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Advances and challenges in uncovering cold tolerance regulatory mechanisms in plants

Author for correspondence:
Shuhua Yang
Tel: +86 10 62734838
Email: yangshuhua@cau.edu.cn

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Yanglin Ding*, Yiting Shi* and Shuhua Yang 

State Key Laboratory of Plant Physiology and Biochemistry, College of Biological Sciences, China Agricultural University, Beijing 100193, China

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Summary

Cold stress is a major environmental factor that seriously affects plant growth and development, and influences crop productivity. Plants have evolved a series of mechanisms that allow them to adapt to cold stress at both the physiological and molecular levels. Over the past two decades, much progress has been made in identifying crucial components involved in cold-stress tolerance and dissecting their regulatory mechanisms. In this review, we summarize recent major advances in our understanding of cold signalling and put forward open questions in the field of plant cold-stress responses. Answering these questions should help elucidate the molecular mechanisms underlying plant tolerance to cold stress.

I. Introduction

The average minimum temperature over most (*c.* 64%) of the total land area on Earth is $< 0^{\circ}\text{C}$ (Rihan *et al.*, 2017). However, many crops, including rice (*Oryza sativa*), maize (*Zea mays*), tomato (*Solanum lycopersicum*), soybean (*Glycine max*) and cotton (*Gossypium hirsutum*), lack the ability to acclimate to cold temperatures and can only grow in tropical or subtropical regions (Chinnusamy *et al.*, 2007). Thus, cold stress adversely affects plant growth and development, limits the geographical distribution of plant species, and decreases crop yields worldwide (Pearce, 2001). Plants have evolved sophisticated mechanisms to withstand cold stress. One such mechanism is cold acclimation, a process by which plants

acquire increased freezing tolerance upon prior exposure to nonlethal low temperature (Guy, 1990; Thomashow, 1999).

During this process, a series of comprehensive physiological and biochemical events take place. At the physiological level, many substances or protective proteins are synthesized in plants, such as soluble sugars, proline and cold-resistance proteins (Kaplan & Guy, 2004; Kaplan *et al.*, 2007). These substances are involved in regulating osmotic potential, ice crystal formation, the stability of cell membranes and reactive oxygen species (ROS) scavenging in plants subjected to cold stress (Lee *et al.*, 2002; Dong *et al.*, 2009). Over the past two decades, numerous components, including messenger molecules, protein kinases and phosphatases, and transcription factors have been identified in cold-stress signalling pathways. The best characterized of these is the CBF-COR signalling pathway. *C-REPEAT BINDING FACTOR/DEHYDRATION-RESPONSIVE ELEMENT-BINDING PROTEIN1 (CBF/DREB1)*

*These authors contributed equally to this work.

genes are rapidly induced under cold stress and they play crucial roles in cold acclimation of plants (Stockinger *et al.*, 1997; Liu *et al.*, 1998). *COR* refers to a class of genes regulated by cold stress such as *COLD REGULATED (COR)*, *LOW TEMPERATURE INDUCED (LTI)* and *COLD INDUCIBLE (KIN)*, some of which encode osmolyte and cryoprotective proteins to protect plant from freezing injury (Yamaguchi-Shinozaki & Shinozaki, 1994; Shi *et al.*, 2018). CBF proteins can directly bind to the promoters of *CORs* and induce their expression, thereby enhancing freezing tolerance (Stockinger *et al.*, 1997; Liu *et al.*, 1998). CBF-COR regulatory signalling pathway is highly complex and requires further in-depth study.

Understanding how plants respond to cold stress will provide valuable information and genetic resources for improving cold-stress tolerance in crops. In this review, we summarize recent developments in our understanding of the regulatory mechanisms underlying cold-stress tolerance and explore open questions that should be the focus of future work.

II. Cold stress and physiological responses in plants

Stress refers to any substance or stimulus that restricts plant metabolism, growth, development and crop productivity, including biotic and abiotic stresses (Lichtenthaler, 1998). Cold stress, including chilling (0–15°C) and freezing (<0°C), is an abiotic stress that adversely affects the growth and agricultural productivity of plants (Guo *et al.*, 2018; Liu J. *et al.*, 2018). Chilling stress usually restricts plant growth and development, and has several major effects on plant cells. First, chilling stress affects membrane rigidification in plant cells, which is considered to be the primary event that triggers downstream cold-stress responses in plants (Orvar *et al.*, 2000). Second, chilling stress disturbs the stability of proteins or protein complexes and reduces the activities of enzymes such as ROS scavenging enzymes. These processes result in photo-inhibition and impaired photosynthesis, as well as considerable membrane damage (Siddiqui & Cavicchioli, 2006; Ruelland *et al.*, 2009). Third, chilling stress affects gene expression and protein synthesis, as it favours the formation of secondary structures in RNA (Rajkowsch *et al.*, 2007; Ruelland *et al.*, 2009). Freezing stress is more damaging to plants than chilling stress, and may even cause plant death. Under natural conditions, freezing damage begins with extracellular ice nucleation (Pearce, 2001). Once ice nuclei form, they grow and form ice crystals, which spread into the apoplast where they induce water efflux, leading to cell dehydration. Irreversible damage occurs when ice crystals spread into cells (Dowgert & Steponkus, 1984; Pearce, 2001).

Plants have evolved sophisticated mechanisms that limit cold-induced damage. For instance, cold acclimation is a process in which plants that are exposed to nonlethal low temperatures for a few days develop an enhanced ability to resist subsequent freezing stress (Guy, 1990; Thomashow, 1999). During this process, plants increase their tolerance to cold stress by synthesizing numerous protective substances (e.g. soluble sugars, proline) and proteins (e.g. LEA, AFP, CSP) (Steponkus *et al.*, 1998; Thomashow, 1999; Kaplan & Guy, 2004, 2005; Kaplan *et al.*, 2007).

Soluble sugars, proline and other lower-molecular-weight solutes function as osmolytes to protect plants from damage caused by cold stress (Ruelland *et al.*, 2009). The accumulation of protective proteins including LATE EMBRYOGENESIS ABUNDANT (LEA) proteins, ANTI-FREEZING PROTEINS (AFPs) and COLD SHOCK PROTEINS (CSPs) during cold acclimation is important for freezing tolerance in plants (Ruelland *et al.*, 2009). LEA proteins have been referred to as hydrophilins, because they have common structural features such as high hydrophilicity. Most LEA proteins are predicted to belong to intrinsically disordered protein (Battaglia *et al.*, 2008). COR15A is the best-characterized LEA protein that resides at the membrane surface during dehydration and stabilizes cell membranes under freezing stress (Bremer *et al.*, 2017a,b). Several other LEA proteins were identified in different plant species, and proved that they are important factors in regulating plant chilling or freezing tolerance (Houde *et al.*, 2004; Qiu *et al.*, 2014; Sasaki *et al.*, 2014; Liu *et al.*, 2015). AFPs bind to specific surfaces of growing ice crystals and inhibit their growth (Wen *et al.*, 2016). AFPs have two kinds of activities in fish and insects. One is the ability to lower the freezing point of water (known as thermal hysteresis), the other is ice recrystallization inhibition (IRI) (Liu *et al.*, 2016; Wen *et al.*, 2016). Plant AFPs have high IRI activity, suggesting that the activity of IRI may be more important for AFPs in plants (Gupta & Deswal, 2014). Some AFPs have been reported to function as important regulators in plants freezing tolerance (Holmberg *et al.*, 2001; Zhang *et al.*, 2010). CSPs are composed of a single cold shock domain (CSD) and function as RNA chaperones in bacterial and plants (Xia *et al.*, 2001; Nakaminami *et al.*, 2005). Arabidopsis CSP2 and CSP3 are important regulators in freezing tolerance (Kim *et al.*, 2009; Sasaki *et al.*, 2013).

III. Sensing of cold signals in plants

Unravelling the mechanism by which plants perceive cold signals is essential for understanding how plants avoid damage caused by low temperatures. Researchers have long focused on the fundamental issue of how plants sense cold signals. Such studies have implied that cold is not sensed by a single protein, but is instead perceived at different sensory levels.

1. The cell membrane fluidity hypothesis

The reduction of cell membrane fluidity following exposure to cold stress is widely considered to be one of cold perception mechanisms, as it is the first line of defence against cold stress. This hypothesis comes from the evidence based on pharmacological studies. Pharmacological assays demonstrated that *COR* expression is induced by membrane rigidification at 25°C, whereas it is inhibited by membrane fluidization at 4°C (Orvar *et al.*, 2000; Sangwan *et al.*, 2001). Diacylglycerol kinase (DAGK) activity, a very early event occurring within seconds of chilling exposure, is associated with membrane fluidity (Miquel *et al.*, 1993; Vaultier *et al.*, 2006). Moreover, the researchers invented a new method to measure membrane fluidity and, further, provided new evidence for the change of membrane fluidity in response to chilling

temperatures in plant cells (Martiniere *et al.*, 2011). Plasma membrane fluidity is correlated with the proportion of desaturated fatty acids. *FATTY ACID DESATURATION2* (*FAD2*) encodes the oleate desaturase essential for membrane fluidity. Mutation of *FAD2* impairs some physiological responses to chilling stress, including leaf number and hypocotyl length (Martiniere *et al.*, 2011). These findings support the notion that reduction of cell membrane fluidity represents an important mode of sensing cold signals.

The plant cytoskeleton is changed upon low temperature (Pokorna *et al.*, 2004). Drugs that stabilize microtubules and filaments inhibit the expression of *COR* gene *BN115*, whereas drugs that destabilize microfilaments promote *BN115* expression (Orvar *et al.*, 2000). Studies also showed that the change of microfilament structure induced by cold is upstream of calcium/ Ca^{2+} influx into the plant cells (Mazars *et al.*, 1997; Orvar *et al.*, 2000). Considering the close link of the cytoskeleton with the plasma membrane, it is possible that changes in the cytoskeleton are involved in early event of cold signalling.

2. Calcium channels

Low temperatures are perceived by TRANSIENT RECEPTOR POTENTIAL (TRP) ion channels in mammals (Bautista *et al.*, 2007; Dhaka *et al.*, 2007); however, these channels are not present in plants. Ca^{2+} is an important second messenger in plant response to environmental changes. Cytosolic Ca^{2+} concentration is increased very rapidly via Ca^{2+} channels after cold treatment in both plants and animals, which is considered as one of the earliest cold signalling events (Knight *et al.*, 1996; Plieth *et al.*, 1999; Knight & Knight, 2012). Interestingly, cold-induced *COR* is dependent on Ca^{2+} (Knight *et al.*, 1996). Therefore, it is possible that ion channels (i.e. Ca^{2+} channels) and electrophysiological responses mediate low-temperature sensing in plants as well. The cyclic nucleotide-gated channels (CNGCs) in Arabidopsis and moss are essential for thermal sensing and thermotolerance (Finka *et al.*, 2012). The plasma membrane and endoplasmic reticulum-localized G-protein regulator CHILLING TOLERANCE DIVERGENCE1 (*COLD1*) coupled with RICE G-PROTEIN α SUBUNIT1 (*RGA1*) was recently shown to be involved in cold sensing by modulating calcium signals and electrophysiological responses in rice (*Oryza sativa*) (Ma *et al.*, 2015). The *COLD1*-*RGA1* complex mediates the cold-induced influx of intracellular Ca^{2+} , leading to the activation of *COR* genes (Ma *et al.*, 2015) (Fig. 1). It would be interesting to investigate whether *COLD1* functions as a Ca^{2+} -permeable channel or as a mediator facilitating Ca^{2+} -permeable channel activity and to determine how *COLD1* transduces cold signals to the nucleus to activate the cold-induced expression of *DREB1s* in rice (*OsDREB1s*).

3. Phytochrome

Two breakthrough studies have demonstrated that temperature-sensitive changes in the protein state of the photoreceptor PHYTOCHROME B (*phyB*) are involved in ambient temperature perception (10–30°C), with *phyB* changing from the active Pfr

state to the inactive Pr state. *phyB* directly binds to the promoters of key target genes in a temperature-dependent manner, and *phyB* null mutants exhibit a constitutive warm-temperature response (Jung *et al.*, 2016; Legris *et al.*, 2016) (Fig. 1). These findings demonstrate that *phyB* governs photomorphogenesis under different temperatures by perceiving light and ambient temperatures. Whether *phyB* also participates in cold sensing needs further investigation.

IV. Messenger molecules involved in cold signal transduction

In addition to Ca^{2+} , emerging evidence suggests that other messenger molecules such as ROS and nitric oxide (NO) are involved in regulating plant response to cold stress. ROS, including superoxide ($\text{O}_2^{\cdot-}$), hydroxyl radicals (OH^{\cdot}), and hydrogen peroxide (H_2O_2) are produced in plants in response to various stresses (Tyystjarvi, 2013). ROS play dual roles in plant cells: on the one hand, they induce gene expression and protein synthesis to protect cells from stress; on the other, they induce oxidative stress (Heidarvand & Maali-Amiri, 2013; Qi *et al.*, 2018). Upon cold stress, plants accumulate H_2O_2 , and excessive H_2O_2 has a deleterious effect on plant cells. As a result, the H_2O_2 scavenging system is activated through the conversion of GSH (reduced glutathione) to GSSG (oxidized glutathione) in plants (Kocsy *et al.*, 2001). Moreover, several defence genes contain antioxidant-responsive elements or GSSG binding sites in their promoter regions (Kocsy *et al.*, 2001), suggesting that the redox signalling chain might regulate gene expression in response to cold stress. Therefore, it will be important to identify *COR* genes that function downstream of redox signalling. Some evidence suggests that there is a close linkage between Ca^{2+} and ROS. For instance, low ROS concentrations promote Ca^{2+} influx into the cytoplasm (Rihan *et al.*, 2017). In addition, Ca^{2+} regulates ROS production in plants under various stimuli, such as drought and high relative humidity stress (Wang *et al.*, 2016). It is necessary to dissect the exact roles of Ca^{2+} and ROS, as well as their relationship, in regulating plant cold signalling.

A crucial role in transducing developmental and environmental cues in plants is played by the gaseous molecule NO (Besson-Bard *et al.*, 2008; Wilson *et al.*, 2008). Cold induces NO production in plants; this is considered to be a general response that takes place in various plant species and organs (Zhao *et al.*, 2009). Pharmacological and genetic studies have shown that nitric reductase (NR)-dependent NO concentrations are positively correlated with cold acclimation and freezing tolerance in Arabidopsis (Zhao *et al.*, 2009; Cantrel *et al.*, 2011; Costa-Broseta *et al.*, 2018); however, a recent study showed that NO is a negative regulator of constitutive freezing tolerance in Arabidopsis (Costa-Broseta *et al.*, 2018).

S-nitrosylation mediated by NO represents a key process in plant growth and development, as well as responses to environmental changes (Hess *et al.*, 2005; Kovacs & Lindermayr, 2013; Hu *et al.*, 2017; Zhan *et al.*, 2018). Cold-induced modifications of S-nitrosylation proteins have been identified in various plant species, such as *Brassica juncea* and Arabidopsis (Abat & Deswal, 2009; Sehrawat *et al.*, 2013; Puyaubert *et al.*, 2014). Many cold-

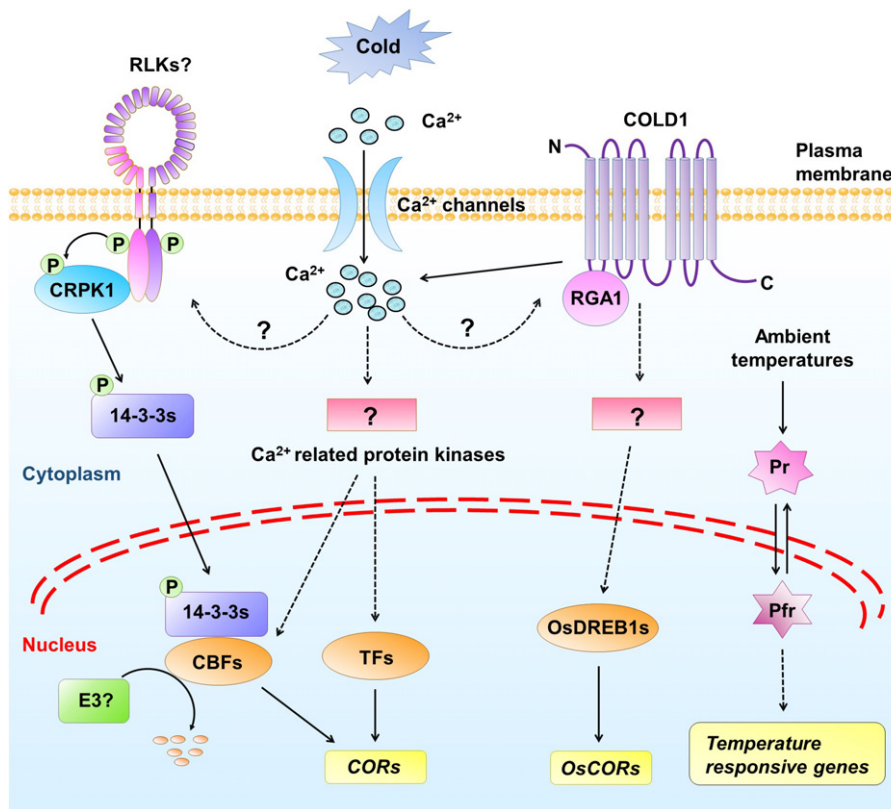


Fig. 1 Overview of cold signal sensing and transduction in Arabidopsis and rice. Cold temperature triggers plasma membrane rigidification and Ca^{2+} channel activation, leading to increased Ca^{2+} concentrations in the cytosol and in turn activation of Ca^{2+} -related protein kinases (i.e. Ca^{2+} -DEPENDENT PROTEIN KINASEs (CDPKs), B-LIKE CALMODULIN BINDING PROTEINs (CBLs) and CBL-INTERACTING PROTEIN KINASEs (CIPKs)). In Arabidopsis, an unknown receptor-like kinase pair might sense cold signals and phosphorylate COLD-RESPONSIVE PROTEIN KINASE1 (CRPK1). Cold-activated CRPK1 interacts with and phosphorylates 14-3-3 proteins. The phosphorylated 14-3-3 proteins shuttle from the cytosol to the nucleus, where they interact with C-REPEAT BINDING FACTOR (CBFs) and promote their degradation via the 26S proteasome pathway. In rice, cold signals are sensed by the plasma membrane-localized protein CHILLING TOLERANCE DIVERGENCE1 (COLD1). The COLD1/RGA1 (RICE G-PROTEIN α SUBUNIT1) protein complex activates the cold-induced influx of intracellular Ca^{2+} , leading to the activation of the cold signalling pathway.

induced S-nitrosylated proteins identified to date are involved in primary metabolism, especially photosynthesis (Puyaubert *et al.*, 2014). NO-mediated S-nitrosylation of iron-containing superoxide dismutase also is important for preventing chilling injury in *B. juncea* (Sehrawat *et al.*, 2013). Moreover, NO depletion diminishes the cold-induced expression of *CBF1/3* and CBF regulons such as *COR15a*, *LTI30* and *LTI78* (Cantrel *et al.*, 2011). It remains to be determined whether S-nitrosylation is a general mechanism for regulating cold-stress responses and whether NO mediates the S-nitrosylation of proteins involved in the CBF-COR signalling pathway.

V. Cold signal transduction in plants

1. Transcriptional regulation of CBF genes

The CBF/DREB1-dependent cold signalling pathway has been studied extensively over the past two decades. The story begins with the important discovery of a novel *cis*-acting element, C-repeat/dehydration response element (CRT/DRE), which is responsive to drought, cold and high-salt stress (Yamaguchi-Shinozaki & Shinozaki, 1994). Since this discovery, CBF proteins have been

isolated sequentially by screening for DNA-binding proteins that bind to the CRT/DRE motif using yeast one-hybrid assays (Stockinger *et al.*, 1997; Liu *et al.*, 1998).

Arabidopsis contains three cold-induced CBF genes, *CBF1–3* (*CBF1/DREB1B*, *CBF2/DREB1C* and *CBF3/DREB1A*), which are arranged in tandem on chromosome IV. In *Arabidopsis*, there is another CBF gene (*CBF4*) that is not induced by cold; however, overexpression of *CBF4* enhances plant freezing and drought tolerance (Haake *et al.*, 2002). CBF1–3 are APETALA2/ETHYLENE-RESPONSIVE (AP2/ERF1)-type transcription factors that directly bind to the conserved CRT/DRE motifs in the promoters of COR genes (known as CBF regulons) and activate their expression under cold conditions (Gilmour *et al.*, 1998; Liu *et al.*, 1998; Medina *et al.*, 1999). Transgenic Arabidopsis plants overexpressing *CBF1* display increased COR expression and enhanced freezing tolerance (Jaglo-Ottosen *et al.*, 1998). CBF orthologues have been isolated in many plant species, including rice, tomato, wheat (*Triticum aestivum*), barley (*Hordeum vulgare*) and maize (Shi *et al.*, 2018). Heterologous expression of Arabidopsis CBFs enhances freezing tolerance in various species, and heterologous expression of CBFs from other plant species enhances freezing tolerance in Arabidopsis (Gilmour *et al.*, 2000; Zhang

et al., 2004; Savitch *et al.*, 2005). However, it is worth noting that cold-sensitive tomato (*Lycopersicon esculentum*) has *CBF* genes, only *LeCBF1* is found to be cold-inducible and functional (Zhang *et al.*, 2004). Overexpression of *LeCBF1* confers tomato freezing tolerance; however, overexpression of cold-tolerant Arabidopsis *CBF3* in tomato plants do not exhibit freezing tolerance, because there are different CBF regulons in tomato and Arabidopsis (Zhang *et al.*, 2004). These studies indicate that the biological function of *CBF1–3* in modulating freezing tolerance is not only highly conserved among plants, but also species-specific.

As *CBF1–3* loci are located adjacent to each other on the same chromosome, it is challenging to generate *cbf1,2,3* triple mutant lines by traditional genetic crossing. Two laboratories have recently succeeded in generating single, double and triple mutants of *CBF* genes using CRISPR/Cas9 technology (Jia *et al.*, 2016; Zhao *et al.*, 2016). The *cbf* triple mutants are the most sensitive to freezing stress of these different mutants under cold-acclimation treatment (Jia *et al.*, 2016; Zhao *et al.*, 2016). RNA-seq analysis of the triple mutants revealed that the expression of c. 10–20% of *COR* genes is CBF-dependent (Jia *et al.*, 2016; Zhao *et al.*, 2016). These findings support the notion that CBFs are key regulators that play redundant roles in cold acclimation in plants.

The expression of *CBF* genes is induced rapidly by cold stress and is both positively and negatively controlled by various transcription factors (Fig. 2). INDUCER OF *CBF* EXPRESSION (*ICE1*), a MYC-type bHLH transcription factor, is the best-characterized transcriptional activator of *CBF* genes to date (Chinnusamy *et al.*, 2003). *ICE1* activates the expression of *CBF* genes by directly binding to their promoters under cold stress. Mutation of *ICE1* impairs cold-induced *CBF* expression and decreases freezing tolerance (Chinnusamy *et al.*, 2003; Ding *et al.*, 2015). *ICE2*, a homologue of *ICE1*, also plays a positive role in regulating *CBF* expression and freezing tolerance (Fursova *et al.*, 2009).

Early studies have shown that Ca²⁺-responsive protein calmodulins (CAMs) are induced by low temperature and CAM activity is essential for *COR* gene expression (Polisensky & Braam, 1996; Tahtiharju *et al.*, 1997). CALMODULIN-BINDING TRANSCRIPTION ACTIVATORS (CAMTAs), which harbour conserved CAM-binding sites, also activate *CBF* expression.

CAMTA3 activates *CBF2* expression, whereas CAMTA1 and CAMTA2 activate *CBF1–3* expression (Doherty *et al.*, 2009; Kim *et al.*, 2013). A recent study showed that CAMTA proteins (CAMTA1–5) positively regulate *CBF1* and *CBF2* expression (Kidokoro *et al.*, 2017). Additionally, CAMTA3 and CAMTA5 regulate *CBF1* expression in response to rapidly (but not slowly) decreasing temperatures (Kidokoro *et al.*, 2017). More importantly, an interesting result showed that the CRT/DRE motif is regulated by Ca²⁺ (Whalley *et al.*, 2011). These studies provide a possible link between calcium and cold signalling.

A member of the R2R3 subfamily of MYB15 transcription factors negatively regulates the expression of *CBFs* through directly binding to the conserved MYB motif in their promoters (Agarwal *et al.*, 2006). OsMYBS3 inhibits cold-induced expression of *OsDREB1B* and negatively regulates chilling tolerance in rice (Su *et al.*, 2010). PHYTOCHROME-INTERACTING FACTOR3/4/7 (*PIF3/4/7*) transcription factors, which function in light signalling, also are involved in negatively regulating *CBF* expression (Franklin & Whitelam, 2007; Lee & Thomashow, 2012; Jiang *et al.*, 2017).

The expression of *CBFs* is gated by the circadian clock (Fowler *et al.*, 2005). CIRCADIAN CLOCK-ASSOCIATED1 (*CCA1*) and LATE ELONGATED HYPOCOTYL (*LHY*), two core components of the circadian clock, are positive regulators of *CBF* expression and plant freezing tolerance (Dong *et al.*, 2011). *CCA1* has two splice variants, *CCA1α* and *CCA1β*. *CCA1β* interacts with *CCA1α* and inhibits its DNA binding activity. Low temperature inhibits *CCA1β* production, thus releasing the inhibition of *CCA1α* activity by *CCA1β* (Seo *et al.*, 2012). Other circadian clock components, namely PSEUDO RESPONSE REGULATORS (*PRRs*), negatively modulate *CBF* expression and freezing tolerance in plants (Nakamichi *et al.*, 2009).

Apart from the above regulators, various hormone-signalling components also orchestrate *CBF* expression. ETHYLENE INSENSITIVE3 (*EIN3*) is a key transcription factor involved in ethylene signalling that is a negative regulator of *CBF* expression and freezing tolerance (Shi *et al.*, 2012). Two F-box proteins, *EIN3*-BINDING F-BOX 1/2 (*EBF1/2*), mediate the degradation of *EIN3* and *PIF3* via the 26S proteasome pathway, thereby

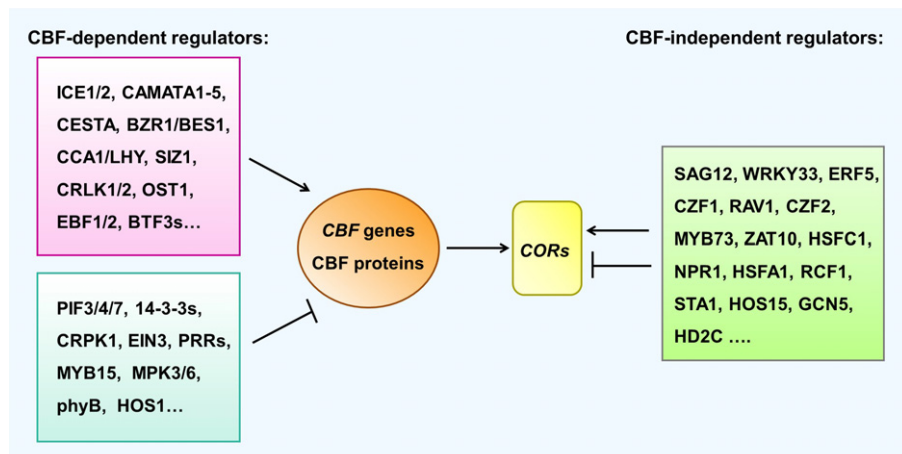


Fig. 2 Positive and negative regulators of the C-REPEAT BINDING FACTOR (*CBF*)-dependent pathway in Arabidopsis. The expression of *CBFs* and the protein stability of *CBFs* are governed by positive or negative regulators, including transcription factors, protein kinases, E3 ubiquitin ligases and SUMO ligases. Additionally, *COLD REGULATED* (*COR*) gene expression is regulated by various *CBF*-independent regulators.

activating *CBF* expression under cold stress (Jiang *et al.*, 2017). JASMONATE ZIM-DOMAIN PROTEIN1/4 (*JAZ1/4*) proteins, which function as repressors of the jasmonic acid (JA) signalling pathway, interact with *ICE1/2* and regulate their transcriptional activities and *CBF* expression (Hu *et al.*, 2013). Brassinosteroids (BRs) also play a role in regulating freezing tolerance. Mutations of *BIN2* and its homologs lead to increased freezing tolerance in plants (Li *et al.*, 2017b). BRASSINAZOLE-RESISTANT1 (*BZR1*) and *BRI1-EMS-SUPPRESSOR1* (*BES1*) are downstream transcription factors of BRASSINOSTEROID-INSENSITIVE2 (*BIN2*) that positively regulate freezing tolerance by partially regulating expression of *CBF1* and *CBF2* via binding to E-box binding sites in their promoters (Li *et al.*, 2017b). The transcription factor *CESTA* downstream of *BIN2* binds to the G-box motif in the promoters of *CBF* genes and regulates their expression (Eremina *et al.*, 2017). These findings suggest that plants integrate hormone and cold signalling pathways for better adaptation to cold stress.

Notably, overexpression of *CBFs* leads to growth retardation and reduced plant biomass (Jaglo-Ottosen *et al.*, 1998; Gilmour *et al.*, 2000; Achard *et al.*, 2008). Conversely, *cbf* triple mutants are larger than the wild-type (WT) under chilling stress (Jia *et al.*, 2016). Therefore, it is possible that *CBFs* are important regulators in the trade-off between plant growth and cold responses. This fine-tuned regulation by *CBFs* might represent an important strategy balancing plant growth and cold tolerance.

2. Transcriptional and post-transcriptional regulation of *COR* genes

The regulation of *COR* genes is important for their functions in cold acclimation; however, only c. 10–20% of *COR* genes are regulated by *CBFs* (Park *et al.*, 2015; Jia *et al.*, 2016; Zhao *et al.*, 2016). Thus, it is important to investigate how the remaining *CORs* are regulated. The cold-induced transcription factor *ZAT12* controls the expression of 24 *COR* genes (Vogel *et al.*, 2005). In addition to *CBF*-dependent *CORs*, *BZR1* also modulates other *COR* genes uncoupled with *CBFs*, such as *WRKY6*, *PYR1-LIKE 6* (*PYL6*), *SUPPRESSOR OF OVEREXPRESSION OF CO1* (*SOC1*), *JASMONIC ACID CARBOXYL METHYLTRANSFERASE* (*JMT*) and *SENESCENCE-ASSOCIATED GENE 12* (*SAG12*), to regulate plant freezing tolerance (Li *et al.*, 2017b). Several other cold-induced transcription factors function in a similar manner to *CBFs* to induce the expression of *COR* genes under cold stress, including *WRKY33*, ETHYLENE RESPONSIVE ELEMENT BINDING FACTOR5 (*ERF5*), *CZF1*, RELATED TO *ABI3/VP1* (*RAV1*), *CZF2*, *MYB73*, *ZAT10* and HEAT SHOCK TRANSCRIPTION FACTOR C1 (*HSFC1*) (Park *et al.*, 2015).

Recently, HEAT SHOCK TRANSCRIPTION FACTOR1 (*HSFA1*) was found to positively regulate cold acclimation by inducing expression of heat stress-responsive genes, which are also one type of *COR* genes, in a *CBF*-independent manner (Olate *et al.*, 2018). *HSFA1* transcription activity is activated by NON-EXPRESSION OF PATHOGENESIS-RELATED GENES 1 (*NPR1*), an SA receptor (Ding *et al.*, 2018b; Olate *et al.*, 2018). Low temperatures induce cytosolic *NPR1* monomerization and

nuclear import, as does SA during the pathogen response (Tada, 2009; Olate *et al.*, 2018). However, unlike the plant pathogen resistance mechanism, *NPR1* interacts with *HSFA1* in the nucleus to modulate the expression of *HSFA1*-regulated genes independently of SA or TGA transcription factors (Olate *et al.*, 2018).

Post-transcriptional regulation also is important for *COR* gene function. *REGULATOR OF CBF GENE EXPRESSION1* (*RCF1*), encoding a DEAD-box RNA helicase, helps ensure the proper pre-mRNA splicing of many *COR* genes under cold stress (Guan *et al.*, 2013). *STABILIZED1* (*STAI*) encodes a pre-mRNA splicing factor that controls the pre-mRNA splicing and mRNA turnover of *COR* genes (Lee *et al.*, 2006). Nevertheless, our knowledge of the mechanisms regulating *COR* gene expression is limited, and future work should aim to identify novel regulators of *COR* genes.

Alternative splicing (AS) is an important post-transcriptional regulation that is required for reprogramming gene expression under stress conditions. Previous study showed an extensive AS in core clock genes in Arabidopsis. AS of these genes such as *LHY* and *CCA1* is temperature-dependent (James *et al.*, 2012). A recent study of genome-wide AS profiling analysis showed a massive and rapid wave of AS coincident with the transcriptional response and identified hundreds of genes such as *RCF1* and *STAI* that have dramatically altered AS in the first few hours of cold treatment ('early AS' genes) (Calixto *et al.*, 2018). This study demonstrates that plants may fine-tune gene expression via AS pathway in response to temperature change (Calixto *et al.*, 2018).

3. Epigenetic regulation of the *CBF*–*COR* pathway

Aside from the roles of transcriptional and post-transcriptional regulation in the *CBF* signalling pathway, epigenetic regulation also plays a role in modulating gene expression under cold stress. MicroRNAs (miRNAs) are involved in cold-stress responses (de Lima *et al.*, 2012; Megha *et al.*, 2018). Analysis of the first small RNA libraries showed that miR393 expression is upregulated, and miR319c and miR398a expression is downregulated under cold stress (Sunkar & Zhu, 2004). Since this initial study, some cold-regulated miRNAs in Arabidopsis have been identified. For instance, Arabidopsis harbours two isoforms of miR397 (miR397a and miR397b) (Sunkar & Zhu, 2004). Arabidopsis plants overexpressing miR397a show increased tolerance to chilling and freezing stress (Dong & Pei, 2014). The expression levels of *CBFs* and their target *COR* genes are higher in miR397a-overexpressing plants than in the WT. Although the cold tolerance conferred by miR397a is at least partially dependent on the *CBF*–*COR* module, it remains unknown whether *CBFs* and *CORs* are direct targets of miRNAs (Dong & Pei, 2014). A proline-rich protein SICKLE (*SIC*) co-localizes with the miRNA biogenesis component *HYL1* and regulates the biogenesis of some miRNAs and degradation of some spliced introns (Zhan *et al.*, 2012). The *sic-1* mutant shows increased sensitivity to chilling and salt stress (Zhan *et al.*, 2012).

A recent study reported a cold-responsive long noncoding RNA, named *SVALKKA*, plays a role in regulating *CBF1* expression. Interestingly, the expression of *SVALKKA* is increased after 4 h of

cold treatment when *CBF1* expression is decreased (Kindgren *et al.*, 2018). RNA Polymerase II read-through transcription of *SVALKKA* results in the production of *asCBF1*, a cryptic antisense transcript overlapping *CBF1* to repress cold-induction of *CBF1* gene (Kindgren *et al.*, 2018). This study provides a new regulatory mechanism of *CBF1* expression under cold stress.

Post-translational histone modifications, along with DNA methylation, are associated with gene expression levels in response to cold stress (Kim *et al.*, 2015). Histone acetylation/deacetylation catalyzed by histone acetyltransferases (HATs) and histone deacetylases (HDAs) plays a role in cold responses in plants (Kim *et al.*, 2015). Arabidopsis *HISTONE DEACETYLASE6* (*HDA6*) is upregulated by cold stress and positively regulates freezing tolerance (To *et al.*, 2011). *HDA6*s also are upregulated by cold stress in maize, leading to global deacetylation at H3 and H4 (Hu *et al.*, 2011). Under cold stress, *HDA6*s appear to directly activate maize *DREB1* (*ZmDREB1*) expression and histone hyperacetylation. DNA demethylation occurs in the *ZmICE1* binding region (Hu *et al.*, 2011). Interestingly, *ICE1* demethylation may have been responsible for the expansion of crofton weed (*Ageratina adenophora*) northward into China (Xie *et al.*, 2015). GENERAL CONTROL NON-DEREPRESSIBLE5 (*GCN5*) is a HAT that positively regulates freezing tolerance in Arabidopsis (Vlachonasios *et al.*, 2003). The induction time of *COR* genes is delayed and their expression levels are reduced in the *gcn5* mutant compared to the WT, indicating that *GCN5* positively regulates freezing tolerance by modulating the histone acetylation of *COR* genes (Vlachonasios *et al.*, 2003) (Fig. 3). Indeed, cold-induced *COR* expression is associated with histone modification levels. In Arabidopsis, H3K27me3 levels in two *COR* genes, *COR15A* and *GOLS3*, decrease gradually upon cold treatment (Choi, 2010). Histone acetylation of *OsDREB1b* in rice and *ZmDREB1A* and *ZmCOR413* in maize is induced by cold stress (Hu *et al.*, 2011; Roy *et al.*, 2014). RNA-DIRECTED METHYLATION4 (*RDM4*) protein was

reported to function in RNA-directed DNA methylation (RdDM) by working with RNA polymerases Pol V and Pol II in Arabidopsis (He *et al.*, 2009). Under cold stress, *RDM4* is important for Pol II occupancy at the promoters of *CBF2* and *CBF3* genes (Chan *et al.*, 2016).

Arabidopsis *HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENE15* (*HOS15*) encodes a WD40-repeat protein involved in histone deacetylation and cold tolerance (Zhu *et al.*, 2008). However, little is known about how *HOS15* regulates *COR* expression and freezing tolerance. A recent study showed that *HOS15* interacts with and works together with HISTONE DEACETYLASE 2C (*HD2C*) to regulate the expression of *COR* genes, including *COR47* and *COR15A*, by directly binding to their promoters (Park *et al.*, 2018) (Fig. 3). Under warm temperatures, the *HOS15*-*HD2C* complex occupies the promoters of *COR* genes and induces the hypoacetylation of *COR* chromatin, leading to the inhibition of *COR* gene expression. Upon cold stress, *HOS15* functions as an E3 ubiquitin ligase by recruiting *CUL4* (*CULLIN4*) to degrade *HD2C*. This process results in the hyperacetylation of H3 on *COR* chromatin, which consequently enhances the ability of *CBFs* to bind to *COR* promoters (Park *et al.*, 2018) (Fig. 3). These findings suggest that epigenetic regulation is an important mechanism for plant responses to cold stress.

4. Post-translational regulation in cold signalling pathway

Besides transcriptional and post-transcriptional regulation, emerging evidence has been shown that post-translational modifications also are important for plant cold responses, including protein phosphorylation/desphosphorylation, ubiquitination, sumoylation and myristoylation.

Protein kinases and phosphatases have been reported to be key regulators of cold-stress responses in prokaryotes and eukaryotes. In the unicellular cyanobacterium *Synechocystis*, the histidine kinase

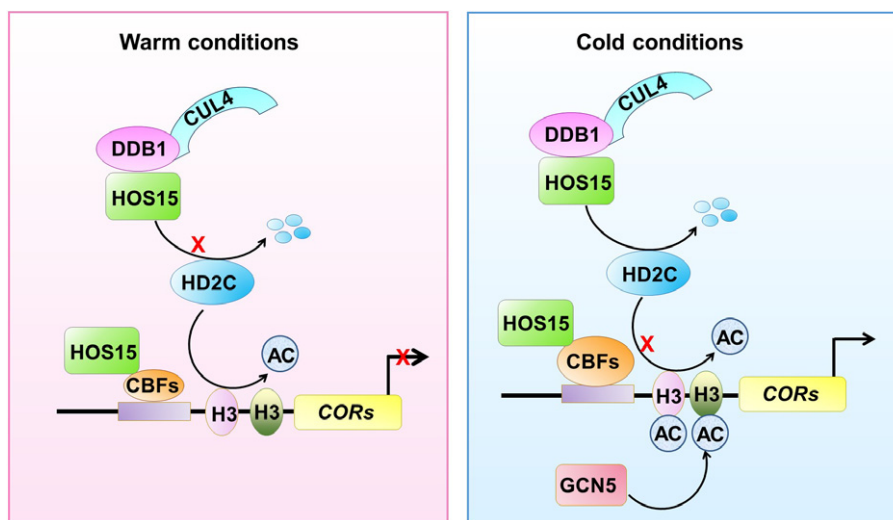


Fig. 3 Epigenetic regulation of *COLD REGULATED* (*COR*) genes in Arabidopsis. Under warm temperatures, *HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENE15* (*HOS15*) forms a complex with *HD2C* that represses *COR* expression via hypoacetylation of *COR* chromatin. Under cold conditions, *HOS15* acts as an E3 ubiquitin ligase in association with *DNA DAMAGED BINDING PROTEIN1* (*DDB1*) and *CULLIN4* (*CUL4*) that degrades *HISTONE DEACETYLASE 2C* (*HD2C*), thereby leading to the hyperacetylation of histone H3 on *COR* chromatin. This renders *CBF* proteins to bind to the *COR* promoters via *HOS15* and activates *COR* expression. Moreover, *GENERAL CONTROL NON-DEREPRESSIBLE5* (*GCN5*) modulates the histone acetylation of *COR* genes.

Hik33 acts as a cold sensor (Suzuki *et al.*, 2000a,b). In *Bacillus subtilis*, the histidine kinase DesK functions in cold signal perception (Aguilar *et al.*, 2001; Albanesi *et al.*, 2004, 2009). Interestingly, DesK can have both protein kinase and phosphatase activities under different temperature conditions (Aguilar *et al.*, 2001; Albanesi *et al.*, 2004, 2009). At warm temperatures, DesK acts as a phosphatase that removes the phosphoryl group from DesR. With the decrease in temperature, the protein structure of DesK changes and it acquires histidine kinase activity. Activated DesK phosphorylates the downstream regulator DesR, thereby activating its target gene, *Des*, to maintain membrane fluidity under cold stress (Albanesi *et al.*, 2009; Cybulski *et al.*, 2015). There is currently no evidence that a protein with both kinase and phosphatase activity functions in plant responses to cold stress. In Arabidopsis, receptors of the plant hormone cytokinin that function as histidine kinases are negative regulators of freezing tolerance (Jeon *et al.*, 2010). It will be interesting to explore whether the mechanism of thermo-sensing by histidine kinases is conserved in the plant kingdom, and whether there is a kinase + phosphatase pair responsible for cold perception.

Pharmacological experiments have demonstrated that protein kinases and phosphatases are involved in plant responses to cold stress. In alfalfa (*Medicago sativa*), the protein phosphatase inhibitor induces the expression of *CAS15*, a *COR* gene, at warm temperatures; however, the protein kinase inhibitor suppresses its induction under cold stress (Monroy *et al.*, 1998). Furthermore, cold stress inhibits the activity of phosphatase 2A in alfalfa (Monroy *et al.*, 1998).

Several key protein kinases and phosphatases have been shown to be involved in cold signal transduction in plants (Fig. 4). We demonstrated previously that OPEN STOMATA1 (OST1), a member of the SNF1-related protein kinase family, positively regulates freezing tolerance in Arabidopsis. The kinase activity of OST1 is activated by cold stress. Cold-activated OST1 interacts with and phosphorylates ICE1 to promote its protein stability and binding activity to the *CBF3* promoter, thereby enhancing freezing tolerance (Ding *et al.*, 2015). Our further study demonstrated that OST1 also interacts with and phosphorylates BASIC TRANSCRIPTION FACTOR3s (BTF3s), β -subunits of a nascent polypeptide-associated complex (NAC) proteins, and facilitates their interaction with CBF proteins, and thus stabilize CBF proteins under cold stress (Ding *et al.*, 2018a). The type 2C phosphatase, ABA INSENSITIVE1 (ABI1), partially inhibits OST1 activity under both abscisic acid (ABA) treatment and cold stress (Ding *et al.*, 2015). The gain-of-function mutant *abi1-1* (in the Col-0 background) shows reduced freezing tolerance, whereas loss-of-function *abi1 abi2 hab1* triple mutants show enhanced tolerance to freezing stress (Ding *et al.*, 2015). Recently, we found that protein phosphatase CLADE E GROWTH-REGULATING2 (EGR2) is a crucial component regulating the activity of OST1 in response to cold stress (Ding *et al.*, 2019). Myristoylation of EGR2 catalyzed by NMT1, an *N*-myristoyltransferase, under warm conditions is important for its interaction with and inhibition of OST1. Under cold stress, the interaction of EGR2 and

NMT1 is attenuated, leading to the accumulation of newly synthesized unmyristoylated EGR2 (u-EGR2). The u-EGR2 shows decreased binding ability to OST1, and it also interrupts the interaction of EGR2 and OST1, thereby releasing OST1 inhibition from EGR2 and increasing freezing tolerance (Ding *et al.*, 2019).

Upon cold stress, cytosolic Ca^{2+} concentrations are dramatically and rapidly increased (Knight *et al.*, 1996). Ca^{2+} -DEPENDENT PROTEIN KINASES (CDPKs), Calcineurin B-like proteins (CBLs), and CBL-INTERACTING PROTEIN KINASES (CIPKs) are shown to modulate freezing or chilling tolerance in Arabidopsis, rice and wheat (Kim *et al.*, 2003; Abbasi *et al.*, 2004; Komatsu *et al.*, 2007; Li *et al.*, 2008; Boudsocq & Sheen, 2012). A recent study reported that in rice, Ca^{2+} -DEPENDENT KINASE24 (OsCPK24) may regulate glutathione concentrations by phosphorylating a glutathione-dependent thioltransferase Grx10 (GLUTAREDOXIN4), and therefore confers chilling tolerance (Liu Y. *et al.*, 2018). However, it is not clear whether or not Ca^{2+} transduces cold signals in plant cells by acting in concert with these protein kinases.

CRLK1 and CRLK2, two calcium/calmodulin-regulated receptor-like kinases, positively regulate freezing tolerance by inhibiting the cold-induced activity of MAP KINASE3/6 (MPK3/6) (Yang H. *et al.*, 2010; Yang T. *et al.*, 2010a; Zhao *et al.*, 2017). In Arabidopsis, MPK6 phosphorylates MYB15 and reduces its binding to the *CBF* promoters to release its inhibitory effect on *CBF* expression (Kim S. H. *et al.*, 2017). However, two recent back-to-back reports provide strong genetic and biochemical evidence showing that Arabidopsis MPK3/6 are negative regulators of the CBF signalling pathway (Li *et al.*, 2017a; Zhao *et al.*, 2017). In Arabidopsis, cold-activated MPK3/6 interact with and phosphorylate ICE1 to reduce its stability and its binding activity to the *CBF3* promoter, leading to reduced freezing tolerance (Li *et al.*, 2017a; Zhao *et al.*, 2017). Moreover, the MAPK/ERK KINASE KINASE1-MAP KINASE KINASE2-MAP KINASE KINASE4 (MEKK1-MEK2-MPK4) cascade positively regulates *CBF* gene expression and freezing tolerance by antagonizing the MKK4/5-MPK3/6 pathway (Zhao *et al.*, 2017). In rice, OsMPK3 phosphorylates OsICE1 and disrupts its interaction with the E3 ubiquitin ligase OsHOS1. This, in turn, enhances OsICE1 protein stability and its ability to bind to *TREHALOSE-6-PHOSPHATE PHOSPHATASE1* (*OsTPP1*), which encodes a key enzyme catalyzing the biosynthesis of the sugar trehalose, thereby enhancing chilling tolerance (Zhang *et al.*, 2017). These findings indicate that MAPK3 plays diverse roles in cold tolerance in different species and that Ca^{2+} and the MAPK cascade are important transducers of cold signals.

Direct evidence for the transduction of cold signals from the plasma membrane to the nucleus was obtained through the discovery of COLD-RESPONSIVE PROTEIN KINASE1 (CRPK1). This cytoplasmic receptor-like kinase is localized at the plasma membrane (Liu *et al.*, 2017). After CRPK1 is activated by cold, it phosphorylates 14-3-3 proteins, which causes their translocation from the cytosol to the nucleus, where they interact with CBF proteins and promote their degradation (Liu *et al.*, 2017). Several important aspects of CRPK1 merit further investigation. For example, how is CRPK1 activated by cold stress? Is

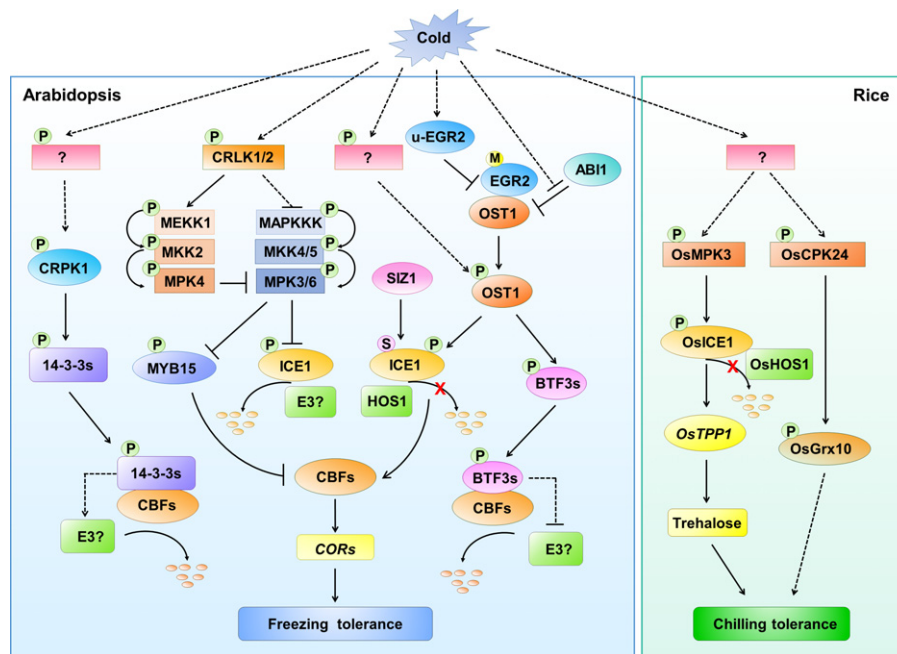


Fig. 4 Regulatory network of protein kinases in cold signalling in Arabidopsis and rice. In Arabidopsis, OPEN STOMATA1 (OST1) activity is inhibited by phosphatases CLADE E GROWTH-REGULATING2 (EGR2) and ABA INSENSITIVE1 (ABI1) under normal temperatures. Upon cold stress, EGR2 myristoylation is suppressed, the resulting unmyristoylated EGR2 shows decreased affinity to OST1, and it also interrupts the EGR2–OST1 interaction, thus releasing OST1 activity. Cold-activated OST1 interacts with and phosphorylates INDUCER OF *CBF* EXPRESSION1 (ICE1), which prevents ICE1 degradation mediated by HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENE15 (HOS1), thus promotes its stability and binding ability to *CBF* promoters. OST1 also phosphorylates BTF3s and promotes their interaction with C-REPEAT BINDING FACTORS (CBFs), which enhances *CBF* stability under cold stress. Cold stress also can activate MPK3/6, which are inhibited by cold-activated CALCIUM/CALMODULIN-REGULATED RECEPTOR-LIKE KINASE1/2 (CRLK1/2) and the MAPK/ERK KINASE KINASE1-MAP KINASE KINASE2-MAP KINASE KINASE4 (MEKK1-MKK2-MPK4) cascade. MAP KINASE KINASE3/6 (MPK3/6) phosphorylate ICE1 and promote its degradation, thereby inhibiting its binding to *CBF* promoters. Conversely, MPK6 phosphorylates MYB15 to decrease its binding to the promoters of *CBFs*. Plasma membrane-localized COLD-RESPONSIVE PROTEIN KINASE1 (CRPK1) is activated by cold stress via an unknown mechanism. Cold-activated CRPK1 phosphorylates 14-3-3 proteins and promotes their accumulation in the nucleus, thus facilitating *CBF* protein degradation by the 26S proteasome pathway. In rice, OsMPK3 phosphorylates OsICE1, suppressing its degradation and thereby increasing trehalose production and enhancing chilling tolerance. OsCPK24 (Ca^{2+} -DEPENDENT PROTEIN KINASE24) phosphorylates glutathione-dependent thioltransferase OsGrx10 (GLUTAREDOXIN4) and positively regulates chilling tolerance in rice.

there a receptor-like protein kinase pair as a partner working together with CRPK1? Is calcium signal involved in CRPK1-mediated cold signalling?

In addition to phosphorylation, the role of protein degradation mediated by the ubiquitination pathway in cold signalling has been extensively studied (Fig. 4). To date, the best-characterized E3 ubiquitin ligase in the *CBF* signalling pathway is HIGH EXPRESSION OF OSMOTICALLY RESPONSIVE GENE1 (HOS1) (Ishitani *et al.*, 1998; Dong *et al.*, 2006). The *HOS1* locus was first identified using a forward genetic screen in Arabidopsis (Ishitani *et al.*, 1998). HOS1 interacts with ICE1 in the nucleus and promotes its degradation under cold stress (Dong *et al.*, 2006). Transgenic plants overexpressing *HOS1* show increased freezing sensitivity (Dong *et al.*, 2006). Intriguingly, OST1-mediated phosphorylation of ICE1 interferes with its interaction with HOS1 (Ding *et al.*, 2015). Like ICE1 in Arabidopsis, OsICE1 in rice also is degraded by OsHOS1 (Zhang *et al.*, 2017). However, the degradation of OsICE1 is inhibited rather than promoted by phosphorylation mediated by OsMPK3 (Zhang *et al.*, 2017).

Sumoylation mediated by SUMO E3 ligases usually protects proteins from degradation. In Arabidopsis, *SIZ1* (*SAP* and *Miz*)

encodes a SUMO E3 ligase that is required for freezing tolerance (Miura *et al.*, 2007). *SIZ1* sumoylates and stabilizes ICE1, thereby promoting *CBF* expression (Miura *et al.*, 2007). The *siz1* null mutant exhibits impaired cold-induced *CBF* expression and freezing tolerance (Miura *et al.*, 2007). *CBF* proteins were recently found to be degraded by the 26S proteasome pathway (Liu *et al.*, 2017; Ding *et al.*, 2018a); however, the E3 ligase remains unknown. Future study will identify the E3 ubiquitin ligase(s) that mediate *CBF* degradation.

Autophagy is another important protein degradation system that removes damaged or toxic components from cells (Liu & Bassham, 2012). ATG proteins are key components of autophagy (Liu & Bassham, 2012; Michaeli *et al.*, 2016). Some *ATGs* are regulated by cold stress in various plant species. For instance, *CaATGs* are upregulated by low temperatures in bell pepper (*Capsicum annuum*), and many *NtATGs* are downregulated in tobacco (*Nicotiana tabacum*) (Zhou *et al.*, 2015; Zhai *et al.*, 2016). *ATG8* family genes contain cold-responsive elements and are induced more rapidly by cold than by other abiotic stresses in wheat (Pei *et al.*, 2014). These findings point to a tight link between

cold responses and autophagy. It would be interesting to investigate the role of autophagy in cold tolerance in more detail.

VI. Conclusions and perspectives

Although the cold signalling pathway in plants has been extensively studied during the past two decades, we are still far away from understanding the molecular mechanism underlying cold signal perception and transduction in plants.

Cold-induced membrane rigidification is thought to represent a primary cold-sensing event (Orvar *et al.*, 2000). However, the protein(s) involved in sensing membrane rigidification are unknown. Calcium channels might be involved in low-temperature sensing in plants. Ca^{2+} -mediated signal transduction within plant cells is relayed to downstream protein kinases and transcription factors (Kudla *et al.*, 2018). Moreover, cold-induced inactivation of the protein phosphatase, PP2A, is mediated by Ca^{2+} influx (Monroy *et al.*, 1998). Thus, future study will identify downstream regulators of Ca^{2+} in the cold signalling pathway, especially protein kinases and protein phosphatases.

Another important issue is how specific plant tissues or organelles respond to cold stress. A recent breakthrough study showed that chilling stress induces the death of columella stem cell daughters and in turn induces DNA damage in root stem cells (their early descendants), which prevents the further division of columella stem cells. This protective mechanism improves the root's ability to overcome cold stress (Jing *et al.*, 2017). In roots, vascular tissue might be an important site for plant responses to cold stress, because the crucial cold-signalling regulators such as OST1 and BTF3s are localized to the vasculature in roots and leaves (Mustilli *et al.*, 2002; Ding *et al.*, 2018a). Stomata also are regarded as important tissues in cold stress responses. Chilling-tolerant species have a higher stomatal index and stomatal frequency than nontolerant species (Palta & Li, 1979), and low temperature induces stomatal closure in the cold-tolerant species *Commelina communis* (Wilkinson *et al.*, 2001). Moreover, oscillations of Ca^{2+} concentrations in Arabidopsis guard cells are induced by cold stress, resulting in stomatal closure (Allen *et al.*, 2000). Intriguingly, the protein kinase OST1 and the transcription factor ICE1 are predominantly localized to stomata (Mustilli *et al.*, 2002; Kanaoka *et al.*, 2008). OST1 is a key regulator of stomatal movement in response to ABA (Mustilli *et al.*, 2002). Further study will focus on the following questions: (1) which organs or tissues predominantly perceive and transduce the cold signal? (2) Is cold-induced stomatal closure regulated by OST1?

Epigenetic regulation is important for plant responses to cold stress. Previous interesting results indicate that chromatin remodelling mediated by H2A.Z is responsible for thermomorphogenesis and the thermosensory activation of flowering (Kumar & Wigge, 2010; Kumar *et al.*, 2012; Tasset *et al.*, 2018). The cold-induced expression of *CBFs* is rapid and transient, peaking at 2–3 h after cold treatment (Thomashow, 1999). Moreover, CBF proteins peak at 6 h (Liu *et al.*, 2017; Ding *et al.*, 2018a); however, the expression of *CORs* peaks at 24 h after cold treatment (Thomashow, 1999). Therefore, there might be an epigenetic switch on the promoters of

CBFs and *CORs*, which might be identified by chromatin structural analysis of these genes and CHIP-seq assays of the whole genome.

Emerging evidence indicates that cold response is tightly associated with defence responses. In Arabidopsis, R proteins confer low-temperature-induced cell death in Arabidopsis (Huang *et al.*, 2010; Yang T. *et al.*, 2010b; Wang *et al.*, 2013; Bao *et al.*, 2014). Cold-regulated transcription factors such as NAC WITH TRANSMEMBRANE MOTIF1 (NTM1)-LIKE6 (NTL6) and ZAT6 are positive regulators of *PR* expression and pathogen resistance (Seo *et al.*, 2010; Shi *et al.*, 2014). Moreover, the transcription factor CAMTA3 directly modulates expression of *CBF2* and genes involved in salicylic acid, a key plant hormone in pathogen resistance (Du *et al.*, 2009; Kim Y. S. *et al.*, 2017). However, the regulation of CAMTA3 during cold stress and pathogen infection is quite different (Zhang *et al.*, 2014; Kim Y. S. *et al.*, 2017). A recent study reported that the SA receptor NPR1 plays an important role in freezing tolerance in plants independently of SA (Olate *et al.*, 2018). These findings suggest that the pathogen and cold responses share many common components. It will be interesting to explore the evolutionary link between these two pathways, whether the same protein (e.g. NPR1) senses both cold and pathogen signals by changing its own conformation, and how the same protein is differentially regulated under different stress conditions.

Although global temperature is increasing, cold extremes and abnormal weather have been observed to increase all over the world (Gupta & Deswal, 2014; Horton *et al.*, 2015). Meanwhile, to extend planting area to high latitude and high altitude, there is an urgent need to develop and cultivate cold-resistant crop varieties. Genetic modification of previously reported cold-stress regulators is an important strategy for enhancing cold tolerance in crops. In addition, identifying quantitative trait loci using high-density mapping populations and genome-wide association studies is a worthwhile approach for exploring novel genes involved in plant resistance to low temperatures.

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ORCID

Shuhua Yang  <https://orcid.org/0000-0003-1229-7166>

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