OPINION PAPER

Understanding plant cold hardiness: an opinion

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How plants adapt to freezing temperatures and acclimate to survive the formation of ice within their tissues has been a subject of study for botanists and plant scientists since the latter part of the 19th century. In recent years, there has been an explosion of information on this topic and molecular biology has provided new and exciting opportunities to better understand the genes involved in cold adaptation, freezing response and environmental stress in general. Despite an exponential increase in our understanding of freezing tolerance, understanding cold hardiness in a manner that allows one to actually improve this trait in economically important crops has proved to be an elusive goal. This is partly because of the growing recognition of the complexity of cold adaptation. The ability of plants to adapt to and survive freezing temperatures has many facets, which are often species specific, and are the result of the response to many environmental cues, rather than just low temperature. This is perhaps underappreciated in the design of many controlled environment experiments resulting in data that reflects the response to the experimental conditions but may not reflect actual mechanisms of cold hardiness in the field. The information and opinions presented in this report are an attempt to illustrate the many facets of cold hardiness, emphasize the importance of context in conducting cold hardiness research, and pose, in our view, a few of the critical questions that still need to be addressed.

Introduction

They sought it with thimbles, they sought it with care;
They pursued it with forks and hope;
They threatened its life with a railway share
They charmed it with smiles and soap – Lewis Carroll “The Hunting of the Snark”

The term cold hardiness is used to represent in a general sense, the ability of a plant to adapt to and withstand freezing temperatures. Collectively, the mechanisms associated with this ability are quite diverse ranging from properties that exist at a structural (whole-plant) level (e.g. deep supercooling of xylem tissues and the existence of preferred sites of ice formation) to cellular adaptations that include specific metabolites, proteins and changes in membrane structure. Superimposed on these aspects of cold hardiness are physiological processes that reflect the interaction of the plant with its environment. These include but are not limited to source-sink relationships, dormancy, and factors that determine rates of deacclimation and the ability to reacclimate. Further complicating the picture is the fact that mechanisms responsible for cold hardiness can often differ within the same plant over a relatively small distance and in some cases between adjacent cells. For example, bark tissues vs xylem tissues in woody plants, crown vs. leaf tissue in cereal plants,

Abbreviations – GC, growth chambers; GE, genetic enhancement; ROS, reactive oxygen species.
and root vs stem tissues can all differ significantly in-planta in how they adapt and respond to freezing temperatures. This implies that the stresses faced by these specific tissues, and hence their adaptations, can vary dramatically. By extension, the imposed stresses and the adaptation of different plants and/or plant parts to those stresses can vary depending on the time of the stress. Early, mid, and late winter freezing stresses can each represent distinct challenges and necessitate the need for specific adaptations. Finally, the challenge of withstanding an acute stress (e.g. a single freezing event) vs a chronic stress (a prolonged period of freezing stress or ice encasement) can require very different adaptive mechanisms.

Based on the above description, perhaps it is no wonder that the ability to provide a measureable and reliable improvement in cold hardiness to agronomic and horticultural crops under field conditions either through conventional breeding or genetic engineering without impacting other important cultural traits such as growth, or yield, has proved to be, like the Snark of Lewis Carroll’s poem, elusive. This statement is not intended to diminish any of the multitude of accomplishments reported in the literature but only to suggest that commercial successes have not been forthcoming. An additional conclusion to draw from the introductory comments is the importance of context. Any research designed to explore and understand cold hardiness should be verified in the context of the physiology, growth habit and life cycle of the plant grown under natural conditions. Experimental protocols and a discussion of the data should reflect that understanding. While the complexity of cold hardiness is apparent, science is developing the technologies to study and address this complexity and most importantly is devising analytical methods to integrate, evaluate and interpret information obtained from many different approaches including molecular, genetic, biochemical, physiological and ecological. The achievement of this level of integration would represent a more holistic form of systems biology (Keurentjes et al. 2011) and could lead to many different opportunities for developing approaches to ameliorate freezing stress and injury in plants.

**Plant adaptation to freezing temperatures**

How plants adapt to freezing temperatures and acclimate to survive the formation of ice within their tissues has been a subject of study for botanists and plant scientists since the latter part of the 19th century (Molisch 1897, reviewed by Wisniewski et al. 2003). Historically, the mechanisms associated with freezing tolerance have been placed into the categories of avoidance and tolerance (Levitt 1980). Mechanisms associated with freeze avoidance, other than life cycle adaptations, have been typically associated with physical attributes of a plant that determine if, when, and where ice forms within the plant, while mechanisms associated with freezing tolerance generally refer to biochemical adaptations brought about or regulated by a specific set of genes (Table 1). With the advent of modern day molecular biology and genomics, recent cold hardiness research has been dominated by the study of the genes, proteins and metabolites involved in the acquisition of freezing tolerance while the study of freeze avoidance mechanisms has greatly diminished (Wisniewski et al. 2009). This has led to thinking of the two categories as mutually exclusive in terms of the role they play in determining whether or not a plant survives an episode of freezing. Additionally, the use of model systems (e.g. cell and tissue cultures, *Arabidopsis*) to understand the process of cold acclimation and general responses to cold temperature (signaling pathways) while providing a wealth of information (e.g. see the review by Ahuja et al. 2010) has perhaps led to an over extrapolation of the data that ignores the context of the whole plant and its interaction with the environment.

In actuality, tolerance and avoidance of freezing are not mutually exclusive categories and both may play a role in determining the ‘hardness’ of any specific plant species or genotype exposed to any particular freezing stress scenario. Both are under genetic control and have evolved in response to selection pressures. The ability of a plant to segregate ice into specific areas of its tissue where it will not do harm may be as critical as the ability of its cells to withstand the dehydrative stress associated with the formation and presence of that ice. In addition, the presence or absence of intracellular ice nucleators can play an important role in ensuring that intracellular ice formation does not occur, especially if water loss is inhibited in some manner and there is lag in the time it takes for the water potential of the cell to come into equilibrium with the vapor pressure of the extracellular ice. In contrast

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Table 1. Categories of mechanisms associated with the adaptation of plants to freezing temperatures.
to the freezing of a body of free water, ice crystals growing within plant tissues do not develop freely in all directions. Tissue structure, compartmentalization, cell walls and membranes represent obstacles to the spread of ice. Levitt (1980) reported that ice formation is initiated in the apoplast or within the conducting elements of vascular tissues. Once initiated, ice spreads rapidly along continuous columns of water. Linear rates of ice growth in woody tissues of lemon (Lucus 1954), mulberry (Kitaura 1967), orange (Yelenosky 1991), and peach (Anderson and Smith 1989) at rates between 7 and 74 cm/min have been reported. Peach blossoms froze within 30 s of the time that the ice front passed the point of blossom attachment (Anderson and Smith 1989). In herbaceous species, rates of longitudinal and lateral ice propagation of 1–4 cm s\(^{-1}\) and 0.3 cm s\(^{-1}\), respectively have been reported in leaves of barley (Hordeum vulgare) by Pearce and Fuller (2001).

Extracellular water or apoplastic water only represents a fraction of total water in a plant. The rate of symplastic water loss from cells depends upon the water potential gradient, cell wall and membrane permeability and cell surface area. Cells will maintain equilibrium with extracellular ice, however, if the cooling rate is very rapid, cells cannot maintain this equilibrium and the symplastic water, which is in a supercooled state, is susceptible to intracellular ice formation from either intracellular ice nucleation (Levitt 1980) or seeding from extracellular ice (Morris and McGrath 1981, Dowgert and Steponkus 1984).

Not all cells in frozen plant tissue are exposed to or are in direct contact with extracellular ice. Ice crystals are often unevenly distributed and in contact with only portions of a cell or are further removed (Wiegand 1906, Quamme 1978, Ishikawa and Sakai 1985, Ashworth et al. 1988, Ashworth 1992). The distance between the intracellular solution and extracellular ice can be several cell diameters or more and the diffusion of water could be through either the liquid or vapor phase. If the tissue is predominantly dry in the intercellular spaces water will then move through the vapor phase. Cortical cells adjacent to extracellular ice collapse more than cells within the periderm, phloem and cambium (Ashworth et al. 1988, Pearce 1988, Pearce and Ashworth 1992).

Despite its potential role in specific aspects of cold hardiness, research on factors that determine how, when and where ice is initiated in plants has greatly decreased over the last 10 years. Intrinsic vs extrinsic nucleators, antinucleators, plant antifreeze proteins, deep supercooling can all be crucial determinants of survivability, especially under spring frost conditions when many plants have deacclimated and have very little freezing tolerance (Wisniewski et al. 2009). Yet our understanding of these factors is lacking. Beyond a descriptive characterization, the identification of the actual components responsible for nucleation and antinucleation activity is needed as well as a realistic assessment of their impact on survivability. A sampling of recent reports on these topics emphasize their potential contribution to plant cold hardiness (Wisniewski et al. 2002, McCully et al. 2004, Taschler et al. 2004, Moffat et al. 2006, Kasuga et al. 2008, 2010, Walters et al. 2009, Aryal and Neuner 2010, Zhang et al. 2010, Hacker et al. 2011).

While the ability to survive a short- or long-term episode of freezing can be dependent on a hierarchy of mechanisms ranging from the molecular to whole plant, it is also important to note that cold hardiness has an ecological context. In nature, plants are subjected to a range of environmental conditions and stress that result in an integrated response leading to cold acclimation. Such integration has been evidenced by the elucidation of the contribution of different signaling pathways to cold hardiness. Additionally, other essential developmental parameters associated with the life cycle of plants are often influenced and/or regulated by similar environmental cues such as flowering, dormancy and seed germination. Interestingly, transcription of genes related to many of these developmental changes also appear to be influenced by circadian clock genes (Fowler et al. 2005, Ibañez et al. 2010).

Another aspect of cold hardiness that is often overlooked is deacclimation. The ability to either maintain a significant level of freezing tolerance despite exposure to temperatures more conducive to growth or the ability to quickly re-acclimate after losing a significant amount of frost tolerance is under genetic control and can be an integral part of the overall cold hardness of a particular species or genotype within a species (Kalberer et al. 2006). As energy is necessary to drive acclimation and sugars play an essential role in freezing tolerance, the resiliency of photosynthesis under stress conditions and how photosynthates are utilized (growth vs acclimation) also need to be better understood.

During cold acclimation, changes in gene expression and protein accumulation occur (Guy 1990, Janská et al. 2010), enabling the plant to tolerate the dehydrative stress associated with freezing temperatures and the presence of extracellular ice. Common proteins associated with low temperature include the dehydrins (Choi et al. 1999, Hara 2010, Hanin et al. 2011), \textit{Wheat Cold Specific} (Houde et al. 1992) proteins and \textit{Cold Responsive} (Gilmour et al. 1992) proteins. All of these categories or types of protein increase in response to low temperature exposure and correlate to some degree with the level of freezing tolerance. In most cases, the
function of these proteins is yet to be conclusively shown but it is generally assumed, based on their structure and composition, that they are cryoprotective and prevent the denaturation of various cellular components under desiccated conditions (Burchett et al. 2006). It has also been suggested that these types of proteins may interact with sugars within a cell in the formation of stable glasses that prevent freeze-induced desiccation (Wolkers et al. 2001).

There is a strong correlation between the development of freezing tolerance and the accumulation of sugars (Öquist et al. 1993, Hurry et al. 1994, Dahal et al. 2012). Plants do not effectively cold acclimate in the dark at low temperatures even though cold associated genes are upregulated (Wanner and Juntila 1999). Schilling (2004) also reported that proteins work in concert with sugars to establish freezing tolerance. The main difference in the ability of winter cereals to cold acclimate to low temperatures (e.g. \( LT_{50} = -25^\circ C \)) in contrast to spring cereals (e.g. \( LT_{50} = -9^\circ C \)) is the ability of winter cereals to effectively accumulate sugars under cold acclimating conditions while spring cereals do not. During cold acclimation of winter annuals there is an increase in the light saturation rates of \( \text{CO}_2 \) assimilation which is correlated with a stimulation of carbon metabolism as a result of an increase in key regulatory photosynthetic enzymes (Stitt and Hurry 2002) as well as higher resistance to low temperature-induced photooinhibition (Hüner et al. 1993). Unlike winter cereals, spring cereals only develop limited freezing tolerance (e.g. \( LT_{50} = -9^\circ C \)) to an acute short-term freeze (2° C h\(^{-1}\) cooling rate) but do not develop resistance to a chronic, prolonged freezing (Gusta et al. 2009). At low non-freezing temperatures winter cereals develop a prostrate growth habit which is positively correlated with increased sugars and anthocyanins (Hu et al. 2001). In contrast, spring cereals have an erect growth habit and are susceptible to low temperature-induced photooinhibition of photosynthesis (Hüner et al. 1993, Dahal et al. 2012).

One of the major advances in the past decade of freezing tolerance research was the discovery of the cold-inducible \( CBF \) or \( DREB \) transcriptional activators which have been shown to regulate a number of genes associated with low temperature response in plants (Thomashow et al. 2001, Shinozaki et al. 2003). Regulation of these genes is brought about through the binding of \( CBF \) protein to a specific motif (C-repeat) present in the promoter region of the genes that form the \( CBF \) regulon. While it is recognized that \( CBF \) is responsible for only a portion of the total gene set associated with cold acclimation, numerous genetic transformation studies have showed a clear induction of freezing tolerance in non-acclimated plants overexpressing \( CBF \). The discovery of \( CBF \) was made in \( Arabidopsis \); however, its existence and importance has been established in many other species through heterologous gene expression studies where \( Arabidopsis \) \( CBF \) genes have been expressed in other species or \( CBF \) genes from other species have been expressed in \( Arabidopsis \).

The overexpression of \( CBF \) genes has also been associated with other aspects of plant development. A significant reduction in growth is one of the common responses observed but overexpression of \( CBF \) has also been shown to have an effect on the onset of leaf senescence and dormancy (Wisniewski et al. 2011), as well as parameters such as plant architecture, leaf anatomy and photosynthetic performance (Dahal et al. 2012). While these additional effects on plant development illustrate the level of integration that has evolved in plants regarding their response to and use of environmental cues, it also highlights a major question. Namely, is the acquisition of a significant level of freezing tolerance, as represented by the gene set associated with cellular adaptation to dehydrative stress, compatible with growth? While a review of the literature suggests this is possible and reports of new genes conferring abiotic stress tolerance are rapidly increasing, most of the studies have been conducted in \( Arabidopsis \) and rarely have the plants, \( Arabidopsis \) or otherwise, been tested in the field under a wide array of environmental conditions. Varshney et al (2011) have highlighted this shortcoming in their review on the potential of agricultural biotechnology being used to improve plant performance in a variable environment. These authors have emphasized the need to adapt and use precise and large-scale phenotyping based on a good understanding of the key processes for drought and heat tolerance either alone or in combination. This is sage advice as well for those working on the development of plants with improved freezing tolerance.

**Protocols for inducing cold acclimation and assessing injury**

**Natural vs artificial acclimation**

Lack of progress in improving the frost tolerance of crop plants may be partially because of the difficulty in artificially producing the complex environmental conditions present when plants acclimate in nature (Gusta et al. 2009). There are significant differences between natural and artificial cold acclimation conditions and so plants that are cold acclimated in growth chambers (GC) may respond differently to those acclimated naturally (Robertson et al. 1994, Wisniewski et al. 2006,
Field grown plants are exposed to light intensities 4 to 12 times greater than those typically used in a GC and the light spectrum varies from summer to autumn compared with the constant spectrum in a GC. Varying diurnal temperatures that produce mixed messages are common in the field in contrast to constant temperatures in a GC. Plants in the field are often exposed to strong winds from different directions that affect gene expression and plant structure (e.g. secondary wall development). Because of the large mass and high specific heat of soil, roots cool slowly in the field in autumn compared to roots of plants in pots in a GC. Moving potted plants from a warm environment directly to a GC may induce a cold shock rather than an acclimation response resulting in erroneous data that reflects the specific experimental conditions rather than normal cold acclimation. Photoinhibition and the production of reactive oxygen species (ROS) resulting from a rapid move from growth conditions to low temperature in a GC may also play a significant role in GC-based studies compared with natural conditions where plants can slowly develop resistance to photoinhibition over time.

Wisniewski et al. (2006) reported enhanced expression of a peach dehydrin in response to low temperatures in a GC; however, 3 weeks of short days at 20°C had no impact on expression. In contrast, under natural conditions prior to any low temperatures, the expression of the dehydrin gene was first observed in August and increased during the autumn. These results provide an example of how gene expression by conditions such as the autumn light spectrum and/or day/night temperature cycling rather than short days alone may play a role in inducing freezing tolerance. Gray and Heath (2005) observed that Arabidopsis leaves that developed solely at low temperatures have a different metabolome compared with leaves shifted to a low temperature. In comparison, no correlation was observed between nine Arabidopsis accessions varying in frost tolerance when they were cold acclimated in GC.

Several differences were observed in a metabolomic study of winter cereals acclimated in a GC compared with naturally acclimated seedlings. Svec and Hodges (1972) compared metabolite changes in winter cereal seedlings cold acclimated in either controlled or natural environments. Total soluble carbohydrates, reducing sugars, total soluble nitrogen, and amino acids were two to fourfold higher in naturally acclimated plants. Total lipids were twofold lower in plants from the GC. Dhanaraj et al. (2007) documented a large number of genes that were induced in a GC but not under field conditions. In that study, genes related to light stress that were induced in plants in the field were not induced in the GC.

In autumn, plants receive mixed messages because of exposure to large temperature variations inherent in normal weather patterns. Temperatures may be 2°C one day and then 20°C for a few days before returning back to 2°C. In contrast, plants in a GC are often exposed to a constant low temperature. Under field conditions in the autumn, seedlings of a spring cultivar of Brassica napus cold acclimated to an LT50 of −7°C compared with −18°C at 2°C in a GC (Schilling 2004). This is comparable to the freezing tolerance of the hardiest winter canola and suggests that the major genes for cold acclimation are present in spring canola but what is different is how the two types perceive the environmental signal(s) for acclimation.

**Artificial freeze tests**

Factors such as the rate and duration of freezing and whether or not plants are wet or dry can play an important role in assessing freeze-induced injury. The time required for the cytoplasm to attain equilibrium with extracellular ice at a specific temperature may require days. For example, Gusta et al. (1997) reported that fully cold acclimated Norstar winter wheat survived −24°C when cooled at 2°C h⁻¹ but was killed at −12°C when held at that temperature for 15 days. Fully acclimated spring wheat can tolerate −9°C when cooled at 2°C h⁻¹; however, spring wheat succumbs to −3°C when it is held at that temperature for 48 h. This has led to the suggestion that an artificial freeze test that employs a cooling rate of 2°C/h or faster may not be sensitive enough to detect small differences in the freezing tolerance of winter annuals (Waalen et al. 2011). More importantly, it highlights the difference between an acute vs a chronic stress. If prolonged exposure to freezing temperature is critical to the plant species being studied, it is important that this be reflected in the design of the freezing test being utilized. In the case of deciduous woody species that deep supercool, the size of the low temperature exotherm decreases when the tissue is held at −20°C or below for an extended period of time suggesting a very slow movement of intracellular water to extracellular ice (Gusta et al. 1983). Franks (1985) has emphasized that the kinetics of various biophysical and biochemical processes need to be considered when studying freezing tolerance in organisms, especially when there is the possibility of undercooling (supercooling) to occur. Fuller and Le Grice (1998) specifically designed an environmental chamber to simulate radiative frost conditions in order
to better understand how plants respond to natural frost events.

**Manifestation of injury – field vs controlled environments**

Often, artificial freeze tests only measure the freezing tolerance of excised leaves without considering the whole plant or the environmental conditions following a freezing event. This may or may not correlate with whole plant survival. Factors such as plant part, plant age and plant morphology (stems, rhizomes, crowns, etc.) need to be considered when assessing the level of cold hardiness of a plant species, especially when trying to identify approaches to improvement by either conventional breeding or genetic enhancement (GE).

In nature, episodic, radiative frosts occur on clear windless nights just prior to sunrise. Under these conditions plants rapidly loses heat to the night sky which is approximately −90°C. After sunrise, frosted plants can be subsequently exposed to light intensities of 600–2000 μmol m⁻² s⁻¹ which may result in photobleaching. The appearance of these plants stand in stark contrast to the dull green water soaked plants observed in artificial freeze tests that are thawed in the dark and maintained at low light intensities (100 μmol m⁻² s⁻¹ or less). Electrolyte leakage is often employed to measure frost tolerance; however, in nature, plants may also be injured by the photobleaching that occurs following a frost. This type of injury only manifests itself after 3 or 4 days under natural conditions of full sunlight. As the electrolyte leakage test is generally conducted the day after an artificial freeze, injury related to photobleaching will not be detected nor will its effect on survival be taken into account.

Canopy structure and leaf orientation can also impact frost injury. Exposed horizontal leaves tend to lose heat faster than erect leaves whereas the leaves within a canopy are somewhat protected by the heat released from the soil. The relative humidity of the atmosphere increases to saturation and water condenses as dew on leaves that are colder than ambient air temperature. Water droplets on leaves can either act as a nucleating agent or activate other extrinsic ice nucleators present on the leaf surface. Dry leaves will supercool to a much greater extent than wet leaves (Wisniewski et al. 2002, Gusta et al. 2004). Non-wetted, cold-hardened canola leaves readily supercool to temperatures as low as −13°C, whereas wetted leaves supercool to only −3°C to −5°C (Gusta et al. 2004). The addition of ice to dry leaves to initiate freezing may not be effective and the plants may remain in a supercooled state.

Wisniewski et al. (1999) have suggested that in order for extrinsic nucleators to induce freezing of plants, ice must physically grow into the leaf interior, either through a stomate, a crack in the cuticle, or through a broken epidermal hair cell. This implies that plants with a thick waxy cuticle, and/or an upright growth habit that can readily shed water may avoid or escape an episodic frost because extrinsic nucleators are not activated (Aryal and Neuner 2010). Wisniewski et al. (2002) showed that tomato plants supercooled to −7°C or lower if their leaves were coated with a hydrophobic particle film that prevented wetting. If we are to select and produce superior frost tolerant plants consideration of these factors may be beneficial.

The roots of winter cereals are killed when exposed to −5 to −9°C; however, the apical meristem tolerates −20 to −30°C in mid-winter (Chen et al. 1983). In the absence of adequate moisture in the spring, seedlings may die before establishing a root system. Following a controlled freeze, Chen et al. (1983) observed that root initiation was more sensitive to freezing than shoot regrowth. The majority of crown cells, as determined by viability tests, were alive, however, the cells for root initiation were impaired. This was further verified using wheat cell callus cultures which were able to grow and divide following a controlled freeze test but were unable to produce roots for survival. Thus, in the field, the lack of root initiation rather than just the death of a proportion of cells is the main cause of winter kill.

**Genomics, biotechnology and systems biology**

Recent advances in the availability of sequenced plant genomes and the ability to economically sequence entire transcriptomes and genomes using ‘next generation sequencing’ has added a valuable asset to the study of trait inheritance. The use of association mapping in combination with genome-wide identification of single nucleotide polymorphisms within specific genotypes, has increased the ability to identify the alleles of genes associated with a specific trait, whether the inheritance is complex involving the contribution of many genes or relatively straight forward involving only a single or a few genes. Using these approaches, recent reports utilizing various *Arabidopsis* genotypes planted in common gardens distributed in distinct climatic settings have identified loci and the alleles of genes within those loci that confer adaptation to specific climates (Fournier-Level et al. 2011, Hancock et al. 2011). Molecular breeding using specific trait markers, therefore, has great potential for providing cultivars with increased freezing tolerance despite whether the
trait exists at a biochemical, structural or regulatory level.

Biotechnology may offer the opportunity for more rapid advances when improvements require the introduction of heterologous genes. However, this statement is tempered by the complexity of stress responses and their integration with other physiological and developmental processes. Sinclair (2011) has noted concerns about using a ‘bottom-up’ approach, starting at the molecular level given the vast number of interacting biochemical networks and the damping of any single molecular change when scaling-up the physiological hierarchy leading to yield improvement. Additionally, Varshney et al. (2011) have noted that while GE technology offers great potential, acceptance of GE-technology is far from universal and political will to facilitate acceptance of GE-crops has not been greatly forthcoming. While admittedly this has nothing to do with understanding plant cold hardiness, it underscores the need for a GE-crop to offer a significant and consistent improvement in stress tolerance and yield improvement if it is to garner commercial investment to clear regulatory hurdles and widespread acceptance by growers and the general public.

Plants are complex organisms composed of multiple cell types, tissues, and organs, as well as a myriad of components within a cell. This complexity results in a multitude of different responses to the environment and results in a great variety of changes within a plant that may limit plant growth. Systems biology, which attempts to integrate the vast amount of data obtained through recent advances in transcriptomic, proteomic and metabolomic technologies, has allowed us to obtain a more comprehensive picture of the response of plants to abiotic stress (Cramer et al. 2011). Systems biology is being used to increase our knowledge of gene regulatory networks, signaling, and cross talk between different biotic and abiotic stress responses. A key to gaining a more holistic understanding of abiotic stress response in plants will be the development of new bioinformatic approaches that allow us to integrate many levels of data, recognize patterns in complex data sets, and develop new models of how biological systems function. In this regard, the word biological cannot be overemphasized.

**Summary and future directions**

We have attempted to illustrate the complexity of the adaptive mechanisms associated with freezing tolerance and highlight the difficulties associated with studying and assessing cold hardiness in plants. We have also suggested that this may be one of the primary reasons for the lack of significant and reliable improvements in freezing tolerance under field conditions. Like the Hunting of the Snark in Lewis Carroll’s poem, freezing tolerance is an elusive goal that has been pursued with many different approaches. It is our opinion, that only by taking into account the whole plant and its environment can we understand what aspect of the plant needs to be addressed in order to improve cold hardness and what protocols should be used to best study and identify sources of improvement. A true systems biology approach that utilizes information from all of the ‘omic’ technologies as well as plant physiology, morphology, and ecology will be required (Cramer et al. 2011). Bioinformatic approaches that are able to integrate information from these various levels will be a great asset and provide the potential for significant advances. Additionally, it is important to stress that a major portion of understanding freezing tolerance revolves around the biology of water and its interactions with cellular components at low temperature (Olien and Livingston 2006). This is where plant structure and morphology play an important role. How ice forms and the kinetics of water movement play an important role in plant freezing response. An understanding of how water interacts with macromolecules within the cell lies at the core of understanding freezing tolerance.

The last two decades has seen an exponential growth in the information we have about how plants respond to low temperature, and the genes and biochemical components that may potentially play a role in adaptation to freezing stress. Fundamental questions, however, remain to be addressed. While not meant to be complete, a sample of these questions is posed below in an attempt to stimulate further research.

1. What determines the specific set of environmental conditions that triggers cold acclimation and what determines the rate of cold acclimation? As discussed, plants in the field respond to multiple environmental cues. How these cues interact to inhibit growth and initiate cold acclimation will greatly increase our ability to identify important genes and gene networks that can be manipulated for increased cold hardiness (Cramer et al. 2011).

2. What features are responsible for differences in cold hardness within a species (e.g. why is one cultivar killed at −20°C while another at −25°C? Often times the level of expression of a specific gene, protein or metabolite can appear to explain levels of cold hardness within a species in a qualitative manner but the correlation weakens when one examines a range of genotypes. Understanding the interplay of factors that contribute to the variety of aspects of cold hardness may help to explain differences
in cold hardiness within a species in a quantititative manner.

3. What are the features that allow some species in a genus to survive $-196\,^\circ\text{C}$ when other species within that genus are killed at $-15\,^\circ\text{C}$ (e.g. Cornus serica vs C. florida, respectively)? Adaptation of plants to adverse environments has evolved over many millennia. Understanding the relationship of plant form, physiology and biochemistry to environmental stress resistance requires a broad perspective that takes into account the ecology and evolution of a plant species. This is especially true when trying to assign function to a specific gene, protein or metabolite.

4. What features of the plant allow some plants to supercool to $-20\,^\circ\text{C}$ vs $-40\,^\circ\text{C}$ and why are some species killed at $-60$ or $-80\,^\circ\text{C}$ even though no measurable water can be detected by nuclear magnetic resonance? The properties of a cell, tissue or organ that allow it to deep supercool remain enigmatic despite the widespread prevalence of this ability in many plant species. A critical understanding of deep supercooling is needed in order to take advantage of an important mechanism of cold hardiness; freeze avoidance.

5. How are rates of deacclimation and the ability to reacclimate genetically regulated? One of the greatest challenges in producing crop plants that are stress resistant in a time of global climate change will be to tailor them to respond to erratic and dramatic shifts in temperature without adversely impacting yield. Some species deacclimate very quickly whereas others deacclimate very slowly when exposed to warm temperatures. The ability to manipulate when plants begin to deacclimate and allow them to quickly reacclimate if needed will be necessary to address this challenge.

6. What features are responsible for tolerance to an acute vs a chronic freezing stress? As discussed, the ability of a plant to survive an episodic stress vs a long-term stress can be substantially different, e.g. winter vs spring cereals. Most studies have evaluated the ability of a plant to survive a single freezing event and use these data to generate an LT$_{50}$, presumed to be indicative of field survival. The genetic factors that enable plants to survive a prolonged stress (days to weeks) are still not well understood.

7. How does vernalization control the flow of photosynthates for the process of cold acclimation in winter cereals? When plants are exposed to acclimating temperatures, sugars can be utilized for either growth (biomass) (e.g. spring cereals) or as cryoprotective compounds (e.g. winter cereals). How vernalization regulates the flow of sugars to these two parameters will greatly enhance our understanding of cold hardiness in spring vs winter genotypes of cereal crops.

8. If growth cessation is required for full acclimation what level of cold hardiness can we expect in plants that are growing? Is freezing tolerance compatible with growth? Growth cessation is considered to be a requirement for achieving maximum cold hardiness. In fact, resistance to many environmental stresses may involve some level of tradeoff between resistance and biomass accumulation. Understanding the balance between these two parameters (stress resistance vs growth) for specific mechanisms of stress resistance will be essential to produce new genotypes of crop plants that are high yielding under a wide variety of stressful and non-stressful environments.

It is perhaps most appropriate to end this opinion by quoting a statement made by Felix Franks (1985) in his book on the biophysics of water at low temperature regarding cryobiology and cryotechnology and applying it to the study of freezing tolerance. Too frequently experimental observations on highly complex systems are based on measurements performed under non-equilibrium conditions and rationalized in term of elementary textbook science. The degree of undercooling, the mechanism of ice nucleation, the growth and type of crystals, their size and distribution, the flow properties of the unfrozen matrix and any long-term changes because of ageing need to be taken into account. Fundamental studies of such processes are unglamorous, tedious, and time-consuming, but they form the scientific base of low temperature technology, the understanding and exploitation of which is still in its infancy, even among those who should know better.

**References**


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