Chapter 2
Facing the Cold Stress by Plants in the Changing Environment: Sensing, Signaling, and Defending Mechanisms

Prince Thakur and Harsh Nayyar

1   Introduction

Growth constraints and stress result in significant crop losses and therefore the mechanisms underlying endurance and adaptation to these changes have long been the focus of intense research (Bray 2004). Kültz (2005) elaborated two types of responses to a particular kind of stress (1) stress specific adaptive responses and (2) general responses that confer basic protection. Temperature is one of the important factors, which determine the distribution of plants geographically in an optimum environment where they can survive and complete their life cycle. Chilling stress (<20 °C) is a direct result of low temperature effects on cellular macromolecules, which leads to slowing of metabolism, solidification of cell membranes, and loss of membrane functions (Jewell et al. 2010). Chilling has been known to severely inhibit plant reproductive development in many crop plant species such as rice displaying sterility when exposed to chilling temperatures during anthesis (Jiang et al. 2002). The sudden changes in the plant's environment also lead to the slower growth and low yield because of the shunting of the resources from reproductive processes to metabolic process to achieve tolerance (Smith and Stitt 2007). Chilling stress effects include reduced leaf expansion and growth (Sowinski et al. 2005; Rymen et al. 2007), wilting (Bagnall et al. 1983), chlorosis (Yoshida et al. 1996), and may lead to necrosis and impaired development of reproductive components, restricted seed, and pod development in sensitive plants species (Kaur et al. 2008; Ohnishi et al. 2010; Kumar et al. 2011), which ultimately reduces the yield of grain crops (Suzuki et al. 2008).

The plants are of sessile nature and so they have developed some specific mechanisms to deal with temperature changes in their environment. Hällgren and Öquist

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(1990) have divided the plants into chilling sensitive, chilling resistant, and freezing tolerant types; however the term chilling resistant may be treated as a misnomer because it implies that the these plants are able to regulate their temperature. However, the plants are poikilotherms so the term may be modified to a more elaborative 'chilling tolerant'. In the first category, 'chilling sensitive', the plants show metabolic dysfunctions at the exposure of temperatures slightly below the optimum. The chilling tolerant plants are those ones, which survive the lower range of temperatures but nonfreezing, than optimum. The freezing tolerant plants also survive the freezing conditions and are most hardy of the above classes of plants.

Plants experience a wide range of temperature fluctuations in natural environments. Thus, they have evolved mechanisms to minimize cellular damage at temperature extremes. Growth at low temperatures (cold acclimation) enables plants to initiate signaling cascades and metabolic alterations, which enhance tolerance to freezing temperatures (Chinnusamy et al. 2003).

Temperature change in the micro or macro-environment is a very critical factor, which determines the growth, development, and physiology of the plant. Some of the alterations are visible to us as cold stress symptoms but the main role players are always behind the curtain, which take part in various biochemical and molecular processes in response to cold temperature exposure. These processes together can be termed as 'low temperature induced signal transduction (LTST). These processes are decidedly beneficial to the plant because these are the strategy measures to cope with the stress conditions. Besides the plants also get a very useful character out of these processes i.e. stress memory or cold stress acclimation. The LTST leads to the expression of certain genes of interest in the nucleus, which through central dogma results in the synthesis of some specific proteins. These proteins either structural or enzymes work for the survival of plant during stress conditions and the plant acquires stress tolerance. All these behind the curtain processes are described in detail in the coming heads of this review.

Chilling has been known to cause disruption of DNA strands, reductions in enzymatic activity, rigidification of membranes, destabilization of protein complexes, stabilization of RNA secondary structure, accumulation of reactive oxygen intermediates (ROIs), impairment of photosynthesis, and leakage across membranes ((Nayyar et al. 2005a, b, c, d) also (Nayyar and Chander 2004). Different methods have been used to quantify the cold tolerance in plants like electrolyte leakage (Patterson et al. 1976; (Nayyar et al. 2005a, b, c, d), LT_{50}, percent survival, and chlorophyll fluorescence imaging (Ehlert and Hincha 2008). It has been reported in different scientific writings that cold tolerance in plants comes from two ways (1) it is inherent and (2) after cold acclimation. For a better understanding of the cold tolerance in plants through cold acclimation, a detailed knowledge of biochemical and molecular methods involved in low temperature sensing and signal transduction is required, which is the earliest and most important stage in cold acclimation and development of cold tolerance. The main aim of this review is to discuss the mechanisms of cold sensing mechanisms in plants, the signaling processes and their components, which commence thereafter and the resulting tolerance mechanisms. Baena-Gonzalez (2010) has reviewed the various mechanisms that subsequently become engaged upon exposure of plants to stress to modulate gene expression in response to energy signals (Fig. 2.1).
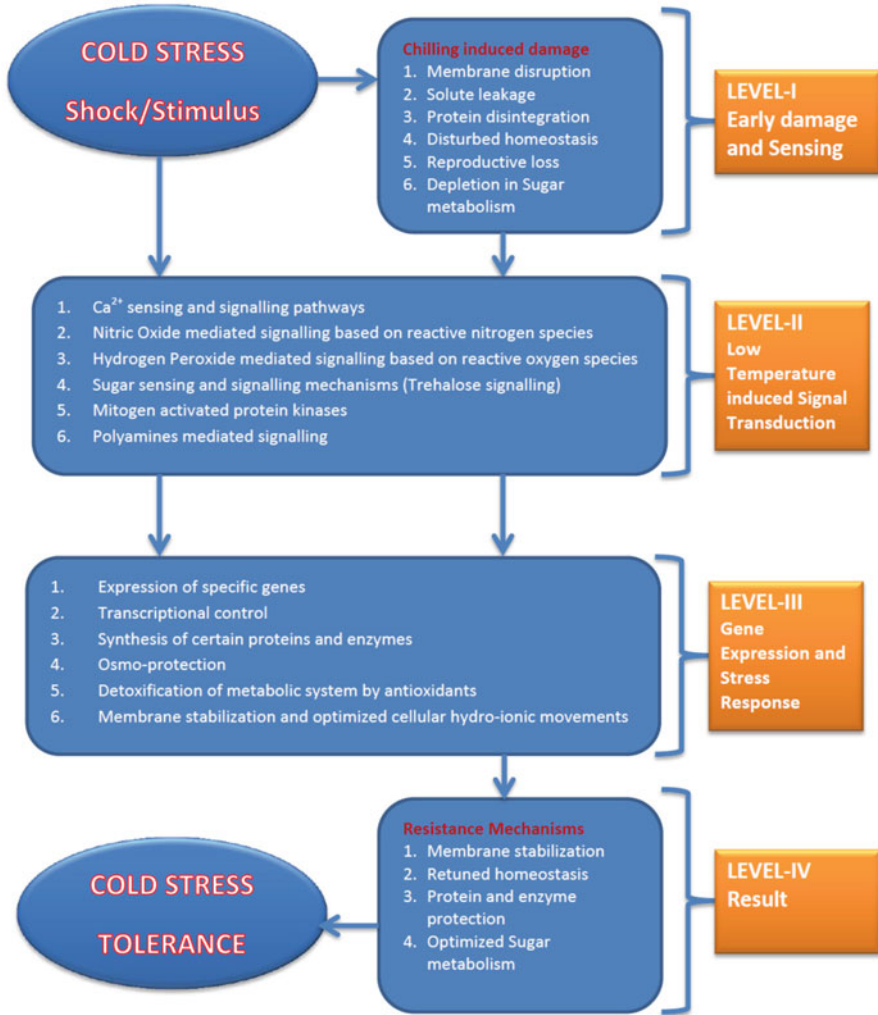
Fig. 2.1 A schematic four tier mechanism of cold stress response and acquired tolerance in plants. Cold tolerance is also known as cold hardening or cold acclimation and it is described as the development or increase in tolerance to cold temperatures over time by means of adaptive and resistive cellular mechanisms, which are activated in response to stressful cold temperature conditions

2   Low Temperature Sensing

Any type of environmental stimulus is sensed by the receptor/osmosensor molecules, which perceive the signal and transmit it to the suitable signal transduction pathways. In plants, the identified receptor/osmosensor molecules include ROP10 (a small G protein from ROP family) (Zheng et al. 2002), ATHK1 (a homologue of yeast SLN1) (Urao et al. 1999), NtC7 (a membrane protein) (Tamura et al. 2003)

and Cre1 (a cytokinin sensor and histidine kinase). But the exact sensor for the perception of low temperature is still elusive. The research during the last decade has indicated that the sensor may be located in the plasma membrane. The signal is then transferred through several components of cascade of transduction pathways.

Temperature is a key abiotic signal that regulates plant function throughout development (Penfield 2008). Alterations in growth temperature act as a stimulus to initiate metabolic changes and promote developmental switches. In the first sight a simple question arises—How do plants sense change in temperature or more specifically how is lower than optimum temperature sensed by plants? Do they have a single thermo-sensor or multiple thermo-sensing mechanisms? The answer lies in the fact that plants are having special temperature preceptor organs, which are highly sensitive to sense a slight negative or positive change in its environment. These receptors not only sense the change in temperature but also inform the cellular headquarters (the nucleus) about the temperature-change condition. Following subheads explain about different mechanisms by means of which the plant senses temperature and subsequently frames strategy to cope up with the conditions.

2.1 Membrane Rigidification

The cellular membrane is the outermost living part of plant cell. The cellular membrane model as suggested by (Singer and Nicholson 1972) gives us much narrowed down information and more appropriately clues about the way of sensing temperature through cell membrane. The cellular membrane is fluid-mosaic in nature and is formed of a bilayer of phospholipids, which is sandwiched between the proteins. The phospholipid bilayer is interspersed by globular proteins, large tunnel proteins, and carbohydrates. The membrane is flexible and semipermeable in nature. Each movement in plasma membrane is by means of its own activation energy i.e. temperature dependence. As the membrane is exposed to temperature below optimum, it undergoes phase transition from liquid crystalline to gel phase. This causes the membrane movements to slow down and the membrane becomes more static than dynamic or rigid (Vigh et al. 2007). Therefore, it may be implicated that plasma membrane is a highly organized system, which plays an important role as communication interface between the cell and extracellular environment. Generally, chilling stress results in loss of membrane integrity and solute leakage. During the last few years, these observations have been documented as the same responses can be mimicked by plants in response to certain agents like DMSO at the ambient temperature. It has also been observed that the membrane fluidizing chemicals like benzyl alcohol, inhibit the responses of plants at considerably low temperatures also (Orvar et al. 2000; Sangwan et al. 2001, 2002; Vaultier et al. 2006). Therefore, it may be suggested here that the primary reception or perception is at the membrane level (Örvar et al. 2001). Injuries due to low temperature are mostly due to decrease in membrane fluidity; this is called rigidification (Hayashi and Maeda 2006). Alterations in the membrane fluidity have been demonstrated to

initiate temperature-signaling pathways in a variety of organisms, tempting speculation that similar mechanisms may operate in plants (reviewed in Samach and Wigge 2005). The effects of low temperature on plasma membrane have been demonstrated by many authors in different experiments and in different organisms e.g. in fish (Cossins et al. 1978; Pehowich et al. 1988), in bacteria (Sinensky 1974), and in blue green algae (reviewed in Mikami and Murata 2003; Los et al. 2010). This results in considerable reduction in growth rate and increase in electrolyte leakage (Nayyar et al. 2005a, b, c, d, 2007) and leaf chlorosis (Murata 1989). Wada et al. (1990) have studied the role played by membrane rigidification in cold stress by cloning the desaturase gene *desA*ftoma from chilling tolerant cyanobacterium *Synechocystis* PCC6803, and then transferring it into the chilling sensitive cyanobaeterium *Anacyslisnidulans*. The activity of this gene caused membrane lipid desaturation in the sensitive species subsequently causing an increase in low temperature tolerance. Therefore, it may be anticipated that saturation of membrane lipids is expected to rigidify the membranes. It has also been postulated in this context that the variations in the membrane phospholipids leads to the generation of a signal phosphatidic acid (PtdOH) within the first one minute of cold exposure as was observed by (Ruelland et al. 2002) in *Arabidopsis thaliana* culture. This phosphatidic acid (PtdOH) formation is one of the earliest response of plants to cold stress and it acts as a signaling molecule in response to cold stress mediating the NO signaling cascade (Fig. 2.3) (Testerink and Munnik 2005). This leads to the conclusion that membrane rigidification activates the downstream low temperature induced signaling pathways (Suzuki et al. 2000a, b).

2.2   Configurational Changes in Proteins

The changes in the membrane fluidity also cause confirmatory changes in the membrane proteins, which starts the signaling cascade. The temperature downshift causes unfolding of proteins (Pastore et al. 2007). Xue (2003) has observed that DNA-binding activity of *CBF2* (*CBF* proteins are transcription factors) in barley (*Hordeum vulgare*) is also temperature dependent and CBF/CRT regulon is a major genetic regulon in cold response by plants (Nakashima et al. 2009; Ruelland et al. 2002). Bae et al. (2003) found 54 nuclear proteins in *Arabidopsis thaliana* and Cui et al. (2005) spotted 60 proteins, which are up- or down-regulated by cold temperature exposure in rice.

2.3   Changes in Cytoskeleton

The low temperature has also been known to affect the multimeric polypeptides. It was reported a long time ago that a drop in temperature causes depolymerization of microtubules and actin microfilaments (Ilker et al. 1979). Pokorna et al. (2004)
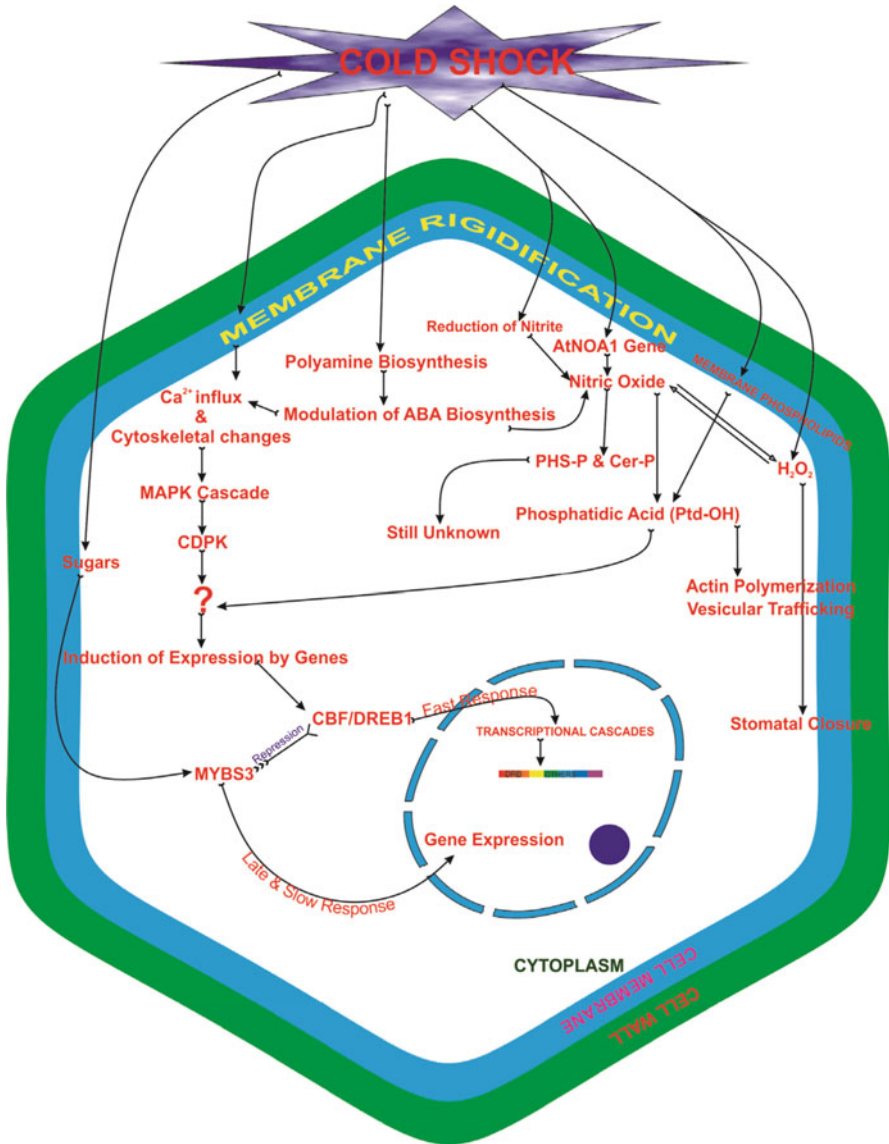
Fig. 2.3 The schematic representation showing interplay of different signaling pathways in response to cold stress in plants. Cod shock in plants starts signaling cascades in plants which also cross talk among themselves. The figure above shows the major pathways like Ca^{2+} signaling which is started as the membrane phase transition occurs. Polyamines are important osmoprotectants which act as signaling compounds and stimulate the ABA synthesis in response to cold exposure. Increased ABA biosynthesis increases the Ca^{2+} influx and biosynthesis of nitric oxide (NO) in turn. NO starts a signaling cascade involving the production of phosphatidic acid (PtdOH) which interacts with Ca^{2+} signaling pathway at some unknown point (?). H_2O_2 also interplays with NO in the web of signaling induced by cold stress exposure. Major converging point of different signaling pathways is CBF/DREB1 which is suppressed by MYBS3 induced in sugar signaling. The gene expression is ultimate and thus cold acclimation is achieved

observed that microtubules disassemble after an exposure of 0 °C for only 20 min. In *Medicago sativa* cells calcium influx and *cas30* expression at 4 °C were also prevented by jasplakinolide (an actin stabilizer) but induced at 25 °C by cytocalacin D a microfilament destabilizer (Orvar 2000). Therefore, this implies that the cytoskeleton assembly is necessary for defending the cold response (Sangwan et al. 2001; Al-Fageeh and Smales 2006)

2.4   Sugar Sensing in Cold Signaling

Sugars play a central regulatory role in many vital processes of photosynthetic plants besides serving the energetic function and are considered as important signals which regulate plant metabolism and development. Plants have the capacity to sense the presence as well as levels of sugars through various pathways that directly or indirectly recognize trehalose, fructose, glucose, or sucrose (Rolland et al. 2006). The basic mechanism behind the sugar sensing phenomena is still not clearly understood. The research in this area has led to the recognition of a hexokinase from *Arabidopsis thaliana* (AtHXK1) which is supposed to be a core component in plant sugar sensing and signaling pathways and plays vital functions as the glucose sensor that integrates the nutrient and hormone signals to govern the gene expression and plant growth in response to environmental aberrations such as cold (Moore et al. 2003; Cho et al. 2006). Cho et al. (2006) have elucidated that AtHXK1 functions to mediate the sugar repression like the photosynthetic *CAB* genes. Independent of the signaling function of HXK1, the metabolism of glucose through it induces the expression of defense-related genes (Xiao et al. 2000). We have recently reviewed the sugar sensing with respect to stressful conditions in grain crops (Thakur et al. 2010).

2.5   Reactive Species' (ROS and RNS) Role in Sensing

Reactive oxygen species (ROS) are toxic oxygen free radicals, which are produced in the plants out of photoreactions and cellular oxidation byproducts under normal conditions (Finkel and Holbrook 2000). One of the earliest responses of plant cells under various abiotic and biotic stresses is the generation of the oxidative burst, during which large quantities of ROS like superoxide, hydrogen peroxide, hydroxyl radicals, peroxy radicals, alkoxy radicals, singlet oxygen, etc. are generated (Bhattacharjee 2005). They are having the potential to cause cellular damage when they accumulate to certain toxic levels. However, these ROS are also having an important role as their accumulation activated defense-signaling pathways thus mitigating cellular damage. It has been estimated that both resistance responses to stresses and normal physiological metabolism can lead to ROS production (Van Breusegem et al. 2001). These beneficial reactive species include nitric oxide and hydrogen peroxide and both of these are involved in stress response in plants.

2.6 Low Temperature Induced Signal Transduction

According to Kultz (2005) two types of stress responses exist, specific and general; the specific ones are against some unique stressful condition like lowered oxygen tension characteristic of hypoxic stress in flooded roots (Magneschi and Perata 2009) and general responses include signals and signaling components which are shared by multiple pathways (Bowler and Fluhr 2000; Kultz, 2005). Duie to this reason, the acclimation to one type of stress in plants may also confer tolerance to other types of stresses also. In plants, the homeostasis is constantly under threat by environmental variables. Hence, for the adaptation and survival, the plants have evolved sensitive and complex mechanisms, which modify their growth and metabolic patterns since to achieve the target of acclimation it must be immediate to reestablish homeostasis, repair damaged cellular components, and reprogram the altered metabolic system (Wang et al. 2003).

The earlier signaling events start with slight perturbation in optimal environment. In order for a plant to respond to low temperature stress conditions, the plant must have the ability to sense the slightest temperature change in the environment so that it may prepare for the larger change in temperature conditions that may follow and which may cause irreversible damage. There are two components of this LTST (1) a mechanism of sensing the low temperature i.e. sensing mechanism and (2) a series of events that transmit the information from sensor to the nucleus, where specific genes need to be activated (Zeller et al. 2009) (Fig. 2.2). The key to understanding plant cold response lies in the identification of new components involved in those processes and the elucidation of the signaling pathways.

3 Signal Transduction Mechanism

As stated earlier, the membranes are primary receptors for the low temperature signal and the proteins embedded in the plasma membrane transmit these signals to cellular machinery through signaling cascade. This ultimately results in gene expression (Murata and Loss, 1997; Loss and Murata, 2000) so this implies that physical state of plasma membrane lipids also regulates the activities of membrane proteins (Sukharev et al. 1999), receptor-associated protein kinases (Wood 1999; Hohmann, 2003) and sensor proteins (Tokishita and Mizuno 1994; Sugiura et al. 1999). Monroy et al. (1998) have elucidated the LTST in six steps, (1) sensing of low temperature (2) transduction of signal into biochemical processes via secondary messengers such as Ca^{2+}, (3) activation/deactivation of kinases and phosphatases (4) transfer of signal to the nucleus (5) activation of specific genes in response to signal {more accurately cold acclimation specific genes *cas* genes and (6) development of cold/freezing tolerance. Mostly these events are studied in isolation for simplicity but a complex set of biochemical and molecular reactions is activated in response to the input signal, which in turn activates many signaling pathways and these pathways
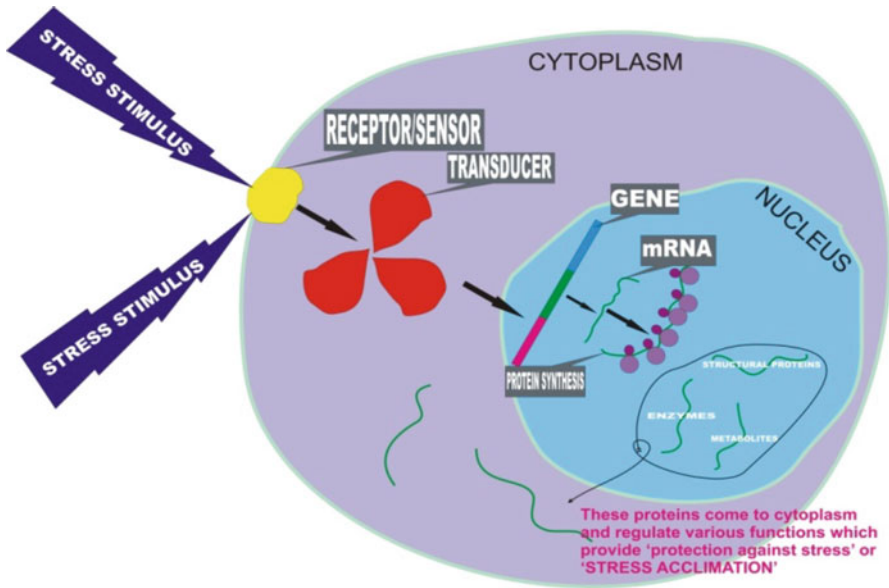
Fig. 2.2 A generalized and simplified scheme of stress-induced signal transduction consequently providing stress tolerance or stress acclimation

cross talk with each other i.e. these are interrelated to each other at various components and stages (Jenkins 1998; Trewavas and Malhó 1998).

Two methods are generally adopted for studying LTST. In the first method, the early signaling events, which take place upon exposure to cold, are studied and the cold inducible genes are investigated as end-point markers to understand the overall progress. In the second method, the mutants involved in the low temperature induced signaling pathway are identified and the role of each component and its sequence in the cascade is established.

3.1 Role of Ca²⁺ in Low Temperature Induced Signal Transduction

Ca^{2+} has been reported to regulate several important cellular functions. It also acts as secondary messenger in the signal transduction system. Whenever Ca^{2+} homeostasis is disturbed inside the cytoplasm, it leads to interference with cellular response, even when Ca^{2+} does not play a direct role in the mediation of cellular processes. The inward flow of Ca^{2+} into the cytosol has been reported to play a crucial role in signal transduction, where it acts as secondary messenger. In plant cells, Ca^{2+} is largely stored in the apoplast, where its concentration is at least 10^{-5} to 10^{-4} M (Cleland et al. 1990; Evans, et al. 1991). Regulation of many protein kinase activities occurs through binding of Ca^{2+} to key regulatory proteins like Ca^{2+}-dependent

protein kinases (CDPKs) (Roberts and Harmon 1992; Cheng et al. 2002), Ca^{2+}/CaM-dependent protein kinases (Shimazaki et al. 1992; Pandey et al. 2002), Ca^{2+}/phospholipid-dependent kinases (Nickel et al. 1991), and a homologue of Ca^{2+}-dependent protein phosphatase have also been identified in plants (Kudla et al. 1999). It has been reported that the cytosolic Ca^{2+} increases in response to cold shock (Knight et al. 1996). This increase in cytosolic Ca^{2+} amplifies the stimulus signal perceived by the plant. Ca^{2+}has been reported to be involved in a variety of stimulus–response pathways., the elicitation of a specific response from a general signal can be explained by means of variations in the amplitude, duration, frequency, and location of the Ca^{2+} signal, as well as in the interactions of this signal with other components of the pathway (McAinsh et al. 1997; McAinsh and Hetherington, 1998). It has also been observed that calcium is required for the total expression of some cold-induced genes like *COR6* and *KIN1* genes of *Arabidopsis thaliana* (Monroy et al. 1993; Monroy and Dhindsa 1995; Knight et al. 1996). Monroy and Dhindsa (1995) elaborated that a gene *Cas15* was not fully expresses due to the chelation of Ca^{2+} in alfalfa and thus the plant could not acclimate to the cold conditions. Later on when the plant was treated with A23187 (A Ca^{2+} ionophore which increases the influx of Ca^{2+}), the expression of *Cas15* was achieved even at very high stressful temperature. This shows that Ca^{2+} is very important in temperature induced gene expression. Some workers have suggested that exposure to one kind of or a specific amount of stress leads to a specific Ca^{2+} influx and signal kinetics, but subsequent exposure to some different amount of stress causes a different Ca^{2+} signal kinetics, than observed previously. This establishes the hypothesis of "stress memory" which modulates plant stress responses. The strength of stimulus response is determined by the extent of Ca^{2+} influx which may be responsible for the specificity of the response. Another factor which confers specificity to the response is the destination of Ca^{2+} influx. Ca^{2+} sensors are also important as they couple the extracellular signaling to intercellular responses and comprise calmodulin- and CaM-related proteins (Snedden and Fromm 2001),calcineurin B-like proteins (Kudla et al. 1999) and CDPKs (Harmon et al. 2000). It has been noted by many authors that cytosolic calcium is involved in signaling pathways induced by various kinds of stresses like heat, cold, drought, and salinity etc. (Trewavas and Malhó 1998). Hence, it may be concluded that the cytosolic calcium acts as a convergence point and plays a central role in the integration of different signal transduction pathways.

3.2 Role of Nitric Oxide

Nitric oxide (NO) has emerged as a key signaling molecule in animals as well as plants during the last decade and its role has been implicated in number of physiological and developmental processes as well as response to abiotic stresses including heat and cold stress (Qiao and Fan 2008; Corpas et al. 2001). In recent years, NO has been shown to be involved in seed germination and reduction of seed dormancy (Bethke et al. 2006a, b, 2007; Libourel et al. 2006), photo-morphogenesis, leaf expansion,

root growth, regulation of plant maturation and senescence (Mishina et al. 2007), suppression of floral transition (He et al. 2004), phytoalexin production (Noritake et al. 1996; Beligni and Lamattina 2000) and as an intermediate downstream of ABA signaling (Bright et al. 2006; Garcia-Mata and Lamattina 2007). NO is a free radical reactive gas with many physiological functions (Besson-Bard et al. 2008a, b; Neill et al. 2008a, b). It has been recognized as an important biological mediator in animals because of its role in certain important functions like neurotransmission, inflammatory responses, and relaxation of smooth muscles (Schmidt and Walter 1994). But its role in plant metabolic system was very much unknown till recently. Kopyra and Gwó (2004) have reported that NO alleviates the deleterious effects of ROS and establishes a stress resistance response. Corpas et al. (2008) have observed the involvement of NO as reactive nitrogen species in case of pea. They found that pea plants in response to stressful conditions activated the metabolism of reactive nitrogen species and that low and high temperature, continuous and high light intensity induced the overproduction of these reactive nitrogen species thereby consequently causing nitrosative stress which is although a cytotoxic effect of NO. The recent investigations on its relative role in plant regulatory and signaling mechanisms have spanned the part of the fissure, and the picture that came out of these investigations shows that it has got many important functions to play in plant system like ubiquitous signal involved in diverse physiological processes that include germination, root growth, stomatal closing, and adaptive response to biotic and abiotic stresses (reviewed in Stuehr et al. 2004; Besson-Bard et al. 2008a, b). But its generation mechanism in plant system is still controversial (Corpas et al. 2004, 2006; Crawford 2006; Zemojtel et al. 2006; Neill et al. 2008a, b). Zhao et al. (2009a, b) has also demonstrated that nitric oxide production in plants is involved in acquiring cold acclimation or cold tolerance. Guillas et al. (2011) have evidenced that NO is produced immediately as a plant response to cold stress and it participates in the regulation of cold-responsive gene expression. They also showed the presence of a novel downstream elements which were identified as phosphosphingolipid metabolic species i.e. phytosphingosine-phosphate (PHS-P) and a ceramide phosphate (Cer-P). Cantrel et al. (2011) have also stated that PHS-P and a Cer-P are transiently synthesized upon chilling. They also stressed that these two phosphosphingolipid species are negatively regulated by NO. NO mediates signaling in response to various abiotic stresses by involving ABA, calcium, and hydrogen peroxide which are also suggested to function in cold response too. The involvement of NO in imparting cold tolerance has been indicated by its exogenous application in certain cold-sensitive plant species such as maize and tomato (Neill et al. 2003). Its mechanism in providing protection against cold has been attributed to its antioxidative feature and suppression of peroxidative metabolism caused by stress (Neill et al. 2002). The role of NO in cold signaling has recently received attention. The cellular metabolism can be affected by NO through S-nitrosylation of protein thiols to form S-nitrosothiols and moreover it can lead to activation or inhibition of protein functions. In one of the studies, a brief cold stress (1–6 h) to *Brassica juncea* seedlings generated many S-nitrosothiols resulting in proteins modifications involving those in antioxidant metabolism (Abat and Deswal 2009). NO levels are reported to be restricted by non-symbiotic haemoglobins

(nHb) that can scavenge NO and keeps its levels below toxic range to act as signaling molecule in cold response (Dordas et al. 2003a, b; Gupta et al. 2011a, b). Additionally, NO may mediate lipid-based signaling in cold response. The sphingolipids produced during cold stress are transiently phosphorylated while NO may prevent this step to facilitate lipid-based cold signaling. It has been reported that NO transduces signals through cGMP as its downstream mediator and also it may interact with other signaling molecules such as H_2O_2, Ca^{2+}, and salicylic acid directly or indirectly (Neil et al. 2003; Lamotte et al. 2004; Wendehenne et al. 2006). Recently, it has been found that NO downstream cascade involves the cytoskeletal proteins as these proteins are involved in many processes regulated by NO in plants (Yemets et al. 2011). Besides the roles of NO as signaling molecule it has also been reported to be a regulator in gene expression (Kopyra and Gwozdz 2004).

3.3 Role of Polyamines

As we know that the plants which are having inherent characteristic of low temperature tolerance, in response to low temperature exposure, start regulatory and molecular mechanisms that are triggered to optimize the metabolic parameters which make sure the survival of the plant under suboptimal temperatures (Stitt and Hurry 2002). Polyamines are the low molecular weight organic polycations having two or more amino ($^-NH_2$) groups. The role of polyamines has been implicated in growth and developmental processes in higher plants especially in response to stressful conditions like senescence and biotic or abiotic stresses. As such they have been reported to encourage DNA replication, transcription, and translation. It has been observed in different plants species hat during exposure to stressful conditions polyamines' biosynthesis is enhanced. Polyamines have also been known to be involved in the plant defense system against environmental changes (reviewed in Alcázar et al. 2006; Groppa and Benavides 2008; Liu and Moriguchi 2007; Hussain et al. 2011). Because polyamines act as the scavengers of ROIs so these confer the protection from the oxidative stress. In low temperature stress the role of polyamines has been studied in detail by many workers in different plant species (Nayyar and Chander 2004). It has been shown that putrescine accumulates in plants under cold stress regimes (Kaplan et al. 2004; Cook et al. 2004; Cuevas et al. 2008) and it is very important for their survival as the *Arabidopsis* mutants with defective putrescine synthesis were having reduced cold tolerance than wild ones. They also demonstrated that alterations in the levels of ABA caused depletion in the putrescine levels which was drastic to plant survival in cold stress.

Plant polyamines have also been known to function as secondary messengers and modulate various anatomical, biochemical, physiological, and molecular processes in intracellular as well as extracellular areas under stress (Kuznetsov et al. 2006; Alcázar et al. 2006, 2010; Cavusoglu et al. 2008). The polyamine-related metabolic enzymes are also associated with cell wall including apoplast, where lignification, suberization, and wall stiffening occur (Kujnetsov et al. 2006). Aronova et al. (2005) have elucidated that polyamines in apoplast are also related to the

generation of H_2O_2 in the apoplast, where it is required for the formation of suberin, lignin, and oxyproline proteins. The role of polyamines has also been reported to be very important and it has been demonstrated that it functions during environmental insult as a part of antioxidative system and protects the membranes from oxidative damage (Kim et al. 2002; Verma and Mishra 2005; Kuznetsov et al. 2006; Shevyakova et al. 2006). In recent years, it has also been proved that polyamines are also having an important role in the regulation of structure and function of photosynthetic apparatus under low temperature stress conditions (Urao et al. 1999).

As already stated that polyamine biosynthesis is increased during exposure to stressful conditions including cold stress so it may be implicated that the initiation of polyamine biosynthesis requires a stress signal in the form of stimulus (Imai et al. 2004). The higher endogenous levels of polyamines may be positively correlated with the increased amount of antioxidants so it can be suggested that polyamines accumulation is able to optimize the metabolic rate and subsequently ensuring the growth and survival of the plant under stress i.e. it increases the stress tolerance of plants (Alcázar et al. 2006). But the exact mechanisms through which the polyamines act as a defense line against stress are still ill defined (Nayyar and Chander 2004; Aronova et al. 2005). Nayyar and Chander (2004) have found beneficial the effects of exogenous application of polyamines on chickpea (*Cicer arietinum* L.) in low temperature stress conditions. Kovacs et al. (2010) observed in case of wheat the effects of cold stress, osmotic stress, and abscisic acid (ABA) on polyamine accumulation and it was found that the levels of putrescine and spermidine levels were higher during the exposure to above stated stress conditions whereas the ABA treatment increased the levels of cadverine. Cuevas et al. (2008) have established that in response to low temperature stress putrescine is synthesized in plants and it modulates ABA biosynthesis at transcriptional levels and demonstrated that polyamines function as regulators of phytohormone biosynthesis. Despite all these findings which put forth a protective role for polyamines, it has still not been established how polyamines modulate biosynthesis of phytohormones like ABA and more studies are needed in this context.

3.4   Role of Trehalose

Trehalose is a nonreducing disaccharide in which the two glucose units are linked in an α, α-1, 1-glycosidic linkage. Although there are three possible anomers of trehalose, that is, α,β-1,1-, β,β-1,1-, and α,α1,1-, only the α,α-trehalose has been isolated from and biosynthesized in living organisms. It is synthesized in two steps (1) trehalose-6-phosphate synthase synthesizes trehalose-6-phosphate (T6P) and (2) T6P is converted to trehalose by trehalose6-phosphate phosphatase. It has also been reported to serve as a signaling molecule to direct or control certain metabolic pathways or even to affect growth in plants and yeast. Besides this, trehalose has also been reported to serve as protective guard for proteins and cellular membranes from inactivation and denaturation by various kinds of environmental constraints

including cold stress as it accumulates in traces in plants in response to stress (Ramon and Rolland 2007; Paul et al. 2008; Paul 2008). Still the accurate role of trehalose in plants is still unclear and needs further research (Fernandez et al. 2010). Its role has also been implicated in the mediation of sugar metabolism in plants as it controls the rate of starch synthesis by means of redox modification of ADP-glucose pyrophosphorylase (Kolbe et al. 2005; Lunn et al. 2006). Trehalose may also stabilize cell membranes whose fluidity decreases during temperature downshift. And thus exogenous application of trehalose has also been observed to confer stress tolerance against cold temperatures (Su et al. 2010). As we have already reviewed that trehalose accumulates rapidly in response to cold shock in plants. This is followed by the transient induction of *TPP* activity (Pramanik and Imai 2005). *TPP* overexpression boosts the trehalose accumulation and confers cold tolerance (Jang et al. 2003; Ge et al. 2008).

3.5   *Mitogen-Activated Protein Kinases*

These are the proteins which catalyze reverse phosphorylations, which is very necessary for relaying signals. The MAPKKKs (MAPK kinase kinase) function by means of cascades which involves the sequential phosphorylation of a kinase by its upstream kinase (Xiong and Shitani 2006). MAPK pathways are activated by various abiotic stresses (Ligterink and Hirt 2001) and they also introduce the characteristic of specificity into the system. In *Arabidopsis thaliana,* three kinds of MAPKKKs have been found (1) CTR1 (2) ANP1-3 and (3) AtMEKK. Out of these, three AtMEKK are expressed in response to different abiotic stresses including cold (Knight and Knight 2001).

3.6   *Transcription Factors*

The process of acquiring tolerance to chilling (freezing or nonfreezing) temperatures can be achieved by exposure of plants to positive low temperatures. This is called cold acclimation. However, it has been experimentally proved that the cold acclimation can also be achieved by exposure to drought or application of ABA (Thomashow 1999). This is because many genes that are induced by cold temperatures are also expressed by application of ABA or exposure to drought stress. Moreover, these genes encode for proteins, which provide tolerance against both drought induced dehydration as well as cold stress. One of these common cold and drought-regulated genes is *RD29A* in *Arabidopsis thaliana.* This gene has been found to contain DRE or CRT (drought-responsive or C-repeat element) in their promoters (Kasuga et al. 2004). It has been noticed in *Arabidopsis thaliana* that two groups of transcription factors are present (1) *DREB1* (also called CBF) and (2) *DREB2.* These transcription factors induce the expression of specific genes for cold

stress and other drought or salinity stress respectively. This also makes it clear that DRE transcription factor is a point at which different stress (cold drought or salt) induced pathways converge (Fig. 2.3). So it can be said that DRE has the capability to integrate the information from two or more stress stimuli and it plays an important role in cross talk of stress signaling pathways.

Transcript profiling experiments revealed that multiple regulatory pathways are activated during cold acclimation, and that one such important pathway involves the c-repeat binding factor (CBF) regulon (Thomashow 1999, 2001). The c-repeat/dehydration-responsive-element binding factor genes (*CBF1-3*) are transcriptional activators involved in governing the plant's responses to low temperatures (Schwager et al. 2011). These include *CBF1*, *CBF2*, and *CBF*3 (Gilmour et al. 2004). Several studies have reported that ectopic overexpression of some CBFs resulted in both activation of target genes and enhanced freezing, salt, or dehydration tolerance of transgenic plants (Jaglo-Ottosen et al. 1998; Liu et al. 1998; Kasuga et al. 1999; Haake et al. 2002). CBF pathway is a central component of cold response, but CBF-independent pathways might also be necessary for the cold stress response (Zhu et al. 2004). Hsieh et al. (2002) suggested that overexpression of *CBF1* increased chilling tolerance in tomato by enhancing *CATALASE1* gene expression and enzyme activity, and oxidative stress tolerance (Hsieh et al. 2002). Direct evidence exists for the activities of some cold-regulated transcription factors (TFs) not participating in the CBF cold-response pathway (Fowler and Thomashow 2002), which suggests that TFs play a crucial role in controlling downstream gene expression as well as the regulation of cross talk between different signaling pathways (reviewed in Knight and Knight 2001). Over-expression of AtCBF1/3 enhanced tolerance against cold, drought, and salt stress in *Brassica* species (Jaglo et al. 2001), wheat (Pellegrineschi et al. 2004) and rice (Oh et al. 2005).

Another transcription factor termed as inducer of CBF expression 1 (ICE1) acts as a key regulator of cold-induced gene expression and is present upstream of CBF. ICE 1 is an *MYC*-type basic helix-loop-helix (bHLH) transcription factor that binds to MYC-cis element in the CBF 3 promoter and may be able to activate the expression of CBF3 upon cold stress. The constitutive expression of *ICE1* enhanced the expression of *CBFs* and *COR* genes leading to increased cold tolerance (Chinnusamy et al. 2003). On the other hand, *ice1* mutant showed impaired chilling tolerance as well as cold acclimation. Moreover in such mutants, a large number of cold-induced genes were either not induced or their induction was 50 % than that of wild-type plants. These findings indicated that ICE1 acts as a key regulator of several cold-responsive CBF-dependent and independent regulons.

3.7 Role of Abscisic Acid

ABA is a phytohormone critical for plant growth and development and plays an important role in integrating various stress signals and controlling downstream stress responses. ABA has been reported to act as an endogenous messenger and

regulates the water status of the plant (Swamy and Smith 1999). As various stresses have been known to induce ABA synthesis, it is now considered as a plant stress hormone (Swamy and Smith 1999; Mahajan and Tuteja 2005). Because the phyto-hormones mainly function as the regulators of adaptive response, the main function of ABA is to maintain and to optimize the plant water status (Swamy et al. 1999) by means of acting as endogenous messenger. In an experiment involving ABA deficient mutants of *Arabidopsis thaliana,* it was found that these mutants wilt and die readily under stress as compared to their wild counterparts (Shinozaki and Yamaguchi-Shinozaki 2000). Very recently (Nguyen et al. 2009) established by their experiments on maize (*Zea mays*) low temperature response that the genes induced by low temperature stress (*ZmCOI6.1, ZmACA1, ZmDREB2A, and ZmERF3*) are also induced by ABA application so it may be implicated that ABA synthesis regulates the induction and expression of specific cold-responsive genes in plants. Low temperatures have been reported to exert their effect on gene expression in ABA-independent pathways (Thomashow 1999; Shinozaki and Yamaguchi-Shinozaki 2000). Genetic analytical studies have shown that there is no clear line of demarcation between ABA-dependent and ABA-independent pathways and the components involved may often cross talk or even converge in the signaling pathway. Ca^{2+} has been found to mediate this cross talk (Fig. 2.3).

The expression of CBF1, CBF2, and CBF3 genes is induced by ABA but to a lower extent than that caused by cold acclimation (Knight et al. 2004). ABA has been also reported to induce the expression of *ICE1* (Chinnusamy et al. 2003). In this way, ICE 1 can also govern the ABA-mediated expression of *CBF* genes. Since cold-induced expression of *CBFs* is transient, ABA may activate ICE1-CBF-dependent and independent pathways that may be required to maintain the expression of *COR* genes during prolonged cold conditions. It has been reported that both ABA-independent and dependent pathways regulate cold-responsive genes (Xiong et al. 1999). ABA-dependent gene expression is regulated by transcription factors that belong to bZIP (ABRE-binding factors or AREB's), MYC and MYB families. *ABRE*-binding factor 1 (*ABF1*) was cloned from *Arabidopsis* (Choi et al. 2000) while its target genes are not known. However, ABAE elements can regulate the *COR* gene expression by involving a C2H2-type zinc finger protein which activates a bZIP transcription factor. *COR* gene expression can also be mediated by ABA by involving a cold inducible bZIP transcription factor in case of soybean (Kim et al. 2001). Cold stress has also been known to affect the auxin transport system in plants and inhibit basipetal auxin transport by blocking the intracellular trafficking of auxin efflux carrier PIN2 (Shibasaki et al. 2009).

3.8   Role of H_2O_2

The univalent reduction of $O_2^{\cdot-}$ produces H_2O_2. Hydrogen peroxide is considered a versatile actor of plant metabolic system. Because it plays dual roles as per its concentration, at low concentration it acts as mediator of signaling pathways leading

to stress acclimation and at higher concentration it orchestrates the cellular damage and death. Low temperature stress has also been shown to induce H_2O_2 accumulation in cells (O'kane et al. 1996). H_2O_2 was disregarded as a cellular toxic metabolite for many years is a ROS, because its accumulation causes oxidative stress and it can lead to damage as well as death of plant. Plants were able to achieve a high degree of control over H_2O_2 accumulation in due course of evolution (Dröge 2002). But it has come to be known now that H_2O_2 is a key signaling molecule in plants under stressful conditions and modulates the expression of various genes (Neill et al. 2002). During the last decade H_2O_2 has been given due attention as a kind of reaction oxygen species which acts as secondary messenger in stress signaling pathways as it is having a long life and high permeability across membranes (Neill et al. 2002; Huang et al. 2002; Yang and Poovaiah 2002). Dat et al. (2000) demonstrated that H_2O_2 plays an important role in plants during biotic and abiotic stress conditions whereas Laloi et al. (2004) have observed that hydrogen peroxide is produced in plants in response to various biotic as well as abiotic stresses. Many physiological as well as biochemical processes in plants including systemic acquired resistance (SAR) and hypersensitive resistance (HR) (Melillo et al. 2006) senescence (Hung et al. 2006), programmed cell death (Houot et al. 2001), stomatal movements (Pei et al. 2000; Zhang et al. 2001; Bright et al. 2006), gravitropism property of roots (Joo et al. 2001), development of lateral secondary and tertiary roots (Su et al. 2006), cell wall formation (Potikha et al. 1999), and pollen–pistil interactions (Mcinnis et al. 2006a, b). Now it has been experimentally proved that proteins functioning in metabolism, energy movement, protein translocation and transport, cellular organization and defense and transcription are encoded by transcripts induced by H_2O_2 (Desikan et al. 2001). Studies have provided evidence that H_2O_2 itself is a key signal molecule, which mediates a series of responses (Desikan et al. 2003) and activates many other important signal molecules such as Ca^{2+}, salicylic acid, ABA, jasmonic acid, ethylene and nitric oxide of plants (Liu et al. 2004; Desikan et al. 2004; Wendehenne et al. 2004). H_2O_2 has also been reported to work in coordination with NO, ABA, jasmonic acid, and ethylene in response to cold stress. Especially, in cold response, ROS such as H_2O_2 may alter calcium expression (signatures) and activate mitogen protein kinases (MAPK) and redox-responsive transcription factors. The expression of *COR* (cold-responsive) genes is reported to be regulated by ROS (Lee et al. 2002). Under cold stress, ROS activate the AtMEKK1/ANP (MAPKKK)-AtMKK2(MAPKK)-AtMPK4/6 (MAPK) MAPK cascade that is imperative for cold acclimation in plants (Teige et al. 2004)

3.9 Role of Cytoskeleton

The eukaryotic cytoskeleton consists of tubulin dimers which form microtubules (MTs), actin monomers which form actin microfilaments (AFs) and vimentin and related proteins that constitute intermediate filaments. MTs and

AFs are both implicated in signaling, and are discussed in the following sections.

3.10 Microtubules

Microtubules are thought to transmit signals from the receptor to the nucleus, since they span the cell from the nucleus to the plasma membrane (Gundersen and Cook 1999). The minus ends of microtubules associate with the microtubule organizing center (MTOC, or centrosome in most animal cells) near the nucleus, and the plus ends terminate near the plasma membrane. This gives microtubules a defined polarity and enables directional transport via the motor molecules kinesin and dynein. As the microtubules provide a surface area ten times larger than the nuclear envelope, there is ample space for protein–protein interactions on their surface. So it should not be surprising that microtubules have been associated with various signaling pathways (Volkmann and Baluska 1999). Microtubules act as a scaffold, bringing components of the signaling pathways together. Another example of microtubules' involvement in signaling is the interaction between microtubules and ERK1/2, both in vitro (Mandelkow et al. 1992) and in vivo (Reszka et al. 1995*;* Morishima-Kawashima and Kosik 1996; Reszka et al. 1997), where microtubule association could retain some activated MAPKs in the cytoplasm. Another MAPK, ERK5, possesses C terminal sequences that suggest that it may a1so be targeted to the cytoskeleton (Zhou et al. 1995). In the G-protein signaling pathway, tubulins have been identified as secondary substrates for G-protein-coupled receptor kinases (Haga et al. 1998; Pitcher et al. 1998). However, no single mechanism for the modulation of G-protein signaling has been identified, since breakdown of microtubules and G-protein subunit-microtubule interactions leads to a multiplicity of events.

Spatial orientation of microtubules is generated by their interaction with proteins such as those found in MTOCs (Marc 1997; Vaughn and Harper 1998). However, since centrosomes are not found in higher plants (Vaughn and Harper 1998), the origin, identity, and precise locations of MTOCs is not known. Microtubules have been shown to play a role in growth orientation in plants (Williamson 1991; Joshi 1998). Mathur and Chua (2000) using transgenic plants expressing a fusion of green fluorescent protein and microtubule-associated protein 4 have shown that MT stabilization leads to growth reorientation in *Arabidopsis* trichomes. The role of MTs in Ca^{2+} channel opening was examined by Thion et al. (1996). When cold-shocked *Nicotiana plumbagnifolia* protoplasts were treated with oryzalin and cytochalasin D, destabilizers of MTs and actin microfilaments, respectively, a synergistic increase of Ca^{2+} influx was observed (Mazars et al. 1997*).* Thus, both MTs and actin microfilaments are speculated to be involved in Ca^{2+} influx in cold signaling.

3.11 Actin Microfilaments

Actin occurs in plant cells in two forms: globular actin (G-actin), which comprises actin monomers, and filamentous actin (F-actin), which consists of assemblies of G-actin and other proteins. Plant actin gene families are more abundant and diverse than those found in other organisms. It has been demonstrated that *Arabidopsis* has ten genes which code for actin (McDowell et al. 1996) whereas *Vicia faba* has five isoforms of actin gene (Janben et al. 1996).

The cell signaling processes are thought to be mediated by the balance between F and G-actin, alterations in the relative amounts of actin binding proteins and their binding abilities, and formation of actin-associated myosin filaments. Recent studies have shown that dynamic interconversions of F- and G-actin play a major role in the regulation of ion channels in the plasma membrane, controlling osmoregulation (Schwiebert et al. 1994; Tilly et al. 1996), as well as cell polarity (Drubin and Nelson 1996), cell growth and proliferation, secretion and cell wall interactions (Grabski et al. 1998).

Plasma membrane-associated actin is involved in the phosphoinositide signaling pathway (Tan and Boss 1992). Actin also plays a role in intracellular movement, including the endocellular localization of ER and Golgi elements, which are fully under F-actin control (Lichtscheidl et al. 1990).

4 Cold Stress Defense/Tolerance Mechanisms in Plants

The outcome of the signal perception, transduction and transcriptional up or down regulation of genes is the production of some metabolites which have plant protection, repair, and stabilizing functions. All these result into acquired tolerance against one or more abiotic stresses. Cold acclimation also known as cold hardening is one such responses that refers to increase in tolerance over time to cold temperatures and results from changes in gene expression and physiology (Xin and Browse 2000; Kalberer et al. 2006).

4.1 Cold Stress Proteins

Proteomic studies have revealed differential expression of proteins in some plant species exposed to cold stress. In pea mitochondria, 33 proteins showed either up-or down regulation under different stress conditions, 20 of which appeared to respond to low temperature of 4 °C for 36 h (Taylor et al. 2005). In rice anthers, a cold treatment for 4 days at 12 °C induced differential expression of 70 proteins out of which 47 were up-regulated, 12 were new, and 11 were down-regulated with a positive identification for 18 of them (Imin et al. 2004). In leaves of poplar seedlings

subjected to 4 °C for 2 weeks, 26 proteins were identified that were COR proteins of which 21 were overexpressed and 5 were repressed (Renaut et al. 2004). Broadly, the types of proteins expressed in response to cold stress are antifreeze proteins (AFP's), dehydrins and late embryogenesis abundant (LEA) proteins, heat shock proteins (HSPs), chaperonins, pathogenesis-related (PR) proteins and those related to transduction, transcription and signaling pathways.

AFP's lower the freezing temperatures in cold acclimated leaves and after the leaves have been frozen and also prevent the growth of ice crystals by binding with them (Griffith et al. 2005). Thus, these proteins protect the cells from the mechanical injury by preventing the size of the individual ice crystals to increase as well as to inhibit the growth of ice crystals into the intercellular spaces. Though these proteins are primarily extracellular in location and activity but intracellular dehydrin in case of peach was found to have AFP proteins like activity (Wisniewski et al. 1999). AFP's are suggested to be homologous of PR proteins such as B 1, 3 glucanases, chitinases or thaumatin like proteins (Griffith and Yaish 2004).

LEA proteins though originally shown to be accumulated in plant embryos during the later stages of embryogenesis (Dure 1993) but now have been found to be expressed in response to osmotic stress, cold and ABA (Wise and Tunnacliffe 2004). These proteins have subclasses and many roles have been suggested for them such as chaperones, DNA-binding and repair, being a structural component of cytoskeleton. Dehydrins, a subgroup of LEA proteins are stable to heat, rich in glycine are expressed in response to abiotic stresses causing dehydration have role in stabilization of membranes and protection of other proteins from denaturation due to water loss induced by the stresses (Allagulova et al. 2003). Dehydrins have been reported to be accumulated due to cold stress in case of herbs and woody plants (Wisniewski et al. 2004). In poplar, the expression of a single 100 kDa LEA protein was documented (Renaut et al. 2004).

HSPs is another category of stress proteins which though originally discovered for their expression in response to heat stress are now reported to be generated due to drought, salt, and cold stresses (Sabehat et al. 1998). HSP's are also referred to as stress-related molecular chaperones. Especially, families of HSP90, HSP70 and small HSP's have been shown to accumulate due to cold stress (Lopez-Matas et al. 2004). HSP's have a role in translation, translocation into organelles, refolding of denatured proteins, prevention of aggregation of denatured proteins and protection of membranes (Tsvetkova et al. 2002).

Pathogenisis-related (PR) proteins, which are expressed due to pathogenic attack, are also produced in response to mechanical injury, xenobiotic compounds, and environmental stresses. There are 14 groups of PR proteins identified that represent B 1,3 glucanases, chitinases, thumatin-like proteins, and lipid transfer proteins (Liu et al. 2003). They are speculated to have a role in signal transduction pathway in reaction to abiotic stresses including cold stress (Hoffmann-Sommergruber 2000).

Besides these proteins, the expression and activity of several enzymes pertaining to various metabolic pathways are either up-regulated or down-regulated depending upon the severity and duration of the cold stress (Hurry et al. 1995). Among several enzymes, those related to photosynthesis (rubisco subuits, rubisco activase,

polypeptides of the PSII O$_2$ evolving complex), carbohydrate metabolism (sucrose phosphate synthase, invertase, sucrose synthase, and enolase), and detoxification enzymes (antioxidants), those of proline metabolism (proline dehydrogenase), and lignin metabolism (caffeic acid 3-*O*-methyltransferase).

4.2   Metabolic Modifications

Exposure of plants to stress conditions results in the alteration of their metabolic activities. This happens by means of two ways (1) adjustment/restoring of the low temperature induced alterations in metabolic parameters like the structure and functional catalytic properties of enzymes by regulatory mechanisms as soon as it occurs (Schwender et al. 2004; Fernie et al. 2004) and (2) modification of the metabolic parameters according to the stress conditions (adaptive mechanisms) such as the production of some metabolites, osmolytes, and phytohormones have been reported to increase during stress conditions (Nayyar 2003a, b; Nayyar et al. 2005a, b, c, d, 2007; Farooq et al. 2008; Kaur et al. 2011). These include sugars, amino acids, organic acids, polyamines and lipids (Nayyar and Chander 2004; Nayyar et al. 2005a, b, c, d; Farooq et al. 2009; Kaur et al. 2011), which eventually assist in cellular protection from cold-induced damage by various mechanisms.

4.3   Antioxidant Systems

Much of the injury to plants caused by chilling stress is associated with oxidative damage at cellular level (Bowler et al. 1992). Inherent metabolic homeostasis of plants is disturbed due to adverse environmental factors, which results in the production of ROS (Suzuki and Mittler 2006).

Protective mechanisms against stressful low temperature conditions can be divided into two separate categories, those involved in removing reactive oxygen intermediates and those involved in reducing production of reactive oxygen intermediates. Generally, the defense system against reactive oxygen intermediates in plant cells is a net result of suppression mechanisms, scavenging, and repair systems. Higher plants have active oxygen scavenging systems consisting of several antioxidant enzymes, such as superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), glutathione reductase (GR), and some non-enzymatic antioxidants, such as ascorbic acid, α-tocopherols, phenolic compounds, and reduced glutathione (Bowler et al. 1992). In recent years, it has become apparent that plants actively produce ROIs as signaling molecules to control processes such as programmed cell death, abiotic stress responses, pathogen defense, and systemic signaling. Higher plants contain numerous enzymatic and non-enzymatic reactive oxygen intermediate scavengers and antioxidants, both water- and lipid-soluble, localized in different cellular compartments. Non-enzymatic antioxidants include

(1) pigments, (2) reduced glutathione (GSH), (3) ascorbate (AsA), (4) vitamin E, and many others.

α-Tocopherol is one of the most acknowledged antioxidant (Polle and Rennenberg 1994). α-Tocopherol is the most abundant tocopherol of the four forms found in plants (α-, β-, γ-, and δ-tocopherol). Its main location is within the chloroplast. Ascorbate, and enzymes that metabolize AsA-related compounds, are involved in the control of several plant growth processes (Cordoba and Gonzalez-Reyes 1994). The most abundant thiol in higher plants is glutathione (Foyer and Halliwell 1976; Foyer 1997; Mullineaux and Creissen 1997). The general picture is that the levels of glutathione in its reduced form (GSH) increase several fold during the chilling conditions in evergreens (Wingsle and Hällgren, 1993; Wildi and Lütz 1996). Many factors, including low temperature and other environmental stresses have been shown to change the ratio or redox status of glutathione (GSH/(GSSG+GSH)) (Karpinski et al. 1997).
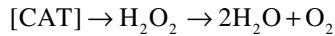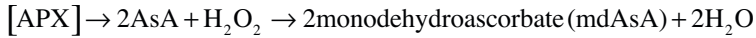
4.4   Enzymatic Antioxidants

In plant cells the enzymatic scavenging system for reactive oxygen intermediates consists of such enzymes as, SOD, CATs, APX, monodehydroascorbatereductase (MDAR), dehydroascorbatereductase (DHAR), glutathione peroxidase (GPX), and glutathione reductase (GR) (Inzé and Montagu 1995; Foyer et al. 1997; Mullineaux and Creissen 1997). Following are some examples where the expression of these antioxidants has been genetically engineered to achieve cold tolerance (Table 2.1).

The enzyme SOD can be taken as an example of the complexity in studying the role of the enzymatic defense system. Different SOD isoforms in plants are differentially expressed and also localized in different compartments within and outside the cell (Schinkel et al. 1998). SOD mRNA levels have been observed to increase during recovery from naturally established winter stress, a combination of high light and low temperature stress (Karpinski et al. 1993, 1994). SOD isoforms are differentially expressed during recovery from winter stress. A comparison of chloroplastic and cytosolic CuZn-SOD mRNA levels showed a 4-fold higher transcript level for the chloroplastic form until mid-May (Karpinski et al. 1993). This higher transcript level was also associated with a higher chloroplastic CuZn-SOD activity. Transcript levels were reduced for both chloroplastic and cytosolic CuZn-SODs and reached similar low levels after the repair process of the photosynthetic apparatus was completed and photosynthetic capacity had fully recovered from winter stress (Karpinski et al. 1993, 1994). In alfalfa plants, Camp et al. (1994) demonstrated that Fe-SOD and Mn-SOD have different protective properties in response to chilling treatment.

In Arabidopsis, a network of at least 152 genes has been reported to be involved in managing the level of H_2O_2 (Davletova et al. 2005). The key enzymes involved in H_2O_2 scavenging are CAT and APX, which catalyze the following reactions:

Table 2.1 Genetic engineering approaches to achieve cold tolerance by overexpression of antioxidant enzymes

Enzyme	Reaction catalyzed	Transgenic plant against cold stress	Reference
Superoxide dismutase (SOD)	$O_2^- + O_2^- + 2H^+ \leftrightarrow 2H_2O + O_2$	Cu/Zn-SOD from *Pisum sativum* to *Nicotiana tobaccum*	Gupta et al. (1993)
		Fe-SOD from *Arabidopsis thaliana* to *Madicago sativa*	McKersie et al. (2000)
		Mn-SOD in *Gossypium hirsutum*	Payton et al. (2001)
Catalase (CAT)	$2H_2O_2 \leftrightarrow 2H_2O + O_2$	Rice (*Oryza sativa*)	Matsumura et al. (2002)
Glutathione reductase (GR)	NADPH+GSSG↔NADP +2GSH	Tobacco (*Nicotiana tabacum*)	Le Martret et al. (2011)
		From *Arabidopsis thaliana* to *Gossypium hirsutum*	Payton et al. (2001); Kornyeyev et al. (2003b)
Dehydroasco-rbate reductase (DHAE)		From human to Tobacco (*Nicotiana tabacum*)	Kwon et al. (2003)
		Tobacco(*Nicotiana tabacum*)	Le Martret et al. (2011)
Ascorbate peroxidase (APX)	AA+ $H_2O_2 \leftrightarrow$ DHA+$2H_2O$	From *Pisum sativum* to *Gossypium hirsutum*	Kornyeyev et al. (2001, 2003a, b)
		From *Spinacea oleracea* to *Nicotiana tobaccum*	Yabuta et al. (2002)
		From *Pisums sativum* to *Lycopersicon esculentum*	Wang et al. (2005)
		Tomato (*Lycopersicum esculentum*) *StAPX*gene in Tobacco(*Nicotiana tabacum*)	Sun et al. (2010)

$$[APX] \rightarrow 2AsA + H_2O_2 \rightarrow 2monodehydroascorbate\,(mdAsA) + 2H_2O$$

$$[CAT] \rightarrow H_2O_2 \rightarrow 2H_2O + O_2$$

CAT acts as a major scavenger of H_2O_2 generated during mitochondrial electron transport, β-oxidation of the fatty acids, and most importantly in photorespiratory oxidation (Scandalios et al. 1997). GPX has generated much attention as an important enzyme in the scavenging of H_2O_2 or the products of lipid peroxidation. The role and function of the chloroplastic GPX during cold hardening and low temperature-induced oxidative stress in trees is under investigation. Expression of genes encoding different isoforms of the same ROI scavenging enzyme are regulated differently in response to low temperature-induced oxidative stress (Karpinski et al. 1993). CATs have also received much attention in respect of plants response to chilling and are thought to play a major role in inducing chilling tolerance (Prasad 1996).

4.5   Other Involvements (Compatible Solutes, Phytohormones, and Others)

In response to almost all the stresses, the increase has been observed in the levels of compatible solutes, which implies that they are having significant role in stress defense/tolerance. The compatible solutes are organic compounds belonging to a chemically diverse small group and are highly soluble. These are also known as osmolytes. These molecules are considered perfectly compatible to cellular functioning, since these do not interfere with cellular metabolism, even at higher concentrations (reviewed in Sung et al. 2003). Proline is one of the most studied and extensively reported cryo- and osmoprotectant, and has been found to accumulate in response to almost all the kinds of abiotic stress conditions like drought, salinity, high tempera-ture, chilling, UV radiation, and heavy metals (Rhodes and Hanson 1993; Nayyar and Walia 2003). In case of *Arabidopsis,* it has been seen that proline levels are increased and accumulated to considerable level during the stress conditions. In our case of review, the integrity of plasma membrane is vital for the low temperature tolerance and it has been suggested that proline may interact with the enzymes to protect the membrane-proteins' structure and activity (Hamilton and Heckathorn 2001). Proline accumulation has been experimentally observed in cold-shocked greenbean plants along with ornithine-δ-aminotransferase and proline dehydrogenase enzymes (Ruiz et al. 2002).

Glycine betaine is another osmolyte coming from the group of betaines (the quarternary ammonium compounds in which the nitrogen atom is fully methylated). In higher plants glycine betaine is synthesized from choline using two enzymes (1) choline monoxygenase and (2) betaine aldehyde dehydrogenase (Rathinasabapathi et al. 1997). Glycinebetaine has been seen to be synthesized at increased levels and accumulated in many plant species in response to various stresses and thereby

providing tolerance to the stress (Hincha et al. 2006), although its presence in plants is not universal as it is not reported to be accumulated in *Arabidopsis,* rice, and tobacco. Transgenics having over-accumulation of glycine betaine have been reported to have tolerance against different stresses including chilling (Sakamoto and Murata 2002). Its exogenous application has also been known to confer stress tolerance and increase growth and survival (Chen et al. 2011). Trehalose is also a compatible solute but its roles are still not much unblemished in plant exposed to cold shock. Some authors have reviewed that it may be considered as a double-faced molecule with both negative as well as positive effects (Fernandez et al. 2010)

Compatible solutes also include sugar alcohols that are acyclic polyols containing three or more hydroxyl groups, which include erythritol, ᴅ-arabitol, ribitol, xylitol, sorbitol, ᴅ-mannitol, galactinol, and rhamnitol (Ahmad et al. 1979).

5   Modification in Gene Expression Pattern and Synthesis of Stress Responsive Genes

At low temperature conditions the plants reorganize their patterns of gene expression and try to maintain homeostasis for obtaining cold stress tolerance (Cook et al. 2004). A number of genes have been identified and reported which express during low temperature stress conditions (Mantri et al. 2007 reviewed in Yadav 2010). The recent DNA microarray technique has made it possible to analyze large scale gene expression and in last few years numerous stress-induced genes have been identified in different crops not only in chilling stress but in other abiotic stresses also (Bray 2004; Maruyama et al. 2004; Seki et al. 2004; Vogel et al. 2005; Mantri et al. 2007). Some drawbacks of microarray like analysis of arbitrarily selected gene segments have been overcome by another technique i.e. serial analysis of gene expression. This technique allows the identification of novel genes under various physiological states of plants. These two methods have helped us to reveal that under stress conditions, some new genes are expressed and in some cases the expression patterns of some genes are altered. Either now they produce the protein products, which directly take part in processes against stress, or they regulate the expression of other genes. Based on these documentations the product proteins can also be classified into two types. First are those which are involved directly in the processes against the stress e.g. LEA proteins, antifreezins, osmotins, chaperones, mRNA binding proteins, enzymatic proteins for osmolytes (proline, trehalose, transporter proteins for proline, sugars, and lipids), detoxification processes and fatty acid metabolism, proteinase inhibiter proteins, and water channel proteins(Kreps et al. 2002; Seki et al. 2002). The functioning of these genes has been proved as in case of some transgenic plants in which these genes are over expressed are considerably stress tolerant (Cushman and Bohnert 2000). The second type of proteins are those which themselves do not take part directly in the stress tolerance mechanisms but further regulate the other signal transduction pathways. The examples of these types of proteins are some transcription factors

(Seki et al. 2003). These transcription factors are involved in the regulation of expression of other stress responsive genes. Some other examples of stress responsive proteins are kinases, phosphatases, calmodulin binding proteins, and 14-3-3 proteins. It has been elaborated by some authors that transgenics having these genes overexpressed in them are tolerant to stress conditions (Zhang et al. 2004; Tester and Bacic 2005; Vinocur and Altman 2005).

The expression of these stress responsive genes (genes expressed in response to cold stress are called cold-responsive genes) is vital for the tolerance and acclimation to the low temperature conditions. The vitality of these genes has been proved by the help of producing transgenics with overexpression of these genes (Ma et al. 2009; Sanghera et al. 2011).

6   Genetic Engineering Against Cold Stress

On exposure of plants to low temperature, a series of genes are induced, the products of which may either directly protect against stress or further control the expression of other target genes (Yamaguchi-Shinozaki and Shinozaki 2006). Transgenic plants have also been and are also being prepared against cold stress i.e. to achieve cold tolerance. These plants have one or more alien genes from stranger or their wild relatives, which over-express and regulate the functioning of metabolic process in a positive manner against stressful temperatures. The analysis of transgenic plants overexpressing one or other genes provides us an understanding of basic mechanism of functioning of stress genes during cold stress exposure (Tayal et al. 2005). (Table 2.2)

7   Conclusion

The study of plant temperature interactions is of great relevance with respect to the global climate change. Even after two decades of molecular and biochemical plant metabolomics research we are not yet able to clearly identify the plant thermo-sensors. However, we have considerably grown in the field of knowledge of various cross talking signaling pathways and responses of plants in respect of changes in their micro- as well as macro environments. The deeper analysis of these responses will bring new insights about the thermo-sensing mechanisms in these sessile poikilotherms. The observable phenological changes are very informative about the small periodic responses of plants to the temperature changes. These phenological alterations must be studied as a link to the temperature changes to facilitate the molecular, physiological, and biochemical studies related to cold tolerance. These will help to reveal about the probable stimulating inputs of the temperature. The genetic studies besides this will help to produce the computer models to understand the problem in digitized way, which will take the comprehensive approaches in hypothesis making

Table 2.2 Shows a list of transgenic plants produced for cold tolerance

Transgenic crop	Gene engineered	Effect of the gene engineering	Reference
Nicotiana tobaccum	wheat *TaSOD1.1* and *TaSOD1.2* genes	Increased SOD activities and decreased MDA content, lessened degree of over-oxidation of the cellular membrane system, the enhancement of physiological functions	Hai Na et al. (2009)
N. tobaccum	*OsSPX1*	Better seedling survival and reduced cellular electrolyte leakage, decreased total leaf Pi content and accumulation of free proline and sucrose	Zhao et al. (2009a, b)
Arabidopsis thaliana	Coda	Accumulation of glycinebetaine	Hayashi et al. (1997)
N. tobaccum	(*AtP5Cs* and *VacP5Cs* for Δ1-pyroline-5-carboxylate synthetase production) from *Arabidopsis* or *Vigna SacB for* levansucrase from *Bacillus subtilis* or the *codA* gene coding for choline oxidase from *Arthrobacter globiformis*	Accumulation of osmoprotectants like proline, fructan or glycine betaine	Konstantinova et al. 2002
Oryza sativa	TERF2	Increased accumulation of osmotic substances and chlorophyll, reduced ROS & MDA content and decreased electrolyte leakage	Tian et al. (2011)
Triticum aestivum	DRE-binding transcription factor gene, *GhDREB* from *Gossypium hirsutum*	Improved tolerance to drought, high salt, and freezing stresses through accumulating higher levels of soluble sugar and chlorophyll in leaves after stress treatments	Gao et al. (2009)
O. sativa	*OsRAN2*	maintained cell division, decreased proportion of cells with intranuclear tubulin and formation of a normal nuclear envelope under the cold condition	Chen et al. (2011)

Table 2.2 (continued)

Transgenic crop	Gene engineered	Effect of the gene engineering	Reference
O. sativa	MYBS3, Single DNA-binding repeat MYB transcription factor	Repressed the well-known DREB1/CBF-dependent cold signaling pathway in rice, and the repression appears to act at the transcriptional level	Su et al. (2010)
O. sativa	OsMYB3R-2 transcription factor	higher transcript levels of several G2/M phase-specific genes, including OsCycB1;1, OsCycB2;1, OsCycB2;2, and OsCDC20.1 increased cell mitotic index, level of cellular free proline was increased	Ma et al. (2009)
O. sativa	OsMYB3R-2	Stress tolerance	Dai et al. (2007)
E. Coli	OtsA/OtsB	Ability to synthesize trehalose	Kandror et al. (2002)
O. sativa	OsTPP1	Trehalose synthesis	Ge et al. (2008)
N. tobaccum	FAD7	ω-3-fatty acid desaturase gene; survival in chilling conditions	Khodakovskaya et al. (2006)

and testing to new horizons. Our group is working to explore these issues based on study of phenology, physiology, biochemistry, and molecular biology of different crops under thermal stresses.

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