

What happens in plant molecular responses to cold stress?

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Abstract Low temperature is one of the major abiotic stresses limiting the productivity and the geographical distribution of many important crops. Many plants increase in freezing tolerance in response to low temperatures. This phenomenon needs a vast reprogramming of gene expression which results in the adjusted metabolic-structural alterations. However, the efficient adjustments are dependent on proper cold signal transduction. The first stage is cold stress signal perception which is carried out by different pathways. Transcriptional cascades are next players which operate through ABA-dependent and ABA-independent pathways to induce cold-regulated (*COR*) gene expression and the result is increasing in the levels of hundreds of metabolites, which some of them are known to have protective effects against the damaging effects of cold stress and some like soluble sugars, reactive oxygen species and photosynthetic metabolites are thought to act as signaling molecules and regulate special *COR* genes. The different aspects of these events are discussed in detail below.

Keywords Cold stress · Signal transduction · *COR* genes · Metabolites

Introduction

Low temperature (LT) is one of the most important abiotic factors limiting growth, productivity and geographical

distribution of agricultural crops. Every year significant losses can result from sudden frosts in fall and from unusual freezing temperatures in winter and late cold spring events. Many plants increase in freezing tolerance (FT) in response to LT, a phenomenon known as cold acclimation (CA). CA is the process that allows hardy plants to develop essential tolerance for cold stress survival through multiple levels of biochemical and cell biological changes. These responses are due to reprogramming of gene expression which results in the adjusted metabolic alterations. The first step in switching on such molecular responses is to perceive the stress as it occurs and to relay information about it through a signal transduction pathway.

Since cold stress shares some common characteristics with other stresses such as drought and salinity, it is important to distinguish the events that take place through cold stress signal transduction from those related to a general stress response. Also to establish a good improvement strategy in crop breeding projects related to cold resistance, it is essential to understand the mechanisms involved in the CA process and development of FT.

Our knowledge about the signaling pathways leading from stimulus to end response in plants has increased over recent years. This review summarizes different aspects of cold stress signaling and plant molecular responses to cold stress.

Cold signal perception

The response of plants to any environmental signal is mediated by a series of reactions, collectively referred to as signal transduction. LT signal transduction starts with the perception of the cold signal. How LT is perceived is a question of central importance. To date, little is known about cold sensors in plants and details of the early

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LT-signaling pathway are missing (Beck et al. 2004, 2007). Several data have suggested a role of membrane physical state on cold transduction. Murata and Wada (1995) and Murata and Los (1997) indicated that a putative sensor protein detects physical phase transitions in microdomains of the plasma membrane as a result of temperature shifts. Örvar et al. (2000) and Sangwan et al. (2001) showed that membrane rigidification, coupled with cytoskeletal rearrangements, triggers LT responses in plants at 25°C. In *Arabidopsis*, by analyzing diacylglycerol kinase (DAGK) activation, a very early event occurring within seconds of cold exposure, it was shown that the activation of the DAGK pathway following a temperature decrease correlates with changes in membrane fluidity (Vaultier et al. 2006). Ding and Pickard (1993) have shown that the tension-dependent activity of a mechanosensitive Ca^{2+} channel (MCC) increases when temperature is lowered and this Ca^{2+} channel could be good candidate for calcium entry which is necessary to activate the DAGK pathway (Vaultier et al. 2006).

The cytoskeleton may act as a LT sensor in plants (Thion et al. 1996; Dodd et al. 2006). The close relationship of the plant cytoskeleton with the plasma membrane, the major platform for signal perception and transduction, suggests that microtubules and microfilaments are downstream targets of various signaling pathways including cold stress. They appear to play a central role in cold signaling and CA. The role of microtubules and actins in opening the Ca^{2+} channel (Thion et al. 1996; Örvar et al. 2000) further support this hypothesis.

Some structural cell wall proteins have been proposed to be possibly involved in sensing LT. The cell wall kinases, the extensins as a hydroxyproline-rich glycoproteins and arabinogalactan proteins (AGPs), are good candidates for linking the extracellular matrix to the inside of the cell and microtubules and mediating stress responses. AGPs are cell wall proteoglycans that are involved in linking the cell wall–plasma membrane and microtubules, and participate in controlling the flow of information between these elements (Nguema-Ona et al. 2007). AGP can be induced by ionic changes and mechanical stimulation, and may be involved in stress sensing or signaling cascades (Humphrey et al. 2007). However, there is no direct evidence for these cell wall proteins' (AGPs) involvement in cold signaling and all data just indicate their possible role.

Phospholipase D (PLD) is also involved in the transduction of stress signals. PLD is one plasma membrane protein that may be responsible for linking cortical microtubules to the plasma membrane (Gardiner et al. 2001; Dhonukshe et al. 2003; Drøbak et al. 2004; Hong et al. 2008) and is therefore suggested to act as a structural and signaling link between the plasma membrane and the cytoskeleton in tobacco and in *Arabidopsis* (Gardiner et al.

2003). The overexpression of a member of *PLD* family has been shown to lead to enhanced FT (Li et al. 2004). Vergnolle et al. (2005) have shown that the inhibition of the production of phosphatidic acid by PLD also inhibited the induction of some specific cold-regulated (*COR*) genes. PLD controls cytoskeleton stability and linkage to the plasma membrane in a Ca^{2+} -dependent and stress-activated manner (Gardiner et al. 2001; Dhonukshe et al. 2003).

Ca^{2+} , a second messenger

Ca^{2+} is an important second messenger in plant cells and a change in the cytosolic concentration of it is an important component of the signaling network by which plant cells respond to environmental and developmental stimuli (Sanders et al. 2002; Dodd et al. 2006; Klimecka and Muszynska 2007). Each stimulus (e.g., LT) can elicit a characteristic Ca^{2+} signature that is recognized by different calcium sensing or response elements (Knight et al. 1996; Plieth et al. 1999; Knight and Knight 2001; Sanders et al. 2002; Klimecka and Muszynska 2007). Calcium sensors then transduce calcium signatures into downstream effects, including altered protein phosphorylation, cytoskeletal rearrangements and modified gene expression patterns (Sanders et al. 2002; McAinsh and Pittman 2009).

Three main groups of Ca^{2+} -permeable channels have been electrophysiologically characterized in the plasma membrane of plant cells. These are the MCC, the depolarization-activated Ca^{2+} channel (DACC) and the hyperpolarization-activated Ca^{2+} channel (HACC) (Sanders et al. 2002; McAinsh and Pittman 2009). These channels function as detectors of chemical and physical stimuli, such as heat and cold, ABA and reactive oxygen species (ROS) and mechanical forces (Ding and Pickard 1993; Chinnusamy et al. 2004; Carpaneto et al. 2007; McAinsh and Pittman 2009). *Arabidopsis* mutants defective in the activation of the molybdenum cofactor of abscisic aldehyde oxidase, namely *aba3/freezing sensitive 1 (frs1)*, also known as *los5 (low expression of osmotically responsive genes 5)*, exhibit hypersensitivity to freezing stress. The *Arabidopsis fro1 (frostbite1)* mutant, which constitutively accumulates high levels of ROS, exhibits impaired expression of *COR* genes and hypersensitivity to chilling and freezing (Chinnusamy et al. 2007).

In addition to plasma membrane, there are other Ca^{2+} -permeable channels that have been identified in the vacuolar membrane, like inositol 1,4,5-trisphosphate (InsP3)- and cyclic ADP-ribose (cADPR)-gated channels (Sanders et al. 2002; McAinsh and Pittman 2009). Pharmacological studies have shown that cADPR-gated Ca^{2+} channels are involved in abscisic acid (ABA)-induced expression of cold-regulated genes in tomato and *B. napus*.

Inositol 1,4,5-trisphosphate (IP₃)-gated Ca²⁺ channels have been implicated in dehydration- and salt stress-induced cytosolic Ca²⁺ elevations (Chinnusamy et al. 2004). Genetic analysis of the *FRY1* locus of *Arabidopsis* suggested the involvement of IP₃ (which, in turn, is expected to generate cytosolic Ca²⁺ oscillations) in ABA, salt and cold stress signaling. *FRY1* encodes an inositol polyphosphate 1-phosphatase, which catabolizes IP₃. An ABA-induced IP₃ transient is altered in the *fryl* mutant, which leads to a more sustained accumulation of IP₃ and a plant that is hypersensitive to ABA, cold and salt stresses (Chinnusamy et al. 2004).

Ca²⁺ efflux transporters

Plants, like all eukaryotes, have two main pathways for cytosolic Ca²⁺ removal: high-affinity Ca²⁺-ATPases and lower-affinity Ca²⁺ exchangers. These two active transporters drive Ca²⁺ out of the cytosol against a steep electrochemical gradient at the plasma membrane and the endomembranes. It has been shown that Ca²⁺-ATPases are transcriptionally regulated by cold stress and knock out studies have indicated a role in vegetative development and therefore, in cold tolerance (George et al. 2008).

Ca²⁺ decoders

It has been indicated that the elevated Ca²⁺ in the nucleus (either diffused from the cytosol or released from nuclear Ca²⁺ reservoirs in response to stimuli) binds directly to transcription factors and modulates their activity (Kim et al. 2009). For example, the first identified Ca²⁺-binding transcription factor, designated AtNIG1 (*Arabidopsis thaliana* NaCl-inducible gene 1), was described in plants (Kim et al. 2009). AtNIG1 is a basic helix-loop-helix-type transcription factor that contains an EF-hand motif. The bacterially expressed recombinant AtNIG1 protein binds to the canonical E-box element (CANNTG), which is found in the promoter region of *CBFs*. *AtNIG1* loss-of-function mutant plants are more sensitive to salt stress and ABA (Kim et al. 2009). However, there is still no data about the involvement of this factor in cold stress signaling by regulating the expression of cold stress-responsive genes.

The direct action of Ca²⁺ cannot be adequate for such complex and systematic modifications occurring due to shaping Ca²⁺ signatures through the cells. The messages of Ca²⁺ signatures should be decoded into proper cellular responses to different stimuli. Ca²⁺ decoders are responsible for translating these secret messages.

Calmodulin (CaM), as one of the most conserved Ca²⁺ binding proteins in eukaryotes, plays a role in the mediation of cellular responses to developmental and

environmental stimuli via regulation of gene expression (Kim et al. 2009).

CaM antagonist prevents CA and reduces expression of cold-regulated genes, supporting a role for CaM in LT signaling (Monroy et al. 1998; Tähtiharju and Palva 2001). However, overexpression of CaM in *Arabidopsis* has been shown to cause reduction in cold-responsive gene expression (Townley and Knight 2002), implying that CaM might have a role as a negative regulator during CA.

Studies suggest that, in plants, CaM also participates indirectly in the regulation of gene expression by acting through a CaM-binding protein kinase and a CaM-binding protein phosphatase (Liu et al. 2007, 2008).

Calcium-dependent phosphorylation/de-phosphorylation processes seem to be involved in activating the CA process (Guy 1999). Calcium-dependent protein kinases (CDPKs) are specifically expressed in plants and are implicated as important sensors in response to abiotic stresses, including cold stress (Chinnusamy et al. 2004; Klimecka and Muszyńska 2007). Monroy and Dhindsa (1995) showed that the strong induction of two *CDPKs* happened, earlier than two cold-regulated genes examined, in a cold treatment.

Another group of proteins identified as interacting with Ca²⁺ includes serine/threonine phosphatases (PPases). The *Arabidopsis* protein phosphatase 2C, AtPP2CA, is cold-inducible, reaching a maximum level by 12 h and remaining high thereafter (Tähtiharju and Palva 2001). In contrary, in alfalfa cells, cold-induced inactivation of protein phosphatase 2A (PP2A) is controlled by Ca²⁺ influx (Monroy et al. 1998), all indicating the role of protein phosphatases in response to cold stress.

MAPKs (mitogen-activated protein kinases) are serine/threonine protein kinases. MAPK cascades are activated by numerous abiotic stresses but they can introduce specificity into the system (Knight and Knight 2001; Chinnusamy and Zhu 2002; Ichimura et al. 2002; Colcombet and Hirt 2008). MAP kinase activity has been shown to be enhanced by cold in alfalfa (Jonak et al. 1996; Sangwan et al. 2002) and *Arabidopsis* (Ichimura et al. 2000). MAPK cascades are able to induce or activate different transcription factors and regulate specific gene expression (Chinnusamy and Zhu 2002; Zhu et al. 2007). *Arabidopsis* mutants, *mkk2*, that cold and salt activation of MPK4 and MPK6 are impaired, are hypersensitive to cold stresses and obviously *MKK2* over-expressor plants displayed increased FT (Teige et al. 2004).

Regulation of gene expression in response to low temperature

Cold acclimation causes an increase in sub-zero FT and gene-expression changes coincident with CA involve several gene-signaling pathways. Cold-responsive genes

encode a diverse array of proteins such as enzymes involved in respiration and metabolism of carbohydrates, lipids, antioxidants, molecular chaperones, antifreeze proteins, and others with a presumed function in tolerance to freezing. Koo et al. (2008) have shown that, based on the different sensitivity of barely cultivars to non-acclimated freezing, genes with greater differential expression in tolerant varieties would either be directly involved in non-acclimated FT or could serve as biomarkers of the differential response to freezing.

The expression of *COR* genes are regulated by both ABA-independent and ABA-dependent pathways (Chinnusamy et al. 2004; Knight et al. 2004). Here, we review some of the aspects of these pathways.

ABA-independent cold signal pathway

Promoter analysis of the *COR* genes has shown that they contain sequence elements that mediate the stress induction of the genes. There are some transcription factors which identify these elements and bind it.

CBF pathway

A family of transcription factors known as C-repeat binding factors (CBFs) (Stockinger et al. 1997) or dehydration-responsive element binding factors (DREB1s) that control ABA-independent expression of *COR* genes in response to cold stress has been identified in Arabidopsis. These transcription factors belong to the ethylene-responsive element binding protein/APETALA2 (EREBP/AP2) family (Stockinger et al. 1997) and DREB subfamily (Chen et al. 2009). This group of subfamily was further divided into six subgroups (A-1 to A-6), among which *DREB1/CBF*-like genes, belonging to the A-1 subgroup, are induced by LT and activate the expression of many cold stress-responsive genes, whereas *DREB2*-like genes, belonging to the A-2 subgroup, are mainly involved in osmotic stress-responsive gene expression (Shinozaki and Yamaguchi-Shinozaki 2007; Nakashima and Yamaguchi-Shinozaki 2006; Nakashima et al. 2009; Chen et al. 2009). Three cold-inducible *CBF/DREB1* genes, *CBF1/DREB1b*, *CBF2/DREB1c* and *CBF3/DREB1a*, have been identified in Arabidopsis (Thomashow 2001). These factors bind to the cold- and dehydration-responsive DNA regulatory elements (DREs), also termed C-repeats (CRTs). CRT/DRE elements contain the conserved CCGAC core sequence, which is sufficient to induce transcription under cold stress.

Studies on A-5 subgroup members, such as *PpDBF1*, *GmDREB2*, and *GhDBF1* (Chen et al. 2007, 2009), suggested that the A-5 subgroup, like the A-1 subgroup, is an

important genetic resource, potentially useful for the improvement of crop stress tolerance. Recently, a *DREB* orthologue, *GmDREB3*, belonging to the A-5 subgroup of the *DREB* subfamily, was isolated from soybean using the RACE method. Northern blot analysis showed that expression of *GmDREB3* in soybean seedlings was induced following cold stress treatment for 0.5 h and was not detected after 3 h. However, it was not induced by drought and high salt stresses or by ABA treatment. Studies showed that *GmDREB3* might be involved in the early cold response compared with *GmDREB2* (Chen et al. 2009). In wheat, Badawi et al. (2008) have identified several *CBF* genes that are subdivided into ten different groups. Several *CBF* groups are amplified only in Pooideae and most of these amplified groups are expressed at a higher level in the freezing tolerant cultivar.

Playing important role in plant cold stress responses, *CBFs* have been greatly studied and impressive amount of information about their regulation pathways has been prepared. Several reports identified direct regulators of *CBF/DREB1* expression.

A transcription factor gene, *Inducer of CBF Expression 1 (ICE1)*, encoding a MYC-like basic helix-loop-helix (bHLH) protein, binds specifically to MYC *cis*-elements (CANNTG) in the *DREB1a/CBF3* promoter region and increases the expression of *DREB1a/CBF3*, which, in turn, activates expression of many downstream genes, leading to a significantly enhanced tolerance to chilling and freezing (Chinnusamy et al. 2003; Chen et al. 2009). Overexpression of the wheat *ICE* genes enhanced FT in the heterologous Arabidopsis system (Badawi et al. 2008). The *ice1* mutation blocks the expression of *CBF3* and decreases the expression of many genes downstream of *CBFs*, which leads to a significant reduction in plant chilling and FT in Arabidopsis (Chinnusamy et al. 2003).

Moreover, *DREB1c/CBF2* negatively regulates the expression of *DREB1b/CBF1* and *DREB1a/CBF3* (Novillo et al. 2004; Chinnusamy et al. 2007). Arabidopsis mutant, *cbf2* (in which the *CBF2/DREB1c* gene was disrupted), have higher capacity to tolerate freezing than wild type ones before and after CA and are more tolerant to dehydration and salt stress. Also, the reduced expression of *CBF3* in the *ice1* mutant is accompanied by an enhanced expression of *CBF2* (Chinnusamy et al. 2007).

The other direct regulator of *CBF/DREB1* expression is MYB15 (an R2R3-MYB family protein in Arabidopsis) which binds to *CBF/DREB1* promoter regions to repress its expression and negatively regulate FT. Interestingly, ICE1 can negatively regulate MYB15 as indicated from the increased *MYB15* transcript level in *ice1* mutant compared with wild-type plants under cold stress (Chinnusamy et al. 2003). ICE1 physically interacts with the Myb domain protein 15 (MYB15) and may attenuate directly (through

binding to *MYB15* promoter) or indirectly (through its downstream genes) *MYB15* expression in response to cold (Badawi et al. 2008). *Myb15* T-DNA knockout mutant plants show enhanced expression of *CBFs* during CA and enhanced FT, whereas transgenic *Arabidopsis* over-expressing *MYB15* shows a decreased expression of *CBFs* and a reduction in FT.

ICE1 is constitutively expressed and localized in the nucleus, but it induces expression of *CBFs* only under cold stress. Also, it has been indicated that *MYB15* is expressed even in the absence of cold stress (Chinnusamy et al. 2007). Using Microarray technology, Koo et al. (2008) showed that *MYB* and *ICE*-like probesets did not show significantly higher transcript levels in cold temperature treatments. Badawi et al. (2008) also showed that the pattern of mRNA expression was not significantly altered by LT treatment in both winter and spring wheat, demonstrating that *ICE1* mRNA expression is constitutive.

Forward genetic analysis in *Arabidopsis* identified two transcription factors: high expression of osmotically responsive genes 9 (*HOS9*) and *HOS10*, that are required for basal FT. The *HOS9* and *HOS10* genes encode homeodomain and an R2R3-type MYB (*AtMYB8*) transcription factors, respectively, and their transcript levels are not cold responsive. Loss-of-function mutations in these genes cause significant decreases in basal and acquired FT. Interestingly, the mutants show stronger or earlier cold induction of several CBF-target genes, such as *RD29A* and *COR15A*, but no effects on the expression of *CBFs*. It seems that *HOS10* might regulate ABA-mediated CA (Chinnusamy et al. 2007).

ZAT12

In addition to CBF pathway, one representative of the small group of genes that responds similar to many different environmental stress conditions is the C₂H₂ zinc-finger protein *Zat12* (*At5g59820*). *Zat12* was found to respond at the steady-state transcript level to different stresses including cold stress. Analysis of *Zat12* using transgenic plants suggested a role for *Zat12* in CA and in the response of plants to oxidative stress (Vogel et al. 2005). The broad response of the *Zat12* gene to different stresses might result from the activation of *Zat12* expression by ROS such as H₂O₂ that accumulate in cells in response to almost all stress conditions (Dat et al. 2000; Apel and Hirt 2004; Mittler et al. 2004; Davletova et al. 2005).

Zat12 contains an EAR-motif-like sequence that may function as a repression domain. Transcriptome analysis of *ZAT12*-overexpressing *Arabidopsis* revealed that the *ZAT12* regulon consists of at least 24 COS (cold standard set) genes, of which nine are cold-induced and 15 are

cold-repressed genes (Vogel et al. 2005; Chinnusamy et al. 2007).

The *ZAT12* cold-response pathway interacts with the CBF cold-response pathway. There is overlap in the genes that comprise the *ZAT12* and CBF regulons, four genes were up-regulated and three down-regulated in response to both transcription factors and LT. Thus, the *ZAT12* and CBF cold-response pathways appear to coordinately regulate the expression of certain cold standard genes. Moreover, constitutive *ZAT12* expression was found to dampen the induction of the *CBF1-3* genes in response to LT. Thus, the *ZAT12* regulon appears to be involved in negative regulation of the CBF cold-response pathway (Vogel et al. 2005; Chinnusamy et al. 2007).

ABA-dependent cold signal pathway

When plants are exposed to LT, for many species, there is a transient rise in ABA content both herbaceous and woody plants. This spike in ABA is associated with the onset of CA and the induction of FT. Exogenous application of ABA at normal growth temperature leads to increased chilling and FT (Guy 1999) and de novo protein synthesis as well as induction of a subset of cold-responsive genes. The promoters of these genes have ABA response elements (ABREs). The ABREs, *cis*-elements with a consensus sequence C/TACGTGGC, confer ABA responsiveness to many genes when more than one copy is present. The class of bZIP transcription factors, ABRE binding proteins (AREBs or ABFs), can bind to ABRE and activate ABA-dependent gene expression. The genes encoding ABFs (*ABF1–4*) are themselves induced by ABA and show differential regulation by various environmental stresses; *ABF1* is induced by cold, *ABF2* and *ABF3* by high salt concentration and *ABF4* by cold, high salt concentration and drought (Choi et al. 2000). The differential regulation of ABFs suggests that separate ABFs are likely to function in these signal transduction cascades through common ABREs. Genetic analysis of the ABA-deficient mutants *los5/aba3* of *Arabidopsis* showed that ABA plays a pivotal role in osmotic stress-regulated gene expression. However, these mutants [also named *aba3/freezing sensitive 1 (frs1)*] also exhibit hypersensitivity to freezing stress. *los5* mutant plants show a significant reduction in the expression of cold and osmotic stress induction of genes (Chinnusamy et al. 2004, 2007).

Cross-talk

The cross-talk between ABA-dependent and ABA-independent regulatory systems has been suggested by genetic and molecular analyses (Kreps et al. 2002). Cross-talk

occurs at the interaction between different *cis*-acting elements. Many cold- and drought-inducible genes contain both DRE/CRT and ABRE elements in their promoter. These *cis*-acting elements are thought to function independently suggesting that cross-talk occurs between the ABRE and DRE. For example, genetic evidence indicates that a stress signaling pathway for the activation of *LEA*-like genes that was completely independent of ABA may not exist (Xiong et al. 2002). This suggestion was verified by a study demonstrating that *CBFs* are also induced by ABA (Knight et al. 2004).

Metabolic and structural adjustments

Cold stress causes dramatic changes to plant metabolism, as a result not only of general reductions in enzyme activities and reaction rates in the cold but also of active reconfiguration of the metabolome (Zhu et al. 2007; Guy et al. 2008). The investigations in *Arabidopsis thaliana* showed that the physiological process of CA significantly influenced the concentration of three-quarters of the >400 metabolite peaks that were detected by GC–TOF MS, and that the levels of most of these peaks were influenced by the CBF-mediated cold-response pathway (Last et al. 2007). Here, we briefly indicate some of these alterations.

Proteins

The proteins are from those metabolites which are differentially expressed in response to cold stress. These proteins are involved in several processes, i.e., signal transduction, RNA processing, translation, protein processing, redox homeostasis, photosynthesis, photorespiration, and metabolisms of carbon, nitrogen, sulfur and energy (Yan et al. 2006). Some of cold associated and inducible proteins are dehydrins (DHNs), antifreeze proteins (AFPs), heat shock proteins (HSPs), cold-shock domain proteins (CSDPs) and enzymes like alternative oxidase, desaturases, etc.

Dehydrins

Dehydrin proteins [also known as group 2 late embryogenesis abundant (LEA) proteins] are produced in plant cells in response to environmental stimuli with a dehydrative component, including LT, drought and salinity, and developmental stages such as seed and pollen maturation (Close 1996; Rorat 2006; Kosova et al. 2007). DHNs are characterized by three highly conserved domains known as the K, Y, and S segments. The number and order of the Y, S, and K segments define different DHN subclasses: Y_nSK_n , Y_nK_n , SK_n , K_n , and K_nS . The SK_n - and K-type of

DHNs seem to be directly involved in CA processes (Rorat 2006). Three *COR* genes encoding, *Wcs120* (K6), *Wcor410* (SK3), and *Wcor14*, well-identified dehydrins, in wheat genotypes have been studied. *Wcs120* and *Wcor14* have CBF in their promoter regions and are activated by the CBF transcription factors. It has been shown that the initial response to LT was accompanied by a burst in *Wcs120*, *Wcor 410*, and *Wcor14* expression that decreased with time (Ganeshan et al. 2008).

In barley, 13 dehydrin genes, *dhn1* to *dhn 13*, have been described to date. Under cold stress, the induction of *dhn5* and *dhn8* genes has been detected at the transcription level. In barley, intermediate (I) and winter (W) cultivars showed a higher level of DHN5 (K9) accumulation and FT than the spring (S) cultivars, which exhibited a lower level of accumulated DHN5 and FT (Kosova et al. 2008).

The exact function of dehydrins is still unclear, but based on their sequence features, they have been postulated to stabilize cell structures against dehydration (Danyluk et al. 1998; Kovacs et al. 2008). In several plant species, DHNs have been shown to possess in vitro cryoprotective activity and in vivo antifreeze activity. Also, they may function as possible osmoregulators or as radical scavengers. Purification of *Arabidopsis* DHNs revealed strong binding to copper-charged columns. Since DHNs do not possess any known metal binding motif, the strong binding observed was suggested to be due to complexes formed by histidine residues within DHNs. Metal binding activity suggests that they may reduce metal toxicity in plant cells under water-stressed conditions (Hara et al. 2005).

ERD10 (SK3) and ERD14 (SK2) (for early response to dehydration) proteins are members of the DHN family, in *Arabidopsis thaliana* and it is shown that they have chaperone activity. ERD10 and ERD14 are able to prevent the heat-induced aggregation and/or inactivation of various substrates, such as lysozyme, alcohol dehydrogenase, firefly luciferase, and citrate synthase. It is also demonstrated that ERD10 and ERD14 bind to acidic phospholipid vesicles without significantly affecting membrane fluidity. Membrane binding is strongly influenced by ionic strength. All these findings provide the rationale for the mechanism of how these proteins avert the adverse effects of dehydration stresses (Kovacs et al. 2008).

Antifreeze proteins

Antifreeze proteins, another LT-responsive gene family, were first discovered and characterized in polar fishes. AFPs are present in relatively high concentration and have the ability to inhibit the growth of an ice crystal by preventing the accretion of water molecules to the growing faces of the crystal by a non-colligative mechanism. AFPs

adsorb onto the surface of ice crystals and modify their shape and growth in a beneficial manner: instead of one large single ice crystal, more but smaller and slower-growing ones develop. During thawing, AFPs may inhibit recrystallization and formation of larger ice crystals. Larger ice crystals increase the possibility of physical damage within frozen plant tissue (Griffith et al. 1997). AFPs also exhibit a property of thermal hysteresis and are frequently synonymous with thermal hysteresis proteins (THPs) (Guy 1999; Houde et al. 2006). The activity of AFPs is present only in the apoplastic extracts of freezing tolerant monocotyledons and dicotyledons after CA.

Analysis of amino-terminal sequences revealed that these polypeptides are all similar to plant pathogenesis-related (PR) proteins (Hon et al. 1995). Some reports have shown that a group of PR proteins, including endo- β -1,3-glucanases, endochitinases and thaumatin-like proteins, are synthesized in overwintering monocots under cold stress and exhibit antifreeze activities (Hon et al. 1995; Atici and Nalbantoglu 2003; Seo et al. 2008).

It seems that these AFP/PR proteins actually have a dual function to protect the plants against ice recrystallization and pathogen attack during the unfavorable conditions of winter. CA of many freeze-tolerant grasses enhances resistance to snow molds, powdery mildews, leaf spots, and rusts (Hon et al. 1995; Guy 1999).

Another group of AFPs is the wheat TaIRI (ice recrystallization inhibition) proteins which have a peculiar bipartite structure. They show a leucine-rich repeat receptor domain of receptor-like protein kinases at their N terminus and ice recrystallization inhibition domains at their C terminus (Ouellet 2007).

It was found that the hormone ethylene, salicylic acid, ABA, and drought are involved in regulating antifreeze activity in response to cold (Atici and Nalbantoglu 2003).

Cold-shock domain proteins

A superfamily of proteins in eukaryotic organisms contains the cold-shock domain (CSDP), and is found in proteins that bind nucleic acids. They were first described as Y-box binding proteins that bind to the Y box and are considered to function as a RNA chaperone (Guy 1999; Somerville 1999). These proteins are conserved from bacteria to animals and higher plants. It was shown that *AtCSPs* gene expression patterns are strongly correlated to various stages of plant development. Using mutant plants it was shown that mutation in the CSDP did not have a major effect on gene and protein expression under normal conditions; however, the expression of several genes was altered under stress conditions. In bacteria, CSDPs relax mRNA secondary structures and play a crucial role for subsequent translation of proteins under LT stress (Nakaminami et al.

2009). Although some plant CSPs have been correlated to abiotic stress conditions, high levels of *AtCSPs* expression were clearly detected in meristem tissues under non-stress conditions (Nakaminami et al. 2009; Park et al. 2009). Therefore, plant CSDPs probably possess multi-functions containing an RNA chaperone activity that may be important under both stress and non-stress conditions. However, the precise biological functions of CSDPs in plants remain largely unknown.

Heat shock proteins

Heat shock proteins are commonly associated with response to high temperatures, but evidence indicates that they can also respond to LT. These HSPs such as Hsps104 and 90, two members of the Hsp70 family, and two members of the smHsp group have been identified through different studies (Guy 1999; Ukaji et al. 1999). A comprehensive survey of the Arabidopsis Hsp70 family revealed that genes encoding four out of five cytoplasmic and two mitochondrial proteins were up-regulated in response to cold temperature. Renaut et al. (2008) showed that two Hsp70-like proteins were up-regulated in response to LT in peach. Two other Hsp70 family members similar to the Arabidopsis chloroplast Hsp70s were also up-regulated at 5°C. HSPs act as molecular chaperones, and their up-regulation may play a pivotal role in preventing aggregation of the denatured proteins, facilitating the refolding under chilling stress (Yan et al. 2006) and aiding protein translocation into organelles (Nover and Scharf 1997).

Plasma membrane

There is a general consensus that the plasma membrane is the primary site of freezing injury (Webb and Steponkus 1993; Uemura et al. 1995, 2006; Ouellet 2007). Environmental changes affect the functions of cell membranes and as a result either the cell adapts to these changes or it would kill it. For adapting to these changes, organisms use factors by which the cell membrane modulates its physical state, for example, changes in lipid head group, in fatty acyl chain length, in fatty acid isomerisation, in proportion of *cis-trans* fatty acids (Uemura et al. 1995, 2006) and the level of unsaturated fatty acids (FAs) (Somerville and Browse 1991; Wada et al. 1994). In many plant species examined, the increase in lipid is primarily a result of an increase in the proportion of unsaturated molecular species of phosphatidylcholine and phosphatidyl ethanolamine, two major phospholipid classes in the plasma membrane in response to cold stress. In addition, there is a decrease in the

proportion of cerebrosides occurring over a wide range of plants (Uemura et al. 2006).

At LT, desaturases provide enhanced molecular motions to the membrane lipids by introducing double bonds into FAs. Desaturases are found in almost all organisms with the exception to some bacteria, such as *E. coli* (Heath and Rock 1996). Acyl-lipid desaturases, one of the important classes of desaturases, introduce double bonds into FA moieties (Maali et al. 2007) that have been esterified to glycerolipids. This takes place in both the plastidial and the endoplasmic reticulum (ER) membranes through two different pathways of FA desaturation (Jin et al. 2001). This type desaturase is the most efficient regulator of the unsaturation level of membrane lipids in response to temperature change (Murata and Wada 1995). The introduction of an appropriated number of double bonds into the fatty acid moieties of membrane glycerolipids decreases the temperature for the transition from the gel (solid) to the liquid-crystalline phase and provides membranes with the necessary fluidity (Russel 1984). Fluidity of the membrane is important for maintaining the barrier properties of the lipid bilayer and for the activation and functioning of certain membrane-bound enzymes (Thomson 1989).

It was shown that the overexpression of the cyanobacterial *desA* gene from *Synechocystis* sp. PCC 6803 encoding for acyl-lipid Δ 12-desaturase in potato plants changed FAs composition and elevated the content of UFAs in leaves of various transgenic lines (Maali-Amiri et al. 2007). It is clear that increase in the polyunsaturated fatty acids in the cell membrane structure causes the stability of cellular membranes in transgenic plants, and as result act as a factor of cold tolerance in plants (Ishizaki-Nishizawa et al. 1996). Increased cold tolerance was also related to improved plant tolerance to oxidative stress induced by hypothermia in potato plants (Maali-Amiri et al. 2007).

Vaultier et al. 2006 have shown that in *fad2* mutant the activation of diacylglycerol kinase (DAGK), which is dependent on membrane rigidification, occurs at higher temperatures (18°C) as compared with the wild type (14°C) and transgenic *FAD3+* (12°C). Thus, one of the cold signal perception pathways works with delay.

Membrane damages can be due to the dehydration that occurs during the freeze–thaw cycle. Elegant experiments with protoplasts isolated from leaves of various herbaceous plant species before and after CA have shown that freezing-induced destabilization of the plasma membrane involves different types of lesions (Uemura et al. 1995; Webb et al. 1996). The incidence of lesions depends on the stage of CA and the extent of freeze-induced dehydration (Uemura et al. 1995, 2006).

In addition to lipid composition alterations, sucrose and other simple sugars (Thomashow 2001; Uemura and Steponkus 2003; Yano et al. 2005) and proteins such as

dehydrins (such as ERD 14) (Uemura et al. 2006) and lipocalins (Charron et al. 2005; Uemura et al. 2006) accumulate at the plasma membrane upon LT exposure and are likely to also play a role in the stabilization of membranes. Sugars stabilize the membranes by interacting with polar groups of phospholipids and establishing hydrophobic bond interaction with proteins, thus supporting the native structure and functioning state of membranes including chloroplasts and mitochondria, which are the major suppliers of active forms of oxygen. Sugars can also act as low-molecular weight antioxidants. It was shown that changes in sugar ratio produced by yeast invertase in potato plants cause a higher tolerance to LT (Deryabin et al. 2005).

Metabolomes as regulating signals

The active reconfiguration of the metabolome is achieved by cold-regulated gene expression changes, which, in turn, are controlled by low-temperature signaling. Metabolism is not, however, a passive target of cold signaling, it can also regulate cold signaling and cold-responsive gene expression (Svensson et al. 2006; Zhu et al. 2007), in fact acts as signal molecules. At least three types of metabolic signals might be important for cold signaling

Soluble sugar

Regulation of acclimation to stress is often highly complex and sugar-signaling is involved in this complexity (Rekarte-Cowie et al. 2008). As sugar metabolism is affected by various abiotic stresses, plants can use sugar status as a signal to modulate growth and development in response to abiotic stresses (Uemura et al. 2006), including cold stress. During CA, sugars, including sucrose and raffinose, accumulate in plants and the genes with roles in sugar synthesis, sugar metabolism, and sugar transport such as β -amylases and Suc synthase are up-regulated in response to LT (Kaplan and Guy 2004; Rekarte-Cowie et al. 2008) and as a signal molecule sugars, can regulate expression of some *COR* genes like β -amylase (Kaplan and Guy 2004). Affymetrix Gene-Chip analysis of gene expression has confirmed that glucose induces changes in gene expression that are typical of developmental leaf senescence (Pourtau et al. 2006). Rekarte-Cowie et al. (2008) have tested the regulatory role of sucrose in the CA of Arabidopsis plants by testing its effect on cold-responsive gene expression as exemplified by *COR78* in Arabidopsis. They have shown that the *COR78* transcript abundance increases in response to sucrose treatments during CA. However, precise understanding of different aspects of sugar-signaling pathways and their role in plant CA regulation remains to be determined.

Chloroplast signals

Exposure of plants to cold stress may induce high excitation pressure and create an energy imbalance. Since there is a delicate equilibrium between the energy that is harvested and used, it has been suggested that the redox status of photosynthesis could be an important signaling mechanism especially during cold stress. However, photosynthetic organisms have evolved a number of mechanisms such as increasing photosynthetic capacity or intensification of protective non-photochemical mechanisms to dissipate excess excitation energy as heat, to improve such conditions (Ensminger et al. 2006).

The tetrapyrrole intermediate Mg-protoporphyrin (Mg-ProtoIX) was found to accumulate in plants under cold conditions and binds to the translational elongation factor 2 protein. Arabidopsis mutants that are deficient in Mg-ProtoIX are impaired in cold-responsive gene expression and have reduced FT. In barley, mutants that are affected in chloroplast development are impaired in cold-responsive gene expression and are completely frost susceptible (Zhu et al. 2007). Also, using four non-allelic *albina* and *xanth* mutants characterized by a block in subsequent steps of the chloroplast development, Svensson et al. (2006) have shown that about 67% of wild-type cold-regulated genes were not regulated by cold in any mutant (chloroplast-dependent cold-regulated genes) but *cbf* genes were equally induced in mutants and wild-type plants chloroplast independent cold-regulated genes. The lack of cold regulation in the mutants is due to the presence of signaling pathway(s) normally cold-activated in wild type but constitutively active in the mutants, as well as to the disruption of low-temperature signaling pathway(s) due to the absence of active chloroplasts.

Also, accumulation of COR14b protein is evidence of a direct interaction between the cold-regulated gene expression originating from a cold-induced signaling pathway and the redox state of the chloroplast. Accumulation of COR14b in barley seems to be important for resistance to combined freezing and high-light tolerance, but not for FT per se (Ensminger et al. 2006; Rapacz et al. 2008). An efficient communication channel must therefore exist between the nucleus and chloroplast to ensure the proper metabolic adjustment needed for the development of FT (Ouellet 2007).

Reactive oxygen species

Under a variety of environmental stress conditions, including LT, drought and salinity, when molecular oxygen is partially reduced, reactive oxygen species (ROS) are generated. Studies suggest a dual role for ROS in plant biology as both toxic byproducts and signal molecules

(Apel and Hirt 2004; Mittler et al. 2004; Hung et al. 2005; Ouellet 2007). In Arabidopsis, a network of at least 152 genes is involved in managing the level of ROS. This network is highly dynamic and redundant, and encodes ROS-scavenging and ROS-producing proteins (Mittler et al. 2004). Using cDNA microarray technology provided evidence that the expression of some genes is up-regulated or some genes are repressed by H₂O₂ (a member of ROS) (Desikan et al. 2001; Hung et al. 2005). The Arabidopsis *fro1* (frostbite1) mutant, which constitutively accumulates high levels of ROS, exhibits impaired expression of *COR* genes and hypersensitivity to chilling and freezing. *FRO1* encodes the Fe-S subunit of complex I (NADH dehydrogenase) of the respiratory electron transfer chain in mitochondria, and its disruption leads to high levels of ROS generation (Lee et al. 2002; Chinnusamy et al. 2007).

Another Arabidopsis mutant, *chyl1*, which is defective in a peroxisomal β -hydroxyisobutyryl-CoA hydrolase needed for fatty acid β -oxidation and valine catabolism, also accumulates high levels of ROS. The *chyl1* mutant shows a reduced cold induction of *CBF* genes, and is defective in chilling and FT (Zhu et al. 2007).

Downstream signaling events associated with ROS sensing involve Ca²⁺ and Ca²⁺-binding proteins, such as calmodulin, the activation of G-proteins, and the activation of phospholipid signaling, which results in the accumulation of phosphatidic acid (Mittler et al. 2004) and activation of polyunsaturated FA peroxidation in membrane lipids (Orlova et al. 2003; Maali-Amiri et al. 2007). ROS signals can also exert their effects directly through the activation of redox-responsive proteins, such as transcription factors and protein kinases (Hung et al. 2005; Chinnusamy et al. 2007).

Studies have provided additional evidence for the regulation of hyper polarization-activated Ca²⁺ channels (McAinsh and Pittman 2009), the activation of Zat12 (Dat et al. 2000; Mittler et al. 2004; Davletova et al. 2005) and the activation of MAPK cascade (Knight and Knight 2001; Mittler et al. 2004; Hung et al. 2005; Colcombet and Hirt 2008) by ROS. ROS is possibly a ubiquitous metabolic signal that modulates many cellular processes, including cold responses (Renaut et al. 2008).

Since oxidative stress is accompanied with cold stress, the ability to activate protective mechanisms, such as an increase in the activity of scavenging enzymes, is vital for plant cold tolerance. For example, a decrease in the expression and activity of cytochrome c oxidase (COX) followed by the accumulation of ROS and increasing alternative oxidase (AOX) induction and activation has been shown (Matos et al. 2007). Overexpression of alternative oxidase has been shown to alleviate oxidative stress in transgenic *A. thaliana* under LT (Sugie et al. 2006). However, this suggests that different parameters involve in

cold tolerance independently and more efficiency or deficiency in one cannot influence some others.

In addition to its role in oxidative stress, Yoshida et al. (2007) have indicated that AOX can dissipate the excess reducing equivalents, which are transported from the chloroplasts, and serve in efficient photosynthesis. Therefore, the up-regulation of AOX can also be a result of excess light energy during cold stress. Another participant in protective mechanism against oxidative stress and was found to be important for chilling tolerance is vitamin E as vitamin-E deficient *Arabidopsis* mutants are chilling sensitive because of defective export of photoassimilate (Maeda et al. 2006; Zhu et al. 2007).

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