Insights into the regulation of C-repeat binding factors in plant cold signaling

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Abstract Cold temperatures, a major abiotic stress, threaten the growth and development of plants, worldwide. To cope with this adverse environmental cue, plants from temperate climates have evolved an array of sophisticated mechanisms to acclimate to cold periods, increasing their ability to tolerate freezing stress. Over the last decade, significant progress has been made in determining the molecular mechanisms underpinning cold acclimation, including following the identification of several pivotal components, including candidates for cold sensors, protein kinases, and transcription factors. With these developments, we have a better understanding of the CBF-dependent cold-signaling pathway. In this review, we summarize recent progress made in elucidating the cold-signaling pathways, especially the C-repeat binding factor-dependent pathway, and describe the regulatory function of the crucial components of plant cold signaling. We also discuss the unsolved questions that should be the focus of future work.

INTRODUCTION

Cold stress, including chilling (cold temperatures of above 0°C) and freezing (below 0°C) stress, restricts the growth, development, and geographic distribution of plants. Chilling stress induces membrane rigidification, reactive oxygen species (ROS) accumulation, protein (or protein complex) destabilization, and metabolic disequilibrium (Chinnusamy et al. 2007). Freezing stress results in the formation of ice crystals within apoplast, causing dehydration and the deterioration of the cell membrane and cell wall (Mahajan and Tuteja 2005). Major crop species that originated from tropical and subtropical regions, such as maize (Zea mays), rice (Oryza sativa), and potato (Solanum tuberosum), are chilling sensitive, whereas temperate crops, including common wheat (Triticum aestivum), rye (Secale cereale) and barley (Hordeum vulgare), have evolved various mechanisms to adapt to low temperatures and can, therefore, survive freezing temperatures (Chinnusamy et al. 2007; Dhillon et al. 2010).

During cold acclimation, plants exposed to chilling temperatures, over a period of several days or weeks, can become more tolerant to freezing temperatures (Thomashow 1999), a process which involves many physiological and metabolic changes (Chinnusamy et al. 2007). A set of COR (Cold-regulated) genes, also known as LTI (Low temperature-induced), KIN (Cold-induced), RAB (Responsive to abscisic acid), or ERD (Early responsive to dehydration), are induced by cold stress, and their proteins act to stabilize cell membrane structures, activate the ROS scavenging system, and produce cryoprotective proteins and metabolites (osmolytes), resulting in an increase of plant tolerance.

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The accumulation of COR proteins and osmolytes, such as sucrose, glycine, betaine, and proline, helps to maintain the integrity of membranes and cellular organelles in plant cells during cold stress (Gilmour et al. 1998; Cook et al. 2004; Guy et al. 2008). Recent studies have shown that LEA (Late embryogenesis abundant) proteins protect enzyme activity to provide freezing tolerance of common wheat and Arabidopsis thaliana (Sasaki et al. 2014; Popova et al. 2015). Using yeast one-hybrid assays, three transcription factors, the CBFs (C-repeat binding factors; also known as dehydration-responsive element binding factor 1 (DREB1)), were isolated as transcriptional activators of a subset of COR genes (Stockinger et al. 1997; Gilmour et al. 1998; Liu et al. 1998), and emerging evidence has demonstrated that the CBFs play key roles during cold acclimation (Chinnusamy et al. 2007). This review summarizes the functions of the CBFs in cold acclimation, and our current understanding of the CBF-mediated cold-signaling pathway, at both the transcriptional and post-translational levels.

FUNCTIONS OF THE CBF FAMILY IN COLD ACCLIMATION

In Arabidopsis, the CBF family includes three members, CBF1/DREB1b, CBF2/DREB1c, and CBF3/DREB1a, which are paralogs arranged in tandem on chromosome IV (Gilmour et al. 1998; Medina et al. 1999). The CBFs are a sub-family of the AP2/ERF (APETALA 2/ethylene-responsive) multi-protein family, which comprises approximately 145 transcription factors with one or more AP2/ERF DNA-binding domains (Ohme-Takagi and Shinshi 1995; Okamura et al. 1997; Riechmann and Meyerowitz 1998). The CBF transcription factors bind to the conserved CRT/DRE (C-repeat/dehydration response element) motif CCGAC (Baker et al. 1994; Stockinger et al. 1997), which is present in some COR gene promoters (Medina et al. 2011).

CBF proteins harbor only one AP2 domain and two conserved signature motifs: PKKP/PKKPAGR (RAGRxxKFxETRHP) and DSAWR. Mutations in the PKKPAGR motif, located upstream of the AP2 domain, compromise the ability of CBF1 to bind with its target gene, COR15a, thereby decreasing COR15a expression under cold treatment (Canella et al. 2010), which suggests that the PKKPAGR motif is important for the biological function of CBF proteins. Expression of the CBF genes is rapidly induced, within 15 min, when plants are exposed to low temperatures, reaching a maximum level within 3 h (Gilmour et al. 1998; Novillo et al. 2004; Medina et al. 2011). Furthermore, genetic analyses revealed that overexpressing the CBF genes promotes induction of their target genes during cold treatment, enhancing the freezing tolerance of these plants (Stockinger et al. 1997; Gilmour et al. 1998; Jaglo-Ottosen et al. 1998; Liu et al. 1998; Kasuga et al. 1999; Gilmour et al. 2004).

Microarray analyses of CBF-overexpressing plants suggested that over 130 COR genes are significantly induced in warm temperatures (Fowler and Thomashow 2002). Transgenic plants with reduced CBF1 and/or CBF3 transcripts by RNA interference and antisense approaches showed significantly decreased freezing tolerances compared with the wild type after cold acclimation (Novillo et al. 2007). Conversely, the cbf2 T-DNA insertion null mutant displayed enhanced freezing tolerance, with higher levels of CBF1 and CBF3 expression (Novillo et al. 2004), suggesting that a negative feedback loop may exist among the CBF genes.

As no CRT/DRE binding sites are present in the CBF1/ CBF3 promoters, the regulation of CBF1 and CBF3 by CBF2 may be indirect (Novillo et al. 2004; Novillo et al. 2007; Medina et al. 2011). Novillo et al. (2007) also showed that expression of the CBF genes was tissue-specific during Arabidopsis development; CBF1 and CBF3 were identically expressed, mainly in the roots, hypocotyls, and cotyledons, whereas CBF2 was mainly expressed in the hypocotyls, cotyledons, and the first and second pairs of leaves (Novillo et al. 2007). After cold treatment, the shared expression pattern of CBF1 and CBF3 still differed from CBF2; CBF1 and CBF3 were mainly expressed in the leaves, sepals, and siliques, but CBF2 was expressed in these tissues as well as in the stems (Novillo et al. 2007). Furthermore, the transcriptional patterns of the CBFs also differ: CBF1 and CBF3 expression reaches a maximum level after 1 h of cold treatment, while the peak of CBF2 expression was delayed until after 2 h (Novillo et al. 2004; Novillo et al. 2007; Medina et al. 2011).

As described above, researchers have made significant progress in dissecting the biological functions of the CBFs, using overexpression, RNAi, and antisense approaches; however, only limited numbers of loss-of-function cbf
mutants are available. Using CRISPR/Cas9 technology, two groups recently succeeded in generating single and multiple mutants of the CBF genes (Jia et al. 2016; Zhao et al. 2016). The triple mutants showed little to no freezing sensitivity in non-acclimated conditions, but were extremely sensitive to freezing stress after cold acclimation (Jia et al. 2016; Zhao et al. 2016), supporting the key role of the CBFs in cold acclimation. RNA-seq analyses of the triple mutants and the wild type under normal and low-temperature conditions led to the discovery that about 10%–20% of COR genes are CBF-dependent.

Intriguingly, the cbf2 mutants generated by CRISPR/Cas9 are more sensitive to freezing than the wild type, in contrast to the phenotype of the cbf2 T-DNA insertion mutant (Novillo et al. 2004; Zhao et al. 2016). The cbf1 cbf3 double mutants also showed contradictory phenotypes; Jia et al. (2016) reported that cbf1 cbf3 was more freezing-sensitive than the wild type but less so than the cbf triple mutants, whereas Zhao et al. (2016) reported that the cbf1 cbf3 mutant had a significantly enhanced freezing tolerance, with two-fold upregulation of CBF2 expression.

These conflicting phenotypes may be due to different genetic materials and different CRISPR/Cas9 targets being chosen; Zhao et al. used the Col-0 background to generate their cbf1 cbf3 CRISPR/Cas9 mutant, in which the whole coding region of CBF1 and the partial coding region of CBF3 were deleted, whereas Jia et al. used CRISPR/Cas9 to mutate CBF1 in an existing cbf3 T-DNA insertion mutant. Another possibility is that the truncated CBF1 protein generated by Jia et al., which lacks the AP2 domain, may interrupt the function of the other CBF proteins.

Although the phenotypes of the cbf single and double mutants are complex, the cbf triple mutant phenotypes are clear and consistent, verifying the positive role of the CBF proteins in cold acclimation. The precise function of each CBF protein in regulating COR genes expression has been reported recently (Shi et al. 2017), and their interconnection should be further explored.

The function of the CBFs in regulating cold acclimation is highly conserved among various plant species, such as canola (Brassica napus), rice, maize, and poplar (Populus balsamifera) (Qin et al. 2004; Savitch et al. 2005; Benedict et al. 2006; Ito et al. 2006). Moreover, heterologous expression of the Arabidopsis CBFs can enhance freezing tolerance in other plant species (Jaglo et al. 2001; Hsieh et al. 2002; Kasuga et al. 2004). These studies reveal the conserved function of the CBFs in regulating plant freezing tolerance.

The various natural populations of Arabidopsis have different freezing tolerances (Hannah et al. 2006; Zuther et al. 2012), which were shown to arise from the natural variation in the promoters and coding regions of the CBFs (Kang et al. 2013; Gehan et al. 2015). For instance, a single base mutation in the trans-activation domain of CBF2 results in a dysfunctional CBF2 protein in ecotypes from warm habitats, and several fragment insertions in the CBF3 promoter results in decreased CBF3 expression in these genotypes (Kang et al. 2013). A short fragment insertion in the C-terminus of CBF2 inhibits its transcriptional activity, resulting in a lower expression of most CBF-regulon genes in the relatively freezing-sensitive ecotypes (Gehan et al. 2015). These studies suggest that natural variation in the CBF locus plays an important role in the adaptive evolution of Arabidopsis.

CBF-INDEPENDENT PATHWAYS IN COLD ACCLIMATION

Although the CBFs play important roles in cold acclimation, less than 20% of COR genes are regulated by the CBFs, suggesting the involvement of CBF-independent transcription factors in the regulation of their expression (Park et al. 2015; Shi et al. 2015; Jia et al. 2016; Zhao et al. 2016). A microarray analysis led to the identification of five transcription factor genes, HSFC1 (Heat shock transcription factor c1), ZAT12 (Zing finger of Arabidopsis thaliana 12), ZF (Zinc finger), ZAT10, and SZF2 (Salt-inducible zinc finger 2), that are co-expressed in parallel with CBF2, and which positively regulate COR gene expression and freezing tolerance (Park et al. 2015).

In Arabidopsis, ESK1 (Eskimo1) encodes a DUF231 domain protein with unknown functions (Xin et al. 2007). The mutation of ESK1 led to constitutively freezing tolerance in Arabidopsis, with altered expression of genes that are independent of the CBF pathway (Xin and Browse 1998; Xin et al. 2007). HOS9 is a homeodomain transcription factor, and the hos9 mutants showed decreased freezing tolerance without affecting the expression of CBFs and downstream COR genes (Zhu et al. 2004). Low temperature induces the expression of Gigantea, which is involved in flowering and circadian clock progress (Fowler et al. 1999; Cao et
The mutant of gi-3 showed reduced freezing tolerance and impaired cold acclimation ability, while the expression of CBFs and their target genes in gi-3 mutant are not affected (Cao et al. 2005).

A key regulator of photomorphogenesis, HY5 (Elongated hypocotyl5), was reported to positively regulate COR gene expression and freezing tolerance in a CBF-independent manner (Catala et al. 2011); however, more recently, HY5 was shown to bind to the promoters of CBF1, CBF2, CBF3, and COR15a and to regulate the expression of CBF3 during the day (Noren et al. 2016). Whether HY5 plays a role in CBF-dependent pathway remains unclear. These data suggest that CBF-independent transcription factors modulate the expression of the COR genes during cold acclimation. It would be interesting to identify novel transcription factors that regulate the CBF-independent COR genes during cold acclimation.

TRANSCRIPTIONAL REGULATION OF THE CBFs IN THE COLD-SIGNALING PATHWAY

ICE1 acts as a positive regulator of the CBFs

To date, several transcriptional regulators of the CBFs have been identified, including ICE1/2 (Inducer of CBF expression), MYB15, CAMTA3 (Calmodulin-binding transcription activator 3), EIN3 (Ethylene insensitive 3), SOC1 (Suppressor of overexpression of co 1), PIF3/4/7 (Phytochrome-interacting factors), CCA1 (Circadian clock associated 1), LHY (Late elongated hypocotyl), BZR1 (Brassinazole-resistant 1)/BES1 (BRI-EMS suppressor 1), and CESTA (Chinnusamy et al. 2003; Agarwal et al. 2006; Doherty et al. 2009; Fursova et al. 2009; Seo et al. 2009; Dong et al. 2011; Lee and Thomashow 2012; Shi et al. 2012; Eremina et al. 2016; Jiang et al. 2017; Li et al. 2017b) (Figure 1).

ICE1, a basic helix-loop-helix (bHLH) transcription factor, was the first CBF transcriptional activator to be identified. The ice1 mutant was isolated in a screen of EMS-mutagenized pCBF3:LUC plants, in which cold-induced CBF expression was blocked (Chinnusamy et al. 2003). The loss of ICE1 function in Arabidopsis reduces plant tolerance to chilling and freezing stress, both with or without cold acclimation (Chinnusamy et al. 2003; Ding et al. 2015). ICE1 binds to the MYC binding sites (CANNTG) of the CBF1-3 promoters and activates their expression under cold treatment (Chinnusamy et al. 2003; Ding et al. 2015).

The function of ICE1 is conserved in many plant species, and the heterogeneous expression of the rice, maize, or tomato ICE1 orthologs in Arabidopsis enhanced plant freezing tolerance (Nosenko et al. 2016; Deng et al. 2017; Lu et al. 2017). ICE2, a paralog of ICE1, is also involved in regulating CBF expression and freezing tolerance (Fursova et al. 2009). Considering that ICE1 and ICE2 are also key regulators of stomatal development (Kanaoka et al. 2008), it is likely that stomata play an important role in the plant response to cold stress.

Post-translational modification of ICE1

ICE1 expression is not responsive to cold treatment (Chinnusamy et al. 2003); therefore, post-transcriptional regulation is likely to be important for its function. The ICE1 protein is regulated by HOS1 (High expression of osmotically responsive gene 1) (Dong et al. 2006), SIZ1 (SAP and Miz 1) (Miura et al. 2007), OST1 (Open stomata 1)/SnRK2.6 (Ding et al. 2015), and MPK3/6 (Zhao et al. 2016; Li et al. 2017a) (Figure 1).

HOS1 is an ubiquitin E3 ligase with a RING finger domain that directly interacts with ICE1 and mediates its degradation both in vitro and in vivo (Dong et al. 2006). Mutations in HOS1 were shown to impair the cold-induced degradation of ICE1, whereas overexpression of HOS1 promoted the degradation of ICE1 under both normal and low temperatures (Dong et al. 2006). SIZ1, a SUMO E3 ligase, is required for the sumoylation of ICE1, which increases its stability under cold stress, resulting in increased CBF expression and enhanced freezing tolerance (Miura et al. 2007).

Recently, cold-activated OST1, a pivotal protein kinase in the ABA pathway (Mustilli et al. 2002), was reported to interact with and phosphorylate ICE1 at Ser278 under cold stress, enhancing its stability and transcriptional activity (Ding et al. 2015). Furthermore, the interaction of OST1 and ICE1 compromised the HOS1-ICE1 interaction, thereby preventing the HOS1-mediated degradation of ICE1.

Consistently, ost1 mutants are sensitive to freezing, whereas OST1-overexpressing plants are freezing tolerant (Ding et al. 2015). Interestingly, cold-induced OST1 activity is independent of ABA, but dependent on the PP2C-type phosphatase ABI1 (Abscisic acid insensitive 1) (Yoshida et al. 2006; Ding et al. 2015). These findings not only reveal the important role of OST1 in the cold-signaling pathway, but also suggest a crosstalk between...
the cold- and ABA-signaling pathways (Dong et al. 2006; Miura et al. 2007; Ding et al. 2015).

Recently, two companion papers reported that MPK3 and MPK6 mediated ICE1 phosphorylation and inactivation in response to cold stress (Zhao et al. 2016; Li et al. 2017a). This interaction promotes the ICE1 stability by inhibiting its HOS1-mediated degradation. As a SUMO E3 ligase, SIZ1 mediates sumoylation of ICE1 and suppresses its HOS1-mediated degradation. Meanwhile, MPK3 and MPK6 phosphorylate ICE1, at Ser94/203/403 and Thr366/382/384, and promote the ICE1 protein degradation, via unknown E3-mediated 26S proteasome pathway. Thus, OST1 and MPK3/6 fine tune the function of ICE1, thereby antagonistically regulating the cold-induced CBF gene expression and plant freezing tolerance. CBF transcription factors, including CBF1, CBF2 and CBF3, are directly regulated by some transcription factors. The positive regulators of CBFs are in green, including ICE1/2, CAMTA3, BZR1/BES1, CESTA, CCA1, and LHY proteins. The negative regulators of CBFs are in red, including MYB15, PIF3/4/7, EIN3, and SOC1. Arrows and T bars indicate activation and inhibition, respectively. Lines and dotted lines indicate direct and indirect regulation, respectively.

Genetic evidence suggests that MPK3 and MPK6 act upstream of ICE1 to regulate the CBF-dependent cold-signaling pathway (Li et al. 2017a). Six (Ser94/203/403 and Thr366/382/384) and three (Ser94/403 and Thr366) MAPK phosphorylation sites were identified in the ICE1 protein in the Li et al. (2017a) and Zhao et al. (2016) studies, respectively. Using phosphosite mutagenesis analyses, MPK3/MPK6-mediated ICE1 phosphorylation was shown to promote ICE1 degradation in cold conditions, thus attenuating ICE1-induced CBF expression and freezing tolerance (Li et al. 2017a).

MPK4 activity was also induced by cold treatments, consistent with previous studies (Teige et al. 2004; Furuya et al. 2013), and positively regulated freezing
tolerance by constitutively suppressing the activities of MPK3 and MPK6 (Zhao et al. 2017). CRLK1 (Calcium/calmodulin-regulated receptor-like kinase 1) and CRLK2 interacted with MEKK1, a kinase upstream of MPK4, and inhibited the activities of MPK3 and MPK6 (Zhao et al. 2017).

These studies suggest that the CRLK1/2-MEKK1-MKK1/2-MPK4 cascade antagonizes the MPK3/MPK6 pathway to enhance the plant response to cold stress. Most recently, rice OsMPK3 was shown to phosphorylate OsbHLH002/OsICE1, thereby preventing the degradation of OsICE1 by OsHOS1 and enhancing its transcriptional activity during chilling stress. Thus, OsMPK3 plays a positive role in OsICE1-mediated cold-signaling in rice (Zhang et al. 2017). These findings suggest that ICE1, as a master regulator of cold signaling, is precisely and tightly regulated by multiple post-translational modifications, and the same modification of ICE1 in different plant species may have different effects.

Other positive transcriptional regulators of the CBFs
In addition to ICE1, the calmodulin-binding transcription activators (CAMTAs) have been identified as CBF transcriptional activators. CAMTA proteins are conserved in plants, and there are six members in Arabidopsis (Finkler et al. 2007). An analysis of the CBF2 promoter sequence revealed seven conserved motif (CM) binding sites that function in CBF2 transcriptional regulation. Using an electromobility shift assay, CAMTA1, CAMTA2, CAMTA3, and CAMTA5 were shown to bind to the CM2 motif to promote CBF1, CBF2, and ZAT12 expression under low temperatures (Doherty et al. 2009).

The camta3 mutant had about a 50% reduction in CBF1 and CBF2 transcript following cold treatment, and further studies showed that CAMTA1 and CAMTA2, the two most closely related proteins to CAMTA3, also function in regulating CBF expression (Doherty et al. 2009; Kim et al. 2013). The double mutants camta1 camta2, camta1 camta3, and camta2 camta3 showed growth defects and decreased freezing tolerance (Kim et al. 2013). The CBF1, CBF2 and CBF3 genes were downregulated in these double mutants, but the reduction of CBF3 expression was not as great as for CBF1 and CBF2, suggesting that the CAMTAs play major roles in regulating CBF1 and CBF2.

Recent studies showed that CAMTA3 and CAMTA5 function in activating CBF expression during rapid temperature decreases, but not during the response to a gradual decrease in temperature (Kidokoro et al. 2017); thus, the CAMTA proteins function redundantly in CBF-dependent cold signaling to control the freezing tolerance of plants. Cold stress induces a rapid increase in cytoplasmic calcium levels, which is required for the proper induction of some COR genes (Knight et al. 1996). It is possible that calmodulins bind to this increased calcium, which would affect the interaction of calmodulins with CAMTA3 and consequently regulate its activity and resulting CBF expression (Doherty et al. 2009).

The components of BR (brassinostroid) signaling are reported to positively regulate the cold-induced transcription of the CBFs (Eremina et al. 2016; Li et al. 2017b). BZR1, BES1, and CES (CESTA) are the key positive regulators of the BR response (He et al. 2005; Yin et al. 2005), and recent studies revealed that these transcription factors directly bind to the E-box and BRRE binding sites of the CBF1 and CBF2 promoters, positively regulating their expression (Eremina et al. 2016; Li et al. 2017b).

The gain-of-function BZR1, BES1 and CES mutants in Arabidopsis had significantly enhanced freezing tolerances, whereas the ces-2 bee1 bee2 bee3 quadruple mutant was hypersensitive to freezing (Eremina et al. 2016). Biochemical evidence indicated that cold treatment induced the accumulation of non-phosphorylated BZR1 protein in plants (Li et al. 2017b). Consistently, the loss-of-function mutant of the GSK3-like kinases responsible for phosphorylating BZR1, bin2 bil1 bil2, showed increased freezing tolerance (Li et al. 2017b).

RNA-seq analysis revealed that BZR1 and CES also regulate CBF-independent gene expression to regulate plant freezing tolerance (Eremina et al. 2016; Li et al. 2017b). These results indicate that some components in BR signaling are involved in regulating cold stress response in both CBF-dependent and CBF-independent manners.

Negative transcriptional regulators of the CBFs
Cold-induced CBF expression decreases after prolonged cold treatment (about 3 h), suggesting that transcriptional repressor(s) may be involved in reducing CBF expression over time. MYB15, an R2R3 type MYB transcription factor, directly binds to the MYB-binding site in the CBF promoters, inhibiting the cold-induced expression of each CBF (Agarwal et al. 2006). Over-expression of MYB15 caused decreased freezing
tolerance, whereas a deficiency of MYB15 activity promoted freezing tolerance through increased CBF and COR expression, suggesting that MYB15 negatively regulates CBF-COR gene expression and plant freezing tolerance (Agarwal et al. 2006). Further study showed that ICE1 interacts with MYB15 to attenuate the expression of MYB15 (Agarwal et al. 2006). ICE1 was also found to act upstream of MYB15 to regulate the expression of PHO1/H3 and the accumulation of inorganic phosphate under Zn deficiency (Pal et al. 2017). These studies indicate that ICE1 may function upstream of MYB15 to regulate CBF-COR gene expression.

A recent study showed that MPK6 phosphorylated MYB15, which in turn compromised its binding to the CBF3 promoter (Kim et al. 2017). Considering that mpk6 mutants are freezing tolerant (Li et al. 2017a; Zhao et al. 2017), there might be some unknown mechanisms by which MPK6-MYB15 regulates plant freezing tolerance, requiring further study.

Jasmonate ZIM-domain (JAZ) proteins function as repressors of the jasmonate (JA) signaling pathway, and interact with transcription factors to inhibit their transcription activity (Wasternack and Song 2017). Currently, JAZ proteins have been shown to interact with bHLH- and MYB-type transcription factors to repress their activity during JA signaling (Boter et al. 2004; Fernandez-Calvo et al. 2011; Qi et al. 2011; Song et al. 2011).

JAZ1 and JAZ4 interact with ICE1 to repress CBF-COR gene expression, thereby negatively regulating freezing tolerance in plants (Hu et al. 2013). Cold treatment promotes JA accumulation, and the exogenous application of JA promoted the freezing tolerance of Arabidopsis. Consistently, the overexpression of JAZ1 or JAZ4 in Arabidopsis inhibited CBF and COR gene expression and repressed freezing tolerance (Hu et al. 2013), which suggests that a JAZ-ICE1 interaction modulates the JA-mediated plant response to cold stress.

EIN3/EIL1 are key transcription factors in the ethylene signaling pathway (Chao et al. 1997). The ein3 eil1 double mutants showed increased freezing tolerance, whereas EIN3 overexpression conferred freezing sensitivity (Shi et al. 2012). Consistent with the freezing phenotypes, CBF expression was upregulated in the ein3 eil1 double mutants, suggesting that EIN3 negatively regulates cold-induced CBF expression. Further study showed that EIN3 directly binds to the EIN3-binding sites (EBS) in the CBF promoters; therefore, EIN3 at least partially negatively regulates freezing tolerance by repressing the cold-induced expression of the CBF genes in Arabidopsis (Shi et al. 2012).

By contrast, another study showed that ethylene positively regulates plant freezing tolerance, and the ACS (1-aminocyclopropane-1-carboxylate synthase) mutants are sensitive to freezing both with and without cold acclimation (Catala et al. 2014; Catala and Salinas 2015). Indeed, several studies showed that ethylene levels were regulated in response to cold stress in different plant species; for example, cold temperatures induced ethylene accumulation in tomato (Ciardi et al. 2014) and beans (Phaseolus vulgaris) (Guye et al. 1987), but inhibited ethylene production in Arabidopsis and barrel clover (Medicago truncatula) (Shi et al. 2012; Zhao et al. 2014). Although ethylene plays a role in the plant freezing tolerance, its exact function requires further verification.

Flowering time is coordinated by temperature, light, and other environmental factors (Boss et al. 2004; Kim et al. 2004; Balasubramanian et al. 2006). Low temperatures delay flowering (Blazquez et al. 2003), suggesting that cold signaling plays a crucial role in flowering regulation. SOC1 encodes a MADS-type transcription factor, which directly binds to the CArG motif in the CBF promoters to repress their expression and consequently that of the COR genes. The soc1 null mutant displayed enhanced freezing tolerance, whereas SOC1-overexpressing plants were sensitive to freezing stress (Seo et al. 2009), indicating the negative role of SOC1 in freezing tolerance.

Overexpression of the CBFs in Arabidopsis delayed flowering by promoting the expression of FLC (Kim et al. 2004); however, whether FLC is a direct target of the CBFs remains unknown (Seo et al. 2009). The flowering times of the fca (flowering time control protein), fve, and svp (short vegetative phase) mutants were insensitive to low temperatures (Blazquez et al. 2003; Halliday et al. 2003; Lee et al. 2007). Under normal conditions, the expression of FLC and the COR genes was upregulated in fve, a mutant with late flowering and increased freezing tolerance (Kim et al. 2004). These studies suggest that SOC1 and FVE constitute genetic links between flowering and the CBF-signaling pathway.

The circadian clock also affects the expression of CBF and some COR genes (Harmer et al. 2000; Bieniawska et al. 2008; Espinoza et al. 2008; Mikkelsen and...
The expression of the CBFs peaks about 8 h after dawn (zeitgeber time 8; ZT8) and drops to a minimum at ZT20 under normal temperatures (Dong et al. 2011), indicating that the circadian clock precisely regulates CBF expression. Furthermore, the cold-temperature induction of CBF expression is gated by the circadian clock (Fowler et al. 2005). Several core circadian regulators were reported to be involved in the cold-stress response, including CCA1, LHY, PRR (Pseudo-response regulator) and TOC1 (Timing of CAB expression 1). CCA1 and LHY expression peaks after dawn, whereas the expression of TOC1 reaches a maximum in the early evening (Harmer et al. 2000).

These three genes comprise a feedback loop that establishes the circadian clock of plants. CCA1 and LHY encode MYB-type transcription factors (Schaffer et al. 1998; Wang and Tobin 1998). The expression of the CBF and COR genes was significantly decreased in the cca1 lhy double mutant, which showed decreased freezing tolerance, and the circadian oscillation of these genes was largely reduced (Dong et al. 2011). Further study demonstrated that CCA1 and LHY directly bind to the EE (Evening elements) and CBS (CCA1-binding sites) of the CBF promoters to upregulate their expression (Dong et al. 2011). The splicing alternation of CCA1 has also been reported to contribute to freezing tolerance (Seo et al. 2012); low temperatures decrease the proportion of CCA1β transcripts, which inhibit the DNA-binding activity of CCA1α through direct interaction. Accordingly, CCA1α-overexpressing plants showed enhanced freezing tolerance, whereas the CCA1β-overexpressing plants were sensitive to freezing (Seo et al. 2012).

The PRR proteins play negative roles in regulating cold stress response. The prr5 prr7 prr9 triple mutant showed enhanced freezing tolerance, and had a constitutively high level of CBF expression (Nakamichi et al. 2009; Nakamichi et al. 2012). In addition, the PRRs also function in regulating the expression of RAV1 (Related to ABI3/VP1 1) and ZAT12, which encode transcription factors that function in parallel with the CBFs. In cotton (Gossypium hirsutum) and soybean (Glycine max), circadian rhythms are involved in chilling and freezing tolerance (Couderchet and Koukkari 1987; Rikin et al. 1993); however, the molecular mechanism by which CBF expression is regulated by the circadian clock in these species remains to be determined.

Light and temperature are two environmental factors that affect plant growth and development, and the integration of these two vital signals is essential for plant survival. Previous studies reported that light quality and photoperiod are involved in the plant freezing response (Franklin and Whitelam 2007; Lee and Thomashow 2012). Red (R) and far-red (FR) light are required for the induction of CBF expression by cold treatment (Kim et al. 2002). Under cool temperatures (16°C), a low R/FR ratio significantly promoted the expression and oscillation of the CBF regulon, enhancing the freezing tolerance of plants (Franklin and Whitelam 2007).

PIFs (Phytochrome-interacting factors) are pivotal transcription factors involved in light signaling and the repression of photomorphogenesis. Under short-day conditions, plants showed increased freezing tolerance with enhanced CBF expression, whereas under long-day conditions, PIF4 and PIF7 directly bind to the G-box and E-box motifs of the CBF promoters and inhibit their expression, decreasing plant freezing tolerance (Lee and Thomashow 2012). In addition, PIF7 was shown to bind to G-box motifs in the CBF2 promoter to down-regulate its expression during the evening (Kidokoro et al. 2009). PIF3 protein is degraded by an E3 ubiquitin ligase, LRB (Light-response bric-a-brack/tramtrack/broad), in the light (Ni et al. 1998; Leivar and Monte 2014).

A recent study showed that PIF3 directly inhibits the expression of the cold-induced CBF genes, so as to negatively regulate cold acclimation (Jiang et al. 2017). Moreover, two F-box proteins, EBF1 (EIN3-binding F-box 1) and EBF2, negatively regulate PIF3 protein stability, via the 26S proteasome pathway (Dong et al. 2017; Jiang et al. 2017). Low temperatures decreased the accumulation of EBF1/2, thereby enhancing the stability of PIF3 (Shi et al. 2012; Jiang et al. 2017). These studies suggest that light signaling plays an important role in regulating the cold response in plants, and that PIF proteins are the key factors integrating light and temperature cues to balance the growth and cold tolerance of plants.

In summary, the identification of these CBF transcriptional regulators highlights the complexity of the CBF-dependent pathway and raises some interesting questions: Is there interaction among these regulators, and what is the significance of their interaction? Improving our understanding of the mechanisms used by these CBF regulators, as well as identifying novel components in the CBF-dependent and independent
pathways, will help to elucidate the regulatory networks of plant cold signaling.

**POST-TRANSLATIONAL MODIFICATION OF THE CBFS**

Besides transcriptional regulation of the CBF genes, recent studies have shown their encoding proteins are modulated by post-translational modifications (Liu et al. 2017; Ding et al. 2018). The CBF proteins confer degradation by the 26S proteasome pathway (Liu et al. 2017). A recent study showed that substrates of OST1, BTF3 (Basic transcription factor 3) and BTF3L (BTF3-like protein), positively regulate freezing tolerance of Arabidopsis. BTF3s are the β-subunits of nascent polypeptide-associated complex. OST1 phosphorylates BTF3s, which enhances the interaction between BTF3s and CBF proteins and, consequently, prevents CBF proteins from ubiquitin-mediated degradation under cold stress (Ding et al. 2018). On the contrary, CBF protein stability is negatively regulated by CRPK1 (cold-responsive protein kinase 1) and 14-3-3 proteins (Liu et al. 2017). Cold-activated CRPK1 phosphorylates 14-3-3 proteins and promotes their translocation from the cytoplasm to the nucleus. In the nucleus, these phosphorylated 14-3-3s interact with CBF1 and CBF3 to facilitate their degradation via the 26S proteasome pathway. Consistently, mutations in CRPK1 and 14-3-3ΔK conferred an enhanced freezing tolerance, suggesting a negative role for the CRPK1-14-3-3 module in regulation CBF protein stability and plant freezing tolerance (Liu et al. 2017). Because the CBF proteins are regulated by the 26S proteasome pathway, the identification of an E3 ligase involved in CBF degradation is the next goal. Answering these questions will provide a new perspective on the CBF-signaling pathway and cold acclimation in land plants.

**COLD-SENSING MECHANISMS IN PLANTS**

To better understand the transduction of cold signals in plants, the cold sensors must be identified; however, this is a challenging task. In rice, COLD1 is a G-protein regulator, localized on the plasma membrane and endoplasmic reticulum, that acts as a cold sensor to regulate chilling tolerance (Ma et al. 2015). COLD1 interacts with the GTPase, RGA1 to enhance its enzymatic activity, and the COLD1-RGA1 complex facilitates the cold-induced influx of Ca^{2+} into cells (Ma et al. 2015).

Recently, PhyB (phytochrome B) was reported to be a photoreceptor and thermosensor that integrates light and temperature signals (Jung et al. 2016; Legris et al. 2016). In another study, a blue light receptor, phototropin, was shown to be involved in low-temperature sensing in the liverwort (Marchantia polymorpha) (Fujii and Kodama 2017); however, whether these photoreceptors are involved in cold sensing in higher plants is unclear (Figure 2).

As mentioned above, CRPK1 negatively regulates the CBF-signaling pathway through phosphorylating 14-3-3 protein (Liu et al. 2017). CRPK1 is a receptor-like cytoplasmic kinase that is localized at the plasma membrane and activated under cold stress. The CRPK1 protein lacks a transmembrane domain, which suggests the existence of a cold-activated receptor-like kinase (RLK) that forms a complex with CRPK1 to perceive the cold signal (Figure 2). The identification of this RLK would improve our understanding of the mechanism involved in CRPK1-mediated cold sensing. In addition, a calcium/calmodulin-regulated receptor-like kinase, CRLK1, was found to be a positive regulator of the cold-signaling pathway (Yang et al. 2010 [Figure 2]). The expression of several cold-responsive genes, including CBF1, RD29A, COR15a, and KIN1, was delayed in the crlk1 knock-out mutants, which had an enhanced sensitivity to chilling and freezing temperatures.

A recent study showed that CRLK1 and its closest homolog, CRLK2, inhibited the activities of MPK3 and MPK6, thereby positively regulating the expression of the CBFs (Zhao et al. 2017). MPK4 was also activated by cold stress to constitutively inhibit the phosphorylation of MPK3 and MPK6, and thus positively regulates the plant freezing tolerance (Zhao et al. 2017). As the CRLK proteins are regulated by calmodulin, future studies should investigate the role of the Ca^{2+} signal in the CRLK-mediated freezing tolerance.

**FUTURE PERSPECTIVES**

Much progress has been made in unraveling the mechanisms involved in the cold response in plants, but many challenging and interesting questions still
need to be addressed. Although important components of the CBF-dependent cold-signaling pathway have been identified, many COR genes are not direct targets of the CBF transcription factors. The development of efficient gene-editing technologies would benefit the identification of novel CBF-dependent and independent transcription factors involved in plant cold signaling.

The identification of COLD1 as the cold sensor in rice provides new directions for research into its cold-sensing mechanism (Ma et al. 2015); however, whether this sensory mechanism exists in other plant species remains to be explored. Given that COLD1 helps to regulate the cytosolic Ca$^{2+}$ influx under cold stress, the Ca$^{2+}$-related protein kinases, such as the CDPKs (Ca$^{2+}$-dependent protein kinases) and CIPKs (CBL-interacting protein kinases), might function in the cold-signaling pathway. The functional study of these kinases will help us understand the mechanism of Ca$^{2+}$ signaling in the cold response.

Two recent studies reported that phytochromes function as thermosensors to regulate the light and temperature response (Jung et al. 2016; Legris et al. 2016). Many components of light signaling play roles in the cold-signaling pathway; therefore, it is important to determine whether phytochromes truly sense the cold in plants, and we must elucidate whether the phytochromes and other photoreceptors are involved in the CBF-mediated cold signaling pathway.

Recently, a new “sacrifice-for-survival” mechanism was reported to improve chilling survival in Arabidopsis roots (Hong et al. 2017). Chilling stress can preferentially induce cell injury in the columella stem cell daughters of the Arabidopsis root tip, re-establishing the auxin maximum in the quiescent center and preventing the further division of the columella stem cells (Hong et al. 2017). This protective mechanism enables Arabidopsis roots to withstand chilling stress and balances growth with the survival of environmental stresses. It will therefore be interesting to explore...
whether the CBF-COR pathway is involved in this survival mechanism.

Phosphorylation plays an important role in the transduction of the cold signal in plants. The OST1 and MPK3/6 protein kinases are activated by cold stress, and can directly phosphorylate and regulate ICE1 stability. Notably, the phosphorylation of ICE1 at different phosphosites appears to exert varying functions. The Ser278 site in ICE1 is phosphorylated by OST1, while MAPKs phosphorylate Ser94/203/403 and Thr366/382/384. The patterns of cold-induced kinase activity are also different; OST1 is rapidly activated by cold stress (within about 30 min), while MPK3 and MPK6 are activated after 1 h of cold treatment. OST1 may therefore function in the early stages of the cold-stress response to inhibit ICE1 degradation and induce CBF-COR expression, whereas MPK3 and MPK6 function later to promote ICE1 degradation, thereby fine-tuning the turnover of ICE1. The mechanisms by which these kinases are activated by cold stress and the interconnection between them awaits further investigation.

ACKNOWLEDGEMENTS

This work was supported by grants from the National Natural Science Foundation of China (31730011 and 31700214). We apologize to all colleagues whose relevant work could not be cited owing to space limitations.

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