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Research

Rapid report

The *cbfs* triple mutants reveal the essential functions of *CBFs* in cold acclimation and allow the definition of CBF regulons in *Arabidopsis*

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Summary

- In Arabidopsis, the C-repeat binding factors (CBFs) have been extensively studied as key transcription factors in the cold stress response. However, their exact functions in the cold response remains unclear due to the lack of a null *cbf* triple mutant.
- In this study, we used CRISPR/Cas9 technology to mutate *CBF1* or *CBF1/CBF2* in a *cbf3* T-DNA insertion mutant to generate *cbf1,3* double and *cbf1 cbf2 cbf3* (*cbfs*) triple mutants.
- The response of the *cbfs* triple mutants to chilling stress is impaired. Furthermore, no significant difference in freezing tolerance was observed between the wild-type and the *cbf1,3* and *cbfs* mutants without cold acclimation. However, the *cbfs* mutants were extremely sensitive to freezing stress after cold acclimation, and freezing sensitivity ranking was *cbfs* > *cbf1,3* > *cbf3*. RNA-Seq analysis showed that 134 genes were CBF regulated, of which 112 were regulated positively and 22 negatively by CBFs.
- Our study reveals the essential functions of CBFs in chilling stress response and cold acclimation, as well as defines a set of genes as CBF regulon. It also provides materials for the genetic dissection of components in CBF-dependent cold signaling.

Introduction

Low temperature is one of the important environmental factors that constrains plant growth, development, and geographical distribution. To overcome this constraint, plants have evolved sophisticated mechanisms, one of which is cold acclimation (Thomashow, 1999), whereby plants acquire freezing tolerance after being exposed to nonfreezing temperatures. Cold acclimation is a complicated process that involves many changes, ranging from gene expression to physiological and biochemical processes (Hua, 2009; Knight & Knight, 2012; Shi *et al.*, 2015). C-repeat binding factors (CBFs), also known as dehydration-responsive element (DRE) binding factor 1 (DREB1), are thought to be key transcription factors involved in cold acclimation (Stockinger *et al.*, 1997; Liu *et al.*, 1998). *CBF* genes are induced rapidly by cold stress, and in turn activate downstream cold responsive gene (*COR*) expression to increase plant freezing tolerance (Stockinger *et al.*, 1997; Liu *et al.*, 1998; Thomashow, 1999). Several transcriptional factors, including ICE1 (inducer of CBF expression 1), MYB15, CAMTA3 (calmodulin-binding transcription activator 3) and EIN3 (ethylene insensitive 3) are shown to regulate the expression of *CBFs* by binding to their promoters (Chinnusamy *et al.*, 2003; Dong *et al.*, 2006; Doherty *et al.*, 2009; Shi *et al.*, 2012). Besides, *CBF* genes are also modulated indirectly by other proteins, such as LOS4 (low expression of osmotically responsive gene 4) (Gong *et al.*, 2002), FIERY2 (Li *et al.*, 2013), and CRLK1 (calcium/calmodulin-regulated receptor-like kinase 1) in Arabidopsis (Yang *et al.*, 2010), and COLD1 in rice (Ma *et al.*, 2015).

There are three *CBF* genes in *Arabidopsis*, known as *CBF1/DREB1B*, *CBF2/DREB1C* and *CBF3/DREB1A*, which belong to the APETALA2/ethylene-responsive factor (ERF) superfamily. CBF/DREB1 proteins bind to C-repeat/DRE *cis*-elements in *COR* gene promoters to regulate their expression (Stockinger *et al.*, 1997; Gilmour *et al.*, 1998; Liu *et al.*, 1998). Overexpression of *CBFs* in *Arabidopsis* or other species, such as *Brassica napus*, wheat (*Triticum aestivum*), rye (*Secale cereale*), and tomato, causes the constitutive expression of downstream *COR* genes and increases plant freezing tolerance, indicating the important role of CBFs in cold acclimation

(Stockinger *et al.*, 1997; Jaglo-Ottosen *et al.*, 1998; Liu *et al.*, 1998; Gilmour *et al.*, 2000; Jaglo *et al.*, 2001). Characterization of loss-of-function mutants of *CBF* genes may shed more light on the exact functions of these genes. However, these three *CBF* genes are located in tandem on chromosome 4, which makes it impossible to generate a *cbfs* triple mutant by recombination segregation. Rather, by using RNA interference and antisense approaches, *CBF1* and/or *CBF3* were knocked down, resulting in down-regulation of downstream target genes under cold stress and impaired cold acclimation (Novillo *et al.*, 2007). The *cbf2* null mutant showed enhanced freezing tolerance with and without cold acclimation, and up-regulation of *CBF1* and *CBF3* genes, suggesting that *CBF2* negatively regulates the expression of *CBF1* and *CBF3* (Novillo *et al.*, 2004). In addition, a recent study reported that by overexpressing the dominant negative *CBF2* form (*CBF2ΔC*), the induction of CBF regulons is impaired, leading to hypersensitivity to freezing stress after cold acclimation (Park *et al.*, 2015).

The target genes of CBFs have been identified using multiple approaches. GeneChip arrays have revealed that overexpression of *CBF1*, *CBF2* and *CBF3* activates a common set of 30 genes (Gilmour *et al.*, 2004). A recent study using Affymetrix ATH1 DNA chip analysis showed that 133 genes are regulated by three *CBFs* (Park *et al.*, 2015). However, overexpression of *CBFs* driven by the *35S* promoter leads to ectopic expression, which may cause nonspecific binding. Therefore, generation of a *cbfs* triple mutant is required to define the target genes of CBFs, as well as the exact contribution of *CBFs* in the cold stress response. Given that obtaining a triple mutant by traditional genetic approaches is impossible, the newly developed CRISPR/Cas9 technology may solve this problem. This technology was recently reported to specifically mutate *Arabidopsis* genes, and the mutations are successfully inherited by progeny (Mao *et al.*, 2013; Feng *et al.*, 2014).

In this study, we used the CRISPR/Cas9 system to mutate *CBF1* and *CBF1/CBF2* in a *cbf3* T-DNA insertion mutant to generate *cbf1 cbf3* and *cbf1 cbf2 cbf3* (*cbfs*) mutants. We analyzed the responses of these mutants to chilling and freezing stresses. Our results showed that *cbfs* mutant fails to respond to chilling stress. Consequently, the *cbf1 cbf3* and *cbfs* mutants are hypersensitive to freezing stress after cold acclimation. We further defined the CBF-regulated genes by RNA-Seq assay.

Materials and Methods

Plant materials and growth conditions

Arabidopsis thaliana (L) Heynh plants were grown on ½ Murashige and Skoog (MS) medium at 22°C under a 16 h : 8 h, light:dark photoperiod. The T-DNA insertion mutant *cbf3* (SAIL_244_D02) used in this study was obtained from Arabidopsis Biological Resource Center (Columbus, OH).

Generation of *cbf1,3* and *cbfs* mutants

To generate *cbf1,3* double and *cbfs* triple mutants, a *CBF1* specific target and three *CBF1*/2 conserved targets were selected as the targets for Cas9 to mutate *CBF1* and *CBF1/2*, respectively. Vector construction and mutant identification were performed as described (Xing *et al.*, 2014). The targets were cloned into the pHSE401 vector, named C1-pHSE401 for *CBF1*, and C1/2-1-pHSE401 and C1/2-2-pHSE401 for *CBF1/2*. The constructs were introduced into the *cbf3* mutant by floral dip (Clough & Bent, 1998). The resulting transgenic T_1 seeds were screened on ½ MS medium containing 25 mg l^{-1} hygromycin. Genomic fragments covering the mutation sites were amplified from the T_1 transgenic plants by PCR and sequenced. T_1 heterozygous mutants were kept for the T_2 generation to identify homozygous mutants. The mutant seeds were harvested from individual lines to obtain T_3 seeds. The seeds were screened with hygromycin, and nonhygromycin resistant seeds were used for the following experiments.

Plasmid construction and plant transformation

To generate *pCBFs::CBFs-Myc*, The genomic fragments of *CBF* genes containing 1.52-, 1.78- and 1.72-kb DNA sequences upstream of *CBF1*, *CBF2* and *CBF3* were amplified and cloned into pCAMBIA1300 vector with a Myc tag. The resulting vectors were transformed into the wild-type Col plants by floral dip (Clough & Bent, 1998). The homozygous transgenic plants were crossed into *cbfs-1* to generate *cbfs pCBFs::CBFs-Myc* plants.

Chilling stress response assay

For plants grown on soil, 14-d-old seedlings grown on ½ MS plates at 22°C were transplanted onto soil; after 3-d recovery at 22°C, the pots were transferred to a 4°C growth chamber and kept for the indicated time. For plants grown on plates, seeds of the wild-type, *cbf1,3* and *cbfs* were sown on ½ MS plates and kept at 22°C for 3 d, and then transferred to 4°C and maintained for the indicated time. At least three independent experiments were done and each experiment was performed with three technical replicates.

Assays for freezing tolerance, electrolyte leakage, and proline content

Freezing tolerance was assessed as described (Shi *et al.*, 2012). Briefly, 2-wk-old plants grown at 22°C on ½ MS plates were treated with or without cold acclimation (4°C for 3 d) and then subjected to freezing assay. The program was set at 0°C and dropped 1°C h^{-1} to desired temperatures. After freezing treatment, the plants were put at 4°C in the dark for 12 h and then transferred to 22°C for an additional 3 d. The survival rates were counted. Electrolyte leakage assays were performed as described (Ding *et al.*, 2015). Proline content was measured as described (Bates *et al.*, 1972). At least three independent experiments were done and each experiment was performed with three technical replicates.

High-throughput mRNA sequencing analysis

Fourteen-day-old seedlings grown on ½ MS medium at 22°C were treated at 4°C for 0, 3 or 24 h. Total RNA was extracted

and 3 μg of RNA for each sample was used for library construction and subsequent RNA-deep sequencing on the Illumina Hiseq 2500 platform. RNA-Seq data were collected from two independent experiments. The adaptor sequences and low quality sequences were removed. Approximately 4.0 GB of clean reads were generated from each sample. The clean reads were mapped to the Arabidopsis reference genome (TAIR10) using TopHat v.2.1.0 (Trapnell *et al.*, 2009) with TAIR10 gene annotation as the transcript index. The minimum and maximum intron lengths were set to 40 and 5000 separately (Kim *et al.*, 2013). Cufflinks v.2.2.1 (Roberts *et al.*, 2011) was used to assemble the new transcripts (Trapnell *et al.*, 2013). HTSeq v.0.6.0 (Anders *et al.*, 2015) was used to calculate the raw read counts for each gene. Gene expression normalization among samples was performed by using DESeq2. The different gene expression data were collected from the comparison with a fold change ≥ 2 and a false discovery rate (FDR, Benjamini–Hochberg adjusted *P* value) ≤ 0.01 (Love *et al.*, 2014).

RNA extraction and qRT-PCR

Total RNA was extracted from 14-d-old seedlings using the RNAprep pure Plant Kit (Tiangen, Beijing, China), and HiScript II Q RT SuperMix (Vazyme, Nanjing, China) was used for reverse transcription. Quantitative real-time PCR (qRT-PCR) was performed with a SYBRGreen PCR Master Mix kit (Takara, Kusatsu, Japan). Relative expression levels were calculated as described previously (Shi *et al.*, 2012), and the specific primers used are listed in Supporting Information Table S1.

Results and discussion

Generation of *cbf1,3* and *cbfs* mutants in Arabidopsis

To explore the contributions of *CBF* genes to freezing tolerance, a *cbf3* T-DNA insertion mutant (SAIL_244_D02) was obtained from ABRC. Sequencing analysis showed that the T-DNA was located in the 22 bp after ATG (Figs 1a, S1a),

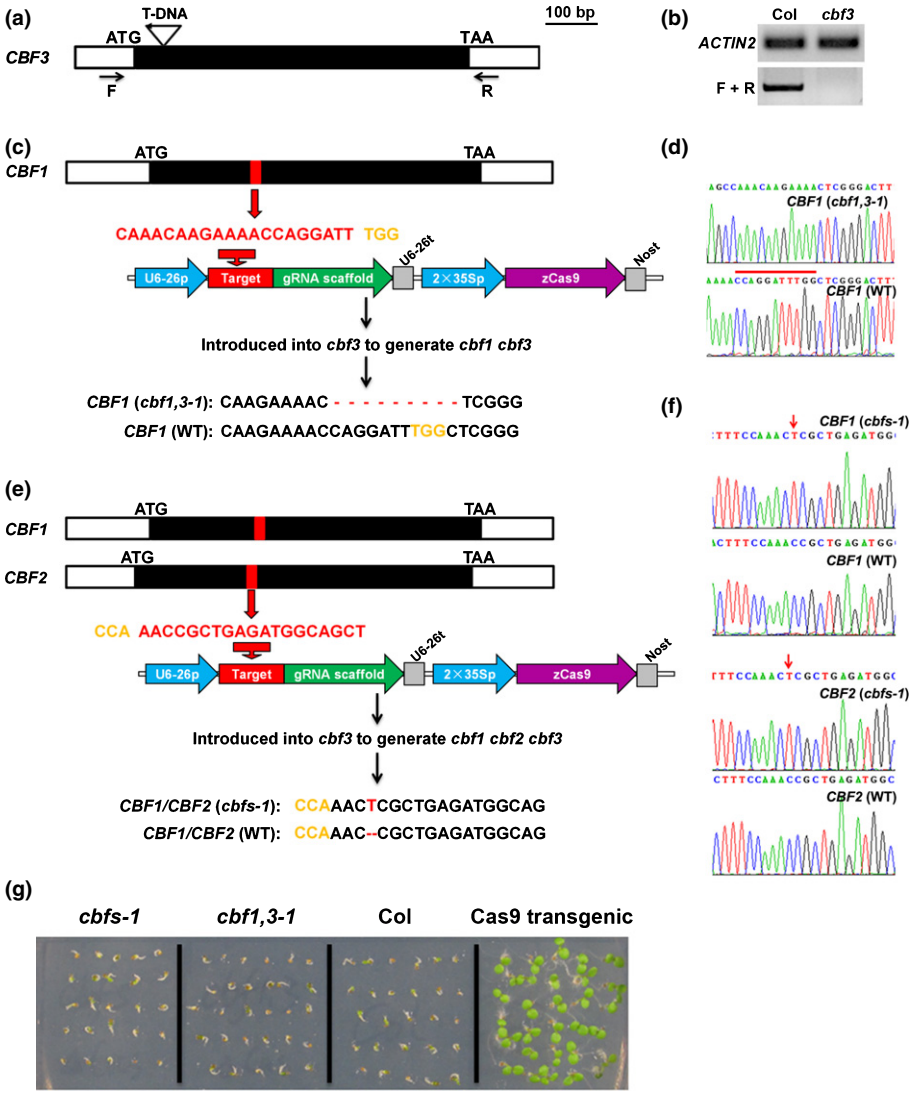

Fig. 1 The generation of *cbf1,3-1* double and *cbfs-1 Arabidopsis* triple mutants. (a) Schematic of the T-DNA insertion mutant *cbf3*. (b) RT-PCR analysis of *cbf3* mutant. The seedlings were grown on ½ MS medium for 14 d and subsequently treated at 4°C for 3 h. RT-PCR was performed using *CBF3* specific primers. *ACTIN2* was used as a control. (c) Schematic of the *cbf1,3-1* double mutant. (d) SANGER sequencing chromatography showing the deletion in the *cbf1,3-1* double mutant. (e) Schematic of the *cbfs-1* triple mutant. (f) SANGER sequencing chromatography showing the mutations in *cbfs-1* triple mutant. (g) Separation of the *Cas9* gene from the mutant. The details of target construction and mutant generation are described in the Materials and Methods section.

which is presumed to produce a stop codon after the seventh aa. qRT-PCR showed that full length of *CBF3* gene was not expressed in the *cbf3* mutant (Fig. 1b). Based on freezing assay, there was no significant difference between wild-type Col and *cbf3* under nonacclimated conditions (Fig. S1b), whereas the *cbf3* mutant showed slightly decreased freezing tolerance compared to the wild-type under cold-acclimated conditions (Fig. S1c).

The gRNA target for *CBF1* was cloned into pHSE401 and subsequently transformed into a *cbf3* mutant to generate *cbf1 cbf3* double mutants, *cbf1,3-1* and *cbf1,3-2* (Figs 1c, S2a). We identified homozygous *cbf1,3-1* and *cbf1,3-2* double mutants lines among T_2 progeny by sequencing. An 11 bp deletion (from 210 to 220 bp after ATG) and 2 bp deletion (from 212 to 213 bp after ATG) in the coding region of *CBF1* were found in *cbf1,3-1* and *cbf1,3-2*, respectively (Figs 1c,d, S2a). The deletions result in reading frame shifts, thus producing translation stop at the AP2 domain. By the same approach, three different common gRNA targets for both *CBF1* and *CBF2* were designed and subsequently transformed into the *cbf3* mutant to generate *cbf1 cbf2 cbf3* (*cbfs*) triple mutants, *cbfs-1* and *cbfs-2* (Figs 1e, S2b). In the *cbfs-1* triple mutant, a T was inserted into the Cas9 editing targets, which are located at 236 bp and 245 bp in the coding regions of *CBF1* and *CBF2*, respectively, leading to reading frame shifts (Fig. 1e,f). In the *cbfs-2* triple mutant, 77 bp deletions were found in the coding regions of *CBF1* and *CBF2* (from 174 to 250 bp for *CBF1* and from 165 to 241 for *CBF2* after ATG) (Fig. S2b). As a result, the potential transcripts of *CBF1* and *CBF2* would harbor stop codons in *cbfs-1* and *cbfs-2* mutants. To exclude the effect of the *Cas9* gene, we isolated *cbf1,3-1* and *cbfs-1* mutants by screening for hygromycin resistance (Fig. 1g). The nonhygromycin resistant lines were identified and used for further study.

The triple mutant has impaired response to chilling stress

To assess the phenotypes of *cbf1,3* and *cbfs* under normal and chilling conditions, wild-type, *cbf1,3-1* and *cbfs-1* mutants were grown on soil at 22°C or 4°C. The *cbf1,3-1* and *cbfs-1* mutants did not show any detectable abnormalities during the vegetable or reproductive stages (Fig. 2a,b). Under chilling stress, the *cbf1,3-1* mutant resembled wild-type plants; however, the size of *cbfs-1* mutant was much bigger than that of the wild-type after transferring to 4°C for 50 d (Fig. 2c). A similar phenomenon was observed in seedlings grown on ½ MS medium plates (Fig. 2d). When grown on ½ MS medium for 8 d, root length of *cbfs-1* mutant was *c.* 25% shorter than that of the wild-type at 22°C (Fig. 2e). However, when grown at 4°C for 45 d, this difference was nearly abolished (Fig. 2e,f). These results indicate that both the shoots and the roots of *cbfs-1* mutants are less affected by cold stress, suggesting that *CBF* genes are required for plant response to chilling stress and CBF target genes might be involved in plant growth repression. Consistent with this notion, overexpression of *CBFs* results in growth retardation (Jaglo-Ottosen *et al.*, 1998; Kasuga *et al.*, 1999; Gilmour *et al.*, 2004). It has been shown that the retarded growth of a *CBF1*-overexpressing plant is partially due to the accumulation of DELLA proteins, which mainly act as transcriptional repressors in GA signaling (Achard *et al.*, 2008). We speculate that the reduced accumulation of DELLA at least partially contributes to the non-response of *cbfs* to chilling stress.

CBF genes are required for cold-acclimation process to withstand freezing stress

We further examined the effect of the loss of *CBF* function on freezing tolerance. No significant difference in freezing tolerance was observed between *cbf1,3-1*, *cbfs-1* and the wild-type without acclimation (Fig. 3a,b). Occasionally, the *cbfs-1* mutant showed slightly but not significantly freezing sensitivity compared to the wild-type. An indicator of plasma membrane damage caused by cold stress, electrolyte leakage was comparable among *cbf1,3-1*, *cbfs-1* and the wild-type (Fig. 3c). These results demonstrate that the *CBF* genes do not play important roles in regulating the basal freezing tolerance of plants.

After cold acclimation, *cbf1,3-1* and *cbfs-1* showed enhanced freezing sensitivity, and the *cbfs-1* mutant showed strikingly more sensitivity than *cbf1,3-1* to freezing stress (Fig. 3a). When treated at −9°C for 1 h, *c.* 95% of the wild-type and 80% of *cbf1,3-1* survived, while only 10% of *cbfs-1* plants survived. When treated at −10°C for 1 h, *c.* 80% of the wild-type and 58% of *cbf1,3-1* survived, but nearly all *cbfs-2* plants were dead (Fig. 3d). Consistent with these results, electrolyte leakage was mildly increased in *cbf1,3-1* and dramatically increased in *cbfs-1* compared to the wild-type (Fig. 3e). Furthermore, we tested the content of the cryoprotectant proline in *cbfs-1*. No significant difference in proline content was observed in *cbfs-1* vs the wild-type at 22°C; however, *cbfs-1* accumulated much less proline than the wild-type under chilling stress (Fig. 3f).

To confirm that the freezing sensitive phenotypes were caused by the mutations in the *CBF* genes, we analyzed the freezing sensitivity of the second alleles of *cbf1,3* and *cbfs*. The acclimated *cbf1,3-2* displayed mild but significant sensitivity to freezing stress compared to the wild-type (Fig. S3a–c), and the acclimated *cbfs-2* mutants showed extremely sensitive phenotype to freezing stress (Fig. S3d–f). Next we introduced the Myc-tagged CBFs under the control of their native promoters (*pCBFs::CBFs-Myc*) into the *cbfs-1* triple mutant. qRT-PCR analysis showed that *CBFs-Myc* were expressed in the *cbfs-1* triple mutant (Fig. S4a). Freezing tolerance assay showed that each of *CBF* genes could complement the freezing sensitive phenotype of the *cbfs-1* triple mutant after cold acclimation (Fig. S4b–e). Taken together, these results strongly demonstrate that *CBF* genes play redundant and crucial roles in cold acclimation.

The freezing sensitive phenotypes of *cbf1,3* double mutants with and without cold acclimation are consistent with the previous study obtained from RNAi or antisense transgenic lines showing the important role of *CBF1* and *CBF3* genes in cold acclimation (Novillo *et al.*, 2007). As the *cbfs* mutants are much more sensitive than the *cbf1,3* mutants after cold acclimation, implying the importance of CBF2 in cold acclimation. *CBF2* was shown to negatively regulate the expression of *CBF1* and *CBF3* (Novillo *et al.*, 2007). It is also possible that there is other feedback regulation among *CBF* genes. qPCR analysis showed that though

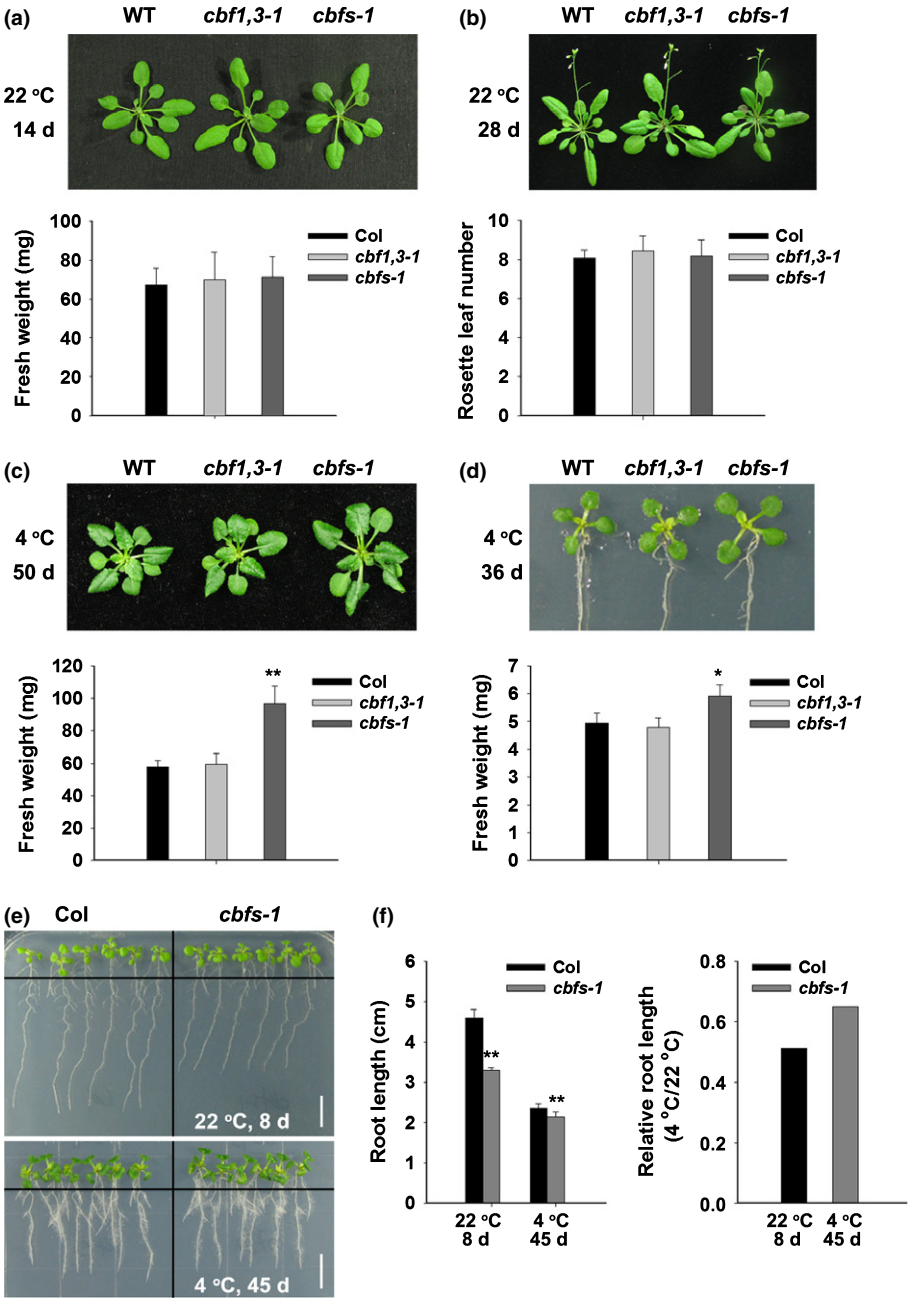

Fig. 2 Loss-of-function of *CBFs* results in an impaired response to chilling stress in *Arabidopsis*. (a, b) The growth phenotypes of wild-type Col (WT), *cbf1,3-1* and *cbfs-1* during vegetative (a) and reproductive (b) stages. Fourteen-day-old seedlings grown on ½ MS plates were transplanted to soil and grown at 22°C with 16 h:8 h, light:dark. Photographs were taken 2 wk after transplanting (a, upper panel), and the fresh weight was measured (a, lower panel). For reproductive growth, photographs were taken 4 wk after transplanting (b, upper panel) and the rosette leaf number was counted (b, lower panel). (c, d) Shoot growth of WT, *cbf1,3-1* and *cbfs-1* on soil (c) or ½ MS medium plates (d) at 4°C. Fourteen-day-old seedlings were transplanted to soil, moved to a 4°C growth chamber and grown for 50 d before photographs were taken (c, upper panel). Fresh weight was measured subsequently (c, lower panel). Seeds were sown on ½ MS medium and germinated at 22°C for 3 d before being transferred to a 4°C growth chamber and kept for 36 d (d, upper panel); fresh weight was subsequently measured (d, lower panel). (e, f) Root length of the WT and *cbfs-1* grown at 22°C or 4°C. Five-day-old seedlings were transplanted to ½ MS medium containing 1% agar and grown at 22°C for an additional 8 d or 4°C for an additional 45 d before photographs were taken. Root length and relative root length were subsequently measured. Bars, 1 cm. Data are mean of three technical replicates ± standard deviation (SD) ($n = 30$ for each replicate: $^*P < 0.05$; $^{**}P < 0.01$; Student's *t*-test). All experiments described earlier were carried out three times with similar results.

the basal *CBF2* expression is higher in *cbf3* mutant than the wild-type without cold treatment, the expression of *CBF1* and *CBF2* in *cbf3* single mutant was comparable to the wild-type after cold treatment (Fig. S5). In addition, the cold-induction of *CBF2* in the *cbf1,3* mutants was indistinguishable from the wild-type (Fig. S5), suggesting that *CBF1* and *CBF3* do not have significant feedback regulation on *CBF2*.

Transcriptome analysis of *cbfs* by RNA-sequencing

To further investigate the effect of *CBFs* on the transcriptome profile, we performed RNA-Seq analysis in 14-d-old wild-type and *cbfs-1* seedlings treated at 4°C for 0, 3, or 24 h. Two independent experiments were carried out, and differentially expressed genes were analyzed using HTSeq and DESeq2 software. At 3 h after cold treatment, 664 genes were up-regulated ($\log_2 \geq 1$, FDR ≤ 0.01), and 187 genes were down-regulated ($\log_2 \leq -1$, FDR ≤ 0.01) in the wild-type. When cold treatment was extended to 24 h, 1460 genes were up-regulated and 1466 genes were down-regulated (Fig. 4a,b; Table S2). A total of 1816 genes were up-regulated and 1530 genes were downregulated in wild-type plants under cold stress (Fig. 4a,b; Table S2).

In the *cbfs-1* mutant, the up-regulated genes numbered 609 and 1375, respectively, at 3 h or 24 h of cold treatment, and the down-regulated genes numbered 163 and 1349, respectively (Fig. 4a,b; Table S3). A total of 1731 genes were up-regulated

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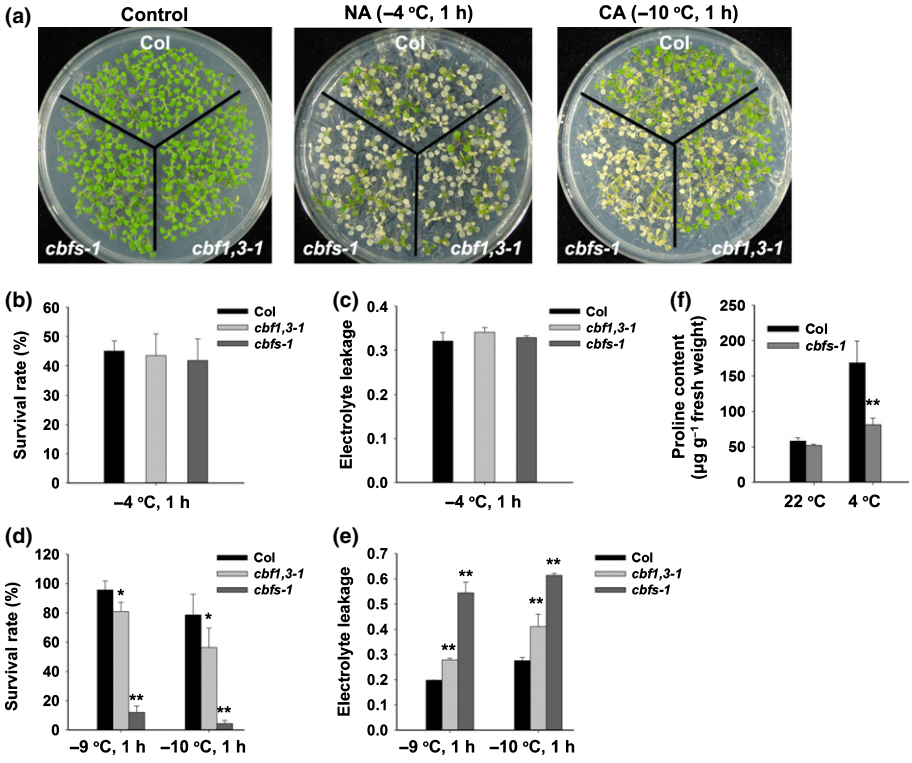

Fig. 3 Freezing tolerance of *cbf1,3-1* and *cbfs-1 Arabidopsis* mutants. (a–e) The freezing assay of *cbf1,3-1* and *cbfs-1* mutants. Two-week-old plants grown at 22°C were treated in a freezing chamber from 0°C and cooled at −1°C h^{-1}. The seedlings were kept in the chamber until −4°C for 1 h (non-acclimated, NA) or −9°C and −10°C for 1 h (cold-acclimated, CA). Photographs were taken after 3 d of recovery at 22°C (a). Survival rates (b, d) and electrolyte leakage (c, e) were measured in the plants described earlier. The data are the means of three technical replicates ± SD ($n = 35$ for each replicate: $^*P < 0.05$; $^{**}P < 0.01$; Student's *t*-test). Three independent experiments were carried out with similar results. (f) Proline content of the WT and *cbfs-1* at 22°C or 4°C. Fourteen-day-old seedlings with or without 3-d cold acclimation were used for proline content measurement. The data are the means of three technical replicates ± SD ($^{**}P < 0.01$; Student's *t*-test). At least three independent experiments were done with similar results.

and 1403 genes were down-regulated in the *cbfs-1* mutant under cold stress. Moreover, a total of 1394 cold-induced genes and 1113 cold-repressed genes were identified in both the wild-type and *cbfs-1* mutant (Fig. 4a,b). These results suggest that a large number of *COR* genes are not affected in the *cbfs-1* mutant under cold stress.

Further comparison between wild-type and *cbfs-1* was performed to reveal CBF-regulated genes, which were selected by the following criteria: genes are up- or down-regulated (fold change ≥ 2, FDR ≤ 0.01) in the *cbfs-1* mutant compared to the wild-type at permissive conditions (22°C) or under cold stress. Under permissive conditions, only three genes were up-regulated (named CBF-repressed genes) and six genes were down-regulated (named CBF-activated genes) in *cbfs-1* compared to the wild-type (Table S4). After cold treatment, five genes were up-regulated and 27 genes were down-regulated in *cbfs-1* compared to the wild-type at 3 h (Table S5), and these numbers expanded to 19 and 101, respectively, at 24 h of cold treatment (Table S6). Heat-maps of the two time points also clearly show the expression difference (Fig. 4c, d). Therefore, a total of 134 genes were defined as CBF-regulated genes, of which 112 were CBF-activated genes and 22 were CBF-repressed genes (Table S7).

The number of differentially expressed genes ($\log_2 \geq 1$, FDR ≤ 0.01) under cold stress (24 h) in wild-type in this study was similar to those in a previous study (Park *et al.*, 2015) (up-regulated genes: 1460 vs 1256, down-regulated genes: 1466 vs 1381). Approximately 38% (51 out of 133) of the genes induced in *CBF*-overexpressing lines (Park *et al.*, 2015) are repressed in *cbfs-1* (Fig. S6a). Furthermore, *c.* 74% (20 out of 27) of the genes repressed in the *CBF2ΔC* plants are also repressed in *cbfs-1* (Park *et al.*, 2015) (Fig. S6b). We further checked our RNA-Seq data using lower criteria (fold change > 1.5, $P < 0.05$), a total 89 genes out of 133 in *CBF*-overexpressing plants and all 27 genes identified in *CBF2ΔC* plants were also down-regulated in the *cbfs-1* mutant under cold stress (Table S8). The reasons that these results do not overlap with each other totally are likely due to the use of different accessions (Col vs Ws), sequencing methods (RNA-Seq vs Affymetrix ATH1 DNA chip), growth conditions (16 h:8 h light:dark cycle vs constant light), and nonspecific binding effect of overexpression (Park *et al.*, 2015). Nevertheless, by using the *cbfs-1* triple mutant, our study covers most CBF-regulated genes identified by overexpression study. More importantly, more new CBF-regulated genes were identified in the *cbfs-1* triple mutant, which helps us to better understand the role of CBFs in the cold stress response.

Further cluster analyses indicated that the CBF-regulated genes are not only involved in cold stress response, but also implicated in drought and salt stress response, hormone response, and carbohydrate metabolism (Fig. S7; Table S9). For example, *ERD10* is also induced by water deprivation (Kiyosue *et al.*, 1994), *HVA22D* and *HVA22E* are induced by ABA (Chen *et al.*, 2002), *LTI65/RD29B* is activated in response to high-salt (Yamaguchi-Shinozaki & Shinozaki, 1993). Besides, some of these genes also contribute to carbohydrate metabolism and anthocyanin biosynthesis, like *GolS3*, *G6PD3* and *UF3GT*, suggesting that CBFs also regulate plant metabolism by controlling the expression of these genes under cold stress. Furthermore, some transcription factors and kinases, such as *RAP2.1*, *ERF4*, *RLP32* and *CIPK25*, are also regulated by *CBFs*. The inhibition of low temperatures on plant growth is partially due to changed hormone biosynthesis or signaling (Scott

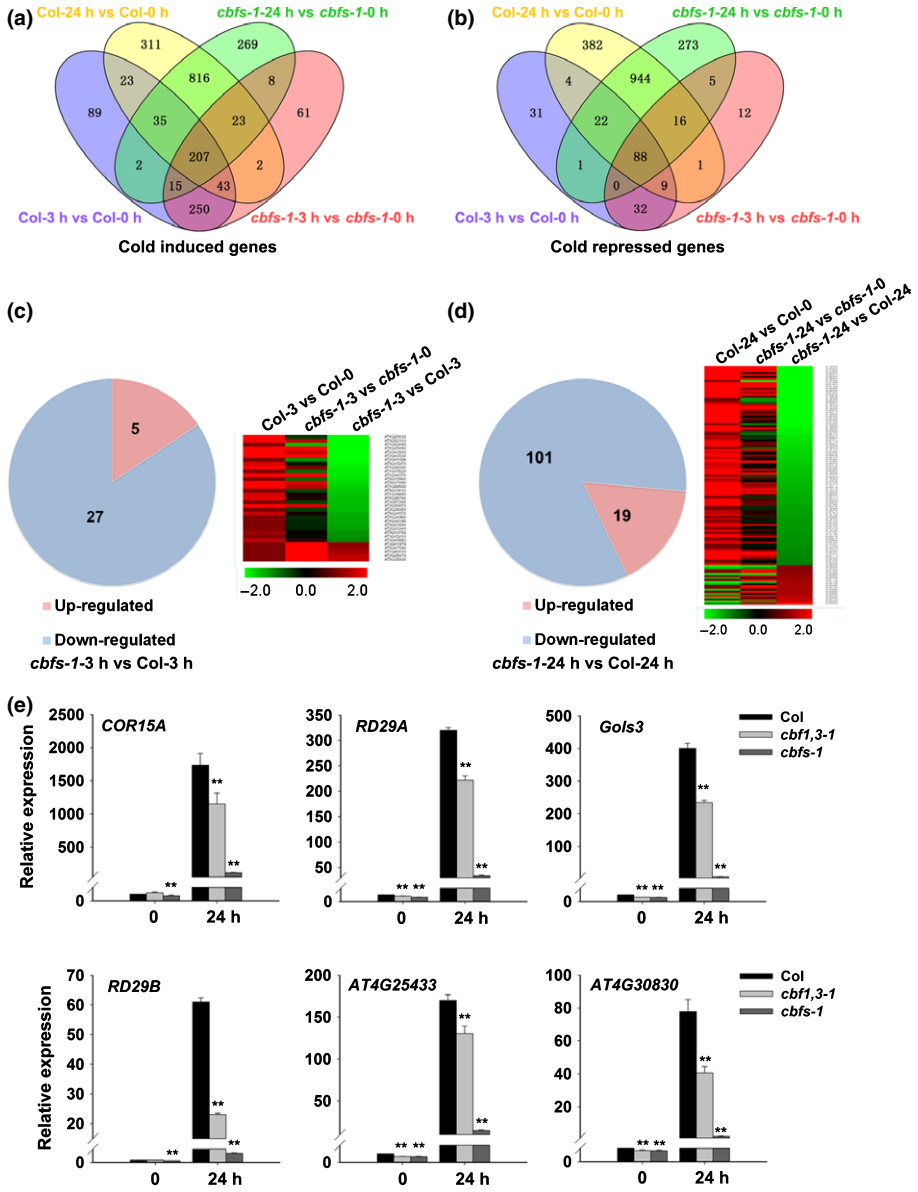

Fig. 4 Transcriptome analysis of wild-type Col and *cbfs-1 Arabidopsis* mutant. (a, b) The cold induced (a) or repressed (b) genes expression in WT Col and *cbfs-1*. Fourteen-day-old seedlings were treated at 4°C for 0, 3, 24 h for RNA-Seq assay. Each sample was compared with 0 h time point to choose the cold induced ($\log_2 \geq 1$, FDR ≤ 0.01) or repressed ($\log_2 \leq -1$, FDR ≤ 0.01) genes. The differentially expressed genes were picked out to draw the Venn diagram on the website: http://bioinfogp.cnb.csic.es/tools/venny/. (c, d) The differentially expressed genes between *cbfs-1* and Col with 3 h or 24 h cold treatment. The genes were selected with the criteria $\log_2 \geq 1$ or ≤ -1, FDR ≤ 0.01. The heat-map was drawn with the software MultiExperiment Viewer (MEV). (e) Expression of CBF regulon genes in Col, *cbf1,3-1* and *cbfs-1*. Six genes were chosen from the 112 CBF-induced genes. Fourteen-day-old seedlings were treated with 4°C for 0 or 24 h. The gene expression was measured by qRT-PCR. The data are the means of three technical replicates ± SD (**$P < 0.01$; Student's *t*-test). Three independent experiments were carried out with similar results.

et al., 2004; Achard *et al.*, 2008). Intriguingly, the *cbfs* mutants are much bigger than the wild-type under chilling stress and some hormone related genes are down-regulated in *cbfs-1* mutant, suggesting that CBFs might be involved in hormone biosynthesis or signaling pathways. Further dissection of hormone responses of this mutant will shed light on the molecular regulation mechanisms of hormone and cold signaling pathways.

Furthermore, we analyzed the promoter regions of these genes. Of the 112 CBF-induced genes, 82 contain one or more CBF conserved binding motif (CCGAC) in their promoters, whereas the other 30 do not include this motif in their promoters. These data suggest that 82 genes are directly regulated by CBFs, while the other 30 genes are indirectly regulated by CBFs (Table S10).

To confirm the RNA-Seq data, six genes were selected to perform qRT-PCR. These genes included the previously reported CBF target genes, *COR15A*, *GolS3*, *RD29A* and *RD29B*. The cold-induction of these genes in the *cbfs-1* mutant was drastically reduced compared to that in the wild-type (Fig. 4e). The cold-induction of two genes, including *At4 g25433* and *At4 g30830*, was also impaired in the *cbfs-1* mutant, which is consistent with the RNA-Seq results. We also examined the expression of these genes in the *cbf1,3-1* mutant. The cold-induction of the earlier *COR* genes was lower than in the wild-type but higher than in the *cbfs* mutant (Fig. 4e), which correlates with the freezing phenotypes. The results demonstrate that CBFs are required for the regulation of *COR* genes.

It is noteworthy that, CBFs only regulates *c.* 7% of *COR* genes, but they greatly contribute to cold acclimation, suggesting that these *COR* genes play important roles in plant responses to cold stress. However, the *cbfs* mutants do not totally abolish cold acclimation, as the acclimated *cbfs* mutants can withstand lower freezing temperatures than the nonacclimated ones, supporting the notion that CBF-independent pathways exist. A number of transcription factors, including *ZAT12*, *HSFC1*, *RAV1*, *MYB73*, *MYB44*, *CRF2*, *WRKY33*, *ERF6*, *CRF3* and *RVE2*, which are considered first-wave transcription factors (Park *et al.*, 2015), are

also comparably induced in the *cbfs-1* mutant at 3 h of cold treatment (Table S3), suggesting that these CBF-independent transcription factors play important roles in cold acclimation and need further investigation.

In conclusion, we took advantage of CRISPR/Cas9 technology to generate *cbfs* triple mutants. Our study not only reveals the essential functions of CBFs in response to chilling and freezing stress, but also provides materials to study the genetic interaction of components in CBF-dependent cold signaling.

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Author contributions

These authors made the following contributions to the manuscript: Y.J. and S.Y. planned and designed the research. Y.J. and Y.D. performed the experiments. Y.J., Y.D., Y.S., X.Z., Z.G. and S.Y. analyzed and discussed the data. Y.J., Y.D. and S.Y. wrote the manuscript.

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Supporting Information

Additional Supporting Information may be found online in the Supporting Information tab for this article:

Fig. S1 The freezing assay of *cbf3 Arabidopsis* mutant.

Fig. S2 The generation of *cbf1,3-2* double and *cbfs-2* triple *Arabidopsis* mutants.

Fig. S3 Freezing phenotype of *cbf1,3-2* double and *cbfs-2* triple *Arabidopsis* mutants.

Fig. S4 Freezing phenotype of *cbfs-1* triple *Arabidopsis* mutant expressing one of *CBF* genes.

Fig. S5 Expression of *CBF1* and *CBF2* genes in *cbf3* and *cbf1,3-1 Arabidopsis* mutants under cold stress.

Fig. S6 The comparison between the previously reported CBF-induced genes and the results from this study in *Arabidopsis*.

Fig. S7 Functional classification of CBF-regulated genes in *Arabidopsis*.

Table S1 Gene-specific primers used in this study

Table S2 Cold induced and repressed genes in *Arabidopsis* wild-type plants

Table S3 Cold induced and repressed genes in *cbfs-1 Arabidopsis* mutant

Table S4 Differentially expressed genes between *cbfs-1 Arabidopsis* mutant and Col at 3 h of cold treatment

Table S5 Differentially expressed genes between *cbfs-1 Arabidopsis* mutant and Col at 3 h of cold treatment

Table S6 Differentially expressed genes between *cbfs-1 Arabidopsis* mutant and Col at 24 h of cold treatment

Table S7 List of CBF-regulated genes in *Arabidopsis*

Table S8 The comparison of CBF-activated genes with the former reported data in *Arabidopsis*

Table S9 Cluster analysis of the CBF-activated genes in *Arabidopsis*

Table S10 The promoter analysis of the CBF-activated genes in *Arabidopsis*

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