4 Freezing Tolerance of Plant Cells: From the Aspect of Plasma Membrane and Microdomain

Daisuke Takahashi, Matsuo Uemura, and Yukio Kawamura

Abstract

Freezing stress is accompanied by a state change from water to ice and has multiple facets causing dehydration; consequently, hyperosmotic and mechanical stresses coupled with unfavorable chilling stress act in a parallel way. Freezing tolerance varies widely among plant species, and, for example, most temperate plants can overcome deleterious effects caused by freezing temperatures in winter. Destabilization and dysfunction of the plasma membrane are tightly linked to freezing injury of plant cells. Plant freezing tolerance increases upon exposure to nonfreezing low temperatures (cold acclimation). Recent studies have unveiled pleiotropic responses of plasma membrane lipids and proteins to cold acclimation. In addition, advanced techniques have given new insights into plasma membrane structural non-homogeneity, namely, microdomains. This chapter describes physiological implications of plasma membrane responses enhancing freezing tolerance during cold acclimation, with a focus on microdomains.

Keywords

Plant · Cold acclimation · Plasma membrane · Microdomain · Freezing tolerance · Proteome

Abbreviations

ACBP	Acyl-coenzyme A-binding protein
ASG	Acylated sterylglycoside
BCB	Blue-copper-binding protein
BI	Bax inhibitor
BRI	Brassinosteroid insensitive
CBF	C-repeat-binding factor
CPK	Calcium-dependent protein kinase
Cryo-SEM	Cryo-scanning electron microscopy
DRM	Detergent-resistant membrane
DRP	Dynamin-related protein
FLA	Fasciclin-like arabinogalactan protein
FLOT	Flotillin
FS	Free sterol
GH17	O-glycosyl hydrolase family 17

D. Takahashi
Central Infrastructure Group Genomics and Transcript Profiling, Max-Planck-Institute of Molecular Plant Physiology, Potsdam, Germany
e-mail: dtakahashi@mpimp-golm.mpg.de

M. Uemura
United Graduate School of Agricultural Sciences and Department of Plant-biosciences, Faculty of Agriculture, Iwate University, Morioka, Japan

Y. Kawamura (✉)
Cryobiofrontier Research Center and Department of Plant-biosciences, and United Graduate School of Agricultural Sciences, Iwate University, Morioka, Iwate, Japan
e-mail: ykawa@iwate-u.ac.jp

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GIPC	Glycosyl inositol phosphoryl ceramide
GPDL	Glycerophosphoryl diester phosphodiesterase-like protein
GPI	Glycosylphosphatidylinositol
H_{II}	Hexagonal II
HIR	Hypersensitive-induced reaction
LCB	Long-chain base
LCBK	LCB kinase
LTP	Lipid transfer protein
PDCB	Plasmodesmata callose-binding protein
PHS-P	4-Hydroxy-sphinganine-phosphate
PLD	Phospholipase D
PTM	Posttranslational modification
SG	Sterylglycoside
SGT	Sterol glycosyltransferase
SLAH	Slow anion channel 1 homolog
SLD	Sphingolipid Δ8 LCB desaturase
SYP	Syntaxin of plants
SYT	Synaptotagmin

4.1 Introduction

As immovable organisms, plants must continually monitor ambient conditions and properly respond to environmental changes. In spite of this limitation, plants have adapted to extreme environments ranging from aquatic habitats to alpine areas and constitute one of the most successful groups of organisms worldwide. In various environments, plants suffer from different abiotic stresses that homogenously, extensively, and species-nonspecifically influence plant growth and survival. Abiotic stresses include unfavorable temperatures, water unavailability, high salinity, inadequate light, and physical pressures. Among these stresses, freezing stress has the most critical effect on plant survival. Freezing stress is accompanied by a state change from water to ice and has multiple facets causing dehydration; consequently, hyperosmotic and mechanical stresses coupled with unfavorable chilling stress act in a parallel way (Steponkus 1984). While most temperate plants can overcome deleterious effects caused by freezing temperatures in winter, tropical and some temperate species cannot withstand such temperatures. Freezing tolerance thus varies widely among plant species and sometimes even within natural accessions of a single species.

One obvious question is what the key factor is for understanding freezing tolerance in plants. The most crucial component of plant cells under freezing conditions is the plasma membrane (Steponkus 1984). The disruption of the plasma membrane, which delineates extra- and intracellular environments, leads directly to cell death. Plasma membrane stability and flexibility are therefore deduced to be directly related to plant survival under freezing temperatures (Steponkus 1984). In addition, freezing stress is multifactorial: cold temperatures disrupt enzymatic activities and the physicochemical behavior of the plasma membrane, while extracellular freezing induces water migration from within the cell to extracellular space, increases osmotic concentration in unfrozen water, and puts mechanical pressure on the plant cell surface. Taken together, freezing stress is accompanied by cold, dehydration, osmotic, and mechanical stresses, all of which are more or less associated with plasma membrane function (Takahashi et al. 2013c). The plasma membrane is thus a key factor for overcoming complex freezing injury.

Because freezing tolerance is enhanced by cold acclimation using nonfreezing low temperatures such as 4 °C, the mechanisms of freezing tolerance have traditionally been studied by comparing plant samples before and after cold acclimation. In addition, comparisons between strongly and weakly freezing-tolerant plants, such as rye (*Secale cereale*) and oat (*Avena sativa*), have yielded a better understanding of plant freezing tolerance (Uemura and Yoshida 1984; Uemura and Steponkus 1989; Webb et al. 1994; Takahashi et al. 2013a). Recent advancements in proteomic and lipidomic technologies and the development of the lipid raft model, a new modification of the fluid mosaic model, have provided important information on complex changes of the plasma membrane caused by physiological shifts under stressed conditions. Genetic and physiological approaches using model plants have also unveiled sophisticated and subtle strategies used by plants to adapt to severe freezing.

In this review, general features of cold acclimation and freezing tolerance in plants are first

summarized. After describing advances in plasma membrane studies, various aspects raised by these studies and future perspectives in plant low-temperature biology are then discussed.

4.2 General Features of Cold Acclimation and Freezing Tolerance

Freezing stress is accompanied by several stress factors, all of which must be overcome by plants. Plant cells will otherwise be disrupted, leading in turn to death of the cell and eventually the individual organism. Detailed mechanisms of cold acclimation and freezing tolerance as survival strategies against severe freezing are discussed in this section.

4.2.1 Cold Acclimation as a Process Toward Adaptation to Freezing

To withstand severe freezing stress, temperate plants have developed a set of adaptation mechanisms referred to as cold acclimation. Cold acclimation is principally achieved via nonfreezing low temperatures and short-day conditions. The maximum freezing tolerance and optimal duration of cold acclimation vary with plant species. For example, oat and rye achieve their maximum freezing tolerances under cold acclimation treatment for 4 weeks, but their tolerances are different (−10 °C for winter oat and −15 °C for winter rye) (Webb et al. 1994). The model plant *Arabidopsis* also has the capacity for cold acclimation. The maximum freezing tolerance of *Arabidopsis*, −10 °C, is attained by cold acclimation treatment for 7 days (Uemura et al. 1995). Among natural accessions, however, the maximum freezing tolerance varies considerably, ranging from −8 to −14 °C (Hannah et al. 2006).

During cold acclimation, solutes, including sugars, amino acids, and specific proteins (e.g., dehydrin), accumulate to prevent membranes from undergoing freezing-induced denaturation and disruption (Koster and Lynch 1992; Welin et al. 1994; Danyluk et al. 1998; Wanner and Junttila 1999; Kosová et al. 2008). This process is regulated by the expression of specific genes such as C-repeat-binding factors (CBFs), which quickly increase in the first step of cold acclimation (Thomashow 1998, 1999). For instance, rye, a monocot plant, accumulates a variety of solutes, including proline, soluble sugars, and glycine betaine, during cold acclimation (Koster and Lynch 1992), and repartitions fructans and simple sugars within lower and upper crown tissues during freezing at −3 °C (Livingston et al. 2006). In *Arabidopsis*, several sugars, such as glucose, fructose, and sucrose, clearly increase during cold acclimation and decrease rapidly during de-acclimation (Zuther et al. 2015). Contents of these sugars as well as transcript abundances of *CBF1* and *CBF2* under cold acclimation have been found to be correlated with freezing tolerance in each natural accession of *Arabidopsis* (Zuther et al. 2015).

Remodeling of cell wall composition and structure has also been observed during the cold acclimation process in several species. Studies based on cryo-scanning electron microscopy (Cryo-SEM) have provided information on the cell wall as a barrier against extracellular freezing (Pearce 1988; Yamada et al. 2002). Changes in cell wall components such as crude cell wall, pectin, hemicellulose, and lignin are actually induced by cold acclimation treatment (Zabotin et al. 1998; Kubacka-Zębalska and Kacperska 1999; Solecka et al. 2008; Amid et al. 2012; Domon et al. 2013; Livingston et al. 2013; Baldwin et al. 2014; Ji et al. 2015). In winter oat, concentrations of apoplastic fructan, glucose, fructose, and sucrose increase during cold acclimation and sub-zero temperature acclimation immediately after cold acclimation (Livingston and Henson 1998). This response may also contribute to the prevention of ice crystal growth and propagation during freezing.

4.2.2 The Plasma Membrane as a Primary Site of Freezing Injury

In addition to the effects of chilling stress induced by low temperatures, freezing stress caused by sub-zero temperatures (<0 °C) has diverse impacts on plant cell survival (Levitt 1980; Steponkus 1984). For example, exposure to freezing temperatures is attended by a risk of an intracellular state change from water to ice, a lethal event. In plant cells that survive under freezing, this intracellular freezing does not generally take place, and ice nucleation occurs preferentially in extracellular space. Nevertheless, a difference in chemical potential is established between the extracellular ice and the intracellular solution, consequently leading to cellular dehydration. Plants must therefore overcome extracellular freezing-induced cellular dehydration. Because ice crystals exclude solutes, this extracellular ice formation additionally leads to increased concentrations of solutes in unfrozen portions of extracellular and/or intracellular solutions. Greatly concentrated solutes may cause osmotic (and/or salinity) stress in plant cells. Furthermore, physical pressure from enlarged extracellular ice crystals is deleterious to plant cells (Levitt 1980; Steponkus 1984). Freezing stress therefore has multiple aspects, including cold, dehydration, osmotic, and mechanical stresses (Fig. 4.1).

In any of the freezing-induced stresses, the plasma membrane is the most important factor determining plant cell survival. In particular, the plasma membrane acts as a barrier against invasion of extracellular ice into intracellular space during extracellular freezing-induced dehydration and mechanical stress. Intracellular freezing would otherwise occur and lead to cell death. Furthermore, the plasma membrane defines cell shape, surface area regulation, and trafficking between intracellular and extracellular spaces. Plasma membrane behavior and function may be very important for dealing with freezing-induced dehydration and hyperosmotic stresses in plant cells. Extended dehydration and osmotic stresses can prevent proper functioning of the plasma membrane through their harmful effects on physicochemical characteristics of plasma membrane components (Steponkus 1984). In addition, dehydration results in interbilayer fusion between the plasma membrane and other intracellular membranes, leading to injuries referred to as fracture-jump lesions and lamellar-to-hexagonal-II (H_{II}) phase transitions (Steponkus 1984; Gordon-Kamm and Steponkus 1984a; Webb and Steponkus 1993; Webb et al. 1993, 1994). When endomembranes are in close apposition to the plasma membrane, fracture-jump lesions or H_{II} phase transitions are thought to arise by structuration between lipid bilayers of both membranes. H_{II} phase transitions, in particular, are accompanied by the formation of a long cylinder-like structure with circularly arranged polar head groups of the membrane. These freezing-induced membrane structures are considered to trigger demixing and lateral segregation of principal membrane components such as lipids and proteins, thereby disrupting normal functions of the plasma membrane.

4.2.3 Significance of the Plasma Membrane in the Enhanced Freezing Tolerance After Cold Acclimation

Most of the cold acclimation-related events mentioned above are strongly associated with the enhancement of plasma membrane stability. For example, accumulations of sugars and dehydrins are important events that prevent interbilayer fusion of the plasma membrane with other endomembranes (Gordon-Kamm and Steponkus 1984a, b; Steponkus et al. 1988; Uemura and Steponkus 1989; Webb and Steponkus 1993; Webb et al. 1993, 1994, 1995; Danyluk et al. 1998; Ashraf and Foolad 2007; Eriksson et al. 2011; Rahman et al. 2013; Thalhammer et al. 2014). These solutes can interact with the plasma membrane under freezing-induced dehydration conditions and maintain a certain distance between the plasma membrane and intracellular organelle membranes. Alternatively, sugars and amino acids such as prolines contribute to

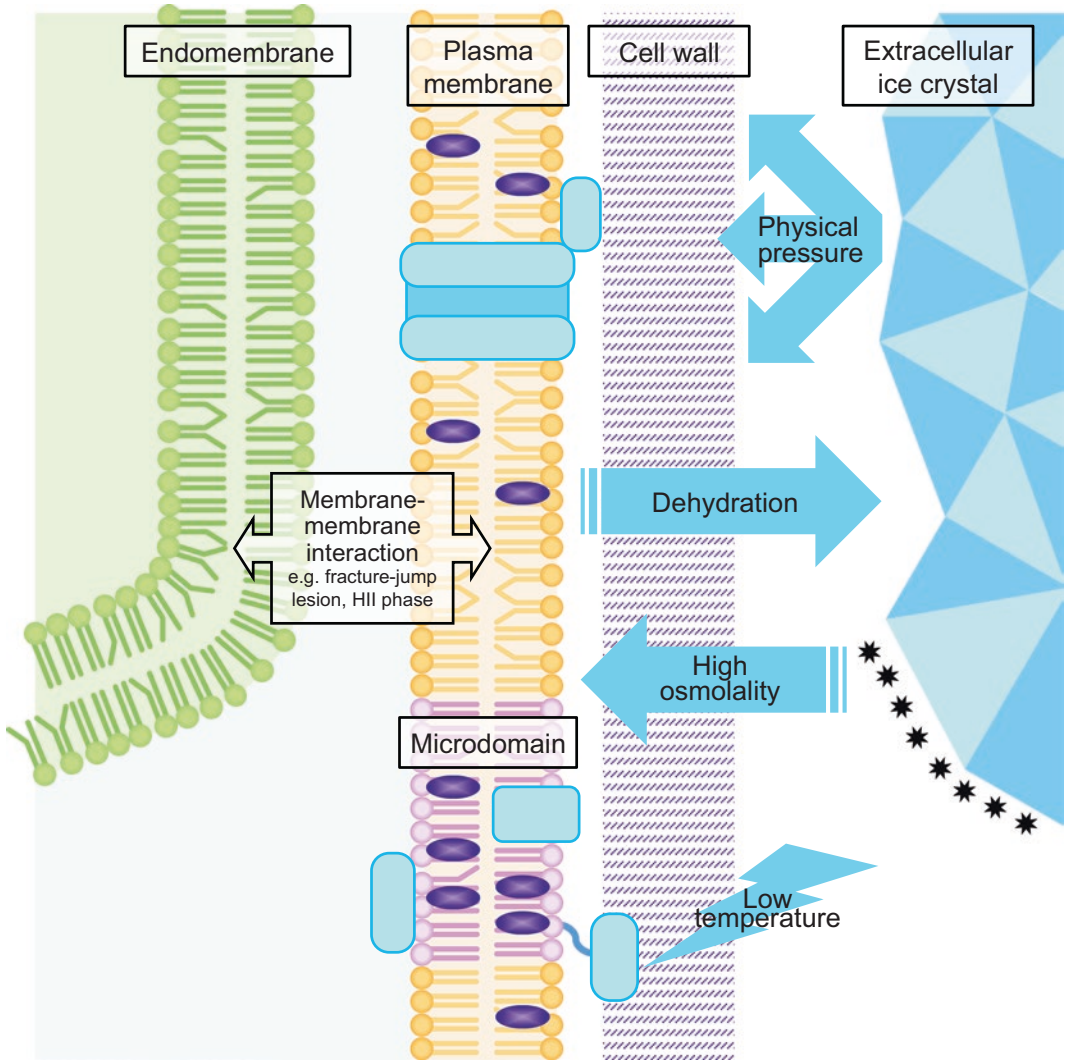

Fig. 4.1 Schematic illustration of freezing injury. Low temperatures influence the functions of plasma membrane-associated proteins. Extracellular ice formation induces water absorption from intracellular space. Expanded ice crystals impose physical pressure on the plasma membrane surface. Eventually, the plasma membrane can be fused with closely positioned endomembranes. Condensed solutes in extracellular unfrozen water lead to osmotic stress and dysfunction of the plasma membrane

scavenging of free radicals, osmotic adjustment, and buffering of cellular redox potentials. Although the extracellular matrix is not critical to plant survival under freezing conditions, since protoplast has a substantial freezing tolerance (Gordon-Kamm and Steponkus 1984a, b; Steponkus et al. 1988; Uemura and Steponkus 1989), extracellular responses such as modification of cell walls and accumulation of apoplastic solutes may help prevent penetration of ice from extracellular space through the plasma membrane into the cell (Yamada et al. 2002). On the basis of all this evidence, the plasma membrane is inferred to be the principal site affected by changes in intracellular and extracellular compartments in response to cold acclimation. In other words, protection of the plasma membrane takes highest priority under freezing conditions, and the stability of the plasma membrane determines plant freezing tolerance.

4.3 Changes of the Plasma Membrane in Response to Cold Acclimation

As mentioned above, the plasma membrane is the most crucial component for plant survival under freezing temperatures. The plasma membrane is composed of lipids and proteins, both of which should be affected both quantitatively and qualitatively to enhance freezing tolerance. In other words, cold acclimation alters lipid and protein compositions and functions to enhance plasma membrane integrity and stability under freezing temperatures. Modifications that take place after translation, such as glycosylation, may also be important factors that change plasma membrane properties. In addition, the structural model of the plasma membrane has been updated as the result of new findings. Along with technological developments, changes of the plasma membrane in response to cold acclimation have thus been studied from various angles.

4.3.1 Components and Structure of the Plasma Membrane

The two major components of the plasma membrane are lipids and proteins. Plasma membrane lipids are mainly composed of phospholipids, sphingolipids, and sterols. Each lipid class contains a wide range of members. For instance, more than 300 different types of sphingolipids have been estimated in various kinds of organisms (Hannun and Luberto 2000). Basically, the predominant structure of the plasma membrane is the lipid bilayer. Most plasma membrane lipids have both a hydrophilic head and a hydrophobic tail in one molecule; the exception is sterol lipids, which contain a very hydrophobic ring structure and, in some cases, sugar and acyl moieties. Head groups are always oriented to the outside of the bilayer, while tails face each other toward the center of the two-layered sheets. Head groups have specific structures and diverse properties. Because it can determine physical and chemical properties of the plasma membrane surface, the molecular assembly of head groups plays an important role in overall cell functionalities and characteristics.

In addition to lipids, the plasma membrane contains a remarkably diverse population of proteins. Tanz et al. (2013) confirmed at least 4000 proteins in *Arabidopsis* as plasma membrane-localized proteins by GFP tagging and mass spectrometry. There are several categories of plasma membrane proteins. For example, some plasma membrane proteins are integrated into the lipid bilayer, while others are weakly attached to the surface. These proteins play important roles in transportation of ions and small molecules, signal transduction, synthesis of extracellular components, secretions of proteins and other molecules, and intracellular and/or intercellular vesicle trafficking.

Some of these membrane proteins are dynamically modified after translation (i.e., posttranslational modification or PTM). More than 300 different PTM mechanisms have been reported (Zhao and Jensen 2009; Kline-Jonakin et al. 2011), with the ones most highly emphasized in plasma membrane studies being phosphorylation, acetylation, glycosylation, and oxidation. These PTMs possibly regulate protein activity, stability, localization, and interactions with other molecules (Yadeta et al. 2013). Furthermore, lipid-anchored proteins, such as glycosylphosphatidylinositol (GPI)-anchored, myristoylated, palmitoylated, and prenylated proteins, are covalently bound to plasma membrane lipids. Some PTMs such as glycosylation and lipid modification can be further diversified by various bonding partners.

Modern biochemical and cell biological techniques have yielded new insights into plasma membrane structure. In the past, the structure of the plasma membrane was described in terms of the fluid mosaic model proposed by Singer and Nicolson (1972). In the fluid mosaic model, components of the plasma membrane such as proteins and lipids are considered to be laterally diffused at random. This model is still widely supported by many studies based on animal, plant, yeast, and artificial liposome systems. More recently, however, several studies have uncovered the existence of microdomains formed

in the plasma membrane by lateral segregation of specific components such as sphingolipids, sterols, and certain proteins (Simons and Ikonen 1997). These microdomains are of various sizes (Kusumi et al. 2005) and have been implicated in many plant cell physiological processes, such as pollen tube tip growth, intercellular virus movement, responses to iron deficiency, and regulation of various membrane proteins including brassinosteroid insensitive1 (BRI1), hypersensitive-induced reaction (HIR) proteins, Rac/ROP small GTPase (Rac1), and respiratory burst oxidase homologs (Raffaele et al. 2009; Liu et al. 2009; Hao et al. 2014; Wang et al. 2015; Nagano et al. 2016; Gutierrez-Carbonell et al. 2016; Lv et al. 2017).

Along with the current consensus that microdomain components are mainly composed of hydrophobic molecules, microdomains are thought to be obtainable as nonionic detergent-resistant membrane (DRM) fractions at chilling temperatures. This latter postulation, however, is still debatable. On the one hand, DRMs do not always reflect the structure and nature of membrane microdomains (Tanner et al. 2011; Malinsky et al. 2013). On the other hand, remorin, a major DRM protein, is becoming recognized as a microdomain marker because it organizes patch-like structures in the plasma membrane that can be disrupted by treatment with a sterol chelator (methyl-β-cyclodextrin) (Kierszniowska et al. 2009; Raffaele et al. 2009). At a minimum, however, analysis of DRM fractions on a large scale seems to be a suitable first step to uncover the association between specific nanostructures of the plasma membrane and various physiological processes.

4.3.2 Lipidomic Changes of the Plasma Membrane During Cold Acclimation

As mentioned above, lipids are a principal component of the plasma membrane. The molecular composition of plasma membrane lipids and their molecular changes during cold acclimation have been investigated in many studies of various plant species. In early studies, both compositional analysis of lipids and comparative analysis before and after cold acclimation were performed using plasma membrane fractions (Steponkus 1984; Yoshida and Uemura 1984; Uemura and Yoshida 1984; Ishikawa and Yoshida 1985; Palta et al. 1993; Uemura and Steponkus 1994; Uemura et al. 1995). Following the incorporation of the microdomain or lipid raft model into the fluid mosaic model of the plasma membrane, several reports emerged of changes to microdomain lipid composition during cold acclimation (Örvar et al. 2000; Minami et al. 2009; Degenkolbe et al. 2012; Takahashi et al. 2016a). More recently, improvements to lipidomic analysis techniques (mainly driven by advances in mass spectrometric methods) have allowed more in-depth analysis of membrane lipids (Degenkolbe et al. 2012; Vu et al. 2014; Tarazona et al. 2015; Takahashi et al. 2016a).

4.3.2.1 Phospholipids

Early studies dealing with plasma membrane lipids and cold acclimation focused on lipid class composition and the degree of unsaturation of plasma membrane lipids. For example, Uemura and Steponkus (1994) initially revealed that proportions of phospholipids and of unsaturated ones (mainly phosphatidylcholine and phosphatidylethanolamine) significantly increased in the plasma membrane over 4 weeks of cold acclimation in both rye and oat. These trends were also observed in *Arabidopsis* leaves (Uemura et al. 1995). Increases in the proportion of highly hydrated lipids such as phospholipids and the degree of unsaturation decrease hydrophobicity at the membrane surface and increase membrane fluidity, respectively. Even if plant cells are extremely dehydrated by freezing, the plasma membrane can consequently be kept spatially separate from intracellular membranes because of water molecules bound to the surface of the plasma membrane. The most significant impact of these changes is thus considered to be a decrease in the freezing-induced formation of the H_{II} phase; this is because the lamellar-to-H_{II} transition is an interbilayer event that occurs at

the site of closely apposed membranes derived from freezing-induced dehydration.

In DRM fractions, however, the propensity toward cold acclimation-induced changes observed in the plasma membrane is not applicable (Minami et al. 2009; Takahashi et al. 2016a). For example, phospholipid proportions in DRMs are slightly decreased by cold acclimation treatment in *Arabidopsis* (Minami et al. 2009) and are relatively steady during cold acclimation in rye (Takahashi et al. 2016a). These results indicate that microdomains have different properties than other plasma membrane areas and function as a scaffold for various physiological responses during cold acclimation.

4.3.2.2 Sphingolipids

Sphingolipids, which are a putative major component of plant microdomains, are also affected by cold acclimation treatment. Glucocerebrosides, one of the major sphingolipid classes, generally decrease in both monocot and dicot plants to prevent undesirable phase transitions under freezing temperatures (Lynch and Steponkus 1987; Uemura and Steponkus 1994; Uemura et al. 1995). In DRM fractions, however, similar to phospholipids, few changes take place in glucocerebroside proportions during cold acclimation (Minami et al. 2009; Takahashi et al. 2016a); this suggests that the structure of the sphingolipid-enriched microdomain is preserved, with its function consequently partially maintained and/or fulfilled even during cold acclimation when the total sphingolipid proportion in the plasma membrane decreases. Glycosyl inositol phosphoryl ceramide (GIPC) is another predominant sphingolipid class (Markham et al. 2006) in addition to glucocerebrosides but still remains poorly characterized (Buré et al. 2014). No information therefore exists about the changing patterns of GIPC and its significance in the plasma membrane and microdomains during cold acclimation.

Long-chain base (LCB), a major component of sphingolipid molecules, plays an important role in plant cold response. Cold treatment induces transient formation of 4-hydroxy-sphinganine-phosphate (PHS-P), a phosphorylated LCB, via enhanced activity of LCB kinase 2 (LCBK2) (Dutilleul et al. 2012). LCBK1 also potentially determines plant freezing tolerance through regulation of reactive oxygen species homeostasis (Huang et al. 2017). Sphingolipid Δ8 LCB desaturase 1 (SLD1) is associated with response to cold temperatures together with Bax inhibitor-1 (BI-1) (Nagano et al. 2014). Interestingly, BI-1 determines the abundance of representative DRM proteins, flotillin homolog (FLOT), and hypersensitive-induced reaction protein 3 (HIR3) in *Arabidopsis* DRM fractions (Ishikawa et al. 2015). Recent studies have also demonstrated that SLD is a protein associated with chilling injury of chloroplasts in tomato (Zhou et al. 2016). Although many studies have revealed that the metabolism of sphingolipid and its derivatives is important for cold response, the involvement of the sphingolipid-enriched microdomain itself in freezing tolerance and cold acclimation has not yet been fully characterized.

4.3.2.3 Sterols

Sterols are another component of the plasma membrane and microdomains. Previous studies of *Arabidopsis*, oat, and rye (Uemura and Steponkus 1994; Uemura et al. 1995) have noted a decrease in glycosylated sterols such as sterylglycosides (SGs) and acylated sterylglycosides (ASGs) and an increase in free sterols (FSs). Because of the hydrophobicity of sterol and acyl moieties in the ASG molecule, lowering of the proportion of ASGs would be expected to help prevent dehydration-induced formation of the H_{II} phase under freezing-induced dehydration conditions (Webb et al. 1995).

Sterol compositions of DRM fractions of different plant species show specific responses to cold acclimation (Minami et al. 2009; Takahashi et al. 2016a), which suggests that sterols embedded in the lipid bilayer of microdomains have physiological significance related to modulation of activities of microdomain-recruited proteins in association with freezing tolerance. Interestingly, transfer of the sterol glycosyltransferase (SGT) gene isolated from *Withania somnifera* to *Arabidopsis* confers salt, heat, and cold stress tolerance (Mishra et al. 2013, 2015). Analysis of knockout lines of the

Arabidopsis SGT gene, *TTG15/UGT80B1*, has revealed that cold-acclimated plants, but not non-acclimated ones, have a decreased survival rate after freeze-thawing (Mishra et al. 2015). A decrease in ASGs and an increase in FSs may therefore improve membrane stability under freezing conditions; at the same time, these glycosylated sterols are important for freezing tolerance acquisition and potentially contribute to microdomain functions such as assisting cellulose production during cold acclimation (Endler and Persson 2011).

4.3.3 Proteomic Changes of the Plasma Membrane During Cold Acclimation

Changes in the proteomic profile of the plasma membrane during cold acclimation have been characterized in *Arabidopsis*, orchard grass, oat, and rye. Two pioneer studies (Uemura and Yoshida 1984; Yoshida and Uemura 1984) reported compositional changes of plasma membrane proteins during cold acclimation by SDS-PAGE analysis. Mass spectrometric approaches have contributed to the identification of plasma membrane proteins isolated from *Arabidopsis* (Kawamura and Uemura 2003; Minami et al. 2009). Shotgun proteomic analysis has allowed high-throughput identification and quantification of plasma membrane proteins isolated from several plant species (Li et al. 2012a; Takahashi et al. 2012, 2013a, 2016b). In addition, advances in proteomic technologies, including increased mass spectrometer sensitivity, have allowed the detection of less abundant proteins in plant cells. DRM proteins are generally extracted in small amounts (only roughly 10–20% of total plasma membrane proteins in tobacco, *Arabidopsis*, oat, and rye) (Mongrand et al. 2004; Minami et al. 2009; Takahashi et al. 2012). Several early studies relying on classical gel-based proteomics have characterized the compositions of DRM proteins in various plant species (Mongrand et al. 2004; Borner et al. 2005; Morel et al. 2006; Laloi et al. 2006; Lefebvre et al. 2007; Krügel et al. 2008; Minami et al. 2009; Fujiwara et al. 2009). Taken together, these results suggest that plant DRM fractions typically accumulate various functional proteins, such as P-type ATPase, aquaporin, remorin, tubulin, leucine-rich repeat receptor like kinase, NADPH oxidase, hypersensitive-induced reaction proteins (Band 7 family proteins), and glucan synthase (reviewed in Takahashi et al. (2013b)). Recent studies using Orbitrap technologies have revealed an even greater diversity of plasma membrane proteins accumulating in DRM fractions; these proteins include several GPI-anchored proteins, arabinogalactan proteins, and heat shock proteins (Kierszniowska et al. 2009; Takahashi et al. 2012, 2013a, 2016b; Gutierrez-Carbonell et al. 2016).

Some DRM proteins have been characterized with a focus on the effects of sterol depletion on their distribution into DRM fractions. As studied by Kierszniowska et al. (2009) and reviewed by Tapken and Murphy (2015), proteomes of typical DRM proteins, such as fasciclin-like arabinogalactan proteins, FLOTs, and glycosyl hydrolases, show responses to sterol depletion treatments, consistent with the concept of microdomains. Not all DRM-enriched proteins, however, are affected by sterol depletion treatments, which suggest that various kinds of microdomains exist in the plasma membrane. On the other hand, some DRM proteins have been identified as actual microdomain components of the plasma membrane in plant cells. Remorin, the best-characterized DRM protein, is recognized as a lipid raft marker because it localizes as a patch-like structure on the plasma membrane (Raffaele et al. 2009; Furt et al. 2010; Jarsch et al. 2014; Bozkurt et al. 2014; Konrad et al. 2014; Frescatada-Rosa et al. 2014). Slow anion channel 1 homolog 3 (SLAH3) co-localizes with calcium-dependent protein kinase 21 (CPK21) and remorin 1.3 on the plasma membrane (Demir et al. 2013). FLOTs, KAT1 (K^+ channel), plasma membrane intrinsic protein2;1 (PIP2;1), and syntaxin of plants 21 (SYP21) also exhibit patch-like distributions on the plasma membrane (Bhat et al. 2005; Li et al. 2011, 2012b; Jarsch et al. 2014). The auxin transporter PIN1 interacts with ABCB19 in the microdomain compartment on

the plasma membrane, and sphingolipid and sterol biosynthesis is correlated with *abcb9* phenotypes, which suggests that sphingolipid- and sterol-enriched microdomains have significant roles in fundamental physiological processes (Titapiwatanakun et al. 2009; Yang et al. 2013). Consequently, lipid remodeling in the plasma membrane during cold acclimation is deduced to be closely related to microdomain dynamics and lateral distribution of plasma membrane proteins.

Taking into consideration all the results of proteomic studies of cold acclimation, the following events can be hypothesized to occur during cold acclimation in DRM proteins: (1) an increase in P-type H^+-ATPases, (2) disassembly of cytoskeletal components (such as tubulin) in the juxtamembrane region, (3) rearrangement of vesicle trafficking proteins, and (4) accumulation of membrane protection proteins on the plasma membrane surface. Furthermore, the DRM proteins synaptotagmin 1 (SYT1), dynamin-related protein 1E (DRP1E), and phospholipase D (PLD), all of which increase during cold acclimation, have been functionally studied in detail.

SYT1 has been identified as a cold acclimation-induced protein in DRM fractions (Kawamura and Uemura 2003; Minami et al. 2009; Li et al. 2012a). SYTs were originally identified as exocytosis-related proteins in animal cells (Südhof 2002). SYTVII in animals is related to membrane resealing, which takes place via calcium-dependent exocytosis following mechanical disruption of the plasma membrane (Reddy et al. 2001; McNeil and Kirchhausen 2005). Both immunological and genetic approaches have demonstrated that plant SYT1 is also involved in the resealing of plasma membranes damaged by freezing-induced mechanical stress (Yamazaki et al. 2008). During injury, for example, the following series of events takes place: (1) ice crystals spread into the extracellular space and (2) physically press against the plasma membrane; (3) the plasma membrane is eventually mechanically punctured; (4) calcium influx occurs from the extracellular space into the cytoplasm through the damaged sites; and (5) endomembranes are fused at the site of the damaged plasma membrane via calcium-binding SYT1. These events eventually seal the damaged site and decrease the occurrence of freezing injury.

Cold acclimation induces an increase in endocytosis-related proteins of the DRP family and clathrin heavy chains in DRM fractions (Minami et al. 2009, 2010). In these proteins, DRP1E is also transcriptionally regulated by low temperature, whereas other DRP genes are not greatly induced by cold treatment (Minami et al. 2015). A genetic approach using *drp1e* mutants has demonstrated that DRP1E does not affect freezing tolerance before cold acclimation but is needed for full development of freezing tolerance afterwards. According to microscopic analysis, DRP1E localizes nonuniformly in specific areas of the plasma membrane; furthermore, dot-like GFP–DRP1E signals are observed that do not move horizontally but instead appear and disappear from the cell surface. These results support the hypothesis that DRP1E functions to accelerate endocytotic events on the plasma membrane, rearranges plasma membrane components during cold acclimation via the clathrin-dependent endocytosis pathway, and eventually enhances freezing tolerance (Minami et al. 2015).

PLDδ, a lipid modification enzyme, increases in DRM fractions during cold acclimation (Kawamura and Uemura 2003; Minami et al. 2009). Several recent studies mainly using genetic approaches have revealed that PLDs are also strongly associated with plasma membrane stability and freezing tolerance during cold acclimation. The regulation of phospholipid metabolism during cold acclimation is one of the most well-studied mechanisms of plasma membrane lipids. PLD produces phosphatidic acid via hydrolysis of membrane phospholipids. Experimental evidence exists that PLDδ acts as a positive regulator of plant freezing tolerance. Although cold-induced PLDδ seems to be involved in neither the expression of various cold-regulated proteins nor an increase in sugars, which are known to be important components in freezing tolerance acquisition in plants, experimental evidence exists that PLDδ acts as a positive regulator of plant freezing tolerance (Li

et al. 2004). Cytosolic acyl-coenzyme A-binding protein 6 (ACBP6) enhances freezing tolerance in conjunction with the accumulation of some phosphatidic acid species and an increase in *PLDδ* expression, which suggests that ACBP6 is involved in the induction of the *PLDδ* gene (Chen et al. 2008). The *pldα1* mutant, however, increases freezing tolerance, most likely as the result of higher accumulation of COR47, COR78, and osmolytes during cold acclimation (Rajashekar et al. 2006). These observations may reflect the potential roles of PLDs in cold acclimation processes, including phosphatidic acid signaling.

4.3.4 GPI-Anchored Proteins Responded to Cold Acclimation

GPI-anchored proteins are a group of lipid-modified plasma membrane proteins that are anchored to the membrane via glycolipids. Changes in plasma membrane lipids during cold acclimation should thus directly influence the behavior and function of GPI-anchored proteins. GPI-anchored proteins are synthesized in the ER lumen and are always transferred to the extracellular leaflet of the plasma membrane via the vesicular transport system (Udenfriend and Kodukula 1995; Eisenhaber et al. 1999; Ferguson 1999). Diverse responses of GPI-anchored proteins in the juxtamembrane have recently been elucidated (Takahashi et al. 2016b). The abundances of 44 of 163 GPI-anchored proteins are significantly increased in the plasma membrane but are quite stable in DRMs, which suggests that a GPI-anchored protein–microdomain interaction exists in the plasma membrane and that the contact dynamics of these two factors can be changed by cold acclimation treatment.

In regard to the functions of cold acclimation-responsive GPI-anchored proteins, these proteins are more or less associated with cell wall organization and remodeling (Takahashi et al. 2016b). The importance of cell wall characteristics during cold acclimation and freezing stress, especially compositions and pore sizes, has been discussed earlier, as ice nucleation starts in intercellular space (Pearce 1988; Pearce and Fuller 2001; Yamada et al. 2002; Rajashekar et al. 2006). Increases in pectin content and the degree of methyl esterification have been observed in cell walls of oilseed rape and pea (Kubacka-Zębalska and Kacperska 1999; Solecka et al. 2008; Baldwin et al. 2014). Associations of hemicellulose and cuticular wax with cold acclimation and freezing tolerance have also been reported (Zabotin et al. 1998; Amid et al. 2012; Domon et al. 2013). Blue-copper-binding protein (BCB), another cold acclimation-inducible GPI-anchored protein (Takahashi et al. 2016b), has been demonstrated to regulate freezing tolerance via modulation of lignin biosynthesis (Ji et al. 2015). New candidate GPI-anchored proteins identified as cold acclimation-induced plasma membrane proteins (e.g., lipid transfer proteins, LTPs; fasciclin-like arabinogalactan proteins, FLAs; glycerophosphoryl diester phosphodiesterase-like proteins, GPDLs; and O-glycosyl hydrolase family 17 proteins, GH17s) may therefore connect compositional changes of plasma membrane proteomes/lipidomes and cell wall remodeling during cold acclimation via microdomain-dependent or microdomain-independent regulation.

These GPI-anchored cell wall-related proteins are associated with cuticle layer formation (LTPs), cell wall organization and biomechanics (FLAs), cellulose deposition and pectin network formation (GPDLs), and callose turnover (GH17) (Johnson et al. 2003; Levy et al. 2007; Hayashi et al. 2008; DeBono et al. 2009; MacMillan et al. 2010; Kim et al. 2012). As mentioned above, the microdomain component sitosterol-β-glucoside is considered to be a primer of cellulose synthesis on the plasma membrane or a regulating factor of cellulose synthase and its activity, as these activities are enriched in DRM fractions (Peng 2002; Schrick et al. 2004; Endler and Persson 2011). These GPI-anchored cell wall-related proteins may therefore regulate cell wall dynamics via remodeling of the plasma membrane environment and/or microdomains during cold acclimation, an idea based on studies of cellulose synthase. Yeast PER1, required for

lipid remodeling of GPI, is, in fact, important for appropriate targeting of GPI-anchored proteins to microdomains, which suggests that the membrane environment in which GPI is anchored may play significant roles in the function of GPI-anchored proteins (Fujita et al. 2006).

We have identified several GH17s potentially GPI-anchored to the plasma membrane that are induced during cold acclimation in several plant species (Li et al. 2012a; Takahashi et al. 2013a, 2016b). One of these GH17 proteins, AtBG_ppap, is essential for callose turnover and is a key component for regulation of plasmodesmal movement and cell-to-cell communication (Levy et al. 2007). Grison et al. (2015) have recently reported that the plasmodesmata membrane is enriched in sterols and sphingolipids, reminiscent of lipid profiles of DRM fractions. Although not all plasmodesmata-localizing proteins are fractioned to DRM proteins, at least one, namely, plasmodesmata callose binding 1 (PDCB1), is partitioned to DRMs, and its localization is influenced by sterol depletion. Specific partitioning of GPI-anchored proteins to DRMs has not been confirmed (Takahashi et al. 2016b), but these proteins may be connecting with microdomain-enriched plasmodesmata at appropriate times. Interestingly, GH17s in poplar degrade plasmodesmal neck callose to release cell-to-cell communications toward bud dormancy release after winter freezing (Rinne et al. 2001, 2011). Callose-dependent regulation of intercellular communication via plasmodesmata might be coordinated with lipid remodeling of the plasma membrane and/or microdomains during cold acclimation and freezing stress.

4.4 Future Perspectives

Technical advances in omics research should help facilitate the discovery of new aspects of compositional changes of the plasma membrane during cold acclimation. Careful observations and refined analyses of physiological and genetic studies of plasma membrane-associated proteins using their mutants have unveiled the impact of lipidomic and proteomic changes and the importance of the plasma membrane during cold acclimation and freezing (Table 4.1).

Table 4.1 Representative plasma membrane-associated proteins characterized by previous studies

Name	Change[a]	Function	Phenotype[b]		References
			Loss of function	Gain of function	
LCB kinase 1 (LCBK1)	→	Kinase activity of sphingolipid LCB	↓ freezing tolerance		Huang et al. (2017)
			↓ sugar and proline content	↑ freezing tolerance	
			↑ ROS level	↑ sugar and proline content	
			↓ ROS-associated genes		
LCB kinase 2 (LCBK2)	→	Kinase activity of sphingolipid LCB	↓ cold-induced PHP-P synthesis	N/A	Dutilleul et al. (2012)
			↑ root growth at 12 °C		
			↓ DELLA and RGL3 genes		
Sphingolipid Δ8 LCB desaturase (SLD1)	↑ CA3d	Desaturase activity of sphingolipid LCB	↓ Δ8 unsaturation of LCB	N/A	Chen et al. (2012)
			↓ tolerance against prolonged chilling stress		Nagano et al. (2014)
			↓ total sphingolipid and root growth under cold temperature (sld1sld2)		

(continued)

Table 4.1 (continued)

Name	Change[a]	Function	Phenotype[b]		References
			Loss of function	Gain of function	
Sterol glycosyltransferase (SGT)	N/A	Glycosylation of sterol molecules	↓ freezing tolerance	↑ cold tolerance	Mishra et al. (2013), (2015)
			↓ FS and SG	↑ *RD29a* and *RD29b* genes (*A. thaliana* overexpressing *W. somnifera SGT* family gene)	
Lipocalin	↑ CA1d	Transport of small and hydrophobic molecule	N/A	↑ freezing tolerance (protoplast)	Charron et al. (2002)
					Uemura et al. (2006)
Protein phosphatase 2C (PP2C)	↑ CA1d	Serine/threonine phosphatase activity	↑ freezing tolerance	N/A	Tähtiharju and Palva (2001)
		Negative regulator of ABA			
Synaptotagmin 1 (SYT1)	↑ CA1d	Calcium-induced membrane-membrane fusion	↓ freezing tolerance	N/A	Yamazaki et al. (2008)
Dynamin-related protein 1E (DRP1E)	↑ CA12h	Scission vesicle from the plasma membrane	↓ freezing tolerance	N/A	Minami et al. (2015)
Cold-responsive protein kinase 1 (CRPK1)	→	Phosphorylation of 14-3-3 protein and negatively regulates CBFC-repeat binding factor (CBF) expression	↑ freezing tolerance	N/A	Liu et al. (2017)
Phospholipase Dδ (PLDδ)	↑ CA3d	Hydrolyze phospholipids to phosphatidic acid and head group	↓ freezing tolerance	↑ freezing tolerance	Li et al. (2004)
Phospholipase Dα1 (PLDα1)	N/A	Hydrolyze phospholipids to phosphatidic acid and head group	↑ freezing tolerance	N/A	Welti et al. (2002)
			↑ COR47 and COR78		Rajashekar et al. (2006)
			↑ raffinose content		
Blue-copper-binding protein (BCB)	↑ CA7d	Regulator of lignin biosynthesis	↑ freezing tolerance	N/A	Ji et al. (2015)
			↓ phenylalanine ammonia-lyase genes (PALs)		
			↓ lignin content		
Lipid transfer proteins (LTP)	↑ CA3h	Lipid transport	No changes	↑ freezing tolerance	Guo et al. (2013)
				↑ soluble sugar content	

[a]Arrows indicate expression trends at the mRNA and/or protein level during cold acclimation (CA)
[b]Phenotypes of knockdown/knockout or overexpression mutants

On the other hand, most of the previous studies employed single condition for cold acclimation treatment. However, cold acclimation conditions (e.g., processing temperature, cooling rate, and light conditions) can influence plasma membrane changes and eventual plant freezing tolerance. Furthermore, plant freezing tolerance can be determined by cellular responses to not only cold acclimation but also freezing and post freeze-thawing processes. Omics studies of the plasma membrane should therefore be focused not only on cold acclimation-induced changes but also on changes during acclimation, freezing, thawing, and recovery.

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