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

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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 hardness, primarily due to the large increase in phospholipids. The fluidity of the plasma membrane, as determined by fluorescent polarization technique, increased with hardness. 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. cv 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 × 1.5 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 H2O 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 mM 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 SHAM2, 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 3,600g for 10 min and 156,000g for 20 min. The 3,600g pellet was discarded and the 156,000g pellet was used for the isolation of the plasma membrane. The 156,000g pellet was washed once by suspending in 0.5 M sorbitol-10 mM K-phosphate (pH 7.8) and then centrifuged at 156,000g 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 400g 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.

Abbreviations: SHAM, salicylhydroxamic acid; PMSF, phenylmethylsulfonyl fluoride; PEG 4000, polyethylene glycol (mol wt 3340); BHT, butylated hydroxytoluene.

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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 x 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. Sterolglycosides 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% H2SO4, 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 Mg2+-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 105.000g 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

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**Table 1. Distribution of Various Enzyme Markers after Phase Partitioning of Crude Membranes in 5.6% Dextran and 5.6% PEG**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Mg2+-ATPase</th>
<th>Acid Phosphatase</th>
<th>NADH Cyt Red</th>
<th>IDPase</th>
<th>Cyt c ox</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µmol/h</td>
<td>µmol/10 min</td>
<td>µmol/h</td>
<td>µmol/10 min</td>
<td></td>
</tr>
<tr>
<td>Upper phase (plasma membrane)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5.6 (9.5)</td>
<td>34.4 (19.5)</td>
<td>12.5 (9.9)</td>
<td>1.2 (3.0)</td>
<td>4.6 (13.6)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Lower phase (mixture of endomembranes)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>52.8 (90.5)</td>
<td>141.7 (80.5)</td>
<td>113.6 (90.1)</td>
<td>41.5 (97.0)</td>
<td>29.0 (86.4)</td>
<td>72.6 (100)</td>
</tr>
</tbody>
</table>

**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.
brane phospholipids.

unsaturated/saturated ratio refers to the values after controlled hardening of excised twigs at 0°C for 3 weeks. G.S. and DEF represent the periods for growth cessation and defoliation, respectively.

FIG. 2. Seasonal changes in phospholipids and sterols in mulberry plasma membranes. (■—■), mol ratio of free sterols to phospholipids; (▲—▲), molar ratio of steryl glycosides to phospholipids; (●—●), molar ratio of total sterols to phospholipids; (○—○), freezing tolerance. H corresponds to the values after controlled hardening of excised twigs at 0°C for 3 weeks.

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; (○—○), unsaturated/saturated ratio (18:1 ± 18:2 + 18:3) / (16:0 + 18:0 + 20:0). H refers 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°C (scale B) and 25°C (scale A), respectively; (▲—▲), activation flow energies of anisotropy parameter values after the Arrhenius plots; (●—●), 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 200 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.

Fluorescence Polarization Measurement. The fluorescent hy-
drocarbon 1,6-diphenyl-1,3,5-hexatriene (DPH) was used as a probe for monitoring the relative fluidity of the isolated plasma membranes (28). Final concentration of the fluorophore was $10^{-4}$ M/100 $\mu$g of membrane protein in 0.5 M sorbitol-5 mM K-phosphate (pH 7.3). The steady state fluorescence polarization ratio was expressed as the fluorescence anisotropy, $r$, and as the anisotropy parameter, $[(r_l/r)-1]^{-1}$, as reported previously (28). The anisotropy parameter is inversely correlated with the relative fluidity of the membrane lipid-bilayers.

**Electron Microscopy.** Plasma membrane fractions were fixed in 3% glutaraldehyde in 0.5 M sorbitol-66 mM K-phosphate buffer (pH 7.3) for 1 h at 0°C and 1 h at 21°C. Fixed membrane materials were pelleted by centrifugation, washed in cold buffer solution, post-fixed in cold 2% OsO$_4$, and dehydrated in an increasing concentration of ethanol and finally in n-butylglycidyl ether. The dehydrated pellets were embedded in epoxy resin and sectioned. The sections were stained either with uranyl acetate-lead citrate solution or with periodic acid-chromatic acid-phosphotungstic acid (PACP-staining) (11) and viewed with a JEM 100C electron microscope as reported before (20, 30).

**RESULTS**

**Assessment of Purity of Isolated Plasma Membranes.** According to our previous studies (20, 30), plasma membranes can be identified by the following criteria: (a) PACP staining, (b) specific NPA (N-Naphthalpylmalic acid) binding capacity, (c) specific partitioning into the PEG-enriched upper phase in an aqueous two-polymer phase system containing NaCl, (d) stability in the presence of Zn$^{2+}$ ions and at a pH less than 5, (e) orthovanadate sensitive Mg$^{2+}$-ATPase.

The plasma membranes isolated from living bark cells of mulberry trees were preferentially partitioned into the upper PEG-enriched phase in the aqueous two-polymer phase system and stained positive with PACP-staining. As presented in Figure 1, electron micrographs of plasma membrane samples isolated from living bark cells of mulberry trees collected in different seasons revealed a high proportion of PACP-stained smooth vesicles, indicating high purity of plasma membranes. The specificity of the PACP-staining for the plasma membranes was also determined on intact cortical parenchyma cells of mulberry tree (data not shown).

No aggregation was observed in the presence of more than 5 mM ZnCl$_2$ or at a pH lower than 5. This membrane fraction also contained an orthovanadate sensitive Mg$^{2+}$-ATPase with an optimum pH at 7 (about 50% inhibition by 50 $\mu$M of vanadate). Endomembranes such as chloroplasts, mitochondria, and ER were preferentially partitioned into the lower phase and readily aggregated in the presence of 5 mM ZnCl$_2$ and at a pH less than 5.

As shown in Table I, most of the NADH Cyt reductase, Cyt oxidase, and IDPase preferentially partitioned into the lower phase after phase partitioning of the crude membranes (3,500–156,000 g pellets) in an aqueous PEG-dextran polymer two phase system. About 20% of the total ATPase activity in the crude membranes was recovered in the upper phase. The specific ATPase activity in the upper phase was 2 times higher than that in the lower phase. Most of the ATPase activity which partitioned into the lower phase does not appear to be of plasma membrane origin, based on the amount of phosphotungstic acid-CrO$_3$ positive vesicles in this fraction (data not shown). According to prior studies using seedlings of orchard grass (30) and winter rye (20), considerable amounts of ATPase were not associated with the plasma membrane partitioned into the lower phase. Based on the above evidence, it appears that the plasma membranes iso-
related from mulberry trees are of high purity.

**Lipid Composition.** The seasonal fluctuations in phospholipids and sterols in mulberry plasma membranes are presented in Figure 2. Total phospholipid content per mg protein increased dramatically from summer to winter. A small peak in late September was observed at the time the trees stopped growth. The increase in plasma membrane phospholipid content from October to December was closely associated with the development of cold hardness. A significant increase in the phospholipid content was also demonstrated after controlled hardening at 0°C for 3 weeks of excised twigs collected in middle and late October.

Plasma membranes isolated from mulberry twigs were highly enriched in sterols, especially during the growing season (Fig. 2). The free and glycosylated sterols occurred in roughly equal amounts throughout the period studied. The total amount of sterols on a mg protein basis was relatively stable until mid-September, and thereafter declined by about 20% during the period of cessation of growth and defoliation. As a result of the reversed changes in quantities of sterols and phospholipids, the ratio of sterols to phospholipids decreased from early September to December.

The seasonal changes in plasma membrane phospholipid composition are shown in Figure 3. The major phospholipids were phosphatidyl choline and ethanolamine with phosphatidyl glycerol in lesser amounts. Phosphatidyl choline declined linearly from September to December while phosphatidyl ethanolamine conversely increased during this period. Phosphatidyl serine and phosphatidyl inositol increased from summer to late October and then plateaued. There were no detectable changes in phosphatidyl glycerol during this period of study.

The seasonal changes in fatty acid composition of phospholipids from plasma membranes and endomembranes are shown in Figures 4 and 5, respectively. Plasma membrane phospholipids are characterized by a relatively lower level of unsaturation as compared to the endomembranes. In summer-collected twigs, the ratio of unsaturated to saturated fatty acids extracted from endomembranes was high, being comparable to the ratio for plasma membranes isolated from winter twigs. The content of linoleate increased linearly from early autumn to mid winter in both membranes while the palmitate content decreased gradually towards winter. The content of linolenate in both membranes decreased from summer to late autumn, reached a minimum level by the middle of October and increased towards winter. The ratio of unsaturated to saturated fatty acids in both membranes increased abruptly from the middle of October to December as the cold hardness increased. After controlled hardening of excised twigs at 0°C for 3 weeks, the ratio of unsaturated to saturated fatty acids increased slightly.

**Fluidity Changes.** The seasonal changes in the relative fluidity of the plasma membranes were monitored by determining the fluorescent polarization ratio of the embedded hydrocarbon fluorophore, DPH (Fig. 6). The anisotropy parameter, \((r_s/r-1)^{-1}\),

![Figure 8. Electrophoretograms of plasma membrane polypeptides. Plasma membranes were solubilized in a buffer system containing 2% SDS, 5% β-mercaptoethanol, 10% glycerol, and 0.0625 M Tris-HCl buffer (pH 6.8) with heating for 3 min. The solubilized and denatured polypeptides were electrophoresed on a slab gel with discontinuous 0.1% SDS-buffer system. Polypeptides were visualized with Coomassie blue stain and destain procedure (6). Lanes I to VI correspond to sampling date, August 27, September 7, 30, October 15, 30, and December 25, respectively.](image-url)
calculated from the polarization ratio, is inversely correlated with the relative fluidity of membrane bilayers. The fluidity was dependent on temperature, i.e. the lower the temperature, the lower the fluidity. The fluidity of the plasma membranes increased abruptly from the middle of October to December in parallel with the increase in cold hardiness. If partially hardened excised twigs were cold hardened under controlled conditions, there was a concomitant increase in the fluidity. The activation flow energies calculated from the Arrhenius plots of the anisotropy parameter values increased from August to the middle of October and then reached a plateau.

**Electrophoresis of Proteins.** The electrophoretograms of plasma membrane proteins solubilized with 0.1% SDS at 0°C without the addition of SH reducing agents is shown in Figure 7. Bands 7, 9, 10, and 11 increased abruptly from middle to late October. A significant difference in the electrophoretic patterns was observed before and after late October. A gradual increase in band 5 was also noted from September to December. In December, remarkable increase in high mol wt proteins, bands 1 and 2, were observed. Band 7 increased in staining intensity with Coomassie blue from the middle of October to December, while bands 9, 10, and 11 decreased from late October to December. Band 5 steadily increased in intensity from September to December. Bands 1 and 2 were most prominent in samples collected in December and also after controlled hardening of excised twigs at 0°C for 3 weeks. In samples collected on September 7, two or more bands located near band 1 and 2 were evident. However, in later sampling dates these bands were no longer detectable.

Electrophoresis of plasma membrane proteins solubilized by heating in the presence of 2% SDS and 5% mercaptoethanol revealed approximately 30 bands (Fig. 8). The relative intensities of the bands were determined by densitometry (Fig. 9). Bands D (74 kD), G (61 kD), H (53 kD), J (45 kD), K (42 kD), L (36 kD), N (34 kD), and R (26 kD) were relatively stable throughout the season, whereas bands B (100 kD), E (71 kD), F (66 kD), I (50 kD), and M (35 kD) decreased in intensity of staining with Coomassie blue after late September. The lower mol wt bands S (25 kD), T (23 kD), and U (20 kD) increased from fall to winter. More than twenty glycopeptide bands were visualized by the Con-A-peroxidase method (19) (Fig. 10). According to densitometric analysis, bands d (110 kD), c (100 kD), g (85 kD), and h (65 kD) decreased from August to late September at the time of growth cessation (Fig. 11). After the time of growth cessation, there was a marked decrease in bands a (200 kD), b (185 kD), i (60 kD), m (31 kD), and n (30 kD). On the last sampling date, December 25, the glycopeptide band f (90 kD) and j (55 kD) appeared to increase as compared to the September 8 to October 30 samples, while the other glycopeptides decreased, especially in bands d, m, and n.

**DISCUSSION**

Many of the theories on cold acclimation and cold injury are in one way or another centered on membranes, primarily the plasma membrane. Due to the difficulty in extracting sufficient quantities of highly pure plasma membranes for chemical analysis, previous studies have relied on total lipid extracts or total membrane preparations. In the present study, a plasma membrane-enriched fraction of high purity was obtained from living bark tissues of mulberry trees using an aqueous two-polymer phase system containing NaCl (20, 30). The plasma membrane-enriched fraction after repartition was identified by PACP staining, orthovanadate-sensitive MgATPase, and stability in the presence of Zn$^{2+}$ ion and at a pH less than 5.0 (20, 30), and was found to be in a high purity.

The total lipids of plant membranes during cold acclimation have been shown to become more unsaturated; however, there does not appear to be a simple relationship between lipid composition and cold hardiness (14, 24). No change was detected in the fatty acid composition and sterol content of plasma membranes extracted from cold acclimating seedlings of orchard grass (31), winter rye (21), and tubers of Jerusalem artichoke (Ishikawa and Yoshida, unpublished). However, there was a marked increase in the phospholipid content during the hardening period which was closely associated with the development of cold hardiness. In the present study using mulberry trees, which are distinctly more cold hardy than the above herbaceous tissue, there were significant changes during cold acclimation in the fatty acid composition of plasma membrane phospholipids. From August to the beginning of October, there was a marked increase in linoleate and a marked decrease in linolenate with little change in cold hardness. During this period, palmitate remained relatively constant. Since these are the three major fatty acids found in the plasma membranes of mulberry trees, the ratio of unsaturated to saturated fatty acids did not change during this period. After growth cessation, there was a dramatic increase in cold hardness. During this period, the content of linolenate continued to increase while the content of palmitate started to decrease and linolenate increased slightly. This resulted in a large increase in the ratio of unsaturated to saturated fatty acids. An analogous situation occurred in the endomembranes which partitioned into the lower phase.

After controlled hardening at 0°C of excised twigs in October, the ratio of unsaturated to saturated fatty acids increased to a lesser extent compared with naturally hardened trees cold hardy to the same temperature. The results suggest that, in part, the degree of lipid unsaturation may depend upon the growing temperature.

The plasma membranes of mulberry trees were highly enriched in sterol compounds, particularly during the active growing season. Free sterols and glycoside sterols fluctuated similarly during the period of study. Sterols, expressed on a mg protein basis, were relatively stable until mid September but had de-
creased approximately 20% by November and December. Due to a large increase in phospholipids from October to November, the sterol to phospholipid ratio decreased with time.

Changes in phospholipids in plasma membranes closely paralleled changes in cold hardiness. There was also a dramatic increase in phospholipids in excised twigs hardened at 0°C for 3 weeks. An enrichment in total phospholipid content with increasing cold hardiness has been demonstrated in a wide range of plants (6, 16, 17, 23, 26, 29). Recently, we have demonstrated an increase in phospholipid during cold acclimation in plasma membranes isolated from herbaceous tissue (21, 30). Thus, the phospholipid-enrichment in plasma membrane seems to be a general phenomena related to development of cold hardiness in plants. This fact may also be supported by alterations in the population of plasma membrane inner particles during cold acclimation and deacclimation of some plant species. In freeze fracture studies, Sugawara and Sakai (19) showed that the inner membrane particles at the fracture face E were reduced after cold acclimation of Jerusalem artichoke callus. Parish (13) reported that there was an increase from winter to spring in the inner-membrane particles of plasma membrane of cambial-zone cells of Salix.

Vigh et al. (22) using ESR techniques, reported that the plasma membranes in protoplasts isolated from winter wheat leaves became more fluid with cold acclimation. In our study, a marked change in plasma membrane fluidity occurred with cold acclimation, as determined by fluorescent polarization techniques. The difference in fluidity between summer and winter plasma membranes of mulberry tissue is equivalent to about a 10°C in temperature. Although the change in fluidity from summer to the middle of October was small, the activation energies as calculated from the Arrhenius plots of the anisotropy parameters demonstrated a marked increase. This would suggest that qualitative alterations occur during this period which affect membrane fluidity. For example, changes in the ratio of sterols to phospholipids, changes in sterol composition and lipid-protein interactions would have affected the fluidity. Interestingly, in all the herbaceous plants we have tested so far, little or no change was
observed in this fluidity of the plasma membrane during cold acclimation (21, 31). In these herbaceous tissues, only minor changes in fatty acid composition occurred during cold acclimation.

Changes in plasma membrane proteins, particularly the glycoproteins, were observed during the early fall to winter season. Most of the polypeptide changes, however, occurred near the period of growth cessation. Since the plasma membrane is involved in cell wall biosynthesis, active transport and numerous other physiological functions, it is not too surprising that changes would occur as the tissue matures in the fall. These prominent changes in plasma membrane proteins during the early fall may in turn provide a reason why growth cessation is a prerequisite for the development of cold hardiness in woody species.

After growth cessation, certain polypeptide changes did occur in the plasma membrane, primarily in the high mol wt proteins. Significant changes in plasma membrane proteins during cold acclimation have also been observed in orchard grass (31), winter rye (21), and Jerusalem artichoke tubers (Ishikawa, Yoshida, unpublished data), although changes were slight in lipid components.

As reported earlier, a phase transition of plasma membrane bilayers under freezing temperatures was closely related to freezing injury of plant cells (28). As the hardness increased, the transition temperatures shifted downward. The transition, however, appeared to depend not on the lipids per se, but primarily on the membrane proteins (27, 28). In herbaceous plants which are not capable of cold hardening to the same level as extremely hardy woody species, such as mulberry tree, the transition temperature of plasma membrane would have been compensated both through quantitative and qualitative changes in the proteins and in turn changes in the protein-lipid interaction. In plant species with extreme hardness, lipid unsaturation and decrease in the relative amounts of sterols would have an additional role to determine the thermotropic properties of plasma membranes. Future work is required to establish how wide spread this is.

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