Chapter 3
Cold Tolerance

Mike Humphreys and Dagmara Gasior

Abstract Considerations of preparations for a changing climate will generate thoughts of mitigating a rise in temperature and greenhouse gas emission and a change in water availability. Accordingly, reduced prioritization on future research objectives aimed at crop adaptations sufficient to instigate and sustain cold tolerance or winter hardiness expression at any specific location might be deemed by some as the logical outcome. However, such a conclusion would be a grave mistake. With increasing frequency, crops of high agricultural value are being grown at locations beyond their natural ranges of adaptation, a consequence in part of farmers attempting to seize new opportunities to exploit some positive scenarios of climate change that might provide more profitable agricultural output. A second and even greater driver is man's response to the ever-increasing requirement to feed a growing global population, and with only limited and finite land available that is deemed suitable for agricultural use. For the latter, there is increased use of marginal locations for agricultural production, which will include those locations at high altitude where temperatures are frequently suboptimal for crop production and, in many cases, likely to challenge crop persistency over winter months. In certain temperate locations, where winter temperatures are considered generally moderate, crop growing seasons are becoming extended, encouraged frequently by national policy makers seeing economic advantages in management practices that can achieve an all-year-round cropping potential, but with a great risk. The maintenance of crop growth is the consequence of failure, at least in part, of the initiation and subsequent expression of the appropriate adaptive responses necessary to assure a high probability of winter survival which include growth cessation. Such scenarios place crops at risk of total collapse following any sudden temperature drop and especially onsets of frost conditions. In situations of fluctuating winter temperatures, assured crop survival requires the maintenance of the required adaptive response in place until such time as there is little likelihood of

M. Humphreys (✉) • D. Gasior
IBERS, Aberystwyth University, Gogerddan, Aberystwyth SY23 3EE, Ceredigion, Wales, UK
e-mail: mkh@aber.ac.uk

C. Kole (ed.), *Genomics and Breeding for Climate-Resilient Crops*, Vol. 2,
DOI 10.1007/978-3-642-37048-9_3,

any further incidence of frosts. For optimal crop production, the subsequent appropriate timing of the cessation of the adaptive responses is also essential to enable crop growth to proceed fully as soon as possible, once growth advantageous spring conditions arise.

Cold tolerance and winter survival are complex traits, each having distinct genetic controls and involving responses to the many interacting stresses, their relative importance dependent on crop location. Frost tolerance is considered the trait of main priority with the understanding and manipulation of the factors necessary to optimize initiation of the appropriate cold acclimation responses sufficient to retain cell membrane integrity and prevent desiccation, the most appropriate objectives in crop improvement. Gene expression sufficient to initiate frost tolerance has many equivalent requirements and responses to those required to combat other abiotic stresses that can induce cell desiccation such as prolonged exposures to conditions of drought or salinity. Some of the major aspects and their relative importance are reviewed herein.

3.1 Introduction

Unlike animals that can seek shelter during winter, land plants are stationary and exposed to all that the weather might throw at them; to survive, they must either adapt to the climate native to their specific location or alternatively avoid the worst conditions via their ontogeny either through entry into vegetative dormancy for perennial species or in the case for summer annuals, by completing their life cycles and reproductive phase prior to the full onset of winter conditions, overwintering as seed.

Greaves (1996) defines suboptimal temperature stress as "*any reduction in growth or induced metabolic, cellular or tissue injury that results in limitations to the genetically determined yield potential caused as a direct result of exposure to temperatures below the thermal thresholds for optimal biochemical and physiological activity or morphological development*."

The human population is increasing at an alarming rate and is anticipated to rise globally to nine billion by 2050, while at the same time agricultural productivity in many regions is decreasing due to the effects of increasing environmental stresses. While the impact of droughts on crop yields receives with some justification particular attention, cold stress is also a serious threat to the sustainability of crop yields and can lead to major crop losses that can include detrimental effects on their quality and post-harvest life.

With population growth, a demand for increased food production will follow, and already an increasing use of marginal lands for agriculture is being observed together with the frequent use of crop species not adapted ideally to the growth conditions they are likely to encounter. It would be incorrect to assume that cold stress was an issue restricted only to agricultural systems in extreme northern and southern latitudes. In countries where land suitable for agriculture is limited or fully

utilized, increasing use is being made of alternative marginal regions at altitudes higher than previously employed for agriculture where low temperatures are encountered that may and frequently do provide constraints to efficient crop persistency and production.

At higher latitudes, low temperature is the most important limiting factor and is especially important in northern regions such as Norway where forage species are the major components of agricultural land. In such locations, winters are long and growing seasons very short. In less extreme conditions than those found in northern Europe, the increased use of marginal regions for crop production has led to the expanded use of crops such as maize and rice. In part, this has been encouraged by farmers making use of rising temperatures having resulted from climate change to grow crops considered previously completely unsuitable to their farming locations. For example, in northern Britain, increased temperatures have led to the use of forage maize in areas close to and beyond their safe margins of acclimatization bringing potential risk as frequently temperature extremes and weather patterns fluctuate wildly in such locations over very short time periods. Any small deterioration in anticipated weather conditions can result in potentially total crop failures. In addition, with expanded use of certain crops and due to rising temperatures, plant pathogens previously uncommon in certain locations are becoming more prevalent and elsewhere appearing earlier in the growing season than in previous years, e.g., crown rust in northern Britain (Roderick et al. 2003). Alternative biotic and abiotic stresses interact frequently to exacerbate crop loss. Furthermore, the growth of "new" crops by farmers previously untrained and unfamiliar to their use and their requirements for optimal growth will only further exacerbate through poor farming practice, the dangers to crop production.

Chilling or cold stress (temperatures 0–15 °C) and frost stress (<0 °C) are distinct from one another and should be considered, at least in part, as different stresses as they harbor alternative mechanisms for plant resistance, and these controlled unsurprisingly by different genetic determinants. Rice may suffer chilling injury even at temperatures as high as 15 °C. Protection against cold stress can be achieved through the use of the correct agronomic methodologies and also by breeding and the use of the best adapted varieties. One example of the use of appropriate farming practice is the late sowing of warm-season crops to avoid encountering the lowest expected temperatures at the germination stage when crops are especially vulnerable and, in reverse, a strategy that uses early varieties to avoid an encounter with cold at their maturation stage (Caradus and Christie 1998).

Most temperate plants acquire chilling and freezing tolerance upon prior exposure to sublethal low temperature and reduced day-length, a process called cold acclimation (CA). Many physiological and biochemical changes occur during CA including slowed or arrested growth; reduced water content; protoplasm viscosity; alterations to photosynthetic pigments; reduced ATL levels (Levitt 1980); transient increases in abscisic acid (ABA) (Chen et al. 1983); changes in membrane lipid composition (Uemura and Steponkus 1994); accumulation of compatible solutes

including proline, betaine, polyols, and soluble sugars; and the accumulation of antioxidants (Tao et al. 1998).

Considerable resources are necessary to sustain and protect plant metabolism under low temperature stress and for subsequent recovery following the onset of more benign growth conditions. Our best known temperate grasses, cereals, and many other crops like artichokes, asparagus, leeks, and onions all store fructans, a soluble polymer of fructose molecules capable of rapid polymerization and depolymerization. The partitioning of solutes is important because survival from freezing depends on survival of apices, particularly the lateral buds rather than mature leaf tissue (Eagles et al. 1993).

Many agronomically important crops are incapable of CA. Cold stress affects virtually all aspects of cellular function in plants. The cold stress signal is transduced through several components of complex signal transduction pathways. The main components are calcium, reactive oxygen species, protein kinase, protein phosphatase, and lipid signaling cascades. The plant hormone abscisic acid (ABA) also mediates the response of cold stress and functions in many plant developmental processes including bud dormancy. It also has a major role in drought tolerance where it is produced in plant roots in response to the onset of decreased soil water potential. The cold stress signal leads to regulation of transcription factors and effector genes known collectively as cold-regulated (*COR*) genes. The effector genes encoding proteins under this category include chaperones, late embryogenesis abundant (LEA) proteins, osmotin, antifreeze proteins (AFPs), mRNA-binding proteins, and key enzymes for osmolyte biosynthesis such as proline, water channel proteins, sugar and proline transporters, detoxification enzymes, enzymes for fatty acid metabolism, proteinase inhibitors, ferritin, and lipid-transfer proteins. The transcription factors involved during cold stress response are inducers of C-repeat binding factor expression-1, C-repeat binding factors, myeloblasts, and mitogen-activated protein kinase. Analyses of the expression of *COR* genes indicate the presence of multiple signal transduction pathways between the initial stress signals and the outcome of gene expression. Use of these genes and transcription factors in genetic modification of agricultural crops can improve cold tolerance and productivity.

Various phenotypic symptoms in response to cold stress include poor germination, stunted seedlings, yellowing of leaves (chlorosis), reduced leaf expansion, and wilting, and these may lead to death of tissue. Cold stress also severely hampers the reproductive development of plants.

3.2 Winter Hardiness

Winter hardiness in temperate plant species is a complex trait comprising the ability to survive a range of abiotic and biotic stresses, which include freezing, ice encasement, waterlogging, soil heave, desiccation by dry winds, starvation, and snow mold. Winter hardiness in a plant has three components: acclimation,

midwinter hardiness, and de-acclimation. The acclimation process is triggered late in the growing season by decreasing photoperiods as day-lengths shorten and as temperatures decline. These environmental cues induce physiological and biochemical changes in the plant that then result in greater cold tolerance.

In short, plant winter hardiness is the outcome of a seasonal shift between growth, quiescence, and assimilate storage in response to a cool temperate climate. Its level of effectiveness will vary and will be dependent on the location and on genotype × environment (G × E) interactions. As with breeding for drought resistance, prior to any accurate assessment of crop performance and winter hardiness, it is necessary that new varieties should be assessed under all or as many as practical the environmental scenarios they are likely to encounter if they are to be used widely commercially. G × E interactions are complex. For example, a winter hardy plant growing in a maritime environment will not necessarily have an equivalent adaptation when transferred to a continental climate and vice versa.

It should be noted that combinations of fluctuating temperatures and changes to light intensities common to what are considered mild maritime climates such as those found in the UK can be as hazardous to a plant's survival as those that are encountered following persistent severe subzero temperatures such as those found commonly in extreme northern or southern latitudes and will require an alternative adaptive response. However, as a general requirement when plants are exposed to subzero temperature, there is a need for resistance to desiccation through the maintenance of the integrity of the plant cell membrane.

After CA, certain species can withstand extreme low temperatures. Non-acclimated (NA) birch and dogwood are injured at −10 °C but after CA can survive experimental freezing to −196 °C and survive −40 °C to −50 °C under natural conditions. NA wheat and rye are killed at −5 °C to −10 °C but after CA, wheat can survive to −15 °C and rye to −30 °C (Thomashow 1990). Among grasses, the most freezing-tolerant forage grass used in European agriculture is Timothy (*Phleum pratense*) whose minimum temperature survival has been measured as −25 °C.

Possibly the most important component of winter hardiness in Northern Europe is the ability to withstand periods of ice encasement. Ice-encasement tolerance expressed as LD_{50} (lethal dose for 50 % of plants) varied from 50 days at −1 °C in Berings hairgrass (*Deschampsia beringensis*) to 2 days in a cultivar of orchard grass (*Dactylis glomerata*) (Humphreys and Humphreys 2005).

In general, a tolerance to freezing temperatures is the most important component for winter survival, but also of considerable importance as described earlier is the capability to withstand combinations of stresses due to desiccation, which is influenced also by wind and occurrence of ice encasement. Other factors affecting winter survival is resistance to mechanical heaving and also low light, snow cover, winter pathogens, and fluctuating temperatures, with the relative importance of each depending on the location.

Cold and freezing tolerance are complex traits governed by many gene loci of greater or lesser importance and involving epigenetic and pleiotropic effects. It has been demonstrated that in certain crops such as the outbreeding forage grasses of the *Lolium–Festuca* complex, the potential of a plant genome for cold-tolerance

expression may in certain cases not be achieved fully until obstacles to their full expression are removed or suppressed (Rapacz et al. 2005). In this case, through gene segregation at meiosis, cultivars and plant genotypes that were previously considered to have low winter hardiness and poor snow mold resistance were able to produce novel progeny by androgenesis that expressed both traits with extreme efficiency to extents well in excess of their parent genotypes. In another study, the authors proved that freezing tolerance (FT) was only loosely correlated with tolerance of ice encasement in ten forage grass species (Gudleifsson et al. 1986).

In Icelandic grasslands, the physical properties of the soils affect survival rates; snow mold was not considered as important a factor as was ice encasement, which was the main cause of plant damage (Gudleifsson 1971). Andrews and Gudleifsson (1983) found no correlation between FT and ice-encasement tolerance in cultivars of Timothy and concluded that the greater winter hardiness of Timothy relative to other species was due to its high resistance to ice encasement. Spring ground cover studies in Nordic countries (Nordic Gene Bank 1996) showed that Timothy cultivars were more damaged by ice (~50 % damage) than snow cover (~25 % damage), with frost alone causing intermediate damage (~30 %).

The rate and extent of de-hardening is a critical factor in winter survival. Overwintering plants are particularly susceptible to freezing damage in the spring if the de-acclimation process occurs either prematurely or too rapidly, or if unpredictable temperature fluctuations occur (Levitt 1980; Gay and Eagles 1991). Eagles (1989) suggested that the nature of an adaptive CA process will vary with the stability and predictability of winter conditions in a particular environment. In stable and predictably cold continental climates where the onset of freezing temperatures is rapid, a photoperiod-triggered and rapid acclimation process is desirable, while in the more variable and less severe conditions of a maritime climate, a temperature-dependent response might enable plants to exploit a mild autumn or spring by continuing to grow. However, in cultivars adapted to maritime climates, de-acclimation may occur in response to fluctuations in winter and spring temperature with a risk of damage by subsequent frosts (Eagles 1994).

A changing climate that generates a change in plant ontogeny will require a change in strategy by the plant breeder and will require a complex and holistic approach to counter interacting plant stresses. At locations where winter temperatures are increasing as a consequence of climate change, so that continued plant growth is encouraged, where previously it was prevented due to low temperatures that induced winter dormancy, and also where precipitation is decreasing, that priorities for crop designs for resistance to stresses other than those associated normally with winter such as drought-tolerance, previously considered important only at other times of the year and at alternative growth stages, will become major requirements necessary to safeguard crop yields. A decrease in winter rainfall that causes unseasonal winter droughts is becoming more commonplace and this will require a new breeding strategy that encompasses a common stress tolerance to what was considered previously solely as winter and summer stress factors.

Simulations predict the following changes in the Norwegian climate towards the year 2100: changes in mean yearly temperatures of 2.5–3.5 °C, most pronounced in

continental and northern regions; milder winters with increased minimum temperatures of 2.5–4 °C, more frequent incidents of freezing-thawing cycles; and less snow cover in continental regions (Rognli pers. com–RegClim project). The increasing mean temperatures will lead to prolonged growth seasons and give increased biomass production, especially in annual crop plants but also in perennial crops provided that they are well adapted to the changing winter climates of which increased freezing stress possibly will be the most challenging.

Ice encasement, waterlogging, and soil compaction all include a component of hypoxic or anoxic stress. In waterlogged soils, respiration by roots and soil microflora depletes dissolved oxygen and toxic by-products of anaerobic respiration accumulate (Pulli 1989). In colder climates, soil and root respiration is slowed but freezing and ice encasement may completely seal soil and plant surfaces against penetration by oxygen. Injury to cells may result from ethanol self-poisoning, cytoplasmic acidosis, insufficient adenosine triphosphate (ATP) generation, and metabolic lesions caused by reentry of oxygen (Vartapetian and Jackson 1997). The shoot suffers an impeded supply of water, minerals, and root hormones.

Metabolic adaptations are important in short-term survival of anaerobic stress (Vartapetian and Jackson 1997). Increased activity in both the glycolytic and fermentation pathways is observed, along with increased expression of enzymes in this pathway.

Avoidance of cytoplasmic acidosis by early switching from production of acidic lactate to neutral ethanol may underlie root tolerance of reduced oxygen (Davies 1980). In this model, transient lactate fermentation acidifies the cytoplasm at the onset of anaerobiosis, suppresses lactate dehydrogenase (LDH) activity, and triggers pyruvate decarboxylase (PDC). Transient acidosis of the cytoplasm has been shown in excised maize root tips within 20 min of transfer to anaerobic conditions (Roberts et al. 1982), and direct manipulation of cell acidity substantiated the idea of a pH switch (Fox et al. 1995).

Anatomical adaptations in roots may promote survival by allowing avoidance of anoxia (Jackson 1990) and include root aerenchyma, which transports oxygen to, and removes volatiles from, the roots. Dormancy is exhibited by shoot tissue of many species exposed to prolonged anaerobic conditions as in the rhizomes of wetland species (Brändle 1991). Stomatal closure, epinastic leaf curvature, slowed leaf expansion, and enhanced leaf senescence are also triggered by soil waterlogging, as a means of reducing transpiration (Else et al. 1995).

3.3 Genetic Adaptations for Cold Acclimation and Improved Winter Hardiness

Altered gene expression during CA (Guy et al. 1985) has been demonstrated in a range of crop species (Hughes and Dunn 1996) and the model species *Arabidopsis thaliana* (Thomashow 1998). Cold-responsive genes are involved in biochemical

and physiological changes required for growth and development at low temperature or are directly involved in freezing tolerance (FT) (Thomashow 1998).

The major negative effect of cold stress is that it induces severe membrane damage due largely to the acute dehydration associated with freezing. Cold stress is perceived by the receptor at the cell membrane. Then a signal is transduced to switch on the cold-responsive genes and transcription factors for mediating stress tolerance. Understanding the mechanism of cold stress tolerance and genes involved in the cold stress signaling network is a vital step for crop improvement.

Several studies have established that major genes, or gene clusters, involved in the control of frost and drought tolerance are located on a region of the long arm of Triticeae group 5 chromosomes. Traits like winter hardiness (Hayes et al. 1993; Pan et al. 1994), vernalization response and frost tolerance (Sutka and Snape 1989; Galiba et al. 1995; Laurie et al. 1995), cold- and drought-induced ABA (abscisic acid) production (Galiba et al. 1993; Quarrie et al. 1997), and osmotic stress tolerance (Galiba et al. 1992) have all been mapped to this region. Across the grasses and cereals, this chromosome region has been a major focus for genome study and for crop improvement.

Development of winter hardiness requires exposure of plants to low nonfreezing temperatures, typically 0–10 °C, and a shortened photoperiod. The majority of research studies for crop winter survival have focused on genetic changes that affect the key stage of CA either by natural breeding, often employing gene transfers from wild crop relatives (e.g., Humphreys et al. 2007). For many countries, due to concerns with the use of genetic modification (GM) technologies, plant breeding offers the only opportunity for an improved crop design, but transgenic technologies may be employed either as "proof of principle" of gene function or directly in crop improvement when restrictions on the use of GM technologies are loosened (Sanghera et al. 2011). In both approaches, efforts have concentrated on inclusion of functional genes necessary for the induction of appropriate physiological mechanisms required to withstand exposures to freezing temperatures. For all crops to survive the winter, plants must engage mechanisms whereby sensitive tissues can avoid freezing or undergo cold hardening compatible with the normal variations of the local climate, coordinate the induction of the tolerance at the appropriate time, maintain adequate tolerance during times of risk, and properly time the loss of tolerance and resumption of growth when the risk of freezing has passed (Guy 1990). For some locations and latitudes, should winter temperatures continue to rise following climate change, it may be necessary for breeding efforts to concentrate more on adaptation to short day-length rather than to a tolerance of low temperature in order to both achieve winter hardiness and also to avoid a vernalization requirement otherwise necessary for flower induction, of course an essential prerequisite for seed production and a crop yield.

Considerable resources are necessary to sustain and protect plant metabolism under low temperature stress and for recovery subsequent to the onset of more benign growth conditions. CA and freezing tolerance are the result of a complex interaction between low temperature, light, and photosystem II (PSII) excitation pressure. The redox state of PSII reflects fluctuations in the photosynthetic energy

balance and so acts as a sensor of any environmental stresses that disturb that balance. Changes to the redox state of PSII, triggered by a low-temperature shift, were proposed to be a temperature-sensing mechanisms involved in cold acclimation (Rapacz et al. 2004). Humphreys et al. (2006) reported how non-photochemical quenching (NPQ) mechanisms for expulsion of excess light energy during CA are found in the forage grass species *Festuca pratensis*, which is adapted to northern latitudes, but are not expressed similarly in its close relatives, the major agricultural grasses *Lolium perenne* and *Lolium multiflorum,* which are adapted to lower latitudes. As will be described below, alien gene transfers between *F. pratensis* and *Lolium* spp. have enhanced PSII adaptation to freezing temperatures and have led to improved CA efficiency and to freezing tolerance (Humphreys et al. 2007).

3.3.1 A Novel Introgression-Mapping Approach for PSII Adaptations to Freezing

Modern breeding methods can make use of evolved adaptations to cold stress in wild-type relatives to improve their freezing tolerance (FT). The technique introgression mapping is employed currently in the *Lolium–Festuca* complex for trait "dissection" and transfers of key alleles from donor to recipient species by natural plant breeding. The grass complex provides the main grass species used for livestock agriculture and offers excellent opportunities for analyzing the genetic determinants of both simple (e.g., Moore et al. 2005) and complex traits (e.g., Humphreys et al. 2005). The highly heterogeneous genotypes and phenotypes typical of *Lolium* and *Festuca* species are a consequence of excessive genome reorganization due to the promiscuous chromosome recombination that occurs in these obligate outbreeding species. Their heterogeneous nature has provided *Lolium* and *Festuca* species with considerable allelic variation and adaptive capabilities for the successful colonization and establishment as distinct ecotypes evolved to growth in contrasting climates within temperate grasslands throughout the world. *Festuca* species are generally better adapted than *Lolium* to marginal areas where they may be exposed to the more extremes of abiotic stresses (Humphreys et al. 1998). *F. pratensis* (meadow fescue) is a particularly good source of genetic variants for cold tolerance (Alm et al. 2011). In terms of CA, a very important recent development was the discovery of a potential role for photoreceptors in *Lolium* and *Festuca* responses to low temperatures (Rapacz et al. 2004).

Cold acclimation and freezing tolerance (FT) are the results of a complex interaction between low temperature, light, and photosystem II (PSII) excitation pressure. At low temperatures, plants have two principal difficulties: the maintenance of cell membranes in a fluid state, and the thermo-dependency of photosynthetic electron transport and carbon fixation which are slowed at low temperature (Guy 1990; Huner et al. 1996; Thomashow 1999). The PSII reaction center is the

key site for regulation of light energy and also the main site of photoinhibitory damage. The D1/D2 protein dimer at the core of PSII appears to be crucial in maintaining the integrity of the complex (Mattoo et al. 1989). Indeed, Canter et al. (2000) demonstrated in *F. pratensis* that repair measures to the chloroplast are CA induced. A representation difference analysis was used to amplify selectively upregulated cDNA fragments from cold-induced *F. pratensis* seedlings. The gene *psba,* which codes for the D1 protein of PSII, was prominent among the upregulated cDNA fragments and was recovered following 4 days of CA.

Photoinhibition (the light-induced reduction in the photosynthetic capacity) is related to the redox state of PSII expressed as excitation pressure. The redox state of PSII reflects fluctuations in the photosynthetic energy balance and so acts as a sensor of any environmental stresses. Changes that disturb that balance include those triggered by a low-temperature shift and have been proposed to be one of the temperature-sensing mechanisms involved in CA (Rapacz 2002).

During autumn and winter, plants are subjected to excess light compared to the energy demand of dark photosynthetic reactions. This can cause photoinhibition. When the rate of PSII damage exceeds the rate of repair, photoinhibition occurs reflected in decreased F_v/F_m (maximum quantum yield of PSII). Rapacz et al. (2004) using plant populations derived from *L. multiflorum* × *F. pratensis* cultivars reported a relationship between cold tolerance and F_v/F_m. There was a strong negative correlation between maximum quantum yields of PSII (F_v/F_m) before winter and winter survival, with plants with higher F_v/F_m having lower winter survival. It was found among *L. multiflorum* × *F. pratensis* hybrid plants that those that were winter hardy were also more resistant to cold-induced inactivation of PSII. This was due primarily to increases in non-photochemical quenching (NPQ) during CA where excess energy was dissipated by heat via the xanthophyll cycle. Humphreys et al. (2006) reported a significant increase in NPQ activity by *F. pratensis* genotypes in response to CA conditions, a response that was not repeated by *Lolium* species thereby providing one possible explanation why *F. pratensis* has more efficient CA capabilities.

A set of seven chromosome substitution lines where *F. pratensis* chromosomes replaced homoeologous *Lolium perenne* chromosomes (King et al. 2002a, b) in an otherwise undisturbed *Lolium* genome were used by the current authors to locate the source of the *F. pratensis* genes responsible for NPQ expression. The substitution lines were assessed for changes in PSII during a CA period of 2 weeks and for NPQ at two contrasting light intensities (PAR 175 μmol m^{-2} s^{-1}, and PAR 250 μmol m^{-2} s^{-1}). All CA substitution lines were subsequently freeze tested and the LT_{50} (freezing temperature necessary to induce 50 % tiller mortality) determined.

The chlorophyll fluorescence studies on the different chromosome substitution lines demonstrated that *L. perenne* in combination with different *F. pratensis* chromosomes responded to CA and light of different intensities, in different ways. However, it was the PSII response by the *F. pratensis* chromosome 4 substitution line that was associated primarily with efficient induction of NPQ expression under high light intensity, and this corresponded with enhanced freezing

tolerance. In support of this new research outcome, Humphreys et al. (2006) provided preliminary evidence that the presence of an *F. pratensis* translocation at the end of *L. multiflorum* chromosome 4 led to increased CA-induced NPQ, and this was frequently associated with an improvement in freezing tolerance. Simple sequence repeat (SSR) markers were associated with the alien gene transfers suitable for future use in marker-assisted breeding (Mike Humphreys, unpublished).

Using the extensive macrosynteny between related grass crops (rice, Triticeae) among the Pooideae, a comparative crop species study for mapping orthologous gene loci led to the publication of the first genetic linkage map in *F. pratensis* for quantitative trait loci (QTL) associated with abiotic stress resistance (Alm et al. 2011). The study showed that major structural differences exist between *Lolium*/*Festuca* and *Triticeae* chromosomes 4 and 5, which are especially relevant to the acquisition of freezing tolerance. It is considered that two major frost tolerance and winter survival QTLs on *Festuca* chromosome 5 correspond to the *Fr-A1* and *Fr-A2* loci on homoeologous Group 5A in wheat (Sutka and Snape 1989; Vagujfalvi et al. 2003). However, an important QTL for frost tolerance was located at a terminal region of *Lolium*/*Festuca* chromosome 4, which contains the vernalization locus *Vrn-1* (Jensen et al. 2005), which in the Triticeae is located on chromosome 5. In a study of near-isogenic lines (NILs) of wheat, it was shown that the *Vrn-1-Fr-1* interval accounted for 70–90 % of the difference in the FT of the NILs substantiating the importance of this locus in CA (Storlie et al. 1998). An interaction between the vernalization and CA regulatory gene networks are thought to operate by extending the CA period and increasing the frost tolerance by delaying the induction of *Vrn-1* and thereby the transition to the reproductive stage (Galiba et al. 2009). The structural difference between chromosomes 4 and 5 of *Festuca* and Triticeae makes it possible to separate the effects of vernalization and frost tolerance/winter survival.

The identification of *F. pratensis* genes on chromosome 4 thought to also contain the *Vrn-1* locus and now with proven involvement in a CA response by PSII for the first time provides us with both a mechanism for improved FT, and through introgression mapping, the means by which to access the *F. pratensis* genes responsible to improve winter hardiness and freezing tolerance in *Lolium*.

3.3.2 Cold-Regulated Genes

The C-repeat binding factor (*CBF*) genes are key regulators of the expression of *COR* (cold-regulated genes), which are conserved among diverse plant lineages such as dicots and monocots. The CBF transcription factors recognize the cis-acting CRT/DRE (C-repeat/dehydration-responsive element) element in the regulatory regions of *COR* genes (Stockinger et al. 1997). Twenty *CBF* genes have been identified in barley (*Hordeum vulgare*), of which 11 are found in two tight tandem clusters on the long arm of chromosome 5H in the same region as the *Fr-H2* frost

tolerance locus (Skinner et al. 2006; Francia et al. 2007). An orthologous genomic region in *Triticum monococcum* contains similar *CBF* gene clusters located at the *Fr-A*^{m}*2* frost tolerance QTL (Vagujfalvi et al. 2003; Miller et al. 2006). In *Lolium perenne,* Tamura and Yamada (2007) mapped four *LpCBF* genes in a short interval on *Lolium* LG5 most likely syntenic with regions on Triticeae group 5 chromosomes. Studies of the organization of the *CBF* cluster in barley and wheat have shown that the number of *CBF* genes at the *Fr-H2*/*Fr-A1* locus may vary among cultivars with winter forms having a higher copy number of some *CBF*s (Francia et al. 2007; Knox et al. 2010). The cosegregation of the *CBF* gene clusters with the barley *Fr-H2* and wheat *Fr-A*^{m}*2* frost tolerance loci, their role in cold acclimation (Stockinger et al. 1997), and the association of transcript levels of *CBF* genes with *FT* loci (Vagujfalvi et al. 2003) make them obvious candidates for one of the two major frost tolerance QTLs on Triticeae group 5 chromosomes. The locations of two frost tolerance/winter survival QTLs on the chromosome 5F of the forage grass *Festuca pratensis* correspond most likely to the *Fr-A1* and *Fr-A2* loci on wheat homoeologous group 5A chromosomes; one of these QTLs (*QFt5F-2*/*QWs5F-1*) has *FpCBF6* as a candidate gene and shown to be rapidly upregulated during CA (Alm et al. 2011).

Using a targeted approach of achieving understanding of key regulatory mechanisms through crops of common ancestry and using conserved genome regions and synteny, knowledge achieved through studies made in fully sequenced model crops and organisms such as rice, *Brachypodium,* or *Arabidopsis* may be used in crop genome studies where researchers lack access to equivalent resources.

A similar approach may be applied to the photoperiodic response where Triticeae Group 1, 2, and 7 chromosomes have known genes and QTLs for photoperiodic response (Welsh et al. 1973; Law et al. 1978; Scarth and Law 1984; Hayes et al. 1993; Pan et al. 1994; Laurie et al. 1995; Bezant et al. 1996; Sourdille et al. 2000) influencing adaptation and survival and which may be sought in other crop species.

As implied earlier, from a plant "perspective," many stresses that contribute to suboptimal growth, yield and persistency, and the relevant plant-initiated responses aimed at mitigating their most damaging effects should not be considered in isolation, even though many researchers frequently do just that by becoming specialists focused in aspects of a single abiotic stress without due consideration of other contributory stress factors. Aspects of stresses such as drought, cold, or salinity have a similar detrimental effect on the plant and they or indeed others may interact to enhance a stress effect and complicate all endeavors to initiate an adaptive response. Abiotic stresses, notably extremes in temperature, photon irradiance, and supplies of water and inorganic solutes, frequently limit growth and productivity of major crop species such as wheat. These often operate in conjunction: extreme temperature and high photon irradiance often accompany low water supply, which can in turn be exacerbated by subsoil mineral toxicities that constrain root growth. Furthermore, one abiotic stress can decrease a plant's ability to resist a second abiotic, or indeed a biotic, stress.

Most cereals are moderately sensitive to a wide range of abiotic stresses, and variability in the gene pool generally appears to be relatively small providing only few opportunities for major step changes in tolerance. Although of only moderate benefit, examples exist where cold tolerance has been enhanced following introgression from tolerant landraces into commercial lines using marker-assisted breeding (Dubcovsky 2004). The exploitation of fully sequenced model grass crops such as rice and *Brachypodium* provides candidate genes for transfers to enhance stress tolerance. Taken together with steadily increasing transformation frequencies for many grasses, the functional genomics approach to the study and manipulation of abiotic stresses in grasses is becoming a feasible objective. Need for use of the pioneer model plant *Arabidopsis thaliana* for such work is decreasing steadily as more relevant knowledge is becoming obtainable, firstly through model crops of greater synteny with the target crops, and finally of course from genomic studies made on those crops themselves. In other words, for forage grasses and cereals, model grasses *Brachypodium distachyon* and rice are more relevant for genetic studies of abiotic stress resistance than the dicot model species *Arabidopsis thaliana*. Of course, the reverse would apply for dicotyledonous crops such as those among the Brassiceae. Many of the mechanisms of tolerance to abiotic stresses can have fundamentally different characteristics between monocots and dicots, so transferring knowledge from *Arabidopsis* to the major crops are often of limited value. Having said that, a large part of the fundamental research concerning plant cold stress response and FT was carried out using the model species, *Arabidopsis thaliana,* as key mechanisms and transcription factors are found both in dicots and monocots and are involved in the regulation of expression of many cold (and drought) stress response genes (Gilmour et al. 1998; Ito et al. 2006). In the dicot model plant *Arabidopsis,* three *CBF* genes exist (Shinwari et al. 1998), while in the monocot cereal species, 10–20 *CBF* genes have been identified (Miller et al. 2006; Skinner et al. 2006; Francia et al. 2007). Even though dicot and monocot *CBF* gene function is conserved in the sense that *CBF* genes all appear to be involved in abiotic stress response pathways, there have been divergence in the *CBF* family size and hence probably also *CBF* gene function during the evolution of dicot and monocot lineages (Sandve and Fjellheim 2010). Such lineage-specific evolution can provide a direct inference of gene function based on homology between model plants and agriculturally important species. Moreover, neither *Arabidopsis* nor rice is adapted to a perennial life in extreme winter climates. This is important because adaptation to a perennial life history in harsh winter climates must have required changes at the genetic level, which cannot be studied using an annual model species. Hence, if we only use model plants such as *Arabidopsis* or rice to do research on cold and frost stress response, this might provide only limited insights into the genetic mechanisms underlying FT in important agricultural species.

The Pooideae is a large and economically important subfamily including cereals (Triticeae tribe) and forage grasses (Poaeae tribe). Divergence of temperate grasses from the most recent common ancestor shared with rice is thought to have happened ~46–42 million years ago (Gaut 2002; Sandve et al. 2008). Parallel to the origin and early evolution of the Pooideae group, the global climate became gradually cooler

(Zachos et al. 2001). As opposed to rice, which is adapted to warm and humid environments, Pooideae grasses radiated in cooler environments (Barker et al. 2001). This is reflected by the present distribution of Pooideae species which is extremely skewed towards cooler environments (Hartley 1973). Thus, evolution of cold and frost stress responses, either through fine tuning of ancient abiotic stress responses or evolution of novel adaptations to cold environments, must have been central for adaptation, colonization, and speciation in the Pooideae subfamily.

3.4 Effects of Freezing Temperatures on Plant Physiology

Each plant has an optimum set of temperatures for its growth and development, but these will differ from one species or species-ecotype to another. Plants native to warm habitats such as maize (*Zea mays*), soybean (*Glycine max*), cotton (*Gossypium hirsutum*), and tomato (*Lycopersicon esculentum*) exhibit symptoms of chilling injury upon exposure to even nonfreezing temperatures below 10–15 °C (Guy 1990; Lynch 1990).

Cold stress-induced injury in plants may appear after 48–72 h of stress exposure. Plants exposed to cold stress show various phenotypic symptoms that include reduced leaf expansion, wilting, and chlorosis (yellowing of leaves) and may lead to necrosis. Cold stress also severely affects the reproductive development of plants, and this has been seen in rice plants at the time of anthesis and leads to sterility in flowers. The effects of cold stress at the reproductive stage of plants delay heading and result in pollen sterility, which is thought to be one of the key factors responsible for the reduction in grain yield of crops (Suzuki et al. 2008). As mentioned earlier, the major adverse effect of cold stress in plants has been seen in terms of plasma membrane damage. This has been documented due to cold stress-induced dehydration (Steponkus 1984; Steponkus et al. 1993). The plasma membrane is made up of lipids and proteins. Lipids in the plasma membrane are made up of two kinds of fatty acids: unsaturated and saturated fatty acids. Unsaturated fatty acids have one or more double bonds between two carbon atoms, whereas saturated fatty acids are fully saturated with hydrogen atoms. Lipids containing more saturated fatty acids solidify faster and at temperatures higher than those containing unsaturated fatty acids. Therefore, the relative proportion of these two types of fatty acids in the lipids of the plasma membrane determines the fluidity of the membrane (Steponkus et al. 1993). At the transition temperature, a membrane changes from a semifluid state into a semicrystalline state. Cold-sensitive plants usually have a higher proportion of saturated fatty acids in their plasma membrane. Therefore, cold-sensitive plants have a higher transition temperature. On the other hand, cold-resistant plants have a higher proportion of unsaturated fatty acids and thereby a lower transition temperature.

Ice formation is the major cause of plant damage. Ice formation in plant tissues during cold stress leads to dehydration. Ice is formed in the apoplast, the free diffusional space outside the plasma membrane, because it has relatively lower

solute concentration. It is known that the vapor pressure of ice is much lower than water at any given temperature. Therefore, ice formation in the apoplast establishes a vapor pressure gradient between the apoplast and surrounding cells. As a result of this gradient, the cytoplasmic water migrates down the gradient from the cell cytosol into the apoplast. This adds to existing ice crystals in the apoplastic space and causes a mechanical strain on the cell wall and plasma membrane, leading to cell rupture (McKersie and Bowley 1997; Olien and Smith 1997; Uemura and Steponkus 1997).

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

Recently, attempts have also been directed towards analyzing the effect of cold stress on the whole-plant metabolome. Metabolic profiling of *Arabidopsis* plants revealed that CA increases 75 % of the 434 analyzed metabolites (Cook et al. 2004; Kaplan et al. 2004). The role of such metabolites in plants has been known as osmoprotectants. In addition to their role as osmoprotectants and osmolytes, certain metabolites (individual metabolites or the redox state) induced during CA might act as signals for reconfiguring gene expression. For example, cold stress induces the accumulation of proline, a well-known osmoprotectant. Microarray and RNA gel blot analyses have shown that proline can induce the expression of many genes, which have the proline-responsive element sequence ACTCAT in their promoters (Satoh et al. 2002; Oono et al. 2003).

3.4.1 Impacts of Cold Stress on Crop Production

Each crop has an optimal thermal threshold. As mentioned earlier, tropical and subtropical crops may be damaged at above 0 °C temperatures, while temperate crops depending on species and ecotype can acclimatize and resist temperatures from −5 °C to −30 °C (Thomashow 1998). In temperate crops, suboptimal temperatures during spring lead to decreased productivity and yield stability (Stamp 1984).

3.4.2 Chilling Injury (Temperatures 0–15 °C)

Resistance to low but nonfreezing temperatures takes the form of physiological and morphological adaptations that allow plants to tolerate or avoid cold stress and may arise either at the cellular, tissue, or whole plant level. Cold avoidance mechanisms are found in both annual and perennial plants and include seed production or vegetative dormancy, the latter also an avoidance strategy in temperate frost tolerant crops such as many tree and forage species.

For crops grown outside their regions of evolved adaptation such as maize, exposure to low temperatures may cause reductions in growth or induce damage in some form. Maize being moderately sensitive to chilling temperatures may have cellular or tissue injury on exposure to temperatures below 5 °C and reduced growth at temperatures below 15 °C. Chilling stress can be assessed at different developmental stages such as at germination and during growth and also in terms of photosynthetic activity, development of reproductive organs, and fruit ripening (Blum 1988).

Cold tolerance as with drought tolerance can alter at different stages of plant ontogeny. For example, in rice, cold tolerance may reduce at the reproductive growth stages compared with the vegetative when demands on plant resources in terms of grain filling reduce resilience against low temperatures. In maize, a susceptibility to low temperatures early in the life cycle may be explained by injuries incurred during seed, embryo, and seedling development. For maize, temperature for germination should not fall below 10 °C (Herczegh 1970). Normal leaf development in maize requires temperatures above 15 °C (Nie et al. 1992). Other crops such as tomato may suffer reduced growth and fruit formation and over more prolonged periods of death following exposures to low temperatures (Nieuwhof et al. 1997).

In all cases plant ontogeny affects tolerance to cold (and especially freezing temperatures). Seed imbibition is very sensitive to chilling. In maize, chilling results in exudation of amino acids and carbohydrates from kernels as a consequence of cell membrane damage (Miedema 1982). Low temperatures not only affect germination rate but also subsequent seedling growth. The spring temperatures of more northerly latitudes are usually too low for the favorable germination of crops such as maize. Most maize plants germinate poorly at temperatures below 6 °C (Eagles 1982).

Soil temperatures are also critical to plant growth with any decrease of potential damage to root growth and water and nutrient uptake. A small reduction of 2–3 °C soil temperatures may in maize affect root growth. The effect of root temperature on growth in maize is complex and may also affect shoot growth, but after tassel formation and stem elongation, shoot growth is affected more by air rather than soil temperature (Ellis et al. 1992). As a breeding strategy, reliable germination and rapid growth in cold soils should raise crop performance across environments and extend crop range and should facilitate early sowing. In general leaf extension is controlled more by the temperature at the shoot apex rather than at the root (Watts

1972). After emergence, leaves appear successively, but young leaves are more susceptible to cold than mature leaves.

Cold sensitivity can be manipulated by changes in the levels of desaturation of fatty acids in membrane lipids. The importance of the role of membrane lipid desaturation in cold tolerance has been demonstrated in transgenic experiments (Nishida and Murata 1996). Protective proteins are induced by cold temperatures (Guy 1990). The effects of low temperatures on protein synthesis associated with cell membrane protection has been studied in maize and shown to be high in winter but to subsequently decline. Similar responses have been reported in many other crops such as spinach, rapeseed, and rice (Guy et al. 1985; Meza-Basso et al. 1986; Hahn and Walbot 1989). Proline has been shown to improve CT and aid cell structure protection in many crops, such as maize (Xin and Li 1993), potato, wheat and barley, and in *L. perenne* was shown to improve osmotic adjustment during CA (Thomas and James 1993).

Cold phases cause a reaction in the plant that prevents sugar entry into the pollen and as a consequence no subsequent starch accumulation to provide the energy necessary for pollen germination thereby limiting pollination and seed set.

3.5 Biotechnology

As an alternative strategy to plant breeding, biotechnological approaches, although not suitable for all countries due to current restrictions in their use, offer new strategies that can be used to develop transgenic crop plants with improved tolerance to cold stress and also if plant hybridization is possible, a means to test the efficacy of candidate genes for use in plant breeding programs. As cold tolerance is a quantitative trait, the transfer of individual gene variants will in many cases have only a limited benefit and will not reproduce generally the overall tolerance observed in the donor genotype. However, rapid advance in recombinant DNA technology and development of precise and efficient gene transfer protocols have resulted in efficient transformation and generation of transgenic lines in a number of crop species (Wani et al. 2008). A number of genes have been isolated and characterized that are responsive to freezing stress.

When a plant is subjected to chilling or freezing stress, a suite of genes are engaged, resulting in increased levels of several metabolites and proteins, some of which may be responsible for conferring a certain degree of protection. Since many aspects of CA are under transcriptional control, transcription regulatory factors are considered suitable for use in transgenic technologies where they may "trigger" a stress response and provide some tolerance. As mentioned previously, modifications in lipid composition that stabilize cell membranes and prevent cellular leakage lead to CA. Therefore, the overexpression of glycerol-3-phosphate acyltransferase led to the alteration to the unsaturation of fatty acids and conferred chilling tolerance in transgenic rice (Ariizumi et al. 2002) and tomato (Sui et al. 2007) plants.

Over the last 20 years, advancement in plant biotechnology has led to the identification and isolation of a number of transcription factor(s) related to cold stress tolerance and in many cases aided understanding of those genes with major relevance to the acquisition of cold and freezing tolerance. These include encoding enzymes that are required for the biosynthesis of various osmoprotectants for modifying membrane lipids, late embryogenesis abundant (LEA) proteins, and detoxification enzymes. In these studies, either a single gene for a protective protein or an enzyme was overexpressed under the control of the constitutive 35S cauliflower mosaic virus (CaMV), promoter in transgenic plants, although several genes have been shown to function in environmental stress tolerance and response (Shinozaki et al. 2003).

The C-repeat binding factor (CBF) genes represent one of the most significant discoveries in the field of low-temperature adaptation and signal transduction and are present in all major crops. The expression of many low temperature-inducible genes is regulated by *CBF*/*DREB*1 (dehydration-responsive element binding) transcription factors. Three *CBF*/*DREB*1 genes (*CBF3*/*DREB1a*, *CBF1*/*DREB1b*, and *CBF2*/*DREB1c*) belonging to the *AP2*/*DREBP* family of DNA-binding proteins have been identified in *Arabidopsis* (Jaglo-Ottosen et al. 2001). Transgenic *Arabidopsis* plants constitutively overexpressing a cold-inducible transcription factor (*CBF1*; *CRT*/*DRE*-binding protein) showed tolerance to freezing without any negative effect on the development and growth characteristics (Jaglo-Ottosen et al. 1998). Furthermore, overexpression of *Arabidopsis CBF1* has been shown to activate *COR* (cold-regulated) homologous genes at non-acclimating temperatures (Jaglo-Ottosen et al. 2001). Several stress-induced *COR* genes such as *rd29A*, *COR15A*, *kin1*, and *COR6.6* are triggered in response to cold treatment (Thomashow 1998).

Various *COR* genes isolated from *Arabidopsis* have protective roles against dehydration. Overexpression of *CBF1*/*DREB1b* and *CBF3*/*DREB1a* enhances cold tolerance by inducing *COR* genes (Jaglo-Ottosen et al. 1998; Liu et al. 1998). It also leads to the accumulation of sugar and proline (Gilmour et al. 2000). *CBF*/*DREB1* genes are thought to be activators that integrate several components of the CA response by which plants increase their tolerance to low temperatures after exposure to low but nonfreezing conditions. In recent times, extensive research efforts have been undertaken to identify and characterize *COR* genes, and a number of orthologs of the *Arabidopsis CBF* cold-response pathway have been found (Yamaguchi-Shinozaki and Shinozaki 2006). Many of these putative orthologs have been structured, analyzed, and functionally tested. The expression patterns of the *CBFs* and *CORs* in response to low temperature are similar in a variety of plants species, involving rapid cold-induced expression of the *CBF*s followed by expression of *CBF*-targeted genes that increase freezing tolerance.

Recently, a *CBF1* gene was introduced into tomato under the control of a CaMV35S promoter and that resulted in transgenic plants showing improved tolerance to chilling and higher activity of superoxide dismutase (SOD), higher non-photochemical quenching (NPQ), and lower malondialdehyde (MDA) content.

This would suggest that CBF1 protein plays an important role in protection of PSII and PSI during low temperature stress (Zhang et al. 2011). The relevance in forage grasses of CA adaptations for freezing tolerance that led to high NPQ expression was described earlier (Rapacz et al. 2005; Humphreys et al. 2007).

The importance of *CBF*-independent pathways is also supported by analysis of mutants that have increased freezing tolerance, for example, mutations in eskimo1 (*ESK1*), a protein of unknown function, and result in constitutive freezing tolerance. The *Eskimo1* mutation was first identified as a mutation conferring frost survival without recourse to a CA period (Xin and Browse 1998). Subsequently, Bouchabke-Coussa et al. (2008) demonstrated that *ESKIMO1* mutants are more tolerant to freezing, but only after acclimation. The genes that are affected by the *ESK1* mutation are distinct from those of the *CBF* regulon (Xin et al. 2007).

Various studies using transcriptome data have demonstrated that additional cold-regulatory pathways exist in addition to those cold-responsive genes regulated by *CBFs* (Flower and Thomashow 2002; Kreps et al. 2002). At least 28 % of the cold-responsive genes are not regulated by *CBF* transcription factors indicating that these genes are members of different low-temperature regulons, including 15 encoding known or putative transcription factors (Vogel et al. 2005).

The expression of related cold shock proteins (CSPs) from bacteria, *CspA* from *Escherichia coli,* and *CspB* from *Bacillus subtilis* promotes stress adaptation in multiple plant species. Transgenic rice plants expressing *CspA* and *CspB* show improved stress tolerance against various stresses, including cold, heat, and drought.

When overexpressed in *Arabidopsis* and tobacco, the soybean gene *SCOF-1*, which encodes for a zinc-finger protein, can activate *COR* gene expression and increase freezing tolerance in non-acclimated transgenic plants. The *SCOF-1* gene may regulate the activity of SGBF-1 as a transcription factor in inducing *COR* gene expression and interacts with a G-box binding *bZIP* protein, *SGBF-1*. The *SGBF-1* protein can activate ABRE-driven reporter gene expression in *Arabidopsis* leaf protoplasts (Kim et al. 2001).

Forward genetic analysis in *Arabidopsis* identified two transcription factors and high expression of osmosis-responsive genes, *HOS9* and *HOS10*, which are required for basal freezing tolerance (Zhu et al. 2004, 2005). Similarly, microarray analysis led to the identification of the cold stress-inducible *AP2* family transcription factor gene related to *ABI3/VP1* (*RAV1*) (Flower and Thomashow 2002; Vogel et al. 2005) that might regulate plant growth under cold stress.

The overexpression of genes encoding *LEA* proteins can improve the stress tolerance of transgenic plants. For example, the freezing tolerance of strawberry leaves was enhanced by expression of the wheat dehydrin gene *WCOR410* (Houde et al. 2004). Trehalose is a nonreducing disaccharide that is present in diverse organisms ranging from bacteria and fungi to invertebrates, in which it serves as an energy source, osmolyte, or protein/membrane protectant. Various studies have revealed regulatory roles of trehalose-6-phosphate, a precursor of trehalose, in sugar metabolism and growth and development in plants (Iordachescu and Imai 2008). Trehalose levels are generally quite low in plants but may alter in response to

environmental stresses. Although the involvement of trehalose metabolism in stress tolerance is indubitable, our understanding of how it exactly interacts and acts upon stress pathways is far from complete. Studies of individual trehalose biosynthesis genes will help us to precisely assess their specific roles in the abiotic stress context and may enable us to develop new strategies to enhance abiotic stress tolerance of crop plants. Plants synthesize trehalose in a pathway that is common to most organisms, through the production of the intermediate trehalose-6-phosphate.

3.5.1 Microbial Trehalose Biosynthesis Genes

Major advances in the study of trehalose biosynthesis in plants have been made in the past decade. Transgenic plants overexpressing microbial trehalose biosynthesis genes have been shown to contain increased levels of trehalose and display drought, salt, and cold tolerance. In silico expression profiling of all *Arabidopsis* trehalose-6-phosphate synthases (TPSs) and trehalose-6-phosphate phosphatases (TPPs) revealed that certain classes of TPS and TPP genes are differentially regulated in response to a variety of abiotic stresses. These studies point to the importance of trehalose biosynthesis in stress responses. Since trehalose is an osmoprotectant (Műller et al. 1995) and membrane and protein integrity protectant (Crowe et al. 1984), different groups with varied success attempted to create stress tolerant plants by introducing microbial trehalose biosynthetic genes by use of transgenic technologies (Iordachescu and Imai 2008). Pramanik and Imai (2005) and Shima et al. (2007) isolated and characterized two rice TPPs (*OsTPP1* and *OsTPP2*). Both rice TPPs were induced transiently by cold, salt, and drought stress, as well as exogenous ABA applications; however, transient induction of *OsTPP1* occurred generally earlier than that of *OsTPP2* suggesting a tight regulation of trehalose biosynthesis in response to multiple abiotic stresses (Shima et al. 2007). Trehalose was also transiently induced following chilling stress, its increase being correlated with the increase of *OsTPP1* transcript and OsTPP1 activity (Pramanik and Imai 2005). Moreover, accumulation of trehalose in response to chilling stress coincided with the phase change of glucose and fructose levels (Pramanik and Imai 2005).

Many temperate crops such as the forage ryegrasses accumulate fructan as their main vegetative carbon and energy reserve, and it is a major nutrient for livestock on grazed pastures and receiving conserved feeds. Accumulation of fructan, rather than starch, has been suggested to have evolved to meet a need to adapt to cold winters and dry summers. Consequently, fructans are widely believed, and very frequently quoted, to be important in determining tolerance to environmental stresses such as cold and drought. Carbohydrates are well recognized as having multiple roles in integrating a wide range of plant growth and environmental responses. However, many correlations between fructan and stress tolerance are circumstantial, and evidence of direct relationships is poor. It has been widely reported that fructan content increases during exposure to many abiotic stresses, but accumulation purely as a by-product of a reduced growth rate when photosynthesis

remains unaffected is very different from an active, functional role in stress tolerance. Prior to winter, temperate plant species acclimatize, during which their metabolism is redirected towards synthesis of cryoprotectant molecules such as soluble sugars (saccharose, raffinose, stachyose, trehalose), in addition to sugar alcohols (sorbitol, ribitol, inositol) and low molecular weight nitrogenous compounds (proline, glycine betaine). These, in conjunction with dehydrins, COR proteins and heat-shock proteins (HSPs) act to stabilize both membrane phospholipids and proteins, and cytoplasmic proteins, maintain hydrophobic interactions and ion homeostasis, and scavenge reactive oxygen species (ROS); other solutes released from the symplast serve to protect the plasma membrane from ice adhesion and subsequent cell disruption (Hare et al. 1998; Iba 2002; Wang et al. 2003; Gusta et al. 2004; Chen and Murata 2008). The process of solute release, especially of vacuolar fructans to the extracellular space, is vesicle-mediated and tonoplast-derived (Valluru and Van den Ende 2008). Fructans are transported to the apoplast by postsynthesis mechanisms, probably in response to cold stress (Valluru et al. 2008). The activity of fructan exohydrolase, which generates increased sugar (glucose, fructose, sucrose) content, is an important part of the hardening process. Symplastic and apoplastic soluble sugar—not only fructan precursors but also trehalose, raffinose, as well as fructo- and gluco-oligosaccharides—contributes directly to membrane stabilization (Livingston et al. 2006).

In barley, trehalose induces the expression and activity of fructan biosynthesis enzymes. However, for fructan accumulation, glucose or mannitol is also required (Wagner et al. 1986; Műller et al. 2000). From a microarray analysis following trehalose treatment, Bae et al. (2005) showed that the expression of a wide range of other genes was also influenced by trehalose. A role for trehalose and trehalose-6-phosphate in abiotic and biotic stress signaling has been confirmed by the observation that coordinated changes occur in transcript levels of the enzymes involved in their metabolism, especially after exposure to cold, osmotic, and salinity stresses (Iordachescu and Imai 2008).

3.6 The *CBF/DREB* Pathway

As previously mentioned, the *CBF/DREB*-responsive pathway provides a major route for the production of cold-responsive proteins. CBF1, 2, and 3 are all responsive to low temperature, and their encoding genes are present in tandem on *Arabidopsis* chromosome 4. *CBF2/DREB1C* is a negative regulator of both *CBF1/DREB1B* and *CBF3/DREB1A*. *CBF3* is thought to regulate the expression level of *CBF2* (Novillo et al. 2004; Chinnusamy et al. 2006). Thus, the function(s) of *CBF1* and *CBF3* differs from those of *CBF2* and act additively to induce the set of *CBF*-responsive genes required to complete the process of CA (Novillo et al. 2007). Upstream of *CBF* lie both *ICE1* (inducer of *CBF* expression), a positive regulator of *CBF3*, and *HOS1* (high expression of osmotically sensitive), a negative regulator of *ICE1*. Because of the rapid induction of *CBF* transcripts following plant exposure

to low temperature, *ICE1* is unlikely to require fresh synthesis, but is already present in the absence of cold stress and is only activated when temperature is lowered (Chinnusamy et al. 2003). The *LOS1* (low expression of osmotically responsive genes) product is a translation elongation factor 2-like protein, which negatively regulates *CBF* expression.

The likely regulators of *CBF1* and *CBF2* are bHLH proteins other than ICE1. In addition to *ICE1*, a further positive regulator of *CBF* expression is LOS4, an RNA helicase-like protein (Gong et al. 2002). *CAX1* (cation exchanger), which plays a role in returning cytosolic Ca_2+ concentrations to basal levels following a transient increase in response to low-temperature stress, is a negative regulator of CBF1, 2, and 3 (Hirschi 1999; Catalá et al. 2003).

Knowledge gained from the study of *Arabidopsis* has proven to be largely, but not completely transferable to crop plants. A better model for cereal and grass crop species of the Pooideae will be rice and especially *Brachypodium* with which they share closer syntenic relations (Moore et al. 1995). In barley, the *CBF* genes *HvCBF3*, *HvCBF4,* and *HvCBF8* are all components of the frost resistance QTL located on chromosome 5H (Francia et al. 2004).

A contrasting approach has targeted comparisons between spring- and winter-sown cereal cultivars. For example, Monroy et al. (2007) observed that spring and winter wheat share the same initial rapid expression of cold-inducible genes, but that their transcriptional profiles diverge widely during CA. While in winter cultivars the expression of CA genes continues over time, in spring cultivars, their levels of expression decline and the CA process is overridden by the transition from the vegetative to the reproductive stage.

3.7 Antifreeze Proteins and Ice Recrystallization Inhibition Genes

Antifreeze proteins were first isolated from Antarctic fish and were shown to depress the freezing point of blood serum to −2 °C, one degree below that at which the serum melts (Duman et al. 1993). This noncolligative effect, termed thermal hysteresis (TH), is evident in other aqueous solutions and appears to result from the adsorption of the proteins to the surface of ice crystals and inhibition of further crystal growth.

In general, plant AFPs exhibit low levels of TH and are present at much lower concentrations than fish AFPs (Duman 1994; Hon et al. 1995). AFPs may modify the activity of ice nucleators. Indeed, AFPs infiltrated into the extracellular space of leaves of *Solanum tuberosum*, *Brassica napus,* and *Arabidopsis* depressed the freezing point by up to 1.8 °C (Cutler et al. 1989). No ice crystals were formed showing that the AFP acted as an anti-ice nucleator.

Functional molecular studies support a link between ice recrystallization inhibition (*IRI*) genes and adaptation to cold stress (John et al. 2009). Transcription of the

IRI genes is controlled by exposure to cold temperature, and they encode proteins that bind to ice crystals to change their ability to increase in size in vitro. In vivo protein function is however still debated. The current leading hypothesis is that IRI activity hinders ice expansion in the apoplast and thus protects plant cells from mechanical damage, elevating plant frost tolerance. But *IRI* genes also encode an LRR domain that is homologous to pathogenesis-related proteins, and this raises the question if IRI proteins have dual functions in cold stress response.

Frost tolerance adaptations are, in many organisms, associated with the evolution of AFPs (Zachariassen and Kristiansen 2000). AFPs can affect freezing and ice crystallization-related stress via different mechanisms. TH depresses the freezing point at which ice crystallization initiates, which renders it possible for organisms to remain unfrozen yet survive under freezing temperatures. IRI on the other hand does not hinder ice crystallization but manipulates the growth of the ice crystals such that small ice crystals grow at the expense of larger ice crystals. Even though the functional significance of IRI in vivo has been demonstrated, IRI proteins are believed to prevent or minimize the cellular damage in plants (Smallwood and Bowles 2002).

Animal AFPs generally possess high TH characteristics and lower ice crystallization initiation temperatures by 1–5 °C (Barrett 2001; Griffith and Yaish 2004). Plant AFPs on the other hand have low TH activity but exhibit strong ice recrystallization inhibition (IRI) activity (Griffith and Yaish 2004). AFPs have been isolated from different plants and there are at least five isoforms of AFPs within the cold tolerant grasses; full-length nucleotide sequences are available for genes encoding five AFPs (Griffith and Yaish 2004; Middleton et al. 2009). In addition, AFPs have been identified in carrot (Worrall et al. 1998). Pooideae subfamily-specific *IRI* gene homologs have so far been identified and isolated in perennial ryegrass (Sandve et al. 2008), wheat (Tremblay et al. 2005), and Antarctic hair grass (John et al. 2009). Most likely these genes have evolved from a common ancestor gene of the leucine-rich repeat phytosulfokine receptor kinase (*LRR-PSR*)-like genes present in rice and *Arabidopsis* (Sandve et al. 2008).

3.8 Conclusion

To summarize all the above, a simple illustration is presented (Fig. 3.1) of the impacts of subzero temperatures on a cell of a non-acclimated or cold-sensitive grass genotype and the response by an equivalent genotype once acclimated fully having achieved a frost tolerance sufficient to safeguard against cell damage by the winter stresses endured.

By 2050, it is estimated that the Earth's human population will be 9.07 billion. Of these, 62 % will live in Africa, Southern Asia, and Eastern Asia; numerically the same as if the world's current population lived solely in these regions. These overpopulated regions will suffer the greatest from the outcomes of climate change and in particular high temperatures and shortage of nonsalinized water suitable for

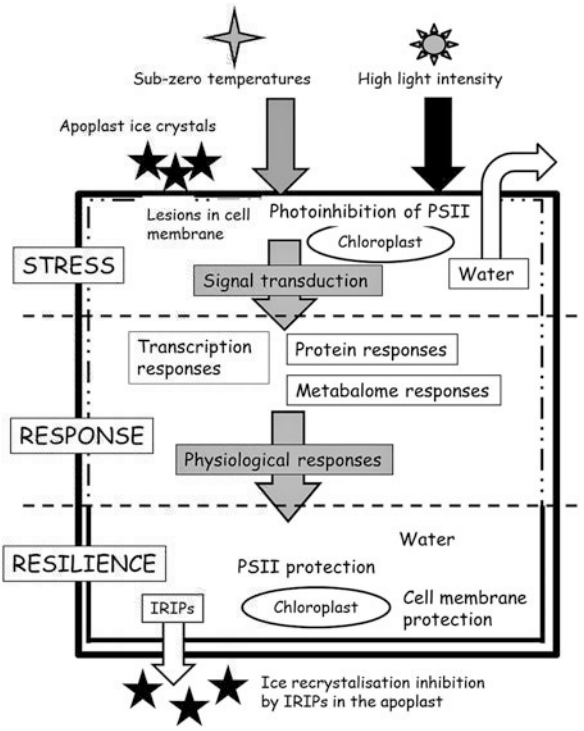

Fig. 3.1 The consequences on a non-acclimated plant cell when exposed to freezing temperatures, and high irradiance, and the resilience found to safeguard cellular integrity in an equivalent adapted and cold-acclimated genotype

human consumption and food production. In more northern and southern regions, climate predictions indicate opportunities for some increases in crop yields will likely following climate change. However, it will still require plant breeders to provide the crops best adapted to these regions. Weather extremes fluctuate with increasing frequency and crop design must respond to include some plasticity to provide appropriate responses to the diverse stresses crops will likely encounter.

In Ireland, where livestock agriculture predominates, increasing temperatures have led to national policies aimed at an all-year growing season. Such policies in locations prone to fluctuating weather patterns bring obvious dangers to sustainable crop production, as does the cultivation of marginal regions such as uplands and mountain regions subject to low temperatures and usage of non-frost adapted high-yielding crop species.

The cold tolerance trait is complex but many attributes of stress tolerance require common gene complexes for resistance to desiccation, and as described herein, these will often span requirements to resist freezing, drought, salinity, and high winds. Specific to cold tolerance, it will be necessary to target those genes that control the main regulatory systems and in particular those concerned with cold acclimation and photoperiod response.

In summary, while growth of many crops must from necessity be restricted to their current climatic zones of adaptation, for others including many of the forage

and energy crops and cereals, a holistic approach to plant breeding is now required that provides crops with the capability to withstand a wide range of abiotic and biotic stresses. New high throughput genotype and phenotype technologies such as the incorporation of genome-wide selection in crop improvement programs and use of hyperspectral imaging phenomics facilities provide opportunities to assemble correct crop genotypes for a predictable and desirable phenotype. Such technologies for the first time make possible complex redesigns of crops to provide where deemed necessary new adaptive variation derived either directly from wild-type relatives or via transgenic technologies. To increase crop production, the incorporation of nonnative and even tropical crops, including C_4 plants with more efficient photosynthesis, in cooler temperate regions will in some cases become a feasible option. Such strategies require careful preparation by the plant breeder to ensure sufficient and appropriate adaptive variation is available. A good example at IBERS Aberystwyth University is the development and use of the biomass elephant grass (*Miscanthus giganteus*). This fast growing C_4 biomass plant, a native to Eastern Asia, is grown commercially as a natural triploid hybrid generated from progenitor species *M. sinensis* and *M. sacchariflorus*. Ecotypes of the progenitor species' show vast diversity in location and in biomass and adaptive traits, and by careful plant collection and selection of the progenitor species, new synthetic *M. giganteus* hybrids are being developed to include winter adaptive traits that match the growth conditions they will encounter in Europe far from their natural habitat (Robson et al. 2012). While equivalent breeding strategies may not be available to all food crops, it will require to feed future generations ingenuity such as this to achieve the maximum possible crop growth potential in all those marginal locations available for agricultural use.

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