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Research Article

The precise regulation of different *COR* genes by individual CBF transcription factors in *Arabidopsis thaliana*[oo]

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Abstract The transcription factors *CBF1/2/3* are reported to play a dominant role in the cold responsive network of *Arabidopsis* by directly regulating the expression levels of *cold responsive* (*COR*) genes. In this study, we obtained CRISPR/Cas9-mediated loss-of-function mutants of *cbf1∼3*. Over 3,000 *COR* genes identified by RNA-seq analysis showed a slight but significant change in their expression levels in the mutants compared to the wild-type plants after being treated at 4 °C for 12 h. The C-repeat (CRT) motif (5′-CCGAC-3′) was enriched in promoters of genes that were up-regulated by CBF2 and CBF3 but not in promoters of genes up-regulated by CBF1. These data suggest that CBF2 and CBF3 play a more important role in directing the cold response by regulating different sets of downstream *COR* genes. More than 2/3 of *COR* genes were co-regulated by two or three CBFs and were involved mainly in cellular signal transduction and metabolic processes; less than 1/3 of the genes were regulated by one CBF, and those genes up-regulated were enriched in cold-related abiotic stress responses. Our results indicate that CBFs play an important role in the trade-off between cold tolerance and plant growth through the precise regulation of *COR* genes in the complicated transcriptional network.

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INTRODUCTION

Arabidopsis thaliana is a short-lived annual plant that is distributed worldwide (Koornneef et al. 2004; Weigel 2012). Temperature is one of the most important environmental factors that limits the growth and distribution range of *Arabidopsis*. The freezing tolerance of *A. thaliana* can be significantly improved after the plants are exposed to low, but non-freezing, temperatures for a short period of time; this phenomenon is known as cold acclimation (Gilmour et al. 1988; Browse and Xin 2001). Understanding the molecular mechanisms of the cold responsive network in *A. thaliana* will help to elucidate the mechanisms underlying this plant adaptation at the molecular level.

In recent years, there have been many studies on the different components that play a role in the cold responsive network in *Arabidopsis*. The *Arabidopsis* genome encodes three *CBF* transcription factors, *CBF1/DREB1B*, *CBF2/DREB1C* and *CBF3/DREB1A*, which respond to low temperatures. CBF1∼3 belong to the AP2/ERF transcription factor family (Stockinger et al. 1997), and the three *CBF* genes are tandemly clustered in an 8.7 kb region in chromosome 4. The three *CBF* genes are paralogous genes sharing high sequence similarity. They are induced specifically and rapidly by low temperature, and they recognize and bind to *cis*-elements in the promoters of *cold-responsive* (*COR*) genes. The CBF-mediated pathway is considered one of the most important signaling pathways involved in cold

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acclimation (Medina et al. 2011). CBFs are also major players in determining the freezing tolerance of plants (Gilmour et al. 2004; Vogel et al. 2005; Park et al. 2015; Shi et al. 2015).

It has been reported that CBF1∼3 play a major role in cold acclimation (Alonso-Blanco et al. 2005; Lin et al. 2008; McKhann et al. 2008; Zhen and Ungerer 2008a, 2008b; Chinnusamy et al. 2010; Zhao et al. 2016). Constitutive overexpression of each *CBF* in plants resulted in enhanced cold tolerance (Gilmour et al. 2000), while the knockdown of *CBF1* and *CBF3* by RNAi endowed plants with compromised cold tolerance (Novillo et al. 2007). Although other factors such as ethylene (Shi et al. 2012), abscisic acid (ABA) (Lee and Seo 2015) and *FLC* (Lee and Park 2015) might also directly or indirectly contribute to cold tolerance, CBFs are the central components in the complex cold responsive network. For instance, a transcriptome analysis of *CBF* overexpression lines and *CBF2* dominant-negative lines showed that about one-third of the cold-induced *COR* genes are dependent on a functional CBF regulatory module (Park et al. 2015). A recent study showed that the three CBFs were indeed essential for cold acclimation and that CBF2 was more important than CBF1 and CBF3 for the cold response in *Arabidopsis* (Zhao et al. 2016). But a comparison of expression profiles of three single knockout mutants generated by the same method at the same time has not been reported.

CRISPR/Cas9 systems are adaptive defense systems that prevent invasion from alien nucleic acids in prokaryotic organisms (Barrangou et al. 2007) and have been recently modified and used to efficiently edit the genome in many eukaryotic organisms, including humans (Horvath and Barrangou 2013), mice (Ma et al. 2014; Shen et al. 2013), zebra fish (Chang et al. 2013), invertebrates (Friedland et al. 2013; Yu et al. 2013), yeast (DiCarlo et al. 2013) and plants (Feng et al. 2013; Miao et al. 2013; Shan et al. 2013). In this study, we successfully obtained three *cbf* single mutants using CRISPR/Cas9 technology and conducted an RNA-seq analysis of each of these three loss-of-function mutants. The result showed that about half of the *COR* genes were up- or down-regulated in a similar manner in both the single mutant and wild type (WT) plants, while a large majority of genes were only slightly affected in the *cbf* single mutants, suggesting that there is a functional redundancy among these genes and that other components exist in the cold responsive pathways. The C-repeat (CRT) motif (5′-CCGAC-3′) was enriched in the promoters of CBF2- and CBF3-up-regulated genes but not in promoters of CBF1-up-regulated genes or the genes of the single mutants that were down-regulated. Genes up-regulated in the single mutant plants were tightly associated with the cold responsive network and may be involved in the interplay between the CBF-dependent pathway and the CBF-independent hormone pathways. The genes co-regulated by two or three CBFs were mainly involved in cellular signal transduction and metabolic processes. Our results indicate that CBFs play an important role in the trade-off between cold tolerance and plant growth through the precise regulation of *COR* genes.

RESULTS

Generation of *cbf* single mutants using the CRISPR/Cas9 system

To obtain knockout mutants of the three *CBFs*, we utilized the CRISPR/Cas9 system, which has been developed in recent years (Feng et al. 2013; Miao et al. 2013; Shan et al. 2013). We made double-stranded breaks in the target genes *CBF1*, *CBF2* and *CBF3* and obtained inheritable homozygous single mutants. A single base was introduced to the coding region of three *CBF* genes, respectively, so that a premature stop of translation occurred, leading to a large deletion of the AP2 DNA-binding domain (Figure 1). No significant morphological changes were observed in the three single mutants under normal growth conditions.

We conducted freezing tolerance assays with two-week-old seedlings of the three single mutants and found that there were no significant differences in cold tolerance among the three single mutants and WT plants (Figure 2). These results are consistent with the previous reports that these three CBFs are functionally redundant.

Transcriptome profiles of cold responsive genes in WT and three *cbf* single mutants

It was reported that CBF1∼3 were possibly under different regulatory controls (Novillo et al. 2007; Achard et al. 2008; Lee and Seo 2015) and that there could be an interaction between the three CBFs (Novillo et al. 2004; Shi et al. 2015). Therefore, we hypothesized that each

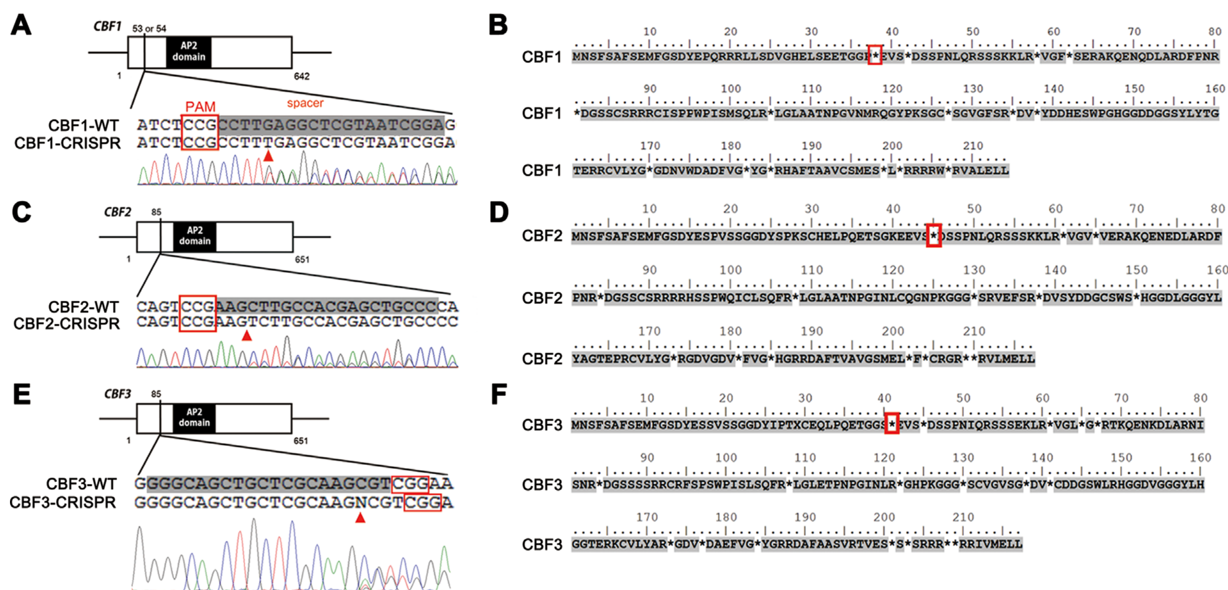

Figure 1. Genomic editing in *CBF1-3* using CRISPR-Cas9 in *Arabidopsis thaliana*
(**A**, **C**, and **E**) A schematic drawing displaying the structure of three *CBF* genes and the red arrowhead showing the locations of a single nucleotide insertion by the CRISPR-Cas9 system in *CBF1*, *CBF2* and *CBF3*, respectively. Protospacer adjacent motif (PAM) sequence is framed in the red box followed by the grey-shaded spacer sequence designed in this study. The "N" in CBF3 means two types of nucleic acids (A and T) were observed at the same time in the T1 generation, but only T observed in T2 generation. The number above indicates the position in the gene where a nucleoside is inserted, and two numbers below indicate the first and last amino acid residues respectively. The confirmation of inserted nucleotide is shown by the sequencing map in which each peak is in accordance with the base in the sequence above. (**B**, **D**, and **F**) The amino acid sequences of the *cbf1∼3* mutants respectively. Asterisks indicate the positions of the stop codons with the first stop codon marked by a red box.

CBF precisely regulates a different set of downstream cold responsive genes. To test this hypothesis, we performed an RNA-seq analysis of two-week-old seedlings of WT (Col-0) and *cbf1∼3* single mutants at room temperature (22 °C) and seedlings exposed to 4 °C for 2 h or 12 h. High-quality RNA-seq data were obtained (Figures S1, S2), and the expression of several well-studied cold-induced *COR* genes such as *COR6.6*, *COR47*, *ERD10*, *KIN1*, *LTI78*, *CBF1*, *CBF2* and *CBF3* were confirmed by quantitative real-time polymerase chain reaction (PCR) (Figures 3, S3).

Because CBFs are transcription factors with strong activation activities (Kang et al. 2013), we used a four-fold change rather than a two-fold change as a filter to identify the differentially expressed *COR* genes after normalization with the Cuffdiff package. We identified 3,766 *COR* genes in the WT plants after treating them at 4 °C for 12 h. Our results indicated that 1,920 of these genes were up-regulated, and 1,846 were down-regulated after treatment at 4 °C for 12 h (Table S1). Gene Ontology (GO) analysis revealed that many of these cold-induced *COR* genes were implicated in the response to an abiotic stimulus (GO: 0009628, $P = 5.5 \times 10^{-26}$) and an ABA stimulus (GO: 0009737, $P = 3.6 \times 10^{-16}$). Some *COR* genes were enriched in the responses to different stresses such as water deprivation (GO: 0009414, $P = 6.8 \times 10^{-14}$), salt stress (GO: 0009651, $P = 1.5 \times 10^{-10}$), cold (GO: 0009409, $P = 3.3 \times 10^{-7}$), cold acclimation (GO: 0009631, $P = 1.9 \times 10^{-7}$), and hyperosmotic stress (GO: 0006972, $P = 1.5 \times 10^{-5}$). However, the cold-repressed *COR* genes were enriched in only the regulation of transcription (GO: 0045449, $P = 1.5 \times 10^{-3}$), gibberellin biosynthetic process (GO: 0009686, $P = 1.5 \times 10^{-3}$), oxidation reduction (GO: 0055114, $P = 2.6 \times 10^{-3}$), response to hormone stimulus (GO: 0009725, $P = 8.2 \times 10^{-3}$) and endogenous stimulus (GO: 0009719, $P = 2.5 \times 10^{-2}$) (Table S2), rather than in the biological processes mentioned earlier. Our results indicated that when the cold responsive pathways were activated, some other pathways were inhibited.

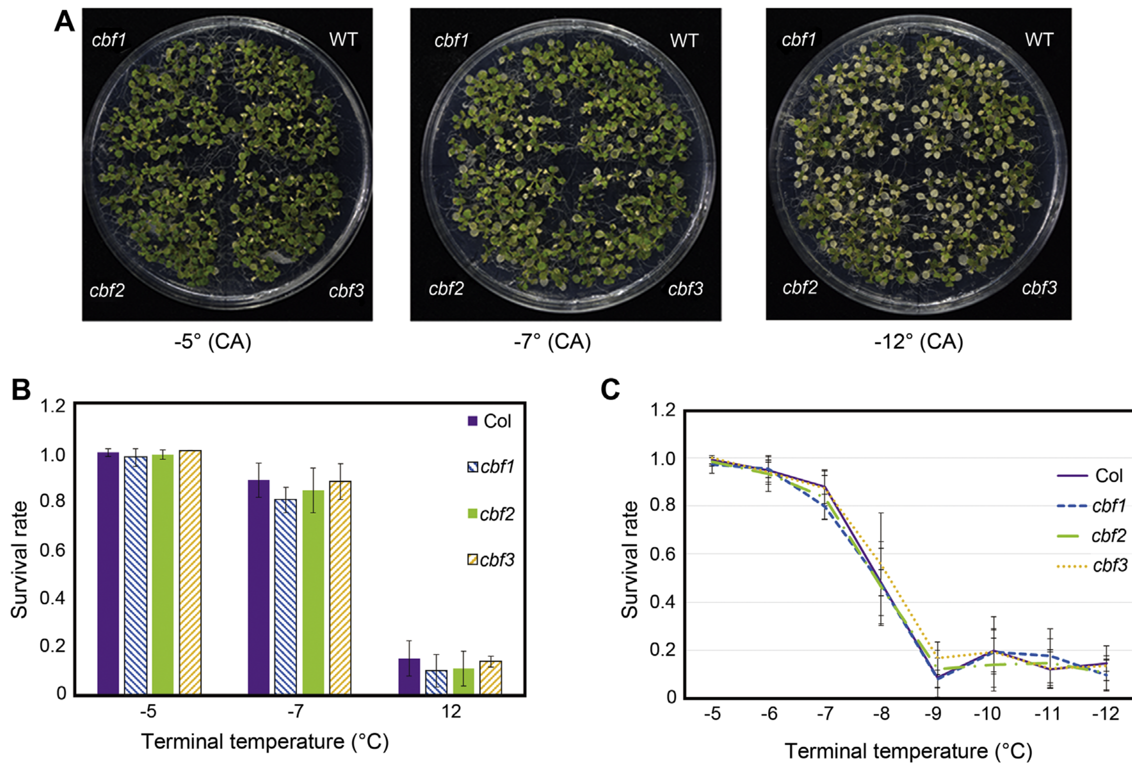

Figure 2. Freezing tolerance of three single *cbf* mutants compared with WT
(**A**) Performance of wild type (WT) and *cbf1*∼*3* mutant plants at different freezing temperatures. (**B**) Survivorship of WT and *cbf1*∼*3* mutant plants at different freezing temperatures. (**C**) Comparison of freezing tolerance of WT and three single mutants after cold acclimation. The survival rate was calculated when the plants were moved to normal growth conditions for 2 d after treatment at freezing temperatures. The individuals with green cotyledons and true leaves were identified as survivors. The freezing tolerance was tested at different terminal temperatures. Each assay was conducted at least on three plates as replicates at a time with 30 individuals in each plate. Error bars indicate the standard deviation.

We then identified differentially expressed genes in the three single mutants *cbf1*, *cbf2* and *cbf3* using the same criteria that was used to identify the *COR* genes in the WT plants. Totals of 1,990, 2,031 and 1,948 genes were up-regulated, and 1,739, 1,515 and 1,833 genes were down-regulated, respectively, in the *cbf1*, *cbf2* and *cbf3* mutants after treatment at 4 °C for 12 h (Table S3). We found that more than half of the cold-induced and nearly half of cold-repressed *COR* genes were shared in all three single mutants (Figure 4), revealing again the functional redundancy among the three CBFs. These *COR* genes could be regulated by any of the three CBFs, or could be independent of the three CBFs.

A set of genes regulated by individual CBFs (a CBF regulon) was identified by expression profiles of *Arabidopsis* (Ws-2) transgenic lines constitutively overexpressing *CBF1*, *CBF2* and *CBF3* respectively (Gilmour et al. 2004; Park et al. 2015). About 30% (383/1,256) of cold-induced *COR* genes and 17% (236/1,381) of cold-repressed *COR* genes identified by Park et al. (2015) in Ws-2 overexpression lines were also identified in our study using Col-0 ecotype as material. Furthermore, about 72% (96/133) of up-regulated CBF regulon genes in Ws-2 were also up-regulated at least by one CBF in Col-0, and about 31% (12/39) of down-regulated CBF regulon genes were down-regulated at least by one CBF in Col-0. The difference in these two datasets may possibly result from the differences of the experimental materials (Ws-2 vs Col-0), treatment time (24 h for Ws-2 vs 12 h for Col-0 at 4 °C), and/or methodology (microarray for Ws-2 vs RNA-seq for Col-0; and twofold change of differential expressed genes in Ws-2 vs fourfold change in Col-0 after cold treatment).

Hierarchical clustering of *COR* genes in WT and three single *cbf* mutants

To investigate which groups of genes are regulated in the single *cbf* mutants, we further analyzed the *COR* genes under cold treatment for 0 h (room

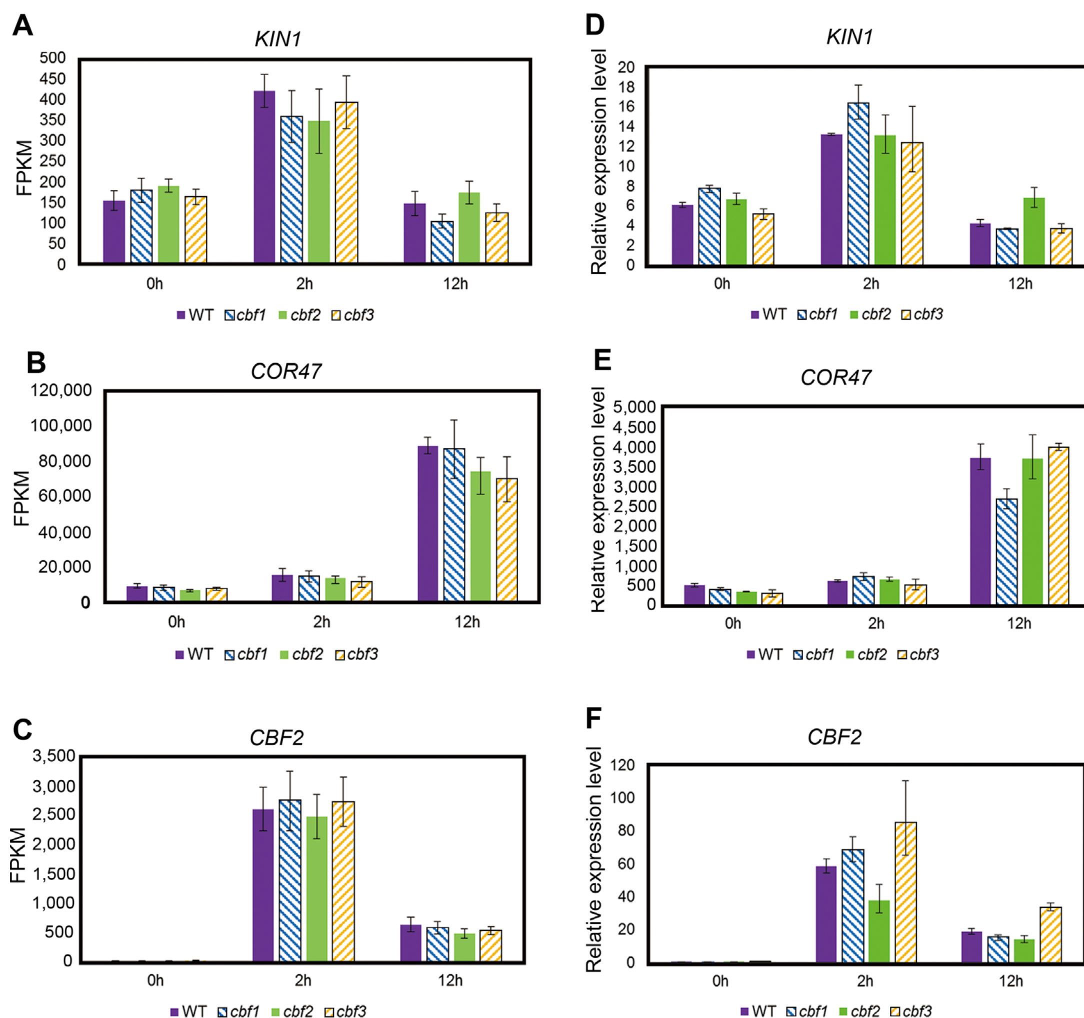

Figure 3. Validation of the RNA-seq results by quantitative real-time PCR
Genes with different expression pattern to cold stress, such as *KIN1*, *COR47* and *CBF2* were selected. (**A–C**) are RNA-seq results, and (**D–F**) are quantitative real-time polymerase chain reaction (PCR) data. The average number of reads of each gene for the three biological repeats was used for comparison in the real-time PCR results. Error bars indicate the standard deviation.

temperature), 2 h and 12 h. In contrast to the 2 h cold treatment, most of the *COR* genes were only significantly up- or down-regulated by the 12 h treatment, which suggests a downstream response leading to cold tolerance (Figure 5). The up-regulated *COR* genes could be grouped into two clusters. Cluster A contains 1,033 genes that were up-regulated in both WT plants and the three single *cbf* mutants. Cluster B contains 807 genes that were up-regulated in only WT plants but did not respond to cold treatment in at least one of the three single *cbf* mutants (Figure 5). This result suggests that, in spite of functional redundancy, the lack of one single *CBF* could change the expression patterns of some downstream *COR* genes (*i.e.*, Cluster B genes).

Most of the genes in Cluster A exhibited a similar (yellow color) or decreased (green color) expressional level in the single *cbf* mutant plants (Figure 6), suggesting that the response of Cluster A genes to cold treatment was slightly impaired in single *cbf* mutants. A more quantitative comparison made use of both the up-regulated and down-regulated genes in the WT and three single mutants as shown in the Venn diagram with undetectable or low (Fragments Per Kilobase Million (FPKM) < 0.1) mean expression excluded. We found that the three groups of differentially expressed genes exhibited significant linear correlations between the WT and *cbf* mutant plants ($R^2_{WT\text{-}cbf1} = 0.7832$, $R^2_{WT\text{-}cbf2} = 0.7541$, $R^2_{WT\text{-}cbf3} = 0.8103$, Figure 7). The slopes of the linear regression fit curve

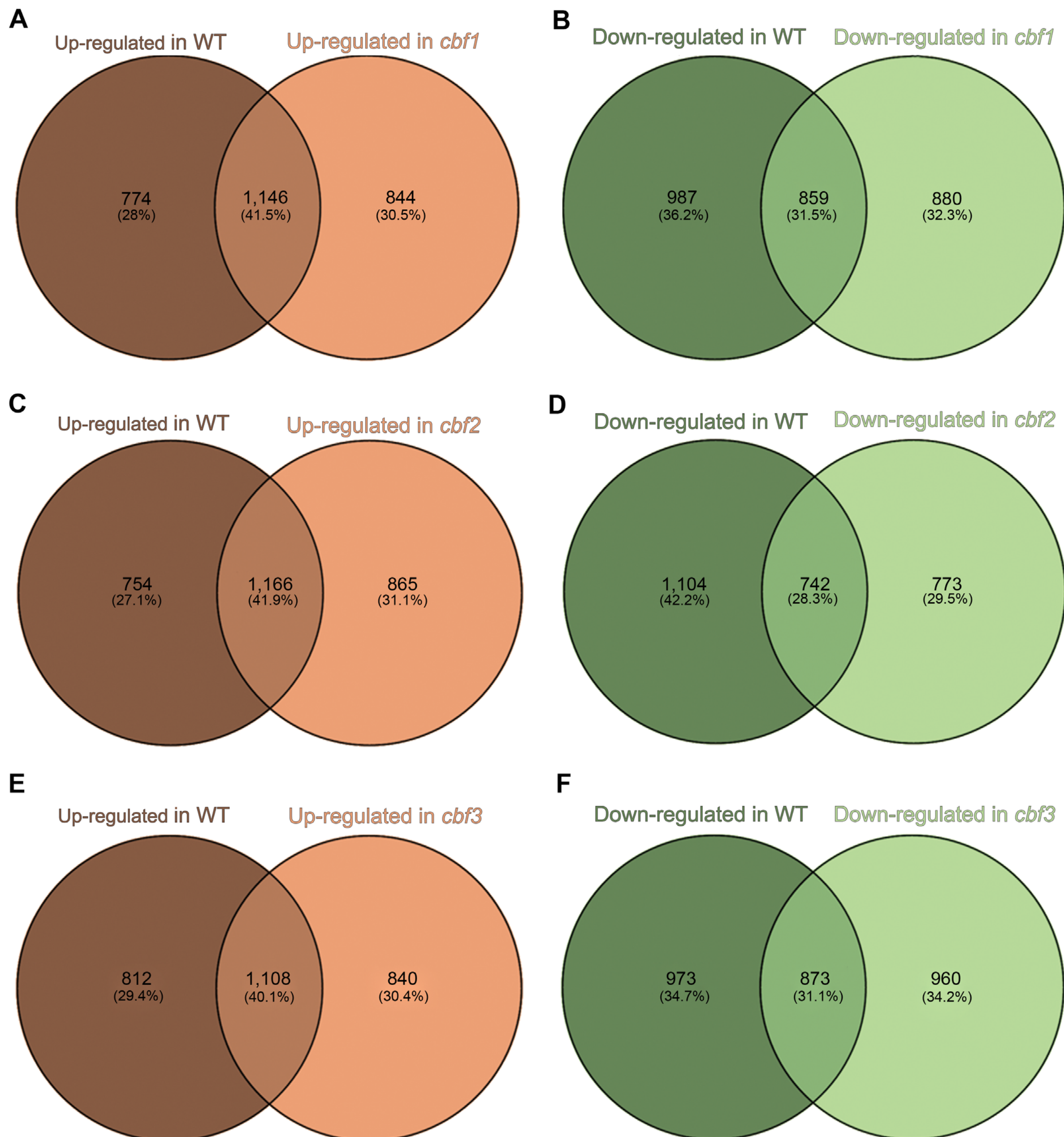

Figure 4. Venn diagrams of the genes up-regulated or down-regulated in WT and *cbf1~3* after cold treatment for 12 h
(**A**, **B**) The number of up-regulated and down-regulated genes in wild type (WT) and *cbf1* respectively, and the number in the overlapped regions are genes shared by WT and *cbf1*. (**C**, **D**) The number of up-regulated and down-regulated genes in WT and *cbf2* respectively, and the number in the overlapped regions are genes shared by WT and *cbf2*. (**E**, **F**) The number of up-regulated and down-regulated genes in WT and *cbf3* respectively, and the number in the overlapped regions are genes shared by WT and *cbf3*.

were slightly less than 1, 0.9158, 0.8719, and 0.9101, in the *cb1*, *cbf2*, and *cbf3* mutants, respectively. This suggests that although the single *cbf* mutants had a response to the cold stress and that most of the *COR* genes were regulated in a pattern similar to the genes in WT plants, the intensity of the cold response was slightly affected. This is consistent with the fact that the three single mutants share a similar level of cold tolerance (Figure 2).

Identification of genes regulated by a single CBF

To understand the different roles of each *CBF* in the cold responsive network, we conducted an in-depth analysis

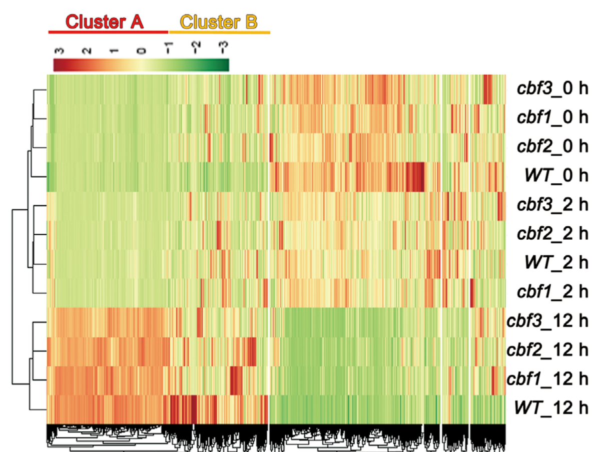

Figure 5. Hierarchical clustering of all *COR* genes in the WT and three single *cbf* mutants after treatment with cold for 0 h, 2 h and 12 h ($n = 3,766$)

Cluster A refers to the group of genes up-regulated in all samples after treatment with cold for 12 h. Cluster B contains genes that were up-regulated in wild type (WT) at 12 h but had a different pattern in at least one of three single *cbf* mutants. The average expression level of three biological repeats in each sample was used. R pheatmap parameters: cutree_rows = 3, scale = "row".

of the genes that were differentially regulated in the *cbf* mutants. The reason we compared the genes expressed at 12 h of cold treatment is because that most *COR* genes previously identified were up-regulated at 12 h when the expression of three *CBF*s already decreased. Therefore, the change of gene expression at 12 h would reflect more direct regulation by CBFs than that at 24 h. If the

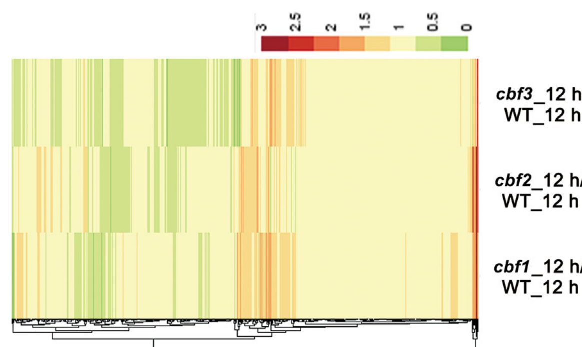

Figure 6. Hierarchical clustering of relative expression of the 1,033 *COR* genes in Cluster A

The values of relative expression were calculated by dividing the expression level of a single mutant by that of wild type (WT) at 12 h after cold treatment. R pheatmap parameters: scale = "row".

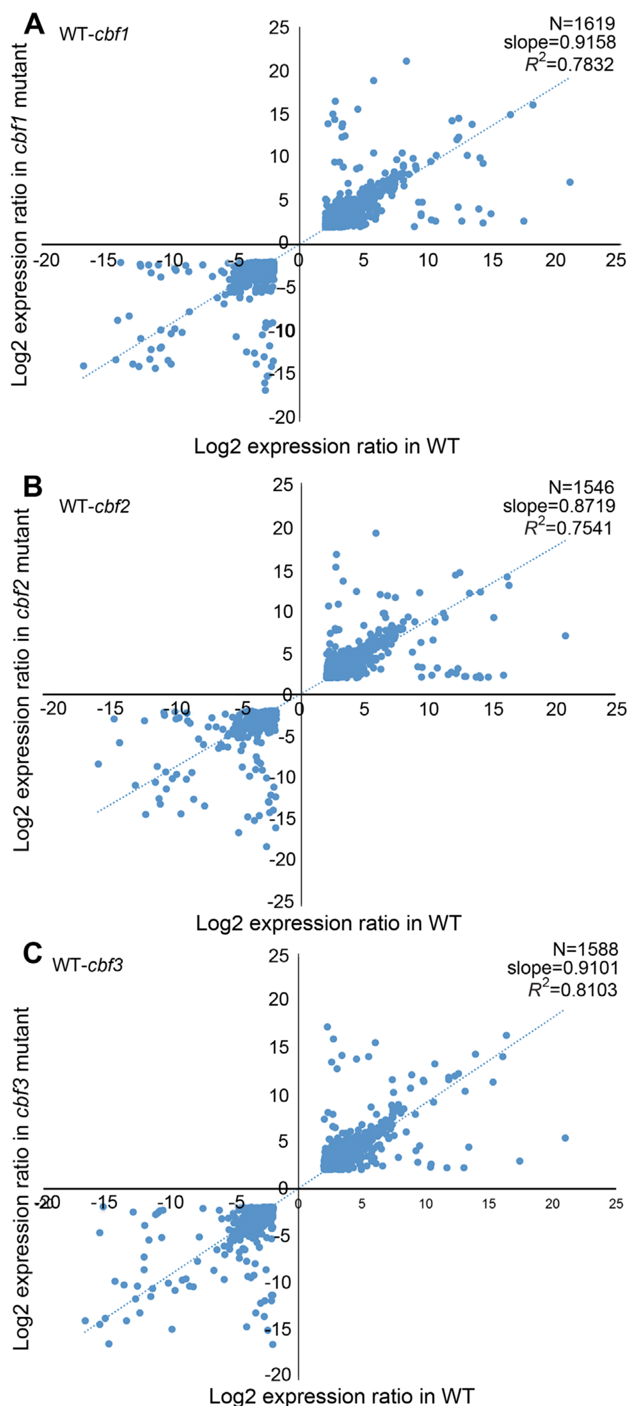

Figure 7. Comparison of expression levels of the genes that were both up-/down-regulated in the wild type (WT) versus *cbf1* (A), *cbf2* (B) or *cbf3* (C).

The expression ratio of a gene was calculated as the log2 ratio of cold-treated over non-cold-treated expression levels. Only ratios higher than 1 or less than −1 and mean expression levels greater than zero were considered. Dotted lines indicated linear regression fit lines based on $N = 1,619$ data points in WT versus *cbf1*, $N = 1,546$ data points in WT versus *cbf2*, and $N = 1,588$ data points in WT vs *cbf3*.

expression of a gene was changed after cold treatment in the WT plants but not in one of the single *cbf* mutants, the gene could be a downstream gene specific to one of the *CBF* genes, regardless of the function of the other two *CBFs*. These types of genes were designated as single CBF-regulated genes (Figure 4 and Cluster B in Figure 5). We found that CBF1 regulated approximately 1,761 genes (774 cold-induced and 987 cold-repressed), CBF2 regulated approximately 1,858 genes (754 cold-induced and 1,104 cold-repressed), and CBF3 regulated approximately 1,785 genes (812 cold-induced and 973 cold-repressed) (Figure 4; Table S4).

Promoter motif enrichment analysis on the single CBF-regulated genes

To investigate the abundance of potential target genes of CBFs, we analyzed the enrichment of the CBF-binding site, 5′-CCGAC-3′ (CRT/DRE motif), in the promoters of all six groups of single CBF-regulated genes. Motif enrichment in a group of genes was tested against a background distribution generated by 2,000 random samplings from all genes in the genome of *Arabidopsis*. The results showed that this motif was enriched in the promoters of CBF2- and CBF3-up-regulated genes, but not in the promoters of CBF1-up-regulated genes ($P_{CBF1} = 0.1695 > 0.05$, $P_{CBF2} = 0.0149 < 0.05$, $P_{CBF3} = 0.0066 < 0.01$), suggesting that compared with CBF1, more *COR* genes were directly regulated by CBF2 and CBF3. It is also consistent with the previous reports that *CBF2* and *CBF3* play a more important role than *CBF1* in the adaptation to low temperature in natural populations (Alonso-Blanco et al. 2005; Kang et al. 2013; Oakley et al. 2014; Gehan et al. 2015) and the fact that the genes regulated by CBF2 and CBF3 under the 12 h cold treatment were clustered together, distinct from genes regulated by CBF1 (Figure 5). In contrast, the CRT motif was significantly less enriched in the promoters of single CBF-down-regulated genes than the average enrichment in the whole genome ($P_{CBF1} = 0.0023 < 0.01$, $P_{CBF2} = 0.0003 < 0.01$, $P_{CBF3} = 0.0001 < 0.01$), suggesting that the repression of these genes was not due to direct regulation by the three CBFs.

Genes regulated by individual CBFs were controlled by a complex transcriptional network

Because there were overlaps in either the three single CBF-up-regulated genes or in the three single CBF-down-regulated genes, we made a further comparison between them. In the 1,089 up-regulated genes, 443 genes were co-regulated by three CBFs, 365 genes by two CBFs and 281 genes by one single CBF (Figure 8A). In the 1,379 down-regulated genes, 618 genes were co-regulated by three CBFs, 449 genes by two CBFs and 312 genes by one single CBF (Figure 8B). The numbers of genes regulated by the different combinations of CBFs (three, or two CBFs) and by one CBF were in a similar pattern, both in the up-regulated and down-regulated genes. More genes were co-regulated by two or three CBFs than those only regulated by one CBF (Figure 8C–E).

A GO analysis on the genes regulated by different CBFs revealed that in the single CBF-up-regulated genes, a large number of genes were enriched in the following ontologies: response to abiotic stimulus (GO: 0009628, $P = 2.8 \times 10^{-7}$), hormone stimulus (GO: 0009725, $P = 4.4 \times 10^{-6}$), abscisic acid stimulus (GO: 0009737, $P = 1.5 \times 10^{-4}$), salt stress (GO: 0009651, $P = 3.8 \times 10^{-4}$), water deprivation (GO: 0009414, $P = 2.1 \times 10^{-3}$), and temperature stimulus (GO: 0009266, $P = 2.8 \times 10^{-2}$) (Table S5). This result suggests that these single CBF-regulated genes are closely related to the cold-related regulatory network and may be involved in the interplay of the CBF-dependent pathway and CBF-independent pathways involved in phytohormones. However, the genes regulated by three or two CBFs were not significantly enriched in the gene ontology response to abiotic stimulus ($P = 9.8 \times 10^{-2} > 0.05$). Instead, they were enriched in the following gene ontologies: response to organic substance (GO: 0010033, $P = 1.3 \times 10^{-2}$), regulation of transcription (GO: 0045449, $P = 4.0 \times 10^{-3}$), protein transport (GO: 0015031, $P = 3.9 \times 10^{-2}$), and messenger RNA (mRNA) transport (GO: 0051028, $P = 7.7 \times 10^{-3}$) (Table S6), suggesting that the genes co-regulated by CBFs are mainly involved in cellular signal transduction and metabolic processes associated with cold stress. In contrast, the single CBF-down-regulated genes were not related to the ontology of abiotic stress response; instead, they were implicated in the following gene ontologies: regulation of transcription (GO: 0045449, $P = 8.5 \times 10^{-3}$), chromatin assembly or disassembly (GO: 0006333, $P = 1.8 \times 10^{-4}$), protein-DNA complex assembly (GO: 0065004, $P = 1.0 \times 10^{-3}$), embryonic development ending in seed dormancy (GO: 0009793, $P = 3.3 \times 10^{-3}$) and fruit development (GO: 0010154, $P = 1.4 \times 10^{-2}$) (Table S7), suggesting that plant growth

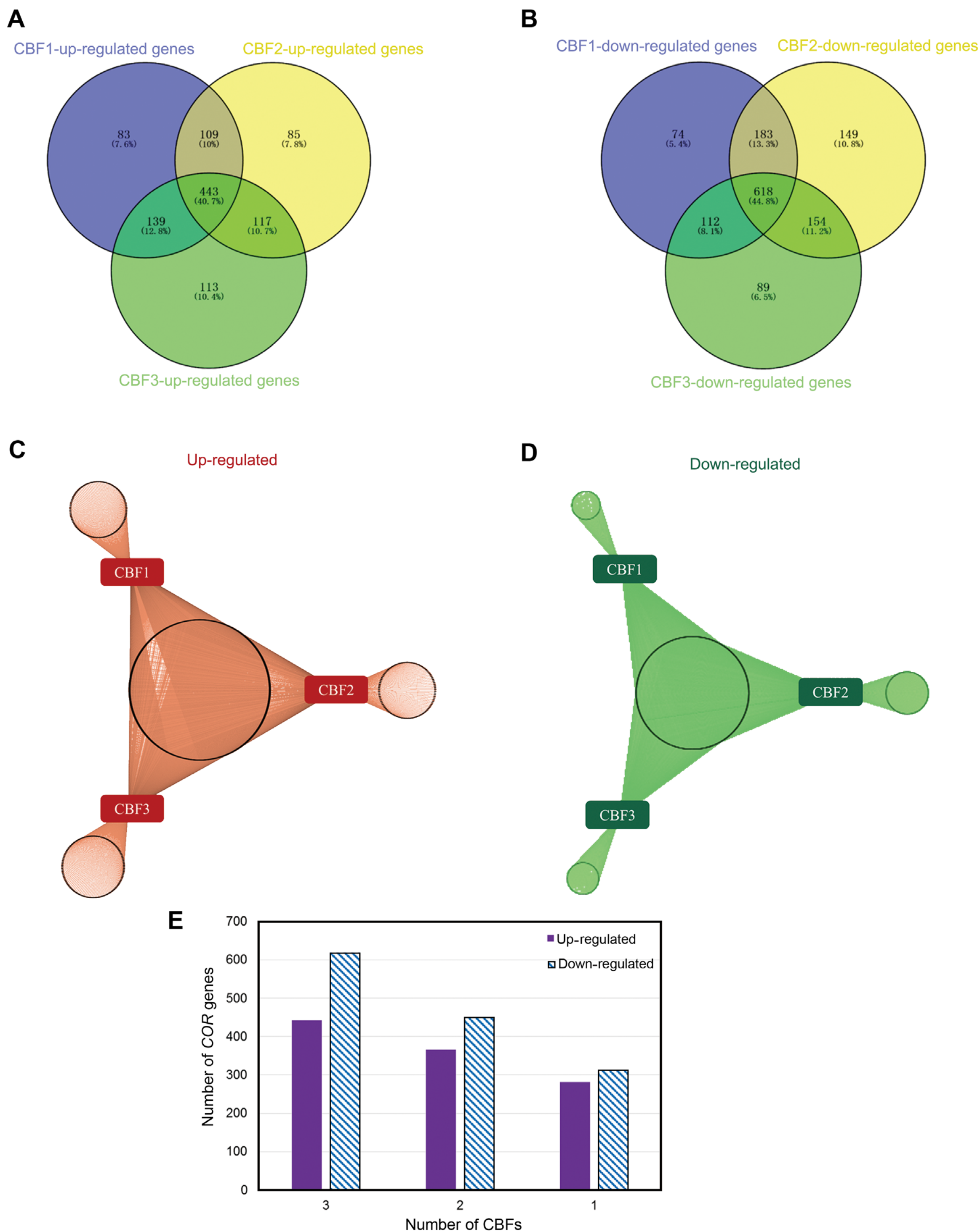

Figure 8. Transcriptional regulatory networks revealed by the gene expression pattern in three single *cbf* mutants
(**A**, **B**) Venn diagrams showing the number of genes specifically up-regulated and down-regulated respectively by single CBFs, overlapped by different combinations of two CBFs and by all three CBFs. Numbers in different sectors indicate the number of genes regulated by different single and combinations of CBFs (overlapped areas). (**C**, **D**) The up-regulated network and down-regulated network respectively. Black dots around the small circles represent genes regulated by single CBFs. Black dots around in the central big circles represent genes co-regulated by two or three CBFs. The lines connecting the dots and CBF1, CBF2, and/or CBF3 represent the regulation relationship. (**E**) The number of genes regulated by one, two, and three CBFs, respectively.

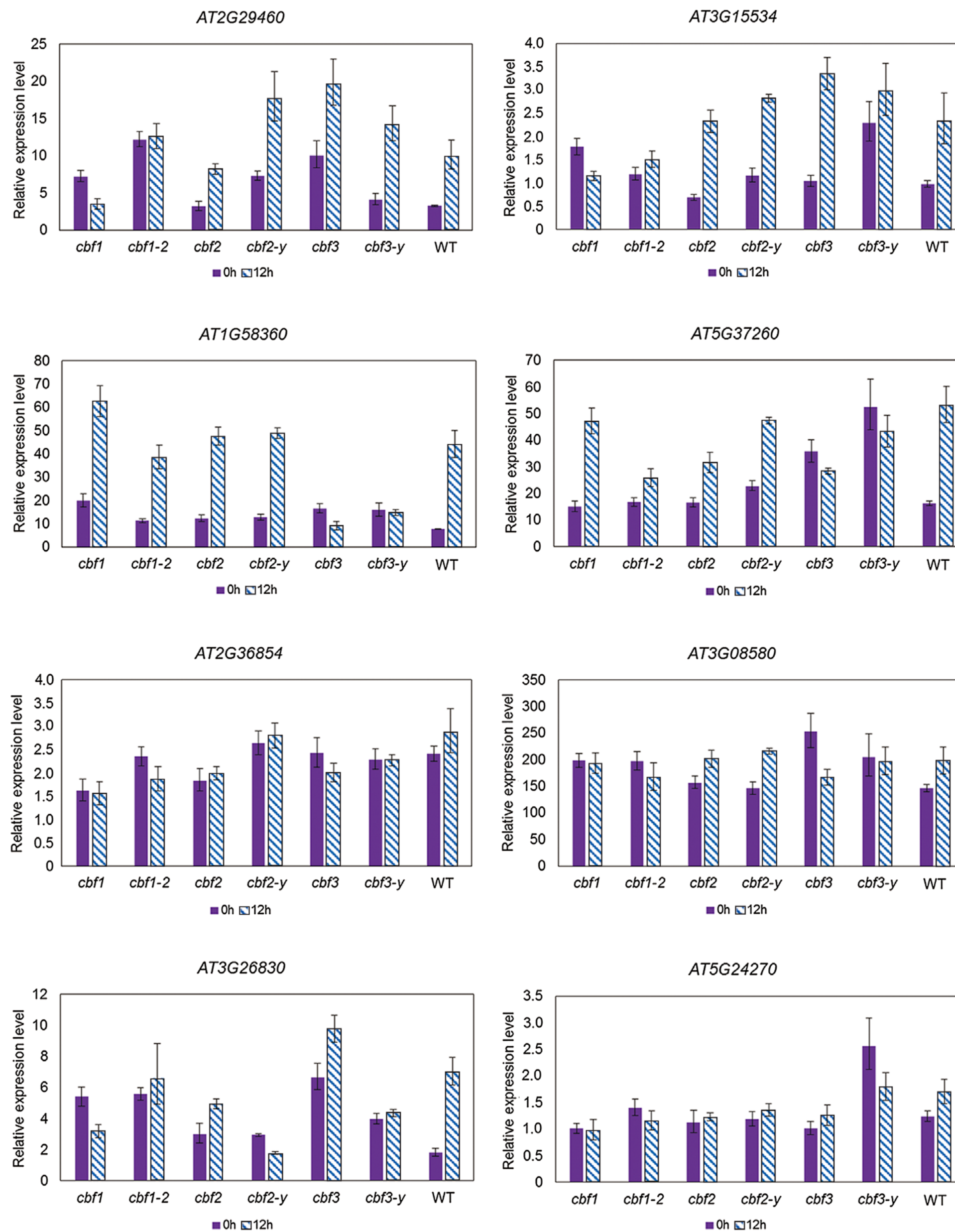

Figure 9. The confirmation of differentially expressed genes in different *cbfs* lines by quantitative real-time PCR
RNAs of 2-week-old seedlings of wild type (WT) and six mutant lines (*cbf1*, *cbf1-2*, *cbf2*, *cbf2-y*, *cbf3*, *cbf3-y*) growing at room temperature (22 °C) and treated at 4 °C for 12 h were extracted respectively for quantitative real-time polymerase chain reaction. Fourteen genes were randomly picked from the up-regulated genes in seven sectors in Figure 8A (two from each sector), together with two well-known *COR* genes as control. Error bars indicate the standard deviation.

and metabolism are compromised by cold stress. There may exist a trade-off between the benefit of acquisition of cold tolerance and cost of growth and metabolism, which is consistent with the reports that over-expression of CBF genes resulted in dwarfness and early flowering phenotypes (Achard et al. 2008; Park et al. 2015).

Because the single *cbf* mutants in this study were generated by CRISPR/Cas9 technology, there is a possibility that the *CBF*-specific single-guide RNAs also target other genes. To clarify this, we compared the expression levels of some CBF differentially regulated genes in two independent lines for each of the three single mutants: two independent lines for *cbf1* and one

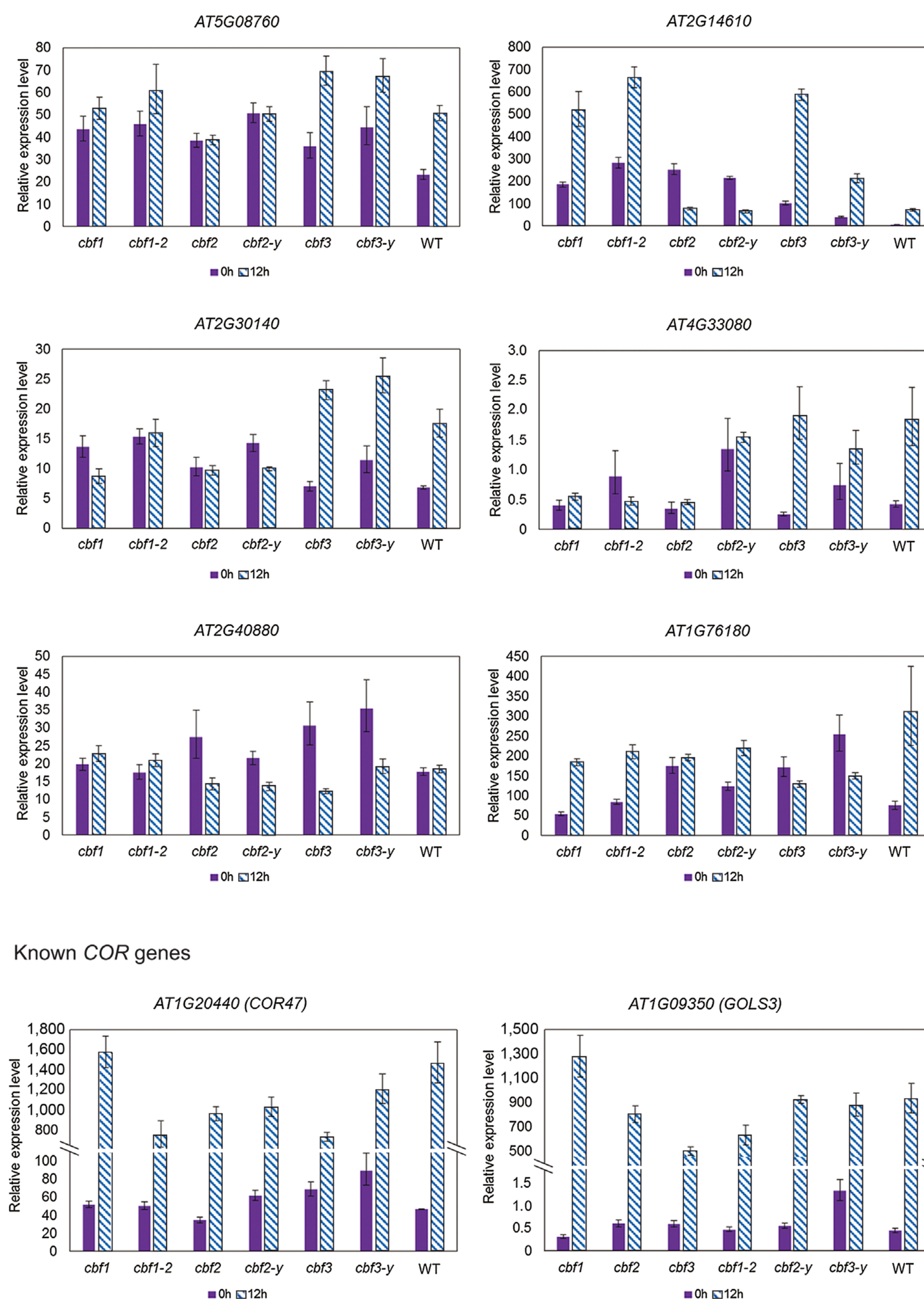

Figure 9. Continued.

line for *cbf2* and *cbf3* respectively, generated in this study by CRISPR/Cas9 technique, and one T-DNA insertion line for each of *cbf2* (*cbf2-y*, Novillo et al. 2004) and *cbf3* (*cbf3-y*, Jia et al. 2016). Fourteen genes were picked from the up-regulated genes in Figure 8A (two were randomly picked from each of seven sectors), together with two well-known *COR* genes as control to confirm the reliability of our results. Quantitative real-time PCR was performed to examine the expression levels of these genes in different lines (Figure 9). The results showed that the expression patterns of these genes were generally consistent with the RNA-seq results (Figure 9).

DISCUSSION

In recent years, CRISPR/Cas9 technology has been developed into a powerful tool to specifically knock out

target genes in many organisms including plants. Compared to the conventional T-DNA insertional mutagenesis in plants, the CRISPR/Cas9 system is specifically useful in knocking out multiple genes that are closely clustered and even tandemly arranged in one locus of a chromosome. CBF transcription factors are reported to play central roles in the cold response of plants. However, the three homologous *CBF* genes are tandemly clustered together in a single locus on chromosome 4, which has made it difficult to obtain single loss-of-function mutants for each of the three CBF genes with the traditional methods. Taking advantage of the CRISPR/Cas9 technology, we have successfully obtained homozygous single mutants *cbf1*, *cbf2* and *cbf3* with a frame shift mutation before their DNA-binding domain; therefore, we were able to analyze the genes that are regulated by each CBF transcription factor. These single mutants could be important materials, together with the double and triple mutants, for further dissecting the distinct role of each CBF in the cold responsive network and other regulatory networks.

In this study, we identified more than 3,000 *COR* genes, and many of the genes that were up-regulated were associated with the plant response to cold-related abiotic stimuli. However, the loss-of-function of single CBF genes did not result in much change in the expression patterns of more than half of these *COR* genes. This is consistent with the recent report that only *cbf1*, *cbf2*, *cbf3* triple mutants and *cbf2*, *cbf3* double mutants exhibited higher cold sensitivity (Zhao et al. 2016). Our results also showed that, first, these three CBF transcription factors are functionally redundant in the plant response to cold since no significant decrease in cold tolerance was observed in any of the *cbf* single mutants. However, our result is different from some previous reports. Novillo et al. found that a *cbf2* T-DNA insertion mutant displayed an increased cold tolerance (Novillo et al. 2004), while Alonso-Blanco and his colleagues reported that a deletion in *CBF2* promoter in Cvi ecotype led to a reduced freezing tolerance (Alonso-Blanco et al. 2005). A recent work on the loss-of-function mutant of *CBF2* generated by CRISPR/Cas9 revealed an increase of sensitivity to freezing temperature (Zhao et al. 2016). It seems that different genetic backgrounds or different experimental conditions could affect the regulation efficiency of three CBFs. Therefore, more detailed analysis on the single mutants, different combination of double mutants, and triple mutant under the same condition is needed to clarify the discrepancies.

Second, the three CBF transcription factors have different roles. In previous studies, CBF2 and CBF3 were found to play a critical role in the adaptation of natural populations of *Arabidopsis* to different habitats (Alonso-Blanco et al. 2005; Kang et al. 2013; Oakley et al. 2014; Gehan et al. 2015). Although the three single *cbf* mutants showed slightly reduced number of *COR* genes with differential expression in response to cold, we found, by motif enrichment analysis, that up-regulated *COR* genes were enriched in CBF-binding motifs in CBF2- and CBF3-up-regulated genes, but not in CBF1-up-regulated genes, suggesting that more *COR* genes were direct targets of CBF2 and CBF3. These results are consistent with the results from many other studies (Vogel et al. 2005; Novillo et al. 2007; Zhen and Ungerer 2008a; Kang et al. 2013; Oakley et al. 2014; Gehan et al. 2015).

Third, the single CBF-regulated genes might be more directly related to the plant response to cold, as well as to plant hormones according to GO analysis in this study. However, the genes that were co-regulated by two or three CBFs might be more directly involved in cellular signal transduction and metabolic processes, which may indirectly affect the ability of the plant to properly respond to cold. In the present study, we are only focused on which of the *COR* genes were regulated by single CBFs, and inferred the co-regulated genes by identifying the genes which share the similar expression pattern in more than one single mutant. The mechanism of how those single CBF-regulated genes are differentially regulated by each CBF transcription factor needs to be investigated in the future.

The identified single-CBF-regulated genes are only parts of the whole CBF regulon. The expressional change of some *COR* genes caused by loss-of-function mutation of a single CBF should also be examined in double or triple mutants since many *COR* genes were reported to be tightly co-regulated by CBFs. Some *COR* genes require normal functions of all three CBFs, and other *COR* genes are co-regulated by different combinations of two CBFs and are only down-regulated in double or triple mutants (Novillo et al. 2007). Therefore, more genes, especially those co-regulated by two or three CBFs, are involved in the transcription network of CBF regulation.

Plant response to cold is a complicated and tightly regulated process. On one hand, the cold responsive pathway was activated, whereas the metabolism-related pathways were repressed, in which three CBFs played a central role. On the other hand, each CBF could regulate different sets of downstream genes despite their functional redundancy. The studies on the triple mutants of CBFs generated by CRISPR/Cas9 (Jia et al. 2016; Zhao et al. 2016) provided very important information about the cold regulatory network in *Arabidopsis*, but further transcriptome and proteomic analyses of the different combinations of the double and triple mutants are required to dissect a more precise role for each of the CBF transcription factors in the cold responsive network of *A. thaliana*.

MATERIALS AND METHODS

Plant materials and growth conditions

The seeds of Col and *cbf* single mutants of *Arabidopsis thaliana* were sterilized with 15% NaClO for 15 min, washed with sterile water five times, plated on half-strength Murasshige and Skoog (MS) media (Sigma) with 0.7% agarose, and stratified for 3 d at 4 °C. Then, the plates were placed under normal growth conditions for 1 week with a 16 h photoperiod that had a minimum illumination of 120 μmol/m^2/s at 22 $\pm$ 2 °C. Seedlings were harvested for DNA extraction and mutation identification after growing for 3–4 weeks in sterilized soil. Plants used for the RNA-seq analysis and quantitative real-time PCR were grown in plates for one more week followed by treatment at 4 °C for 0 h, 2 h and 12 h.

Single *cbf* mutants generated using the CRISPR/Cas9 system

The spacer sequence was designed as complementary oligonucleotides based on the sequences of the targeted genes, *CBF1*, *CBF2* and *CBF3*. They were annealed with overhangs specific to the *Bbs*I restriction enzyme site and ligated into the linearized vector harboring AtU6-26 promoter. The fragment-containing spacers driven by the AtU6-26 promoter were recovered using the restriction endonucleases *Kpn*I and *Sal*I. This fragment was cloned into the vector pCAMBIA1300, which harbors the Cas9-coding sequence driven by the CaMV 35S promoter. The destination plasmid was transformed into agrobacterium, and the Col plants were transformed using the floral dip method (Feng et al. 2013; Miao et al. 2013).

The positive-transformed T1 plants were screened by sowing the seeds of the transformed plants on the MS media with hygromycin. Then, the genomic DNA from the T1 plants was extracted, and the target genes were amplified by PCR. The amplified products were sequenced to examine if mutations were introduced into the targeted sites.

T1 plants with single-mutated *CBF1*, *CBF2*, and *CBF3* were grown under normal conditions and self-fertilized for homozygous T2 mutants.

Freezing tolerance test

The freezing tolerance of the mutants was examined as described by Kang et al. (2013) and Jia et al. (2016) with some modifications. Two-week-old plants in Petri dishes with cold acclimation (at 4 °C for 3 d, abbreviated as CA) were transferred into a low-temperature growth chamber (Percival Intellus Environmental Controller) at −1 °C (dark). After the temperature of the entire plate fell to −1 °C, the plates were incubated at −1 °C for 16 h. Then, the temperature in the chamber was decreased by 1 °C/h to the desired temperatures (−5, −6 and −12 °C, which were set based on preliminary experiments exploring the full range of tolerance). After being kept at the desired temperature for 1 h, the plates were removed from the chamber and incubated at 4 °C for 24 h in the dark and then placed under normal growth conditions for recovering. The survival rate was calculated when the plants were moved to normal growth conditions for 2 d after freezing treatment. The individuals with green cotyledons and true leaves were identified as survivors. Each assay was conducted using replicates of at least three plates at a time with 30 individuals in each plate.

Expression levels of target genes measured by quantitative real-time PCR

The seedlings were harvested for total RNA extraction after they were treated at 4 °C for 2 and 12 h. The plants grown under normal conditions were used as control. Total RNA was extracted using TRIzol reagent (Invitrogen, Cat. # 15596026) and reverse-transcribed by RTase (Promega, Cat. # A5003). The complementary DNA (cDNA) was used as the template for quantitative real-time PCR.

Quantitative real-time PCR was conducted in an ABI 7500 Fast Sequence detector using SYBR Green Master mix (Toyobo, Cat. # QPK-201). All primer sequences are listed in the Supporting Information Table S4. Quantitative real-time PCR amplifications were performed in a total volume of 20 μL, including 2 μL of gene-specific primers (5 μmol/L) for 5′ and 3′ extension, 1 μL of cDNA, 10 μL of reaction mixtures of enzymes and fluorescent dyes, and 5 μL of double-distilled water. The PCR-cycling conditions were performed as follows: an initial polymerase activation step at 94 °C for 5 min, followed by 40 cycles of 94 °C for 30 s, 59 °C for 30 s, and 72 °C for 30 s. The data were collected after each extension step (30 s at 72 °C). For a negative control, 1 μL of cDNA was replaced by the equal amount of double-distilled water. Comparative ΔΔCt method was used to calculate the relative expression level of a gene. The relative gene expression values were standardized to the expression level of the *EF1A* gene (*At5g60390*) coding for the translation elongation factor EFTu/EF1A, which has been demonstrated to be not influenced by cold stress. For each sample, two replicates were carried out each time. The cycle threshold (Ct) values for genes were calculated by the formula POWER (2, −Ct) in Excel, and the obtained Ct values of target genes were divided by the value of *EF1A* to get the relative expression level.

RNA-seq analysis

RNA was extracted from four samples (WT, *cbf1*, *cbf2* and *cbf3*) with three biological repeats after 2-week-old seedlings were treated at 4 °C for 0 h, 2 h and 12 h, and the cDNA libraries were then constructed. Samples were sequenced with an Illumina Hi-seq 4000 sequencer.

Raw reads including the adapters and low-quality data (if there were more than 76 bp with each sequencing base quality lower than five in the reads) were filtered. To obtain the differentially expressed genes in different lines, we adopted the method of Kim et al. (2013). Tophat (version 2.0.14) (Kim et al. 2013) was used to align the clean reads with the *A. thaliana* genome in TAIR10 (http://www.arabidopsis.org/download_files/Genes/TAIR10genomerelease/TAIR10chromosomefiles/TAIR10_chr_all.fas) with parameters: −i 36 −I 20000 −p 5 −r 20–mate-std-dev 50. The parameters used in this study were consistent with a recent report (Huang et al. 2015), confirming the accuracy and integrity of our analysis. The Cuffdiff (version 2.2.1; Trapnell et al. 2012) (parameters: default) was used for stringent statistical analysis to normalize and find the differential expression levels of the RNAs using the FPKM values and the *P*-values in the output bam files of each single mutant. Venn diagrams were made using VENNY (http://bioinfogp.cnb.csic.es/tools/venny/).

The library pheatmap of R package (version 3.2.5) was used to generate all heatmaps, and each one except the diagram in Figure 6 was made using the parameter scale = "row" to scale the expression level in the row. Euclidean distance was adopted for hierarchical clustering in both row and column.

Gene Ontology annotation was performed with DAVID (Database for Annotation, Visualization and Integrated Discovery) (Huang et al. 2009a, 2009b) (https://david.ncifcrf.gov/) using default parameters.

Promoter motif enrichment analysis

The DNA motifs were counted within the region 1 kb upstream of the translation start codon based on TAIR version 10 annotation (Arabidopsis Information Resource, http://www.arabidopsis.org/). Motif enrichment in a group of genes was tested against a background distribution generated by 10,000 times of picking up every 2,000 random samplings as a group to calculate the enrichment of CRT motif from all genes in the genome and the significance of enrichment was presented as *P*-value.

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AUTHOR CONTRIBUTIONS

Y.S. designed some of the experiments, performed most of the research and drafted the manuscript. J.H. carried out RNA-seq data alignment and preliminary data analysis. T.S. contributed to the freezing tolerance assay. X.W., C.Z. and Y. A. participated in the identification of single mutants using CRISPR/Cas9 system. H.G. initiated the project, designed some of the experiments, supervised the study and revised the manuscript.

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article: http://onlinelibrary.wiley.com/doi/10.1111/jipb.12515/suppinfo

Figure S1. Base percentage composition along clean reads in all samples

Figure S2. Distribution of average qualities along reads in all samples

Figure S3. Validation of RNA-seq results by comparing with quantitative real-time PCR results

Table S1. All *COR* genes identified in Col

Table S2. GO analysis on all *COR* genes in Col

Table S3. All differentially expressed genes in single *cbf* mutants

Table S4. Single CBF-regulated genes

Table S5. GO analysis on *COR* genes up-regulated only by one CBF

Table S6. GO analysis on *COR* genes up-regulated by 2 or 3 CBFs

Table S7. GO analysis on all single CBF-down-regulated genes

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