FUNCTIONALITY OF SOYBEAN CBF/DREB1 TRANSCRIPTION FACTORS

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ABSTRACT

Soybean (*Glycine max*) is considered to be cold intolerant and is not able to significantly acclimate to cold/freezing stress. In most cold tolerant plants, the C-repeat/DRE Binding Factors (CBF/DREBs) are critical contributors to successful cold-responses; rapidly increasing following cold treatment and regulating the induction of many cold responsive genes. In soybean vegetative tissue, we found strong, transient accumulation of CBF transcripts in response to cold stress; however, the soybean transcripts of typical cold responsive genes (homologues to Arabidopsis genes such as dehydrins, *ADH1, RAP2.1*, and *LEA14*) were not significantly altered. Soybean CBFs were found to be functional, as when expressed constitutively in Arabidopsis they increased the levels of *AtCOR47* and *AtRD29a* transcripts and increased freezing tolerance as measured by a decrease in leaf freezing damage and ion leakage. Furthermore the constitutive expression of *GmDREB1A;2* and *GmDREB1B;1* in Arabidopsis led to stronger up-regulation of downstream genes and more freezing tolerance than *GmDREB1A;1*, the gene whose transcript is the major contributor to total CBF/DREB1 transcripts in soybean. The inability for the soybean CBFs to significantly up regulate the soybean genes that contribute to cold tolerance is consistent with poor acclimation capability and the cold intolerance of soybean.

KEYWORDs: soybean; *Glycine max*; cold tolerance; CBF; DREB1

ABBREVIATIONS:

1. INTRODUCTION

Environmental stresses such as cold, drought, and salinity are serious problems for plants. Soybean is chilling and cold/freezing intolerant [1-4] with severe damage occurring at temperatures proximal to freezing. In the field, little growth occurs at temperatures below 6-7 °C [5], and cool temperatures at the end of the growing season are a major limiting factor in soybean yield and production [6]. Unlike soybean, *Arabidopsis thaliana* is able to cold acclimate and thus survive under severe cold-stress conditions; e.g., -8 to 10 °C [7, 8]. In Arabidopsis, the C-repeat response element binding factors (CBFs) play a key, though not exclusive role [9, 10], as positive regulators in an ABA-independent cold responsive pathway functioning to up-regulate many cold-regulated genes and thereby are critical contributors to cold/freezing tolerance [11]. CBF genes are members of the DREB (dehydration responsive element binding protein) family transcription factors that contain AP2 DNA-binding domains. *AtCBF1*, *AtCBF2,* and *AtCBF3* (*DREB1B*, DREB*1C,* and DREB*1A;* respectively) are up regulated significantly and rapidly by cold stress specifically, but much less so by ABA or dehydration [12, 13]. In contrast, *AtCBF4 (DREB1D)* and *AtDREB2* genes are more specifically regulated by ABA, salinity, and dehydration but not by cold stress [12, 14, 15].

Both the DREB1 and DREB2 family of proteins have binding activity with elements containing the core nucleotide sequence CCGAC [12, 13, 16]. Genes up-regulated by constitutive expression of *AtCBF3 (DREB1A)* contain ACCGAC significantly more

frequently than GCCGAC and TGTCGG in their promoters; while ACCGAC, CCGACT and GTCGGT are present in similar frequency in promoters of genes up-regulated by AtDREB2A [17, 18]. Overexpression of AtDREB1A or AtDREB2A in Arabidopsis transgenic plants induces the expression of cold responsive genes such as *RD29a* and *COR47* without exposure to low temperature [18-20]. The accumulation of downstream genes mediated by CBF/*DREB1* genes, increases abiotic stress tolerances to freezing, dehydration, and high salt [14, 20-24]. In cold-intolerant tomato, the transgenic expression of AtCBF1 gene increased cold regulated genes [25] [26, 27] and the expression of AtCBF1 in tomato increases freezing tolerance [26-28]. However, overexpressing tomato CBF (LeCBF1) in tomato did not result in increased freezing tolerance. The genomic organization of the CBF genes is quite different in soybean. In Arabidopsis and tomato the CBF genes are located in a cluster (neighboring genes) on a single chromosome, while in soybean the four CBF homologs are on four different chromosomes. Soybean genes, SCOF-1 and GmDREB2 (Arabidopsis DREB2-like) are transcription factors that respond to cold stress and the transgenic expression enhances freezing tolerance of Arabidopsis plants [29, 30]. Transgenic sweet potato plants expressing SCOF-1 also showed increased low temperature stress tolerance [31]. Despite the presence of SCOF-1 and CBF-like DREB1 transcription factors, soybean is cold-intolerant [7, 8, 32]; genes homologous to key Arabidopsis cold responsive genes, such as vegetative dehydrins and ADH, are not accumulated in response to cold stress [33, 34].

During the latter course of the described work, a comprehensive examination of the regulation of soybean CBF-like transcription factors by heat, cold, and drought stress, the functionality of several GmDREB1s in Arabidopsis and soybean, and the regulation by GmDREB1B;1 suggested the participation of DREB1 transcription factors in a variety of abiotic stress conditions [35]. These results and others are compared to those described in the present report (Table S1). The present report extends these findings by reporting on genes for GmCBF-like proteins (including the most highly constitutive and cold-induced expressed, CBF-like DREB1, GmDREB1A;1) that were not examined previously as well as providing a comprehensive RNASEQ analysis of cold regulated DREBs and the examination of the kinetics of CBF responses to cold. We further examined the responsiveness of soybean to cold by examining the kinetics of responsiveness of soybean CBF-like, DREB1 transcription factors, the functionality of these factors, and the changes in transcript levels of potential cold responsive genes in soybean. It was observed that several soybean CBF-like (GmDREB1) transcription factors can function to up-regulate characterized CBF responsive Arabidopsis genes and further can confer cold-tolerance when expressed transgenically in Arabidopsis. This paper supports a hypothesis that soybean lacks an appropriate transcriptional response to cold despite the fact that the cold sensing and initial portions of the CBF/DREB1 dependent cold signaling pathway are functional.

2. MATERIALS AND METHODS

2.1 Growth conditions

Seeds for *Glycine max*, 'Young' (PI 508266) were generously provided by Tommy Carter (USDA-ARS, NC State University, Raleigh, NC). 'Young' was used for all experiments except RNASEQ analysis which used 'Williams 82' (PI518671). Seeds were soaked overnight in water at room temperature and then sown in pots (16.5 cm diameter x 11 cm tall) containing a potting soil composed of a mixture of peat, perlite, and vermiculite (PRO-MIX BX, Premier Tech Horticulture) at a depth of approximately 2 cm. Seedlings were grown and watered regularly in a plant growth chamber with 18 h light/6 h dark at 22 °C. Lighting was 175 to 225 μmole m^{-2} sec^{-1}. Plants were not inoculated with Rhizobium nor were they fertilized for these short-term experiments. Each replicate was created by combining plant organs from at least 4 plants (of similar developmental stage) from a minimum of 2 different pots. Cold treatment was for 2 days at 4 °C. Cold treatment (4 °C) was started at 4 h after the lights turned on (Zeitgeber Time, ZT4 h) on day 10. Seedlings or unifoliate leaves and hypocotyls were harvested by freezing in liquid nitrogen followed by storage at -80 °C for later transcript analysis. Arabidopsis plants were grown under the same conditions as soybean and were typically used at four weeks old unless otherwise noted. For chemical rescue of growth phenotype, Arabidopsis transgenic plants grown on soil were treated by spraying 10^{-4} M of Gibberellin A3 (ACROS organics #119860050) once a week starting with 10 day old seedlings until phenotype observation.

2.2 Creation of transgenic Arabidopsis plants expressing *GmDREB1* genes

The coding regions for *GmDREBs* were PCR amplified from *Glycine max*, 'Young' (PI 508266) cDNA with PfuUltra II Fusion HS DNA Polymerase (Agilent Technology Cat. No. 600852) and primers were designed to add appropriate restriction enzyme sites for cloning (Table S2). The PCR products were cloned into pCR®-blunt (Invitrogen. Cat. No. K270020) and the sequence was confirmed. *GmDREB1A;1*, *GmDREB1B;1*, and *GmDREB1B;2* sequences were identical to the sequence of the c.v. 'Williams 82' soybean sequence in the Phytozome site (Phytozome v9.1: http://www.phytozome.net/). *GmDREB1A;2* had a single nucleotide difference (T instead of C) at 548 position resulting in the predicted amino acid methionine in 'Young' rather than threonine found in 'Williams 82'. The coding region of *GmDREB1 was* cloned into pCambia1302 (replacing GFP) following the *CaMV35S* promoter. The Nos poly-A site of pCambia1302 was used as 3'UTR. The construct was transformed into *Agrobacterium* (GV3101) and then introduced into Arabidopsis (Col-2) WT plants by the floral dip method [36]. Hygromycin B resistant (15 μg/mL) seedlings were selected by a long hypocotyl phenotype [37], and the hygromycin resistant phenotype was calculated on the T2 generation and T3 generation to confirm the presence of a single insertion and homozygous plants. Four-week-old transformed Arabidopsis seedlings were harvested by freezing in liquid nitrogen followed by storage at -80 °C for transcript analysis.

2.3 Transcript analysis

For RT-qPCR, RNA from 100 mg of pulverized tissue (Arabidopsis seedlings/soybean leaves or stems) in liquid nitrogen was obtained with the RNeasy Plant Mini kit (Qiagen Cat. No. 74903). RNA treated with DNase (Qiagen Cat. No. 79254) during isolation, was then eluted from the column with water, and quantified by absorbance at 260nm. Complementary DNA was synthesized with 500 ng RNA, oligo dT primers and SuperScript® III First-Strand Synthesis System (Invitrogen. Cat. No. 18080051) in a 20 μL reaction volume. Complementary DNA was diluted 4-fold (1 μL represents 6.25 ng RNA). One μL of 4-fold diluted cDNA was analyzed with 400 or 500 nM of each primer (Table S2) and 10 μL Power SYBR® Green Master mix (Applied Biosystems®) for a final 20 μL total volume. Standard curves were created using the highest input cDNA ensuring all quantified values of samples were within the linear range. These data (figures 2, 3 5) were first normalized to actin levels and then plotted relative to 0 time controls. For absolute quantitation (absolute amount of cDNA), a PCR product was obtained by amplification from each respective gene, which was then purified and quantitated by UV absorbance and then used as template in PCR reactions to create linear standard curves (typically 10 attomoles to 40 femtomoles). This analysis allows direct comparison of the absolute levels of the distinct transcripts. All samples for quantification were in the linear range of the standard curve. PCR efficiencies were calculated and all data was within 85% to 105% efficiency (R^2>0.99). Dissociation curves were analyzed for all qPCR products and a single PCR product was confirmed.

2.4 RNASEQ analysis

Unifoliate leaves from cold-treated soybean (c.v. 'Williams 82') seedlings (2 weeks old) were harvested at 0 h, 1 h and 24 h. All treatments were performed in triplicate (with $n \geq 6$ plants per replication). RNA was isolated as described above and three libraries for each condition were created. RNASEQ analysis was performed by CGB genomics service facility, Indiana University (Bloomington, IN). Reads were mapped to the 68,552 transcript assembly using Bowtie 2.0 and then filtered to retain only the best alignments for each read. Reads were counted as the number of reads mapping to any transcript of that gene. Read counts were normalized across samples and adjusted P-value were obtained using the DESeq package (version 1.12) in R/Bioconductor. Normalized transcript count for each gene in the cold treatments were compared to control to analyze differential expression pattern.

2.5 Analysis for whole plant freezing tolerance

Potted Arabidopsis plants, four to five weeks old, were transferred to -4.5 °C for 24 h in the dark followed by 4 °C for 24 h in the dark and then returned to normal growth conditions for three days for recovery. The damage scale was scored after three days of the recovery, based on area and extent of damage on leaves (Figure S4).

2.6 Electrolyte leakage

Aerial portions of whole Arabidopsis plants, four to five weeks old, were harvested and transferred into 16 x 100 mm glass test tubes. Plants were kept at -1.0 °C for one h, then an ice crystal was added. Plants were treated sequentially for 2 h at each target

temperature, then transferred into 4 °C in the dark for 12 h. Treated plants were incubated with 3 mL deionized water for at least 6 h with shaking. Conductivity was measured with a portable conductivity and TDS meter (Milwaukee Model MW301 EC meter). One hundred percent electrolyte leakage was determined following freezing of plants at -80 °C overnight.

3. RESULTS

3.1 Identification of soybean CBF homologs

Soybean *DREB1* genes were found by searching for the presence of an AP2 DNA binding domain in the soybean genomic sequences (Phytozome v9.1: http://www.phytozome.net/). At least 44 *DREB* candidates (Figure 1) were found including those previously described such as *GmDREB2* [30] and *GmDREB2A;2* [24]. The most similar genes to Arabidopsis CBFs (*AtDREB1*s) were *GmDREB1A;1, GmDREB1A;2, GmDREB1B;1, GmDREB1B;2, GmDREB1C;1, GmDREB1D;1* and *GmDREB1D;2*. In this group of *CBF/DREB1*-like genes, *GmDREB1A;2, GmDREB1B;1, GmDREB1B;2* and *GmDREB1D;1* were reported to be up-regulated at the transcript level in response to cold stress; the *GmDREB1B;1* transcript additionally accumulated in response to dehydration [17]. While most of these genes exhibited strong responses to cold stress, they were also variably responsive to heat, salt and dehydration [35]. The *GmDREB1A;1* homolog was reported to accumulate in response to dehydration stress in wild soybean [38]. All of these genes have very similar Nuclear Localization Signals (NLS) and AP2 domains followed by an acidic region at C-terminus. Unlike *CBF1, 2* and *3* of Arabidopsis and tomato [13, 28], the

soybean *CBF* genes are not found as tandem arrays on the same chromosome (Chromosome 4 in Arabidopsis, Chromosome 3 in tomato), but rather were scattered among seven different soybean chromosomes. Soybean is a tetraploid plant, with about 75% of the genes having multiple copies [39]. Sequences of the gene pairs *Glyma09g27180: Glyma16g32330, Glyma20g29410: Glyma10g38440 and Glyma17g14111: Glyma05g03560* are very similar to each other, suggesting duplication of these genes; while *Glyma01g42500* does not have an apparent paralog.

3.2 GmDREB1A;1 (Glyma09g27180) and *GmDREB1A;2 (Glyma16g32330)* are strongly up-regulated in response to cold temperature.

Of the most closely related CBF-like genes, *GmDREB1A;1, GmDREB1A;2, GmDREB1B;1* and *GmDREB1B;2*, only *GmDREB1A;1* was not previously reported as a cold-inducible gene at 24 h by microarray analysis [17]. However in Arabidopsis, *AtCBF* genes are strongly and transiently up-regulated; peaking at 2-4 h following cold treatments and returning to much lower levels by 24 h [40]. To determine whether cold-induced increases in the *GmCBFDREB1* genes were transient, we examined transcript levels of *GmDREB1A;1* and *GmDREB1A;2* and a representative of another known cold-inducible transcription factor *SCOF-1* (Glyma17g35430) [29] during early cold stress. The *GmDREB1A;1* transcript was massively increased in leaves and stems following 3 h of cold treatment (Figure 2). The transcript of the paralog gene, *GmDREB1A;2*, was also accumulated, but somewhat less so (Figure 2). Interestingly, while *GmDREB1A;2* was relatively more cold responsive in stem than in leaves, *GmDREB1A;1* transcript was more strongly accumulated in leaves.

Following strong accumulation in initial response to cold, both *GmDREB1A;1* and *GmDREB1A;2* were significantly decreased by 48 h. These rapid, large and transient accumulations in response to cold stress are very similar to the transient expression pattern of Arabidopsis CBF genes [13]. While the level of the *SCOF-1* transcript was also strongly increased in response to cold stress, the transient change (Figure 2) was much less than that observed in the DREB1A transcripts.

3.3 GmCBF/DREB1 and *GmDREB2* transcript changes in response to cold: RNASEQ analysis

To determine the global transcriptional responses of soybean to cold, RNASEQ analysis was performed. We focus here on the analysis of the transcript level of primary transcription factors such as *DREB* and *SCOF-1* related genes, and transcript levels of several soybean genes homologous to Arabidopsis cold responsive genes (Figure 3, 4, and S1). This experiment was designed to distinguish immediate responses (1 h) and later responses (24 h) to cold. In unifoliate leaves from young soybean seedlings, transcripts of the *GmCBF-like* genes, [*GmDREB1A;1 (Glyma09g27180), GmDREB1A;2 (Glyma16g32330), GmDREB1B;1 (Glyma20g29410),* and *GmDREB1B;2 (Glyma10g38440);* as well as *GmDREB1C;1 (Glyma01g42500), GmDREB1D;2 (Glyma17g14111),* and *GmDREB1D;1 (Glyma05g03560)]* were significantly accumulated by 1 h after cold treatment (adjusted P-value <0.001) (Figure 3). By 24 h of cold treatment, transcripts of four *GmCBF* genes (*GmDREB1A;1, GmDREB1A;2, GmDREB1C;1* and *GmDREB1D;1*) had significantly decreased compared to 1 h but still sustained levels substantially

above the 0 h time control level (adjusted P-value <0.001). None of the soybean genes similar to *AtDREB2* or *3* were up-regulated at 1 h cold treatment, indicating that the *GmCBF/DREB1* gene group are the immediate cold responsive genes acting as primary cold transcription factors (Figure 3). By 24 h of cold treatment, transcripts of four *DREB2*-like genes (*Glyma18g43750, Glyma14g06080, Glyma02g42960*, and *Glyma10g07756*) were significantly accumulated, consistent with the previous observations of *GmDREB2A;1* (*Glyma02g42960*) and *GmDREB2A;2 (Glyma14g06080)* cold responses [24]. The cold transcription factors, *SCOF-1* (*Glyma17g35430*) and *SCOF-1* like genes (*Glyma06g04840, Glyma04g04760, Glyma20g26940, Glyma14g09760* and *Glyma10g40400*) were also significantly up-regulated within 1 h of cold treatment and most sustained a significantly high level of transcript at 24 h (Figure S1).

3.4 Transcriptional changes of dehydrins and other potential cold responsive soybean genes.

Dehydrins and alcohol dehydrogenase (ADH) are rapidly responding cold-inducible genes in Arabidopsis (e.g., *AtCOR47* and *AtADH1*). Previously, of the 10 dehydrin genes identified in the soybean genome; 2 KS-type dehydrins (*Glyma17g24193* and *Glyma16g04190*) and *GmERD14 (Glyma04g01130)* were highly expressed in soybean leaves; while *Mat9* (*Glyma09g31740*), *Glyma08g05361, Glyma04g01181*, *Glyma12g36430* and a KS-type dehydrin (*Glyma17g24193*) were highly expressed in mature seeds [33]. Only the KS genes appeared cold-responsive, however; it was concluded that these genes responded primarily in a CBF/DREB1-independent

(ABA-dependent) mode (Figure S2) as they lacked a putative CRT/DRE promoter element and they were responsive to exogenously applied ABA [33]. In the RNASEQ analysis (Figure 4), the two KS-type dehydrins (*Glyma17g24193* and *Glyma16g04190*), as well as *Mat9 (Glyma09g31740)* and *Mat1* (*Glyma07g10030*) were up-regulated by 24 h in the cold (adjusted P-value <0.001); and the most abundant acidic dehydrin transcript in vegetative tissues under non-stressed conditions, *GmERD14* (*Glyma04g01130*), was not up-regulated (Figure 4), consistent with the previous report by Yamasaki et al [33]. Soybean homologs of Arabidopsis cold responsive genes, *GmADH10 (Glyma14g27940.1)*, *AtRAP2* homolog (*Glyma14g09320.1*) and *AtLEA14* homolog (*Glyma09g38990.1*) were examined (Figure S2). Despite the presence of putative abiotic stress responsive motifs in the promoters of *GmRAP2* and *GmLEA14*, neither of these was significantly up-regulated.

3.5 Regulation of Arabidopsis cold-responsive genes by *GmCBF/DREB1s*

Cold-treated, wild-type Arabidopsis plants show a typical high level of accumulation of *AtCBF3* transcripts by 1-4 h of cold-stress, which then decreases to substantially lower levels by 24 h (Figure S3). In response to CBF accumulation, Arabidopsis *COR47, RD29a*, and *ADH1* transcripts accumulated to their greatest level by 4 h of cold stress (Figure 5), consistent with previous observations in Arabidopsis [21, 41]. Additionally, it has been shown, in the absence of any cold treatment, that the constitutive expression of *AtCBF1-3* transcription factors increased the transcript levels of *AtRD29a*, *AtCOR47*, and *AtADH-1* [21, 41].

The responsiveness of these known Arabidopsis CBF regulated genes was utilized to evaluate the functionality of the soybean CBFs. Constructs containing *GmDREB1A;1*, *GmDREB1A;2*, *GmDREB1B;1,* or *GmDREB1B;2* were introduced and constitutively expressed in Arabidopsis under the control of the cauliflower mosaic virus *35S* promoter (Figure 5). Non-transformed wild-type plants and homozygous transgenic plants expressing *GmDREB1A;1* (3 lines), *GmDREB1A;2* (2 lines), *GmDREB1B;1* (2 lines), *GmDREB1B;2* (2 lines) and GFP (1 line) were examined for the induction of several predicted responsive genes. Transgenic lines expressing high amounts of *GmDREB1A;1* (09B-1-6, 09C-6-2), *GmDREB1A;2* 16A-5-4) transcripts (Figure 6), showed strong accumulation of *AtCOR47* and *AtRD29a* (Figure 5). It was interesting that only the overexpression line of *GmDREB1B;1* led to significant accumulation of AtADH1 transcript. Overall these data indicate that *GmDREB1A;1*, *1b*, and *2a* can function to up-regulate the Arabidopsis cold responsive genes, *AtCOR47* and *AtRD29a*.

3.6 GmCBF/DREB1 expression increases freezing stress tolerance.

Non-acclimated wild-type and transgenic Arabidopsis plants treated at -4.5 °C for 24 h were examined for damage. The extent of damage was estimated based on visible chlorophyll bleaching and leaf collapse (Figure S4). The wild-type plants and the transgenic plants expressing *GFP* were severely damaged, with component scale averages of 3.17 and 3.06, respectively (Table 1). The plants with high transcript levels of *GmDREB1A;1*, *GmDREB1A;2*, or *GmDREB1B;1* were not damaged (component scale values of 0 to 0.1) indicating increased freezing tolerance of the

plants (Table 1 and Figure 7). Two low level expresser lines of *GmDREB1A;1*(09B-2-2) and *GmDREB1A;2* (16A-5-4) were heavily injured similar to wild-type line (Table 1). However, *GmDREB1B;1* (20C-4-3), *GmDREB1B;2* (10A-5-1 and 10A-4-1) had slightly less injury. To obtain a more quantitative evaluation of freezing tolerance, the high expression lines (09B-1-6, 09C-6-2, 16A-1-5 and 20B-1-5) were tested using the electrolyte leakage assay (Figure 8). All transgenic lines expressing high levels of soybean CBF/DREB1s showed significantly less leakage compared to wild-type and the GFP expression line. The lines of *GmDREB1A;2* (16A-1-5) and *GmDREB1B;1* (20B-1-5) were the most freezing tolerant based on the electrolyte leakage assay. These data indicate that the soybean *GmDREB1A;1*, *GmDREB1A;2* and *GmDREB1B;1* are able to confer freezing tolerance in Arabidopsis by transcriptionally activating CBF targets, as long as a sufficient level of *GmCBF/DREB1* transcript is present.

3.7 Transgenic expression of the *GmCBF/DREB1* genes in Arabidopsis impacts the size of plants

Arabidopsis plants over-expressing *GmCBF/DREB1s* (Figure 6) are dwarfed, a phenotype attributed to the suppression of the genes controlling plant growth and bolting [12, 22, 23]. To obtain a quantitative estimate of CBF/DREB1 transcript levels and to be able to quantitatively compare the levels of the distinct transcripts in different transgenic plants; the copy number of transcripts (contained in 6.25 ng total RNA) for the *GmDREB1* genes in the transgenic plants and non-transformed plants (4 weeks old) were absolute-quantified by Real-Time qPCR analysis (Figure 6 and

Figure 6S). The two lines of *GmDREB1A;1* (09B-1-6 and 09C-6-2), one line of *GmDREB1A;2* (16A-1-5), and one line of *GmDREB1B;1* (20B-1-5) all of which had the highest levels of the transcript (> 10^7 copies per 6.25 ng RNA) were dwarfed; while the lines expressing significantly less than 10^7 copies, one line of *GmDREB1A;1* (09B-2-2), one line of *GmDREB1A;2* (16A-5-4), one line of *GmDREB1B;1* (20C-4-3) and two lines of *GmDREB1B;2* (10A-5-1 and 10A-4-1) appeared normal in size (Figure 6). Since the dwarfed phenotype of constitutively expressing *AtCBF1* in tomato plants were complemented by application of GA3 [26]; we tested for the chemical rescue of the dwarf phenotype caused by *GmCBFDREB1*s. After six weeks of GA treatment, the inflorescence flowering structure of the dwarf plants, two lines of *GmDREB1A;1* (09B-1-6 and 09C-6-2), one line of GmDREB1A;2 (16A-1-5), and one line of *GmDREB1B;1* (20B-1-5) were partially complemented (size and branching, Figure S5). This suggested that the level of *GmCBF/DREB1* expression in Arabidopsis affected GA biosynthesis or GA stability, leading to dwarfism [42].

4. Discussion

4.1 Comparison of cold-inducible *CBF/DREB1* genes in soybean with those in other plants

Based upon protein sequence similarity of soybean *DREB1* genes to Arabidopsis *CBF* genes; GmDREB1A;1, GmDREB1A;2, GmDREB1B;1, GmDREB1A;2, GmDREB1C;1, GmDREB1D;1, and GmDREB1D;2 (*Glyma09g27180, Glyma16g32330, Glyma20g29410, Glyma10g38440, Glyma01g42500, Glyma05g03560 and Glyma17g14111* respectively) were hypothesized to be the most likely cold inducible soybean CBF-like genes (Figure 1). It is shown here that this hypothesis was clearly supported as these CBF-like transcripts accumulated rapidly, strongly, but transiently in response to cold in soybean. Further, several of these soybean CBF-like genes, when transgenically expressed, were capable to up-regulate typical downstream genes leading to the acquisition of cold tolerance in Arabidopsis. In contrast, none of the remaining DREB1 genes nor any of the twenty DREB2 genes responded after 1 h of cold and only two increased significantly by 24 h, with six decreasing in response to cold. Consistent with these observations, some members of the DREB2 family, both in Arabidopsis [12] and in soybean [24], are more strongly up-regulated in response to dehydration and salt stress than cold stress.

The Arabidopsis *CBF 1-3* genes are closely-linked, tandemly arranged genes located on chromosome 4 [13] and likewise the *CBF1-3* genes of tomato (*S. lycopersicum*, *S. pimpinellifolium* and *S. habrochaites*) and potato (*S. tuberosum* and *S. commersonii*) are also tandem-linked genes. In potato (*S. tuberosum* and *S.*

commersonii), an additional set of tandem-linked *CBF* genes (*CBF4-5*) were found as orthologs to *S. tuberosum* and *S. commersonii CBF1-3* [43]. Unlike the Arabidopsis, tomato, and potato CBF genes, the soybean *GmCBF* genes are distributed on distinct chromosomes. The protein sequences of all *GmCBF-like/DREB1* genes are most similar to that of *AtCBF4* (*AtDREB1d*) sequence (Figure 1). *AtCBF4* is thought to be derived from same ancestor as *AtCBF1-3* based on coding sequence and promoter sequence similarity between *AtCBF4* and *AtCBF1-3* [14]. There is a greater degree of synteny in the neighboring genes to *GmDREB1A* and *1B* gene*s* to the region surrounding *AtCBF4* than to the region surrounding the tandem-linked *AtCBF1-3* (data not shown); suggesting that the *GmCBF/DREB1* genes may be derived more recently from the same ancestor as *AtCBF4*. Since soybean is thought to have duplicated its genome twice, at about 59 and 13 million years ago [39], we suggest that the genes pairs of GmDREB1A;1-GmDREB1A;2, GmDREB1;1-GmDREB1B;2, and GmDREB1D;1-GmDREB1D;2 may have originated through a series of duplications from one ancient *CBF4*-like gene.

4.2 GmCBF-like DREB1s as primary cold responsive transcription factors

GmDREB1A;1 (*Glyma09g27180*) transcripts were accumulated from 400 to greater than 1,000-fold in leaves and 250 to 400-fold in stems by 1 h of cold treatment (Figure 2 and 3). *GmDREB1A;2* (*Glyma16g32330*) transcript was accumulated 10 to 40-fold and 30 to 140-fold in leaves and stems, respectively by 4 h of cold treatment (Figure 2). These responses are comparable to those found in Arabidopsis, where *AtCBF3* transcript was up-regulated 300-fold within 1 h of cold treatment (Figure S3).

In Arabidopsis, *CBF1-3* are the primary cold inducible transcription factors [12, 13], with *AtCBF3* and *AtCBF1-2* being first transcriptionally activated by *ICE-1* and *CAMTA*, respectively [44-46]. There are five potential elements (myc-recognition sites, CANNTG) present in the promoter (~1kb from the ATG) of *AtCBF3* [44, 47] to which ICE1 could bind. The promoter of *GmDREB1A;1* (*Glyma09g27180*) gene similarly has 2 and 9 myc-recognition sites within 1kbp and 1.5kbp, respectively while *GmDREB1A;2* (*Glyma16g32330*) gene has 3 and 4 Myc-recognition sites in 1kbp and 1.5kbp, respectively. These potential ICE responsive elements are consistent with the hypothesis that soybean and Arabidopsis share initial steps in their cold-signaling mechanisms.

All of the seven genes encoding *GmCBF/DREB1* proteins are up-regulated after 1 h cold (Figure 3), while none of the *GmDREB2* or *3* genes were significantly up-regulated at 1 h. These suggest that the entire up-stream portion of the soybean CBF/DREB1 cold signaling pathway, including *ICE1* and *CAMTA*, is functional to induce the expression of *GmCBF/DREB1* genes leading to transcript accumulation. However, this activation is not sufficient to induce cold tolerance in soybean plants. In Arabidopsis, the accumulation of *CBF* causes up-regulation of a downstream regulon [17, 21, 23, 48, 49]. In Arabidopsis the CRT/DRE is the most common element in cold inducible promoters; however, in soybean the most common element in the promoters of cold-induced genes are the ABRE and T/G box [17]. This further suggests that the soybean cold responsive pathway utilizing CBF is not functioning in the same way as in Arabidopsis. Nevertheless, in at least one case it has been

demonstrated that GmDREB1B;1 can directly interact with the promoter of a stress responsive soybean promoter [35]. Of further interest, with regard to cold signaling, is the strong cold-induced increase of SCOF1-like transcripts. Transgenic expression of GmSCOF1 enhances cold tolerance (Kim et al., 2011) and likely indirectly activates genes by increasing SGBF-1 binding to ABRE sites in Arabidopsis (Kim et al 2001). However, the soybean SCOF1-like sequences are most similar to the Arabidopsis AZF and STZ, both of which act as negative regulators of gene expression in Arabidopsis. It remains to be shown whether SCOF1-like proteins are negative or positive regulators of gene activity in soybean following cold treatments.

4.3 Functionality of *GmCBF/DREB1* genes depends on sufficient level of expression

AtCBF1-3 accumulation in response to cold stress, induces the expression of the CBF regulon by binding CRT/DRE elements (A/GCCGAC) in the cold-responsive promoters [50]. Typically, overexpression of *AtCBF1-3* increases the expression of *AtRD29a* and *AtCOR47*, utilizing 4 and 3 CRT/DRE elements present within 1kb upstream of the ATG, respectively [21, 41]. Arabidopsis plants overexpressing *AtCBF1-3* have a dwarf phenotype due to regulation of GA biosynthesis pathway by CBF genes [22, 23, 51]. Exogenous GA3 application returns the normal growth habit to transgenic tomato plants expressing AtCBF1 [26]. Here a range of constitutive levels of *GmCBF/DREB1* transcripts in transgenic Arabidopsis was obtained (Figure 6 and Figure S5). A *GmDREB1* expression difference of approximately ten-fold (Figure S6) seems sufficient to induce dwarfism (Figure 6 and Figure7), to determine

the on or off status of cold-regulated genes (Figure 5 and Figure S5), and to determine freezing or non-freezing tolerance (Figure 7 and Figure 8).

4.4 GmDREB1B;1 has a regulatory pattern distinctive from *GmDREB1A;1* and *GmDREB1A;2.*

The downstream activated gene targets of GmDREB1B;1 have been carefully characterized [35]. When compared on a limited scale here, with other CBF-like DREB1's it was observed that the line with the highest level of *GmDREB1B;1* (20B-1-5) transcript, showed an increase of *AtADH1* transcript greater than 3-fold; while the *GmDREB1A;1* (09B-1-6 and 09C-6-2) and *GmDREB1A;2* (16A-1-5) had less than a 2-fold increase (Figure 5 and S5). *AtADH1* was reported to be a cold, dehydration, hypoxia, and ABA-inducible gene [52, 53]. Regulation of the *ADH1* gene in Arabidopsis by *AtCBF2* is suggested by experiments, which alter accumulation of *CBF2* [54-56] and overexpression of *CBF2* [57]. Maruyama et al [17] suggested that the specific promoter elements regulated by DREB2 are ACCGAC, GTCGGT and CCGACT, while DREB1A/CBF3 regulated primarily ACCGAC. In the Arabidopsis ADH promoter, a GTCGGT sequence is found rather than the ACCGAC sequence. Perhaps in transgenic Arabidopsis plants, *GmDREB1B;1* may better recognize the GTCGGT element (in AtADH) and thereby induce the expression of *AtADH1*.

4.5 GmDREB1A;2 and *GmDREB1B;1* expressing Arabidopsis plants have more freezing tolerance than those expressing *GmDREB1A;1*.

Based upon electrolyte leakage (Figure 8) the constitutive *GmDREB1A;2* and *GmDREB1B;1* expression lines (16A-1-5 and 20B-1-5, respectively) were more freezing tolerant than *GmDREB1A;1* lines (09B-1-6 and 09C-6-2), even though all of the lines showed similar levels of accumulation of *AtCOR47* and *AtRD29a* (Figure 5 and Figure S6). This may mean that distinctive promoter specification, perhaps impacting distinct genes regulated by *GmDREB1A;2* or *GmDREB1B;1* but not by *GmDREB1A;1*, results in more freezing tolerance.

4.6 Soybean dehydrins are not directly regulated by the CBF pathway during cold stress

The RNASEQ analysis indicated the KS-type dehydrins, *Mat9* (*Glyma09g31740*), and *Mat1* (*Glyma07g10030*) transcripts were accumulated in response to cold stress, but the rest of the dehydrins were not (Figure 4). It is likely the dehydrin genes are not directly regulated by the CBF-like DREB1s. The KS-type dehydrin, *Glyma17g24193* contains ABRE sites (and no CRT/DRE), and is up-regulated by exogenous ABA treatment [33]. *Mat9* (*Glyma09g31740*) and *Mat1* (*Glyma07g10030*) are dehydrins, which are primarily expressed in seeds and contain many potential ABRE elements [58, 59]. Maruyama et al [17] showed a high frequency of ABREs in the promoters of cold inducible genes in soybean. This indirect regulation of dehydrin genes by cold is consistent with the demonstration of GmDREB1B:1 activation of ABRE-mediated gene expression [35]. It is likely that soybean has a robust ABA-dependent, cold response.

5.0 SUMMARY

Overall these findings confirm the initial steps in the CBF/DREB1 pathway in soybean are responsive to cold stress culminating in the accumulation of transcripts of *GmCBF/DREB1* genes. Since the cold signal transduction pathway in soybean is appropriately functional, from the perception of cold to the elevation of *GmCBF/DREB1* transcripts; the downstream components may be deficient in their cold response. Possible contributions to the lack of an appropriate cold response may include insufficient levels of *GmCBF/DREB1* transcripts and/or protein, that are insufficiently accumulated to up-regulate downstream genes, absence of co-activators or presence of strong negatively acting transcriptional regulators, or the lack of appropriate CBF/DREB1 responsive elements in the promoters of critical cognate soybean genes. Most of this study was performed at a development stage (approximate 2 week old seedlings) and in tissues (unifoliates) that are most likely to be exposed to cold damage. However, the general mechanism (or deficiencies) controlling these regulatory circuits have yet to be shown to be active in other tissues or other developmental stages of soybean (e.g., mature tissues, such as trifoliate leaves and flowers, etc.).

FIGURE LEGENDS

Figure 1. Sequence similarity of Arabidopsis and soybean DREB-related proteins. GmDREB1 and 2 were collected from a BLASTP search from phytozome using Arabidopsis DREB protein sequences. The Arabidopsis and soybean DREB 1 and 2

amino acid sequence were aligned and clustered by clustalW. The phylogenetic tree with bootstrap value and distances between branches was constructed with MEGA6.0 [60] using a neighbor-joining method based on the Jones-Tylor-Thornton model with 1,000 bootstraps. Glyma indicates *Glycine max* (soybean) DREB genes, all others are Arabidopsis genes. The dotted line separates the branch containing the GmDREBs most similar to the AtCBF1-4 genes. The bar indicates the number of substitutions per site.

Figure 2. Transcript levels of *GmDREB1A;1* & *GmDREB1A;2* (*Glyma09g27180* & *Glyma16g32330*, respectively) and *SCOF-1* (*Glyma17g35430*) in response to cold stress (c.v. 'Young') normalized by *Actin11* expression. Open circles indicate transcript levels in soybean leaves, closed squares in soybean stems. Fold changes shown are relative to 0 time. Biological replications (1 and 2) represent RNA isolation performed on different experimental plants (at least 4 plants per replicate) at different times (0, 1, 4, and 48 h following cold treatment).

Figure 3. Changes in transcript levels of *GmDREB* genes in response to cold stress. RNASEQ analysis was performed in triplicate at all time points (log 2 transcript reads). Each replicate was composed of 6 plants. Standard deviation is shown. Black bar, grey and striped bar indicate 0, 1 and 24 h of cold stress, respectively. Adjusted p-value < 0.001 indicated by asterisks are significantly different reads compared to non-cold samples (0 h). *GmDREB1/CBF* is the subset of *DREB1*s that are most

similar to the *AtCBF*s. The sequence relationships of the "other" DREBs is shown in Figure 1.

Figure 4. Changes in transcript levels of soybean dehydrins in response to cold stress. RNASEQ analysis was performed in triplicate at all time points (log10 scale for transcript reads). Black bar, grey and striped bar indicate 0, 1, and 24 h of cold stress, respectively. Adjusted p-value < 0.001 indicated by asterisks are significantly different reads compared to non-cold samples (0 h).

Figure 5. Transcript levels of *AtCOR47, AtRD29a*, and *AtADH1* in Arabidopsis transgenic plants (4 weeks old). *CBF* regulated genes (*AtCOR47, AtRD29a* and *AtADH1*) in cold-treated wild type and in non-cold treated transgenic Arabidopsis expressing *GmCBF*-like *DREB*s. Fold-change of transcript accumulation, determined by RT-qPCR, are compared to transcript levels in the 0 h (no cold) wild-type plants. Expression of sufficient levels of *GmDREB1*s in the transgenic lines shows accumulation of *AtCOR47* and *AtRD29a* transcript without cold stress treatment, similar to cold accumulations due to *AtCBF* regulation in response to cold. Check mark (√) indicates those lines showing a dwarf phenotype.

Figure 6. Arabidopsis transgenic plants constitutively expressing *GmDREB1* genes. Three-week-old transgenic plants were photographed. Transcript levels of each *GmDREB1* in four week old seedlings was absolute quantified to show the number of transcripts in 6.25 ng total RNA by RT-qPCR analysis.

Figure 7. The transgenic plants expressing a sufficient level of *GmDREB1* have freezing tolerance. One-month-old transgenic plants were treated in -4.5 °C for 24 h in dark followed by 4 °C for 24 h in the dark and then returned to normal growth conditions for two days for recovery. The photographs were taken after recovery.

Figure 8. Transgenic Arabidopsis expressing *GmDREB1* genes show enhanced freezing tolerance as determined by electrolyte leakage.

Table 1. Transgenic expression of *GmDREB1* genes in Arabidopsis confers enhanced freezing tolerance. Freezing treatment was at -4.5°C for 24 h on 5 week-old plants followed by 4°C for 24 h in the dark and then returned to normal growth conditions for two days for recovery. The damage scale was scored after three days of the recovery, based on area and extent of damage on leaves (Figure S4). A higher number indicates greater damage.

Supplemental Figures and Tables

Figure S1. Changes in transcript levels of *SCOF-1* genes in response to cold stress. RNASEQ analysis was performed in triplicate at all time points (log2 scale); each replicate was composed of 6 plants. Standard deviation is shown. Black bar, white and striped bar indicate 0, 1 and 24 h of cold stress, respectively. Asterisk indicates significant difference (adjusted P-value < 0.001) as compared to 0 h cold.

Figure S2. (A) Transcript levels of soybean homologs to cold responsive genes for two dehydrins (KS-dehydrin and *GmERD14*), alcohol dehydrogenase (ADH), *RAP2.1*, and *LEA14*. Cold treatment was initiated with 10-day-old seedlings at ZT-4 h (4 h after dawn, 18 h light / 6h dark). Samples were harvested at 0, 1, 4, and 48 h after initiation of cold (4°C) treatment. Total RNA was isolated, treated with DNase, reverse transcribed with oligo-dT as primer, then analyzed by semi-quantitative RT-PCR. Samples previously used for dehydrin transcript analysis (KS-dehydrin: *Glyma17g24193.1* and GmERD14: *Glyma04g01130.1*; Figure 7, Yamasaki et al., 2013) were compared to *ADH-1, RAP2.1*, and *LEA14*. (B) The locations of predicted core elements, ABRE and CRT/DRE, in the promoter regions are shown.

Figure S3. Accumulation of *AtCBF3* transcript in Arabidopsis in response to cold stress. Cold treatment (4 °C) on Col-2 (wild-type) started at ZT-4h (18 h day/ 6 h

night). Fold-change ($\log_{10}$) of transcript accumulation was calculated by comparison to transcript levels in the 0 h (no cold) wild-type plants.

Supplement Figure S4**.** Damage scale (0 to 6) for intact Arabidopsis plants following freezing treatment.

Supplement Figure S5. The reduced flowering structure of Arabidopsis expressing *GmDREB1*s is partially complemented by GA3. Transgenic plants containing GmDREB1 gene were treated with and without GA3 treatment (10^{-4} M) once a week starting at 10 days old. A (non-transformed wild-type, Col-II), B (1302C-1-4: *35S::GFP*), C (09B-1-6: *35S::GmDREB1A;1*), D (09B-2-2: *35S::GmDREB1A;1*), E (09C-6-2: *35S::GmDREB1A;1*), F (16A-1-5: *35S::GmDREB1A;2*), G (16A-5-4: *35S::GmDREB1A;2*), H (20B-1-5: *35S::GmDREB1B;1*), I (20C-4-3: *35S::GmDREB1B;1*), J (10A-5-1: *35S::GmDREB1B;2*), K (10A-4-1: *35S::GmDREB1B;2*). Photographs were taken at 6 weeks.

Supplement Figure S6. The correlation between absolute levels of transgenic *GmDREB1s* and relative levels of downstream transcripts; *AtCOR47, AtRD29a*, and *AtADH1*. Each data point represents a distinct transformant with different expression levels of the *GmDREB1*. Transcript amount of *GmDREB1*s are shown as $\log_{10}$.

Table S1. Summary of Environmental Regulation of GmDREB1, 2, and 3 genes. Up-regulation in response to the following abiotic conditions is reported; before cold stress. Arrows indicate an increase or decrease following stress, N.S. indicates no significant change. C1 and C24 indicate time course significantly changed in response to cold relative to transcript level. C, cold; D, Dehydration; S, Salinity; H, Heat; A, ABA. References are 1, Kidokoro et al., 2015; 2, Mizoi et al., 2013; 3, Chen et al 2007, 4, Chen et al., 2008.

Table S2. List of primers for Real Time-qPCR analysis, creation of *DREB1* constructs and semi-qPCR analysis.

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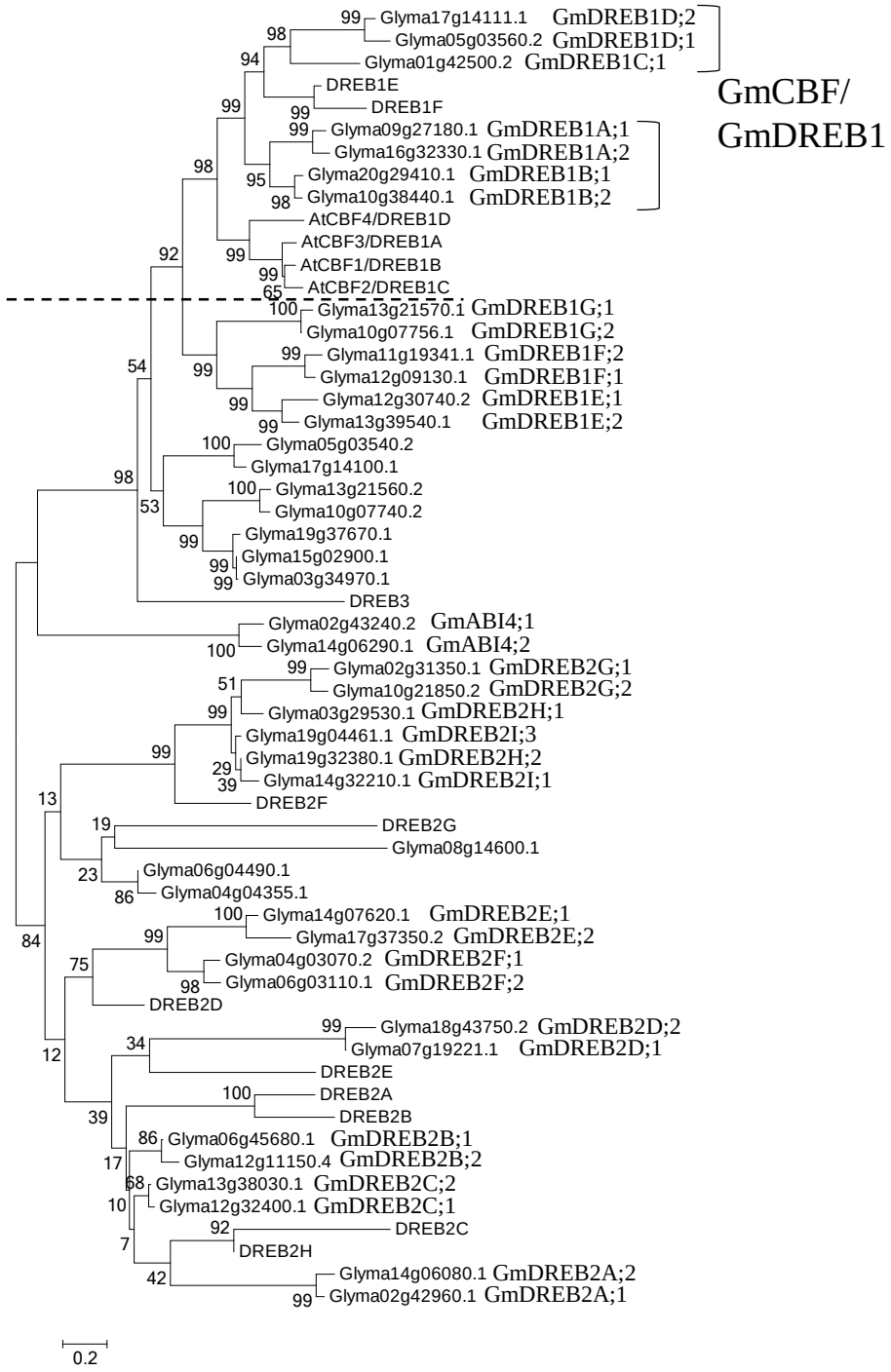


Figure 1. Sequence similarity of Arabidopsis and soybean DREB-related proteins. GmDREB1 and 2 were collected from a BLASTP search from phytozome using Arabidopsis DREB protein sequences. The Arabidopsis and soybean DREB 1 and 2 amino acid sequence were aligned and clustered by clustalW. The phylogenetic tree with bootstrap value and distances between branches was constructed with MEGA6.0 [60] using a neighbor-joining method based on the Jones-Tylor-Thornton model with 1,000 bootstraps. Glyma indicates *Glycine max* (soybean) DREB genes, all others are Arabidopsis genes. The dotted line separates the branch containing the GmDREBs most similar to the AtCBF1-4 genes. The bar indicates the number of substitutions per site.

Tamura K, Stecher G, Peterson D, Filipski A, and Kumar S (2013) MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. Molecular Biology and Evolution:30 2725-2729.

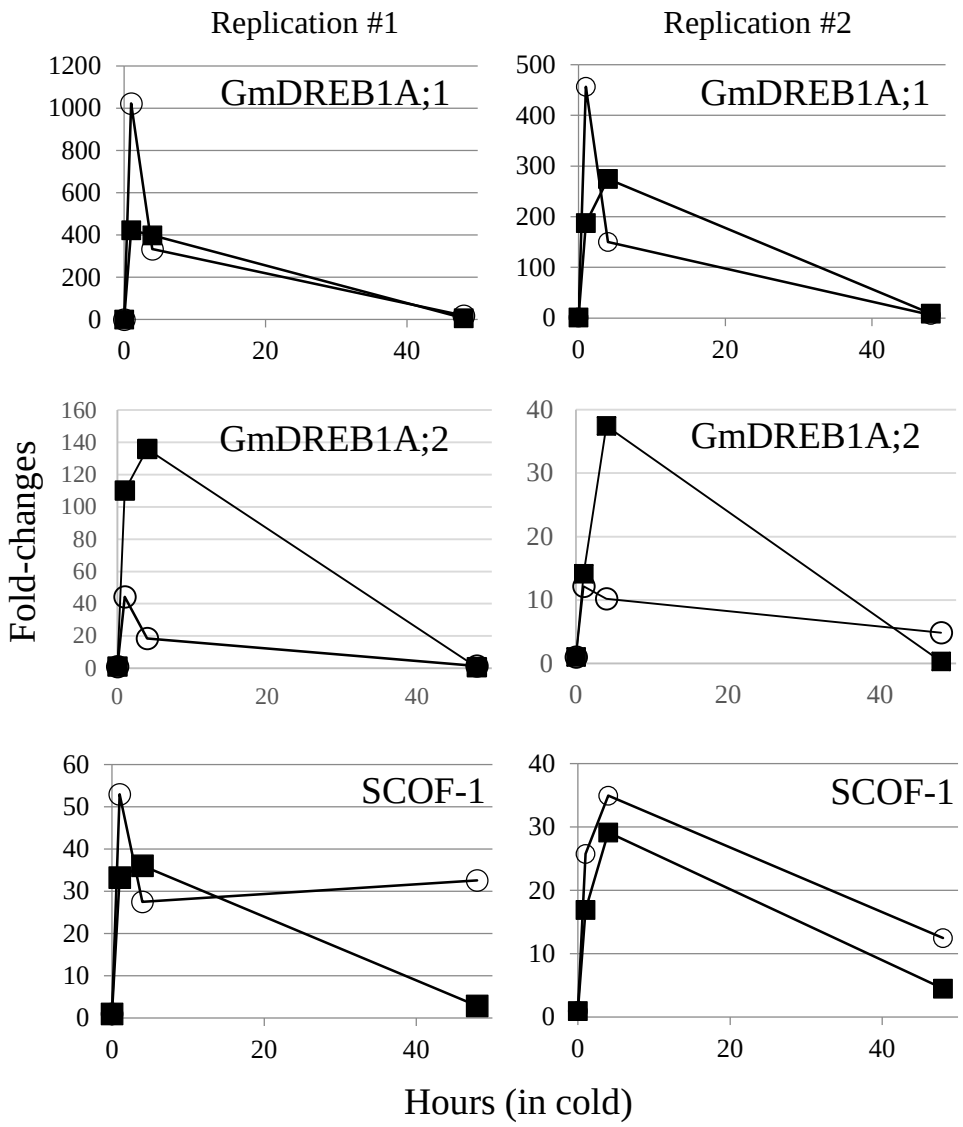


Figure 2. Transcript levels of *GmDREB1A;1* & *GmDREB1A;2* (*Glyma09g27180* & *Glyma16g32330*, respectively) and *SCOF-1* (*Glyma17g35430*) in response to cold stress (c.v. 'Young') normalized by *Actin11* expression. Open circles indicate transcript levels in soybean leaves, closed squares in soybean stems. Fold changes shown are relative to 0 time. Biological replications (1 and 2) represent RNA isolation performed on different experimental plants (at least 4 plants per replicate) at different times (0, 1, 4, and 48 h following cold treatment).

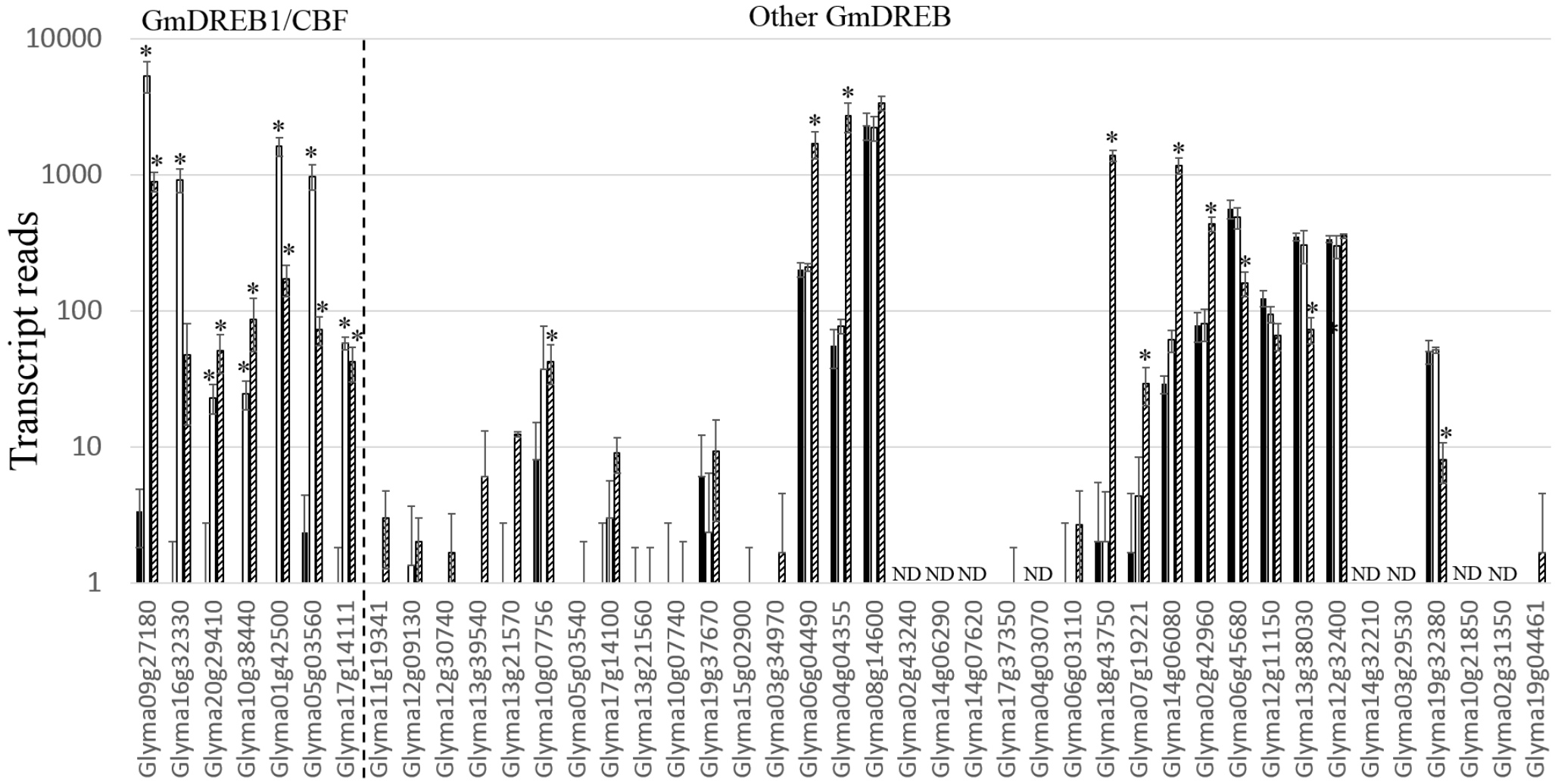


Figure 3. Changes in transcript levels of *GmDREB* genes in response to cold stress. RNASEQ analysis was performed in triplicate at all time points (log 2 transcript reads). Each replicate was composed of 6 plants. Standard deviation is shown. Black bar, grey and striped bar indicate 0, 1 and 24 h of cold stress, respectively. Adjusted p-value < 0.001 indicated by asterisks are significantly different reads compared to non-cold samples (0 h). *GmDREB1/CBF* is the subset of *DREB1*s that are most similar to the *AtCBF*s.

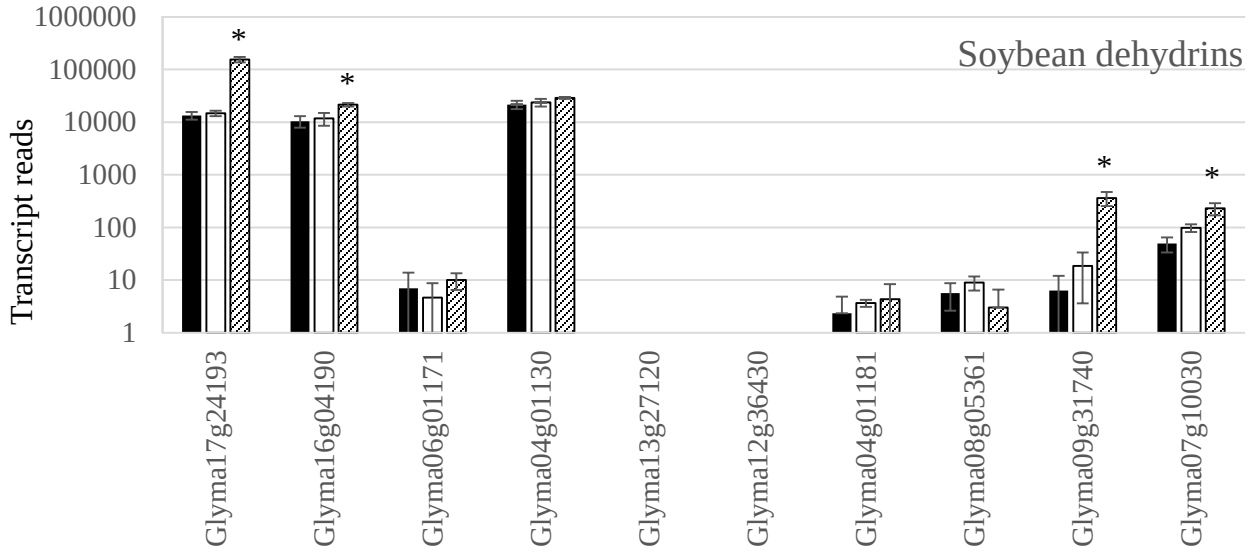
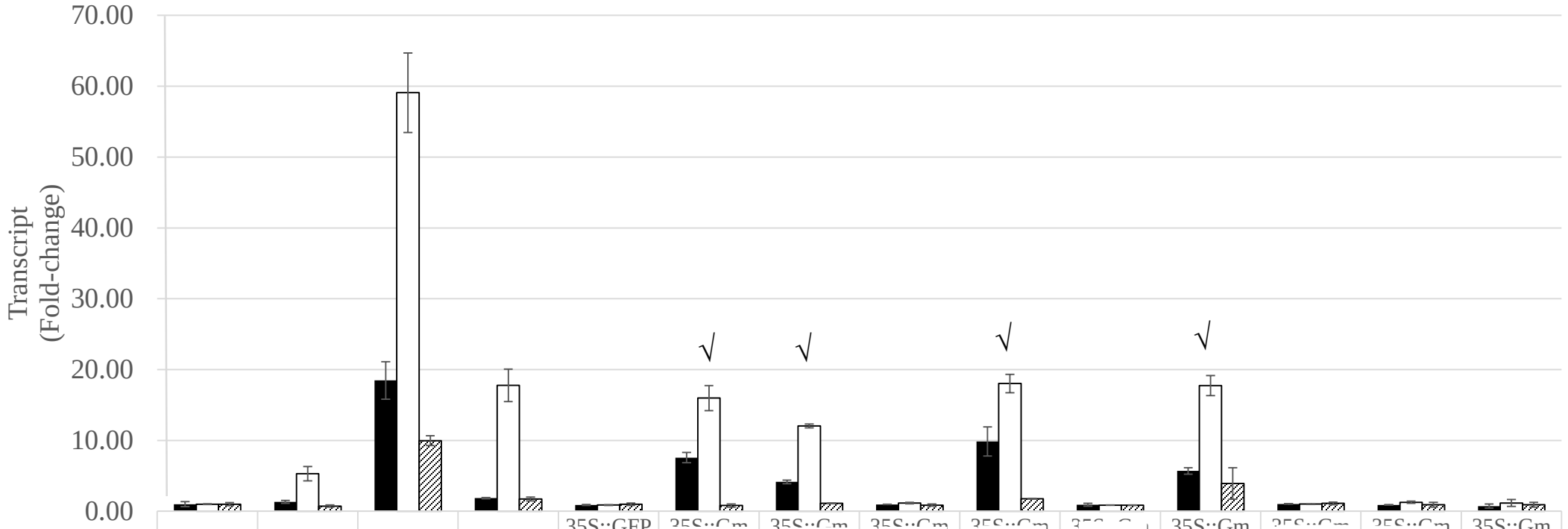
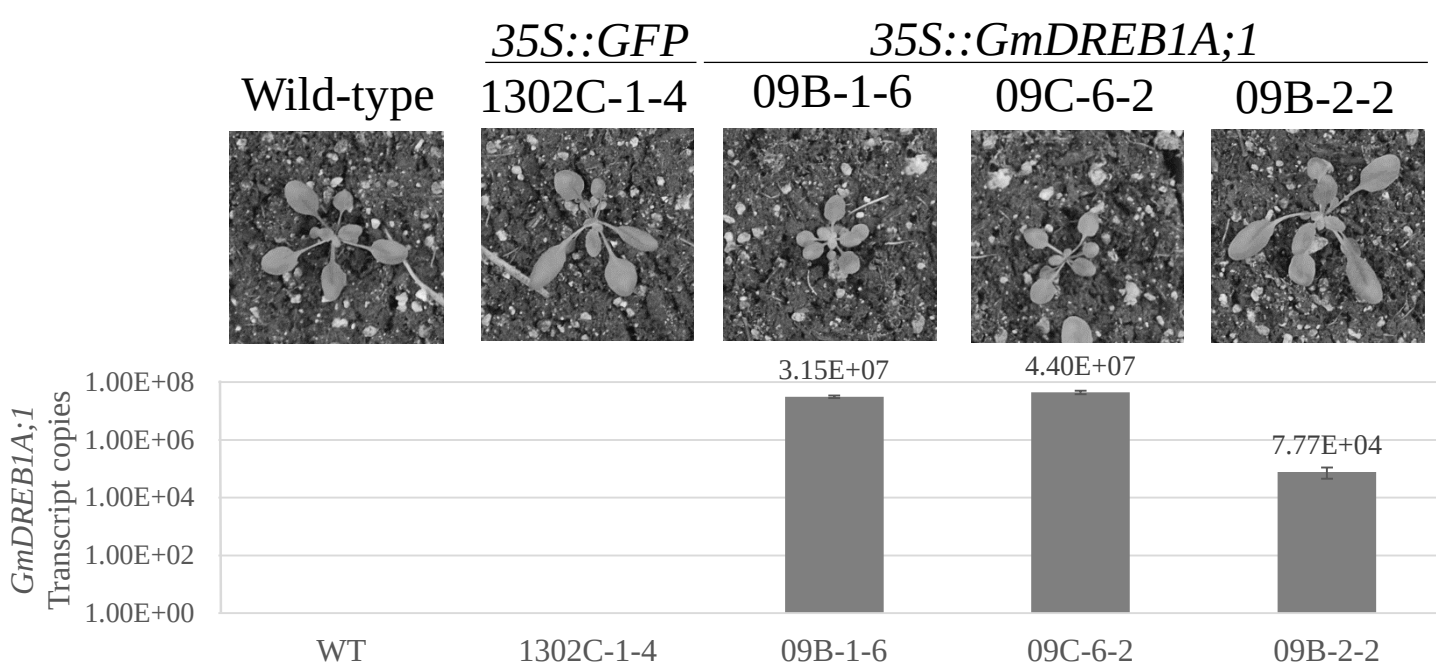


Figure 4. Changes in transcript levels of soybean dehydrins in response to cold stress. RNASEQ analysis was performed in triplicate at all time points (log10 scale for transcript reads). Black bar, grey and striped bar indicate 0, 1, and 24 h of cold stress, respectively. Adjusted p-value < 0.001 indicated by asterisks are significantly different reads compared to non-cold samples (0 h).



	WT cold 0h	WT cold 1h	WT cold 4h	WT cold 24h	35S::GFP (1302C-1-4)	DREB1A;1 (09B-1-6)	DREB1A;1 (09C-6-2)	DREB1A;1 (09B-2-2)	DREB1A;2 (16A-1-5)	DREB1A;2 (16A-5-4)	DREB1B;1 (20B-1-5)	DREB1B;1 (20C-4-3)	DREB1B;2 (10A-5-1)	DREB1B;2 (10A-4-1)
■ AtCOR47	1.00	1.33	18.48	1.85	0.89	7.58	4.15	0.97	9.86	0.92	5.70	1.03	0.90	0.73
□ AtRD29a	1.00	5.30	59.09	17.77	0.87	15.98	12.03	1.15	18.03	0.86	17.74	1.02	1.27	1.17
▨ AtADH1	1.00	0.73	9.96	1.75	0.99	0.82	1.12	0.87	1.77	0.87	3.91	1.11	0.93	0.92

Figure 5. Transcript levels of *AtCOR47, AtRD29a*, and *AtADH1* in Arabidopsis transgenic plants (4 weeks old). *CBF* regulated genes (*AtCOR47, AtRD29a* and *AtADH1*) in cold-treated wild type and in non-cold treated transgenic Arabidopsis expressing *GmCBF*-like *DREB*s. Fold-change of transcript accumulation, determined by RT-qPCR, are compared to transcript levels in the 0 h (no cold) wild-type plants. Expression of sufficient levels of *GmDREB1*s in the transgenic lines shows accumulation of *AtCOR47* and *AtRD29a* transcript without cold stress treatment, similar to cold accumulations due to *AtCBF* regulation in response to cold. Check mark (√) indicates those lines showing a dwarf phenotype.



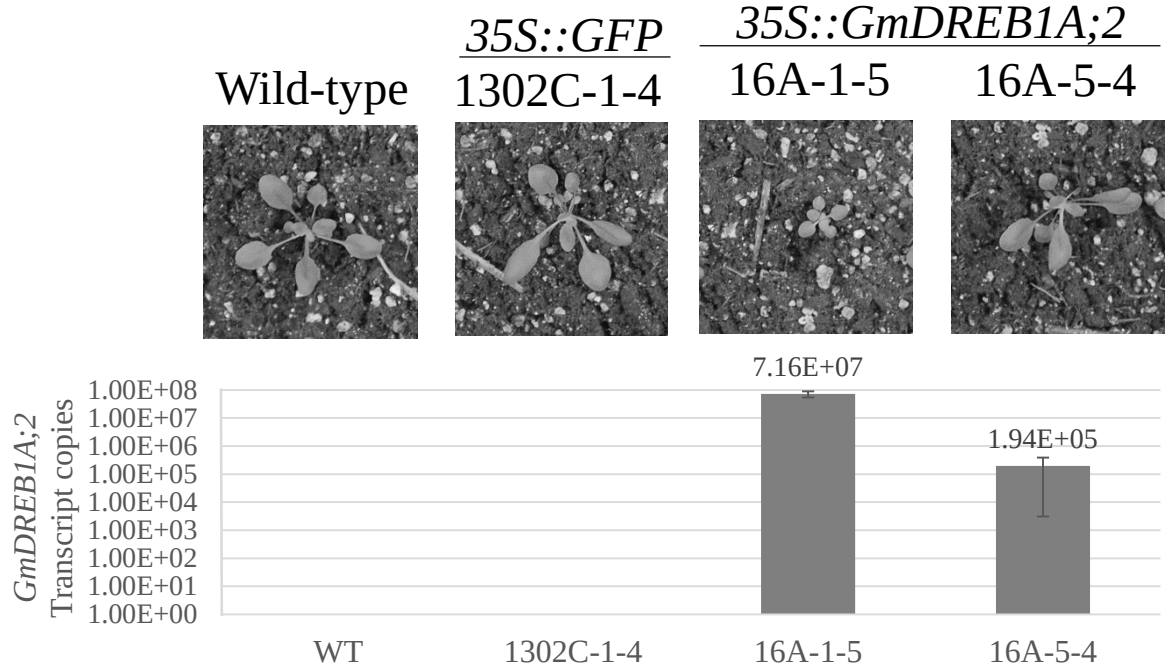


Figure 6. Arabidopsis transgenic plants constitutively expressing *GmDREB1* genes. Three-week-old transgenic plants were photographed. Transcript levels of each *GmDREB1* in four week old seedlings was absolute quantified to show the number of transcripts in 6.25 ng total RNA by RT-qPCR analysis.

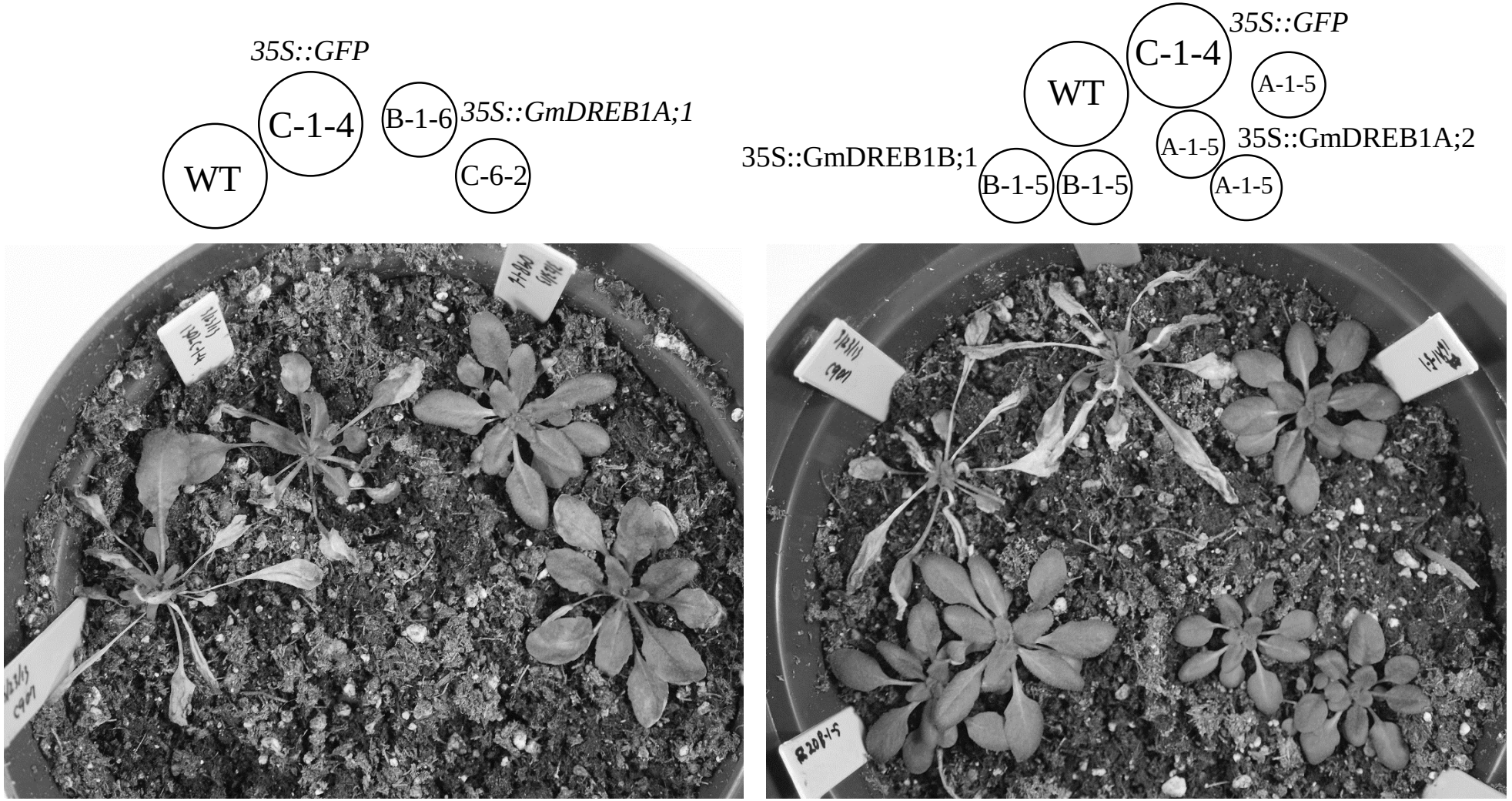


Figure 7. The transgenic plants expressing a sufficient level of *GmDREB1* have freezing tolerance. One-month-old transgenic plants were treated in -4.5 °C for 24 h in dark followed by 4 °C for 24 h in the dark and then returned to normal growth conditions for two days for recovery. The photographs were taken after recovery.

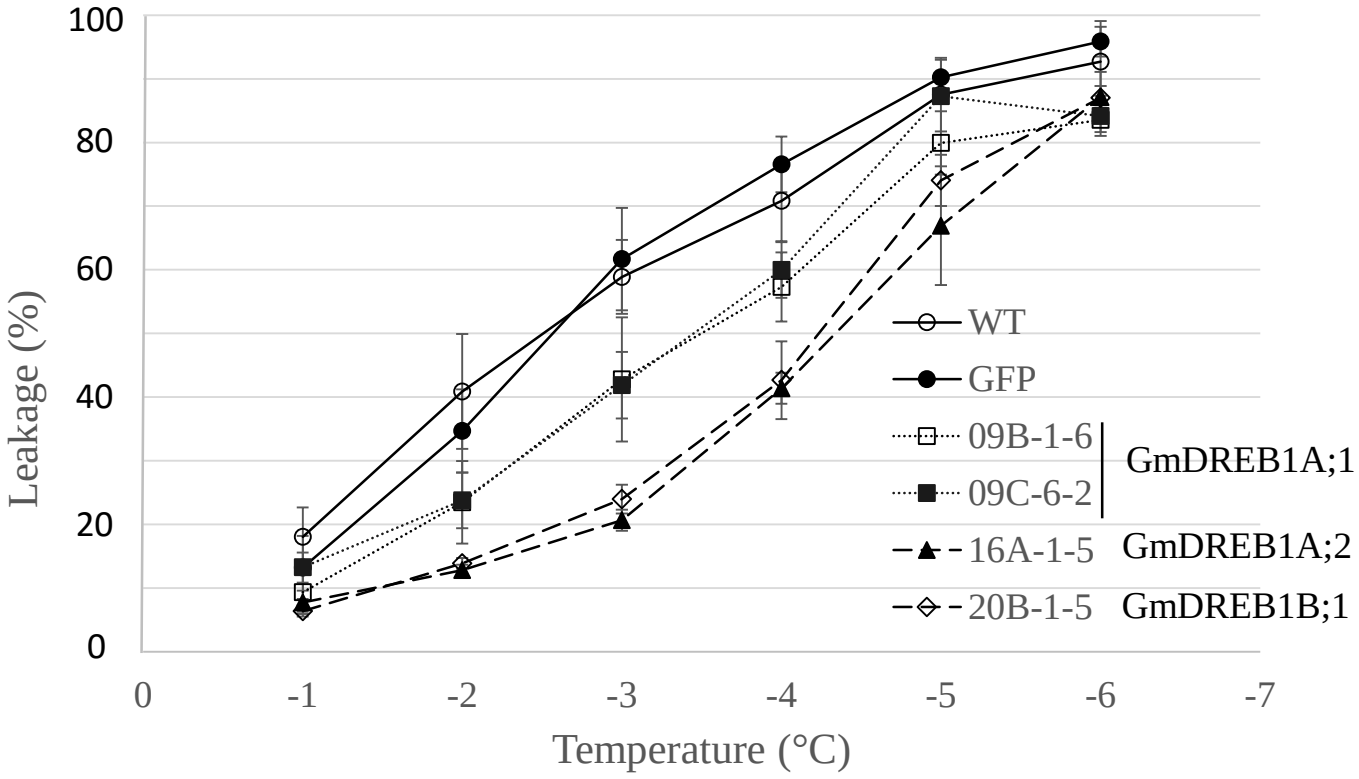


Figure 8. Transgenic Arabidopsis expressing GmDREB1 genes show enhanced freezing tolerance as determined by electrolyte leakage.

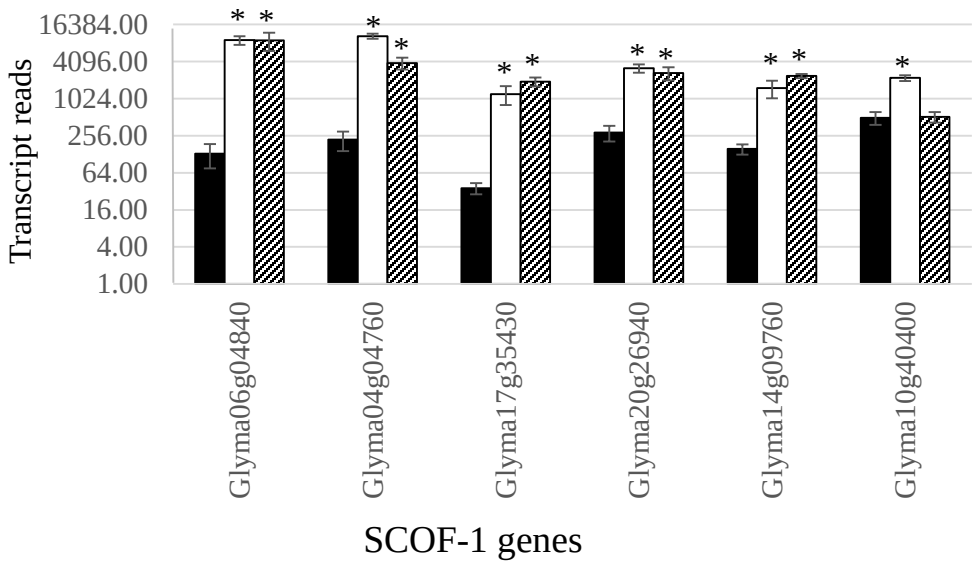


Figure S1. Changes in transcript levels of SCOF-1 genes in response to cold stress. RNASEQ analysis was performed in triplicate at all time points (log2 scale); each replicate was composed of 6 plants. Standard deviation is shown. Black bar, white and striped bar indicate 0, 1 and 24 hours of cold stress, respectively. * indicates significant difference (adjusted P-value < 0.001) as compared to 0 hour cold.

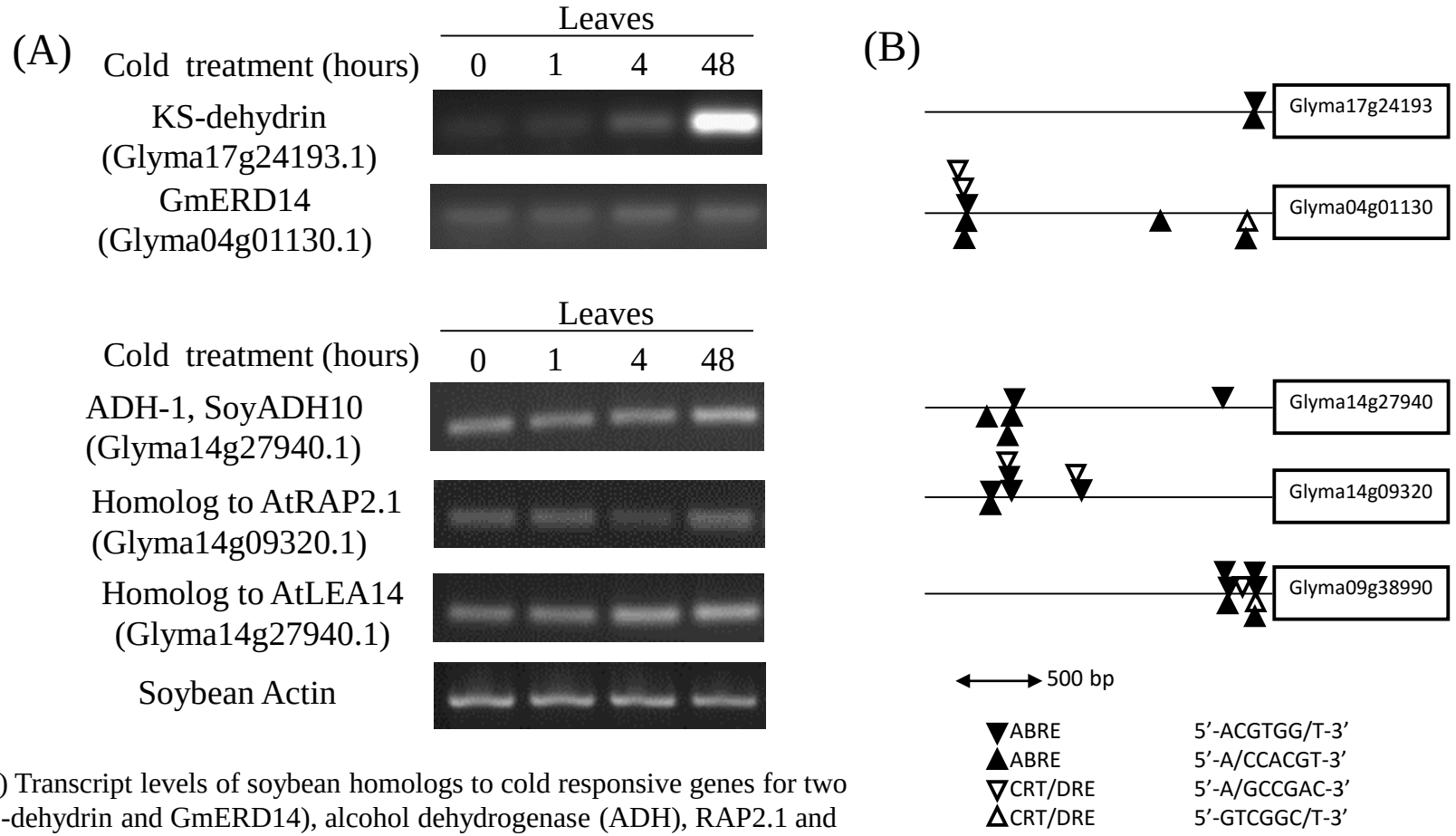


Figure S2 . (A) Transcript levels of soybean homologs to cold responsive genes for two dehydrins (KS-dehydrin and GmERD14), alcohol dehydrogenase (ADH), RAP2.1 and LEA14. Cold treatment was initiated with 10 day-old seedlings at ZT-4 h (4h after dawn, 18h light / 6h dark). Samples were harvested at 0h, 1h, 4h and 48h after initiation of cold (4°C) treatment. Total RNA was isolated, treated with DNase, reverse transcribed with oligo-dT as primer, then analyzed by semi-quantitative RT-PCR. Samples previously used for dehydrin transcript analysis (KS-dehydrin: Glyma17g24193.1 and GmERD14: Glyma04g01130.1; Figure 7, Yamasaki et al., 2013) were compared to ADH-1, RAP2.1, and LEA14. (B) The locations of predicted core elements, ABRE and CRT/DRE, in the promoter regions are shown.

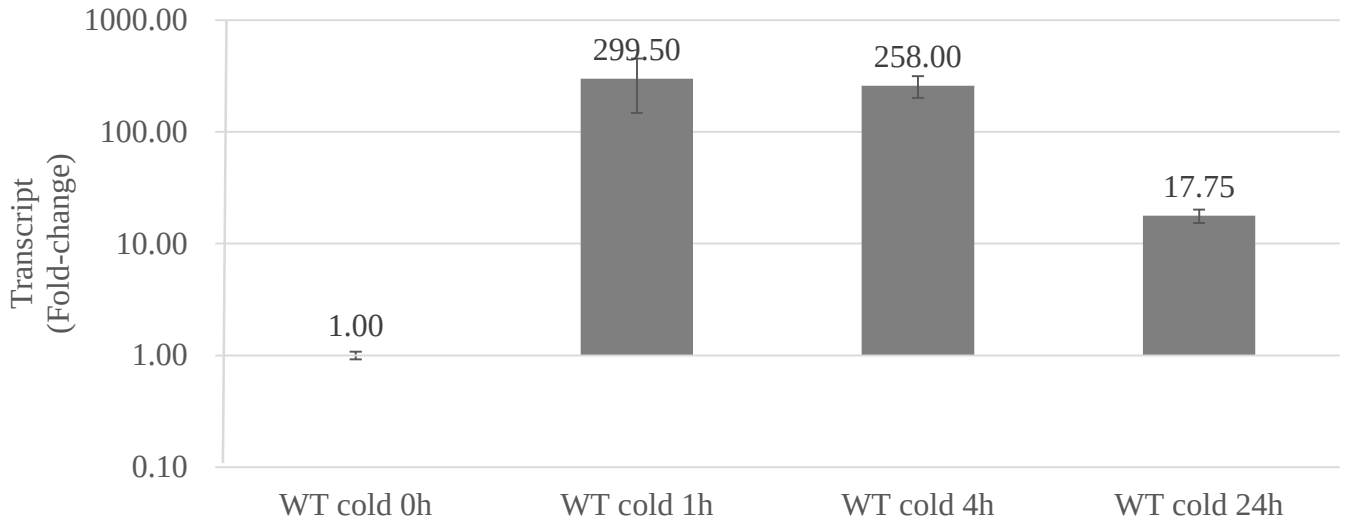


Figure S3. Accumulation of AtCBF3 transcript in Arabidopsis in response to cold stress. Cold treatment (4°C) on Col-2 (wild-type) started at ZT-4h (18 hours day/ 6 hours night). Fold-change ($\log_{10}$) of transcript accumulation was calculated by comparison to transcript levels in the 0 hour (no cold) wild-type plants.

Damage 0
No damage on any leaves

Damage 1
Minor damage (bleached) at the edge of a few leaves

Damage 2
Heavily damaged (wrinkled or leaf form collapsed) on single leaf or minor damage on less than 50% leaves

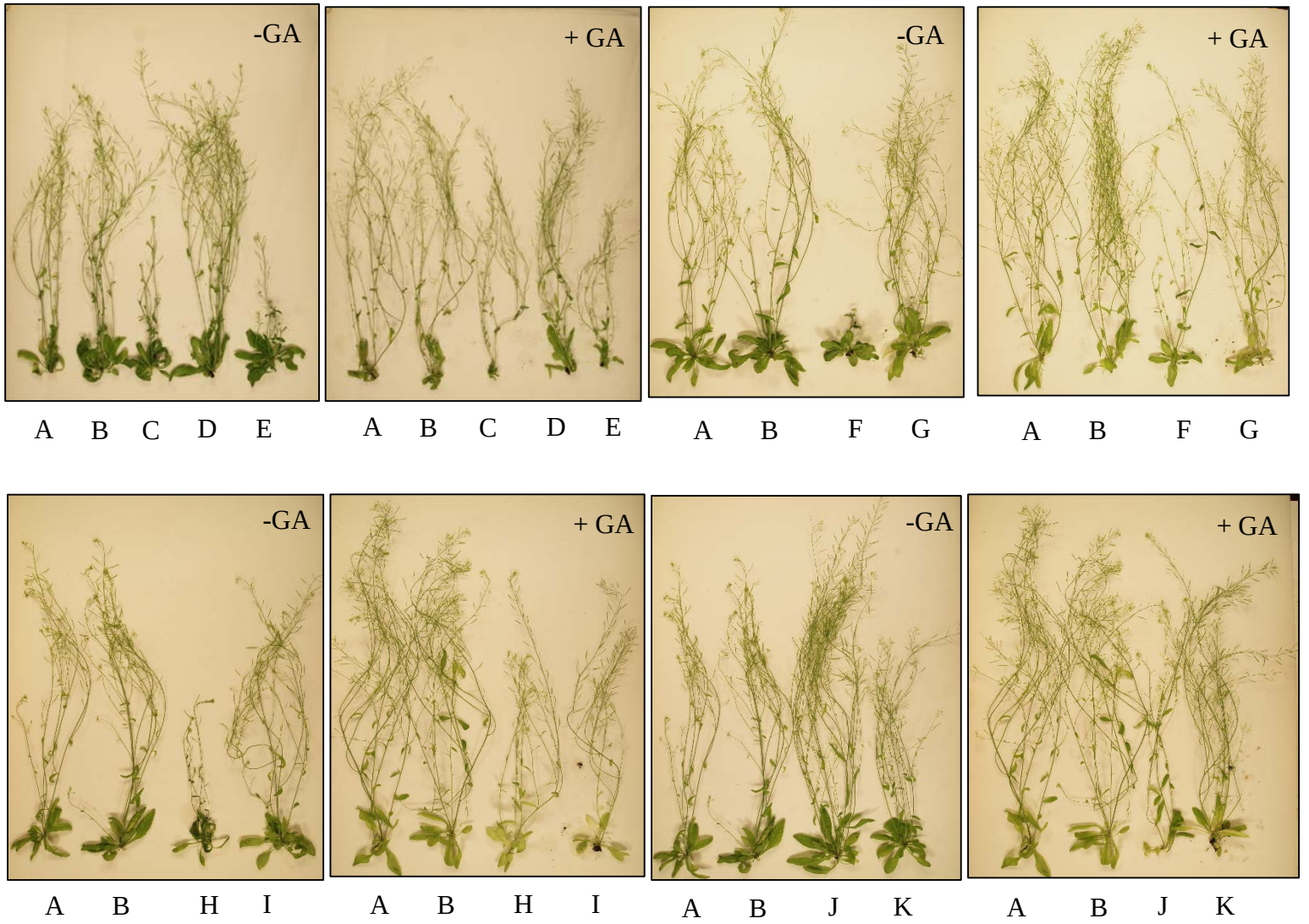
Damage 3
Heavily damaged on multiple leaves or minor damage on more than 50~75% leaves

Damage 4
Heavily damaged on 50% of leaves of a plant and minor damage on more than 50~75% leaves

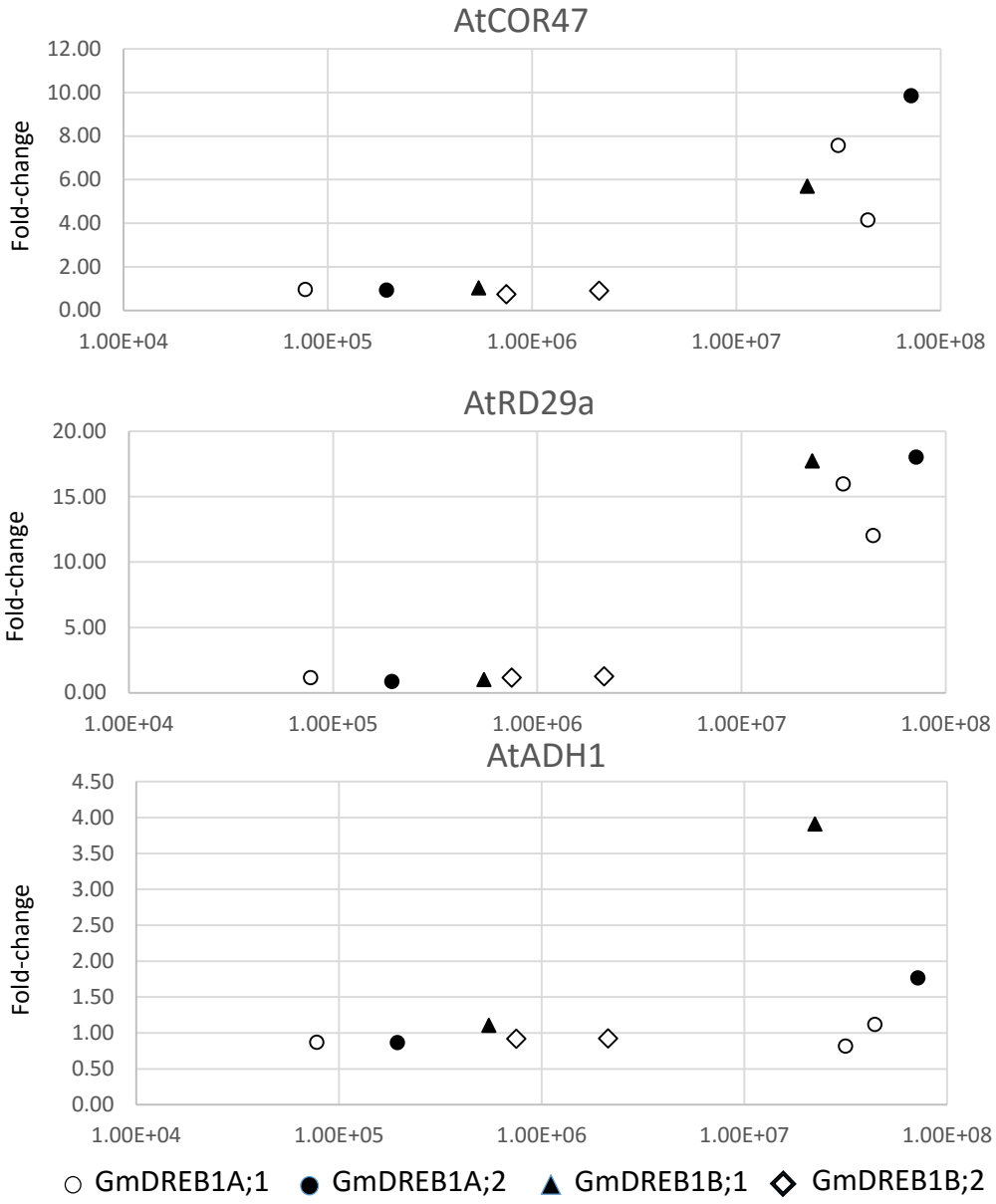
Damage 5
Heavily damaged on most leaves but still remaining green tissue, minor damage (bleached) on more than 75%~ leaves

Damage 6
100% area of a plant bleached or dehydrated

Supplement Figure S4. Damage scale for intact Arabidopsis plants following freezing treatment.



Supplement Figure S5. The reduced flowering structure of Arabidopsis expressing GmDREB1s is partially complemented by GA3. Transgenic plants containing GmDREB1 gene were treated with and without GA3 treatment (10^{-4} M) once a week starting at 10 days old. A (non-transformed wild-type, Col-II), B (1302C-1-4: *35S::GFP*), C (09B-1-6: *35S::GmDREB1A;1*), D (09B-2-2: *35S::GmDREB1A;1*), E (09C-6-2: *35S::GmDREB1A;1*), F (16A-1-5: *35S::GmDREB1A;2*), G (16A-5-4: *35S::GmDREB1A;2*), H (20B-1-5: *35S::GmDREB1B;1*), I (20C-4-3: *35S::GmDREB1B;1*), J (10A-5-1: *35S::GmDREB1B;2*), K (10A-4-1: *35S::GmDREB1B;2*). Photography was taken at 6 weeks.



Supplement Figure S6. The correlation between absolute levels of transgenic GmDREB1 and relative levels of downstream transcripts; AtCOR47, AtRD29a, and AtADH1. The level of transcript of the transgenic GmDREB1 relative to the DREB-regulated transcript change. Each data point represents a distinct transformant with different expression levels of the GmDREB1. Transcript amount of GmDREB1s are shown as $\log_{10}$.

Absolute transcript levels of transgenic soybean DREB1 genes in Arabidopsis (number of transcripts per 6.25 ng RNA)

Gene Name	Gene Locus (Wm82.a1.v1.1)	Previously published	Ref	This work
GmDREB1A;1	Glyma09g27180			C1,24⇧
GmDREB1A;2	Glyma16g32330	C⇧, D⇧, S⇧, H⇧	1	C1⇧
GmDREB1B;1	Glyma20g29410	C⇧, D⇧, S⇧, H⇧	1	C1,24⇧
GmDREB1B;2	Glyma10g38440	C⇧, D⇧, S⇧, H⇧	1	C1⇧
GmDREB1C;1	Glyma01g42500	C⇧, D⇧, S⇧, H⇧	1	C1,24⇧
GmDREB1D;1	Glyma05g03560	C⇧, D⇧, S⇧, H⇧	1	C1,24⇧
GmDREB1D;2	Glyma17g14111			C1,24⇧
GmDREB1F;2	Glyma11g19341			C (N.S.)
GmDREB1F;1	Glyma12g09130	C⇧, D⇧, S⇧, H⇧	1	C (N.S.)
GmDREB1E;1	Glyma12g30740			C (N.S.)
GmDREB1E;2	Glyma13g39540			C (N.S.)
GmDREB1G;1	Glyma13g21570			C (N.S.)
GmDREB1G;2	Glyma10g07756			C (N.S.)
GmDREB1	Glyma05g03540			C (N.S.)
GmDREB1	Glyma17g14100			C (N.S.)
GmDREB1	Glyma13g21560			C (N.S.)
GmDREB1	Glyma10g07740			C (N.S.)
GmDREB1	Glyma19g37670			C (N.S.)
GmDREB1	Glyma15g02900			C (N.S.)
GmDREB1	Glyma03g34970			C (N.S.)
GmDREB2	Glyma06g04490	C⇧, D⇧, S⇧, A⇧	3	C24⇧
GmDREB2	Glyma04g04355			C24⇧
GmDREB2	Glyma08g14600			C (N.S.)
GmABI4;1	Glyma02g43240			C (N.S.)
GmABI4;2	Glyma14g06290			C (N.S.)
GmDREB2E;1	Glyma14g07620			C (N.S.)
GmDREB2E;2	Glyma17g37350			C (N.S.)
GmDREB2F;1	Glyma04g03070			C (N.S.)
GmDREB2F;2	Glyma06g03110			C (N.S.)
GmDREB2D;2	Glyma18g43750			C24⇧
GmDREB2D;1	Glyma07g19221			C24⇧
GmDREB2A;2	Glyma14g06080	C⇧, D⇧, S⇧, H⇧	2	C24⇧
GmDREB2A;1	Glyma02g42960	C⇧, D⇧, S⇧, H⇧	2	C24⇧
GmDREB2B;1	Glyma06g45680	D⇧, S⇧	2	C24⇩
GmDREB2B;2	Glyma12g11150	D⇧, S⇧	2	C (N.S.)
GmDREB2C;2	Glyma13g38030			C24⇩
GmDREB2C;1	Glyma12g32400			C (N.S.)
GmDREB2I;1	Glyma14g32210			C (N.S.)
GmDREB2H;1	Glyma03g29530			C (N.S.)
GmDREB2H;2	Glyma19g32380			C24⇩
GmDREB2G;2	Glyma10g21850			C (N.S.)
GmDREB2G;1	Glyma02g31350			C (N.S.)
GmDREB2I;3	Glyma19g04461			C (N.S.)
GmDREB3	Glyma17g05240	C⇧	4	C1⇧

Table S1. Summary of Environmental Regulation of GmDREB1, 2, and 3 genes.
Up-regulation in response to the following abiotic conditions is reported; before cold stress.
C, cold; D, Dehydration; S, Salinity; H, Heat; A, ABA.
Arrows indicate an increase or decrease following stress, N.S. indicates no significant change.
C1 and C24 indicate time course significantly changed in response to cold relative to transcript level

References are 1, Kidokoro et al., 2015; 2, Mizoi et al., 2013; 3, Chen et al 2007, 4, Chen et al., 2008.

Target for transcript for Real-qPCR	Primer name	Primer sequence (5'→3')	Annealing temperature (°C)
GmDREB1A;1	Glyma09g27180.1Real-TimeU527	CAACGGCGACGGCACAAG	63
	Glyma09g27180.1Real-TimeL574	AAGCAACTCAGGCATATCCAACAC	
GmDREB1A;2	Glyma16g32330.1+391RT-PCR-U	GCCACTGCTAACGCAAAGGAT	60
	Glyma16g32330.1+461RT-PCR-L	CACTCTTGCTTTGTATTCGTATTTTCTAAGG	
GmDREB1B;1	Glyma20g29410.1+502RT-PCR-U	CCGGAGTATCTGAGGAACATGGT	60
	Glyma20g29410.1+583RT-PCR-L	ACAGAGAAACTTCAGCATCGTCAAA	
GmDREB1B;2	Glyma10g38440.1+459RT-PCR-U	GGCGACGGAGCGTGAAG	57
	Glyma10g38440.1+514RT-PCR-L	CGGCGACATGAGCACCAT	
SCOF-1	Glyma17g35430.1Real-TimeU	CGTTCTCGCGACCATCCTTCT	64
	Glyma17g35430.1Real-TimeL	TGACGTGGCGGTTGTTGAC	
GlymaActin	GlymaACT11Real-U	ATCTTGACTGAGCGTGGTTATTCC	60
	GlymaACT11Real-L	GCTGGTCCTGGCTGTCTCC	
AtCBF3	AtCBF3+578qPCR-U	TTCCGTCCGTACAGTGGAAT	58
	AtCBF3+625qPCR-L	AACTCCATAACGATACGTCGTC	
AtCOR47	AtCOR47+642qPCR-U	CGGTACCAGTGTCGGAGAGT	60
	AtCOR47+749qPCR-L	ACAGCTGGTGAATCCTCTGC	
AtEF1α	Atef1α+354qPCR-U	CACCACTGGAGGTTTTGAGG	60
	Atef1α+572qPCR-L	TGGAGTATTTGGGGGTGGT	
AtADH1	AtADH1+33qPCR-U	AGCTGCTGTGGCATGGGA	63
	AtADH1+214qPCR-L	TCTGCGGTGGAGCAACCT	
AtRD29a	AtRD29A+311qPCR-U	GCACCAGGCGTAACAGGTAAAC	63
	AtRD29A+467qPCR-L	AAACACCTTTGTCCCTGGTGG	
Creation for 35S::GmDREB1	Primer name	Primer sequence (5'→3')	Annealing temperature (°C)
GmDREB1A;1	Glyma09g27180.1U-NcoI	ccatggtaATGTTTACCTTGAATCATTCTTCT	52
	Glyma09g27180.1L-PmlI	cacgtgTTAAATTGAGAAATTCCATAGTGA	
GmDREB1A;2	Glyma16g32330.1U-NcoI	ccatggtaATGTATACCTTGAACCACTC	55
	Glyma16g32330.1L-PmlI	cacgtgTTAAATTGAGAAACTCCATAGG	
GmDREB1B;1	Glyma20g29410.1U-NcoI	ccatggtaATGTTTTCCATCAATCATTTCT	50
	Glyma20g29410.1L-BstEII	ggtcaccaataactgagttaaaTTAAATG	
GmDREB1B;2	Glyma10g38440.1U-NcoI	ccatggtaATGTTTTCCATCAATCATTTCT	51
	Glyma10g38440.1L-BstEII	ggtcaccTTAAATGGAGTAACTCCACAAC	
Target for transcript for semi-qPCR	Primer name	Primer sequence (5'→3')	Annealing temperature (°C)
SoyADH10 (Glyma14g27940.1)	Glyma14g27940.1+21UpRT	GACCATCAAGTGCAAAGCTG	59
	Glyma14g27940.1+1264LowRT	AATGCGAGGAAACAATGGAG	
Homolog to AtRAP2.1 (Glyma14g09320.1)	Glyma14g09320.1+290UpRT	GCCTTAACTTCCCCGAACTC	60
	Glyma14g09320.1+487LowRT	AGTCGGGCTTGAGATTGAGA	
Hommolog to LEA14 (Glyma09g38990.1)	Glyma09g38990.1+37UpRT	GCGGAGAAAGTCACCAACAT	59
	Glyma09g38990.1+174LowRT	ACAAATGGGAATGGAAGTGG	
GmActinV00450	rtGmActinV00450U	CCAAGCTGTTCTCTCCTTGTATG	58
	rtGmActinV00450L	CCAGACTCATCATATTCACCTTTAG	

Table S2. List of primers for RealTime-qPCR analysis, creation of DREB1 constructs and semi-qPCR analysis.