Plant Physiol. (1993) 103: 793–803

Plasma Membrane Lipids Associated with Genetic Variability in Freezing Tolerance and Cold Acclimation of *Solanum* Species[1]

Jiwan P. Palta*, Bruce D. Whitaker, and Laurie S. Weiss

Department of Horticulture, University of Wisconsin, 1575 Linden Drive, Madison, Wisconsin 53706 (J.P.P., L.S.W.); and Horticultural Crops Quality Lab, United States Department of Agriculture/ Agricultural Research Service, Beltsville Agricultural Research Center-West, Beltsville, Maryland 20705 (B.D.W.)

Simultaneous comparisons were made between a freezing-tolerant, cold-acclimating (CA) wild potato species (*Solanum commersonii*) and a freezing-sensitive, nonacclimating (NA) cultivated species (*Solanum tuberosum*). Comparative studies allowed differentiation of plasma membrane lipid changes associated with increased freezing tolerance following CA from lipid changes that can result from metabolic adjustment to reduced temperature during CA. Following CA treatment lipid changes found in both the NA and CA species included a decrease in palmitic acid, an increase in unsaturated to saturated fatty acid ratio, an increase in free sterols, an increase in sitosterol, and a slight decrease in cerebrosides. Lipid changes detected only in the acclimating species included an increase in phosphatidylethanolamine, a decrease in sterol to phospholipid ratio, an increase in linoleic acid, a decrease in linolenic acid, and an increase in acylated steryl glycoside to steryl glycoside ratio. These changes were either absent or opposite in the NA species, suggesting an association of these lipid changes with CA. Furthermore, the lipid changes associated with increased freezing tolerance during CA were distinct from lipid differences between the two species in the NA state.

The plasma membrane is regarded as a key site of injury during freeze-thaw stress in herbaceous plants (Palta and Li, 1980; Steponkus, 1984; Arora and Palta, 1988; Iswari and Palta, 1989; Palta, 1989). Because CA results in increased freezing tolerance in many plant species, it follows that the plasma membrane must undergo changes to tolerate a higher degree of freeze-thaw stress (Levitt, 1980). Change in membrane lipid composition during CA has been studied for the last 20 years in early studies investigating PL fatty acid composition. Some studies reported an increase in the proportion of unsaturated fatty acids (de la Roche et al., 1972; Grenier et al., 1972; Willemot, 1975, 1977). However, others failed to detect such an increase in fatty acid unsaturation during CA (de la Roche et al., 1975; de la Roche, 1979; Vigh et al., 1985). The contradictory results from these studies can be explained in part by the fact that many researchers have analyzed the lipid composition of whole tissues, or crude membrane or organelle preparations, rather than that of purified plasma membrane. In addition, comparative studies aimed at establishing a relationship between freezing or chilling stress resistance and lipid composition have utilized unrelated plant species.

In the early 1980s, changes in plasma membrane lipid composition associated with CA were studied in several plant species and contradictory results were obtained. In woody tissues such as mulberry bark, plasma membrane PL underwent a dramatic increase in the level of unsaturation during CA in fall and winter, primarily due to an increase in the proportion of 18:2 (Yoshida, 1984). In addition, a decrease in the sterol to PL ratio was noted during CA in this species. However, the same researchers found no significant changes in plasma membrane PL or sterol content or composition during CA of the herbaceous species winter rye and orchard grass (Uemura and Yoshida, 1984; Yoshida and Uemura, 1984). In contrast, following CA of winter rye, Lynch and Steponkus (1987) found substantial changes in the molecular species of PL and an increase in FS (primarily β-sitosterol) balanced by decreases in ASG and SG in plasma membrane isolated from leaf tissue. The contradictory results from the two studies using winter rye can be explained, in part, by the differences in the tissues used and the CA protocols employed. In one study, 20-d-old seedlings were CA at 5:2°C (day:night with a 12-h photoperiod) for 10 to 30 d (Uemura and Yoshida, 1984). In the other study, 2-week-old seedlings were first transferred to 13:7°C (day:night with an 11.5-h photoperiod) for 1 week and then to continuous 2°C (10-h photoperiod) for an additional 4 to 6 weeks (Lynch and Steponkus, 1987). Thus, about 2-week-old plants were compared with 6- to 8-week-old acclimated plants. Winter rye continued to grow and develop, albeit slowly, at CA temper-

[1] This work supported by the College of Agriculture and Life Sciences, University of Wisconsin-Madison, and by a U.S. Department of Agriculture competitive grant to J.P.P. (Agreement No. 85-CRCR-1-1673).

* Corresponding author; fax 1–608–262–4743.

Abbreviations: ASG, acylated steryl glycoside; BHT, butylated hydroxytoluene; CA, cold acclimation, (ed), (ing); DGDG, digalactosyldiacylglycerol; FAME, fatty acid methyl ester(s); FID, flame ionization detector; FS, free sterol(s); LPC, lysophosphatidylcholine; LPE, lysophosphatidylethanolamine; MGDG, monogalactosyldiacylglycerol; NA, nonacclimation, (ed), (ing); NL, neutral lipid(s); PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PL, phospholipid(s); SHAM, salicylhydroxamic acid; 16:0, palmitic acid; 16:1, palmitoleic acid; 18:0, stearic acid; 18:1, oleic acid; 18:2, linoleic acid; 18:3, linoleic acid; 20:0, arachidonic acid; 22:0, behenic acid.

atures (Griffith and McIntyre, 1993). During this period, rye leaves became progressively more tolerant to freeze-thaw stress. Thus, the relatively long CA periods in these studies present further difficulty in distinguishing lipid changes that might be associated with aging.

One weakness of the elegant study of Lynch and Steponkus (1987) is the inability to distinguish lipid changes that are related to CA per se (i.e. development of increased freezing tolerance) from the lipid changes that result from low temperature adjustment of plant metabolism. Such changes in membrane lipids from lowered growth temperatures are well documented (Lynch and Thompson, 1984; Kinney et al., 1987).

We have addressed these problems by comparative study of lipid changes in genetically related CA and NA potato species (*Solanum commersonii* and *Solanum tuberosum*, respectively). The acclimating potato species attained maximum freezing tolerance in 10 to 12 d and ceased growth during acclimation treatment, thus minimizing the confounding effects of growth and aging at low temperature. Furthermore, by looking at lipid changes in purified plasma membrane from these two species under identical conditions, we attempted to sort out lipid changes that might be directly associated with CA.

MATERIALS AND METHODS

Plant Material

Solanum tuberosum L. cv Red Pontiac and *Solanum commersonii* Dun. (PI 472834) were maintained as stem cultures (Steffen and Palta, 1986) on Murashige and Skoog medium (1962). Plantlets were potted in Jiffy Mix (JPA, West Chicago, IL) in 8-L pots and grown for 8 to 10 weeks at 20:18°C (day:night) with a 14-h photoperiod of 350 to 400 μmol s^{-1} m^{-2} from cool white fluorescent light. Hoagland and Arnon (1950) nutrient solution modified by 5-fold increased zinc sulfate and addition of 85 μM potassium chloride was used at one-quarter strength and delivered to excess four times daily. For CA, 8- to 10-week-old plants were transferred to 4:2°C (day:night) with a 14-h photoperiod of 75 to 100 μmol s^{-1} m^{-2} from cool white fluorescent light for 10 d. Within 10 d under these conditions, *S. commersonii* reached maximal freeze-thaw tolerance, whereas *S. tuberosum* showed no increase in freeze-thaw tolerance. Excised leaflets were subjected to a simulated natural freeze-thaw protocol (Steffen et al., 1989). Freeze tolerance was determined by electrolyte leakage as described previously (Steffen and Palta, 1986).

Preparation of Plasma Membrane

Plasma membrane was purified according to Iswari and Palta (1989) with slight modifications as follows. Twenty-five grams of youngest fully expanded leaflets were excised, ground with a mortar and pestle in 37 mL of homogenizing buffer (0.5 M Suc, 75 mM Mops-NaOH, 5 mM EGTA, 1 mM PMSF, 2 mM SHAM, 2.5 mM sodium bisulfite, 1.5% [w/v] PVP, 0.5% [w/v] BSA, 10 μg mL^{-1} BHT, pH 7.6), and filtered through four layers of cheesecloth. The filtrate was centrifuged at 8,000*g* for 20 min, and the resulting supernatant was then pelleted at 48,000*g* for 2.5 h. This total membrane pellet was resuspended in 10 mM potassium phosphate buffer (pH 7.3) containing 0.53 M Suc. Total membranes were partitioned in a two-phase system of 5.6% (w/v) each dextran (average mol wt 500,000) and PEG (mol wt 3,500) containing 0.35 M Suc, 10 mM potassium phosphate buffer (pH 7.3), and 30 mM NaCl. After centrifugation at 2,500*g* for 10 min, the plasma membrane-rich upper phase was removed and transferred to a fresh lower phase, mixed, and centrifuged again. This second upper phase was removed, diluted 4-fold with phase-diluting buffer (0.53 M Suc, 5 mM Mops-NaOH, 1 mM EGTA, 10 mM KCl, 0.2 mM PMSF, 1 mM DTT, pH 7.6), and centrifuged for 2.5 h at 48,000*g*. The membrane pellet was resuspended in 2 mL of phase-diluting buffer, resulting in a protein concentration of about 1 to 2 mg mL^{-1}. Potato leaf membranes, prepared in this manner, are highly enriched in plasma membranes and virtually free of chloroplast, mitochondria, ER, tonoplast, and Golgi membranes (Iswari and Palta, 1989).

Protein Concentration

Protein concentrations of the plasma membrane fractions were assayed using the method of Markwell et al. (1978).

Lipid Extraction

Plasma membrane volumes for lipid extraction were normalized based on total membrane protein. Volumes corresponding to 0.5 mg of membrane protein were extracted for polar lipid fatty acid analysis. Volumes corresponding to 2.0 mg of membrane protein were extracted for total lipid fractionation. Lipids were extracted from membrane samples by sequential addition of 5 mL of boiling isopropanol, 5 mL of methanol, 20 mL of chloroform, and 45 μL of 0.5% (w/v) BHT in chloroform. The sample and solvents were sealed under nitrogen and agitated for 60 min at 4°C. Nine milliliters of 0.8% (w/v) aqueous KCl were added, the mixture was vortexed vigorously and centrifuged, and the aqueous upper phase was discarded. The chloroform phase was mixed with 9 mL of methanol:water (1:1, v/v) and centrifuged, and the aqueous phase was again discarded. The lipid-containing chloroform phase was dried under nitrogen and resuspended in 0.5 mL of chloroform (for polar lipid fatty acid analysis) or 1.0 mL of chloroform (for total lipid fractionation).

Polar Lipid Fatty Acid Analysis

Polar lipids were separated from the total lipid by column chromatography using a 2-mL bed of 100 to 200-mesh silica gel (Sigma) packed in glass columns. Columns loaded with extracted lipid in 0.5 mL of chloroform were washed with 6 mL of chloroform to remove neutral lipids, and polar lipids were then eluted with 8 mL of methanol. The polar fraction was dried under nitrogen and resuspended in 0.2 mL of chloroform. Fatty acids were released from polar lipids by alkaline hydrolysis with 2 mL of 0.6 N methanolic NaOH for 90 min at room temperature. After neutralization with 2 mL of 0.6 N HCl, the FAME were extracted into hexane concentrated by nitrogen evaporation and analyzed by GLC (Shimadzu model 9AM, Shimadzu Scientific Instruments Inc.,

Columbia, MD) using an FID and a 1.6-m glass column packed with 10% SP-2330 on 100/120 Chromosorb WAW (Supelco, Bellefonte, PA). Column temperature was programmed to rise from 180 to 220°C at 6°C min^{-1} after 1 min at the initial temperature. Injector and detector temperatures were 250°C and 300°C, respectively. Carrier gas was nitrogen at 50 cc min^{-1}. Individual FAME were identified by comparison with known standards (Sigma) and were quantified as the weight percent of the total FAME.

Fractionation of Total Lipids

From total lipid samples in 1 mL of chloroform, two 20-μL aliquots were removed for assay of total lipid phosphorous according to Ames (1966). Ten micrograms of lathosterol (cholest-7-en-3β-ol; Sigma) were added to the remaining sample to serve as an internal FS standard. These total lipid samples were then loaded on a 5-mL bed of silicic acid (100- to 200-mesh Biosil A; Bio-Rad, Richmond, CA) in chloroform in a 1-cm diameter glass column, and NL, glycolipids, and PL were eluted sequentially with chloroform:acetone (20:1, v/v), acetone:methanol (20:1, v/v), and methanol:water (10:1, v/v). Lipid column fractions were dried under nitrogen at 40°C and stored in chloroform at −80°C prior to subsequent fractionation and analyses.

Isolation and Analysis of FS

NL were dissolved in 250 μL of ethanol:acetone (1:1, v/v), and FS were precipitated by adding an equal volume of 80% ethanol containing 2 mg mL^{-1} digitonin (Sigma). FS were recovered from digitonides and analyzed by GLC on a Perkin-Elmer 8320 gas chromatograph with a 30-m × 0.25-mm i.d. SPB-1 capillary column (Supelco) and an FID (Whitaker and Wang, 1987; Whitaker and Lusby, 1989). Identifying the major peaks as cholesterol, campesterol, stigmasterol, sitosterol, and isofucosterol was confirmed by GC-MS as described in Whitaker and Lusby (1989).

Isolation and Analysis of Glycolipids

Glycolipid fractions dissolved in 0.5 mL of chloroform were applied to 20 × 20 cm glass TLC plates coated with 0.25 mm of silica gel 60 (EM Science, Gibbstown, NJ). Individual glycolipids, including MGDG, DGDG, glucocerebrosides, SG, and ASG were separated by TLC in a solvent mixture of chloroform:methanol:ethyl acetate:acetic acid:water (60:20:10:10:2) and identified by co-chromatography with authentic standards and by reaction with acidic $FeCl_3$ reagent (Lowry, 1968). Glycolipid spots were visualized with iodine vapor, scraped into tubes, and extracted with 3 mL of chloroform:methanol (2:1, v/v) and 1 mL of 0.8% (w/v) NaCl. Samples were vortexed and cetrifuged, and the glycolipid-containing chloroform phase was recovered and dried under nitrogen. Galactolipid fractions (MGDG and DGDG) and a portion of the cerebroside fraction were each dissolved in 0.1 mL of *N,N*-dimethylformamide and quantified spectrophotometrically after addition of 0.4 mL of 5% (w/v) phenol and 2 mL of concentrated H_2SO_4 (Roughan and Batt, 1968). Aliquots of ASG and SG were used for spectrophotometric determination of sterol by a modification of the method of Zlatkis et al. (1953) as described by Whitaker et al. (1990). Aliquots of cerebrosides, SG, and SG derived from ASG by deesterification were also quantified by HPLC (model 600, Waters Associates, Milford, MA) using UV detection at 205 nm. Cerebroside and SG were separated on a 15-cm × 4.6-mm i.d. C_6 reversed-phase column with 5-μm Spherisorb packing (Chromanetics, Malaga, NY) by elution with a linear gradient of acetonitrile and water ranging from 55 to 80% acetonitrile, delivered at 1.0 mL min^{-1} (Kesselmeier et al., 1985; Whitaker et al., 1990). Sterol composition of ASG and SG was determined by hydrolyzing the steryl glycoside with 2 N TFA in dioxane:water (1:1, v/v), precipitating the liberated sterols with digitonin after hexane extraction, and analyzing the FS by GLC (Whitaker and Lusby, 1989).

Isolation and Analysis of PL

The PL fraction required a clean-up step involving washing with 10 mL of methanol:water (1:1) on C_{18} reversed-phase Sep-Paks (Waters Associates) to remove PEG contamination (residual from plasma membrane isolation), which co-eluted with the PL during column chromatography. PL were then separated by TLC on 10 × 20 cm glass plates coated with 0.25 mm of Silica Gel 60 in the solvent mixture of chloroform:methanol:acetic acid:water (85:15:12.5:3.5). Individual PL (PE, PC, PA, PI, PG, LPE, and LPC) were identified by co-chromatography with authentic standards (Sigma) and by reaction with spray reagents specific for choline, ethanolamine, or inositol (Kates, 1986). PL spots were visualized with iodine vapor, scraped, and eluted as described for the glycolipids, then quantified using the method of Ames (1966) for organic phosphate.

Fatty Acid Analysis

Fatty acids esterified to Glc on ASG were converted to FAME with 0.6 N methanolic KOH, and the FAME were recovered by hexane extraction as described for total polar lipids. Fatty acids on PC and PE were analyzed by capillary GLC with FID as described in Whitaker (1992).

RESULTS

Plasma Membrane Lipid Changes following CA Treatment in CA *S. commersonii* and NA *S. tuberosum*

CA treatment resulted in an increased freeze tolerance from −4.5 to −9°C in the wild potato species *S. commersonii*, but there was no change in *S. tuberosum*, which was killed at approximately −2.5°C both before and after CA treatment.

Following CA treatment, total lipid per mg membrane protein did not change significantly in *S. tuberosum*, but increased by about 17% in *S. commersonii* (Table I). No consistent changes in the recovery of plasma membrane protein (from tissue on a fresh weight basis) were found before and after CA treatment in the two species (data not shown). PL content increased slightly following acclimation treatment in *S. commersonii* but not in *S. tuberosum*. Sterol to PL remained similar following CA treatment in *S. tuberosum*, whereas *S. commersonii* showed a slightly lower sterol-to-PL ratio that decreased further following acclimation (Table I).

Table I. *Lipid composition of the plasma membrane isolated from NA and CA-treated S. tuberosum and S. commersonii leaf tissue*

Values ± SE of three separate samples (three independent experiments) are expressed as μmol mg^{-1} membrane protein and as mol percent of total lipid.

Species	Lipid	NA	CA	NA	CA
		μmol mg^{-1} protein		*mol percent of total lipid*	
S. tuberosum	PL	0.53 ± 0.05	0.55 ± 0.02	46.4 ± 1.5	46.8 ± 1.2
	Total sterols	0.52 ± 0.03	0.55 ± 0.05	45.0 ± 1.1	46.2 ± 1.7
	Cerebroside	0.07 ± 0.01	0.06 ± 0.01	6.5 ± 1.3	5.0 ± 0.6
	MGDG	0.01 ± 0.00	0.01 ± 0.00	0.9 ± 0.2	0.9 ± 0.3
	DGDG	0.01 ± 0.00	0.01 ± 0.00	1.3 ± 0.3	1.1 ± 0.1
	Total	1.15 ± 0.08	1.19 ± 0.06		
	Sterol/PL	0.96	0.99		
S. commersonii	PL	0.41 ± 0.03	0.50 ± 0.03	48.3 ± 2.0	51.1 ± 1.4
	Total sterols	0.36 ± 0.07	0.42 ± 0.06	41.6 ± 2.8	40.7 ± 2.0
	Cerebroside	0.05 ± 0.00	0.05 ± 0.00	6.1 ± 0.5	4.9 ± 0.8
	MGDG	0.02 ± 0.00	0.02 ± 0.00	2.2 ± 0.2	1.9 ± 0.1
	DGDG	0.02 ± 0.00	0.01 ± 0.00	1.8 ± 0.2	1.4 ± 0.2
	Total	0.86 ± 0.11	1.01 ± 0.10		
	Sterol/PL	0.88	0.81		

PL

The PL content and composition was virtually unchanged in *S. tuberosum* following the CA treatment (Table II). In contrast, *S. commersonii* showed a 25% increase in PL on a per mg protein basis. This increase was due primarily to increased PE from 0.15 to 0.21 μmol mg^{-1} membrane protein following acclimation. On a mol percent basis, there was an increase in PE and a slight decrease in PC in both species following the CA regime (Table II). However, these changes in PE and PC were more pronounced in *S. commersonii*.

The polar lipid fatty acid composition in *S. commersonii* showed a significant increase in 18:2 with concurrent decrease in 16:0 (as weight percent) following acclimation treatment (Table III). In *S. tuberosum*, however, there was no change in 18:2, a slight decrease in 16:0, and a small increase in 18:3. The increase in 18:2 in *S. commersonii* PL was most dramatic in PC, whereas the proportion of 18:2 decreased slightly in *S. tuberosum* PC (Table IV). The weight percent of 18:2 in PE increased in both species following CA treatment, but was consistently greater in PE from *S. commersonii* (Table IV). Thus, considering the increase in PE in *S. commersonii* after CA (Table II), the overall increase in 18:2 in PE and PC in *S. commersonii* polar lipids can be attributed to the specific increase of 18:2 in PE and PC, plus the increased amount of PE carrying a greater proportion of 18:2. The ratio of 18:2 to 18:3 in the total polar lipids decreased in *S. tuberosum* and increased in *S. commersonii* following acclimation treatment (Table III). Among the individual PL, the change in the ratio of 18:2 to 18:3 was most pronounced in PC from *S. commer-*

Table II. *PL composition of the plasma membrane isolated from NA and CA-treated S. tuberosum and S. commersonii leaf tissue*

Values ± SE of three separate samples (three independent experiments) are expressed as μmol mg^{-1} membrane protein, as mol percent of total lipid, and as mol percent of total PL.

Species	Lipid	NA	CA	NA	CA	NA	CA
		μmol mg^{-1} protein		*mol percent total lipid*		*mol percent PL*	
S. tuberosum	PC	0.20 ± 0.01	0.20 ± 0.00	17.7 ± 0.1	17.2 ± 0.7	38.4 ± 1.4	36.6 ± 0.7
	PE	0.20 ± 0.02	0.21 ± 0.01	16.6 ± 0.3	17.6 ± 0.4	35.7 ± 0.6	37.6 ± 0.1
	PA	0.06 ± 0.01	0.06 ± 0.00	4.7 ± 0.8	4.7 ± 0.3	10.2 ± 1.5	10.0 ± 0.4
	LPC	0.02 ± 0.00	0.02 ± 0.00	2.0 ± 0.3	2.1 ± 0.2	4.3 ± 0.5	4.4 ± 0.4
	LPE + PI	0.04 ± 0.01	0.04 ± 0.00	3.5 ± 0.4	3.6 ± 0.1	7.5 ± 0.6	7.7 ± 0.1
	PG	0.02 ± 0.00	0.02 ± 0.00	1.8 ± 0.1	1.7 ± 0.2	3.9 ± 0.2	3.6 ± 0.5
	Total	0.53 ± 0.05	0.55 ± 0.02	46.4 ± 1.5	46.8 ± 1.2		
	PE/PC	0.93	1.03				
S. commersonii	PC	0.18 ± 0.01	0.19 ± 0.01	20.6 ± 0.9	18.5 ± 1.0	42.6 ± 0.2	36.3 ± 1.5
	PE	0.15 ± 0.01	0.21 ± 0.01	17.5 ± 0.9	21.1 ± 0.9	36.2 ± 0.6	41.2 ± 0.8
	PA	0.04 ± 0.00	0.05 ± 0.01	4.6 ± 1.0	4.9 ± 1.0	9.5 ± 1.7	9.6 ± 2.0
	LPC	0.01 ± 0.00	0.02 ± 0.01	1.2 ± 0.2	1.7 ± 0.4	2.6 ± 0.6	3.4 ± 0.8
	LPE + PI	0.02 ± 0.00	0.03 ± 0.01	2.6 ± 0.1	3.0 ± 0.3	5.5 ± 0.5	3.4 ± 0.8
	PG	0.02 ± 0.00	0.02 ± 0.01	1.8 ± 0.4	1.9 ± 0.4	3.7 ± 0.9	3.7 ± 0.8
	Total	0.41 ± 0.03	0.51 ± 0.04	48.3 ± 2.0	51.1 ± 1.4		
	PE/PC	0.85	1.14				

Table III. *Polar lipid fatty acid composition of the plasma membrane isolated from NA and CA-treated S. tuberosum and S. commersonii leaf tissue*

Values ± SE of three separate samples (three independent experiments) are expressed as weight percent of polar lipid fatty acids.

Fatty Acid	*S. tuberosum*		*S. commersonii*	
	NA	CA	NA	CA
	wt percent of total fatty acids		*wt percent of total fatty acids*	
16:0	34.0 ± 0.4	31.4 ± 0.8	33.3 ± 0.4	29.8 ± 0.7
16:1	1.3 ± 0.1	1.1 ± 0.1	1.7 ± 0.2	1.3 ± 0.1
18:0	2.9 ± 0.1	3.0 ± 0.1	4.3 ± 0.1	3.6 ± 0.2
18:1	2.3 ± 0.1	1.7 ± 0.2	7.6 ± 0.6	6.5 ± 0.8
18:2	36.5 ± 1.0	35.5 ± 1.3	32.0 ± 0.2	39.6 ± 0.9
18:3	18.2 ± 1.3	21.8 ± 2.2	17.3 ± 0.8	15.6 ± 0.3
20:0	2.4 ± 0.1	2.3 ± 0.1	2.3 ± 0.1	1.9 ± 0.5
22:0	1.3 ± 0.1	1.9 ± 0.3	1.7 ± 0.2	1.8 ± 0.2
18:2/18:3	2.01	1.62	1.85	2.55
18:2/16:0	1.07	1.13	0.96	1.33
Unsaturated/saturated	1.39	1.56	1.41	1.70

sonii (Table IV). The ratio of unsaturated to saturated fatty acids in PC and PE increased in both species following acclimation treatment, but this ratio was consistently higher in PC and PE from *S. commersonii* (Table IV).

Steryl Lipids

Following acclimation treatment, there were changes in steryl glycosylation and acylation as well as in sterol composition in plasma membrane from both species. In both *S. tuberosum* and *S. commersonii* there was a significant increase in FS when expressed as either μmol per mg protein or mol percent of total lipids (Table V). However, changes in the amount of ASG and SG following acclimation treatment differed in the two species. Expressed on a per mg protein basis, the amounts of ASG and SG changed very little in *S. commersonii*, whereas in *S. tuberosum* there was an increase in SG and a decrease in ASG (Table V). The ratio of ASG to SG was higher in *S. commersonii* than in *S. tuberosum* prior to acclimation treatment and was much higher in *S. commersonii* following CA treatment (Table V).

A reduction in the proportion of cholesterol content in ASG, SG, and FS was evident in both species after acclimation treatment but was more dramatic in *S. commersonii* (Tables VI–VIII). The proportions of sitosterol and isofucosterol in FS increased in both species following acclimation treatment (Table VIII). The most prominent changes in ASG fatty acid composition were an increase in 18:2 in *S. commersonii* and an increase in 18:3 in *S. tuberosum* (Table VI). The ratio of 18:2 to 18:3 in ASG decreased in *S. tuberosum* and increased in *S. commersonii* (Table VI).

Plasma Membrane Lipid Composition Comparison between Freeze-Tolerant *S. commersonii* and Freeze-Sensitive *S. tuberosum* under Normal Growing Conditions

S. commersonii is a freeze-tolerant (to −4.5°C) wild potato species, whereas *S. tuberosum* is a freeze-sensitive (−2.5°C) cultivated potato species. The leaf plasma membrane lipids of *S. commersonii* and *S. tuberosum* showed qualitative similarity but differed in quantitative respects (Tables I–VIII).

In both species, lipids comprised primarily PL (46–48 mol

Table IV. *Fatty acid composition of the plasma membrane PC and PE isolated from NA and CA-treated S. tuberosum and S. commersonii leaf tissue*

Values are expressed as weight percent of total PC or PE fatty acids.

Fatty Acid	*S. tuberosum*				*S. commersonii*			
	PC		PE		PC		PE	
	NA	CA	NA	CA	NA	CA	NA	CA
16:0	36.5	32.0	39.9	36.8	28.2	24.1	31.7	29.5
16:1	0.6	0.6	0.5	0.4	0.7	0.7	0.5	0.9
18:0	4.3	6.1	3.6	3.8	4.2	3.8	3.2	2.8
18:1	4.7	6.5	2.2	2.7	12.3	12.1	5.2	5.0
18:2	34.4	33.3	35.9	38.1	33.3	39.8	40.5	43.5
18:3	18.4	19.9	15.1	15.9	19.0	17.1	17.0	16.7
20:0	0.7	1.1	2.4	1.9	1.7	1.8	1.5	1.3
18:2/18:3	1.88	1.67	2.38	2.40	1.75	2.33	2.38	2.60
18:2/16:0	0.94	1.04	0.90	1.04	1.18	1.65	1.28	1.47
Unsaturated/saturated	1.38	1.54	1.17	1.34	1.92	2.35	1.74	1.97

Table V. *Steryl lipid composition of the plasma membrane isolated from NA and 10-d CA-treated S. tuberosum and S. commersonii leaf tissue*

Values ± SE of three separate samples (three independent experiments) are expressed as μmol mg^{-1} membrane protein, as mol percent of lipids, and as mol percent of total steryl lipid.

Species	Lipid	NA	CA	NA	CA	NA	CA
		μmol mg^{-1} protein		*mol percent of total lipids*		*mol percent of total steryl lipid*	
S. tuberosum	FS	0.05 ± 0.00	0.10 ± 0.01	4.15 ± 0.22	8.22 ± 0.47	9.3 ± 0.7	17.8 ± 1.3
	SG	0.10 ± 0.01	0.15 ± 0.01	8.91 ± 0.86	12.31 ± 0.56	19.7 ± 1.5	26.6 ± 0.9
	ASG	0.36 ± 0.03	0.31 ± 0.03	31.91 ± 0.45	25.70 ± 1.44	71.0 ± 0.8	55.5 ± 1.0
	ASG/SG	3.61	2.09				
	SG/FS	2.14	1.50				
	(ASG + FS)/SG	4.07	2.76				
S. commersonii	FS	0.01 ± 0.00	0.05 ± 0.01	1.69 ± 0.93	4.83 ± 0.18	4.1 ± 0.1	12.0 ± 1.0
	SG	0.07 ± 0.01	0.05 ± 0.01	7.46 ± 0.59	4.88 ± 0.86	17.9 ± 0.8	11.9 ± 1.5
	ASG	0.28 ± 0.06	0.31 ± 0.05	32.47 ± 2.23	30.95 ± 1.39	78.0 ± 0.8	76.2 ± 1.0
	ASG/SG	4.00	6.20				
	SG/FS	4.52	1.05				
	(ASG + FS)/SG	4.56	7.16				

percent) and sterols (42–45 mol percent). Small amounts of cerebrosides (6.1–6.5 mol percent) and galactolipids (2–4 mol percent) were also present (Table I). PL were mainly PC, PE, and PA with small amounts of LPC, LPE, PG, and PI also present (Table II). Polar lipid fatty acids included mainly 16:0, 18:2 and 18:3 (Table III). Small quantities of 16:1, 18:0, 18:1, 20:0, and 22:0 were also present. PC and PE had 16:0 and 18:2 as the major fatty acids (Table IV). Overall unsaturated to saturated fatty acid ratio was higher in *S. commersonii* than in *S. tuberosum* (Tables III and IV). Sterols included cholesterol, campesterol, stigmasterol, sitosterol, and other undetermined sterols as FS, SG, or ASG (Tables V–VIII). FS also included isofucosterol (Table VIII). The steryl lipid composition of both species was dominated by ASG with proportionately much smaller amounts of SG and FS (Table V). Fatty acids on the ASG were primarily 16:0 and 18:2 (Table VI).

S. tuberosum showed greater total lipid (PL, steryl lipid, glycolipid, and cerebroside) per mg membrane protein than did *S. commersonii*: 1.15 μmol compared with 0.86 μmol, respectively, in the NA state (Table I). Under normal growing conditions, there was a slightly higher proportion of PL in *S. commersonii* compared with *S. tuberosum* (Table I). The ratio of total sterol to PL was about 11% lower in *S. commersonii* than in *S. tuberosum*. *S. commersonii* had a greater percent composition of cholesterol and a lower percent composition of stigmasterol in ASG, SG, and FS than did *S. tuberosum* (Tables VI–VIII).

DISCUSSION

The two tuber-bearing potato species used in the present study proved to be very useful for understanding plasma membrane lipid changes during CA that may be associated

Table VI. *ASG sterol and fatty acid compositions of the plasma membrane isolated from NA and 10-d CA-treated S. tuberosum and S. commersonii leaf tissue*

Values ± SE of three separate samples (three independent experiments) are expressed as the weight percent of sterol or total fatty acid in the ASG.

Sterol/Fatty Acid	*S. tuberosum*		*S. commersonii*	
	NA	CA	NA	CA
	wt percent of total ASG sterol		*wt percent of total ASG sterol*	
Cholesterol	6.1 ± 0.3	5.1 ± 0.3	11.0 ± 0.8	8.6 ± 0.7
Campesterol	2.0 ± 0.1	1.9 ± 0.1	2.8 ± 0.1	2.8 ± 0.1
Stigmasterol	19.3 ± 1.0	19.2 ± 0.4	13.0 ± 0.5	13.5 ± 0.8
Sitosterol	57.8 ± 0.6	60.6 ± 1.7	58.4 ± 1.9	61.8 ± 1.6
Other sterols	14.8 ± 0.7	13.3 ± 1.9	14.8 ± 1.3	13.3 ± 1.4
	mol percent of total ASG fatty acids		*mol percent of total ASG fatty acids*	
16:0	66.2 ± 0.4	64.2 ± 0.9	64.2 ± 1.7	63.3 ± 1.3
18:0	7.4 ± 0.5	7.9 ± 0.3	8.1 ± 0.3	7.7 ± 0.1
18:1	1.8 ± 0.1	1.5 ± 0.3	4.3 ± 0.2	3.4 ± 0.1
18:2	16.1 ± 0.7	16.3 ± 0.6	14.3 ± 1.3	17.7 ± 0.9
18:3	5.1 ± 0.2	7.0 ± 0.3	4.8 ± 0.7	4.2 ± 0.5
20:0	2.7 ± 0.2	2.7 ± 0.1	2.4 ± 0.1	2.4 ± 0.1
18:2/18:3	3.16	2.33	2.98	4.21

Table VII. *SG sterol class composition of the plasma membrane isolated from NA and 10-d CA-treated S. tuberosum and S. commersonii leaf tissue*

Values ± SE of three separate samples (three independent experiments) are expressed as the weight percent of total sterol in the total SG.

Sterol	*S. tuberosum*		*S. commersonii*	
	NA	CA	NA	CA
	wt percent of total SG sterol		*wt percent of total SG sterol*	
Cholesterol	6.6 ± 0.2	4.8 ± 0.4	14.0 ± 0.6	9.0 ± 0.8
Campesterol	2.2 ± 0.1	1.8 ± 0.1	3.2 ± 0.3	3.1 ± 0.2
Stigmasterol	20.2 ± 0.6	18.9 ± 0.4	11.5 ± 1.0	13.5 ± 0.6
Sitosterol	60.2 ± 0.5	62.1 ± 0.9	58.4 ± 1.4	58.9 ± 1.7
Other sterols	10.9 ± 1.3	12.4 ± 0.6	13.0 ± 0.7	15.5 ± 1.2

with increased freezing tolerance. The freezing-tolerant, acclimating *S. commersonii* showed several plasma membrane lipid changes following CA treatment that did not occur in the freezing-sensitive, NA *S. tuberosum*. It has not been possible in previous studies to differentiate the lipid changes related to the development of increased freezing tolerance during CA from the changes that result from metabolic adjustment to low, nonfreezing temperatures. Following growth at lowered temperature, numerous membrane lipid changes have been observed that do not necessarily result in increased freezing tolerance (Lynch and Thompson, 1984; Kinney et al., 1987). Most research to date on CA herbaceous material has utilized monocotyledonous species. Winter rye and orchard grass have been investigated for plasma membrane lipid alteration during cold acclimation (Uemura and Yoshida, 1984; Yoshida and Uemura, 1984; Lynch and Steponkus, 1987). These monocotyledonous plants continue to grow and develop, albeit slowly, at CA temperatures (Griffith and McIntyre, 1993). Thus, in studies using these plants it is difficult to discern lipid changes that may be specifically associated with the development of freezing tolerance during acclimation from those required for low-temperature growth. Furthermore, in the studies by Lynch and Steponkus (1987) and Yoshida and Uemura (1984), the long CA periods (4–9 weeks) introduce the added difficulty of distinguishing those lipid changes that might be associated with aging. The CA potato species, *S. commersonii*, completely acclimates in 10 to 12 d, and thus the confounding effects of growth and aging at low temperature are minimal.

An increase in PL content appears to be associated with increased freezing tolerance following acclimation treatment. This increase was found only in *S. commersonii*, the species able to cold acclimate (Table I). An increase in the plasma membrane PL (per mg protein) during CA has been reported for herbaceous (Uemura and Yoshida, 1984; Yoshida and Uemura, 1984; Ishikawa and Yoshida, 1985; Lynch and Steponkus, 1987) and overwintering woody species (Yoshida, 1984). Our results showed that the increase in PL in *S. commersonii* was primarily due to an increase in PE (Table II), which is consistent with the results reported on plasma membrane from leaf (Lynch and Steponkus, 1987) and crown tissue (Uemura and Yoshida, 1984) of rye. We also found that as PE increased (mol percent), there was a proportionate decrease in PC (mol percent) following acclimation treatment (Table II). The fact that PE increased and PC did not when expressed on a membrane protein basis, and the fact that these changes were noted only in *S. commersonii*, may indicate that increased synthesis of PE occurs during CA.

We found a correlation between a decrease in sterol to PL ratio and an increase in freezing tolerance following CA treatment (Table I). This decrease was due primarily to an increase in PL (mol percent of total lipids) and was noted only in the acclimating species, *S. commersonii*. These results are consistent with those reported in the plasma membrane of many plant tissues (Uemura and Yoshida, 1984; Yoshida, 1984; Yoshida and Uemura, 1984; Lynch and Steponkus, 1987). A lower sterol-to-PL ratio has been associated with higher fluidity (Yoshida and Uemura, 1990). Membrane fluidity has been found to increase during CA (Yoshida, 1984). Recently, plasma membrane of freezing-tolerant cell lines of eukalyptus was found to be more fluid than in freezing-sensitive cell lines (Leborgne et al., 1992). Thus, a decrease in the ratio of sterol to PL may be a parameter involved in CA. It is not fully understood if different steryl lipids (FS, SG, and ASG) exert different effects on membrane fluidity; however, it has been suggested that SG and ASG could alter lipid bilayer fluidity through less orderly packing in the bilayer as compared with FS (Catz et al., 1985). However, with the addition of FS, SG, or ASG, overall similar effects

Table VIII. *FS composition of the plasma membrane isolated from NA and 10-d CA-treated S. tuberosum and S. commersonii leaf tissue*

Values ± SE of three separate samples (three independent experiments) are expressed as the weight percent of total FS.

Sterol	*S. tuberosum*		*S. commersonii*	
	NA	CA	NA	CA
	wt percent of FS		*wt percent of FS*	
Cholesterol	15.5 ± 0.6	8.4 ± 0.3	19.4 ± 0.7	7.6 ± 0.3
Campesterol	2.4 ± 0.0	1.7 ± 0.2	3.1 ± 0.8	2.2 ± 0.3
Stigmasterol	25.2 ± 0.9	23.4 ± 0.8	17.6 ± 1.0	17.6 ± 0.6
Sitosterol	36.6 ± 0.5	40.9 ± 1.4	39.5 ± 1.0	44.0 ± 1.8
Isofucosterol	16.4 ± 1.0	22.8 ± 1.1	16.9 ± 0.6	26.2 ± 1.3
Other sterols	4.0 ± 0.2	2.7 ± 0.4	3.4 ± 1.1	2.4 ± 0.2

on phase-transition properties of dipalmitoyl PC have been reported (Mudd and McManus, 1980).

Our results show an increase in the proportion of 18:2 in plasma membrane lipids following CA only in the acclimating species *S. commersonii* (Tables III, IV, and VII). This increase in 18:2 was evident in the total polar lipids as well as in individual PC, PE, and ASG fractions. Conflicting results on the changes in the PL fatty acid composition have been reported. Yoshida and Uemura (1984) found no significant change in the plasma membrane fatty acid composition in crown tissue of orchard grass following CA. In another study the same researchers found a decrease in 18:2 and an increase in 18:3 in early stages of CA of rye crown tissue (Uemura and Yoshida, 1984). However, in the plasma membrane of overwintering mulberry bark cells, a dramatic increase in 18:2 and a decrease in 18:3 was found during fall and mid-winter (Yoshida, 1984), but these changes were observed to reverse during deacclimation in spring (Yoshida, 1986). Similar results have been reported for pine needles (Sutinen et al., 1989), cranberry leaves (Abdallah and Palta, 1989), and apple bud tissue (Wang and Faust, 1990). Yoshida and Uemura (1990) have concluded from their studies that in herbaceous plants such as orchard grass, winter rye, and Jerusalem artichoke, there seems to be no correlation between lipid unsaturation and the development of freezing tolerance. Our results in the present study (Tables III, IV, and VI) do not support this conclusion.

The increase in the proportion of 18:2 in plasma membrane PL following CA treatment in *S. commersonii* occurred at the expense of 16:0 and 18:3 (Tables III and IV). This is consistent with the PL fatty acid changes reported to occur in several woody species during fall and winter (Yoshida, 1984, 1986; Sutinen et al., 1989; Wang and Faust, 1990). However, our data showed that a decrease in 16:0 following CA treatment also occurred even in the NA species *S. tuberosum* (Tables III and IV). These data suggest that a decrease in 16:0 does not play a role in CA, whereas a decrease in 18:3 may be of interest. This conclusion is supported by opposite changes in the 18:2/18:3 ratio in the two species following CA treatment (Table III).

From a detailed analysis of plasma membranes of rye leaves, Lynch and Steponkus (1987) reported an increase in the diunsaturated molecular species of PL following CA. Results of our study show that an increase in 18:2 and a decrease in 18:3 is associated with CA. It is interesting to note that major molecular species in potato tuber microsomes were found to be those containing at least one of the acyl chains as 18:2; 18:3/18:2, 18:2/18:2, 18:2/16:0 were 71% of the total (Demandre et al., 1987). Thus, it appears that the increase in 18:2 found in our study following CA may be due to an increase in the PL molecular species containing 18:2 and may suggest a specific increase in 18:2/18:2 molecular species, since this is known to increase in rye (Lynch and Steponkus, 1987).

Results from the present study suggest that 18:2 may play a specific role in increasing freezing tolerance of herbaceous plants (potato) during CA. With regard to possible membrane phase change at freezing temperatures, there is an advantage to increasing 18:2 as compared with other fatty acids, including the more unsaturated 18:3. For example, the phase-transition temperature of 18:0/18:2 PC is −16°C, but it is −13°C for 18:0/18:3 PC (Marsh, 1990). The transition temperature for 18:0/18:1 PC is 5°C and for 16:0/16:0 PC it is 41°C. Major molecular species in plasma membrane include 16:0/18:2 and 18:2/18:2 (Lynch and Steponkus, 1987). The phase-transition temperatures of 16:0/18:2 PC and 18:2/18:2 PC were found to be −20 and −53°C, respectively, in the hydrated state (Lynch and Steponkus, 1989). Thus, the ability of a plant to specifically increase 18:2 could be advantageous in terms of maintaining plasma membrane lipid in a liquid-crystalline phase, avoiding the gel-crystalline state. It is important to emphasize that such simplistic one-temperature phase-change phenomena are unlikely to occur in the complex mixtures of various lipid components that make up biological membranes. Generally, complex lipid mixtures tend to show broad temperature transitions that appear to be inconsistent with the relatively narrow temperature range corresponding to freeze-killing temperatures. However, there are also specific associations of individual lipids with integral membrane proteins, and alteration of the required fluidity or a change to the gel state of this annular lipid may disrupt protein function, resulting in damage at freezing temperatures. It is possible that the increase in 18:2 during CA is preferentially associated with key plasma membrane functions that are subject to disruption by freezing stress.

Recent studies provide evidence that lipid compositional changes in plasma membrane can account for increased cryostability of this membrane following CA (Steponkus et al., 1988; Lynch and Steponkus, 1989). Following fusion with mono- or diunsaturated species of PC, NA protoplasts behaved like acclimated protoplasts in response to cryostress (Steponkus et al., 1988). The increased 18:2 found in our study may provide similar cryostability to the plasma membrane during freeze-thaw stress.

We found that an increase in the ratio of unsaturated:saturated fatty acid is not necessarily related to CA. This ratio was found to increase in both species, but only one species increased freezing tolerance following CA treatment (Tables III and IV). However, the increase in the unsaturated-to-saturated ratio was, in general, more pronounced in the acclimating species, *S. commersonii*. The increase in this ratio resulted from an increase in 18:2 in the acclimating species and from an increase in 18:3 in the NA species. Thus, despite the increase in the unsaturation of the plasma membrane lipids due to the increased proportion of 18:3, there was no increase in freezing tolerance in *S. tuberosum*.

Our results show that an increase in FS following CA treatment is not alone sufficient to account for an increase in freezing tolerance (Table V). We found this increase in both the acclimating and NA species, although increases were more dramatic in the acclimating species. FS have been found to increase (mol percent total lipid) in response to CA in rye plasma membrane (Lynch and Steponkus, 1987). Uemura and Yoshida (1984) found no significant change in FS (per mg basis) in the plasma membranes of rye crown tissue during acclimation. In potato leaf plasma membrane, FS were found to be a relatively small proportion (4–18%) of the total sterol compared with the values of 63 to 87% in plasma membrane of rye (Lynch and Steponkus, 1987). In potato leaf plasma membrane, ASG was the dominant form of sterol,

whereas ASG was found to be a relatively small proportion (8%) of the total sterol in rye plasma membrane. High levels of ASG are characteristic of solanaceous species (Duperon et al., 1984, Whitaker, 1988).

In potato species, the ratio of ASG to SG appears to be related to the development of freezing tolerance during CA (Table V). This ratio increased dramatically in the acclimating species and decreased dramatically in the NA species (Table V). This may be a unique feature of potato plasma membrane, since in rye plasma membrane FS increased at the expense of both ASG and SG following CA (Lynch and Steponkus, 1987). Changes in the ASG/SG ratio or an increase in the proportion of ASG plus FS at the expense of SG could be important in modulating the phase behavior of plasma membrane PL during acclimation and could have impact on the physical properties of the plasma membrane. Our results of a near doubling the ASG + FS to SG ratio in acclimating species and a significant decrease in the NA species (Table V) suggest that these changes in sterol conjugation may play a role in developing freezing tolerance during CA of potato leaf tissue.

Our results show that the increase in sitosterol concurrent with reduced cholesterol and other sterols (expressed as wt percent) may not be important in the development of freezing tolerance during acclimation because similar changes in sterol composition were observed in both acclimating and NA species (Tables VI–VIII). An increase in the proportion of β-sitosterol following CA has been found in rye leaf and crown tissue plasma membrane (Uemura and Yoshida, 1984; Lynch and Steponkus, 1987). From our study it appears that β-sitosterol (and its precursor isofucosterol) increases in response to lowered growth temperature, but this may not be related to increased freezing tolerance following CA.

Cerebrosides have been implicated as a possible source of lipid solidification during low temperature due to their high melting point (Curatolo, 1986). A 2- to 4-fold decrease in cerebrosides was found following CA in rye leaf plasma membrane (Lynch and Steponkus, 1987). However, cerebrosides constituted a relatively small percentage of the total lipids in potato leaf plasma membrane; this percentage did not significantly change after acclimation treatment in either species (Table I). It appears that cerebrosides may not have a significant role in potato leaf freezing tolerance.

Leaf plasma membrane lipid composition in these two species differed in some respects from the plasma membrane of other plant species. The potato leaf plasma membrane contained nearly even amounts (w/w) of protein and total lipid, and the protein-to-lipid ratio was greater than reported in the plasma membrane of winter rye seedlings (Lynch and Steponkus, 1987) or orchard grass (Uemura and Yoshida, 1984) but was comparable to that in oat root plasma membrane (Norberg et al., 1991). In potato leaf, the plasma membrane lipid was over 90 mol percent PL and sterol with the remaining <10% glycolipid and cerebroside (Table I). Both potato species showed the high levels of sterols characteristic of plasma membrane in comparison with endo membranes. The sterols of potato leaf plasma membrane were present mainly as ASG and SG (Table V), whereas plasma membrane of other species studied have included primarily FS (Uemura and Yoshida, 1984; Lynch and Steponkus, 1987). In agreement with our results, high levels of ASG are reported to be characteristic of solanaceous species in the genera *Solanum* and *Lycopersicon* (Duperon et al., 1984; Whitaker, 1988).

As discussed above, several plasma membrane lipid composition changes were found to be specifically associated with CA, i.e. the development of freezing tolerance in *S. commersonii*. However, there appears to be only small differences in plasma membrane lipid composition that can be correlated to the differences in freezing tolerance between the two potato species in the NA state. The wild *S. commersonii* has a higher degree of freezing tolerance (−4.5°C) compared with the cultivated *S. tuberosum* (−2.5°C) under normal growing conditions (NA state). Comparing the plasma membrane from leaves of NA plants, a lower sterol-to-PL ratio, a higher proportion of ASG (mol percent), and a lower proportion of FS (mol percent) were evident in *S. commersonii*, and thus may be related to greater freezing tolerance under normal growing conditions. No significant differences in the PL head group or fatty acid composition were found between the two species under NA conditions. These results suggest that the differences in freezing tolerance in the NA state and the ability to increase freezing tolerance during CA cannot be explained by the same biochemical and/or genetic mechanisms. We have recently obtained evidence in support of this conclusion. In segregating *Solanum* populations we have found that NA freezing tolerance and acclimation capacity are controlled by separate sets of genes (Stone et al., 1992, 1993).

Following CA treatment, we found several lipid changes unique to the CA species *S. commersonii* and several lipid changes common to both acclimating and NA species. We recognize that the changes common to both species may be necessary but not sufficient (in themselves) to provide enhanced freezing tolerance following CA. These changes may be necessary for metabolic adjustment to low temperature, which may be the first step for CA. From our studies we have been able to sort out these changes from the lipid changes that may be directly related to the development of greater freezing tolerance (CA). The results presented here suggest that the lipid changes unique to CA species may have a direct role in freezing tolerance, yet the unequivocal role of these lipids in freezing tolerance and CA remains to be established.

Received March 8, 1993; accepted June 24, 1993.
Copyright Clearance Center: 0032-0889/93/103/0793/11.

LITERATURE CITED

Abdallah AY, Palta JP (1989) Seasonal changes in membrane polar lipid fatty acid composition coincide with initiation of fruit ripening and changes in freezing stress resistance of leaves of cranberry (abstract No. 595). Plant Physiol **89:** S-100

Ames BN (1966) Assay of inorganic phosphate, total phosphate and phosphatases. Methods Enzymol **8:** 115–118

Arora R, Palta JP (1988) *In vivo* perturbation of membrane-associated calcium by freeze-thaw stress in onion bulb cells. Simulation of this perturbation in extracellular KCl and its alleviation by calcium. Plant Physiol **87:** 622–628

Catz DS, Tandecarz JS, Cardini CE (1985) Steryl glucoside and acyl

steryl glucoside formation in the amyloplast membrane during the development of potato tuber. Plant Sci **38:** 179–184

Curatolo W (1986) The interactions of 1-palmitoyl-2-oleylphosphatidylcholine and bovine brain cerebroside. Biochim Biophys Acta **861:** 373–376

de la Roche AI (1979) Increase in linolenic acid is not a prerequisite for development of freezing tolerance in wheat. Plant Physiol **63:** 5–8

de la Roche AI, Andrews CJ, Pomeroy MK, Weinberger PA, Kates M (1972) Lipid changes in winter wheat seedlings at temperatures inducing cold hardiness. Can J Bot **50:** 2401–2409

de la Roche AI, Pomeroy MK, Andrews CJ (1975) Changes in fatty acid composition in wheat cultivars of contrasting hardiness. Cryobiology **12:** 506–512

Demandre C, Justin AM, Nguyen XV, Gawer M, Trémolières A, Mazliak P (1987) Molecular species of phosphatidylcholine in plants: biosynthesis and role in oleate desaturation or freezing resistance. *In* PK Stumpf, JB Mudd, WD Ness, eds, The Metabolism, Structure and Function of Plant Lipids. Plenum Press, New York, pp 273–282

Duperon R, Thiersault M, Duperon P (1984) High level of glycosylated sterols in species of *Solanum* and sterol changes during the development of the tomato. Phytochemistry **23:** 743–746

Grenier G, Tremolieres A, Therrien HP, Willemot C (1972) Changements dans les lipides de la luzerne en conditions menant à l'edurcissement au froid. Can J Bot **50:** 1681–1689

Griffith MG, McIntyre HCH (1993) The interrelationship of growth and frost tolerance in winter rye. Physiol Plant **87:** 335–344

Hoagland DR, Arnon DI (1950) The water culture method of growing plants without soil. Calif Agric Exp Stn Circ 337

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

Iswari S, Palta JP (1989) Plasma membrane ATPase activity following reversible and irreversible freezing injury. Plant Physiol **90:** 1088–1095

Kates M (1986) Techniques in Lipidology, Ed 2. Elsevier, Amsterdam, The Netherlands

Kesselmeier J, Eichenberger W, Urban B (1985) High performance liquid chromatography of molecular species from free sterols and steryl glycosides isolated from oat leaves and seeds. Plant Cell Physiol **26:** 463–471

Kinney AJ, Clarkson DT, Loughman BC (1987) Phospholipid metabolism and plasma membrane morphology of warm and cool rye roots. Plant Physiol Biochem **25:** 769–774

Leborgne N, Dupou-Cézanne L, Teulières C, Canut H, Tocanne J, Boudet AM (1992) Lateral and rotational mobilities of lipids in specific cellular membranes of *Eucalyptus gunnii* cultivars exhibiting different freezing tolerance. Plant Physiol **100:** 246–254

Levitt J (1980) Responses of Plants to Environmental Stresses, Ed 2. Academic Press, New York

Lowry RR (1968) Ferric chloride spray detector for cholesterol and cholesterol esters on thin layer chromatograms. J Lipid Res **9:** 397

Lynch DV, Steponkus PL (1987) Plasma membrane lipid alterations associated with cold acclimation of winter rye seedlings (*Secale cereale* L. cv Puma). Plant Physiol **83:** 761–767

Lynch DV, Steponkus PL (1989) Lyotropic phase behavior of unsaturated phosphatidylcholine species: relevance to the mechanism of plasma membrane destabilization and freezing injury. Biochim Biophys Acta **984:** 267–272

Lynch DV, Thompson GA (1984) Chloroplast phospholipid molecular species alterations during low temperature acclimation in *Dunaliella*. Plant Physiol **74:** 198–203

Markwell MK, Haas SM, Beiber LL, Tolbert NE (1978) A modification of the Lowry procedure to simplify protein determinations in membrane lipoprotein samples. Anal Biochem **87:** 206–210

Marsh D (1990) CRC Handbook of Lipid Bilayers. CRC Press, Boca Raton, FL

Mudd JB, McManus TT (1980) Effect of steryl glycosides on the phase transition of dipalmitoyl lecithin. Plant Physiol **65:** 78–80

Murishige T, Skoog F (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol Plant **15:** 473–497

Norberg P, Mansson JE, Liljenberg C (1991) Characterization of glucoceramide from plasma membranes of plant root cells. Biochim Biophys Acta **1066:** 257–260

Palta JP (1989) Plasma membrane ATPase as a key site of perturbation in response to freeze-thaw stress. *In* DD Randall, DG Blevins, eds, Current Topics in Plant Biochemistry and Physiology, Vol 8. University of Missouri, Columbia, MO, pp 41–68

Palta JP, Li PH (1980) Alterations in membrane transport properties by freezing injury in herbaceous plants: evidence against the rupture theory. Physiol Plant **50:** 169–175

Roughan PG, Batt RD (1968) Quantitative analysis of sulfolipid (sulfoquinovosyl diglyceride) and galactolipids (monogalactosyl and digalactosyl diglycerides) in plant tissues. Anal Biochem **22:** 404–411

Steffen KL, Arora R, Palta JP (1989) Relative sensitivity of photosynthesis and respiration to freeze-thaw stress in herbaceous species. Importance of realistic freeze-thaw protocols. Plant Physiol **89:** 1372–1379

Steffen KL, Palta JP (1986) Effect of light on photosynthetic capacity during cold acclimation in a cold-sensitive and cold-tolerant potato species. Physiol Plant **66:** 353–359

Steponkus PL (1984) Role of the plasma membrane in freezing injury and cold acclimation. Annu Rev Plant Physiol **35:** 543–584

Steponkus PL, Uemura M, Balsamo RA, Arvinte T, Lynch DV (1988) Transformation of the cryobehavior of rye protoplasts by modification of the plasma membrane lipid composition. Proc Natl Acad Sci USA **85:** 9026–9030

Stone JM, Palta JP, Bamberg JB, Weiss LS (1992) Freezing tolerance and cold acclimation capacity are genetically distinct quantitative traits in a segregating *Solanum* population (abstract No. 749). Plant Physiol **99:** S-126

Stone JM, Palta JP, Bamberg JB, Weiss LS, Harbage JF (1993) Inheritance of freezing resistance in tuber-bearing *Solanum* species: evidence for independent control of nonacclimated freezing tolerance and cold acclimation capacity. Proc Natl Acad Sci USA (in press)

Sutinen ML, Rybarczyk SJ, Palta JP (1989) Seasonal changes in polar fatty acid composition coincide with changes in freezing stress resistance in pine needles. *In* DD Randall, DG Blevins, eds, Current Topics in Plant Biochemistry and Physiology, Vol 8. University of Missouri, Columbia, MO, p 286

Uemura M, Yoshida S (1984) Involvement of plasma membrane alterations in cold acclimation of winter rye seedlings (*Secale cereale* L. cv Puma). Plant Physiol **75:** 818–826

Vigh L, Horvath I, van Hasselt PR, Kuiper PJC (1985) Effect of frost hardening on lipid and fatty acid composition of chloroplast thylakoid membranes in two wheat varieties of contrasting hardiness. Plant Physiol **79:** 756–759

Wang SY, Faust M (1990) Seasonal changes of membrane lipids in apple shoots. J Am Soc Hortic Sci **115:** 462–467

Whitaker BW (1988) Changes in the steryl lipid content and composition of tomato fruit during ripening. Phytochemistry **27:** 3411–3416

Whitaker BW (1992) Glycerolipid-fatty-acid desaturase deficiencies in chloroplasts from fruits of *Capsicum annum* L. Planta **187:** 261–265

Whitaker BW, Lee EH, Rowland RA (1990) EDU and ozone protection: foliar glycerolipids and steryl lipids in snapbean exposed to O_3. Physiol Plant **80:** 286–293

Whitaker BW, Lusby WR (1989) Steryl lipid content and composition in bell pepper fruit at three stages of ripening. J Am Soc Hortic Sci **114:** 648–651

Whitaker BW, Wang CY (1987) Effects of paclobutrazol and chilling on leaf membrane lipids in cucumber seedlings. Physiol Plant **70:**

404–411

Willemot C (1975) Stimulation of phospholipid biosynthesis during frost hardening of winter wheat. Plant Physiol **55:** 356–359

Willemot C (1977) Simultaneous inhibition of linolenic acid synthesis in winter wheat roots and frost hardening by BASF 13-338, a derivative of pyridazinone. Plant Physiol **60:** 1–4

Yoshida S (1984) Chemical and biophysical changes in the plasma membrane during cold acclimation of mulberry bark cells (*Morus bombysis* Koidz. cv Goroji). Plant Physiol **76:** 257–265

Yoshida S (1986) Reverse changes in plasma membrane properties upon deacclimation of mulberry trees (*Morus bombysis* Koidz.). Plant Cell Physiol **27:** 83–89

Yoshida S, Uemura M (1984) Protein and lipid compositions of isolated plasma membranes from orchard grass (*Dactylis glomerata* L.) and changes during cold acclimation. Plant Physiol **75:** 31–37

Yoshida S, Uemura M (1990) Responses of the plasma membrane to cold acclimation and freezing stress. *In* C Larsson, IM Møller, The Plant Plasma Membrane. Springer-Verlag, Berlin, pp 293–319

Zlatkis A, Zak B, Boyle AJ (1953) A new method for the direct determination of serum cholesterol. J Lab Clin Med **41:** 486–492