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Full Length Article

Expression Pattern of Abscisic Acid Insensitive 3 (*ABI3*) in Soybean (*Glycine max*) and its Interaction Mechanism between Storage Protein Gene Promoter

RuoChen Du[1], Ajab Khan[1], YongGang Qiao[2], Yanyan Yi[1], Li Wang[2], XiaYu Liu[2], HongQuan Li[1*] and JinSheng Wang[2*]
[1]*College of Animal Science and Veterinary Medicine, Shanxi Agriculture University, Taigu, Shanxi 030801, PR China*
[2]*College of Life Science, Shanxi Agriculture University, Taigu, Shanxi 030801, PR China*
[*]For correspondence: livets@163.com; edu_sxndwjs@126.com

Abstract

Among the transcription factors of *Arabidopsis*, abscisic acid insensitive 3 (ABI3) was mediated in accumulating of seed protein. In soybean (*Glycine max* L. Merr.), the mRNA expression content of *ABI*3-like (two members: *GmABI*3-8 and *GmABI*3-18) and storage protein genes are significantly correlated. However, the expression of *ABI*3-like in different plant tissues or during seed germination as well as the interaction mechanism between *ABI*3-like and the storage protein gene still not known. In current research, the transcriptional expression of *ABI*3-like was studied supplementary, and the interaction mechanism with the storage protein gene was demonstrated in the yeast one-hybrid system. The results demonstrated that the mRNA content of soybean *ABI*3-like in the seeds were significantly higher than those of other plant tissues ($P<0.05$) and decreased with time during seed germination. In the yeast one-hybrid system, *GmABI*3-8 could bind to the RY motif located in the promoter of the storage protein gene and positively regulated its expression. The RY motif as a transcriptional activity regulation factor was related to the expression of storage protein genes in soybean. Our results showed that *ABI*3-like has a crucial function in storage proteins accumulation and may facilitate further molecular breeding methods for developing high-protein soybean varieties. © 2018 Friends Science Publishers

Keywords: Abscisic acid insensitive 3; Soybean; Interaction mechanism; mRNA expression pattern; Storage protein gene

Introduction

The abscisic acid insensitive 3 (ABI3) and its homologous transcription factors exhibited the major function in the seeds development of *Arabidopsis* and maize, and also mediated in seeds dormancy and reserve protein expression (Suzuki and Mccarty, 2008; Grimault *et al.*, 2015; Mao and Sun, 2015). In soybean (*Glycine max* L. Merr.), *ABI*3-like includes two members: *GmABI*3-8 and *GmABI*3-18.

The RY motif is a seed-specific cis-acting element located in the promoter of storage protein gene (Nielsen *et al*., 1989). Indeed, The RY motif is the only commonly regulatory sequence of soybean storage protein gene promoters and exists in two forms: one located in the legumin-box and one that is free (Li and Zhang, 2011). Previous research have been shown that the storage protein accumulation and dormancy process were regulated by the interaction of ABI3 with the RY motif in *Arabidopsis* seeds (Sakata *et al.*, 2010; Delahaie *et al.*, 2013).

Our previous study reported that the transcriptional expression content of soybean *ABI*3-like and storage protein genes are significantly correlated during developing seed (Du *et al*., 2016). Refer to soybean, nevertheless, the transcriptional expression features of *GmABI*3-8 and *GmABI*3-18 in different plant tissues or during seed germination, as well as the underlying molecular mechanism is still unknown. Thus, the objectives of this study were to investigate the transcriptional pattern of soybean *ABI*3-like within different soybean plant tissues and at different germination stages as well as the interaction mechanism between *ABI*3-like and seed storage protein gene. The elucidation of these mechanisms could render an enlightening direction for molecular breeding of high protein soybean.

Materials and Methods

Plant Materials

Soybean seeds (*Glycine max* L. Merr., NC111-1) were provided by Shanxi Agricultural University, China, and planted in Jinzhong City, Shanxi Province. Soybean flowering was set as d 0. The roots, stems, leaves, flowers, and seeds of at least 10 plants were collected at 40 d post

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flowering. More than 100 seeds were collected at maturity and stored (Fehr *et al*., 1971). After 6 h of imbibition, mature soybean seeds were cultivated under hydroponic conditions (solution (1000 mL): water 1000 mL, $Ca_2SO_4 \cdot 2H_2O$ 460 mg, K_2HPO_4 136 mg, $MgSO_4$ 60 mg, KCl 75 mg, $FeC_6H_5O_7$ 75mg, $Ca(NO_3)_2 \cdot 4H_2O$ 30 mg, H_3BO_3 2860 mg, $MnSO_4$ 1810 mg, $ZnSO_4 \cdot 7H_2O$ 220 mg, $CuSO_4 \cdot 5H_2O$ 800 mg, $MoO_4 \cdot H_2O$ 20 mg; Sigma-Aldrich, Shanghai, China) in darkness and the constant temperature of 25°C, and the hypocotyledonary axis and cotyledon were collected four times every 24 h. All samples were preserved at -80°C after treated with liquid nitrogen.

Quantitative Real-time PCR (qRT-PCR)

Total RNA and DNA were extracted using the RNA Isolation Kit (Huayueyang Bio, Beijing, China) and the DNA Isolation Kit (TianGen Biotech, Beijing, China), following the manufacturers' protocols. Total RNA was quantified by the Nano-Drop (Wilmington, DE, USA) ND-1000 spectrophotometer and cDNA synthesis was performed using the PrimeScript RT Master Mix Kit (TaKaRa, Dalian, China). The product with a standardized concentration was stored at -20°C. The primers used for qRT-PCR were designed using Primer Premier 5.0 (Premier Biosoft, Palo Alto, CA, USA) (Table 1). qRT-PCR was carried out with the SYBR Premix Ex Taq^{TM} II Kit (TaKaRa) using Mx3000PTM (Stratagene, Santa Clara, CA, USA), according to the manufacturer's protocol. Water was used as a negative control and the expression of each gene was tested in triplicate. The relative gene expression content was calculated by the ΔΔCt method (Pfaffl, 2001) using *CYP*2 as the internal control (Table 1).

Amplification of the Target Fragment and Construction of the Vector

PCR primers (containing restriction sites) were also designed by Primer Premier 5.0 (Table 1). PCR products were sequenced and checked using BLAST preceded by 3% agarose gel electrophoresis. The target fragment pGADT7 and the pHIS2 vector (BioVector NTCC, Beijing, China) were digested with EcoRI/NdeI and MluI/SacI and then, connected. Connection products were transferred to DH5α (TransBionovo, Beijing, China), containing ampicillin for pGADT7 and kanamycin for pHIS2. Plasmids were extracted using the HiPure Plasmid Micro Kit (Magen Bio, Guangzhou, China) and then, sequenced. Based on sequencing results, the *Gy*2-Defect (*Gy*2-D) promoter was artificially synthesized by removing two RY repeat elements. The *Gy*2-D promoter was constructed into the pHIS2 vector through the same process.

Yeast One-hybrid

A mixture of 10 μL mixed plasmids, 600 μL PEG/LiAc, and 10 μL pre-denatured salmon sperm was added to 100 μL AH109 yeast cells (BioVector NTCC) and cultured at 30°C with an agitation of 200 rpm for 30 min. Then, the mixture was placed in a constant temperature of 42°C for 15 min after the addition of 70 μL DMSO, cooled on ice for 5 min, and centrifuged at 8000 rpm for 30 s. The precipitant was suspended, coated on a medium, and cultured for 4 d.

Statistical Analysis

The analysis of data were performed using SPSS 19.0 (IBM, Armonk, NY, USA), whereas the generation of graphs were done by SigmaPlot 12.5 (Systat Software, San Jose, CA, USA). The data were expressed as the mean ± standard error ($\bar{x}$± SE), and $P < 0.05$ was considered to be significant.

Results

Expression Profiles of *GmABI*3-8 and *GmABI*3-18 in Different Tissues

At 40 d post flowering, the expression level of mRNA for *GmABI*3-8 and *GmABI*3-18 were the highest in the seed, followed by those in the flower, stem, root and leaf (Fig. 1). The expression level of both genes in seed were significantly ($P < 0.05$) higher than other tissues, but no differences were identified among the non-seminal tissues.

***ABI*3-like Expression Patterns in Hypocotyledonary Axis and Cotyledon during Seed Germination**

During seed germination, the transcriptional levels of two mentioned genes decreased with time both in hypocotyledonary axis and cotyledon, 0 h was significantly ($P < 0.05$) higher than that in other periods (Fig. 2). Additionally, the mRNA expression level of *GmABI*3-18 decreased faster than that of *GmABI*3-8.

Gene Amplification and Vector Construction

The results of gene amplification (Fig. 3) showed that the size of *GmABI*3-8 was approximately 2,300 bp and that of the *Gy*2 promoter approximately 850 bp. The homology between sequencing results and BLAST results were found to be over than 95%.

*GmABI*3-8 was constructed using the pGADT7 vector, whereas *Gy*2 and the *Gy*2-D promoter using the pHIS2 vector (Fig. 4). The homology between sequencing results and BLAST results were found to be over than 95%.

Interaction between *GmABI*3-8 and *Gy*2 (*Gy*2-D) in Yeast One-hybrid System

On a culture medium without Trp and Leu (Fig. 5a), the yeast co-transformed with pGADT7-*GmABI*3-8 and pHIS2-*Gy*2, pGADT7 and pHIS2-*Gy*2, pGADT7-*GmABI*3-8 and pHIS2-*Gy*2-D, and pGADT7 and pHIS2-*Gy*2-D grew normally.

Table 1: Primers used in qRT-PCR and gene amplification

Gene	Accession No.	Primer sequences (5′-3′)	Product (bp)
*GmABI*3-8	XM_003532261.2	F: TTTCTTTGACCCTGCCTCTATC; R: CACCATTGCATGTTGCTCTTC	91
*GmABI*3-18	XM_006602493.1	F: CAGCAAGGGAGAACAGAAGAG; R: CATCGTCTACTCCAACACCATC	55
*Gy*2	D00216	F: CGGGTTGTCCTAGCACTTATC; R: CTCTCTGAAGCGATGTACCTTT	79
*CYP*2	TC224926	F: CGGGACCAGTGTGCTTCTTCA; R: CCCCTCCACTACAAAGGCTCG	154
*GmABI*3-8	XM_003532261.2	F: ATGGAGTGTGAAGTTGAATTACAAG; R: TATTAATTTACTATTTTTTCAGATTTG	2367
*Gy*2	D00216	F: GGAATTCAATAATTTCTTGGATAGAGAAT; R: ACGCGTCATGGTGATGAGTGTTCAAAG	1388

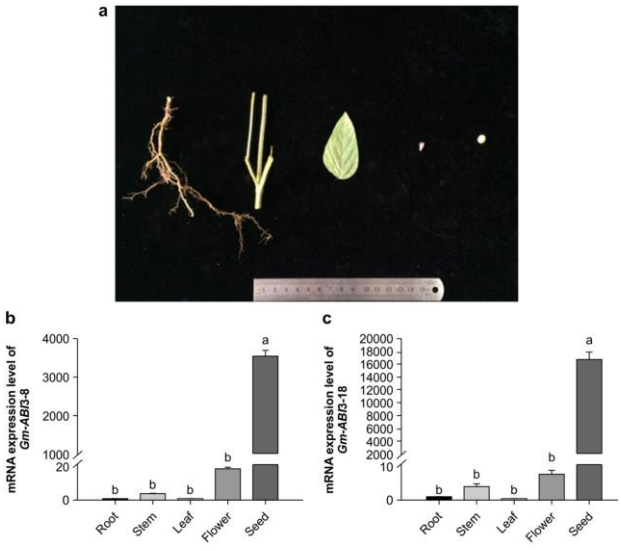

Fig. 1: The mRNA expression pattern of *GmABI*3-8 and *GmABI*3-18 in different plant tissues at 40 d post flowering. (a) From left to right: soybean root, stem, leaf, flower, and seed. (b) The mRNA expression pattern of *GmABI*3-8. (c) The mRNA expression pattern of *GmABI*3-18. Each value is the average of three datasets. Standard error is denoted by error bars. Means followed by the different letters within an accession are significantly different at $P< 0.05$

On a culture medium without Trp, Leu and His (Fig. 5b), the yeast co-transformed with pGADT7-*GmABI*3-8 and pHIS2-*Gy*2 grew normally; that co-transformed with pGADT7-*GmABI*3-8 and pHIS2-*Gy*2-D grew relatively slow; whereas, that co-transformed with pGADT7 and pHIS2-*Gy*2 or pGADT7 and pHIS2-*Gy*2-D was unable to grow.

Discussion

Many studies have shown that *ABI*3 plays a major function in regulating the growth of *Arabidopsis thaliana* seed (Chiu *et al*., 2012). Our data showed that the transcriptional levels of *GmABI*3-8 and *GmABI*3-18 in seed were significantly higher than those in other tissues (Fig. 1). Therefore, *GmABI*3-8 and *GmABI*3-18 might be specifically expressed in the seed and their main function might be to regulate seed metabolism. These findings were in accordance with those described in peanut (Guo *et al*., 2006) and in pea (Gagete *et al*., 2009). In the present study, *GmABI*3-8 and *GmABI*3-18 showed a little expression in the flower (Fig. 1), probably because we used the whole flower as an experimental material. Soybean is a cleistogamous species with a synchronous flowering and seed development, and thus, qRT-PCR could detect a small amount of fluorescence signal. However, further research is needed to investigate the mRNA contents of the two *ABI*3-like in different flower organs.

In *A. thaliana*, *ABI*3 is participated in the dormancy and dehydration of seeds, since the *ABI*3 mutant showed a precocious germination phenotype (Sugliani *et al*., 2010). However, similar results have not been reported in other species. In the present study, the transcriptional levels of *ABI*3-like decreased with time both in the hypocotyledonary axis and the cotyledon (Fig. 2). Additionally, the mRNA contents of the two in the hypocotyledonary axis and the cotyledon were relatively high after 6 h of imbibition, and no differences were identified compared with those in mature seeds (Fig. 2). Therefore, imbibition did not affect *GmABI*3-8 and *GmABI*3-18's expression; however, the expression levels significantly decreased at 1 d after germination and reached non-expression levels at 4 d after germination (Fig. 2). Thus, the function of *GmABI*3-8 and *GmABI*3-18 might be the promotion of seed dormancy and the inhibition of seed germination. However, the underlying mechanisms that control the decrease in the transcriptional levels of two *ABI*3-like during the transition from seed dormancy to seed germination remain unclear. Thus, further research is needed to investigate the expression pattern of *GmABI*3-8 and *GmABI*3-18 from d 0 to 1 d after germination in order to identify the factors that control seed dormancy and germination.

The evolution of homologous genes has been studied extensively in various species, such as tobacco and rice (Jiang *et al*., 2013). In our previous study (Du *et al*., 2016), we reported that the expression curves of *GmABI*3-8 and *GmABI*3-18 had two peaks each; one located in the storage protein accumulation stage and another in the dehydration stage. However, the *GmABI*3-18's expression content was more active during the dehydration stage, whereas that of *GmABI*3-8 was more active during the storage protein accumulation stage. Additionally, *GmABI*3-8 was more highly correlated with the storage protein genes at the mRNA expression level than *GmABI*3-18. This study shows the changes in mRNA expression profile of *GmABI*3-18 were more obvious than those of *GmABI*3-8 in different tissues or during seed germination (Fig. 2).

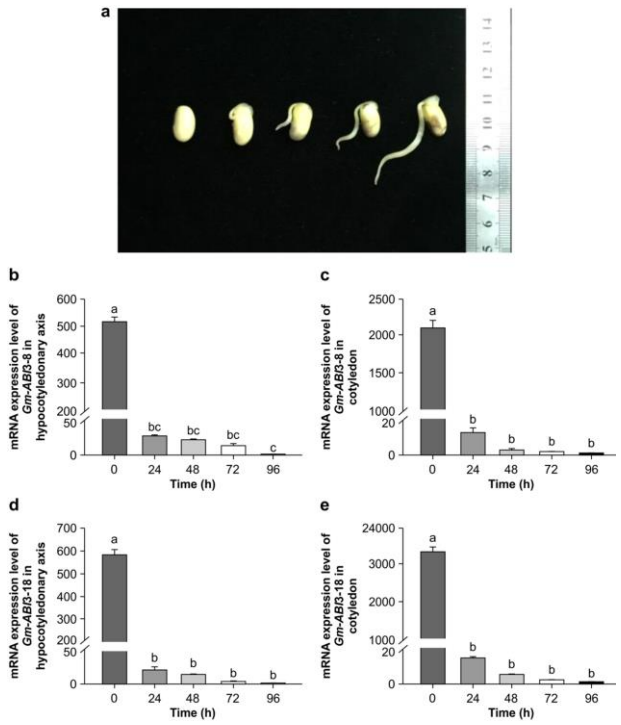

Fig. 2: The mRNA expression pattern of *GmABI*3-8 and *GmABI*3-18 in hypocotyledonary axis and cotyledon during seed germination. (a) Hypocotyledonary axis and cotyledon at 0 h, 24 h, 48 h, 72 h, and 96 h post imbibition for 6h. (b) The mRNA expression pattern of *GmABI*3-8 in the hypocotyledonary axis. (c) The mRNA expression pattern of *GmABI*3-8 in the cotyledon. (d) The mRNA expression pattern of *GmABI*3-18 mRNA in the hypocotyledonary axis. (e) The mRNA expression pattern of *GmABI*3-18 mRNA in the cotyledon. Each value is the average of three datasets. Standard error is denoted by error bars. Means followed by the different letters within an accession are significantly different at $P< 0.05$

Therefore, we concluded that the homologous genes soybean *ABI*3-like differed in the expression localization and timing, probably because the former was more closely related to storage protein synthesis, whereas the latter more closely associated with seed dehydration and inhibition of seed germination. We assumed that the expression patterns of these homologous genes changed when their function became more precise after soybean polyploidization and thus, might be of great significance for the study of gene evolution in soybean.

Lelievre *et al.* (1992) reported that the binding activity of the promoter reduced approximately 10-fold in tobacco, when the RY repeat element was deleted; however, the transcription factor that interacted with the RY repeat element was not identified. In the present study, similar results were obtained by the yeast one-hybrid system and showed that *GmABI*3-8 is a transcription factor that could be combined with the RY motif (Fig. 5).

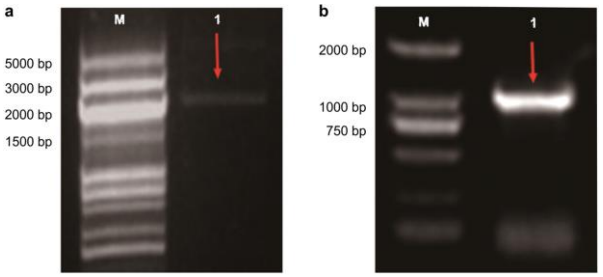

Fig. 3: Agarose gel electrophoresis of *GmABI*3-8 and *Gy*2 promoter. (a) Agarose gel electrophoresis of *GmABI*3-8. Lane M is a 5,000 bp DNA marker. Lane 1 is *GmABI*3-8. (b) Agarose gel electrophoresis of *Gy*2 promoter. Lane M is a 2,000 bp DNA marker. Lane 1 is the *Gy*2 promoter

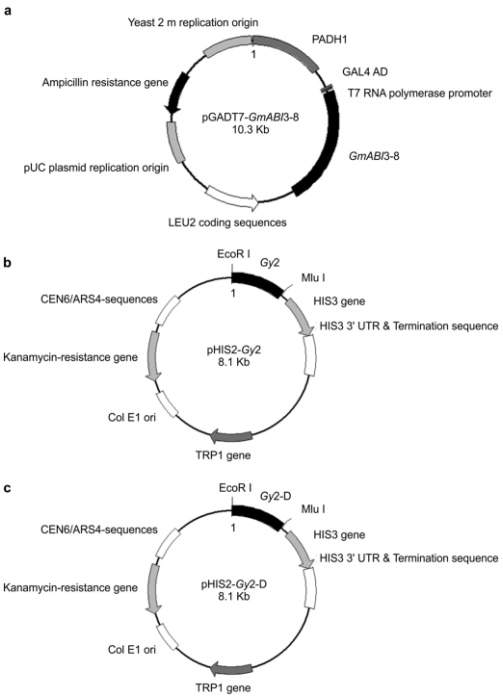

Fig. 4: Vectors used in the yeast one-hybrid system. (a) pGADT7-*GmABI*3-8 vector. (b) pHIS2-*Gy*2 vector. (c) pHIS2-*Gy*2-D vector

Compared with the *Gy*2 promoter, the *Gy*2-D promoter lacked two RY motifs that directly reduced the binding activity of the *Gy*2 promoter with *GmABI*3-8, leading to a significant reduction in expression. These results confirmed that the RY motif regulated rather than activated the expression of *Gy*2, which could be still expressed even when the promoter did not contain any RY motifs. Since the RY motif existed in all soybean storage protein gene promoters, we assumed that *GmABI*3-8 positively regulated the expression of storage protein gene by combining with the RY motif. *GmABI*3-8 might be also associated with other cis-acting elements that activated gene expression; however, the underlying mechanism remains to be further studied.

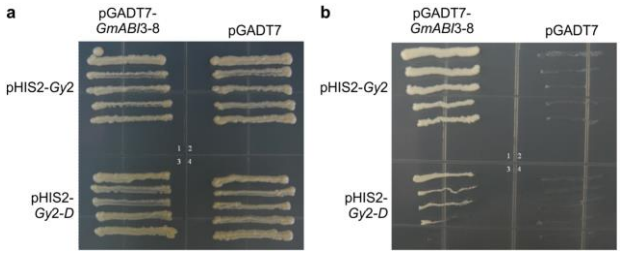

Fig. 5: The growth of yeast co-transformed with different vectors. (a) Control group. Culture medium without Trp and Leu. Sections 1 to 4 contain pGADT7-*GmABI*3-8 and pHIS2-*Gy*2, pGADT7 and pHIS2-*Gy*2, pGADT7-*GmABI*3-8 and pHIS2-*Gy*2-D, and pGADT7 and pHIS2-*Gy*2-D, respectively. (b) Treatment group. Culture medium without Trp, Leu and His. Sections 1 to 4 contain pGADT7-*GmABI*3-8 and pHIS2-*Gy*2, pGADT7 and pHIS2-*Gy*2, pGADT7-*GmABI*3-8 and pHIS2-*Gy*2-D, and pGADT7 and pHIS2-*Gy*2-D, respectively

The promoter of *Gy*2 included two types of RY motifs (Shirsat *et al*., 1990): one of 28 bp located in the legumin-box that only exists in 11S storage protein gene promoters and one that is free and exists in 7S and 11S storage protein gene promoters. Yoshino *et al*. (2006) reported that the activity of the *CG-alpha*-1 (7S gene) promoter was highly related to the integrity of the RY motif. Our results determined that the free RY motif could bind to the transcription factor and that the deletion of RY motifs significantly reduced the activity of the *Gy*2 promoter. It could be assumed that the loss of free RY motifs resulted in decreased binding activity, whereas the loss of the RY motif in the legumin-box did not affect the binding activity, or that the loss of RY motifs in the legumin-box resulted in the structural damage of legumin-box, reducing the binding activity. To clarify this hypothesis, promoters containing only one type of RY motif need to be developed and compared in a yeast one-hybrid system.

Due to the low correlation between the mRNA expression level of *GmABI*3-18 and that of the storage protein genes, we could not verify the binding activity of *GmABI*3-18. However, the homology between *GmABI*3-18 and *GmABI*3-8 was more than 91%, and thus, *GmABI*3-18 might be able to interact with the RY motif. To better understand the regulatory mechanism of the storage protein genes, we need to further study: 1) the different effects of *GmABI*3-18 and *GmABI*3-8 on the same storage protein genes by investigating the interaction between *GmABI*3-18 and the *Gy*2 promoter; 2) the simultaneous interaction of *GmABI*3-8 and *GmABI*3-18 with the *Gy*2 promoter and confirm whether they have a competitive or synergistic effect; and 3) the interaction between *GmABI*3-8 and *GmABI*3-18 with other storage protein gene promoters to identify the regulation of the same transcription factors on different storage protein genes. In addition, *ABI*3 was related to seed dehydration and drought resistance (Chiu *et al*., 2012; Zeng *et al*., 2013). Therefore, the interaction between *ABI*3-like and *ABI*5-like and the effect on ABRE might further reveal its functions related to the role of dehydration.

Our results indicated that the production of high protein soybean germplasm might be possible by improving the expression level of *ABI*3-like using molecular breeding technology. To this end, *ABI*3-like needs to be transferred to *ABI*3 deficient A. thaliana to analyze the effect of over expression in *ABI*3-like and the function of protein synthesis at the phenotypic, RNA, and protein level. Next, the above experiments will be carried out in soybean, and high protein soybean lines with stable inheritance will be obtained through screening. However, some technical problems might occur related to the involvement of *ABI*3-like in the complex regulatory network of seed maturation (Fatihi *et al*., 2016). Therefore, further research is necessary to find whether the unilateral increase of *ABI*3-like expression is related to transcription factors, such as *FUS*3, *LEC*1 and *LEC*2, as well as whether the increase in *ABI*3-like expression levels results in the early dehydration of the seed and the shortening of the whole growth cycle.

Conclusion

We obtained the complete transcriptional profiles of *GmABI*3-8 and *GmABI*3-18 in different plant tissues, at seed germination, and the whole seed development cycle combined with previous results. Our results revealed that the functions of *GmABI*3-8 and *GmABI*3-18 were participate in regulating the storage protein synthesis, dehydration, and germination of seed. *GmABI*3-8 transcription factor could regulate the expression of storage protein gene by binding to the RY motif in the promoter of the storage protein gene.

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