Arabidopsis CBF1 and CBF3 have a different function than CBF2 in cold acclimation and define different gene classes in the CBF regulon

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The C-repeat-binding factor (CBF)/dehydration-responsive element-binding factor (DREB1) proteins constitute a small family of *Arabidopsis* transcriptional activators (CBF1/DREB1B, CBF2/DREB1C, and CBF3/DREB1A) that play a prominent role in cold acclimation. A fundamental question about these factors that remains to be answered is whether they are functionally equivalent. Recently, we reported that CBF2 negatively regulates *CBF1* and *CBF3* expression, and that *CBFs* are subjected to different temporal regulation during cold acclimation, which suggested this might not be the case. In this study, we have analyzed the expression of *CBF* genes in different tissues of *Arabidopsis*, during development and in response to low temperature, and characterized RNA interference (RNAi) and antisense lines that fail to accumulate *CBF1* or/and *CBF3* mRNAs under cold conditions. We found that *CBF1* and *CBF3* are regulated in a different way than *CBF2*. Moreover, in contrast to CBF2, CBF1 and CBF3 are not involved in regulating other *CBF* genes and positively regulate cold acclimation by activating the same subset of CBF-target genes. All these results demonstrate that CBF1 and CBF3 have different functions than CBF2. We also found that the CBF regulon is composed of at least two different kind of genes, one of them requiring the simultaneous expression of both *CBF1* and *CBF3* to be properly induced. This indicates that CBF1 and CBF3 have a concerted additive effect to induce the whole CBF regulon and the complete development of cold acclimation.

freezing tolerance | low temperature | DREB1 | abiotic stress | cold signaling

The identification of the C-repeat-binding factors (CBF1–3) (1–3), also named dehydration-responsive element-binding factors (DREB1B, -1C, and -1A, respectively) (4), represented a significant step toward the understanding of how gene expression is regulated during cold acclimation, the adaptive response whereby many plants increase their freezing tolerance in response to low nonfreezing temperatures (5). The CBFs/DREB1s belong to the AP2/EREBP family of transcription factors (6) and bind to the cold- and dehydration-responsive DNA regulatory element designated C-repeat (CRT)/dehydration response element (DRE) (7, 8). CRT/DRE elements contain the conserved CCGAC core sequence, which is sufficient to activate gene transcription under cold stress (7, 8) and is present in the promoters of many cold-inducible genes (9). The *CBF/DREB1* genes do not contain the CCGAC sequence in their promoters but are also induced by low temperature. This induction is transient and precedes that of cold-inducible genes with the CRT *cis* element. The expression of *CBF/DREB1* genes, however, is not activated by dehydration and salt stress (2–4).

An important issue in the study of CBFs is their individual function. Despite extensive research carried out on these transcriptional activators, whether they have overlapping function(s) has not yet been conclusively established. Constitutive overexpression of each *CBF* in *Arabidopsis* results in similar expression of CRT-containing target genes and increased freezing tolerance under control conditions (4, 10–13), indicating that CBFs play an important and equivalent role in cold acclimation. The *CBF* genes are organized in tandem on chromosome 4 of *Arabidopsis* (2–4), in all likelihood arising from gene duplication events followed by selection. However, despite their high sequence similarity (±85%), several differences, namely amino acid substitutions and insertions/deletions, are present throughout the CBF proteins, suggesting that each CBF may have a distinct function. In addition, Chinnusamy *et al*. (14) identified a mutation in an *Arabidopsis* gene encoding a MYC-like basic helix–loop–helix transcriptional activator called ICE1, that results in the failure of *CBF3* to be induced in response to low temperature but has little effect on the cold induction of *CBF1* and *CBF2*. Although the significance of this observation in regard to cold acclimation and freezing tolerance remains to be determined, it also suggests that the three CBFs may not play an equivalent role.

In a previous work (15), we reported that the expression of *CBF1* and *CBF3* during cold acclimation precedes that of *CBF2*, reinforcing the idea that the CBF proteins may have different functional activities. Furthermore, we isolated an *Arabidopsis* null mutant, *cbf2*, in which the *CBF2* gene was disrupted by a T-DNA insertion (15). Unexpectedly, *cbf2* plants were not impaired in their capacity to cold acclimate but, on the contrary, they showed increased freezing tolerance before and after cold acclimation, as well as increased tolerance to dehydration and salt stress. Characterization of mutant plants revealed that CBF2 negatively regulates *CBF1* and *CBF3* expression, ensuring that it is transient and tightly controlled, which, in turn, guarantees the correct induction of downstream genes and the accurate development of *Arabidopsis* tolerance to freezing and related stresses (15). To establish the function of CBF1 and CBF3 and to further understand the contribution of each individual CBF to cold acclimation response, we have analyzed the expression of the *CBF* genes at the tissue level during *Arabidopsis* development and in response to low temperature. In addition, we have characterized RNAi lines that do not display cold induction of *CBF1* or *CBF3* expression and transgenic lines that fail to simultaneously accumulate both *CBF1* and *CBF3* mRNAs in response to low temperature. Here, we show that *CBF1* and *CBF3* have different expression patterns and, therefore, are regulated in a different way than *CBF2*. Furthermore, we demonstrate that, in contrast to CBF2, CBF1 and CBF3 are not

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involved in regulating other *CBF* genes and act as positive regulators of cold acclimation by activating the same subset of CBF-target genes. Our results reveal that CBF1 and CBF3 have an additive effect and are concertedly required for the induction of all CBF-target genes and the complete development of cold acclimation response in *Arabidopsis*. In fact, we provide evidence that the CBF regulon is constituted by at least two subsets of genes, and one of them requires the simultaneous expression of both *CBF1* and *CBF3* to be properly induced. On the basis of these data, the function of CBFs in cold acclimation and the induction of the CBF regulon in response to low temperature are discussed.

Results

CBF1 and _CBF3_ Display Different Expression Patterns than _CBF2_ During _Arabidopsis_ Development and in Response to Low Temperature. As a first step to assess the role of individual CBFs in cold acclimation, the expression patterns of *CBF1*, *-2*, and *-3* during *Arabidopsis* development and in response to low temperature were determined. With this aim, we obtained *Arabidopsis* transgenic plants containing chimeric genes consisting of promoter fragments (≈1 kb) from each individual *CBF* fused to the *uidA* (*GUS*) reporter gene. Four independent transgenic lines for each construct, all containing a single copy of the transgene in homozygosity, were analyzed. In all cases, the levels of *GUS* mRNAs increased markedly in response to low temperature, mirroring the expression patterns of endogenous *CBFs* (Fig. 1*A*). These data indicate that, as described (16, 17), the proximal regions of the promoters contain all *cis*-acting elements required for cold responsiveness of *CBFs*.

Once it was proved that the isolated *CBF* promoter fragments were able to drive the transcription of *GUS* in a similar way as the transcription of endogenous *CBF* genes, this reporter gene assay was used to follow the expression of each *CBF* at tissue level during *Arabidopsis* development and in response to low temperature. Transgenic seeds did not exhibit GUS staining in any case (Fig. 1*B*). During germination and early stages of development, transgenic lines containing *CBF1*::*GUS* and *CBF3*::*GUS* fusions showed an identical pattern of GUS activity, the staining being restricted to roots, hypocotyls, and cotyledons (Fig. 1*B*). *CBF2*::*GUS* seedlings disclosed GUS activity in hypocotyls and cotyledons but also in the first and second pairs of leaves (Fig. 1*B*). They did not display, however, any GUS staining in roots (Fig. 1*B*). Remarkably, GUS activity in all transgenic seedlings decreased gradually during development, completely disappearing 4 weeks after germination (Fig. 1*B*).

Fully developed transgenic lines containing *CBF1*::*GUS* and *CBF3*::*GUS* constructs also exhibited identical patterns of GUS activity. Under control conditions, they did not show any GUS staining (Fig. 1*C*). When exposed to low temperature, *CBF1*::*GUS* and *CBF3*::*GUS* transgenic lines disclosed GUS staining in leaves, sepals, and siliques (Fig. 1*C*). Unstressed *CBF2*::*GUS* adult plants, however, presented GUS activity in sepals and in the abscission zone of the siliques (Fig. 1*C*). In the case of *CBF2*::*GUS* adult plants growing under cold conditions, GUS activity was observed in leaves, sepals, siliques, and also in stems (Fig. 1*C*). Taken together, all these results demonstrate that *CBF1* and *CBF3* genes have the same expression pattern during *Arabidopsis* development and in response to low temperature. Moreover, this pattern is different from that exhibited by *CBF2*, indicating that CBF1 and CBF3 may have the same function, and that it can be different than that of CBF2.

Cold Acclimation Response Is Impaired in _CBF1_ and _CBF3_ RNAi Lines. To establish the individual function of CBF1 and CBF3, we searched for mutants in the collections of T-DNA lines that are available. Because we did not find any insertion that abolished the expression of *CBF1* or *CBF3*, we decided to use an RNAi approach. RNAi constructs designed to target the last 173 and 250 nt of *CBF1* and *CBF3* transcripts, respectively, were used to transform *Arabidopsis*, and several independent transgenic lines for each RNAi construct were obtained. Homozygous lines containing a single transgene insertion were selected, and the effect of RNAi constructs on *CBF1* and *CBF3* transcript accumulation was assessed by RNA gel blot hybridization with specific probes (3). As shown in Fig. 2, different *CBF1* and *CBF3* RNAi lines were identified that exhibited undetectable levels of *CBF1* and *CBF3* transcripts, respectively, after being exposed to low temperature. Cold induction of *CBF2* and *CBF3* in *CBF1* RNAi lines and *CBF1* and *CBF2* in *CBF3* RNAi lines was essentially as in wild-type plants, confirming the gene specificity of the RNAi constructs. *CBF1* RNAi lines 2 and 4 and *CBF3* RNAi lines 1 and 4 were chosen for further studies (Fig. 2). No significant morphological differences were found in these lines compared with wild-type *Arabidopsis* (data not shown).

The role of CBF1 and CBF3 in freezing tolerance and cold acclimation was determined by examining the freezing tolerance of 3-week-old *CBF1* and *CBF3* RNAi lines before and after cold acclimation (4°C, 7 days). Plants were exposed for 6 h to different

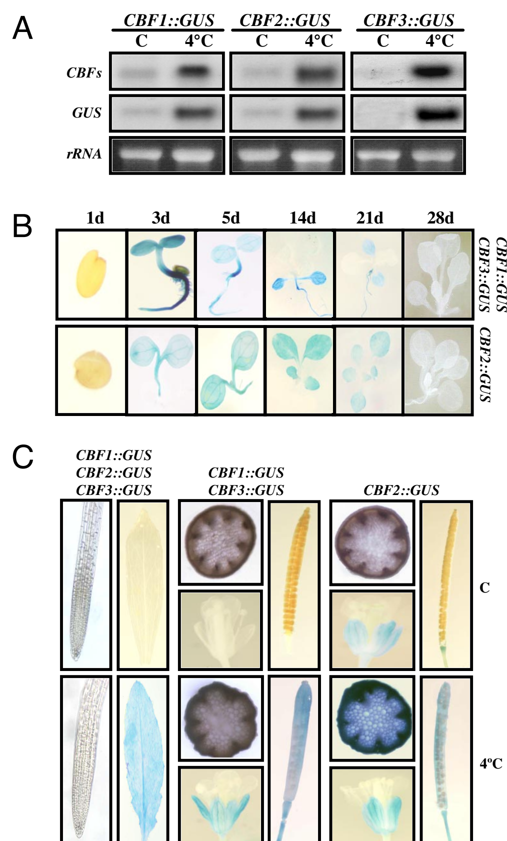

Fig. 1. Histochemical localization of *GUS* activity in transgenic *Arabidopsis* containing *CBF* promoters::*GUS* fusions. (*A*) Transcript levels of endogenous *CBF* genes and *GUS* reporter gene in transgenic plants containing *CBF1*, *CBF2*, and *CBF3* promoter::*GUS* fusions grown under control conditions (C) or exposed to 4°C for 3 h (4°C). Similar RNA loading was verified by rRNA staining with ethidium bromide. (*B*) GUS staining of transgenic seedlings containing *CBF1* and *CBF2* promoter::*GUS* fusions 1, 3, 5, 14, 21, and 28 days after germination. Seedlings containing the *CBF3*::*GUS* fusion exhibited identical patterns of GUS activity as *CBF1*::*GUS* transgenic seedlings. (*C*) GUS staining of different organs (roots, stems, leaves, flowers, and siliques) from adult transgenic plants containing *CBF1* and *CBF2* promoter::*GUS* fusions grown under control conditions (C) or exposed to 4°C for 3 h (4°C). In all cases, *CBF3*::*GUS* adult plants displayed the same patterns of GUS activity as the transgenic plants containing the *CBF1*::*GUS* construct.

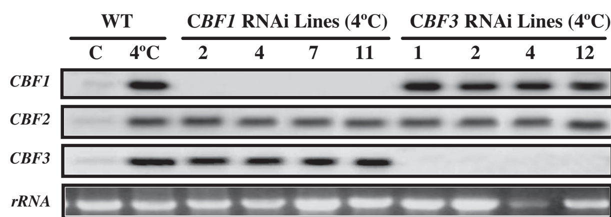

Fig. 2. Expression of *CBF* genes in *CBF1* and *CBF3* RNAi lines. Transcript levels of endogenous *CBF1*, *CBF2*, and *CBF3* genes in Col plants (WT) and different *CBF1* and *CBF3* RNAi lines grown under control conditions (C) or exposed to 4°C for 3 h (4°C). Similar amounts of RNA were present in each sample, as confirmed by ethidium bromide staining of rRNA.

freezing temperatures, and survival was scored after 7 days of recovery under controlled growth conditions. Fig. 3*A* shows that RNAi lines and wild-type plants had similar levels of freezing tolerance before cold acclimation, the temperature that causes 50% lethality (LT_{50}) value being around −7.0°C in both cases. However, *CBF1* and *CBF3* RNAi plants were significantly less freezing tolerant than the wild type after cold acclimation (Fig. 3*B*). The LT_{50} values of all RNAi lines were very similar (−8.5°C and −8.7°C for *CBF1* RNAi lines and −8.9°C and −8.8°C for *CBF3* RNAi lines) and higher than that of wild-type plants (−9.7°C). The freezing-tolerance phenotypes of representative nonacclimated and cold-acclimated wild-type plants and RNAi lines are shown in Fig. 3 *C* and *D*, respectively. These data demonstrate that *CBF1* and *CBF3* RNAi lines are defective in cold acclimation, which indicates that CBF1 and CBF3 act as positive regulators of this adaptive response.

***CBF1* and *CBF3* RNAi Lines Exhibit Reduced Induction of a Subset of CBF-Target Genes in Response to Low Temperature.** To understand why the silencing of *CBF1* or *CBF3* impairs cold acclimation, we investigated the transcript levels of several well known cold-inducible genes in the *CBF1* and *CBF3* RNAi lines. The genes analyzed included *LTI78*, *COR15A*, *COR47*, *ERD10*, *KIN1*, *KIN2*, and *COR15B*, which have been described as part of the CBF regulon (13, 18, 19), as well as *RCI2A*, *CHS*, and *RAB18*, which are not considered CBF targets (13, 18, 19). Because *CBF1* RNAi lines 2 and 4 displayed identical expression patterns, only the results obtained with line 2 are presented (Fig. 4). Under control conditions, *CBF1* RNAi line 2 and wild-type plants always showed very similar expression patterns of the genes examined (Fig. 4). In response to low temperature, the levels of all messengers increased in both *CBF1* RNAi line 2 and wild-type plants (Fig. 4). Nevertheless, in the case of CBF-target genes *LTI78*, *COR15A*, *COR47*, and *ERD10*, these levels were considerably lower in the *CBF1* RNAi line 2 than in the wild type (Fig. 4). Interestingly, the cold induction of genes *KIN1*, *KIN2*, and *COR15B*, which are also CBF targets, was similar in the *CBF1* RNAi line 2 and wild type (Fig. 4). As expected, the transcript levels of non-CBF-target genes *RCI2A*, *CHS*, and *RAB18* in response to low temperature were the same in the *CBF1* RNAi line 2 and wild-type plants (Fig. 4). Remarkably, the expression patterns exhibited by *CBF3* RNAi lines 1 and 4, under both control and cold conditions, were almost undistinguishable from those described for *CBF1* RNAi line 2. The results obtained with *CBF3* RNAi line 4 are presented in Fig. 4. In summary, the cold induction of several CBF-target genes is reduced in *CBF1* and *CBF3* RNAi lines, which most likely accounts for their defect in cold acclimation. We conclude that CBF1 and CBF3 positively regulate the induction of a subset of genes from the CBF regulon in response to low temperature and are required for the complete development of cold acclimation.

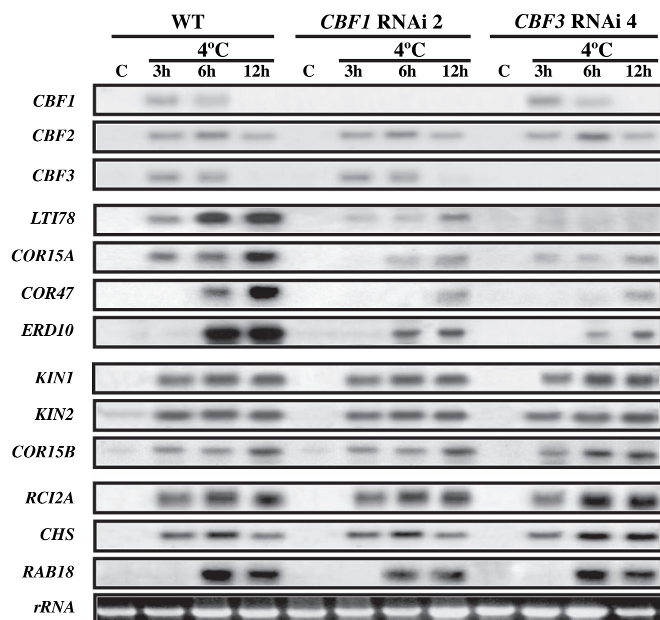

Fig. 4. Expression of cold-inducible genes in *CBF1* and *CBF3* RNAi lines. RNA-blot hybridizations were performed with total RNA isolated from Col (WT) and *CBF1* (line 2) and *CBF3* (line 4) RNAi plants grown under control conditions (C) or exposed to 4°C at the indicated times. Transcript levels of CBF-target genes *LTI78*, *COR15A*, *COR47*, *ERD10*, *KIN1*, *KIN2*, and *COR15B*, and non-CBF-target genes *RCI2A*, *CHS*, and *RAB18* are presented. Transcript levels of *CBF1*, *CBF2*, and *CBF3* genes are shown as internal controls. Similar amounts of RNA were present in each sample as confirmed by ethidium bromide staining of rRNA.

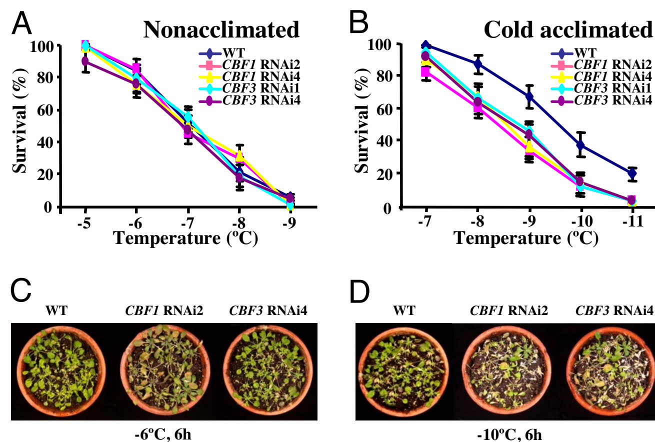

Fig. 3. Freezing tolerance of *CBF1* and *CBF3* RNAi lines. Three-week-old plants from Col (WT) and *CBF1* RNAi and *CBF3* RNAi lines were exposed to different freezing temperatures for 6 h. Freezing tolerance was estimated as the percentage of plants surviving each specific temperature after 7 days of recovery under control conditions. (*A*) Tolerance of nonacclimated plants. (*B*) Tolerance of cold-acclimated (7 d at 4°C) plants. (*C*) Representative nonacclimated plants from WT, *CBF1* RNAi line 2, and *CBF3* RNAi line 4, 7 days after being exposed to −6°C for 6 h. (*D*) Representative cold-acclimated plants from WT, *CBF1* RNAi line 2 ,and *CBF3* RNAi line 4, 7 days after being exposed to −10°C for 6 h. In *A* and *B*, data are expressed as means of three independent experiments with 50 plants each. Error bars indicate SE.

***Arabidopsis* Deficient in Both *CBF1* and *CBF3* Transcripts Show Lower Capacity to Cold Acclimate than *CBF1* and *CBF3* RNAi Lines.** Results described above demonstrate that the expression patterns of *CBF1* and *CBF3* are identical, that the lack of *CBF1* or *CBF3* transcripts has a similar effect on cold acclimation, and that

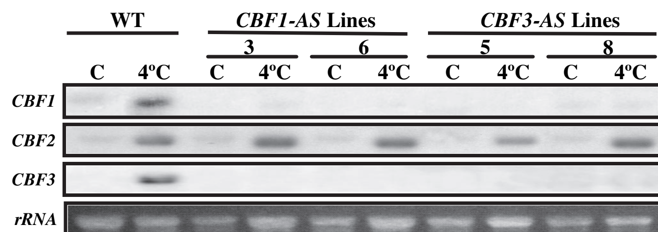

Fig. 5. Expression of *CBF* genes in *CBF1* and *CBF3* antisense transgenic lines. Transcript levels of endogenous *CBF1*, *CBF2*, and *CBF3* genes in Col plants (WT) and different *CBF1* and *CBF3* antisense transgenic lines (*CBF1-* and *CBF3-AS*, respectively) grown under control conditions (C) or exposed 3 h to 4°C (4°C). Similar amounts of RNA were present in each sample, as confirmed by ethidium bromide staining of rRNA.

CBF1 and CBF3 regulate the same target genes, strongly suggesting that these factors have the same function in cold acclimation. Intriguingly, however, the characterization of RNAi lines also demonstrated that the absence of CBF1 is not compensated by CBF3 and vice versa, which is unexpected for functionally redundant proteins. To further investigate the function of CBF1 and CBF3, transgenic lines were obtained that expressed *CBF1* or *CBF3* cDNAs in antisense orientation under the control of CaMV 35S promoter (*CBF1-* and *CBF3-AS*, respectively). Among the lines containing a single copy of each transgene in homozygosity, several were identified by RNA-blot hybridizations in which both cold-induced *CBF1* and *CBF3* transcripts were almost undetectable, whereas *CBF2* mRNA levels were similar to those of wild-type plants. Four of these lines (*CBF1-AS3*, *CBF1-AS6*, *CBF3-AS5*, and *CBF3-AS8*) were selected for additional analysis (Fig. 5).

The freezing tolerance of antisense lines was determined as described for RNAi lines (see above). Fig. 6*A* shows that nonacclimated *CBF1-* and *CBF3-AS* lines had similar levels of freezing tolerance to wild-type plants. The LT_{50} values of the wild-type and antisense lines were estimated to be approximately −6.9°C. In the case of cold-acclimated plants, *CBF1-* and

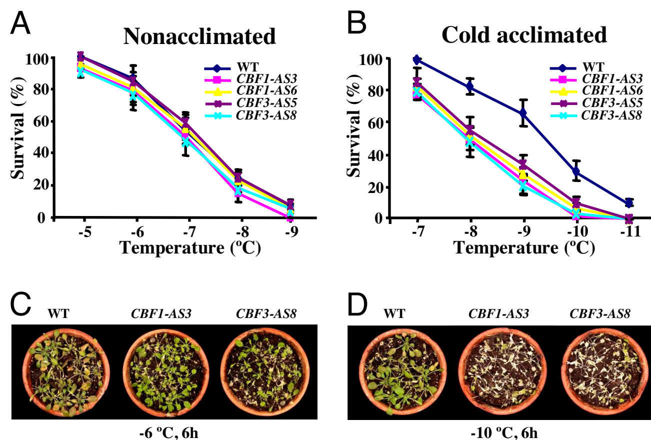

Fig. 6. Freezing tolerance of *CBF1* and *CBF3* antisense transgenic lines. Three-week-old plants from Col (WT) and *CBF1* and *CBF3* antisense transgenic lines (*CBF1-* and *CBF3-AS*, respectively) were exposed to different freezing temperatures for 6 h. Freezing tolerance was estimated as for RNAi lines. (*A*) Tolerance of nonacclimated plants. (*B*) Tolerance of cold-acclimated (7 d at 4°C) plants. (*C*) Representative nonacclimated plants from WT, *CBF1-AS* line 3 and *CBF3-AS* line 8, 7 days after being exposed to −6°C for 6 h. (*D*) Representative cold-acclimated plants from WT, *CBF1-AS* line 3, and *CBF3-AS* line 8, 7 days after being exposed to −10°C for 6 h. In *A* and *B*, data are expressed as means of three independent experiments with 50 plants each. Error bars indicate SE.

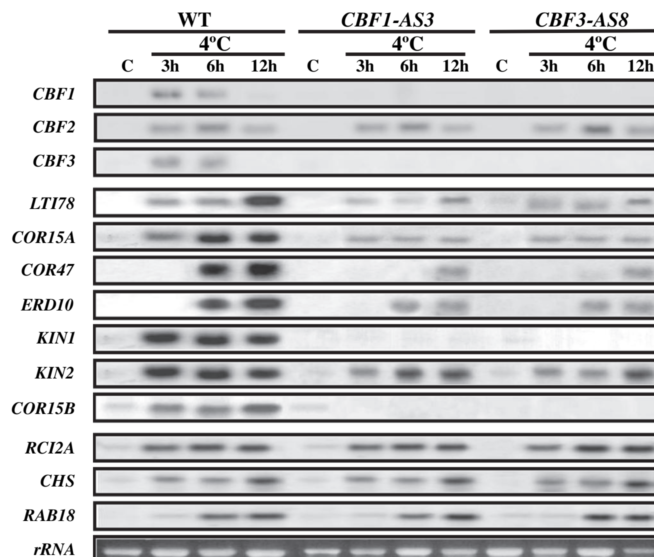

Fig. 7. Expression of cold-inducible genes in *CBF1* and *CBF3* antisense transgenic lines. RNA-blot hybridizations were performed with total RNA isolated from Col (WT) and *CBF1* (line 3) and *CBF3* (line 8) antisense transgenic plants (*CBF1-* and *CBF3-AS*, respectively) grown under control conditions (C) or exposed to 4°C at the indicated times. Transcript levels of CBF-target genes *LTI78*, *COR15A*, *COR47*, *ERD10*, *KIN1*, *KIN2*, and *COR15B*, and non-CBF-target genes *RCI2A*, *CHS*, and *RAB18* are presented. Transcript levels of *CBF1*, *CBF2*, and *CBF3* genes are shown as internal controls. Similar amounts of RNA were present in each sample as confirmed by ethidium bromide staining of rRNA.

CBF3-AS lines exhibited significantly reduced freezing tolerance compared with wild types (Fig. 6*B*). The LT_{50} values of antisense lines were very uniform (between −7.9°C and −8.2°C) and statistically higher than the LT_{50} value of wild-type plants (−9.6°C). The freezing tolerance phenotypes of representative nonacclimated and cold-acclimated wild-type plants and antisense lines are shown in Fig. 6 *C* and *D*, respectively. Interestingly, the LT_{50} values of cold-acclimated antisense lines were substantially higher than those of *CBF1* and *CBF3* RNAi lines (see above), revealing that the simultaneous absence of *CBF1* and *CBF3* transcripts reduces the capacity of *Arabidopsis* to cold acclimate more than the individual silencing of *CBF1* or *CBF3*.

***CBF1/CBF3* Antisense Lines Are Impaired in Cold Induction of More CBF-Target Genes than *CBF1* and *CBF3* RNAi Lines.** To study the molecular basis of freezing tolerance phenotypes displayed by antisense lines, *CBF1-AS3* and *CBF3-AS8* lines were monitored for the transcript levels corresponding to the same genes analyzed in RNAi lines (see above). Under control conditions, all transcripts were at similar levels in wild-type and *CBF1-AS3* plants (Fig. 7), as occurred in RNAi lines (Fig. 4). However, after low-temperature treatment, the induction of all CBF targets analyzed was clearly lower in *CBF1-AS3* than in wild type (Fig. 7), which is in contrast to what was observed in RNAi lines where the induction of *KIN1*, *KIN2*, and *COR15B* was as in the wild type (Fig. 4). The transcript levels of non-CBF-target genes *RCI2A*, *CHS*, and *RAB18* in response to low temperature were the same in *CBF1-AS3* and wild-type plants (Fig. 7). The expression patterns displayed by antisense line *CBF3-AS8*, under control and cold conditions, were almost identical to those described for *CBF1-AS3* (Fig. 7), consistent with the resembling phenotypes of freezing tolerance exhibited by both antisense lines (Fig. 6). We conclude that the induction of the CBF regulon after low-temperature treatment is more affected in *Arabidopsis* that are simultaneously deficient in both *CBF1* and *CBF3* transcripts

than in plants just impaired in *CBF1* or *CBF3* mRNAs, which should account for their lower capacity to cold acclimate.

Discussion

Over the last years, experimental strategies based on ectopic overexpression have been used to examine the role of individual CBFs in cold acclimation (4, 10–13). From these studies, it has been proposed that the three CBFs have redundant functional activities. Nevertheless, loss-of-function analysis to definitively establish the specific contribution of each factor has only been recently carried out for CBF2 (15). Results indicated that CBF2 negatively regulates *CBF1* and *CBF3* expression, and that *CBF2* is induced later than *CBF1* and *CBF3* during cold acclimation (15), suggesting that the three CBFs may not have an equivalent function. In this study, we have used a *GUS* reporter gene assay as well as RNAi and antisense approaches to determine the specific role of CBF1 and CBF3. Our data demonstrate that CBF1 and CBF3 have a different function than CBF2, and that they are concertedly required to induce the whole CBF regulon and the complete development of cold acclimation response.

In agreement with previous reports (16, 17), we show that the *cis*-acting elements involved in cold induction of *CBFs* are contained within the proximal regions (≈1 kb) of their promoters, and that this induction is regulated at the transcriptional level. Histochemical determination of GUS activity in transgenic plants containing *CBF*::*GUS* fusions revealed that *CBF1* and *CBF3* have an identical expression pattern during *Arabidopsis* development and in response to low temperature. Moreover, this pattern is clearly different than the one exhibited by *CBF2*. These data are consistent with the fact that *CBF1* and *CBF3* are induced at the same time and earlier than *CBF2* during cold acclimation (15) and suggest that CBF1 and CBF3, but not CBF2, have the same function. Interestingly, *CBF* promoters provide constitutive *GUS* expression in seedlings during the first weeks after germination, indicating that *CBFs* are tightly regulated and may have an important role throughout the early stages of *Arabidopsis* development. It is tempting to speculate that at this period, when plants are more fragile, constitutive expression of *CBFs* allows them to overcome unexpected freezing temperatures and other related stresses. Zarka *et al*. (17) reported that the three *CBF* promoters, when fused to *GUS* in *Arabidopsis* transgenic plants, responded to low temperature in Northern blot analysis. However, in contrast to our results, the GUS activity in histochemical assays did not reflect the differences in *GUS* transcript levels (17). The reason for these different results may be due to differences in the developmental stages of the plants used in the respective works.

Additional evidence that CBF1 and CBF3 may have the same function, and that this function is different than that of CBF2, was obtained from the characterization of RNAi lines with silenced *CBF1* or *CBF3* genes. First, *CBF1* and *CBF3* RNAi lines seem to be specifically affected in cold induction of *CBF1* and *CBF3*, respectively. This indicates that, contrary to CBF2, which was shown to negatively regulate the expression of *CBF1* and *CBF3* (15), CBF1 and CBF3 are not involved in regulating the expression of other *CBF* genes. The expression of *CBF4*, a homolog of *CBF1*, *CBF2*, and *CBF3* (20), is also not affected in RNAi lines (F.N. and J.S., unpublished results). Furthermore, *CBF1* and *CBF3* RNAi lines are impaired in cold induction of the same CBF-target genes, revealing that CBF1 and CBF3 positively control the expression of a subset of genes from the CBF regulon during cold acclimation. As expected from these results, *CBF1* and *CBF3* RNAi lines are defective in cold acclimation but are not affected in their constitutive freezing tolerance. These data demonstrate that, in contrast to CBF2 that has been described to negatively regulate freezing tolerance and cold acclimation (15), CBF1 and CBF3 are positive regulators of this adaptive response but are not involved in the constitutive freezing tolerance of *Arabidopsis*.

It is intriguing, however, that *CBF1* and *CBF3* RNAi lines are defective in low-temperature-induced gene expression and cold acclimation, indicating that CBF1 and CBF3 do not compensate the absence of each other as expected for functionally redundant proteins. The characterization of *Arabidopsis* transgenic plants that fail to simultaneously accumulate both *CBF1* and *CBF3* transcripts in response to low temperature uncovered that, remarkably, they exhibit impaired cold induction of all CBF-target genes examined, including those that are not affected in RNAi lines. This reduced induction should account for the impaired capacity of *CBF1*/*CBF3* antisense lines to cold acclimate that is lower than that of RNAi lines. These results indicate that, although CBF1 and CBF3 seem to transactivate the same CBF-target genes in response to low temperature, both factors are required in concert for the correct induction of the whole CBF regulon and the complete development of cold acclimation. Indeed, our results reveal that the CBF regulon consists of at least two different subsets of genes. Whereas one needs the expression of either *CBF1* or *CBF3* to be properly induced during cold acclimation, the other needs the simultaneous expression of both *CBF1* and *CBF3*. Interestingly, the genes examined that belong to the first subset (*KIN1*, *KIN2*, and *COR15B*) contain fewer CRT/DRE motives (one or two) in their promoters (defined as the 1,200 bp of the sequence preceding the ATG codon from each gene) than the genes belonging to the second subset (*COR15A*, *LTI78*, *COR47*, and *ERD10*) (three or four), allowing one to speculate that the number of CRT/DRE motives may condition the number of CBF factors necessary for a CBF-target gene to be adequately induced during cold acclimation.

The characterization of *CBF1*/*CBF3* antisense transgenic lines also uncovered that, as in the case of RNAi lines, they exhibit normal induction of *CBF2* in response to low temperature, confirming that, in contrast to CBF2, CBF1 and CBF3, are not implicated in regulating the expression of other *CBF* genes. Moreover, the antisense transgenic lines show a clear induction of the CBF targets analyzed when exposed to 4°C. This induction may indicate that, in addition to regulating *CBF1* and *CBF3* expression (15), CBF2 is also directly involved in activating the CBF regulon during cold acclimation, which is consistent with its capacity to activate transcription (4). The possibility that the induction of the CBF targets detected in cold-treated antisense lines could be mediated by CBF-independent pathways cannot, however, be completely excluded at present. The *CBF1*/*CBF3* antisense transgenic lines, like the RNAi lines, are also not affected in their constitutive capacity to tolerate freezing, corroborating that CBF1 and CBF3 do not play any role in the constitutive freezing tolerance of *Arabidopsis*.

In summary, we conclude that the three CBFs do not have fully overlapping functions. CBF1 and CBF3 play a different role than CBF2 in both constitutive freezing tolerance and cold acclimation. Furthermore, although CBF1 and CBF3 seem to transactivate the same targets, both factors have an additive effect and are concertedly required to induce the whole CBF regulon and the complete development of the adaptive response. From the results presented in this work, we propose that a certain level of CBF factors would be necessary for the induction of all CBF targets and the accurate activation of cold acclimation in *Arabidopsis*, and that this level would be attained only when the *CBF1*, *CBF2*, and *CBF3* genes are properly and coordinately induced. This assumption is consistent with the results obtained from our RNAi and antisense transgenic lines, and with those reported from *Arabidopsis* constitutively overexpressing individual *CBFs* (4, 10–13). Thus, in the RNAi lines analyzed, the required level of CBF factors could not be attained because *CBF1* or *CBF3* are silenced. As a consequence, only a subset of CBF-target genes would be adequately induced in response to

low temperature, and the cold acclimation response would not reach its complete development. In the case of antisense transgenic lines, under low-temperature conditions, they cannot accumulate CBF1 and CBF3, which would result in a deficient induction of the whole CBF regulon and, therefore, a lower capacity to cold acclimate than RNAi lines. Constitutive overexpression of each *CBF* gene in *Arabidopsis* grown under control conditions has been described to induce the CBF regulon and increase freezing tolerance as after cold acclimation (13). When cold acclimated, the freezing tolerance of *CBF*-overexpressing plants increases further (12, 13). We suggest that the levels of each overexpressed CBF in the corresponding nonacclimated transgenic plants are constitutively so high that, despite the absence of the two other CBFs, the CBF regulon is induced, and cold acclimation can proceed. In the case of cold-acclimated *CBF*-overexpressing plants, their increase in freezing tolerance would be due, at least in part, to the very high levels of CBF factors that are attained in these plants because of the overexpression of each *CBF* and the induction of the endogenous *CBFs*. Although the identification and characterization of *Arabidopsis* plants with additional combinations of silenced *CBFs* are needed to have a complete scenario of how CBFs function, our findings provide insight to better understand the molecular basis of freezing tolerance and cold acclimation response.

Materials and Methods

Plant Materials, Growth Conditions, and Treatments. Seeds from *Arabidopsis thaliana* (L.) Heynh, ecotype Columbia (Col), were purchased from Lehle Seeds. Plant growth conditions and treatments were as described (15).

Generation of Transgenic Plants Containing *CBF* Promoters::*GUS* Fusions and Histochemical Localization of GUS Activity. Fragments covering 1.04, 0.86, and 1.24 kb immediately upstream of the *CBF1*, *CBF2*, and *CBF3* coding regions, respectively, were amplified by PCR by using genomic DNA as template and primers indicated in supporting information (SI) Table 1. The three promoter fragments were cloned into the HindIII and ScaI sites of pBI101.2 (21) to yield the *CBF1*::, *CBF2*::, and *CBF3*::*GUS* fusions. These fusions, once verified by DNA sequencing, were transferred to *Agrobacterium tumefaciens* LBA4404 (22). Transformation of *Arabidopsis* was performed following the floral dip method (23). The histochemical localization of GUS activity was carried out as described (24).

Generation of *CBF1* and *CBF3* RNAi Lines. To obtain *CBF1* RNAi lines, specific 173-bp fragments from the 3′ untranslated region of *CBF1* were amplified by PCR by using genomic DNA as template and primers described in SI Table 1. The PCR products generated to form the sense and antisense fragments were cloned into the BamHI-HindIII and KpnI-XhoI sites of the pKannibal vector (25), respectively. Subsequently, the NotI fragment containing both fragments in opposite orientation separated by an 800-bp intron sequence was subcloned into the binary vector pART27 containing the 35S promoter (26). After we verified the NotI fragment by DNA sequencing, this plasmid was transferred to *A. tumefaciens* LBA4404 (22). Transformation of *Arabidopsis* was performed as described above. To obtain *CBF3* RNAi lines, we followed the same strategy described for *CBF1* RNAi lines but using specific 250-bp fragments from the 3′ untranslated region of *CBF3* that were amplified by PCR with primers indicated in SI Table 1.

Generation of *CBF1* and *CBF3* Antisense Transgenic Lines. To obtain *CBF1* antisense lines, a fragment covering 545 bp of the *CBF1* coding region was amplified by PCR by using genomic DNA as template and primers described in SI Table 1. The fragment was cloned into the SacI-BamHI sites of the pROK2 binary vector under the control of the 35S promoter (27) in antisense orientation and subsequently sequenced. This plasmid was transferred to *A. tumefaciens* LBA4404 (22), which was used to transform *Arabidopsis* as described above. The strategy to obtain *CBF3* antisense lines was identical to that described for *CBF1* antisense lines but using a fragment covering 478 bp of the *CBF3* coding region. Primers used to amplify this fragment are indicated in SI Table 1.

Molecular Biology Methods. Total RNA was isolated as described (28). Restriction digestions, cloning, and RNA-blot hybridizations were performed following standard protocols (29). Specific probes for the *CBF* genes have been described (3). The *GUS* probe was as reported in Llorente *et al.* (30). Specific probes for *LTI78*, *COR15A, COR47, KIN-1*, and *RCI2A* have also been described (15). Probes for *ERD10*, *KIN2*, *COR15B*, and *RAB18* were obtained by PCR amplification of 610-, 735-, 690-, and 498-bp genomic fragments, respectively, using primers described in SI Table 1. The probe used for *CHS* was its 413-bp HindIII genomic fragment. Equal RNA loading (20 μg) in the experiments was monitored by rRNA staining. RNA samples for each experiment were analyzed in at least two independent blots, and each experiment was repeated at least twice.

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