Genetic analysis reveals a complex regulatory network modulating CBF gene expression and Arabidopsis response to abiotic stress

Fernando Novillo¹, Joaquín Medina², Marta Rodríguez-Franco³, Gunther Neuhaus³ and Julio Salinas¹,*

¹ Departamento de Biología Medioambiental, Centro de Investigaciones Biológicas-Consejo Superior de Investigaciones Científicas, Ramiro de Maeztu 9, 28040 Madrid, Spain
² Departamento de Biotecnología INIA, Centro de Biotecnología y Genómica de Plantas, Campus de Montegancedo, 28223 Madrid, Spain
³ Faculty of Biology, Cell Biology, Freiburg University, Schänzlestr. 1, D-79104 Freiburg, Germany

* To whom correspondence should be addressed. E-mail: salinas@cib.csic.es

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Abstract

Arabidopsis CBF genes (CBF1–CBF3) encode transcription factors having a major role in cold acclimation, the adaptive process whereby certain plants increase their freezing tolerance in response to low non-freezing temperatures. Under these conditions, the CBF genes are induced and their corresponding proteins stimulate the expression of target genes configuring low-temperature transcriptome and conditioning Arabidopsis freezing tolerance. CBF2 seems to be the most determinant of the CBFs since it also regulates CBF1 and CBF3 expression. Despite the relevance of CBF genes in cold acclimation, little is known about the molecular components that control their expression. To uncover factors acting upstream of CBF2, mutagenized Arabidopsis containing the luciferase reporter gene under the control of the CBF2 promoter were screened for plants with de-regulated CBF2 expression. Here, the identification and characterization of five of these mutants, named acex (altered CBF2 expression), is presented. Three mutants show increased levels of cold-induced CBF2 transcripts compared with wild-type plants, the other two exhibiting reduced levels. Some mutants are also affected in cold induction of CBF1 and CBF3. Furthermore, the mutants characterized display unique phenotypes for tolerance to abiotic stresses, including freezing, dehydration, and high salt. These results demonstrate that cold induction of CBF2 is subjected to both positive and negative regulation through different signal transduction pathways, some of them also mediating the expression of other CBF genes as well as Arabidopsis responses to abiotic stresses.

Key words: Abiotic stress, Arabidopsis mutants, CBFs, cold acclimation, dehydration, freezing tolerance, low temperature, salt stress, signal transduction.

Introduction

Freezing temperature is a major environmental factor that affects growth and development of plants, and limits their geographical distribution and crop yield. Plants from temperate regions have evolved an adaptive process to increase their freezing tolerance after being exposed to low, non-freezing temperatures. This process, called cold acclimation (Guy, 1990), involves several physiological and biochemical changes, most of them controlled by low temperature through changes in gene expression (Salinas, 2002). Recent global expression analyses in Arabidopsis have shown that >1500 genes are induced or repressed in response to low temperature (Matsui et al., 2008; Zeller et al., 2009), suggesting that cold acclimation is mediated by different signal transduction pathways. Interestingly, a number of these genes are also regulated by other abiotic stresses such as drought and high salt (Matsui et al., 2008; Zeller et al., 2009), which indicates that plant responses
to abiotic stresses are related and share common signalling pathways.

A significant step toward the understanding of how gene expression is regulated during cold acclimation was the identification of the Arabidopsis C-repeat-binding factors (CBF1–CBF3) (Gilmour et al., 1998; Medina et al., 1999), also termed dehydration-responsive element-binding factors (DREB1B, 1C, and 1A, respectively) (Liu et al., 1998). These factors bind to the low-temperature-responsive DNA regulatory elements designated as C-repeat (CRT)/dehydration response element (DRE) (Stockinger et al., 1997; Gilmour et al., 1998; Liu et al., 1998). CRT/DRE motifs contain the conserved CCGAC core sequence, which is sufficient to activate gene transcription under cold stress (Baker et al., 1994; Yamaguchi-Shinozaki and Shinoaki, 1994) and is present in the promoters of many cold-inducible genes (Thomashow, 1999). The CBF genes do not contain the CCGAC sequence in their promoters but are also induced by low temperature. This induction is transient, rapid, and not caused by dehydration and salt stress (Gilmour et al., 1998; Liu et al., 1998; Medina et al., 1999). The CBFs regulate the expression of ~12% of the Arabidopsis cold-inducible genes (Fowler and Thomashow, 2002), suggesting that they have an important role in cold acclimation. In fact, constitutive overexpression of CBF genes activates the expression of genes containing the CRT/DRE element in their promoters at control temperature, which results in constitutive freezing tolerance and enhanced tolerance to dehydration and high salt (Jaglo-Ottosen et al., 1998; Kasuga et al., 1999; Gilmour et al., 2004). Transgenic Arabidopsis overexpressing the CBF genes also display dwarf phenotypes as well as late flowering, low number of seeds, and leaf senescence (Kasuga et al., 1999; Gilmour et al., 2004; Sharabi-Schwager et al., 2010), which indicates that the expression of these genes must be subjected to a tight regulation. According to this presumption, different transcription factors have been reported to interact directly with the promoters of the CBF genes and regulate their induction. ICE1, a basic helix–loop–helix (bHLH) transcription factor, has been described to bind to the CBF3 promoter and activate the expression of CBF3 in response to low temperature (Chinnusamy et al., 2003). ICE2, an ICE1 homologue, is involved in regulating the cold induction of CBF1 (Fursova et al., 2009). An R2R3-MYB transcription factor, MYB15, was shown to interact with ICE1 and to negatively regulate the cold induction of the three CBF genes through the MYB elements located in their promoters (Agarwal et al., 2006). Vogel et al. (2005) reported a zinc finger, ZAT12, that also negatively regulates the expression of the CBF genes. Recently, different members of calmodulin-binding transcription activators (CAMTAs) have been uncovered that bind to the CBF2 promoter inducing the expression of CBF2 (Doherty et al., 2009). Finally, the bHLH factor PIF7 has been found to bind to the G-box element present in the CBF2 promoter and to function as a repressor in transient assays (Kidokoro et al., 2009). Furthermore, in addition to these transcription factors, during the last years other proteins that are also implicated in controlling CBF expression have been identified (for reviews see Chinnusamy et al., 2007; Medina et al., 2011).

Consistent with the fact that the expression of CBF genes is tightly regulated, the physiological and molecular characterization of an Arabidopsis cbf2 null mutant revealed that the absence of CBF2 provokes an increase in the accumulation of CBF1 and CBF3 transcripts under both control and low temperature conditions, indicating that CBF2 negatively modulates the expression of CBF1 and CBF3 (Novillo et al., 2004). This increase correlates with higher levels of transcripts corresponding to CBF target genes and an enhancement of Arabidopsis tolerance to freezing temperature, before and after cold acclimation, as well as to dehydration and high salt (Novillo et al., 2004). On the other hand, the characterization of Arabidopsis plants with reduced induction of CBF1 and/or CBF3 in response to low temperature revealed that CBF1 and CBF3 function additively in cold acclimation and differently from CBF2 (Novillo et al., 2007). Indeed, low levels of CBF1 or CBF3 cause a decrease in the capacity of Arabidopsis to cold acclimate, though to a lesser extent than the absence of CBF1 and CBF3 simultaneously. As expected, these effects on cold acclimation correlate with low levels of mRNAs corresponding to CBF target genes (Novillo et al., 2007). All these data strongly suggest that CBF2 represents a unique regulon for low temperature-regulated gene expression, different from those of CBF1 and CBF3.

Unfortunately, despite the relevance of CBF2 in cold acclimation, little is known about the molecular components that control its expression which should constitute crucial upstream intermediates in cold signalling. As mentioned above, some transcription factors, including MYB15, CAMTAs, and PIF7, have been shown to modulate CBF2 expression by binding to its promoter (Agarwal et al., 2006; Doherty et al., 2009; Kidokoro et al., 2009). Additional proteins involved in regulating CBF2 expression, however, remain to be found. In an attempt to identify new molecular components controlling the expression of CBF2, a population of ethyl methanesulphonate (EMS)-mutagenized Arabidopsis carrying the firefly luciferase (LUC) reporter gene under the control of the CBF2 promoter was screened for plants with altered CBF2 expression (acex). Here, the characterization of five acex mutants exhibiting different patterns of cold-induced CBF2 expression is reported. The results demonstrate that the induction of CBF2 in response to low temperature is subjected to both positive and negative regulation through several signal transduction pathways, some of them also mediating the expression of other CBF genes as well as Arabidopsis response to abiotic stress.

Materials and methods

Plant materials, growth conditions, and treatments

Arabidopsis thaliana (L.) Heynh, ecotype Columbia (Col-0), was used in this study. Plants were grown at 20 °C under a long-day photoperiod (16 h of cool-white fluorescent light, photon flux of 90 μmol m⁻² s⁻¹) in pots containing a mixture of organic substrate...
results

isolation of arabidopsis mutants de-regulated in cbf2 expression

To identify mutants de-regulated in CBF2 expression, _Arabidopsis_ transgenic lines containing a single copy of a fusion between the CBF2 promoter and the LUC reporter gene were generated (Fig. 1A). The CBF2 promoter used (~870 to ~10) contains all regulatory elements necessary to confer expression in response to low temperature (Novillo et al., 2007; Doherty et al., 2009; Kidokoro et al., 2009). A line homozygous for the insertion [hereafter referred to as the wild type (WT)], which showed a clear and homogenous induction of LUC activity under cold conditions, was selected for EMS mutagenesis. Morphologically, this line was identical to the Col-0 parental ecotype.

A primary screening was conducted with 25 000 2-week-old M2 seedlings for mutants with altered LUC activity after low temperature treatment. Seedlings displaying constitutive luminescence (seven), or lower (34) or higher (54) LUC activity than the WT line were selected and allowed to self-pollinate. From them, only two, 17, and 29 plants belonging to the different classes mentioned above, respectively, survived and set seeds. The progeny of those plants that set enough seeds were subjected to a secondary screening to eliminate false positives. At the end, five mutants having a clear altered LUC activity in response to cold were selected. Three of them (lines 8, 48, and 84) exhibited higher activity than the WT line, while the other two (lines 55 and 506) disclosed lower activity (Fig. 1B).

To establish whether the five mutants selected on the basis of their LUC activity phenotypes were actually affected in the expression of the endogenous _CBF2_ gene, RNA-blot hybridizations were performed with WT plants and the mutant lines grown under control conditions or exposed to 4°C for different times. The results revealed that in the five

and vermiculite (3:1, v/v) or in Petri dishes containing MS medium (Murashige and Skoog, 1962) or GM medium (MS medium supplemented with 1% sucrose) solidified with 0.8% (w/v) agar.

LUC analysis was performed with 2-week-old plants grown in Petri dishes with MS medium. LUC activity in response to low temperature was detected after exposing plants to 4°C in a growth chamber for 24 h. Low temperature treatments for expression analysis were performed by transferring 4-week-old pot-growing plants to a growth chamber set to 4°C for different periods of time, under the photoperiodic conditions described above and a light intensity of 45 μmol m⁻² s⁻¹. After treatments, plants were immediately frozen in liquid N₂, and stored at ~80°C until their use. Freezing assays were carried out in a temperature-programmable freezer. Non-acclimated or cold-acclimated (7 d at 4°C) 3-week-old pot-grown plants were exposed to 4°C for 30 min in darkness and subsequently the temperature was lowered by 1°C h⁻¹. The final desired freezing temperature was maintained for 6 h and then the temperature was increased again to 4°C at the same rate. After thawing at 4°C for 24 h in the dark, plants were returned to their original growth conditions (see above). Tolerance to freezing was determined as the capacity of plants to resume growth after 10 d of recovery under control conditions. Dehydration tolerance was analysed on 2-week-old plants grown in Petri dishes containing GM medium. Tolerance was determined, after removing plants from the medium, placing them on a dry filter paper, and allowing them to develop for 1 d without watering, as the percentage of initial fresh weight (FW) that remained following the treatment. Salt stress was accomplished by transferring 2-week-old plants vertically grown in Petri dishes containing GM medium to new dishes supplemented with 125 mM NaCl. Tolerance was estimated by determining the root elongation and the FW of plants after 7 d of treatment.

Generation of CBF2::LUC transgenic plants

To generate the _CBF2_ promoter::LUC fusion, a _CBF2_ promoter region (~870 to ~10) was placed in front of the _Tobacco mosaic virus_ 5‘-untranslated Ω leader sequence (Gallie et al., 1987) fused to the firefly _LUC_ gene coding sequence (Millar et al., 1992) and the _Cauliflower mosaic virus_ (CaMV) 35S polyadenylation sequence (Topfer et al., 1988). The cassette was subsequently cloned into the plant transformation vector pBIN19 (Bevan, 1984) to yield the CBF2::LUC construct, which was transferred to _Agrobacterium tumefaciens_ C58C1 (Debouma et al., 1985). Transformation of _Arabidopsis_ was performed by vacuum infiltration (Clough and Benf, 1998) and plants homozygous for one copy of the CBF2::LUC transgene were selected by segregation analysis.

Mutagenesis, LUC imaging screening, and genetic analysis

EMS mutagenesis was performed on 60 000 seeds from a selected transgenic line containing a single copy of the CBF2::LUC fusion in homozygosis. Seeds were incubated in 100 ml of 0.3% EMS, 0.5% Triton X-100 for 12 h on a rotary shaker, and then washed 15 times with 250 ml of sterile water. Mutagenized (M1) seeds were divided into 48 pools, sown in pots, and the resulting plants allowed to self-pollinate. M2 seeds from each pool were collected independently, sterilized, and plated in Petri dishes containing MS medium.

For luminescence imaging, 2-week-old M2 plants grown at 20°C or exposed for 24 h at 4°C were sprayed with 1 mM luciferin and then kept in the dark for 5 min to avoid fluorescence interference. Luminescence images were then collected to identify de-regulated _CBF2_ expression mutants. All images were acquired with 10 min exposure time, using an intensified CCD camera 3200 LN/C system (Astromed Ltd, Cambridge, UK) (Kost et al., 1995). Putative mutants were transferred to soil and allowed to self-pollinate. The M3 progeny were re-examined for altered LUC activity as described above to discard false positives.

The character of selected mutations was determined by crossing the mutant lines with wild-type plants. In all crosses, wild-type plants were used as recipients and mutant lines as pollinators. The resulting _F_1 plants and their corresponding _F_2 families were analysed for LUC activity in response to low temperature as described above. For allelism tests, the selected mutants were crossed reciprocally and the _F_1 progeny analysed for their cold-induced luminescence.

Molecular biology methods

Total RNA was isolated from 4-week-old wild-type and mutant plants according to the method described by Logeman et al. (1987). Restriction digestions, cloning, and RNA-blot hybridizations were performed following standard protocols (Sambrook et al., 1989). Specific probes for _CBF1_, _CBF2_, _CBF3_, _COR15A_, _COR47_, _KIN1_, _LT78_, and _RCI2A_ have been described before (Novillo et al., 2004). Similar RNA loading in the experiments was monitored by rRNA staining with ethidium bromide. In some cases, the intensity of hybridization bands was quantified by densitometry with the ImageJ image processing program and corrected for the differences detected in RNA loading. RNA samples from each experiment were analysed in at least two independent blots, and each experiment was repeated at least twice.
mutants the induction of $CBF2$ was de-regulated and correlated with the LUC activity (Fig. 1C). The induction of $CBF2$ in mutants with increased LUC activity was significantly higher and more sustained than in WT plants, particularly in the mutant H84. In the case of mutants having reduced LUC activity, the induction of $CBF2$ was significantly lower than in the WT line, especially in the mutant L55 (Fig. 1C). All these mutants with altered $CBF2$ expression were designated as $acex$. Compared with the WT line, $acex$ mutants did not present any obvious morphological or developmental abnormality (data not shown).

Genetic characterization of $acex$ mutants

In all cases, the F1 plants resulting from crosses between the $acex$ mutants and the WT line displayed wild-type LUC activity in response to low temperature (Table 1). Furthermore, the progeny of these heterozygous F1 plants always

![Fig. 1. Selected mutants showing altered $CBF2$ gene expression. (A) Schematic representation of the $CBF2$:LUC fusion. The LUC gene was placed under the control of a $CBF2$ promoter fragment (-870 to -10). The position of the translational enhancer (Ω) and termination (TER) sequences is indicated. (B) Luciferase activity in mutants with high (H, left panel) or low (L, right panel) luminescence compared with the WT line. Thirteen-day-old plants grown in MS medium at 20 °C and exposed for an additional 1 d at 4 °C. (C) $CBF2$ expression patterns in H (left panel) and L (right panel) mutants. Total RNA was prepared from 4-week-old WT and mutant plants exposed to 4 °C for the indicated times, and hybridized with a $CBF2$-specific probe. Histograms represent the relative quantification of the hybridization signals as obtained by densitometric analysis after correction for the RNA loading differences detected by rRNA staining. In the histogram corresponding to H mutants, data are expressed as means of nine or three independent quantifications (from independent RNA-blot hybridizations) for the WT and mutants, respectively. In the histogram corresponding to L mutants, data are expressed as means of six or three independent quantifications (from independent RNA-blot hybridizations) for the WT and mutants, respectively. Bars indicate the SE. R.U., relative units.](image-url)
Table 1. Genetic characterization of acex mutations

| Crosses | F1 (WT:MUT) | F2 (WT:MUT) | χ² |
|---------|-------------|-------------|----|
| WT × acex8 | 9:0 | 127:35 | 0.99 |
| WT × acex48 | 8:0 | 162:48 | 0.51 |
| WT × acex84 | 13:0 | 128:34 | 1.39 |
| WT × acex55 | 12:0 | 147:39 | 1.61 |
| WT × acex506 | 15:0 | 145:41 | 0.86 |

a WT, wild-type LUC activity; MUT, mutant LUC activity. acex8, acex48, and acex84 are mutants showing lower LUC activity than the WT line. acex55 and acex506 are mutants showing lower LUC activity than the WT line. Values of χ² < 3.84 correspond to a 3:1 segregation.

Table 2. Allelism analysis of acex mutations

| Crosses | F1 (WT:MUT) |
|---------|-------------|
| acex8 × acex48 | 9:0 |
| acex8 × acex84 | 11:0 |
| acex48 × acex84 | 11:0 |
| acex55 × acex506 | 9:0 |

WT, wild-type LUC activity; MUT, mutant LUC activity. acex8, acex48, and acex84 are mutants showing lower LUC activity than the WT line. acex55 and acex506 are mutants showing lower LUC activity than the WT line.

exhibited a segregation of their LUC activity phenotypes of ~3:1 between WT and mutant (Table 1). These data indicated that each one of the five selected acex mutants was caused by a recessive mutation in a single nuclear gene.

Allelism analyses revealed that the five acex mutants belonged to five different complementation groups. In fact, all F1 plants obtained from crosses between mutants disclosed wild-type LUC activity in response to low temperature (Table 2), demonstrating that the selected acex mutations were not allelic.

Physiological characterization of acex mutants

The physiological characterization of the acex mutants was carried out by analysing their sensitivity to freezing and other related abiotic stresses such as dehydration and high salt. Freezing tolerance was determined in non-acclimated and cold-acclimated (7 d at 4 °C) plants as their capacity to resume growth after being exposed for 6 h to different freezing temperatures when returned to control conditions. Figure 2A shows that the three mutants with high CBF2 induction (acex8, acex48, and acex84) had similar levels of freezing tolerance to the WT plants when non-acclimated, the temperature that causes 50% lethality (LT₅₀) being around –7.0 °C in all cases. Nevertheless, mutants acex8 and acex48 were significantly more freezing tolerant than the WT line after cold acclimation (Fig. 2B). The LT₅₀ values of the mutants were very similar (~10.3 °C) and lower than that of WT plants (~9.4 °C). Mutant acex84 did not present any difference from the WT line regarding its freezing tolerance (Table 2). Thus, in the mutant acex8 the induction of CBF1 phenotypes of non-acclimated and cold-acclimated WT and acex55 plants, respectively.

Dehydration was induced by maintaining plants on a dry filter paper for 1 d without watering. The rate of dehydration was determined as the percentage of initial FW that remained following the treatment. Wild-type and acex plants did not present significant differences in their initial FW values (data not shown). After dehydration, acex8 and acex48 plants maintained an average of 22.5% and 20% of their initial FW, respectively, whereas WT plants maintained only 17% (Fig. 4A). Mutant acex84 did not show a significant difference in its remaining FW with respect to WT plants (Fig. 4A). Mutants acex55 and acex506, in turn, were significantly more sensitive to dehydration than the WT line, only maintaining an average of 9% and 7.5% of their initial FW, respectively, after treatment (Fig. 4A). Figure 4B displays the dehydration phenotypes of mutants acex8 and acex55 as representative examples of tolerant and sensitive mutants, respectively, compared with WT plants.

The tolerance to salt stress was estimated by determining the root elongation in acex and WT plants after growing for 7 d in a medium containing 125 mM NaCl. The FW of the plants after treatment also proved to be an estimate of their salt tolerance. WT and acex plants had similar root elongation and FW values under control conditions (data not shown). All mutants, except acex8, exhibited the same levels of salt tolerance as the WT line. acex8 plants subjected to salt stress, however, showed increased root elongation (20%) and remaining FW (23%) compared with WT plants (Fig. 4C). These significant differences among acex8 and WT plants were clearly apparent at the phenotypical level (Fig. 4D).

Molecular characterization of acex mutants

The characterization of the acex mutants was completed by analysing the expression of CBF1 and CBF3, as well as of different genes whose transcripts accumulate in response to low temperature through CBF-dependent (COR15A, COR47, KIN1, and LT78) and CBF-independent (RC12A) pathways (Novillo et al., 2004, 2007). In mutants acex8, acex48, and acex84, the expression levels of all genes analysed under control conditions were the same as in the WT plants (Fig. 5). However, when exposed to 4 °C, several differences in the expression patterns of some genes were observed between mutants acex8 and acex48, and the WT line. Thus, in the mutant acex8 the induction of CBF1
and CBF3 was slightly higher and more sustained than in the WT. In addition, COR15A and KIN1 also showed higher induction than in WT plants (Fig. 5). The mutant acex48 displayed increased induction levels of all genes except RCI2A. Furthermore, as in the case of mutant acex8, the induction of CBF1 and CBF3 was a little more sustained than in the WT line (Fig. 5). In the case of mutant acex84, the induction levels of all genes analysed were unaffected (Fig. 5).

The molecular characterization of the acex mutants with reduced cold induction of CBF2, acex55 and acex506, revealed that they had similar expression patterns of the genes analysed both under control conditions and in response to low temperature (Fig. 6). Mutants acex55 and acex506 did not present increased expression of CBF1, CBF3, and CBF target genes as did the cbf2 null T-DNA mutant (Novillo et al., 2004), in all likelihood because they are caused by trans-acting mutations. Instead, at 20 °C, the transcript levels of all genes were very much alike in the mutants and WT plants (Fig. 6). Furthermore, when exposed to cold, acex55 and acex506 exhibited a decreased induction of CBF1 and CBF3, as well as of the CBF target genes COR15A and COR47. The other genes had the same induction levels as in the WT line (Fig. 6).

Discussion

In an attempt to uncover molecular components acting upstream of CBF2, an essential gene in cold acclimation, Arabidopsis mutants in which its expression is de-regulated have been isolated. The screening procedure was based on transgenic Arabidopsis plants containing the LUC reporter gene under the control of a CBF2 promoter fragment that includes all the elements needed to confer CBF2 cold expression (Novillo et al., 2007; Doherty et al., 2009; Kidokoro et al., 2009). This experimental strategy, namely using promoter::LUC constructs to screen for Arabidopsis mutants affected in stress-regulated gene expression, has been previously carried out by several laboratories (Ishitani et al., 1997; Foster and Chua, 1999; Chinnusamy et al., 2003; Medina et al., 2005; Dong et al., 2009) and has been crucial to reveal signalling intermediates underlying plant responses to different adverse environmental situations. In this work, the characterization of five mutants with altered CBF2 expression is reported. These mutants, named acex, account for five different loci. Three of them, acex8, acex48, and acex84, show higher induction of CBF2 than WT plants in response to low temperature. The other two, acex55 and acex506, display lower induction. Under control conditions, the expression of CBF2 in all mutants is as in the WT line.

The identification of Arabidopsis mutants with increased or reduced cold induction of CBF2 indicates the existence of, at least, one signal transduction pathway that negatively modulates the expression of CBF2 in response to low temperature and another pathway that promotes it. The
The fact that the selected *acex* mutations are not allelic indicates that each of them defines a different component in the signalling cascades that mediate CBF2 induction under cold conditions, confirming that the expression of CBF2 is highly regulated. Mutants with constitutive expression of CBF2 were not isolated. Similar studies conducted to identify mutants de-regulated in cold induction of CBF3 did not allow the isolation mutants having constitutive expression of this gene (Chinnusamy et al., 2003; Dong et al., 2009).

Since the constitutive expression of CBF genes originates dwarf phenotypes and a low number of seeds (Kasuga et al., 1999; Gilmour et al., 2004), most probably mutants with constitutive CBF2 expression are difficult to detect or do not set enough seeds for the screening.

To characterize the *acex* mutants molecularly, the impact of the corresponding mutations on the expression of CBF1 and CBF3, as well as on the expression of different cold-regulated genes whose induction is mediated (COR15A, COR47, KIN1, and LTI78) or not (RCI2A) by the CBFs (Novillo et al., 2004, 2007) was analysed. Under control conditions, the expression of CBF1 and CBF3 was not detected in any mutant and, consequently, not the expression of CBF target genes either. Accordingly, the WT phenotype that is present in the *acex* mutants is consistent with the absence of CBF gene expression under unstressed conditions. Interestingly, however, in response to low temperature, the induction patterns of CBF1 and CBF3 are altered in all mutants except in *acex*84. In fact, paralleling the levels of CBF2 transcripts, the induction of CBF1 and CBF3 is increased in mutants *acex*8 and *acex*48 and reduced in mutants *acex*55 and *acex*506, indicating that the proteins identified by these mutations, in addition to modulating the cold induction of CBF2, are also involved in regulating the expression of CBF1 and CBF3 under cold conditions. In mutant *acex*84, however, only the levels of CBF2 transcripts are higher than in the WT when exposed to low temperature, which indicates that the corresponding protein specifically regulates the cold induction of CBF2. Taken together, all these data suggest that, as already described for CBF3 regulation (Chinnusamy et al., 2003; Agarwal et al., 2006), the expression of CBF2 is also mediated through both specific and non-specific signalling pathways.

Remarkably, mutants *acex*8 and *acex*48, both of them having high induction levels of all CBF genes, exhibit different expression patterns of CBF target genes in response to low temperature. In the mutant *acex*8, only COR15A and KIN1 are more induced than in the WT line. However, in the mutant *acex*48 the induction of all CBF targets analysed is increased. This difference may be due to the variation that exists between *acex*8 and *acex*48 in the levels of CBF1 and CBF3 transcripts. In this regard, it has been proposed that the induction of CBF target genes depends on the amount of total CBFs (Novillo et al., 2007). In contrast to *acex*8 and *acex*48, the mutant *acex*84, which shows WT induction levels of CBF1 and CBF3 transcripts when exposed to cold, also has the same cold induction levels of the CBF target genes.
genes as the WT plants. Probably, the amount of total \textit{CBF} transcripts in this mutant would not be sufficient to promote an increase in the induction of the \textit{CBF} targets. As for the mutants \textit{acex55} and \textit{acex506}, although they have low induction levels of all \textit{CBF} genes when exposed to cold, they are only affected in the induction of \textit{COR15A} and \textit{COR47}. Again, this expression pattern might be determined by the amount of \textit{CBFs}. Nonetheless, it cannot be excluded that, in addition to regulating the levels of \textit{CBF} transcripts in response to low temperature, the signalling intermediates defined by the \textit{acex} mutations could also function in controlling the cold induction of \textit{CBF} target genes through \textit{CBF}-independent pathways. Indeed, various studies have described that genes belonging to the \textit{CBF} regulon are also induced by cold in a \textit{CBF}-independent way (Baker \textit{et al.}, 1994; Wang and Cutler, 1995; Zhu \textit{et al.}, 2004; Vogel \textit{et al.}, 2005; Yoo \textit{et al.}, 2007). All \textit{acex} mutants show induction levels of \textit{RCI2A} in response to low temperature identical to those of the WT plants, confirming that this gene does not belong to the \textit{CBF} regulon and demonstrating that the \textit{acex} mutations do not affect any intermediate step involved in regulating the cold induction of \textit{RCI2A}.

Mutants \textit{acex8}, \textit{acex48}, \textit{acex84}, and \textit{acex506} are not altered in their constitutive capacity to tolerate freezing, which is consistent with their WT gene expression profiles under control conditions. In contrast, mutant \textit{acex55}, which also has WT gene expression patterns, is impaired in its constitutive freezing tolerance. The corresponding mutation, therefore, should uncover a positive regulator of the \textit{Arabidopsis} constitutive freezing tolerance that would function through a \textit{CBF}-independent signalling pathway. Regarding the freezing tolerance of \textit{acex} mutants after cold acclimation, \textit{acex8} and \textit{acex48} plants display a higher capacity to cold acclimate than the WT line, whereas \textit{acex55} and \textit{acex506} plants are impaired in their cold-induced freezing tolerance. These tolerance phenotypes are consequent on the induction levels of \textit{CBF} genes and \textit{CBF} target genes in these mutants in response to low temperature. The mutant \textit{acex84}, in turn, is not affected in its freezing tolerance after cold acclimation. Considering that \textit{acex84} has increased induction levels of \textit{CBF2} when exposed to cold, but WT induction levels of \textit{CBF1}, \textit{CBF3}, and \textit{CBF} target genes, the results reported here suggest that the amount of \textit{CBFs} in \textit{acex84} in response to low temperature should be insufficient to promote an increase in the induction levels of \textit{CBF} targets, as already mentioned, and, therefore, in its cold-induced freezing tolerance.

In addition to causing an increase in the capacity of \textit{Arabidopsis} to cold acclimate, the \textit{acex8} mutation also provokes higher tolerance to dehydration and salt stress compared with the WT line. However, the \textit{acex48} mutation...
that also increases freezing tolerance after cold acclimation gives rise to higher tolerance to dehydration but not to salt stress. The acex84 mutation, that does not affect the cold-induced freezing tolerance of Arabidopsis, does not alter the tolerance to dehydration and salt stress either. In the case of mutants acex55 and acex506, an increased sensitivity to
dehydration but a tolerance to salt stress like the WT line was observed. From these results, it can be concluded that
the factors defined by the identified mutations not only play
an important role in the freezing tolerance of *Arabidopsis* but
are also involved in the tolerance of *Arabidopsis* to other
related abiotic stresses such as dehydration and high salt.
This illustrates, once more, that the signalling pathways that
mediate plant responses to low temperature, dehydration,
and salt stress converge at different points like those defined
by the *acex* mutations.

As already mentioned, some factors have been reported
to regulate the expression of *CBF2* (Agarwal *et al.*, 2006;
Chinnusamy *et al.*, 2007; Doherty *et al.*, 2009; Kidokoro
*et al.*, 2009; Medina *et al.*, 2011). Although the possibility
that some of the *acex* mutants correspond to genes already
described as *CBF2* regulators cannot be excluded, from the
data available it is highly unlikely. In fact, when compared
with the previously isolated mutants affected in *CBF2*
expression, the *acex* mutants have very different pheno-
types. The *acex* mutants are morphologically, physiologi-
cally, and/or molecularly different from the mutants
previously described as having altered *CBF2* expression
(Agarwal *et al.*, 2006; Chinnusamy *et al.*, 2007; Doherty
*et al.*, 2009; Kidokoro *et al.*, 2009; Medina *et al.*, 2011),
which strongly indicates that they are not allelic and identify
new molecular components controlling *CBF2* expression.
Based on the phenotypes displayed by the *acex* mutants,
a working model is proposed for the function of the gene
products identified in regulating *CBF2* expression and
*Arabidopsis* tolerance to abiotic stress (Fig. 7). According
to this model, proteins ACEX8, ACEX48, and ACEX84 would
act, directly or indirectly, as negative regulators of *CBF2*
induction in response to low temperature. Furthermore,
ACEX8 and ACEX48 would also modulate, negatively, the
cold induction of *CBF1* and *CBF3*, and, most probably
through the *CBF* targets, the capacity of *Arabidopsis* to cold
acclimate. Since the mutant *acex84* is not affected in its
capacity to cold acclimate, the ACEX84 protein, that would
specifically regulate the induction of *CBF2* under cold
conditions, would not play an apparent role in cold
acclimation. ACEX8, moreover, would have a function, as
a negative regulator, in *Arabidopsis* tolerance to dehydra-
tion and salt stress. ACEX48, however, would only be
involved in the tolerance of *Arabidopsis* to dehydration.
On the other hand, proteins ACEX55 and ACEX506
would positively regulate the induction of the three *CBF*
genomes and some target genes by low temperature and,
therefore, the capacity of *Arabidopsis* to cold acclimate.
ACEX55 would also control, in a positive way, the
conservative freezing tolerance of *Arabidopsis* through a *CBF-
*independent signalling cascade. Additionally, both ACEX55
and ACEX506 proteins would act as positive modulators of
*Arabidopsis* tolerance to dehydration. These results demon-
strate the complexity of the molecular mechanisms plants
have evolved to respond and adapt to their environment. The
molecular identification of *acex* mutations and the subsequent
functional characterization of the corresponding factors will
contribute to further understanding of the role of *CBF2* in
cold acclimation and the intricate signalling networks that
regulate *CBF* genes expression and *Arabidopsis* response to
abiotic stresses.

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