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Role of biomolecules in sensing, signal transducing and acclimation in plants against frost induced low temperature stress - A Review

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Abstract – Frost induced freeze damages are more common in subtropical plant species. *Many evergreen plants get drastically damaged while certain manage to tolerate this low temperature stress by way of developing acclimation against this stress. This review discusses the recent developments in cold stress sensing, receptors involved and the perception of the stress by the plants. Together with this it also elaborate several signalling pathways that trigger the synthesis of cold responsive proteins in the plant system. Biochemical and physiological approaches defining components of low temperature tolerance has also discussed. Role of reactive oxygen species in peroxidation of lipids which play pivotal in membrane fluidity has also been elucidated. Role of carbohydrates in tolerance through reabsorption of water and other mechanism, lignins in cytoskeltal rearrangements and TCF mediated signalling is also key factor in plant survival under low temperature stress. Cold acclimation and gene regulation at transcription level has opened new horizon for technology development for protection of agricultural/horticultural plant species against frost and low temperature stress.*

KEY WORDS: Frost - freeze damage, Membrane rigidification, CBFs/ DREBs

INTRODUCTION

Among various environmental stresses, low temperature is one of the most important stress limiting the productivity and distribution of plants across the globe. Low temperature induces two types of stresses, the chilling stress (0-15°C) which results from temperatures cool enough to produce injury without forming ice crystals in plant tissues and the freezing stress (<0^0C) which leads to ice formation within plant tissues. Low temperature stress is common in nature especially for evergreens species in the subtropical regions where it causes considerable damage to the plant species which lack the intrinsic mechanism to fight this stress. In order to cope with such conditions, some plant species have the ability to increase their degree of freezing tolerance in response to low temperatures, a phenomenon known as cold acclimation. It is well established that there occur some of the molecular and physiological changes during cold acclimation which impart the ability for plant cold tolerance [1, 2]. The process of cold tolerance is more complex in plants with evergreen leaves dominated in the subtropical regions where unfavourable temperature season are relatively short, the retention of evergreen leaves is beneficial to carbon fixation. Long lived leaves allow the plant to take advantage of every favourable and unfavourable opportunity for dry matter production and growth. However, leaf longevity requires various protecting mechanisms in order to survive the manifold dangers. The toughening and hardening of leaves will clearly make the foliage liable to resist climatic impairments and also defend itself against herbivores and pathogen stress [3].

Dehydration during winter is the cause of death among broadleaf evergreens. Low temperature has a huge impact on the survival and geographical distribution of plants. It affects a range of cellular metabolisms in plant system depending on the intensity and duration of the stress. As a result of exposure to low temperatures, many physiological and biochemical cell functions have been correlated with visible symptoms (wilting, chlorosis, or necrosis) [4, 5]. Often, these adverse effects are accompanied by changes in cell membrane structure and lipid composition [5, 6], cellular leakage of electrolytes and amino acids, a diversion of electron flow to alternate pathways [7], alterations in protoplasmic streaming and redistribution of intracellular calcium ions [8, they also involve changes in protein content and enzyme activities [4] as well as ultrastructural changes in a wide range of cell components, including plastids, thylakoid membranes and the phosphorylation of thylakoid proteins, and mitochondria [9]. Brief exposures to low temperatures may only cause transitory changes, and plants generally revive. However, prolonged exposure to stress causes plant necrosis or death. To overcome stresses generated by exposure to low non-freezing temperatures, plants can trigger a cascade of events that

cause changes in gene expression and thus induce biochemical and physiological modifications that enhance their tolerance [2, 10]. This phenomenon is known as chilling or cold acclimation. As membranes are the primary site of low temperature stress adaptation of living cells to chilling temperatures is a function of alteration in the membrane lipid composition by increased fatty acid unsaturation. Exposure of plants to temperature stress leads to the modification of metabolism in two ways: firstly, 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 and membrane metabolite transporters [11]. Interestingly, regulatory mechanisms of plants become active and function to restore normal metabolite levels, and most importantly, metabolic fluxes [12, 13] 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 induce stress tolerance have long been linked to stress responses [14, 15]. 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 [16, 17]. Phytohormones and salicylic acid have been suggested to play important roles in sustaining the growth and development of plants at cold temperature [18]. In the recent past great attention has been paid towards studying plant cold acclimation through introduction of molecular and genetic technologies and also towards elucidation of intricate signal transduction pathways responsible for low temperature response [19]. The review discussed here has focused mainly on addressing the questions: How do plant sense the decreasing temperature, What are the signalling cascades that transduce low temperature cue to cell nucleus and brings about change in gene expression and what role the bio-molecules play in cold acclimation. A thorough understanding on this subject matter can provide a fillip to the process of technology development for protecting economic plant species against this severest weather related stress.

1. Cold stress sensing, receptors and perception

Despite the extensive studies on cold acclimation pathways, the mechanism that how plant sense the low temperature is unclear. Low temperature triggers production of secondary messengers, such as Ca^{2+} which are perceived by secondary sensors in plants to cold stress responses. One possible primary cold signal sensors in the plasma membrane is Ca^{2+} channel. Calcium acts as a mediator of stimulus–response coupling in the regulation of plant growth, development, and responses to environmental stimuli [20, 21]. Cold stress-induced rigidification of plasma membrane micro domains can cause actin cytoskeletal rearrangement. This may be followed by the activation of Ca^{2+}channels and increased cytosolic Ca^{2+} levels, which may be involved in the cold acclimation process [22, 23]. The Ca^{2+} released from internal cellular reserves, mediated by inositol triphosphate, is upstream of the expression ofCBFs (C-repeat binding factors) and COR (cold responsive) genes in the cold-signalling pathway(s) [24, 25]. Recently, Doherty et al [26] provided more evidence for a link between calcium signalling and cold induction of the CBF pathway, showing that calmodulin binding transcription activator (CAMTA) factors bind to a regulatory element in the CBF2 gene promoter. As the CAMTA proteins are calmodulin binding transcription factors, they may act directly in the transduction of LT-induced cytosolic calcium signals into downstream regulation of gene expression [26]. Similarly, CRLK1, a novel calcium/calmodulin-regulated receptor-like kinase, was reported to be crucial for cold tolerance in plants [27]. In plants cellular Ca^{2+} dynamics are detected in response to cold with in 40s through a novel aequorin based Ca^{2+}signalling mechanisms [28]. Recent studies on *Arabidopsis* and moss indicted that the cyclic nucleotide gated calcium channels (CNGC) is important for thermal sensing and thermo-tolerance [29]. Currently, one widely purpose hypothesis is that the reduction in membrane fluidity caused by cold stress appears to be primary events of cold perception to activate the Ca^{2+} channels in plants. Secondly, the lipid composition of plant has been shown to play a pivotal role in plant response to cold stress. Arabidopsis fad-2 mutant is defective in oleate desaturase and shows an irregular membrane composition and membrane rigidification, which results in lethality at low temperature [30]. Activity of diacylglycerol kinase (DAGK) can be used to monitor membrane rigidification. The enzyme is activated in the fad 2 mutant but activated at 18^0C in the fad2 mutant but activated at 14^0C in wild type plants [31]. Moreover ADS2 (Acyl lipid desaturase 2) plays an essential role in chilling and freezing tolerance in Arabidopsis by adjusting the composition of organelle membrane lipids [32]. Interestingly, SFR2 encodes a galactolipid remodelling enzymes localized on outer membrane stabilization during freezing [33]. Recent studies have shown that cold induces proteolyitic activation of a membrane anchored NAC transcription factor NTL6 [7]. This proteolytic processing of NTL6 is also promoted in the fad3 fad7 fad8 triple mutants, which exhibit membrane rigidification may regulate NTL6 processing [7]. These finding support the hypothesis that plant cell can perceive cold stress via membrane rigidification. 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. Thus far, no plant sensors for low temperature have been identified. Multiple primary sensors are thought to be involved in stress sensing. Each sensor may perceive a specific

aspect of the stress and may be involved in a distinct branch of the cold signalling pathway. Plants may sense low temperature through changes in the physical properties of membranes, because membrane fluidity is reduced during cold stress [34]. plasma membrane rigidification raised by a membrane rigidifier, dimethyl sulfoxide (DMSO), can induce the expression of *COR* (cold-responsive) genes, even at normal growth temperatures, whereas the application of a membrane fluidizer, benzyl alcohol, prevents the induction of *COR* gene expression at low temperatures [22, 34]. Membrane rigidification induces cytosolic $Ca2^+$ signatures, and the transient increase in $Ca2^+$ regulates COR gene expression. Because COR gene expression was impaired by gadolinium, a mechano-sensitive $Ca2^+$ channel blocker, it is suggested that mechanosensitive $Ca2^+$ channels may be involved in the perception of cold-induced membrane rigidification [35]. The $Ca2^+$ signal can be transduced into the nucleus. Nuclear $Ca2^+$, which is monitored by a chimera protein, formed by the fusion of aequorin to nucleaoplasmin, is also transiently increased after cold shock, and the peak of nuclear $Ca2^+$ is delayed at 5 to 10 s, compared to the peak of cytosolic $Ca2^+$ [36]. In animal cells, the increase in nuclear $Ca2^+$ is caused by nuclear envelope, which is continuous with the endoplasmic reticulum, one of major $Ca2^+$ stores [37]. The increase in nuclear $Ca2^+$ can be propagated by cytosolic $Ca2^+$ transients via the nuclear pore complexes [37]. Because the architecture of nuclear envelope in plants is similar to that described in animal cells with the presence of numerous nuclear pore complexes [38], the nuclear $Ca2^+$ signal may be initiated from nuclear envelope and propagated by cytosolic $Ca2^+$ transients in plants. Because nuclear $Ca2^+$ signalling is also important to control gene transcription in plants [39], as well as animal cells [37], identification of transporters, which are localized to plasma membrane or membrane of nuclear envelope and are involved in regulation of cold-inducible Ca2+ transients, may elucidate the detail mechanisms how Ca^{2+} signal regulates cold signalling. The cold stress-induced Ca2+ signature can be decoded by different pathways. Plants possess groups of $Ca2^+$ sensors, including CaM (calmodulin) and CMLs (CaM-like), CDPKs ($Ca2^+$-dependent protein kinases), CCaMK ($Ca2^+$-and $Ca2^+$/CaM-dependent protein kinase), CAMTA (CaM-binding transcription activator), CBLs (calcineurin B-like proteins) and CIPKs (CBL-interacting protein kinases). Genetic analysis demonstrated that CDPKs work as positive regulators [40], but calmodulin-3 is a negative regulator of gene expression and cold tolerance in plants [41]. CBLs relay the Ca2+ signal by interacting with and regulating the family of CIPKs. As the cbl1 mutant exhibits a chilling sensitive phenotype, CBL1 regulates cold response by interacting with CIPK7 [42]. CAMTA3 has been identified as a positive regulator of CBF2/DREB1C expression through binding to a regulatory element (CG-1 element, vCGCGb) in its promoter [26]. The camta2 camta3 double mutant plants are sensitive to freezing temperatures. The expression of CBF3/DREB1A is not regulated by CAMTA, because there is no CG-1 element in its promoter [26]. In addition to the plasma membrane, chloroplast may also play a role in sensing ambient temperature. Under low temperature, an imbalance between the capacity to harvest light energy and the capacity to dissipate this energy through metabolic activity causes excess photosystem II (PSII) excitation pressure, leading to generation of reactive oxygen species (ROS). The phosphorylation of proteins in response to cold and the suppression of protein phosphatase activity may also provide a means for the plant to sense low temperature. The MPK (mitogen-activated protein kinase) cascade is implicated in the regulation of cold signalling and cold tolerance. Arabidopsis MPK4 and MPK6 are phosphorylated by MKK2 (MAP kinase kinase2) when exposed to cold stress, and constitutively activated MKK2 over expressing plants exhibit cold tolerance and the up-regulation of CBF/DREB1s [CRT (C-repeat)/DRE (dehydration responsive-element) binding proteins] [43]. The cold activation of SAMK, an alfalfa MPK, requires membrane rigidification, and the activation of SAMK by low temperatures is inhibited by blocking the influx of extracellular Ca2+ and is prevented by an antagonist of CDPKs, suggesting that membrane rigidification, Ca2+ fluxes and CDPKs are required for the activation of MPK cascades in alfalfa [44]. Together with these results, several signalling pathways are triggered to promote the production of COR (cold-responsive) proteins. Environmental stresses including cold stress are first perceived by the receptors present on the membrane of the plant cells. The signal is then transduced downstream and many signalling 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 signalling cascades. It is believed that specificity is achieved by the combination and timing of the activation of different signalling 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 unfavourable conditions [45, 46, 47 and 48]. 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 participating 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 signalling pathway. Additionally, several other cellular components are also involved in the stress signal transduction mechanism. These accessory molecules may not directly participate in signalling but participate in the modification or assembly of major signalling components. Mainly such components are protein modifiers and act in post-translational modification of signalling proteins. Such modifiers are involved in myristoylation, glycosylation, methylation

and ubiquitination of signalling proteins [47, 48]. Lipid molecules are also very important in signal transduction during cold stress. Though lipid signalling 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 [49]. 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 [49, 50]. 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 signalling cascades [51]. Abscisic acid is an important phytohormone that plays a crucial role in several plant stress responses, including cold stress [48, 52]. Furthermore, phospholipaseD has been linked to reactive oxygen species, which are known to be involved in abscisic acid and in cold stress responses [48, 53]. In *Arabidopsis*, different phospholipasesD such as *AtPLDa1* and *AtPLDd* have been implicated in both the production of and responses to reactive oxygen species [54, 55]. The *AtPLDd* expression was shown to be induced by abscisic acid and cold in *Arabidopsis* plants [56]. Cold-induced freezing tolerance is reportedly impaired in *Atpldd* T–DNA knock-out *Arabidopsis* mutant plants and enhanced in *AtPLDd*-overexpressing plants, which also display decreased and increased freezing-induced phosphatidic acid production, respectively [57]. As a whole, it seems that phospholipases D are involved in multiple aspects of both the overlapping and distinct signalling networks that are activated by cold stress. Interestingly, histidine kinases (HKs) are one important class of hormone receptors in plants. There are 10 putative HKs in Arabidopsis, which are known as ethylene receptors and nonethylene receptors. The ethylene receptors have been found to be localized in the endoplasmic reticulum (ER) membrane [58, 59]. Among these receptors, subfamily I members ETR1 (ethylene response 1) and ERS1 (ethylene response sensor 1) have histidine kinase activity, while subfamily II members ETR2, ERS2 and EIN4 (ethylene insensitive 4) lack amino acid residues critical for this enzymatic activity. The subfamily II receptors are generally thought to function as Ser/Thr kinases [60, 61, 62 and 63]. The ethylene receptors function in concert with the physically associated Raf-like kinase CTR1 (constitutive triple response 1) to block the signal transduction pathway [64]. Genetic studies show that gain-of-function mutations in ethylene receptors of different subfamily, such as etr1-1 and ein4-1, exhibit enhanced freezing tolerance, whereas ctr1-1 loss-of-function mutant shows decreased freezing tolerance [65]. These results suggest that ethylene receptors are positive regulators of freezing tolerance. ER membrane-located cytokinin (CK) receptors Arabidopsis histidine kinase 2 (AHK2), AHK3 and AHK4/CRE1 (cytokinin receptor 1) belong to the nonethylene receptors. In Arabidopsis, CK signalling pathway is a two-component system, which consists of AHKs, Arabidopsis histidine phosphotransfer proteins (AHPs), and Arabidopsis response regulators (ARRs). Upon activation, AHKs transmit the signals via AHPs to ARRs through the phosphorelay cascade in Arabidopsis [66]. A recent study showed that the ahk2-2 ahk3-2 and ahk3-2 cre1-12 double mutants display enhanced freezing tolerance without affecting the CBF expression, indicating that CK receptors function as negative regulators in the plant responses to low temperatures [67]. However, cold stress has no obvious effect on the expression of AHK2, AHK3 and AHK4/CRE1 [67], which indicates that cold stress might alter CK receptor activity through an unknown mechanism. Type-A ARR genes are rapidly induced by CKs, and they are shown to regulate the activity of type-B ARRs via a negative feedback loop [68, 69] Among 10 type-A ARRs in Arabidopsis, ARR5, ARR6, ARR7 and ARR15 are rapidly induced upon cold stress [67, 70]. Consistently, our group found that transgenic Arabidopsis plants overexpressing ARR5, ARR7 and ARR15 promote freezing tolerance while the expression level of CBF slightly changes in transgenic lines [65]. By contrast, another study reported that overexpression of type-A ARR7 causes hypersensitivity to freezing, whereas arr5, arr6, and arr7 mutants show enhanced freezing tolerance [67]. Although the mechanisms underlying type-A ARR genes in cold response have not been revealed, we assume that ARR5, ARR7 and ARR15 are a group of rapid cold-responsive regulators that contribute to freezing tolerance. However, the molecular mechanisms of type-A ARRs action might be different from those of well-known COR genes. Compared with the traditional COR genes, the cold-induction of ARRs only goes up to five-fold. One possibility is that the negative regulatory role of CK signalling in freezing tolerance is at least partially dependent on ABA signalling [67]. Therefore, type-A ARRs might act as key players to directly integrate cytokinin, cold and ABA signalling. Furthermore, EIN3, the key transcription factor of ETH signalling, was shown to negatively regulate the expression of CBFs and type-A ARRs by directly binding to their promoters [65]. This study expands our knowledge of the network of ETH and CK signalling in plant responses to environmental stresses. It will be very interesting to identify novel cold response components that are directly regulated by the two component system signalling pathways.

2. Biochemical and physiological approaches

When exposed to stress plants have different ways of dealing with it to compensate due to lack of their mobility. A plant response to low temperature varies from species to species and even within the same family. Every plant has an optimum set of temperature for its normal growth and development. It is general notice that plant growing in warm habitats exhibit symptoms of injury when exposed to low non- freezing temperatures. However, appearance of injury depends upon the sensitivity of plant to low temperature stress and varies from plant to plant. Low temperature stress result in poor growth, yellowing of leaves withering and reduced tillering. Low temperature may result in pollen sterility due to low temperature during reproductive stages of plant which is thought to be one of the key factors responsive for reduction in yield [71]. The main major adverse effect of low temperature stress in plants has been seen in terms of plasma membrane damage due to cold stress induced dehydration [72]. Regardless of complexity one major common mechanism used by plants to deal with cold stress is to change in membrane lipid composition to protect its membrane stability and integrity. The plasma membrane of plants is made up of phospholipids and proteins. Lipids in the plasma membrane of plants are made up of two types of fatty acids First, unsaturated fatty acid and second saturated fatty acid. Unsaturated fatty acids have one or more double bond between two carbon atoms whereas saturated fatty acids are fully saturated with hydrogen atom. Lipids containing more saturated fatty acids solidify faster at temperature higher than those containing unsaturated fatty acids and the relative proportion of the two type of fatty acid in lipids of plasma membrane determine the fluidity of the membrane [72]. It is known to great extent that one of the membrane lipid response actions when exposed to low temperature would be increase in unsaturation of fatty acids compared to fatty acid in plant under normal conditions [73, 74]. However this ratio differ substantially from plant to plant and even the same plant can act differently depending on the way of exposure to low temperature i.e. acclimated (3-4°C) vs. Non acclimated plant (22-24°C) [75]. The plant species which can withstand even during the freezing temperature of late spring or early fall frost can be used more successfully for cultivation during low temperature conditions. Therefore the selection of low temperature tolerant plant species is very important for the sustainability of the important plant species. Additionally, the understanding of how cold stress induces its injurious effects on plants is crucial for the development of frost tolerant crops. Fatty acids are the major constituents of membrane glycerolipids. Most of the fatty acids in biological membranes are desaturated, with one or more double bonds in their fatty-acyl chains. The physical properties of membrane lipids depend on the number of double bonds in the constituent fatty acids. Unsaturated fatty acids are synthesized from saturated fatty acids by fatty acid desaturases that convert single bonds to double bond [76]. The extent of desaturation of individual fatty acids is regulated genetically and environmentally, and temperature is a critically important environmental factor that regulates the extent of desaturation [77, 78]. The cold-induced expression of genes for fatty acid desaturases (*des* genes) increases the extent of desaturation of fatty acids at low temperatures in cyanobacteria [77,78 and 79]

2.1 Low temperature induced Reactive Oxygen Species (ROS) and lipid peroxidation: One of the stress response in the plants is the stimulated production of reactive oxygen species (ROS) e.g., OH·, O_2·, H_2O_2 etc. These species cause considerable damage through peroxidation of membrane lipid components and also through direct interaction with various macromolecules. Cells have adapted different mechanisms to keep the ROS level in check. However, low ROS concentration participates in signal transduction mechanism. These ROS are scavenged by low molecular weight antioxidative metabolites e.g., glutathione, ascorbic acid, α-tocopherol and antioxidative enzymes e.g., catalase, ascorbate peroxidase and superoxide dismutase. However, under different stress conditions the free radical generation exceeds the overall cellular antioxidative potential leading to oxidative stress, which contributes to adverse effects on plant growth.One of the consequences of uncontrolled oxidative stress is cells, tissues, and organs injury caused by oxidative damage. The role of ROS in abiotic stress management has become a subject of considerable research interest, particularly since ROS have been reported to be involved in processes leading to plant stress acclimation [80]. This finding indicates that ROS are not simply toxic by-products of metabolism, but act as signalling molecules that modulate the expression of various genes, including those encoding antioxidant enzymes and modulators of H_2O_2 production [80, 81]. In addition, LT stress has been reported to cause significant increases in the levels of the non-enzymatic antioxidants ascorbate and glutathione, as well as the activity of the main NADPH-generating dehydrogenases [82]. It has long been recognized that high levels of free radicals or reactive oxygen species (ROS) can inflict direct damage to lipids. The primary sources of endogenous ROS production are the mitochondria, plasma membrane, endoplasmic reticulum, and peroxisomes [83] through a variety of mechanisms including enzymatic reactions and/or autooxidation of several compounds, such as catecholamines and hydroquinone. The two most prevalent ROS that can affect profoundly the lipids are mainly hydroxyl radical (HO·) and hydroperoxyl ($HO^{2\cdot}$). The hydroxyl radical (HO·) is a small, highly mobile, water-soluble, and chemically most reactive species of activated oxygen. This short-lived molecule can be produced from O_2 in cell metabolism and under a variety of stress conditions. A cell produces around 50 hydroxyl radicals every second. In a full day, each cell would generate 4 million hydroxyl radicals, which can be neutralized or attack biomolecules [84]. Hydroxyl radicals cause oxidative damage to cells because they un-specifically attack biomolecules [85] and located less than a few nanometres from its site of generation. It is generally assumed that HO· in biological systems is formed through redox cycling by Fenton reaction, where free iron (Fe^{2+}) reacts with hydrogen peroxide (H_2O_2) and the Haber-Weiss reaction that results in the production of Fe^{2+}. The hydroperoxyl radical (HO_2·) plays an important role in the chemistry of lipid peroxidation. This

protonated form of superoxide yields H_2O_2 which can reacts with redox active metals including iron or copper to further generate HO· through Fenton or Haber-Weiss reactions. The HO_2· is a much stronger oxidant than superoxide anion-radical and could initiate the chain oxidation of polyunsaturated phospholipids, thus leading to impairment of membrane function [86, 87 and 88]. Lipid peroxidation can be described generally as a process under which oxidants such as free radicals or non radical species attack lipids containing carbon-carbon double bond(s), especially polyunsaturated fatty acids (PUFAs) that involve hydrogen abstraction from a carbon, with oxygen insertion resulting in lipid peroxyl radicals and hydroperoxides as described previously [89]. Glycolipids, phospholipids (PLs), and cholesterol (Ch) are also well-known targets of damaging and potentially lethal peroxidative modification. Lipids also can be oxidized by enzymes like lipoxygenases, cyclooxygenases, and cytochrome P450. In response to membrane lipid peroxidation, and according to specific cellular metabolic circumstances and repair capacities, the cells may promote cell survival or induce cell death. Under physiological or low lipid peroxidation rates (subtoxic conditions), the cells stimulate their maintenance and survival through constitutive antioxidants defence systems or signalling pathways activation that regulates antioxidants proteins resulting in an adaptive stress response. By contrast, under medium or high lipid peroxidation rates (toxic conditions) the extent of oxidative damage overwhelms repair capacity, and the cells induce apoptosis or necrosis programmed cell death; both processes eventually lead to molecular cell damage which may facilitate development of various pathological states and accelerated aging. The impact of lipids oxidation in cell membrane and how these oxidative damages are involved in both physiological processes and major pathological conditions have been analysed in several reviews [90, 91]. The overall process of lipid peroxidation consists of three steps: initiation, propagation, and termination [89] In the lipid peroxidation initiation step, prooxidants like hydroxyl radical abstract the allylic hydrogen forming the carbon-centred lipid radical (L·). In the propagation phase, lipid radical (L·) rapidly reacts with oxygen to form a lipid peroxy radical (LOO·) which abstracts a hydrogen from another lipid molecule generating a new L· (that continues the chain reaction) and lipid hydroperoxide (LOOH). In the termination reaction, antioxidants like vitamin E donate a hydrogen atom to the LOO· species and form a corresponding vitamin E radical that reacts with another LOO· forming nonradical products. Once lipid peroxidation is initiated, a propagation of chain reactions will take place until termination products are produced. Review with extensive information regarding the chemistry associated with each of these steps is available [89]. In addition the low temperature induced harmful effects in lipid composition of bio membranes effects their fluidit additional factors also contribute to damage induced by cold stress including synthesis and accumulation of compatible solutes and cold acclimation induced proteins [92] changes in carbohydrate metabolism [93, 94] and boosting of radical scavenging potential of cell [95, 96]. Thus cold stress results in loss of membrane integrity leading to solute leakage. Further, cold stress disrupts the integrity of intracellular organelles leading to loss of compartmentalisation resulting in impairment of photosynthesis, proteins assembly and general metabolic processes. Cold acclimation in Arabidopsis revealed that upon cold acclimation the amount of metabolites increased [97, 98] role of these metabolites in plant is called osmoprotectants. In addition to their role as osmolytes certain metabolites induced during cold acclimation might acting as signal for reconfiguring the gene expression e.g proline is induced during various abiotic stresses including low temperature. Low temperature thus affects all aspects of cellular functions in plants and one of the major influences of cold induced dehydration is membrane disintegration thus causing adverse effect on the growth and development of plants.

2.2 Mechanisms of acclimation to low temperatures

The primary mechanisms involved in cold acclimation are related to a number of processes. These include molecular and physiological modifications occurring in plant membranes, increased levels of ROS and the activation of ROS scavenger systems, changes in the expression of cold related genes and transcription factors, alterations in protein and sugar synthesis, proline accumulation, and biochemical changes that affect photosynthesis. Membranes are a primary site of cold-induced injury Several studies have demonstrated that membrane rigidification, coupled with cytoskeletal rearrangements, calcium influxes, and the activation of MAPK cascades, triggers LT responses [34, 45 and 99]. The lipid composition of the plasma membrane and chloroplast envelopes in acclimated plants changes such that the threshold temperature for membrane damage is lowered relative to that for non-acclimated plants [5]. This is achieved by increasing the cold adapted membranes' unsaturated fatty acid content, which makes them more fluid [100]. The process of cold acclimation promotes the stabilization of membranes, which prevents damage leading to cell death. The acclimation process also activates mechanisms that protect membrane fluidity by ensuring the optimal activity of associated enzymes [6]. At the physiological level, photosynthesis is strongly affected by exposure to cold. The cessation of growth resulting from cold stress reduces the capacity for energy utilization, causing feedback inhibition of photosynthesis [4]. In cold-acclimated winter annuals, photosynthetic activity is maintained by increases in the abundance and activity of several Calvin cycle enzymes [101]. This recovery is associated with elevated levels of thylakoid plastoquinone A and a concomitant rise in the apparent size of the intersystem electron donor pool to PSI [102]. Consequently, non-photochemical quenching increases in cold-stressed leaves in parallel with increased zeaxanthin levels to compensate for the reduced electron consumption by photosynthesis. Zeaxanthin protects the PSII reaction centres from over-excitation

[103]. Nevertheless, Ruelland and Zachowski (2010) reported that energy dissipation via nonphotochemical quenching (NPQ) and electron transport was not only enhanced following cold acclimation but also contributed to protection from oxidative damage. Xanthophylls are not considered photosynthetic pigments per se, the xanthophylls (notably, violaxanthin, antheraxanthin, and zeaxanthin) help in protecting the photosystems and their abundance increases at low temperatures [104]. Xanthophylls have structural roles and act as natural antioxidants, quenching triplet Chl and singlet oxygen, which are potentially harmful to the chloroplast [105, 106]. It has also been postulated that unbound zeaxanthin and other carotenoids may also stabilize thylakoid membranes against putative peroxidative damage and heat stress [107]. Flavonoids accumulate in leaves and stems in response to low temperatures. They are synthesized via the phenylpropanoid pathway, which is controlled by key enzymes, including phenylalanine ammonia-lyase and chalcone synthase [108]. Recently, it has been reported that cold stress induces transcriptomic modifications that increase flavonoid biosynthesis, including reactions involved in anthocyanin biosynthesis and the metabolic pathways that supply it [109]. In response to cold and other osmotic stresses, plants accumulate a range of compatible solutes including cererosides, free sterols, sterol glucosides and acylatedsterols, glucosides, raffinose, arbinoxylans, and other soluble sugars. In addition, plants accumulate other solutes such as glutamic acid, amino acids (alanine, glycine, proline, and serine), polyamines and betaines [110, 111]. These different molecules, which are often degraded once the stress has passed, are referred to as osmolytes, osmoprotectants or compatible solutes.

2.3 Carbohydrate metabolism under low temperature stress

Carbohydrate metabolism has been reported to have greater instantaneous low temperature sensitivity than other components of photosynthesis [112]. Although the precise function of soluble sugars remains to be determined, their accumulation in cold-acclimated plants suggests roles as osmoregulators, cryoprotectants or signalling molecules [113]. Sugars play multiple roles in low temperature tolerance. As typical compatible osmolytes, they contribute to the preservation of water within plant cells, thereby reducing water availability for ice nucleation in the apoplast [114]. Sugars might protect plant cell membranes during cold-induced dehydration, replacing water molecules in establishing hydrogen bonds with lipid molecules [114, 115]. Moreover, carbohydrates may also act as scavengers of reactive oxygen species and contribute to increased membrane stabilization [116]. Sugar not only acts as an energy and carbon source but also functions as a signalling molecule in a variety of physiological processes, and plays versatile roles in plant growth and development [117, 118]. Accumulating evidences suggest that sugar functions as an osmotic substance, a membrane stabilizer and might be an antioxidant in cold stress responses [119, 120 and 121].The metabolism of sugar is a dynamic process regulated by numerous enzymes whose expression and activities change in response to LT stimulation [122]. Additionally, sugar exhibits flexible homeostasis between source-and-sink tissues or organs due to cell-to-cell and long-distance transport mediated by various sugar transporters [123]. Recent studies indicated that sugar acts as a potent signal molecule in plant growth and development regulations [117, 124]. Based on these convoluted regulatory interactions with sugar, the integration of environmental stimuli with its allocation is crucially important for stress responses in plants. The genomics era has led to discoveries regarding the mechanisms of sugar metabolism and its ability to facilitate cold tolerance. Recent studies focusing on the responses of sugar metabolism, transportation and signalling under stress conditions have broadened our knowledge of the roles of sugar in plant cold hardiness. Transcriptome profiling experiments performed in *Arabidopsis* have identified numerous sugar-related genes involved in sugar synthesis, transportation and signalling, which showed differential expressions after LT treatment [125]. The expression patterns of the most key enzymes and their activities have been explored in recent studies upon LT stress, for instance, sucrose (Suc) synthase (*SUS*) and invertase (*INV*) in Suc metabolism [118, 126], raffinose (Raf) synthase (*RS*) and galactinol synthase (*GolS*) in Raf metabolism [127, 128, 129 and 130], and trehalose (Tre)-6-phosphate synthase (*TPS*) in Tre metabolism [131]. The contributions of these genes to cold tolerance have been verified in different species. Several reports show that LT induces starch degradation, and the involved main enzymes coding genes in this pathway including glucan water dikinase (*GWD*), beta-amylase (*BAM*) as well as its downstream genes, such as maltose transporter (maltose excess1, *MEX1*) and disproportionating enzyme 2 (*DPE2*), are differentially regulated and facilitate plant cold responses [132, 133, 134, 135 and 136]. For instance, *PtrBAM1* has been identified as a CBF regulon, which functions under cold conditions [137]. Additionally, sugar transporters, such as Suc transporter (SUT) and sugars will eventually be exported transporters (SWEET), can also be altered by cold stress [138, 139 and 140]. Additionally, hexokinase (HXK) and Suc nonfermenting1-related protein kinase 1 (SnRK1) are known to participate in sugar signal transduction by modulating the abundances of diverse gene transcripts and integrating stress response substrates, including abscisic acid (ABA) and ethylene [117, 124, 141 and 142]. In this respect, sugar metabolism, transport and signalling could participate in the plant cold response. Sugar signalling is also closely associated with hormone signalling, the control of growth and development, and stress responses in plants [143]. Depending on the plant species, various forms of soluble sugars are involved in physiological reactions to cold stress. For example, treatment of rice seedlings with fructose or glucose prior to LT treatment increases their resistance to cold. Cotton cotyledon discs floating on a sucrose solution in the dark were less injured by cold than those on non-sugar solutions [144]. The oligosaccharides raffinose and stachyose are especially associated with cold

hardiness, low temperature and dormancy [144, 145]. Moreover, the concentration of sucrose, the most easily detectable sugar in cold-tolerant species, increases several fold during exposure to LT [146]. The accumulation of sucrose in cane sugar exposed to salt stress or to LT stress supports the role of this sugar as an osmoprotectant that stabilizes cellular membranes and maintains turgor [147]. In addition, high sucrose levels correlate with the priming of defence responses in rice that overexpresses the PRms gene from maize, which encodes a PR-1 type protein [148]. Trehalose is a non-reducing disaccharide of glucose that is found in a variety of organisms including bacteria, yeast, fungi, insects and invertebrates, where it serves as a stress protectant and/or a reserve carbohydrate [149, 150]. Although increased levels of trehalose are associated with abiotic stress tolerance in transgenic plants expressing heterologous microbial genes, the function of endogenous trehalose in higher plants remains unclear. This sugar possesses the unique capacity for reversible water absorption and appears to be superior to other sugars in protecting biological molecules from desiccation-induced damage [150]. Further, transgenic *A. thaliana* plants that accumulated trehalose displayed significantly enhanced freezing tolerance [151]. Increases in trehalose concentration may also be involved in starch accumulation [150]. During exposure to LT, starch content typically declines following hydrolysis, and there is a corresponding increase in the concentration of free saccharides [152, 153]. However, in several cases, increases in the levels of both soluble sugars and starch have been reported during cold acclimation. For instance, in cabbage seedlings grown at 5^0C, the concentrations of starch and all soluble sugars (myo-inositol aside) in the leaves increased gradually during cold acclimation [154]. However, the induced freezing tolerance was lost after only 1 day of acclimation at control temperatures and this change was associated with a large reduction in sugar content. Carbohydrate accumulation at LT may be explained through the activation of specific enzymes [144, 153]. This suggests that although LT inhibits sucrose synthesis and photosynthesis, various biochemical and physiological adaptations to LT counteract these effects. These adaptations include the post-translational activation and enhanced expression of enzymes involved in the sucrose synthesis pathways and those of Calvin cycle—in particular, the cytosolic enzymes fructose-1,6-bisphosphatase, sucrose phosphate synthase and sucrose synthase [155].

2.3.1 Compatible osmolytes other than sugars

(a) Proline: Apart from acting as osmolyte for osmotic adjustment, proline contributes to stabilizing sub-cellular structures (e.g., membranes and proteins), scavenging free radicals and buffering cellular redox potential under stress conditions. It may also act as protein compatible hydrotrope alleviating cytoplasmic acidosis and maintaining appropriate $NADP^+$/NADPH ratios compatible with metabolism.In many plant species, proline accumulation under salt stress has been correlated with stress tolerance, and its concentration has been shown to be generally higher in salt tolerant than in salt sensitive plants.[Its accumulation normally occurs in cytoplasm where it functions as molecular chaperons stabilizing the structure of proteins and its accumulation buffer cytosolic pH and maintains cell redox status. It has also been proposed that its accumulation may be part of stress signal influencing adaptive responses The positive correlation between the accumulation of endogenous proline and improved cold tolerance has been found mostly in LT-insensitive plants such as barley, rye, winter wheat, grapevine, potato, chickpea and A. thaliana [156, 157 and 158]. Proline plays multiple roles in plant stress tolerance, as a mediator of osmotic adjustment, a stabilizer of proteins and membranes, an inducer of osmotic stress-related genes, and as a scavenger of ROS [156, 157 and 159]. The most probable roles of proline are to (1) regulate cytosol acidity, (2) stabilize the NAD/NADH ratio, (3) increase the photochemical activity of the photosystem II in thylakoid membranes and (4) decrease lipid peroxidation [160]. Most chilling-sensitive plants that accumulate Proline under LT conditions do not acquire cold tolerance [161], unless a high concentration of Proline was applied prior to stress [162]. It appears therefore that proline possesses the potential to alleviate LT injury in chilling-sensitive plants, but for some reason this system fails under natural conditions.

(b) Glycine Betaine: The accumulation of glycine betaine (GB) usually correlates with the plant's level of stress tolerance. Both the genetically engineered biosynthesis of GB in plants that do not naturally accumulate GB and the exogenous application of GB enhance the tolerance of such plants to various abiotic stresses [163]. Possible roles for GB include stabilization of the transcriptional and translational machinery. GB stabilizes protein complexes and membranes in vitro and may indirectly induce H2O2-mediated signalling pathways. Plants adapted to low temperature conditions accumulate certain molecules that have a cryoprotective role [164]. Glycine betaine is one such cryoprotective solute, which protects the activities of enzymes and proteins, stabilizes membranes [165] and photosynthetic apparatus [166, 167] under chilling and freezing temperatures. Cold tolerance has been associated with accumulation of glycine betaine in several plant species [168]. Moreover, genetically manipulated plants for higher glycine betaine production show enhancement of stress tolerance [169] that provides ample evidence for its involvement in defense response. In addition, exogenous application of glycine betaine has been reported to induce cold tolerance [170]. Possible roles for glycine betaine in stress tolerance include stabilization of complex proteins and membranes in vivo, protection of transcriptional and translational machinery, and acting as a molecular chaperone in the refolding of enzymes [165]. It also reduces the peroxidation of membrane lipids and protects electron transport via complex II in mitochondria [171]. Plant species vary in their capacity to synthesize glycine betaine and some plants, such as spinach and barley, accumulate relatively high levels of glycine betaine in their chloroplasts while others, such as *Arabidopsis* and tobacco, do not effectively synthesize this compound

[164]. Exogenously applied glycine betaine or the expression of transformed genes for endogenous glycine betaine synthesis increases cold tolerance [172] in glycine betaine non accumulating plants.

2.4 Lignin in cold acclimation

It is well known that lignin fills the spaces in the cell wall to reduce water permeability and increase the stiffness of the cell wall. Extensive cellular studies have shown that freezing tolerance is directly related to cell permeability and cell wall properties, in particular lignin content, so that water outflows and ice forms in the extracellular spaces without damaging cellular structure. The plant cell wall is the extracellular matrix consisting of cellulose, hemicelluloses and lignin. It plays essential role in plant growth and adaptive responses to adverse environmental conditions [173, 174 and 175]. The cell wall integrity and structure are dynamically regulated during plant development and are capble of being remodelled in response to various environmental stresses [176, 177, 178 and 179] Fine tuning regulation of the proportion and the amount of each matrix component wit in the cell wall determines its nature and function. Remarkably, deposition of lignin phenylpropanoid polymers, which is highly hydrophobic in the cell wall determines cell wall stiffness and permeability to water [180, 181 and 182]. Previous studies have shown that the expression of genes related to cell wall biosynthesis and remodelling is dramatically altered under cold treatment [183]. Cryo-scaning electron microscopy (cryoSEM) revealed that both cell membrane and cell wall properties play equally important roles in cold acclimation and freezing tolerance [184]. Lignin is a major component of the plant secondary cell wall, and the amounts of lignin are altered after cold treatment in various species [185, 186]. In the past decades, some genes that regulate lignin biosynthesis have been identified [187, 188 and 189]. Among them, Phenylalanine ammonia-lyase 1–4 (PAL) encoding the enzymes that catalyze the first step in the phenylpropanoid pathway regulate biosynthesis of lignin and secondary metabolites (e.g. flavonoids and salicylic acid) in Arabidopsis thaliana [190, 191 and 192]. Arabidopsis thaliana blue copper binding gene (BCB) is another positive regulator of lignin synthesis, and AtBCB overexpression substantially increases lignin content in Arabidopsis roots [193]. It has been shown that PAL1-PAL4 and BCB genes are responsive to a variety of environmental stimuli, including pathogen infection, wounding, nutrient depletion, UV irradiation, and extreme temperature, etc. [193] suggesting their roles in plant stress resistance. However, it remains unknown how these genes mediate plant responses to biotic and abiotic stresses. Recently Hangtao et al., 2015 [194] reported that specifically cold induced nuclear protein, Tolerant to chilling and freezing 1 (TCF1) interacts with chromatin containing a target gene Blue Copper Binding Protein (BCB), encoding a glycosylphosphatidylinositol anchored protein that regulate lignin biosynthesis. These evidences shows that reduction in lignin content dramatically increases plant freezing tolerance, while lignin maintenance required for cold acclimation is regulated by TCF mediated signalling. This finding provides the first direct molecular evidence that freezing tolerance is directly related to cell wall properties during cold acclimation.

3. Cold acclimation signalling pathways

Currently, the best understood cold acclimation signalling pathway is the ICE1-CBF-COR transcriptional cascade. In this pathway, C-repeat (CRT)-binding factors (CBFs)/dehydration responsive element binding factors (DREBs) are rapidly induced by cold, and bind to the promoter regions of COR genes to activate their transcription [195]. Emerging evidence has shown that CBF dependent pathway is regulated by many important regulators at transcriptional, posttranscriptional and posttranslational levels. The ICE–CBF transcriptional cascade is acknowledged to modulate freezing tolerance. In this pathway, CBFs/ DREBs are rapidly induced by cold. CBFs/DREBs can bind to CRT/DRE cis-elements in the promoter regions of COR genes and activate their transcription [196, 197]. There are three CBF genes (CBF1/DREB1b, CBF2/DREB1c and CBF3/DREB1a) in the Arabidopsis genome. Overexpression of CBF genes in Arabidopsis results in enhanced freezing tolerance [198], whereas knockdown of CBF1 and/or CBF3 increases plant sensitivity to freezing stress after cold acclimation [199]. However, the cbf2 mutant shows a freezing tolerance phenotype with or without cold acclimation [200]. Gene expression analysis indicated that CBF2 plays a negative role in the expression of CBF1 and CBF3 [200], suggesting the existence of a negative feedback regulatory network in the cold stress response. The CBF signalling pathway is conserved. Expression of Arabidopsis CBF genes in many species can enhance cold tolerance [201].

3.1 CBF-dependent pathway of cold signalling [ICE-CBF/DREB1 Pathway and Cold-Responsive Gene Regulation]

ICEs (Inducer of CBF Expressions) Are Transcription Factors Controlling Cold Signalling through the Regulation of CBF/DREB1s**.** The ICE–CBF transcriptional cascade is acknowledged to modulate freezing tolerance. In this pathway, CBFs/ DREBs are rapidly induced by cold. CBFs/DREBs can bind to CRT/DRE cis-elements in the promoter regions of COR genes and activate their transcription [197, 204 and 205]. There are three CBF genes (CBF1/DREB1b, CBF2/DREB1c and CBF3/DREB1a) in the Arabidopsis genome. Overexpression of CBF genes in Arabidopsis results in enhanced freezing tolerance [198], whereas knockdown of CBF1 and/or CBF3 increases plant sensitivity to freezing stress after cold acclimation [199]. However, the cbf2 mutant shows a freezing tolerance phenotype with or without cold acclimation [200]. Gene expression analysis indicated that CBF2 plays a negative role in the expression of CBF1 and CBF3 [200], suggesting the existence of a negative feedback regulatory network in the cold stress response. The CBF signalling pathway is

conserved in different species. Expression of Arabidopsis CBF genes in many species can enhance cold tolerance [201]. Natural variation analyses in Arabidopsis accessions with different levels of freezing tolerance further supports the notion that CBF genes play essential roles in the basal freezing tolerance [202, 203]. It is noteworthy that CBF genes are induced by cold rapidly and transiently (induce within minutes, reach the maximum within 1–3 h, and decrease rapidly afterwards), while cold-induction of COR genes (starts after several hours, and reaches the maximum at up to 24 h) is much slower than that of CBF genes. It is possible that under cold stress, CBF proteins accumulate to a certain extent to induce COR gene expression. Alternatively, CBF proteins are modified or have partners that are activated by cold slowly. Therefore, it is important to examine the dynamics of CBF proteins under cold stress, and identify CBF-associated proteins to unravel the molecular regulation of CBF proteins in cold stress responses.

3.1.1 Regulation of the CBF pathway at transcription level

The low temperature induced expression of CBFs is positively regulated by several transcription factors among which ICE1 (inducer of CBF expression 1) encodes a MYC-type bHLH transcription factor that can bind to the CBF3 promoter, thereby activating CBF3 gene expression [206]. The ice1 mutant is defective in cold-induced CBF3 expression and shows reduced chilling/freezing tolerance, whereas overexpression of ICE1 increases freezing tolerance [206]. ICE2, a homolog of ICE1, positively regulates CBF1 expression and enhances freezing tolerance [207]. CBF2 is activated when the transcription factor CAMTA3 (calmodulin-binding transcription activator 3) binds to the CBF2 promoter [26]. Cold induction of CBF2 and some COR genes is impaired in the camta3 mutant, and the camta1 camta3 double mutant is hypersensitive to freezing stress [26]. Moreover, a Ca2+-binding calmodulin like receptor protein kinase, CRLK1, can enhance cold tolerance by regulating CBF regulons [27]. CBF1 and its downstream genes are downregulated in crlk1 loss-of-function mutant-under cold stress [27]. Further studies showed that CRLK1 mediates cold stress responses by interacting with MEKK1 and repressing MAPK kinase activity [27]. Because calcium influx is an early event in the cold signalling, these studies suggest a possible link between Ca2+-dependent MAPK cascade and cold signalling. Besides positive transcriptional regulators, there are also several transcription factors that negatively regulate the expression of CBFs and their downstream genes [208, 209 and 210]. A member of R2R3-MYB family of transcription factor MYB15 can bind to MYB recognition elements in the promoters of CBF genes. The myb15 mutant displays enhanced cold-induction of CBFs and freezing tolerance, whereas MYB15-overexpressing plants are defective in CBF expression and thus hypersensitive to freezing. Interestingly, MYB15 can interact with ICE1 [209] however, the biological significance of this interaction remains unclear. In addition, Arabidopsis ZAT12, a C2H2 zinc finger protein, has a negative effect on the expression of CBFs [208]. Recently, EIN3, one of the transcription factors in the ETH signalling pathway, has been identified as a repressor of CBFs during cold acclimation [210]. EIN3 directly binds to the promoters of CBFs and negatively regulates expression of downstream cold-induced genes. In Arabidopsis, the endogenous ETH content is reduced after 1–3 h of cold treatment in Arabidopsis, which appears to be consistent with the results that the cold-induced expression of CBF genes peaks at 1–3 h and decreases at 6 h after cold treatment. Interestingly, cold treatment induces the accumulation of EIN3 protein in the nucleus after 6 h in an EIN2-dependent manner [210, 211]. It is possible that the high level of EIN3 is necessary for the repression of CBF genes after their rapid induction and a transcriptionally antagonistic interplay exists between CBF and ETH signalling pathways. In the absence of the ethylene signal, EIN3 protein is targeted by EBF1/EBF2 complexes and degraded by the 26S proteasome [212]. These results prompt us to assume that cold might block the degradation of EIN3. Consistent with this notion, the EBF1 protein is degraded after 6 h of cold treatment [210]. The antagonistic regulation of the CBF signalling pathway by cold and ethylene signalling might be a balancing mechanism between establishment of the appropriate stress tolerance and minimal effects on plant growth and development. Under normal growth conditions, EIN3 suppresses the transcription of CBFs, thereby repressing expression of downstream COR genes. At early stages of cold stress, decreasing in endogenous ethylene inactivates the transcriptional repression of CBFs by ETH signalling and triggers CBF dependent cold acclimation. Later on cold stress promotes EIN3 accumulation to prevent over accumulation of CBFs. Further studies on how ETH signalling responds to cold temperature and then regulates CBF signalling pathway this help us to fully understand the regulatory mechanisms of freezing tolerance.

3.2 Regulation of the CBF pathway at post translational level

Post-transcriptional mechanisms based on alternative splicing, pre-mRNA processing, RNA stability, RNA silencing and export from the nucleus play critical roles in cold acclimation and cold tolerance. Pre-mRNA processing and export are important processes for the regulation of gene expression in eukaryotes Plants regulate the stress-dependent export of mRNA from the nucleus and the selective translation of stress-associated genes and increase the stability of related transcripts. CBF genes regulate cold acclimation by modulating CBF pathway at posttranslational level also. The RING finger E3 ligase HOS1 (high expression of osmotically responsive genes1) interacts with and ubiquitinates ICE1, which leads to ICE1 degradation via the 26S proteasome pathway [213]. In the hos1 mutant, CBFs and their target genes are hyper-induced during cold treatment, whereas over expression of HOS1 reduces cold-induction of CBFs and freezing

tolerance [213, 214]. In contrast, a small ubiquitin-related modifier (SUMO) E3 ligase, SIZ1 (SAP and Miz 1), mediates sumoylation of ICE1, which reduces the polyubiquitination of ICE1 to enhance its stability [215]. Consistently, the siz1 mutation causes reduced cold-induction of CBFs and freezing tolerance, whereas the transgenic plants overexpressing SIZ1 display enhanced freezing tolerance [215]. Thus, two different modifications of ICE1 render opposite effects on ICE1 stability and CBF3 expression. One puzzle is the fact that continuous cold treatment promotes ICE1 protein degradation rather than stabilizes it in Arabidopsis [213]. We proposed that at an early stage of cold stress, ICE1 induces downstream COR gene expression. Certain important regulators may function at this stage to prevent the degradation of ICE1. A recent study showed that repressors of jasmonate acid (JA) signalling—the bHLH-interacting proteins JAZ1/4—interact with ICE1/2 and repress ICE1 transcriptional activity, thereby modulating CBF expression and freezing tolerance (Hu et al. 2013). Although there is no direct evidence showing that low temperature can inhibit the activity of the JA receptor COI1, it was indeed shown that cold rapidly induces the accumulation of JA, which can be sensed by COI1 and then recruits JAZ proteins for degradation, leading to activation of ICE1 [216]. Combined with previous studies that EIN3/EIL1 interact with JAZ1 to mediate jasmonate-regulated responses [217, 218], it is possible that JAZs are antagonistic or synergistic to EIN3/EIL1 and ICE1, and modulate the CBF signalling pathway in multiple regulatory levels in early cold response.

3.4 Post-transcriptional regulation of COR genes

Pre-mRNA processing and exports constitute important mechanisms of regulation of gene expression in eukaryotes. Pre-mRNA undergoes various nuclear processes such as the addition of a 5′ methyl cap and poly(A) tail and intron splicing. Splicing is necessary to remove introns and to synthesize translationally competent mRNAs. Primary transcripts with more than one intron can undergo alternative splicing to produce functionally different proteins from a single gene. Molecular analysis has revealed the importance of the posttranscriptional regulation of cold-response genes, including premRNA processing and mRNA export from the nucleus. For example, Arabidopsis FIERY2 (FRY2), which encodes an RNA polymerase II C-terminal domain (CTD) phosphatase, functions in mRNA processing [219]. Mutation of FRY2 results in mRNA retention during pre-mRNA processing, especially under stress conditions [220]. fry2 mutants show increased expression of CBF genes; however, they are hypersensitive to freezing stress [219]. Similarly, the rcf1 (regulator of CBF gene expression1) mutant was recently identified to exhibit hypersensitivity to cold stress albeit with high cold-induction of CBF genes. RCF1 encodes a cold-inducible DEAD-box RNA helicase, which is essential for maintaining the proper pre-mRNA splicing of many COR genes under cold stress [221]. Another pre-mRNA splicing factor, STA1 (stabilized 1), is required for pre-mRNA splicing and mRNA turnover of COR genes. Mutation of STA1 renders plants defective in COR15A gene splicing and hypersensitive to chilling stress [222]. The nuclear pore complex (NPC) is composed of nucleoporins (NUPs) and is involved in the export of mRNAs or small RNAs to the cytoplasm [223]. In Arabidopsis, a mutation in nup160 causes a decrease in poly(A) mRNA export at low temperature, which causes reduced CBF expression and chilling/freezing-sensitive phenotypes [224]. LOS4 encodes a DEAD-box RNA helicase that plays a crucial role in temperature responses. The los4-1 mutant exhibits a chilling-sensitive phenotype with reduced expression of CBF3 and delayed expression of CBF1/2, whereas los4-2 is cold tolerant but heat sensitive. Accordingly, mRNA export is blocked in los4-1 under both normal and cold conditions. However, in los4-2 mutants, mRNA export is normal under cold stress but is defective at high temperatures [225]. Taken together, these results suggest the important role of pre-mRNA processing and mRNA export in cold stress responses.

3.5 CBF-independent pathway of cold signalling

Transcriptome analysis indicated that only 12% of the cold responsive genes are controlled by CBFs [226] so, some CBF-independent components must function in cold signalling like Arabidopsis esk1 mutant, shows constitutive freezing tolerance that is independent of the CBF regulon [227]. Loss of HOS9, a homeobox transcription factor, causes reduced freezing tolerance without affecting the expression of CBFs and their target genes [228]. In addition, GIGANTEA (GI), which encodes a nuclear-localized protein involved in flowering and the circadian clock, is induced by low temperature. The gi-3 mutant shows both decreased constitutive cold tolerance and impaired cold acclimation ability without affecting CBF expression [229]. Among CBF-independent cold signalling pathways, ABA dependent cold signalling pathway has been studied for many years. Transcriptome analysis revealed that 10% of ABA responsive genes are also responsive to cold stress [230]. some COR genes, such as RD29A, RD22, COR15A and COR47, their promoters not only contain the CRT/DRE motif but also harbor ABA response (ABRE) cis-elements that can be activated by ABRE-binding proteins/factors (AREBs/ABFs) [231]. Although it was shown that ABA levels increase slightly in response to low temperature [232], genetic studies indicate that ABA biosynthesis and signalling components are important for the expression of COR genes [233, 234]. One of the ABRE-binding proteins, ABI3, was shown to function in the cold stress response. Ectopic expression of the seed-specific ABI3 confers ability to express COR genes in vegetative tissues and enhances freezing tolerance in Arabidopsis [235]. One study showed that ABF2 interacts with CBF3 in vitro [236], this suggest that both these pathways are not entirely independent of each other.

CONCLUSION

Frost induced freezing is a major abiotic stress for evergreen plant species. Plants exposed to low temperatures often show a common response in the form of oxidative stress. However, the extent of damage caused by freeze depends greatly on the biomolecular constitution of the plants. There is ample need to develop temperature tolerance in crop plants by exploring suitable strategies. Numerous research findings support the notion that Ca^{2+} induced rigidification of plasma membrane can cause active cytoskeletal rearrangement which helps the plants in developing acclimation against low temperature stress. One of the common mechanism used by plants to deal with clod stress is to change membrane lipid composition to protect its membrane stability and integrity. The strategies resisting ROS induced lipid peroxidation can serve as key mechanism for protection of plants against frost induced freezing damage. Trehalose sugars have the unique capacity for reversible water absorption and appear to be superior to other sugars in protecting biomolecules from freeze induced desiccation damage. Elaborated findings on TCF mediated signalling of lignin biosynthesis for modifying cell membrane and cell wall properties can play a great role for tolerance development against low temperature stress in plants. Over-expression of CBF genes resulting in enhanced freezing tolerance needs to be worked out beyond *Arbidopsis* for avoiding economic losses. Therefore, more advanced research focusing on the development of plants those restrain genes which promote the accumulation/synthesis of beneficial biomoleclues. Considering these facts, a well organized approach should combine to investigate the molecular, physiological, and metabolic aspects of low temperature stress tolerance both at the cellular and the whole plant level.

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