Chapter 17
ABA Regulation of the Cold Stress Response in Plants

Yiting Shi and Shuhua Yang

Abstract Low temperature is a major environmental factor that limits plant growth, productivity, and distribution. To ensure optimal growth and survival, plants must respond and adapt to cold stress using a variety of biochemical and physiological processes. Currently, the most thoroughly understood cold-signalling pathway is the C-repeat binding factor/DRE-binding factor (CBF/DREB) transcriptional regulatory cascade. Abscisic acid (ABA) is an important stress hormone in plants that has been demonstrated to be involved in the cold stress response through regulation of a set of specific stress-responsive genes. The current consensus is that both ABA-dependent and ABA-independent pathways are involved in plant responses to cold stress. This chapter summarises recent progress made in our understanding of cold signalling and the role of ABA in cold stress, and we also address cross talk between ABA and several classical phytohormones that integrate with cold signalling.

Keywords Cold signalling · ABA regulation · Plant responses · Cross talk

17.1 Introduction

As sessile organisms, plants suffer from a variety of abiotic environmental stresses, including low temperature, which can limit plant growth and the geographic distribution of a plant species, potentially impacting the reproduction of economically important crops. To adapt to adverse environments, plants have

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evolved a number of complex mechanisms to avoid or tolerate cold stress. Cold acclimation is one of the most thoroughly understood mechanisms contributing to increased cold tolerance in plants. During cold acclimation, plants modulate multiple morphological and physiological processes in order to establish a new state of cellular and metabolic homeostasis, which also involves in changes in phytohormone homeostasis, allowing for adaptation to stressful conditions (Hirayama and Shinozaki 2010; Theocharis et al. 2012; Zhu et al. 2007).

Abscisic acid (ABA) is a well-known stress hormone that plays a crucial role in dehydration stress. However, it has also been suggested that ABA plays a role in cold acclimation by triggering specific cellular and molecular osmotic responses. This chapter highlights the latest advances in our understanding of cold-response mechanisms that are either directly or indirectly influenced by ABA signalling. In particular, we focus on the regulatory networks used for signal perception, signal transduction, and the regulation of stress-responsive genes. Furthermore, both antagonistic and synergistic cross talk between ABA and other plant hormones involved in cold signalling will be discussed.

17.2 Cold-Signal Sensing and Transduction Pathways

Low temperatures can severely disrupt the metabolism and physiological homeostasis of plant cells and can even lead to plant death. Cold stress can be divided into non-freezing chilling stress (below 15 °C and above freezing point) and freezing stress (below freezing point). Chilling stress inhibits the activities of enzymes involved in photosynthesis, respiration, and biochemical processes such as reactive oxygen species (ROS) scavenging, leading to oxidative damage that can result in toxic compounds accumulation and the inhibition of metabolic reactions (O'Kane et al. 1996; Yang et al. 2005). Freezing stress results in the formation of intracellular ice crystals that induce cellular dehydration and osmotic stress, leading to membrane damage, and ultimately, to death of tissues (Uemura et al. 1995; Webb and Steponkus 1993). Most temperate plants are able to tolerate freezing stress following prior exposure to chilling, non-freezing temperatures, which is referred to as cold acclimation (Thomashow 1999). During cold acclimation, plants initiate global transcriptome changing and become tolerant to freezing temperatures by increasing the accumulation of osmolytes (such as soluble sugars) and antifreezing proteins, as well as by altering membrane composition, which together protect plant cells from dehydration and metabolic disruption (Yamada et al. 2002).

It has been noted that a reduction in the fluidity of the plasma membrane appears to be a primary event for the cold signalling sensing (Yamada et al. 2002). For example, in *Synechocystis*, histidine kinases (Hiks) are used to percept decreased levels of unsaturated fatty acids in the plasma membrane and activate the cold-induced *des* genes, which are responsible for the feedback maintenance of membrane lipid composition to modulate cold tolerance (Suzuki et al. 2000).

Lipid composition of membranes, especially the portion of galactolipids containing unsaturated fatty acids, is crucial for high plant species during cold acclimation. In *Arabidopsis*, the expression of *FAD2*, which encodes an enzyme that is essential for polyunsaturated lipid synthesis. *fad2* mutants show irregular membrane composition and cannot survive at low temperature (Miquel et al. 1993). Similar chilling-sensitive phenotypes are also observed in loss-of-function *fad5* (Hugly and Somerville 1992). A recent study showed that a lipid desaturase, acyl-lipid desaturase2 (ADS2), is required for chilling and freezing tolerance in *Arabidopsis* and functions by altering membrane lipid composition (Chen and Thelen 2013). Furthermore, the *Arabidopsis* sensitive to freezing2 (SFR2) protein was identified as a galactolipid-remodelling enzyme that is localised to the outer chloroplast membrane, and it is essential for membrane lipid remodelling of chloroplast envelope under freezing stress (Moellering et al. 2010; Fourrier et al. 2008).

Under cold stress, membrane rigidity triggers second-messenger molecules such as Ca^{2+} and to activate complex signaling pathways involved in protein kinases or transcription-factor cascades (Viswanathan and Zhu 2002). However, the underlying mechanisms that plants use to perceive and transduce cold signals remain elusive. Currently, the most thoroughly understood cold-signalling pathway is the ICE–CBF–COR transcriptional cascade, which plays a crucial role in the activation of multiple downstream *cold-regulated* (*COR*) genes (Thomashow 2010) (Fig. 17.1). Acting as central nodes in the cold acclimation pathway, the C-repeat binding factors (CBFs) gene family is conserved in many plant species. In *Arabidopsis*, three CBF transcription factors belonging to the AP2/ERF (apetala 2/ethylene-responsive factor) superfamily have been identified. CBFs are also known as dehydration-responsive element binding factors (DREBs), which recognise the conserved cold- and dehydration-responsive C-repeat/DRE DNA motifs found within the promoter regions of *COR* genes (Liu et al. 1998). *CBF1* overexpression triggers constitutive expression of the *COR* genes and induces freezing tolerance in *Arabidopsis* (Jaglo-Ottosen et al. 1998). Expression of the *CBFs* is rapidly induced by cold temperatures, and a growing number of studies have demonstrated that transcriptional regulation of the *CBF* genes is controlled by multiple mechanisms. In *Arabidopsis*, CBF2 is a negative regulator of the expression of *CBF1* and *CBF3*, and it plays an important role in freezing tolerance (Novillo et al. 2004; Novillo et al. 2007). To date, several key upstream regulators of the *CBF* genes have been identified and characterised. CAMTA3 encodes a calmodulin-binding protein and acts upstream to activate *CBF2* expression by binding to the CG-element in its promoter region in response to cold signals (Doherty et al. 2009). The MYC-type, basic helix-loop-helix transcriptional activator inducer of CBF expression 1 (ICE1) has been identified as a transcriptional activator that binds to MYC *cis*-elements within the *CBF3* promoter, and the expression of *CBF3* and its target *COR* genes is impaired in *ice1*-mutant plants during cold acclimation. In addition, overexpression of ICE1 in *Arabidopsis* results in increased freezing tolerance, supporting a pivotal role for ICE1 in the cold stress response (Chinnusamy et al. 2003). The transcription

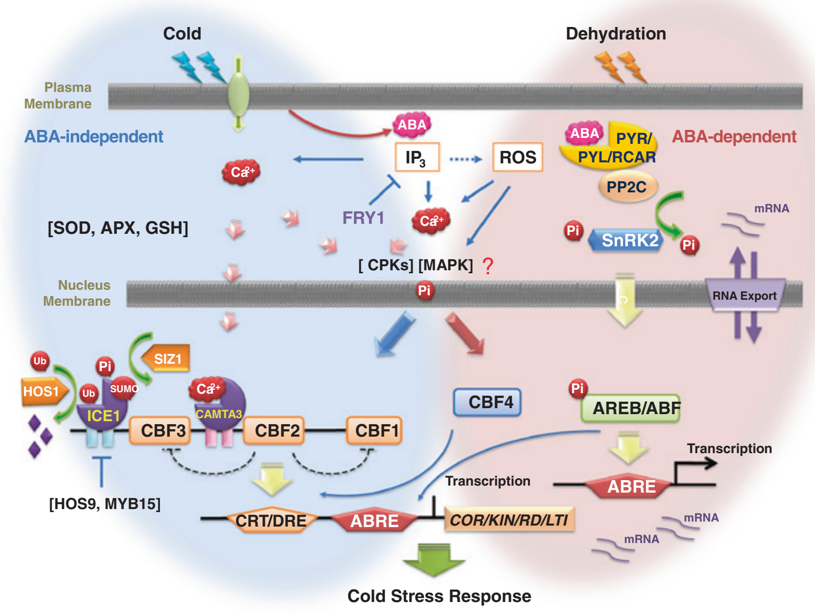

Fig. 17.1 Schematic illustration of the cold and dehydration response regulatory networks in *Arabidopsis*. IP_3, Ca^{2+}, and ROS act as second messengers in signalling networks to transduce signals through protein kinases or transcription-factor cascades. CBFs and AREB/ABFs transcription factors are responsible for the regulation of *COR* genes containing CRT/DRE (CCGAC) and ABRE (ACGT) motifs in their promoters, respectively. In *Arabidopsis*, approximately 10 % of the cold-induced transcriptome contains both CRT and ABRE motifs in their promoter regions, which are upregulated by both cold and dehydration stresses. CBFs are activated by ICE1 and CAMTA transcription factors, whereas repressed by MYB15. HOS1 and SIZ1 encode RING E3 ligase and SUMO E3 ligase, respectively, which antagonistically regulate the abundance of ICE1 protein. Cold activates ICE1 protein and AREB/ABFs are induced by ABA-mediated dehydration signalling pathway. *IP_3* inositol 1,4,5-triphosphate, *ROS* reactive oxygen species, *CPK* calcium-dependent protein kinase, *MAPK* Ras-mitogen-activated protein kinase, *Ub* ubiquitin moiety, *SUMO* Small ubiquitin-related modifier, *Pi* phosphoryl group

factor EIN3 was shown to negatively regulate expression of the *CBFs* by directly binding to *CBF* promoters to modulate the cold stress response in plants. Furthermore, a loss-of-function *ein3* mutant shows enhanced freezing tolerance, whereas overexpression of *EIN3* leads to decreased freezing tolerance (Shi et al. 2012). Using a genetic screening strategy involving changes in the expression of a luciferase construct under the control of a stress-inducible *RD29A* promoter, high expression of osmotically responsive genes1 (HOS1), which encodes a RING-type ubiquitin E3 ligase, was isolated as an upstream negative regulator of the *CBFs*. HOS1 interacts with and ubiquitinates ICE1 to negatively regulate the stability of the ICE1 protein (Dong et al. 2006). The R2R3-type MYB transcription factor AtMYB15 (MYB domain protein 15) was found to physically interact with ICE1. *MYB15* overexpression leads to reduced expression of the *CBF* genes, whereas a*myb15* mutant shows increased *CBF* expression. MYB15 binds to the *CBF3* promoter to repress its *CBF* expression and negatively regulates freezing tolerance (Agarwal et al. 2006). Subsequently, a SUMO E3 ligase, SAP and Miz (SIZ1), was identified as a positive regulator of the ICE1 protein. SIZ1 can sumoylate ICE1 and repress the polyubiquitination of ICE1, which, in turn, enhances ICE1 stability (Miura et al. 2007). Therefore, regulation of the ICE1-CBF cascade at both transcriptional and post-translational levels demonstrates the existence of a complex network of CBF-dependent cold-signalling pathways.

17.3 The Role of ABA Biosynthesis and Signalling in Cold Stress

Investigations into the role of ABA in cold stress were originally based on observations made in the 1960s in woody species, showing that application of the gibberellic acid (GA) inhibitor dormin—which was later identified as ABA-resulted in increased freezing tolerance in trees, equivalent to that observed in plants that had undergone cold acclimation (Chrispeels and Varner 1967; Thomas et al. 1965). It has been shown that cold tolerance is usually accompanied by increased endogenous ABA levels in various plant species (Daie and Campbell 1981; Lang et al. 1994; Mantyla et al. 1995). Continuous application of ABA induces chilling tolerance in chilling-sensitive plant species, such as maize, rice, cucumber, and pepper. Furthermore, exogenous ABA application in temperate plants such as poplar, barely, wheat, and *Arabidopsis* can partially mimic cold acclimation and enhance freezing tolerance (Kadlecová et al. 2000; Smoleńska-Sym et al. 1995; Zhu et al. 2000; Thomashow 1999). Transcriptome analyses in *Arabidopsis* showed that a number of ABA-responsive genes can be induced by cold treatment (Zeevaart and Creelman 1988). However, it has also been shown that ABA application in several plant species has little effect on freezing tolerance, or if there are any changes, they are much less significant than those induced by cold acclimation (Gusta et al. 1982; Fayyaz et al. 1978; Holubowicz et al. 1982), suggesting the natural diversity of ABA responses in different plant species.

The primary source of increased ABA levels under stress conditions is a de novo biosynthesis pathway that converts carotenoids into bioactive ABA. ABA biosynthesis pathway may be required for full development of the cold response, as defects in both basal and acquired freezing tolerance have been observed in ABA-deficient mutants. For instance, *ABA1* and *ABA3* are identified as genes encoding enzymes involved in ABA biosynthetic pathway. Cold induction of the *COR* genes is reduced in the *aba3* mutants *aba3/los5/frs1* (Llorente et al. 2000;

Xiong et al. 2002b). Cold acclimation was shown to be impaired in an *aba1* mutant, which has also reduced expression of specific *COR* genes (Mantyla et al. 1995). The expression of none of the ABA biosynthesis genes is affected by cold treatment in *Arabidopsis* (Lee et al. 2005). Therefore, ABA biosynthesis is not an early event in response to cold stress. Consistent with this, foliar application of ABA to a wide range of plant species does not induce cold hardness or freezing tolerance (Gusta et al. 2005). A recent study showed that cold stress specifically activate the expression of ABA biosynthesis genes in reproductive organs, such as the inflorescence meristem, with only slightly increased expression in the leaves and vegetable organs (Baron et al. 2012). This finding supports the notion of ABA function in stimulating and hastening plant harvests under adverse environmental conditions. Taken together, ABA may function late in the development of cold-induced metabolic changes and is required for determining the maximum levels of cold tolerance in plants.

ABA activates a wide array of genes associated with stress response, seed dormancy, and stomatal movement through the ABA signalling pathway (Yamaguchi-Shinozaki and Shinozaki 2006). The PYR/PYL/RCAR family of proteins are ABA receptors that can inhibit the activity of the type-2C protein phosphatase PP2C in the presence of ABA, relieving the repression of the SnRK2 kinases activity, which in turn phosphorylates other downstream regulators (Furihata et al. 2006; Ma et al. 2009; Park et al. 2009). Overexpression of *TaSnRK2.3* in *Arabidopsis* results in enhanced root-system architecture and significantly enhances the tolerance of this species to drought, salt, and freezing stresses (Tian et al. 2013), whereas downregulation of PP2CA proteins increases ABA sensitivity and accelerates cold acclimation in transgenic plants (Tahtiharju and Palva 2001). Promoter analysis found that some DRE/CRT motif-containing *COR* genes are induced by cold and dehydration and impaired in ABA-deficient mutants (Thomashow 1999; Shinozaki and Yamaguchi-Shinozaki 2000). As mentioned earlier, CBFs/DREB1s and their homolog, CBF4, were shown to bind to CRT/DRE motifs in vivo. *CBF4* expression is induced by drought and ABA, but not by cold, which is quite distinctive from *CBF1–CBF3*. Overexpression of *CBF4* results in increased freezing and drought tolerance (Haake et al. 2002). It appears that at least three separate regulatory signalling pathways exist that are mediated by the binding of transcription factors to CRT/DRE motifs. Among these, cold-induced CBF1–CBF3 and drought-induced DREB2 are the primary transcription factors that control gene expression through the ABA-independent pathway, whereas CBF4 acts as a regulator of the ABA response (Thomashow 1999; Shinozaki and Yamaguchi-Shinozaki 2000). In addition to the CRT/DRE motif, many cold-response gene promoters harbour ABRE *cis*-elements that can be activated in vivo by ABA through direct binding of bZIP transcription-factor proteins (AREBs/ABFs, ABRE-binding proteins/factors) (Uno et al. 2000). This evidence explains that expression of the *COR* genes is regulated by both ABA-dependent and ABA-independent pathways (Fig. 17.1).

17.4 ABA-Dependent Signalling Pathway for Cold Acclimation

Genetic and physiological analyses indicate that ABA-dependent signalling pathway plays an essential role in stress-responsive gene expression during cold-induced osmotic stress. ABA biosynthesis genes contribute to the enrichment of *COR* genes, such as *RD29A*, *RD22*, *COR15A*, *COR47*, and *P5CS* via activity of their ABRE *cis*-element (Xiong et al. 2001a, 2002b). In the study on *Arabidopsis*, four AREB/ABF transcription factors are found to be required for the activation of ABA-mediated signalling (Uno et al. 2000). Among them, *ABF1* expression is significantly induced by cold, but not by osmotic stress (Choi et al. 2000), whereas *AREB1/ABF2*, *AREB2/ABF4*, and *ABF3* are specifically induced by ABA and drought stress (Abdeen et al. 2010; Kang et al. 2002; Fujita et al. 2005). AREB proteins do not appear to play a role in CBF-dependent cold signalling; however, one study showed that ABF2, ABF3, and ABF4 interact with DREB2C in vitro (Lee et al. 2010). In addition, approximately 10 % of ABA-responsive genes are also responsive to cold stress (Kreps et al. 2002). Thus, although ABA- and cold stress-induced transcription factors show distinct regulation, some of the *COR* genes appear to be induced by both of these stimuli. Therefore, one could speculate that limited, cold-induced ABA production could enhance *COR* gene activation through the ABA-dependent pathway during cold acclimation.

In addition to AREBs, other bZIP transcription factors belonging to the ABI5 subfamily have been characterised as ABRE-binding proteins and were shown to affect seed germination as well as the cold stress response. For example, ectopic expression of the seed-specific transcriptional activator *ABI3* confers the ability to express *COR* genes in vegetative tissues and enhances freezing tolerance in *Arabidopsis* (Tamminen et al. 2001). ABI5 has been shown to act downstream of and interact with ABI3 (Suzuki and McCarty 2008; Finkelstein and Lynch 2000, and it can be sumoylated and degraded by the SUMO E3 ligase SIZ1, which attenuates ABA signalling (Miura et al. 2009). Indeed, SIZ1-mediated SUMO conjugation is also required for the expression of *CBF3* by modulating the activity of ICE1 (Miura et al. 2007). *siz1* mutants are impaired in cold acclimation and are sensitive to ABA during seed germination (Miura et al. 2007, 2009). A recent study revealed that SIZ1 is able to sumoylate and stabilise the negative regulator of ABA signalling MYB30 (Zheng et al. 2012). Therefore, SIZ1 appears to be an integration node that mediates ABA and cold responses through post-transcriptional modifications.

Several MYC and MYB family genes have been reported to be important regulators of ABA-responsive gene expression under cold stress. For instance, MYB96 is induced by ABA and drought, and it enhances ABA-mediated drought and freezing tolerance (Guo et al. 2013; Seo et al. 2009). Expression of *OsMYB3R-2* in rice is induced by cold, drought, and salt stresses, and overexpression of *OsMYB3R-2* increases tolerance to freezing, drought, and salt stresses in transgenic *Arabidopsis* (Dai et al. 2007). Recent studies suggest that some NAC-domain family members are involved in stress responses (Olsen et al. 2005). For example, expression of a rice NAC gene, *OsNAC5*, is induced by osmotic stress and ABA, and overexpression of *OsNAC5* increases stress-induced proline and soluble-sugar levels and enhances tolerance to cold, salt, and drought stresses (Takasaki et al. 2010; Song et al. 2011). Thus, it can be assumed that cold induction of some ABA-responsive genes is regulated by different kinds of transcription factors.

17.5 Second Messengers Integrate Cold and ABA Signalling

Both cold stress and ABA treatment induce the production and accumulation of IP3, Ca^{2+}, and ROS inside the cell. These molecules act as second messengers in signalling networks to amplify and transduce signals through activating protein kinases or transcription-factor cascades. Increased inositol 1,4,5-triphosphate (IP_3) levels, which are catalysed by phospholipase C (PLC) under cold stress, are known to release Ca^{2+} from vacuoles into the cytosol (Allen and Sanders 1995; Munnik et al. 1998). In *Arabidopsis*, *FRY1* encodes an inositol polyphosphate 1—phosphatase that is involved in IP_3 metabolism. *fry1* mutant plants show significantly higher IP_3 levels when treated with ABA, and they display defects in cold acclimation and hypersensitivity to osmotic stress, with lowered expression of certain stress-responsive genes (Xiong et al. 2001b).

In plants, Ca^{2+} is a widely used and important signalling molecule during early responses to abiotic stresses, as it functions by activating protein kinase cascades, which in turn can activate transcription factors and stress-responsive genes. More specifically, a transient influx of cytoplasmic Ca^{2+} occurs in response to cold shock, and it has been suggested that low-temperature-induced changes in membrane fluidity are the primary thermosensor signal that activates the Ca^{2+} influx in plants (Knight et al. 2004). Furthermore, calcium is required for the full expression of the *COR* genes in *Arabidopsis*. *CAX1*, which encodes a vacuolar membrane-located Ca^{2+}/H^{+} antiporter, participates in the regulation of cold acclimation. Loss-of-function mutations in *CAX1* increase *CBF* expression and enhance freezing tolerance following acclimation in *Arabidopsis* (Catala et al. 2003). One calmodulin proteins in *Arabidopsis*, AtCML9, has recently been demonstrated to be responsive to ABA, cold, and salt stress through an ABA-dependent pathway. The *cml9* mutant exhibits hypersensitivity to ABA and enhanced tolerance to stress and drought (Magnan et al. 2008). CaM methylation is also involved in the early growth of *Arabidopsis* and responds to ABA, cold, and heat stress during germination (Banerjee et al. 2013). As mentioned earlier, the calmodulin-binding transcriptional activator *CAMTA3* is involved in the regulation of freezing tolerance (Doherty et al. 2009). More importantly, a recent study suggested that calcium channels may involve in temperature perception in land plants, based on the observation that plants lacking the membrane protein cyclic nucleotide gated calcium channel (CNGC) showed constitutively high levels of Ca^{2+} influx and impaired thermoperception in a moss and in *Arabidopsis* (Finka et al. 2012). Moreover, a homologue of the synaptic Ca^{2+}-binding protein synaptotagmin (SYT1), which is a calcium sensor that can prevent Ca^{2+}-dependent membrane, damages in *Arabidopsis* protoplasts under freezing conditions (Yamazaki et al. 2008). These results suggest the central role of cytosolic calcium to trigger cold signalling. Ca^{2+} may function through calmodulin or Ca^{2+} sensors to modulate the activation of downstream calcium-dependent protein kinases (CPKs). Calcineurin B-like proteins (CBLs), one kind of calcium sensors, were also shown to regulate expression of the *CBF* genes, based on the observations that overexpression of *CBL1* resulted in reduced freezing tolerance, whereas *cbl1* null mutants showed increased cold-induced expression of stress genes and enhanced freezing tolerance (Albrecht et al. 2003; Cheong et al. 2003).

Several CBL-interacting protein kinases (CIPKs) and CPKs have been implicated in the responses to ABA, cold, and high-salt stress. In *Arabidopsis*, the expression of *CIPK3* is strongly induced by cold stress and ABA treatment, but not by drought stress. In addition, the expression of various cold-induced marker genes is all significantly delayed in a *cipk3* mutant, and the induction of ABA-responsive genes is also significantly inhibited (Kim et al. 2003). Therefore, CIPK3 may function as a cross talk node between signalling pathways for ABA, cold, and other abiotic stresses. It has also been suggested that CIPK1 is a convergence point for the ABA-dependent and ABA-independent stress responses. For example, CIPK1 can interact with CBL1 and CBL9, resulting in the formation of CBL1/CIPK1 and CBL9/CIPK1 complexes that mediate the ABA-independent and ABA-dependent responses, respectively, during osmotic stress (D'Angelo et al. 2006). Rice *OsCDPK7* is a cold- and salt-inducible gene, and overexpression of *OsCDPK7* confers cold and salt/drought tolerance on rice plants (Abbasi et al. 2004). Collectively, these results indicate a central role for calcium in sensing and transducing the cold and ABA signals.

17.6 ABA and ROS Under Cold Stress

The induction of ROS-scavenging systems is common to many stress pathways including ABA and cold stress (Fujita et al. 2006). The roles of ROS in cold stress are considered in two ways. On the one hand, excessive ROS levels are toxic and can lead to lipid peroxidation, and ultimately, to oxidative stress. On the other hand, increased ROS levels can also play a benefit signalling role and activate scavenging enzyme systems to eliminate excess ROS in plants subjected to low temperatures. Numerous studies have shown that ROS-scavenging enzymes such as superoxide dismutase (SOD), ascorbate peroxidise (APX), catalase (CAT), and glutathione peroxidise (GPX) are required for protection against cold-induced oxidative damage (Burdon et al. 1996; O'Kane et al. 1996). For example, overexpression of antioxidant-defensive genes enhances chilling tolerance in maize and soybean (Van Breusegem et al. 1999; Kocsy et al. 2001), whereas repression of catalase gene expression reduces chilling tolerance (Kerdnaimongkol and Woodson 1999). *Arabidopsis chs* (*chilling-sensitive*) mutants display increased sensitivity to chilling temperature, which is at least partially due to the excessive accumulation of H_2O_2 in *chs* mutant seedlings (Huang et al. 2010a, b; Wang et al. 2013; Yang et al. 2010). Recent studies also show that ROS act as signal molecules mediating cold stress-signal transduction. For instance, the *Arabidopsis* mutant *frostbite1* (*fro1*) has a defective form of mitochondrial complex I NADH dehydrogenase, leading to constitutively lower ROS levels compared with the wild type, and this mutant shows reduced expression of cold stress-responsive genes and decreased cold acclimation (Lee et al.2002). *ZAT12* encodes a ROS-responsive zinc-finger transcription factor that functions as a positive regulator of the cold-response pathway. Overexpression of *ZAT12* results in enhanced freezing and oxidative tolerance (Davletova et al. 2005), which suggests that ROS signalling plays a beneficial role in mediating cold acclimation in plants.

It has been shown that the application of exogenous ABA effectively alleviates the symptoms of cold injury in many species. One explanation for this phenomenon is that ABA signalling is able to induce the transcription of ROS-scavenging enzymes. Indeed, one study showed that stress-induced ABA accumulation stimulates ROS scavenging to help maintain the cellular redox state (Guan et al. 2000). Furthermore, ABA-dependent proline accumulation under abiotic stress is regulated by the ROS-scavenging-mediated cellular redox state, which is also important for the cold response (Kishor et al. 2005). In particular, ABA treatment enhances ROS accumulation by activating plasma membrane-bound NADPH oxidases (Kwak et al. 2003). *ABO5* and *ABO6*, which encode a PPR (pentatricopeptide repeat) protein and DEXH box RNA helicase, respectively, are required for ABA-induced ROS production in mitochondria (He et al. 2012; Liu et al. 2010). The activities of ABI1 and ABI2 are inhibited by H_2O_2 in vitro (Meinhard and Grill 2001; Meinhard et al. 2002), and further studies demonstrated that ABI2 interacts with glutathione peroxidase 3 (GPX3), which is an ROS-scavenging enzyme that functions as a redox transducer and scavenger to modulate ABA signalling (Miao et al. 2006). Glucosamine (GlcN) is a naturally occurring amino sugar that inhibits plant growth by significantly increasing the production of ROS. Ectopic overexpression of GlcN induces cell death, whereas scavenging of endogenous GlcN can enhance tolerance to oxidative, drought, and cold stresses in *Arabidopsis* (Chu et al. 2010).

As well-known signalling components downstream of ROS, several mitogen-activated protein kinases (MAPKs) have been shown to be activated by cold and ABA. ABA application induces MAPK activation within only a few minutes (Knetsch et al. 1996). The MAPK cascade is an important signalling pathway that enables the transmission of environmental and hormone signals to activate regulatory components within the cytoplasm and initiate cellular-responsive processes.

In vivo assays data revealed that the MKK2–MPK4/MPK6 complex functions downstream of MEKK1 during salt and cold stress. MPK4 and MPK6 are strongly phosphorylated by MKK2 in response to cold and salt treatment, and null allele of *mkk2* is impaired in ability of cold acclimation (Teige et al. 2004). Direct evidence of MAPK activation by ABA was observed in guard cells. MPK9 and MPK12 function downstream of ROS as positive regulators of ABA signalling, and *mpk9 mpk12* double mutants are partially but specifically impaired in cold-induced stomatal closure, suggesting that they play a role in the cold-mediated ABA signalling cascade (Jammes et al. 2009). *Arabidopsis* NDP kinase 2 (NDPK2) was shown to interact with two oxidative stress-related MAPKs, MPK3, and MPK6, and overexpression of *NDPK2* enhances cold tolerance (Moon et al. 2003). Taken together, these findings raise the possibility that the MAPK cascade acts downstream of ROS in cold and ABA signalling.

17.7 The Effects of Circadian Signals on Cold and ABA Signalling

The circadian clock is regulated by a central oscillator consisting of transcription/translation feedback loops that are entrained to light and temperature cues. The MYB transcription factors circadian clock associated1 (CCA1) and late elongated hypocotyl (LHY) can directly bind to the promoters of the evening-loop components *timing of CAB expression1* (*TOC1*), *pseudo response regulator* (*PRR7*), and *PRR9* to repress their expression after dawn; conversely, PRR7 and PRR9 act as negative regulators of *CCA1* and *LHY* expression in the morning (Harmer 2009). Interestingly, *CBF* gene expression is under circadian regulation, and the cold induction of *CBFs* is gated by the circadian clock (Fowler et al. 2005). It has been shown that CCA1 and LHY are positive regulators of *CBF* expression that directly bind to *CBF* promoters (Lee and Thomashow 2012; Dong et al. 2011). In the morning, *CBF* expression increases following induction of *CCA1* and *LHY*, and *CBF* transcript levels peak shortly after those of *CCA1* and *LHY*. It has also been suggested that CAMTA3 and ICE1 act synergistically with CCA1 and LHY to induce *CBF* expression during the day (Dong et al. 2011). During the evening, the evening element TOC1 interacts with phytochrome interacting factor 7 (PIF7) to repress expression of *CBF2* by directly binding to its promoter. Therefore, the circadian-gated regulation of the *CBF* genes appears to involve the action of CCA/LHY complex and PIF7/TOC1 evening complex (Dong et al. 2011). A recent study showed that TOC1 functions as a molecular switch that connects the circadian clock to the ABA-mediated drought stress response (Legnaioli et al. 2009). ABA signalling is closely related with circadian oscillator period, for example, ABA-mediated stomatal movement varies during day and night (Tallman 2004). It was proposed that the circadian clock may modulate stomatal closure under high-temperature conditions when water supplies are limited in order to optimise water-usage efficiency (Robertson et al. 2009). TOC1 has been found to negatively regulate the expression of ABA-related genes by directly interacting with ABI3 (Kurup et al. 2000). Moreover, TOC1 can also bind to the promoter of *ABAR*, a chloroplastic ABA binding protein, to control its circadian expression, and the gated-induction of *TOC1* by ABA is abolished in *ABAR* RNAi plants. Furthermore, overexpression of *TOC1* causes significant changes in plant responses to ABA and drought stress, which are indicative of a feedback mechanism linking the circadian clock to the ABA response (Legnaioli et al. 2009). Collectively, these findings illustrate the mechanisms by which the circadian clock regulates the cold and ABA responses.

17.8 Post-Transcriptional Modification Mediates Cold and ABA Signalling

RNA splicing and transport can lead to tissue-specific differences in gene expression as well as affect mRNA stability and turnover via nonsense-mediated decay (McGlincy and Smith 2008). By performing a genetic screen for deregulated expression of *RD29A* promoter-driven luciferase reporter gene, several genes encoding RNA processing proteins were identified. *Loss of osmotic responsiveness 4* (*LOS4*) encodes a putative DEAD-box RNA helicase that has been implicated in nucleo-cytoplasmic mRNA transport. The recessive mutations *los4-1* and *los4-2* affect cold-induced *CBF* transcription and lead to changes in chilling and freezing tolerance (Gong et al. 2002, 2005), suggesting that mRNA translocation may also be a control point in cold acclimation. It was also found that a *los4-2* mutant was hypersensitive to ABA (Gong et al. 2005). A recent study identified a *regulator of CBF2 gene expression1* (*rcf1-1*) mutant that was hypersensitive to cold stress. *RCF1* also encodes a cold-inducible DEAD-box RNA helicase. However, unlike LOS4, RCF1 maintains the proper splicing of *COR* pre-mRNAs under cold stress conditions (Guan et al. 2013). Another pre-mRNA splicing factor *Stabilized1* (*STA1*) which is required for pre-mRNA splicing and mRNA turnover is upregulated by cold stress, but not by ABA or NaCl (Lee et al. 2006). A *sta1* mutant displays a chilling-sensitive phenotype and hypersensitivity to ABA-induced root growth, as well as defects in splicing of the cold-induced *COR15A* gene (Lee et al. 2006). *Arabidopsis FIERY2* (*FRY2*), which encodes an RNA polymerase II C-terminal domain (CTD) phosphatase, may function in mRNA processing. FRY2 plays a negative role in regulating salt stress and the ABA response during seed germination, and it has been shown to be a repressor of the CBF transcription factors. However, a *fry2* mutant was shown to be hypersensitive to freezing stress (Xiong et al. 2002a). Sensitive to ABA and drought2 (SAD2) was found to be involved in nucleo-cytoplasmic trafficking during ABA treatment, and *sad2* mutant plants show a hypersensitive response to ABA and enhanced expression of stress genes in response to ABA, salt, and low temperatures (Verslues et al. 2006). Therefore, post-transcriptional regulation also serves as a major mechanism in the global control of the ABA and cold responses.

17.9 Epigenetic Modifications in Cold and ABA Signalling

DNA and histones are epigenetically modified through acetylation and methylation following exposure to stress (Kim et al. 2010; Feng and Jacobsen 2011). *HOS15* encodes a protein similar to the human WD-40 repeat protein TBL1 (transducin-like protein-1), which is involved in histone deacetylation. Both ABA and cold induce the expression of *HOS15*, and *hos15-1* mutants exhibit hypersensitivity to ABA-induced inhibition of germination and freezing stress. Furthermore, HOS15 interacts with histone H4, and levels of acetylated histone H4 are higher in *hos15* mutants than in the wild type (Zhu et al. 2008). Therefore, HOS15 is involved in the regulation of ABA and the cold stress response through H4 deacetylation-dependent chromatin remodelling in *Arabidopsis*. Consistently, increased levels of acetylated histone H4 are consistently associated with increased expression of *RD29A* in a *hos15* mutant compared with the wild type under cold stress. On the other hand, histone deacetylation results in a non-permissive chromatin conformation that represses transcription. In *Arabidopsis*, histone deacetylase 6 (HDA6) has been reported to be involved in the ABA response and low-temperature-mediated flowering (Luo et al. 2012; Chen and Wu 2010). In *Arabidopsis*, *HDA6* encodes an RPD3-type histone deacetylase, and a loss-of-function mutation in*HDA6*, *axe1–5*, displays hypersensitivity to ABA and salt stress (Luo et al. 2012). Interestingly, HOS1 was discovered as a chromatin remodelling factor for cold-induced transcriptional silencing of the *FLC* gene through an interaction with HDA6, thereby antagonising the actions of FVE and resulting in the inhibition of flowering in these plants (He and Amasino 2005; Jung et al. 2013). These findings suggest that plant flowering or stress response processes might be epigenetically controlled through similar mechanisms.

17.10 Cross Talk Between Phytohormones and the Cold Response

Emerging evidence indicates that cold acclimation is influenced by changes in the homeostasis of various hormones and that hormone signalling is crucial for regulation of the CBF pathway. Genetic analyses of *Arabidopsis* mutants with compromised ABA biosynthesis or signalling have identified a complex interplay between ABA and other phytohormone-signalling pathways. Generally, when plants are challenged by stress conditions, the stress hormones ABA and jasmonate acid (JA) inhibit growth by modulating the actions of auxin, GA, and cytokinin (Achard et al. 2006; Wolters and Jurgens 2009; Peleg and Blumwald 2011). The growth inhibition decreases the capacity for energy utilisation, which in turn, results in cold acclimation processes.

17.10.1 GA and Cold Stress

As an important plant phytohormone, GA affects plant abiotic stress responses, including those to salt, oxidative, and cold stresses during germination and seedling development. Acting as GA-signalling repressors, DELLA proteins have been identified as the central modulators of growth under a variety of stress conditions (Achard et al. 2006). ABA promotes the accumulation of DELLA proteins, which induces growth repression in plant lateral roots under salt stress through an ABA-dependent pathway (Achard et al. 2006). Intriguingly, GA treatment rescues normal growth and abolishes the late-flowering phenotype of *CBF1-ox* plants (Achard et al. 2008). Further investigation revealed that cold stress causes increased expression of the biosynthetic enzyme genes *AtGA2ox3* and *AtGA2ox6*, which reduce endogenous levels of bioactive GA. Consistently, overexpression of *CBFs* represses plant growth via the accumulation of DELLA proteins (Achard et al. 2008). ABA is known to act as an antagonist of GA by inhibiting the expression of *GA20ox* and *GA3ox* to reduce GA levels during seed germination (Razem et al. 2006) (Fig. 17.2a). It has been demonstrated that the existence of a novel mechanism through which CBFs may regulate dormancy in parallel to their functions in CBF-mediated cold signalling, by regulation of *DOG1* expression, which is a positive regulator of GA catabolism and ABA biosynthesis that promotes seed maturation at cool temperatures (Chiang et al. 2011; Kendall et al. 2011). A recent study showed that ABI3 and ABI5 interact with DELLA proteins to inhibit seed germination by repressing the expression of high-temperature-induced genes (Lim et al. 2013). Thus, DELLA-dependent growth restraint is necessary for cold acclimation and temperature-mediated seed germination.

17.10.2 Cytokinin and Cold Stress

Cytokinin signalling belongs to the two-component signalling system, which involves plant adaptation to environmental stress (Argueso et al. 2009; Nishimura et al. 2004). The accumulated body of evidence indicates that cytokinin acts as an antagonist of ABA during environmental stress responses (Peleg and Blumwald 2011; Hwang et al. 2012). Based on studies involving mutants or transgenic plants with altered cytokinin biosynthesis, it has been postulated that cytokinin acts as a negative regulator to modulate abiotic stress signalling (Nishiyama et al. 2011). In *Arabidopsis*, the cytokinin receptor histidine kinases AHK2, AHK3, and CRE1 have been shown to play important roles in the regulation of plant abiotic

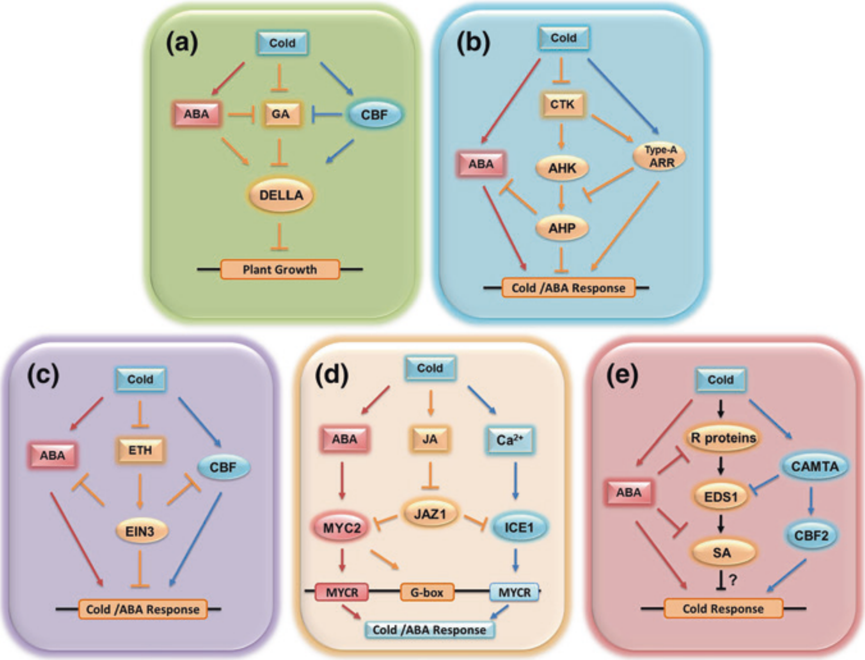

Fig. 17.2 Models of multiple interactions between ABA and other plant hormones in cold stress response. There are multiple points of interaction between ABA and other plant hormones including GA (**a**), cytokinin (**b**), ethylene (**c**), JA (**d**), and SA (**e**) in regulation of plant response to cold stress. *CTK* cytokinin, *ETH* ethylene, *DELLA* DELLA protein, *AHK Arabidopsis* histidine kinase, *AHP Arabidopsis* histidine phosphor-transferase, *ARR Arabidopsis* response regulator, *EIN3* Ethylene insensitive3, *JAZ* jasmonate-zim-domain protein, *MYC2* MYC-related transcriptional activator2

responses in both ABA-dependent and ABA-independent signalling pathways (Tran et al. 2007); on the other hand, the homologous gene *AHK1* was identified as an osmotic stress sensor that positively regulates abiotic stress (Wohlbach et al. 2008; Kumar et al. 2013; Tran et al. 2007). Increasing evidence suggests a negative role for cytokinin signalling in cold- and ABA-mediated abiotic stress. For instance, *ahk2 ahk3* double mutants are highly tolerant to cold, drought, and salt stress and show strong expression of ABA-responsive genes (Jeon et al. 2010; Tran et al. 2007). However, whether cytokinin receptors can perceive stress signals is still unclear. AHP2, AHP3, and AHP5 function as redundant negative regulators of the drought stress response in both ABA-dependent and ABA-independent manners (Nishiyama et al. 2013). These studies support the notion that AHK2–AHK4 and AHPs play negative roles under unfavourable environmental conditions and may integrate with ABA signalling to modulate stress responses (Fig. 17.2b). Type-A *ARR* genes are negative response regulators in cytokinin signalling pathway. They are induced by cytokinin as well as by cold stress (To et al. 2004).

Overexpression of type-A *ARRs* enhances freezing tolerance in *Arabidopsis* (Shi et al. 2012). In contrast, another study demonstrated that type-A ARRs are negative regulators of the cold response (Jeon et al. 2010). Although it can be assumed that the cytokinin signalling pathway plays a negative regulatory role in freezing tolerance, at least partially through inhibition of the ABA response, further study will be necessary to define the exact role of cytokinin signalling, particularly for the ARRs, in the regulation of cold signalling.

17.10.3 Ethylene and Cold Stress

Ethylene plays an important role in regulating plant responses to drought, flooding, and biotic stress (Wilkinson and Davies 2010). However, the function of ethylene in plant responses to cold stress appears to be complex and species dependent. Increased ethylene biosynthesis has been correlated with enhanced chilling and freezing tolerance in several plant species, including tomato (*Lycopersicones culentum*), cucumber (*Cucumis sativus*), and tobacco (*Nicotiana tabacum* cv. NC89) (Wang and Adams 1982; Ciardi et al. 1997; Zhang and Huang 2010). In contrast, improved cold tolerance was associated with the suppression of ethylene biosynthesis in mung bean (*Vignaradiata*) and *Arabidopsis* (Collins et al. 1995; Shi et al. 2012). In *Arabidopsis*, application of the ethylene precursor ACC decreases freezing tolerance, whereas application of the ethylene biosynthesis inhibitor AVG and the ethylene receptor antagonist $AgNO_3$ promote freezing tolerance. Consistent with these findings, several ethylene-insensitive mutants, including *etr1-1*, *ein4-1*, *ein2-5*, *ein3-1*, and *ein3 eil1*, exhibit enhanced freezing tolerance. Genetic and biochemical analyses revealed that ethylene negatively regulates cold signalling, at least partially, through direct transcriptional control of the COR *CBFs* and type-A *ARR* genes via EIN3 (Shi et al. 2012) (Fig. 17.2c).

It is known that ethylene can promote seed germination and repress seed dormancy by antagonising ABA (Wilkinson and Davies 2010). Previous studies indicated that *etr1*-1 and *ein2-5* mutants show enhanced seed dormancy, and their germination is hypersensitive to ABA (Chiwocha et al. 2005; Wang et al. 2007). As ABA accumulates in *etr1* and *ein2* mutants (Ghassemian et al. 2000; Chiwocha et al. 2005; Wang et al. 2007), ethylene may negatively regulate ABA biosynthesis during germination. Consistent with this notion, microarray data showed that a group of genes involved in ABA signalling are upregulated in an *ein3 eil1* double mutant (Shi et al. 2012). Interestingly, a recent study revealed the mechanism of how ABA inhibits root growth by increasing ethylene biosynthesis in Arabidopsis. ABA-activated calcium-dependent protein kinases, CPK4 and CPK11 can phosphorylate ethylene biosynthesis synthase ACS6 and enhance its stability, thus promoting ethylene production (Luo et al. 2014). Although the mechanisms underlying the complex cross talk between ethylene, ABA, and cold signalling remain unclear, it appears likely that the upregulation of ABA-responsive genes also contributes to the freezing tolerance of ethylene-insensitive mutants.

17.10.4 JA and Cold Stress

JA has recently been found to positively modulate cold tolerance. In *Arabidopsis*, exogenous application of JA significantly improves freezing tolerance, whereas blockage of JA biosynthesis and signalling decreases freezing tolerance. Further study revealed that JAZ1 and JAZ4 proteins physically interact with the ICE1-proteins to repress their transcriptional activity, thereby repressing expression of *CBF*s and their regulons (Hu et al. 2013) (Fig. 17.2d). Therefore, it appears that JA modulates cold stress primarily through an ABA-independent pathway. However, an antagonistic interaction between ABA and JA signalling has also been reported. In particular, the transcription factor MYC2 was shown to act as a node for the integration of ABA and JA signalling. MYC2 can directly interfere with defence signalling through JAZ-mediated repression, whereas MYC2 acts as an important positive regulator of the ABA-dependent signalling pathway (Abe et al. 2003; Fernandez-Calvo et al. 2011) (Fig. 17.2d). Furthermore, recent studies showed that an ABA-inducible bHLH transcription factor, JAM1, acts as a repressor that negatively regulates JA signalling in *Arabidopsis* (Nakata et al. 2013).

17.10.5 SA and Cold Stress

Salicylic acid (SA) is important for the activation of plant defence responses (Raskin 1992). Multiple studies have demonstrated a link between cold signalling and the SA-mediated defence response. Cold stress induces the accumulation of SA in *Arabidopsis*, whereas SA-deficient mutants show increased growth in response to cold (Scott et al. 2004). Gain of function of R/R-like protein mutants that overproduce SA, such as*chs1*, *chs2*, and *chs3*, display chilling-sensitive phenotypes that are partially dependent upon SA (Huang et al. 2010a; Yang et al. 2010; Wang et al. 2013). Indeed, there appears to be an optimal threshold of endogenous SA that is crucial for the chilling-dependent inhibition of plant growth. Nevertheless, the exact role of SA in freezing tolerance remains unclear. For example, several SA-overproducing mutants, including *cpr1*, *cpr5*, and *slh1*, show enhanced freezing tolerance (Yang et al. 2010), whereas overexpression of DEAR1 (DREB and EAR motif protein) induces SA accumulation and freezing sensitivity in *Arabidopsis* (Tsutsui et al. 2009). The master regulator of cold signalling, ICE1, was shown to directly regulate the expression of *BON1-associated protein* (*BAP1*), which encodes a C2-domain protein that negatively mediates the SA-dependent defence responses (Zhu et al. 2011; Yang et al. 2006). CAMTA3/AtSR1 recognises the *CBF2* promoter to positively regulate *CBF2* expression during cold stress, but it also interacts with the *EDS1* promoter to repress SA-dependent disease resistance (Kim et al. 2013; Doherty et al. 2009; Du et al. 2009).

ABA has been shown to play a broad role in the regulation of plant defence responses. For example, ABA acts a positive regulator of the defence response during pathogen invasion, whereas it acts as a negative regulator of the defence response following pathogen invasion (Yasuda et al. 2008; Ton et al. 2009; Robert-Seilaniantz et al. 2011). A recent study revealed that ABA deficiencies antagonise the high-temperature inhibition of disease resistance by enhancing the nuclear accumulation of the resistance proteins SNC1 and RPS4 in *Arabidopsis* (Mang et al. 2012). Therefore, ABA and SA may act antagonistically to affect plant development and the defence response, as well as the cold stress response (Fig. 17.2e).

17.11 Conclusions

Cold acclimation is a complex physiological process that is affected by various developmental and environmental factors, including growth stage, tissue type, photoperiod, hydration status, and low temperatures. ABA has long been thought to play an important role in mediating the cold response in plants, and accumulation of ABA may indeed be required for full cold acclimation. However, the extent of its role in vivo has been controversial. It appears that ABA biosynthesis is not a major aspect of the early cold stress response but rather that it contributes to the maximum induction of cold-responsive genes during later stages. It is also possible that ABA plays diverse roles in the regulation of multiple physiological processes that are affected by chilling or freezing stress. However, the majority of the molecular mechanisms underlying how ABA affects freezing tolerance remain to be investigated. In particular, elucidating whether the key components of ABA signalling are involved in CBF-regulated cold acclimation should be a priority for those studying the role of ABA in cold signalling. In addition, understanding the signalling networks between ABA and the other relevant plant hormones during the cold stress response will be crucial for determining any potential applications for ABA in enhancing plant tolerance to cold stress.

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