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Chemical and Biophysical Changes in the Plasma Membrane during Cold Acclimation of Mulberry Bark Cells (*Morus bombycis* Koidz. cv Goroji)[1]

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ABSTRACT

The lipid and protein composition of the plasma membrane isolated from mulberry (*Morus bombycis* Koidz.) bark cells was analyzed throughout the cold acclimation period under natural and controlled environment conditions. There was a significant increase in phospholipids and unsaturation of their fatty acids during cold acclimation. The ratio of sterols to phospholipids decreased with hardiness, primarily due to the large increase in phospholipids. The fluidity of the plasma membrane, as determined by fluorescent polarization technique, increased with hardiness. Electrophoresis of plasma membrane proteins including glycoproteins revealed change in banding pattern during the early fall to winter period. Some of the protein changes could be related to growth cessation and defoliation. However, minor changes in proteins also occurred during the most active period of hardening. Changes in glycoproteins were coincident both with changes in growth stages and with the development of cold hardiness.

Following a lethal frost, there is often a loss in the semipermeability properties of the plasma membrane in plants. This had led to the belief that the plasma membrane is the primary site of freezing injury (9, 12, 18). Most perennial plants growing in temperate climates develop some degree of cold hardiness with the onset of winter. It follows, therefore, that during cold acclimation the plasma membrane may be altered in order to tolerate extracellular freezing.

Many plants capable of cold hardening exhibit an increase in lipid unsaturation (24) and an increased level of phospholipids during hardening (6, 16, 17, 23, 26, 29). This has led to the speculation that the enhanced fluidity of the membrane by lipid unsaturation is related to cold hardiness. However, no preferential increase in phospholipid unsaturation could be detected in black locust (17) and poplar (29) which are both extreme cold hardy species. These findings have led to the conclusion that membrane unsaturation or fluidity is not related to the development of cold hardiness (6, 14, 17).

In many of the studies relating lipid unsaturation to hardiness, total lipids were investigated rather than the plasma membrane which is considered to be the primary site of injury. This has been primarily due to the lack of methods to isolate sufficient quantities of pure plasma membrane from plant tissues. Recently, we have developed a method of isolating plasma membrane from plant tissues utilizing an aqueous PEG-dextran two-polymer phase system containing NaCl (20, 30). In this report, we followed the changes in lipids, proteins, and fluidity in plasma membranes of mulberry trees during cold acclimation.

MATERIALS AND METHODS

Plant Materials. Living bark tissues from current twigs of mulberry trees (*Morus bombycis* Koidz. vc Goroji) were used in the present study. Frost hardiness was evaluated by measuring the electrolyte leakage after freeze thawing of the tissues. Living bark tissues (500 mg) were cut into pieces (1.0 × 0.5 cm) and frozen in test tubes (1.5 × 15 cm) at −3°C for 2 h with small pieces of ice. Thereafter the tissues were cooled by 5°C increments at 1-h intervals. After holding at the desired temperature for 16 h, the frozen tissue pieces were then thawed at 0°C. Upon thawing, the tissues were immersed in 5 ml of distilled H_2O and incubated at 25°C for 4 h with a gentle shaking and subjected to measurement of the conductivity. Cold hardiness was expressed as the temperature which resulted in a 50% leakage as compared with completely killed tissues by fast freezing in liquid nitrogen.

Isolation of Plasma Membranes. The living bark tissues were removed from the twigs, cut into small pieces and homogenized for 75 s at 0°C with a polytron PT20 at the medium speed setting. The homogenizing medium contained 0.5 M sorbitol, 100 mM Tris-HCl, 5 mM EGTA neutralized with Tris, 3% PVP (mol wt 24,500), 0.5% defatted-BSA, 2.5 mM potassium metabisulfite, 2 mM SHAM[2], and 1 mM PMSF (pH 7.8). Sixty g of tissues were homogenized in 300 ml of the above medium. The homogenate was squeezed through four layers of gauze and then through two layers of 'Miracloth', and then subjected to differential centrifugation successively at 3,600*g* for 10 min and 156,000*g* for 20 min. The 3,600*g* pellet was discarded and the 156,000*g* pellet was used for the isolation of the plasma membrane. The 156,000*g* pellet was washed once by suspending in 0.5 M sorbitol-10 mM K-phosphate (pH 7.8) and then centrifuged at 156,000*g* for 20 min. The pellet was resuspended in the same buffer system used for washing. The membrane suspension was added to a phase system containing 5.6% (w/w) of Dextran T500 and 5.6% (w/w) of PEG 4000 made up in 0.5 M sorbitol, 10 mM K-phosphate (pH 7.8) and 30 mM NaCl. The phase mixture containing the membrane suspension was thoroughly mixed by several inversions and centrifuged at 400*g* for 3 min to hasten the phase setting. All procedures were done at 0°C. The upper phase was removed and subjected to a repartition by mixing with a newly synthesized lower phase. The upper phase after the repartition was highly enriched in plasma membranes.

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[2] Abbreviations: SHAM, salicylhydroxamic acid; PMSF, phenylmethylsulfonyl fluoride; PEG 4000, polyethyleneglycol (mol wt 3,340); BHT, butylated hydroxytoluene.

FIG. 1. Electron micrographs of isolated plasma membranes. Plasma membranes were isolated from mulberry twigs sampled on September 7 (1) and on December 28 (2). A and B refer to the conventional stain with uranyl acetate-lead citrate (× 15,000) and with periodic acid-chromic acid-phosphotungstate (× 15,000), respectively.

Table I. *Distribution of Various Enzyme Markers after Phase Partitioning of Crude Membranes in 5.6% Dextran and 5.6% PEG*

Crude membranes were prepared from living bark tissues of mulberry trees (240 g fresh wt) and subjected to phase partition system as described in "Materials and Methods." Parentheses indicate per cent recovery of total ezyme activity in each fraction.

	Protein	Mg^{2+}-ATPase	Acid Phosphatase	NADH Cyt Red	IDPase	Cyt *c* ox
	mg	*µmol/h*		*µmol/10 min*	*µmol/h*	*µmol/10 min*
Upper phase (plasma membrane)	5.6 (9.5)	34.4 (19.5)	12.5 (9.9)	1.2 (3.0)	4.6 (13.6)	0 (0)
Lower phase (mixture of endomembranes)	52.8 (90.5)	141.7 (80.5)	113.6 (90.1)	41.5 (97.0)	29.0 (86.4)	72.6 (100)

Lipid Extraction from Membranes. Total lipids were extracted from membrane samples according to Bligh and Dyer (3) except 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 of Kieselgur Type 60, 70–230 mesh; Merck). Elution of each lipid class was performed as reported previously (31).

Quantitative analysis of phospholipids was performed according to Marinetti (10) with a slight modification (29). Quantitative analysis of sterols were performed according to Zlatkis *et al.* (32). Cholesterol was used as the standard. Sterylglycosides were analyzed using the anthrone method (2). Briefly, the glycosides were dissolved in 20 µl of methanol, added to 3 ml of 2% anthrone in 70% H_2SO_4, and then 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 a 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 a solvent mixture of chloroform-methanol-acetic acid (65:25:8, v/v/v).

Enzyme Assays. Activities of Mg^{2+}-ATPase, antimycin A-insensitive NADH Cyt *c* reductase, Cyt *c* oxidase, acid phosphatase, and IDPase were assayed as reported earlier (30). Protein was quantified by the Coomassie Brilliant Blue-dye binding technique of Bradford (4).

SDS Slab PAGE of Plasma Membrane Proteins. A plasma membrane preparation equivalent to 500 µg of protein was resuspended in 0.2 M KCl, 10 mM Tris-maleate (pH 7.3), 1 mM EDTA, 10 µg/ml of BHT, and centrifuged at 105,000*g* for 40 min. The pellet was suspended in 10 mM Tris-HCl (pH 6.8), 10 µg/ml of BHT, and recentrifuged at the same speed as described

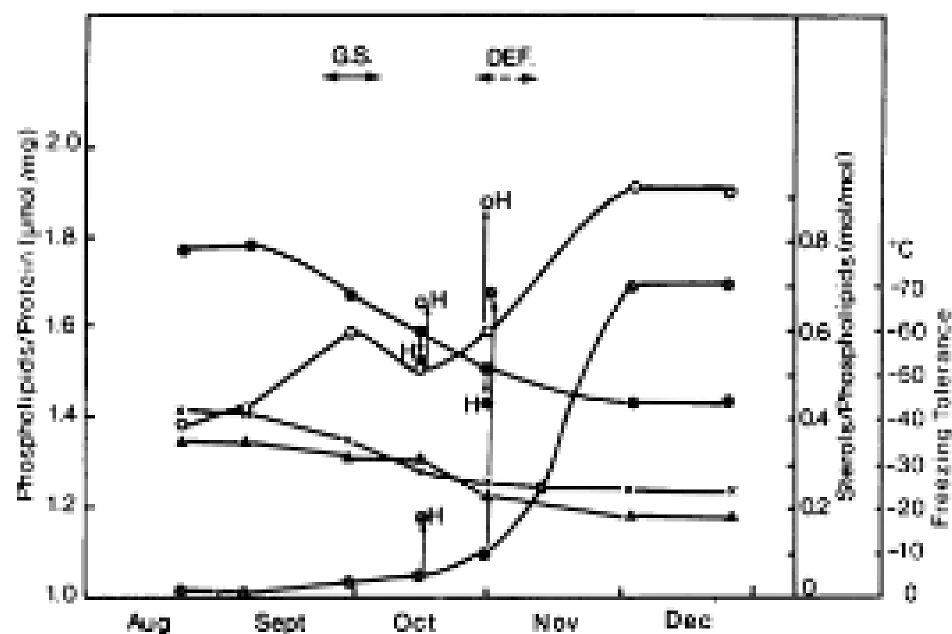

FIG. 2. Seasonal changes in phospholipids and sterols in mulberry plasma membranes. (○——○), µmol phospholipid/mg membrane protein; (▲——▲), molar ratio of free sterols to phospholipids; (×——×), molar ratio of steryl glycosides to phospholipids; (●——●), molar ratio of total sterols to phospholipids; (**O**——;**O**), freezing tolerance. H corresponds to the values after contolled hardening of excised twigs at 0°C for 3 weeks. G.S. and DEF represent the periods for growth cessation and defoliation, respectively.

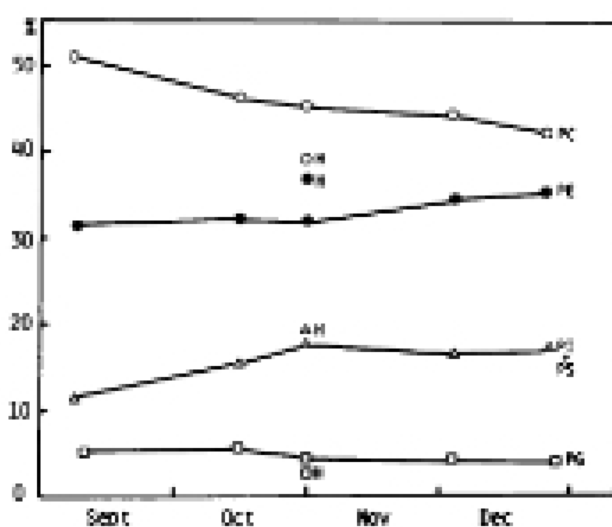

FIG. 3. Seasonal changes in phospholipid composition of mulberry plasma membranes. (○——○), phosphatidyl choline (PC); (●——●), phosphatidyl ethanolamine (PE); (□——□), phosphatidyl glycerol (PG); (△——△), phosphatidyl inositol (PI) plus phosphatidyl serine (PS). H corresponds to the values after controlled hardening at 0°C for 3 weeks.

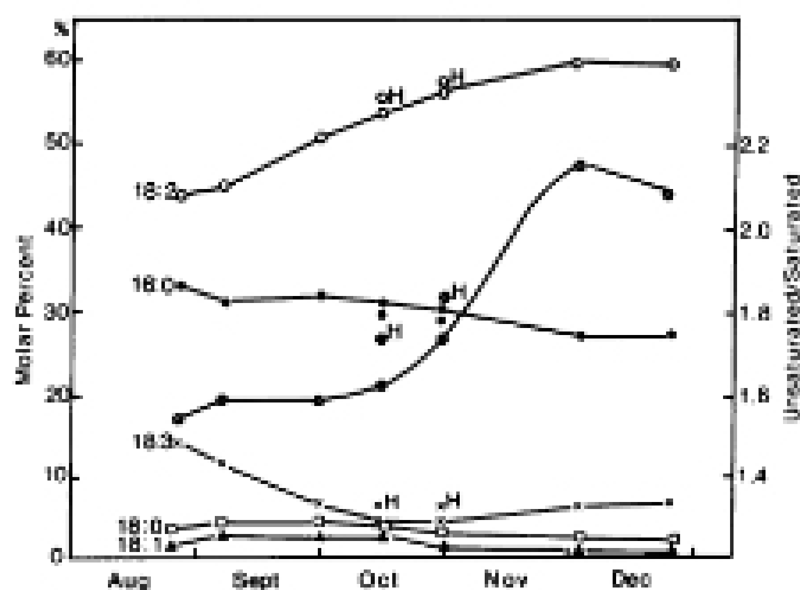

FIG. 4. Seasonal changes in fatty acid composition of plasma membrane phospholipids. (●——●), palmitate; (□——□), stearate; (▲——▲), oleate; (○——○) linoleate; (×——×), linolenate. (**O**——**O**), unsaturated/saturated ratio (18:1 ± 18:2 + 18:3) / (16:0 + 18:0 + 20:0). H refer to Figures 1 and 2.

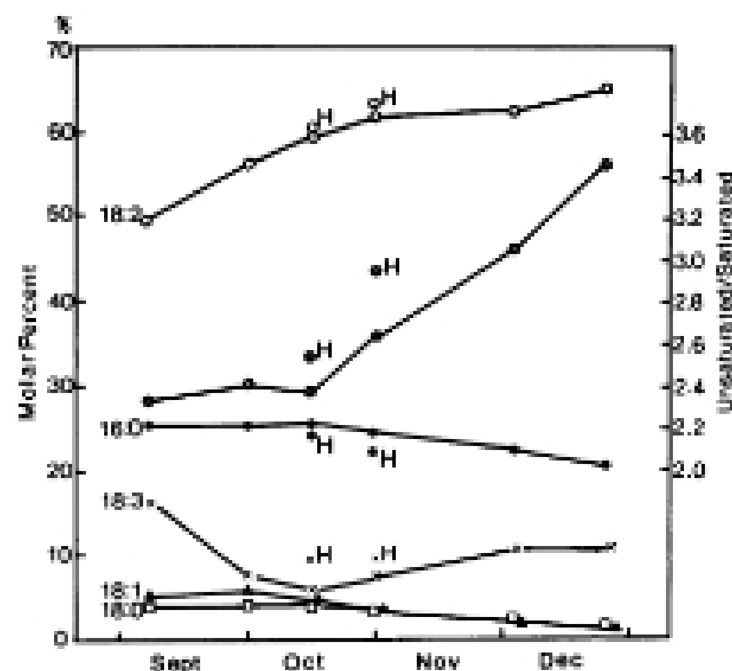

FIG. 5. Seasonal changes in fatty acid compositions of phospholipids in endomembrane mixtures. Endomembrane mixtures were recovered from the lower phase of the phase partitioning system. Marks refer to Figure 4.

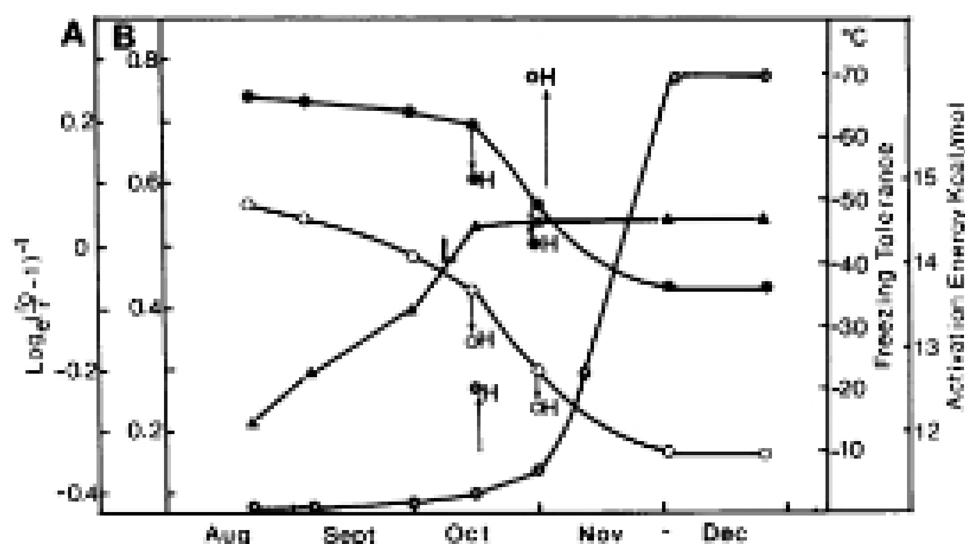

FIG. 6. Changes in relative fluidity of mulberry plasma membranes with season. (●——●) and (○——○), plasma membrane fluidities at 5 (scale B) and 25°C (scale A), respectively; (▲——▲), activation flow energies of anisotropy parameter values after the Arrhenius plots; (**O**——**O**), freezing tolerance. Marks H refer to Figures 1 to 4.

above. The membrane pellets were solubilized in 250 µl of 0.0625 M Tris-HCl (pH 6.8), 2% SDS, 10% glycerol (w/v), 5% 2-mercaptoethanol, and 0.001% bromophenol blue by heating for 3 min in a boiling water bath.

The discontinuous SDS buffer system of Laemmli (8) was used for the SDS-PAGE slab. A 8.5% SDS-PAGE slab, 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 (TI), mol wt 21,500; albumin from bovine serum, mol wt 68,000; RNA-polymerase from *E. coli* (core enzyme), α, β, γ subunits; mol wt 39,000, 155,000, and 165,000 D, 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 (25) was used.

For the analysis under relatively mild denaturing conditions, the plasma membrane proteins were solubilized in 0.1% SDS, 0.0625 mM Tris-HCl (pH 7.3), 10% glycerol at 0°C for 1 h. Electrophoresis was performed at 6°C with continuous buffer system containing 0.1% SDS at a constant current of 5 mamp.

Fluorescence Polarization Measurement. The fluorescent hy-