Available online at www.sciencedirect.com
ScienceDirect
Progress in Natural Science 18 (2008) 579–583
Progress in Natural Science
www.elsevier.com/locate/pnsc

Short communication

Cis-regulatory element-based genome-wide identification of DREB1/CBF targets in *Arabidopsis*

Huan Zhao [a], Weizhong Liu [a,b], Yong Hu [a], Xianglin Liu [a], Yikun He [a,*]

[a] *College of Life Science, Capital Normal University, Beijing 100037, China*
[b] *College of Life Sciences, Shanxi Normal University, Linfen 041004, China*

Received 10 September 2007; received in revised form 21 October 2007; accepted 23 October 2007

Abstract

Microarray analysis is used to identify transcriptional targets. However, the direct targets of transcription factors cannot be distinguished from indirect ones; further, genes with low-expression levels cannot be identified by this method. In the present study, we exploit the *cis*-element dehydration-responsive element (DRE) that is known to be responsible for the transcription of DRE binding factor 1 (DREB1) targets in the promoter region of all *Arabidopsis* genes. Putative targets whose promoters contain the elements were verified by both microarray and reverse transcription-polymerase chain reaction (RT-PCR) analysis. Five new DREB1/CBF direct targets were identified. Compared with traditional microarray analysis, our method is convenient and cost-effective for identifying the downstream targets of transcription factors.

Keywords: *Arabidopsis*; *Cis*-element; Transcription factor; DREB1/CBF; Stress

1. Introduction

The DREB1/CBF family of transcription factors is responsible for activating the expression of many cold resistance genes induced during cold acclimation in *Arabidopsis* [1,2]. It is important to identify and characterize genes that are controlled by these transcription factors to reveal the molecular mechanisms of freezing tolerance. Microarrays have been used to identify the downstream targets of DREB1/CBF [3–5]. The transcriptional profiles of both wild-type and transgenic *Arabidopsis* plants that overexpress these transcription factors were compared, and the genes that exhibited changes in the transcription levels were selected for further characterization. However, most targets identified by this method were found to be regulated indirectly by the DREB1/CBF transcription factors. Furthermore, genes with low-expression levels could not be identified by microarray analysis.

A variety of methods have been developed to identify the transcriptional regulatory regions in genomic sequences [6–10]. However, these have not been widely used in identifying the downstream targets of transcription factors. Genome-wide analysis of abscisic acid (ABA)-responsive elements (ABRE) and their coupling elements was used to identify ABA-responsive genes in *Arabidopsis* [11]. But, since ABA induces multiple and complex gene expression in *Arabidopsis*, it is difficult to determine which proteins bind to ABRE and promote gene expression, and thus, it is difficult to predict the regulatory pathways or mechanisms underlying the expression of ABA-responsive genes.

Previous studies were unable to clearly elucidate the regulation of ABA-responsive genes. In the present study, we aimed to identify target genes of DREB1/CBF that binds to a 9-bp conserved *cis*-element DRE with the sequence

* Corresponding author. Tel.: +86 10 68902345; fax: + 86 10 68981191.
E-mail address: yhe@mail.cnu.edu.cn (Y. He).

1002-0071/$ - see front matter © 2007 National Natural Science Foundation of China and Chinese Academy of Sciences. Published by Elsevier Limited and Science in China Press. All rights reserved.
doi:10.1016/j.pnsc.2007.10.014

TACCGACAT [12,13]. The genes that contain DRE in their promoter region were predicted to be candidate downstream targets of DREB1/CBF. To verify this, the transcriptional levels of candidate genes in both transgenic and wild-type plants, determined by microarray analysis and reverse transcription-polymerase chain reaction (RT-PCR), were compared. Direct targets of DREB1/CBF that were previously undetected were identified by this method.

2. Materials and methods

2.1. Genome-wide scanning

The DRE search sequence was retrieved from the *Arabidopsis* genome database TAIR (ftp://ftp.arabidopsis.org/home/tair/Genes/). The program Element Finder was developed and used in this study to scan the genome for DREB1/CBF binding sites. The program can be downloaded from our website http://life.cnu.edu.cn/soft/Element_Finder.rar. In all *Arabidopsis* genes, regions extending 2000 bp upstream of the first translation start codon were analyzed for the presence of the DRE sequence TACCGACAT. The genes that contain DRE in their promoters were output as candidate downstream targets of DREB1/CBF.

2.2. Plant material and growth conditions

Arabidopsis (ecotype ws-2) and its *DREB1b*/*CBF1* overexpression line [14] were kindly provided by M.F. Thomashow (Michigan State University, USA). Seeds were surface-sterilized and then spread on petri plates containing MS medium (Sigma, USA) solidified with 0.8% agar. Immediately after plating, the seeds were stratified for 4 days at 4 °C to ensure uniform germination. Plants were grown in controlled-environment chambers under the conditions of 16 h light/8 h darkness at 22 °C for 14 days. For cold treatments, the plates containing the plants were maintained at 4 °C for 4 h, and tissue samples were harvested at 2, 5, and 8 h later.

2.3. RNA isolation

The aerial parts of 50–150 plants were frozen in liquid nitrogen after treatment. Total RNA was extracted using TRIZOL reagent according to the manufacturer's instructions (Sigma, USA).

2.4. Microarray analysis

Biotinylated target RNA was prepared from total RNA using the procedure described by the manufacturer of the *Arabidopsis* Gene-Chip (Affymetrix, Santa Clara, CA) and hybridized to *Arabidopsis* genome ATH1 microarrays, according to the protocol provided by the manufacturer (Affymetrix), in a hybridization oven (model 640, Affymetrix). The microarrays were washed and stained with streptavidin–phycoerythrin using a Microarray Fluidics Station model 400 and then scanned with a Gene Array Scanner (Hewlett-Packard, Palo Alto, CA).

The results were quantified and analyzed using MicroArray Suite 5.0 software (Affymetrix, Inc.). First, genes with reliable signal levels (detection p-value, <0.05) were given detection calls of "P" (present) and others were called as "A" (absent). Next, changes in RNA levels in the *DREB1b*/*CBF1* overexpression line were assessed using Wilcoxon's signed rank test as described (Affymetrix: Statistical Algorithms Reference Guide). When the increase in the signal level in the *DREB1b*/*CBF1* overexpression line was significant (change p-value, <0.0045), a call of "I" (increase) was given. When the decrease in signal was significant (change p-value, >0.9955), a call of "D" (decrease) was given.

Genes in the *DREB1b*/*CBF1* transgenic samples with an absolute call of present, difference calls of increase, and an at least twofold change in expression were selected as genes upregulated by *DREB1b*/*CBF1* overexpression.

2.5. RT-PCR

The total RNA from each sample was reverse-transcribed into single-stranded cDNA using oligo (dT) 15 primers and Superscript II reverse transcriptase (Invitrogen, USA). Each single-stranded cDNA was diluted for subsequent PCR amplification. Specific primers for PCR were designed according to the sequences of the candidate downstream genes of DREB1/CBF and were analyzed by BLAST search to ensure that the primers would not mismatch with other genes. PCR was carried out under normal conditions, with adjustments of the cycle number and annealing temperature according to the gene expression level and the melting temperature (T_m) of the primers. Three independent experiments were performed. The RT-PCR fragments were fully sequenced. *eIF* and *Actin* were used as internal quantitative controls in every PCR.

3. Results and discussion

3.1. DREB1/CBF downstream targets identification

The promoter regions of all *Arabidopsis* genes were scanned for the DRE sequence and the binding site of DREB1, using the program Element Finder as described above. Eighty genes containing the DRE sequence in their promoters were identified. Among them, three genes have been reported being DREB1b/CBF1 targets: *At5g52310, At5g1596*, and *At5g15970* [12–14]. This demonstrated that the program was effective in identifying *cis*-regulatory elements that are known to be responsible for the transcription of target genes.

3.2. Gene expression level determined by microarray analysis

The ATH1 Affymetrix chips containing more than 22,000 probe sets corresponding to 24,000 genes were used to identify genes controlled by the DREB1b/CBF1 tran-

scription factor. The mRNAs extracted from transgenic *Arabidopsis* plants that overexpressed *DREB1b/CBF1* and wild-type control plants were used.

The Affymetrix Microarray Suite software (MAS version 5.0) was used to analyze the signals for each probe set. The genes that exhibited calls of increase and at least twofold changes in expression were considered as genes responsive to DREB1b/CBF1 (Table 1).

The *Arabidopsis* genome contains a total of approximately 25,500 protein coding genes [15]. The ATH1 microarray contains probe sets corresponding to 24,000 genes. Approximately 6% of all *Arabidopsis* genes were undetected (no probe sets). A total of 303 genes (1.2% of all genes) were upregulated twofold or more in the *DREB1b/CBF1* overexpression line, and 291 (1.1%) were downregulated twofold or more (Fig. 1(b)). Of the candidate DREB1/CBF targets, five genes (6.3%) (*At5g44600*, *At1g34050*, *At1g70390*, *At2g20825*, and *At3g01940*) were undetected by microarray analysis, and eight genes (10%) (*At2g15970*, *At2g47180*, *At3g25140*, *At3g45600*, *At5g15960*, *At5g19570*, *At5g49910*, and *At5g52310*) were upregulated twofold or more. No candidate DREB1/CBF targets were downregulated by more than twofold (Fig. 1(a)).

Ten percent of the candidate target genes of DREB1/CBF were upregulated in the transgenic line. It is difficult to identify direct targets of DREB1b/CBF1 among these genes. However, most of these genes are regulated by DREB1b/CBF1 because only 1.2% of all *Arabidopsis* genes were upregulated, and the proportion of target genes predicted to be upregulated was higher than this value. Therefore, this method is effective in identifying direct targets of transcription factors.

However, the transcription level of other candidate genes was not influenced by the overexpression of CBF1/DREB1B. This suggests that the DRE element is not enough for the expression of candidate downstream genes even if CBF/DREB1 can bind to the element in the promoter region. Some specific sequences or DNA secondary structures near the DRE element may influence gene transcription.

3.3. The expression level of candidate DREB1/CBF downstream genes determined by RT-PCR

The transcriptional levels of genes that contain the DRE element in their promoters were evaluated by RT-PCR in the *DREB1b/CBF1* overexpression line subjected to cold conditions (Fig. 2). Eleven genes that were upregulated in the *DREB1b/CBF1* overexpression line were also upregulated after cold treatment.

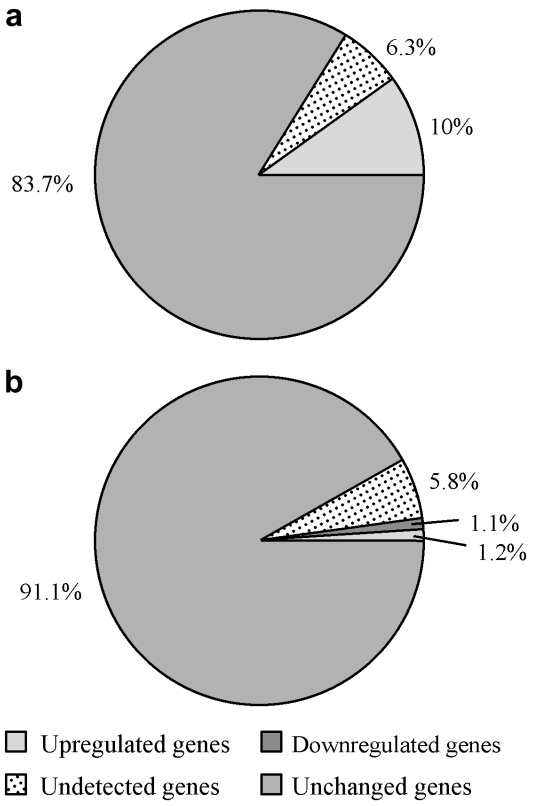

Fig. 1. Pie chart of gene expression in transgenic plants. (a) The proportion of up/downregulated and undetected genes and genes with unchanged expression among the candidate DREB1b/CBF1 targets; (b) the proportion of up/downregulated and undetected genes and genes with unchanged expression among all *Arabidopsis* genes.

Among the 11 genes, the RT-PCR results of 6 genes (*At5g15960*, *At5g15970*, *At5g52310*, *At2g47180*, *At3g45600*, and *At5g49910*) were consistent with the results of the microarray analysis of these genes. Two low-expression genes (*At1g60470* and *At1g14780*) were not detected ("Absent") by the microarray analysis. This indicated that genes with low levels of expression, which were not identified by microarray analysis, could be detected by RT-PCR. Similar to the results of the microarray analysis, the expres-

Table 1
Genes upregulated in DREB1b/CBF1 overexpression transgenic line detected by microarray analysis

Gene	Function or comment	Change (fold)
At2g15970	Cold-acclimation protein, putative (FL3-5A3), similar to cold acclimation WCOR413-like protein	2.4
At2g47180	Galactinol synthase, putative, similar to galactinol synthase	2.1
At3g25140	Glycosyl transferase family 8 protein	2.1
At3g45600	Senescence-associated family protein, similar to senescence-associated protein 5	2
At5g15960	Stress-responsive protein (KIN1)/stress-induced protein (KIN1)	3.4
At5g15970	Stress-responsive protein (KIN2)/stress-induced protein (KIN2)/cold-responsive protein (COR6.6)/cold-regulated protein (COR6.6)	3.4
At5g49910	Heat shock protein 70/HSP70 (HSC70-7)	2.1
At5g52310	Low-temperature-responsive protein 78 (LTI78)/desiccation-responsive protein 29A (RD29A)	45.2

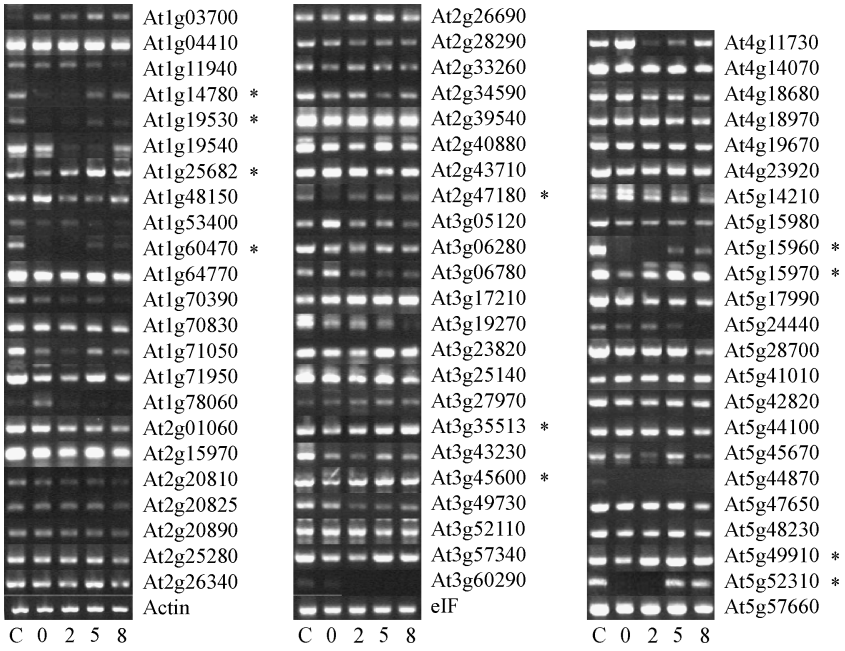

Fig. 2. RT-PCR results of the candidate DREB1/CBF downstream genes. The RNA of 35S:*DREB1b*/*CBF1* transgenic plants (C), untreated wild-type plants (0), or cold-treated wild-type plants (cold treatment for 2, 5, and 8 h) was reverse transcribed to cDNA, and the expression of candidate DREB1/CBF downstream genes was detected by using primers specific to these genes. The asterisks indicate the genes regulated by DREB1b/CBF1 during cold stress. *Actin* and *eIF* were used as internal quantitative controls.

sion level of *At2g15970* and *At3g25140* was found to be upregulated in the transgenic line by RT-PCR. However, the expression of these genes was not affected by the cold treatment, suggesting that these were indirectly regulated by DREB1b/CBF1.

3.4. New *DREB1b*/*CBF1* target genes

According to the microarray and RT-PCR analysis, eight genes (*At1g14780*, *At1g60470*, *At2g47180*, *At3g45600*, *At5g15960*, *At5g15970*, *At5g49910*, and *At5g52310*) could be considered as genes directly regulated by DREB/CBF1. Four genes (*At5g15960*, *At5g15970*, *At5g49910*, and *At5g52310*) encoding LEA/COR or heat shock protein that are thought to play an important role in freezing tolerance [16] were identified. Other genes included those encoding enzymes related to carbohydrate metabolism (*At1g60470* and *At2g47180*), a gene associated with senescence (*At3g45600*), and a gene with unknown function (*At1g14780*). These genes, except for *RD29*, *Kin1*, and *Kin2* (*At5g52310*, *At5g15960*, and *At5g15970*) [14], have not been reported before as DREB1/CBF targets. The relationship between the five novel DREB1/CBF targets and stress tolerance has yet to be elucidated and should be analyzed in the future.

In summary, *cis*-element-based identification of transcription factor targets is an effective way to identify direct downstream genes of transcription factors; the identification of these genes has not been possible by microarray analysis. More DREB1/CBF direct targets were identified by this method. Moreover, this method is convenient and cost-effective compared with microarray analysis.

Acknowledgements

This work was supported by the National High-Tech Research Development Program (No. 2002AA2Z1001) and the National Natural Science Foundation of China (Grant No. 30328003), and PHR (IHLB). We thank M. Thomashow for providing seeds of *Arabidopsis* (ecotype ws-2) and its *DREB1b*/*CBF* overexpression line.

References

[1] Jaglo-Ottosen KR, Gilmour SJ, Zarka DG, et al. *Arabidopsis* CBF1 overexpression induces *COR* genes and enhances freezing tolerance. Science 1998;280:104–6.
[2] Kasuga M, Liu Q, Miura S, et al. Improving plant drought, salt, and freezing tolerance by gene transfer of a single stress-inducible transcription factor. Nat Biotechnol 1999;17:287–91.
[3] Maruyama K, Sakuma Y, Kasuga M, et al. Identification of cold-inducible downstream genes of the *Arabidopsis* DREB1A/CBF3 transcriptional factor using two microarray systems. Plant J 2004;38:982–93.
[4] Fowler S, Thomashow MF. *Arabidopsis* transcriptome profiling indicates that multiple regulatory pathways are activated during cold acclimation in addition to the CBF cold response pathway. Plant Cell 2002;14:1675–90.
[5] Seki M, Narusaka M, Abe H, et al. Monitoring the expression pattern of 1300 *Arabidopsis* genes under drought and cold stresses by using a full-length cDNA microarray. Plant Cell 2001;13:61–72.
[6] Papatsenko D. ClusterDraw web server: a tool to identify and visualize clusters of binding motifs for transcription factors. Bioinformatics 2007;23:1032–4.

[7] Waleev T, Shtokalo D, Konovalova T, et al. Composite Module Analyst: identification of transcription factor binding site combinations using genetic algorithm. Nucleic Acids Res 2006;34:W541–5.
[8] Alkema WB, Johansson O, Lagergren J, et al. MSCAN: identification of functional clusters of transcription factor binding sites. Nucleic Acids Res 2004;32:W195–8.
[9] Sinha S, Liang Y, Siggia E. Stubb: a program for discovery and analysis of *cis*-regulatory modules. Nucleic Acids Res 2006;34:W555–9.
[10] Sosinsky A, Bonin CP, Mann RS, et al. Target Explorer: an automated tool for the identification of new target genes for a specified set of transcription factors. Nucleic Acids Res 2003;31:3589–92.
[11] Zhang W, Ruan J, Ho TH, et al. *Cis*-regulatory element based targeted gene finding: genome-wide identification of abscisic acid- and abiotic stress-responsive genes in *Arabidopsis thaliana*. Bioinformatics 2005;21:3074–81.
[12] Liu Q, Kasuga M, Sakuma Y, et al. Two transcription factors, DREB1 and DREB2, with an EREBP/AP2 DNA binding domain separate two cellular signal transduction pathways in drought- and low-temperature-responsive gene expression, respectively, in *Arabidopsis*. Plant Cell 1998;10:1391–406.
[13] Stockinger EJ, Gilmour SJ, Thomashow MF. *Arabidopsis thaliana* CBF1 encodes an AP2 domain-containing transcriptional activator that binds to the C-repeat/DRE, a *cis*-acting DNA regulatory element that stimulates transcription in response to low temperature and water deficit. Proc Natl Acad Sci USA 1997;94:1035–40.
[14] Gilmour SJ, Zarka DG, Stockinger EJ, et al. Low temperature regulation of the *Arabidopsis* CBF family of AP2 transcriptional activators as an early step in cold-induced *COR* gene expression. Plant J 1998;16:433–42.
[15] Initiative The Arabidopsis Group. Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. Nature 2000;408:796–815.
[16] Thomashow MF. Plant cold acclimation: freezing tolerance genes and regulatory mechanisms. Annu Rev Plant Physiol Plant Mol Biol 1999;50:571–99.