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Identification of *Cis*-Acting Promoter Elements in Cold- and Dehydration-Induced Transcriptional Pathways in Arabidopsis, Rice, and Soybean

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Abstract

The genomes of three plants, Arabidopsis (*Arabidopsis thaliana*), rice (*Oryza sativa*), and soybean (*Glycine max*), have been sequenced, and their many genes and promoters have been predicted. In Arabidopsis, *cis*-acting promoter elements involved in cold- and dehydration-responsive gene expression have been extensively analysed; however, the characteristics of such *cis*-acting promoter sequences in cold- and dehydration-inducible genes of rice and soybean remain to be clarified. In this study, we performed microarray analyses using the three species, and compared characteristics of identified cold- and dehydration-inducible genes. Transcription profiles of the cold- and dehydration-responsive genes were similar among these three species, showing representative upregulated (dehydrin/LEA) and downregulated (photosynthesis-related) genes. All ($4^6 = 4096$) hexamer sequences in the promoters of the three species were investigated, revealing the frequency of conserved sequences in cold- and dehydration-inducible promoters. A core sequence of the abscisic acid-responsive element (ABRE) was the most conserved in dehydration-inducible promoters of all three species, suggesting that transcriptional regulation for dehydration-inducible genes is similar among these three species, with the ABRE-dependent transcriptional pathway. In contrast, for cold-inducible promoters, the conserved hexamer sequences were diversified among these three species, suggesting the existence of diverse transcriptional regulatory pathways for cold-inducible genes among the species.

Key words: plant genome; *cis*-acting promoter elements; cold; dehydration; microarray

1. Introduction

Low temperature and dehydration affect plant growth and productivity, and many genes respond to both stressors at the transcriptional level, and their gene products function in stress tolerance and response.[1–4] These genes include key metabolic enzymes, late embryogenesis-abundant (LEA) proteins, detoxification enzymes, chaperones, protein kinases and transcription factors.[1–4] The *cis*-acting

elements that function in stress-responsive gene expression have been analysed to elucidate the molecular mechanisms of gene expression in response to these stresses.[5] The dehydration-responsive element (DRE), containing the core sequence A/GCCGAC, is a *cis*-acting element that regulates cold- and dehydration-responsive gene expression in Arabidopsis (*Arabidopsis thaliana*).[6] A similar motif was identified as the C-repeat and low temperature-responsive promoter element.[7,8] The abscisic acid (ABA) -responsive element (ABRE), containing the core sequence ACGTGG/T, is a *cis*-acting element that regulates dehydration- and high salinity-responsive gene expression in Arabidopsis and rice (*Oryza sativa*).[5,9] ABA-responsive gene expression requires multiple ABREs or an ABRE with a coupling element as a functional promoter.[10–15]

Arabidopsis cDNAs encoding the ethylene-responsive element-binding factor/APETALA2-type DRE-binding (DREB) proteins (CBF1, DREB1A, and DREB2A) have been isolated by one-hybrid screening in yeast,[16,17] and they specifically bind to the DRE/C-repeat sequence to activate gene transcription in Arabidopsis. Arabidopsis has three DREB1/CBF proteins encoded by genes that lie in tandem on chromosome 4 in the order *DREB1B/CBF1*, *DREB1A/CBF3*, and *DREB1C/CBF2*.[17,18] Arabidopsis also has DREB2A and DREB2B proteins.[17] Although expression of all three *DREB1/CBF* genes is induced by cold but not by dehydration, both *DREB2* genes are induced by dehydration and high salinity.[17] Both DREB1/CBF and DREB2 proteins bind to DRE, but DREB1/CBFs function in cold-responsive gene expression, and DREB2s function in dehydration-responsive gene expression.

CaMV *35S* promoter-driven overexpression of DREB1/CBFs increases stress tolerance to freezing, dehydration, and high salinity in transgenic Arabidopsis.[17,19,20] More than 100 downstream targets of DREB1A/CBF3 have been identified by microarray analysis.[21–25] Overexpression of the constitutively active form of *DREB2A* (*35S:DREB2A-CA*) significantly increases dehydration tolerance but only slightly increases freezing tolerance.[26] Microarray analyses of *35S:DREB2A-CA* plants revealed that DREB2A regulates expression of many dehydration-responsive genes; however, some genes regulated by DREB1A are not regulated by DREB2A.[26]

Promoter analysis of DREB1A- and DREB2A-regulated genes and gel mobility shift assays revealed that DREB1A has the highest affinity for A/GCCGACNT, and DREB2A preferentially binds ACCGAC,[23,26] thus controlling the induction of different downstream genes. DREB2A expression also is regulated by heat-shock stress, and thermo-tolerance is significantly increased in *35S:DREB2A-CA* plants.[27] Heat shock-inducible proteins and transcription factors are upregulated in *35S:DREB2A-CA* plants but not in *35S:DREB1A* plants. DREB2A-regulated genes are involved in dehydration, high salinity, and heat-shock stress tolerance but not freezing stress.[27] DREB1A and DREB2A downstream gene products mostly have similar functions, but they encode different enzymes for carbohydrate metabolism.[25]

ABRE-binding (AREB) proteins or ABRE-binding factors (ABFs) have been isolated using ABRE sequences as baits in one-hybrid screens in yeast.[28,29] AREB/ABFs encode basic-domain leucine zipper (bZIP) transcription factors, and they comprise a family with nine members in Arabidopsis.[28–35] Among the nine members, *AREB1/ABF2*, *AREB2/ABF4*, and *ABF3* are induced by dehydration and high salinity in vegetative tissues,[36] and their gain-of-function mutants show enhanced dehydration stress tolerance.[36–38] The *areb1 areb2 abf3* triple mutant displays enhanced ABA insensitivity and reduced dehydration stress tolerance compared with control plants, and these effects are accompanied by disruption of dehydration-responsive gene expression.[39]

In this study, the promoter sequences in cold- and dehydration-inducible genes of Arabidopsis, rice, and soybean (*Glycine max*) were analysed. Previously, we reported cold- and dehydration-responsive genes in Arabidopsis.[25] In the current study, we used oligo microarrays to identify cold- and dehydration-responsive genes in rice and soybean. The observed frequencies of all ($4^6 = 4096$) hexamer sequences in cold- and dehydration-inducible promoters were compared with standardized promoters to estimate conserved sequences and to determine representative cold- and dehydration-responsive transcriptional pathways in Arabidopsis, rice, and soybean.

2. Materials and methods

2.1. Plant materials and growth conditions

Wild-type rice plants (*Oryza sativa* L. cv. Nipponbare) were grown in plastic pots filled with nutrient soil for 2 weeks under flooded lowland conditions with a 12-h light (28°C)/12-h dark (25°C) regimen (50 ± 10 μmol photons/m^2/s). The plants were then: (i) transferred from 28 to 10°C and grown for 1 day for the cold treatment; or (ii) grown for 3 days at 28°C without watering to a 15.6% (SD 1.1) (w/w) soil moisture content for the dehydration treatment. Wild-type soybean plants (*Glycine max* (L.) Merr. cv. Nourin No. 2) were grown in plastic pots filled with nutrient soil for 3 weeks under a 12-h light/12-h dark regimen (50 ± 10 μmol photons/m^2/s) at 28°C. The plants were then: (i) transferred from 28 to 4°C and grown for 1 day for the cold

treatment; or (ii) grown for 4 days at 28°C without watering to <10% (w/w) soil moisture content for the dehydration treatment.

2.2. Microarray analyses

A rice oligo microarray (RAP-DB) was used for transcriptome analysis (Agilent Technologies). We constructed a new oligonucleotide microarray for soybean transcriptome analysis capable of detecting 43 640 protein-coding genes using eArray software with soybean protein-coding genes.[40] Oligo microarray construction was performed by Agilent Technologies Inc. The RNAs are labelled with a Low RNA Input Linear Amplification/Labeling Kit™ (Agilent Technologies) according to the manufacturer's instructions. Aliquots of Cy5-labelled cRNA of the samples and Cy3-labelled cRNA of the control samples are used for hybridization in the microarray. Biological and technical (dye swap) replicate sample sets are analysed. After hybridization, microarray slides are scanned (scanner model G2505C with scan control software, version A.8.5.1; Agilent Technologies) and the data are analysed using Feature Extraction software, version 10.10.1.1 (Agilent Technologies). All microarray data analyses were performed according to the Agilent methodology. Raw data were analysed by GeneSpring GX software, version 11.5.1 (Agilent Technologies) and normalized using the Lowess normalization method. Expression log ratios and Benjamini and Hochberg false discovery rate *P*-values of the cross-gene error model were also calculated by GeneSpring GX. Microarray design and data were deposited at MIAMExpress (accession numbers E-MEXP-2294, E-MEXP-2295, E-MEXP-2725, E-MEXP-2726, and E-MEXP-3164).

2.3. Measurement of ABA levels

The ABA level in 100 mg of each plant was quantified as described[41] using a liquid chromatography-mass chromatography system (UPLC/Quattro Premier XE; Waters) with an ODS column (AQUITY-UPLC BEH-C 18, 1.7 μm, 2.1 × 100 mm; Waters). Reproducibility was assessed using three biological replicates in each experiment.

3. Results

3.1. Identification of cold- and dehydration-responsive genes in rice and soybean

Microarray analyses were used to identify cold- and dehydration-responsive genes in rice and soybean. The 389-Mb rice genome and the 1115-Mb soybean genome have been sequenced, and 37 544 and 46 430 protein-coding genes, respectively, have been predicted.[40,42] A rice oligo microarray (RAP-DB) including 29 690 rice genes was used for transcriptome analysis (Agilent Technologies). In rice plants exposed to cold or dehydration, 4022 (cold) and 4632 (dehydration) genes were significantly upregulated [Benjamini and Hochberg false discovery rate (FDR): $P < 0.05$; fold change (FC): >2], and 4704 (cold) and 5189 (dehydration) genes were significantly down-regulated (FDR: $P < 0.05$; FC: <0.5) (Supplementary Tables S1 and S2). Because a soybean oligo microarray was not available, we constructed a new oligo microarray for transcriptome analysis capable of detecting 43 640 protein-coding genes using eArray software with soybean protein-coding genes.[40] In soybean plants exposed to cold or dehydration, 5993 (cold) and 4433 (dehydration) genes were significantly upregulated (FDR: $P < 0.05$; FC: >2), and 6350 (cold) and 5098 (dehydration) genes were significantly downregulated (FDR: $P < 0.05$; FC: < 0.5) (Supplementary Tables S3 and S4).

3.2. Molecular function of cold- and dehydration-responsive genes in Arabidopsis, rice, and soybean

We annotated cold- and dehydration-responsive genes in rice and soybean with Gene Ontology (GO) data, and we re-annotated these genes in Arabidopsis. First, all amino acid sequence data were downloaded from TAIR10,[43] RAP-DB,[44] and Phytozome (http://www.phytozome.net/), and motifs were searched using the HMMER program.[45] Pfam entries were then converted to GO terms according to the methods of Hunter *et al.*[46] Secondly, data for Enzyme Commission (EC) numbers were downloaded from KEGG,[47] AraCyc,[48] Plantcyc,[49] and Kappa-view[50] and then converted to GO terms.[51] Thirdly, we downloaded data for GO terms from TAIR10, RAP-DB, Phytozome, AgriGO,[52] GRAMENE,[53] and AmiGO.[54] Based on the GO data, 3717 molecular functions were categorized into 27 classes according to the KEGG PATHWAY. Among the genes detected by the oligo microarrays, 10 005 (Arabidopsis), 11 271 (rice), and 17 416 (soybean) genes were annotated in the databases (Supplementary Tables S5–S9), and our Arabidopsis transcription profile was similar to that of rice and soybean. For each species, dehydrin/LEA- and photosynthesis-related genes were representative of up- and downregulated classes, respectively, whereas helicase genes were representative of a cold-induced upregulated class (Fig. 1).

3.3. Base composition in Arabidopsis, rice, and soybean promoters

To identify conserved sequences in cold- and dehydration-inducible promoters, we collected sequence data for 10 000 promoters of Arabidopsis, rice, and

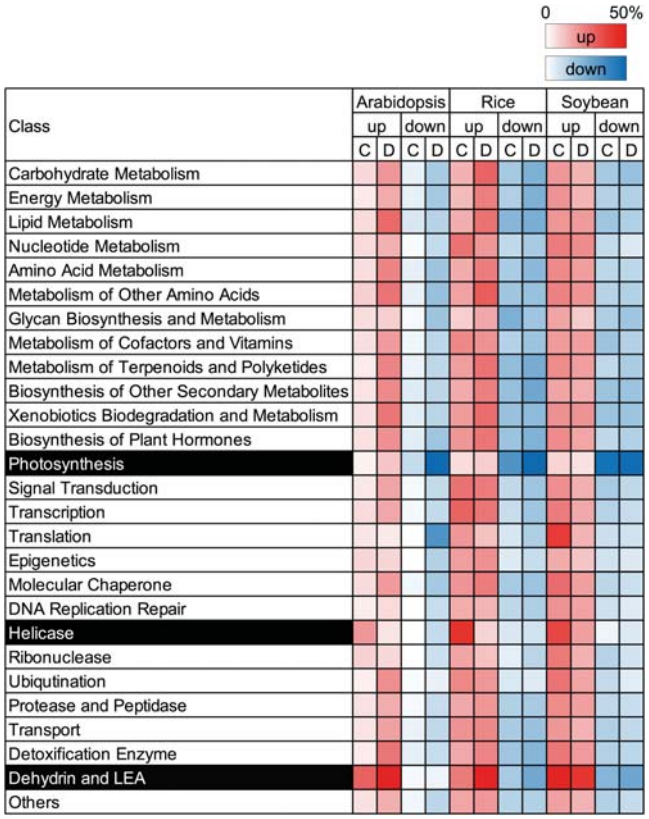

Figure 1. Molecular function of cold- and dehydration-responsive genes in Arabidopsis, rice, and soybean. Heat maps of 27 upregulated (up; red) or downregulated (down; blue) molecular function classes indicating the frequency (%; shading in boxes) in cold-treated (C) and dehydration-treated (D) plants.

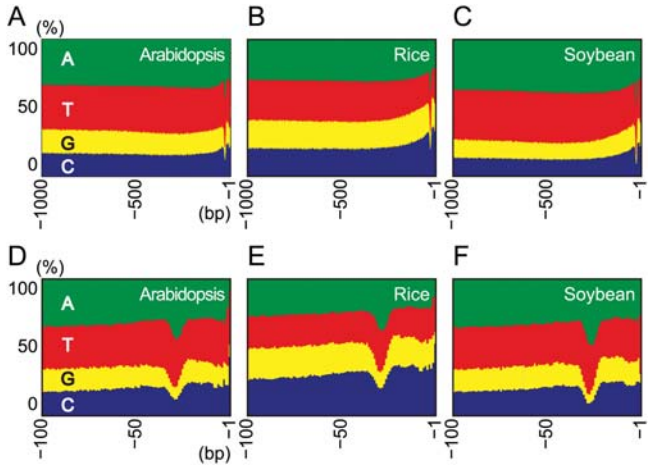

Figure 2. Base composition in promoters of Arabidopsis, rice, and soybean. Adenine (A, green), thymine (T, red), guanine (G, yellow), and cytosine (C, blue) base composition in promoter regions from −1 to −1000 (A–C) or from −1 to −100 (D–F).

soybean genes. As we previously reported 10 000 promoter sequences of Arabidopsis genes,[23] we used these data and also collected promoter sequences of rice and soybean genes. Sequences of 10 000 independent promoters from −1 to −1000 were randomly selected from genomic sequences of rice and soybean genes that have data for the full-length cDNA sequences to obtain the mean value of base compositions. We estimated positions of transcriptional start sites using sequences of more than 10 000 independent full-length cDNA clones for each species, which were previously compiled into databases.[55,56]

To understand aspects of the promoter sequences of each species, we estimated base composition of the promoter sequences. Previously, we analysed base composition in Arabidopsis promoters.[23] In this study, we analysed base composition in rice and soybean promoters, and we re-analysed that in Arabidopsis promoters. To estimate the sample mean of base composition in 10 000 promoters of three species, single random sampling ($n = 100$) was conducted for 1000 replicates, based on the central limit theorem. Base compositions of rice and soybean promoters were compared with Arabidopsis promoters. Promoter base compositions were AT-rich in Arabidopsis (A:C:G:T = 33.7:17.0:16.5:32.8), rice (A:C:G:T = 29.2:22.0:20.4:28.3), and soybean (A:C:G:T = 37.2:14.1:13.2:35.5) (Fig. 2A–C). In addition, the Smirnov–Grubbs test ($P < 0.05$) was used to detect outliers in promoter regions. In each species, high-frequency AT regions were found ~30 bases upstream of the transcriptional start sites, with high-frequency C regions from −1 and −100, except in AT-enriched regions (Fig. 2D–F; Supplementary Tables S10–S12).

3.4. *Frequency of promoter hexamer sequences in Arabidopsis, rice, and soybean*

Several core sequences of *cis*-acting elements are hexamer sequences,[6,7,9,57] including the core sequences of DRE (A/GCCGAC), ABRE (ACGTGG/T), and G box (CACGTG). To estimate biases in the frequency of promoter hexamer sequences, the number of all ($4^6 = 4096$) hexamer sequences was determined for 10 000 independent promoters (−1 to −1,000) of Arabidopsis, rice, and soybean. Single random sampling ($n = 100$) of 1000 replicates was used to determine standardized promoters. We calculated sample means of frequencies of each hexamer sequence in the standardized promoters, and considered those sample means as observed frequencies in the standardized promoters (Supplementary Tables S13–S15). The most-frequent and second most-frequent hexamers were AAAAAA and TTTTTT, respectively, in standardized promoters of each species. The 10 most-frequent hexamers in standardized promoters of Arabidopsis were similar to those

in standardized promoters of rice and soybean, with only W (Weak 2H bonds) residues (Supplementary Tables S13–S15). The 10 least-frequent hexamers in standardized promoters of Arabidopsis and soybean were not similar to those in standardized promoters of rice, and they had only S (Strong 3H bond) residues. The promoter hexamer sequences with the lowest frequency were GGGCGC (Arabidopsis), CGCCCG (soybean), and GCGTTA plus other sequences with W residues (rice) (Supplementary Tables S13–S15).

To estimate over-represented hexamer sequences, first, expected frequencies were calculated using the base compositions of promoter sequences in each species (Supplementary Tables S16–S18). Then, the observed and expected frequencies of all hexamer sequences were compared (*Z*-test: $P < 0.05$). In Arabidopsis, GGCCCA had the highest (5.5) and TGGGCC had the second highest (5.4) fold-change between observed and expected frequencies (Supplementary Table S19). These were representative over-represented promoter hexamer sequences named site IIa, which act as *cis*-acting elements for basic helix-loop-helix or TCP-family transcription factors.[58,59] Additionally, several repeat sequences (TCTCTC, CTCTCT, AAAAAA, TTTTTT, GAGAGA, and AGAGAG) were over-represented in Arabidopsis promoters (Supplementary Table S19). Rice and soybean promoters were similar to those of Arabidopsis, but they also had hexamer sequences with S residues (GGGGGG, CCCCCC, GGGCCC, GCGGCG, GGCGGC, and CGCCGC) (Supplementary Tables S20 and S21).

3.5. *Distribution of promoter hexamer sequences in Arabidopsis, rice, and soybean*

To estimate distribution biases of promoter hexamer sequences, all hexamer sequences were localized in 10 000 independent promoters (from −1 to −1000) in Arabidopsis, rice, and soybean. The number of hexamer sequences was calculated and standardized for every 50 bases within promoters, and two major peaks (from −1 to −50 and from −51 to −100) were found. Distribution patterns of representative hexamer sequences are illustrated by heat maps (Fig. 3A–I).

The over-represented hexamer sequences (from −1 to −50) in Arabidopsis were similar to those in rice and soybean (Supplementary Tables S22–S24). The 10 most-frequent hexamers (Fig. 3A and B; TCTTCT, TATATA, TTCTTC, TCTCTC, CTCTCT, CTTCTT, TATAAA, TCTCTT, TTCTCT, and TTTCTT) in Arabidopsis were classified into two groups, one consisting of W residues (WR) and the other consisting of pyrimidine residues (YR). The frequencies of all possible ($2^6 = 64$) WR (Fig. 3A–C) and YR (Fig. 3D–F)

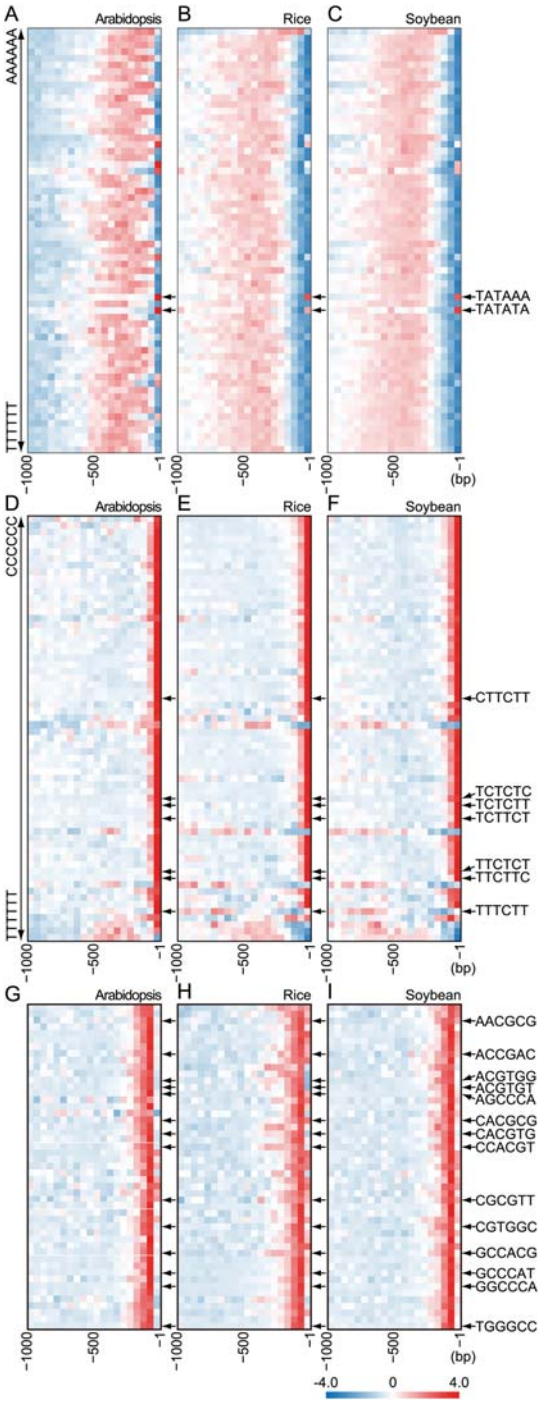

Figure 3. Heat maps of representative sequences in promoters of Arabidopsis, rice, and soybean genes. The number of hexamer sequences was calculated and standardized for every 50 promoter bases to show high (red) and low (blue) frequencies (*Z*-scores) in Arabidopsis, rice, and soybean. All ($2^6 = 64$) W (A–C) or Y (D–F) residue sequences in promoters are shown. Arrows in (A)–(F) indicate the 10 most-frequent hexamers identified (TTCTTC, TCTTCT, CTTCTT, TCTCTT, TTCTCT, TATAAA, ATATAA, TTTCTT, TATATA, TCTCTC). Several *cis*-acting element sequences like DRE (ACCGAC), ABRE (ACGTGG, ACGTGT, ACGTGG, CCACGT, CGTGGC, GCCACG), G box (CACGTG), CE3 (AACGCG, CACGCG), RE1 (GCCCAT), and site IIa (AGCCCA, GGCCCA, TGGGCC) were over-represented between −51 and −100 and are also marked by arrows (G–I).

hexamers in each species were determined and are presented as heat maps. Among the 64 WR in each species between −1 and −50, TATAAA and TATATA were over-represented while the others were under-represented in all species. The peak position of the TATATA sequence in promoters was at −33 (Arabidopsis), −32 (rice), and −31 (soybean) (Supplementary Fig. S1). Most YRs in each species were over-represented between −1 and −50, with peak positions near TATAAA and TATATA sequences. Similar YR hexamers have been reported to be over-represented as plant-specific sequences in Arabidopsis and rice promoters.[60] In each species, the over-represented hexamer sequences between −51 and −100 included several *cis*-acting elements (Fig. 3G–I; Supplementary Tables S25–S27), such as DRE (ACCGAC), ABRE (ACGTGG, ACGTGT, ACGTGG, CCACGT, CGTGGC, and GCCACG), G box (CACGTG), CE3 (AACGCG and CACGCG), RE1 (GCCCAT), and site IIa (AGCCCA, GGCCCA, and TGGGCC). In contrast, these sequences were under-represented between −501 and −1000. We also found several novel over-represented sequences in promoters from −51 to −100 (Fig. 3G–I; Supplementary Tables S25–S27), which were not present in the plant *cis*-acting regulatory DNA elements (PLACE) database.[61]

3.6. *Conserved promoter sequences of downstream genes of DREB1A, DREB2A, and AREB family*

We previously reported downstream genes of the transcription factors DREB1A and DREB2A using an oligo microarray.[23] In *35S:DREB1A* and *35S:DREB2A-CA* plants, 259 and 373 genes were significantly upregulated, respectively (FDR: $P < 0.05$; FC: >2). In our present study, we selected the 100 most highly upregulated downstream genes with promoter information and counted all promoter hexamer sequences. To determine conserved promoter sequences, the observed frequencies of all promoter hexamer sequences of downstream genes of DREB1A or DREB2A were compared with standardized promoters (*Z*-test: $P < 0.05$). The *Z*-scores of all hexamer sequences are illustrated by scatter plots (Fig. 4A; Supplementary Table S28). In promoters of downstream genes of DREB1A, the ACCGAC sequence had the highest *Z*-score (a core sequence of DRE was the most conserved), with 3.8-fold more ACCGAC sequences than in standardized promoters. DRE also had the sequence with the second highest *Z*-score (TGTCGG), and other parts of DRE sequences (GTCGGC and GCCGAC) were found in promoters of downstream genes of DREB1A. The third highest *Z*-score (ACGTGG) was part of ABRE, with 2.4-fold more ACGTGG sequences than in standardized promoters (Fig. 4A; Supplementary Table S28).

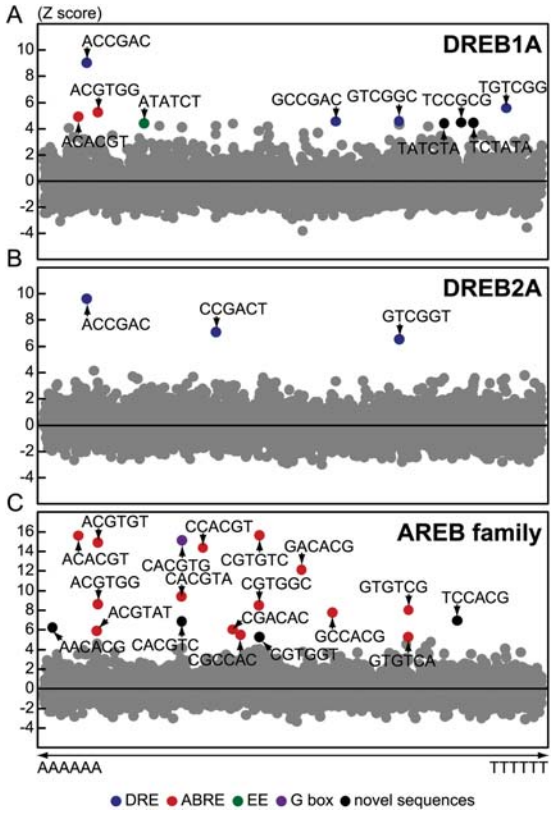

Figure 4. Scatter plots showing *Z*-scores (*y* axes) for the observed frequencies of all hexamer sequences (*x* axes) in promoters of downstream genes of DREB1A (A), DREB2A (B), and the AREB family (C) compared with standardized promoters. The highly conserved sequences DRE (blue), ABRE (red), EE (green), and G box (purple) are shown.

These results confirmed our previous findings showing that DRE and ABRE are conserved in promoters of downstream genes of DREB1A.[23] In addition, we found that a portion of Evening element (EE: ATATCT) was also highly conserved in promoters of downstream genes of DREB1A ((Fig. 4A; Supplementary Table S28). In promoters of downstream genes of DREB2A, DRE had the most conserved sequence (ACCGAC), with 3.9-fold more ACCGAC sequences than in standardized promoters. The second (CCGACT) and third (GTCGGT) highest *Z*-scores were also part of DRE (Fig. 4B; Supplementary Table S29). These results also confirmed our earlier finding that DRE is highly conserved in promoters of downstream genes of DREB2A.[26]

AREB1, AREB2, and ABF3 are members of the AREB family, which is a subgroup of the bZIP-type transcription factors, and they are key transcription factors under conditions of dehydration. Many downstream genes of the AREB family are expressed under dehydration stress in the *areb1 areb2 abf3* triple mutant.[39] In promoters of downstream genes of the AREB family, the most conserved sequence (CGTGTC in the ABRE motif) was 5.4-fold more abundant

than in standardized promoters. ABRE also had the sequence with the second highest *Z*-score (ACACGT), and other parts of ABRE and G box were highly conserved in promoters of downstream genes of the AREB family (Fig. 4C; Supplementary Table S30). Hence, our comprehensive promoter analyses could detect conserved sequences in a select promoter set.

3.7. *Conserved sequences in cold- and dehydration-inducible promoters of Arabidopsis, rice, and soybean*

In cold-inducible promoters of Arabidopsis, the most conserved sequence (ACCGAC in DRE) was 3.6-fold more abundant than in standardized promoters (Fig. 5A; Supplementary Table S31). The second most conserved sequence (AATATC) was part of EE (Fig. 5A; Supplementary Table S31). Several hexamers in DRE (CCGACA and GTCGGT), EE (ATATCT and GATATT), and ABRE (ACGTGG) sequences also were highly conserved in cold-inducible promoters of Arabidopsis (Fig. 5A; Supplementary Table S31). In dehydration-inducible promoters of Arabidopsis, the most conserved sequence (ACACGT), second most conserved sequence (ACGTGT), and other highly conserved sequences were part of ABRE and G box (CACGTG) (Fig. 5B; Supplementary Table S32).

In cold-inducible promoters of rice, the most (CGTACG) and second most (GTAGTA) conserved sequences were 2.8- and 2.1-fold, respectively, more abundant than in standardized promoters (Fig. 5C; Supplementary Table S33). These sequences were not registered with PLACE.[61] Other highly conserved sequences in cold-inducible rice promoters were part of ABRE (ACGTAC and GTACGT) (Fig. 5C; Supplementary Table S33). The dehydration-inducible rice promoters had the most (ACACGT in ABRE) and second most (CACGCG in CE3) conserved sequences, as well as other highly conserved ABRE (CGCCAC), CE3 (ACGCGT and CGCGTC), and novel (CCGATC, CGATCG, and GCACGC) sequences (Fig. 5D; Supplementary Table S34).

The cold-inducible soybean promoters had the most (ACACGT in ABRE; 2.6-fold more abundant than in standardized promoters) and second most (ACGCGT in CE3) conserved sequences, as well as other highly conserved sequences (ACGTGG in ABRE; CGCGTT in CE3; ATGTCG in DRE; and CACGTT, CCGTGT and AACACG sequences) (Fig. 5E; Supplementary Table S35). CACGTT is part of pABRE3, T/G box, QAR, WAR, and OsBP-5 binding site, all of which have been identified as *cis*-acting elements that regulate gene expression in response to ABA[62] or jasmonates.[63–65] The dehydration-inducible soybean promoters had the most

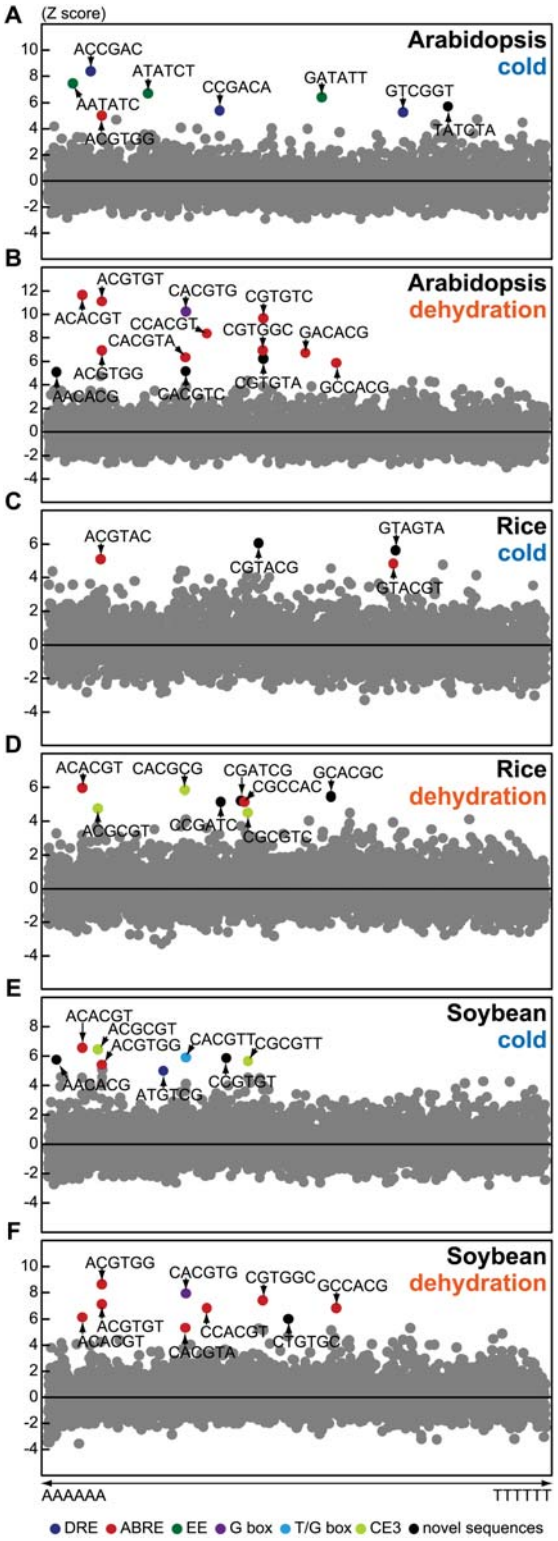

Figure 5. Scatter plots showing *Z*-scores (*y* axes) for the observed frequencies of all hexamer sequences (*x* axes) in cold-inducible (A, C, E) and dehydration-inducible (B, D, F) promoters in Arabidopsis (A, B), rice (C, D), and soybean (E, F) compared with standardized promoters. The highly conserved sequences of DRE (blue), ABRE (red), EE (green), G box (purple), T/G box (light blue), CE3 (light green), and novel sequences (black) are shown.

(ACGTGG in ABRE; 3.5-fold more abundant than in standardized promoters) and second most (CACGTG in G box) conserved sequences, and there were other highly conserved parts of ABRE (Fig. 5F; Supplementary Table S36).

3.8. *Six most-frequent core motifs known as* cis*-acting elements in cold- and dehydration-inducible promoters in Arabidopsis, rice, and soybean*

We found that several *cis*-acting elements were conserved in promoters of cold- and dehydration-inducible genes using our comprehensive promoter analysis. Then, we focused on the six most-frequent *cis*-acting elements (DRE, ABRE, EE, G box, CE3, and T/G box) in cold- or dehydration-inducible promoters and illustrated the *Z*-score of each *cis*-acting element using heat maps.

In cold-inducible Arabidopsis promoters, DRE and EE were highly conserved motifs, and ABRE, G box, and T/G box also were conserved; in contrast, in dehydration-inducible Arabidopsis promoters, ABRE and G box were highly conserved motifs, and DRE, EE, CE3, and T/G box also were conserved. The DRE and EE motifs occurred more frequently in cold-inducible promoters, whereas the ABRE and G box motifs occurred more frequently in the dehydration-inducible promoters (Fig. 6). In cold-inducible soybean promoters, ABRE and T/G box were highly conserved, and DRE, G box, and CE3 also were conserved. The observed frequencies of six representative motifs in dehydration-inducible soybean promoters were similar to those in dehydration-inducible Arabidopsis promoters. In dehydration-inducible soybean promoters, ABRE and G box were highly conserved, and DRE and T/G box also were conserved. ABRE and G box occurred more frequently in dehydration-inducible soybean promoters (Fig. 6). In our promoter analyses, there were no extremely highly conserved motifs in rice promoters similar to DRE or ABRE in cold- or dehydration-inducible Arabidopsis promoters. In cold-inducible rice promoters, ABRE, EE, and CE3 were conserved. In dehydration-inducible rice promoters, CE3 was a highly conserved motif, and DRE, ABRE, and G box were conserved. EE motifs were more frequent in cold-inducible rice promoters, and ABRE, G box, and CE3 motifs occurred more frequent in dehydration-inducible rice promoters (Fig. 6).

The observed frequencies of the six *cis*-acting elements in promoters of downstream genes of DREB1A were similar to those of cold-inducible promoters of Arabidopsis. In promoters of downstream genes of DREB1A, DRE and EE were highly conserved motifs, and ABRE and G box also were conserved. In contrast, the observed frequencies of the six *cis*-acting motifs in promoters of downstream genes of DREB2A were not similar to those of cold- or dehydration-inducible promoters of Arabidopsis. Only DRE was highly conserved in promoters of downstream genes of DREB2A. In promoters of downstream genes of the AREB family, ABRE and G box were highly conserved motifs, and DRE and CE3 were conserved (Fig. 6). The frequencies of the six *cis*-acting elements in promoters of downstream genes of the

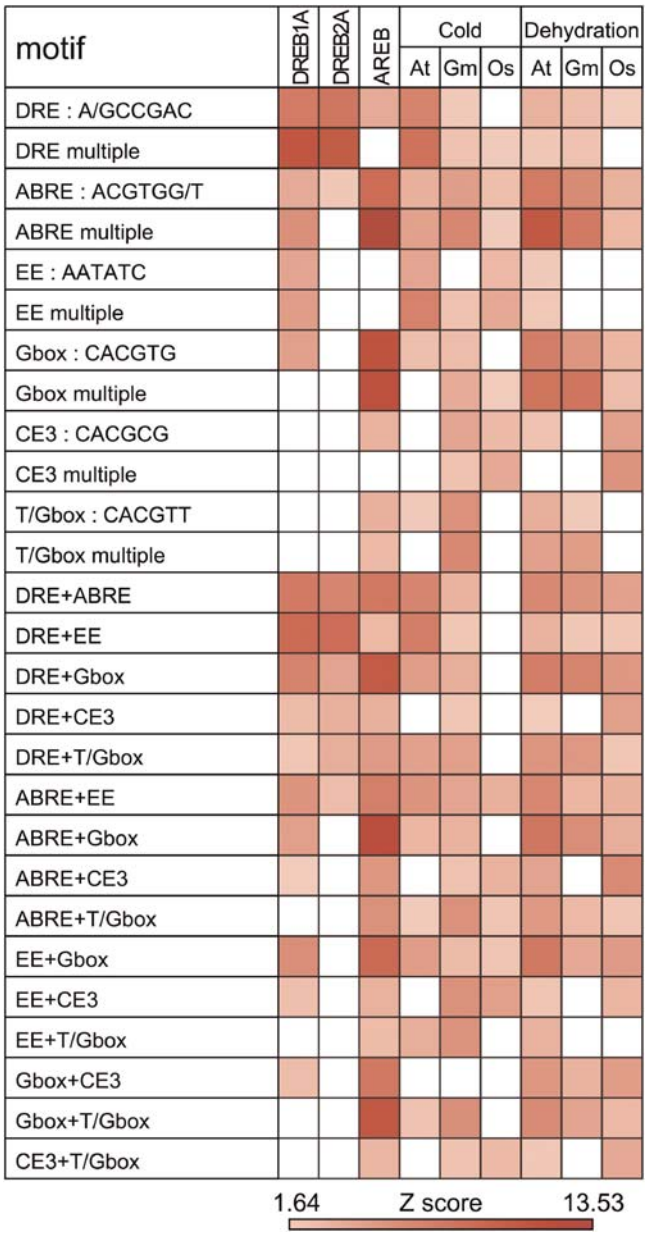

Figure 6. Heat maps of frequencies of the six most-frequent *cis*-acting elements (DRE: A/GCCGAC, ABRE: ACGTGG/T, EE: AATATC, G box: CACGTG, CE3: CACGCG, T/G box: CACGTT) in cold- or dehydration-inducible promoters of Arabidopsis (At), rice (Os), and soybean (Gm). Each small square indicates the conserved level of the *cis*-acting elements. Solid gray and white squares show conserved and non-conserved *cis*-acting elements, respectively (*Z*-test: $P < 0.05$).

AREB family were similar to those of dehydration-inducible promoters of Arabidopsis.

3.9. *Quantification of ABA in Arabidopsis, rice, and soybean exposed to cold or dehydration*

We found that ABRE was highly conserved in cold- and dehydration-inducible promoters of Arabidopsis, rice, and soybean. To clarify relationship between these ABRE sequences and ABA effects on the dehydration- and cold-inducible genes, we measured ABA levels in Arabidopsis, rice, and soybean plants exposed to cold or dehydration. ABA levels in cold-treated Arabidopsis and rice plants were 1.8- and 3.8-fold higher, respectively, than in untreated controls. In contrast, the level of ABA in soybean plants decreased under cold conditions and was 3.7-fold lower than in untreated controls. Under dehydration conditions, ABA levels increased 21.2-, 34.6-, and 5.1-fold in Arabidopsis, rice, and soybean, respectively, when compared with untreated controls (Supplementary Fig. S2).

4. Discussion

In this study, transcriptome and comprehensive promoter sequence analyses demonstrated general characteristics of promoter sequences in cold- or dehydration-inducible genes of Arabidopsis, rice, and soybean. Rice and soybean transcription profiles were similar to those of Arabidopsis, showing representative upregulated (dehydrin and LEA genes) and downregulated (photosynthesis-related genes) classes after exposure to cold or dehydration. In contrast, the profiles for cold- or dehydration-inducible promoter sequences displayed various characteristics in each species.

In our promoter analyses, DRE was a highly conserved motif in cold-inducible Arabidopsis promoters and in promoters of downstream genes of DREB1A. Indeed, 56% of cold-inducible promoters of Arabidopsis contain single or multiple DRE core sequences (25.6% standardized promoters of Arabidopsis contain single or multiple DRE core sequences). In addition, the observed frequencies of the six most-frequent *cis*-acting elements in cold-inducible Arabidopsis promoters were similar to those in promoters of downstream genes of DREB1A (Figs 4A, 5A, and 6). CaMV *35S* promoter-driven overexpression of DREB1A increases freezing stress tolerance in transgenic Arabidopsis.[17,19,20] Microarray analyses identified >100 downstream targets of DREB1A/CBF3, and gel mobility shift assays revealed that DREB1A has the highest affinity for the DRE core sequence.[21–25] These results suggest that the DRE/DREB1A-dependent transcriptional regulatory pathway plays an important role in cold-inducible gene expression in Arabidopsis.

In dehydration-inducible promoters of Arabidopsis and in promoters of downstream genes of DREB2A, DRE also was conserved, revealing that 41% of dehydration-inducible promoters of Arabidopsis contain single or multiple DRE core sequences. In Arabidopsis, overexpression of the constitutively active form of DREB2A (*35S:DREB2A-CA*) significantly increases dehydration tolerance, and microarray analyses revealed that DREB2A regulates the expression of many dehydration-responsive genes[26,27] by preferentially binding ACCGAC.[27] However, the observed frequencies of six representative *cis*-acting elements in dehydration-inducible promoters of Arabidopsis were not similar to those in promoters of downstream genes of DREB2A. These results suggest that DRE/DREB2A-dependent transcriptional regulation has a particular role in the expression of dehydration-inducible genes in Arabidopsis. DRE also was a conserved motif in dehydration-inducible soybean and rice promoters; 22% (soybean) and 49% (rice) of dehydration-inducible promoters contain single or multiple DRE core sequences compared with 12.7 (soybean) and 40.3% (rice) for standardized promoters. Orthologous DREB2A genes in soybean (Glyma06g03110, Glyma17g37350, Glyma14g06080) and rice (Os01g0165000, Os05g0346200) were significantly upregulated in plants exposed to dehydration (Supplementary Tables S2 and S4). These results suggest that DRE-dependent gene expression also occurs in both soybean and rice in response to dehydration.

ABRE was a highly conserved motif in dehydration-inducible promoters of Arabidopsis, soybean, and rice (Figs 5B, D, F and 6) and in promoters of downstream genes of the AREB family (Figs 4C and 6). Notably, 82 (Arabidopsis), 70 (soybean), and 71% (rice) of dehydration-inducible promoters contain single or multiple ABRE core sequences compared with 44.8 (Arabidopsis), 39.5 (soybean), and 53.5% (rice) for standardized promoters. The ABRE motif is a *cis*-acting element for the AREB family transcription factors, including Arabidopsis AREB/ABFs and ABI5 and rice TRAB1 proteins, which can bind to ABRE and activate dehydration-inducible gene expression.[28–35,66] The *areb1 areb2 abf3* triple mutant has impaired dehydration-inducible gene expression and reduced dehydration stress tolerance.[39] The AREB/ABF genes were reported to be induced by dehydration in Arabidopsis plants,[36] and our microarray analyses revealed that AREB/ABF orthologs in soybean (Glyma19g37910, Glyma03g00580, Glyma04g04170, Glyma13g03880, Glyma10g08370) and rice (Os02g0766700: OsAREB1, Os01g0859300: OsAREB3, Os06g0211200: OsAREB8,

Os08g0472000: TRAB1) were significantly upregulated in response to dehydration (Supplementary Tables S2 and S4). Hence, ABRE/AREB family-dependent gene expression likely plays a major role in dehydration-inducible gene expression in Arabidopsis, soybean, and rice.

In our study, ABRE was also conserved in cold-inducible promoters of Arabidopsis, rice, and soybean (Figs 5A, C, E), and 62 (Arabidopsis), 63 (soybean), and 67% (rice) of cold-inducible promoters contain single or multiple ABRE core sequences. Expression levels of several AREB/ABFs and their orthologous genes, including Arabidopsis At1g49720: ABF1 and At4g34000: ABI3 and rice Os02g0766700: OsAREB1 and Os07g0686100: OsAREB7, were slightly increased in response to cold (Supplementary Tables S1 and S3). The ABRE functions as a *cis*-acting element that regulates ABA-responsive gene expression.[5,9] We showed that ABA levels increased significantly in all three species in response to dehydration, but they decreased (soybean) or slightly increased (Arabidopsis and rice) in response to cold (Supplementary Fig. S2). These results suggest that cold-inducible ABRE-dependent gene expression occurs in Arabidopsis, rice, and soybean, but most of this expression is independent of ABA in the three species, especially soybean.

EE was a highly conserved motif in cold-inducible Arabidopsis promoters and had a higher observed frequency in cold-inducible than in dehydration-inducible promoters in all three species (Fig. 6). In Arabidopsis, EE has a *cis*-acting element for CCA1 and LHY transcription factors, which are related to circadian clock-regulated genes that control cold-inducible gene expression.[67–70] Orthologous rice genes for CCA1 and LHY have similar expression patterns as in Arabidopsis.[71] Hence, EE-dependent transcription likely regulates cold-inducible gene expression more than dehydration-inducible gene expression in Arabidopsis, rice, and soybean. We identified novel highly conserved CGTACG and GTAGTA sequences in cold-inducible rice promoters that were not highly conserved in cold- or dehydration-inducible promoters in Arabidopsis or soybean (Fig. 5). These sequences have never been reported as *cis*-acting elements for cold- or dehydration-inducible gene expression, and thus they are novel candidates for *cis*-acting elements involved in cold-inducible gene expression in rice.

Several cold- or dehydration-inducible promoters contained one or more *cis*-acting elements. DRE and ABRE have an interdependent relationship in the *rd29* promoter.[14] We analysed the colocalization of six kinds of *cis*-acting elements in cold- or dehydration-inducible promoters of Arabidopsis, rice, and soybean (Fig. 6). In cold-inducible promoters of Arabidopsis, the most and the second most colocalized motifs were DRE + EE and DRE + ABRE, respectively. In addition, promoters of downstream genes of DREB1A had similar colocalized motifs to cold-inducible promoters of Arabidopsis (Figs 4A and 6). Several downstream genes of DREB1A also are induced by dehydration or regulating by the circadian clock.[23] In cold-inducible gene expression in Arabidopsis, parts of the DRE-dependent transcriptional regulatory pathway overlapped with the ABRE-dependent and EE-dependent pathways. Multiple ABRE motifs are necessary for the expression of ABA-inducible genes in seeds and vegetative tissues. Either an additional copy of ABRE or coupling elements is necessary for ABA-inducible gene expression.[10–15] Most of the known coupling elements are similar to ABREs and contain an ACGT motif. The representative coupling element, CE3, was identified using the promoters of barley or rice ABA-inducible genes.[12,13] In our analyses of dehydration-inducible promoters in Arabidopsis and soybean, the ABRE motif was highly co-localized with its likely coupling elements ABRE, DRE, G box, or EE (Fig. 6). In dehydration-inducible promoters in rice, the ABRE motif was highly co-localized with CE3 (Fig. 6), suggesting that the ABRE coupling elements in monocots differ from those in dicots.

Based on our comprehensive promoter sequence analyses, we can estimate the evolution of dehydration- or cold-responsive transcriptional pathways. The dehydration-inducible promoter sequence profiles displayed similar characteristics in Arabidopsis, rice, and soybean. ABRE is the most conserved sequence in dehydration-inducible promoters of all three species (Figs 5B, D, F and 6). In response to dehydration, ABA levels increased in all three species (Supplementary Fig. S2). It has been reported that most dehydration-inducible genes are also induced by ABA,[3,72] and ABA is thought to be involved in dehydration-inducible gene expression in all land plants.[3,72,73] These results suggest that the ABA-responsive ABRE-dependent transcriptional pathway is fundamental to dehydration-responsive gene expression in land plants, including Arabidopsis, rice, and soybean, and that it arose during land colonization of plants before the divergence of monocots and dicots. In contrast, the cold-inducible promoter sequence profiles displayed different characteristics among the three species we studied (Figs 5 and 6), suggesting that cold-responsive transcriptional pathways diversified after land colonization of plants.

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