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ORIGINAL ARTICLE

The *AtDREB1A* transcription factor up-regulates expression of a vernalization pathway gene, *GmVRN1-like*, delaying flowering in soybean

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Abstract The dehydration-responsive element binding proteins/C-repeat binding factors (i.e., DREBs/CRT) are crucial transcription factors, they can bind the *cis*-elements containing the A/GCCGAC sequence in the promoter region. Overexpression of *DREB* genes enhances the resistance to multiple abiotic stresses, but also causes dwarfism and delayed flowering in many plant species. In this study, constitutive overexpression of *AtDREB1A* in soybean plants caused dwarfism and delayed-flowering phenotypes. The delayed-flowering phenotype was not affected by day length and could not be reversed by exogenous gibberellic acid. The expression levels of flowering-related genes were determined by quantitative real-time RCR. The *Glyma11g13220* (designated as *GmVRN1-like*) expression was strongly induced in transgenic plants. *GmVRN1-like* was homologous to the *Arabidopsis thaliana VRN1* gene (*At3g18990*). Electrophoretic mobility shift assay showed that AtDREB1A could bind the dehydration-responsive element motif, ACCGAC, in a region −157 to −186 bp upstream of *GmVRN1-like*. The motif shared 100 % identity with the DRE sequence present in *RD29A*. Our results imply that the delayed-flowering of *AtDREB1A*-overexpressing plants might be caused by up-regulating of *GmVRN1-like* gene.

Keywords Soybean · *AtDREB1A* overexpression · *GmVRN1-like* · Delayed flowering

Abbreviations

ABA	Abscisic acid
AP2/EREBP	APETALA2/ethylene responsive element binding protein
CBF	C-repeat binding factors
CO	Constans
DAE	Days after emergence
DREB	Dehydration-responsive element binding proteins
EMSA	Electrophoretic mobility shift assay
FLC	Flowering locus C
FT	Flowering locus T
GA	Gibberellic acid
SOC1	Suppressor of constans 1
VRN1	Vernalization 1

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Introduction

Abiotic stresses, including drought, salinity, and temperature have disadvantageous effects on plant development. Many of genes associated with stress resistance have been identified (Kissoudis et al. 2014). Transcription factors can regulate downstream genes by binding the *cis*-elements present in their promoter (Chen and Zhu 2004). The dehydration-responsive element binding proteins (DREBs) transcription factors regulate the stress-inducible genes in an abscisic acid (ABA)-independent pathway (Yamaguchi-Shinozaki and Shinozaki 2006). The DREBs containing a conserved DNA-binding domain belong to the APETALA2/ethylene responsive element binding protein (AP2/EREBP) superfamily. They can regulate the downstream stress-inducible genes through binding the dehydration responsive element (DRE) with core sequence A/GCCGAC present in their promoter region. This interaction regulates genes expression and enhances the tolerance to abiotic stresses (Baker et al. 1994; Yamaguchi-Shinozaki and Shinozaki 1994; Stockinger et al. 1997).

Many studies have found that overexpression of *DREB* genes improve tolerance to multiple abiotic stresses in many plants such as *A. thaliana*, rice, wheat, maize, soybean, barley and tomato (Agarwal et al. 2006; Lata and Prasad 2011; Mizoi et al. 2012). The *A. thaliana DREB* genes have been well-studied. Six genes encoding CBF/DREB1 proteins (*DREB1a*, *DREB1b*, *DREB1c*, *DREB1d*, *DREB1e*, and *DREB1f*) have been identified (Sakuma et al. 2002; Gilmour et al. 2004). The expression of *DREBs* is up-regulated by low temperature and transiently contributes to enhanced tolerance (Agarwal et al. 2006). Multiple mechanisms, such as soluble sugars and proline accumulation, cold-regulated genes regulation, have been identified to be responsible for increasing freezing tolerance (Thomashow 1998, 1999; Gilmour et al. 2004).

Overexpression of *DREB* genes also cause dwarfism and delayed flowering (Liu et al. 1998; Gilmour et al. 2000; Hsieh et al. 2002; Dubouzet et al. 2003; Kasuga et al. 2004; Ito et al. 2006; Achard et al. 2008; Huang et al. 2009; Li et al. 2012; Suo et al. 2012). Some cases of dwarfism can be reversed by exogenous gibberellic acid (GA) treatments (Hsieh et al. 2002; Magome et al. 2004; Achard et al. 2008; Huang et al. 2009; Suo et al. 2012). Studies have also shown that dwarfism is mediated by a GA metabolic pathway (Magome et al. 2004; Achard et al. 2008; Huang et al. 2009; Suo et al. 2012). However, some instances of dwarfism cannot be reversed (Magome et al. 2004). Some delayed-flowering phenotypes can be rescued or partially rescued using exogenous GA (Achard et al. 2008; Magome et al. 2008; Huang et al. 2009). This implies that the delayed-flowering phenotype is GA-pathway dependent. However, in some cases, delayed flowering cannot be rescued by exogenous GA treatments (Tong et al. 2009; Suo et al. 2012). Little is known at the molecular level regarding how overexpression of *DREB* genes causes delayed flowering. Previous studies showed that overexpression of *DREB* genes causes delayed flowering through activating the floral suppressor, *FLOWERING LOCUS C* (*FLC*) (Seo et al. 2009).

Flowering is the most critical event in the life cycle of angiosperm.To live and propagate, plants have evolved complicated and coordinated genetic networks responding to exogenous and endogenous signals to make sure flowering at the right time (Boss et al. 2004; Baurle and Dean 2006). In *A. thaliana*, there are at least four pathways regulating flowering time, including the GA, autonomous, photoperiod, and vernalization pathways (Simpson and Dean 2002; Boss et al. 2004; Bernier and Perilleux 2005; Baurle and Dean 2006). The photoperiod and vernalization pathways regulate flowering time through sensing environmental signals related to day length and low temperature, respectively. In contrast, the GA and autonomous pathways are controlled by internal signals in response to flowering (Srikanth and Schmid 2011). However, there is increasing evidence that these pathways may not act independently of each other. There exists extensive cross-talk among different pathways, ultimately affecting the downstream floral integrators including *FLOWERING LOCUS T* (*FT*), *SUPPRESSOR OF CONSTANS 1* (*SOC1*), and *LEAFY*. The *CONSTANS* (*CO*) and *FLC* regulate these integrator genes antagonistically (Mouradov et al. 2002). *CONSTANS* acts as a floral activator, whereas *FLC* acts as a floral repressor (Michaels and Amasino 1999; Lee et al. 2000; Mouradov et al. 2002).

Soybean is a major oilseed leguminous crop that has unique vegetative and floral characteristics. However, information regarding the molecular mechanisms of flower initiation and development is limited. There is increasing evidence of conservation of flowering pathways among many plant species (Hecht et al. 2005). Comparative genomic analyses demonstrated that there are conserved genes between soybean and *A. thaliana* (Jung et al. 2012; Kim et al. 2012). Additionally, 491 putative soybean flowering regulatory genes have been characterized (Jung et al. 2012). Previously, we observed dwarfism and delayed flowering in *AtDREB1A* overexpression soybean plants. The dwarfism could be rescued by GA, but delayed flowering could not (Suo et al. 2012). According to these results, we speculate that the regulatory mechanisms of dwarfism and delayed flowering are relatively independent. In this study, we investigated the response of *AtDREB1A* to photoperiod in transgenic and wild-type (WT) plants. We also evaluated the transcriptional levels of homologs of *A.*

thaliana genes related to flowering using quantitative real-time polymerase chain reaction (qRT-PCR). Finally, an electrophoretic mobility shift assay (EMSA) was used to analyze the target gene of *AtDREB1A*. The results indicated that up-regulation of *GmVRN1-like* may be responsible for inducing delayed flowering in *AtDREB1A*-overexpressing plants.

Materials and methods

Plant materials and phenotypic analysis

Transgenic lines of soybean cultivar Huachun 5 overexpressing *AtDREB1A* were developed using an *Agrobacterium tumefaciens*-mediated method described in the previous study (Suo et al. 2012). Because the L2 plants displayed obvious phenotypic changes, the T_3 plants of L2 were selected for subsequent studies. The transgenic L2 and WT soybean seeds were first germinated in sand until cotyledons emerged (about 5 days). Healthy and uniform seedlings were transferred to pots with one plant per pot and a 5-day recovery period. The seedlings were grown in growth chambers with three photoperiods, corresponding to 16, 12, and 8 h of light. The temperatures during growth were 28 and 24 °C during the light and dark periods. There were three replicates for each photoperiod condition. The numbers of trifoliate leaves and flowering time were recorded during the growth period.

Transcriptional analyses

To examine the transcriptional levels of soybean flowering-related genes under different day length conditions, fully expanded leaves from transgenic L2 and WT plants were sampled at 12 h after dawn during the fourth trifoliate leaves stage. The detailed sampling days were listed in Online Resource 1. Total RNA of soybean was isolated using TRIzol reagent (Invitrogen, USA). 1 mg total RNA was treated with RNase-free DNase (TaKaRa, Japan) and then was reversed using the oligo (dT) primer and M-MLV reverse transcriptase (Invitrogen, USA). The qRT-PCR was performed with a SYBR Green I kit (Bio-Rad, USA) using a CFX96 system (Bio-Rad, USA). Three biologically independent RNA samples were analyzed by qRT-PCR in triplicate. The primer efficiency have been determined with the range of 90.2–109.0 % and used for calculated gene expression. The gene *β-tubulin* was used as internal control (Wang et al. 2012; Lü et al. 2015). The $2^{-\Delta\Delta Ct}$ method was used to detect the relative expression levels of flower genes (Livak and Schmittgen 2001). According to the Bio-Rad CFX96 manufacturer's instructions, we used the calibration sample as the control, which consists of mixed-samples cDNA, the *tubulin* primer as well as SsoFast EvaGreen Supermix kit (Bio-Rad), the other samples were treatments. Eighty-five genes homologous to *A. thaliana* flowering time regulators were selected based on published information (Srikanth and Schmid 2011; Jung et al. 2012; Kim et al. 2012; Watanabe et al. 2012; Blumel et al. 2015) (Table 1). Gene information and primer sequences are provided in Online Resource 2.

Electrophoretic mobility shift assay

We generated a glutathione S-transferase (GST)-AtDREB1A recombinant protein. Previous reports indicated that the DREB protein is capable of interacting with the DRE motif (A/GCCGAC) present in *rd29A* (Magome et al. 2008). Therefore, the promoter of *rd29A* was also prepared as an experimental control.

The length of 663 bp *AtDREB1A* open reading frame (ORF) was obtained from *A. thaliana* cDNA. The *AtDREB1A* ORF digested with *Bam*HI/*Eco*RI was cloned into the pGEX-4T-2 vector. After sequencing to confirm, the vector was transformed into *E. coli* BL21. Expression of the AtDREB1A-GST fusion protein was induced using 50 nmol isopropyl β-D-1-thiogalactopyranoside at 37 °C for 12 h with shaking (150 rpm). Proteins were extracted with 4 ml B-PER™ protein extraction reagent (Pierce, USA). We used 5′-Cy5-labeled double-stranded oligonucleotides as gel shift assay probes. The probe sequences containing the core sequence ACCGAC were as follows:

VRN1-like: 5′-ACTAGTTGTCT*ACCGAC*ATGCATGT ACGTG-3′

Mutant-VRN1-like: 5′-ACTAGTT G TCT*ACTTAT*ATG CATGTACGTG-3′

rd29A: 5′-GATATACT*ACCGAC*ATGAGTTCCAAAA AGC-3′

Mutant-rd29A: 5′-GATATACT*ACTTAT*ATGAGTTCC AAAAAGC-3′ (Magome et al. 2008)

For competition experiments, the competitive probes were added at a 100-fold molar excess. The EMSA was performed with the LightShift Chemiluminescent EMSA kit (Pierce).

Results

Overexpression of *AtDREB1A* in soybean caused severe dwarfness and delayed flowering

Transgenic lines of soybean cultivar Huachun 5 overexpressing *AtDREB1A* were developed using an *Agrobacterium tumefaciens*-mediated method described previously (Suo et al. 2012). The transgenic lines showed dwarfism

Table 1 Information regarding selected flowering-related genes

Arabidopsis locus ID	Abbreviation	Function	Pathway	Soybean homologous genes
AT1G65480/ AT4G20370	FT	PEBP (phosphatidylethanolamine-binding protein) family protein	Integrator	Glyma02g07650,Glyma08g28470, Glyma08g47810, Glyma08g47820
				Glyma16g04830,Glyma16g04840, Glyma16g26660, Glyma16g26690
				Glyma19g28390, Glyma19g28400,Glyma18g53670
AT3G57390	AGL18	AGAMOUS-like 18	Integrator	Glyma02g33040
AT2G45660	SOC1	AGAMOUS-like 20	Integrator	Glyma03g02200,Glyma07g08830, Glyma09g40230, Glyma18g45780
				Glyma05g03660
AT5G10140	FLC, AGL25	K-box and MADS-box transcription factor family protein	Vernalization, autonomous	Glyma05g28130
AT1G69120	AP1 AGL7	K-box and MADS-box transcription factor family protein	Photoperiod	Glyma01g08150,Glyma02g13420, Glyma08g36380, Glyma16g13070
AT4G36920	AP2, FL1	Integrase-type DNA-binding superfamily protein	Photoperiod	Glyma01g39520,Glyma03g33470, Glyma05g18170, Glyma10g22390
				Glyma11g05720, Glyma17g18640, Glyma19g36200
AT2G28550	RAP2.7	related to AP2.7	Photoperiod	Glyma02g09600,Glyma11g15650, Glyma12g07800, Glyma13g40470,
				Glyma15g04930
AT5G62430	CDF1	cycling DOF factor 1	Photoperiod	Glyma06g20950
AT4G08920	CRY1	cryptochrome 1	Photoperiod	Glyma04g11010, Glyma06g10830, Glyma13g01810, Glyma14g35020
AT1G04400	CRY2	cryptochrome 2	Photoperiod	Glyma02g00830,Glyma10g32390, Glyma18g07770, Glyma20g35220
AT5G03840	TFL	PEBP family protein	Photoperiod	Glyma03g35250,Glyma09g26550, Glyma16g32080, Glyma19g37890
AT1G22770	GI	gigantea protein (GI)	Photoperiod	Glyma09g07240, Glyma10g36600, Glyma20g30980
AT4G16280	FCA	RNA binding; abscisic acid binding	Autonomous	Glyma17g03960
AT3G10390	FLD	Flavin containing amine oxidoreductase family protein	Autonomous	Glyma02g18610
AT2G43410	FPA	RNA binding	Autonomous	Glyma11g13490, Glyma12g05490, Glyma13g42060, Glyma15g03330
AT2G19520	FVE	Transducin family protein/WD-40 repeat family protein	Autonomous	Glyma09g07120,Glyma13g42660, Glyma15g02770, Glyma15g18450
AT5G13480	FY	Transducin/WD40 repeat-like superfamily protein	Autonomous	Glyma13g26820, Glyma15g37830
AT4G02560	LD	Homeodomain-like superfamily protein	Autonomous	Glyma03g36970, Glyma19g39620
AT3G18990	VRN1	AP2/B3-like transcriptional factor family protein	Vernalization	Glyma01g11670, Glyma04g43620, Glyma07g21160, Glyma08g44640
				Glyma08g44650, Glyma09g18790, Glyma09g20280, Glyma11g13210
				Glyma11g13220, Glyma20g01130, Glyma20g24220,Glyma12g05250
				Glyma16g05110, Glyma19g27950
AT3G24440	VRN5	Fibronectin type III domain-containing protein	Vernalization	Glyma05g35280, Glyma07g09800, Glyma08g04440, Glyma09g32010
AT5G57380	VIN3	Fibronectin type III domain-containing protein	Vernalization	Glyma17g07000
AT4G16845	VRN2		Vernalization	Glyma11g03960,Glyma01g41460,

and delayed-flowering phenotypes. The dwarfism was recovered after treated with 144 μM GA once a week for 3 consecutive weeks or 60 μM GA three times in 1 week (Suo et al. 2012). However, the delayed-flowering phenotype could not be rescued (Online Resource 3). The delayed flowering of the transgenic plants was regardless of day length. Additionally, compared with WT soybean plants, the transgenic plants had fewer leaves under all day length conditions (Fig. 1). Under short day length conditions, WT plants flowered at 32 DAE with 13 leaves, while transgenic plants flowered at 46 DAE with 8 leaves. Under intermediate and long day length conditions, WT plants flowered at 60 DAE with 42 leaves and 53 DAE with 18 leaves, respectively (Table 2). However, the transgenic plants did not flower until 60 DAE under the intermediate and long day length conditions (Table 2).

Fig. 1 Phenotypes of *AtDREB1A*-overexpressing transgenic and WT soybean plants grown under different day length conditions. **a** Phenotypes of transgenic and WT soybean plants grown under 8 h light/16 h dark conditions at 35 days after emergence (DAE). **b**, **c** Magnified view of (**a**), flower buds formed in WT plants, but transgenic plants maintained their vegetative growth. **d** Phenotypes of transgenic and WT soybean plants under 12 h light/12 h dark conditions at 60 DAE. **e**, **f** Magnified view of (**d**), WT plants flowered, but transgenic plants maintained their vegetative growth. **g** Phenotypes of transgenic and WT soybean plants under 16 h light/8 h dark conditions at 54 DAE. **h**, **i** Magnified view of (**g**), WT plants flowered, but transgenic plants maintained their vegetative growth

Table 2 Number of leaves in transgenic and WT soybean plants grown under different day length conditions

DAE	8 h light/16 h dark		12 h light/12 h dark		16 h light/8 h dark	
	WT	L2	WT	L2	WT	L2
11	1.34 ± 0.33a	1.00 ± 0.00a	1.67 ± 0.33a	1.00 ± 0.00a	1.34 ± 0.33a	1.00 ± 0.00a
18	2.34 ± 0.33b	2.00 ± 0.00b	3.67 ± 0.33a	2.67 ± 0.33b	2.67 ± 0.33b	2.00 ± 0.00b
25	4.33 ± 0.33bc	3.33 ± 0.33d	6.67 ± 0.33a	4.67 ± 0.33b	4.00 ± 0.00bc	3.33 ± 0.33d
32	7.00 ± 0.58b	4.67 ± 0.33d	12.30 ± 0.33a	6.00 ± 0.00bc	7.00 ± 0.58b	5.33 ± 0.33d
39	13.00 ± 0.58a(F)	6.67 ± 0.33c	14.30 ± 0.33a	7.67 ± 0.33bc	13.00 ± 0.58a	9.00 ± 0.58b
46	13.00 ± 0.58c	7.67 ± 0.33f	22.30 ± 0.88a	8.67 ± 0.33ef	15.00 ± 0.00b	10.30 ± 0.88d
53	13.00 ± 0.58c	8.00 ± 0.00d(F)	31.70 ± 0.88a	12.00 ± 0.00c	17.30 ± 0.88b(F)	13.00 ± 0.58c
60	13.00 ± 0.58d	8.00 ± 0.00e	42.30 ± 0.88a(F)	25.30 ± 0.67b	17.30 ± 0.88c	14.70 ± 0.67d

Values are the means of three biological replicates ± standard error. *Different letters* in each row indicate significant differences as determined by the analysis of variance, $p < 0.05$

DAE days after emergence, *F* flowering

AtDREB1A overexpression affects the expression levels of flowering-related genes

Overexpressing *DREB* genes often result in delayed flowering, but the molecular mechanism involved in this is little known (Huang et al.2009; Tong et al. 2009). In our study, the delayed-flowering phenomenon could not be recovered by the GA treatment, implying this flowering delay is GA-independent pathway. Thus, the transcriptional levels of major flowering-related genes of the other three flowering pathways and key flowering integrator genes were detected under different day length conditions in WT and transgenic plants, and 26 genes displayed differential expression patterns (Fig. 2).

In the vernalization pathway, the differentially expressed genes included the *AtVRN1* homologs *Glyma11g13210*, *Glyma11g13220*, *Glyma20g01130*, *Glyma07g21160*, and *Glyma12g05250*. We also analyzed the homologs of *AtVRN2* (*Glyma01g41460*), *AtVRN3* (*Glyma17g07000*), and *AtVRN5* (*Glyma09g32010* and *Glyma07g09800*). Compared with WT plants, the transcriptional level of each of these genes was much higher in transgenic plants regardless of day length conditions. Especially, the transcriptional level of *Glyma11g13220* (designated as *GmVRN1-like*) was pronouncedly up-regulated, about 27-fold, 50-fold, 20-fold higher in transgenic soybean plants than that in WT plants under long, intermediate and short day condition, respectively. These results suggest a potential relationship between *GmVRN1-like* and *AtDREB1A* in terms of the delayed-flowering phenotype (Fig. 2a).

In the autonomous flowering pathway, we identified the *A. thaliana FVE* homologs *Glyma09g07120* and *Glyma15g18450*. We also examined the transcriptional levels of *FCA* homologs (*Glyma12g05490*, *Glyma17g03960*, and *Glyma15g03330*) and *FY* homologs (*Glyma13g16820*, *Glyma15g37830*, and *Glyma19g39620*). Compared with that in WT plants, these genes showed higher transcriptional levels in transgenic plants under all 3-day length conditions (Fig. 2b). The photoperiod floral meristem genes, including *Glyma15g04930*, *Glyma13g40470*, *Glyma02g09600*, and *Glyma05g18170* of the AP2 family, were more highly expressed in transgenic soybean plants than in WT plants (Fig. 2c).

We also detected the transcriptional levels of soybean floral integrators, including a floral repressor (*FLC*) and floral activators (*FT* and *SOC1*). The *FLC* had a higher, while the *FT* and *SOC1* showed lower transcriptional levels in transgenic plants than those in WT plants (Fig. 2d).

GmVRN1-like may be a direct downstream target of *AtDREB1A* in soybean

The DREB transcription factors can specifically bind the DRE *cis* element with a core A/GCCGAC sequence presenting in the promoter of downstream genes (Stockinger et al. 1997; Gilmour et al. 1998; Liu et al.1998). The higher expression level of *GmVRN1-like* in transgenic plants than in WT plants, suggested that *GmVRN1-like* may be the downstream target of *AtDREB1A*. There is a motif with a core sequence of ACCGAC in the *GmVRN1-like* promoter region, 168 bp upstream of the start codon (Lü et al. 2015). This motif has 100 % identity to the DRE *cis*-element present in the promoter of *RD29A* (Maruyama et al. 2004).

To confirm whether AtDREB1A can bind DRE motif in the *GmVRN1-like* promoter, EMSA experiments were conducted. Shifted bands were observed under the conditions of GST-AtDREB1A recombinant protein combined with *rd29A* or *GmVRN1-like* probes. No shifted bands were observed under the conditions of probes only or probes combined with GST control protein (Fig. 3). Both of cold and mutated probes impaired the signal in a small degree. It turns out that AtDREB1A interacts with core sequence of

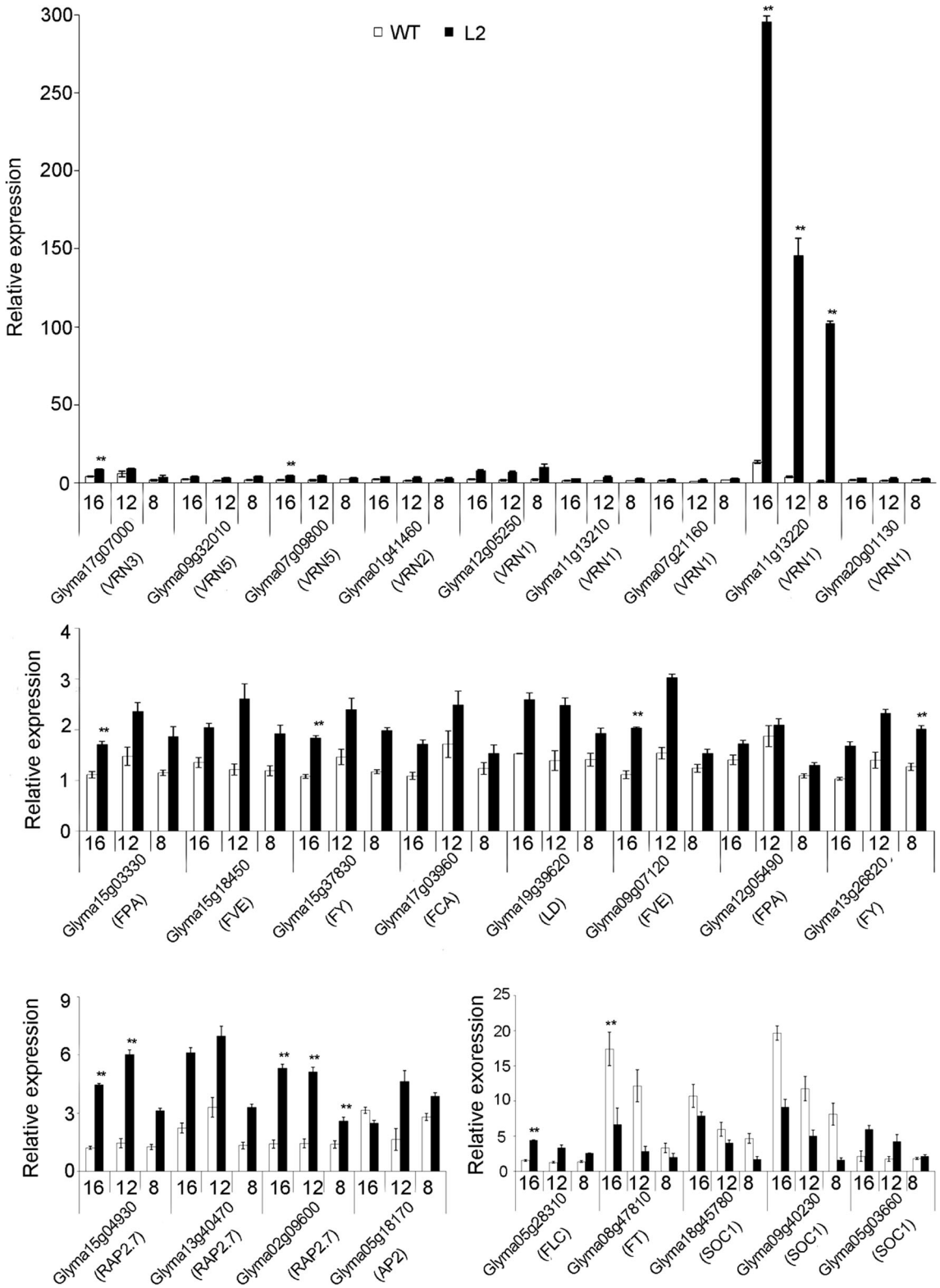

Fig. 2 Transcriptional levels of flowering-related genes **a** transcriptional levels of genes in the vernalization pathway. **b** Transcriptional levels of genes in the autonomous pathway. **c** Transcriptional levels of genes in the photoperiod pathway. **d** Transcriptional levels of floral integrators. *β-tubulin* was used as an internal control. Values are the mean of three biological replicates ± standard error. Student's *t* test, $^{**}p < 0.01$

DRE element present in the promoter of *GmVRN1-like* in vitro (Fig. 3).

Discussion

The delayed-flowering phenotypes caused by overexpression of *AtDREB1B*, *GhDREB1*, and *AtDREB1F* were rescued by GA treatments (Achard et al. 2008; Magome et al. 2008; Huang et al. 2009), suggesting that they are GA-metabolism dependent. However, delayed flowering cannot be reversed by exogenous GA treatment caused by overexpression of *AtDREB1A* or *DgDREB1A* (Tong et al. 2009; Suo et al. 2012), which means that other mechanisms may regulate the flowering of transgenic plants. In our previous study, overexpression of *AtDREB1A* gene in soybean resulting transgenic plants exhibited dwarfism with no observable internodes, darker green and had smaller leaves and seeds compared with the WT plants. The abnormal phenotypic characteristics could be reversed after exogenous GA treatments, we speculated that *AtDREB1A* mediates GA metabolism by regulating genes in GA synthesis and deactivation pathways (Suo et al. 2012).

Previous studies regarding delayed flowering focused only on the expression of key flowering regulators, including *FLC*, *FT*, and *CO*. For example, with respect to *DgDREB1A*- overexpressing *A. thaliana* plants, *CO* and *FT* were down-regulated, while the expression level of *FLC* was unaffected. This suggested that delayed flowering is associated with the photoperiod pathway (Sun et al. 2013). Moreover, *FLC* and *CO* were affected in *A. thaliana* plants overexpressing *GhDREB1*, indicating that delayed flowering may be the result of changes to multiple flowering pathways (Huang et al. 2009). However, flowering is a complex process that is regulated by numerous genes. In soybean, 491 flowering regulatory genes belonging to the photoperiod, vernalization, autonomous, and GA flowering pathways have been identified by comparing the soybean and *A. thaliana* genomes (Jung et al. 2012). Thus, we analyzed the expression levels of major flowering genes from the photoperiod, vernalization, and autonomous pathways along with flowering integrators in *AtDREB1A*-overexpressing transgenic and WT soybean plants. The expression level of *GmVRN1-like*, which is a homolog of the *A. thaliana* flowering vernalization gene (*VRN1*), was significantly ($P < 0.01$) higher in transgenic plants than that of WT plants (Fig. 2). Additionally, EMSA results revealed that *GmVRN1-like* is a direct downstream target of *AtDREB1A* (Fig. 3). The *FLC* expression level was activated, while the *FT* and *SOC1* expression levels were inhibited in transgenic plants (Fig. 2). This indicates that the late flowering of transgenic soybean may be linked to changes in the vernalization flowering pathway, despite soybean being a photoperiod-sensitive plant.

The overexpression of *DREB* genes causes delayed flowering because of the up-regulation of *FLC* expression. However, little is known about how *DREB* genes regulate *FLC* expression (Seo et al. 2009). In this study, there was no evidence that *AtDREB1A* directly regulates *FLC* expression, but the EMSA results indicated that *AtDREB1A* regulates *GmVRN1-like* expression by binding the cold response elements in the *GmVRN1-like* promoter region. Intermittent cold treatment is known to delay flowering through up-regulation of *FLC* expression (Kim et al. 2012). The overexpression of *AtDREB1A* may simulate exposure to cold stress, thus activating the *GmVRN1-like* gene, which belongs to the vernalization flowering pathway.

In *A. thaliana*, FLC chromatin was inactivated initially by VIN3 production through histone modification (Sung and Amasino 2004). Thereafter, FLC chromatin structure

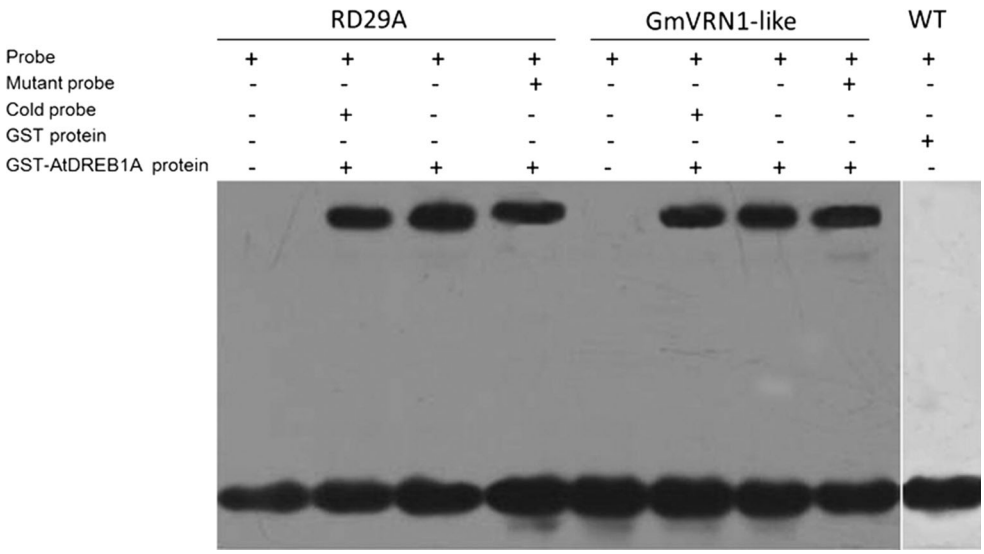

Fig. 3 EMSA results indicating AtDREB1A specifically bound to the DRE in vitro. Biotin-labeled DNA probes (1 nmol) were combined with purified protein (5 μg). The *rd29A* labeled probe efficiently binds to the GST-AtDREB1A recombine protein. The *GmVRN1-like* labeled probe have shown the same results with that of *rd29A* labeled probe, which indicated the AtDREB1A can bind the DRE *cis-elemnt* present in the promoter region

was permanently inactivated by VRN1, VRN2, and LHP1 via heterochromatin formation (Bastow et al. 2004; Sung and Amasino 2004; Seo et al. 2009). The *FLC* gene is regulated by many pathways, which are associated with different chromatin pathways and co-transcriptional mechanisms related to antisense transcripts called COOLAIR (cold-induced long antisense intragenic RNA) (Swiezewski et al. 2009; Sun et al. 2013). In soybean, we completed two-hybrid assays to preliminarily study the relationship between GmVRN1-like and GmFLC, but no interactions were observed (data not shown).

There has been limited research on soybean *VRN1* genes. In *A. thaliana*, VRN1 encodes a plant-specific protein (Levy et al. 2002). Overexpression of *VRN1* causes early flowering in *A. thaliana*. However, *vrn1* mutants exhibit decreased vernalization response rather than delayed flowering (Levy et al. 2002). Low homology between *GmVRN1-like* and *AtVRN1* suggests there are many differences in their functions (Lü et al. 2015). We have cloned *GmVRN1-like* and overexpressed it in *A. thaliana* and found it promotes flowering in transgenic plants (Lü et al. 2015). This further confirms the likely role of *GmVRN1-like* in the regulation of flowering.

Soybean is typically a photoperiod-sensitive plant. Comparative genomic analyses revealed that vernalization pathway genes are present in the soybean and *A. thaliana* genome (Schmutz et al. 2010; Jung et al. 2012). This indicates their potential role in soybean flowering. Our findings demonstrate that the overexpression of *AtDREB1A* activates *GmVRN1-like* expression in transgenic soybean plants. In *A. thaliana*, brief exposures to cold conditions before vernalization lead to CBF-activated *FLC* expression, which delays flowering and increases freezing tolerance (Seo et al. 2009). In wheat, *VRN-1* negatively regulates the cold acclimation pathway (Chew and Halliday 2011). In our study, overexpression of *AtDREB1A* might mimic prolonged exposure to cold stress, which triggers the protective vernalization pathway to ensure flowering is arrested until more favorable conditions return. The vernalization pathway may be an alternative flowering pathway in soybean that is activated in specific situations, including exposure to stress.

Conclusions

Constitute overexpression of *AtDREB1A* result in soybean plants delayed flowering. The up-regulation of *GmVRN1-like* expression may be responsible for this phenomenon. Our results suggest that although soybean is not a vernalization crop, the vernalization pathway may serve as an alternative flowering pathway that is activated in specific conditions.

Author contribution statement Conceived and designed the experiments: HCS, JL, HN. Performed the experiments: HCS, JL, QBM, CYY, XXZ, XM, SZH. Analyzed the data: HCS, JL, XM. Contributed reagents/materials/analysis tools: QBM, CYY, XXZ. Wrote the paper: HCS, JL, QBM, HN, SZH.

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