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RESEARCH PAPER

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

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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

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 N_2, 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 (Deblaere *et al.*, 1985). Transformation of *Arabidopsis* was performed by vacuum infiltration (Clough and Bent, 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 (M_1) seeds were divided into 48 pools, sown in pots, and the resulting plants allowed to self-pollinate. M_2 seeds from each pool were collected independently, sterilized, and plated in Petri dishes containing MS medium.

For luminescence imaging, 2-week-old M_2 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 M_3 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*, *LTI78*, 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.

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 homogeneous 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 M_2 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 endogeneous *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

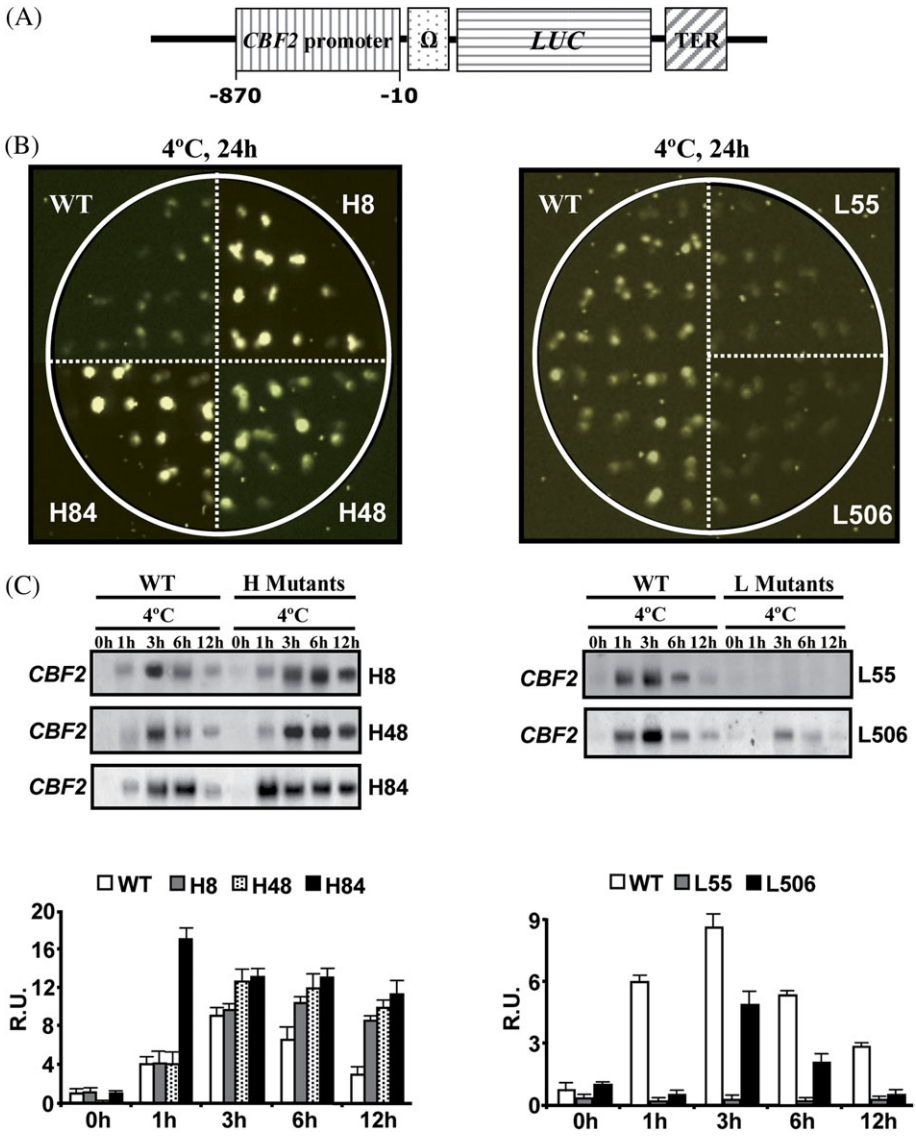

Fig. 1. Selected mutants showing altered *CBF2* gene expression. (A) Schematic representation of the *CBF2*::*LUC* fusion. The *LUC* gene was placed under the control of a *CBF2* promoter fragment (–870 to –10). The position of the translational enhancer (Ω) and termination (TER) sequences is indicated. (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.

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 F_1 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 F_1 plants always

Table 1. Genetic characterization of *acex* mutations

Crosses	F_1 (WT:MUT)[a]	F_2 (WT:MUT)	χ^2
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×*cex506*	15:0	145:41	0.86

[a] WT, wild-type LUC activity; MUT, mutant LUC activity.
acex8, *acex48*, and *acex84* are mutants showing higher LUC activity than the WT line. *acex55* and *acex506* are mutants showing lower LUC activity than the WT line.
Values of χ^2 <3.84 correspond to a 3:1 segregation.

Table 2. Allelism analysis of *acex* mutations

Crosses	F_1 (WT:MUT)
acex8×*acex84*	9:0
acex8×*acex48*	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 higher 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 recesive mutation in a single nuclear gene.

Allelism analyses revealed that the five *acex* mutants belonged to five different complementation groups. In fact, all F_1 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 (LT_{50}) 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 LT_{50} 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 capacity to cold acclimate (Fig. 2B). The freezing tolerance phenotypes of non-acclimated and cold-acclimated WT and *acex8* plants are displayed in Fig. 2C and D, respectively, as a representative example. As for the mutants having low induction of *CBF2*, while the mutant *acex55* exhibited a significant decreased freezing tolerance when non-acclimated, the LT_{50} of mutant and WT plants being –6.5 °C and –7.2 °C, respectively, the mutant *acex506* behaved like the WT line (Fig. 3A). After being cold acclimated, however, both mutants were significantly impaired in their capacity to tolerate freezing. The LT_{50} values of *acex55* and *acex506* mutants were –8.0 °C and –8.1 °C, respectively, while that of WT plants was –9.5 °C (Fig. 3B). As a representative example, Fig. 3C and D shows the freezing tolerance 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 *LTI78*) and CBF-independent (*RCI2A*) 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*

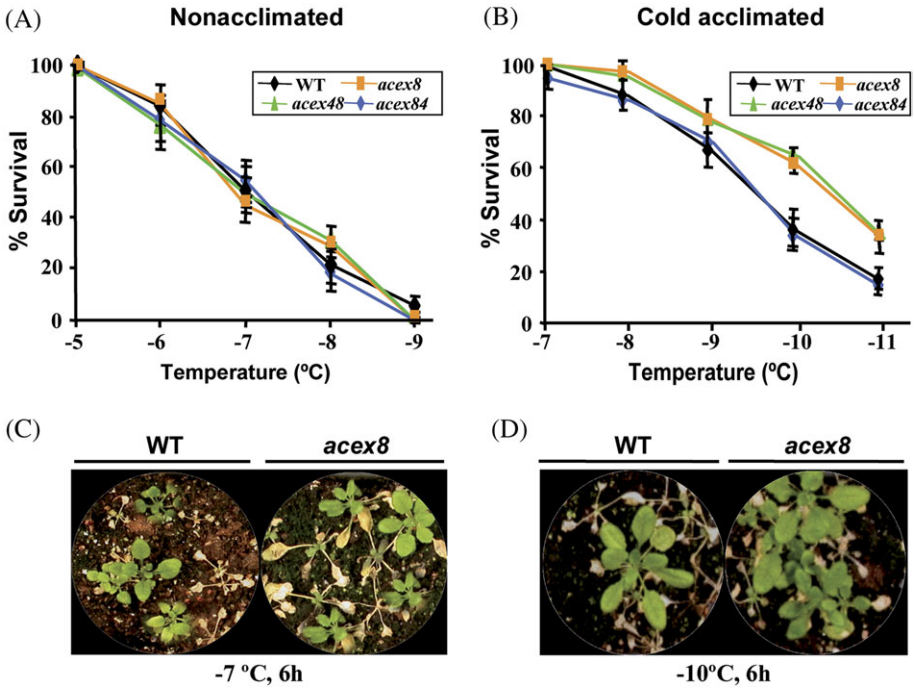

Fig. 2. Freezing tolerance of *acex* mutants showing increased induction of *CBF2* in response to low temperature. Non-acclimated and cold-acclimated (7 d, 4 °C) 3-week-old WT and mutant (*acex8*, *acex 48*, and *acex 84*) plants were exposed to different freezing temperatures for 6 h. Freezing tolerance was estimated as the percentage of plants surviving each specific temperature after 10 d of recovery under control conditions. (A) Freezing tolerance of non-acclimated plants. (B) Freezing tolerance of cold-acclimated plants. (C) Representative non-acclimated WT and *acex8* plants 10 d after being exposed to −7 °C for 6 h. (D) Representative cold-acclimated WT and *acex8* plants 10 d after being exposed to −10 °C for 6 h. In A and B, data are expressed as the means of three independent experiments with at least 50 plants each. Bars indicate the SE.

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

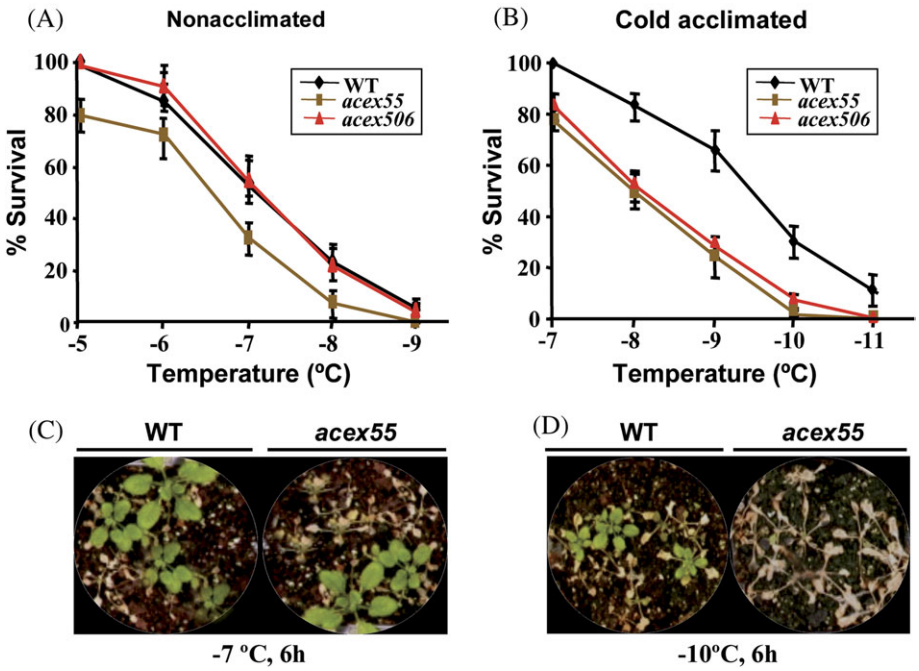

Fig. 3. Freezing tolerance of *acex* mutants showing reduced induction of *CBF2* in response to low temperature. Non-acclimated and cold-acclimated (7 d, 4 °C) 3-week-old WT and mutant (*acex55* and *acex506*) plants were exposed to different freezing temperatures for 6 h. Freezing tolerance was estimated as the percentage of plants surviving each specific temperature after 10 d of recovery under control conditions. (A) Freezing tolerance of non-acclimated plants. (B) Freezing tolerance of cold-acclimated plants. (C) Representative non-acclimated WT and *acex55* plants 10 d after being exposed to –7 °C for 6 h. (D) Representative cold-acclimated WT and *acex55* plants 10 d after being exposed to –10 °C for 6 h. In A and B, data are expressed as the means of three independent experiments with at least 50 plants each. Bars indicate the SE.

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 *acex84*. In fact, paralleling the levels of *CBF2* transcripts, the induction of *CBF1* and *CBF3* is increased in mutants *acex8* and *acex48* and reduced in mutants *acex55* and *acex506*, 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 *acex84*, 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 *acex8* and *acex48*, 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 *acex8*, only *COR15A* and *KIN1* are more induced than in the WT line. However, in the mutant *acex48* the induction of all CBF targets analysed is increased. This difference may be due to the variation that exists between *acex8* and *acex48* 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 *acex8* and *acex48*, the mutant *acex84*, 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

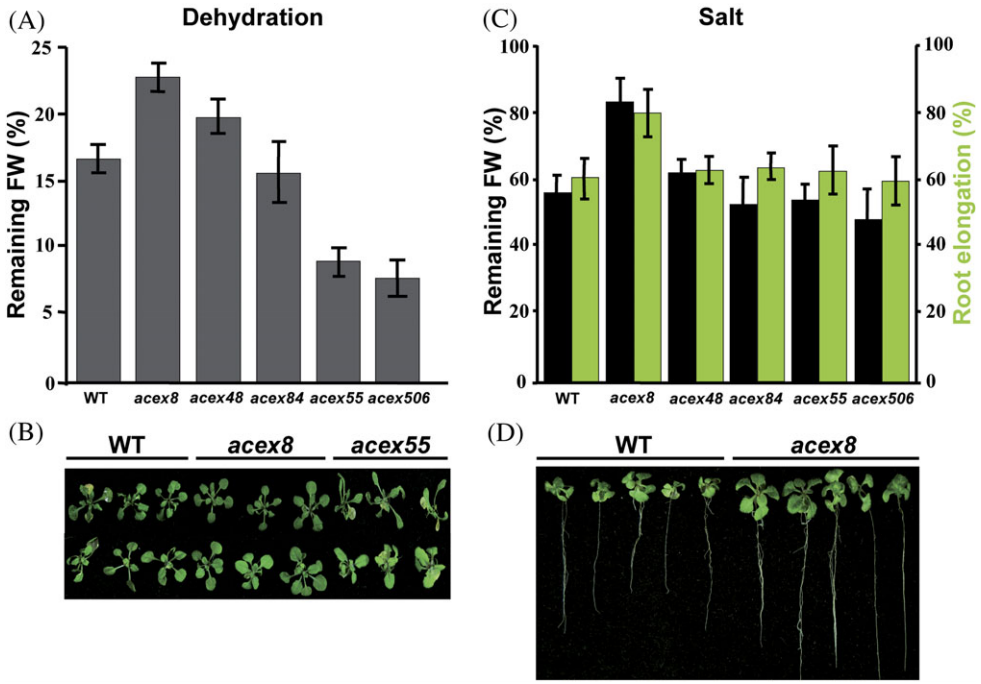

Fig. 4. Tolerance to dehydration and salt stress of *acex* mutants. (A) Dehydration tolerance of WT and *acex* plants. Tolerance was estimated as the percentage of initial FW that remains after transferring 2-week-old plants to a dry filter paper and allowing them to develop for 1 d without watering. (B) Representative WT, *acex8*, and *acex55* mutant plants after dehydration treatment. (C) Salt tolerance of WT and *acex* plants. Tolerance was estimated by determining the root elongation (green bars) and remaining FW (black bars) of 2-week-old plants transferred to a medium containing 125 mM NaCl for 7 d. (D) Representative WT and *acex8* mutant plants after salt treatment. In A and C, data are expressed as means of three independent experiments with at least 20 plants each. Bars indicate the SE. In A, values obtained from the WT and *acex8*, *acex48*, *acex55*, and *acex506* mutants were significantly different (P <0.05), as determined by Student's test, except in the case of mutant *acex84*. In C, values obtained from the WT and the *acex8* mutant were significantly different (P <0.05).

genes as the WT plants. Probably, the amount of total *CBF* transcripts in this mutant would not be sufficient to promote an increase in the induction of the CBF targets. As for the mutants *acex55* and *acex506*, although they have low induction levels of all *CBF* genes when exposed to cold, they are only affected in the induction of *COR15A* and *COR47*. Again, this expression pattern might be determined by the amount of CBFs. Nonetheless, it cannot be excluded that, in addition to regulating the levels of *CBF* transcripts in response to low temperature, the signalling intermediates defined by the *acex* mutations could also function in controlling the cold induction of CBF target genes through CBF-independent pathways. Indeed, various studies have described that genes belonging to the CBF regulon are also induced by cold in a CBF-independent way (Baker *et al.*, 1994; Wang and Cutler, 1995; Zhu *et al.*, 2004; Vogel *et al.*, 2005; Yoo *et al.*, 2007). All *acex* mutants show induction levels of *RCI2A* in response to low temperature identical to those of the WT plants, confirming that this gene does not belong to the CBF regulon and demonstrating that the *acex* mutations do not affect any intermediate step involved in regulating the cold induction of *RCI2A*.

Mutants *acex8*, *acex48*, *acex84*, and *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 *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 *Arabidopsis* constitutive freezing tolerance that would function through a CBF-independent signalling pathway. Regarding the freezing tolerance of *acex* mutants after cold acclimation, *acex8* and *acex48* plants display a higher capacity to cold acclimate than the WT line, whereas *acex55* and *acex506* plants are impaired in their cold-induced freezing tolerance. These tolerance phenotypes are consequent on the induction levels of *CBF* genes and CBF target genes in these mutants in response to low temperature. The mutant *acex84*, in turn, is not affected in its freezing tolerance after cold acclimation. Considering that *acex84* has increased induction levels of *CBF2* when exposed to cold, but WT induction levels of *CBF1*, *CBF3*, and CBF target genes, the results reported here suggest that the amount of CBFs in *acex84* in response to low temperature should be insufficient to promote an increase in the induction levels of CBF targets, as already mentioned, and, therefore, in its cold-induced freezing tolerance.

In addition to causing an increase in the capacity of *Arabidopsis* to cold acclimate, the *acex8* mutation also provokes higher tolerance to dehydration and salt stress compared with the WT line. However, the *acex48* mutation

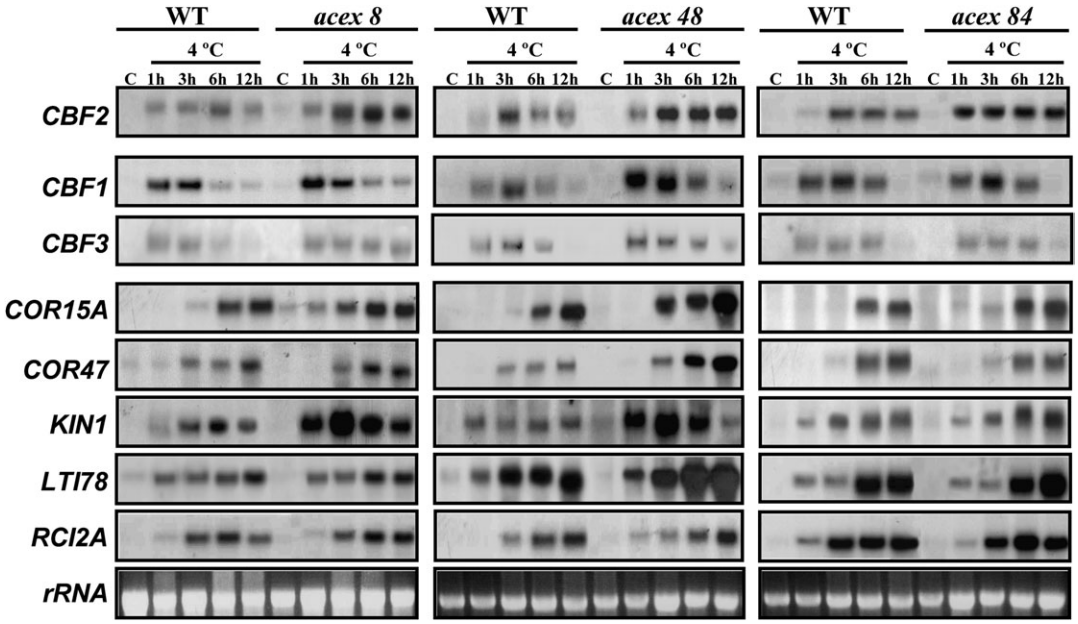

Fig. 5. Transcript levels of *CBF1*, *CBF3*, and different cold-inducible genes in *acex8*, *acex48*, and *acex84* mutants. RNA-blot hybridizations were performed with total RNA isolated from 4-week-old Col (WT) and mutant plants grown under control conditions (C) or exposed to 4 °C for the indicated times. Specific probes for *CBF1* and *CBF3*, as well as for CBF target genes *COR15A*, *COR47*, *KIN1*, and *LTI78*, and the non-CBF-target gene *RCI2A* were used for hybridizations. The transcript levels of *CBF2* in the mutants are shown as internal controls. Similar amounts of RNA were present in each sample as confirmed by ethidium bromide staining of *rRNA*.

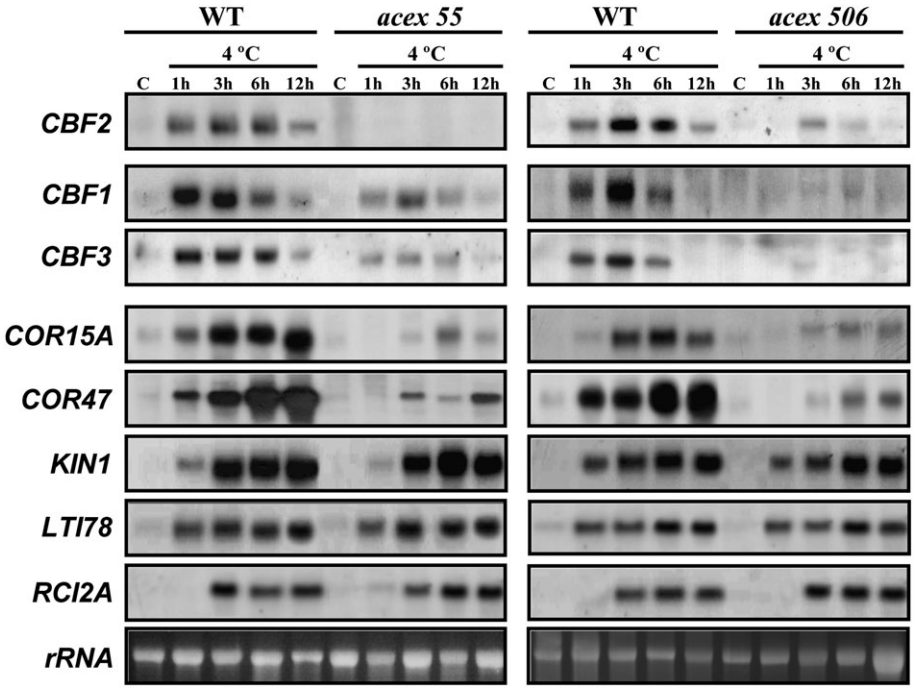

Fig. 6. Transcript levels of *CBF1*, *CBF3*, and different cold-inducible genes in *acex55* and *acex506* mutants. RNA-blot hybridizations were performed with total RNA isolated from 4-week-old Col (WT) and mutant plants grown under control conditions (C) or exposed to 4 °C for the indicated times. Specific probes for *CBF1* and *CBF3*, as well as for CBF target genes *COR15A*, *COR47*, *KIN1*, and *LTI78*, and the non-CBF-target gene *RCI2A* were used for hybridizations. The transcript levels of *CBF2* in the mutants are shown as internal controls. Similar amounts of RNA were present in each sample as confirmed by ethidium bromide staining of *rRNA*.

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

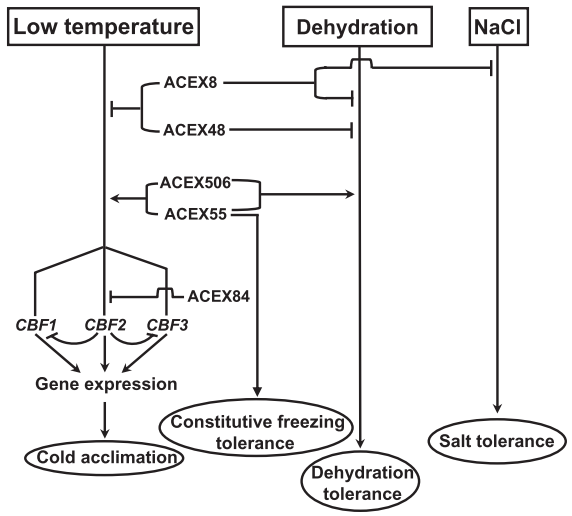

Fig. 7. Proposed model for the function of identified ACEX factors in regulating *CBF2* expression and *Arabidopsis* tolerance to abiotic stresses. Gene products defined by the selected *acex* mutants are shown to positively (arrowheads) or negatively (end lines) regulate the expression of *CBF* genes in response to low temperature as well as the tolerance of *Arabidopsis* to freezing, before and after cold acclimation, dehydration, and high salt.

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 phenotypes. The *acex* mutants are morphologically, physiologically, 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 dehydration 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* genes 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 consitutive 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 demonstrate 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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