




# Responses of the Plant Cell Wall to Sub-Zero Temperatures: A Brief Update

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Our general understanding of plant responses to sub-zero temperatures focuses on mechanisms that mitigate stress to the plasma membrane. The plant cell wall receives comparatively less attention, and questions surrounding its role in mitigating freezing injury remain unresolved. Despite recent molecular discoveries that provide insight into acclimation responses, the goal of reducing freezing injury in herbaceous and woody crops remains elusive. This is likely due to the complexity associated with adaptations to low temperatures. Understanding how leaf cell walls of herbaceous annuals promote tissue tolerance to ice does not necessarily lead to understanding how meristematic tissues are protected from freezing by tissue-level barriers formed by cell walls in overwintering tree buds. In this mini-review, we provide an overview of biological ice nucleation and explain how plants control the spatiotemporal location of ice formation. We discuss how sugars and pectin side chains alleviate adhesive injury that develops at sub-zero temperatures between the matrix polysaccharides and ice. The importance of site-specific cell-wall elasticity to promote tissue expansion for ice accommodation and control of porosity to impede ice growth and promote supercooling will be presented. How specific cold-induced proteins modify plant cell walls to mitigate freezing injury will also be discussed. The opinions presented in this report emphasize the importance of a plant's developmental physiology when characterizing mechanisms of freezing survival.

**Keywords:** Abiotic stress • Cell wall • Cold acclimation • Dehydration • Freezing tolerance • Supercooling

## Introduction

At northern latitudes, a lack of adequate winter hardiness is the main environmental factor limiting plant distributions.

Overwintering success is contingent on whether plant tissues can tolerate the formation of tissue ice or supercool to avoid freezing injury. Exposure to sub-lethal temperatures or endogenous abscisic acid (ABA) induces a cold acclimation response that alters numerous physiological traits. The up-regulation of several C-repeat binding factor/dehydrative response element binding transcription factors promotes the downstream modification of the plasma membrane and cell wall, the accumulation of cryoprotectant metabolites and proteins and the reduction of tissue water (see Uemura et al. 2006, Knight and Knight 2012, Wisniewski et al. 2014, 2020, Panter et al. 2020 for comprehensive reviews). Exposure to non-lethal freezing temperatures ( $-3^{\circ}\text{C}$ ) can induce an additional sub-zero acclimation response (Trunova 1965, Olien and Smith 1977, Livingston and Henson 1998, Takahashi et al. 2019, 2020). In acclimated plants, the disruption of the plasma membrane from freeze-thaw injury is generally accepted as the primary cause of lethal injury (Sakai and Larcher 1987, Olien and Livingston 2006, Uemura et al. 2006, Arora 2018). There is less agreement on the importance of the cell wall in mitigating plasma membrane damage.

Early experiments involving isolated plant protoplasts reported no significant difference (Siminovitch et al. 1978) or a higher freezing tolerance (Murai and Yoshida 1998) when compared to intact cells. This work drew two major conclusions that the firm attachment of the plasma membrane to the cell wall enhances freezing injury and the cell wall provides minimal tolerance to freezing. In contrast, observations with artificial matrices and intact plant tissues support the theory that the cell wall is an integral barrier to ice nucleation and propagation (Olien 1974, Ashworth and Abeles 1984, Wisniewski 1995, Yamada et al. 2002, McCully et al. 2004). Recent studies highlight the importance of tissue-specific variability in cell-wall elasticity as a mechanism to cope with tissue ice or promote supercooling (Neuner et al. 2019, Willick et al. 2019, Stegner et al. 2020, Steiner et al. 2020).

During cold and sub-zero acclimation, the cell wall and extracellular space, referred to as the apoplast, experience significant remodeling that leads to the acquisition of freezing tolerance and avoidance (Knight and Knight 2012, Panter et al. 2020). Treatment of Arabidopsis (*Arabidopsis thaliana*) at 4°C for 12 to 48 h downregulate cell-wall genes that promote cell expansion (Hannah et al. 2005). Prolonged exposure to 4°C (for days) upregulates transcripts signaling the accumulation of matrix polysaccharides, and wall remodeling enzymes that minimize matrix porosity, depending on the tissue, enhance or reduce cell-wall rigidity (Panter et al. 2020). Numerous studies report on the higher accumulation or specific activity of expansins, glycoside hydrolases (GHs), pectin methylesterases (PMEs) or xyloglucan endotransglucosylase/hydrolases (XTHs) in response to declining temperatures (Takahashi et al. 2016, 2019, Duruflé et al. 2017). These cell-wall modifications promote the development of tissue-level barriers responsible for the degree and depth of freezing survival.

The cell wall is not only a barrier against ice ingress to the plasma membrane, a primary site of freezing injury, but it also controls the propagation of ice. This mini-review will consider how low-temperature acclimation modifies the apoplast and discuss the significance of these changes in enhancing freezing tolerance and avoidance in herbaceous and woody plants.

### Ice Nucleation and the Role of the Cell Wall in Tolerating Freezing Dehydration Injury

The attraction of water molecules to cell-wall polymers is a major determinant in the freezing behavior of individual cells. Under normal atmospheric pressures, a decline in temperature decreases the kinetic energy of water molecules. Water molecules cooled below 4°C have lower kinetic energy than what is required for the formation of hydrogen bonds. At 0°C, hydrogen bonds form more frequently resulting in metastable clusters of regularly ordered water molecules. A gradual reduction in temperature enhances the likelihood of a stable nucleus and the development of an extended ice lattice. Pure water can supercool to –38°C before undergoing homogeneous ice nucleation (Franks 1982). However, an ice nucleator can serve as a template for a stable nucleus and promote heterogeneous ice nucleation at temperatures close to 0°C. Common sources of ice nucleation include apoplastic bacterial epiphytes, intrinsic plant-derived substances, extrinsic ice nucleation active microorganisms and soil particulates (reviewed by Wisniewski et al. 2014).

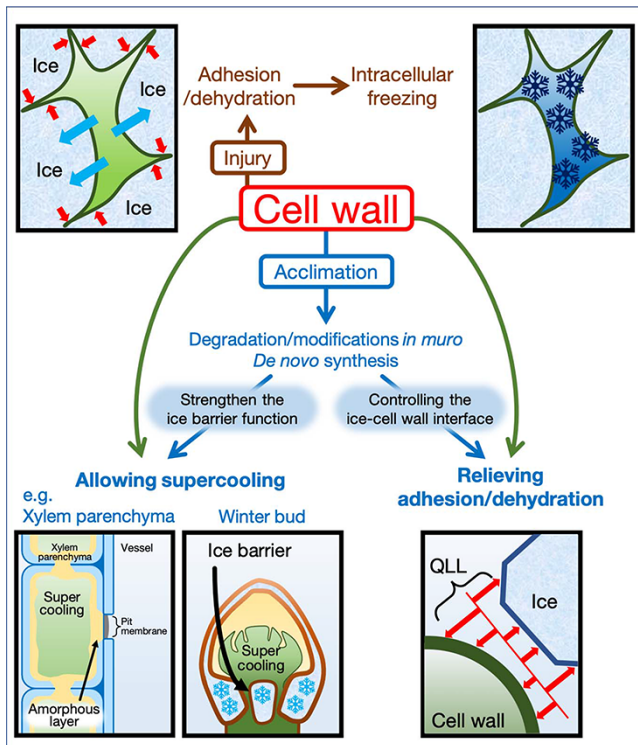
Under natural conditions, herbaceous freezing-tolerant plants cool at slow rates (<3°C h<sup>-1</sup>) and initiate ice nucleation in extracellular spaces between –0.5 and –4°C (Arora 2018). The ensuing equilibrium freezing is a result of small displacements in the energy required for water molecules to join (freeze) or escape (melt) the extracellular ice lattice. A gradual reduction in temperature promotes the diffusion of intracellular water to extracellular ice until the energy required for freezing

and melting attains a new equilibrium. Injury from equilibrium freezing arises from interactions among the ice aggregates, the cell-wall matrix and the adjoining plasma membrane.

Below 0°C, there is a competitive interaction between ice and hydrophilic cell-wall polymers for a thin layer of intervening water molecules known as a quasi-liquid layer (QLL) (Olien 1974, Slater and Michaelides 2019). Equilibrium freezing reduces the kinetic freedom of water molecules (Franks 1982) while enhancing the adhesive tension of the ice lattice and the QLL. At sub-zero temperatures, a sufficient reduction in QLL thickness promotes adhesions of ice with cell-wall polymers (Olien 1974). This injurious stress can result in significant distortions of cell integrity that ruptures the plasma membrane as temperature-induced intracellular desiccation ensues (Olien and Smith 1977). Interestingly, between 0 and –100°C, the kinetic energy of adhesion stress is greater than the kinetic energy associated with freezing (Olien and Livingston 2006). One would think that this is the primary cause of freezing injury. Yet freezing-tolerant herbaceous plants experience lethal injury at –10 to –30°C from equilibrium freezing-induced dehydration (Olien 1974, Olien and Smith 1977, Olien and Livingston 2006, Wisniewski et al. 2020). Dehydration and adhesion likely occur simultaneously within adjacent tissues (Fig. 1).

One proposed mechanism for the alleviation of adhesive stress is that plants exude water-soluble solutes into the apoplast to enhance the width of QLL (Fig. 1). During exposure to mild frost (–2 to –5°C), winter cereals hydrolyze fructan oligosaccharides into their constituent hexose sugars (Trunova 1965). Based on this discovery, Olien hypothesized that plants exude soluble sugars into the extracellular space to relieve adhesive stress (Olien and Smith 1977). Subsequent experiments with winter oat and fall rye at –3°C confirmed the presence of extracellular fructan hydrolases and cell-wall invertases that hydrolyze fructan resulting in a concomitant increase in extracellular sucrose, glucose and fructose and a decline in intracellular fructan concentrations (Livingston and Henson 1998). It is, however, an open topic of debate whether sub-zero acclimation induces an increase in hexose sugars solely to minimize adhesions or to lower the availability of free water.

Factors other than simple sugars can mitigate damage caused by ice ingress. Isothermal calorimetric analysis indicated that, unlike the non-acclimated oat crown, the fraction of unfrozen water in cold-acclimated oat crowns did not follow the colligative freezing pattern observed in sucrose solutions (Livingston 2007). This suggests that cold acclimation promotes water sequestration in the intracellular space. Solutes exuded from the ice lattice in affiliation with the cell-wall matrix could serve as a barrier to ice. With magnetic resonance microimaging, Willick et al. (2019) identified a putative cell-level dehydrative barrier between the ice sensitive shoot apical meristem and freezing-tolerant vascularized tissues. In winter oat recovering from freezing, the same tissue region accumulates suberin and hypothetically operates as a physical barrier against secondary pathogenic injury (Livingston et al. 2013).



**Fig. 1** Schematic diagram of the effect of cell wall on freezing tolerance of plants. When plant cells or tissues are exposed to severe freezing. The QLL expands after the incorporation of soluble substances into the apoplastic space to relieve adhesion and dehydration stress. Reduction of the QLL can result in injurious adhesions forming between the cell wall and extracellular ice crystal. Simultaneously, the establishment of a vapor pressure gradient from the intracellular supercooled water to the extracellular ice crystal will promote dehydration injury. Specialized plants can avoid freezing altogether by reducing the porosity of the cell wall (e.g. xylem parenchyma cells) or by developing tissue-level barriers (e.g. winter bud of woody perennials).

Intracellular water molecules will diffuse along the sugar osmotic gradient, through the hydrated cell-wall matrix and QLL toward the extracellular ice aggregate (Wisniewski et al. 2014, 2020). Prolonged dehydration at sub-zero temperatures can weaken cellular integrity through shifts in redox chemistry, protein and lipid denaturation (see review by Arora 2018). Severe dehydration can result in cytorrhysis or the deformation of the cell-wall matrix and separation from the plasma membrane (McCully et al. 2004, Stegner et al. 2020, Steiner et al. 2020). Lethal injury is avoidable if the cell wall can maintain its structure without separating from the plasma membrane or if the cell-wall matrix is elastic enough to allow contraction and expansion during dehydration and rehydration.

Recent evidence suggests that the cold-induced increase in cell-wall rigidity is heterogeneous and that herbaceous plants and algae reinforce the cell-wall matrix at specific junction zones to control the flexibility and direction of cell expansion during freezing (McCully et al. 2004, Stegner et al. 2020, Steiner et al. 2020). Jarvis et al. (2003) stresses the importance of these junctions for growth and cellular integrity and

as reinforcements with various polymer compositions in the primary cell walls and the middle lamella. Thick cell-wall occlusions at cell corners of cold acclimated and frozen streptophyte green algae (*Klebsormidium crenulatum*) that maintain membrane and cell-wall adhesion were hypothesized to reinforce cells against cytorrhysis (Steiner et al. 2020). McCully et al. (2004) postulated that cell-wall juncture zones in the petioles of white clover (*Trifolium repens* L.) and California poppy (*Eschscholzia californica* Cham.) leaves maintain higher flexibility for separation to accommodate large ice lenses. Stegner and colleagues hypothesize a similar theory that glacier buttercup (*Ranunculus glacialis* L.) leaves accommodate ice lenses because of the inherent flexibility of the pectin-rich layer in mesophyll cell walls (Stegner et al. 2020). Herburger and Holzinger (2015) previously reported that callose deposition in cell walls reduced water loss. A similar mechanism could explain the cold-induced callose deposition in streptophyte green algae (Steiner et al. 2020). Additional immunostaining, Raman and Mid-infrared spectroscopy imaging studies will further elucidate the site-specific importance of cell-wall elasticity to better explain how plant cells tolerate the presence of ice within various tissues.

### Remodeling of the Woody Perennial Cell Wall Promotes Freezing Avoidance Mechanisms

Overwintering plants need to protect tissues critical for re-growth. Tissues of woody perennials are classically employed to study the primary adaptations to freezing. Cortical parenchyma cells survive exposure to sub-zero temperatures by tolerating extracellular ice formation, whereas some woody perennials develop xylem parenchyma cells that supercool to avoid freezing (Sakai and Larcher 1987).

The stability at ultra-low temperatures distinguishes deep supercooling from the transitory supercooling in annual plants. Supercooling from  $-1$  to  $-12^{\circ}\text{C}$  enhances the freezing avoidance of annuals that cannot tolerate freezing dehydration stress during the growing season. The depth of this transitory supercooling depends on leaf tissue wettability and the formation of tissue barriers against extrinsic ice nucleation (reviewed by Wisniewski et al. 2014). Some woody perennial leaves, rhizomes, overwintering tree buds and xylem parenchyma cells deep supercool to temperatures ranging from  $-15$  to  $-60^{\circ}\text{C}$  (Quamme et al. 1973, Sakai and Larcher 1987, Kuroda et al. 2003). These plants develop barriers against tissue water loss and ice ingress. The entrapped water remains in a liquid phase due to the lack of heterogeneous ice nucleators or the accumulation of anti-ice nucleating substances (Kasuga et al. 2008, Ishikawa et al. 2015).

Woody perennials that exhibit deep supercooling develop tissues lacking large extracellular spaces in favor of a continuous network of cell-wall microcapillaries (Sakai and Larcher 1987). Studies involving artificial matrices identified that microcapillaries with diameters of less than 100 nm impede the propagation of ice (Olien 1974, Ashworth and Abeles 1984).



Within these microcapillaries, the strength of the adhesive forces between water and cell-wall polymers is greater than the forces of cohesion amongst water molecules (Wisniewski 1995, Slater and Michaelides 2019). In the absence of an intrinsic nucleator, the adhesive tension between the cell-wall matrix and water molecules in small microcapillaries (<4 nm) is sufficient to depress the ice nucleation temperature to  $-25^{\circ}\text{C}$  and inhibit the evaporative loss of water to an adjacent ice sink (Olien 1974, Ashworth and Abeles 1984).

The importance of pore diameter was experimentally confirmed in peach (*Prunus persica* L. cv. Loring) xylem ray parenchyma cells treated with pectinase that resulted in the partial degradation of the cell wall, greater pore size and a reduction in the capacity to deep supercool (Wisniewski 1995). However, the xylem parenchyma cells of apple stems remain supercooled after the disruption of the plasma membrane by freeze-thawing, steam or a chloroform treatment (Quamme et al. 1973) suggesting that the cell wall, irrespective of the properties of the plasma membrane, can promote deep supercooling.

In xylem parenchyma, the largest microcapillaries are reportedly at the pit membranes (Wisniewski 1995). De-acclimation of peach stems at room temperature or treatment with pectinase, ethylene glycol tetraacetic acid (EGTA) and oxalic acid (Wisniewski 1995) reduces deep supercooling capacity, indicating that cold-induced cell-wall remodeling influences xylem pit diameter. Treatment of xylem parenchyma with EGTA reduces pit membrane JIM7 epitope labeling for pectin with 35–90% methyl esters (Wisniewski and Davis 1995), indicating that  $\text{Ca}^{2+}$  cross-linking reduces pore diameter. These examples underscore that homogalacturonan (HG) deposition within the amorphous layer might be related to the ability of xylem parenchyma to supercool (Fig. 1).

Visual observations with infrared video thermography illustrate that xylem parenchyma cells freeze intracellularly as single cells or in small cell groups (Neuner et al. 2010). One proposed mechanism for the maintenance of supercooling is that woody perennials accumulate solutes to depress the ice nucleation temperature. Infiltration of Japanese beech (*Fagus crenata* Blume.), katsura tree (*Cercidiphyllum japonicum* Siebold & Zucc.) and mulberry (*Morus alba* L.) stems with 0.5 M glucose enhances the supercooling capacity of xylem parenchyma cells (Kasuga et al. 2013). Regardless of species or degree of acclimation, infiltration of stems with water reduces the supercooling capacity. Interestingly, summer collected stems infiltrated with 0.5 M glucose have higher supercooling capacities as compared with non-infiltrated cold-acclimated winter stems. Although the cell wall influences the maintenance of supercooling, significant shifts in deep supercooling ( $>10^{\circ}\text{C}$ ) are likely induced by the accumulation of intracellular solutes. Further research will help clarify the role played by cell walls in the seasonal changes in freezing resistance in woody plant species.

Some overwintering buds undergo extraorgan freezing, where water from the floret or primordial meristem migrates

along the vapor pressure gradient to ice aggregates in the subtending bud scales or shoot xylem (Sakai and Larcher 1987). Unlike xylem parenchyma cells, ice nucleation and rapid cooling ( $>5^{\circ}\text{C h}^{-1}$ ) in dormant buds induce the intracellular freezing of the primordia (Sakai and Larcher 1987, Ishikawa et al. 2015). Interestingly, partially dehydrated grape hybrid (*Vitis amurensis* × Seibel 13,053 cv. Yamasachi) bud primordia remain supercooled at high (5 and  $16^{\circ}\text{C h}^{-1}$ ) rates of cooling (Kasuga et al. 2020).

To avoid freezing, dormant buds develop chemical and structural barriers to prevent ice ingress into primordial tissues (Fig. 1). Bud scales coated with hydrophobic compounds including cutin and suberin are likely barriers against extrinsic ice formation (Neuner et al. 2019). Dormant buds of *Pinaceae*, which exhibit extraorgan freezing, develop a crown plate between the bud and subtending shoot. Cells in the crown plate develop thick cell walls and remain tightly packed relative to cells in vascular and primordial tissues (Endoh et al. 2009, Kuprian et al. 2017). Cold acclimation of Norway spruce (*Picea abies* L.) buds promotes pectin accumulation in the crown plates (Kuprian et al. 2017). Independently, Lee et al. (2017) observed that shifting Norway spruce to short day (12 h) conditions induces the de-methyl esterification and  $\text{Ca}^{2+}$  cross-linking of HG in the crown plate. This mechanism was reversible by shifting Norway spruce into long day (24 h) conditions. A similar pattern of cold-induced de-methyl esterification occurs in cold-acclimated peach buds (Wisniewski and Davis 1995). These examples underscore the importance of the cell wall in preventing freezing injury.

### Molecular Implications of *in Muro* Modifications for the Control of Freezing Behavior

Advancements in proteomic technologies have further expanded our understanding of the cold and sub-zero acclimation of plant cell walls. Several apoplastic proteins putatively associated with cell-wall degradation and/or modification *in muro* during cold acclimation treatment (Table 1). In Arabidopsis, XTH and PME/ PME inhibitor (PMEI) show diverse responses during cold and sub-zero acclimation. Loss of function of *XTH21* or *XTH19* has been shown to reduce freezing tolerance (Shi et al. 2014, Takahashi et al. 2020), suggesting that xyloglucan reorganization is linked to freezing tolerance. In cold-acclimated winter rape (*Brassica napus* subsp. *oleifera*), higher leaf tensile strength correlates with enhanced specific activity of PME and the concomitant de-methyl esterification of HG (Solecka et al. 2008). The tissue-specific calcium cross-linking of HG was experimentally observed to inhibit the propagation of ice in xylem parenchyma cells (Wisniewski and Davis 1995) and overwintering buds (Kuprian et al. 2017). A *mur1* mutant, which lacks fucosylation of side chains necessary for efficient borate-diester linkages of pectin rhamnogalacturonan II (RG-II) dimerization, has a lower freezing tolerance as compared with wild-type plants (Panter et al. 2019). These findings suggest that

**Table 1** The effect of cold or sub-zero acclimation on the accumulation of select cell-wall-associated proteins. Listed proteins were retrieved from previous proteome studies

Species	Tissue (fraction)	Treatment	Cell-wall-associated protein <sup>a</sup>	Response <sup>b</sup>	Reference
<i>Arabidopsis thaliana</i> (Col-0)	Leaves (apoplast)	4°C for 7 d (cold) –3°C for 3 d (sub-zero)	Expansin	– (cold)	Takahashi <i>et al.</i> (2019)
			Glycosyl hydrolase	– (sub-zero)	
			Lipid transfer protein (GPI)	+	
			Pectin methylesterase	±	
			Pectin methylesterase inhibitor	+	
			Pectinacetyltransferase	+	
			Xyloglucan endotransglucosylase/hydrolase	±	
			α-L-arabinofuranosidase	– (cold)	
			β-galactosidase	– (cold)	
			Polygalacturonase	±	
<i>Arabidopsis thaliana</i> (Col-0 and Sha)	Leaves (apoplast)	Grown at 15°C (cold) or 22°C (control)	Endo-β-glucuronidase	+	Durruffé <i>et al.</i> (2017)
			Expansin	+	
			Lipid transfer protein (GPI)	–	
			N-acetyl-β-glucosaminidase	–	
			Pectin methylesterase	+	
			β-1,3-glucosidase	–	
			Fasciclin-like arabinogalactan protein (GPI)	+	
			Lipid transfer protein (GPI)	+	
			β-1,3-glucosidase (GPI)	+	
			Caffeoyl-CoA O-methyltransferase	– (tolerant)	
<i>Pisum sativum</i> <i>Brassica rapa</i> var. <i>rapa</i> (susceptible/tolerant cultivars)	Leaves (total) Roots (total)	10°C/4°C for 11 d 10°C/4°C for 48 h, –4°C for 8 h	Polygalacturonase inhibitor 1	– (susceptible)	Dumont <i>et al.</i> (2011) Zeng <i>et al.</i> (2018)
<i>Allium cepa</i>	Bulb scales (total)	Frozen at –4.5°C and then thawed on ice	α-xylosidase-like	– (frozen)	Greene <i>et al.</i> (2012)
			α-L-arabinofuranosidase	– (frozen)	
<i>Secale cereale</i>	Leaves (plasma membrane)	4°C for up to 28 d	Fasciclin-like arabinogalactan protein (GPI)	+	Takahashi <i>et al.</i> (2013)
			β-1,3-glucosidase (GPI)	+	

(continued)

Table 1 (Continued)

Species	Tissue (fraction)	Treatment	Cell-wall-associated protein <sup>a</sup>	Response <sup>b</sup>	Reference
<i>Triticum aestivum</i>	Crown shoot apical meristem (SAM) and vascular tissues (apoplast)	4°C for 21 or 42 d	α-glucosidase Apoplastic invertase Cell-wall-β-glucosidase Fasciclin-like arabinogalactan protein (GPI) Fructan exohydrolase Glucan endo-1,3-β-glucosidase Lipid transfer protein Pectin methyltransferase Xylanase inhibitor α-xylosidase β-D-glucan exohydrolase β-expansin β-galactosidase	+ + + + + (SAM only) + + + + + + ± (susceptible) - (tolerant)	Willick et al. (2018)
<i>Eucalyptus benthamii</i> (tolerant)	Leaves (total)	12.4°C for 20 d	Caffeoyl-CoA O-methyltransferase	- (tolerant)	Oberschelp et al. (2020)
<i>E. grandis</i> (susceptible)	Leaves (total)		Caffeoyl-CoA O-methyltransferase	+ (tolerant)	
			Cinnamyl alcohol dehydrogenase	- (both)	
			Hydroxycinnamoyl transferase	+ (susceptible)	
			Xyloglucan endotransglucosylase	- (tolerant)	
			Xylosidase	-	
			β-glucosidase	+ (winter)	Pagrer et al. (2014)
<i>Hydrangea paniculata</i>	Bark/xylem (total)	Early Jan to mid-Jun	Caffeoyl-CoA O-methyltransferase	+ (winter)	Die et al. (2017)
<i>Rhododendron catawbiense</i>	Leaves (total)	Aug to Dec	Cellulose synthase-like	+	
<i>R. ponticum</i>					
<i>Vitis amurensis</i>	Buds (total)	Late fall and early winter	Proteins associated with phenylpropanoid biosynthesis pathway	±	Masocha et al. (2020)
<i>V. vinifera</i>					

<sup>a</sup>Purative glycosylphosphatidylinositol-anchored proteins denoted by (GPI) in parentheses.

<sup>b</sup>Relative higher (>1.5-fold, +) or lower (<0.67-fold, -) accumulation of cold-induced cell-wall-associated proteins. If the response differs depending on the isoforms or fractions, ± is noted.

xyloglucan reorganization and the cross-linking of HG and RG-II enhances freezing tolerance.

Cold-induced reductions in  $\alpha$ -L-arabinofuranosidases and  $\beta$ -galactosidases abundance (Table 1) correspond with higher concentrations of cell-wall L-arabinose and galactose (Duruffé et al. 2017, Takahashi et al. 2020). These responses are presumably associated with the greater abundance of  $\alpha$ -1,5-arabinan and  $\beta$ -1,4-galactan pectin RG-I sidechains (Montes et al. 2008, Moneo-Sánchez et al. 2018). It is currently unknown how pectin RG-I side chains mitigate freezing injury. In resurrection plants, arabinan side chains and arabinogalactan proteins (AGPs) act as 'plasticizer' to promote the convoluted folding of the cell wall to reduce cell size and maintain turgor (Moore et al. 2006) and after rehydration facilitate the restoration of cell shape by preventing the irreversible adhesion of pectin polysaccharides (Moore et al. 2013). Whereas the accumulation of truncated galactan side chains due to over expression of  $\beta$ -galactosidase in potato (*Solanum tuberosum* L.) causes reduction in the water-binding capacity and a decrease in the tolerance of tubers to dehydration (Klaassen and Trindade 2020).

The biomechanical hotspot model hypothesizes that the extensibility of the cell wall is manipulated at sites of close contact comprised of xyloglucan and possibly RG-I between cellulose microfibrils (Cosgrove 2016). The flexibility of cell walls in dehydrated resurrection plants (Moore et al. 2006, 2013) and the ice-accommodating tissues of herbaceous leaves (McCully et al. 2004) may result from the site-specific manipulation of cell-wall extensibility. Furthermore, desiccation-tolerant plants may utilize cell-wall folding as a general mechanism to tolerate freezing-induced dehydration injury. To accommodate cell wall folding, plants must adjust membrane elasticity and/or the site-specific connections between the cell wall and plasma membrane. In the absence of cold-induced wall folding, RG-I arabinan and galactan side chains could alleviate freezing-induced adhesive injury and play a role in maintaining hydration of the QLL. Additional research is needed to confirm these theories in cold-acclimated plants.

Post-translationally modified membrane-bound glycosylphosphatidylinositol (GPI) anchored proteins maintain important roles in cold-induced cell-wall modification (Table 1). Among them, fasciclin-like AGPs and glycerophosphoryldiester phosphodiesterase-like proteins enhance primary cell-wall adhesion (Shi et al. 2003, Hayashi et al. 2008). Whereas some lipid transfer proteins promote the export and deposition of monomers required for the development of suberin and cuticular wax lipid barriers (Kim et al. 2012). The GPI anchored proteins associate with cell-wall polysaccharides via protein or O-glycan interactions (Yeats et al. 2018) and some of these can covalently bind both pectin and arabinoxylans (Tan et al. 2013). Artificial and seasonal cold acclimation of herbaceous annuals and woody perennials enhances the accumulation of GPI AGPs (Table 1). The mRNA levels of AGPs in the needles of Sitka spruce (*Picea sitchensis* Carr.) (Greene et al. 2012) and buds and roots of hybrid poplar (*Populus simonii* x *P. nigra*) (Zang et al. 2015) reach their highest levels in autumn and decline in the

winter. Plasma membrane and the cell-wall interactions during freezing dehydration and thawing have a significant effect on recovery (Arora 2018). Therefore, the identification of proteins that regulate membrane-to-wall interactions as a mechanism of freezing tolerance in plants is an interesting area of future study.

## Conclusion and Perspectives

Differences in the developmental biology of Arabidopsis as opposed to other herbaceous annuals, grasses or woody perennials will alter the cell-wall chemistry and its role in freezing tolerance or avoidance strategies. For example, fructan exohydrolases and cell-wall invertase-mediated freezing avoidance is proposed for monocotyledonous crops such as wheat (Livingston and Henson 1998), yet no such response is reported in trees. While it is proposed that the remodeling and accumulation of lignocellulosic polysaccharides enhance freezing tolerance in *Rhododendron* (*Rhododendron catawbiense* Michx.) (Wei et al. 2006), mutants of Arabidopsis that accumulate lower abundances of lignin exhibit greater freezing tolerance (Ji et al. 2015). These contrasting results highlight the importance of understanding the developmental history and physiology of your plant of interest. Mechanisms that enhance freezing tolerance or avoidance in a herbaceous annual may not translate into greater overwintering survival for a woody perennial. We must understand the developmental history and physiology of your plant of interest before engaging in cell-wall molecular studies. Without this basic knowledge, it is next to impossible to make any meaningful improvements in plant cold hardiness.

We must also recognize that cell walls are not homogenous structures. Understanding how tissue-specific cell-wall modifications in woody xylem parenchyma, dormant buds, tissues associated with plant meristems in grasses and herbs (Fig. 1) are key to advancing our understanding of plant freezing tolerance and avoidance. We can visualize tissue-level phenotypes using high-definition infrared thermography, nuclear magnetic resonance micro-imaging and cryo-scanning electron. Targeted Raman and Fourier transform infrared spectroscopy imaging techniques with omics approaches will provide deeper insight into mechanisms of freezing tolerance and avoidance.

## Data Availability

Source data for table is provided in the paper.

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## Disclosures

The authors have no conflicts of interest to declare.

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