Title

Lipid profiles of detergent resistant fractions of the plasma membrane in oat and rye in association with cold acclimation and freezing tolerance

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22 **Abstract**

23 Cold acclimation (CA) results in alteration of the plasma membrane (PM) lipid
24 composition in plants, which plays a crucial role in the acquisition of freezing tolerance via
25 membrane stabilization. Recent studies have indicated that PM structure is consistent with the
26 fluid mosaic model but is laterally non-homogenous and contains microdomains enriched in
27 sterols, sphingolipids and specific proteins. In plant cells, the function of these microdomains in
28 relation to CA and freezing tolerance is not yet fully understood. The present study aimed to
29 investigate the lipid compositions of detergent resistant fractions of the PM (DRM) which are
30 considered to represent microdomains. They were prepared from leaves of low-freezing tolerant
31 oat and high-freezing tolerant rye. The DRMs contained higher proportions of sterols,
32 sphingolipids and saturated phospholipids than the PM. In particular, one of the sterol lipid
33 classes, acylated sterylglycoside, was the predominant sterol in oat DRM while rye DRM
34 contained free sterol as the major sterol. Oat and rye showed different patterns (or changes) of
35 sterols and 2-hydroxy fatty acids of sphingolipids of DRM lipids during CA. Taken together,
36 these results suggest that CA-induced changes of lipid classes and molecular species in DRMs
37 are associated with changes in the thermodynamic properties and physiological functions of
38 microdomains during CA and hence, influence plant freezing tolerance.

39 **Keywords**

40 Cold acclimation; Oat (*Avena sativa*); Rye (*Secale cereale*); Freezing tolerance; Plasma
41 membrane; Detergent resistant membrane (Microdomain, Lipid raft); Sterol; Sphingolipid;
42 Phospholipid

43

44 **Abbreviations**

45 **CA** cold acclimation

46 **NA** non-acclimation

47 **PM** plasma membrane

48 **DRM** detergent-resistant membrane fraction of the plasma membrane

49 **PC** phosphatidylcholine

50 **TLC** thin layer chromatography

51 **FS** free sterol

52 **SG** sterylglucoside

53 **ASG** acylated sterylglucoside

54 **GlcCer** glucocerebroside

- 55 **PL** phosholipid
- 56 **PE** phosphatidylethanolamine
- 57 **PC** phosphatidylcholine
- 58 **PG** phosphatidylglycerol
- 59 **PA** phosphatidic acid
- 60 **PS** phosphatidylserine
- 61 **PI** phosphatidylinositol
- 62 **DGDG** digalactosyldiacylglycerol
- 63 **MGDG** monogalactosyldiacylglycerol
- 64 **LysoPE** lyso-phosphatidylethanolamine
- 65 **LysoPC** lyso-phosphatidylcholine
- 66 **LysoPG** lyso- phosphatidylglycerol
- 67 **H_{II}** hexagonal II
- 68 **DOPC** dioleoyl-PC
- 69 **DOPE** dioleoyl-PE
- 70 **UGT** UDP-glucose: sterol glucosyltransferases

- 71 **FAH** fatty acid 2-hydroxylase
- 72 **PLD** phospholipase D
- 73 **d18:1** 8-sphingadienine
- 74 **d18:2** 4,8-sphingadienine
- 75 **t18:1** 4-hydroxy-8-sphingenine
- 76 **ANOVA** analysis of variance

77 **Introduction**

78 Cold acclimation (CA) is one of the most important processes for adaptation to
79 freezing temperatures in plants. During this process, the plasma membrane (PM) composition
80 changes dynamically, which is crucial for acquisition of freezing tolerance. The PM is
81 composed of a variety of proteins and lipids which are considered to be distributed
82 homogeneously and to move relatively freely [40]. However, recent studies have raised the
83 possibility that PM contains microdomains in which components are restricted in their
84 movement. Subsequent biochemical studies proposed that microdomains can be extracted as
85 detergent resistant membrane fractions (DRM) and suggested that DRM-associated proteins and
86 lipids are important for determining the structure and function of microdomains [38,15,54,8,26].
87 In fact, previous studies, using proteomics techniques, identified DRM-enriched proteins in oat
88 and rye PM and revealed their dynamic responses to CA [42,43]. The results suggested that
89 some microdomain proteins associated with CA in the two plant species contribute to their
90 vastly different freezing tolerance.

91 In addition to proteins, many studies have suggested that lipids are necessary for
92 microdomain functions. Simons and Ikonen considered the lateral organization of raft-like lipid
93 microdomains to be due to preferential packing of sphingolipids and cholesterol in specific
94 small regions [38,40]. Sphingolipids, in fact, form nanodomains in phosphatidylcholine

(PC)-based lipid bilayers and cholesterol fills the intermolecular spaces of sphingolipids [35,37].

Thus, microdomain formation and stabilization may be driven by lipid–lipid and lipid–protein

interactions in addition to protein–protein interactions [11,27,38,39]. In plants, some studies

have examined the significance of lipids in microdomains. Borner et al. [4] and Mongrand et al.

[31] first quantified lipid class and fatty acid composition using DRM as microdomain-enriched

fractions. In the same way, the DRM lipid compositions of maize embryos and bean leaves have

been characterized [6,7]. Furt et al. [12] reported that phosphatidylinositol-4,5-bisphosphate

forms a cluster-like structure that is not influenced by sterol depletion, and that

phosphoinositide metabolism-related enzyme activities in DRMs are higher than in the PM.

Lipid changes in DRMs were reported in *Arabidopsis* leaves during CA [30].

Although free sterol proportions changed in DRMs of the *Arabidopsis* PM during CA [30], the

authors performed only lipid composition measurements at the lipid class level, and did not

examine the molecular species in each lipid class. In the present study, we took biochemical and

lipidomic approaches to determine the lipid compositions in oat and rye DRMs during CA.

Considering a previous study that dealt with PM lipid compositions in oat and rye during CA

[44], we aimed to (a) compare lipid compositions between the PM and DRMs, (b) investigate

compositional changes of PM and DRM lipids during CA and (c) compare lipid composition

and CA-induced changes between low freezing-tolerant oat and high freezing-tolerant rye. The

physical properties of membrane lipids influence their stability and are important for the acquisition of plant freezing tolerance during CA [13,41,46]. Therefore, both the physical properties of the PM and microdomains and the biochemical functions of microdomains during CA will be discussed by comparing oat and rye.

Materials and methods

Plant Materials and Isolation of DRM Fractions

Non-acclimated and cold-acclimated oat (*Avena sativa* L. cv. New almighty) and rye (*Secale cereale* cv. Maskateer) were grown under conditions reported by Takahashi et al. [42]. PM and DRM isolation were performed in accordance with Takahashi et al. [42].

Total Lipid Extraction and Thin Layer Chromatography

Extraction of total lipids from PM and DRM fractions was carried out according to the method of Bligh and Dyer [3]. Isopropanol (2.5 mL) was added to 1 mL of PM or DRM suspensions. Subsequently, 1.25 mL of chloroform was added twice and samples in test tubes were mixed well. After adding 1.25 mL of 0.9% (w/v) NaCl, samples were incubated for 15 min

at room temperature. Test tubes were then centrifuged at $196 \times g$ for 5 min to induce phase separation and the lower phase was collected in a new test tube. To re-extract lipids, chloroform (1.5 mL) was added to the remaining upper phase, test tubes were then centrifuged ($784 \times g$ for 10 min) and the lower phase was combined with the previously collected lower phase. After adding 2 mL of chloroform/isopropanol/0.9% (w/v) NaCl (3:47:48, v/v/v) to the combined lower phase, samples were centrifuged at $196 \times g$ for 5 min and the lower phase was collected in a new test tube. The lower phase was then dried at 40°C under N_2 gas flow and after adding an aliquot of chloroform, it was stored at -20°C under N_2 gas until use.

Thin layer chromatography (TLC) analysis of total lipids was conducted using silica gel plates (Silica gel 60, 0.25-mm thickness, Merck, Darmstadt, Germany) with chloroform/methanol/water (65:25:4, v/v/v) as the developing solvent. Subsequently, solvent on thin layer plates was removed completely and lipids were visualized by spraying with 0.1% (w/v) primuline in acetone (Sigma-Aldrich, St Louis, MO, USA).

Quantification of Sterols, Glucocerebrosides and Phospholipids

Silica gel of the spot corresponding to free sterol (FS), sterylglucoside (SG), acylated sterylglucoside (ASG), glucocerebrosides (GlcCer) and phospholipids (PLs) were collected and subjected to quantification of each lipid class.

148 Sterol quantification was carried out according to the method of Zlatkis and Zak
149 (1969) with a slight modification [55]. Briefly, 1.1 mL of acetic acid was added to the silica
150 powder in sample tubes and sonicated. An aliquot (1 mL) of *o*-phthalaldehyde in acetic acid
151 (0.1%, w/v) and 1 mL of concentrated sulfuric acid were added successively and mixed well.
152 These additions generated heat. After cooling the mixture at room temperature for 15 min,
153 sample tubes were centrifuged at $196 \times g$ for 10 min to precipitate silica powder. Absorbance of
154 supernatants at 550 nm was measured. Standard curves were generated from absorbances of
155 cholesterol standards in acetic acid in the range of 0 to 0.15 μ M.

156 GlcCer quantification was performed as sugar determination according to the method
157 of DuBois et al. (1956) [9]. Briefly, 1 mL of water was added to silica powder in sample tubes
158 and then sonicated. Subsequently, 0.5 mL of 5% (w/v) phenol and 2.5 mL of concentrated
159 sulfuric acid were successively added and mixed well. These additions generated heat. After
160 cooling the mixture at room temperature for 15 min, sample tubes were centrifuged at $196 \times g$
161 for 10 min. Supernatants were collected and absorbance at 485 nm was measured. Standard
162 curves were generated from absorbances of glucose standards in the range of 0 to 0.15 μ M.

163 PL was determined as phosphate according to the method of Marinetti (1962) [29].
164 Briefly, 50 μ L of water and 0.5 mL of 70% perchloric acid were added to sample tubes
165 containing silica gel and mixed well. Subsequently, tubes were incubated at 200°C for 30 min.

166 After cooling to room temperature, 3 mL of water and 0.5 mL of 2.5% (w/v) ammonium
167 molybdate, 0.2 mL of Fiske-SubbaRow reagent (30 g of sodium hydrogen sulfite, 1 g of sodium
168 sulfite and 1 g of 1-amino-2-naphthol-4-sulfonic acid in 200 mL of water) were added in
169 sequence and mixed well. Tubes were boiled for 7 min, cooled to room temperature and
170 centrifuged at $196 \times g$ for 10 min. Supernatants were collected and absorbance at 700 nm was
171 measured. Standard curves were generated from absorbances of KH_2PO_4 standards in the range
172 of 0 to 0.25 μM .

173

174 *Determination of Molecular Species of Sterols, Glucocerebrosides and Phospholipids*

175 Total lipid fractions obtained from 50 μg of PM protein were separated into neutral
176 lipid (e.g. FS), glycolipid (SG, ASG and GlcCer), and PLs using silica gel column
177 chromatography according to the method of Lynch and Steponkus [28]. Each separated fraction
178 was dried by blowing N_2 gas, dissolved in 1 mL chloroform/acetic acid (100:1, v/v) and
179 transferred to a Sep-Pak Silica Classic Cartridge (Waters, Milford, MA, USA) coupled to a
180 glass syringe barrel. Neutral lipids were eluted with 10 mL chloroform/acetic acid (100:1, v/v).
181 Glycolipids were then eluted by sequential addition of 5 mL acetone and 5 mL acetone/acetic
182 acid (100:1, v/v). Subsequently, phospholipids were eluted by addition of 7.5 mL
183 methanol/chloroform/water (100:50:40, v/v/v). Water/chloroform (12:9, v/v) was added to

phospholipid fractions and the fractions were centrifuged at $1000 \times g$ for 5 min. The resultant lower phase was collected and dried under N_2 gas. The three lipid fractions were dissolved in chloroform/acetic acid (100:1, v/v) and stored at $-20^\circ C$ under N_2 gas until use.

For determination of molecular species of FS, neutral lipid fractions were dried again and dissolved in 100 μL chloroform and transferred into a vial insert (Shimadzu Scientific, Kyoto, Japan). After evaporating chloroform from the vial insert, lipids were dissolved in 5 μL chloroform. Subsequently, the sample (0.5 μL) was injected into a GC-18A gas chromatograph (Shimadzu Scientific) equipped with a dimethyl polysiloxane coated TC-1 capillary column (0.25 mm $\times$ 15 m; GL Science, Tokyo, Japan). Lipids were detected using a hydrogen flame ionization detector (FID). Temperature of the column was increased at $3^\circ C/min$ from $180^\circ C$ to $240^\circ C$. Both injector and detector temperatures were set at $250^\circ C$. Each sterol species was identified by the retention time of peaks corresponding to the following standards: cholesterol, campesterol, stigmasterol and sitosterol (Wako Chemicals, Tokyo, Japan).

GlcCer molecular species analysis was carried out according to the method of Imai et al [19]. First, total lipid extracts were subjected to mild alkaline hydrolysis to remove glycerolipids. The completely dried total lipid extracts were dissolved in 133 μL of 0.4 M KOH in methanol at $37^\circ C$ for 1 h and neutralized with 5 μL of HCl. Subsequently, 2666 μL of chloroform, 2533 μL of methanol and 1333 μL of water were sequentially added to samples and

202 resultant lower phases were collected. Sample fractions containing GlcCer was transferred to a
203 Sep-Pak Plus Silica Cartridge (Waters, Milford, MA, USA) coupled to a glass syringe barrel
204 and chloroform/acetic acid (100:1, v/v) was passed through the column. The fractions
205 containing GlcCer were then eluted with chloroform/methanol (2:1, v/v) and injected into a
206 LC-10AT pump (Shimadzu Scientific) coupled with ACQUITY TQD tandem quadrupole mass
207 spectrometer (Waters) as described in Watanabe et al. [45]. Samples were separated on a 3 μ m
208 TSKgel ODS-100Z column (Tosoh, Tokyo, Japan) eluted with a gradient from 80% solvent A
209 (methanol/formic acid, 1000/1, v/v)/20% solvent B (water/formic acid, 1000/1, v/v) to 100%
210 solvent A for 30 min and then solvent A for 70 min at a flow rate of 200 μ L/min. The following
211 conditions were used for detection: capillary voltage, 3 kV; desolvation gas flow, 600 L/h;
212 nebulizer gas flow, 50 L/h; source temp., 120°C; and collision gas flow, 0.3 mL/min (4-5 mbar).
213 Identification of various GlcCer molecular species was carried out based on 30 pairs of
214 precursor ions $[M + H]^+$ and product ions of sphingoid base moieties as listed in Watanabe et al.
215 [45] in the positive ionization MRM mode.

216 Determination of molecular species of SG and ASG was carried out by direct-infusion
217 electrospray ionization triple quadrupole mass spectrometry according to Schrick et al. [36] with
218 minor modifications. An aliquot of each sample (0.2 mL) was combined with 0.15 nmol PG
219 (di20:0 [phytanoyl]) as an internal standard in a total sample volume of 1 mL of

220 chloroform/methanol/300 mM ammonium acetate in water (300/665/35, v/v/v). The sample was
221 directly infused at 30 μ L per min. The analytical parameters and data processing were as
222 described previously [36].

223 Determination of phospholipid species was performed using direct-infusion
224 electrospray ionization triple quadrupole mass spectrometry according to the method described
225 in the supplemental data of Xiao et al. [52] with minor modifications. The samples were
226 dissolved in 1 mL chloroform. An aliquot of extract (200 μ L) in chloroform was used. Precise
227 amounts of internal standards, obtained and quantified as previously described [49], were added
228 in the following quantities (with some small variation in amounts in different batches of internal
229 standards): 0.27 nmol PC (di12:0), 0.27 nmol PC (di24:1), 0.27 nmol Lyso PC (13:0), 0.27 nmol
230 Lyso PC (19:0), 0.14 nmol PE (di12:0), 0.14 nmol PE (di23:0), 0.14 nmol Lyso PE (14:0), 0.14
231 nmol Lyso PE (18:0), 0.14 nmol PG (di14:0), 0.14 nmol PG (di20:0 [phytanoyl]), 0.14 nmol
232 Lyso PG (14:0), 0.14 nmol Lyso PG (18:0), 0.10 nmol PI (16:0-18:0), 0.07 nmol PI (di18:0),
233 0.09 nmol PS (di14:0), 0.09 nmol PS (di20:0 [phytanoyl]), 0.14 nmol PA (di14:0), 0.14 nmol PA
234 (di20:0 [phytanoyl]), 0.22 nmol DGDG (16:0-18:0), 0.32 nmol DGDG (di18:0), 0.90 nmol
235 MGDG (16:0-18:0), and 0.18 nmol MGDG (di18:0). The samples and internal standard mixture
236 were combined with solvents composed of chloroform/methanol/300 mM ammonium acetate in
237 water (300/665/35, v/v/v) at a final volume of 0.8 mL. Samples were then directly infused at 30

μL per min. The analytical parameters and data processing protocol were the same as described previously [52]. The mean unsaturation index was calculated as the average number of double bonds per fatty acid present ([the percentage of each lipid] x [number of double bonds/number of fatty acid species per lipid]) as described by Lee et al [24].

Results

Determination of Lipid Profiles in Oat and Rye DRM during CA

DRM fractions were extracted from purified PM fractions by adding Triton X-100 as described in Peskan et al. [34]. A white layer was observed at the interface of the 30% and 35% (w/w) sucrose layers. As previously described [42], the recovery rates of DRMs from PM fractions (based on protein amount) significantly decreased in both oat and rye DRM during CA (from 13.86% to 8.16% in oat and 11.73% to 7.17% in rye).

In the present study, we extracted lipids from PM and DRM fractions corresponding to 100 μg protein each, and subsequently separated the extracted lipids with TLC. Comparing the patterns of PM and DRM lipids with those of commercial marker lipids showed that the PM and DRM fractions contained FS, ASG, SG and PLs (PE, phosphatidylethanolamine; PG,

phosphatidyl glycerol; PA, phosphatidic acid; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol) as major lipid components (Fig. 1). DRM fractions contained smaller amounts of PLs than PM fractions. Three sterol classes (FS, ASG and SG) were more brightly stained in DRMs than the PM. As shown in Fig. 1, ASG and FS seemed to be the major components in oat and rye DRMs, respectively. Sterols, glycolipids and PLs in each spot from the TLC plates were quantified by colorimetric assays for sterol, sugar and phosphate moieties, respectively. PE, PG, PA, PC, PS and PI were combined for quantification because they were difficult to separate into distinct spots.

The quantification of lipids (mol% of total lipids [nmol/100 µg protein]) in NA and CA oat is described in Table 1. The proportions of sterols and PLs were quite different between the PM and DRMs in oat. For sterols, the mol% of sterols in the total lipids accounted for 42.6% and 73.6% in the PM and DRMs, respectively. The major sterol component was ASG (28.5% in PM and 50.3% in DRMs). In contrast to sterols, the proportion of PLs in the PM was greater than in DRMs (41.9% in PM and 11.4% in DRMs). The GlcCer proportion in the PM was similar to that in DRMs (15.5% in PM vs 15.0% DRMs). These results indicate that, in oat DRMs, sterols are enriched and PLs are depleted.

In rye, similar to oat, DRMs contained much higher amounts of sterols and lower amounts of PLs than the PM (Table 2). In rye, however, FS was the major form of sterol in both

the PM and DRMs, accounting for 50.5% of the total lipids in DRMs. The proportion of PLs was lower in DRMs than in the PM (48.0% in PM vs 20.6% in DRMs). Although DRMs tended to contain a slightly higher proportion of GlcCer than the PM (15.6% in DRMs vs 13.4% in PM), the difference was not significant. Overall, rye DRMs had higher sterols and lower PLs than the PM, and had a different sterol composition from oat DRMs.

After CA treatment, the total amount of sterols in the oat PM decreased from 42.6% to 37.7% (Table 1). Sterols in oat DRMs also decreased from 73.6% to 66.0% (Table 1). During CA, the proportions of FS in the oat PM significantly decreased from 10.2% to 7.4%, respectively, while no changes were observed in the rye PM (Tables 1 and 2). In oat DRMs, FS significantly decreased from 21.1% to 14.4% during CA. Similarly, ASG in rye DRMs significantly decreased from 9.6% to 5.8% during CA (Tables 1 and 2). GlcCer decreased from 15.5% to 12.6% and 13.8% to 7.8% in the oat and rye PMs, respectively, during CA (Tables 1 and 2). However, there were no statistically significant changes of GlcCer in either oat or rye DRMs during CA (Tables 1 and 2). Slight increases in PL were observed in PM and DRM in both species. (Tables 1 and 2).

290 Fig. 2 shows the sterol compositions in the oat and rye PM and DRMs during CA. In
291 both the PM and DRMs, the sterol composition in oat was quite different from that in rye. In oat,
292 ASG was the major sterol in both the PM and DRMs while FS was abundant in the rye PM and
293 DRMs. In the oat PM and DRMs, the proportion of FS decreased but that of SG increased
294 during CA. In contrast, the proportion of ASG in rye DRMs decreased from 14.7% to 7.7% but
295 the proportion of SG increased from 5.3% to 10.0%.

296 To obtain detailed FS compositional data, we performed gas chromatography analysis.
297 We examined four major sterol species, sitosterol, stigmasterol, campesterol and cholesterol,
298 and calculated the proportion of each sterol in the total FS (Fig. 3, Supplemental Table 1). Oat
299 FS were mainly composed of sitosterol, stigmasterol and cholesterol. On the other hand, in the
300 rye PM and DRMs, sitosterol and campesterol were the predominant sterol species. Although
301 campesterol, stigmasterol and cholesterol were significantly enriched in rye NA DRMs
302 compared with the rye NA PM (1.14-, 1.53- and 2.54-fold, respectively), the general FS
303 composition was similar between the rye PM and DRMs.

304 For SG and ASG, we used a newly developed quantification method with electrospray
305 ionization tandem (triple quadrupole) mass spectrometry (ESI-MS/MS) as reported by Schrick
306 et al. [36] and calculated the proportions of four major sterol species in the total SG and ASG

fractions (Fig. 4, Supplemental Table 1). Although, we could not make a simple comparison between FS and SG/ASG compositions because of the different analysis methods, the proportions of stigmasterol in oat SG and ASG were 24.9 to 37.3% lower than those in FS. In both oat and rye, the predominant sterol species in SG and ASG was sitosterol and most of the CA-induced changes were common among FS, SG and ASG (Figs. 4A and 4B).

In addition to sterol composition, we determined the compositions of the acyl chains bound to ASGs (Fig. 4C). The predominant acyl species in ASGs was 16:0 in both oat and rye. In both species, DRMs contained significantly higher proportions of 16:0 than the PM. Specifically, rye DRMs had 1.33 and 1.26 times higher proportions of 16:0 than the PM at NA or CA conditions. Although the proportions of unsaturated acyl chains such as 16:1, 18:1, 18:2, 18:3 and 20:1 were higher in the rye PM than the oat PM (32.0% in oat PM vs 43.1% in rye PM), no remarkable changes were observed during CA in either the oat or rye PM. During CA, unsaturated lipid species such as 18:3 and 18:1 slightly increased (1.27-fold) and the proportion of 16:0 decreased (0.89-fold) in rye DRMs.

GlcCer Compositions of the PM and DRMs in Oat and Rye during CA

GlcCer is composed of a C18 sphingoid base and a 2-hydroxy fatty acid. First, we measured the sphingoid base, which contains three major species (d18:1, 8-sphingadienine;

d18:2, 4,8-sphingadienine; t18:1, 4-hydroxy-8-sphingenine) with *cis*-8 (*Z*) and *trans*-8 (*E*) isomers [17], and calculated the sphingoid base compositions against the total sphingoid base content in oat and rye (Fig. 5, Supplemental Table 2). t18:1 (*Z*) was the predominant sphingoid base in the PM and DRMs isolated from both oat and rye. During CA, in both oat and rye DRMs, there were no remarkable changes in sphingoid base composition during CA.

The 2-hydroxy fatty acid analysis revealed that the proportions of two major components, 24h:0 and 24h:1, were different between oat and rye (Fig. 6, Supplemental Table 2). In the NA PM, 24h:0 accounted for a higher proportion in rye than in oat (25.1% and 15.0%, respectively). When compared in the PM and DRMs, oat had a higher proportion of 24h:1 in DRMs than in the PM (65.8% and 47.6%, respectively). On the other hand, rye DRMs contained a higher proportion of 24h:0 than the PM (37.8% and 25.1%, respectively) and the proportion of 24h:1 was lower in DRMs than in the PM (29.0% and 38.3%, respectively). No obvious shifts of 2-hydroxy fatty acid composition were observed in the oat or rye PM during CA. Although the 2-hydroxy fatty acid composition in oat DRMs was unchanged during CA, rye DRMs showed a decrease in 24h:0 (37.8% to 29.9%) and an increase in 24h:1 (29.0% to 41.5%) during CA.

PL Compositions of the PM and DRMs in Oat and Rye during CA

343 We next determined the molecular species composition of PA, PS, PI, PE, PC, PG,
344 LysoPE, LysoPC and LysoPG, and then calculated the proportion of each of these PL classes
345 (Fig. 7, Supplemental Table 3). In all samples, PA and PC were the predominant PLs and
346 accounted for more than 80.3%. The proportion of PC in NA DRMs was lower than that in the
347 PM in both oat and rye (39.4% in oat DRMs and 59.1% in rye DRMs). Conversely, PE was
348 enriched in DRMs and the DRM/PM ratios for PE were 4.6 and 3.8 in oat and rye, respectively.
349 After CA, the PC proportion increased by 1.44-fold and the PA proportion decreased by
350 0.65-fold in oat DRMs. Similarly to oat, the PA and PC proportions changed by 1.28- and
351 0.29-fold, respectively, in rye DRMs.

352 To examine the unsaturation levels of acyl chain in PLs in oat and rye, we computed
353 the proportions of each PL molecular species with 0–6 double bonds (Fig. 8A). In NA oat and
354 rye, the pair of fatty acids in a single PL molecule mainly contained 2, 3, 4, or 5 double bonds
355 with smaller amounts of 0, 1, and 6 double bonds. For example, PC (34:2), PC (34:3) and PC
356 (36:4) in NA oat and PC (34:2), PC (36:4) and PC (36:5) in NA rye were the three most
357 abundant PL species. NA DRMs contained more low-unsaturated PL species than PM such as
358 PLs containing two double bonds in both oat and rye (1.39- and 1.49-fold greater in NA DRMs
359 than in NA PM in oat and rye, respectively). In oat PM and DRM, less unsaturated PLs
360 decreased and higher unsaturated ones increased during CA. For example, the proportion of PLs

361 containing three double bonds in oat was 1.33-fold greater in CA DRM than in NA DRM.

362 However, rye DRM did not show any statistically significant changes in the proportions of each
363 PL during CA.

364 Based on the results in Fig. 8A, we calculated the mean unsaturation index of PLs in
365 each fraction and plant species during CA (Fig. 8B). When comparing the PM and DRMs under
366 NA, mean unsaturation index in the PM were greater than those in DRMs in both oat and rye
367 (1.22- and 1.23-fold in oat and rye, respectively) which means DRMs contain much more
368 saturated PLs than PM. In the PM, the unsaturation index significantly increased in both oat and
369 rye from 1.63 to 1.71 and from 1.70 to 1.74 during CA. In DRMs, however, there were no
370 statistical changes in rye during CA even though oat DRMs showed an increased unsaturation
371 level from 1.33 to 1.42.

372 373 374 **Discussion**

375 Previously, PM lipid compositions in oat (cv. Ogle) and rye (cv. Puma) was
376 determined and discussed in relation to differences in the freezing tolerance of the two species
377 after CA [44]. Webb et al. [48] reported that freeze-induced lesions of the PM occurred at

different temperatures in oat and rye and structural changes of the PM such as lamellar to H_{II} phase transitions were observed under freezing temperature in oat protoplast. In the present study, the lipid composition of microdomain-enriched DRM fractions and the PM was determined in oat and rye both before and after CA and are discussed from the viewpoint of the properties of each lipid species and the influence of these lipids on freezing tolerance with reference to previous studies.

Microdomain Compositions in Oat and Rye before CA

In the present study, DRM fractions were isolated as microdomain-enriched fractions by detergent treatment of the PM and subsequent sucrose-density gradient centrifugation. PM and DRM lipid separation and subsequent quantification revealed that sterols were highly enriched but PLs were depleted in the DRM fractions in both oat and rye before CA treatment (Fig. 1, Tables 1 and 2). Previous studies with *Arabidopsis*, tobacco and leek also showed lower proportions of PLs and higher proportions of sphingolipids and sterols in DRM fractions than PM fractions [4,23,31], which is generally consistent with the present study. In the present study, however, a sphingolipid, GlcCer, did not show statistically significant differences between the PM and DRMs in either oat or rye (Tables 1 and 2). Because sphingolipids can be relatively tightly associated with each other and have a high melting point due to the long chain fatty acid

in their molecular structure and high hydrophobicity, they form a lipid nanodomain with low fluidity that differs from the fluid phase mainly composed of PLs and probably contribute to microdomain formation [38]. On the other hand, sterols are also considered to be a major component of microdomains because they have an affinity to GlcCer and fill the intermolecular spaces of GlcCer [38]. Thus, these results indicate that sterols may be a particularly important constituent of microdomains to determine their functions and structure in oat and rye.

Alterations of PM and Microdomain Compositions in Oat and Rye during CA

The relationship between PM lipid composition and freezing tolerance in oat (cv. Ogle) and rye (cv. Puma) has been investigated and discussed previously [44,48]. These studies demonstrated that freeze-induced dehydration results in ultrastructural changes associated with the PM, fracture-jump lesions and lamellar-to-hexagonal II (H_{II}) phase transitions, and that changes of PM lipid compositions decrease the occurrence of these membrane-associated lesions. Although CA treatment resulted in many compositional changes in the PM, the patterns of PM lipid changes (e.g. an increase of PLs and a decrease of GlcCer in oat) were common in the previous and present studies [44]. In oat and rye, the proportions of PLs and GlcCer in the PM changed, which may influence lipid bilayer hydration because PLs and GlcCer are highly and poorly hydrated lipid classes, respectively [47]. Hydration of the PM surface is important

for preventing H_{II} phase formation, which is an inter-bilayer event that occurs when interaction between the PM and various closely-positioned endomembranes triggered by freeze-induced dehydration becomes stronger [13,48]. Therefore, greater hydration of the PM surface in both oat and rye achieved by increasing PLs and decreasing GlcCer during CA may contribute to increasing PM stability under freezing conditions. Additionally, because the PL/GlcCer ratio was higher in rye (6.68) than in oat (3.94), the rye PM may have a lower incidence of H_{II} phase formation and more stable membrane structure than the oat PM under freezing temperatures.

In oat DRMs, the proportion of sterols significantly decreased (by 7.6%) and the proportion of PLs increased (by 4.3%) during CA, while the GlcCer proportion did not change (Table 1). Conversely, rye DRMs did not show any significant changes in the proportions of sterols, GlcCer or PLs. The proportion of GlcCer in both oat and rye did not change in DRMs during CA, but was decreased in the PM, which may be related to the fact that GlcCer is the main component of microdomains [38]. The same tendency was also observed in *Arabidopsis* DRMs [30]. The proportion of sterols decreased in oat DRMs (73.6% to 66.0%) during CA but did not change in rye DRMs (63.8% to 64.5%). Plant sterols are considered to promote lipid domain formation [53] and also to be important for regulating the temperature sensitivity of raft-like membrane structures [2]. Although the proportions of PLs increased by 4.3% during CA in oat DRMs (11.4% to 15.7%), rye DRMs did not show a change in PL proportion and

maintained a higher proportion of PLs than oat DRMs after CA (20.6% in oat and 21.2% in rye after CA). As described above, PLs are important for maintaining PM stability under freezing conditions [13,48]. Thus, the high proportion of PLs in rye DRMs may contribute to maintain and/or change microdomain properties during freezing.

Alterations of Sterol Compositions in Oat and Rye during CA

ASG and FS were the predominant sterols in the oat and rye PM, respectively, which was also observed in DRM samples (Fig. 2). In DRMs, the proportion of ASG increased in oat (68.2% to 71.7%) but decreased in rye (14.7% to 7.7%) during CA. Webb et al. [46] revealed that ASG is more effective than FS for H_{II} phase formation in lipid mixtures of dioleoyl-PC (DOPC) and dioleoyl-PE (DOPE) under dehydration conditions. ASGs contain a sterol ring, sugar and acyl group, and are more hydrophobic than other sterols [46]. Therefore, the different proportions of ASGs between oat and rye DRM may result in different membrane behavior under freeze-induced dehydration and ultimately influence plant freezing tolerance. The biosynthesis of SG and ASG is catalyzed by UDP-glucose:sterol glucosyltransferases (UGTs, EC 2.4.1.173, 22, 23). Thus, the activity of UGTs possibly contributes to the different ASG proportions in oat and rye.

In addition to sterol lipid classes, the molecular species of sterols were determined

before and after CA (Figs. 3 and 4). In oat and rye, sitosterol was commonly the predominant phytosterol among FSs, SGs and ASGs (Figs. 3, 4A and 4B). Just for a reference, the proportion of each sterol species in total sterol content (FS, SG and ASG in combination) was calculated although it is not scientifically sound to combine the data obtained by different quantification methods for each sterol class (data not shown). Results showed that sitosterol accounted for 58.0% to 81.0% of total sterol content in samples obtained from both oat and rye, which means that biosynthesis of each sterol species is relatively similar in oat and rye while sterol modification processes such as glycosylation and acylation for SG and ASG biosynthesis might be an important difference between the two plant species as we mentioned above. However, Oat FS contained more stigmasterol than rye FS in NA DRMs (38.7% in oat and 4.5% in rye) and DRMs prepared from NA rye had more sitosterol than NA oat DRMs (36.3% in oat and 70.7% in rye) although the SG and ASG sterol compositions were only slightly different between oat and rye. Although no studies have examined the effects of sitosterol on lipid mixtures under freeze-induced dehydration conditions in comparison with stigmasterol, the vast differences in sterol species may contribute to the difference in freezing tolerance between oat and rye.

The acyl chain lipid species of ASGs were also determined (Fig. 4C). The acyl chain compositions in ASGs did not change considerably during CA in oat and rye. DRM ASGs contained more saturated fatty acids (16:0 and 18:0) than PM ASGs in NA oat (68.0% in PM

and 75.8% in DRMs) and the same was true in NA rye (56.9% in PM and 73.6% in DRMs).

Given that microdomains are considered to be enriched in hydrophobic and high-melting point lipids such as sphingolipids and sterols [38], saturated acyl chain ASGs may also tend to gather in microdomain areas in the PM.

Sterols are also important for the modulation of H⁺-ATPase activity [14]. H⁺-ATPase is known as a DRM-enriched protein [30,42,43] and is enriched in microdomains of the yeast *Saccharomyces cerevisiae* [1]. H⁺-ATPase activity is up-regulated by cold and may be involved in the regulation of intracellular pH and membrane potential [20]. Thus, it is possible that differences in sterol composition in microdomains may affect the activity of microdomain-associated proteins, and sterol-enriched microdomains may have an important role for the modulation of protein structure and function as a functional scaffold during CA.

Alterations of GlcCer Compositions in Oat and Rye during CA

Among the two major parts of GlcCer, the C18 sphingoid base showed no significant changes during CA in both oat and rye (Fig. 5). t18:1 was the predominant C18 sphingoid base in both species as reported in *Brassicaceae* [18]. The overall profiles of sphingoid base compositions were similar between oat and rye and the maximum amount of CA-induced changes was less than 4.2% (d18:1[E] in the oat PM; Fig. 5) even though data from Kawaguchi

et al. [21] indicated that higher proportions of t18:1 (Z) were positively correlated with freezing tolerance in grapevine leaves. On the other hand, the 2-hydroxy fatty acid composition was different in oat and rye during CA (Fig. 6). In both oat and rye, 24h:0 and 24h:1 were the major 2-hydroxy fatty acid species. When comparing the PM and DRMs under NA conditions, the 2-hydroxy fatty acid composition in oat DRMs was similar to that in the oat PM. In rye, DRMs contained more 24h:0 and less 24h:1 than the PM. Although the total GlcCer proportions in rye did not differ between the PM and DRMs (Table 2), rye DRMs were enriched in 24h:0-containing GlcCer, which has a relatively higher melting point than 24h:1. During CA, there were no notable changes of 2-hydroxy fatty acid composition in the oat and rye PMs or oat DRMs. Only rye DRMs showed CA-induced changes in 2-hydroxy fatty acid composition (37.8% to 29.9% for 24h:0 and 29.0% to 41.5% for 24h:1). Similar to the acyl chain changes in ASGs, these changes may increase the unsaturation index of fatty acids and affect the physical properties of microdomains in the PM under freezing conditions. Therefore, the 2-hydroxy fatty acid composition of microdomains and its changes during CA may be involved in the higher freezing tolerance in rye.

In addition, sphingolipids have numerous other functions in plant cells. A sphingolipid metabolite, sphingosine-1-phosphate, is responsive to drought-induced signal transduction in guard cells [33,50] and sphingolipid fatty acid 2-hydroxylase (FAH) in *Arabidopsis* is required

504 for oxidative stress responses [32]. The relationship between the 2-hydroxy fatty acid
505 composition in microdomains and many signaling pathways derived from sphingolipids may be
506 involved in the CA mechanism and the acquisition of freezing tolerance. In fact, transient
507 formation of phytosphingosine phosphate is involved in cold response via nitric oxide and
508 AtMPK6 in *Arabidopsis* [5,10].

510 When looking at the composition of PL species in Fig. 7, PC and PA were the two
511 most abundant PL species. However, Uemura and Steponkus reported that PC and PE were the
512 predominant PL species in oat and rye PMs [44]. This difference may have been caused by
513 endogenous phospholipase D (PLD) activity during the PM preparation process. PLD has the
514 ability to produce PA mainly from PC and PE and some PLDs such as PLD γ 1 and PLD δ prefer
515 PE rather than PC [25]. These PLDs require micromolar levels of Ca²⁺ for PA-producing
516 activity and are located in intracellular membranes and the PM [25]. Although we added EGTA
517 to chelate Ca²⁺ and performed PM preparation on ice to repress PLD activity, PL analysis is a
518 long process from PM preparation to PL extraction/quantification. During this process, PE may
519 have been degraded and changed to PA by PLD activity. Nevertheless, PC is still the
520 predominant PL species in both the PM and DRMs (Fig. 7). A previous study of tobacco leaves
521 and maize BY-2 cells revealed that PC is one of the major PL species in both the PM and DRMs
522 [12]. Therefore, PC is universal in monocotyledonous and dicotyledonous plants as the
523 predominant PL in the PM and microdomains. In human cells, extracellular leaflets of the PM
524 are considered to be enriched in sphingomyelin and cytoplasmic leaflets are composed of PC,
525 PE and PS as the major components of lipid rafts [22].

526 PLs containing double or triple unsaturated fatty acids were abundant in both the PM

and DRMs (Fig. 8A). A previous study [44] found that 16:0/18:2 and 16:0/18:3 fatty acid pairs were the major combinations in PC and PE. The present study revealed that PC (34:2) and PC (34:3) were abundant PLs in both oat and rye, accounting for 16.8% and 11.1% of the total PLs, respectively, in the PM fraction prepared from NA oat (Supplemental Table 3). Therefore, the fatty acid composition profile in our study is in agreement with the previous study.

In the PM, PC (36:4) and PC (36:5) accounted for large proportions as major PL species in both NA oat and rye (e.g. 10.9% for PC [36:4] and 8.8% for PC [36:5] in the oat NA PM; Supplemental Table 3). In DRM factions, however, these two PLs were much less abundant (less than 5%) and CA treatment increased their proportions (e.g. 1.63-fold for PC [36:4] and 2.47-fold for PC [36:5] in oat; Supplemental Table 3). Phase transitions of membranes can be promoted by saturated fatty acids at low temperature [16,51] and microdomain-enriched DRMs contained relatively larger proportions of saturated fatty acids than the PM (Fig. 8B). Although saturated fatty acids tend to be associated with sterols and other microdomain components [39,53], plants may be able to decrease saturated fatty acids in microdomain by increase the availability of unsaturated PLs such as PC (36:4) and PC (36:5) in PM during CA in order to avoid phase transitions in microdomains under low temperature. The overall unsaturation index of PLs, in fact, increased in the oat and rye PM and oat DRMs during CA in agreement with the relationship between the physicochemical properties of unsaturated PLs and membrane stability

at low temperature (Fig. 8B). In rye DRMs, however, the mean unsaturation index of PLs did not change during CA. Rye DRMs were rich in FS, which is less effective for freeze-induced phase transitions in membranes than ASG [46], and even the acyl chain of ASGs in rye was relatively enriched in unsaturated species in comparison with oat (Figs. 2 and 4C). Considering these factors in oat and rye, sterol compositions favorable for PM stability at freezing temperatures may be necessary to maintain and/or change physical property and function of microdomain toward acquisition of freezing tolerance during CA.

Supporting information

Supplemental Table 1. Sterol compositions in oat and rye during CA.

Supplemental Table 2. GlcCer compositions in oat and rye during CA.

Supplemental Table 3. PLs compositions in oat and rye during CA.

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References

- [1] M. Bagnat, A. Chang, K. Simons, Plasma membrane proton ATPase Pma1p requires raft association for surface delivery in yeast, *Mol. Biol. Cell.* 12 (2001) 4129–4138.
- [2] J.G. Beck, D. Mathieu, C. Loudet, S. Buchoux, E.J. Dufourc, Plant sterols in “rafts”: a better way to regulate membrane thermal shocks, *FASEB J.* 21 (2007) 1714–1723.
- [3] E.G. Bligh, W.J. Dyer, A rapid method of total lipid extraction and purification, *Can. J. Biochem. Physiol.* 37 (1959) 911–917.
- [4] G.H.H. Borner, D.J. Sherrier, T. Weimar, L.V. Michaelson, N.D. Hawkins, A. MacAskill, J.A. Napier, M.H. Beale, K.S. Lilley, P. Dupree, Analysis of detergent-resistant membranes in *Arabidopsis*: evidence for plasma membrane lipid rafts, *Plant Physiol.* 137 (2005) 104–116.
- [5] C. Cantrel, T. Vazquez, J. Puyaubert, N. Rezé, M. Lesch, W.M. Kaiser, C. Dutilleul, I. Guillas, A. Zachowski, E. Baudouin, Nitric oxide participates in cold-responsive phosphosphingolipid formation and gene expression in *Arabidopsis thaliana*, *New Phytol.* 189 (2011) 415–427.
- [6] L. Carmona-Salazar, M. El Hafidi, C. Enríquez-Arredondo, C. Vázquez-Vázquez, L.E. González de la Vara, M. Gavilanes-Ruíz, Isolation of detergent-resistant membranes from plant photosynthetic and non-photosynthetic tissues, *Anal. Biochem.* 417 (2011) 220–227.

- 592 [7] L. Carmona-Salazar, M. El Hafidi, N. Gutiérrez-Nájera, L. Noyola-Martínez, A.
593 González-Solís, M. Gavilanes-Ruíz, Fatty acid profiles from the plasma membrane and
594 detergent resistant membranes of two plant species, *Phytochemistry*. 109 (2015) 25–35.
- 595 [8] A.D. Douglass, R.D. Vale, Single-molecule microscopy reveals plasma membrane
596 microdomains created by protein-protein networks that exclude or trap signaling
597 molecules in T cells, *Cell*. 121 (2005) 937–950.
- 598 [9] M. DuBois, K.A. Gilles, J.K. Hamilton, P.A. Rebers, F. Smith, Colorimetric method for
599 determination of sugars and related substances, *Anal. Chem.* 28 (1956) 350–356.
- 600 [10] C. Dutilleul, G. Benhassaine-Kesri, C. Demandre, N. Rézé, A. Launay, S. Pelletier, J.-P.
601 Renou, A. Zachowski, E. Baudouin, I. Guillas, Phytosphingosine-phosphate is a signal for
602 AtMPK6 activation and *Arabidopsis* response to chilling, *New Phytol.* 194 (2012)
603 181–191.
- 604 [11] T. Friedrichson, T.V. Kurzchalia, Microdomains of GPI-anchored proteins in living cells
605 revealed by crosslinking, *Nature*. 394 (1998) 802–805.
- 606 [12] F. Furt, S. König, J.J. Bessoule, F. Sargueil, R. Zallot, T. Stanislas, E. Noirot, J. Lherminier,
607 F. Simon-Plas, I. Heilmann, S. Mongrand, Polyphosphoinositides are enriched in plant
608 membrane rafts and form microdomains in the plasma membrane, *Plant Physiol.* 152
609 (2010) 2173–2187.

- 610 [13] W.J. Gordon-Kamm, P.L. Steponkus, Lamellar-to-hexagonal_H phase transitions in the
611 plasma membrane of isolated protoplasts after freeze-induced dehydration, Proc. Natl.
612 Acad. Sci. U. S. A. 81 (1984) 6373–6377.
- 613 [14] A. Grandmougin-Ferjani, I. Schuler-Muller, M.-A. Hartmann, Sterol modulation of the
614 plasma membrane H⁺-ATPase activity from corn roots reconstituted into soybean lipids,
615 Plant Physiol. 113 (1997) 163–174.
- 616 [15] J.M. Holopainen, M. Subramanian, P.K.J. Kinnunen, Sphingomyelinase induces lipid
617 microdomain formation in a fluid phosphatidylcholine/sphingomyelin membrane,
618 Biochemistry. 37 (1998) 17562–17570.
- 619 [16] K. Iba, Acclimative response to temperature stress in higher plants: approaches of gene
620 engineering for temperature tolerance, Annu. Rev. Plant Biol. 53 (2002) 225–245.
- 621 [17] H. Imai, H. Hattori, M. Watanabe, An improved method for analysis of glucosylceramide
622 species having cis-8 and trans-8 isomers of sphingoid bases by LC–MS/MS, Lipids. 47
623 (2012) 1221–1229.
- 624 [18] H. Imai, Y. Morimoto, K. Tamura, Sphingoid base composition of monoglucosylceramide
625 in *Brassicaceae*, J. Plant Physiol. 157 (2000) 453–456.
- 626 [19] H. Imai, K. Yamamoto, A. Shibahara, S. Miyatani, T. Nakayama, Determining
627 double-bond positions in monoenoic 2-hydroxy fatty acids of glucosylceramides by gas

628 chromatography-mass spectrometry, *Lipids*. 35 (2000) 233–236.

629 [20] M. Ishikawa, S. Yoshida, Seasonal changes in plasma membranes and mitochondria
630 isolated from Jerusalem artichoke tubers: possible relationship to cold hardiness, *Plant*
631 *Cell Physiol.* 26 (1985) 1331–1344.

632 [21] M. Kawaguchi, H. Imai, M. Naoe, Y. Yasui, M. Ohnishi, Cerebrosides in grapevine leaves:
633 distinct composition of sphingoid bases among the grapevine species having different
634 tolerances to freezing temperature, *Biosci. Biotechnol. Biochem.* 64 (2000) 1271–1273.

635 [22] V. Kiessling, C. Wan, L.K. Tamm, Domain coupling in asymmetric lipid bilayers, *Biochim.*
636 *Biophys. Acta.* 1788 (2009) 64–71.

637 [23] M. Laloi, A.-M. Perret, L. Chatre, S. Melser, C. Cantrel, M.-N. Vaultier, A. Zachowski, K.
638 Bathany, J.-M. Schmitter, M. Vallet, R. Lessire, M.-A. Hartmann, P. Moreau, Insights into
639 the role of specific lipids in the formation and delivery of lipid microdomains to the
640 plasma membrane of plant cells, *Plant Physiol.* 143 (2006) 461–472.

641 [24] J. Lee, R. Welti, M. Roth, W.T. Schapaugh, J. Li, H.N. Trick, Enhanced seed viability and
642 lipid compositional changes during natural ageing by suppressing phospholipase D α in
643 soybean seed, *Plant Biotechnol. J.* 10 (2012) 164–173.

644 [25] M. Li, Y. Hong, X. Wang, Phospholipase D- and phosphatidic acid-mediated signaling in
645 plants, *Biochim. Biophys. Acta.* 1791 (2009) 927–935.

646 [26] D. Lingwood, K. Simons, Detergent resistance as a tool in membrane research, Nat.
647 Protoc. 2 (2007) 2159–2165.

648 [27] D. Lingwood, K. Simons, Lipid rafts as a membrane-organizing principle, Science. 327
649 (2010) 46–50.

650 [28] D.V. Lynch, P.L. Steponkus, Plasma membrane lipid alterations associated with cold
651 acclimation of winter rye seedlings (*Secale cereale* L. cv Puma), Plant Physiol. 83 (1987)
652 761–767.

653 [29] G.V. Marinetti, Chromatographic separation, identification and analysis of phosphatides, J.
654 Lipid Res. 3 (1962) 1–12.

655 [30] A. Minami, M. Fujiwara, A. Furuto, Y. Fukao, T. Yamashita, M. Kamo, Y. Kawamura, M.
656 Uemura, Alterations in detergent-resistant plasma membrane microdomains in
657 *Arabidopsis thaliana* during cold acclimation, Plant Cell Physiol. 50 (2009) 341–359.

658 [31] S. Mongrand, J. Morel, J. Laroche, S. Claverol, J.-P. Carde, M.-A. Hartmann, P. Moreau,
659 Lipid rafts in higher plant cells: purification and characterization of Triton
660 X-100-insoluble microdomains from tobacco plasma membrane, J. Biol. Chem. 279
661 (2004) 36277–36286.

662 [32] M. Nagano, K. Takahara, M. Fujimoto, N. Tsutsumi, H. Uchimiya, M. Kawai-Yamada,
663 *Arabidopsis* sphingolipid fatty acid 2-hydroxylases (AtFAH1 and AtFAH2) are

664 functionally differentiated in fatty acid 2-hydroxylation and stress responses, *Plant Physiol.*
 665 159 (2012) 1138–1148.

666 [33] C.K. Ng, K. Carr, M.R. McAinsh, B. Powell, A.M. Hetherington, Drought-induced guard
 667 cell signal transduction involves sphingosine-1-phosphate, *Nature*. 410 (2001) 596–599.

668 [34] T. Peskan, M. Westermann, R. Oelmüller, Identification of low-density Triton
 669 X-100-insoluble plasma membrane microdomains in higher plants, *Eur. J. Biochem.* 267
 670 (2000) 6989–6995.

671 [35] M.B. Sankaram, T.E. Thompson, Interaction of cholesterol with various
 672 glycerophospholipids and sphingomyelin, *Biochemistry*. 29 (1990) 10670–10675.

673 [36] K. Schrick, S. Shiva, J.C. Arpin, N. Delimont, G. Isaac, P. Tamura, R. Welti., Steryl
 674 glucoside and acyl steryl glucoside analysis of *Arabidopsis* seeds by electrospray
 675 ionization tandem mass spectrometry, *Lipids*. 47 (2012) 185–193.

676 [37] J.R. Silvius, Cholesterol modulation of lipid intermixing in phospholipid and
 677 glycosphingolipid mixtures. Evaluation using fluorescent lipid probes and brominated
 678 lipid quenchers, *Biochemistry*. 31 (1992) 3398–3408.

679 [38] K. Simons, E. Ikonen, Functional rafts in cell membranes, *Nature*. 387 (1997) 569–572.

680 [39] K. Simons, J.L. Sampaio, Membrane organization and lipid rafts, *Cold Spring Harb.*
 681 *Perspect. Biol.* 3 (2011) a004697–a004697.

- 682 [40] S.J. Singer, G.L. Nicolson, The fluid mosaic model of the structure of cell membranes,
683 Science. 175 (1972) 720–731.
- 684 [41] P.L. Steponkus, Role of the plasma membrane in freezing injury and cold acclimation,
685 Annu. Rev. Plant Physiol. 35 (1984) 543–584.
- 686 [42] D. Takahashi, Y. Kawamura, M. Uemura, Changes of detergent-resistant plasma
687 membrane proteins in oat and rye during cold acclimation: association with differential
688 freezing tolerance, J. Proteome Res. 12 (2013) 4998–5011.
- 689 [43] D. Takahashi, Y. Kawamura, T. Yamashita, M. Uemura, Detergent-resistant plasma
690 membrane proteome in oat and rye: similarities and dissimilarities between two
691 monocotyledonous plants, J. Proteome Res. 11 (2012) 1654–1665.
- 692 [44] M. Uemura, P.L. Steponkus, A contrast of the plasma membrane lipid composition of oat
693 and rye leaves in relation to freezing tolerance, Plant Physiol. 104 (1994) 479–496.
- 694 [45] M. Watanabe, A. Miyagi, M. Nagano, M. Kawai-Yamada, H. Imai, Characterization of
695 glucosylceramides in the *Polygonaceae*, *Rumex obtusifolius* L. injurious weed, Biosci.
696 Biotechnol. Biochem. 75 (2011) 877–881.
- 697 [46] M.S. Webb, T.C. Irving, P.L. Steponkus, Effects of plant sterols on the hydration and
698 phase behavior of DOPE/DOPC mixtures, Biochim. Biophys. Acta. 1239 (1995) 226–238.
- 699 [47] M.S. Webb, T.C. Irving, P.L. Steponkus, Cerebrosides alter the lyotropic and thermotropic

700 phase transitions of DOPE:DOPC and DOPE:DOPC:sterol mixtures, *Biochim. Biophys.*
701 *Acta.* 1326 (1997) 225–235.

702 [48] M.S. Webb, M. Uemura, P.L. Steponkus, A comparison of freezing injury in oat and rye:
703 two cereals at the extremes of freezing tolerance, *Plant Physiol.* 104 (1994) 467–478.

704 [49] R. Welti, W. Li, M. Li, Y. Sang, H. Biesiada, H.-E. Zhou, C.B. Rajashekar, T.D. Williams,
705 X. Wang, Profiling membrane lipids in plant stress responses: role of phospholipase D α in
706 freezing-induced lipid changes in *Arabidopsis*, *J. Biol. Chem.* 277 (2002) 31994–32002.

707 [50] D. Worrall, C.K.-Y. Ng, A.M. Hetherington, Sphingolipids, new players in plant signaling,
708 *Trends Plant Sci.* 8 (2003) 317–320.

709 [51] J. Wu, J. Browse, Elevated levels of high-melting-point phosphatidylglycerols do not
710 induce chilling sensitivity in an *Arabidopsis* mutant, *Plant Cell.* 7 (1995) 17–27.

711 [52] S. Xiao, W. Gao, Q.-F. Chen, S.-W. Chan, S.-X. Zheng, J. Ma, M. Wang, R. Welti, M.-L.
712 Chye, Overexpression of *Arabidopsis* acyl-CoA binding protein ACBP3 promotes
713 starvation-induced and age-dependent leaf senescence, *Plant Cell.* 22 (2010) 1463–1482.

714 [53] X. Xu, R. Bittman, G. Duportail, D. Heissler, C. Vilcheze, E. London, Effect of the
715 structure of natural sterols and sphingolipids on the formation of ordered
716 sphingolipid/sterol domains (rafts): comparison of cholesterol to plant, fungal, and
717 disease-associated sterols and comparison of sphingomyelin, cerebroside, and ceramide, *J.*

718 Biol. Chem. 276 (2001) 33540–33546.

719 [54] D.A. Zacharias, J.D. Violin, A.C. Newton, R.Y. Tsien, Partitioning of lipid-modified
720 monomeric GFPs into membrane microdomains of live cells, Science. 296 (2002)
721 913–916.

722 [55] A. Zlatkis, B. Zak, Study of a new cholesterol reagent, Anal. Biochem. 29 (1969)
723 143–148.

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Figure captions

Fig. 1. TLC patterns of PM and DRM fractions in oat and rye. PM and DRM fractions isolated from oat and rye leaves were examined by TLC with developing solvent composed by chloroform/methanol/water (65:25:4, v/v/v). FS, free sterol; ASG, acylated sterylglucoside; SG, sterylglucoside; GlcCer, glucocerebroside; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PA, phosphatidic acid; PC, phosphatidylcholine; PS, phosphatidylserine; PI, phosphatidylinositol.

Fig. 2. Sterol compositions of PM and DRM fractions in oat and rye. Sterol compositions of PM and DRM were quantified and Y-axis of the graph is mol% of each sterol in the total sterol content. The content of each group was determined colorimetrically after TLC, as shown in Fig. 1. Error bars indicate standard deviations (n=3-5). FS, free sterol; ASG, acylated sterylglucoside; SG, sterylglucoside.

Fig. 3. Molecular species compositions of FS in PM and DRM fractions. Molecular species compositions of FS in PM and DRM were quantified by GC analysis. Y-axis of the graph is proportions of signals derived from each sterol species in total FS signals. Error bars indicate

standard deviations (n=4-8).

Fig. 4. Sterol and fatty acyl compositions of SG and ASG in PM and DRM fractions. SG

and ASG in PM and DRM were quantified by direct infusion MS/MS analysis. The Y-axis of the graph represents the proportion of signals derived from each sterol or acyl species in the total SG or ASG signals. Error bars indicate standard deviations (n=4). Molecular species compositions of the sterol part of SG and ASG are shown in **A** and **B**, respectively. **C** shows molecular species compositions of acyl chains of ASG.

Fig. 5. Compositions of C18 sphingoid bases of GlcCer in PM and DRM fractions.

Compositions of C18 sphingoid bases of GlcCer in PM and DRM were quantified by LC-MS/MS analysis. Y-axis of the graph indicates proportions of signals derived from each sphingoid base species in total GlcCer signals. Error bars indicate standard deviations (n=3). **Z** and **E** mean cis-8 and trans-8 isomers, respectively. d18:1, 8-sphingadienine; d18:2, 4,8-sphingadienine; t18:1, 4-hydroxy-8-sphingenine.

Fig. 6. Compositions of 2-hydroxy fatty acids of GlcCer in PM and DRM fractions.

Compositions of 2-hydroxy fatty acids of GlcCer in PM and DRM were quantified by LC-MS/MS analysis. Y-axis of the graph indicates proportions of signals derived from each 2-hydroxy fatty acid in total GlcCer signals. Error bars indicate standard deviations (n=3).

Fig. 7. PL classes of PM and DRM fractions in oat and rye. PL compositions of PM and DRM were quantified by direct infusion MS/MS analysis and the Y-axis of the graph represents proportions of signals derived from each PL class in total PL signals. Error bars indicate standard deviations (n=4). PA, phosphatidic acid; PS, phosphatidylserine; PI, phosphatidylinositol; PE, phosphatidylethanolamine; PC, phosphatidylcholine; LysoPE, lysophosphatidylethanolamine; LysoPC, lysophosphatidylcholine; LysoPG, lysophosphatidylglycerol; PG, phosphatidylglycerol.

Fig. 8. Unsaturation levels of fatty acid in PLs in PM and DRM fractions. (A) PL molecules were categorized based on the sum of unsaturated bonds in two acyl chains. (B) The Mean unsaturation index was calculated for PM and DRM fractions in oat and rye. Error bars indicate standard deviations (n=4). * $p < 0.01$ by student's t -test.

Table 1

Overall compositions of lipid classes in oat PM and DRM. The results are mol% calculated as (mole of each lipid classes)/(mole of total lipids). The value of nmol lipid/100 µg protein is indicated in parentheses. Each value is the mean ± SD (n=3-5). **p*<0.05 by student's *t*-test comparing NA and CA. FS, free sterol; ASG, acylated sterylglucoside; SG, sterylglucoside; GlcCer, glucocerebroside; PLs, phospholipids.

Lipid	PM		DRM	
	NA (n=3)	CA (n=4)	NA (n=3)	CA (n=5)
Sterols	42.6 ± 3.1 (96 ± 23)	37.7 ± 2.5 (70 ± 10)	73.6 ± 3.8 (175 ± 37)	66.0 ± 3.7* (188 ± 28)
FS	10.2 ± 2.5 (22 ± 4)	7.4 ± 0.7* (14 ± 1*)	21.1 ± 2.5 (50 ± 6)	14.4 ± 2.8* (40 ± 6)
ASG	28.5 ± 2.2 (65 ± 18)	24.8 ± 2.4 (46 ± 8)	50.3 ± 4.2 (121 ± 32)	48.7 ± 2.6 (139 ± 25)
SG	3.9 ± 1.2 (9 ± 4)	5.6 ± 1.1 (10 ± 2)	2.2 ± 0.8 (5 ± 1)	2.9 ± 0.7 (8 ± 3)
GlcCer	15.5 ± 0.7 (35 ± 8)	12.6 ± 0.9* (24 ± 6)	15.0 ± 4.2 (35 ± 10)	18.3 ± 4.7 (53 ± 22)
PLs	41.9 ± 1.2 (94 ± 22)	49.7 ± 2.2* (94 ± 19)	11.4 ± 1.0 (28 ± 11)	15.7 ± 0.8* (46 ± 12)
Total	100 (225 ± 41)	100 (188 ± 29)	100 (238 ± 42)	100 (287 ± 48)

Table 2

Overall compositions of lipid classes in rye PM and DRM. The results are mol% calculated as (mole of each lipid classes)/(mole of total lipids). The value of nmol lipid/100 µg protein is indicated in parentheses. Each value is the mean ± SD (n=3-5). **p*<0.05 by student's *t*-test comparing NA and CA. FS, free sterol; ASG, acylated sterylglycoside; SG, sterylglycoside; GlcCer, glucocerebroside; PLs, phospholipids.

Lipid	PM		DRM	
	NA (n=4)	CA (n=3)	NA (n=4)	CA (n=5)
Sterols	39.2±2.1 (105±16)	40.1±1.8 (122±42)	63.8±4.6 (156±64)	64.5±3.3 (190±37)
FS	32.9±1.9 (87±13)	33.8±5.0 (100±22)	50.5±2.4 (123±48)	51.7±4.1 (153±36)
ASG	2.9±1.5 (8±4)	2.9±2.3 (10±11)	9.6±1.1 (24±10)	5.8±2.3* (16±4)
SG	3.2±1.1 (10±4)	3.4±2.5 (12±12)	3.7±1.5 (9±6)	6.9±1.2* (20±6*)
GlcCer	13.4±1.5 (34±2)	7.8±1.8* (25±14)	15.6±3.7 (37±17)	14.4±3.1 (42±11)
PLs	48.0±0.9 (128±17)	52.1±0.7* (158±52)	20.6±1.0 (48±15)	21.2±3.0 (64±29)
Total	100 (267±28)	100 (305±87)	100 (241±79)	100 (296±61)

Figure(s)

Fig. 1.

Fig. 2 .

Fig. 3 .

Fig. 4.

Fig. 5.

Fig. 6 .

Fig. 7 .

Fig. 8.