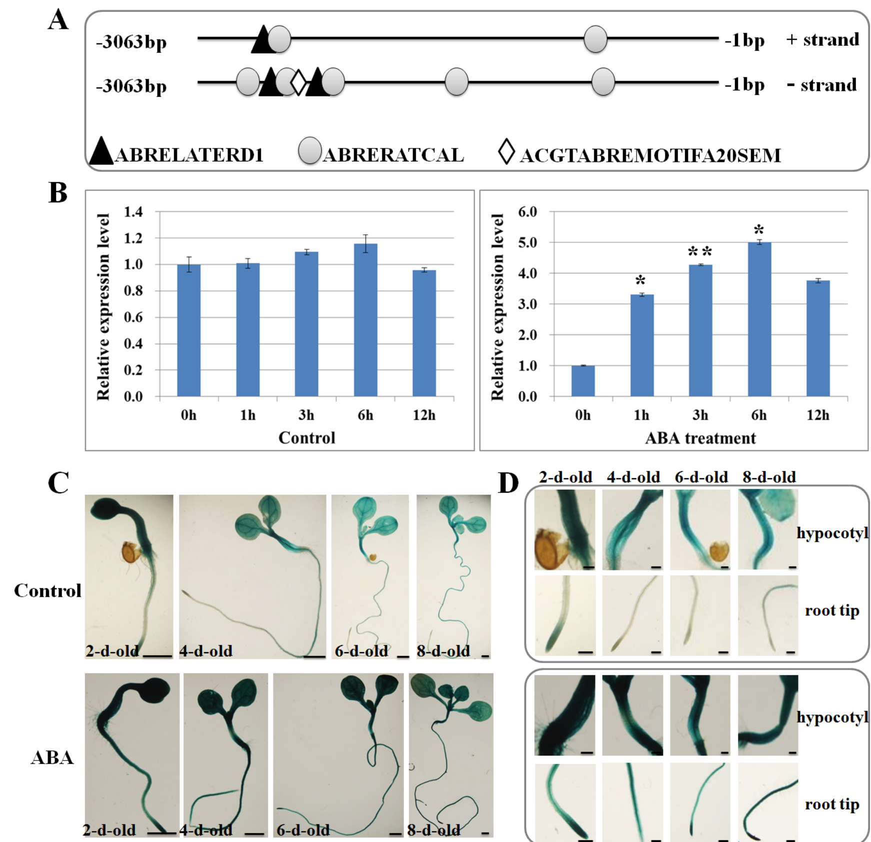

Fig 7. Induced expression of *GsGF14o* in response to ABA stress. (A) The architecture of ABA-responsive cis-elements in *GsGF14o* promoter. (B) Accumulation of *GsGF14o* transcripts in response to ABA stress in wild soybean. *, P < 0.05; **, P < 0.01 by Student's t-test. (C) Evaluation of GUS expression in $P_{GsGF14o}$:GUS transgenic plants in response to ABA stress. Bars are 1mm. (D) GUS expression in the hypocotyls and root tips of transgenic *Arabidopsis* in response to ABA stress. Bars are 200μm.

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GsGF14o Overexpression in *Arabidopsis* Augmented the ABA Inhibition on Seed Germination and Seedling Growth

ABA is an important phytohormone that regulates diverse developmental and physiological processes, for example the inhibition of seed germination and seedling growth [10]. To determine the role of *GsGF14o* in ABA responses, we initially checked the seed germination and seedling growth of the wild type (WT) and *GsGF14o* overexpression (OX) *Arabidopsis* lines (line #1, #4, #9) under ABA treatment.

During the plate seed germination assays, both the WT and OX seeds could germinate and grow well on normal 1/2MS medium (Fig 8A and 8B). However, under 0.6 μM ABA treatment, the WT seeds exhibited much higher germination rates than OX lines (Fig 8B). In details, on the 3rd day after sowing, 87.8% WT seeds could normally germinate, but the germination rates of OX lines were only 35.2% for line 1, 16.7% for line 4, and 25.6% for line 9 (Fig 8B). In addition, ABA application also obviously inhibited the early growth of both WT and OX seedlings (Fig 8A). Consistently, compared with WT, growth of OX seedlings was more severely inhibited by ABA (Fig 8A). Quantification analysis revealed that WT exhibited significantly higher percentages of seedlings with green and open leaves (Fig 8C), and seedlings with four leaves (Fig 8D). In particular, after application of 0.6 μM ABA, almost none of the OX seedlings could develop four leaves, while 25.6% WT seedlings displayed four leaves (Fig 8D). These results

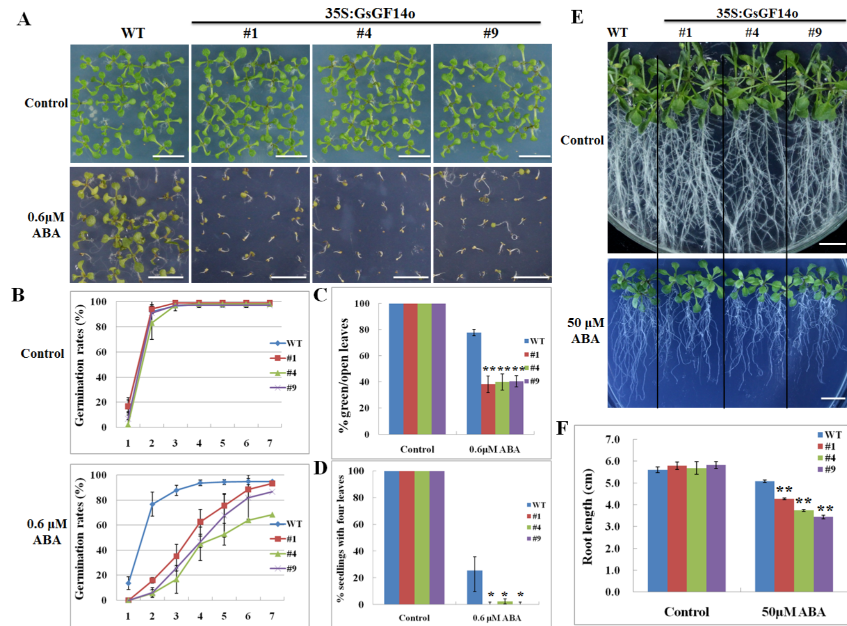

Fig 8. Increased ABA sensitivity of *GsGF14o* transgenic lines during the seed germination and seedling growth stages. (A) The growth performance of WT and *GsGF14o* OX seedlings under ABA stress. Bars are 1cm. (B) Seed germination rates of WT and *GsGF14o* OX lines. (C) Percentage of seedlings with open and green leaves. (D) Percentage of seedlings with four leaves. (E) Phenotypes of WT and OX seedlings. Bars are 1cm. (F) Primary roots of WT and OX seedlings. *, $P < 0.05$; **, $P < 0.01$ by Student's t test.

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suggested that *GsGF14o* OX lines were hypersensitive to ABA stress during the plate seed germination assays.

We further investigated the ABA sensitivity of *GsGF14o* OX lines at the early seedling stage by using the root length assay. To this end, the seven-day-old WT and OX seedlings grown under normal condition were transferred onto 1/2MS medium supplemented with either 0 or 50 μM ABA, and allowed to vertical growth for another 10 days. As shown in Fig 8E, the ABA inhibition on primary root growth of the OX lines was more severe than that of WT (Fig 8E). Statistical analysis also confirmed that the primary roots of the WT seedlings were evidently longer than those of the OX lines under ABA stress (Fig 8F). Taken together, all above results demonstrated that overexpression of *GsGF14o* in *Arabidopsis* resulted in ABA hypersensitivity, and augmented the ABA inhibition effect on seed germination and seedling growth.

GsGF14o Overexpression Promoted ABA Induced Stomata Closure but Not Proline Accumulation

In addition to the inhibition on seed germination and seedling growth, another two typical effects of ABA on plants are the induced stomata closure and proline accumulation [13]. Our previous study showed that overexpression of *GsGF14o* in *Arabidopsis* resulted in smaller stomata, as evidenced by a decrease of the stomata length and width [37]. In order to test whether *GsGF14o* overexpression affected ABA induced stomata closure, we measured the numbers of completely open, partially open and completely closed stomata (Fig 9), respectively, by using scanning electron microscopy. As shown in Fig 9A and 9B, under ABA treatment, the transgenic lines displayed much less open stomata, but more closed stomata than WT. Statistically speaking, the percentage of completely open stomata was 29% for WT, but these values

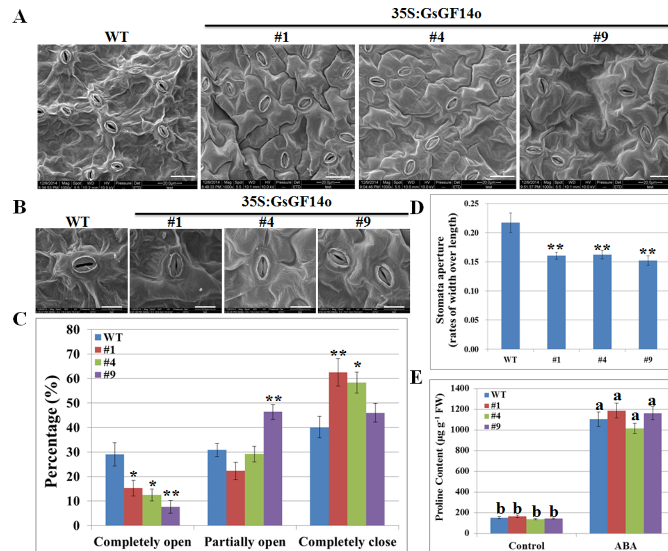

Fig 9. *GsGF14o* overexpression promoted ABA induced stomata closure but not proline accumulation. (A) Stomata from WT and GsGF14o OX lines in response to ABA stress. Bars are 20μm. (B) Presented photos to show stomata from WT and OX lines. Bars are 10μm. (C) Percentage of different stomata in WT and OX lines. (D) Stomata aperture of WT and OX lines. *, $P < 0.05$; **, $P < 0.01$ by Student's t test. (E) Proline content of WT and OX lines. Different letters indicated statistical differences among means by Duncan's Multiple Range Test ($P < 0.05$).

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decreased to 15.2% for line 1, 12.5% for line 4, and 7.9% for line 9. Notably, line 9 displayed higher percentage of partially open stomata than WT, while line 1 and 4 showed more completely closed stomata (Fig 9C). These data illustrated that *GsGF14o* overexpression in *Arabidopsis* promoted ABA induced stomata closure. To confirm this, we further determined stomata apertures (presented by the rates of width over length) of WT and OX lines under ABA treatment. As expected, statistical analysis also suggested that compared with WT, stomata aperture of transgenic lines was more sensitive to ABA (Fig 9D).

In addition to stomata closure, ABA also promotes the accumulation of free proline in plant cells, which may protect plant from adverse environmental challenges. Expectedly, under ABA stress, both WT and OX plants showed a great increase in free proline accumulation. However, beyond our expectation, *GsGF14o* overexpression did not alter the free proline accumulation, as evidenced by similar level of proline content between WT and OX plants under ABA stress (Fig 9E). Taken together, these findings suggested that *GsGF14o* overexpression promoted ABA induced stomata closure, but did not affect ABA induced proline accumulation.

GsGF14o Overexpression Up-Regulated the Expression Levels of ABA Induced Genes

ABA stress is suggested to induce the expression of a number of genes, which are involved in plant responses to environmental stress [52]. In this study, we checked the expression of several ABA induced genes, including *RD29A*, *RD29B*, *RD22*, *COR15A*, *KIN1*, and *RAB18*, all of which have ABRE cis-elements in the promoter region. Quantitative real-time PCR results showed that expression levels of all these genes were greatly and rapidly induced by ABA stress in both WT and OX plants. However, their expression levels in OX plants were significantly higher than that in WT (Fig 10). In conclusion, these results suggested that *GsGF14o* overexpression in Arabidopsis up-regulated the expression levels of ABA induced genes.

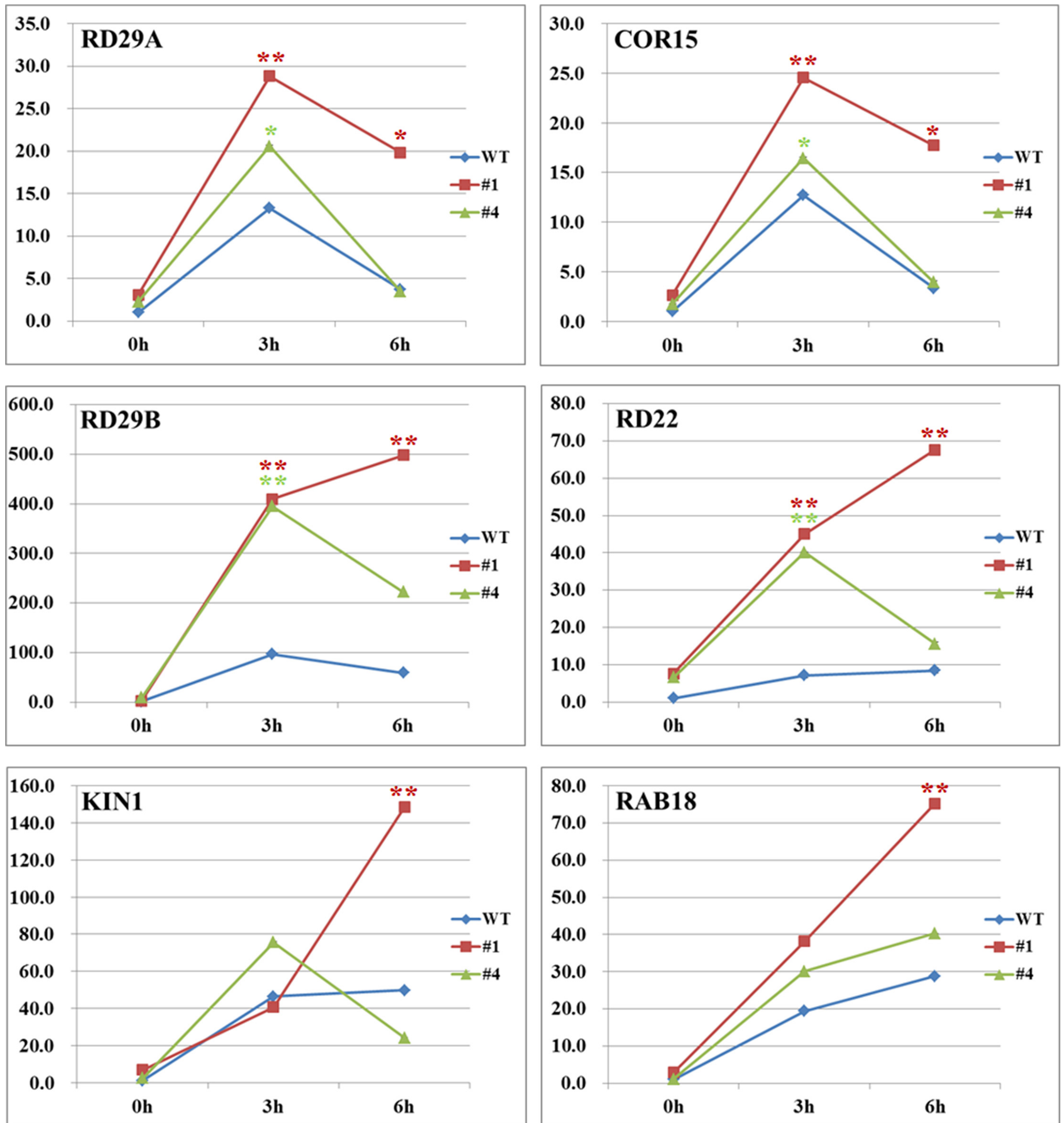

Fig 10. *GsGF14o* overexpression up-regulated the transcript levels of ABA induced genes. Transcription levels of ABA induced genes were determined by quantitative real-time PCR, and *ACTIN2* was used as an internal control. Results were normalized to the corresponding transcript levels of WT plants at 0 h. *, $P < 0.05$; **, $P < 0.01$ by Student's t test.

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GsGF14o Physically Interacted with ABF Transcription Factors in Yeast Cells

Recent studies uncovered that 14-3-3 proteins could physically interact with the AREB/ABF transcription factors, and participate in the ABA signaling transduction in plant cells [29,31,36]. Considering the great changes in expression of these ABRE-containing genes described above (Fig 10), we speculated that GsGF14o might also interact with the AREB/ABF transcription factors. To verify this hypothesis, we used the Y2H (Yeast Two Hybrid) technology to determine whether GsGF14o could interact with AREB/ABFs as described previously [32]. To do this, the *Arabidopsis* ABF1, ABF2, ABF3, ABF4 and ABI5 were in-fused cloned to the pGADT7 vector to express AREB/ABFs at the C-terminus of GAL4 activating domain (Fig 11A). The full-length GsGF14o gene was fused to the GAL4 DNA-binding domain in the pGBKT7 vector, and used as a bait to analyze the protein interaction with AREB/ABFs (Fig 11A). Y2H assays showed that the recombinant yeast cells harboring the pGBKT7-GsGF14o and pGADT7-ABFs vectors could survive well on the SD/-T-L, SD/-T-L-H, and SD/-T-L-H-A selective medium (Fig 11B). However, the yeast cells carrying the pGBKT7-GsGF14o and empty pGADT7 vectors only showed growth on the SD/-T-L medium, but not on the SD/-T-L-H and SD/-T-L-H-A medium. Taken together, these results suggested that GsGF14o did physically interact with AREB/ABFs in yeast cells.

Discussion

14-3-3 proteins are key regulators of multiple signal transduction cascades relating to diverse physiological and biological processes through hundreds of different protein-protein interactions [53–58]. The development of bioinformatics, especially the whole genome sequencing, dramatically assisted for the genome wide survey of plant 14-3-3 proteins, for example in Arabidopsis [59,60], rice [61,62], tomato [63], cotton [64], and populous [47]. As for soybean, Li et al. identified a total of eighteen 14-3-3 genes based on the soybean genome and EST databases [38]. Here, in this study, we further identified the soybean 14-3-3 family proteins based on *Glycine max* genome at Phytozome v10.3 (Table 1), and suggested that soybean 14-3-3 family was highly evolutionary conserved and possessed segmental duplication in evolution.

It is reported that soybean has undergone at least two rounds of genome wide duplications approximately 13 and 59 million years ago, resulting in large-scale duplicated sequences in soybean genome [45]. Very recently, Tian et al. demonstrated the gene duplication of *Populus* 14-3-3 family, and proposed that purifying selection played a pivotal role in the retention and maintenance of *Populus* 14-3-3 family [47]. Consistently, we also observed evidence for the gene duplication of soybean 14-3-3 family (Figs 2–4). Firstly, almost all soybean 14-3-3 genes appeared in pairs in the phylogenetic tree (Fig 1 and Fig 2A), indicating possible gene duplication during evolution of the 14-3-3 family. Secondly, the chromosomal localization and synteny analyses further confirmed that the soybean 14-3-3 family did possess gene duplication (Fig 3). Lastly but most importantly, we confirmed that nine 14-3-3 paralogous gene pairs in soybean genome were generated by segmental duplication based on the locus search at PGDD website (Fig 4). What is more interesting is that two paralogous pairs in group IV (SGF14d/SGF14o, SGF14n/SGF14p) were supposed to originate from a common ancestor, which firstly underwent tandem duplication prior to segmental duplication.

The 14-3-3 family has been well demonstrated to be highly evolutionary conserved in plants [47,64]. As expected, in this study, we also gave three lines of evidence for the evolutionary conservation of soybean 14-3-3 family. Firstly, previous study reported that the 14-3-3 proteins from 27 species of the Viridiplantae kingdom could be clustered into four groups [65]. Here, we also showed that soybean 14-3-3 family was divided into four groups (Fig 2A). However,

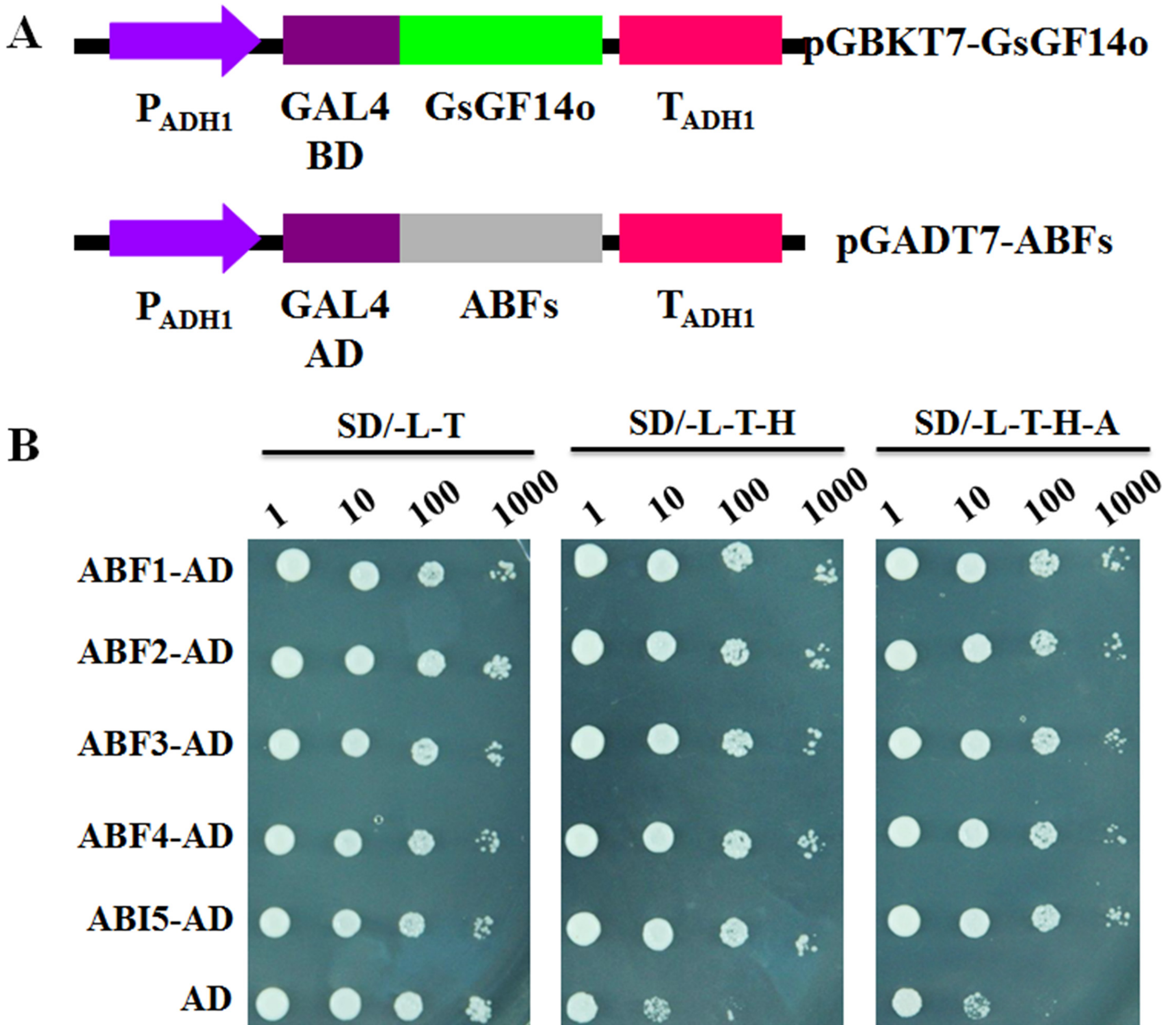

Fig 11. Protein interaction of GsGF14o with ABF transcription factors in yeast cells. (A) Schematic representation of GsGF14o-BD and ABFs-AD fused expression constructors. (B) Yeast two hybrid identification of protein interaction between GsGF14o and ABF transcription factors. The GsGF14o-BD/AD combination was used as a negative control.

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when combined with *Arabidopsis* and rice, there was one more group only containing 14-3-3s from Arabidopsis and rice (Fig 1). Secondly, paralogous 14-3-3 genes within the same group showed highly conserved exon-intron organization (Fig 2B), and similar phenomenon was also found in other species, for example *Populus* [47], and *Mulberry Tree* [66]. Thirdly, soybean 14-3-3s, consisting of nine α-helices (α1 to α9), were highly conserved in amino acid architecture (Figs 5 and 6) [37,38].

An increasing body of work has been done to clarify the roles of 14-3-3s in stress response pathways in plants [67]. For example, very recently, 14-3-3s were reported to be positive regulators of primary root growth under control conditions, but be negative regulators in drought stress [68]. Similarly, in a previous study, we also isolated a *Glycine soja* 14-3-3 family protein GsGF14o, and identified it as a negative regulator of plant responses to drought stress [37]. In this study, we further investigated the expression characteristics of *GsGF14o* in detail, and demonstrated its positive roles in ABA sensitivity. Our previous studies reported the expression patterns of *GsGF14o* in different tissues and organs by using GUS staining assays [37]. We found that GUS expression promoted by *GsGF14o* promoter was relatively higher in the 2-day-old seedlings than 4-, 6-, and 8-day-old plants, which was also observed in this study (Fig 7C). Previous study also showed that expression of *GsGF14o* specifically responded to drought stress, as evidenced by a great increase of *GsGF14o* transcript levels under drought stress, but only a slight increase under salt and cold stresses [37]. Here, we further demonstrated that *GsGF14o* expression was also moderately and rapidly induced by ABA treatment through quantitative real-time PCR analysis (Fig 7B) and GUS activity assays (Fig 7C and 7D). The similar phenomenon was also observed for a *Populus* PP2C gene *PeHAB1*, which was markedly induced by drought but moderately induced by ABA [69]. Consistently, the ABA induced expression was also observed for the homologous 14-3-3 genes in other plant species, including *Brassica napus* [70] and *Oryza sativa* [61,62]. These results strongly suggested that *GsGF14o* might be involved in plant ABA responses and signal transduction.

What is interesting is that *GsGF14o* expression predominantly accumulated in root tips and hypocotyls, especially under ABA stress (Fig 7D). The enriched expression of *GsGF14o* in roots further supported its regulatory role in root development reported by our previous studies [37]. Furthermore, consistent with the obvious accumulation of *GsGF14o* in hypocotyls, we also found that *GsGF14o* overexpression led to longer hypocotyls of transgenic *Arabidopsis* seedlings under white, blue and red light (S3 Fig). This observation was in line with previous researches about 14-3-3 involvement in light signaling [71–73]. Correspondingly, previous studies also gave the direct evidence that 14-3-3 proteins regulated the hypocotyl growth [74]. In addition, *Arabidopsis* 14-3-3s were also reported to regulate root growth and chloroplast development as components of the photosensory system [75]. Considering our previous report about the involvement of *GsGF14o* in root hair formation and stomata development, all these findings strongly suggested the multiple regulatory roles of 14-3-3 proteins in plant growth and development.

Even though researches have revealed the responses of 14-3-3 gene expression to ABA stress, little genetic evidence was given to illustrate the biological function of 14-3-3s in ABA responses. In this study, we gave several lines of genetic and molecular evidence showing the regulatory role of *GsGF14o* in ABA sensitivity. Firstly, *GsGF14o* overexpression in *Arabidopsis* augmented the ABA inhibition on seed germination and seedling growth (Fig 8). One important effect of ABA on plants was the inhibition of seed germination and seedling growth [10]. As expected, *GsGF14o* OX lines displayed much lower germination rates, fewer seedlings with open and green leaves and fewer seedlings with four leaves during the plate seed germination assays (Fig 8A–8D), and exhibited shorter primary roots during the root length assays (Fig 8E and 8F). Secondly, overexpression of *GsGF14o* promoted ABA induced stomata closure (Fig 9A–9D), which might help plant deal with the adverse environment. Thirdly, ABA could induce dramatic changes in the transcriptome of plant cells [16,17]. Expectedly, in this study, we elucidated that *GsGF14o* overexpression also increased the expression levels of the ABA responsive genes (Fig 10). Similarly, previous studies also showed that 14-3-3s affected the expression of ABA-responsive genes [29,76]. Taken together, these results strongly suggested *GsGF14o* was a positive regulator of plant ABA responses.

Until now, several researches have indicated the molecular basis of the 14-3-3s involvement in ABA signal transduction [29–32,76]. It is reported that 14-3-3 proteins could directly interact with the AREB/ABF family transcription factors [29,32], which were the key regulators in ABA responses [9,77,78]. For example, in barley, 14-3-3 proteins were found to regulate ABA stress response through HvABI5 interaction [29,30]. In *Thellungiella salsuginea*, the interaction between 14-3-3 proteins and AREB/ABF transcription factors was also reported [32]. However, there is still no report about the protein interaction of soybean 14-3-3 proteins with AREB/ABFs. In the current study, we showed that GsGF14o could physically interact with AREB/ABFs (ABF1, ABF2, ABF3, ABF4 and ABI5) in yeast cells through Y2H analysis (Fig 11). 14-3-3 interaction with targets was reported to be phosphorylation dependent [36,55,79–82]. However, several studies have identified AREB/ABFs interaction in yeast cells for 14-3-3s from soybean, Arabidopsis, barley and *Thellungiella salsuginea*. Since yeast may not have corresponding kinases as in plants, further researches are required to verify the phosphorylation dependence of 14-3-3s and AREB/ABFs interaction. Anyway, AREB/ABFs could then bind the ABRE elements in the promoter regions, and regulate the expression of ABA induced genes, for example *RD29A*, *RD29B*, *RD22*, *COR15A*, *KIN1* and *RAB18* (Fig 10). Hence, we proposed that GsGF14o participated in plant ABA stress responses partly through interacting with AREB/ABFs and regulated the ABA induced genes.

ABA is generally considered as an important hormone, and helps plants to deal with drought stress [83,84]. However, a serial of researches have suggested that an increased ABA sensitivity was not necessarily accompanied with an increased stress tolerance in plants. Plants showing ABA hypersensitivity may also display hypersensitivity to drought stress, in other words, drought stress responses could be controlled by ABA-dependent or ABA-independent pathway. One of the best studied examples is that the ABA overly sensitive mutant *abo3* displayed decreased drought tolerance [85]. Similar results were also found for *Arabidopsis* histone deacetylase gene *AtHD2C* and Lily ABA-, stress-, and ripening-induced gene *LLA23*, overexpression of which decreased plant ABA sensitivity but increased drought tolerance [86,87]. Very recently, transgenic *Arabidopsis* overexpressing a rice group A PP2C gene *OsPP108* was found to be highly insensitive to ABA but tolerant to drought stress [52]. Similarly, our research also revealed that *GsGF14o* overexpression increased ABA sensitivity, but decreased drought tolerance. Remarkably, expression of the ABA inducible gene *RD29A* in *OsPP108* transgenic lines was down-regulated under ABA treatment, but up-regulated under drought stress. Similarly, our results also showed that expression of the ABA inducible stress responsive genes was up-regulated under ABA treatment (Fig 10), but down-regulated under drought stress [37]. Generally, ABA induced stomata closure is an important adaptive response to drought stress, resulting in reduced water loss [88,89]. The *OsPP108* transgenic lines showed ABA insensitivity, with much more open stomata after ABA treatment than WT; in contrast, the water loss of *OsPP108* transgenic lines obviously decreased under drought stress [52]. Opposite phenomenon was also observed for *GsGF14o*. *GsGF14o* transgenic lines displayed ABA hypersensitivity concerning stomata closure (Fig 9), but exhibited decreased drought tolerance, even though with lower water loss rates [37]. The contradiction in ABA sensitivity and drought tolerance could be explained by the hypothesis that this kind of genes might regulate stress tolerance through ABA-independent mechanism.

According to our results, above the ground, *GsGF14o* overexpression reduced the stomata size [37], and promoted ABA-induced stomata closure (Fig 9), which might help plant to reduce the water loss under drought stress [37]. However, at the same time, these changes in stomata size and stomata movement inhibited the gas exchanges, decreased the photosynthetic activity and thus led to growth penalty under drought stress [37]. What is more important, under the ground, *GsGF14o* overexpression led to less and shorter root hairs, as well as shorter

primary roots under drought stress [37]. As we know, root hairs enable plants to more effectively extract soil moisture by increasing the effective surface area of roots. In conclusion, these morphological changes of *GsGF14o* transgenic plants finally resulted in reduced drought tolerance. Taken together, it is obvious that *GsGF14o* functions in multiple biological processes, including morphological regulation (stomata and root hair), ABA and drought stress responses.

Materials and Methods

Bioinformatic Analysis of the Soybean 14-3-3 Family Genes

To identify the soybean 14-3-3 family genes, a keyword (14-3-3) search against the soybean (*Glycine max* Wm82.a2.v1) genome at Phytozome v10.3 (http://phytozome.jgi.doe.gov/pz/portal.html) was carried out. Multiple sequences alignment of 14-3-3 proteins was performed by using Clustal X, and the maximum-likehood (ML) phylogenetic tree was constructed by using MEGA5.0 with 1000 bootstrap replicates. The exon and intron structures were illustrated by using the Gene Structure Display Server (GSDS, http://gsds.cbi.pku.edu.cn/index.php) [90]. The chromosomal localization of the 14-3-3 family genes was determined by using the MapInspect software. For synteny analysis, the synteny blocks of the soybean genome were downloaded from the Plant Genome Duplication Database (PGDD, http://chibba.agtec.uga.edu/duplication/)[46], and the segmental duplication of soybean 14-3-3 genes was obtained based on the locus search at PGDD website. The three-dimensional structure of GsGF14o protein was predicted by using the I-TASSER software (http://zhanglab.ccmb.med.umich.edu/I-TASSER/)[91]. The cis-elements in *GsGF14o* promoter were predicted by the on-line software PLACE (http://www.dna.affrc.go.jp/PLACE/)[92].

Plant Material, Growth Conditions and Stress Treatments

Wild soybean seeds (*Glycine soja* 50109) were firstly soaked in 98% sulfuric acid (H_2SO_4) for 10 min, washed five times with sterilized distilled water, and then kept in complete darkness with humidity for 2–3 days to promote germination. Germinated seedlings were transferred into 1/4 Hoagland solution and grown at 24–26°C and a 16 h light /8 h dark cycle. For ABA treatment, the roots of 3-week-old seedlings with similar sizes were submerged in 1/4 Hoagland solution supplemented with 100 μM ABA, as described previously [93]. Equal amounts of new-born leaves from 3 individual seedlings were harvested at 0 h, 1 h, 3 h, 6 h and 12 h after ABA application, respectively. Samples were immediately frozen in liquid nitrogen, and then stored at -80°C for RNA extraction.

Seeds of the wild type *Arabidopsis thaliana* (Columbia ecotype) were sterilized with 5% sodium hypochlorite (NaClO) for 6–8 min with shaking, washed with sterilized distilled water for 6–8 times, and then kept at 4°C for 3 days to break seed dormancy. Then *Arabidopsis* were germinated and grown on 1/2MS solid medium or in the standard nutrient solution [94] under controlled environmental conditions (21–23°C, 100 μmol photons m^{-2} s^{-1}, 60% relative humidity, 16 h light/8 h dark cycles). To analyze the expression profiles of ABA induced genes, the 3-week-old WT and transgenic *Arabidopsis* seedlings grown in hydroponic were treated with the standard nutrient solution containing 100 μM ABA. Samples were harvested at 0 h, 1 h, 3 h, and 6 h, and prepared for RNA extraction as descried above.

Quantitative Real-Time PCR Assays

Total RNA was extracted from the 3-week-old wild soybean and/or *Arabidopsis* seedlings by using the EasyPure Plant RNA Kit (Transgen Biotech, China), and then was reversed

Table 2. Gene-specific primers used for quantitative RT-PCR assays.

Gene name	Primer Sequence (5' to 3')
GsGF14o	Forward: CTCCAGTCTCTGGGGGATTTG
	Reverse: CTTGTTCCACGTTTTTGCGG
GAPDH	Forward: GACTGGTATGGCATTCCGTGT
	Reverse: GCCCTCTGATTCCTCCTTGA
ACTIN2	Forward: TTACCCGATGGGCAAGTC
	Reverse: GCTCATACGGTCAGCGATAC
RD29A	Forward: GGCGTAACAGGTAAACCTAGAG
	Reverse: TCCGATGTAAACGTCGTCC
RD29B	Forward: TGAAGGAGACGCAACAAGGG
	Reverse: CAACGGTGGTGCCAAGTGAT
COR15	Forward: AATTTCAAGCACTTAAACTCGT
	Reverse: AGAATGTGACGGTGACTGTG
KIN1	Forward: AACAAGAATGCCTTCCAAGC
	Reverse: CGCATCCGATACACTCTTTCC
RD22	Forward: GGTTCGGAAGAAGCGGAG
	Reverse: GAAACAGCCCTGACGTGATAT
RAB18	Forward: CTTGGGAGGAATGCTTCAC
	Reverse: CTTCTTCTCGTGGTGCTCAC

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transcripted to cDNA by using the SuperScript^{TM} III Reverse Transcriptase kit (Invitrogen, Carlsbad, CA, USA). To exclude genomic DNA contamination before quantitative real-time PCR assays, PCR amplification was carried out with specific primers for the internal reference genes, *GADPH* (in *Glycine soja*) and *ACTIN2* (in *Arabidopsis thaliana*). Quantitative real-time PCR assays were performed using a Stratagene MX3000P real-time PCR instrument and the SYBR Select Master Mix (Applied Biosystems, USA). Three independent biological replicates were carried out and subjected to real-time PCR. Gene specific primers are listed in Table 2.

GUS Histochemical Staining

To explore the GUS expression in the $P_{GsGF14o}$:GUS transgenic *Arabidopsis* in response to ABA, seeds of the T_2 generation transgenic lines (two independent lines) were sowed and grown on normal 1/2MS medium for 2, 4, 6 and/or 8 days, respectively. The young seedlings were moved to 1/2MS liquid medium lacking sucrose for hydroponics for 12 h, and were then transferred to 1/2MS medium supplemented with 100 μM ABA for ABA stress treatment for 6 h. Three or four seedlings from each line and each condition were sampled and subjected to GUS staining. GUS staining was performed by using 5-bromo-4-chloro-3-indolyl-b-D-glucuronide (X-Gluc) as substrate [95].

Seed Germination and Root Length Assays in Response to ABA Stress

In the plate germination assays, WT and OX *Arabidopsis* seeds were germinated and grown on normal 1/2MS medium or 1/2MS medium supplemented with 0.6 μM ABA. The germination rates were recorded for consecutive 7 days after sowing. Pictures were taken on the 7th day to show the growth performance of each line. The numbers of seedlings with open and green leaves and/or seedlings with four rosette leaves were recorded. Ninety seeds of each line were used for each experiment and the experiments were repeated three times.

In the root length assays, the 7-day-old WT and OX seedlings grown on normal 1/2MS medium were transferred to fresh medium without or with 50 μM ABA, and grown vertically for another 10 days. The primary root length of each seedling was measured and recorded. Fifteen seedlings of each line were used for each experiment and the experiments were repeated three times.

Measurement of the Stomata Aperture

To measure the ABA induced stomata closure, rosette leaves of the 3-week-old WT and OX seedlings were detached and floated (abaxial side down) on the solution containing 30 mM KCl, 0.1 mM EGTA, and 10 mM MES-KOH (pH 6.15) under light for 2.5 h, to induce stomatal opening. Then, leaves were transferred to solution containing 30 mM KCl, 0.1 mM $CaCl_2$, 10 mM MES-KOH (pH 6.15), and 10 μM ABA for 2 hours. After that, leaves were fixed in the 0.1 M phosphate buffer (pH 7.4) containing 2.5% glutaraldehyde and 4% para-formaldehyde at 4°C for 3 days, mounted onto standard aluminum stubs for the Hitachi scanning electron microscope, and then sputter-coated with approximately 30 nm gold using a sputter coater (K550, Emitech). The images were then viewed using an S2400 scanning electron microscope (Hitachi) with an accelerating voltage of 1.5 kV. For statistical analysis, 30 stomata were used for each line, and the ratio of length over width was used to represent stomata aperture.

Measurement of Free Proline Content

To analyze the changes of free proline accumulation in response to ABA stress, the 10-day-old WT and OX *Arabidopsis* seedlings grown on normal 1/2MS medium were transferred onto 1/2MS medium with 50 μM ABA for 4 days. Proline content was measured by using the ninhydrin assay as described [96]. Briefly, approximately 0.5 g fresh harvested leaves were homogenized in 3% sulfosalicylic acid, and reacted with acid-ninhdrin and glacial acetic acid for 1 hour at 100°C. The reaction mixture was extracted with toluene, and free proline concentration was calculated by using the values of the absorbance at 520 nm.

Yeast Two Hybrid Assays

The full-length coding region of *GsGF14o* was amplified by using the following primer pairs: 5'-TAAGAATTCATGGCTGCCTCCAA-3' and 5'-TTTGTCGACCACTCTGCCTCCTC-3'. The PCR products were cloned to the pGBKT7 vector to express the GsGF14o-BD fused protein. The full-length coding regions of Arabidopsis ABF transcription factors were amplified by using the following primer pairs: 5'-TCCATCGATACATGGGTACTCAC ATTGAT-3' and 5'-TATCTCGAGACCTTCTTACCACGGACC-3' (for ABF1), 5'-TCCATCGATACATGG ATGGT AGTATGAATTTG-3' and 5'-TATCTCGAGACCAAGGTCCCGACTCTGT-3' (for ABF2), 5'-TCCATCGATACA TGGGGTCTAGATTAAACTTC-3' and 5'-TATCTCGAG ACCAGGGACCCGTCAAT-3' (for ABF3), 5'-TCCATC GATACATGGGAACTCACAT CAAT-3' and 5'-TATCTCGAGACCATGGTCCGGTTAATGT-3' (for ABF4), 5'-TCCAT CGATACATGGTAACTAGAGAAACGAAG-3' and 5'-TATCTCGAGAGAGTGGACAACT CGGGTT-3' (for ABI5). The PCR products were cloned to the pGADT7 vector to express the ABFs-AD fused proteins.

To perform the yeast two hybrid analysis, the pGBKT7-GsGF14o and pGADT7-ABFs constructs were introduced into the yeast strain AH109 using the lithium acetate method, and the culture (OD600 = 0.6) of the PCR-positive transformants were serial diluted (1:10, 1:100, 1:1000), and then pointed onto SD/-Trp-Leu, SD/-Trp-Leu-His, and SD/-Trp-Leu-His-Ade medium.

Supporting Information

S1 Fig. Expression patterns of the soybean 14-3-3 family genes. (A) Expression profiles of soybean 14-3-3s in different tissues. (B) Expression profiles of soybean 14-3-3s in response to alkaline stress (50 mM $NaHCO_3$, pH 8.5) based on the RNA-seq data.
(TIF)

S2 Fig. Sequence identity and alignment of four group I 14-3-3 proteins. (A) Sequence identity among the four group I 14-3-3 proteins. (B) Multiple sequence alignment of the four group I 14-3-3 proteins.
(TIF)

S3 Fig. *GsGF14o* overexpression led to longer hypocotyls of transgenic *Arabidopsis* seedlings. (A) Representative photos to show the hypocotyls from WT and *GsGF14o* OX seedlings under white, blue and red light. (B) Comparison of the hypocotyl length of WT and OX lines.
(TIF)

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Author Contributions

Conceived and designed the experiments: XLS MZS YMZ. Performed the experiments: XLS MZS CC. Analyzed the data: XLS BWJ KJY ZMP YS. Contributed reagents/materials/analysis tools: YMZ ZWQ. Wrote the paper: XLS CMY YS. Read, approved and revised the final manuscript: KJY ZWQ.

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