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Protein and Lipid Compositions of Isolated Plasma Membranes from Orchard Grass (*Dactylis glomerata* L.) and Changes during Cold Acclimation[1]

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ABSTRACT

The chemical composition of plasma membrane fractions isolated from orchard grass seedlings (*Dactylis glomerata* L.) was analyzed and compared with endomembranes. The plasma membrane is characterized by an enrichment of sterols and a lower degree of unsaturation of phospholipids. Steryl glycosides were found to be one of the lipid components of the plasma membrane, but steryl esters and galactolipids were barely detectable. Diphosphatidyl glycerol was characteristically detected in the mitochondrial membrane, but not in the plasma membrane fraction. Plasma mambrane fraction was also characterized by its 'lower fluidity' in comparison with the endomembranes. This may be due to the large amount of sterols and the lower degree of phospholipid unsaturation in plasma membranes.

Electrophoretic comparison of polypeptides was also made between different membranes. The distribution patterns of polypeptides revealed on one- and two-dimensional SDS-slab gels were characteristic for those membranes. The presence of glycopeptide complements was a useful criterion for distinguishing plasma membrane from other membranes. The plasma membrane and the ER + Golgi membranes were enriched in glycopeptides. However, a marked difference was revealed in the total number and the molecular weights of the peptides.

During cold acclimation of orchard grass seedlings, the degree of fatty acid unsaturation increased only slightly in plasma membrane, unlike in endomembranes. The change in sterols-to-phospholipids ratio in plasma membrane was also slight. On the other hand, the phospholipid-to-protein ratio increased significantly in the plasma membrane as cold hardiness increased. A significant change in the polypeptide complements of plasma membrane was also demonstrated during cold acclimation.

Although the plasma membrane has received attention related to many physiological processes of plants, very little information is available as to its chemical composition. The chemical composition of plant plasma membrane is assumed to be, as in microorganisms (22) and animals (19), in a dynamic state in response to environmental factors, wounds, diseases, developmental processes, and so forth. Hodges *et al.* (8) reported on the lipid composition of isolated plasma membrane-enriched fractions from oat roots. The enrichment in sterols was found to be one of the chemical characteristics of plant plasma membranes. Travis *et al.* (24) reported that the lipid and protein composition in plasma membranes of soybean seedlings varied with the developmental stage of the roots.

A major problem encountered by investigators attempting to analyze membrane composition, however, is the difficulty of isolating plant plasma membranes in a high degree of purity and in large quantities needed for detailed studies. Recently we have established a method for isolating plasma membranes of high purity from differentiated higher plants using an aqueous two-polymer phase system containing NaCl (26, 31). The method is applicable to a wide variety of plant species, especially for light-grown green tissues.

In the present study, chemical analysis was made with the isolated plasma membranes, mitochondrial, and ER + Golgi membranes from orchard grass seedlings. A distinct difference was found in both lipid and protein compositions among those isolated membranes. We will also discuss chemical alterations in composition of the plasma membrane as related to the environmental stress of plants.

MATERIALS AND METHODS

Plant Materials. Seedlings of orchard grass (*Dactylis glomerata* L.) growing in the field were used. The basal parts of the seedlings (crown tissues) were cut into the homogenizing medium and disrupted with Polytron PT20 at medium speed setting as reported (31). The basal whitish parts of the tissues were used for the preparation of mitochondria. Freezing tolerance was evaluated by measurement of the ion-leakage after freeze-thawing of the tissues. The tissues were chopped into 1-cm-long pieces and frozen in test tubes (1.5 × 15 cm) at −3°C for 3 h by addition of small pieces of ice. Thereafter the tissues were further cooled down by 5°C steps at 1-h intervals. After holding at the desired temperatures for 2 h, the frozen tissues were thawed at 0°C in air. The thawed tissues were immersed in 10 ml of distilled H_2O and incubated at 25°C for 4 h by gentle shaking before measuring conductivity.

Isolation of Membranes. The homogenizing medium consisted of 0.5 M sorbitol, 50 mM MOPS[2]-KOH, 5 mM EGTA, 2.5 mM potassium metabisulfite, 4 mM salicyl hydroxamic acid, 0.5% defatted BSA, 1 mM PMSF, and 5% soluble PVP (mol wt 24,000). The homogenate was passed through two layers of Miracloth and subjected to differential centrifugations at 4,000*g* for 10 min, 14,000*g* for 15 min, and 156,000*g* for 20 min. The 14,000*g* pellet was washed once by suspending in a medium consisted of 0.5 M sorbitol, 10 mM Tris-maleate, 5 mM EDTA, pH 7.3, and by subsequent centrifugation at 14,000*g* for 20 min. For the isolation of mitochondria, the washed 14,000*g* pellet, suspended in

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[2] Abbreviations: MOPS, 3-(*N*-morpholino)-propanesulfonic acid; PEG 4000, polyethylene glycol (mol wt 3,340); PMSF, phenylmethylsulfonyl fluoride; BHT, butylated hydroxytoluene; TEMED, *N,N,N',N'*-tetramethylethylenediamine.

0.5 M sorbitol, 3 mM Tris-maleate, 1 mM EDTA, pH 7.3, was loaded on a linear sucrose gradient (15–50%, w/w) made up in 5 mM Tris-maleate, 1 mM EDTA, pH 7.3, and centrifuged at 96,000*g* for 3 h at 2°C on Hitachi SW 27 rotor. The mitochondria were banded at the 37% to 40% (w/w) sucrose layers with least contamination by the other membranes as judged by analysis of the marker enzymes such as pH 6.5 ATPase, IDPase, antimycin A insensitive NADH Cyt *c* reductase, catalase, and acid phosphatase. The isolated mitochondria were pelleted at 14,000*g* after dilution with 0.5 M sorbitol in 3 mM Tris-maleate, pH 7.3, and finally suspended in the same medium as used for the dilution.

The ER and Golgi membrane-enriched fraction was isolated from the 156,000*g* pellet by a linear sucrose density gradient as described for the isolation of mitochondria except that the time for centrifugation was 13 h. Before application to the linear sucrose gradient, the 156,000*g* pellet was washed once by suspending and centrifuging at 156,000*g* for 20 min as described above. The pellet was suspended in 10% sucrose (w/w), 5 mM Tris-maleate, 1 mM EDTA, pH 7.3. After the linear sucrose gradient, the ER and Golgi membranes were equilibrated at the 23% to 26% and 24% to 29% sucrose layers, respectively, as judged by the distribution of the membrane markers, *i.e.* antimycin A-insensitive NADH Cyt *c* reductase and latent IDPase activities. Membrane fractions banding at the 23% to 29% sucrose layers were combined and designated as the ER + Golgi membrane-enriched fraction.

Plasma membrane was isolated from the 156,000*g* pellet using an aqueous two-polymer phase system as reported (31). In brief, the 156,000*g* pellet was resuspended in 0.5 M sorbitol, 15 mM Tris-maleate, pH 7.3, and pelleted again at 156,000*g* for 20 min. The pellet was suspended in the same medium as above. The membrane suspension was added to a two-phase system consisting of 5.6% (w/w) of dextran T 500 and PEG 4,000 in a final concentration, 0.5 M sorbitol, 15 mM Tris-maleate, pH 7.3, and 30 mM NaCl. The phase mixture was thoroughly mixed by several inversions and centrifuged at 400*g* for 3 min to hasten the phase setting. All procedures were performed at 0°C. After phase setting, the top phase was removed and subjected to a repartition by mixing with newly synthesized lower phase. The top phase after the repartition was highly enriched in plasma membrane. The purity after the repartition was morphometrically examined according to PTA-CrO_3 stain (16) and also to the specific aggregation tests by $ZnCl_2$ and low pH (4.5) (25), and was found to be much higher (data not shown) than those after a single partition as reported before (31). Nearly the same result as the present study has been previously reported with rye seedlings (25). In the $ZnCl_2$ and low pH tests, the endomembranes other than plasma membrane were easily aggregated both with 3 mM $ZnCl_2$ and low pH at 4.5, whereas plasma membrane was extremely stable against these treatments (25).

Lipid Extraction from Membranes. Total lipids were extracted from membrane samples according to Bligh and Dyer (4) except that isopropanol was used instead of methanol. The total lipid extracts were dissolved in chloroform and subjected to a silicic acid column (3 × 1 cm, equivalent to 1 g Kieselgur Type 60, 70–230 mesh, Merck). Free and esterified sterols were eluted by adding 8 ml of chloroform and then 3 ml of 10% acetone in chloroform. Acylated steryl glycoside and monogalactosyl diglyceride, if present, were eluted by addition of 8 ml of 50% acetone in chloroform. Steryl glycoside and digalactosyl diglyceride, if present, were eluted by addition of 8 ml of acetone. Phospholipids and sulfolipids, if present, were eluted by addition of 8 ml of methanol and then 15 ml of 10% H_2O in methanol.

Quantitative analysis of phospholipids was performed according to Marinetti (14) with a slight modification. Quantitative analysis of sterols were performed according to Zlatkis and Zak (32). Cholesterol was used as the standard. Steryl glycosides were analyzed using the anthrone method (3). For this, the glycosides were dissolved in 20 μl of methanol, and added to 3 ml of 2% anthrone in 70% H_2SO_4, and heated at 90°C for 3 min. The absorbance was measured at 625 nm. Glucose was used as the standard. For the analysis of fatty acids, total phospholipids were transmethylated in 0.5 *N* sodium methoxide solution at 50°C for 15 min. Fatty acid methylesters were analyzed by GC using a G-Scot glass capillary column (FFAP) purchased from Gasukuro Kogyo Co., Ltd.

Each lipid component was identified by co-chromatography with the authentic lipids by TLC. Compositional analysis of phospholipids was performed using one-dimensional TLC with solvent mixture of chloroform-methanol-acetic acid (65:25:8, v/v/v).

One-Dimensional SDS-Slab PAGE of Membrane Preparations. Each membrane preparation equivalent to 1 mg protein was resuspended in 0.2 M KCl, 10 mM Tris-maleate, 1 mM EDTA, 10 μg/ml of BHT, pH 7.6, and centrifuged at 105,000*g* for 40 min. The pellets were suspended in 0.5 M sorbitol-10 mM Tris-maleate, 10 μg/ml of BHT, pH 7.6, and centrifuged at 105,000*g* for 40 min. The membrane pellets were solubilized in 0.0625 M Tris-HCl (pH 6.8), 2% SDS, 10% glycerol (w/v), 5% 2-mercaptoethanol, and 0.001% bromophenol blue with boiling for 3 min.

The discontinuous SDS buffer system of Laemmli (11) was used for the SDS slab-PAGE. A 10% SDS-PAGE slab, about 1 mm thick, 11 cm long, and 15 cm wide was prepared as described by Ames (1), except that the stacking gel (4.4% acrylamide) was 1.5 cm high with 12 sample wells. The final concentration of SDS was 0.1% in both gels and in the electrode buffer. Electrophoresis was run routinely at room temperature at a constant current of 20 mamp. The following proteins were used as the mol wt standards: trypsin inhibitor from soybean (T1), mol wt 21,500; BSA, mol wt 68,000; RNA-polymerase from E. coli (core enzyme), α, β, γ subunits, mol wt 39,000, 155,000, and 165,000, respectively. After electrophoresis, the slab gels were stained and destained according to Fairbanks *et al.* (7). For the determination of glycopeptides, the Con A-peroxidase method (29) was used.

Two-Dimensional SDS-Slab Gel Electrophoresis. Each membrane sample (2 mg protein equivalent) was pretreated with

Table I. *Comparison of Lipid Composition among Different Membranes*

The results given are averages from duplicated determinations.

Membrane Fractions	Phospholipids	Free Sterols	Acylated Steryl Glycoside	Steryl Glycoside	Total Sterols/ Phospholipids
		μmol/mg protein			*mol/mol*
Plasma membranes	1.05	1.15	0.07	0.09	1.25
ER + Golgi membranes	1.11	0.61	0.11	0.10	0.74
Mitochondria	0.44	0.02	ND[a]	ND	0.05

[a] Not determined.

Table II. *Comparison of Phospholipid Composition among Different Membranes*

The results given are averages from duplicated determinations.

Phospholipids	Plasma Membrane	ER + Golgi Membrane	Mitochondria
		mol %	
PC	43.5	45.7	39.8
PE	37.3	27.1	34.7
PI + PS	7.2	10.5	4.5
PG	10.6	14.4	9.9
DPG	0	1.4	8.7
PA	1.3	0.8	2.2

RNase (150 μg in 30 μl of 10 mM Tris-HCl, 1 mM $MgCl_2$, pH 6.8) followed by freezing and thawing in liquid N_2 (three cycles) and then 60 μg of DNase was added. After standing on ice for 10 min, the pretreated membrane samples were diluted with 3 ml of 10 mM Tris-HCl-1 mM $MgCl_2$, pH 6.8, and centrifuged at 105,000*g* for 40 min. The pretreated membrane samples were solubilized in the lysis buffer containing 20 μl of 0.5 M Tris-HCl, pH 6.8, 28 μl of 10% SDS (w/v), 1 μl of 0.1 M $MgCl_2$, 2 μl of BHT-isopropanol solution (10 mg/ml) and water to 138 μl. After boiling for 3 min, 122.4 mg of urea was added. The freshly solubilized membrane proteins were diluted with two volumes of sample dilution buffer containing 9.7 M urea, 5% 2-mercaptoethanol (v/v), 2% Ampholytes (comprising 1.6% pH 5–8 and 0.4% pH 3–10), and 8% Nonidet P-40 as reported by Ames and Nikaido (2). The ratio of the final percentages of Nonidet P-40 to SDS was 8.

Isoelectric focusing gels were made in glass tubing (120 × 1.8 mm i.d.) according to O'Farrell (17) with the following modifications. The 13-ml final volume of gel solution consisted of 7.15 g urea, 1.73 ml of 30% acrylamide monomer-bis stock (30:1.7), 2.60 ml of 10% Nonidet P-40, 0.52 ml of 40% Ampholyte stock (Pharmacia, pH 5–8), 0.13 ml of 40% Ampholyte stock (Pharmacia, pH 3–10), 2.2 ml H_2O and 0.35 ml of riboflavin-TEMED solution (3.5 mg riboflavin-5′-phosphate, 0.25 ml TEMED in 25 ml H_2O). This gel mixture was poured into glass tubes (9.5 cm high), overlayed with 8 M urea, and polymerized under fluorescent light for more than 2 h. After polymerization, the urea-overlay solution was removed and the solubilized membrane samples containing 100 μg proteins were layered on the tube gels and overlayed with 10 μl of solution consisting of 9 M urea and 1% Ampholytes (0.8% pH 5–8 and 0.2% pH 3–10). The tubes were filled with the cathode buffer (20 mM NaOH). The upper reservoir and the lower reservoir were filled with 20 mM NaOH and 10 mM H_3PO_4 solutions, respectively. Electrofocusing was performed at 350 v for 15 h and then at 800 v for 1 h (total 6050 v-h). The gels were removed from the tubes by water pressure from a water-filled syringe attached directly to the gel tube with tygon tubing. One set of gels was cut into 1-cm slices for the determination of the pH gradient and the other set was placed in 15 ml of 2% SDS-equilibration buffer containing 10% (w/v) glycerol, 5% (v/v) 2-mercaptoethanol, 2.3% (w/v) SDS, and 0.0625 M Tris-HCl, pH 6.7 and equilibrated for 30 min by gentle shaking at room temperature as recommended by O'Farrell (17).

The loading of the isoelectrofocusing gels on SDS-slab gel was done as described by O'Farrell (17). The SDS-slab gel used for two-dimensional electrophoresis was basically the same as the one used for one-dimensional electrophoresis except that the stacking gel without sample wells was 2 cm high and the running gel was 11 cm high. Electrophoresis was performed at constant current of 20 mamp for about 3 h at room temperature. After electrophoresis, the peptides were either stained and destained according to Fairbanks *et al.* (7) or stained by the silver-stain method (15).

Fluorescence Polarization Measurement. The fluorescent hydrocarbon 1,6-diphenyl-1,3,5-hexatriene (DPH) was used as a probe for monitoring the relative fluidity of the isolated membranes (20). The final concentration of DPH was 1 μM per 100 μg of membrane protein in 0.5 M sorbitol-5 mM K-phosphate, pH 7.3. Steady state fluorescence polarization was measured with a Elscient Microviscometer mv·/a. DPH embedded in membranes was excited at 375 nm and the emission was detected through a cutoff filter for wavelength below 400 nm. Steady state polarization was expressed as the fluorescence anisotropy, r, and as the anisotropy parameter, $(r_0/r - 1)^{-1}$, which varies proportionally with the rotation relaxation time of the embedded DPH, where r_0 is the maximum limiting anisotropy. The value for r_0 was 0.395 as reported by Kawato *et al.* (9). Within the limitation as reported (13), the anisotropy parameter is empirically related to the relative motional freedom of the embedded DPH molecules, without distinguishing specific mechanisms affecting this depolarizing motion, and provides thereby a comparative index of the fluidity of membrane lipid bilayers. These specific mechanisms included alteration of the rotation rate of the fluorophore and hindered motions owing to structural factors (6, 12).

RESULTS

Lipid Composition. Table I presents the lipid composition of various membrane samples obtained in the purification procedure. Esterified sterols and galactolipids were not detected in these membrane samples, indicating that they were free of contamination by plastid membranes. The plasma membrane fraction was highly enriched in sterols, especially free sterols. Only minor amounts of sterols were detected in mitochondrial fraction. Steryl glycosides were also the lipid components of plasma membrane fraction, while these were relatively enriched in ER + Golgi membranes. The order of enrichment in phospholipids per mg protein basis was ER + Golgi membranes > plasma membrane > mitochondria. The molar ratio of total sterols to phospholipids in plasma membrane fraction was 1.11 to 1.38, being 1.5-fold higher than the ratio in ER + Golgi membranes.

Table II presents phospholipid composition in various membrane samples. Phosphatidyl choline and ethanolamine were the major phospholipids in each membrane sample. The content of phosphatidic acid was less than 2% in the total phospholipids of every membrane sample, indicating that the potent activity of phospholipase D in the cells had been nearly completely controlled during preparation of membranes. Phosphatidyl glycerol was also one of the phospholipid components in plasma mem-

Table III. *Fatty Acid Composition of Phospholipids from Various Membrane Fractions*

The results given are averages from duplicated determinations.

Membrane Fraction	Fatty Acids							Unsaturated/ Saturated
	16:0	16:1	18:0	18:1	18:2	18:3	20:0	
				mol %				
Plasma membrane	25.8	Trace	0.3	2.5	49.1	20.0	2.1	2.53
ER + Golgi	27.1	Trace	0.8	6.6	40.9	23.6	0.9	2.46
Mitochondria	13.6	0.7	0.4	3.9	44.2	35.2	1.8	5.27

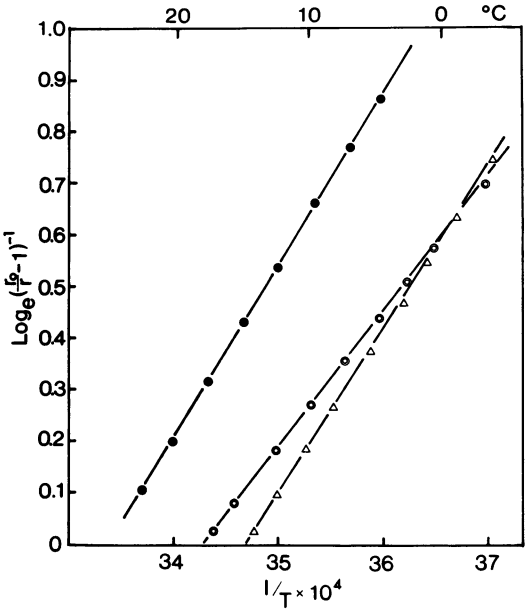

FIG. 1. Arrhenius plots of fluorescence anisotropy parameters of DPH embedded into various membranes. Membranes were isolated from crown tissues of orchard grass seedlings sampled in October. Membranes were labeled with DPH and the fluorescence polarization was measured. Experimental details are described in the text. ●——●, plasma membrane; △——△, ER + Golgi membranes ⊙——⊙, mitochondria.

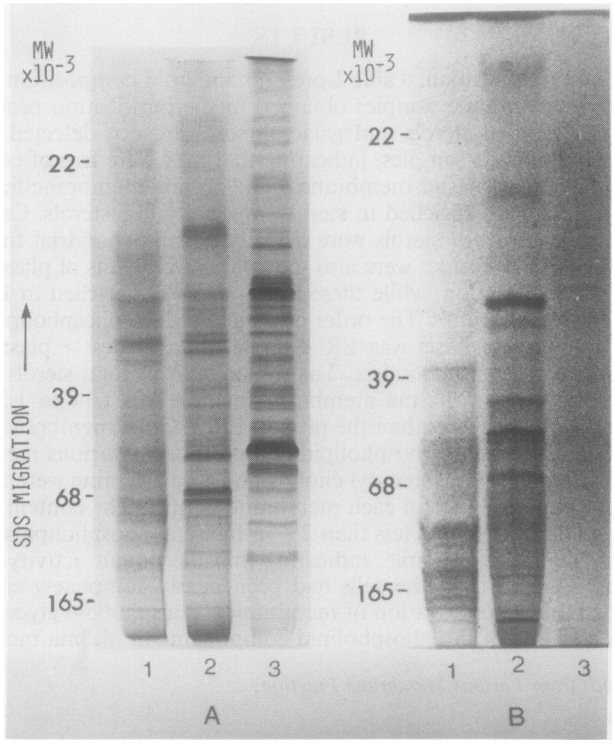

FIG. 2. SDS-PAGE of membrane polypeptides. Plasma membrane (1), ER + Golgi membranes (2), and mitochondria (3) were isolated from orchard crown tissues sampled in October. Each 15 μg of the solubilized and denatured polypeptides was applied on 1-mm thick SDS-slab gels. After electrophoresis, gels were stained with Coomassie briliant blue (A) or stained by the con A-peroxidase procedure (B). Experimental details are described in the text.

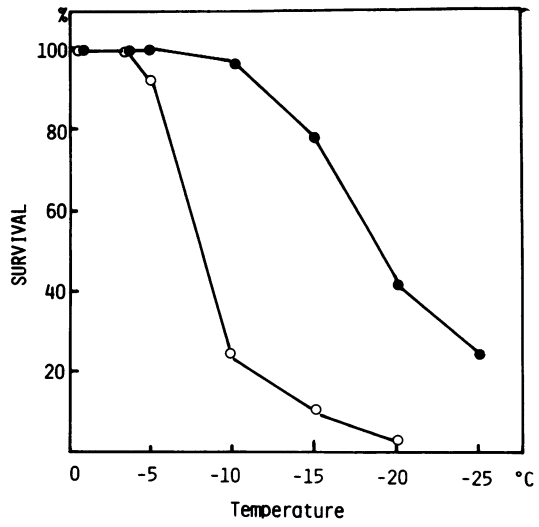

FIG. 3. Freezing tolerance of orchard crown tissues. Tissues were frozen at −3°C for 3 h with ice pieces and then successively cooled down at 2-h intervals to the indicated temperatures. Tissues were kept at the indicated temperatures for 2 h and then thawed at 0°C in air. Survival was evaluated by measuring the electrolyte leakage. O——O, October sample; ●——●, December sample.

brane likely to appear in other membranes. Diphosphatidyl glycerol was characteristically located in mitochondrial fraction.

Table III indicates the fatty acid composition of phospholipids in various membrane samples. Fatty acids of phospholipids in mitochondria were observed to be highly unsaturated. The degree of unsaturation in plasma membrane fraction was nearly the same as in ER + Golgi membranes, but the former membrane was relatively enriched in 18:3 content.

Figure 1 indicates the Arrehnius plots of the anisotropy parameter to compare the relative fluidity between different membranes. At temperatures above 0°C, the relative fluidity, which is the reciprocal of the anisotropy parameter, was in the order, mitochondria > ER + Golgi membranes > plasma membrane. The plasma membrane was relatively low in the fluidity all through the temperature tested. The slopes, in other words the apparent activation energies, were different for the various fractions.

One Dimensional SDS-Slab Gel Electrophoresis. As presented in Figure 2A, there existed a remarkable difference in the polypeptide composition between different membrane samples. Only minor peptide bands in plasma membrane proteins were aparently similar to those of ER + Golgi membranes in their electrophoretic mobilities. These membranes were distinctly different in the composition of glycopeptides (Fig. 2B). Both plasma membrane and ER + Golgi membrane fractions were enriched in glycoproteins. On the other hand, only trace amount of the peptides (near 20 kD) were detectable in mitochondria. In the plasma membrane fraction, the mol wt of the glycopeptides were relatively higher than in the ER + Golgi membrane fraction.

Two-dimensional electrophoretic analysis of the polypeptides solubilized from plasma membrane, ER + Golgi membranes, and mitochondria from orchard grass seedlings revealed a marked difference in both the total number of polypeptides and the relative mobilities (data not shown). Based on these results, it seems likely that a cross-contamination between these membranes is quite low.

Changes in the Chemical Composition of Plasma Membranes during Cold Acclimation. Figure 3 indicates survival curve of freeze-thawed orchard tissues. Fifty per cent survival occurred at −7.8 and −18.0, respectively, in tissue samples harvested on October 6 and December 10. Thus, about a 10°C increment of

Table IV. *Changes in Fatty Acid Composition of Phospholipids after Cold Acclimation*

The results given are averages from duplicated determinations.

Membrane Fraction	Sampling Date	Fatty Acids							Unsaturated/ Saturated
		16:0	16:1	18:0	18:1	18:2	18:3	20:0	
					mol %				
Plasma membrane	Oct 2	26.1	Trace	0.7	7.5	40.6	23.1	1.2	2.54
	Dec 8	26.1	Trace	0.6	6.1	41.5	23.8	1.0	2.57
Endomembranes	Oct 2	23.9	0.4	0.8	9.6	34.4	30.2	0.7	2.92
	Dec 8	21.0	0.7	0.4	6.1	38.6	32.4	0.7	3.49

Table V. *Changes in Lipid Composition of Plasma Membrane after Cold Acclimation*

The results given are averages from duplicated determinations.

	Phospholipids	Total Sterols	Total Sterols/ Phospholipids
		μmol/mg protein	*mol/mol*
Oct 2	1.11	1.10	0.99
Dec 8	1.38	1.22	0.88

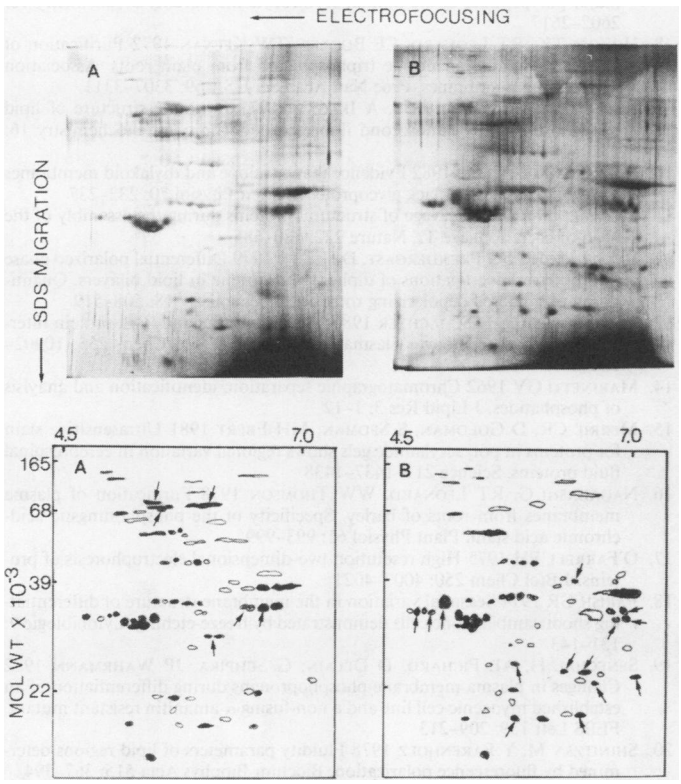

FIG. 4. Comparison of plasma membrane polypeptides between hardy (A) and less hardy (B) orchard crown tissues. The tissues were sampled in early October (less hardy) and in the middle of December (hardy). Two-dimensional SDS-PAGE of the polypeptides and their tracings are presented in the upper and the lower portions, respectively. The gels were stained according to the silver stain procedure described in the text.

cold hardiness was observed from early October to the middle of December in the field.

Table IV presents compositional changes in the fatty acids of phospholipids extracted from different types of membranes. In the plasma membrane fraction, the change in the degree of fatty acid-unsaturation during cold acclimation was very slight. On the other hand, a significant fatty acid-unsaturation during cold acclimation was observed in the endomembranes which were preferentially partitioned into the lower phase with an aqueous two-polymer phase system as described in the text. The change in the sterol-to-phospholipid ratio in plasma membrane was also slight (Table V). The phospholipid-to-protein ratio, however, increased by about 25% upon cold acclimation and thus the plasma membrane became phospholipid enriched.

When a comparison was made with plasma membranes isolated from cold acclimated seedlings, a difference appeared in the polypeptide compositions (Fig. 4). In the present study, there was no serious problem in the separation of the individual polypeptides, unlike the results reported by Booz and Travis (5). After several repetitions, the elctrophoretograms gave nearly the same number and concentration of individual polypeptides. As the cold hardiness increased from −7.8 to −18°C, some polypeptides were decreased in the contents or disappeared; conversely, some new polypeptides appeared. Thus, it is quite likely that the plasma membrane is in a dynamic state, responding to an environmental stress and also to the developmental growth stages.

DISCUSSION

Recently, isolation of plasma membranes from higher plants using an aqueous two-polymer phase system have been achieved in two laboratories (25, 26, 31). The method is basically dependent on the unique surface properties of plasma membranes. Especially, our technique of using a phase partition system containing NaCl is more effective than other methods for the isolation of plasma membranes from a wide variety of plants, mostly light-grown green tissues. In the present study, the chemical composition of isolated plasma membrane was compared with other endomembranes like ER + Golgi and mitochondria. The plasma membrane isolated from orchard grass seedlings was relatively enriched in sterols (1.15 μmol/mg protein). The sterol to phospholipid molar ratio was also high (1.25). This value is comparable to the ratio reported for oat root plasma membrane (8), ranging from 1.07 to 1.21; however, it was considerably higher than the ratio reported for the plasma membrane-enriched fraction from soybean roots (0.29 to 0.78) (24). Sterols were also present in the ER + Golgi fraction; however, the content was relatively low in comparison with the plasma membrane. With other plasma membranes isolated in our laboratory from living bark tissues of mulberry trees (S. Yoshida, unpublished data), rye seedlings (M. Uemura and S. Yoshida, unpublished data), and Jerusalem artichoke tubers (M. Ishikawa and S. Yoshida unpublished data), the sterol to phospholipid molar ratio were observed to range from 0.40 to 1.20. Thus, the ratio is highly dependent on plant species, developmental stages, and possibly on purity of the preparations, and therefore the sterol-to-phospholipid molar ratio is limited in its use as a criterion for identifying plasma membrane from higher plants. In the present study, steryl glycosides were confirmed to be one of the lipid components of plasma membrane of orchard cells, although the content was relatively low in comparison with the content of free sterols. On the other hand, in mulberry plasma membrane, nearly

half the amount of total sterols was comprised of glycosylated sterols (S. Yoshida, unpublished data), therefore the degree of the glycosylation is also dependent on plant species.

The degree of unsaturation of phospholipids was quite high in mitochondrial membranes in comparison with those of plasma membrane and ER + Golgi membranes. The difference in the relative fluidity observed in various types of membranes seems to be related to the sterol to phospholipid ratios and also to the degree of unsaturation of phospholipids in the membranes. The low fluidity of plasma membranes corresponds to the high amount of sterols and relatively low degree of unsaturation.

Electrophoretic analysis of the polypeptides solubilized and denatured from three different membranes revealed marked differences both in the total number and the quality. After two dimensional electrophoresis, it was difficult to detect any polypeptides as being common to all membrane components, especially the major polypeptides. It is also stressed that the composition of glycopeptides were quite different between plasma membrane and ER + Golgi membranes, and that the later membranes were relatively enriched in the glycopeptides with relatively low mol wt. Due to the specificity of Con A binding to the sugar residues, *i.e.* glucose/mannose, a question still remains as to the existence of the other glycopeptides containing other sugar residues such as galactose and *N*-acetyl-β-D-glucosamine. Keegstra and Cline (10) have recently reported that microsomal membranes from pea contain glycopeptides which are accessible to Con A but not to other lectins, such as wheat germ agglutinin and *Ricinum communis* agglutinin.

Our major interest has been concerned with the nature of plasma membrane alterations in response to freezing stress. It is generally considered that plasma membrane has a primary role in determining the freezing injury of plant cells (21). In the present study, changes in the polypeptide complements and the lipid composition were examined. As the cold hardiness increased, some changes in the polypeptide composition both in quality and quantity were observed. Booz and Travis (5) have demonstrated that plasma membranes of soybean roots undergo a significant change in the polypeptide composition during the developmental process. Based on these facts, it may be stated that plant plasma membranes are in a dynamic state, changing their proteins in response to an environmental adaptation of cells and the developmental process.

In many studies relating lipid changes to cold hardiness, total lipid extracts from whole tissues were investigated rather than the plasma membrane which is considered to be the primary site of freezing injury (21). In orchard plasma membrane, only small changes in fatty acid unsaturation and sterol content were observed. The phospholipid-to-protein ratio, however, increased by about 25% as the cold hardiness increased. Similar results also have been obtained in plasma membrane-enriched fractions of mulberry bark tissues (30), winter rye seedlings (25), and Jerusalem artichoke tubers (M. Ishikawa and S. Yoshida, unpublished data).

Sugawara and Sakai (23) have demonstrated by the freeze-fracture method that the inner membrane particles of the plasma membrane, especially on fracture face E, were reduced markedly after hardening of calli of Jerusalem artichoke tubers occurred. Parish (18) has also reported that the inner membrane particles of plasma membranes increased dramatically from winter to spring in cambial cells of *Salix*. The results obtained in the present study may give one of the biochemical bases for explaining those reported morphological changes in plasma membranes during cold acclimation and deacclimation in terms of an alteration in protein concentrations of plasma membranes. Further detailed studies, however, are needed to clarify the causal relation between the development of cold hardiness and those changes observed in the plasma membranes.

In conclusion, the phase partition technique for preparation of pure plasma membranes and more detailed analysis of the isolated membrane components may provide valuable information with respect to the physiological responses of plants to environmental stresses, especially to cold stress.

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