Regulating the Drought-responsive Element (DRE)-mediated Signaling Pathway by Synergic Functions of Trans-active and Trans-inactive DRE Binding Factors in *Brassica napus*

Received for publication, September 26, 2005, and in revised form, February 21, 2006. Published, JBC Papers in Press, February 23, 2006, DOI 10.1074/jbc.M510535200

**Tong-Jin Zhao**†‡, **Shan Sun**†§, **Yang Liu**‡, **Jing-Mei Liu**‡, **Qiang Liu**‡, **Yong-Bin Yang**†‡† and **Hai-Meng Zhou**†‡‡

From the †Department of Biological Sciences and Biotechnology, the ‡State Key Laboratory of Biomembrane and Membrane Biotechnology, and the ‡‡Protein Science Laboratory of the Ministry of Education, Tsinghua University, Beijing 100084, and the ††Yangtze Delta Region Institute of Tsinghua University, Jiaxing 314050, Zhejiang, China

DREB1/C-repeat binding factor (CBF) is a plant-specific family of transcription factors and plays a crucial role in freeze tolerance. In the present work, two groups of drought-responsive element binding factor (DREB)-like genes were isolated from *Brassica napus*, named Group I and Group II. The two groups of genes were both induced by low temperature, but the expression of Group I preceded that of Group II. The Group I DREBs could specifically bind with the DRE cis-acting element and activate the expression of downstream genes, but Group II factors were trans-inactive although they still had the ability to bind with DRE, which was confirmed by electrophoretic mobility shift assay. Fluorescence quenching assays indicated that the DRE binding ability of the two groups was similar. Co-expression of Group II could depress the trans-activation activity of Group I DREBs in a concentration-dependent manner. These results strongly suggested that the trans-active Group I DREBs were expressed at the early stage of cold stress to open the DRE-mediated signaling pathway in cold stress, whereas the trans-inactive Group II DREBs were expressed at the later stage to close the signaling pathway in a competitive manner. The results herein provide a new insight into the regulation mechanisms of the DRE-mediated signaling pathway in response to cold stress.

Low temperature is a major factor limiting the geographical locations of many crops and horticultural plants, and it can usually result in significant losses in plant productivity (1). Many plants have increased tolerance to freezing temperatures after exposure to low non-freezing temperatures, a process known as cold acclimation (2–4). A key function of cold acclimation is to acquire tolerance to multiple forms of membrane damage due to freeze-induced cellular dehydration including expansion-induced-lysis, lamellar-to-hexagonal-II phase transitions, and fracture jump lesions (1, 5–7). A large number of genes have been identified to be cold-inducible and to function in freeze tolerance (1).

In 1994, a drought-responsive element (DRE) was identified to be involved in responsiveness to drought, low temperature, and high salt stress (8), and a similar cis-acting element, C-repeat (CRT), was also reported (9). A family of transcriptional factors known as DREBs or CBFs has been reported to bind with this cis-element and activate the transcription of the downstream genes related to cold, drought, and high salinity (10, 11). Overexpression of DREB1/CBF1 or DREB1A/CBF3 resulted in strong expression of stress-inducible genes, and the transgenic plants acquired higher tolerance to drought, low temperature, and high salinity (11–14). Therefore, DREB1/CBF genes play a central role in tolerance to abiotic stresses.

Since the first report about the cloning of CBF1 from *Arabidopsis thaliana* (10), many studies have been carried out on the regulation mechanisms of this special gene family to promote better understanding of the responses of the plants to abiotic stresses. Genetic screening of *Arabidopsis* plants expressing the firefly luciferase gene driven by the DRE/CRT-containing rd29A promoter (15) has identified several mutants with abnormal expression of cold-inducible genes. Detailed analysis of these mutants revealed that HOS1 (16), LOS1 (17), LOS2 (18), and LOS4 (19) could indirectly affect the expression of DREB1/CBF and thus lead to the deregulated expression of downstream genes. Genetic analysis of the *Arabidopsis* plants expressing the firefly luciferase gene under the control of the *DREB1A/CBF3* promoter indicated that ICE1, a Myc-like basic helix-loop-helix transcriptional activator, could bind with the promoter of *DREB1A/CBF3* and regulate the transcription of *DREB1/CBF* (20). Further evidence of the direct regulation of the DRE/CRT-mediated signaling pathway was that DREB1/CBF2 could function as a negative regulator of CBF1/DREB1B and DREB1A/CBF3 expression (21). These studies contributed much to an understanding of the regulation of the signaling pathway mediated by DREB1/CBF. That is, the expression of stress-response genes (CBF1/DREB1B and DREB1A/CBF3) can be regulated at the transcriptional level. However, it is not clear yet whether or not there is a molecular mechanism to shut down the DREB1/CBF-mediated stress-response pathway at the protein level in higher plants.

In this research, two distinct groups of DREBs were cloned from the cold-induced cDNA library of *Brassica napus* and were named Group I and II, respectively. The two groups of genes had high sequence homology, except that Group I DREBs had two additional insertions in the C-terminal region. Analysis of the expression level and the trans-active

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**8** This work was supported by funds from the National Key Science and Technology Item of China (Grants 2002AA224091 and 2005AA224090), the National Key Basic Research Specific Foundation of China (Grant 1999075607), and the Natural Science Foundation of China (Grants 30221003 and 60401009) and by funds from Jiaxing, Zhejiang. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank/‡EBI Data Bank with accession number(s) AY437878, AY444874, AY444875, AY444877, and AY444876.

1. To whom correspondence may be addressed. Fax: 86-10-6277-1597; E-mail: yiyang@tsinghua.edu.cn.

2. To whom correspondence may be addressed. Fax: 86-10-6277-2245; E-mail: zhm-dbs@tsinghua.edu.cn.

3. The abbreviations used are: DRE, drought-responsive element; DREB, DRE binding factor; CRT, C-repeat; CBF, CRT binding factor; WDR1, wide-type DRE element with the sequence 5’-AGCTTTTTGACCAAGGC-3’; MDRE, mutated DRE element with the sequence 5’-AGCTATTTCATAGGCG-3’; PDI, protein disulfide isomerase; MES, 4-morpholineethanesulfonic acid; 3-AT, 3-amino-1,2,4-triazole; EREBP, ethylene-responsive element-binding protein; SD, synthetic dropout.
activity of these two groups of genes indicated that they functioned in synergy to regulate the DRE-mediated signaling. The trans-active Group I DREBs were expressed at the early stage of cold stress and opened the signal pathway, and then the inactive Group II DREBs were expressed, which competed with the Group I DREBs and closed the stress-induced signaling. The results herein should improve our understanding of the regulation mechanisms of DREB1/CBF-mediated signal transduction pathways at the protein level.

**EXPERIMENTAL PROCEDURES**

**Plant Materials and Stress Treatments**—Seeds of *B. napus* linghong H15 were sterilized and were germinated in germination medium (Murashige and Skoog medium) solidified with 0.8% (w/v) agar. The plants were grown at 25 °C under a long day photoperiod (16 h of cool white fluorescent light). Low temperature treatment was performed on 2-week-old plants. The plants were transferred into a growth chamber set to 4 °C for different periods of time.

**Isolation and Identification of the Two Groups of DREBs**—According to the published DREB1/CBF sequences, nested primers were designed to amplify the sequence of the conserved AP2/EREBP domain from the cold-induced cDNA library of *B. napus*. Then 5'-rapid amplification of cDNA ends and 3'-rapid amplification of cDNA ends were conducted to get the 5'- and 3'-end of the cDNAs according to the manufacturer’s instructions (Takara, Japan). Gene-specific primers were designed to amplify the two groups of DREBs.

**Reverse Transcription and Real-time PCR Analysis**—For RNA analysis, 2-week-old seedlings were subjected to cold stress for various periods. Total RNA was extracted as described previously (22) for preparation of cDNA. I-5 and II-1 (see Fig. 1) were selected to detect the expression levels during cold stress. Actin was amplified as an internal control. The primers used were: BnI-For, 5'-TATGAACCTAGCT-CTACTTCTTGC-3'; BnI-Rev, 5'-CTATAATTCGAGTTTATTGTC-3'; BnII-For, 5'-GATTTAGCCTATTTTTC-3'; BnII-Rev, 5'-ATAACTCAAGGGCACGTC-3'; actin-For, 5'-CATGTTCAGAACCTTGAC-3'; and actin-Rev, 5'-CTTGTATCCATGTCGTTG-3'. The amplified products were separated on 1.2% agarose for quantitative analysis.

Quantitative real-time PCR using SYBR Green I dye was performed on Mx3000PTM (Stratagene, La Jolla, CA). Each sample was run in triplicate. Data were analyzed according to the threshold cycle (Ct) method (23). The amount of the target genes, normalized to the internal reference actin, is given in 2^-ΔΔCt. The amount of I-5 transcripts accumulated after 0.5 h of cold stress was taken as 1.0.

**DNA Binding Analysis of the Two Groups of Genes**—The full-length genes of I-5 and II-1 were fused with protein disulfide isomerase (PDI), a molecular chaperone, to produce I-5-PDI and II-1-PDI fusion proteins, as described previously (24). The recombinant proteins were purified and were demonstrated to be homogenous on 10% SDS-PAGE. Protein concentration was determined according to Bradford (25), using bovine serum albumin as a standard.

Electrophoretic mobility shift assay was carried out using 0.5 μg of DNA elements and 15 μg of PDI fusion proteins. The wild-type DRE (WDRE) and the mutated DRE (MDRE) elements were synthesized as duplex with the following sequences: WDRE, 5'-AGCTACCGACATAAGGC-3', and MDRE, 5'-AGCTATTTTCTACAGGC-3'. The proteins and the cis-element were dissolved in 30 mM Tris-HCl buffer (pH 8.0) and equilibrated at room temperature for 20 min. Then the samples were loaded onto an 8% native polyacrylamide gel. After electrophoresis in 0.5 × Tris-borate-EDTA buffer, the gel was stained with ethidium bromide for visualization of DNA bands.

Quantitative analysis of the binding abilities of I-5-PDI and II-1-PDI was monitored by the quenching of the intrinsic tryptophan fluorescence spectra (24, 26). The samples were prepared according to the same method used for the electrophoretic mobility shift assay analysis samples. Fluorescence spectra were collected on an F-2500 spectrofluorometer using a 1-ml cuvette, with excitation at 280 nm and emission at 300–400 nm. The apparent binding constants of I-5-PDI and II-1-PDI for WDRE were calculated as described previously (26).

** Yeast One-hybrid Assay**—To detect the trans-activation ability of the two groups of DREBs, the coding regions of the genes were cloned into the yeast expression vector YepGAP (11). The constructs were transformed into the yeast harboring the dual reporter genes HIS3 and LacZ under the control of tandem repeats of WDRE or MDRE as described previously (11). The transformants were cultured on SD/-His/-Trp/–Ura with or without 30 mM 3-AT. Colony-lift filter assay and quantitative β-galactosidase activity analysis were carried out according to the *Yeast Protocols Handbook* (36) (Clontech), using o-nitrophenyl p-galactopyranoside as a substrate.

**Co-transformation of I-5 and II-1 in Yeast**—To detect the effect of the expression of II-1 on the trans-activation role of I-5, we constructed a co-transformation yeast one-hybrid system, including two effective vectors, pGBKTK7 (Clontech) and pGBDL, and the reporter yeast (11), as shown in Fig. 4A. pGBKTK7 was constructed by replacing the GAL4-AD of pGADT7 (Clontech) with the GAL4-BD of pGBKTK7. Then genes constructed into these two vectors should be expressed at the same level since they are both fused to GAL4-BD and driven by the promoter of ADH1. The effector plasmids were constructed by inserting I-5 or II-1 downstream of the GAL4-BD of pGBKTK7 or pGBDL. The co-transformants were grown on SD/-His/-Trp/-Leu/–Ura. Quantitative analysis of β-galactosidase activity was performed as described above.

**Western Blot Assay**—I-5 and II-1 were cloned into the pET28a (Novagen) and expressed in *Escherichia coli* BL21(DE3)-pLysS (Stratagene). The expressed proteins were mostly in the inclusion bodies, and the guanidine-denatured proteins were purified by nickel-nitritolriatic acid agarose (Qiagen). The purified proteins were used to prepare mouse monoclonal antibody. Yeast protein preparation was according to the *Yeast Protocols Handbook* (Clontech). Western blot was carried out after 10% SDS-PAGE, and horseradish peroxidase-labeled rabbit anti-mouse antibody was used as the secondary antibody.

**Transient Expression Assay**—To detect the trans-active activity of the two groups of genes, a dual reporter system was constructed, as shown in Fig. 6. For the F reporter construct, the cis-acting element DRE was multimerized three times, placed upstream of the minimal TATA box from the cauliflower mosaic virus 35S promoter, and fused transcriptionally to the firefly luciferase gene (Promega). For the R reporter construct, the β-glucuronidase (GUS) gene in pBI221 (Clontech) was replaced with the *Renilla* luciferase gene (Promega). The tobacco mosaic virus Ω sequence (27) was inserted downstream of the cauliflower mosaic virus 35S promoter in pBI221 to enhance the transcription. For effector plasmids, the construct was similar to that of the R reporter, except that the genes used were I-5 and II-1 instead of *Renilla* luciferase.

The tobacco protoplasts were isolated from the BY2 suspension cultures in a digestion buffer (0.4 M mannitol, 1% cellulase “onozuka” R-10, 0.1% pectolyase, 8 mM CaCl2, 5 mM MES-KOH, pH 5.8). The mixture was shaken at 40–50 rpm in the dark for 4–5 h until most of the cells became single protoplasts. Transient expression assays were performed by polyethylene glycol-mediated DNA transformation as described previously (28). 10 μg of the F reporter, 0.5 μg of the R reporter, and 10 μg of the effector plasmid (if not stated elsewhere) were used for each
FIGURE 1. Sequence analysis of the DREB1/CPF proteins. A, alignment of the amino acid sequences of the seven genes cloned from B. napus. B, a phylogenetic comparison of all known DREB1/CPF proteins. The full length of each protein was used for phylogenetic tree analysis. The GenBank accession numbers of the genes used here are: BnCBF16 (AF499033), BnDREB1-1 (AY437878), BnCBF7 (AF499032), BnDREB1-19 (AY444877), BnDREB1-2 (AY444874), BnDREB1-3 (AY444875), BnCBF5 (AF499031), BnDREB1-23 (AY444876), TdDREB1 (AA506621), BnCBF17 (AF499034), BnCBF (AAL38242), BoCBF1 (AF370731), BoCBF2 (AF370732), BnCBF17 (AA318199), ADREB1C (AB007789), ADREB1A (AB007787), CaCBF5 (AAP35030), CbCBF (AAP35030), ApCBF (ABA42927), ChcbCBF2 (aay21896), AtDREB1B (ab007788), BnCBF1 (ABY443213), EgCBF1A (AB851637), EgCBF1B (AB851638), Avr9-111a (AAG43549), Avr9-111b (AAG43549), MdCBF (ABR88363), MdCBF (AB88400), LeCBF3 (AA577819), VcCBF4 (AAWSB104), GmCBF
transformation. The Renilla and firefly luciferase activities were measured according the manufacturer’s instructions (Promega, Dual-Luciferase reporter assay system).

RESULTS

Sequence Alignment and Phylogenetic Analysis of the DREB1/CBF Proteins—In the present work, seven DREBs were obtained from the library of cold-induced cDNA of B. napus. Sequence alignment (Fig. 1A) showed that the genes could be divided into two groups, Group I (including I-4 and I-5) and Group II (including II-1, II-2, II-3, II-19, and II-23). Group I had a sequence of about 750 bp, whereas Group II had a sequence of about 650 bp. The two groups of genes showed high homology in their sequences, except that Group I had two additional insertions in the C-terminal region. The genes in each group had greater than 90% homology. The genes of Group I are quite similar to the previously known DREB1/CBF proteins (Fig. 1B) and firefly luciferase activities were measured according the manufacturer’s instructions (Promega, Dual-Luciferase reporter assay system).

According to the classification of DREBs based on amino acid sequence (31), these two groups of DREBs belong to the DREB1/CBF subfamily. To fully comprehend the occurrence of these proteins, we searched GenBank™ and made a phylogenetic comparison of all known DREB1/CBF proteins (Fig. 1B). As can be expected, the two groups of genes are classified into two clusters, which are in the same phylogenetic clade containing some other DREB1/CBF genes from crucifer, such as AtDREB1A-C from Arabidopsis, ChCBF25 from shepherd’s purse, and ApCBF from Arabis pumila. It should be noted that in addition to B. napus, some other species also have DREB1/CBF genes belonging to different clusters. In Arabidopsis, AC010795 and AC025417 are in a different phylogenetic clade than AtDREB1A-C. In Oryza sativa, OsDREB1A and OsCBF1 are in a different cluster from OsDREB1E, OsDREB4, and OsCBF1like. In Vitis vinifera, VvCBF4 is in a different phylogenetic clade than the other four VvCBFs. In addition, the DREB1/CBF genes in Hordeum vulgare and Triticum aestivum are also classified into two different clusters. Therefore, the occurrence of these two groups of DREB1/CBF genes might be common in plant, implying that these two groups of genes might have different functions in vivo.

The Expression of Group I Genes in Response to Cold Preceded That of Group II—To explore the expression patterns of the two groups of genes in response to cold stress, total RNAs were extracted from cold-stressed B. napus at different periods. I-5 and II-1 in each group were selected for analysis by reverse transcription (Fig. 2A) and real-time PCR (Fig. 2B), with actin amplified as an internal control. The transcripts of I-5 started to accumulate immediately after cold treatment was begun and reached the maximum at 1.5 h. Subsequently, the transcripts decreased rapidly and disappeared after 4.5 h. II-1 transcripts accumulated at a much slower rate and reached the maximum at about 4–5 h, and then gradually declined. These results indicated that the expression of Group I in response to cold preceded that of Group II, similar to what has been reported in an earlier study of the expression patterns of similar genes (30). A similar phenomenon was reported in another study, in which the expression of Arabidopsis CBF1/DREB1B and DREB1A/CBF3 preceded that of DREB1C/CBF2 (21).
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The Genes of Group I Were Trans-active, whereas Those of Group II Were Inactive—As mentioned above, genes similar to those identified here have been reported to be able to bind with the DRE/CRT element and to activate the downstream genes when they are fused to the activation domain of GAL4 (30). However, it is not clear yet whether these genes are trans-active. Thus a yeast one-hybrid experiment was carried out to test the trans-activation abilities of the two groups of genes (Fig. 3A). The reporter yeasts containing WDRE transformed with I-5 grew well on both 3-AT-containing and -non-containing plates (Fig. 3A and B), and the yeasts showed good expression of the reporter LacZ gene (Fig. 3, C and D). Moreover, the reporter yeasts containing MDRE transformed with I-5 could neither grow on the 30 mM 3-AT medium plates (Fig. 3B) nor show the expression of LacZ (Fig. 3C). These results indicated that I-5 could specifically bind with WDRE and activate the expression of the downstream genes.

In contrast, transformants harboring II-1 could neither grow on the plates containing 3-AT (Fig. 3B) nor show the expression of LacZ (Fig. 3C and D). To confirm that the results here were not specific to II-1, the other members were also tested, and the results were identical to those observed in II-1 (data not shown). Thus from the results above, Group II, in the DRE-specific reporter system, was trans-inactive, but it was not clear whether the trans-inactivation was due to its failure to bind with DRE or lack of trans-active ability. Sequence alignment revealed that the Group II genes contain the conserved DNA binding domain AP2/EREBP, and therefore it is expected that the failure of Group II to activate the downstream genes might be due to the lack of trans-activation activity instead of its inability to bind with DRE. To further confirm this deduction, a chimera IINIC was constructed by fusing the N-terminal region including the AP2/EREBP domain of II-1 with the C-terminal region of I-5 without the AP2/EREBP domain. Similar to the I-5 protein, IINIC also had the ability to bind with the DRE element and activate the expression of its downstream genes, indicating II-1 had the ability to bind WDRE. In fact, II-1, when fused to GAL4-AD, did trans-activate the downstream genes in yeasts (data not shown), which also confirmed that Group II was able to bind with the DRE element. To verify that the inability of II-1 to activate the downstream reporter genes was not due to its failure of expression in the yeasts, Western blot was carried out in yeasts harboring WDRE (Fig. 3D, inset). Due to the high sequence similarity, the monoclonal antibody against I-5 can recognize the II-1 protein and vice versa. The antibody against II-1 was used in the following assay. As is shown in Fig. 3D, inset, II-1, I-5, and the chimera protein IINIC were all expressed in the corresponding yeasts. These results confirmed the deduction that Group II DREBs were able to bind with WDRE but were unable to activate the downstream genes.

I-5 and II-1 Showed Similar Efficiency for Binding with WDRE—Electrophoretic mobility shift assay (Fig. 4A) and fluorescence quenching assay (Fig. 4B) were carried out to characterize the DNA binding ability of I-5 and II-1 more precisely. To increase the solubility of the recombinant proteins, I-5 and II-1 were expressed in the PDI fusion system constructed by us previously (24), and the PDI fusion proteins, I-5-PDI and II-1-PDI, were used in the following assays. Two stranded WDRE and MDRE elements were used to test the binding specificity of the recombinant proteins. As shown in Fig. 4A, both I-5-PDI and II-1-PDI can specifically bind with WDRE. As a control, PDI can bind with neither WDRE nor MDRE. The results here further confirmed the above conclusions that both Group I and Group II had the ability to bind with WDRE.

Fluorescence quenching was carried out to quantify the binding affinities of I-5 and II-1 with wild-type DRE (Fig. 4B). The intrinsic fluorescence of both I-5-PDI and II-1-PDI were quenched gradually with the increase of WDRE until saturation, and finally, about 20% of the intrinsic fluorescence intensity would be quenched. As calculated according to the previous method (26), the apparent binding constants for WDRE of I-5-PDI and II-1-PDI were (2.4 ± 0.4) × 10^7 M⁻¹ and (1.8 ± 0.2) × 10^7 M⁻¹, respectively. These results indicated that the affinity of Group II to bind with WDRE was similar to, or slight weaker than, that of Group I.
Co-expression of II-1 Decreases the Trans-activation Activity of I-5—
The results above clearly indicated that Group II genes were expressed at a later stage upon cold stress (Fig. 2) and were trans-inactive (Fig. 3). Then what is the physiological role of Group II genes? Do the later expressed Group II genes affect the trans-activation activity of the Group I genes? To explore these questions, a yeast co-transformation system was constructed, including two effectors, pGBKTK7 and pGBDL (Fig. 5A). I-5 and II-1 were constructed to pGBKTK7 and pGBDL, named I-5/pGBKTK7 and II-1/pGBDL, respectively. The two constructs were then co-transformed into the reporter yeast harboring the WDRE, and the transformants were grown on SD/-His/-Ura/-Trp/-Leu. Quantitative assay of β-galactosidase activity was performed to investigate the effect of II-1 on the trans-activation activity of I-5 (Fig. 5B). Taking the β-galactosidase activity of the transformants harboring only I-5/pGBKTK7 as 100%, yeasts transformed with II-1/pGBDL showed almost no expression of β-galactosidase, consistent with the results above (Fig. 4B). When the yeasts were co-transformed with II-1/pGBDL and I-5/pGBKTK7, the activity of β-galactosidase decreased to 35.9% (Fig. 5B). To confirm that the inhibition effect was due to the expression of II-1, pGBDL was co-transformed with I-5/pGBKTK7, and the results showed that the β-galactosidase activity decreased only a little (Fig. 5B). The GAL4-BD fusion proteins were detected in the yeasts harboring different constructs. As shown in Fig. 5B, inset, GAL4-BD fusion proteins of I-5 and II-1 were almost equally expressed in the yeast transformed with II-1/pGBDL and I-5/pGBKTK7. Therefore, it can be concluded that the co-expression of II-1 decreased the trans-activation activity of I-5. This conclusion was also confirmed by the results that co-expressed II-1/pGBKTK7 could still depress the trans-activation activity of I-5/pGBDL (data not shown).

FIGURE 5. Co-expression of II-1 decreased the trans-activation activity of I-5. A, the yeast co-transformation system. pGBKTK7 and pGBDL are the two effector plasmids used here. The WDRE-containing yeasts were used here, as mentioned in the legend for Fig. 3. B, quantitative β-galactosidase activity assay of the yeasts transformed by the corresponding constructs. The trans-activation activity of I-5/pGBKTK7 was taken as 100%. The inset shows Western blot analysis of the expression of the GAL4-BD fusion proteins in yeasts transformed with corresponding constructs.

To further confirm the inhibition effect of II-1 on the trans-activation of I-5, a dual reporter system was constructed for transient expression assay in tobacco BY2 protoplasts, consisting of the F reporter, R

FIGURE 6. Transient expression assay of the trans-activation activity of the two groups of genes. A, the dual reporter system constructed for transient expression assay, including R reporter (containing Renilla luciferase (R Luc reporter gene), F reporter (containing firefly luciferase (F Luc) reporter gene), and the effector plasmids. B, co-expression of II-1 decreased the trans-activation activity of I-5 in tobacco BY2 suspension cells. 35S-I was the control effector plasmid without DREBs. I-5 and II-1 represent the effector plasmids containing I-5 and II-1, respectively. 1:0.1, 1:0.2, 1:0.5, and 1:1 are the ratios of I-5 effector plasmid and II-1 effector plasmid used in the transient expression assay.

FIGURE 4. Electrophoretic mobility shift assay (A) and fluorescence quenching (B) analysis of the DRE binding affinities of I-5 and II-1. A, WD and MD are synthesized duplexes of WDRE and MDRE, respectively. B, recombinant proteins I-5-PDI (closed circles) and II-1-PDI (open circles) were used for fluorescence quenching with WDRE.
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DISCUSSION

Although much research has been conducted since the cis-acting element DRE was first identified (8), detailed mechanisms by which the DREB1/CBF family is regulated are still unclear. The induction of these genes has been proposed to be regulated at the transcriptional level, and it is possible that they are not subject to autoregulation (14, 32). Recently, a transcriptional activator, named ICE1, was identified to be the upstream gene of DREB1A/CBF3 (20). It has also been reported that the DREB1/CBF gene expression is controlled, at least in part, by the DREB1/CBF themselves because DREB1/CBF2 has been identified to function as a negative regulator of CBF1/DREB1B and DREB1A/CBF3 expression (21). In addition, the transcription of DREB1/CBF has also been suggested to be under feedback repression by its own gene product or its downstream target gene products (17). According to the sequence features (Fig. 1) and the response to cold stress (Fig. 2), the two groups of genes reported here and the previously reported BnCBFs (21, 22) belong to the DREB1/CBF family. Also, the occurrence of these two groups of genes seemed be common in plants according to phylogenetic analysis because the DREB1/CBF genes reported in other species can also be divided into two different groups similar to the two groups defined here. Since the DREB1/CBF-mediated signal pathway is highly conserved in plants (1, 33–35), the findings here provide another possible regulatory mechanism of DREB1/CBF.

Since the initial cloning of DREB1/CBF and DREB2 from Arabidopsis plants (10, 11), many genes have been cloned from various plant species. Among all the DREB-like genes, only DREB2A of Arabidopsis, which was induced by salt and drought, was reported to show quite weak or no trans-active ability (11), but there has been no such report about genes in the DREB1/CBF family. Here for the first time, we reported the trans-inactive Group II and studied its functions in the DRE-mediated signal pathway. Although the two groups of genes were demonstrated to have similar binding affinity for WDRE, they exhibited quite different trans-activation activity; Group I was trans-active, whereas Group II was similar binding affinity for WDRE, they exhibited quite different trans-activation activity. Although the two groups of genes were demonstrated to have similar binding affinity for WDRE, they exhibited quite different trans-activation activity; Group I was trans-active, whereas Group II was trans-inactive. Group II was quite similar to what has been reported in DREB1/CBF2, CBF1/DREB1B, and DREB1A/CBF3 (21). Therefore, Group II could possibly function in a pattern similar to DREB1/CBF2 to act as a negative regulator of Group I. However, there must be some other roles of Group II genes since they are trans-inactive, unlike the trans-active DREB1/CBF2 (11). Then what is the significance of the expression of the trans-active Group I at the early stage of cold stress, whereas the trans-inactive Group II is expressed at a later stage?

Through co-expression of these two groups of genes in yeast and tobacco BY2 cells, we found that the expression of Group II could depress the trans-activation activity of Group I in a concentration-dependent manner (Figs. 5 and 6). Since both Group I and II DREBs had the ability to bind with WDRE and the binding affinities were similar (Figs. 2 and 3), it is most likely that the two groups of genes function in a competent pattern. The Group I factors expressed early can efficiently bind with the WDRE cis-element on the promoters of the target genes, activate their expression, and open the DREB1/CBF-mediated signal pathway. The Group II factors that are expressed later will compete with Group I to bind with the DRE elements on the promoters of the target genes, prevent their activation, and thus close the signal pathway. The expression of the trans-inactive Group II might be an important form on the regulation of the DRE-mediated signaling pathway, and the two groups of genes might function in synergy to switch on/off this special pathway.

Combining the observations here and the previous findings, we propose a hypothetical model for the roles of the two groups of genes in the regulation of the cold-inducible DRE-mediated signaling pathway, as presented in Fig. 7. When plants are subjected to cold stress, Group I genes are rapidly induced by its upstream transcription factors, like ICE1 (20). Then the expressed trans-active Group I factors bind efficiently with DRE on the promoters of the downstream genes, activate the transcription of these genes, and thus switch on the DRE-mediated signaling pathway to increase the resistance of plants to cold stress. When the proteins of Group I reach a certain level, the trans-inactive Group II is induced by some unknown mechanism, perhaps activated by Group I (21). Then the expressed Group II proteins compete with Group I to bind with DRE elements on the promoters of the target genes and decrease their expression. Group II might also function as a negative regulator of Group I to depress the expression of Group I factors (21). With the decrease in Group I and the increase in Group II protein levels, the activation of DREB1/CBF target genes are gradually prevented. In this way, the cold-inducible DRE-mediated signaling pathway is switched off.
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