Chapter 1

Gene Regulatory Networks Mediating Cold Acclimation: the CBF Pathway

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Abstract

Under low non-freezing temperature conditions, plants from temperate climates undergo physiological and biochemical adjustments that increase their tolerance to freezing temperatures. This response, termed cold acclimation, is largely regulated by changes in gene expression. Molecular and genetic studies have identified a small family of transcription factors, called C-repeat binding factors (CBFs), as key regulators of the transcriptomic rearrangement that leads to cold acclimation. The function of these proteins is tightly controlled and an inadequate supply of CBF activity may be detrimental to the plant. Accumulated evidence has revealed an extremely intricate network of positive and negative regulators of cold acclimation that coalesce at the level of *CBF* promoters constituting a central hub where multiple internal and external signals are integrated. Moreover, *CBF* expression is also controlled at post-transcriptional and post-translational levels further refining *CBF* regulation. Recently, natural variation studies in *Arabidopsis* have demonstrated that mutations resulting in changes in *CBF* expression have an adaptive value for wild populations. Intriguingly, CBF genes are also present in plant species that do not cold acclimate, which suggest that they may also have additional functions. For instance,

CBFs are required for some cold-related abiotic stress responses. In addition, their involvement in plant development deserves further study. Although more studies are necessary to fully harness CBF biotechnological potential, these transcription factors are meant to be key for a rational design of crops with enhanced tolerance to abiotic stress.

Keywords

Transcription factors, Low temperature, Abiotic stress, Gene regulation, Signaling integration, Hormone signaling, Light signaling.

Abbreviations

ABA (abscisic acid)
AP2/ERF (apetala 2/ethylene response factor)
bHLH (beta helix-loop-helix)
BR (brassinosteroids)
CHIP (chromatin immunoprecipitation)
COR (cold regulated genes)
CRISPR (clustered regularly interspaced short palindromic repeats)
CRT (C-Repeat)
DRE (dehydration responsive element)
EMSA (electrophoretic mobility shift assay)
ET (ethylene)
GA (gibberellins)
H3K27me3 (histone H3 lysine 27 trimethylation)
JA (jasmonic acid)
RNAPol II (RNA polymerase II)

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53 **1.1 Introduction**

54 Plants often face environmental conditions that are detrimental for their growth and
55 development. Because of their sessile nature that prevents them to escape from adverse
56 situations, many plant species have evolved phenotypic plastic responses that allow them to
57 adjust their physiology to an ever-changing environment. One of the major constraints of plant
58 growth is low temperature and, not surprisingly, plants from temperate latitudes have evolved a
59 response that allows them to increase their tolerance to freezing. This response, termed cold
60 acclimation (Levitt 1980), is developed after plants are exposed to low non-freezing temperatures.
61 It comports a variety of structural and metabolic changes including adjustments in membrane
62 composition to prevent damage generated by freezing temperatures, accumulation of stress-
63 related proteins and sugars to avoid the dehydration caused by the immobilization of water
64 around ice nuclei, activation of antioxidant enzymes, and protection of the cold-sensitive
65 photosynthetic machinery (Ruelland et al. 2009; Theocharis et al. 2012). Many years ago, it was
66 found that cold acclimation required changes at the transcriptomic level (Guy et al. 1985). Since
67 then, numerous reports have described that this response involves a comprehensive
68 reprogramming of the transcriptome, modifying the expression of thousands of genes (Hannah et
69 al. 2005; Winfield et al. 2010). Indeed, accumulating evidence indicates that the number and
70 amplitude of the changes in gene expression induced by low temperature correlates positively
71 with the capacity of plants to cold acclimate (Hannah et al. 2006). The study of the molecular
72 mechanisms that govern the expression of cold-regulated (*COR*) genes led to the characterization
73 of a small family of transcription factors, known as C-repeat binding factors (CBFs), which are
74 critical for the accurate development of a cold acclimation response. In this chapter we review the

most recent advances concerning the regulation and function of *CBF* genes and discuss the relevance of the CBF signaling pathway to the acquisition of higher freezing tolerance.

1.2 The Identification of the CBFs

The analysis of cis-elements in the promoter regions of different *COR* genes from the model plant *Arabidopsis thaliana* allowed the identification of a short sequence of 9 bp, named dehydration responsive element (DRE), that was able to confer responsiveness to dehydration, salt and low temperature (Yamaguchi-Shinozaki and Shinozaki 1994). Further analysis enabled to establish a core sequence, CCAGC, called the C-repeat (CRT), which was sufficient for low temperature induction of these genes (Baker et al. 1994). Using a yeast one-hybrid approach, Stockinger and collaborators isolated an *Arabidopsis* gene encoding a transcription factor with capacity to bind to the CRT/DRE sequence and, thus, was termed C-repeat binding factor 1 (CBF1) (Stockinger et al. 1997). Intriguingly, it was shown that constitutive expression of *CBF1* increased the freezing tolerance of plants, even in the absence of a low temperature stimulus, and this increase was associated with a rise in the expression of *COR* genes (Jaglo-Ottosen et al. 1998). Soon, it was found that CBF1 was a member of a small family of three transcription factors (CBF1-3) arrayed in tandem (Gilmour et al. 1998; Liu et al. 1998; Medina et al. 1999), all of them having the ability to bind to CRT/DRE boxes in many *COR* genes activating their transcription. These results suggested a relevant role for CBFs in the development of cold acclimation. The first genetic evidence of CBF involvement in this stress response came with the functional characterization of *cbf2* mutants (Novillo et al. 2004; Alonso-Blanco et al. 2005), showing that disruption of *CBF2* expression altered the capacity of *Arabidopsis* to cold acclimate. Later on, the generation of RNAi lines with knocked down *CBF1* and *CBF3* expression demonstrated the involvement of all three CBF proteins in cold acclimation (Novillo et al. 2007). However, the degree of redundancy among the *Arabidopsis* CBFs,

as well as the relationship among them, has not been clarified until the recent isolation of single, double and triple *CBF* mutants generated by means of CRISPR/Cas technology (Zhao et al. 2016; Jia et al. 2016; Shi et al. 2017). In summary, these studies have revealed that the three CBFs are positive regulators of cold acclimation, controlling the expression of a significant number of genes that mediate the development of this response. The CBFs show a substantial degree of functional redundancy as both *cbf1* and *cbf3* single CRISPR mutants can cold acclimate as a wild-type (Zhao et al. 2016; Jia et al. 2016; Shi et al. 2017), and the transcriptome profiling of single mutants display considerable overlap among the target genes regulated by each CBF (Shi et al. 2017). Since their identification in *Arabidopsis*, *CBF* genes have been also identified in a wide range of plant species in both dicot (Zhang et al. 2004; Benedict et al. 2006; Pennycooke et al. 2008; Welling and Palva 2008) and monocot species (Xue 2003; Dubouzet et al. 2003; Qin et al. 2004; Vágújfalvi et al. 2005).

1.3 The Relevance of CBFs

CBF proteins regulate one of the best characterized cold signaling pathways, but the real relevance of this pathway to the cold acclimation process has only recently begun to be realized. Thus, a transcriptome profiling study of *Arabidopsis* transgenic plants over-expressing *CBF* transcription factors found that 11% of all *COR* genes identified were controlled by, at least, one *CBF* (Park et al. 2015). A similar figure was obtained when analyzing an *Arabidopsis* CRISPR mutant devoid of any *CBF* expression, which shows altered expression patterns for about 10% (414) of all *COR* genes in that work (Zhao et al. 2016). Importantly, this alteration results in a severely reduced cold acclimation capacity for this triple mutant (Zhao et al. 2016). An independent study also found that abrogation of *CBF* expression in *Arabidopsis* resulted in defective cold upregulation of *COR* genes with profound consequences in the capacity to cold acclimate (Jia et al. 2016). Nonetheless,

both studies revealed that triple *cbf* mutants still retained some capacity to cold acclimate. All these results manifest that CBFs control a significant portion of the *COR* gene expression.

Further evidence on the relevance of the CBF pathway in cold acclimation has been provided through the study of natural variation for freezing tolerance in *Arabidopsis*. Natural populations of *Arabidopsis* are found in a wide range of climates, and substantial diversity in freezing tolerance among different accessions has been reported (Hannah et al. 2006; Zhen and Ungerer 2008). Intriguingly, several papers have described mutations that result in changes in *CBF* expression as a source of natural variation in this species for cold acclimation capacity (Alonso-Blanco et al. 2005; Kang et al. 2013; Oakley et al. 2014; Gehan et al. 2015; Monroe et al. 2016). It is worth mentioning that some of these studies also concluded that, while having fully functional CBF proteins has an adaptive value in cold climates, it provokes deleterious effects in warmer climates where freezing temperatures are uncommon (Oakley et al. 2014; Gehan et al. 2015; Monroe et al. 2016). In addition, CBF function has been found to significantly contribute to winter hardiness in temperate cereals. In both barley and wheat, variation in copy number of *CBF* genes can be associated with tolerance to freezing temperatures (Knox et al. 2010; Würschum et al. 2017). Taken together, all these findings establish that CBF proteins are major contributors to freezing tolerance in cold-acclimating species.

1.4 The *CBF* Promoters Constitute Central Hubs That Integrate Multiple External and Internal Signals

Overexpression of *CBF* genes, besides increasing freezing tolerance, also results in growth reduction, producing dwarf plants that flower later and have a prostrate growth habit (Gilmour et al. 2004). Interestingly, CBF-loss-of function results in a similar phenotype (Zhao et al. 2016; Jia et al. 2016), suggesting that any deviation from the appropriate level of *CBF* expression is detrimental

for the plant. This is further supported by the observation mentioned above that a functional CBF pathway has deleterious effects on warm climates where freezing temperatures are uncommon (Oakley et al. 2014; Gehan et al. 2015; Monroe et al. 2016). It is not surprising, therefore, that *CBF* expression is tightly regulated (Barrero-Gil and Salinas 2017). *CBF* genes are expressed at low levels in warm conditions but their expression increase transiently by several orders of magnitude within minutes of cold exposure, returning to resting levels after a few hours at low temperature (Gilmour et al. 1998; Liu et al. 1998; Medina et al. 1999). The expression of *CBF* genes has been the object of intense research in the past two decades revealing an intricate network of different transcription factors involved in their regulation (Table 1; Figure 1.1). From these results, a fascinating picture emerges where *CBF* promoters constitute central hubs that integrate not only information about temperature but multiple signaling pathways from both external and internal cues. This section below describes the different signaling pathways that converge on the regulation of *CBF* transcription.

The CBF promoters mediate low temperature signaling through a beta Helix-Loop-Helix (bHLH) transcription factor called INDUCER OF CBF EXPRESSION1 (ICE1). The existence of this factor was postulated to explain the rapidity with which *CBF* genes are upregulated by low temperatures, anticipating that there should be a factor already present at warm temperatures that would be activated by cold to trigger the induction of *CBFs* (Gilmour et al. 1998). A genetic screen designed to identify such a regulator identified ICE1 as a factor that bound to an E-box element in the *CBF3* promoter inducing its expression (Chinnusamy et al. 2003). ICE1 is activated by cold to relieve the *CBF* repression mediated by the factor MYELOBLASTOSIS15 (MYB15), which binds to the MYB sequences in the promoters of *CBFs* to restrain their expression (Chinnusamy et al. 2003; Agarwal et al. 2006). Recently, it has been demonstrated that ICE1 and its close homolog ICE2 can bind to E-box elements in the promoter region of all *CBFs* to activate their expression

(Kim et al. 2015). The CALMODULIN-BINDING TRANSCRIPTIONAL ACTIVATORS (CAMTA) represent another group of transcription factors that, under cold conditions, bind to the promoters of *CBF1* and *CBF2*, in particular to their CGCG motifs, to induce their expression and transduce the cold stimulus (Doherty et al. 2009; Kidokoro et al. 2017).

In addition to low temperature signals, *CBF* promoters also integrate environmental information about light quality, daylight length and internal signals from the circadian clock. In this context, the photoreceptor PHYTOCHROME B (PHYB) determines the photoperiodic regulation of *CBF* transcript levels most likely through its interaction with PHYTOCHROME-INTERACTING FACTOR4 (PIF4) and PIF7, which bind to the G- and E-boxes in the *CBF* promoters to repress their expression (Lee and Thomashow 2012). In a similar manner, both PHYB and PHYD perceive changes in light quality and modulate *CBF* expression, which is considered a mechanism that anticipates temperature seasonal changes (Franklin and Whitelam 2007). Furthermore, the expression of *CBFs* is also regulated by the circadian clock in a PIF-independent manner. Some years ago, it was shown that the cold induction of *CBFs* is gated by the clock so that it is much higher if the cold stimulus occurs at ZT4 than at ZT16 (Fowler et al. 2005). Remarkably, the gating coincides with high levels of the circadian oscillator component CIRCADIAN CLOCK-ASSOCIATED1 (CCA1) (Alabadi et al. 2001), which binds directly to the *CBF* promoters and activates cold acclimation (Dong et al. 2011). Other components of the circadian clock also regulate *CBF* expression negatively, including PSEUDO-RESPONSE REGULATOR5 (PRR5), PRR7, PRR9 and TIMING OF CONSTANS1 (TOC1) (Nakamichi et al. 2009, 2012; Keily et al. 2013).

The *CBF* promoters have also the ability to respond to endogenous signals from multiple hormonal pathways (Shi et al. 2015; Eremina et al. 2016a). For instance, the brassinosteroid signaling transcription factors BRASSINAZOLE RESISTANT1 (BZR1) and CESTA (CES) mediate the activation of the *CBFs* pathway and, consequently, of cold acclimation (Eremina et al. 2016b; Li et

al. 2017). Although further study is necessary to elucidate the precise role played by brassinosteroids (BRs) during the cold response (Barrero-Gil and Salinas 2017), compelling data show that transcription factors BRZ1 and CES can directly bind to CBF promoters and activate their transcription (Eremina et al. 2016b; Li et al. 2017). These findings strongly suggest that *CBF* transcription is determined by BRs levels. The levels of jasmonic acid (JA) also play a positive role on the CBFs pathway. Indeed, JA accumulates early during the development of cold acclimation, relieving the repression of ICE1 by the JASMONATE ZIM-DOMAIN1 (JAZ1) repressor and allowing the induction of *CBF* expression (Hu et al. 2013). The gaseous phytohormone ethylene (ET), on the contrary, functions as a negative regulator of the CBFs pathway and of cold acclimation. Despite conflicting reports about the accumulation of ET in response to low temperature, which may be ascribed to different experimental conditions (Shi et al. 2012; Catalá et al. 2014), conclusive evidence has been presented that cold fosters the disappearance of ET-signaling negative regulators EIN3-BINDING F-BOX1 (EBF1) and EBF2 (Jiang et al. 2017). This, in turn, results in the accumulation of ETHYLENE INSENSITIVE3 (EIN3) and PIF3 transcription factors, that would bind to the *CBF* promoters to repress their expression (Shi et al. 2012; Jiang et al. 2017) (Figure 1.1).

Low temperature reduces the levels of bioactive gibberellins (GAs) in a CBF-dependent manner, which results in an increase of DELLA proteins that promote cold acclimation (Achard et al. 2008; Richter et al. 2013). The precise mechanism whereby DELLA proteins enhance this stress response remains to be elucidated but it may be related to their capacity to regulate *CBF* expression since the accumulation of DELLA proteins generates an increase in *CBF* transcripts (Richter et al. 2013). This increase requires the function of two GATA transcription factors, GATA NITRATE-INDUCIBLE CARBON-METABOLISM INVOLVED (GNC) and GNC-LIKE (GNL), which repress the transcription of *SUPPRESSOR OF CONSTANS1 (SOC1)* (Richter et al. 2013). This negative regulation has important consequences for *CBF* expression and cold acclimation as it attenuates

freezing tolerance either by antagonizing the ICE1 transcription factor (Lee et al. 2015) or repressing *CBF* transcription through direct binding to *CBF* promoters (Seo et al. 2009). In addition, crosstalk with other hormone signaling pathways may account for the implication of DELLA proteins in the development of cold acclimation. For instance, DELLAs interact with the brassinosteroid mediator BZR1 to repress its transcriptional activity and promote photomorphogenesis (Gallego-Bartolome et al. 2012). Likewise, DELLA proteins modulate JA signaling through their interaction with the JAZs negative regulators in several physiological processes, including defense against pathogens (Hou et al. 2010). DELLA proteins have also been shown to interact with the ET-signaling mediator EIN3, antagonizing its transcriptional activity during apical hook development (An et al. 2012). Finally, DELLA proteins interact with PIFs to repress growth during photomorphogenesis (de Lucas et al. 2008). Since most of these DELLA interactors are involved in the establishment of cold acclimation response through their capacity to directly bind to *CBF* promoters regulating their expression (Lee and Thomashow 2012; Shi et al. 2012; Hu et al. 2013; Jiang et al. 2017; Li et al. 2017), it is tempting to speculate that DELLAs might modulate the function of CBFs in cold response as well.

Beside BRs, JA, ET and GAs, it is likely that *CBF* promoters also respond to signals from additional hormones. In this regard, although the cold induction of the *CBFs* was originally defined as independent of abscisic acid (ABA), new data is challenging this notion. First, *CBF* gene expression is induced by exogenous application of ABA (Knight et al. 2004). Second, the induction of *CBF* genes in response to low temperature is reduced in an ABA-insensitive mutant (Ding et al. 2015). Third, several mediators of ABA signaling, including HEPTAHELICAL PROTEIN1 (HHP1), HHP2, HHP3, and OPEN STOMATA1 (OST1), positively regulate *CBF* gene expression under cold conditions by acting on upstream regulators of the CBFs pathway ICE1, ICE2 and CAMTA3 (Ding et al. 2015; Lee and Seo 2015) (Figure 1.1). However, neither the kinase activity of OST1 nor the cold-

induced *CBF* expression appear to be altered in *Arabidopsis* plants with reduced levels of ABA (Ding et al. 2015; Lee and Seo 2015). It has been argued that, perhaps, some components of the ABA-signaling pathway may have an ABA-independent role during the development of cold response (Ding et al. 2015). Nonetheless, ABA levels have been shown to increase transiently during cold response (Lang et al. 1994) and the cold induction of some *CBF*-target genes is reduced in ABA-deficient mutants (Nordin et al. 1993; Xiong et al. 2001; Cuevas et al. 2008). Thus, further research is necessary to determine the effect of ABA in the elicitation of the *CBFs* pathway.

What the study of *CBF* expression is revealing is an intricate network of regulatory events, where, in addition to cold signaling, hormone and light signaling pathways coalesce at the promoters of *CBFs* to tightly control their expression. The numerous negative regulatory loops in this network further support the assumption that inadequate levels of *CBF* proteins are highly detrimental for plant performance. By means of the complex network described in Figure 1.2, a fine-tuning of *CBF* expression is achieved to increase freezing tolerance minimizing adverse effects for growth and development.

1.5 Additional Layers of Regulation of *CBF* Expression

The preceding section describes the complex transcriptional regulation of *CBFs* mediated by a collection of transcription factors that can bind to the *CBF* promoters. Nonetheless, other elements also play important roles in the transcriptional regulation of *CBF* expression (Figure 1.3). For instance, it has been reported that RNA polymerase II binding to *CBF* promoters is mediated by the epigenetic silencing factor RNA-DIRECTED DNA METHYLATION4 (RDM4) (Chan et al. 2015). The molecular mechanism of this control, however, awaits further study. Moreover, transcriptional regulation of *CBF* genes also seems to be determined by histone post-translational modifications. In this sense, the loss-of-function of FVE, a subunit of the polycomb repressor complex 2

responsible for H3K27 histone trimethylation (H3K27me3), results in increased *CBF* mRNA levels (Kim et al. 2004). Intriguingly, a genome-wide analysis of histone modifications in *Arabidopsis* revealed an enrichment of the repressive H3K27me3 mark in the *CBF* promoters at warm temperatures (Sequeira-Mendes et al. 2014). Additional research is needed to determine if this epigenetic landscape varies in response to low temperature and plays some role in regulating *CBF* transcription.

The expression of *CBFs* is also controlled downstream at post-transcriptional and post-translational levels. Thus, several reports indicate that the coordination of mRNA export to the cytoplasm determines *CBF* expression. The lack of either LOW EXPRESSION OF OSMOTICALLY RESPONSIVE GENES4 (*LOS4*) or NUCLEOPORIN160 (*NUP160*), proteins that are located in the nuclear pore rim, results in aberrant nucleo-cytoplasmic mRNA partition, decreased *CBF* induction by low temperature, and reduced cold acclimation capacity (Gong et al. 2002, 2005; Dong et al. 2006). Furthermore, *CBF* expression might be also modulated through the control of mRNA stability. Although there is no evidence of such a regulation yet, it has been reported that *CBF* transcripts have a remarkably short half-life at warm temperatures (Zarka et al. 2003), which suggests there may be some factor(s) regulating their stability. In fact, recent studies have demonstrated that this is an important layer of gene expression regulation, specifically during cold acclimation (Perea-Resa et al. 2016). Hence, it would be worthwhile to study the stability of *CBF* transcripts.

On the other hand, multiple lines of evidence show that cold acclimation is regulated by post-translational mechanisms (Barrero-Gil and Salinas 2013) and two post-translational modifications affecting *CBF* proteins, namely phosphorylation and ubiquitination, have been reported to date. A high-throughput phosphoproteomic analysis identified *CBF1* and *CBF3* as substrates of MITOGEN-ACTIVATED PROTEIN KINASE4 (*MPK4*) and *MPK6* kinases in plants growing

under control conditions (Popescu et al. 2009). The putative effect of this phosphorylation on CBF1 or CBF3 function, however, remains unknown. In a recent work, Liu and colleagues (Liu et al. 2017) have demonstrated that CBFs are subjected to degradation by the 26S proteasome and that 14-3-3 proteins positively regulate this process (Liu et al. 2017). From these results a working model has been proposed in which low temperature promotes 14-3-3 protein migration to the nucleus where they would interact with CBFs to stimulate their binding to an unknown E3 ubiquitin ligase, targeting them for degradation by the 26S proteasome pathway (Liu et al. 2017).

1.6 The Structure and Mode of Action of CBFs

CBF genes share significant sequence identity (approximately 85%) and are characterized by an APETALA2/ETHYLENE-RESPONSE-FACTOR (AP2/ERF) domain that connects them to the superfamily of AP2/ERF transcription factors, which is composed in Arabidopsis by 145 proteins. The AP2/ERF domain is, approximately, 60 amino acids long and originates a structure consisting of a three-stranded beta sheet parallel to an alpha helix that binds to GC-rich boxes in promoter sequences. Moreover, sequence analysis has identified short fragments flanking the AP2/ERF domain that are also required to bind to the CCGAC motif of the CRT/DRE sequence characteristic of the CBF targets. Other important domains within the CBF sequences include an aminoterminal nuclear localization signal and an acidic domain that is important for their transcriptional activation function (Stockinger et al. 1997). The study of the latter revealed another important feature concerning CBFs function. Since acidic domains can interact with histone acetyltransferases, the ability of Arabidopsis CBF1 to interact with these chromatin modifiers was analyzed, revealing that some subunits of the SAGA histone acetyltransferase complex, including ALTERATION/DEFICIENCY IN ACTIVATION2A (ADA2A), ADA2B and GENERAL CONTROL NONDEREPRESSIBLE5 (GCN5), can interact *in vitro* with this CBF (Stockinger et al. 2001). In

addition, it was subsequently demonstrated that these proteins are required for inducing CBF-regulated gene expression (Vlachonasios et al. 2003). Indeed, histone acetylation status has repeatedly been shown to be important for cold-induced gene expression and the cold acclimation process. Whereas histone deacetylation at the cold-inducible promoters leads to gene repression (Kim et al. 2004; Jeon and Kim 2011), histone acetylation is associated with transcriptional activation (Pavangadkar et al. 2010). Thus, these results suggest that the function of CBFs as transcriptional activators may be mediated through the modification of chromatin structure, though the molecular details of this process need further research.

1.7 The Function of the CBFs in Cold Response

The role of CBFs in cold acclimation is to integrate a series of environmental cues and initiate, if appropriate, a transcriptional response that allows the plant to increase its freezing tolerance. As already mentioned, CBF proteins perform this function through their binding to a C-repeat motif, CCGAC, present in the promoter of many cold-induced genes, activating their transcription. Transcriptional activation, in turn, requires histone acetylation and depletion of nucleosomes (Venkatesh and Workman 2015). CBFs can interact with members of the SAGA complex to increase the acetylation levels of CBF-target genes around the C-repeat DNA binding sites, a post-translational modification that is necessary but not sufficient to activate their gene expression (Pavangadkar et al. 2010). It remains to be elucidated the role of CBF binding in nucleosome occupancy. After chromatin remodeling, RNA polymerase II (RNAPol II) needs to be recruited to activate transcription. Recent studies have characterized a multiprotein complex named mediator that plays a fundamental role in RNAPol II recruitment to promoter sequences and activation of transcription in plants (Bäckström et al. 2007). It has been demonstrated that mediator subunits MED2, MED14 and MED16 are required to recruit RNAPol II to CBF-responsive promoters

(Hemsley et al. 2014). These particular mediator subunits bind to C-repeat containing promoters in response to low temperature and, although still is not known which factors determine this binding, CBFs are strong contenders.

Transcriptomic analysis of Arabidopsis triple *cbf* mutants has shown that loss-of-function of all CBF proteins affects the cold regulation of 447 genes (Zhao et al. 2016; Jia et al. 2016). Of these, 363 genes are upregulated while 84 genes are downregulated (Zhao et al. 2016; Jia et al. 2016). Interestingly, 229 out of the 363 (63%) CBF-upregulated genes contain a CCGAC box in their promoter sequences. The remaining 134 genes either contain an unknown cis-element to which CBF proteins can bind or they represent CBF indirect targets. In contrast, only 21 out of the 84 (25%) CBF-downregulated genes contain the CCGAC cis-element. These data suggest that the primary role of CBF proteins during the cold response is to activate gene expression. The functional classification of the CBF-upregulated genes provides interesting insights into the processes regulated by this cold signaling pathway (Zhao et al. 2016; Jia et al. 2016). Indeed, 34 transcription factors are found in this list, revealing the amplificative nature of the CBFs function in cold acclimation. Furthermore, 32 genes are related to carbohydrate metabolism, 24 to lipid metabolism and 16 to cell wall modification (Zhao et al. 2016; Jia et al. 2016), which illustrates some of the metabolic and biochemical adjustments mediated by CBFs to increase freezing tolerance. In this sense, metabolic profiling of Arabidopsis plants have identified increased levels of sucrose and raffinose during this adaptive response (Kaplan et al. 2007), and several genes encoding enzymes involved in the biosynthesis of these sugars are among the set of CBF-inducible genes (Zhao et al. 2016; Jia et al. 2016). Consistent with these results, the over-expression of *CBFs* results in plants that accumulate these sugars even at warm temperatures (Gilmour et al. 2004). Similarly, it is well established that cold exposure induces proline accumulation in Arabidopsis (Kaplan et al. 2007) and a gene that encodes one of the key enzymes for the synthesis of this

amino acid *DELTA-1-PYRROLINE-5-CARBOXYLATE SYNTHASE2 (P5CS2)* is regulated by the CBF transcription factors (Zhao et al. 2016; Jia et al. 2016). Again, the over-expression of *CBF* genes results in plants that accumulate proline even at warm temperatures (Gilmour et al. 2004). Cold acclimation is also associated with changes in lipid membrane composition (Uemura et al. 1995) to prevent damage induced by freezing temperatures (Ruelland et al. 2009). It is likely that some of the CBF-inducible genes related to lipid metabolism are involved in this process. Another well documented response to cold in plants is the accumulation of hydrophilic proteins (Late-embryogenesis abundant proteins and dehydrins) (Ruelland et al. 2009). The Arabidopsis CBF transcription factors are responsible for the cold induction of some of these proteins, including *COLD INDUCIBLE1 (KIN1)*, *KIN2*, *LATE EMBRYOGENESIS ABUNDANT4-5 (LEA4-5)*, *LOW TEMPERATURE-INDUCED30 (LTI30)*, *COLD REGULATED47 (COR47)* and *EARLY RESPONSIVE TO DEHYDRATION10 (ERD10)* (Zhao et al. 2016; Jia et al. 2016).

As discussed above, *CBF* expression is tightly regulated. *CBF* transcripts accumulate quickly after cold exposure, eliciting a transcriptional cascade that results in increased freezing tolerance. However, the cold induction of *CBFs* is transient and the levels of the corresponding mRNAs and proteins return to resting levels after a few hours into the cold response (Novillo et al. 2007; Liu et al. 2017). It has been proposed that the cold induction of *CBFs* needs to be transient since constitutive high levels of *CBF* transcripts lead to growth retardation, dwarfism and delayed flowering in plants growing at warm temperature (Liu et al. 1998; Gilmour et al. 2004). A molecular mechanism for this phenotype was outlined when it was discovered that constitutive overexpression of *CBF1* led to accumulation of DELLA proteins (Achard et al. 2008), which are well known for restraining plant growth (Harberd et al. 2009). In this context, it was shown that overexpression of *CBF1* increases the expression of two genes, *GIBBERELLIN 2-OXIDASE3 (GA2ox3)* and *GA2ox6*, encoding enzymes that deactivate gibberellins, causing the accumulation of DELLAs

(Achard et al. 2008). From these results, it was proposed that one of the functions of CBFs was to restrain plant growth through negative regulation of gibberellin synthesis, which would contribute to increase survival to freezing temperatures (Achard et al. 2008). Intriguingly, neither *GA2ox3* nor *GA2ox6* are affected in mutant plants lacking CBF function and, more important, these mutants also show retarded growth (Zhao et al. 2016; Jia et al. 2016). These observations suggest that CBFs should also play a role in plant development.

Finally, an important question regarding the function of CBFs is whether or not all of them play the same role in the development of freezing tolerance during cold acclimation. Based on a number of observations, it has been proposed that CBFs function is largely redundant. First, similar sets of genes are upregulated in transgenic lines over-expressing different *CBF* genes (Gilmour et al. 2004) as well as in single CRISPR *cbf* mutants (Shi et al. 2017). Second, transgenic lines over-expressing different *CBF* genes show a similar profile of metabolites with high accumulation of proline and sugars (Gilmour et al. 2004). Third, the double *cbf1cbf3* mutant but not the single mutants *cbf1* and *cbf3* show an altered capacity for cold acclimation (Zhao et al. 2016; Jia et al. 2016). Fourth, many of the *CBF* regulators described so far can bind to any of the *CBF* promoters, indistinctively in EMSA or CHIP assays, which suggest a similar regulation for the *CBF* genes (Table 1). Other findings, however, suggest that there may be some divergence in the function of CBFs. Thus, (i) some experiments have revealed subtle differences in expression patterns between *CBF1* and *CBF3* on one side and *CBF2* on the other side, with the latter showing a slower cold-upregulation and wider tissue expression pattern (Novillo et al. 2007); (ii) while CRISPR *cbf1* and *cbf3* single mutants display WT-like capacity to cold acclimate, the capacity of the CRISPR *cbf2* single mutant is significantly reduced compared to WT plants (Zhao et al. 2016); (iii) as already mentioned, *CBF1* and *CBF3*, but not *CBF2*, are targets of MAP-kinases and, furthermore, are subjected to 14-3-3 protein regulation (Popescu et al. 2009; Liu et al. 2017); (iv) a survey of natural

variation for freezing tolerance in 477 European populations of *Arabidopsis* found frameshifts and premature stop codon mutations associated with adaptation to warmer climates in the sequences of *CBF2* and *CBF3* genes, but remarkably not in that of *CBF1* (Monroe et al. 2016). Indeed, some authors have suggested that *CBF2* may have a different function than *CBF1* and *CBF3* (Novillo et al. 2004; Zhao et al. 2016).

Despite the intense research carried out during the last decades, the particular function of each CBF and the interactions among them are not clear yet because there are conflicting results regarding the phenotype of mutant lines. While a T-DNA inserted in the promoter of *CBF2* causes upregulation of *CBF1* and *CBF3* genes, and an enhancement of cold acclimation capacity (Novillo et al. 2004), frameshift mutations created using CRISPR technology in the *CBF2* sequence cause a reduction of cold acclimation capacity without significant impact on the expression of *CBF1* or *CBF3* (Zhao et al. 2016). A similar situation has been observed when analyzing the effect of *cbf1cbf3* double mutations. It has been reported that a large deletion encompassing *CBF1* and *CBF3* loci leads to an increase of *CBF2* expression in response to low temperature and a concomitant increase in freezing tolerance (Zhao et al. 2016). In contrast, a frameshift mutation of *CBF1* combined with a T-DNA insertion in the *CBF3* coding sequence did not increase *CBF2* expression after cold exposure and resulted in a reduced ability to cold acclimate (Jia et al. 2016). These conflicting results reveal that the molecular nature of *CBF* mutant alleles has a definitive influence on the regulation of *CBF* genes. Additional studies are needed to elucidate the complex relationships between the *CBF* genes.

1.8 The Role of CBFs in Plant Response to Cold-Related Abiotic Stresses

Heretofore we have discussed the role of CBF proteins in the context of cold acclimation. Indeed, since their original identification, the *CBF* genes were characterized as specifically induced by cold

but not by other cold-related abiotic stresses like drought, salt or high osmotic conditions (Gilmour et al. 1998; Liu et al. 1998; Medina et al. 1999). However, early studies also demonstrated that overexpression of *CBFs* increases tolerance to salt and drought stresses in *Arabidopsis* (Liu et al. 1998; Kasuga et al. 1999), which suggested that *CBFs* might also have a role in other abiotic stress responses apart from cold acclimation. Additional experiments provided support to this idea by demonstrating that ABA induces *CBF* expression in certain conditions (Knight et al. 2004) since it is well known that ABA increases in response to different abiotic stresses in addition to low temperature, including drought, salt and high osmotic conditions (Walton 1980; Lang et al. 1994; Perea-Resa et al. 2016). Genetic evidence on the implication of *CBFs* in salt and drought tolerance was supplied by the phenotypic analysis of the effects of a T-DNA insertion in the *CBF2* promoter which revealed that, besides increasing *CBF1* and *CBF3* expression as well as freezing tolerance, this mutation also boosted salt and dehydration tolerance (Novillo et al. 2004). Lastly, recent reports have shown that loss-of-function of all three *CBFs* in *Arabidopsis* results in decreased salt tolerance (Zhao et al. 2016). These results firmly establish that *CBFs* play a role in other abiotic stress responses beside cold acclimation, which, after all, is not surprising considering that plant responses to cold, salt and water stresses are quite related (Chinnusamy et al. 2004) and several works have found a significant crosstalk among these responses (Nakashima et al. 2014). Nonetheless, the precise contribution of *CBFs* to these stress responses as well as the specific mechanisms controlled by these transcription factors remain to be determined.

1.9 The Role of *CBFs* in Plants That Do Not Cold Acclimate

We have mentioned earlier that *CBF* genes have been identified in a wide range of plants, including some species that are freezing-sensitive and cannot develop a cold acclimation response, such as rice (Xue 2003), tomato (Zhang et al. 2004) and maize (Qin et al. 2004). These findings

raise the question of what could be the function of CBFs in these organisms. An interesting hypothesis already discussed is that CBFs may have additional roles in plant development (Zhao et al. 2016). Thus, it might be possible that the major role of CBFs in freezing-sensitive species is restricted to their involvement in development. Alternatively, the function of CBFs in freezing-sensitive species might be connected with a role in salt or drought responses.

It is worth noting, however, that a limited but significant capacity to increase constitutive tolerance to chilling temperatures after exposure to suboptimal growth temperatures has been reported in freezing-sensitive species such as maize (Nie et al. 1992), rice (Kuk et al. 2003) and tomato (Barrero-Gil et al. 2016). This response, which has been termed chilling acclimation (Anderson et al. 1994), has been the subject of a recent study in tomato which shows that it shares many characteristics with cold acclimation, including a large transcriptomic rearrangement (Barrero-Gil et al. 2016). Therefore, it is tempting to speculate that CBF proteins in freezing-sensitive species may be responsible for the elicitation of this transcriptomic response in a similar way as they regulate cold acclimation in freezing-tolerant species. Indeed, tomato *CBF* expression is induced by suboptimal growth temperatures (Barrero-Gil et al. 2016). Furthermore, ectopic expression of tomato *SlCBF1* increases freezing tolerance in transgenic *Arabidopsis* (Zhang et al. 2004) and over-expression of *Arabidopsis AtCBF1* increases constitutive chilling tolerance in tomato (Hsieh et al. 2002) and rice (Oh et al. 2005). These findings indicate that tomato and *Arabidopsis* CBF1 homologs have some common functional characteristics. A recent study has shown that *SlCBF1* is a positive regulator of tomato constitutive chilling tolerance (Wang et al. 2015). Thus, it is reasonable to expect that CBFs may have a role in the development of chilling acclimation in tomato and possibly in other freezing-sensitive species.

1.10 Conclusions and Perspectives

The past two decades have witnessed intense research focused on unveiling the molecular mechanism underpinning the cold acclimation response. These efforts have led to the discovery and description of a signaling pathway centered on the function of a small family of transcription factors called CBFs. Natural variation and genetic studies show that these transcription factors are indeed critical for full development of that response. The regulation of *CBF* expression is extremely complex and tightly regulated, positively or negatively, by multiple intermediates and mechanisms operating at many different levels. Our current state of knowledge establishes that CBFs regulate one of the most important signaling pathways controlling cold acclimation.

In spite of all these findings there is a great deal of unknown aspects concerning CBF function. For instance, the regulation of *CBF* expression integrates multiple signaling pathways from internal and external cues but, how this integration is carried out and how it relates to the output of this signaling pathway? Which is the precise role of CBF proteins in plant growth and development? Which are the mechanisms controlled by CBF proteins in abiotic stress responses different from cold acclimation? Which are the specific contributions of each CBF protein to plant development and abiotic stress responses? These questions are of utmost relevance not only for a basic understanding of plant biology but also from an applied perspective. Although more studies are necessary to fully harness CBF biotechnological potential, several field studies have already provided promising data showing that inducible overexpression of *CBF* genes can be a successful strategy to increase crop tolerance to freezing temperatures (Artlip et al. 2014) and drought (Xiao et al. 2009; Datta et al. 2012; Bhatnagar-Mathur et al. 2014). Successful transfer of knowledge from the lab to the field is proving to be slower than what was estimated twenty years ago (Thomashow 2010; Varshney et al. 2011). One of the reasons for this delay is the complexity of abiotic stress responses for which, no doubt, the CBF signaling pathway can serve as a paradigm (Thomashow 2010). However, the degree of knowledge achieved in the past decade combined

with new DNA editing techniques should soon allow for a rational design of crops with enhanced tolerance to abiotic stress. In this scenario, we believe that CBF research is going to play a very important role.

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Figure 1 (117 x 78,9 mm)

Figure 2 (117 x 157,6 mm)

Figure 3 (117 x 126,6 mm)

Table 1: Transcription factors that directly regulate CBF expression. Different cis-elements present in the promoter sequences of CBF genes enable the binding of a collection of transcription factors with positive and negative effects on their transcription. A plus sign indicates the existence of experimental evidence for direct interaction of a transcription factor to a CBF promoter, whereas a negative sign indicates a failure to find support for such binding. CHIP (chromatin immunoprecipitation). EMSA (Electrophoretic mobility shift assay). The effect of the binding of each transcription factor on CBF expression is indicated in the output column

Transcription factor	Signaling pathway	Promoter binding			Techniques	cis -element	Output	References
		CBF1	CBF2	CBF3				
ICE1	cold	+	+	+	CHIP, EMSA	E-box	Positive regulation of CBF1 , CBF2 , CBF3 expression	Kim et al. (2015)
ICE2	cold	+	+	+	CHIP	E-box	Positive regulation of CBF1 , CBF2 , CBF3 expression	Kim et al. (2015)
MYB15	cold	+	+	+	EMSA	MYB	Negative regulation of CBF1 , CBF2 , CBF3 expression	Agarwal et al. (2006)
CAMTA3	cold	+	+	n.d.	EMSA	CGCG box	Positive regulation of CBF1 , CBF2 expression	Doherty et al. (2009); Kidokoro et al. (2017)
CAMTA5	cold	+	+	n.d.	EMSA	CGCG box	Positive regulation of CBF1 , CBF2 expression	Doherty et al. (2009); Kidokoro et al. (2017)
BZR1	BR	+	+	n.d.	CHIP, EMSA	E-box & BRRE	Positive regulation of CBF1 , CBF2 , CBF3 expression	Li et al. (2017)
CES	BR	+	+	+	CHIP, EMSA	G-box & E-box	Positive regulation of CBF1 , CBF2 , CBF3 expression	Eremina et al. (2016)
EIN3	ET	+	+	+	CHIP, EMSA	n.d.	Negative regulation of CBF1 , CBF2 , CBF3 expression	Shi et al. (2012)
PIF3	ET, light	+	+	+	CHIP, EMSA	G-box & E-box	Negative regulation of CBF1 , CBF2 , CBF3 expression	Jiang et al. (2017)
PIF4	light	+	+	+	CHIP, EMSA	G-box & E-box	Negative regulation of CBF1 , CBF2 , CBF3 expression	Lee and Thomashow (2012)
PIF7	light	+	+	+	CHIP, EMSA	G-box & E-box	Negative regulation of CBF1 , CBF2 , CBF3 expression	Kidokoro et al. (2009) ; Lee and Thomashow (2012)
CCA1	circadian	+	+	+	CHIP	EE & CBS	Positive regulation of CBF1 , CBF2 , CBF3 expression	Dong et al. (2011)
TOC1	circadian	-	-	+	CHIP	T1ME, G-box & EE	Negative regulation of CBF1 , CBF2 , CBF3 expression	Keily et al. (2013)
PRR5	circadian	+	+	+	CHIP-seq	T1ME & G-box	Negative regulation of CBF1 , CBF2 , CBF3 expression	Nakamichi et al. (2009); Nakamichi et al. (2012)
PRR7	circadian	+	+	n.d.	CHIP	T1ME & G-box	Negative regulation of CBF1 , CBF2 , CBF3 expression	Nakamichi et al. (2009); Nakamichi et al. (2012)
PRR9	circadian	+	+	n.d.	CHIP	T1ME & G-box	Negative regulation of CBF1 , CBF2 , CBF3 expression	Nakamichi et al. (2009); Nakamichi et al. (2012)
SOC1	Flowering	+	+	+	CHIP	CArG	Negative regulation of CBF1 , CBF2 , CBF3 expression	Seo et al. (2009)