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Physiological, Molecular and Genetic Perspectives

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Emeritus Professor Peter Langridge FTSE

Peter was born in Adelaide in 1953 to a Czech mother and a New Zealand father. He was brought up in Canberra where he studied at the Australian National University. When he graduated, he took up a job in Germany at the University of Freiburg. During his 4 years in Germany, he also met and married his German wife, Ursula, who is also a scientist. In 1984, he moved to the University of Adelaide. He became a Professor in 1996 and from 1998 was the inaugural Research Director of the Cooperative Research Centre for Molecular

Plant Breeding. In 2003, Peter became the Chief Executive Officer and Director of the Australian Centre for Plant Functional Genomics (ACPFG) when it was established and remained in this role until 2014. ACPFG was a major research centre based in Adelaide set up by the Australian Federal Government through the Australian Research Council and the Grains Research and Development Corporation. When he left ACPFG, Peter was appointed Emeritus Professor at the University of Adelaide; he is also an Honorary Professor at the Kazakh National Agrarian University. He is a Fellow of the Australian Academy of Technological Sciences and Engineering and an Honorary Fellow of Food Standards Australia and New Zealand (FSANZ) and James Hutton Institute, UK.

Since 2011, Peter has been chair of the Scientific Board of the Wheat Initiative. The Wheat Initiative was established by the G20 group of countries to provide global coordination of wheat research. The secretariat moved from Paris to Berlin at the beginning of 2018. Peter also chairs several science advisory committees for research organisations in Europe and North America. He chaired the steering committee for the CGIAR Research Program on Dryland Cereals and led a major review of biotechnology capabilities across the CGIAR system. In 2011, he chaired an expert scientific panel for the Australian Government on "Food security in a changing world". Peter is Editor-in-Chief of the Journal Agronomy (MDPI Publishers, Switzerland) and associate editor of eight other

journals. In 2011, he was selected as the South Australian Scientist of the Year, and he has received other awards in Australia and Europe.

Peter's research has focused on plant molecular biology and the science of plant breeding, and he has published over 300 research papers, books and reviews.

Preface

Human population is increasing at an alarming pace and believed to exceed 9.7 billion by 2050, whereas at the same time the agricultural productivity is decreasing due to the growing environmental constraints as a result of global climate change. Cold stress is one of the widespread abiotic stresses affecting crop productivity particularly in temperate regions. Plants have developed various anatomical, physiological and genetic strategies to cope with the cold stress. Conventional breeding methods have resulted in inadequate success in improving the cold tolerance of vital crop plants through inter-specific or inter-generic hybridization. Therefore, it is of the essence to speed up the efforts for unraveling the biochemical, physiological and molecular mechanisms underlying cold stress tolerance in plants. While quite a few programs have been taken up in leading global research institutes but the pace of development of cold stress tolerant cultivars is not up to the mark when compared to ever-increasing pressure of abiotic stresses including cold stress due to global climate change. Moreover, the intricate genetic mechanisms involved in plant adaptation to cold stresses have been a key obstacle for crop improvement using conventional plant breeding tools. Omics technologies including genomics, transcriptomics and proteomics have facilitated elucidation of complex mechanisms involved in plant adaptation to cold stress. Through this book "Cold Tolerance in Plants - Physiological, Molecular and Genetic Perspectives", we have tried our best to include chapters unfolding the implication of cold stress in plants under climate change scenario and the eventual scientific advancements being applied utilizing the existing high throughput omics technologies to come up with novel strategies to mitigate cold stress by unraveling molecular mechanisms responsible for cold stress in plants.

This book provides systematic and comprehensive reference material for researchers, teachers, and graduate students involved in abiotic stress tolerance studies in plants particularly cold stress using physiological, molecular and genomic tools by unfolding principles and application of recently developed technologies and their application in development of stress resilience in plants against cold stresses. The chapters are written by globally reputed researchers and academicians

in the field of plant stress biology. We express sincere thanks and gratefulness to our revered authors, without their untiring efforts this book project would not have been possible. We are also thankful to Springer Nature for providing such opportunity to complete this book project. We are also thankful to all our family members for their support during the entire book project completion.

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Chapter 1
Cold-Induced Injuries and Signaling Responses in Plants

Jigeesha Mukhopadhyay and Aryadeep Roychoudhury

1.1 Introduction

The phenotypic manifestations of cold injury in plants are highly variable. Both low temperature and rapid fluctuations between heat and cold can severely affect the physiology of plants (Miura and Furumoto 2013). Cold stress inflicts damages to fruit trees, horticultural and landscape plants, as well as crop plants, posing a major threat to sustainable agriculture. Commercially important crop plants have been targeted for stress alleviation in order to increase productivity and yield through interspecific and intergeneric breeding which resulted in limited success; however, transgenic approaches to engineer cold-tolerant plants by manipulation of the key genes of the transcriptional and metabolic cascades have contributed to tolerance mechanisms in affected plants to some extent (Rihan et al. 2017). The present chapter highlights the physiological effects of cold stress and gene regulations on perceiving cold stress signals. Finally, the chapter discusses on the cold tolerance mechanisms, genetic engineering for tolerance, and acclimation that allows adaptation and successful breeding strategies for sustainable growth of plants in the face of cold injuries (Sanghera et al. 2011).

1.2 Cold Injuries: Chilling, Frost, and Freeze

Winter injury as well as freeze and frost injury are often synonymous. Cold injuries, however, are more severely manifested due to extreme temperature fluctuation, rather than prolonged low temperature conditions. Sudden temperature fluctuations

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like rapidly falling temperature and hard freeze can result in stress development and injury in plants that have acquired dormancy, but have not yet fully acclimated (Guy 1990). Acclimation to below-freezing conditions can successfully occur, only if the temperature fall is gradual, whereas deacclimation can occur if extended periods of mild winter occur, and this poses a massive threat to plants if they are suddenly exposed to extremely low temperature conditions. Such deacclimated plants are vulnerable to tissue injury and cold stress (Kalberer et al. 2006). However, prolonged low temperatures, viz., during winter, can also severely damage plants, mainly when the temperature drops below a certain tolerance limit. Plants that are already physiologically weak may be, due to previous stress exposures or due to lack of hardiness and adaptability to the harsh conditions of a specific geographical locale, are more prone to suffer from winter injury (Arora and Rowland 2011). The manifestation of winter injury is highly variable, though buds show maximum susceptibility (Fig. 1.1).

Chilling injury can be defined as damage incurred to plants due to temperature exceeding the freezing point (32°F or 0°C). Maximum susceptibility to chilling injury is shown by plants inhabiting tropical or subtropical climes. Flowers, fruits, and leaves are affected in the sensitive species, and manifestation in the form of purple or reddish wilting leaves is common. Frost and freeze injury are closely related since both lead to membrane damage due to osmotic shock, dehydration stress, and ice crystal formation. Frost damage occurs during radiation freeze,

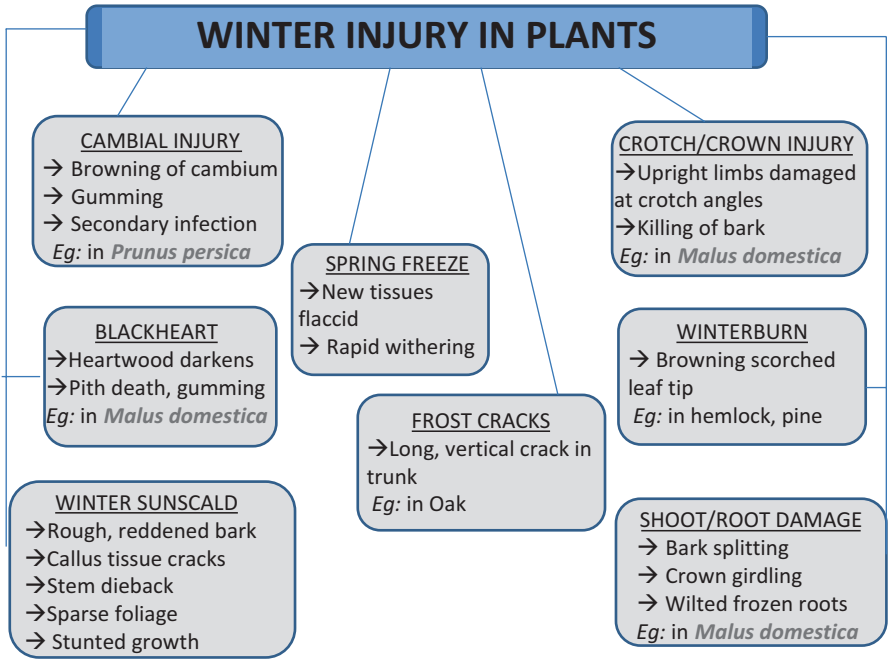

Fig. 1.1 Common manifestations of cold injury in the form of damages to root, shoot, and cambium in chilling-sensitive plants

mainly on calm and clear nights, when plants give off more heat than can be compensated for by the heat received by plants. Thus, it results in a temperature inversion, wherein cold air nearer to the ground is trapped by the warmer air layer above it (air temperature increases with altitude). When the air temperature at the plant level is near or below freezing, the temperature of the plant inevitably is lower than the ambient conditions. Freeze damage, on the other hand, occurs due to advective freezes, when an air mass with below-freezing temperature moves into and occupies an area, displacing warmer air. This causes the temperature of plants low enough to form ice crystals, hence damaging the tissues.

1.2.1 Freezing Injury in Plants

To generalize the term freezing injury, it is mainly concerned with the dysfunctions in physiology of the plant due to freezing of the water contained in plant tissues due to late spring and early fall frosts, low midwinter minima, and rapid temperature fluctuations. Freezing of tissue water is inevitably accompanied by ice formation, which may be intracellular or extracellular (Pearce 2001). Intracellular ice formation may be due to the following: (i) internal nucleation (large polysaccharides or proteins may act as nucleating agents for ice formation) and (ii) penetration (external ice crystals may penetrate into plant cells). Two types of freezing usually occur in plant cells and tissues: (i) vitrification (when rapid freezing of cells to very low temperatures causes the cellular content to get solidified into noncrystalline or amorphous state) and (ii) crystallization (ice crystallization due to gradual drop in temperature may be intracellular or extracellular). The more severe and damaging of the two is intracellular freezing, since it disrupts membrane integrity and can be lethal. Intracellular ice formation in susceptible tender plants is common; however, hardy plants before acclimation may also be affected. Intracellular ice may be formed spontaneously from centers of nucleation in the cytoplasm or may form in cell walls adjacent to intercellular spaces (apoplasm). Sometimes, ice may spread from cell to cell through plasmodesmatal connections. The plasmalemma can serve as a barrier to the entry of ice and hence can partially prevent dehydration, but cells and organelle tend to shrink and succumb to freezing injury to some extent. Thick cuticle can also serve as an effective shield that protects seedlings from external ice. Tissue damages due to freezing injury are characterized primarily by loss of membrane integrity, leakage of metabolites, and perturbations in plasmolysis as well as deplasmolysis.

1.2.1.1 Supercooling and Ice Nucleation

Some “deep supercooled” tissues in hardy plants may also show intracellular freezing. Deep supercooling is a mechanism by which plants avoid freezing injuries. The phenomenon by which water below freezing temperature still maintains its liquid

state is known as supercooling. Supercooling can occur in plants when the liquid held in the intercellular spaces does not make the transition from liquid to solid phase, and hence plants can avoid ice crystallization (Wisniewski et al. 2008). Some fruit trees and hardwoods are capable of supercooling down to –35°C; however, below –40°C, ice crystallization is spontaneous. Smaller crystals formed due to rapid freezing usually melt before causing cold injuries. Thus, water can indefinitely remain in supercooled state, unless the temperature falls below this homogeneous ice nucleation temperature or frost and soil ice invade plants through natural openings like stoma, lenticels, and wound sites. If external ice achieves nucleation, it rapidly spreads through vascular tissues, and the number and localization of nucleations depend on the initial extent of supercooling achieved by the plant. If supercooling is sufficient, multiple ice nucleation sites are available for the external ice to intrude.

Deep supercooling is achieved in some woody plants which involves supercooling of an aqueous fraction which is considerably isolated from seedling by an ice layer and is also divided into distinct compartments (Nuener et al. 2010). This compartmentalized pure water spontaneously freezes at –38°C. The presence of solutes depresses the spontaneous nucleation point as seen in lowering of supercooling by experimental addition of solutes to exotherm of shagbark hickory. In xylem parenchyma and flower bud tissues, ice penetration from adjacent frozen tissues is prevented by a barrier formed by undifferentiated cells between floral primordia in bud and nearby frozen stem tissues. Such barriers which prevent propagation of ice into healthy tissues may involve fine microcapillaries of cell wall, in addition to antinucleating chemicals in protoplasm.

1.2.1.2 Mechanism of Injury

(i) Intracellular freezing injury: intracellular freezing is a rapid process which results in flash freezing of cells which then allows ice crystals to propagate throughout protoplast and vacuole. Macromolecular assembly is disturbed due to mechanical tension and dehydration. Membrane integrity is hampered, and cellular compartmentalization is disrupted, resulting in leakage of hydrolyzing enzymes in the affected tissue. (ii) Extracellular freezing injury: extracellular ice imposes desiccation stress on the protoplasm, which is equivalent to drought stress, since water is removed from the cell to the extracellular ice. Dehydration of plant cells due to freezing injury can be lethal, primarily damaging the membrane of frost-injured cells. Following freezing, membrane proteins are rendered insoluble and protein dissociation into subunits occurs resulting in inactivation of enzymes, just as in case of drought stress. Membrane can thus be established as the primary site of desiccation stress. Membrane proteins are denatured due to a number of factors associated with freezing injury like pH imbalance, increased salt concentration, oxidation of sulfhydryl groups, and change in conformation due to water loss.

Tissue shearing in vascular tissues due to ice crystals has been observed in wheat crowns, azalea flower buds, and developing pear fruitlets. Freezing can also lead to shrinkage of protoplasts in injured plants. Lipoprotein membranes show fractures along hydrophobic regions, since intramolecular hydrogen bonds are weakened due to freezing injury.

1.2.2 Chilling Injury in Plants

Chilling injury is the damage incurred to chilling-sensitive plants at temperatures above the freezing point of tissues but lower than 15°C, i.e., injury at low but non-freezing temperature conditions. Plants which show visual manifestations of injury at temperatures exceeding 15°C are referred to as extremely chilling sensitive (Lukatkin et al. 2012). Accordingly, plants can be classified as (i) chilling-sensitive (severely damaged at temperatures above 0°C but below 15°C) and (ii) chilling-resistant (they are able to tolerate low temperature up to a tolerance threshold and show signs of injury only when ice formation occurs).

1.2.2.1 Mechanism of Chilling Injury

The physical phase transition of cellular membranes from flexible liquid crystalline to rigid gel structure at a temperature critical for chilling injury serves as a controlling response. Lowering of temperature in chilling-sensitive species leads to solidification of membrane lipids, which brings about contraction, causing cracks and channels and, consequently, increased permeability. This disturbed regulation of permeability leads to ionic imbalance and ion leakage from tissues. Enzyme activity is also hampered, since suitable temperature condition for optimum activity is not available. The temperature-induced phase change of membrane lipids is reversible till degenerative damage has been caused to the plant (Parkin et al. 1989).

1.3 Alterations in Cell Membrane: Marker for Chilling Stress Injury

The phase transition of cellular membranes from flexible, fluid state to rigidified solid state serves as a marker for detecting chilling-induced injury in plants. Such phase transition is characterized by the appearance of gel-like sites or microdomains in the plane of the lipid bilayer, which are partially or completely protein-free. Multiple membrane changes are detectable in stressed chilling-sensitive plants, viz., decrease in membrane elasticity, reduced compliance, preventing the inclusion of lipids in membrane composition, reduction of fluidity and hence

flexibility of membrane lipids, and inactivation of membrane-bound enzymes, including H^+-ATPase with increased lateral diffusion of phospholipids, sterols, and proteins in the plasma membrane (Kasamo et al. 1992; Kasamo and Noushi 1987).

Membrane functioning under chilling stress is dependent on the membrane lipids (Routaboul et al. 2000). In chilling-sensitive plants, membrane integrity is affected due to chilling-induced degradation of galactolipids and phospholipids, which result in an increased pool of free fatty acids. In stressed plants, a distinct change in molar ratio of sterols is observed, and increase in ratio of sterols/phospholipids resulted in decreased membrane fluidity on lowering of temperature (Whitaker 1993). A marked increase in unsaturated fatty acids, phospholipid accumulation in tissues, and depletion in sterols and sterol esters are physiological manifestations of chilling stress in sensitive species (Kojima et al. 1998; Kaniuga et al. 1999).

Membrane transport is severely affected due to reduced permeability associated with increased viscosity in response to low temperature. Hence, water uptake and sugar translocation were reduced in chilling-sensitive species. Distinct changes are also observed in the membrane proteins exposed to chilling stress (Fig. 1.2). Protein conformation is lost, and the nonprotein components of enzymes are released, resulting in changes in the allosteric control of activity and kinetic parameters. Low temperature-induced enzyme inactivation is also mediated by protein-lipid interactions in the membrane. Molecular ordering of membrane lipids changes due to low

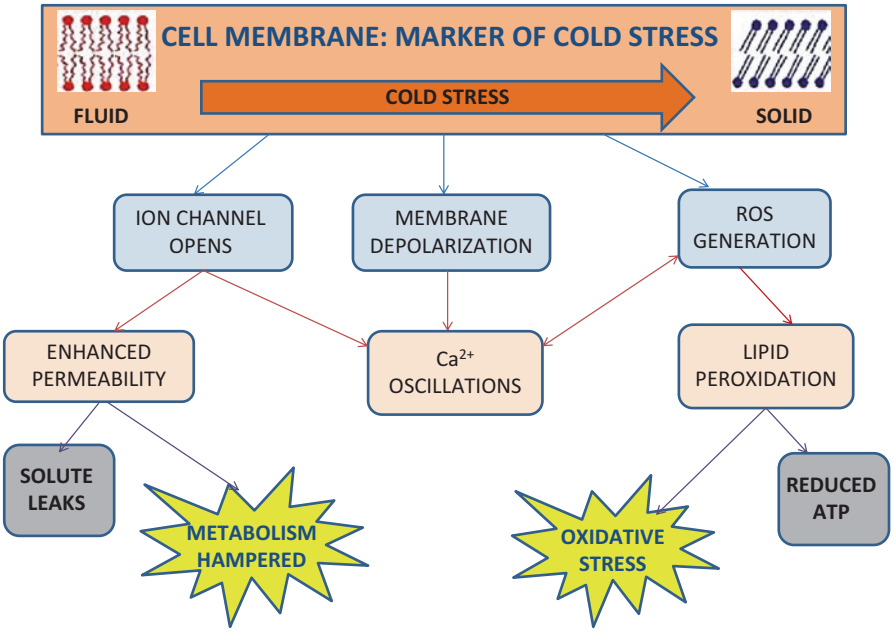

Fig. 1.2 Scheme for initiation of physiological changes on perception of cold stress cues with the plasma membrane serving as the main marker for detection of chilling stress; ROS (reactive oxygen species), ATP (adenosine triphosphate)

temperature exposures. These changes are accompanied by lowered ATP levels and increased membrane permeability. Hence, the membrane is the seat for the detection of chilling-induced injury in plants. Prolonged exposure to chilling stress disrupts membrane integrity and compartmentalization, solute leakage, and increase in the activation energy barrier for membrane-bound enzymes, thus jeopardizing the overall physiological status of the affected species.

1.4 Cold Perception and Downstream Signaling

Environmental cues perceived by the plant result in an intricate network of downstream signaling cascades. Different receptors at the cellular level are involved in receiving the external signals and, in turn, transfer them intracellularly. Plants are sensitive to both magnitude and rate of temperature fluctuations. Thermal responses in plants in the face of cold stress involve a complex intracellular machinery and genetic regulation. There are two principal transcriptional pathways that are activated in response to cold stress, C-repeat (CRT)/dehydration responsive element (DRE)-binding factor (CBF/DREB)-dependent and CBF/DREB-independent. The transcription factor, CBF, acts as a master regulatory player and is induced by the binding of trans-acting factors to the promoter regions of the *CBF* gene (Fowler and Thomashow 2002). The constitutively expressed ICE1 (Inducer of CBF Expression 1) binds to the corresponding cis element on the *CBF* promoter and elicits the ICE1-CBF cold-responsive pathway, which is conserved in diverse plant species (Chinnusamy et al. 2003).

1.4.1 Stress Perception Through Plasma Membrane Rigidification

The physiological responses of plants to stress are variable; however, membrane rigidification is a common response, since rapid fall in temperatures induces membrane to become rigid at microdomains. Signaling pathways involving calcium waves have also been worked out in alfalfa and *Brassica napus*, where cold stress induction led to actin cytoskeletal rearrangement and loss of fluidity of the plasma membrane, activation of Ca^{2+} channels, and, hence, rapid calcium oscillations (Orvar et al. 2000). Increased cytosolic Ca^{2+} levels induce the expression of *cold-responsive (COR)* genes, which can be activated artificially by a membrane rigidifier like dimethyl sulfoxide (DMSO) even at 25°C, while its expression is inhibited by a membrane fluidizer like benzyl alcohol even at 0°C (Sangwan et al. 2001). Ca^{2+} is the ubiquitous second messenger and is a major player in the cold-responsive signaling pathways (Knight et al. 1996), and mechanosensitive calcium channels have been found to be involved in cold acclimation. Intracellular calcium ion

channels implicated in *COR* expression are activated by cyclic ADP-ribose and inositol-1, 4, 5-triphosphate (IP_3). A typical Ca^{2+}-responsive signaling pathway consists of Ca^{2+}-activated phospholipase C and D (PLC, PLD) which produce IP_3 and phosphatidic acid, respectively, and, in turn, activate IP_3-gated calcium channels.

Rise in intracellular calcium levels can be perceived by calcium-dependent protein kinases (CDPKs) and calmodulins (CAMs) and salt overly-sensitive 3-like (SOS3-like) or calcineurin B-like (CBL) proteins. That CDPKs play a functional role in cold stress signaling was proved through a transient expression system in maize leaf protoplasts where a constitutively active form of an *Arabidopsis* CDPK (CDPK1) activated the expression of abscisic acid (ABA)-responsive promoter of *HVA1* gene (Sheen 1998). Hence, CDPKs were proved to have a positive role in mediating cold signaling; however, CAMs, CBLs, and SOS3-like proteins are negative regulators of such signaling cascades.

1.4.2 The CBF-COR Regulon: Transcriptional Machinery

Cold stress response is mediated by a gene regulatory network in which the CBFs are critical transcription factors, as they are involved in the control of the *COLD-REGULATED (COR)* genes through the CBF-COR regulon (Thomashow 1999). CBFs are also involved in the drought and salinity stress-responsive pathways, thereby proving that there exists an intricate cross-talk mechanism between the different forms of abiotic stress. The CBFs belong to the APETALA/ethylene response element-binding protein (AP2/EREBP) transcription factor family (Stockinger et al. 1997) and are modulated by upstream regulators like inducer of CBF expression 1 (ICE1), *high expression of osmotically responsive 1* (*HOS1*) gene, and MYB15 (Agarwal et al. 2006). ICE1 is a constitutively expressed myc-like bHLH (basic helix-loop-helix) transcription factor, which binds to *CBF3* gene promoter, inducing its expression, and is degraded via the ubiquitin-proteasomal pathway through the cold signaling attenuator HOS1, an E3 ubiquitin ligase (Dong et al. 2006).

The promoter regions of *COR* genes consist of one or multiple copies of the C-repeat/DRE with the highly conserved CCGAC core sequence. The CBFs or DREBs control ABA-independent expression of *COR* genes in response to cold stress, which indicates that ABA may be able to potentiate cold-induced CBF signaling, but ABA and cold stimuli may not be concurrent. CBF-DREB1 is involved in transcriptional response to cold as well as osmotic stress-regulated genes, whereas CBF/DREB2 is exclusively responsive to cold stress, and not to salinity or osmotic stress conditions, and is controlled by ICE1 transcription factor. This further provides an insight into the cross-talk of abiotic stress-responsive signaling pathways. Microarray analysis of CBF-overexpressing transgenic plants identified several CBF target genes involved in signaling, transcription, osmolyte biosynthesis, reactive oxygen species (ROS) detoxification, membrane transport, hormone metabolism, and stress response and can sufficiently induce cold tolerance in diverse plant species, e.g., *AtCBF1* of tomato enhanced oxidative stress tolerance under chilling