

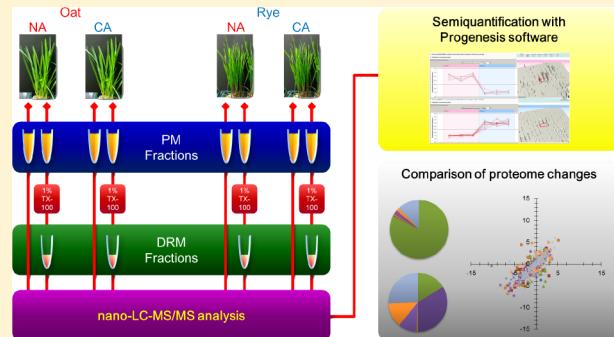
Changes of Detergent-Resistant Plasma Membrane Proteins in Oat and Rye during Cold Acclimation: Association with Differential Freezing Tolerance

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Supporting Information

ABSTRACT: Cold acclimation (CA) results in an increase in freezing tolerance of plants, which is closely associated to functional changes of the plasma membrane (PM). Although proteomic studies have revealed compositional changes of the PM during CA, there has been no large-scale study of how the microdomains in the PM, which contains specific lipids and proteins, change during CA. Therefore, we conducted semi-quantitative shotgun proteomics using microdomain-enriched detergent-resistant membrane (DRM) fractions extracted from low freezing-tolerant oat and highly freezing-tolerant rye. We identified 740 and 809 DRM proteins in oat and rye, respectively. Among the proteins identified, the abundances of a variety of proteins, such as P-type ATPase and aquaporins, were affected by CA in both oat and rye. Some CA-responsive proteins in the DRM fractions, such as heat shock protein 70, changed differently in oat and rye. In addition, changes in lipocalins and sugar transporters in the DRM fractions were different from those found in total PM fraction during CA. This is the first report to describe compositional changes in the DRM during CA. The proteomic profiles obtained in the present study hint at many possible microdomain functions associated with CA and freezing tolerance.



KEYWORDS: cold acclimation, freezing tolerance, proteomics, nano-LC–MS/MS, plasma membrane, microdomain, detergent-resistant membrane, monocotyledonous plants

INTRODUCTION

Freezing tolerance of temperate plants increases significantly when the plants are exposed to nonfreezing, low temperatures. This is termed cold acclimation (CA), which is one of the abiotic stress adaptation mechanisms of plants. During this process, many intracellular changes occur that enhance plant freezing tolerance in a complex manner and include changes in the expression of transcription factors and their target genes.¹ As a result, cold-induced proteins, such as LEA (late embryogenesis abundant) and COR (cold regulated) proteins, accumulate,^{2,3} and compatible solutes are synthesized.⁴ In addition, compositional and functional changes of the plasma membrane (PM) occur, which together contribute to the increase in freezing tolerance.^{1,5} Under cold conditions, the PM plays important roles in temperature sensing and signal transduction.^{6–8} In addition, the PM behaves as the final barrier against dehydration and invasion of ice crystals from the outside of cells during extracellular freezing, a typical way of freezing in temperate herbaceous plants, and tolerates an extreme change of surface area during a freeze–thaw cycle.^{5,9} Thus, damage to PM function and structure would be lethal to cells, and plants must have mechanisms to protect the PM against stresses imposed by a freeze–thaw cycle. In fact,

manifestation of freezing injury associated with the PM has been frequently reported as ultrastructural changes, such as fracture-jump lesions and hexagonal II phase transitions occurring at freezing temperatures.^{10,11} Thus, it is reasonable to assume that compositional changes in the PM influence directly the development of freezing tolerance during CA. As expected, several studies have shown that the lipid (lipid class and degree of unsaturation of acyl chains in glycerolipids) and protein (both qualitative and quantitative) compositions of the PM change dynamically during CA.^{12–17}

Distribution of lipid and protein components in the PM was thought to be homogeneous and to move dynamically in the membrane, as proposed in the fluid-mosaic model.¹⁸ However, the concept of a microdomain that is enriched in specific lipids and proteins with restricted movement was recently proposed.¹⁹ Microdomains contain sphingolipids and sterols, both of which tend to gather together because of their hydrophobic characteristics^{20–22} and specific functional proteins. In animal cells, microdomains act as scaffolds in association with

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membrane trafficking, signal transduction, and the endocytosis and exocytosis pathways. Microdomains can be biochemically extracted as detergent-resistant membranes (DRMs), which is a fraction resistant to nonionic detergent treatment on ice.^{19,20,23} In plants, DRMs have been used widely for microdomain research in several species, including tobacco, *Arabidopsis thaliana*, leek, *Medicago truncatula*, potato, rice, oat, and rye.^{24–33} Plant microdomains are also suggested to be involved in pollen tube tip growth and intercellular movement of viruses.^{34,35}

The relationship between CA and PM microdomains has been studied recently in *Arabidopsis* cells using isolated DRM fractions.^{32,36} During CA, the lipid composition of *Arabidopsis* DRM fractions changed considerably but differently from those of the total PM fraction. Furthermore, DRM-enriched proteins such as H⁺-ATPases, aquaporins, clathrins, and dynamin-related proteins showed significant changes during CA. These results suggest that microdomains have specific roles in the CA mechanism. We previously characterized DRM fractions prepared from oat (*Avena sativa*) and rye (*Secale cereale*) grown under nonacclimated (NA) conditions. The protein compositions of DRM fractions in these two monocotyledonous plants were similar, and some proteins were conserved in oat, rye, and *Arabidopsis*.³³ We selected these two monocotyledonous plants because these are closely related phylogenetically but have vastly different freezing tolerances after CA.³⁷ Therefore, it is reasonable to hypothesize that the protein compositions of oat and rye change differently during CA, permitting us to discuss the roles of DRM proteins in the mechanisms of CA and freezing tolerance in plants.

Because of plant immobility, plants have to recognize, respond, and adapt to environmental stimuli at a cellular level. Thus, elucidation of cellular factors involved in stress adaptation is essential for production of stress-tolerant crops and stable food supply. In the present study, we performed shotgun proteomic analyses of DRMs isolated from the PMs of leaves of oat and rye before and after CA. In addition, label-free semiquantification of DRM proteins was carried out to further profile how DRM proteins change during CA. To the best of our knowledge, this is the first large-scale analysis of DRM proteins associated with an abiotic environmental stress response in a plant system using label-free shotgun proteomics. On the basis of these results, we will discuss possible functional contributions of the DRM fraction to CA in plants. Our results will contribute to a better understanding of how plants can adapt to survive at freezing temperatures from the aspects of the involvement of PM and DRM proteome changes in crop species and provide valuable information for molecular breeding of cold-tolerant crops. The development of stress-tolerant crops will expand cultivated land area and mitigate loss of crop production in global climate change era.

■ EXPERIMENTAL PROCEDURES

Plant Materials

Seeds of oat (*Avena sativa* cv. New Almighty) and rye (*Secale cereale* cv. Maskateer) were sown in vermiculite supplemented with Hoagland solution at 18 °C with a 16 h photoperiod (90 μmol/m²/s). After 12–14 days, leaves were harvested for experiments as NA plants. To obtain CA plants, we further grew NA oat and rye plants at 2 °C with a 12 h photoperiod (100 μmol/m²/s) for 4 weeks. We could not see any growth

and developmental changes in both oat and rye plants during low-temperature treatment for 4 weeks.

Evaluation of Freezing Tolerance

The electrolyte leakage method has been used widely for evaluation of plant freezing tolerance^{38–40} and was employed in the present study. Harvested leaves were washed with precooled water and dried with Kimtowel. The leaves were cut into 1 cm pieces, put into glass tubes with aliquots of water (100 μL), and cooled in an alcohol bath (NCB-3400, EYELA, Tokyo, Japan) at –2 °C for 15 min. After ice nucleation by the addition of ice droplets (50 μL in volume) and equilibration of the temperature at –2 °C for 2 h, samples were cooled further at a rate of –2 °C/h. At specified temperatures, samples were transferred to 4 °C. After incubation overnight for thawing, 4 mL of water was added and samples were shaken for 150 min. Then, a conductance meter (Twin Cond, HORIBA, Kyoto, Japan) was used to measure electrolytes that had leaked from cells during freeze–thawing. Subsequently, samples were boiled for 20 min and shaken for 150 min. Then, electrolytes fully leaked from cells were measured again. Using the two measured values, the ratio of electrolyte leakage caused by freeze–thawing was calculated (leakage at the first measurement/leakage at the second measurement).

Isolation of Plasma Membrane and Detergent-Resistant Membrane Fractions

PM preparation was performed using a polyethylene glycol–dextran aqueous two-phase partition system, as previously described.^{16,33} All procedures were conducted at 4 °C or below. Leaves were chopped and homogenized in a medium composed of 0.5 M sorbitol, 50 mM MOPS/KOH (pH 7.6), 5 mM EGTA, 5 mM EDTA, 5% (w/v) polyvinylpyrrolidone (MW: 40 000), 0.5% (w/v) BSA, 2.5 mM phenylmethane-sulfonyl fluoride, 4 mM salicylyhydroxamic acid, and 2.5 mM dithiothreitol using a polytron (Kinematica PT10-35, Brinkmann Instruments, Westbury, NY). After filtering with gauze, homogenates were centrifuged at 10 000g for 15 min and subsequently at 231 000g for 50 min. The pellets obtained were suspended in a microsome suspension medium (0.25 M sucrose and 10 mM KH₂PO₄/K₂HPO₄ [pH 7.8]) and centrifuged again as above. The resultant pellets were resuspended in a two-phase partition solution consisting of 5.8% (w/w) polyethylene glycol (MW: 3350; Sigma-Aldrich, St. Louis, MO), 5.8% (w/w) dextran (Sigma-Aldrich), 0.25 M sucrose, 30 mM NaCl, and 10 mM KH₂PO₄/K₂HPO₄ (pH 7.8). The two-phase system was mixed well and centrifuged at 650g for 5 min. The resultant upper phase was collected and added to a newly prepared lower phase, mixed, and then centrifuged as above. These processes were repeated three times to increase the purity of the PM. The final upper phase was collected, diluted with a PM suspension medium (0.25 M sucrose, 10 mM Mops/KOH (pH 7.3), and 2 mM EGTA), and precipitated by ultracentrifugation as previously described. This process was repeated twice to remove the polymers completely.

Preparation of DRM fractions was carried out according to the method of Peskan et al.²⁴ Purified PM pellets were resuspended in 2.7 mL of TED buffer consisting of 50 mM Tris-HCl (pH 7.4), 3 mM EDTA, and 1 mM dithiothreitol. An aliquot of 10% (w/v) Triton X-100 in TED buffer (300 μL) was then added to PM suspension and the PM-TX-100 mixture was incubated for 30 min on ice. To the PM-TX-100 mixture, 12 mL of 65% (w/w) sucrose in TED buffer was immediately added and mixed well. Then, 48, 35, 30, and 5% (w/w)

sucrose-TED solutions were subsequently overlaid on the PM-TX-100 mixture and centrifuged at 141 000g for 20 h at 4 °C using a swing-type rotor P28S (Hitachi Koki, Tokyo, Japan) to obtain DRM fractions. After centrifugation, a white band at the interface of the 30 and 35% (w/w) sucrose layers (Supplemental Figure 1 in the Supporting Information) was collected, diluted with PM suspension medium, and centrifuged at 231 000g for 50 min. This fraction was designated DRM. Protein content of PM and DRM suspensions were measured using the Bradford assay (Bio-Rad, Munich, Germany) with BSA as a standard.

One-Dimensional SDS-PAGE

PM and DRM samples (equivalent to 1 µg protein) were suspended in an equal volume of sodium dodecyl sulfate (SDS) sample buffer (2% [w/v] SDS, 50 mM Tris-HCl [pH 6.8], 6% [v/v] β-mercaptoethanol, 10% [v/v] glycerol, and bromophenol blue) and denatured by heating to 95 °C for 20 min. Proteins were separated on a 10% [w/v] polyacrylamide gel with a 4.5% [w/v] stacking gel and visualized by silver staining.¹⁷

Sample Preparation and Data Acquisition for Nano-LC–MS/MS Analysis

Protein samples (50 µg protein) were subjected to in-solution tryptic digestion for nano-LC–MS/MS analysis according to the protocol of Takahashi et al.³³ Membrane fractions were pelleted by centrifugation and dissolved in a detergent mixture (MPEX PTS Reagents for MS, GL Science, Tokyo, Japan). Proteins (5 µg) determined with the Pierce BCA Protein Assay Kit (Pierce, Rockford, IL) were digested by trypsin according to instruction manual of MPEX PTS Reagents provided by the manufacturer. The peptide samples were desalted with SPE C-TIP (AMR, Tokyo, Japan), and the volume was adjusted to 15 µL with 0.1% [v/v] trifluoroacetic acid. Peptide solutions were subjected to nano-LC–MS/MS analysis.³³ The data acquisition steps using ADVANCE UHPLC system (MICHRON Bioresources, Auburn, CA) and LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Waltham, MA) are described in Takahashi et al.³³ Peptide solutions were concentrated with a trap column (L-column Micro 0.3 × 5 mm; CERI, Japan) on an ADVANCE UHPLC system (MICHRON Bioresources, Auburn, CA). Peptides eluted with 0.1% (v/v) formic acid in acetonitrile were separated with a Magic C18 AQ nano column (0.1 × 150 mm; MICHRON Bioresources) using a linear gradient of acetonitrile (from 5 [v/v] to 45% [v/v]) at a flow rate of 500 nL/min. Peptides were then ionized by an ADVANCE spray source (MICHRON Bioresources) with spray voltage of 1.8 kV. Mass analysis was performed using an LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific, Waltham, MA) equipped with Xcalibur software (version 2.0.7, Thermo Fisher Scientific). Full-scan mass spectra were obtained in the range of 400 to 1800 *m/z* with a resolution of 30 000. Collision-induced fragmentation was applied to the five most intense ions at a threshold above 500. These experiments were repeated four times with samples collected from biologically independent plants.

Analysis of Nano-LC–MS/MS Data

Raw files of MS/MS spectra were converted to the mgf format using Proteome Discoverer (ver. 1.1.0.263, Thermo Fisher Scientific) under the following parameters: precursor mass range, *m/z* 350–5000; highest and lowest charge state, 0; lower

and upper RT limit, 0; the minimum total intensity of a spectrum, 0; and the minimum number of peaks in a spectrum, 1. Peptide data obtained were searched and proteins were identified using the MASCOT search engine (version 2.3.02, Matrix Science, London, U.K.) searching against the NCBInr Green Plants database (version 20121002, comprising 1 095 445 sequences) for quantification of DRM fraction proteins according to the following parameters: number of missed cleavage, 1; fixed modifications, carbamidomethylation (C); variable modifications, oxidation (M); peptide mass tolerance, 5 ppm; MS/MS tolerance, 0.6 Da; and peptide charges, +1, +2, and +3. The false discovery rate (FDR), which is based on a search of the Mascot decoy database, was less than 5%. Definitions of identified proteins were based on the following filters: including at least one unique top-ranking peptide; ion score cut off *p* ≤ 0.05; and identified two or more times in four repeated experiments. If a peptide was assigned to multiple proteins, the highest-scoring protein was selected in the list. In semiquantitative analysis, raw files were subjected to Progenesis LC–MS software (version 4.0, Nonlinear Dynamics, New Castle, U.K.). Peptides were assigned to proteins by MASCOT searching as previously described. Finally, significant proteins were filtered with ANOVA (*p* < 0.05) and fold change (>2.0) according to normalized peptide intensity.

Topology and Post-Translational Modification Prediction

Acquired proteins were used for prediction of transmembrane domains, signal peptides, cellular locations, sites of glycosyl-phosphatidylinositol (GPI) modification, and myristylation using the following online tools: transmembrane domains, SOSUI engine version 1.10 (<http://bp.nuap.nagoya-u.ac.jp/sosui/>); cellular locations, TargetP 1.1 server (<http://www.cbs.dtu.dk/services/TargetP/>); signal peptides and membrane anchor peptides, SignalP 3.0 server (<http://www.cbs.dtu.dk/services/SignalP/>); GPI modification, Big-PI Plant Predictor (http://mendel.imp.ac.at/gpi/plant_server.html); and N-terminal myristylation, Plant-Specific Myristylation Predictor (<http://plantsp.genomics.purdue.edu/myrist.html>).

RESULTS AND DISCUSSION

Freezing Tolerance in Oat and Rye

Freezing tolerance of oat and rye leaves was measured during CA by the electrolyte leakage method (Figure 1). Various evaluation methods have been reported for determining plant freezing tolerance, such as electrolyte leakage measurement, the regrowth method and photosynthesis activity measurement.^{38,41,42} The electrolyte leakage method, which measures conductivity derived from electrolytes in medium leaked from the cytoplasm by a freeze-thawing, determines the extent of freezing injury directly occurring on the PM, which is the primary site of the freezing injury.^{5,39} Thus, we believe that the electrolyte leakage measurement is suitable for characterizing PM proteome changes during CA.

In NA plants, the temperature at which 50% of electrolyte leakage occurs (LT_{50}) was -5 °C in oat and -8 °C in rye. CA decreased the LT_{50} in both oat and rye. In oat, CA treatment for 1 week increased freezing tolerance to the LT_{50} of -8 °C, but the LT_{50} did not change further after CA for 2 weeks or longer. The LT_{50} in rye decreased to -16 to -18 °C after CA for 1 week and was further lowered with prolonged CA; after CA for 4 weeks, the LT_{50} was -20 °C. These results showed that freezing tolerances of oat and rye are different in NA plants, and the difference was further enhanced after CA. Our

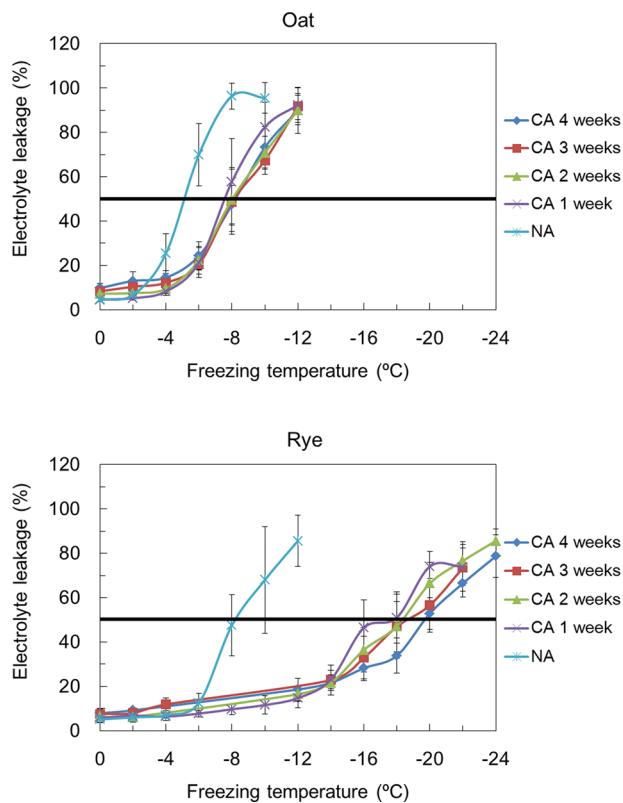


Figure 1. Freezing tolerance of oat and rye leaves. Freezing tolerance is expressed as the percentage of electrolyte leakage, which reflects the extent of freezing injury of the PM. In this experiment, oat and rye were cold acclimated for 0, 1, 2, 3, and 4 weeks; then, the electric conductance was measured after subjecting them to a freeze–thaw cycle ($n = 3\text{--}12$). The temperature at which 50% electrolyte leakage (indicated by a heavy horizontal line) occurs is designated as LTS0 in the text.

observations are consistent with results of a previous study.³⁷ Additional increases in freezing tolerance were not observed after CA for 5 weeks in both oat and rye;³⁷ therefore, we used oat and rye leaves before and after 4 weeks of CA for PM and DRM proteomic studies.

Isolation and Characterization of PM and DRM Fractions

The overall workflow of the present study is described in Supplemental Figure 2 in the Supporting Information. PM fractions were prepared from leaves of oat and rye before and after CA with four biological independent plant samples, and a half of each fraction was used for extraction of the DRM fraction. All of these fractions were subsequently subjected to nano-LC–MS/MS analysis and identification of compositional differences among the samples under the same conditions.

When purified PM samples were incubated with Triton X-100 on ice and subsequently subjected to sucrose density gradient centrifugation, a white band that is considered to contain DRM was visible at the interface of the 30 and 35% sucrose layers for both oat and rye (Supplemental Figure 1 in the Supporting Information). In our previous study, the recovery of DRM from PM fractions (based on protein amount) in NA samples was 10–20% and 8–12% for oat and rye, respectively.³³ This tendency was the same, and the recovery was less in the CA samples than in the NA samples: the recovery of DRM fraction from the PM was 6–12% in oat and 5–8% in rye (data not shown). The decrease in the

recovery of DRM fractions after CA was also reported for PMs of *Arabidopsis*.³² This might reflect differences in lipid and protein compositions in DRMs in leaves before and after CA,^{32,36} which may change the resistance to detergent treatment.

One-dimensional SDS-PAGE revealed considerable differences in protein compositions between the DRM and PM in both NA and CA samples as well as between NA and CA in both PM and DRM fractions (Figure 2). Some proteins were highly enriched in the DRM, and some of the DRM fraction proteins changed during CA. Several proteins were enriched in DRM in both species before and after CA (i.e., No. 4 in oat and No. 2 in rye in Figure 2). These results are consistent with previous studies in *Arabidopsis* and tobacco,^{25–27} in which the authors reported vast differences in protein compositions between the DRM and PM.

Furthermore, in both oat and rye, some proteins increased or decreased in DRM and PM fractions during CA (Figure 2). Although some proteins changed during CA in both PM and DRM fractions (e.g., No. 2 in CA oat PM and DRM and No. 3 and No. 4 in CA rye PM and DRM), there were proteins that changed only in the DRM fraction but not in the PM fraction (e.g., No. 5 in CA oat DRM and No. 6 in CA rye DRM). CA-induced changes in DRM protein composition have been reported in *Arabidopsis*.³² When CA-induced changes in the DRM were compared between oat and rye, some proteins were found to change in both species (e.g., No. 2 in CA oat DRM and No. 3 in CA rye DRM); however, changes of some proteins were species-dependent (e.g., No. 5 in CA oat DRM and No. 9 in rye DRM). Collectively, these results suggest that the microdomain has specific functions in the PM and has different effects on the CA mechanism depending on plant freezing tolerance.

Identification and Functional Categorization of DRM proteins

PM and DRM proteins were subjected to LTQ Orbitrap XL mass spectrometry analysis after digestion with trypsin. We identified 304 PM proteins and 227 DRM fraction proteins in oat and 329 PM proteins and 255 DRM fraction proteins in rye under NA conditions (Supplemental Tables 1 and 2 in the Supporting Information). Furthermore, 283 PM proteins and 199 DRM fraction proteins in oat and 314 PM proteins and 256 DRM fraction proteins in rye were identified under CA conditions. All detected peptides were exported and formatted for Excel in Supplemental Tables 5–12 in the Supporting Information. Identified proteins were classified into 13 functional categories according to Bevan et al. In particular, cell-structure-related proteins, signal transduction-related proteins, and transporters were frequently identified in both the PM and DRM in oat and rye. For example, 21% of identified DRM fraction proteins in NA rye were transporters.

Signal intensities, which are related to the extent of accumulation of each peptide, were then calculated and normalized using Progenesis LC–MS software. In this software, quantification steps were based not on fragment ion mass, but on peptide mass signals.⁴⁴ Protein identification processes then were conducted using multiplex mass data sets derived from PM and DRM analysis under NA and CA conditions in each of four replicates to minimize data loss and increase proteome coverage. From these analyses, we identified 740 and 809 DRM fraction proteins in oat and rye, respectively. Among them, 136 and 220 DRM proteins responded to CA treatment in oat and

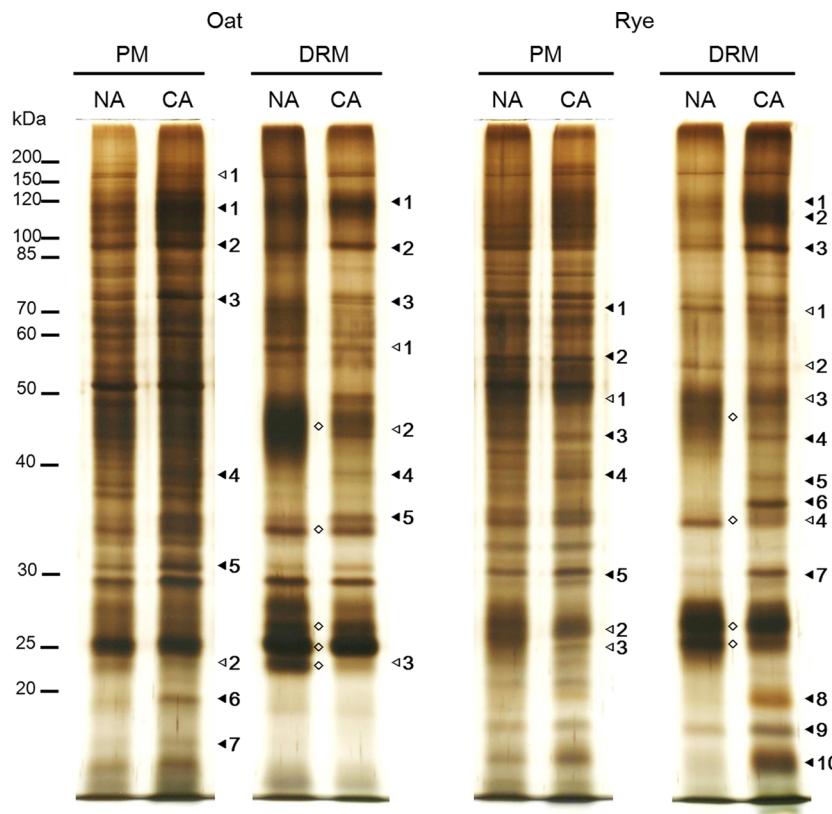


Figure 2. One-dimensional SDS-PAGE profiles of PM and DRM proteins in oat and rye during CA. PM and DRM proteins prepared from NA and CA oat and rye (1 μ g protein equivalent) were separated using SDS-PAGE and visualized by silver-staining. Diamonds indicate highly accumulated DRM fraction proteins. Filled and open triangles indicate CA-increased and CA-decreased proteins, respectively.

rye (fold change >2.0 , ANOVA $p < 0.05$) and were analyzed further to elucidate the function of DRM in CA (Supplemental Table 3 in the Supporting Information).

The accumulation of each protein in the PM and DRM fractions was first compared to reveal the enrichment in the DRM before and after CA. In the quantification process, the DRM/PM ratio of proteins was calculated using the normalized abundance of each protein. Subsequently, DRM-enriched (DRM/PM > 2.0 , ANOVA $p < 0.05$), DRM-nonenriched proteins (DRM/PM < 0.5 , ANOVA $p < 0.05$), and non-preferentially partitioned proteins (DRM/PM < 2.0 or >0.5 , ANOVA $p > 0.05$) were classified into 13 functional categories based on Bevan et al.⁴³ (Figure 3). These results clearly showed that specific protein groups were enriched in DRM fractions. In NA samples, the proportion of transporters in the DRM-enriched proteins was greater (by 7.3 times in oat and 6.7 times in rye) than in DRM-nonenriched proteins (Figure 3A,C). Proteins classified in the category of energy, metabolism, and protein synthesis were not so enriched in the DRM. We found that there are differences between oat and rye in the proportion of DRM-enriched disease/defense-related proteins. Oat contained a significantly higher proportion of DRM-enriched disease/defense proteins than rye did based on Fisher's exact test ($p < 0.01$). In oat, the proportions of disease/defense proteins were less in DRM-nonenriched proteins than in DRM-enriched ones.

Considering CA conditions, the proportions of each category of DRM-enriched and nonenriched proteins such as energy, protein synthesis, disease/defense, and transporters did not change after CA. Although cell structure-related proteins such as actins and tubulins are also known as DRM-enriched

proteins and are CA-responsive,^{8,17,45–47} proportional changes of these proteins during CA were not significant in either species. These results indicate that CA does not affect the DRM proteome qualitatively. Many not-preferentially partitioned proteins, which were not affected by detergent treatment on ice, were also identified in oat and rye. While proteins related to protein synthesis and signal transductions tended not to be enriched in or were removed from DRM fractions, transporters were considerably underrepresented in nonpreferentially partitioned proteins than in DRM-enriched proteins after CA. These results indicate that transport proteins are susceptible to the effects of lateral segregation on the PM.

Next, we calculated the CA/NA ratio of proteins in the DRM and PM to reveal what proteins significantly increased and decreased or did not change after CA (Figure 4). Changes in PM and DRM protein compositions during CA seemed to be similar in both oat and rye. For example, in all fractions examined, disease/defense- and signal-transduction-related proteins increased in the DRM and PM after CA, and protein synthesis and transport-related proteins decreased after CA. When looking at the data in detail, the decrease in the proportion of protein synthesis proteins mostly represented decreases in ribosomal proteins in the fraction. For example, the CA/NA ratios of ribosomal protein L12 (gil3986695) in oat and rye PM were 0.27 and 0.45, respectively (Supplemental Table 3 in the Supporting Information). Ribosomal proteins are related to protein translation and are located in the endoplasmic reticulum (ER).⁴⁸ In barley, CA resulted in an induction of the transcript level of ribosomal proteins.⁴⁹ Considering these results, changes to the PM–ER interaction

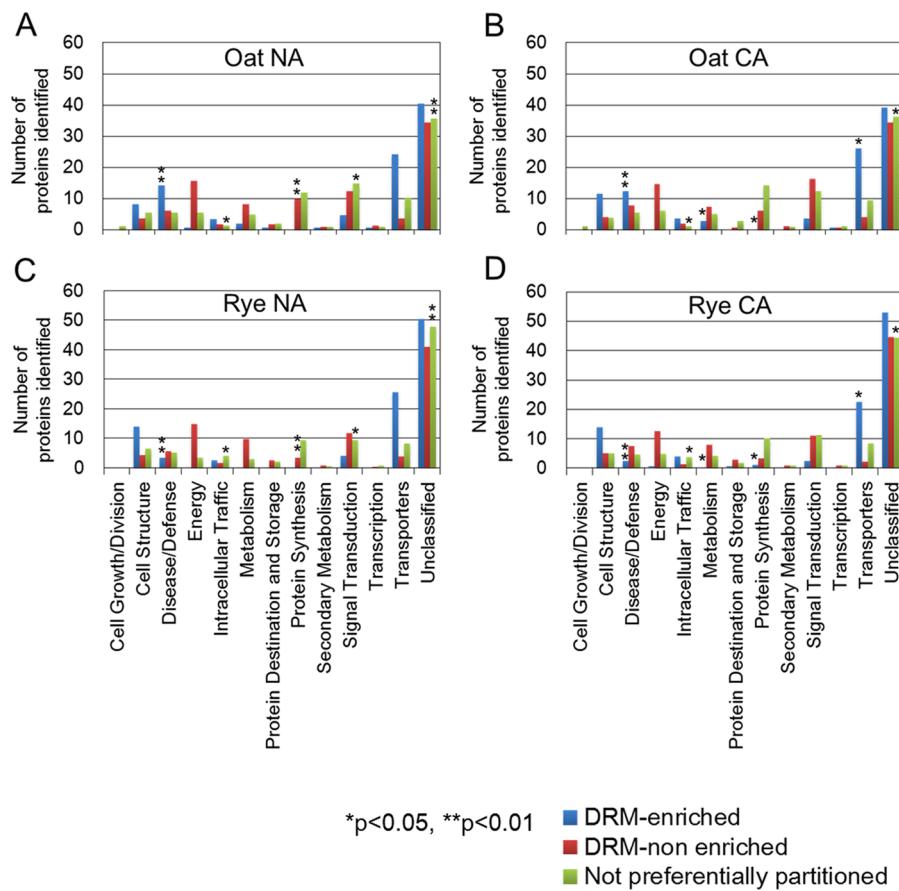


Figure 3. Functional categorization of proteins differentially contained in the PM and DRM. Peptides were selected according to the following criteria using Progenesis LC–MS software: ANOVA $p < 0.05$; DRM/PM ratio, >2.0 or <0.5 ; expect value cut off, >0.05 . Proteins were then identified using the Mascot server and classified into 13 functional categories based on Bevan et al.⁴³ Panels A–D show distribution of DRM-localized and nonlocalized proteins in the NA oat, CA oat, NA rye, and CA rye, respectively. Statistical significance (*, $p < 0.05$; **, $p < 0.01$) between oat and rye during CA, which was calculated with Fisher's exact test based on the number of identified proteins, is shown.

may occur during CA and result in decreased ribosomal proteins in the PM.

We also found that some transporters decreased after CA in the PM and DRM fractions, including many V-type ATPases and aquaporins. V-type ATPase is known to be located in various membranes, including the PM, in plant cells, in addition to tonoplast,^{50–52} and responds to salt stress⁵³ and CA.¹⁷ Thus, V-type ATPase may be involved in the CA mechanism through intracellular pH regulation. The number of CA-increased disease/defense-related proteins accounted for 13% of total increased proteins in the PM and 24% of total increased proteins in the DRM in oat, whereas they accounted for 11% of total increased proteins in the PM and 7.6% of total increased proteins in the DRM in rye. The components of the proteins in this category were quite different between oat and rye. Oat had several CA-increased heat shock proteins, especially in DRM fractions. There were a variety of cold-stress-related proteins such as dehydrins/LEA proteins and lipocalins in both the PM and DRM of rye. In particular, dehydrin is well known to have multiple functions to enhance cold tolerance, such as cryoprotective and antifreeze activities.⁵⁴ Dehydrin was observed to bind lipid vesicle and has important roles for protection and stabilization of lipids and proteins under freezing temperature.^{55,56} Thus, it may be reasonable to hypothesize that rye has a number of cold-stress-related proteins that protect the microdomain and hence increase

freezing tolerance during CA. Collectively, the differences in alterations in PM and DRM proteome described above suggest that cold responses in the PM are different, both inside of the DRM (e.g., microdomains) and outside of the DRM.

Abundance-Based Analysis of DRM-enriched Proteins in Oat and Rye

We further calculated the normalized abundance of each of DRM-enriched protein using Progenesis software and the proportions of the proteins in each functional category (Figure 5). In NA DRM samples, transporters accounted for 63 and 66% of proteins by abundance in oat and rye, respectively (Figure 5A,B). Almost all of the transporters enriched in the DRM were ATPases and aquaporins (Supplemental Table 3 in the Supporting Information). ATPase has been demonstrated to accumulate in lipid rafts in yeast cells.⁵⁷ In addition, sterols, which are enriched in microdomains and the proportion and the class of which differ considerably in oat and rye DRM fractions,⁵⁸ are able to regulate ATPase activity.⁵⁹ Taken together, these results suggest that microdomains are associated with a sterol–ATPase regulation mechanism as a scaffold and that sterol compositions affect intra- and extracellular pH and membrane potential, which would ultimately influence freezing tolerance of plants.

The second predominant category in NA DRM samples was disease/defense- and cell-structure-related proteins in oat and rye, respectively (Figure 5A,B). Cell-structure-related proteins

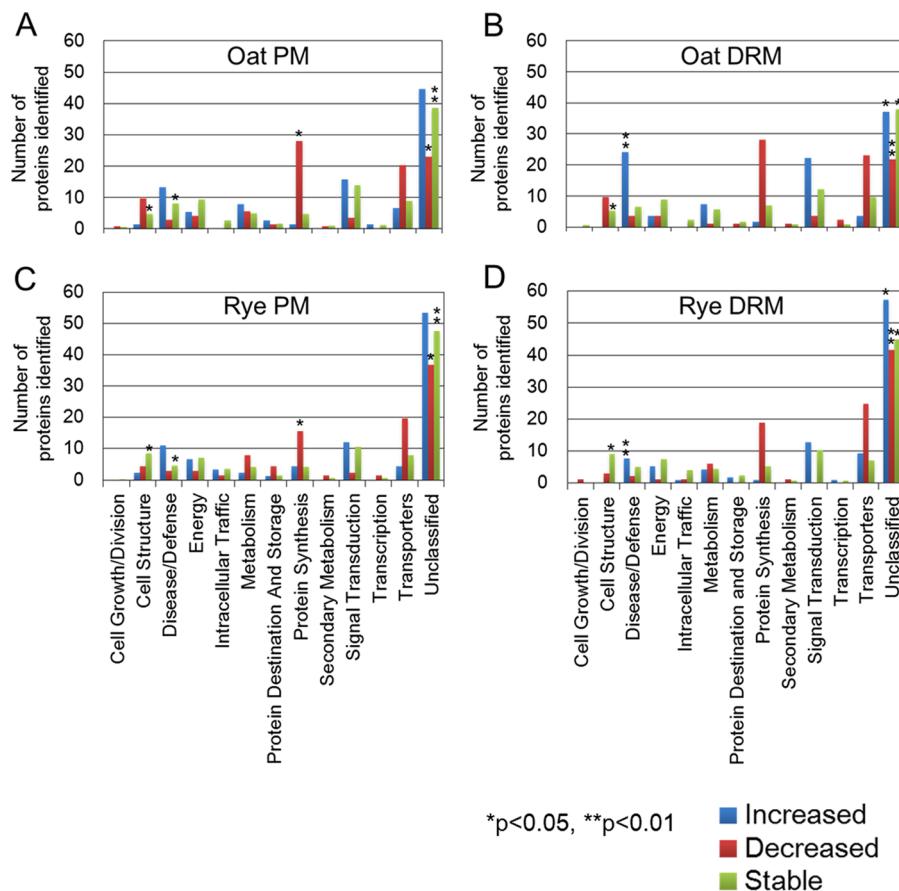


Figure 4. Functional categorization of proteins significantly changed during CA. Peptides were selected according to the following criteria, using Progenesis LC–MS software: ANOVA $p < 0.05$; CA/NA ratio, >2.0 or <0.5 ; expect value cut off, >0.05 . Proteins were then identified using the Mascot server and classified into 13 functional categories based on Bevan et al.,⁴³ as described in Figure 3. Panels A–D show distribution of CA-increased and CA-decreased proteins in the oat PM, oat DRM, rye PM, and rye DRM, respectively. Statistical significance (*, $p < 0.05$; **, $p < 0.01$) between oat and rye during CA, which was calculated with Fisher's exact test based on the number of identified proteins, is shown.

(such as actin) enriched in rye DRM fractions are considered to be closely associated with microdomains and restrict PM protein mobility cooperatively with microdomains in animal cells.^{60–63} Thus, the functional roles of microdomains purified as DRM fractions may be different between oat and rye. However, no substantial changes of any functional categories during CA were found in either species. This result suggests that the global functional balance of DRM and PM proteins in each functional category is not affected by CA treatment. Figure 3 also shows the enrichment of each functional category of during CA.

Remarkable differences between oat and rye DRMs were observed in the proportions of disease/defense- and cell-structure-related proteins (Figure 5). Oat DRMs contained a considerable amount of hypersensitive-induced reaction (HIR) proteins classified into disease/defense proteins (e.g., the DRM/PM ratios of gil23345042 were 5.1 and 3.2 in NA and CA, respectively). Some HIR proteins are proposed as PM-associated regulators of hypersensitive cell death and interact with leucine-rich repeat (LRR) proteins.^{64–67} HIR proteins were also identified as DRM-related proteins^{31,32} and stress responsive proteins.^{68,69} However, the functions of these proteins in freezing tolerance or CA remain unknown. Information about the relationship between CA and the functions of HIR proteins is required.

In contrast, rye DRMs contained a large amount of cell structure-related proteins, such as tubulins. Tubulin, a cytoskeleton component, is dynamically reconstructed during the CA process in rye,⁴⁵ and a decline of tubulins is reported to be important for CA performance in wheat.⁴⁷ Microdomains and tubulin and actin function cooperatively, and their cooperation has important roles in the localization and movement of proteins in animal cells.⁷⁰ Interestingly, microtubule depolymerization enhances cold shock-induced calcium channels.⁷¹ Similarly, reorganization of the actin cytoskeleton is induced by membrane rigidification and reacts to low-temperature signal transduction.⁸ Thus, it is possible that tubulins and actins are associated with microdomain functions as an integral component of PM–cytoskeleton interactions during CAs, and these regulatory mechanisms influence freezing tolerance in rye.

Abundance-Based Analysis of CA-responsive DRM Fraction Proteins in Oat and Rye

We then determined quantitative changes of CA-responsive DRM proteins in oat and rye (the difference of normalized abundance between CA and NA samples). When the proteins were categorized based on their functions according to Bevan et al.,⁴³ there were significant differences between oat and rye in the functional categories into which CA-responsive DRM proteins (ANOVA $p < 0.05$; CA/NA ratio, >2.0 or <0.5) were classified. Among CA-decreased DRM fraction proteins,

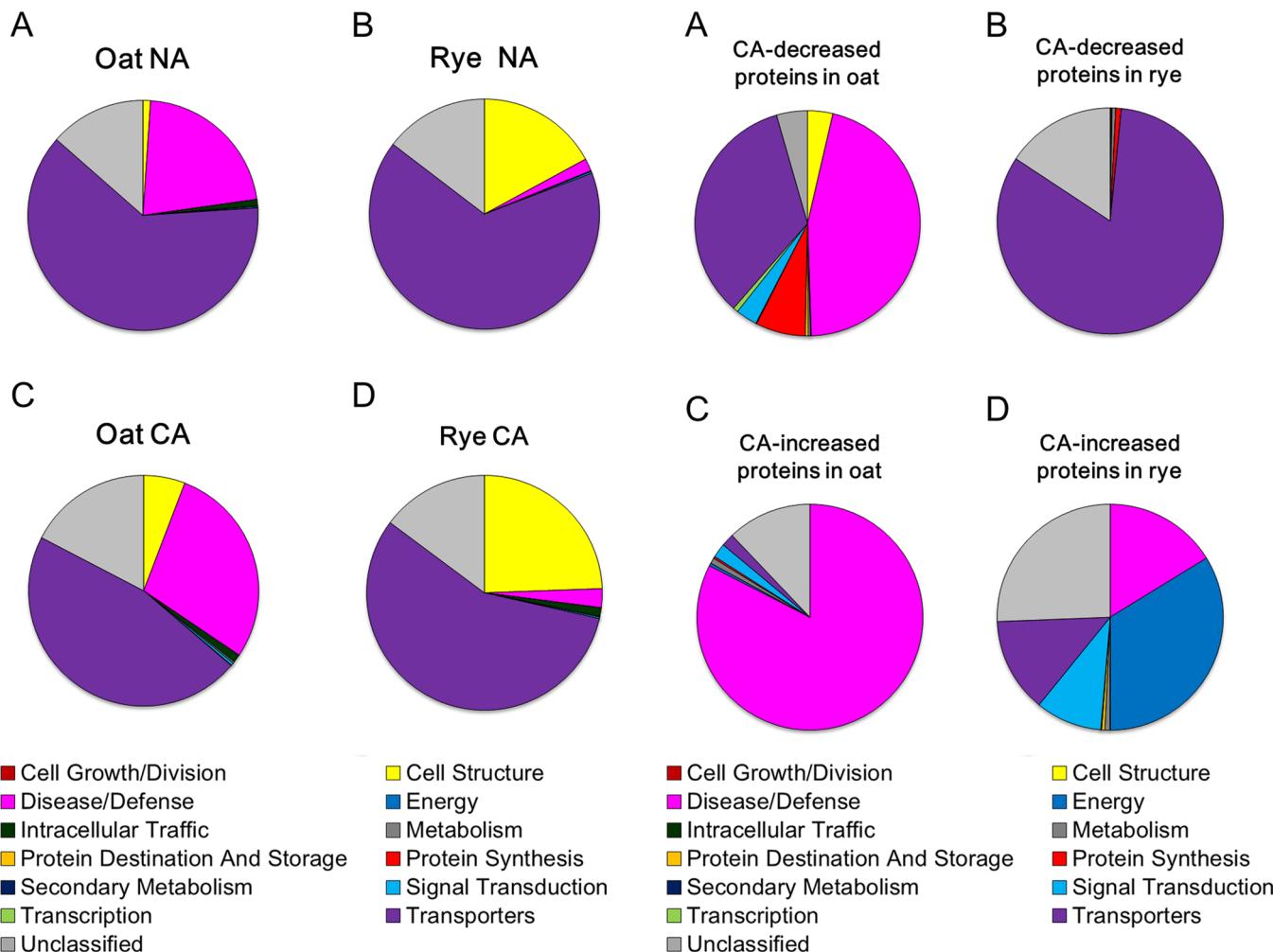


Figure 5. Functional categorization of DRM-enriched proteins in NA and CA samples based on protein abundance. Peptides were selected according to the following criteria using Progenesis LC–MS software: ANOVA $p < 0.05$; DRM/PM ratio, >2.0 ; expect value cut off, >0.05 . Proteins were then identified using the Mascot server and classified into 13 functional categories based on Bevan et al.,⁴³ as described in Figure 3. Percentage shown is based on normalized abundance of proteins classified in each category. Panels A–D show abundance of DRM-enriched proteins in each functional category of the NA oat, NA rye, CA oat, and CA rye, respectively.

disease/defense-related proteins and transporters were abundant in oat (Figure 6A), while almost all CA-responsive proteins were transporters (except for proteins in unclassified category) in rye (Figure 6B). Meanwhile, 83% of CA-increased proteins were disease/defense-related proteins in oat (Figure 6C) and, in rye, 16, 34, and 14% of CA-increased proteins were disease/defense-related, energy-related, and transporter proteins, respectively (Figure 6D).

In rye DRMs, 83% of CA-decreased proteins were transporters, most of which were aquaporins (Supplemental Table 3 in the Supporting Information). It has been reported that overexpression of a *Rhododendron catawbiense* aquaporin, *RcPIP2*, led to a decrease in freezing tolerance of transgenic *Arabidopsis* plants.⁷² The authors considered that these results reflected a low-level freeze-induced desiccation and faster rehydration during thawing. Improper water transport under CA conditions may result in severe dehydration caused by extracellular ice formation. Supplemental Table 3 in the

Figure 6. Functional categorization of CA-responsive proteins based on protein abundance. Peptides were selected according to the following criteria using Progenesis LC–MS software: ANOVA $p < 0.05$; CA/NA ratio, >2.0 or <0.5 ; expect value cut off, >0.05 . Proteins were then identified using the Mascot server and classified into 13 functional categories based on Bevan et al.,⁴³ as described in Figure 3. Percentage shown is based on normalized abundance of proteins in each functional category. Panels A–D show abundance of CA-decreased proteins in oat, CA-decreased proteins in rye, CA-increased proteins in oat, and CA-increased proteins in rye, respectively.

Supporting Information shows that aquaporins were highly enriched in the DRM (e.g., DRM/PM of gil162460423 was 3.2 in NA oat and gil68533200 was 3.9 in NA rye) and a remarkable decline of aquaporin abundance was found, particularly in rye DRM, during CA (e.g., CA/NA of gil115334277 was 0.3 in rye DRM). Thus, decline of aquaporins in rye DRM may be one mechanism for avoiding injury during thawing after freezing. Furthermore, the activities of some of PM aquaporins can be regulated by phosphorylation⁷³ and are affected by heteromerization. Thus, segregation of aquaporins into microdomains may modulate water transport activity through regulation of aquaporin activity and contribute to quantitative and functional changes of aquaporins during CA.

In oat DRMs, CA resulted in an increase in heat shock protein 70 (HSP70), which is classified into the disease/defense category (e.g., gil356505100 identified in DRM, was 2.9 times higher in CA than in NA, see Supplemental Table 3 in the Supporting Information). HSP70 plays important roles in

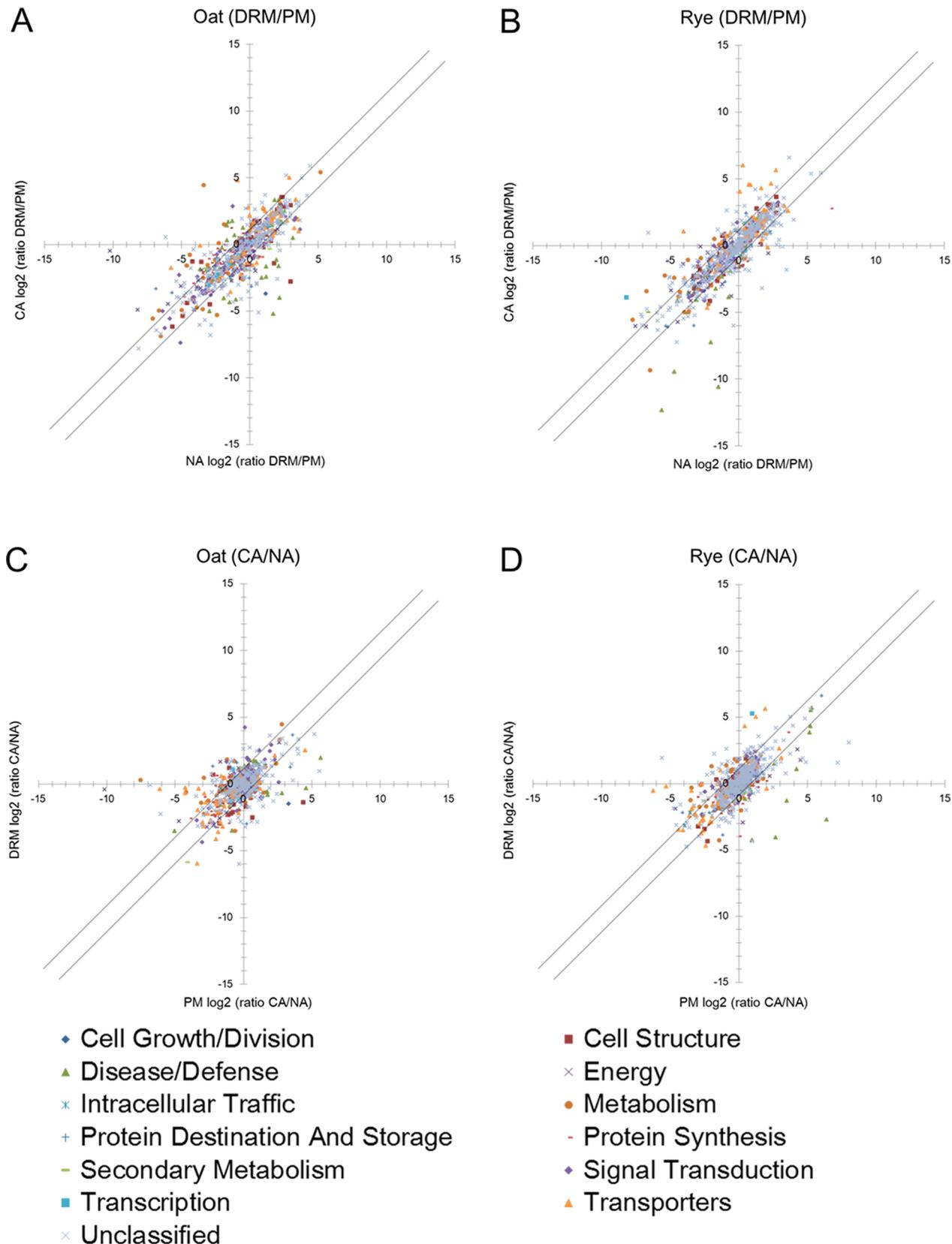


Figure 7. Scatter plot of identified proteins in oat and rye. Normalized abundance of all identified proteins was calculated using Progenesis LC–MS software. The log₂ value of the signal abundance of each protein in two determinations (i.e., DRM vs PM in NA and CA samples and CA vs NA in PM and DRM samples) was calculated and plotted. (A,B) log₂ values of DRM/PM in NA on the x axis and DRM/PM in CA on the y axis in oat (A) and rye (B). (C,D) Log₂ value of CA/NA in PM on the x axis and CA/NA in DRM on the y axis in oat (C) and rye (D).

protein folding and protein transport and responds to low temperature.^{74,75} In fact, a positive correlation between HSP70 accumulation and CA was found in soybean, spinach, and poplar.^{76–78} In our previous study, DRMs in oat were shown to comprise lipids with high melting temperatures that may cause liquid crystal-gel phase transition under freezing temperatures.⁵⁸ These data may support the view that oat micro-domains tend to denature proteins and is one of the vulnerable domains at freezing temperatures. In addition, lipid raft-HSP interactions in animal cells have been discussed in association with the signaling platform.⁷⁹ Therefore, it is possible that oat HSP70 facilitates protein refolding or other unknown processes in plant microdomains during CA and freeze/thawing. In rye DRMs, the noticeable CA-increased proteins were transporters, mostly represented by induction of P-type ATPase (Supplemental Table 3 in the Supporting Information). As previously described, ATPases are predicted to be located in micro-domains, and their activity is affected by surrounding microdomain-enriched sterol molecules. In addition to sterol changes in the DRM during CA, an increase in ATPases in rye DRMs may support the hypothesis that cooperative changes of ATPase and sterols in rye microdomains result in changes in the modulation of intra- and extracellular pH and membrane potential, ultimately contributing to enhanced freezing tolerance.

Changes in the Ratio of CA/NA and DRM/PM in Oat and Rye

To see whether there are changes in the partitioning of each protein into DRMs during CA and if there are any effects of CA on the distributions of specific protein groups in DRM, \log_2 values of the DRM/PM ratio of NA samples were plotted against \log_2 values of DRM/PM ratio of CA samples for each protein (Figure 7A,B). In these panels, the proteins with lower DRM/PM values under NA and higher DRM/PM values under CA (proteins plotted in the upper-left quarter) indicate that enrichment of the protein into DRMs increased during CA. Conversely, the proteins with a combination of higher \log_2 DRM/PM values in NA and lower \log_2 DRM/PM values in CA (proteins plotted in the lower-right quarter) indicate that partitioning of the protein into DRM decreased during CA. In general, in both oat and rye, the DRM/PM ratio of each protein did not change during CA, and there were no significant differences in the enrichment in the DRM of proteins, regardless of functional categories that the protein belongs to. These data indicate that the distribution of each protein in the PM (i.e., inside and outside the DRM) is not significantly affected by CA treatment.

In both oat and rye, however, we observed that some proteins were found to behave differently from the majority of proteins. For example, in oat, focusing on proteins that increased their enrichment in the DRM during CA, a predicted protein (gil326519689) classified into unclassified proteins was plotted on the point ($-0.6, 1.9$) (Figure 7A). The DRM/PM ratio in NA and CA was 0.7 ($p > 0.05$) and 3.8 ($p < 0.0005$), respectively (Supplemental Table 3 in the Supporting Information). This means that the relative abundance of this protein in the DRM or partitioning of this protein into the DRM increased after CA. Putative LRR domains were detected in the amino acid sequence of this protein, which is similar to the LRR sequence found in *Medicago truncatula* proteins (Blast score: 633). LRR receptor-like kinases were also detected and enriched in *Arabidopsis* DRMs.⁸⁰ LRR is one of representative

motifs of amino acid sequence structure that are included in a broad range of functional proteins and is often important for protein–protein interaction.⁸¹ Changes of affinity of the LRR-containing proteins for DRM may suggest that LRR protein–microdomain interactions are regulated by CA treatment and hence affect protein function through changes in protein–protein accessibilities.

In rye, there are proteins for which enrichment in the DRM increased during CA. Two sugar-transporting proteins, gil357115762 and gil357158410, changed from non-DRM-enriched proteins in NA to DRM-enriched proteins in CA (Figure 7B). The DRM/PM ratio increased for gil357115762 from 1.1 ($p > 0.05$) in NA to 16 ($p < 0.005$) in CA. For gil357158410, the DRM/PM ratio increased from 1.8 ($p > 0.05$) in NA to 23 ($p < 0.005$) in CA. These results clearly indicate that sugar transporters become enriched in the DRM after CA. Intracellular sugar concentration is one of the most important factors for acquiring freezing tolerance during CA. Intracellular ice formation is lethal for plant survival;⁸² therefore, solute accumulation inside the cell is necessary to increase osmotic concentration, which results in lowering of the freezing point of the cell, thus decreasing the probability of the cell freezing. In addition, sugars can act as cryoprotective solutes to prevent macromolecules (e.g., proteins) from denaturing during freeze-induced stresses. Actually, rye accumulates a variety of solutes, including glycinebetaine, proline, and soluble sugars, during CA⁴ and relocates fructans and simple sugars in the apical region and crown tissues during freezing at $-3\text{ }^{\circ}\text{C}$.⁸³ Thus, repartitioning of sugar transporters to the DRM in the PM after CA may indicate that microdomain-dependent regulation of sugar transportation is involved in proper solute modulation at the cellular or tissue level.

The \log_2 values of the CA/NA ratio of PM samples were then plotted against \log_2 values of the CA/NA ratio of DRM samples for each of protein (Figure 7C,D). In oat, we found interesting behavior of temperature-induced lipocalins in the PM and DRM during CA (Figure 7C, Supplemental Table 3 in the Supporting Information). Four distinct lipocalins were identified in oat, and, among them, two lipocalins (gil18650668 and gil77744845) statistically increased after CA in the PM (by 3.2- and 3.5-fold, respectively, $p < 0.001$). In the DRM during CA, however, there was no statistical change in gil18650668 and only a slight decrease in gil77744845 (CA/NA: 0.6 ; $p < 0.05$). Lipocalins are widely distributed in plant, animal, and microbial species and are characterized by three highly conserved domains.⁸⁴ *Arabidopsis* temperature-induced lipocalin 1 (*TIL1*) is responsive to heat stress, and a *TIL1*-knockout mutant (*til1-1*) is vulnerable to heat stress because *TIL1* is essential for protecting lipids and proteins from oxidation induced by heat stress.⁸⁵ In addition, NA protoplasts isolated from *Arabidopsis* *lipocalin*-overexpressing mutants showed enhanced freezing tolerance.⁸⁶ Therefore, lipocalins may help to acquire freezing tolerance during CA through an association with the PM. Lipocalins may have different interaction behaviors between microdomain and nonmicrodomain areas during CA because of differences in the lipid environments between the inside and outside of the microdomain or changes in the lipid environment during CA, resulting in different patterns of change between the PM and DRM.

In Supplemental Table 4 in the Supporting Information, the DRM-enriched proteins that were found in NA samples and that statistically changed, either increased or decreased, during

CA are listed. Twenty-four and 37 DRM-enriched proteins were identified as CA-responsive proteins in oat and rye, respectively. Interestingly, many DRM-enriched proteins statistically decreased after CA. In both oat and rye, aquaporins were commonly identified as DRM-enriched and CA-decreased proteins. Additionally, there were other common proteins in oat and rye, such as cellulose synthase and V-type proton ATPase. Decreases in PM proteins are, at least partially, considered to be caused by endocytosis. In animal cells, raft-dependent endocytosis is observed, and there are various endocytic pathways mediated by raft-enriched caveolins and dynamins.⁸⁷ Some of the PM modifications during CA may be caused by microdomain-dependent endocytosis, and this process may result in the removal of specific DRM-enriched proteins, such as aquaporins, from the PM bilayer.

Changes of Post-Translationally Modified Proteins during CA in DRM Fractions

In animal cells, post-translational modifications of proteins are important for defining protein functions in microdomains.⁸⁸ Among them, the addition of a GPI anchor is associated with microdomains.^{88,89} GPI is embedded in the exoplasmic leaflet of the PM and is bound to protein.⁹⁰ Some GPI-anchored proteins were observed to be localized in a cholesterol-dependent microdomain-like structure with 70 nm diameter by fluorescence resonance energy transfer analysis.⁹¹ When the Big-PI plant predictor predicted GPI-anchored proteins, we found more GPI-anchored proteins in the DRM of rye ($n = 21$) than in oat ($n = 9$) (Figure 8A). CA-induced changes in the abundance of GPI-anchored proteins in the DRM were significant in rye (Figure 8B). Interestingly, in rye, all of the CA-responsive GPI-anchored proteins in the DRM increased during CA. Among them, two GPI-anchored proteins, gil326502418 and gil326494592, increased in rye DRM by 12- and 2.6-fold, respectively. These proteins are structurally similar to 1,3- β -glucosidase-like proteins (Blast scores 606 and 764, respectively). In *Arabidopsis*, the β -glucosidase-like-protein encoded by the *SFR2* gene, which is located in chloroplast outer envelope, has an ability to protect chloroplasts against freezing.⁹² *SFR2* protein results in changes of the ratio of lipids in chloroplast envelope and stabilization of membranes during freezing.⁹³ Furthermore, other GPI-anchored proteins, such as fasciclin-like proteins (FLAs), were identified in the rye PM and increased after CA (e.g., gil115349890 and gil115349914, Supplemental Table 3 in the Supporting Information). These proteins are cell-adhesion molecules and are responsive to several environmental stresses. In *Arabidopsis* roots, FLA was suggested to be related to responsiveness to salt stress through cell adhesion.⁹⁴ In wheat, some FLAs were induced by CA treatment.⁹⁵ Taken together, the stress responsiveness of GPI-anchored proteins, including 1,3- β -glucosidase-like proteins and FLAs, may be important and significant during plant CA. Thus, further studies to reveal the functional involvement of these GPI-anchored proteins in CA are clearly warranted.

Another important post-translational modification of membrane proteins is myristylation, which plays roles in localization of proteins to microdomains.⁹⁶ Myristylation consists of a C14 saturated fatty acid bound to a specific protein. When the Plant-Specific Myristylation Predictor predicted myristylated proteins (Figure 8C,D), the number of putative myristylated proteins identified was similar in oat ($n = 31$) and rye ($n = 27$) (Supplemental Table 1 in the Supporting Information). In oat and rye DRMs, several myristylated

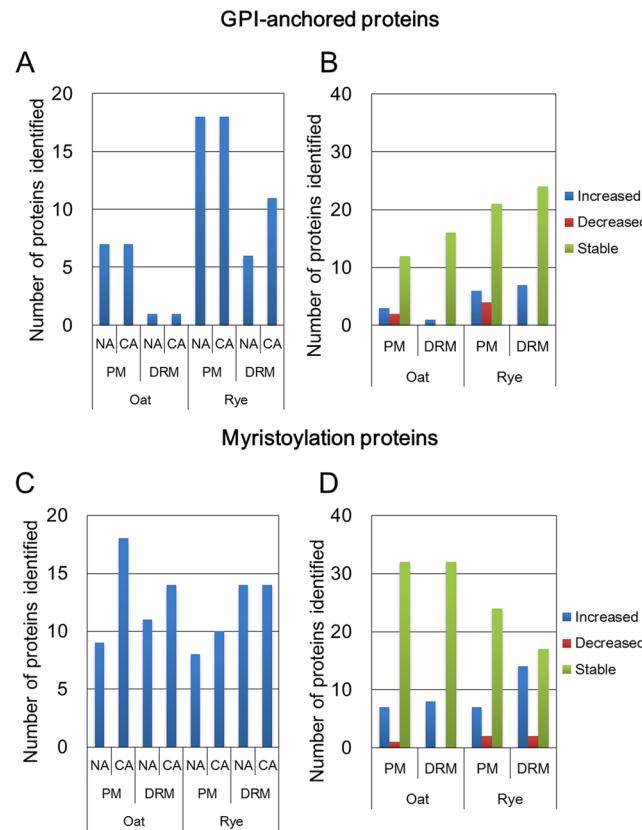


Figure 8. Identification of the putative GPI-anchored (A,B) and N-terminal myristoylated proteins (C,D) in oat and rye. Putative GPI-anchored proteins and myristoylated proteins were predicted by Big-PI plant predictor and Plant-Specific Myristylation Predictor, respectively. (A,C) Number of potential GPI-anchored proteins and myristoylated proteins in each experimental section, respectively. (B,D) Number of CA-increased (ANOVA $p < 0.05$; CA/NA ratio >2.0), -decreased (ANOVA $p < 0.05$; CA/NA ratio <0.5), and stable (unchanged) GPI-anchored proteins and myristoylated proteins, respectively.

proteins increased after CA, including signal transduction-related proteins, such as calcium-dependent kinase (gil84626055) and receptor-like kinase (gil255537473). Signal transduction, which is a putative microdomain function, may support smooth reconstruction of CA cells in plants via myristoylated protein–microdomain interactions.

CONCLUSIONS

We found in the present study that DRM proteomes responded to CA dynamically in both oat and rye. CA-induced changes in some DRM proteins, including P-type ATPase, aquaporin, and several cytoskeleton components, occurred commonly in the two species. These proteins may contribute to adjust and optimize PM function to freezing temperature in both oat and rye, which is thought to be a common requirement to increase tolerance to freezing stress. In addition, there is another aspect that changes of DRM proteome were considerably different between low-tolerant oat and high-tolerant rye. In oat DRM, CA resulted in an increase in HSP70 during CA, while in rye, ATPases considerably increased during CA. HSP may prevent other membrane proteins from denaturing or destabilization under freezing temperature. Rye may maintain membrane potential and intracellular pH with modulating DRM-localized ATPases during CA. In addition to this change, in rye, sugar

transporters were differentially partitioned into DRM fraction in the PM before and after CA. Intra- and extracellular sugars are thought to be important for increasing freezing tolerance in plants as cryoprotective solutes. Thus, particularly in rye, microdomain and sugar transporters may act cooperatively for effective and suitable sugar transporting at cellular or tissue levels during CA. These results indicate that oat and rye have both common and different ways to adapt to freezing temperature through changes of microdomain protein compositions and properties of proteins between the inside and outside of microdomain. Therefore, microdomain proteins are thought to have an important role for CA and affect plant freezing tolerance. Results in the present study warrant further studies of DRM proteins to understand how these proteins are working in CA process.

■ ASSOCIATED CONTENT

§ Supporting Information

Supplemental Table 1. Identified PM and DRM fraction Proteins in Oat and Rye; Supplemental Table 2. Functional Categorizations of Identified PM and DRM fraction Proteins in Oat and Rye; Supplemental Table 3. Identified CA-responsive Proteins and Peptides in Oat and Rye as Determined by Progenesis Software; Supplemental Table 4. DRM-enriched and CA-responsive Proteins in Oat and Rye; Supplemental Tables 5-12. Identified Peptides Lists in Each Fractions and Experiments; Supplemental Figure 1. Detergent-resistant Membrane after Sucrose Density Gradient Centrifugation; Supplemental Figure 2. Work flow of Large-Scale Quantitative Proteomics of DRM Fractions in Oat and Rye. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS:

PM, plasma membrane; DRM, detergent-resistant membrane; NA, nonacclimated or nonacclimation; CA, cold-acclimated or cold acclimation; ANOVA, analysis of variance; GPI, glycosylphosphatidylinositol

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