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Sudesh Kumar Yadav. Cold stress tolerance mechanisms in plants. A review. *Agronomy for Sustainable Development*, 2010, 30 (3), 10.1051/agro/2009050 . hal-00886535

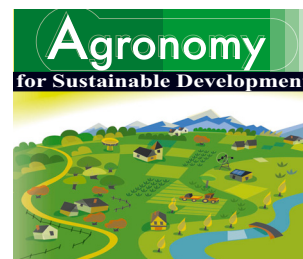
**HAL Id: hal-00886535**

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Submitted on 11 May 2020

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## Review article

# Cold stress tolerance mechanisms in plants. A review

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(Accepted 8 October 2009)

**Abstract** – The human population is increasing at an alarming rate, whereas at the same time agricultural productivity is decreasing due to the effect of various environmental problems. In particular, cold stress is a serious threat to the sustainability of crop yields. Indeed, cold stress can lead to major crop losses. Various phenotypic symptoms in response to cold stress include poor germination, stunted seedlings, yellowing of leaves (chlorosis), reduced leaf expansion and wilting, and may lead to death of tissue (necrosis). Cold stress also severely hampers the reproductive development of plants. The major negative effect of cold stress is that it induces severe membrane damage. This damage is largely due to the acute dehydration associated with freezing during cold stress. Cold stress is perceived by the receptor at the cell membrane. Then a signal is transduced to switch on the cold-responsive genes and transcription factors for mediating stress tolerance. Understanding the mechanism of cold stress tolerance and genes involved in the cold stress signaling network is important for crop improvement. Here, I review cold stress tolerance mechanisms in plants. The major points discussed are the following: (1) physiological effects of cold stress, (2) sensing of cold temperatures and signal transduction, and (3) the role of various cold-responsive genes and transcription factors in the mechanism of cold stress tolerance.

**cold stress / signal transduction / cold-responsive genes / transcription factors**

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## 1. INTRODUCTION

One-third of the total land area is considered as potentially suitable for arable agriculture. However, only some 10% of the world's 13 billion hectares are farmed. This is because abiotic stress in one form or another limits production on most of the world's 1.4 billion farmed hectares of land. This is a problem that is not going away. Among abiotic stresses, low and high temperature stress is very critical for determining the agricultural production. Plants exhibit a maximum rate of growth

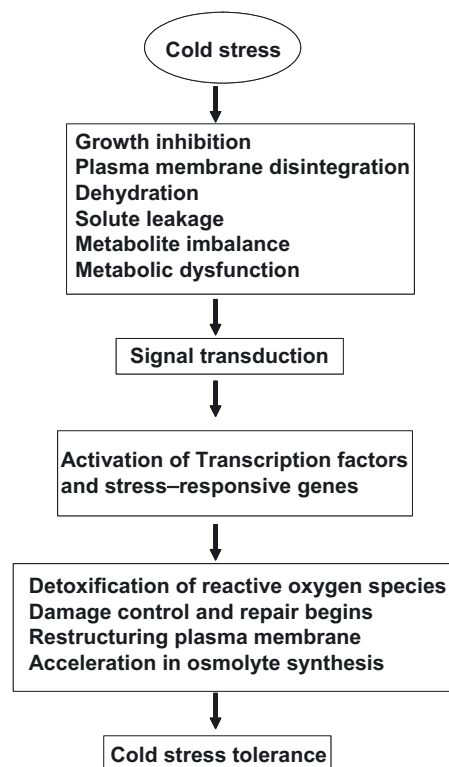
and development at an optimum temperature or over a diurnal range of temperatures (Fitter and Hay, 1981). When ambient temperature deviates from optimal, physiological, biochemical, metabolic and molecular changes occur within plants. This is an effort of plants to maximize growth and developmental processes and to maintain cellular homeostasis during such adverse conditions. Under increasingly stressful conditions, plants experience progressively more abnormal, impaired or dysfunctional cellular and whole-plant processes until the cardinal temperatures for survival are reached (Fitter and Hay, 1981). At the extremes of the natural temperature range of a plant, the degree of physiological, cellular, metabolic and molecular dysfunction becomes so severe that it leads to death.

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Plants feel stress under high as well as low temperature exposure. Mesophilic temperate plants possess inducible temperature-stress tolerance. During high temperature exposure, stress tolerance can be induced by exposure to short-term elevated temperature and this is known as acquired thermotolerance (Kotak et al., 2007), while at lower temperatures, stress tolerance can be induced by exposure to reduced temperature and is known as chilling tolerance and/or cold acclimation. Chilling tolerance is the ability of a plant to tolerate low temperatures (0–15 °C) without injury or damage (Somerville, 1995), while cold acclimation is an enhanced tolerance to the physical and physiochemical vagaries of freezing stress (Guy, 1990; Thomashow, 1999). Both cold acclimation and chilling tolerance involve an array of biochemical, molecular and metabolic processes (Thomashow, 1999; Larkindale et al., 2005; Kotak et al., 2007; Zhu et al., 2007).

Exposure of plants to temperature stress leads to the modification of metabolism in two ways. First, plants try to adjust their cellular metabolism that altered due to rising or falling of temperatures. Temperature stress changes the structure, catalytic properties and function of enzymes (Kubien et al., 2003) and membrane metabolite transporters. Interestingly, regulatory mechanisms of plants become active and function to restore normal metabolite levels, and most importantly, metabolic fluxes (Schwender et al., 2004; Fernie et al., 2005). Secondly, the modifications of metabolism in response to temperature stress are mainly linked to enhanced tolerance mechanisms. Many metabolites thought to have important properties that could contribute to induced stress tolerance have long been linked to stress responses (Guy, 1990; Thomashow, 1999; Nayyar et al., 2005). Particular interest has been focused on metabolites that can function as osmolytes. Osmolytes are involved in the regulation of cellular water relations and reduce cellular dehydration. Their compatible solute behavior allows them to function to stabilize enzymes, membranes and other cellular components. Osmolytes are also involved in retailoring of membrane lipid composition to optimize the liquid/crystalline physical structure necessary for proper membrane function and energy sources. Such stress-responsive metabolites particularly include soluble sugars, amino acids, organic acids, polyamines and lipids (Levitt, 1972; Guy, 1990; Nayyar et al., 2005; Farook et al., 2009).

Plants experience cold or chilling stress at temperatures from 0–15 °C. Under such situations, plants try to maintain homeostasis to acquire freezing tolerance and this involves extensive reprogramming of gene expression and metabolism (Thomashow, 1999; Cook et al., 2004). In the recent past, great attention has been paid towards elucidation of the intricate signal transduction pathways responsible for low-temperature response (Lee et al., 2005). Fundamental responses of plants during cold stress exposure and acclimation mechanisms are presented in Figure 1. Through work carried out in the last decade or so, several key components of signaling pathways and regulatory mechanisms contributing to freezing tolerance have been identified. This article summarizes the latest work that addresses the following questions: what are the physiological effects of cold stress in plants? How are cold temperatures sensed by plants? How is a cold stress signal transduced

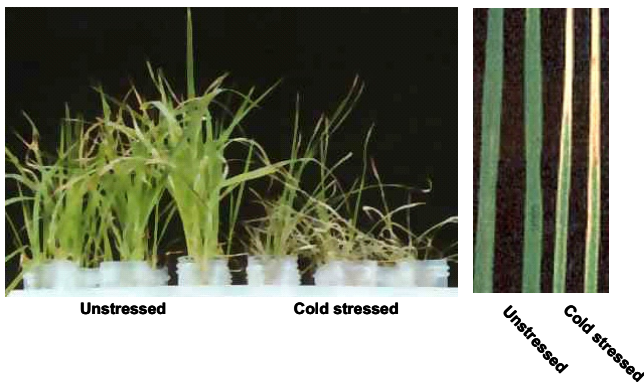


**Figure 1.** Fundamental responses of plants during cold stress exposure. Cold stress exposure causes various physiochemical disturbances, leading to growth inhibition. Cold stress response is perceived by plants through a signal transduction that leads to the activation of transcription factors and cold-responsive genes. Such transcription factors and genes control the damage due to cold stress and help in providing tolerance to plants.

to the nucleus to regulate gene expression? What are different genes and transcription factors involved in the mechanism of low temperature tolerance?

## 2. EFFECT OF COLD ON PLANT PHYSIOLOGY

Each plant has an optimum set of temperatures for its proper growth and development. A particular set of temperature conditions, which are optimum for one plant, may be stressful for another plant. It has generally been noticed that plants native to warm habitats exhibit symptoms of injury upon exposure to low non-freezing temperatures (Lynch, 1990). For example, plants such as maize (*Zea mays*), soybean (*Glycine max*), cotton (*Gossypium hirsutum*), tomato (*Lycopersicon esculentum*) and banana (*Musa sp.*) show signs of injury upon exposure to temperatures below 10–15 °C (Lynch, 1990; Guy, 1990; Hopkins, 1999). However, the appearance of injury symptoms depends upon the sensitivity of a plant to cold stress and varies from plant to plant. Cold stress-induced injury in plants may appear after 48 to 72 h of stress exposure. Plants exposed to cold stress show various phenotypic symptoms that include reduced leaf expansion, wilting and chlorosis (yellowing of leaves) and may lead to necrosis (death of



**Figure 2.** Effect of cold stress on rice seedlings. Rice seedlings exposed to  $-2^{\circ}\text{C}$  for 12 h and then allowed to recover for 1 week. Damage due to cold stress is seen as retardation in growth, reduced leaf expansion, wilting, chlorosis (yellowing of leaves) and necrosis (death of tissue). Picture on right hand side presents a closer view of the unstressed and cold stress-exposed leaves of rice.

tissue). The phenotypic symptoms of rice seedlings upon exposure to cold stress are shown in Figure 2. Cold stress also severely affects the reproductive development of plants and this has been seen in rice plants at the time of anthesis (floral opening), which leads to sterility in flowers (Jiang et al., 2002).

Cold stress generally results in poor germination, stunted seedlings, yellowing of leaves, withering and reduced tillering. The effects of cold stress at the reproductive stage of plants delay heading and result in pollen sterility, which is thought to be one of the key factors responsible for the reduction in grain yield of crops (Suzuki et al., 2008). The major adverse effect of cold stress in plants has been seen in terms of plasma membrane damage. This has been documented due to cold stress-induced dehydration (Steponkus, 1984; Steponkus et al., 1993). The plasma membrane is made up of lipids and proteins. Lipids in the plasma membrane are made up of two kinds of fatty acids: unsaturated and saturated fatty acids. Unsaturated fatty acids have one or more double bonds between two carbon atoms, whereas saturated fatty acids are fully saturated with hydrogen atoms. Lipids containing more saturated fatty acids solidify faster and at temperatures higher than those containing unsaturated fatty acids. Therefore, the relative proportion of these two types of fatty acids in the lipids of the plasma membrane determines the fluidity of the membrane (Steponkus et al., 1993). At the transition temperature, a membrane changes from a semi-fluid state into a semi-crystalline state. Cold-sensitive plants usually have a higher proportion of saturated fatty acids in their plasma membrane. Therefore, cold-sensitive plants have a higher transition temperature. On the contrary, cold-resistant plants have a higher proportion of unsaturated fatty acids and hence a lower transition temperature.

The agricultural crops which can withstand even during the freezing temperatures of late spring or early fall frost can be used more successfully for cultivation during cold stress. Therefore, selection of low temperature-tolerant crops is very

important for the sustainability of agriculture. Additionally, understanding of how cold stress induces its injurious effects on plants is crucial for the development of frost-tolerant crops. It has been noticed that cold-induced ice formation is the real cause of plant damage. Ice formation in plant tissues during cold stress leads to dehydration. Ice is formed in the apoplastic space of a plant tissue because that has relatively lower solute concentration. It is known that the vapor pressure of ice is much lower than water at any given temperature. Therefore, ice formation in the apoplast establishes a vapor pressure gradient between the apoplast and surrounding cells. As a result of this gradient, the cytoplasmic water migrates down the gradient from the cell cytosol to the apoplastic space. This adds to existing ice crystals in the apoplastic space and causes a mechanical strain on the cell wall and plasma membrane, leading to cell rupture (McKersie and Bowley, 1997; Olien and Smith, 1997; Uemura and Steponkus, 1997).

In addition to the well-established harmful effect of cold stress alterations in lipid composition of the biomembranes, affecting their fluidity (Senser and Beck, 1982; Quinn, 1985; Williams, 1990; Welti et al., 2002), certain additional factors may also contribute to damage induced by cold stress. This includes synthesis and accumulation of compatible solutes, the synthesis of cold acclimation-induced proteins (Close, 1997; Shinozaki and Yamaguchi-Shinozaki, 2000), changes in the carbohydrate metabolism (Hansen and Beck, 1994; Hansen et al., 1997; Liu et al., 1998; Frankow-Lindberg, 2001) and the boosting of the radical scavenging potential of the cells (Tao et al., 1998; Hernández-Nistal et al., 2002; Baek and Skinner, 2003). Taken together, cold stress results in loss of membrane integrity, leading to solute leakage. Further, cold stress disrupts the integrity of intracellular organelles, leading to the loss of compartmentalization. Exposure of plants to cold stress also causes reduction and impairing of photosynthesis, protein assembly and general metabolic processes.

Recently, attempts have also been directed towards analyzing the effect of cold stress on the whole-plant metabolome. Metabolic profiling of *Arabidopsis* plants revealed that cold acclimation increases 75% of the 434 analyzed metabolites (Cook et al., 2004; Kaplan et al., 2004). However, metabolite profiles in *Arabidopsis* do not appear to correlate with cold acclimation capacity (Hannah et al., 2006). The role of such metabolites in plants has been known as osmoprotectants. In addition to their role as osmoprotectants and osmolytes, certain metabolites (individual metabolites or the redox state) induced during cold acclimation might act as signals for reconfiguring gene expression. For example, cold stress induces the accumulation of proline, a well-known osmoprotectant. Microarray and RNA gel blot analyses have shown that proline can induce the expression of many genes, which have the proline-responsive element sequence ACTCAT in their promoters (Satoh et al., 2002; Oono et al., 2003).

Cold stress affects virtually all aspects of cellular function in plants. One of the major influences of cold stress-induced dehydration is membrane disintegration. Such changes caused by cold stress adversely affect the growth and development of plants.

### 3. SENSING AND SIGNAL TRANSDUCTION DURING COLD STRESS

Plants are sessile in nature. Therefore, the only way for them to survive under adverse environmental conditions is to adapt to changing surroundings quickly and efficiently. The plastic nature of higher plants makes them able to react to different stresses with specific responses in growth, development and metabolism. In view of this, understanding of such plant responses could be of great importance for agriculture. This might be helpful in developing stress-tolerant crop varieties. Cold stress exposure reduces the fluidic nature of cellular membranes and increases their rigidity. This documents that the primary site of cold stress sense in plants could be associated with membrane fluidity, protein and nucleic acid conformation, metabolite concentration, and cellular redox status.

Cold stress is sensed by plant cells through its membrane rigidification effect. Rigidification of the plasma membrane during cold exposure has been shown to induce cold-responsive genes that help in cold acclimation in alfalfa and *Brassica napus* (Orvar et al., 2000; Sangwan et al., 2001). The *Arabidopsis* *fad2* mutant defective in oleate desaturase exhibits membrane rigidification and cold stress sensitivity, suggesting the relation of cold stress sense to plasma membrane rigidification (Vaultier et al., 2006). Additionally, cold stress exposure in plants has been found to increase cytosolic calcium levels. This increase in calcium is mediated through membrane rigidification-activated mechano-sensitive or ligand-activated calcium channels. The higher level of calcium in the cytosol leads to signal amplification through phospholipids (Vergnolle et al., 2005; Williams et al., 2005; Chinnusamy et al., 2006; Komatsu et al., 2007).

Environmental stresses including cold stress are first perceived by the receptors present on the membrane of the plant cells. The specific receptor for cold stress is not yet known. The signal is then transduced downstream and many signaling pathways are activated. Studies have shown that such pathways are often activated in concert. The various components are calcium, reactive oxygen species, protein kinase, protein phosphatase and lipid signaling cascades. It is believed that specificity is achieved by the combination and timing of the activation of different signaling pathways. The change in cytosolic calcium level is sensed by calcium-binding proteins. These calcium-binding proteins do not possess enzymatic activity but undergo conformation changes in a calcium-dependent manner. The change in calcium-binding proteins makes them interact with other proteins and often initiates a phosphorylation cascade. Through this cascade, plant cells could target major stress-responsive genes or the transcription factors. Transcription factors also regulate the expression and function of genes, which ultimately leads to plant adaptation and survival during unfavorable conditions (Shinozaki and Yamaguchi-Shinozaki, 2000; Shinozaki et al., 2003; Mahajan and Tuteja, 2005; Yamaguchi-Shinozaki and Shinozaki, 2006).

Individual plant cells respond to the environmental stress in the way described above and then the whole plant acts synergistically. The change in gene expression governed by the signal cascade mechanism also induces changes in genes par-

ticipating in the formation of plant hormones such as abscisic acid, salicylic acid and ethylene. These hormones may amplify the same cascade or may initiate a new signaling pathway. Additionally, several other cellular components are also involved in the stress signal transduction mechanism. These accessory molecules may not directly participate in signaling but participate in the modification or assembly of major signaling components. Mainly such components are protein modifiers and act in post-translational modification of signaling proteins. Such modifiers are involved in myristoylation, glycosylation, methylation and ubiquitination of signaling proteins (Mahajan and Tuteja, 2005; Yamaguchi-Shinozaki and Shinozaki, 2006).

Lipid molecules are also very important in signal transduction during cold stress. Though lipid signaling is relatively less studied, phosphatidic acid produced by both phospholipase D and the concerted action of phospholipase C and diacylglycerol kinase has been proposed as a membranous secondary messenger molecule. Phosphatidic acid is rapidly and transiently generated in response to various stresses and has been proposed to function as a second messenger (Meijer and Munnik, 2003). This phosphatidic acid constitutes a minor portion of membrane lipids under control conditions, but its levels significantly increased upon exposure to numerous stresses including cold stress (Munnik, 2001; Meijer and Munnik, 2003). Several modes of action for phosphatidic acid in signal transduction can be imagined. Its functions are mainly based on the recruitment and regulation of enzymatic activity of target proteins. The recruitment of soluble proteins to particular membranes can have numerous effects, such as localization of a protein to the site where it is active, localization of a protein to a site where it is modified and sequestration of a protein from the site where it is active. Alternatively, phosphatidic acid could influence the enzyme activity of proteins already residing in the membrane or of recruited proteins or protein complexes. A plethora of phosphatidic acid-binding proteins have been found in different organisms, and many have been proposed to function in signaling cascades (Testerink and Munnik, 2005).

Abscisic acid is an important phytohormone that plays a crucial role in several plant stress responses, including cold stress (Verslues and Zhu, 2005; Mahajan and Tuteja, 2005). Furthermore, phospholipase D has been linked to reactive oxygen species, which are known to be involved in abscisic acid and in cold stress responses (Laloi et al., 2004; Mahajan and Tuteja, 2005). In *Arabidopsis*, different phospholipases D such as *AtPLD $\alpha$ 1* and *AtPLD $\delta$*  have been implicated in both the production of and responses to reactive oxygen species (Zhang et al., 2003, 2005). The *AtPLD $\delta$*  expression was shown to be induced by abscisic acid and cold in *Arabidopsis* plants (Katagiri et al., 2001). Cold-induced freezing tolerance is reportedly impaired in *Atpldd* T-DNA knock-out *Arabidopsis* mutant plants and enhanced in *AtPLD $\delta$* -overexpressing plants, which also display decreased and increased freezing-induced phosphatidic acid production, respectively (Li et al., 2004). As a whole, it seems that phospholipases D are involved in multiple aspects of both the overlapping and distinct signaling networks that are activated by cold stress. Cold stress-induced

increase in phospholipases D is also involved in changes in membrane fluidity and osmotic balance.

Secondary signals, such as abscisic acid and reactive oxygen species, can also induce calcium signatures that impact cold signaling. *Arabidopsis* mutants defective in the activation of the molybdenum cofactor of abscisic aldehyde oxidase, namely *aba3/freezing sensitive 1 (frs1)* (Llorente et al., 2000), also known as *los5* (low expression of osmotically responsive genes 5) (Xiong et al., 2001), exhibit hypersensitivity to freezing stress. The *los5* mutant plants show a significant reduction in the expression of cold- and osmotic stress-induced genes (Xiong et al., 2001). Reactive oxygen species accumulate in plant cells during exposure to various abiotic stresses, and they appear to have a strong influence on cold regulation of gene expression. The *Arabidopsis fro1* (frostbite1) mutant, which constitutively accumulates high levels of reactive oxygen species, exhibits impaired expression of cold-responsive genes and hypersensitivity to chilling and freezing. The *FRO1* gene 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 reactive oxygen species generation (Lee et al., 2002). Besides their effect on calcium signatures, reactive oxygen species signals can also exert their effects directly through the activation of redox-responsive proteins, such as transcription factors and protein kinases.

Ultimately, cold acclimation involves precise regulation of expression of transcription factors and effector genes, collectively known as cold-regulated genes (Thomashow, 1999; Viswanathan and Zhu, 2002). Significant progress has been made in identifying transcriptional, post-transcriptional and post-translational regulators of cold-induced expression of cold-regulated genes. Promoters of many of the cold-regulated genes contain cis-elements such as dehydration-responsive elements/C-repeat elements (A/GCCGAC), an abscisic acid-responsive element (PyACGTGGC), and myeloblastosis recognition sequences (C/TAACNA/G) and/or myelocytomatosis recognition sequences (CANNTG) (Yamaguchi-Shinozaki and Shinozaki, 2005). Additionally, abiotic stresses including cold stress regulate the expression and activity of various kinases of mitogen-activated protein kinase pathways. Under cold stress, reactive oxygen species activate the AtMEKK1/ ANP1 (MAPKKK)-AtMKK2 (MAPKK)-AtMPK4/6 (MAPK) mitogen-activated protein kinase cascade that is necessary for cold acclimation in plants (Kovtun et al., 2000; Teige et al., 2004). This suggests that mitogen-activated protein kinase cascades act as a converging point in abiotic stress signaling.

Cold stress is sensed by a yet unknown receptor. The signal is then transduced through several components of signal transduction pathways. The components of signal transduction pathways discussed here are also involved in the transduction of other environmental stress signals, suggesting a common pathway for several responses in plants. The cold stress signal leads to changes in the expression of cold-regulated genes and the level of expression determines the fate of plants under such stress.

#### 4. ROLE OF COLD-RESPONSIVE GENES AND TRANSCRIPTION FACTORS

Developing transgenic plants for cold tolerance is a fast and effective biotechnological tool to improve agricultural crops. Transgenic approaches have been used to improve cold tolerance in rice. Various studies on crop improvement have indicated that screening for genes involved in cold tolerance is a crucial initial step for crop improvement strategy using engineering (Hsieh et al., 2002; Dubouzet et al., 2003; Ohnishi et al., 2005; Ito et al., 2006). Among the genes identified for cold tolerance, genes encoding transcription factors are found to be better for improving stress tolerance in plants. Most of such transcription factors have been identified in *Arabidopsis* and rice. The functional and biochemical features of specific cell types are determined by their particular gene expression profiles. Such global gene expression patterns can be represented by a “transcriptome”, which reveals the identity and the level of expression of each expressed gene in a defined population of cells (Velculescu et al., 1997; Tuteja and Tuteja, 2004). The transcriptome can be modulated by both external and internal factors, and thereby provide not only a wealth of basic biological insights but also a global view of biological responses to environmental stimuli.

Transcriptome expression profiles of a plant under normal and stressed conditions can be obtained and compared by various methods, such as ribonucleic acid (RNA)-deoxyribonucleic acid (DNA) hybridization measurements, subtractive hybridization, subtraction libraries and differential display (Donson et al., 2002). However, these methods have technical shortcomings and therefore, are unable to provide overall gene expression patterns. The recent DNA microarray technique allows larger-scale quantitative gene expression analysis. Microarray is a very useful technique for transcriptome analysis. This has been used in identifying many stress-inducible genes involved in stress response and tolerance (Shinozaki et al., 2003; Seki et al., 2004). A large number of genes responsive to various abiotic stresses have been identified using various microarrays (Seki et al., 2001, 2002; Fowler and Thomashow, 2002; Kreps et al., 2002; Rabbani et al., 2003; Bray, 2004; Maruyama et al., 2004; Vogel et al., 2005). However, microarray has some innate limitations. It only allows the analysis of arbitrarily chosen genes (Duggan et al., 1999; Jones et al., 2001; Patankar et al., 2001; Lorenz and Dean, 2002; Gibbings et al., 2003). Another technology known as serial analysis of gene expression partially overcomes this limitation (Velculescu et al., 1995). It is an extremely promising, efficient and global approach for analyzing gene expression profiles under different physiological states. It has the advantage over various other techniques that it allows identifying novel genes along with their associated pathways and metabolic circuits. Also, the Affymetrix 22K GeneChip ATH1 has been used recently to identify more stress-inducible genes. The genes identified using the Affymetrix 22K GeneChip ATH1 can be seen at The *Arabidopsis* Information Resource Uniform Resource Locator (<http://www.arabidopsis.org/>).

Upon stress exposure, the expression of various genes alters. Such stress-responsive genes have been found to provide protection to plants in two ways. They protect from stress by producing important metabolic proteins. The products of stress-responsive genes are also involved in regulation of genes of signal transduction pathways. Based upon different functions of proteins encoded by stress-responsive genes, these are classified into two groups (Fowler and Thomashow, 2002; Kreps et al., 2002; Seki et al., 2002). The first group includes proteins that probably function in stress tolerance, such as chaperones, late embryogenesis abundant proteins, osmotin, antifreeze proteins, mRNA-binding proteins, key enzymes for osmolyte biosynthesis such as proline, water channel proteins, sugar and proline transporters, detoxification enzymes, enzymes for fatty acid metabolism, proteinase inhibitors, ferritin, and lipid-transfer proteins. Some of these stress-inducible genes encoding key enzymatic proteins for osmolyte biosynthesis, late embryogenesis abundant proteins, and detoxification enzymes have been overexpressed in transgenic plants. Such transgenics have been found to be stress-tolerant (Cushman and Bohnert, 2000). This has evinced that the gene products of the stress-inducible genes really function in stress tolerance.

The second group contains protein factors involved in further regulation of signal transduction and gene expression that probably function in stress response. This group includes various transcription factors (Seki et al., 2003). These transcription factors regulate various stress-inducible genes cooperatively or separately, and may constitute gene networks. Functional analysis of such stress-inducible transcription factors should provide more information on the complex regulatory gene networks that are involved in responses to stresses including cold stress. There are a few more proteins involved in the complex network of stress regulatory mechanism of plants such as kinases, protein phosphatases, enzymes involved in phospholipid metabolism, calmodulin binding protein and 14-3-3 proteins. The functions of most of these genes are not fully understood yet. Some of these stress-inducible regulatory genes that encode transcription factors have been overexpressed in plants and generated stress-tolerant phenotypes in transgenic plants (Zhang et al., 2004; Tester and Bacic, 2005; Vinocur and Altman, 2005).

Cold stress-induced genes are known as cold-responsive genes. Most of such genes encoding polypeptides are homologs of late embryogenesis abundant proteins and the polypeptides that are synthesized during the late embryogenesis phase (Dure, 1993; Ingram and Bartels, 1996; Close, 1997). These late embryogenesis abundant-like proteins are mainly hydrophilic and have relatively simple amino-acid composition. They are composed largely of a few amino acids with repeated amino acid sequence motifs and are predicted to contain regions capable of forming amphipathic alpha-helices. The examples of cold-responsive genes include *COR15a* (Artus et al., 1996), alfalfa *Cas15* (Monroy et al., 1993) and wheat *WCS120* (Houde et al., 1992). The expression of cold-responsive genes has been shown to be critical for both chilling tolerance and cold acclimation in plants. The identified *Arabidopsis* cold-responsive genes are *COR78/RD29*, *COR47*,

*COR15a* and *COR6.6* (Thomashow, 1999). These genes are induced by cold, dehydration or abscisic acid. A *COR15A* polypeptide encoded by the *COR15a* gene is targeted to the chloroplast. Formation of hexagonal II phase lipids is a major cause of membrane damage in non-acclimated plants during cold stress. However, *COR15a* expression decreases the propensity of the membranes to form hexagonal II phase lipids in response to freezing (Uemura and Steponkus, 1997). This observation suggests the role of *COR15A* polypeptide in providing freezing tolerance.

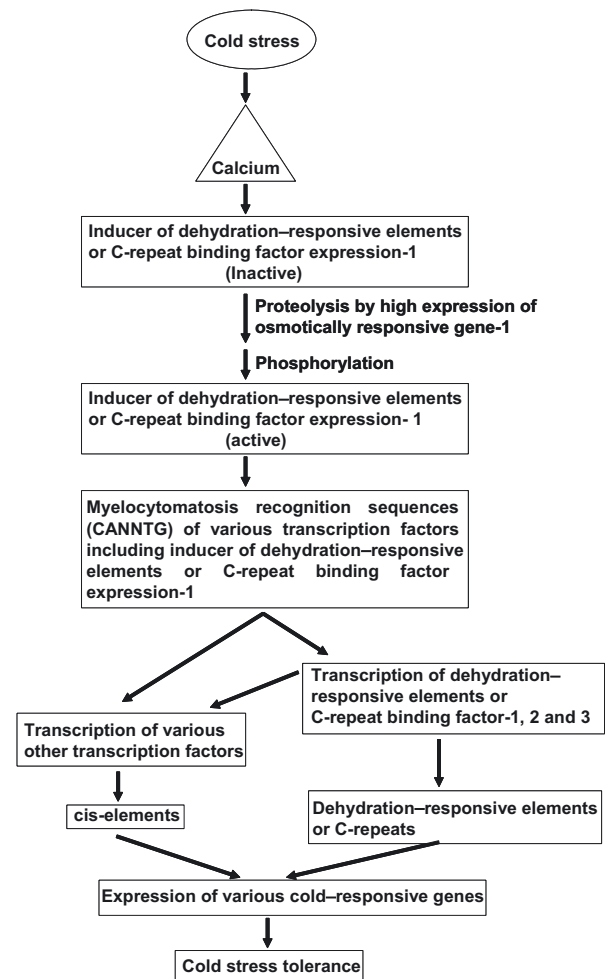
Every gene has promoter sequences upstream of their coding region. The expression of a gene is driven by its promoter. Promoters have regulatory elements in their sequences that control the expression of a gene. In view of this, analysis of the promoter elements of cold-responsive genes revealed that they contain dehydration-responsive elements or C-repeats. Some of the cold-responsive genes also contain an abscisic acid-responsive element in their promoter sequences (Yamaguchi-Shinozaki and Shinozaki, 1994; Stockinger et al., 1997). The expression of cold-responsive genes is induced during cold exposure. Exposure of plants to cold stress also induces the expression of a transcription factor, i.e. dehydration-responsive elements or C-repeat binding factors. This suggests that these binding factors regulate the expression of cold-responsive genes during cold stress. Overexpression of dehydration-responsive elements or C-repeat binding factors led to the induction of cold-responsive genes under unstressed conditions, documenting the regulatory role of binding factors in cold-responsive gene expression (Stockinger et al., 1997). Cold stress induces three dehydration-responsive elements or C-repeat binding factor genes in *Arabidopsis* such as *AtCBF1* (*DREB1B*), *AtCBF2* (*DREB1C*) and *AtCBF3* (*DREB1A*). Dehydration-responsive elements or C-repeat binding factors bind to the promoter of cold-responsive genes and other cold-regulated genes. The overexpression of these regulatory elements not only resulted in increased freezing tolerance but also an increase in drought tolerance (Liu et al., 1998). This finding provides strong support that a fundamental role of cold-responsive genes is to protect the plant cells against cellular dehydration.

The role of these various factors has been substantiated by developing transgenics. Constitutive or stress-inducible overexpression of dehydration-responsive elements or C-repeat binding factor-1 or -3 in transgenic *Arabidopsis* plants resulted in constitutive or enhanced expression of cold-responsive genes. This increased abiotic-stress tolerance, including freezing tolerance, in *Arabidopsis* (Jaglo-Ottosen et al., 1998; Liu et al., 1998; Kasuga et al., 1999). Overexpression of *Arabidopsis thaliana* dehydration-responsive elements or C-repeat binding factor-1 or -3 also enhanced chilling, freezing, drought and/or salt-stress tolerance in *Brassica* (Jaglo et al., 2001), tomato (Hsieh et al., 2002), tobacco (Kasuga et al., 2004), wheat (Pellegrineschi et al., 2004) and rice (Oh et al., 2005). Similarly, overexpression of rice (Dubouzet et al., 2003) and maize (Qin et al., 2004) dehydration-responsive element binding factor-1 in transgenic *Arabidopsis* induced constitutive expression of cold-responsive genes and conferred tolerance to freezing and drought stress. These

studies have documented that dehydration-responsive elements or C-repeat binding factor-dependent gene expression is an important, evolutionarily conserved component of cold acclimation in diverse plant species. However, transcriptome analysis of dehydration-responsive elements or C-repeat binding factors overexpressing transgenic *Arabidopsis* revealed that only ~12% of the cold-responsive genes are induced by dehydration-responsive elements or C-repeat binding factors (Fowler and Thomashow, 2002). This suggests the possibility of other transcriptional activators' or repressors' role in cold acclimation.

To find this, T-DNA insertion mutants of *Arabidopsis* were screened. Genetic analysis of mutants led to the identification of an inducer of dehydration-responsive elements or C-repeat binding factor expression-1 as an activator of dehydration-responsive elements or C-repeat binding factor-3 (Chinnusamy et al., 2003). The inducer of dehydration-responsive elements or C-repeat binding factor expression-1 encoded a transcription factor that specifically recognized myelocytomatosis sequence on the promoter of dehydration-responsive elements or C-repeat binding factor-3. Interestingly, transgenic *Arabidopsis* overexpressing an inducer of dehydration-responsive elements or C-repeat binding factor expression-1 did not express dehydration-responsive elements or C-repeat binding factor-3 at warm temperatures, but showed a higher level of expression for dehydration-responsive elements or C-repeat binding factor-3 as well as other cold-responsive genes such as *RD29* and *COR15a* at low temperatures. This suggests that cold stress induced an inducer of dehydration-responsive elements or C-repeat binding factor expression-1 is a crucial activator of dehydration-responsive elements or C-repeat binding factor-3 in plants. In addition to the role of an inducer of dehydration-responsive elements or C-repeat binding factor expression-1 in regulating dehydration-responsive elements or C-repeat binding factors, homeodomain leucine zipper has also been found to interact with dehydration-responsive elements or C-repeat binding factors. The expression of two dehydration-responsive elements or C-repeat binding factor-1 such as cDNAs CaCBFIA and CaCBFIB in hot pepper was induced in response to low temperature stress (4 °C) (Kim et al., 2004). The induction in expression of homeodomain leucine zipper and its interaction with CaCBFIB during cold stress suggested that homeodomain leucine zipper could also be an important regulon of dehydration-responsive elements or C-repeat binding factors. So far, studies have evinced the existence of one of the major and important molecular mechanisms of cold tolerance in plants, as presented in Figure 3.

One of the largest families of transcription factors, myeloblasts also play a very significant role in regulating gene expression in response to environmental and developmental changes. In this category, myeloblasts are predominant transcription factors. Based on the number of tandem repeats of SANT 'SWI3, ADA2, N-CoR and TFIIB' DNA-binding domains myeloblastosis transcription factors are divided into three subfamilies: myeloblastosis-like proteins (MYB1R factors), R2R3-type myeloblastosis factors and



**Figure 3.** Involvement of various transcription factors in the induction of cold-responsive genes during cold stress. Cold stress exposure in plants induces the calcium signature. This signature then induces an inducer of dehydration-responsive elements or C-repeat binding factor expression-1 in its inactive form. The variant ring finger protein high expression of osmotically responsive gene-1 has been identified as a negative regulator of cold responses. The high expression of osmotically responsive gene-1 is an E3 ligase required for the ubiquitination of an inducer of dehydration-responsive elements or C-repeat binding factor expression-1. High expression of the osmotically responsive gene-1 physically interacts with an inducer of dehydration-responsive elements or C-repeat binding factor expression-1 and mediates the proteolysis and phosphorylation of an inducer of dehydration-responsive elements or C-repeat binding factor expression-1, converting it from inactive to active form. An active inducer of dehydration-responsive elements or C-repeat binding factor expression-1 regulates the expression of dehydration-responsive elements or C-repeat binding factors and other transcription factors by binding to myelocytomatosis recognition sequences in their promoter regions. The dehydration-responsive elements or C-repeat binding factors also regulate the expression of other transcription factors. These various transcription factors then regulate the expression of various cold-responsive genes by acting on cis-elements and C-repeats/ dehydration-responsive elements in their promoter or upstream sequences. Induction in expression of cold-responsive genes determines tolerance during cold stress.



R1R2R3 myeloblastosis (MYB3R) factors with one, two or three repeats, respectively (Rosinski and Atchley, 1998).

In *Arabidopsis*, 198 genes encode myeloblastosis transcription factors and it is one of the largest families among all of the transcription factor families (Yanhui et al., 2006; Pasquali et al., 2008). The function of some of these myeloblastosis transcription factors has been elucidated through T-DNA insertional mutagenesis and transcript expression profiling analysis (Kranz et al., 2000; Stracke et al., 2001; Yanhui et al., 2006). This has revealed the roles of R2R3 myeloblastosis proteins in cell cycle control, secondary metabolism, cellular morphogenesis, meristem formation, hormonal signaling and stress responses (Salomoni et al., 1997; Meissner et al., 1999). Several R2R3 myeloblastosis genes have been found to be involved in the responses to environmental stimuli including cold stress (Yanhui et al., 2006). In this category, high expression of osmotically responsive genes such as *HOS10* encoding a R2R3-type myeloblastosis transcription factor is essential for cold acclimation (Zhu et al., 2005). A myeloblastosis 15 controls the expression of dehydration-responsive elements or C-repeat binding factors and other cold-responsive genes (Agarwal et al., 2006). Overexpression of rice myeloblastosis transcription factors *OsMYB3R-2* and *OsMYB4* in *Arabidopsis* has been found to significantly increase the cold tolerance (Vannini et al., 2004; Dai et al., 2007; Pasquali et al., 2008).

In addition to the role of late embryogenesis abundant-like proteins and myeloblastosis transcription factors in cold acclimation, recent studies have found the essential role of the Zinc-finger group of proteins in cold stress tolerance. Overexpression of Zinc-finger genes such as *OsiSAP8*, *OsCOIN*, *OsISAP1*, *OsbHLH1*, *OsDREB1/CBF*, *ROs-bZIP*, *SNAC2* and *OsNAC6* confers cold stress tolerance at the seedling stage in rice (Wang et al., 2003; Mukhopadhyay et al., 2004; Ohnishi et al., 2005; Ito et al., 2006; Cheng et al., 2007; Liu et al., 2007; Nakashima et al., 2007; Hu et al., 2008; Kanneganti and Gupta, 2008).

The use of advanced biotechnological tools has enabled us to identify the role of several other signaling components and metabolic regulators in stress response. A *OsTPP1* gene encoding trehalose-6-phosphate phosphatase has been documented to confer cold stress tolerance in rice by inducing the expression of cold-responsive genes (Pramanik and Imai, 2005; Shima et al., 2007; Ge et al., 2008). Similarly, hydrophobic proteins encoded by *OsLti6* genes (*OsLti6a* and *OsLti6b*) also enhanced tolerance to cold stress in rice seedlings (Morsy et al., 2005). The *Arabidopsis FAD8* gene (Gibson et al., 1994) encodes a fatty acid desaturase that contributes to freezing tolerance by altering the lipid composition. The cold-responsive genes encoding molecular chaperones, including a spinach heat shock protein gene *hsp70* (Anderson et al., 1994), and a *Brassica napus* heat shock protein gene *hsp90* (Krishna et al., 1995), contribute to freezing tolerance by stabilizing proteins against freeze-induced denaturation.

Stress responses in plants are also mediated by the mitogen-activated protein kinase cascades as described under the signal transduction section. Overexpression of a rice mitogen-activated protein kinase *OsMAPK5* conferred tolerance to various environmental stresses including cold stress in rice

seedlings (Xiong and Yang, 2003). Stress-responsive genes encoding calcineurin B-like protein-interacting protein kinases such as *OsCIPK03* and *OsCIPK12* also play important roles in conferring cold stress tolerance in rice (Xiang et al., 2007). The role of calcium-dependent protein kinases in signal transduction of plants has also been well documented. The calcium-dependent protein kinases encoded by *OsCDPK7* and *OsCDPK13* have been found to be positive regulators for enhancing cold stress tolerance (Saijo et al., 2000, 2001; Wan et al., 2007; Wang et al., 2008). Induction in expression of a rice calcium-dependent protein kinase *OsCDPK13* and accumulation of *OsCDPK13* protein were upregulated in response to cold (Abbasi et al., 2004). A rice calcium-dependent protein kinase *OsCDPK13* overexpressing transgenic rice had higher recovery rates following cold stress in comparison with non-transgenic rice. Cold-tolerant rice varieties exhibited higher expression of a rice calcium-dependent protein kinase *OsCDPK13* than the cold-sensitive ones (Abbasi et al., 2004). These studies have documented the important role of calcium-dependent protein kinases in mediating tolerance in response to cold stress.

For acclimation to cold stress, plants involve precise signaling and regulation of the transcriptome. Among the various reported transcription factors, an inducer of dehydration-responsive elements or C-repeat binding factor expression-1 and dehydration-responsive elements or C-repeat binding factors play pivotal roles in the regulation of cold-responsive genes and providing cold tolerance to plants.

## 5. CONCLUSION

Cold stress is one of the major environmental stresses that limit agricultural crop productivity by affecting their quality and post-harvest life. Most temperate plants acquire chilling and freezing tolerance upon prior exposure to sublethal cold stress, a process called cold acclimation. Still, many agronomically important crops are incapable of cold acclimation. Cold stress affects virtually all aspects of cellular function in plants. Such changes caused by cold stress adversely affect the growth and development of plants. Cold stress is sensed by a yet unknown receptor. The cold stress signal is transduced through several components of signal transduction pathways. Major components are calcium, reactive oxygen species, protein kinase, protein phosphatase and lipid signaling cascades. Abscisic acid also mediates the response of cold stress. The cold stress signal leads to regulation of transcription factors and effector genes, collectively called cold-regulated genes. The effector genes encoding proteins under this category include chaperones, late embryogenesis abundant proteins, osmotin, antifreeze proteins, mRNA-binding proteins, key enzymes for osmolyte biosynthesis such as proline, water channel proteins, sugar and proline transporters, detoxification enzymes, enzymes for fatty acid metabolism, proteinase inhibitors, ferritin, and lipid-transfer proteins. The transcription factors involved during cold stress response are inducers of C-repeat binding factor expression-1, C-repeat binding factors, myeloblasts and mitogen-activated protein kinase. Analyses of the expression

of cold-regulated genes indicate the presence of multiple signal transduction pathways between the initial stress signals and gene expression. Use of these genes and transcription factors in genetic modification of agricultural crops can improve cold tolerance and productivity.

**Acknowledgements:** I am grateful to Dr. P. S. Ahuja, Director, IHB, for his useful suggestions. I would like to acknowledge the Council of Scientific and Industrial Research (CSIR) and Department of Science and Technology (DST), Govt. of India, for their financial help with conducting research in my laboratory.

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