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**Cross-disciplinary insights into the mechanisms of plant cold hardiness: from molecules to ecosystems.**

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Understanding how plants tolerate or avoid metabolic disturbances induced by chilling temperature or by the formation of intrinsic ice has been a subject of interest for scientists since the 19th century. Advancements over the last thirty years have led to exciting observations in how interactions among plant genes, mRNAs, proteins and metabolites, and modifications to the epigenome, enhance freezing tolerance. Unfortunately, this has not yet led to tangible improvements in the capacity of economically important crops to tolerate or avoid chilling and freezing injuries. Underappreciated in the design of many controlled experiments is that species-specific adaptations to chilling or freezing stress are the result of exposure to multiple environmental cues. Data generated from experiments wherein only temperature is manipulated may not reflect field conditions and reduce our ability to translate results observed in the lab to the field.

To further complicate matters, land temperatures have increased globally by 1.1°C over the last 150 years and mean winter temperatures are projected to rise over the next 60 years by an additional 2°C in Central Europe and 2 to 4°C in the Northwest United States and Canada (**Intergovernmental Panel on Climate Change 2022**). Warmer than average fall temperatures will reduce the maximum capacity of plants to survive exposure to sub-zero temperatures. The higher prevalence of mid-winter thawing will melt snow cover that insulates below-ground plant organs from lethal low air temperatures. Exposure to temperatures above 0°C over winter will deharden plant tissues and result in a greater susceptibility to freezing injury and delay chilling exposure for endodormancy release. Meaningful improvements in crop cold hardiness will come from identifying traits associated with how plants increase cold hardiness and maintain it under different winter microclimates.

In December 2021, our scientific community was fortunate to meet online and share the newest knowledge on the plasticity of cold adaptation in plants during the 12th International Plant Cold Hardiness Seminar (IPCHS). Since the first IPCHS in 1977, we have witnessed the development of molecular biological and bioinformatic technologies as well as non-destructive monitoring techniques that have impacted our understanding of how plants survive at severe low temperatures. The 12th IPCHS focused on understanding plant survival strategies from molecular to ecological perspectives. Of the six sessions focusing on these various aspects, the sessions on biophysics of ice nucleation and the molecular response of plants to low temperatures were dedicated to the memory of Charles Robert Olien and Dirk Hincha, who have made significant contributions to our understanding and appreciation of plant cold hardiness. Three sessions on ecophysiology and plant-microbe interactions at low temperatures were organized to discuss a variety of topics beyond molecular biology under controlled environmental conditions.

Discussions included perspectives from the outdoor environment and provided an opportunity to rediscover how plants live at low and sub-zero temperatures.

This special issue collected 16 papers based on the presentations of the 12th IPCHS (**Charrier 2022; Cvetkovska et al. 2022; Hamilton et al., 2023; Khatun et al. 2023; Kutsuno et al. 2023; Lintunen et al. 2022; Mura et al. 2022; Nassuth and Jesionowska 2022; North et al. 2022; Sasaki and Imai 2023; Stegner et al. 2022, 2023; Vyse et al. 2022a, 2022b; Watanabe et al. 2022; Willick and Lowry 2022**). The following papers presented in this special issue reflect a wide array of structural, phenological, biochemical and molecular strategies that have evolved in plants to survive at sub-zero temperatures.

Under subzero temperatures, the way plant tissues freeze determines whether the plant survive this stressful event. As observed for most stress, resistance can be split into two main processes: avoidance and tolerance. Avoidance is the ability to inhibit ice propagation from the surface of the plant towards inner tissues. **Hamilton et al. (2023) studied, in different maize (*Zea mays*) genotypes, the role of epicuticular waxes in forming a physical barrier to ice propagation. Chilling conditions preceding a frost modified the molecular and thickness of the leaf cuticle, suggesting that this dynamic response could be a focus in the selection of frost-avoiding corn genotypes.** **Stegner et al. (2023)** explored whether the freezing-induced separation of the mesophyll from vascular tissue in the endodermis of mountain pine (*Pinus mugo* Turra) needles impacted ice management and photosynthesis. Cryo-microscopy was used to visualize that ice in the endodermis was confined to the central vascular cylinder. Lignified tangential cell walls were hypothesized to impede ice propagation into the non-frozen mesophyll capable of gas exchange.

The extent of dehydration in mesophyll cells in response to the accumulation of extra-tissue ice varies from functioning cells containing supercooled water to cells under cytorrhysis. To assess the structural factors associated with injury manifesting from freezing dehydration, **Stegner et al. (2022)** characterized the tissue structure in 11 species. Analysis of tissue traits identified that species with thick cell walls and small intercellular spaces promote supercooling. In contrast, species with high levels of freezing dehydration contained large intercellular spaces that could accommodate ice.

Ice propagation within the xylem sap creates a phase separation between the solid ice, water vapour and the remaining fraction of supercooled liquid water. Under low water potential, gas forms bubbles within the ice lattice that, upon thawing, coalesce to form an embolism of the whole xylem conduit. It is unknown how bubble characteristics are determined in the frozen xylem. To help address this question, **Lintunen et al. (2022)** provided a quantitative assessment of the bubble volume in frozen sap of *Betula pendula*, highlighting the role of vessel size, water status and bark permeability in the air-to-ice ratio. They

report that bubble size increased with the volume of the xylem conduit, but also with lower water potential and bark permeability. These factors controlling the development of winter embolisms are important drivers of woody species distribution at high elevations (**Charrier et al. 2013**).

Plants can enhance their capacity to survive harsh winter conditions by transiently increasing their level of freezing tolerance through a process known as cold acclimation. **Willick and Lowry (2022)** explored the genotypic variations required to induce cold acclimation in three switchgrass (*Panicum virgatum* L.) ecotypes. This work identified that populations originating from more northern latitudes initiated cold acclimation at 7°C warmer temperatures than populations originating from more southern latitudes. An earlier response to declining temperatures was hypothesized to provide plants with additional time to prepare for winter and cope with rapid changes in fall temperatures associated with frosts.

Exposure to low threshold temperatures induces bud endodormancy to limit the exposure of tender organs to harsh winter conditions. However, the current models used to predict the onset of endodormancy vary depending on the study (reviewed in **Charrier 2022**). By integrating the effect of temperature, photoperiod and their interaction, **Charrier (2022)** explored which models were the most efficient to predict endodormancy release and bud break in walnut (*Juglans regia* L. cv Franquette). Most of the models tested provided an efficient prediction of phenology and the inclusion of photoperiod in the model improved its accuracy.

Exposure to threshold temperatures and photoperiod cues results in the activation of an Inducer of CBF Expression (ICE), also known as a regulator of stomatal development, but the role of ICE in cold acclimation was recently questioned in *Arabidopsis thaliana*. The induction of CBFs, also known as dehydration response elements (DRE), will in turn activate the cold-regulated (COR) genes. **Nassuth and Jesionowska (2022)** analyzed sequence data for ICE and its related regulators, STOMATA 1 (OST1) and MITOGEN ACTIVATED PROTEIN KINASE 3/6 (MPK3/6). Bryophytes, brown algae and ferns have one type of ICE protein, while angiosperms have two types of ICE proteins, each with characteristic interaction motifs. OST1 ortholog was conserved in all plant species, including algae, whereas regulatory sequence motifs for the OST1 in the ICE proteins were less conserved in the non-angiosperms. MPK3/6 motifs were conserved in both types of ICE proteins in angiosperms. These data indicate that the overlap between stomatal regulation and cold acclimation mechanisms needs further investigation in an evolutionary context.

The initial exposure to cold acclimating temperatures serves as a priming event for plants. Primed freezing tolerance in plants can be improved by exposure to further acclimation treatment (triggering) after a lag phase. **Vyse et al. (2022a)** conducted a comprehensive study using metabolomic and transcriptomic analysis during cold priming and triggering. Genes encoding Late Embryogenesis Abundant

(LEA) proteins, antifreeze proteins, or reactive oxygen species (ROS) detoxifiers showed higher expression in triggering phase compared to priming phase. Some transcription factors and other cold-responsive genes were strongly induced during triggering, suggesting the existence of a unique mechanism for fine-tuning plant response to temperature fluctuation.

Downstream of the cold acclimation response pathway, cold-induced and/or dehydration-induced synthesis of specific compounds stabilize cell membranes, proteins and nucleic acids. Soluble sugars and ABA synergistically inhibit growth and tolerance to dehydration in the gemma of liverwort (*Marchantia polymorpha*) (Akter et al. 2014). Using a line expressing a genome-edited liverwort ABA DEFICIENT 1 (MpABA1), **Khatun et al. (2023)** observed that endogenous ABA regulates a positive feedback loop for sugar-induced sugar accumulation, thus promoting dehydration tolerance.

Another noticeable change that occurs in cold acclimating plants is the modification in membrane composition, which protects against freeze-induced dehydration, oxidative and freeze-thaw injury. Previously, **Minami et al. (2015)** identified that the cold-induced DYNAMIN-RELATED PROTEIN 1E (DRP1E) regulates cold-induced plasma membrane compositional modifications through endocytosis. Proteome analysis of cold-acclimated Arabidopsis by **Watanabe et al. (2022)** identified 81 differentially regulated proteins in wild-type and 71 proteins in a *drp1e* knockdown mutant suggesting that DRP1E contributes to the reconstruction of the plasma membrane during cold acclimation.

When cold-acclimated plants are exposed to warmer temperatures, they undergo physiological dehardening through a process known as deacclimation. **Vyse et al. (2022b)** identified during the initial stages of deacclimation that Arabidopsis undergoes a hypoxia-like response resulting in increased alcohol dehydrogenase activity and an upregulation of hypoxia gene markers. Mutant lines lacking a hypoxia response revealed slower rates of deacclimation. Observations from RNA sequencing identify that the regulation of deacclimation is as complex as cold acclimation and finely controlled to maintain the rate and onset of deacclimation.

The cell wall provides mechanical resistance to the volumetric expansion of the extracellular ice. Cell wall composition and thickness are therefore key components in the time course of cold acclimation and deacclimation. **Kutsuno et al. (2023)** investigated how cell wall monosaccharide composition shifts in response to temperature cues. During cold acclimation, an increased proportion of arabinogalactan proteins, pectic arabinan and galactan, and xyloglucan were observed in the cell wall. However, during deacclimation, cell wall modification was not a reversible process as several components in deacclimated plants did not return to non-acclimated level. **Kutsuno et al. (2023)** hypothesized that deacclimation does

not restore the cell wall to its non-acclimated stage but rather the cell wall is modified to promote growth under warming conditions.

After the release of endodormancy, woody perennials deacclimate in response to warm temperatures. **North et al. (2022)** observed in grapevines (*Vitis* spp.) an interacting effect of chill unit accumulation and current temperature on deacclimation. This work hypothesizes that the dynamic of cold deacclimation can represent an alternative method to forcing tests used to assess the cryptic transition between endodormancy and ecodormancy.

Low temperature stress in the natural environment is inseparable from the influence of snow cover and the microorganisms that lurk there. Prolonged snow cover protects plants from freezing injury but exposes plants to low light, chilling and high humidity conditions that provide optimal conditions for psychrophilic 'snow mold' pathogens. **Sasaki and Imai (2023)** reviewed recent progress in differentiating between the molecular mechanisms of pathogen-induced and cold-induced disease resistance.

While plants are negatively impacted by excessive snow cover, the loss of snow cover and exposure to warming winter conditions can negatively impact plants. The earlier budbreak observed by **Mura et al. (2022)** in black spruce (*Picea mariana* [Mill.] BSP) at more Northern latitudes increases the risk of a false spring *i.e.* when growth resumption happens before the last freezing event. This work highlights that under warming winter conditions, black spruce populations from the most northern part of their range are more responsive to temperature fluctuation, which increases their vulnerability to freezing injury.

Many cold-water algae are psychrophilic that have adapted to growth under low temperature conditions and cannot proliferate at temperatures higher than 20°C. Therefore, rising temperatures in polar lakes have left many psychrophilic algae vulnerable to heat stress. **Cvetkovska et al. (2022)** reviewed how characterizing the genome of Antarctic psychrophile *Chalmydomonas priscuii* and its relative *Chalamydomas* sp. ICE-L has given rise to a plethora of new tools to characterize the genetic basis of psychrophily.

Our journey to understand the complexity of the freezing process requires novel means to assess how plants tolerate and avoid the formation of ice within their tissues. While we have learned much over the last thirty years about how plants respond to cold at different levels, it is still not clear how multiple environmental cues observed in the field influence the cold hardy phenotype. We hope that from this special issue, readers can gain some insight into the current understanding of how plants respond to low temperatures and that this work will spark future research to expand our understanding of plant behaviour under adverse growth conditions. It is also our hope that future cross-disciplinary insights into

the mechanisms of plant cold hardiness will accelerate discussions on the impact of warming winter conditions on how plants survive at sub-zero temperatures.

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