The Chemical Composition of Membrane Lipids in Acclimation to Chilling of Squash (*Cucurbita moschata* Duch. cv Shirogikuza) Seedlings

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(Abstract)

Chilling-tolerance of squash (*Cucurbita moschata* Duch. cv Shirogikuza) cotyledons was measured after 3 d of cold acclimation at 12°C. Squash seedlings grown at 12°C (chilling-treated, CT) and 25°C (non-treated, NT) were analyzed to examine whether the chilling treatment affected lipid composition and content. A slight decrease of sterol lipid/glycerophospholipid ratio was observed in CT seedlings, and although the levels of unsaturated fatty acids in phosphatidylglycerol were not significantly different between the two seedlings, the level of 3-*E*-hexadecenoic acid increased slightly in CT seedlings. In phosphatidylcholine, the level of linolenic acid slightly increased in the CT seedlings. In ceramide monohexoside, the levels of 8-*Z*- and 8-*E*-stereoisomerism of 4-hydroxy sphingoid bases were essentially the same between the two seedlings, while a slightly increased level of 2-hydroxy palmitic acid was observed in CT seedlings. The involvement of lipids in the change to chilling sensitivity is discussed in this experiment.

Key words: ceramide monohexoside; chilling acclimation; *Cucurbita*; phosphatidylcholine; phosphatidylglycerol

Abbreviations: CMH, ceramide monohexoside; CT, chilling-treated; GC, gas chromatography; GL, glyceroglycolipid; HPLC, high-pressure liquid chromatography; LHCII, light harvesting chlorophyll *a/b* protein associated with photosystem II; NT, non-treated; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PL, glycerophospholipid; SL, sterol lipid; TLC, thin-layer chromatography. For sphingoid bases: d18:2(4*E*,8*E*) or (4*E*,8*Z*), 4-*E*, 8-*E*- or 4-*E*, 8-*Z*-sphingadienine; t18:1(8*E*) or (8*Z*), 4-hydroxy-8-*E*- or 4-hydroxy-8-*Z*-sphingenine. Nomenclature for fatty acids: X:Y or Xh:Y, normal or 2-hydroxy fatty acid containing X carbon atoms with Y double bonds.

Introduction

When cultivating greenhouse vegetable crops, chilling temperature (0–15°C) is a strong stress that causes plants to exhibit a variety of physiological dysfunctions. For this reason, studies on acclimation to chilling are very important for ensuring crop quality. Whereas there are many reports that chilling acclimation involves a number of changes in plant cellular metabolism,[1,2] few studies have centered on the changes in chilling acclimation of chilling-sensitive vegetable crops cultivated in greenhouses. In this experiment, we have characterized such a chilling-inducible phenomenon into a typical genotype of chilling sensitive squash (*Cucurbita moschata* Duch. cv Shirogikuza). The squash cultivar, Shirogikuza, is resistant to harmful insects, such as *Melitta satyriniformis* and *Anasa tristis*, and to soil pathogens. In addition, Shirogikuza is more resistant to high humidity and temperature than a number of lines of *C. maxima*. Shirogikuza is therefore used as the rootstock of several crops of Cucurbitaceae in Japan.

The thermotropic lipid phase-transition hypothesis, i.e. the primary event in chilling injury is the formation of a phase separation of non-fluid membrane components, was proposed by Lyons[3] and Raison.[4] This hypothesis has led to correlations between chilling sensitivity of plants and the composition of membrane lipids being studied in many plants.[5-7] It was suggested that ceramide monohexoside (CMH) may act in plants' physiological reaction to low temperature, a response noted in the chilling sensitivity of mung bean hypocotyls [8] and the cryostability of rye plasma membranes.[9] CMH, a representative sphingolipid in plants, is localized in plasma membranes and tonoplasts as a major lipid class.[10] We have shown that the structure of the sphingoid bases of plant sphingolipids is more complicated than that of animal sphingolipids[11] and found, using differential scanning calorimetry, that CMH species having 8-*Z*-unsaturated sphingoid bases have a lower phase transition temperature than those having 8-*E*-forms.[12] Continuing this research, we previously speculated that the degree of 8-*Z*-unsaturation of the sphingoid base would be different between chilling-resistant and chilling-sensitive plants. Although we recently discovered that the degree of 8-*Z*-unsaturation in leaf CMHs was not correlated with the chilling sensitivity of higher plants, chilling-resistant plants had more t18:1(8*Z*) components than t18:1(8*E*).[7]

It was reported that chilling sensitivity is correlated with the extent of fatty acid unsaturation of phosphatidylglycerol (PG) of chloroplast membranes in genetically engineered tobacco plants.[13] In that study, a gene for glycerol-3-phosphate acyltransferase from the squash genotype, Shirogikuza, was analyzed as a representative sample of chilling-sensitive plants.

We found that cotyledons of the Shirogikuza seedlings were severely affected by chilling treatments for 2 d, which resulted in known chilling injury symptoms after 2 d of post-chilling recovery at 25°C. However, the level of injury to the cotyledons treated at 7°C was significantly lower when the seedlings were pre-treated at a moderate chilling

temperature of 12°C for 3 d. These observations were different to those noted on the primary leaves. We also examined the lipid and fatty acid composition of CT and NT seedlings to determine if the observed changes in the chilling sensitivity of the cotyledons and the primary leaves were related to alterations in membrane lipid composition and content.

Materials and Methods

Plant material and cultivation of the seedlings: Squash seeds were obtained from Watanabe Saishujo Co. (Miyagi Prefecture, Japan) and stored at 4°C. The seeds were then germinated in moist vermiculite and grown for 12 d in a controlled environment at 25°C under continuous fluorescent illumination (20 μE m^{-2} s^{-1}). NT seedlings were maintained in this environment for an additional 3 d prior to the extraction of lipids. CT seedlings were treated by transferring them to 12°C for 3 d under the same illumination. Cotyledons and primary leaves were cut at the bases and immediately dipped in boiling isopropanol.

Visual assessment of chilling injury: All of the following procedures were performed under continuous illumination at 20 μE m^{-2} s^{-1}. Plants were germinated at 25°C and grown at a standard growth condition as described above for 12 d. Seedlings were divided into four groups of 30–40 plants each. One set of seedlings was transferred to 3°C for 2 d, and the second set transferred to 12°C for 3 d before undergoing the cold treatment of 3°C for 2 d. The third set was transferred to 7°C for 2 d and the fourth set transferred to 12°C for 3 d before undergoing the cold treatment at 7°C for 2 d. Each set of seedlings was visually assessed for chilling injury symptoms after 2 d of post-chilling recovery at 25°C. The evaluation of plant injury was based on total deterioration of the cotyledons or primary leaves.

Extraction and fractionation of lipids: Lipids were extracted according to the method of Bligh and Dyer.[14] Total lipids were separated by ion-exchange column chromatography on DEAE-Sepharose CL-6B (Amersham Pharmacia Biotech), column chromatography on silica gel (Wakogel C-200, Wako Pure Chemical Industries, Osaka) and thin-layer chromatography (TLC) with precoated silica gel plates (Wakogel B-10) as described previously.[15] Experimental procedures for isolation and analyses of CMH were undertaken using the method of Ohnishi et al.[16]

Quantification of lipids: Acyl lipids were separated from total lipids by two-dimensional TLC on a precoated silica gel plate. A solvent system of chloroform:methanol:water (65:25:4, by volume) was used in the first development, and chloroform:acetone:methanol:acetic acid:water (10:4:2:2:1, by volume) was used in the

second. Separated lipids were visualized on the plates in UV light after spraying with 0.001% (w/v) of primuline in 80% (v/v) acetone, and the spots of individual acyl lipids scraped off. Absolute amounts of fatty acids were estimated using a defined amount of pentadecanoic acid as an internal standard. Component sterol content of sterol lipids was assayed using cholestane as a standard[17], and sugar content of glyceroglycolipid (GL) and CMH quantified using mannitol as a standard.[16]

Analysis of fatty acids: Phospholipid samples were subjected to methanolysis to obtain fatty acid methyl esters. These products were analyzed with a Shimadzu GC-9A gas chromatograph equipped with a hydrogen flame-ionization detector. The fatty acid methyl esters were separated on a fused silica capillary column (0.25 mm × 50 m) coated with nitorile silicone (ULBON HR-SS-10, Shinwa Chemical Industries, Japan). The column temperature was programmed from 40–60°C at 10°C min^{-1} and from 140–200°C at 2°C min^{-1}. Injector and detector temperatures were maintained at 250°C. Fatty acid methyl esters were identified by comparing their retention times with those of reference standards. Diacyglycerol species composition of phosphatidylcholine (PC) and PG was analyzed by selective hydrolysis at the C-3 position of sn-glycerol moiety with phospholipase C. The resulting 1, 2-diacylglycerols were converted to 2, 4-dinitrobenzoyl (DNB) derivatives using DNB-chloride.[18] Their products were analyzed using reversed-phase high-pressure liquid chromatography (HPLC) according to the previously described method.[19]

CMH was methanolyzed with 3% (w/v) HCl (gaseous) in dry methanol at 100° C for 3 h to analyze the component 2-hydroxy fatty acids. After adding water, the resultant total fatty acid methyl esters were extracted with n-hexane, dried under nitrogen, then separated into the 2-hydroxy and the non-hydroxy fatty acid fractions by silica gel TLC using n-hexane:diethyl ether (17:3, v/v) as a mobile phase. 2-Hydroxy fatty acid methyl esters were converted to trimethylsilyl (TMS) ether derivatives, then analyzed by gas chromatography (GC) (model 163, Hitachi, Tokyo, Japan) equipped with a hydrogen flame ionization detector and a glass column (2 m × 3 mm; internal diameter) packed with 3% SE-30 on Chromosorb WAW-DMCS. The column temperature was programmed from 180–290°C at 2°C min^{-1}. 2-Hydroxy fatty acid methyl esters were identified by comparing their retention times with those for authentic standards.

Analysis of sphingoid bases: CMH was hydrolyzed with 1 M HCl in aqueous methanol at 70°C for 18 h [20] and the reaction mixture washed with *n*-hexane. The residue of the methanol phase was then adjusted to pH 9.6 with 6 N KOH. The component sphingoid bases were extracted with diethyl ether, evaporated under nitrogen and converted to fatty aldehydes by $NaIO_4$ oxidation. The resulting fatty aldehydes were analyzed by capillary GC under the same conditions as described above.[15] Fatty aldehydes were identified by comparing their retention times with those of authentic standards.

Results and Discussion

Injury and acclimation of seedlings at chilling temperatures: Chilling injury was measured in the cotyledons and the primary leaves of squash seedlings. The cotyledons displayed a notable deterioration when exposed to a temperature of 3°C (Fig. 1a). The levels of chilling damage were not reduced when the cotyledons were exposed to 12°C for 3 d before chilling at 3°C. In contrast, the level of injury was almost five-fold lower in cotyledons pre-treated at 12°C then exposed to 7°C compared with the levels in Non cotyledons. This result indicated that an acclimation to chilling at 7°C occurred in the cotyledons pre-treated at 12°C. On the other hand, the primary leaves exposed at 7°C showed similar low levels of chilling injury for both pre-treated and non-treated seedlings (Fig. 1b), which suggested that 7°C is not cool enough to cause much chilling damage to primary leaves. Although the four independent experiments in the seedlings pre-treated at 12°C showed a wide divergence of chilling injury levels, the levels were somewhat lower for pre-treated than non-treated primary leaves.

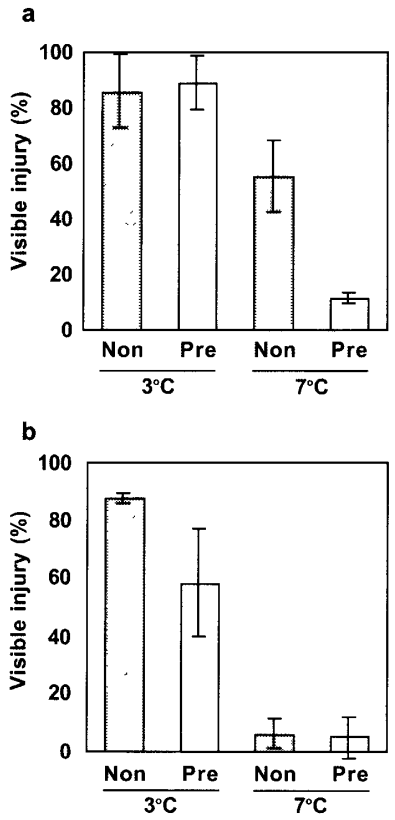

Fig. 1. Chilling-induced visible injury of cotyledons (a) and primary leaves (b) of squash seedlings.

Plants, grown at 25°C for 12 d, were transferred to 3 or 7°C for 2 d and returned to 25°C (Non). Plants, grown at 25°C for 12 d, were exposed to 12°C for 3 d, then transferred to 3 or 7°C for 2 d and returned to 25°C (Pre). Values for percent injury are the means ± SD of 30 to 40 seedlings (n=4).

Effect of chilling acclimation on lipid composition: In cotyledons grown at 25°C (NT), GL, glycerophospholipids (PL), and sterol lipids (SL) were major polar lipid components that amounted to 51%, 26% and 19%, respectively. CMH was a minor component comprising 4% of the total polar lipids (Table 1). Cotyledons grown at 12°C (CT) had slightly increased levels of PL and decreased levels of SL. This resulted in a slight decrease of the SL/PL ratio. Primary leaves grown at 25°C (NT) showed similar proportions of the polar lipids, amounting to 52%, 31%, 13% and 4%, in GL, PL, SL and CMH, respectively. The overall proportions of polar lipids in the primary leaves was not significantly altered by the chilling treatment at 12°C.

An increase in the level of PL and a decrease in the level of SL in membrane lipids during cold acclimation have been reported in many plants, such as oat and rye leaves [21], *Arabidopsis thaliana* leaves [22] and cucumber roots. [23] These changes in PL and SL levels result in a decreased SL/PL ratio, similar to the CT cotyledons of Shirogikuza. In addition, primary leaves grown at either 25°C or 12°C have lower SL/PL ratios than the ratio from the cotyledons grown at 12°C. These observations, together with our data that injury levels in the primary leaves grown at 7°C were very low in both pre-treated and non-treated seedlings (Fig. 1b), suggest that lowering the SL/PL ratio might be involved in chilling tolerance.

The levels of CMH in Shirogikuza cotyledons and primary leaves were not affected by a chilling treatment at 12°C for 3 d (Table 1). In contrast, decreased levels of CMH during cold acclimation are observed in several chilling-resistant plants. [21, 22, 24] It has been reported that the level of CMH in primary leaves of wheat seedlings changes significantly during the first four days of chilling acclimation [24], suggesting a specific role for CMH in initiating chilling tolerance. In squash cotyledons, an acclimation process by the chilling treatment would be associated with some factors other than the level of CMH.

Table 1 Lipid Composition (wt %) in Cotyledons and Primary Leaves of NT and CT Growing Seedlings of Squash

Lipid	Cotyledons		Primary Leaves	
	NT[a]	CT[b]	NT	CT
GL	51	51	52	51
PL	26	29	31	32
SL	19	16	13	13
CMH	4	4	4	4
SL/PL	0.73	0.55	0.42	0.41

[a] Non-treated seedlings. [b] Chilling-treated seedlings.

Effect of chilling acclimation on fatty acid composition in PL classes: The composition of fatty acids in PC, PE, and PG is shown in Table 2. In cotyledons, decreased proportions of 16:0 and 18:2 in PC of CT seedlings were balanced by an elevated proportion of 18:3. Accordingly, the estimated average number of double bonds per acyl chain (i.e., unsaturation index) increased. In PE of the CT seedlings, a lower level of 18:2, but not 16:0, was accompanied by a higher level of 18:3, whereas the unsaturation indices of PE in NT and CT seedlings were very similar. Although there were no clear distinctions in proportions of individual fatty acids of PC and PE between CT and NT seedlings, a slightly higher proportion of 18:3 was observed in the PC of CT seedlings. An increase in 18:3 has been observed in several plants exposed to chilling [25, 26], but the role of 18:3 content in extraplastidial PL observed in acclimated plants remains unclear. The transgenic tobacco plants, which contain increased levels of 18:3 through the introduction of a construct with ω-3 fatty acid desaturase gene, showed that an increase in the 18:3 level in PL is not directly involved in compensation for the diminution in growth or membrane properties observed under low temperatures. [27]

For PG in the cotyledons of CT seedlings, a decreased proportion of 16:0 was accompanied by an enhanced proportion of 16:1(3*E*). The level of 16:0 in CT primary leaves decreased and was somewhat compensated for an enhanced proportion of 16:1(3*E*). The data for CT seedlings, regarding a higher proportion of 16:1(3*E*) in PG, suggested that turnover of 16:0 unsaturation increased in both cotyledons and primary leaves of CT seedlings. Xu and Siegenthaler reported that low temperature treatments induce increases

Table 2 Fatty Acid Composition (mol%) of PC, PE, and PG in Cotyledons and Primary Leaves of NT and CT Growing Seedlings of Squash

Fatty Acid	PC				PE				PG			
	Cotyledons		Primary Leaves		Cotyledons		Primary Leaves		Cotyledons		Primary Leaves	
	NT[a]	CT[b]	NT	CT	NT	CT	NT	CT	NT	CT	NT	CT
16:0	36.9	34.2	33.3	32.5	41.3	40.6	40.6	40.6	72.2	67.7	64.8	60.9
16:1(3*E*)	0	0	0	0	0	0	0	0	15.5	19.7	18.4	22.8
18:0	4.6	3.8	4.7	4.1	2.7	3.4	2.4	1.8	6.4	5.5	5.1	7.1
18:1	0.8	1.2	3.2	1.9	1.0	1.4	1.8	1.1	2.1	2.2	2.6	1.3
18:2	12.4	9.1	13.7	12.4	15.1	10.6	12.0	11.9	1.5	2.0	3.1	1.8
18:3	45.3	51.7	45.1	49.1	39.9	43.0	43.2	44.6	2.3	2.9	6.0	6.1
UI[c]	1.62	1.75	1.66	1.74	1.51	1.52	1.54	1.59	0.12	0.15	0.27	0.23

[a] Non-treated seedlings. [b] Chilling-treated seedlings.
[c] Unsaturation index = [% of 18:1+(% of 18:2)×2+(% of 18:3)×3]/100.

in 16:1(*E*) and 18:3 levels in thylakoid membrane PG of squash cotyledons.[28] However, in this study, the content of C_{18} fatty acids in PG was not significantly different between NT and CT seedlings. On the other hand, a decreased proportion of 16:1(3*E*) in PG during cold acclimation has been observed in several plants. [29-31] Hunter et al. reported that low temperature induces a specific decrease in 16:1(3*E*) content, which influences light harvesting chlorophyll *a/b* protein associated with photosystem II (LHCII) organization in winter rye. [32] As the changes in extent of the *E*-unsaturation of 16:0 in the present case were very minor, it is unlikely that increased levels of 16:1(3*E*) are influenced to LHCII.

Effect of chilling acclimation on PL molecular species composition: Table 3 contains a comparison of the diacylglycerol species of PC and PG. In regards to PC, an increase in 18:3 was compensated for the enhanced proportion of 18:3/18:3 species higher in cotyledons than in primary leaves between NT and CT seedlings. In contrast, 16:0/18:3 was similar in the cotyledons and primary leaves between the two seedlings. In addition, the data indicated that the unsaturation of 18:2 is induced by chilling treatment to produce 18:3/18:3 and 16:0/18:3 from 18:3/18:2 and 16:0/18:2, respectively. In PG, the proportion of 16:0/16:1(3*E*) increased, and that of 16:0/16:0 decreased, in both cotyledons and primary leaves. The total levels of saturated and E-monounsaturated molecular species,

Table 3 Diacylglycerol Species Composition (mol%) of PC and PG in Cotyledons and Primary Leaves of NT and CT Growing Seedlings of Squash

Diacylglycerol Species [a]	PC				PG			
	Cotyledons		Primary Leaves		Cotyledons		Primary Leaves	
	NT[b]	CT[c]	NT	CT	NT	CT	NT	CT
18:3/18:3	14	22	14	18	0	0	0	0
18:2/18:3	11	9	11	9	0	0	0	0
18:2/18:2d	7	3	5	4	0	0	0	0
16:0/18:3	47	49	43	45	5	6	10	12
16:0/18:2	12	9	15	14	3	4	6	3
16:0/16:1(3*E*)	0	0	0	0	28	38	29	36
16:0/16:0	0	0	0	0	49	40	37	31
16:0/18:0	0	0	0	0	9	5	5	5
Others	9	8	12	10	6	7	13	13

[a] Positional isomers are not regarded. [b] Non-treated seedlings.
[c] Chilling-treated seedlings. [d] Including 18:1/18:3 species.

such as 16:0/16:0, 16:0/16:1(3*E*), and 16:0/18:0, in PG accounted for 86% and 71% in cotyledons and primary leaves of NT seedlings, respectively. In CT seedlings, the total levels of these molecular species in PG slightly decreased to 83% in cotyledons; the same levels as observed in the primary leaves.

Effect of chilling acclimation on sphingoid base and fatty acid compositions in CMH: The composition of 2-hydroxy fatty acids and sphingoid bases of CMH is shown in Table 4. Nine types of fatty acids with chain lengths from C_{16} to C_{26} were found, and 16h:0 and 24h:0 were major components in both the cotyledons and primary leaves. The proportion of 16h:0 was slightly higher in CT seedlings, and was balanced by a decreased proportion of 24h:0 in both organs. In sphingoid bases, the proportion of d18:2(4*E*,8*E*) was slightly higher in CT seedlings, and was accompanied by a lower proportion of t18:1(8*E*). It could be inferred that 16h:0 is preferably attached to d18:2(4*E*,8*E*) and 24h:0 is attached to either d18:2(4*E*,8*E*) or geometrical isomers of t18:1(8*E* or 8*Z*). [12]

It was reported that CMH species combining 4-hydroxy base components (e.g., t18:1(8*Z*) and (8*E*)) and 2-hydroxy fatty acids with very long chains such as C_{22}, C_{24} and C_{26} would set the phase transition temperature in a liposome system of unsaturated phospholipids. [8] In addition, we have found by chilling resistant soybean plants that 8-Z-sphingoid bases [d18:2(8*Z*) and t18:1(8*Z*), especially the latter component] are greatly

Table 4. Composition (mol%) of 2-Hydroxy Fatty Acids and Sphingoid Bases of CMH in Cotyledons and Primary Leaves of NT and CT Growing Seedlings of Squash

Component	Cotyledons		Primary leaves	
	NT[a]	CT[b]	NT	CT
2-Hydroxy fatty acid				
16h:0	49.2	53.2	53.2	55.2
22h:0	10.0	10.5	12.3	13.2
24h:0	27.2	24.2	24.5	22.6
26h:0	4.6	4.0	5.1	4.2
Others	8.9	8.1	5.8	4.8
Sphingoid base				
d18:2(4*E*,8*E*)	71.2	75.2	68.1	68.8
d18:2(4*E*,8*Z*)	0.7	0.5	2.4	3.2
t18:1(8*E*)	19.4	16.6	15.2	12.8
t18:1(8*Z*)	7.5	6.4	8.8	7.8
Others	1.2	1.3	5.5	7.4

[a] Non-treated seedlings. [b] Chilling-treated seedlings.

increased as a result of chilling stress. [11] As 4-hydroxy bases are usually bonded to very long chain 2-hydroxy fatty acids, we hypothesized that 8-*Z*- and 8-*E*-stereoisomerism of the 4-hydroxy sphingoid bases would be involved in a mechanism of resistance to low temperature. In a comparison of the proportion of t18:1(8*E*) to t18:1(8*Z*), we have suggested that the more tolerant a grapevine species is to low temperature, the more t18:1(8*Z*) it has in CMH. [33] We calculated that the proportions of t18:1(8*E*) to t18:1(8*Z*) in cotyledons and primary leaves were 2.59 and 1.73, respectively. These proportions, however, were essentially the same between NT and CT seedlings. This result suggests that the change in chilling sensitivity in the current experiments was associated with some mechanisms other than 8-*Z*- and 8-*E*-stereoisomerism of the 4-hydroxy sphingoid bases in CMH.

We also noted in tobacco leaves that the level of 16h:0 increased as a result of chilling stress (data not shown). Recently, we studied the effects of plant CMH on the fluidity of phospholipid liposomes by fluorescence depolarization of the hydrophobic fluorescence probe, and found that the addition of rice bran CMH species containing dihydroxy sphingoid bases exhibited a lower decrease in fluidity compared to those containing trihydroxy bases (unpublished data). It is presumed, therefore, that increases of the 16h:0 and d18:2 levels in CT seedling CMH, similar to the increases in 18:3-containing PC species, would be partially effective in maintaining membrane fluidity under low temperatures. For this reason it is of interest to further examine the importance of the length of 2-hydroxy fatty acids in chilling tolerance.

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References

1) Nishida, I. and Murata, N., Chilling sensitivity in plants and cyanobacteria: the crucial contribution of membrane lipids. *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, **47**, 541-568 (1996).
2) Prasad, T. K., Anderson, M. D., Martin, B. A. and Stewart, C. R., Evidence for chilling-induced oxidative stress in maize seedlings and a regulatory role for hydrogen peroxide. *The Plant Cell*, **6**, 65-74 (1994).
3) Lyons, J. M., Chilling injury in plants. *Annu. Rev. Plant Physiol.*, **24**, 445-466

(1973).

4) Raison, J. K., The influence of temperature-induced phase changes on kinetics of respiratory and other membrane-associated enzymes. *J. Bioenerg.*, **4**, 258-309 (1973).
5) Murata, N., Molecular species composition of phosphatidylglycerols from chilling-sensitive and chilling-resistant plants. *Plant Cell Physiol*, **24**, 81-86 (1983).
6) Roughan, P. G., Phosphatidylglycerol and chilling sensitivity in plants. *Plant Physiol.*, **77**, 740-746 (1985).
7) Imai, H., Ohnishi, M., Hotsubo, K., Kojima, M., and Ito, S., Sphingoid base composition of cerebrosides from plant leaves. *Biosci. Biotechnol. Biochem.*, **61**, 351-353 (1997).
8) Yoshida, S., Washio, K., Kenrick, J. and Orr, G., Thermotropic properties of lipids extracted from plasma membrane and tonoplast isolated from chilling-sensitive mung bean (*Vigna radiata* [L.] Wilczek). *Plant Cell Physiol.*, **29**, 1411-1416 (1988).
9) Lynch, D. V. and Steponkus, P. L., Plasma membrane lipid alterations associated with cold acclimation of winter rye seedlings (*Secale cereale* L. cv. Puma). *Plant Physiol.*, **83**, 761-767 (1987).
10) Yoshida, S. and Uemura, M., Lipid composition of plasma membranes and tonoplasts isolated from etiolated seedlings of mung bean (*Vigna radiata* L.), *Plant Physiol.*, **82**, 807-812 (1986).
11) Ohnishi, M. and Ito, S., Structutral Characterization of plant sphingolipids in relation to chilling and freezing tolerance (in Japanese). *Jpn. J. Oli Chem. Soc.*, **46**, 1213-1225 (1997).
12) Ohnishi, M., Imai, H., Kojima, M., Yoshida, S., Murata, N., Fujino, Y. and Ito, S., Separation of cerebroside species in plants by reversed-phase HPLC and their phase transition temperature. ***In*** "The 19th World Congress of The International Society for Fat Research (ISF) and The 27th Annual Meeting of The Japan Oil Chemists' Society (JOCS) II", The Japan Oil Chemists' Society, Tokyo, pp. 930-935 (1988).
13) Murata, N., Ishizaki-Nishizawa, O., Higashi, S., Hayashi, H., Tasaka, Y. and Nishida, I., Genetically engineered alteration in the chilling sensitivity of plants. *Nature*, **356**, 710-713 (1992).
14) Bligh, E. G. and Dyer, W. J., A rapid method of total lipid extraction and purification. *Can. J. Biochem. Physiol.*, **37**, 911-917 (1959).
15) Mano, Y., Kawaminami, K., Kojima, M., Ohnishi, M. and Ito, S., Comparative composition of brown rice lipids (lipid fractions) of indica and Japonica rices. *Biosci. Biotechnol. Biochem.*, **63**, 619-626 (1999).
16) Ohnishi, M., Ito, S. and Fujino, Y., Characterization of sphingolipids in spinach leaves. *Biochim. Biophys. Acta*, **752**, 416-422 (1983).
17) Fujino, Y. and Ohnishi, M., Isolation and structure of diglycosylsterols and triglycosylsterols in rice bran. *Biochim. Biophys. Acta*, **574**, 94-102 (1979).

18) Kito, M., Takamura, H., Narita, H. and Urade, R., A sensitive method for quantitative analysis of phospholipid molecular species by high-performance liquid chromatography. *J. Biochem.*, **98**, 327-331 (1985).

19) Mano, Y., Nishiyama, S., Kojima, M., Ohnishi, M. and Ito, S., Analysis of the molecular species of glycerolipids from rye grains by reversed-phase high-performance liquid chromatography. *Cereal Chem.*, **68**, 280-284 (1991).

20) Gaver, R. C. and Sweeley, C. C., Method for mathanolysis of sphingolipids and direct determination of long-chain bases by gas-liquid chromatography. *J. Am. Oil Chemist's Soc.*, **42**, 294-298 (1965).

21) Uemura, M. and Steponkus, P. L., A contrast of the plasma membrane lipid composition of oat and rye leaves in relation to freezing tolerance. *Plant Physiol.*, **104**, 479-496 (1994).

22) Uemura, M., Joseph, R. A. and Steponkus, P. L., Cold acclimation of *Arabidopsis thaliana*. *Plant Physiol.*, **109**, 15-30 (1995).

23) Bulder, H. A. M., den Nijs, A. P. M., Speek, E. J., van Hasselt, P. R. and Kuiper, P. J. C., The effect of low root temperature on growth and lipid composition of low temperature tolerant rootstock genotypes for cucumber. *J. Plant Physiol.*, **138**, 661-666 (1991).

24) Bohn, M., Heinz, E. and Dorffling, K., Changes in the sphingolipid and fatty acid composition of winter wheat plasma membranes induced by low temperature. ***In*** "Advanced in plant lipid research", eds. Sánchez, J., Cerdá-Olmedo, E., and Martínez-Force, E., Universidad de Sevilla, Sevilla, pp. 503-506 (1998).

25) Smolenska, G. and Kuiper, P. L. C., Effect of low temperature upon lipid and fatty acid composition of roots and leaves of winter rape plants. *Physiol. Plant*, **41**, 29-35 (1977).

26) Clarkson, D. T., Hall, K. C. and Robert, J. K. M., Phospholipid composition and fatty acid desaturation in roots of rye during acclimation of low temperature. *Planta*, **149**, 464-471 (1980).

27) Hamada, T., Kodama, H., Takeshita, K., Utsumi, H. and Iba, K., Characterization of transgenic tobacco with an increased a-linolenic acid level. *Plant Physiol.*, **118**, 591-598 (1998).

28) Xu, Y., and Siegenthaler, P.-A., Low temperature treatments induce an increase in the relative content of both linolenic and Δ3-*trans*-hexadecenoic acids in thylakoid membrane phosphatidylglycerol of squash cotyledons. *Plant Cell Physiol.*, **38**, 611-618 (1997).

29) Norman, H. A., McMillan, C., and Thompson, G. A., Phosphatidylglycerol molecular species in chilling-sensitive and chilling-resistant populations of *Avicennia germinans* (L.). *Plant Cell Physiol.*, **25**, 1437-1444 (1984).

30) Kodama, H., Horiguchi, G., Nishiuchi, T., Nishimura, M. and Iba, K., Fatty acid desaturation during chilling acclimation is one of the factors involved in conferring

low-temerature tolerance to young tobacco leaves. *Plant Physiol.*, **107**, 1177-1185 (1995).

31) Wu, J., Lightner, J., Warwick, N. and Browse, J., Low-temperature damage and subsequent recovery of fab1 mutant Arabidopsis exposed to 2°C. *Plant Physiol.*, **113**, 347-356 (1997).

32) Huner, N. P. A., Krol, M., Williams, J. P., Maissan, E., Low, P. S., Roberts, D. and Thompson, J. E., Low temperature development induces a specific decrease in trans-Δ3-hexadecenoic acid content which influences LHCII organization. *Plant Physiol.*, **84**, 12-18 (1987).

33) Kawaguchi, M., Imai, H., Naoe, M., Yasui, Y. and Ohnishi, M., Cerebrosides in grapevine leaves: distinct composition of sphingoid bases among the grapevine species having different tolerances to freezing temperature. *Biosci. Biotechnol. Biochem.*, **64**, 1271-1273 (2000).