

REVIEW

Temperature-perception, molecules and mechanisms

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Summary

The strategies used by living organisms to survive under low and freezing temperatures reveal the extraordinary adaptability of life on Earth. Understanding the molecular mechanisms underlying cold adaptation and freezing survival will provide new insights into the existing relationships between living organisms and their environment, and the possibility of developing multiple biotechnological applications. In the case of plants, the use of classical genetic and new “omics” approaches is allowing to the identification of new elements involved in regulating the cold acclimation response. The challenge ahead is to determine temperature-perception molecules and mechanisms, to uncover new internodes of multiple responses, and to integrate the regulation not only at the transcriptome but also at proteome and metabolome levels. Attaining these goals will significantly contribute global understanding of the adaptive strategies plants have evolved to cope with hostile environmental conditions, and to the development of biotechnological strategies to improve crop tolerance to freezing and other important abiotic stresses.

Key words: *Arabidopsis*; low temperature response; cold acclimation; freezing tolerance; cold signalling

INTRODUCTION

Low and freezing temperatures severely conditioned life of all organisms in many different ways, from the reduction of biochemical reaction rates or changes in membrane fluidity and protein conformation, to the need for protection against freezing. Regarding plants, freezing temperatures limit their development and geographical distribution, and account for important losses in crop production. In temperate regions, many species have evolved a low

temperature response, known as cold acclimation, whereby they can increase their freezing tolerance after a period of exposure to low-nonfreezing temperatures (Levitt 1980). Understanding the molecular mechanisms governing this adaptive response is important in learning how plants grow and reproduce under hostile conditions, and should help to develop biotechnological strategies to improve crop tolerance to freezing. The process of cold acclimation is quite complex and involves a high number of physiological and biochemical changes, most of them controlled by low temperatures through changes in gene expression, which during cold acclimation is mainly regulated at the transcriptional level (Salinas 2002, Yamaguchi-Shinozaki and Shinozaki 2006, Van Buskirk and Thomashow 2006), although recent findings indicate that regulation at the posttranscriptional, translational and posttranslational levels may also be important (Mazzucotelli et al. 2006, Zhu et al. 2007, Catalá and Salinas 2008). For some years, our lab has been using the model plant

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Arabidopsis and a collection of experimental approaches to uncover signalling intermediates involved in regulating plant responses to low temperature. Some of them resulted in the mediation of metabolic adjustments or changes in the composition of plasma membrane induced by cold conditions. Furthermore, by screening a cDNA library from cold-acclimated seedlings of *Arabidopsis* with a subtracted probe enriched in cold-induced transcripts (Jarillo et al. 1994), we identified a series of Rare Cold Inducible (*RCI*) genes that have been shown to be implicated in cold signalling. The functional characterization of these genes has provided important insights into the molecular mechanisms underlying plant responses to low temperature, including cold acclimation. A number of reviews covering plant responses to low temperature and cold acclimation have been recently published (Mazzucotelli et al. 2006, Van Buskirk and Thomashow 2006, Yamaguchi-Shinozaki and Shinozaki 2006, Zhu et al. 2007, Catalá and Salinas 2008, Penfield 2008). In this paper, we will describe and discuss some of our contributions to the understanding of how plants respond when facing low temperature conditions, and how the process of cold acclimation is regulated.

PHYSIOLOGICAL CHANGES INDUCED BY LOW TEMPERATURE

One of the primary effects of low temperature in cells is the alteration of membrane fluidity properties and fatty acid composition due to an increase in the content of polyunsaturated lipids (Murata and Los 1997, Aguilar et al. 1998, Suzuki et al. 2001, Hayward et al. 2007). In plants, this increase is essential for low-temperature survival and directly affects membrane-bound metabolic processes such as respiration and photosynthesis (Cossins 1994). A shift toward an anaerobic metabolism has been reported in different species (i.e., maize and rice) when exposed to low temperature, which provokes a rapid increase of alcohol dehydrogenase gene (*ADH*) expression and activity (Christie et al. 1991). In *Arabidopsis*, we have demonstrated that *ADH* expression is also induced by cold stress, and that this induction is mediated by abscisic acid (ABA) (Jarillo et al. 1993). Nevertheless, in spite of the induction, our results revealed that *ADH* is not essential for the correct development of cold acclimation response (Jarillo et al. 1993).

Photoinhibition, a reduction of photosynthetic activity that may be caused by a combination of light

and cold, has been directly correlated with low temperature sensitivity due to an increase in photooxidative stress (Harvaux and Kloppstech 2001). Anthocyanins, which function in photoprotection acting as light-screening pigments, accumulate in leaves and stems in response to low temperature and changes in light intensity (Mancinelli 1984, Christie et al. 1994). The synthesis of these pigments is controlled, in part, by the key enzymes phenyl ammonia lyase (*PAL*) and chalcone synthase (*CHS*). In our laboratory, Leyva et al. (1995) reported that the expression of *Arabidopsis PAL* and *CHS* genes is induced in response to cold in a light-dependent manner, and that this induction is regulated at the transcriptional level. As in the case of *ADH*, genetic analysis has revealed that *CHS* is not essential for the increase in freezing tolerance originated by cold acclimation (Leyva et al. 1995). Taken together, all these results suggest that the function of *ADH*, *PAL* and *CHS* would be related more to the response to chilling stress than to cold acclimation. Low temperature also affects the protein composition of photosystems (Gray et al. 1997, Krol et al. 1999). We could show that the expression of *CAB1*, a gene encoding a light-harvesting chlorophyll a/b binding protein, is induced when *Arabidopsis* plants are exposed to 4 °C (Capel et al. 1998). In contrast to *PAL* and *CHS*, the induction of *CAB1* in response to cold is light-independent (Capel et al. 1998), indicating that it is regulated through a different pathway. Interestingly, it has been reported that *Arabidopsis* light-harvesting complex (*LHC*) proteins, including *CAB1*, play an important role in protecting photosystem I from photoinhibition (Alboresi et al. 2009).

In addition of modifying the content of polyunsaturated lipids in the plasma membrane, low temperature also induces changes in the composition of plasma membrane proteins. Kawamura and Uemura (2003) reported the isolation of 38 proteins from *Arabidopsis* plasma membrane whose levels increase or decrease in response to cold. Among them, they identified proteins involved in CO₂ fixation, membrane repair, protection of membrane to osmotic stress, or proteolysis (Kawamura and Uemura 2003). One of these proteins, with function in membrane repairing, *SYT1*, has been demonstrated to play a role in freezing tolerance and cold acclimation (Schapire et al. 2008, Yamazaki et al. 2008). Recently, we described a family of eight *Arabidopsis* small hydrophobic proteins (*AtRCI2A-H*) that are also located in the plasma membrane (Medina et al. 2007). The expression of *AtRCI2* genes is differentially regulated in *Arabidopsis* organs and in response to low temperature (Capel et al. 1997,

Medina et al. 2001, 2007). AtRCI2A-C and AtRCI2H are able to complement for the loss of the yeast AtRCI2A-homologue PMP3 (Medina et al. 2007), which has been involved in the maintenance of plasma membrane potential, suggesting a similar role for AtRCI2 during cold stress (Medina et al. 2007).

It has been well known for several years that exposition to low temperatures also provokes changes in the composition of cell wall components in several plant species. Wei et al. (2006) reported that expression of *C3H*, a *Rhododendron* gene encoding a protein implicated in lignin accumulation in the cell wall, is cold induced, suggesting a possible role of lignin in low temperature responses. On the same way, we reported the identification of *RCI3* gene of *Arabidopsis*, that encodes an active cationic peroxidase, a kind of protein that has been involved in lignin and suberin depositions in the cell wall (Llorente et al. 2002).

THE PROCESS OF COLD ACCLIMATION

As already mentioned, cold acclimation is an adaptive response whereby some plants acquire an increase in freezing tolerance upon a prior low-nonfreezing temperature exposition (Levitt 1980). Plants are able to acclimate to cold by extensively reprogramming their transcriptome, proteome and metabolome. In recent years, the efforts of several laboratories, including our own, have begun to elucidate the regulatory networks that mediate cold acclimation. In the next paragraphs, we intend to present the progress in our understanding of how low temperature signals are perceived and transduced leading to the activation of this adaptive response.

Signal transduction in cold acclimation

The first step in any signalling pathway implies the recognition of the signal by a receptor. It has been proposed that changes in the fluidity of cell membranes could act as a receptor of temperature changes in plant cells (Örvar et al. 2000, Sangwan et al. 2002). Nevertheless, experimental evidence demonstrating that this is the case has not yet been provided, and how plants perceive low temperatures still remains to be elucidated. What seems to be clear is that transient increases in cytosolic calcium concentration ($[Ca^{2+}]_{cyt}$) are essential for plant response to low temperature (Knight 2000). Increases in $[Ca^{2+}]_{cyt}$ mainly result from Ca^{2+} influx through permeable channels in the plasma membrane and/or Ca^{2+} discharge from internal stores (Piñeros and Tester 1997). After Ca^{2+} influx, efflux systems to

internal stores and out of the cell restore $[Ca^{2+}]_{cyt}$ to unstimulated levels via Ca^{2+} pumps and Ca^{2+}/H^{+} exchangers (Knight 2000). One of the *RCI* genes identified in our lab, *RCI4*, turned out to be identical to *CAX1*, an *Arabidopsis* gene encoding a H^{+}/Ca^{2+} antiporter essential for correct regulation of $[Ca^{2+}]_{cyt}$ (Hirschi et al. 1996). *RCI4/CAX1* expression is induced by cold through an ABA-independent pathway (Catalá et al. 2003). Functional analysis revealed that *RCI4/CAX1* is involved in the increase of freezing tolerance that is induced by cold acclimation (Catalá et al. 2003). In fact, *rci4/cax1* mutants show enhanced capacity to cold acclimate, which correlates with a higher induction of *CBF/DREB1* genes and their corresponding targets in response to low temperature (see below) (Catalá et al. 2003). We proposed that *RCI4/CAX1* would function to restore $[Ca^{2+}]_{cyt}$ to unstimulated levels after the increase induced by low temperatures (Fig. 1). The absence of *RCI4/CAX1* activity would result in an extended cold signal that would provoke a higher induction of the *CBFs/DREB1s* and their target genes, and, therefore, an increase in *Arabidopsis* freezing tolerance. These results demonstrate that an accurate $[Ca^{2+}]_{cyt}$ regulation is essential for the correct development of cold acclimation response, and that *RCI4/CAX1* is an essential partner of the regulatory mechanisms controlling $[Ca^{2+}]_{cyt}$ homeostasis in *Arabidopsis*.

Generally, Ca^{2+} signals mediating regulatory pathways are associated with reversible phosphorylation events. Reversible phosphorylation of pre-existing proteins has been demonstrated to be essential for cold acclimation in alfalfa and *Arabidopsis* (Tähtiharju et al. 1997, Monroy et al. 1998). *RCI1A* and *RCI1B* are two Rare Cold Inducible genes of *Arabidopsis* identified in our laboratory that encode two members of the 14-3-3 protein family (Jarillo et al. 1994, Abarca et al. 1999). The 14-3-3 proteins function as dimmers to regulate the activity of other previously phosphorylated proteins to which they interact (Abarca et al. 1999, Roberts et al. 2002). The kinetics of *RCI1A* and *RCI1B* expression in response to low temperature correlates with the increase of freezing tolerance induced by cold acclimation, suggesting a role for these proteins in this adaptive process (Jarillo et al. 1994). The 14-3-3 protein family of *Arabidopsis* includes 12 isoforms, but only *RCI1A* and *RCI1B* are cold inducible (Jarillo et al. 1994, Roberts et al. 2002). Preliminary data with a null mutant for *RCI1A* indicate that the corresponding protein acts as a negative regulator of cold acclimation and is involved in regulating cold-induced gene expression (Catalá et al. unpublished).

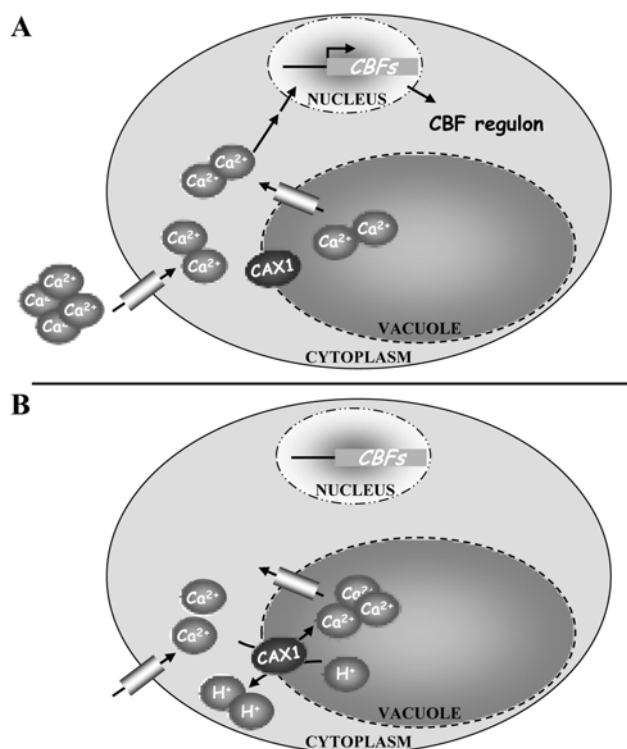


Fig. 1. **Functional model of RCI4/CAX1 during cold acclimation.** A. Exposition to low temperature induces a transient increase in $[\text{Ca}^{2+}]_{\text{cyt}}$ as a consequence of Ca^{2+} influx through permeable channels in the plasma membrane and discharge from internal stores. This increase, conveniently decoded and transduced by downstream effectors, enhances the expression of the CBF genes and their targets, activating the cold acclimation response. B. When the cold signal vanishes, RCI4/CAX1 introduces cytoplasmic Ca^{2+} into the vacuole, contributing, together with other Ca^{2+} transporters, to restore $[\text{Ca}^{2+}]_{\text{cyt}}$ to unstimulated levels. The restoration of $[\text{Ca}^{2+}]_{\text{cyt}}$ levels restrains induction of CBFs .

Regulation of gene expression during cold acclimation

Global expression analyses have revealed that about one thousand genes are regulated by low temperature in *Arabidopsis* (Lee et al. 2005, Matsui et al. 2008, Zeller et al. 2009). Among these genes, one third is repressed, with only one gene encoding a transcription factor, which indicates that gene expression during cold acclimation is mainly activated (Lee et al. 2005). In addition, many early cold-induced genes encode transcription factors or proteins involved in transcription. Thus, more than one hundred genes have been annotated to function in transcription, 95 of them coding for transcription factors (Lee et al. 2005). These results indicate that cold-induced gene expression is regulated through different signal transduction pathways. To date, the most important pathways that have been identified and characterized are those mediated by ABA and the CBF/DREB1 transcription factors, respectively.

ABA and cold-induced gene expression

It is well documented that ABA levels increase in plants in response to different environmental adverse conditions, including low temperatures, and that exogenous treatment with ABA enhances freezing tolerance (Chen and Gusta 1983, Lang et al. 1989). Moreover, it has been described that ABA-defective or -insensitive *Arabidopsis* mutants have a lower capacity to cold acclimate, indicating that ABA is essential for a full cold acclimation response (Heino et al. 1990, Gilmour and Thomashow 1991). In our laboratory, Llorente et al. (2000) isolated a mutant of *Arabidopsis*, named freezing sensitive 1 (*frs1*), that showed reduced freezing tolerance before and after cold acclimation. *frs1* resulted to be an allele of *ABA3*, which encodes an important component of ABA biosynthesis. Actually, *frs1* mutants have lower ABA levels compared to wild-type plants. The fact that *frs1* is affected in its constitutive freezing tolerance indicates that ABA not only plays an

important role in cold acclimation but also has a function in the constitutive tolerance of *Arabidopsis* to freezing temperatures (Llorente et al. 2000). Molecular characterization of *frs1* demonstrated that ABA mediates the induction of gene expression in response to low temperatures (Llorente et al. 2000). Confirming our results, Xiong et al. (2001) reported the identification and characterization of *los5*, which resulted to be another allele of *ABA3*. Similar to *frs1* mutants, *los5* plants have reduced levels of ABA, decreased accumulation of transcripts corresponding to different cold-inducible genes, and reduced tolerance to freezing (Xiong et al. 2001). All these data demonstrate that ABA mediates cold acclimation by controlling cold-regulated gene expression.

The CBF family of transcriptional activators

The best characterized signalling pathway controlling cold-inducible gene expression in *Arabidopsis* is that mediated by the small family of transcriptional activators named C-repeat binding factors/dehydration responsive element binding factors 1 (CBFs/DREB1s). These factors regulate the expression of around 12% of the *Arabidopsis* cold-inducible genes (Fowler and Thomashow 2002), which gives an idea about their significance in cold acclimation. CBF1 was the first to be isolated due to its ability to bind to the *COR15A* promoter and to induce its expression (Stockinger et al. 1997). Subsequently, three different labs, including our own, independently reported that CBF1 belongs to a protein family composed by three members (CBF1-3) (Gilmour et al. 1998, Liu et al. 1998, Medina et al. 1999). CBFs have an acidic C-terminal region that acts as a transcriptional activator motif and an AP2 DNA-binding domain to recognize and interact with the C-repeat (CRT)/dehydration responsive element (DRE) motif (CCGAC) present in the promoters of a number of cold-inducible genes that constitute the CBF regulon (Stockinger et al. 1997, Gilmour et al. 1998, Liu et al. 1998). The three CBF genes are organized in tandem in chromosome 4 of *Arabidopsis* and their expression is rapidly and transiently induced by cold (Gilmour et al. 1998, Liu et al. 1998, Medina et al. 1999). Using a quantitative trait loci (QTL) mapping approach, we determined that the genetic basis of the natural variation for freezing tolerance in *Arabidopsis* is mainly produced by a QTL that colocalize with the CBF cluster (Alonso-Blanco et al. 2005), confirming the relevance of these factors in cold acclimation. Constitutive overexpression of each individual CBF gene in *Arabidopsis* induces the accumulation of mRNAs from genes belonging to the CBF regulon as well as an increase in freezing tolerance, suggesting that the CBFs might be

functionally redundant (Kasuga et al. 1999, Gilmour et al. 2004). Genetic analysis, however, uncovered a different and more interesting scenario. In fact, the molecular and functional characterization of a knock-out mutant for *CBF2* revealed that the absence of CBF2 increases *Arabidopsis* tolerance to freezing temperatures, before and after cold acclimation (Novillo et al. 2004). Furthermore, this increase correlates with a higher accumulation of *CBF1* and *CBF3* transcripts, and, consequently, of mRNAs corresponding to CBF-target genes, under both control and low-temperature conditions (Novillo et al. 2004). These data indicate that CBF2 negatively modulates the expression of *CBF1* and *CBF3*, ensuring the adequate expression of the CBF-regulon during cold acclimation (Novillo et al. 2004). On the other hand, the characterization of *Arabidopsis* plants with reduced induction of *CBF1* or *CBF3* in response to low temperature revealed that, contrary to CBF2, CBF1 and CBF3 are not involved in regulating the expression of other CBF genes, demonstrating that the three CBFs do not have the same function (Fig. 2) (Novillo et al. 2007). Moreover, results showed that although CBF1 and CBF3 seem to positively regulate the induction of the same target genes, they are concertedly required to induce the whole CBF-regulon and, therefore, the complete development of the cold acclimation response in *Arabidopsis* (Novillo et al. 2007).

The results described above evidence the high complexity to which the regulation of CBF expression is subjected, and prompt the importance of an accurate control of their induction levels. Different transcription factors have been reported as interacting with the promoters of the CBF genes and regulating their induction (Fig. 2). Thus, MYB15 binds to the CBF promoters to repress their expression (Agarwal et al. 2006). The ICE1 MYC-like bHLH transcription factor binds to the *CBF3* promoter to activate its expression (Chinnusamy et al. 2003). Moreover, ICE1 interacts to and represses MYB15 (Agarwal et al. 2006). Vogel et al. (2005) reported that the overexpression of an *Arabidopsis* zinc finger, ZAT12, significantly dumped the induction of the CBFs in response to low temperature. Strikingly, ZAT12 does not seem to affect CBF-targeted gene expression, which suggests a complex role for this protein in the regulation of cold-inducible gene expression and cold acclimation (Vogel et al. 2005). ICE2, another MYC-like bHLH transcription factor that activates *CBF1* expression (Fursova et al. 2009), has also been described. In addition to these factors, other proteins involved in regulating the expression of CBFs have been identified (Zhu et al. 2007), confirming that these genes are, in fact, subjected to a tight regulation.

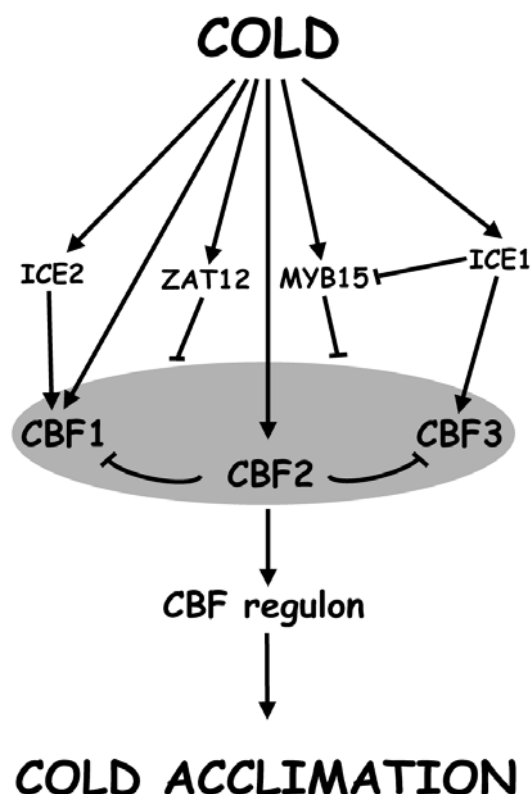


Fig. 2. **CBFs are central factors in cold-acclimation.** *CBFs* are subjected to a tightly regulation. ICE1 and ICE2 have been described to positively regulate *CBF3* and *CBF1* expression, respectively. ZAT12 and MYB15 negatively regulate *CBF* expression. In addition, CBF2 represses *CBF1* and *CBF3* expression. ICE1 has also been described to repress MYB15. The expression of *CBFs* leads to the induction of the CBF regulon and the subsequent activation of cold acclimation response.

Interplay between cold acclimation and plant responses to other abiotic stresses

It has already been mentioned that about one thousand genes are regulated by low temperature in *Arabidopsis* (Lee et al. 2005, Matsui et al. 2008, Zeller et al. 2009). Interestingly, many of these genes are also regulated by salt and water stresses (Matsui et al. 2008, Zeller et al. 2009), indicating that plant responses to abiotic stresses are closely related and share common components. Ishitani et al. (1997), trying to dissect the complex network that regulates *Arabidopsis* response to cold and osmotic stresses, screened for *Arabidopsis* mutants with deregulated expression of a luciferase reporter gene driven by the promoter of RD29A, a CBF-target gene whose expression is induced in response to cold,

dehydration, high salt and ABA. This genetic approach allowed them to isolate and characterize a number of mutants (*hos* and *los*) with altered capacity to cold acclimate. Most of these mutants were also affected in their tolerance to water and/or salt stress (Xiong et al. 2002), supporting the idea that plant responses to low temperature, water stress and high salt have several features in common. Using a similar experimental approach, we identified *Arabidopsis* mutants with high (*hor*) or low (*lor*) expression of *AtRCI2A* (see above) in response to low temperature, dehydration, high salt or ABA (Medina et al. 2005). As in the case of *hos* and *los* mutants, several *hor* and *lor* mutant lines are not only affected in their ability to cold acclimate but also in their sensitivity to dehydration, high salt and ABA treatments (Medina et al. 2005), further supporting the narrow relationship that exists between plant responses to abiotic stresses. Based on the results obtained from the physiological and molecular characterization of the *hor* and *lor* mutants, we proposed a working model that reflects the remarkable complexity of the networks whereby plants appropriately respond when exposed to adverse abiotic environmental conditions (Fig. 3).

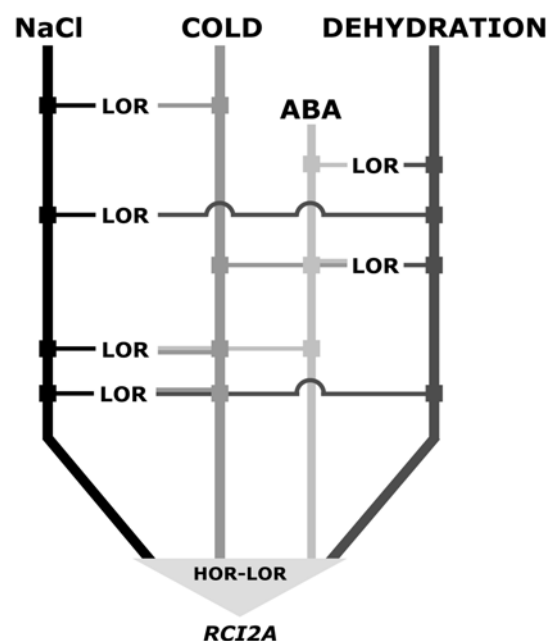


Fig. 3. **Working model for interactions between plant responses to cold, dehydration, high salt and ABA treatments.** From the results obtained when characterizing the *hor* and *lor* mutants, a signalling network involved in abiotic stress response can be proposed. The pathways mediating *AtRCI2A* expression in response to cold, dehydration, salt stress and ABA treatment interact and converge at different levels. Indeed, we found *lor* mutants

affected in the expression of *RCI2A* in response to two (Cold/NaCl; Cold/Dehydration; NaCl/Dehydration; ABA/Dehydration), three (Cold/NaCl/Dehydration; Cold/Dehydration/ABA; Cold/NaCl/ABA) and all treatments. We also identified *hor* mutants altered in *RCI2A* expression by all treatments. The proteins defined by the mutants identified are placed in putative positions in the pathways. Nevertheless, the existence of individual pathways mediating single responses, as well as the possibility that this signalling network interacts with other networks cannot be excluded.

ADAPTIVE RESPONSES TO LOW TEMPERATURE IN OTHER ORGANISMS

Many microorganisms and animals have also evolved adaptive mechanisms to cope with low and freezing temperatures, being able to successfully colonize cold environments, which represent most of the Earth biosphere. Interestingly, a number of these adaptive mechanisms are common with plants. For instance, animals and microorganisms also preserve membrane function and fluidity under low temperature conditions by increasing fatty acid unsaturation (Aguilar et al. 1998, Suzuki et al. 2001, Hayward et al. 2007). Moreover, as in the case of plants, animals and microorganisms produce high levels of compatible solutes, i.e. sugars, amino acids, polyols, to protect cells against the deleterious effects originated by freezing temperatures (Yancey 2005). Intracellular ice formation is lethal and most organisms living in regions exposed to subzero temperatures, including plants, synthesize antifreeze proteins to inhibit, slow down, or control the growth of ice crystals (Venketesh and Dayananda 2008). Antioxidant defense is another common adaptive mechanism evolved by microorganisms, plants and animals to survive at low temperature. Strategies for the detoxification of cold-induced reactive oxygen species that cause membrane damage include the production of high levels of antioxidant enzymes and detoxifying compounds (Schulze et al. 2005, Voituron et al. 2005, Storey 2006, Gocheva et al. 2009).

CONCLUSIONS AND PERSPECTIVES

Low temperatures are one of the most important plant abiotic stress factors. Confronted with them, some plants are able to adapt through mechanisms based on membrane composition changes, protein synthesis and activation of active oxygen scavenging systems.

One of the most interesting adaptive responses to low temperature which plants have evolved is the so called process of cold acclimation, whereby they acquire an increase in freezing tolerance upon a prior low-nonfreezing temperature treatment. This adaptive mechanism mainly relies on gene induction, although recent reports indicate postranscriptional, translational and posttranslational regulation is also involved and therefore identification of intermediaries in protein and mRNA transport and stability will be essential in order to completely understand the regulation of the cold acclimation process. How plants perceive the cold signal and how the signal is transduced to accurately activate plant responses, including the process of cold acclimation, still remains largely unknown. Recent studies in our own and others' laboratories have identified signalling components that mediate cold acclimation. Interestingly, some of these works have reported that several regulators are shared with other abiotic environmental responses. These discoveries reveal the complexity of molecular mechanisms underlying the adaptation of plants to their environment, raising the question of how plants coordinate the signalling pathways involved in the response to different abiotic stresses.

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