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2.1 Bioprospection

Bioprospection is the exploration of biodiversity for new biological resources of social and economic value (Beattie et al., 2010). It is carried out by a wide variety of industries that include pharmaceuticals, botanical medicines, crop protection, cosmetics, horticulture, agricultural seeds, environmental monitoring, manufacturing, and construction (Beattie et al., 2010). Bioprospection incorporates two fundamental goals, (1) the sustainable use through biotechnology of biological resources and their conservation, and (2) the scientific and socio-economic development of source countries and local communities (Sittenfeld, 1996). Bioprospection initially focused on the potential undiscovered economic value of biodiversity, especially with regards to the pharmaceutical industry (Simpson et al., 1996). Pharmaceutical bioprospecting has been sharply criticized for what has been known as ‘biopiracy’ (Mgbeoji, 2006) in which large international pharmaceutical corporations make use of local medicinal knowledge without acknowledging that it is indigenous intellectual property. Also, global decline of biodiversity has affected the development of valuable products including medicines, industrial processes and crop varieties. Thus, various treaties/policies/laws have been enacted to control the use of intellectual property and to establish equitable benefit sharing as mentioned below:

2.1.1 International Policies/Treaties Governing Bioprospection

2.1.1.1 The Convention on Biological Diversity (CBD):

In 1992, the Convention on Biological Diversity (CBD) was signed by 150 nations for global commitment towards conservation of biological diversity and sustainable and equitable sharing of its benefits arising from the use of genetic resources. It was the first international treaty for conservation and sustainable use of biodiversity. The main objectives of CBD are laying down broad goals, objectives and general principles, which are to be implemented by contracting parties through measures at national level. The CBD framed out some measures relating to conservation and sustainable use of biodiversity and to technology transfer and benefit sharing for fulfilling above objectives (Lohan and Johanson, 2003). The convention clearly establishes the control and sovereignty of local agency over the biological resources and its diversity (Kumar and Tarui, 2004).
2.1.1.2 International Cooperative Biodiversity Group (ICBG):

The International Cooperative Biodiversity Group (ICBG) was launched in 1991 and was initiated in 1992 in a collaborative effort of U.S. National Institutes of Health, National Science Foundation and U.S. Agency for International Development. This program focused on the potential relationships between drug development, biological diversity, and sustainable economic growth (Rosenthal, 1998). The main goal of ICBG was the discovery and development of pharmaceutical and other useful agents from natural products and to conserve the biological resources from which these products are derived. Countries that are hosting the searches can expect fair rewards and benefits.

2.1.1.3 World Intellectual Property Organization (WIPO):

The World Intellectual Property Organization (WIPO) administers 23 international treaties dealing with different aspects of intellectual property protection. It covers a broad range of intellectual property rights, including copyright, trademarks, geographical indications, trade secrets, and patents. This program focused on the protection of intellectual property and standardizing intellectual property systems around the world (Lohan and Johanson, 2003).

2.1.1.4 International Treaty on Plant Genetic Resources for Food and Agriculture:

The International Treaty on Plant Genetic Resources for Food and Agriculture was adopted in November 2001. Its main objectives are conservation and sustainable use of plant genetic resources for food and agriculture and the fair and equitable sharing of benefits arising out of their use for sustainable agriculture and food security. It also contains provisions for facilitating access to genetic resources and sharing benefits arising from the use of these resources in an equitable and fair manner (Lohan and Johanson, 2003).

A range of documents developed by indigenous communities, researchers, professional associations, and bioprospecting companies has generated a significant shift in the ethical and legal framework within which bioprospecting operates (Beattie et al., 2005). All these programs have been adopted by many research groups that emphasize securing economic benefits for host countries and promotions of biodiversity conservation (Kursar et al., 2006; INBio, 2009). Nevertheless, many issues still remain unresolved (Roe and Elliott, 2010).

Recently, the Millennium Ecosystem Assessment (MEA) (http://www.millenniumassessment.org) provided an opportunity to review ecosystem products and to analyse them in context of bioprospecting. MEA encouraged the expansion of the term bioprospecting beyond pharmaceuticals, to the search for a wide variety of other products including crop pollinators, biological control agents, organisms for biological
monitoring, plant genetic resources for agriculture, horticulture and forestry, species for aquaculture, bioremediation, ecological restoration and biological mining and a wide range of organisms that provide products such as new materials and designs, or intellectual inspiration for applications in engineering, manufacturing and construction. Also, MEA placed biodiversity at the core of many major industries worldwide (Beattie et al., 2010).

The benefits of bioprospection have emerged from a wide range of organisms and environments worldwide and it is not possible to predict that which species or habitats will be critical to society, or industry, in the future. These benefits include an unexpected variety of products that includes chemicals, gene, metabolic pathways, structures, materials and behaviors (Beattie et al., 2010). Bioprospection has multiple goals, including the conservation of biodiversity, the sustainable management of natural resources and economic development.

2.2 Industries Involved in Bioprospection

Bioprospection involves the use of a wide variety of species by many industries (Ten Kate and Laird, 1999; Beattie and Ehrlich, 2004). The examples given here are a small part of a much longer list and have been selected because they are either the subject of major ongoing investments or already a commercial reality.

2.2.1 Pharmaceutical Bioprospecting

Antibiotic resistance in many human pathogens is likely to provoke an increase in pharmaceutical bioprospecting, which remains a vital source for drug discovery (Wessjohann, 2000; McGeer and Low 2003; Newman et al., 2003). Many important drugs, such as aspirin, were derived from natural products (Jack, 1997), the pharmaceutical industry has invested heavily in the exploration of species-rich communities such as rain forests and coral reefs in search of commercially profitable pharmaceuticals (Ismail et al., 1995; Bailey, 2001). For example, Malaria, one of the world’s most deadly diseases, has been treated historically with drugs derived from natural products such as quinine, chloroquine, mefloquine, and doxycycline and today the artemisinins derived from Artemisia annua has been widely used as drug against this parasite. Various compounds from natural resources have been approved for marketing during the 1990s in the United States and various other countries. Various drugs has been derived from wide range of organisms such as bacteria and fungi (both terrestrial and marine), plants, algae, and a variety of invertebrates, including worms, insects and mollusks (Henkel et al., 1999). Natural products are still important sources of novel compounds for pharmaceuticals and about 62% new, nonsynthetic chemical entities have been derived from natural products for cancer research over the period 1982–2002 (Beattie et al., 2005).
2.2.2 Microbial Bioprospection

Microbial biodiversity is also being intensely explored for a wide range of new energy sources including bioethanol, biobutanol, biodiesel, methane, methanol and electricity generating microbial fuel cells, each of them capitalizing on the astonishing diversity of enzymes, metabolic pathways and secondary compounds harboured by microorganisms (Beattie and Ehrlich, 2004). Other organisms broadly categorized as microbes include single-cell algae which are being sought for end-products as diverse as hydrogen, solar cell electronics and bio-inspired artificial photosynthesis (Demain, 2009; Ganapathy et al., 2009). Recent research on some soil microorganisms strongly suggested that antibiotics are generally secreted in sub-lethal concentrations, and hence are important for intra and inter-specific communication, as they are for competitive inhibition, providing powerful insights into combating drug resistance (Mlot, 2009). It was estimated that exploration of microbial diversity, led to the generation of billions of dollars of pharmaceutical, clinical, industrial and agricultural products worldwide annually (Demain, 2000).

2.2.3 Cosmetics Industry

Wild resources are rich source of various important natural products. Cosmetics industries use wild harvested or cultivated products in a wide variety of products, including hair products, baby care, nail care, oral hygiene, deodorants, skin care, and fragrances. Many of the natural products include saponins, flavonoids, amino acids, anti-oxidants, and vitamins (Beattie et al., 2005).

2.2.4 The Botanical Medicine Industry

Botanical medicines are specific biochemical compounds derived from whole plant. Various examples include ginkgo, echinacea, garlic and ginseng (Beattie et al., 2005). Various food ingredients and products have been sold by nutraceuticals industry, which conferred health or medical benefits. These include dietary supplements, individual nutrients, foods enhanced in various biotechnological ways, and fortified foods. Major products of this industry include dietary additives. Other products include teas with added ginseng, probiotic yogurts, fruit juices fortified with calcium, and flour fortified with folic acid. Various companies in the food industry are interested in sugar substitutes such as the sweet-tasting proteins produced by plants such as Dioscoreophyllum cumminisii, Thaumatococcus daniellii, and Richardella dulcifera, all from West Africa, and Capparis masaikai from South China (Beattie et al., 2005). The nutraceuticals market was estimated at $16.7 billion in 1996 (Ten Kate and Laird, 1999) and interest is rapidly growing worldwide.
2.2.5 Ethnobotanical Bioprospecting

Historically, much corporate drug discovery depended on indigenous knowledge delivered to modern science through ethnobotany. Over 50% of modern prescribed medicines were originally discovered in plants, and plants continue to be the source of significant therapeutic compounds (Pearce and Puroshothaman, 1993; Cragg and Newman, 2004). Many were developed because the plants were used in indigenous medicine, and some common drugs were first used only on a local scale. For example, in Europe, aspirin was first isolated from *Filipendula ulmaria* because it had long been used as medicine to treat pain and fevers. Another European drug was derived from *Digitalis purpurea*, the leaves of which were first used to treat congestive heart failure. The active ingredients, digitoxin and dihydroxigenin, remain an important treatment for heart ailments (Beattie *et al.*, 2005). Farnsworth *et al.*, (1985) showed that at least 89 plant-derived medicines used in the industrial world were originally discovered by studying indigenous medicine. Indigenous peoples generally have large pharmacopeias, since plants are often the only source of medicine available to them. A large number of plant species has been used as medicines based on ethnobotanical studies (Cox and Balick, 1994; Balick, 1994; Peters *et al.*, 1989; McCutcheon *et al.*, 1992).

2.2.6 Biological Control and Crop Protection

Biocontrol is an industry that involves bioprospecting activity worldwide, particularly in developing alternatives to chemical pesticides that have severe environmental and occupational safety hazards. Biological control is a more established biodiversity-based industry but it is currently expanding through new knowledge of biodiversity (Bellows, 1999). Many biological control is for crop protection, using predators, parasites, or pathogens or their products to limit pests. Various biological controls used for crop protection includes the control of soil pathogens; the protection of tree crops such as olives, citrus, bananas, and coffee; used in tree plantations, greenhouses, and grape vines; and to control the weeds in both terrestrial and aquatic environments as well as medical and veterinary pests. Bioprospecting for this industry requires study of the diversity both of the organism being controlled as well as the control agent(s). Biological control agents include plants, viruses, bacteria, fungi, insects, nematodes, and many other kinds of invertebrates and have been extremely successful in many parts of the world (Bellows and Fisher, 1999).

The commercial value of biological control come from the crop protection market, from which it is estimated that sales by the top 10 crop protection companies in 1997 totaled $25 billion.
2.2.7 Biomimetics

Biomimetics is the generic name for a wide variety of biologically inspired technologies. The industries involved use the structures and materials of organisms as the models, blueprints, or inspiration for novel materials and manufactured products (Mann et al., 1989). Among the best-known examples are the shell and radula (teeth) of various mollusks that have informed the manufacture of high-tech ceramics and other materials, including car parts and industrial crystals. Another high-profile research program is the application of the properties of spider silk to the manufacture of novel high-tensile fibers (Beattie and Ehrlich, 2004; Mann, 2001; Craig, 2003). At present, the use of biomimetics is scattered throughout a variety of engineering, manufacturing, and construction industries, and it is difficult to identify its commercial worth or to predict its value in the future (Beattie and Ehrlich, 2004).

2.2.8 Biomonitoring

Biological monitoring is an industry developed in response to the necessities of tracking down sources of pollution across large geographical areas. This would normally require vast resources in terms of conventional instrumentation, but the status of the environment can also be monitored by using organisms that routinely “sample” the environment, such as aquatic or marine filter feeding animals. Provided that the species used are both widespread and common and that collection for lab testing does not compromise their populations, there is little need for instrumentation outside the analytical laboratory (Boyle, 1987; Rosenberg and Resh, 1993). Biomonitoring is also applied to the detection of pollutants in soils and may involve a range of selected test organisms, including bacteria, algae, earthworms, and nematodes.

2.2.9 Bioremediation

Bioremediation is an example of the potential for novel biodiversity based industries. This industry is often associated with heavy industry and mining, especially in countries where the law requires restoration of abandoned industrial sites and mines (Crawford and Crawford, 1998). Two common methods are applied by this industry; (i) remediation by altering the site environment to allow resident, beneficial microorganisms to proliferate, (ii) augmentation of the site by the addition of beneficial microbes. Both these methods explore microbial diversity not just for species tolerant to the pollutants concerned but those that metabolize them, either transforming them into less harmful derivatives or sequestering them from other species in the ecosystem (Beattie et al., 2005).
2.2.10 Horticulture and Agricultural Industries

The global horticulture industry is worth many billions of dollars, mainly based on cultivated plants. Although all horticultural species are derived from wild species, current reliance on wild plant biodiversity is limited to a few areas, notably flowers harvested from native plants and genetic material taken from native plants to improve or establish new horticultural varieties (Beattie et al., 2005). This is not to minimize the size or cost of the industrial effort developing new varieties, and the revenues they generate are large. For example, it is estimated that it can cost up to $5 million to develop a single new variety (Ten Kate and Laird, 1999).

The development of new seed varieties for agriculture is a major use of plant biodiversity, some of it from wild, native plants, but much of it from the wealth of crop varieties that have been bred to adapt crops to a host of local conditions worldwide (Brush, 2004). New varieties are developed through traditional plant breeding protocols, genetic engineering, or a combination of these two. For example, the production of one wheat variety may involve thousands of plant breeding crosses and dozens of different individual lines, including wild ones, from many countries and over many centuries (Mujeeb-Kazi et al., 1996; Quick et al., 1996; Cassaday and Smale, 2001). The research budgets for agricultural biotechnology are estimated at $1.95 billion annually but only a small proportion of this involves the harvest of seeds from the wild.

2.2.11 Ecological Restoration

Ecological restoration differs from bioremediation in that it attempts to recreate the ecosystem that once existed. Nevertheless, restoration is a much needed industry worldwide as a result of national and local government legislation requiring the repair of damaged ecosystems such as abandoned industrial and mining sites, eroded agricultural lands, and surface water degraded by a wide variety of human activities. This demand has generated a new industry with active societies such as the Society for Ecological Restoration. The basic resource is biodiversity (Handel et al., 1994) and the species used are most often those from neighboring or comparable ecosystems that can be carefully harvested or grown offsite and moved to the restoration site (Harker et al., 2001; Whisenant, 2001). Effective ecological restoration requires deep knowledge of species, their ecological functions, and their interactions with each other and the environment.

2.3 Recent Industry Trends

Various novel products were identified, developed and produced from biodiversity, and there is geographical mismatch between centers of biodiversity, especially for tropical and
temperate zones (Barbier and Aylward, 1996; Simpson and Sedjo, 1996). Besides, pharmaceutical bioprospecting, various tropical/temperate partnerships have been formed and some developing countries are beginning to enter the industry independently to harness the resources from these regions (Beattie et al., 2005). In recent years, several laboratories and companies have applied natural history knowledge and ecological and evolutionary criteria for discovering new products. For example, Coley et al., (2003) carried out pharmaceutical bioprospecting in the tropical forests of Panama. They isolated a variety of novel molecules from wild plants and tested for activity against cancer, Chargas’ disease, leishmaniasis, malaria and HIV (Coley et al., 2003). Thus, it has been suggested that natural environments along with ecological and evolutionary conditions can be exploited for bioprospecting new species and novel molecules.

2.4 Cold Deserts of Western Himalayas: A Niche for Bioprospection

The Indian Himalayan region (IHR) with 250-300 km across stretches over 2,500 km from Jammu & Kashmir in the west to Arunachal Pradesh in the east spreading between 21°57’ – 37°5’ N latitudes and 72°40’ – 97°25’ E longitudes (Nandy et al., 2006). The region is broadly divided into eastern Himalaya, central Himalaya and western Himalaya, each region has its rich diversity and mountain specificities viz. inaccessibility, fragile, marginality, diversity, niche and adaptability (Jodha, 1992).

Varying altitudes, topography and agro-climatic conditions (from sub-tropical to temperate and cold-alpine and glacial) have resulted in rich and diversified flora along with a large number of medicinal and aromatic plants that are used as raw material for manufacturing a large number of ayurvedic medicines.

High species richness (50 species) and diversity (15 families) has been observed in western Himalayan region (range 3624 m to 4332 m) that includes various threatened, endangered and ecologically important plant species (Singh et al., 2008a). These flora included some common plants with a widespread distribution such as Anaphalis triplinervis, Androsace sempervivoides, Anemone obtusiloba, Lagotis cashmeriana, Polygonum affine, Potentilla argyrophylla, Taraxacum officinale and Tanacetum dolichophyllum; ecologically important plants such as Cassiope festigiata, Chaerophyllum villosum, Corydalis meifolia, Cremanthodium plantagineum, Cynanchus lobatus, Nepeta eriostachya, Pedicularis roylei, Rhododendron anthropogon, Salix flagellaris, Senecio laetus, Thymus linearis and Waldheimia glabra (Singh et al., 2008a).

Though wild species inhabited extreme environments and are uniquely adapted to a range of abiotic stresses including: temperature extremes, drought, salinity, nutrient deprivation and metal toxicity (Sahoo et al., 2004; Ghawana et al., 2010; Bhardwaj et al.,
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LT is a major environmental limitation to crop productivity and to the distribution of wild species (Pearce, 1999). Depending on the severity of LT, the stress could be either, chilling stress at temperatures above zero (0-15°C) or freezing stress at subzero temperatures (Levitt, 1980; Chinnusamy et al., 2007). Many plant species respond to LT, acclimate to cold, and survive temperatures slightly to moderately below freezing (Thomashow, 1999). Yet, temperate and woody perennial plants are distinguished by their ability to survive in regions with truly extreme winter temperatures (below -80 °C; Kramer and Kozlowski, 1979). Hence, plants are divided between three cold stress categories (Pearce, 1999):

1. chill-susceptible: damaged by temperature below 12°C,
2. chill-tolerant but freezing susceptible: able to acclimate to temperature below 12°C but unable to survive freezing,
3. freeze-tolerant: able to acclimate to survive temperature significantly below freezing.

The nature of chill- and freezing-stresses is different. Chill-stress is a direct effect of LT on cells. Freezing, however, often acts indirectly damaging cells by dehydration. Many freezing-tolerant herbaceous plants survive only moderate freezing, between -7°C and -30°C (Sakai and Larcher, 1987). However, there are some much hardier woody species; cutting of some will survive immersion in liquid nitrogen (Pearce, 1999).

2.5 Survival at LT

Plants are able to survive freezing temperatures by two general mechanisms either freeze avoidance or freeze tolerance (Levitt, 1980). Freeze tolerance is the capability of plants to tolerate ice formation in intracellular spaces. This is achieved by undergoing various changes necessary for surviving ice formation within the tissues (Levitt, 1980; Sakai and Larcher, 1987). The mechanisms include; (i) an increase in the concentration of cytoplasmic solutes that act as a cryoprotectants (Levitt, 1980; Thomashow, 1999), and (ii) modulations in the structure and composition of plasma membrane that increase the fluidity and stability of membranes at LTs (Steponkus and Webb, 1992).

2.6 Mechanisms of LT Tolerance

The molecular basis of cold acclimation and acquired freezing tolerance in Arabidopsis and winter cereals has been studied extensively (Chinnusamy et al., 2007). Plants modified their metabolism and growth to adapt to LT by reprogramming gene expression during cold acclimation (Yamaguchi-Shinozaki and Shinozaki, 2007; Chinnusammy et al.,
The molecular mechanisms leading to LT-induced plant adaptive response involves three main steps; (i) perception of LT; (ii) transduction of the signal to activate/repress expression of appropriate genes; and (iii) regulation of the genes (Smallwood and Bowles, 2002).

### 2.6.1 Physiological Changes during LT

The effect of LT depends on the degree of severity and the time of exposure (Solanke and Sharma, 2008). Plants exposed to temperature ranging from 0°C to 10°C causes chilling stress (low non-freezing temperature) whereas exposure to temperature below 0°C causes freezing stress. The potential chilling symptoms are: surface lesion, desiccation, a water-soaked appearance of tissue, discoloration, tissue break down, senescence, ethylene production, shortened shelf life and faster decay due to leakage of plant metabolites. Numerous physiological and biochemical changes are known to occur in plants in response to LT. The most notable changes include changes in membrane lipid composition, reduction in tissue water content, cessation of growth, transient increase in abscisic acid levels, accumulation of compatible osmolytes (proline, betaine, pyrols and soluble sugars) and increased levels of antioxidants (Xin and Browse, 2000). Any of these changes could act as the perception point for LT induced signal cascade, resulting in acquisition of LT tolerance (Smallwood and Bowles, 2002).

### 2.6.2 Cellular Changes during LT

Perception of LT is the preliminary step in LT-signaling cascade. It has been demonstrated that a short exposure to LT results in onset of LT-signaling pathway (Henriksson and Trewavas, 2003). Various changes at cellular level brought about by LT are described in the following section:

#### 2.6.2.1 Changes in Membrane Fluidity

Membranes have been suggested to be the primary target for temperature perception at the cellular level (Minorsky, 1989; Murata and Los, 1997). Decrease in membrane fluidity is the first effect of LT (Murata and Los, 1997). Membrane fluidity is directly and reversibly affected by changes in temperature also in higher plants; an increase in temperature renders the membranes more fluid, whereas a decrease in temperature rigidifies them (Los and Murata, 2004). Many experimental evidences proved that the rigidification of membrane is an early and primary sensors of LT stress (Nishida and Murata, 1996; Murata and Los, 1997). It undergoes qualitative as well as quantitative modification in its lipid composition under cold stress (Wang et al., 2006). The extent of unsaturation of membrane lipids is the major factor that influences the fluidity of membrane lipids. Many plant species change the extent of
membrane lipid unsaturation in response to alteration in growth temperature (Quinn, 1988). In cyanobacteria *Synechocystis* PCC6803, it has been hypothesized that temperature-mediated alteration of membrane fluidity may itself be the primary temperature sensing event, and it was proposed that the same might be true in higher plants (Murata & Los, 1997). The hypothesis of perception by decrease in membrane fluidity was supported by experiments on palladium-catalyzed hydrogenation of unsaturated lipids (resulting in decreased membrane fluidity) in the surface membrane of *Synechocystis* PCC6803 cells at constant temperature. The experiments showed that hydrogenation resulted in activation of the transcription of a fatty acid desaturase A (*desA*) gene (Vigh *et al.*, 1993), which had been previously reported to be induced by LT (Los *et al.*, 1993). Mutation analysis of gene encoding fatty acid desaturase, in *Synechocystis* exhibited plasma membrane rigidification even at room temperature and increased expression of number of cold inducible genes (Inaba *et al.*, 2003). The rigidification enhanced the response of gene expression to cold, whereas there was no effect on heat-responsive gene expression (Inaba *et al.*, 2003). In alfalfa (*Medicago sativa*) suspension cultures, utilization of a membrane-fluidizer benzyl alcohol (BA) prevented cold-induced gene expression and development of cold acclimation even at 4°C, but utilization of a membrane-rigidifier, dimethylsulfoxide (DMSO) resulted in the induction of cold acclimatization-specific (*cas*) genes even at 25°C (Orvar *et al.*, 2000; Sangwan *et al.*, 2002). Furthermore, it has been shown in *Brassica napus* cell cultures that BA prevented the cold inducibility of *BN115* gene (an orthologue of *Arabidopsis Cor15*), whereas DMSO induced the expression even in the absence of a LT treatment (Sangwan *et al.*, 2001). The role of membrane rigidification in cold perception and signal transduction has been suggested in *Arabidopsis* (Voultier *et al.*, 2006). The experiments showed the activation of diacylglycerol kinase, which is very early event occurring within seconds of cold stress (Voultier *et al.*, 2006).

### 2.6.2.2 Cytoskeleton Reorganization

The process of re-organization of actin cytoskeleton occurs downstream of the changes in membrane fluidity but upstream of the calcium ion (Ca$^{2+}$) influx (Orvar *et al.*, 2000; Sangwan *et al.*, 2001, 2002). Numerous studies demonstrated that rearrangement actin cytoskeleton trigger LT responses in tobacco, alfalfa (*Medicago sativa*) and *Brassica napus* (Mazars *et al.*, 1997; Orvar *et al.*, 2000; Sangwan *et al.*, 2001, 2002). A dramatic increase in the cold-induced Ca$^{2+}$ influx was observed in *Nicotiana plumbaginifolia* by the use of microfilament disrupting drug, cytochalasin D (CD) (Mazars *et al.*, 1997). These findings were further strengthened by Orvar *et al.*, (2000) in alfalfa (*Medicago sativa*), through use of stabilizers (jasplakinolide; JK) and destabilizers (cytochalasin D; CD) of the plant actin.
microfilaments. Stabilization of the microfilaments by JK, prevented the induction of cold acclimation specific gene (cas30), Ca\textsuperscript{2+} influx and lowered freezing tolerance, whereas destabilization by CD induced cas30 transcripts and increased Ca\textsuperscript{2+} influx at 25°C (Orvar et al., 2000). In Brassica napus, BN115 promoter-driven GUS reporter gene was shown to be induced at 25°C by treatment with microfilament destabilizer (latrunculin B) and microtubule destablizer (oryzalin/colchicine), while the expression of transgene was abolished even at 0°C in plants treated with JK and a microtubule stabilizer, taxol (Sangwan et al., 2001). It was also shown that the temporal expression of cytoskeletal reorganization is between membrane rigidification and the influx of Ca\textsuperscript{2+} (Orvar et al., 2000; Sangwan et al., 2001), which is known to be required for the acclimation process (Monroy and Dhindsa, 1995). The rapid and enhanced induction of Wcor719 gene encoding an actin binding protein (ABP) in wheat by LT suggested the regulation of the structure of actin cytoskeleton during LT was mediated by ABPs and further strengthened the role of cytoskeleton in LT response (Danyluk et al., 1996).

These findings suggested the role of actin cytoskeleton reorganization in preserving the structural integrity of the cellular membrane during LT. Also, it has been suggested that cytoskeleton might be a platform for several other physiological functions involved in cold acclimation, such as protein trafficking and modulation of protein kinases/phosphatases activities (Orvat et al., 2000).

2.6.3 LT Sensors

The sensor systems responsible for the induction of the various pathways during LT are still poorly understood (Kacperska, 2004). There are some putative LT-sensors suggested by different research groups, but as yet is no direct experimental proof for either of them (Murata and Los, 1997; Los and Murata, 2004). Lots of potential sensor candidates have been proposed to operate in membranes of cold-stressed cells these include phospholipid metabolism, calcium channels (responsible for Ca\textsuperscript{2+} influx), histidine kinase and/or a two-component histidine kinase (Xiong et al., 2002). Some putative sensors of cold are described here:

2.6.3.1 Ca\textsuperscript{2+} Permeable Channels

LT induces an influx of extracellular Ca\textsuperscript{2+} (apoplastic) into the cytoplasm through calcium permeable channels, which has been considered as LT-sensors (Knight et al., 1991, 1996; Monroy et al., 1993; Monroy and Dhindsa, 1995; Plieth et al., 1999; Sanders et al., 2002). The activation of Ca\textsuperscript{2+} by cold is thought to be the result of physical alteration in cellular structure. Various studies have shown LT-stimulation of a plasmalemma mechanosensitive Ca\textsuperscript{2+} channels in onion epidermal prooplasts which had been pre-activated by membrane stretch. These mechanosensory Ca\textsuperscript{2+} channels probably serve to detect not only...
mechanical stress, but also thermal stimuli (Ding and Pickard, 1993). The temperature dependence of the mechanosensitive channels suggests that these might be temperature sensors for a variety of temperature responses, including cold damage and cold acclimation (Ding and Pickard, 1993).

Recently, transient activation of a non-selective Ca\(^{2+}\)-permeable cation channels in response to rapid cooling was identified in Arabidopsis mesophyll cells using patch-clamp technique (Carpaneto et al., 2007). It has been suggested that the cold-induced membrane rigidification (Monroy and Dhindsa, 1995) might be coupled to the opening of mechanosensitive Ca\(^{2+}\) channels that have been shown to be cold-activated in nature (Ding and Pickard, 1993). Studies utilizing the blocking of Ca\(^{2+}\) influx by Ca\(^{2+}\) channel blockers (La\(^{3+}\), verapamil) and calcium chelator 1,2-bis(o-aminophenoxy) ethane N,N,N',N'-tetraacetic acid (BAPTA) in alfalfa cells resulted in inhibition of cas genes at 4°C, and Ca\(^{2+}\) channel agonist (Bay K8644) or Ca\(^{2+}\) ionophore (A23187) induced cas genes even at 25°C (Monroy et al. 1993; Monroy & Dhindsa 1995) and Arabidopsis (Tahtiharju et al. 1997). Similarly in Brassica napus, Gdadinium (Gd\(^{3+}\)), a mechanosensitive Ca\(^{2+}\) blocker and ruthenium red prevented the cold activation of BN115, but Ca\(^{2+}\) ionophore (A23187) and cyclic ADP-ribose (cADPR) activated it at 25°C (Sangwan et al., 2001).

Several studies have suggested that LT-induced Ca\(^{2+}\) influx occurs downstream of membrane rigidification and cytoskeletal reorganization (Orvar et al. 2000; Sangwan et al. 2001). These studies demonstrated a clear correlation between LT and Ca\(^{2+}\) influx into the cells, suggesting that a LT-modulated Ca\(^{2+}\) channel could indeed be involved in temperature sensing.

### 2.6.3.2 Histidine Kinases

The two-component systems (TCS) has been studied in *E. coli* and *B. subtilis* (Aguilar et al., 2001; Stock et al., 2000). TCS consists of a histidine kinase and a response regulator. Hik33 (HikA membrane-bound histidine kinase), was identified as a cold sensor in *Synechocystis*, and it was postulated that Hik33 might detect decrease in temperature by sensing the rigidification of membrane lipids (Suzuki et al., 2000). Moreover, targeted mutagenesis of the *hik33* gene reduced the expression of a subset of cold-inducible genes upon cold shock but not all of them. These findings suggested that Hik33 is involved in LT-sensing but there might be other putative LT-sensor(s) that has not yet been identified in *Synechocystis* (Suzuki et al., 2000; 2001). The experimental evidence suggested that *Bacillus subtilis* histidine kinases DesK, also act as LT-sensors and regulate desaturase gene expression in response to LT (Aguilar et al., 2001).
In higher plants, a TCS similar to that of prokaryotes might be involved in LT sensing. LT-induced expression of TCS (ATRR1 and ATRR2) was shown to be involved in *Arabidopsis* (Urao *et al.*, 1998). The transcripts of *ATHK1* showed enhanced induction in response to high salinity and LT (Urao *et al.*, 1999). The overexpression of hybrid histidine kinase, *ATHK1*, in yeast *sln1* and *sho1* deletion mutants, enabled the mutants to grow normally under high-salinity conditions, suggesting that the *ATHK1* might act as a putative osmosensor in *Arabidopsis* (Urao *et al.*, 1999). A sensory histidine kinase and a response regulator have been reported to be involved in osmosensing in plants (Urao *et al.*, 1999; Urao *et al.*, 2000). Recently, the expression of a rice response regulator, *OsRR6* gene, was shown to be significantly up-regulated by salt, dehydration and LT treatments (Jain *et al.*, 2006) and microarray analysis revealed that 15 TCS elements were differentially expressed in response to salt, dehydration and LT (Jain *et al.*, 2008).

### 2.6.3.3 Receptor Kinases

Receptor-like kinases (RLKs) are large gene family with ~610 reported members from *Arabidopsis* (Shiu and Bleecker, 2003; Shiu *et al.*, 2004) which act as putative LT-sensors. RLKs have membrane spanning domains, which function in transmitting extracellular signals into intracellular target molecules. In *Arabidopsis*, a gene encoding a receptor-like kinase (*RPKI*) was reported to be induced by ABA treatment, or dehydration, high-salt and LT treatments. These reports suggest a possible role of this class of genes in the perception of LT stress (Hong *et al.*, 1997). RLKs have been reported to play major roles in several aspects of plant signaling processes including growth, development and perception of stress stimuli (Clark *et al.*, 1997; Czernic *et al.*, 1999; Scheer and Ryan, 2002; He *et al.*, 2004). However, the receptors in plants response to LT stress are still poorly understood.

### 2.6.3.4 Phospholipases

Alterations in the membrane phospholipids metabolism are implicated in cold response signaling. *Phospholipase* C and D are accumulated as early as 15 seconds after cold treatment (Ruelland *et al.*, 2002). It increases the production of phosphatidic acid (PtdOH) by hydrolyzing membrane phospholipids, which is proposed as membrane based secondary messenger molecule. Phospholipase D anchors the microtubules to “plasma membrane” so its activation can lead to conformational change in the cytoskeleton (Gardiner *et al.*, 2001; Dhonukshe *et al.*, 2003). It further leads to actin filaments rearrangement, thus probably activating stretch-induced Ca$^{2+}$ channels.
2.6.3.5 Photosynthesis As LT Sensor

In response to LT, photosynthetic tissues of higher plants have to adjust their photosynthetic capacity (Ensminger et al., 2006). Since there is a delicate equilibrium between the energy harvested and utilized, it has been suggested that the redox status of photosynthesis could be an important signalling mechanism especially during LT stress (Huner et al., 1998).

Change of light intensity or temperature causes an imbalance between the light energy absorbed during the light phase of photosynthesis, through photochemistry (temperature-independent) and the energy metabolized through dark reactions (temperature-dependent). Exposure of plants to LT might induce high excitation pressure and create an energy imbalance that affects the relative redox state of photosystem II (PSII) (Huner et al., 1998). Alterations in the redox state of PSII, triggered by a LT shift, has been proposed to be one of several potential temperature sensing mechanisms involved in cold acclimation (Huner et al., 1998; Ensminger et al., 2006). It has been proposed that photosynthesis interacts with other cellular processes during cold acclimation involving cross-talk between photosynthetic redox, cold acclimation and sugar-signalling pathways to regulate plant acclimation to LT (Ensminger et al., 2006). The redox state of the components of the photosynthetic electron transport chain therefore serve as LT-sensors and play an important role in the co-ordination of chloroplast and nuclear gene expression (Gray et al., 1997; Fey et al., 2005).

2.6.4 Functional Proteins Involved in Cold Stress

2.6.4.1 Proteins Related to Metabolism

Metabolism is broadly up-regulated in response to LT. Various genes included housekeeping genes such as elongation factor-1α (EF-1α) and β-tubulin, which were up-regulated in maize, Arabidopsis, barley during LT (Berberich et al., 1995; Chu et al., 1993; Dunn et al., 1993). The up-regulation of EF-1α was consistent with the up-regulation of protein synthetic capacity. This was further supported by results from Solanum sogarandinum, which showed up-regulation of genes involved in primary metabolism during LT (Rorat et al., 1997). Several other genes encoding proteins for galactinol synthase, pyrroline-5-carboxylate synthase, alcohol dehydrogenase have been reported from several plants species during LT (Liu et al., 1997; Reimholz et al., 1997; Jarillo et al., 1993). Also, many heat shock proteins were found to be expressed constitutively as well as under stress conditions (Vierling, 1991). For example, HSP70s are essential for protein folding and assembly (Parswell and Lindquist, 1993). The up-regulation of hs704 mRNA and of related sequences was due to higher demand in the cell for folding and assembly of proteins in spinach during cold acclimation (Anderson et al., 1994). This suggested that heat shock proteins have protective role during LT.
2.6.4.2 Boiling Stable Proteins

Late embryogenesis abundant (LEA) proteins are highly hydrophilic and boiling stable (Pearce, 1999). They have been found to be accumulated in vegetative tissues during dehydrative stresses (Bray, 1993). LEA proteins have repeated amino acid sequence motifs separated by less conserved sequences. Their tolerance to denaturing conditions suggested their role in stabilising protein structures in a low water environment (Pearce, 1999). A group 3 LEA, HVA1 has been isolated from barley, which showed up-regulation during LT (Sutton et al., 1993). Also, the expression of HVA1 in rice increased its tolerance of salt and drought stress (Xu et al., 1996). A number of other cold-responsive proteins such as KIN1, COR15, COR24, COR47, LTI67, LTI78 and COR 160 have been reported from Arabidopsis, which are also highly hydrophilic and boiling stable (Kurkela and Franck, 1992; Lin et al., 1990; Nordin et al., 1993). This suggested that these proteins have protective role during LT.

2.6.4.2.1 Dehydrins (Group 2 LEAs)

Dehydrin mRNAs and proteins are most commonly reported cold up-regulated molecular entities (Pearce, 1999). Dehydrins have a lysine-rich motif called K segment (EKKGIMDKIKEKLPG) present in their sequences. Many dehydrins also contained a series of serine residues, known as S segment while other contained one or more Y segment near the amino terminus [(V/T)DEYGNP]. There are some glycine-rich sequences present within amino-terminal region and between K segments. The number of repeats of the Y and K segments and the presence or absence of other sequence features varies widely between different dehydrins (Pearce, 1999).

Three dehydrins namely Wes120, Cor39 and Wcor410 have been reported from wheat responsive to LT (Danyluk et al., 1994; Guo et al., 1992; Houde et al., 1992). Thus it has been suggested that dehydrins, other LEAs or other potentially protective proteins might protect membranes during early step of destabilisation caused by LT.

2.6.4.3 Extracellular Proteins

A notable feature of cold acclimation is the involvement of extracellular proteins. The movement of extracts from the leaf apoplast in plants able them to acclimate to frost and showed the accumulation of specific polypeptides during cold acclimation (Antikainen et al., 1997; Hon et al., 1994).

2.6.4.3.1 Antifreeze Proteins (AFPs)

Several proteins extracted from the leaf apoplast of rye and other cereals have antifreeze activity in vitro (Antikainen et al., 1997; Hon et al., 1994a). These proteins have strong sequence similarity to three types of pathogenesis-related (PR) proteins namely, class I endochitinases, β-1,3-endoglucanases and thaumatin (Hon et al., 1994b). These proteins have
been reported from *Solanum dulcamara* and other plants with antifreeze activity (Duman, 1994; Duann and Olsen, 1993).

### 2.6.4.3.2 Non-specific Lipid Transfer Proteins

Non-specific lipid transfer proteins (nsLTPs) are extracellular proteins, which are synthesized by plants in cuticle (Thoma *et al.*, 1994; Thoma *et al.*, 1993) and wax layer (Pyee *et al.*, 1994). The nsLTP mRNAs have been reported from barley during cold stress (Pearce *et al.*, 1998). This suggested that nsLTP might function as protectant during cold stress but their physiological function remains to be elucidated.

### 2.6.5 Signal Transducers during LT

A number of components have been proposed to be involved in LT signaling, probably due to the fact that LT simultaneously activates different pathways in the cell. Signal transduction normally acts through second messengers, which are either formed or released from intra- or extracellular stores \([\text{Ca}^{2+}], \text{inositol triphosphates (IP}_3\text{)}\) and reactive oxygen species (ROS). Second messengers can modulate intracellular \([\text{Ca}^{2+}]\) levels and the activity of many enzymes. These alterations often initiate a protein phosphorelay or trigger other molecular events that target proteins directly involved in cellular protection or transcription factors controlling specific sets of stress-regulated genes usually by modification of gene expression (Xiong *et al.*, 2002; Chinnusamy *et al.*, 2004).

Cold signal transduction is a complex process in which parallel and branched signalling pathways converge and cross-talk leading to the development of freezing tolerance. The signal transduction network is characterized by multiple points of convergence and divergence that enable signal integration at different levels, and provide the molecular basis for the appropriate downstream responses (Xiong and Zhu, 2001; Xiong *et al.*, 2002; Chinnusamy *et al.*, 2004). Some of the components of signal transduction are described in the following sections.

#### 2.6.5.1 Calcium Dependent Protein Kinases (CDPKs)

LT causes a rapid and transient increase in \([\text{Ca}^{2+}]_{cyt}\) and changes in the phosphorylation status of specific proteins (Monroy *et al.*, 1993; Monroy and Dhindsa, 1995). The major \([\text{Ca}^{2+}]\) sensors in plants are calmodulin (CaM), CaM domain-containing protein kinases (CDPKs), calcineurin B-like proteins (CBLs) and CBL-interacting protein kinases (CIPKs). CDPKs are a family of serine-threonine kinases primarily found in the plant kingdom and likely to function as sensor molecules in pathways implicated as important sensors in calcium-mediated signaling in response to abiotic stresses, including cold (Harmon *et al.*, 2000; Cheng *et al.*, 2002; Ludwig *et al.*, 2004). LT causes a rapid and transient increase in \([\text{Ca}^{2+}]_{cyt}\) and changes in the phosphorylation status of specific proteins (Monroy *et al.*, 1993; Monroy and Dhindsa, 1995).
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1993; Monroy and Dhindsa, 1995). Inhibition of either calcium influx or protein kinase activity (by staurosporine) prevented the cold-induced protein phosphorylation, cas gene expression and the development of freezing tolerance (Monroy et al., 1993; Monroy and Dhindsa, 1995). W7 an antagonist of CDPK and calmodulin (CaM), inhibited the capacity of cell culture to cold acclimation (Monroy et al., 1993). Moreover, the members of a CDPK gene family were shown to be differentially regulated during the early stages of cold acclimation (Monroy and Dhindsa, 1995). Transient transactivation assays of stress-responsive reporter gene constructs in maize (Zea mays) protoplasts transformed with genes encoding both wild-type and a mutated form of CDPKs provided the first evidence of the involvement of a particular CDPK in specific signal/response pathways (Sheen, 1996).

W7 an antagonist of CDPK inhibited kin gene expression and cold acclimation (Tahtiharju et al., 1997). In Arabidopsis, CDPK transcripts have been reported to be elevated after exposure to cold, salt and drought (Urao et al., 1994; Tahtiharju et al., 1997), and overexpression of rice OsCDPK7 yielded cold and salt/drought-tolerant rice plants (Saijo et al., 2000). In rice, a constitutively expressed membrane-bound CDPK showed significantly increased auto-phosphorylation and kinase activity in response to LT suggesting a post-translational regulation of CDPK by LT (Martin and Busconi, 2001).

Recently, CDPK and MAPK pathways were shown to function in a concerted manner to control the response specificity to biotic and abiotic stresses (Ludwig et al., 2004, 2005). The involvement of protein kinase C dependent phosphorylation in cold stress in Brassica juncea was shown by Deswal et al., (2004). Yang et al., (2003) and Abbasi et al., (2004) have reported that OsCDPK13 might be an important signaling component in response of rice to gibberellins and cold stress. Komatsu et al., (2007) demonstrated that over-expression of OsCDPK13 confers cold tolerance in rice. Recently, PaCDPK1 gene from an orchid, Phalaenopsis amabilis, was shown to be transcriptionally activated in response to LT (Tsai et al., 2007). The studies indicated the involvement of calcium signaling in cold acclimation of plants and that CDPKs might couple the Ca$^{2+}$ response to downstream events, leading to metabolic adaptation and development of freezing tolerance.

2.6.5.2 Phosphatases

Protein phosphatases (PP2A, 2B) act as Ca$^{2+}$ sensors and have their role in LT-signaling. The early steps of cold acclimation include a rapid phosphorylation and dephosphorylation of pre-existing proteins (Monroy et al., 1993). In alfalfa, the role of protein phosphorylation in the LT regulation of cas15 revealed that an inhibition of protein phosphatase 2A (PP2A) was necessary for the induction of cas15. Moreover, inhibition of PP2A by okadaic acid (specific protein phosphatase inhibitor) at 25°C resulted in
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the accumulation of cas15 transcripts (Monroy et al., 1998). The Arabidopsis protein phosphatase 2C, AtPP2CA rapidly induced by LT reached to a maximum level by 12 h and remaining high thereafter (Tähtiharju and Palva, 2001). Transgenic plants of Arabidopsis expressing AtPP2CA in antisense orientation showed that regulation of cold-responsive genes (RAB18, RC12A/LTI6, RD29A/LTI78) was cold stress-dependent similar to the wild type, but these were super-induced during cold stress in AtPP2CA antisense plants and conferred better freezing tolerance. Cold-responsive gene expression and cold acclimation were also accelerated in AtPP2CA antisense plants (Tähtiharju and Palva, 2001). Thus, by regulation of the phosphorylation and dephosphorylation equilibrium, the LT signal transduction cascade leads to LT-responsive gene expression and development of freezing tolerance (Monroy et al., 1998).

2.6.5.3 Mitogen Activated Protein Kinase Cascade

Mitogen activated protein kinases (MAPKs) are ubiquitous molecules present in diverse organisms including yeast, plants and humans (Widmann et al., 1999). MAPKs are a family of serine/threonine protein kinases associated with perception and transduction of extracellular, developmental and physiological stimuli (Hirt, 1997). MAPKs are induced by numerous biotic and abiotic stresses such as LT (Jonak et al., 1996; Mizoguchi et al., 1996), heat shock, osmotic shock (Jonak et al., 1996; Droillard et al., 2002), high salinity (Mizoguchi et al., 1996), wounding (Seo et al., 1995; Usami et al., 1995; Stratmann and Ryan, 1997; Bögge et al., 1997), senescence (Berberich et al., 1999) and UV irradiation (Stratmann et al., 2000). MAPKs are involved in various physiological, developmental and hormonal responses. Various defense-related signaling molecules such as salicylic acid (SA) (Zhang and Klessig, 1998), hydrogen peroxide (H2O2) (Desikan et al., 1999), jasmonic acid (JA) and its methyl ester methyl jasmonate (MeJA) (Seo et al., 1999; Kumar and Klessig, 2000) are reported to activate MAPKs. The response is mediated through a phosphorylation cascade resulting in the induction of specific set of genes. The MAPK perform their function as part of protein kinase module and involves a minimal three-component system involving MAPK kinase kinase (MAPKKKs) at the top hierarchal level, MAPK kinase (MAPKKs) in the middle and MAPKs as the last component. MAPKs are activated by dual phosphorylation on their threonine and tyrosine residues located in the activation loop by MAPKKs which in turn are activated by MAPKKKs through phosphorylation of conserved serine and/or threonine residues in their T-loop (Ichimura et al., 2002).

The changes in calcium levels result in alterations in protein phosphorylation within minutes after cold treatment (Monroy et al., 1993) suggesting that calcium acts as a secondary messenger in the signal transduction pathway, together with a MAP kinase cascade. Earlier
evidence of involvement of MAPKs comes from studies of Mizoguchi et al., (1995) in Arabidopsis. An up-regulation of transcripts of AtMEKK1 (MAPKK), AtMPK3 and S6 ribosomal kinase (AtPK19) were demonstrated in response to cold, touch and salinity. It has also been reported that AtPK6 and AtPK19 are up-regulated in response to LT (Mizoguchi et al., 1995). In alfalfa, a closely related homologue, MMK4, was transiently activated by cold and drought stress (Jonak et al., 1996). Significantly, two other alfalfa MAPKs, MMK2 and MMK3, were not activated by LT, suggesting specificity in LT activation of MAPKs (Jonak et al., 1996). Several protein kinases including the Arabidopsis ATMPK4 and ATMPK6 showed rapid and transient activation in response to LT (Ichimura et al., 2000). In alfalfa, cytoskeleton destabilizers (latrunculin B and oryzalin) caused LT- activation of stress-activated protein kinase (SAMK) at 25°C but activation was blocked by stabilizer JK (Sangwan et al., 2002). The activation of SAMK by temperature, chemically modulated membrane fluidity or cytoskeleton destabilizers was inhibited both by blocking extracellular Ca²⁺ influx and using an antagonist (W7) of CDPKs. The results suggested that LT was sensed at the level of plasma membrane and cytoskeleton re-organization and the transduction was effected by Ca²⁺ fluxes and CDPKs into activation of distinct MAPK cascades (Sangwan et al., 2002). Constitutively expressing or over expressing MKK2 in Arabidopsis resulted in elevated MAPK kinase activity and improved freezing and salt tolerance (Teige et al., 2004).

LT-induced enhanced accumulation of MAPK transcripts have been reported in several plant systems for example, OsMAPK4 in rice (Fu et al., 2002), BnMPK3 in Brassica napus (Yu et al., 2005), CbMAPK3 in Chorispora bungeana (Zhang et al., 2006a) and GhMAPK in cotton (Wang et al., 2007a). These studies provide evidence of the potential involvement of MAPK cascades in LT-mediated signal transduction pathway.

### 2.6.6 Sensors of Ca²⁺ Signatures

Although, ‘Ca²⁺ signatures’ are suggested for the stimuli-specific cellular responses, the molecules that “sense” and “interpret” the Ca²⁺ signals (Fig. 2.1) provide additional specificity to the Ca²⁺ signal transduction pathway (Sanders et al., 2002). As a second messenger, Ca²⁺ transmits the primary signal into cellular responses (such as gene expression) most likely through Ca²⁺-regulated proteins that include Ca²⁺ sensors and their targets (Sanders et al., 2002). Different calcium sensors recognize specific calcium signatures and transduce them into downstream effects, including altered protein phosphorylation and gene expression patterns (Sanders et al., 1999, 2002). The major Ca²⁺ sensors in plants which are immediate downstream components after Ca²⁺ changes, include calmodulin (CaM) (Zielinski, 1998; Snedden and Fromm, 2001; Luan et al., 2002), calcium-dependent protein kinases (CDPKs; Harmon et al., 2000; Sanders et al., 2002), calcineurin B–like proteins (CBLs; Luan
et al., 2002) and Ca$^{2+}$-regulated phosphatases. Further, the sensors can be divided into two types, sensor relays and sensor responders. Sensor relays, such as calmodulin, undergo a Ca$^{2+}$-induced conformational change that is relayed to an interacting partner. The interacting partner then responds with some change in its enzyme activity or structure. This type of sensor includes calmodulin and calcineurin B-like proteins (Luan et al., 2002). On the contrary, sensor responders, such as CDPKs, undergo a Ca$^{2+}$-induced conformational change that alters the protein’s own activity or structure.

**Figure 2.1:** Schematic representation of LT-response in a plant cell (adapted from Winfield et al., 2010).

These two different modes of decoding Ca$^{2+}$ signals have been reported extensively in plants and result in multiplicity of Ca$^{2+}$-induced signal transduction pathways to diverse stimuli (Sanders et al., 2002).

### 2.6.6.1 Role of Calcium

Immediate and transient increase in cytosolic calcium ([Ca$^{2+}$]$_{cyt}$) from extracellular or intracellular pools (Fig 2.1) and its involvement in subsequent LT signalling has been well documented (Knight et al., 1991, 1996; Monroy et al., 1993; Monroy and Dhindsa, 1995; Plieth et al., 1999; Orvar et al., 2000; Sangwan et al., 2001). In alfalfa and *B. napus*, Ca$^{2+}$ channel blockers and calcium chelators prevented the expression of *cas* genes at 4°C and...
development of freezing tolerance. \textit{Ca}^{2+} channel agonist or ionophore activated \textit{cas} genes at 25°C, and resulted in development of freezing tolerance (Monroy \textit{et al.}, 1993; Monroy \& Dhindsa 1995; Sangwan \textit{et al.}, 2001). Similar studies in Arabidopsis utilizing \textit{Ca}^{2+} channel blockers and \textit{Ca}^{2+} chelators inhibited the LT activation of (cold-induced) \textit{kin} and \textit{LTI} genes (Knight \textit{et al.}, 1996; Tähtiharju \textit{et al.}, 1997; Hendriksson and Trewavas, 2003). These studies suggested that modulation in [\textit{Ca}^{2+}]_{\text{cyt}} is a component of LT signal transduction. It has also been proposed that calcium functions as a second messenger in response to chilling (Minorsky, 1989; Knight \textit{et al.}, 1991) and cold acclimation (Ding and Pickard, 1993; Monroy \textit{et al.}, 1993).

\subsection{Specificity of Signal ‘Calcium Signature’}

Calcium is a ubiquitous second messenger in eukaryotic signal transduction cascades (Knight \textit{et al.}, 1996; Knight and Knight, 2001; Sanders \textit{et al.}, 2002). In plants, various environmental signals including hormones, light, pathogen elicitors, mechanical and abiotic stress are known to modulate [\textit{Ca}^{2+}]_{\text{cyt}} levels (McAinsh \textit{et al.}, 1997; Sanders \textit{et al.}, 1999; Rudd and Franklin-Tong, 2001; Sanders \textit{et al.}, 2002). In addition, many intrinsic growth and developmental processes, such as elongation of root hairs and pollen tube formation, are regulated by [\textit{Ca}^{2+}]_{\text{cyt}} modulation (Felle and Hepler, 1997; Holdaway-Clarke \textit{et al.}, 1997; Wymer \textit{et al.}, 1997). Because different stress and/or developmental signals often elicit distinct and specific cellular responses, it is important to ask, how cells distinguish the \textit{Ca}^{2+} signals produced by different stimuli?

Unlike most other ions, calcium does not freely diffuse within cells and is stored in multiple organelles, including the apoplast, vacuole, nuclear envelope, endoplasmic reticulum (ER), chloroplasts, and mitochondria (Sanders \textit{et al.}, 2002). Recent studies suggested that each stimuli can elicit [\textit{Ca}^{2+}]_{\text{cyt}} signals that differ in their kinetics, magnitude, frequency and localization of the influx i.e. both spatial and temporal (Malho’ \textit{et al.}, 1998; McAinsh and Hetherington, 1998; Allen \textit{et al.}, 2000, 2001; Rudd and Franklin-Tong, 2001; Plieth, 2005). A combination of changes in all [\textit{Ca}^{2+}]_{\text{cyt}} parameters produced by a particular signal is referred to as a “\textit{Ca}^{2+} signature”.

“\textit{Ca}^{2+} signature” implies that the information necessary to produce a stimuli-specific response is encoded in its temporal (amplitude, duration, frequency) and spatial (subcellular-location, dimension, spread) behaviour. Thus, each “\textit{Ca}^{2+} signature” is proposed to regulate different sub-sets of \textit{Ca}^{2+}-dependent downstream components and hence, specifically induce a specific physiological response (Sanders \textit{et al.}, 2002; Plieth, 2005).

“\textit{Ca}^{2+} signatures” measured in cold-acclimated and non-acclimated plants show a bimodal (two peaks) waveform that is altered in acclimated plants, which show an enhanced
second peak as compared to non-acclimated plants (Knight and Knight, 2000). This alteration was attributed to the cold-induced vacuolar Ca\(^{2+}\) release without any effect on extracellular Ca\(^{2+}\) influx and hence providing more evidence of the involvement of both extracellular and intracellular pools in LT acclimation (Knight et al., 1996; Sangwan et al., 2001).

### 2.6.6.3 Calmodulin (CaM)

CaM, a highly conserved and ubiquitous Ca\(^{2+}\)-binding protein in eukaryotes, is known to be a primary sensor of \([\text{Ca}^{2+}]_{\text{cyt}}\) and undergoes Ca\(^{2+}\)-induced conformational change and regulates activity of target proteins either positively or negatively (Zielinski, 1998; Snedden and Fromm, 2001; Yang and Poovaiah, 2003). These include a wide variety of protein molecules such as enzymes, transcription factors, cytoskeletal proteins, and membrane-bound transporters (Luan et al., 2002; Yang and Poovaiah, 2003). In Arabidopsis and tobacco cells, LT enhanced the transcription of genes encoding CaM and CaM-like proteins (Braam and Davis, 1990; van der Luit et al., 1999). This LT-responsive expression of CaM genes is partially regulated by Ca\(^{2+}\) (Polisensky and Braam, 1996). Studies with alfalfa cells (Monroy et al., 1993) and Arabidopsis (Tāhtiharju et al., 1997) indicated that CaM antagonist prevented cold acclimation and reduced the expression of cold-regulated genes, supporting a role of CaM in LT signalling. In contrast, overexpression of CaM3 in Arabidopsis has been shown to cause reduced level of cold-responsive gene expression (Townley and Knight, 2002), implying that CaM might have a role as a negative regulator during cold acclimation. These findings suggested that CaM can act as both positive and negative regulator of Ca\(^{2+}\)-mediated LT signal transduction through different downstream target components.

### 2.7 Transcriptional Regulation during LT

In 1970, Weiser proposed that changes in gene expression might occur during cold acclimation, and that these gene expression changes might be a key factor in cold acclimation. Transcriptional regulation of the response to LT has been studied extensively. Microarray analysis has been used to examine LT-induced gene expression in Arabidopsis (Fowler and Thomashow 2002; Kreps et al., 2002; Lee et al., 2005; Hannah et al., 2005; Seki et al., 2001). Response to LT is accompanied by modulation of gene expression resulting in up or down-regulation of several genes (Seki et al., 2001, 2002a; Fowler and Thomashow, 2002). Many of the LT-induced genes are also up-regulated by various other abiotic stresses including drought, salt and ABA, suggesting that a common set of signal transduction pathways might be triggered during different stress responses (Thomashow, 1999; Seki et al., 2001, 2002a, 2002b; Kreps et al., 2002; Shinozaki et al., 2003). Recently, microarray studies have revealed that the expression of over 500 genes in Arabidopsis is altered in response to LT (Vogel et al., 2005). Although a great majority of the LT-inducible genes are regulated by transcription
factors CBF and ZAT12, the basis for the regulation of the remaining 70% of the LT-induced genes and 95% LT-repressed genes is unknown (Vogel et al., 2005). Elucidation of the mechanism regulating these LT-responsive genes is yet to be understood.

LT response appears to be complex as revealed by genetic studies using a luciferase gene driven by the COR78/RD29A promoter (Ishitani et al., 1997). A large number of mutants were isolated that were defective in the induction of this fusion gene in response to cold, drought, salinity and ABA treatment. These mutants were classified into three major classes based upon the response of osmotically regulated genes: hos have high expression, los display low expression and cos show constitutive activity of these genes (Ishitani et al., 1997). Molecular cloning of several genes defined by these mutations revealed that cold acclimation in Arabidopsis involves a wide range of key signal and metabolic components (Chinnusamy et al., 2003; Gong et al., 2002; Guo et al., 2002; Lee et al., 2001, 2002a, b; Xiong et al., 2001a, b; Zhu et al., 2004). Taken together, these results demonstrate the complex and interactive relationships among different pathways regulated by LT.

Earlier reports of alteration in gene expression during cold acclimation were reported in spinach (Guy et al., 1985). Since then, various differential screening and cloning studies have led to the identification of cold-regulated (COR) genes in various plant species (Mahopatra et al., 1989; Hajela et al., 1990; Kurkela and Franck, 1992; Cattivelli and Bartels, 1990; Gilmour et al., 1992; Houde et al., 1992; Nordin et al., 1993).

2.7.1 Cold-regulated Genes (COR)

The COR genes have also been designated as LTI (low-temperature induced), KIN (cold-inducible), RD (responsive to dehydration), and ERD (early responsive to dehydration) (Thomashow, 1998). The COR genes encode highly hydrophilic polypeptides that remain soluble in boiling dilute aqueous buffer and have relatively simple amino acid composition (Thomashow, 1998). Constitutive expression of COR15a in chloroplasts of Arabidopsis resulted in enhanced freezing tolerance of protoplasts and chloroplasts (Artus et al., 1996). Gene fusion studies with COR15a (Baker et al., 1994), LTI78/RD29a (Horvath et al., 1993) and COR6.6/KIN2 (Wang and Cutler, 1995) demonstrated that the promoters of these cold-regulated (COR) genes were activated in response to LT and drought. Deletion analysis of the promoters of these genes, including the COR15a (Baker et al., 1994) and LTI78/RD29a genes (Yamaguchi-Shinozaki and Shinozaki, 1994) revealed the presence of multiple copies of a functional cis-acting element, a 5-bp CCGAC core motif known as a CRT/C-repeat/DRE (dehydration responsive element) sequence and was suggested to impart LT and drought responsiveness to these genes (Yamaguchi-Shinozaki and Shinozaki, 1994). The CRT/DRE
cis-acting element has also been identified in the promoter of the *B. napus* gene BN115, where it was named Low Temperature Responsive Element (LTRE; Jiang *et al.*, 1996).

Moreover, some of the COR genes contain ABA-Responsive Elements (ABREs), that mediate the ABA responsiveness of these genes (Seki *et al.*, 2002b). The expression of COR genes is regulated by both ABA-independent and ABA-dependent pathways (Ishitani *et al.*, 1997; Shinozaki and Yamaguchi-Shinozaki, 2000). A transcription factor that binds to the CRT element was identified using yeast one-hybrid system and was named as CRT binding factor 1 (CBF1) (Stockinger *et al.*, 1997).

### 2.7.2 CBF-Dependent Pathway during LT

#### 2.7.2.1 CBF Proteins

Understanding the regulation of COR genes involved identification of cold inducible CBFs in *Arabidopsis* (Liu *et al.*, 1998; Stockinger *et al.*, 1997). CBF proteins belong to a
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A small family of transcription factors that include CBF1, CBF2 and CBF3 (Gilmour et al., 1998; Medina et al., 1999) also known as DREB1b, DREB1c and DREB1a, respectively (Liu et al., 1998). CBF proteins belong to a small family of plant specific transcription factors (AP2/ERF subfamily) and have distinguishing features in their structure that set them apart from most of the other AP2/ERF proteins (Gilmour et al., 1998; Liu et al., 1998; Jaglo et al., 2001). Analysis of the deduced amino acid sequences indicated that these proteins have molecular masses of about 24 kDa, a potential nuclear localization sequence, and a possible acidic activation domain (Stockinger et al., 1997; Gilmour et al., 1998; Liu et al., 1998). The CBF proteins contain a highly conserved 60 amino acids conserved DNA-binding domain (ERF/AP2 domain). Another protein with an AP2/ERF domain, CBF4, was isolated by Haake et al., (2002). CBF4 was 63% identical to the three CBF1–3 proteins and was induced by drought and ABA but not by cold (Haake et al., 2002).

The CBF genes are induced within 15 min of plants being exposed to low non-freezing temperatures and in turn induce the expression of cold-regulated genes that contain the CRT/DRE-regulatory element also referred as the “CBF regulon” i.e. regulated by CBF (Gilmour et al., 1998; Liu et al., 1998; Medina et al., 1999). The CBF1-3 genes have been shown to be not responsive to ABA or dehydration (Medina et al., 1999). It has been suggested that the CBF1, 2 and 3 have redundant functional activities (Gilmour et al., 2004). Similar to CBF1, both CBF2 and CBF3 were shown to activate the expression of reporter genes in yeast that contained the CRT/DRE as an upstream activator sequence (Gilmour et al., 1998). These transcription factors activate the expression of COR genes and are considered as “Master Switches” that trigger a signal transduction cascade leading to enhanced freezing tolerance (Thomashow, 2001).

2.7.2 CBF Cold-response Pathway in Other Plants

CBF-dependent gene expression is an important, evolutionary conserved component of cold acclimation in diverse plant species (Nakashima and Yamaguchi-Shinozaki, 2006). The homologous components of the Arabidopsis CBF cold-response pathway have been found in many plants, including Brassica napus (Jaglo et al., 2001; Gao et al., 2002), Glycine max (soybean; Li et al., 2005), tomato (Zhang et al., 2004a), Prunus avium (cherry; Kitashiba et al., 2004), Triticum aestivum (wheat; Shen et al., 2003; Kume et al. 2005; Badawi et al., 2007), Secale cereale (rye; Jaglo et al., 2001), Zea mays (maize ; Qin et al., 2004) , Oryza sativa (rice; Dubouzet et al., 2003), Betula pendula (birch; Welling and Palva, 2008), Hordeum vulgare (barley; Choi et al., 2002; Skinner et al., 2005), Brassica pekinensis (Chinese cabbage; Zhang et al., 2006b), Eucalyptus gunnii (Eucalyptus; El Kayal et al., 2006), Vitis spp. (grape; Xiao et al., 2006) and Populus spp. (poplar; Benedict et al., 2006). A
comparative analysis of these gene sequences indicated that these sequences might be conserved across different species in the plant kingdom (Jaglo et al., 2001).

On the other hand, homologous CBFs isolated from different crop species differ in activation in response to environmental stresses. For example, in tomato (unable to cold acclimate) functional CBF homologues exist, but a functional CBF regulon is lacking and very few genes are induced by LT (Zhang et al., 2004a). Only one of its three homologous genes, LeCBF1, LeCBF2, and LeCBF3, was activated in response to LT (Zhang et al., 2004a). Thus, the components of CBF regulon are conserved among plant species including those that cannot cold acclimated, but differ in regulation of gene expression in response to environmental stimuli.

2.7.2.3 Over-expression of CBF

 Constitutive over-expression of CBF1 or CBF3 resulted in enhanced freezing tolerance, even without LT stimulus, in Arabidopsis (Jaglo-Ottosen et al., 1998; Kasuga et al., 1999; Liu et al., 1998). Furthermore, transgenic over-expression of Arabidopsis CBFs was sufficient to induce cold tolerance in diverse plant species (Table 2.1). Further, CBF homologs have been identified from several chilling-tolerant and chilling-sensitive plant species and transgenic analysis confirmed their pivotal role in cold acclimation (Table 2.1). This suggested that CBF transcription network plays an essential role in cold acclimation in diverse plant species. The ectopic over-expression of CBF genes from Arabidopsis not only enhanced the freezing tolerance but also resulted in constitutive expression of downstream COR genes and other CRT/DRE containing target genes (Jaglo-Ottosen et al., 1998; Jaglo et al., 2001; Liu et al., 1998; Kasuga et al., 1999; Gilmour et al., 2000). Multiple biochemical changes that are associated with cold acclimation and thought to contribute to increased freezing tolerance (e.g. accumulation of sugars and proline) occurred in non-acclimated transgenic Arabidopsis plants that constitutively expressed CBF3 (Gilmour et al., 2000). It has also been proposed that the CBF genes act to integrate the activation of multiple components of the cold acclimation response and is an important regulator in the cold acclimation response, which in turn promotes freezing tolerance (Gilmour et al., 2000). A dominant ice1 mutation, resulted in almost complete inhibition of CBF3 transcript accumulation in response to LT and decreases the expression of many CBF-targeted genes. ICE1 encodes a MYC-like bHLH protein that binds to a canonical MYC cis-element in the CBF3 promoter of Arabidopsis and has been proposed to play a fundamental role in the regulation of CBF3 (Chinnusamy et al., 2003).
Table 2.1: Abiotic stress tolerance of transgenic plants overexpressing CBFs (adapted from Chinnusamy et al., 2010)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Transgenic plants</th>
<th>Stress tolerance of transgenic plants</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>AtCBF1/2/3</td>
<td>Brassica napus</td>
<td>Constitutive over-expression enhanced both basal and acquired freezing tolerance</td>
<td>Jaglo et al., 2001</td>
</tr>
<tr>
<td>AtCBF1</td>
<td>Tomato</td>
<td>Constitutive ove-expression enhanced oxidative stress tolerance under chilling stress; enhanced tolerance to water-deficit stress</td>
<td>Hsieh et al., 2002a, b</td>
</tr>
<tr>
<td>AtDREB1A/CBF3</td>
<td>Tobacco</td>
<td>Transgenic plants expressing RD29A::DREB1A exhibited enhanced chilling and drought tolerance</td>
<td>Kasuga et al., 2004</td>
</tr>
<tr>
<td>AtDREB1A/CBF3</td>
<td>Wheat</td>
<td>Transgenic plants expressing RD29A promoter::AtDREB1A gene showed delayed water stress symptoms</td>
<td>Pellegrineschi et al., 2004</td>
</tr>
<tr>
<td>AtCBF3</td>
<td>Rice</td>
<td>Constitutive over-expression resulted in enhanced tolerance to drought and high salinity and a marginal increase in chilling tolerance</td>
<td>Oh et al., 2005</td>
</tr>
<tr>
<td>AtDREB1A/CBF3</td>
<td>Maize</td>
<td>RD29A::CBF3 transgenic plants are more tolerant to cold, drought, and salinity</td>
<td>Al-Abed et al., 2007</td>
</tr>
<tr>
<td>AtCBF1</td>
<td>Potato</td>
<td>Constitutive or stress-inducible expression of CBF1 or CBF3 but not CBF2 conferred improved freezing tolerance to frost-sensitive Solanum tuberosum</td>
<td>Pino et al., 2007</td>
</tr>
<tr>
<td>OsDREB1</td>
<td>Arabidopsis</td>
<td>Over-expression in Arabidopsis induced target COR genes and conferred enhanced tolerance to freezing and drought stresses</td>
<td>Dubouzet et al., 2003</td>
</tr>
<tr>
<td>OsDREB1A/B</td>
<td>Rice</td>
<td>Constitutive expression conferred improved tolerance to cold, drought, and salinity</td>
<td>Ito et al., 2006</td>
</tr>
<tr>
<td>ZmDREB1</td>
<td>Arabidopsis</td>
<td>Over-expression in Arabidopsis induced COR genes and conferred tolerance to freezing and drought</td>
<td>Qin et al., 2004</td>
</tr>
<tr>
<td>BnCBF5 and BnCBF17</td>
<td>Brassica napus</td>
<td>Over-expression led to increased constitutive freezing tolerance, increased photochemical efficiency and photosynthetic capacity</td>
<td>Savitch et al., 2005</td>
</tr>
</tbody>
</table>

The icel mutant showed impaired chilling tolerance and cold acclimation, while constitutive expression of ICE1 enhanced the expression of CBFs and COR genes and freezing tolerance of transgenic Arabidopsis. This suggested that cold-induced modification of ICE1 is necessary for activation of its targeted genes (Chinnusamy et al., 2003). The Ice1 mutation also affected the cold-induction of CBF1 and CBF2. However, the ice1 mutation has little effect on CBF2 transcript accumulation under LT indicating that different CBF gene family members are regulated differently. ICE1 is constitutively expressed and over-expression of ICE1 in wild plants increased the LT expression of CBF regulon and improved freezing tolerance. However, this over-expression did not result in the activation of the CBF regulon at ambient temperature (Chinnusamy et al., 2003). This suggested that LT-induced post-
translational modification was necessary for ICE1 to activate downstream genes in plants. Over-expression of \textit{ICE2} (Atgl12860, a homolog of \textit{ICE1}) induced the expression of \textit{CBF1} and conferred freezing tolerance in \textit{Arabidopsis} after cold acclimation (Fursova \textit{et al.}, 2009). In wheat, the \textit{ICE1} homologs \textit{TaICE141} and \textit{TaICE187} were constitutively expressed and activated the wheat CBF group 4 and conferred freezing tolerance. Over-expression of \textit{TaICE141} and \textit{TaICE187} in \textit{Arabidopsis} enhanced \textit{CBF} and \textit{COR} gene expression and enhanced freezing tolerance only after cold acclimation. This suggested that similar to \textit{Arabidopsis} \textit{ICE1}, wheat \textit{ICE1} also needs to be activated by cold acclimation (Badawi \textit{et al.}, 2008). The ability of \textit{ICE1} to activate gene transcription in response to LT was proposed to be dependent on the phosphorylation and dephosphorylation of \textit{ICE1} (Chinnusamy \textit{et al.}, 2003; Chinnusamy \textit{et al.}, 2007; Chinnusamy \textit{et al.}, 2010).

\subsection*{2.7.2.4 Self-regulation}
Initially it was proposed that \textit{CBF} genes might not display self-regulation due to the absence of DRE/CRT elements in their promoters (Thomashow \textit{et al.}, 2001). However, inactivation of \textit{CBF2} gene resulted in constitutive expression of \textit{CBF1} and \textit{CBF3}. Also, \textit{LTI78}, \textit{KIN1} and \textit{COR47} and the \textit{chf2} null mutants exhibited more freezing tolerant than the wild-type plants. Hence, it was proposed that \textit{CBF2} played a crucial role in \textit{Arabidopsis} freezing tolerance by acting as a negative regulator of \textit{CBF1} and \textit{CBF3} (Novillo \textit{et al.}, 2004). \textit{CBF2} might be involved in feedback regulation of \textit{CBF1} and \textit{CBF3} expression during cold acclimation in which \textit{CBF1} and \textit{CBF3} are quickly induced in response to LT followed by the induction of \textit{CBF2} after 1 h. Induction of \textit{CBF2} then leads to down-regulation of \textit{CBF1} and \textit{CBF3} expression (Novillo \textit{et al.}, 2004). In contrast, in plants where the expression of \textit{CBF1} and/or \textit{CBF3} is impaired, the induction of \textit{CBF2} in response to LT was not diminished. Thus, \textit{CBF1} and \textit{CBF3} were not implicated in regulating the expression of \textit{CBF2} genes (Novillo \textit{et al.}, 2007).

\subsection*{2.7.2.5 Negative Regulators}
\textit{MYB15} is another transcription factor that binds to the promoters of \textit{CBFs} (Fig 2.2) and represses their expression (Agarwal \textit{et al.}, 2006). In recent studies, the \textit{myb15} null mutants were shown to be more tolerant to freezing stress than wild-type plants, mainly due to an increase in cold-induction of the CBFs and target genes (Agarwal \textit{et al.}, 2006). It was suggested that \textit{ICE1} might physically interact with \textit{MYB15} and directly repress (through binding to \textit{MYB15} promoter) or indirectly (through its downstream genes) \textit{MYB15} expression in response to LT (Agarwal \textit{et al.} 2006). Thus, \textit{HOS1} and \textit{MYB15} might act as negative regulators while the MYC transcription factor, \textit{ICE1} and \textit{SIZ1} could act as positive regulators to modulate expression of \textit{CBF3} and may be other CBFs, to control plant responses to LT.
2.7.2.6 Ubiquitination of ICE1

It has been suggested that the early signaling components upstream of CBF might be subject of specific ubiquitination-mediated degradation (Lee et al., 2001). HOS1 (Fig. 2.2), which might negatively regulate CBF transcription by inducing the degradation of ICE1, has been shown to be a RING finger protein with ubiquitin E3 ligase activity that interacts with ICE1 and represses the expression of CBFs and their downstream genes (Dong et al., 2006). HOS1 migrates to the nucleus in response to LT treatment and polyubiquitinates ICE1, targeting this transcription factor for proteasome degradation (Lee et al., 2001; Dong et al., 2006). Taken together these results suggested a mechanism by which HOS1 attenuates the LT response by mediating the degradation of ICE1 and possibly other regulators of LT responses through the ubiquitin proteasome pathway (Dong et al., 2006).

2.7.2.7 SUMOylation of ICE1

Small ubiquitin-related modifier (SUMO) conjugation/deconjugation in plants has been implicated in responses to heat shock, oxidative stress, hypoxia, phosphate limitation, ABA, flowering, and pathogen defense (Kurepa et al., 2003; Lois et al., 2003; Murtas et al., 2003; Miura et al., 2005; Yoo et al., 2006; Lee et al., 2007). SUMO conjugation to protein substrates (SUMOylation) is a reversible post-translational modification that is regulated by environmental stimuli in animals and yeasts (Johnson, 2004). The Arabidopsis genome includes only one gene homolog of E3 sumo ligases, SIZ1 (Miura et al., 2007). SIZ1 expression is not induced by cold although mutations in SIZ1 decrease tolerance of Arabidopsis to freezing and chilling temperatures, indicating that SUMOylation also plays an important role in plant response to LT (Miura et al., 2007). SIZ1 has been shown to catalyze the SUMOylation of recombinant ICE1 which reduced the polyubiquitination of ICE1 in vitro (Miura et al., 2007). A possible mechanism suggested is that sumoylation of ICE1 by SIZ1 represses the polyubiquitination of ICE1 and leads to enhanced stability of ICE1 at LT. Thus, SIZ1 has been implicated as a regulator of cold acclimation by controlling ICE1 activity, CBF3 expression and target gene function (Miura et al., 2007). Furthermore, ICE1 SUMOylation also repressed the expression of MYB15, a negative regulator of CBF expression (Miura et al., 2007). Together, these results suggested that SIZ1-mediated SUMO conjugation/deconjugation of ICE1 is a key process that initiates many changes in gene expression that are required for LT tolerance.

2.7.2.8 ICE1-like Regulators

ICE1 and ICE1-like proteins (Fig 2.2) have been proposed to be involved in the LT-responsive CBF-dependent and -independent pathways that induce the expression of COR genes (Chinnusamy et al., 2003, 2006; Zarka et al., 2003; van Buskirk and Thomashow,
The likelihood of presence of other ICE-like proteins was supported by mutational analysis of CBF2 promoter resulting in identification of two regions, ICEr1 and ICEr2. These were only weakly responsive to LT, but in combination imparted a robust LT response (Zarka et al., 2003). ICEr1 sequence (CACATG) includes a consensus recognition site for bHLH proteins (CANNTG). Therefore, ICEr1 was suggested as a potential binding site for the ICE1 protein. Since there are no obvious known transcription factor-binding sites within the ICEr2 sequence, hence it is possible that other ICE1-like transcription factors might be involved in binding to these sites and thus in regulation of CBF response pathways (Zarka et al., 2003).

Stomata play a crucial role in regulating photosynthesis and transpiration. Recently, the scream-D dominant mutant and ice1 mutant were found to be the same as R236H, which resulted in constitutive stomatal differentiation in the epidermis, and the entire epidermis differentiates into stomata. Thus, ICE1 is required for controlled stomatal development. ICE1 formed a dimer with other bHLH transcription factors, SPEECHLESS (SPCH), MUTE, and FAMA, which regulate stomatal development. This is suggested that ICE1 might act as a link between the formation of stomata and the plant response to environmental cues (Kanaoka et al., 2008).

Recently, members of the calmodulin binding transcription activator (CAMTA) family proteins have been identified as transcriptional regulators of CBF2 expression. Cold-induced expression of CBF2 was considerably lower in camta2 mutant as compared to wild type plants. The CAMTA3 protein binds to conserved DNA motifs present in CBF2 promoter and regulates CBF2 expression. The camtal/camta2 double mutant exhibited hypersensitivity to freezing stress as compared to wild type plants. Since CAMTA proteins can interact with calmodulins, cold-induced calcium signals might regulate CBFs expression through CAMTA proteins (Doherty et al., 2009).

2.7.3 CBF-Independent Pathways of LT Response

CBF-independent pathways have been shown to be involved in the complex LT response of plants. DNA microarrays analysis showed that extensive changes occur in the transcriptome of Arabidopsis in response to LT (Seki et al., 2001; Fowler and Thomashow, 2002; Kreps et al., 2002; Seki et al., 2002a; Maruyama et al., 2004; Vogel et al., 2005; Lee et al., 2005). Fowler and Thomashow (2002) surveyed expression of ~8,000 Arabidopsis genes and found that transcripts for ~4% (306) of the genes were responsive to LT, with 3% (218) being up-regulated and 1% (88) being down-regulated. However, only 12% of the LT-responsive genes could be assigned to the CBF regulon and at least 28% of the LT-responsive genes were not affected by expression of the CBF transcription factors, including 15 encoding known or putative transcription factors. Hence, non-CBF transcription factors might play a
role in the regulation of the remaining large portion of cold-responsive genes. It is thus possible that cold acclimation is associated with the activation of multiple LT-regulatory pathways including both CBF-dependent and -independent pathways (Seki et al., 2001; Fowler and Thomashow, 2002; Kreps et al., 2002; Seki et al., 2002a; Maruyama et al., 2004; Vogel et al., 2005).

The importance of CBF-independent pathways was supported by analysis of mutants that have increased freezing tolerance. The eskimol mutant of Arabidopsis described by Xin and Browse (1998) is constitutively more freezing tolerant than wild-type plants, but the COR genes are not constitutively expressed indicating that the mutation activated a freezing tolerance pathway other than CBF-dependent pathway. Mutations in ESKIMO1 (ESK1), a protein of unknown function, resulted in constitutive freezing tolerance, but the genes affected by eskl mutation were demonstrated to be distinct from those of the CBF regulon (Xin et al., 2007).

Additional LT-responsive pathways that involve the ZAT12, HOS9 and HOS10 transcription factors have been suggested to be involved in LT-response of Arabidopsis (van Buskirk and Thomashow, 2006). In Arabidopsis, over-expression of ZAT12 (a zinc-finger protein) induced the expression of 9 genes that were normally induced in response to LT and repressed the expression of 15 genes that were down-regulated in response to LT (Vogel et al., 2005). In addition, the constitutive overexpression of ZAT12 negatively regulated the CBF1 and CBF3 LT-response pathway (Vogel et al., 2005). The CBF2 and ZAT12 regulons were shown to share some common genes that are up-regulated and down-regulated by LT, which suggested that these two pathways overlap with each other. It has been shown that the over-expression of ZAT12, significantly dampened the induction of the CBFs in response to LT but did not seem to affect the CBF-targeted gene expression, which suggested a complex role and may be a distinct regulatory pathway other than CBF, involved in the regulation of LT-responsive gene expression and cold acclimation (Vogel et al., 2005).

Two other transcription factors, HOS9 and HOS10 were also proposed to play important roles in the regulation of COR genes and freezing tolerance, in a CBF-independent pathway (Zhu et al., 2004, 2005). A promoter RD29A::LUC reporter genetic screen led to the identification of a freezing-sensitive hos9 mutant in Arabidopsis. HOS9 encoded a putative homeodomain transcription factor that was constitutively expressed and localized in the nucleus. As compared with the wild-type, the hos9 mutant was hypersensitive to freezing with or without cold acclimation, although LT induction of CBFs was not altered. Furthermore, transcriptome analysis of hos9 mutant plants under LT stress suggested that the HOS9 regulon
is different from that of the CBFs. Thus, Zhu et al., (2004) suggested that HOS9 might play an important role in regulating cold acclimation through a CBF-independent pathway.

In Arabidopsis, HOS10 encodes a R2R3-type MYB transcription factor. The hos10 mutants were characterized by a rapid induction of COR genes in response to LT without affecting the expression of the CBF genes. Moreover, the hos10 mutant was less tolerant to freezing and unable to cold acclimate despite an enhanced expression of some COR genes. In hos10 mutants the expression of NCED3 (a 9-cis-epoxycarotenoid dioxygenase required for ABA synthesis) was repressed that suggested a positive regulation of NCED3 expression (Zhu et al., 2005). Thus, HOS10 was proposed to act as a negative regulator of COR gene expression that acts downstream of the CBF proteins and might be involved in ABA-mediated regulation of cold acclimation in Arabidopsis (Zhu et al., 2005). The exact functioning of HOS9 and HOS10 is yet to be understood completely.

Another transcription factor, LOV1 (long vegetative phase 1) has been reported to regulate the expression of CBF-target genes in a CBF-independent way (Yoo et al., 2007).

LOV1 encodes a NAC-domain containing transcription factor. The lovl-4 null mutants generated by activation tagging were hypersensitive to freezing but were able to cold acclimate, while plants over-expressing LOV1 exhibited a higher constitutive freezing tolerance (Yoo et al., 2007). The freezing tolerance was accompanied by up-regulation of COR genes (COR15A and KIN1) without affecting expression of the CBF family of genes. The cold-induction of COR genes was significantly impaired in lovl-4 as compared to wild-type plants, indicating that LOV1 is a positive regulator of LT response. However, the cold-induction of CBFs was not affected in both lovl-4 and wild-type plants, which suggested that LOV1 might be involved in CBF-independent pathway of LT response (Yoo et al., 2007).

2.7.4 ABA-dependent LT Response

Abscisic acid (ABA) signaling is central to any discussion of stress response since osmotic stress leads to ABA accumulation (Xiong and Zhu, 2001). ABA treatment and drought have been reported to trigger cold acclimation and increase in LT tolerance in many species (Chen and Gusta, 1983; Gusta et al., 2005). Several reports have suggested the involvement of ABA in cold acclimation of plants. Endogenous ABA content has been shown to increase transiently upon exposure to LT in various plants including Arabidopsis (Lang et al., 1989, 1994), tomato (Daie and Campbell, 1981), potato (Chen et al., 1983), and barley
Exogenous application of ABA at normal growth temperature has been shown to cause increased chilling and freezing tolerance in various plants including potato (Chen et al., 1983), maize (Xin and Li, 1992), Brassica napus (Johnson-Flanagan et al., 1991), Arabidopsis (Lang et al., 1989, 1994), chickpea (Kumar et al., 2008). De novo protein synthesis and induction of LT-responsive genes have been shown to occur in response to exogenous ABA (Chen et al., 1983; Robertson et al., 1987; Lang et al., 1989; Lang and Palva, 1992; Mantyla et al., 1995; Seki et al., 2002b). Studies have also demonstrated that ABA can substitute for the cold requirement in inducing freezing resistance (Chen et al., 1983; Chen and Gusta, 1983; Mantyla et al., 1995). The importance of ABA in LT response has been supported by the fact that the expression of many LT-induced genes is modulated by ABA, and many ABA mutants are also impaired in LT-induced gene expression (Heino et al., 1990; Mantyla et al., 1995). ABA-insensitive (abi) and ABA (aba)-deficient mutants have been shown to be impaired in cold acclimation (Heino et al., 1990; Gilmour and Thomashow, 1991; Mantyla et al., 1995). Arabidopsis ABA-null mutants (aba-1) are impaired in their ability to cold acclimate with LT treatment but application of ABA resulted in development of freezing tolerance in the aba mutants (Heino et al., 1990; Mantyla et al., 1995; Llorente et al., 2000), whereas abi-1 mutants did not induce cold acclimation (Mantyla et al., 1995). These observations indicated that there exist both ABA-dependent and ABA-independent stress signaling pathways in response to LT.

ABA has been shown to regulate the transcription of many LT-responsive genes, indicating the presence of promoter elements involved in ABA-induced gene expression, (Lang and Palva, 1992; Shinozaki and Yamaguchi-Shinozaki, 2000). Several ABA-responsive elements (ABREs) have been identified and defined as 8-10 bp consensus sequence C/TACGTGGC, characterized by a core motif ACGT that confers ABA-responsiveness to several genes when present in more than one copy (Busk and Pages, 1998; Leung and Giraudat, 1998; Hattori et al., 2002). Single ABRE motifs were not ABA-responsive (Skriver et al., 1991) and required a second ABRE or “coupling element” (CE) for optimal ABA-responsiveness (Shen and Ho, 1995; Shen et al., 1996). The ABRE motif is highly conserved and has been found in the ABA-responsive promoters of many species, including Arabidopsis, wheat (Guiltinan et al., 1990), rice (Skriver et al., 1991) and barley (Shen and Ho, 1995). ABRE has been identified in the promoters of many ABA-responsive COR genes, including COR15a (Baker et al., 1994) and RD29a (Yamaguchi-Shinozaki and Shinozaki, 1994) genes, which are also controlled by the CRT/DRE element. Interestingly, the CRT/DRE has been implicated as a CE in Arabidopsis (Narusaka et al., 2003).
A small family of ABRE-binding bZIP proteins described as ABFs (ABRE-binding factors) or AREBs (ABA-responsive element-binding proteins) have been isolated using yeast one-hybrid screens (Choi et al., 2000; Uno et al., 2000). These factors possess not only the ABRE-binding activity, but also the capability to transactivate ABRE-containing reporter genes and are reported to be induced by ABA, high salinity, LT or drought (Choi et al., 2000). The genes encoding ABFs (ABF1-4) are themselves induced by ABA and show differential regulation by various environmental stresses; ABF1 is induced by LT, ABF2 and ABF3 by high salt concentration and ABF4 by LT, high salt concentration and drought (Guiltinan et al., 1990; Uno et al., 2000; Choi et al., 2000; Kang et al., 2002). Over-expression of ABF3 and ABF4/AREB2 has been demonstrated to enhance chilling and freezing tolerance capacity of transgenic plants (Kim et al., 2004a). These findings suggest that the ABF/AREB mediated pathway is involved in cold acclimation and development of freezing tolerance in Arabidopsis.

The ABA levels in cold-acclimatizing Arabidopsis have been proposed to be regulated by the HOS10 (Zhu et al., 2005). HOS10 has been proposed to act as a negative regulator of COR gene expression and positive regulator of NCED and thus might be involved in ABA-mediated regulation of cold acclimation in Arabidopsis (Zhu et al., 2005).

The expression of a transcription factor, SCOF1, from soybean has been shown to be specifically induced by LT and ABA but not by dehydration or high salt concentration (Kim et al., 2001a, 2001b). SCOF1 encoded a C2H2-type zinc finger protein and its over-expression resulted in constitutive expression of LT-inducible genes harbouring either DRE/CRT or ABRE in the promoter sequence and enhanced LT tolerance of non-acclimated transgenic Arabidopsis and tobacco plants (Kim et al., 2001a). SCOF-1 was localized to the nucleus but did not bind directly to either DRE/CRT or ABRE elements (Kim et al., 2001a). In vitro studies revealed that SCOF-1 enhanced the DNA binding activity of SGBF-1, a soybean G-box binding bZIP transcription factor to ABRE (Kim et al., 2001a) and interacted with SGBF-1 in a yeast two-hybrid system. SGBF-1 was also shown to induce the ABRE-dependent gene expression, which was enhanced by SCOF-1 (Kim et al., 2001a). These results suggested that SCOF-1 might act as a positive regulator of COR gene expression mediated by ABRE via interaction with SGBF-1.

Interestingly, recent studies have demonstrated that CBFs transcripts are also induced by ABA (Knight et al., 2004; Haake et al., 2002) reported the isolation of CBF4, a CBF homolog in Arabidopsis. The expression of CBF4 was shown to be induced rapidly during drought stress and ABA treatment, but not by LT. Constitutive over-expression of CBF4 resulted in the expression of LT- and drought-induced genes under non-stress conditions. The
transgenic plants exhibited enhanced tolerance to freezing and drought conditions (Haake et al., 2002). These findings suggested the existence of an ABA-dependent pathway for the regulation of genes through the DRE/CRT element.

2.8 Posttranscriptional Gene Regulation during LT

2.8.1 Messenger RNA processing

Pre-mRNA processing and exports constitute important mechanisms of regulation of gene expression in eukaryotes (Chinnusamy et al., 2010). Recent studies have implicated the important role of intron splicing in the regulation of photosynthesis, flowering, grain quality in cereals and plant defense response (Chinnusamy et al., 2010). For example, In wheat, cold stress induction of two early cold-regulated (e-cor) genes coding for a ribokinase (7H8) and a C3H2C3 RING finger protein (6G2) undergo stress-dependent splicing. Both of these genes are regulated by intron retention under cold stress, whereas 6G2 intron retention is also regulated by drought stress. However, homologs of these genes did not show stress-regulated intron retention in Arabidopsis. Interestingly, barley homologs of 7H8 and 6G2 showed stress-dependent intron retention under cold stress, whereas barley albino mutants defective in chloroplast development failed to retain introns in these genes under cold stress (Hu et al., 2008). The Arabidopsis COR15A gene encoding a chloroplast stromal protein with cryoprotective activity plays an important role in conferring freezing tolerance to chloroplasts (Mastrangelo et al., 2005). The Arabidopsis stabilizedl (stal) mutant is defective in the splicing of the cold-induced COR15A pre-mRNA and is hypersensitive to chilling, ABA, and salt stresses. STA1 encodes a nuclear pre-mRNA splicing factor and is upregulated by cold stress. STA1 catalyzes splicing of COR15A, which is necessary for cold tolerance (Nakayama et al., 2007). Hence, the stress regulated alternate splicing machinery may change the splicing pattern of some of the stress-responsive gene.

2.8.2 Small RNAs

Micro-RNAs (miRNAs) and small interfering RNAs (siRNAs), act as ubiquitous repressors of gene expression in animals and plants. Cold stress up-regulated and down-regulated miRNAs have been identified in Arabidopsis. Abiotic stress-induced or up-regulated small RNAs can down-regulate their target genes, which are likely negative regulators and/or determinants of the stress response. In contrast, stress down-regulated small RNAs can up-regulate their target mRNAs, which are likely positive regulators and/or determinants of stress tolerance (Tanabe et al., 2007). Accumulation of ROS is induced by abiotic stresses. Superoxide dismutases (SOD) catalyze conversion of the superoxide radical into H2O2, which is then detoxified by ascorbate peroxidase. The miR398 expression is reduced and that
of its target genes CSD1 and CSD2 enhanced under oxidative stress in *Arabidopsis*. Under normal conditions, miR398 targets the CSD mRNAs for cleavage, and thus stress-induced reduction in miR398 expression results in accumulation of CSD transcripts. Because miR398 and its target sequence on the CSD mRNAs are conserved across plant species, miR398 appears to play a ubiquitous role in ROS detoxification under abiotic stresses (Sunkar *et al.*, 2007).

### 2.9 Reactive Oxygen Species (ROS) during LT

Exposure to several environmental factors such as high light, drought, heavy metals, salt, temperature extremes, UV radiation, herbicides and pathogen attacks disrupt the cellular homeostasis and result in enhanced ROS production (Apel and Hirt, 2004; Mittler, 2002; Gratão *et al.*, 2005). In plants, ROS are produced continuously as by-product of various aerobic metabolic processes occurring in various cellular components, such as chloroplasts, mitochondria, peroxisomes, glyoxysomes, plasma membrane and the apoplastic space (Mittler, 2002; Foyer and Noctor, 2003; Apel and Hirt, 2004; Asada, 2006; Haliwell, 2006; Gechev *et al.*, 2006). Under non-stressed conditions, the ROS are rapidly removed by both enzymatic and non-enzymatic scavenging systems to maintain a normal redox homeostasis (Apel and Hirt, 2004). However, under stress environment cause an imbalance in the redox homeostasis and ROS such as singlet oxygen (\( ^1 \text{O}_2 \)), perhydroxyl radical (\( \text{HO}_2^- \)), superoxide radical (\( \text{O}_2^- \)), hydroxyl radical (\( \text{OH} \)) and peroxides and widely distributed hydrogen peroxide (\( \text{H}_2\text{O}_2 \)) tend to accumulate. However, charged \( \text{O}_2^- \) is impermeable through phospholipid membranes and is relatively nontoxic against biological macromolecules. At physiological pH, the \( \text{O}_2^- \) disproportionates to \( \text{H}_2\text{O}_2 \), a relatively stable form of ROS, and \( \text{O}_2 \), either spontaneously or by the action of superoxide dismutases (SODs). The \( \text{H}_2\text{O}_2 \) passes through membranes and reaches cellular components distant from the initial sites of its generation (Lamb and Dixon, 1997).

Several studies have suggested a dual role for ROS in plant biology as both toxic by-products of aerobic metabolism and key regulators of growth, development and defence pathways (Mittler *et al.*, 2004). The role of ROS as damaging, protective or signaling factors depends on the delicate equilibrium between ROS production and scavenging and on their spatial and temporal regulation (Edreva, 2005).

Exposure of plants to LT has been shown to result in an increase in the concentration of ROS that might damage membrane lipids, proteins and nucleic acids, leading to the cell death (O’Kane *et al.*, 1996; Prasad *et al.*, 1994a, 1994b; Pastori *et al.*, 2000; Mitter, 2002; Apel and Hirt 2004; Suzuki and Mittler, 2006). The chloroplast is a major source of ROS during chilling through inhibition of CO\(_2\) fixation, although, recently, mitochondrial ROS
accumulation during chilling has been demonstrated (Zykova et al., 2002; Foyer and Noctor, 2003; Noctor et al., 2007).

ROS have been shown to influence the expression of a number of genes and signal transduction pathways, suggesting that cells have evolved strategies to utilize ROS as signals that control various biological programs (Dalton et al., 1999; Desikan et al., 2001). ROS have been suggested to be involved in LT regulation of gene expression and various other abiotic stresses (Mittler et al., 2004). ROS may alter Ca$^{2+}$ signatures and activate MAPKs cascade and redox-responsive transcription factors (Desikan et al., 1999; Kveton et al., 2000; Moon et al., 2003; Nakagami et al., 2006).

Molecular analysis of the FROSTBITE1 (FRO1) locus has demonstrated that the expression of COR genes such as RD29a, KIN1 COR15a, and COR47, is regulated by ROS levels (Lee et al., 2002a). The FRO1 encodes a Fe-S subunit of complex I (NADH dehydrogenase) of the electron-transfer chain in the mitochondrion, and its disruption leads to high accumulation ROS (Lee et al., 2002a). The frol mutant, constitutively accumulates high levels of ROS and exhibits impaired expression of COR genes and hypersensitivity to chilling and freezing. It has been suggested that the accumulation of ROS probably triggers Ca$^{2+}$ signaling in the absence of LT, which might desensitize the cells to LT-induced Ca$^{2+}$ signaling. This could be the cause of reduced cold induction of COR genes and reduced cold acclimation in frol mutant plants (Lee et al., 2002a). Besides, ROS can also exert their effects directly through the activation of redox-responsive proteins, such as transcription factors and protein kinases.

2.9.1 ROS Scavenging Systems

ROS scavenging systems (enzymatic and non-enzymatic) maintain the cellular redox homeostasis and thus are involved in protecting plants against ROS toxicity (Pastori and Foyer, 2002; Mittler, 2002). The enzymatic system consists of SOD involved in the water-water cycle (WWC), ascorbate peroxidase (APX) in WWC and ascorbate-glutathione cycle (AGO), mono-dehydroascorbate reductase (MDAR) in AGC, glutathione reductase (GR) in AGC and glutathione peroxidase cycle (GPXC) and catalase (CAT). The non-enzymatic system includes antioxidants, such as ascorbate, glutathione, carotenoids, tocopherol and flavonoids (Prasad, 1996; Mittler, 2002; Apel and Hirt, 2004).

2.9.1.1 Superoxide Dismutase (SOD)

SOD catalyzes the dismutation of superoxide radical in various organisms, including plants. The dismutation of superoxide into H$_2$O and O$_2$ constitute the first line of cellular defence in plants (McCord and Fridovich, 1969, Foyer and Halliwell, 1976). Three types of SOD have been characterized based on the nature of the metal co-factor present at the
catalytic site, i.e. copper/zinc (CuZnSOD), iron (FeSOD), or manganese (MnSOD) SODs. CuZnSOD is generally found in the cytosol and chloroplasts, MnSOD in mitochondria, whereas FeSOD is present in the chloroplasts of some plants (Bannister et al., 1987; Alscher et al., 2002). Transgenic alfalfa plants expressing a tobacco MnSOD exhibited increased vigour after freezing stress and increased winter survival under field conditions (McKersie et al., 1993, 1999). Similarly, overexpression of an Arabidopsis FeSOD in alfalfa resulted in reduced secondary injury symptoms and enhanced recovery from stresses during winter (McKersie et al., 2000). These studies suggested that SOD might play an important role in amelioration of oxidative stress upon exposure to LT.

2.9.1.2 Ascorbate Peroxidase

Ascorbate peroxidase (APX) is a heme-containing protein that along with catalase, plays a central role in scavenging H$_2$O$_2$, therefore protecting plants from oxidative stress (Asada 1992; Shigeoka et al., 2002). In higher plants, APX isozymes are distributed in at least four distinct cellular compartments: stromal APX (sAPX) and thylakoid membrane-bound APX (tAPX) in chloroplasts, microbody (including glyoxysome and peroxisome) membrane-bound APX (mAPX), mitochondrial membrane-bound APX (mitAPX) and cytosolic APX (cAPX) (Asada 1992; Shigeoka et al., 2002). APX haem moiety consists of a protophyrin prosthetic group that is inhibited by cyanide and azide (Shigeoka et al., 1980; Chen and Asada, 1989). They function as scavengers of H$_2$O$_2$ at the expense of ascorbate (as an electron donor) and protect plant cells from the damaging effects of H$_2$O$_2$. Ascorbate (Asc) is the reducing agent in the first reaction catalyzed by APX. Asc is oxidized into MDA that can be regenerated by MDAR using NAD(P)H as a reducing equivalent. Enhanced activity of APX have been reported in response to chilling in cucumber (Lee and Lee, 2000), soybean seedlings (Yadeghari et al., 2008) and in the leaves and roots of cold-acclimated rice (Kuk et al., 2003). In another experiment, prior exposure of rice seedlings to heat-shock resulted in an increased accumulation of APX transcripts and reduced chilling injury (Sato et al., 2001). Overexpression of both CuZnSOD and APX in chloroplasts of transgenic sweet potato plants resulted in enhanced tolerance to methyl viologen-mediated oxidative stress and chilling (Lim et al., 2007).

2.9.1.3 Catalase

Catalase (CAT) is a tetrameric enzyme responsible for dismutation of H$_2$O$_2$ into O$_2$ and water in the peroxisomes and thus protects the cell from the deleterious effects of H$_2$O$_2$ accumulation. Increased activity CAT in cold acclimated seedlings of maize (Prasad, 1997) and gladiolus somaclones (Bettaieb et al., 2007) was proposed to play a major role in inducing chilling tolerance. Expression of E. coli KatE improved the resistance to photo-oxidation
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caus ed by drought stress and high-light intensity in tobacco (Shikanai et al., 1998) and protected transgenic tomato plants from the photo-oxidative stress invoked by paraquat treatment, drought stress and chilling stress (Mohamed et al., 2003). Anti-sense CAT-deficient tobacco plants were demonstrated to be highly susceptible to high light, paraquat, salt and ozone but not to chilling (Willekens et al., 1997). Expression of FaCatl was shown to be up regulated in cold- and salt-stressed leaves (Yang et al., 2006). Overexpression of a wheat CAT improved tolerance against LT stress in transgenic rice due to effective detoxification of H$_2$O$_2$ by the enhanced catalase activities (Matsumura, et al., 2002).

2.9.1.4 Glutathione Reductase

Glutathione reductase (GR) is the last enzyme in the AGC. GR catalyzes the NAD(P)H-dependent reduction of GSSG to maintain the antioxidant GSH in its reduced state. GR is also involved in the GPXC where oxidized GSSG is converted into GSH using NAD(P)H. GR has a central role in maintaining reduced glutathione (GSH) pool during stress (Pastori et al., 2000). The GPXC also detoxifies hydrogen peroxide to water but uses glutathione directly as a reducing agent. GR is mainly localized in chloroplasts, mitochondria, cytosol and peroxisomes (Creissen et al., 1995; del Rio et al., 2002; Rudhe et al., 2004). A dual-targeted GR localized to chloroplasts and mitochondria has also been reported (Mullineaux et al., 1996; Rudhe et al., 2002; Chew et al., 2003). Several studies have shown increased GR activity during cold hardening of conifers, and higher activities throughout winter in dormant tissue (Esterbauer and Grill, 1978; Anderson et al., 1992; Hausladen and Alscher, 1994). Similarly in pea, GR transcript showed enhanced accumulation in response to LT (Romero-Puertas et al., 2006). GR transcripts were strongly induced by various stress treatments including ozone, paraquat, salt, hydrogen peroxide, chilling or ABA but depressed by heat treatment in Brassica campestris (Lee et al., 2002c).

2.9.1.5 Glutaredoxin

Glutaredoxins (GRXs), also known as thioltransferases, are small, ubiquitous, heat-stable proteins of ~12 kDa. GRXs are oxidoreductases of the thioredoxin family that mediate the reversible reduction of intracellular disulfide bonds. GRXs reduce disulfides using conserved cysteines located in the active site motifs, and depend on glutathione (GSH) for reduction of the oxidized form (Rouhier et al., 2004; Fernandes and Holmgren, 2004). Arabidopsis thaliana comprises of 31 GRX family members, which fall into three subgroups (Rouhier et al., 2006), the classical CPYC group, the CGFS group and the CC-type group, which is specific for higher plants (Lemaire, 2004). In Populus trichocarpa, 36 members belonging to the three groups have been identified, in rice, wheat, maize, barley and Pinus taeda, all groups are represented, but there are not as many GRX with a CCxC/S active site in
these organisms whereas they are prominent in *Arabidopsis thaliana* (Rouhier *et al.*, 2004). In plants, very little is known about the function of GRX. They are implicated in many different ways, for example by directly reducing peroxides or dehydroascorbate (DHA), by reducing peroxiredoxins (Prx), and also by protecting thiol groups on other enzymes via glutathionylation/ deglutathionylation mechanisms (Rouhier *et al.*, 2004; Rouhier *et al.*, 2006). Transcripts of *DaGrx* from *Deschampsia antarctica* were shown to be cold-regulated and were speculated to be involved in redox regulation during cold acclimation (Gidekel *et al.*, 2003).

**2.9.1.6 Monodehydroascorbate Reductase (MDAR)**

MDAR is an enzymatic component of the AGC, one of the major antioxidant systems of plant cells for the protection against the damages produced by ROS. The primary product of the APX reaction in AGC is MDA radical which is converted back to ascorbate by MDAR or via a spontaneous dismutation into DHA. The ascorbate regeneration is mediated by DHAR driven by oxidation of glutathione (GSH) to glutathione disulphide (GSSG). MDAR is critical in maintaining the proper concentration of ascorbate in cells by reducing the MDA radical directly to ascorbate at the expense of an NAD(P)H. The MDAR activity has been described in several cellular compartments, such as chloroplasts, cytosol, mitochondria, glyoxysomes, and leaf peroxisomes (Leterrier *et al.*, 2005). In *Parthenium argentatum*, LT was demonstrated to cause significant increase in the contents of monodehydroascorbate as well as the activities of all antioxidative enzymes including MDAR (Sundar *et al.*, 2004). Transcripts of MDAR have been shown to be up-regulated in response to LT in pea (Leterier *et al.*, 2005). Over-expression of tobacco MnSOD in maize resulted in enhanced accumulation of MDAR, DHAR and GR activities (Kingston-Smith and Foyer, 2000).

**2.10 Cross Talk with Other Stresses**

Dehydration is the common physiological change during LT, drought and salt stress (Beck *et al.*, 2007). Decrease in turgour pressure due to freezing is known to induce biosynthesis of plant stress hormone ABA. ABA dependent pathway plays very little but significant role during LT (Gusta *et al.*, 2005). Also, the generation of ROS occurs in almost all abiotic stresses. During signalling processes like Ca\(^{2+}\) influx, signal transducers to transcription factors, cross talk is possible (Knight and Knight, 2001). There are many reviews on cross talk among different abiotic stresses (Knight and Knight, 2001; Zhu, 2001; Xiong *et al.*, 2002; Chinnusamy *et al.*, 2004; Beck *et al.*, 2007; Vij and Tyagi, 2007). Hence, cold signaling might act as a network, in which much more overlap between its branches is possible (Solanke and Sharma, 2008).