

Cold Acclimation of *Arabidopsis thaliana*¹

Effect on Plasma Membrane Lipid Composition and Freeze-Induced Lesions

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Maximum freezing tolerance of *Arabidopsis thaliana* L. Heyn (Columbia) was attained after 1 week of cold acclimation at 2°C. During this time, there were significant changes in both the lipid composition of the plasma membrane and the freeze-induced lesions that were associated with injury. The proportion of phospholipids increased from 46.8 to 57.1 mol% of the total lipids with little change in the proportions of the phospholipid classes. Although the proportion of di-unsaturated species of phosphatidylcholine and phosphatidylethanolamine increased, mono-unsaturated species were still the preponderant species. The proportion of cerebroside decreased from 7.3 to 4.3 mol% with only small changes in the proportions of the various molecular species. The proportion of free sterols decreased from 37.7 to 31.2 mol%, but there were only small changes in the proportions of sterylglucosides and acylated sterylglucosides. Freezing tolerance of protoplasts isolated from either nonacclimated or cold-acclimated leaves was similar to that of leaves from which the protoplasts were isolated (−3.5°C for nonacclimated leaves; −10°C for cold-acclimated leaves). In protoplasts isolated from nonacclimated leaves, the incidence of expansion-induced lysis was ≤10% at any subzero temperature. Instead, freezing injury was associated with formation of the hexagonal II phase in the plasma membrane and subtending lamellae. In protoplasts isolated from cold-acclimated leaves, neither expansion-induced lysis nor freeze-induced formation of the hexagonal II phase occurred. Instead, injury was associated with the “fracture-jump lesion,” which is manifested as localized deviations of the plasma membrane fracture plane to subtending lamellae. The relationship between the freeze-induced lesions and alterations in the lipid composition of the plasma membrane during cold acclimation is discussed.

Membrane destabilization resulting from freeze-induced dehydration is the primary cause of freezing injury in plants (Steponkus, 1984; Steponkus and Webb, 1992). Although all cellular membranes are vulnerable to freeze-induced destabilization, the plasma membrane is of primary importance because of the central role that it plays during a freeze/thaw cycle. During cold acclimation, the cryostability of the plasma membrane is increased; in part, this is a consequence of alterations in its lipid composition

that alter its lyotropic (dehydration-induced) phase behavior. Therefore, the mechanistic significance of changes in membrane lipid composition, ideally at the molecular species level, should be assessed from a perspective of their effect on the lyotropic rather than the thermotropic phase behavior or fluidity of the plasma membrane.

We have previously demonstrated that there are several alterations in the lipid composition of the plasma membrane of winter rye leaves during cold acclimation (Lynch and Steponkus, 1987; Uemura and Steponkus, 1994). The most pronounced changes are (a) an increase in the proportion of PL as a consequence of increases in the proportion of di-unsaturated species of PC and PE and (b) a decrease in the proportion of CER. The increase in the proportion of PL occurs during the first 7 to 10 d of cold acclimation, whereas CER decrease progressively during 4 weeks of cold acclimation, after which winter rye leaves attain the maximum freezing tolerance.

Alterations in the plasma membrane lipid composition during cold acclimation are associated with alterations in the cryobehavior of the plasma membrane (Steponkus et al., 1990, 1993; Steponkus and Webb, 1992). For example, during freeze-induced osmotic contraction, the surface area of the plasma membrane of NA protoplasts of rye is not conserved due to the formation of endocytotic vesicles (Dowgert and Steponkus, 1984). Sufficiently large area re-

Abbreviations: ACC protoplasts, protoplasts isolated from leaves of cold-acclimated seedlings; ASG, acylated sterylglucoside(s); CER, cerebroside(s) [the shorthand designations of molecular species of CER, $x:y(h)-x:y$, refer to (hydroxy)acyl and long-chain base moieties separated by a hyphen, and in both moieties, x represents the number of carbon atoms and y represents the number of double bonds]; d18:1, sphinganine (d, dihydroxy long-chain bases); d18:2, sphingadienine; FS, free sterol(s); H_{II}, hexagonal II phase; ILA, interlamellar attachment(s); IMI, inverted micellar intermediate(s); IMP, intramembrane particles; NA protoplasts, protoplasts isolated from leaves of nonacclimated seedlings; PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PF fracture face, protoplasmic fracture face; PG, phosphatidylglycerol; PI, phosphatidylinositol; PL, phospholipids (the shorthand designations of molecular species of PL, $x:y/x:y$, refer to acyl moieties in the *sn*-1/*sn*-2 positions, x represents the number of carbon atoms and y represents the number of double bonds); PS, phosphatidylserine; SG, sterylglucoside(s); t18:0, 4-hydroxysphinganine (t, trihydroxy long-chain bases); t18:1, 4-hydroxysphinganine; T_{EL50}, temperature at which 50% electrolyte leakage occurred.

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ductions are irreversible and result in one form of injury referred to as expansion-induced lysis. In contrast, the plasma membrane of ACC protoplasts forms exocytotic extrusions during freeze-induced osmotic contraction, and the surface area is conserved such that expansion-induced lysis does not occur (Gordon-Kamm and Steponkus, 1984b). This difference in the cryobehavior of the plasma membrane is also observed with liposomes prepared from the total lipid extracts of the plasma membrane of nonacclimated and cold-acclimated rye leaves, suggesting that the differential cryobehavior of the plasma membrane during osmotic contraction is a consequence of alterations in the lipid composition of the plasma membrane (Steponkus and Lynch, 1989). Direct evidence for the involvement of lipid alterations in the transformation of the cryobehavior of the plasma membrane has come from membrane-engineering studies in which artificial enrichment of the plasma membrane of NA protoplasts of rye with mono- or di-unsaturated species of PC results in a transformation in the cryobehavior of the plasma membrane such that endocytotic vesiculation of the plasma membrane does not occur during freeze-induced osmotic contraction—instead, exocytotic extrusions are formed (Steponkus et al., 1988). This transformation results in an increase in freezing tolerance because of a decrease in the incidence of expansion-induced lysis (Steponkus et al., 1988; Uemura and Steponkus, 1989).

Similarly, alterations in membrane lipid composition during cold acclimation are also responsible for the decreased propensity for the freeze-induced formation of the H_{II} phase. In NA protoplasts and nonacclimated leaves, freeze-induced dehydration results in a lamellar-to- H_{II} phase transition in regions where the plasma membrane is brought into close apposition with subtending endomembranes; however, formation of the H_{II} phase does not occur in ACC protoplasts (Gordon-Kamm and Steponkus, 1984a) or cold-acclimated leaves (Webb and Steponkus, 1993). Severe dehydration results in formation of the H_{II} phase in liposomes formed from the total lipid extract of the plasma membrane of nonacclimated leaves but not in liposomes formed from the total lipids of the plasma membrane of cold-acclimated leaves (Cudd and Steponkus, 1988). In addition, artificial enrichment of the plasma membrane with di-unsaturated species of PC precludes the participation of the plasma membrane in the freeze-induced formation of the H_{II} phase (Sugawara and Steponkus, 1990). Collectively, these studies demonstrate that alterations in the lipid composition of the plasma membrane during cold acclimation are causally related to the increase in the cryostability of the plasma membrane of rye protoplasts.

Subsequent studies have demonstrated that the extreme difference in the freezing tolerance of winter rye and spring oat is associated with a vast difference in the lipid composition of the plasma membrane—both before and after cold acclimation (Uemura and Steponkus, 1994). In nonacclimated leaves, the plasma membrane of oat contains greater proportions of CER (27.2 mol% of total lipids in oat versus 16.4 mol% in rye) and ASG (27.3 mol% in oat versus 2.9 mol% in rye), and lower proportions of FS (8.4 mol% in oat

versus 38.1 mol% in rye) and PL (28.8 mol% in oat versus 36.6 mol% in rye). During cold acclimation, the proportion of di-unsaturated species of PC in the plasma membrane increases in both oat (from 33.4 to 44.2 mol% of total PC) and rye (from 40.4 to 53.5 mol%); there is a progressive decrease in the proportion of CER in the plasma membrane of rye but only a slight decrease in oat; and there are only small changes in the proportions of sterols (both free and glucosylated forms) in both oat and rye. As a result, after cold acclimation, the proportions of both CER (24.2 mol% in oat versus 10.5 mol% in rye) and ASG (22.0 mol% in oat versus 1.4 mol% in rye) remain substantially greater in oat than in rye.

The freeze-induced lesions of the plasma membrane of oat are identical to those of rye both before (expansion-induced lysis and the freeze-induced formation of the H_{II} phase) and after (fracture-jump lesion) cold acclimation, but they occur at significantly higher temperatures in oat (Webb et al., 1994). The increased propensity for the freeze-induced formation of the H_{II} phase in oat is associated with the high proportion of ASG, which more strongly increase the propensity for the lamellar-to- H_{II} phase transition in mixtures of dioleoylphosphatidylcholine and dioleoylphosphatidylethanolamine than do FS (Webb et al., 1995). In addition, lipid mixtures with a low PL:CER ratio undergo the lamellar-to- H_{II} phase transition at a lower osmotic pressure than mixtures with a high PL:CER ratio (Webb et al., 1992).

Recently, we have initiated studies on cold acclimation and freezing injury of *Arabidopsis thaliana*. Although *A. thaliana* is widely used in studies of gene expression and the synthesis of unique proteins during cold acclimation (Thomashow, 1993), there is very little information available on alterations in membrane lipid composition during cold acclimation. Because numerous lipid mutants of *A. thaliana* are available, *A. thaliana* is potentially an ideal species for further studies of lipid alterations during cold acclimation. The primary objective of the present study is to characterize the lipid composition of the plasma membrane isolated from leaves of *A. thaliana* before and after cold acclimation. Preliminary studies of freeze-induced lesions in the plasma membrane of *A. thaliana* are also presented to provide insight into the mechanistic significance of the changes in the lipid composition of the plasma membrane that occur during cold acclimation.

MATERIALS AND METHODS

Plant Materials

Seeds of *Arabidopsis thaliana* L. Heyn (Columbia) were purchased from Lehle Seeds (Tucson, AZ) and planted in moist Terra-Lite Metro-Mix 350 (A.H. Hummert Seed Co., St. Louis, MO), which contains nutrients. Seedlings were grown in a controlled-environment chamber at 23°C under continuous illumination (light intensity, 150 $\mu\text{E m}^{-2} \text{s}^{-1}$ at soil level). Plants were irrigated as necessary with water. Nonacclimated plants remained in this environment for 14 d. Cold acclimation was achieved by transferring 14-d-old plants to 2°C (8-h photoperiod, 125 $\mu\text{E m}^{-2} \text{s}^{-1}$ at soil level)

for up to 14 d. Leaves were excised at soil level and immediately used for experiments.

Plasma Membrane Isolation

Plasma membrane-enriched fractions were isolated using a two-phase partition system of PEG/dextran according to the procedure of Uemura and Yoshida (1983). Leaves were homogenized in a medium (4 mL/g fresh weight leaves) that consisted of 0.5 M sorbitol, 50 mM Mops/KOH (pH 7.6), 5 mM EDTA, 5 mM EGTA, 1.5% (w/v) PVP (molecular weight 40,000), 0.5% (w/v) defatted-BSA, 1 mM PMSF, 4 mM salicylhydroxamic acid, and 2.5 mM potassium metabisulfite with a Polytron (Brinkmann) at a medium speed setting for 30 to 45 s at 0°C. After homogenization, the homogenates were filtered through four layers of cheesecloth. The filtrates were successively centrifuged at 10,000g for 15 min and at 100,000g for 1 h. The microsomal fraction (10,000g to 100,000g fraction) was suspended in a solution of 0.25 M Suc and 10 mM $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ buffer (pH 7.8). The microsomal suspensions were then fractionated with an aqueous, two-polymer, phase-partition system consisting of 6.0% (w/w) PEG 3,350 (Sigma) and 6.0% (w/w) dextran T500 (Pharmacia) in a solution of 0.25 M Suc, 30 mM NaCl, and 10 mM $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$ buffer (pH 7.8). The two-phase partition was repeated three times at 0°C. The upper phase of the final two-phase system, which contained the plasma membrane vesicles, was recovered and diluted with a washing solution of 0.25 M Suc and 10 mM Mops/KOH (pH 7.3). After centrifugation at 156,000g for 30 min to remove the polymers, the plasma membrane-enriched pellet was suspended in the washing solution and immediately used for lipid extraction.

Marker enzyme activities in the microsomal and plasma membrane fractions were determined according to the methods of Uemura and Yoshida (1983) and Yoshida et al. (1986). The marker enzymes determined in this study were vanadate-sensitive ATPase for the plasma membrane, nitrate-sensitive ATPase for the tonoplast, Triton X-100-stimulated UDPase for Golgi bodies, Cyt *c* oxidase for mitochondria, and NADH Cyt *c* reductase for ER. Chl was quantified after extraction with 80% (v/v) acetone according to the method of Arnon (1949). Protein content was determined by a dye-protein binding method using BSA as a standard (Bradford, 1976).

Lipid Analysis

Lipids were extracted from the plasma membrane-enriched fractions according to the procedure of Bligh and Dyer (1959), with the modification that isopropanol was used instead of methanol to minimize phospholipase D activity during the extraction (Uemura and Yoshida, 1984). Total lipid extracts were separated into neutral lipids, glycolipids, and PL on Sep-Pak silica cartridges (Waters) by the procedure of Lynch and Steponkus (1987). Lipid extracts were dissolved in 2 mL of chloroform:acetic acid (100:1, v/v), transferred to the Sep-Pak cartridge, and sequentially eluted with 20 mL of chloroform:acetic acid (100:1, v/v) for neutral lipids, 10 mL of acetone and 10 mL

of acetone:acetic acid (100:1, v/v) for glycolipids, and 7.5 mL of methanol:chloroform:water (100:50:40, v/v/v) for PL. After the sequential elution was completed, 2.25 mL of chloroform and 3 mL of water were added sequentially to the phospholipid-containing eluate to effect a phase separation and facilitate recovery of PL. The recovery of each lipid after chromatography on the Sep-Pak column was determined to be nearly 100%. Separation of individual lipids was performed by TLC (Silica gel 60, 0.25-mm thickness, Merck, Darmstadt, Germany) with the following solvent mixtures: petroleum ether:ethyl ether:acetic acid (80:35:1, v/v/v) for neutral lipids, chloroform:methanol:water (65:25:4, v/v/v) for glycolipids, and chloroform:methanol:acetic acid (65:25:8, v/v/v) for PL. Individual lipids were identified by co-chromatography with authentic standards and the use of specific spray reagents (Kates, 1972).

Quantitative analyses of sterols were performed according to the method of Zlatkis and Zak (1969) using cholesterol as a standard. Lipid sugar content was quantified by the method of Dubois et al. (1956) using Glc as a standard. The amount of ASG and SG assayed by the separate determinations of sterol and sugar content were in good agreement. Lipid phosphorous was quantified according to the method of Marinetti (1962) using KH_2PO_4 as a standard. All procedures for the quantification of each lipid were performed after TLC in the presence of silica gel to minimize loss of lipids during extraction with organic solvent.

Molecular Species Analysis of Lipids

A molecular species analysis of PC and PE was carried out according to the method of Lynch and Thompson (1984). Diacylglycerols, which were obtained by treatment of PC and PE with phospholipase C, were analyzed by GC using a 12-m \times 0.25-mm SP-2330 fused silica capillary column (Supelco) at temperatures programmed from 190 (0.6 min holding) to 250°C at a rate of 20°C min⁻¹. The injector and detector were maintained at 270 and 300°C, respectively. Helium was used as a carrier gas with a split injection. Identification of molecular species was based on comparison of retention times with authentic standards and comparison to the relative retention time factors reported by Myher and Kuksis (1982).

Molecular species of CER were separated and identified by HPLC using a C₁₈ reverse-phase column (Cahoon and Lynch, 1991). Separation of intact, underivatized molecular species was achieved using a LiChrospher 100RP-18e column (25 cm \times 4 mm, 20% carbon bonding, 5- μ m particle size, Merck) and a mobile phase of acetonitrile:methanol (3:2, v/v) at a flow rate of 1.5 mL min⁻¹. CER that were eluted from the column were detected by UV absorption at 210 nm. Identification of CER molecular species was based on comparisons with the retention times reported in Cahoon and Lynch (1991) and Uemura and Steponkus (1994).

Determination of Freezing Tolerance of Leaves

Freezing injury of leaves was assessed by electrolyte leakage. For this, three to six leaves were placed in a test tube (10 \times 100 mm), and the samples were then cooled to

-2°C . After 1 h, ice formation was effected by introducing a small piece of ice into the test tubes. After an additional 2 h of incubation at -2°C , the samples were cooled in decrements of 1°C at 30-min intervals and kept at the specified temperatures for 1 h. After the samples were thawed overnight at 4°C and then incubated with 3 mL of distilled water at room temperature for 2 h, electrolyte leakage from the leaves was measured with a conductivity meter (model 32, Yellow Springs Instrument Co., Yellow Springs, OH). The solution was then removed and the leaves were frozen in liquid nitrogen for 30 min, thawed at room temperature, and then re-incubated with the original solution to obtain a value for 100% electrolyte leakage for each sample. The percentage of electrolyte leakage from leaves was determined by the ratio of electrolyte leakage to 100% electrolyte leakage.

Isolation and Determination of Freezing Tolerance of Protoplasts

Protoplasts were enzymatically isolated from leaves according to the method of Somerville et al. (1981) with slight modifications. Individual leaves were cut into only three pieces and incubated in an enzyme solution consisting of 1.3% (w/v) cellulysin (Calbiochem) and 0.4% (w/v) macerage (Calbiochem) in an isotonic sorbitol solution containing 1 mM CaCl_2 and 10 mM Mes/KOH (pH 5.5) (0.40 osmolal for nonacclimated leaves and 0.60 osmolal for 1-week-acclimated leaves). After the leaves were incubated in the enzyme solution for 2 h at 28°C in the dark, the undigested leaf sections were removed by filtering the suspension through four layers of cheesecloth. The filtrate was centrifuged at 50g for 10 min at 0°C to collect the protoplasts. The pellet was suspended in an isotonic sorbitol solution containing 1 mM CaCl_2 and 10 mM Mes/KOH (pH 5.5) and then washed twice by centrifugation as described above. The washed protoplasts were suspended in the isotonic sorbitol solution containing 1 mM CaCl_2 and 10 mM Mes/KOH (pH 5.5) and kept on ice.

Freezing of protoplasts was performed as described previously (Uemura and Steponkus, 1989). Briefly, an aliquot of the protoplast suspension (0.5 mL, 2×10^5 protoplasts) in a test tube (10 \times 100 mm) was placed in an ethanol bath at -2°C for 15 min prior to ice nucleation. After an additional 30-min incubation at -2°C , the samples were cooled to the specified temperatures at a rate of $0.8^{\circ}\text{C min}^{-1}$. After 30 min at the specified temperatures, the samples were thawed at room temperature and then kept on ice. In studies to determine the incidence of expansion-induced lysis, a hypertonic thaw treatment was used to minimize osmotic expansion of protoplasts during thawing of the suspending medium. For this, the frozen samples were warmed at -2°C for 5 min, after which a hypertonic sorbitol solution containing 1 mM CaCl_2 and 10 mM Mes/KOH (pH 5.5), which was precooled to -2°C , was added to the suspensions. This procedure yielded a final osmolality of 1.08 after melting of ice in the sample. Then the samples were kept on ice. Survival of protoplasts was determined by staining with fluorescein diacetate at a final concentration of 0.001% (w/v) (Widholm, 1972). After the samples

were incubated with fluorescein diacetate for 5 min at room temperature, the number of protoplasts that retained fluorescein was counted in a hemocytometer.

Freeze-Fracture EM

The freeze-fracture EM studies were conducted with protoplasts isolated from leaves. Small aliquots (approximately 2 μL) of the protoplast pellet, which was obtained after centrifugation of the protoplast suspension at 50g for 10 min, were loaded onto a freeze-fracture sample holder and placed in a small well of a copper block that was cooled by a circulating ethanol bath (Neslab ULT-80 [Portsmouth, NH]). Samples were cooled to -2°C for 15 min, after which ice nucleation was effected by touching the droplet with tweezers cooled in liquid nitrogen. The protoplast pellets were frozen at various temperatures over the temperature range of -2 to -16°C for 30 min before cryofixation for freeze-fracture EM. All cooling rates were $0.8^{\circ}\text{C min}^{-1}$. Sample temperature was monitored with a thermocouple placed in an identical position in an adjacent well of the copper block.

Cryofixation for freeze-fracture EM was achieved by plunging the protoplasts into liquid propane supercooled by liquid nitrogen. Samples were fractured on a Balzers 360 (Balzers, Liechtenstein) freeze-fracture device at -102°C and less than 2×10^{-6} torr. Fractured specimens were first coated with 2 nm of platinum and then with 20 nm of carbon, as determined by a quartz-crystal thickness monitor. Replicas were washed overnight with 100% H_2SO_4 and then for several hours in Clorox and examined on a Philips EM300 (Eindhoven, The Netherlands) electron microscope at 80 kV accelerating voltage.

RESULTS

Purity of the Plasma Membrane Fraction

The plasma membrane-enriched fraction isolated from *A. thaliana* leaves was free of contamination with various endomembranes as assessed by the distribution of marker enzyme activities (Table I). The specific activity of ATPase in the presence of Triton X-100 (0.05%, w/v) was higher ($20.53 \mu\text{mol ATP mg}^{-1} \text{protein h}^{-1}$) in the plasma membrane than in the microsomal fraction ($12.81 \mu\text{mol ATP mg}^{-1} \text{protein h}^{-1}$). The inhibition of ATPase activity by vanadate (100 μM), which is an inhibitor of plasma membrane ATPase, was quite high (86% inhibition) in the plasma membrane fraction but less pronounced (43% inhibition) in the microsomal fraction. The ATPase activity in the plasma membrane fraction was not diminished (5% inhibition) by KNO_3 , which is an inhibitor of tonoplast ATPase. In contrast, addition of KNO_3 decreased (36% inhibition) the ATPase activity in the microsomal fraction. The specific activities of marker enzymes such as Cyt *c* oxidase (marker enzyme for mitochondria), NADH Cyt *c* reductase (ER), and Triton X-100-stimulated UDPase (Golgi body) were all low in the plasma membrane fraction in comparison with those in the microsomal fraction, and the total activity of each was also low ($\leq 0.8\%$ of that in the microsomal fraction). In addition, Chl was not detected in

Table I. Marker enzyme activities of the microsomal and plasma membrane fractions isolated from leaves of *A. thaliana*

The plasma membrane fraction was isolated from the microsomal fraction of *A. thaliana* leaves using an aqueous, two-polymer, phase-partition system described in "Materials and Methods." The microsomal fraction contained 8.24 mg of Chl and 13.37 mg of protein (50 g fresh weight leaves)⁻¹; the plasma membrane fraction contained 0.36 mg of protein (50 g fresh weight leaves)⁻¹ and no Chl was detectable.

| Marker Enzyme | Microsomal Fraction | | Plasma Membrane | |
|-----------------------------------|-----------------------------|--------------------------------|-----------------------------|--------------------------------|
| | Total activity ^a | Specific activity ^a | Total activity ^a | Specific activity ^a |
| ATPase (pH 6.5) | | | | |
| + Triton X-100 | 171.33 | 12.81 | 7.33 | 20.53 |
| + Triton X-100 + vanadate | 97.76 | 7.31 | 1.04 | 2.92 |
| + Triton X-100 + KNO ₃ | 109.69 | 8.20 | 6.96 | 19.49 |
| Cyt c oxidase | 100.33 | 7.50 | 0.13 | 0.36 |
| NADH Cyt c reductase | 151.63 | 11.34 | 0.36 | 1.00 |
| Triton X-100-stimulated UDPase | 79.91 | 5.98 | 0.66 | 1.85 |

^a Total and specific activity of the marker enzyme were expressed as μmol substrate (50 g fresh weight leaves)⁻¹ h⁻¹ and μmol substrate mg⁻¹ protein h⁻¹, respectively.

the plasma membrane fraction, indicating that the plasma membrane-enriched fraction was free of contamination with thylakoid membranes.

Plasma Membrane Lipid Composition of Nonacclimated Leaves

The plasma membrane of nonacclimated leaves was characterized by high proportions of PL (46.8 mol% of the total lipids) and FS (37.7 mol%) and relatively low proportions of CER (7.3 mol%), SG (4.9 mol%), and ASG (3.4 mol%) (Table II). Collectively, sterol lipids (FS + SG + ASG) represented 46.0 mol% of the total lipids.

The preponderant PL in the plasma membrane were PC (35.5 mol% of total PL) and PE (38.9 mol%), with lesser proportions of PG (9.0 mol%), PI + PS (10.3 mol%), and PA (6.4 mol%) (Table III). Mono-unsaturated species, such as 16:0/18:2 and 16:0/18:3, were the preponderant molecular species of both PC (60.8 mol%) and PE (67.6 mol%) (Table IV). Di-unsaturated species, such as 18:1/18:3, 18:2/18:2, and 18:2/18:3, occurred in lower proportions (36.9 mol% in PC and 28.6 mol% in PE). The plasma membrane of *A. thaliana* contained very small proportions of di-saturated species of PC and PE, such as 14:0/14:0, 14:0/18:0, 16:0/16:0, and 16:0/18:0 (0.9 mol% in PC and 1.8 mol% in PE).

The majority (82.0 mol%) of the CER species in the plasma membrane of *A. thaliana* leaves contained 16:0h as the acyl moiety and either d18:1, d18:2, t18:0, or t18:1 as the

long-chain base (Table V). Only a small proportion (5.9 mol%) of the molecular species contained 24:1h as the acyl moiety.

Effect of Cold Acclimation on Plasma Membrane Lipid Composition

After cold acclimation for 7 d at 2°C (8-h photoperiod), the proportion of PL in the plasma membrane increased from 46.8 to 57.1 mol% of the total lipids, the proportion of CER decreased from 7.3 to 4.3 mol%, and the proportion of FS decreased from 37.7 to 31.2 mol% with only slight decreases in the proportions of SG (from 4.9 to 4.6 mol%) and ASG (from 3.4 to 2.9 mol%) (Table II). This resulted in a decrease in the total proportion of the sterol lipids (FS + SG + ASG) from 46.0 to 38.7 mol% after cold acclimation.

Although there was an increase in the proportion of PL (expressed as mol% of the total lipids) after cold acclimation, there was little difference in the proportion of the various phospholipid classes (expressed as mol% of total PL) before and after cold acclimation (Table III). There were, however, changes in the proportions of the various molecular species of both PC and PE during cold acclima-

Table II. Lipid composition of the plasma membrane isolated from nonacclimated (NA) and cold-acclimated (ACC) leaves of *A. thaliana*

The results are the average \pm SD of three determinations.

| Lipid | NA | ACC |
|-------|------------------------------|----------------|
| | <i>mol % of total lipids</i> | |
| PL | 46.8 \pm 1.1 | 57.1 \pm 1.5 |
| CER | 7.3 \pm 1.0 | 4.3 \pm 1.4 |
| FS | 37.7 \pm 1.1 | 31.2 \pm 1.9 |
| SG | 4.9 \pm 0.6 | 4.6 \pm 0.9 |
| ASG | 3.4 \pm 0.4 | 2.9 \pm 0.3 |

Table III. PL composition of the plasma membrane isolated from nonacclimated (NA) and cold-acclimated (ACC) leaves of *A. thaliana*

The results are the average \pm SD of three determinations.

| PL | NA | ACC |
|----------------------------------|------------------------------------|-----------------------|
| | <i>mol % of total PL</i> | |
| PC | 35.5 \pm 1.7 (16.6) ^a | 35.9 \pm 2.6 (20.4) |
| PE | 38.9 \pm 3.4 (18.2) | 39.8 \pm 2.5 (22.6) |
| PG | 9.0 \pm 1.2 (4.2) | 8.8 \pm 2.0 (5.0) |
| PI and PS | 10.3 \pm 1.0 (4.8) | 8.8 \pm 1.2 (5.0) |
| PA | 6.4 \pm 1.4 (3.0) | 7.2 \pm 1.7 (4.1) |
| Total PL (mol % of total lipids) | (46.8) | (57.1) |

^a Values in parentheses are the proportions expressed as a mol % of the total lipids.

Table IV. Molecular species of PC and PE of the plasma membrane isolated from nonacclimated (NA) and cold-acclimated (ACC) leaves of *A. thaliana*The results are the averages \pm SD of three determinations.

| Molecular Species | PC | | PE | |
|----------------------------------------------|--------------------------|----------------|--------------------------|----------------|
| | NA | ACC | NA | ACC |
| | <i>mol % of total PC</i> | | <i>mol % of total PE</i> | |
| Di-saturated species | | | | |
| 14:0/14:0 | 0.1 \pm 0.1 | 0.1 \pm 0.1 | 0.2 \pm 0.1 | 0.5 \pm 0.2 |
| 14:0/18:0, 16:0/16:0 | 0.4 \pm 0.2 | 0.4 \pm 0.2 | 0.6 \pm 0.2 | 0.3 \pm 0.1 |
| 16:0/18:0 | 0.4 \pm 0.1 | 0.5 \pm 0.2 | 1.0 \pm 0.2 | 0.7 \pm 0.2 |
| Total | 0.9 | 1.0 | 1.8 | 1.5 |
| Mono-unsaturated species | | | | |
| 16:0/18:1 | 2.9 \pm 0.4 | 2.1 \pm 0.5 | 0.8 \pm 0.2 | 0.8 \pm 0.1 |
| 16:0/18:2 | 32.1 \pm 2.2 | 30.5 \pm 2.6 | 43.0 \pm 3.4 | 38.4 \pm 2.5 |
| 16:0/18:3 | 23.6 \pm 2.0 | 20.7 \pm 2.1 | 21.0 \pm 2.2 | 19.5 \pm 2.1 |
| 18:0/18:1 | 0.6 \pm 0.2 | 0.7 \pm 0.3 | 0.4 \pm 0.1 | 0.2 \pm 0.1 |
| 18:0/18:2 | 0.4 \pm 0.1 | 0.5 \pm 0.2 | 1.5 \pm 0.3 | 1.5 \pm 0.3 |
| 18:0/18:3 | 0.8 \pm 0.3 | trace | trace | trace |
| 22:0/18:2 | 0.4 \pm 0.3 | 0.3 \pm 0.4 | 0.9 \pm 0.3 | 1.0 \pm 0.4 |
| Total | 60.8 | 54.8 | 67.6 | 61.4 |
| Di-unsaturated species | | | | |
| 18:1/18:1 | 2.3 \pm 0.5 | 0.9 \pm 0.2 | 1.2 \pm 0.3 | 1.1 \pm 0.3 |
| 18:1/18:2 | 2.7 \pm 0.4 | 3.4 \pm 0.5 | 2.4 \pm 0.5 | 2.4 \pm 0.4 |
| 18:1/18:3, 18:2/18:2 | 14.8 \pm 1.8 | 18.2 \pm 1.5 | 14.8 \pm 1.4 | 16.6 \pm 1.5 |
| 18:2/18:3 | 13.1 \pm 1.5 | 16.1 \pm 1.8 | 8.6 \pm 1.1 | 12.3 \pm 1.4 |
| 18:3/18:3 | 4.0 \pm 0.6 | 4.2 \pm 0.5 | 1.6 \pm 0.4 | 2.5 \pm 0.5 |
| Total | 36.9 | 42.8 | 28.6 | 34.9 |
| Others | 1.4 | 1.4 | 2.0 | 2.2 |
| Total of all species (mol % of total lipids) | 16.6 | 20.5 | 18.2 | 22.7 |

tion (Table IV). The proportion (mol% of total PC or PE) of di-unsaturated species, such as 18:1/18:3, 18:2/18:2, and 18:2/18:3, increased in both PC (from 36.9 to 42.8 mol%) and PE (from 28.6 to 34.9 mol%), and the proportion of mono-unsaturated species, such as 16:0/18:2 and 16:0/18:3, decreased from 60.8 to 54.8 mol% in PC and from 67.6 to 61.4 mol% in PE. Nevertheless, mono-unsaturated species were still predominant in both PC and PE after cold acclimation.

Although there was a decrease in the proportion of CER in the plasma membrane during cold acclimation, there were only small changes in the proportions of various molecular species (Table V).

Freezing Tolerance of Leaves

When grown under nonacclimating conditions (23°C, continuous light), electrolyte leakage from leaves of *A. thaliana* occurred after freezing to -2°C and reached a maximum ($\geq 95\%$) at -5°C (Fig. 1). $T_{\text{EL}50}$ was approximately -3.5°C . Cold acclimation at 2°C resulted in a substantial increase in the freezing tolerance of leaves, with a large increase occurring during the 1st d of cold acclimation. After 1 d of cold acclimation, electrolyte leakage did not occur until the leaves were frozen to -4°C and the $T_{\text{EL}50}$ decreased to -7°C . The maximum freezing tolerance was attained after 7 d of cold acclimation; at that time, the $T_{\text{EL}50}$ was -10°C . Additional increases in the freezing tolerance were not elicited by 14 d of cold acclimation.

Table V. Molecular species composition of CER of the plasma membrane isolated from nonacclimated (NA) and cold-acclimated (ACC) leaves of *A. thaliana*The results are the average \pm SD of three determinations.

| Molecular Species | NA | ACC |
|-------------------------------------------------------|----------------|---------------------------|
| | | <i>mol % of total CER</i> |
| 16:0h-d18:1 | 7.0 \pm 0.5 | 9.4 \pm 0.6 |
| 16:0h-d18:2 | 3.3 \pm 0.4 | 4.4 \pm 0.3 |
| 16:0h-d18:2(i) ^a ; 16:0-d18:2 ^b | 12.3 \pm 1.5 | 14.4 \pm 1.6 |
| 16:0h-t18:0; 20:1h-t18:1 | 47.9 \pm 3.5 | 44.6 \pm 3.1 |
| 16:0h-t18:1 | 11.5 \pm 2.0 | 12.4 \pm 2.1 |
| 22:0h-t18:1 | 2.0 \pm 0.6 | 3.2 \pm 0.5 |
| 24:0h-t18:1 | 2.6 \pm 0.4 | 2.5 \pm 0.4 |
| 24:1h-d18:2 | 2.0 \pm 0.3 | 2.6 \pm 0.4 |
| 24:1h-d18:2(i); 20:0h-d18:1; 25:1h-t18:1 | 0.7 \pm 0.4 | 1.0 \pm 0.2 |
| 24:1h-t18:1(i) | 3.2 \pm 0.6 | 2.9 \pm 0.5 |
| Others | 7.5 | 2.6 |
| Total of all species (mol % of total lipids) | 7.3 | 4.3 |

^a (i) refers to an isomeric form of long-chain base moiety of either d18:2 or t18:1. In the studies of Cahoon and Lynch (1991), t18:1(i) and d18:2(i) were identified as 4-hydroxy-8-*trans*-sphingene and sphinga-4-*trans*-8-*trans*-dienine, respectively. The other isomeric forms of the base, t18:1 and d18:2, were tentatively identified as 4-hydroxy-8-*cis*-sphingene and sphinga-4-*trans*-8-*cis*-dienine, respectively. ^b If two or more molecular species are present on the line, the corresponding peak separated by HPLC contains a mixture of these molecular species, in which case the molecular species listed first is the major component of the peak and others are minor components.

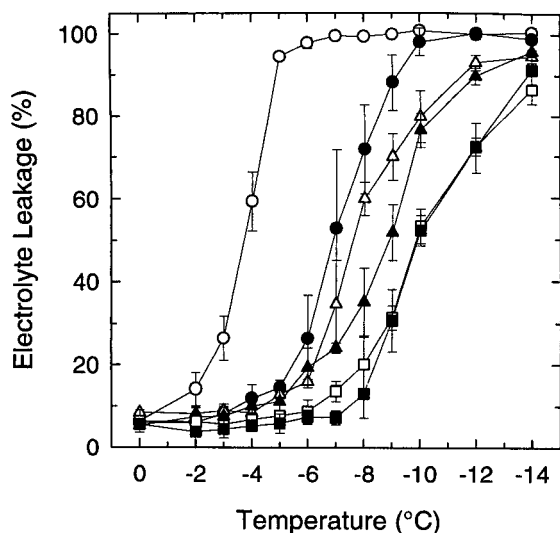


Figure 1. Freezing tolerance of leaves of *A. thaliana* during cold acclimation; survival was determined by measurement of electrolyte leakage from leaves after a freeze/thaw treatment. ○, Nonacclimated leaves; ●, 1-d-acclimated leaves; △, 3-d-acclimated leaves; ▲, 5-d-acclimated leaves; □, 7-d-acclimated leaves; ■, 14-d-acclimated leaves. The results shown are the average and SD of a minimum of three different experiments; two samples were used for a given experiment. If no SD values are seen in the figure, they are smaller than the size of the symbols.

Freezing Tolerance of Protoplasts

Survival of NA protoplasts of *A. thaliana* declined from >90% to <5% over the range of -2 to -6°C with a 50% decrease in survival occurring at -5°C (Fig. 2A). In contrast, survival of 1-week-ACC protoplasts remained at 100% over the range of 0 to -4°C and declined only after a freeze/thaw cycle to -6°C or below (Fig. 2B). A 50% decrease in survival occurred at -9°C . Thus, the freezing tolerance of both NA and ACC protoplasts was similar to that of the leaves from which they were isolated.

Freeze-Induced Lesions Associated with the Plasma Membrane

The large decrease in survival of NA protoplasts that were frozen over the range of -2 to -6°C was not a consequence of expansion-induced lysis (Fig. 2). As previously reported (Steponkus et al., 1988), the difference in survival after a conventional freeze/thaw treatment and a freeze/hypertonic-thaw treatment is attributable to expansion-induced lysis. In NA protoplasts, survival was only slightly greater ($\leq 10\%$) over the range of -2 to -4°C after the freeze/hypertonic-thaw treatment than after the conventional freeze/thaw treatment; at -6°C or below, there was no difference between the two treatments. In ACC protoplasts, there was little difference in survival between the two treatments over the range of 0 to -16°C . Thus, in contrast to rye (Uemura and Steponkus, 1989) and oat (Webb et al., 1994), the incidence of expansion-induced lysis was quite low in both NA and ACC protoplasts of *A. thaliana* at any subzero temperature.

Freeze-fracture EM studies revealed that the freeze-induced formation of the H_{II} phase was the principal ultrastructural manifestation of freezing injury in NA protoplasts of *A. thaliana* (Figs. 3 and 4). The H_{II} phase was observed at a high frequency in protoplasts frozen at -6 , -8 , and -10°C , temperatures at which survival was <5%. The presence of the H_{II} phase in protoplasts frozen at higher temperatures (-2 and -4°C) was not determined in these preliminary studies; hence, the threshold temperature for formation of the H_{II} phase is not known at this time.

Formation of the H_{II} phase is an interbilayer event and involves the participation of two or more bilayers, and the H_{II} phase is a three-dimensional array of inverted cylindrical micelles with water sequestered in the core of each cylinder. Most frequently, the H_{II} phase is observed in regions where the plasma membrane is brought into close apposition with the outer membrane of chloroplasts (Figs. 3 and 4).

Participation of the plasma membrane in the formation of the H_{II} phase is clearly evident in Figure 3A, in which a region of the plasma membrane that is in the lamellar phase appears to be contiguous with the domain of the H_{II} phase. In other instances, domains of the plasma membrane that are characterized by undulating striations and a low density of IMP intergrade between the lamellar regions of the plasma membrane with a normal and random distribution of IMP and the H_{II} domains (Fig. 3, B and C). Although the undulating striations resemble the H_{II} phase, they are less ordered and possibly represent intermediate stages in the lamellar-to- H_{II} phase transition. In addition, lamellar regions of the plasma membrane are either apar-

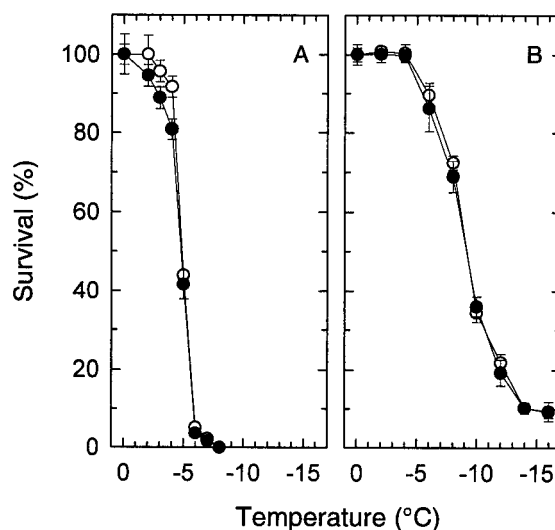


Figure 2. Survival of protoplasts isolated from *A. thaliana* leaves after a conventional freeze/thaw treatment (●) or a freeze/hypertonic-thaw treatment (○). A, NA protoplasts; B, 1-week-ACC protoplasts. Survival was determined by staining with fluorescein diacetate. The results shown (percent survival of unfrozen control) are the average and SD of two different experiments; two samples were used in a given experiment. If no SD values are seen in the figure, they are smaller than the size of the symbols.

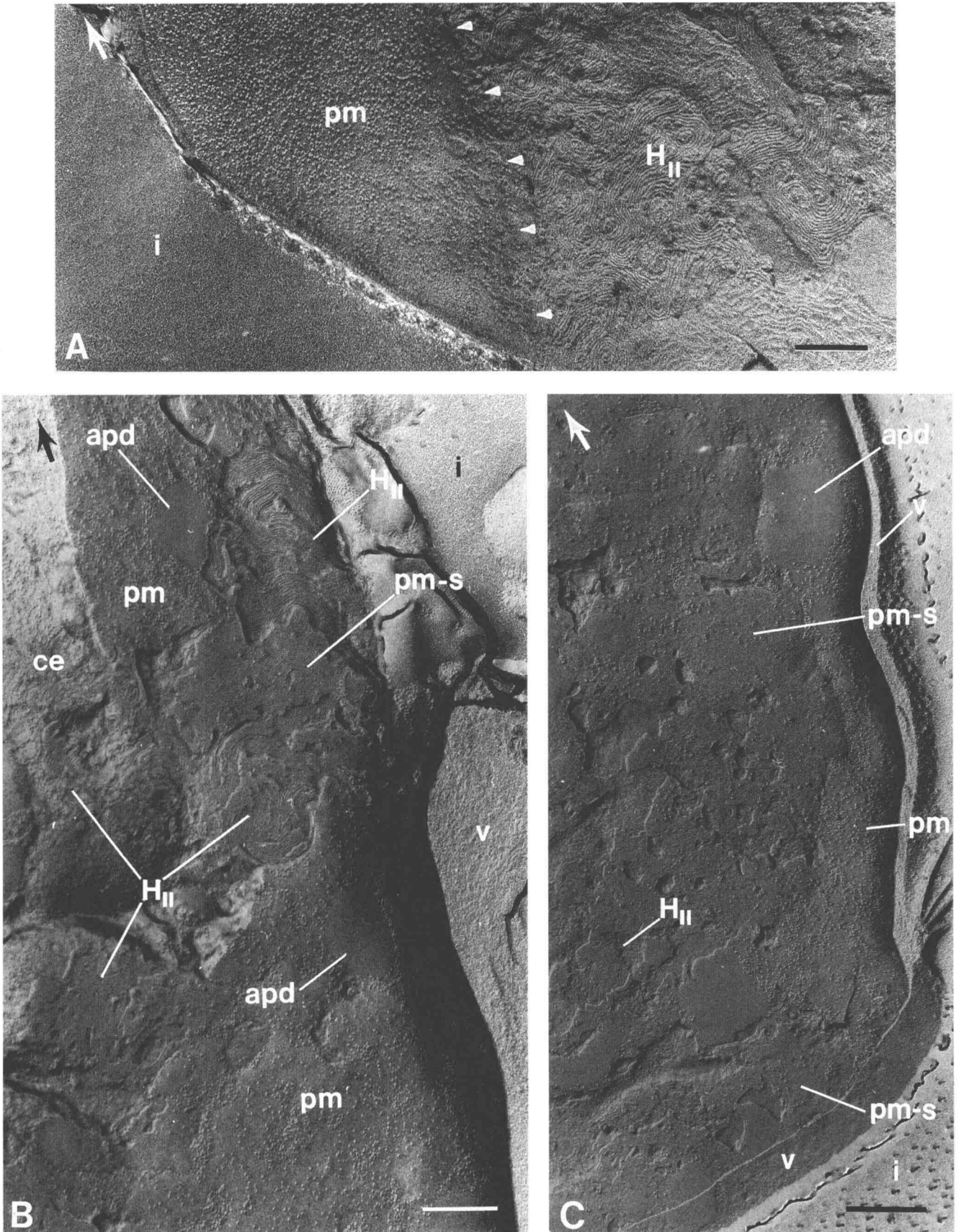


Figure 3. (Legend appears on opposite page.)

ticulate or have a greatly reduced number of IMP (Fig. 3, B and C).

Involvement of the outer membrane of the chloroplast envelope in the freeze-induced formation of the H_{II} phase is shown in Figure 4. Frequently, the entire fracture face of the chloroplast envelope is characterized by the undulating striations and domains of the H_{II} phase (Fig. 4, A and B). Domains of the H_{II} phase are characterized by well-ordered arrays of cylindrical micelles, which are obvious in regions where the cylinders are cross-fractured and establish that such domains are not merely closely appressed lamellae (Fig. 4C). The undulating striations that appear in large regions of the chloroplast envelope vary in appearance: some regions appear to be more ordered and pronounced (Fig. 4D) than others (Fig. 4E), as previously observed in rye (Gordon-Kamm and Steponkus, 1984a) and oat (Webb et al., 1994). The origin of the undulating striations is not known, but they are thought to represent an intermediate stage in the lyotropically induced lamellar-to- H_{II} phase transition. It is possible that the well-ordered striations occur in the lipid monolayer that is in the lamellar form but is situated on the outer layer of the array of inverted cylindrical micelles that constitute the H_{II} phase and is, therefore, the "capstone" of the H_{II} array (Steponkus et al., 1993).

Freeze-induced formation of the H_{II} phase did not occur in protoplasts isolated from leaves of *A. thaliana* that were cold-acclimated for 1 week (i.e. at the maximum freezing tolerance). Instead, freezing injury was associated with the fracture-jump lesion (Fig. 5), which was first reported to occur in rye protoplasts (Fujikawa and Steponkus, 1990) and subsequently was observed in rye leaves (Webb and Steponkus, 1993) and oat protoplasts (Webb et al., 1994). In *A. thaliana*, the fracture-jump lesion was observed, albeit at a low frequency, in protoplasts frozen at -8°C ; however, it was observed at a high frequency in protoplasts that were frozen to -12°C or below.

The fracture-jump lesion is characterized by localized deviations in the fracture-plane of the plasma membrane such that the fracture-plane "jumps" from the plasma membrane to either aparticulate lamellae or aparticulate regions of the subtending lamellae that are in close apposition with the plasma membrane (Fig. 5A) (see Steponkus et al., 1993, for a complete description of the fracture-jump lesion). The endomembranes that are most frequently observed to be involved in the fracture-jump lesion are chlo-

roplast envelopes (Fig. 5B) and, at a lower frequency, the tonoplast and ER. Figure 5A illustrates a high incidence of the fracture-jump lesion in the plasma membrane. Figure 5B illustrates the fracture-jump lesion in a region of the plasma membrane that is overlying a chloroplast; in some regions, at least three different fracture planes are visible and may represent the outer and inner envelopes of the chloroplast beneath the plasma membrane. Figure 6A illustrates the occurrence of the fracture-jump lesion with several layers of lamellae below the primary fracture face, which appears to be through the tonoplast. Figure 6B illustrates the occurrence of the fracture-jump lesion with several layers of lamellae above the primary fracture-face, which is also thought to be through the tonoplast.

DISCUSSION

Characteristics of the Plasma Membrane Lipid Composition

The lipid composition of the plasma membrane and tonoplast of higher plants is substantially different from that of other cellular membranes in that there is a high proportion of sterols (both free and glucosylated forms) and CER, neither of which are present in other cellular membranes (Douce and Joyard, 1990; Larsson et al., 1990). There are, however, considerable differences in the proportion of PL, CER, and sterols in the plasma membrane of different species. For example, in plasma membrane fractions isolated from leaves, the proportion of PL ranges from 28.8 mol% of the total lipids in oat (Uemura and Steponkus, 1994) to 63.9 mol% in spinach (Rochester et al., 1987), CER range from 6.5 mol% in potato (Palta et al., 1993) to 27.2 mol% in oat (Uemura and Steponkus, 1994), FS range from 1.7 mol% in potato (Palta et al., 1993) to 38.1 mol% in rye (Uemura and Steponkus, 1994), ASG range from 0 mol% (not detected) in barley (Rochester et al., 1987) to 32.5 mol% in potato (Palta et al., 1993), and SG range from 0 mol% (not detected) in spinach and barley (Rochester et al., 1987) to 15.1 mol% in rye (Lynch and Steponkus, 1987). Therefore, in comparison to other species, the plasma membrane of *A. thaliana* leaves contains high proportions of PL (46.8 mol%) and FS (37.7 mol%) and low proportions of CER (7.3 mol%), ASG (3.4 mol%), and SG (4.9 mol%).

The plasma membrane lipid composition also varies with different organs from the same plant such as leaves

Figure 3. (On opposite page.) Freeze-fracture electron micrographs of the plasma membrane of protoplasts isolated from leaves of nonacclimated *A. thaliana* seedlings (14 d old); protoplasts were frozen at -6°C , which was the highest temperature that resulted in less than 10% survival. A, PF fracture face of the plasma membrane (pm) showing a typical, lamellar region (left side), which is characterized by a random distribution of IMP, melding into a region where the H_{II} phase has formed (right side). The transition between the lamellar region and the H_{II} domain is delimited by the arrowheads. i, Ice in the suspending medium. Magnification: 42,600 \times . Bar represents 300 nm. Arrow indicates direction of shadowing. B, PF fracture face of the plasma membrane (pm) overlying the chloroplast envelope (ce), with the H_{II} phase appearing in localized regions. Large regions of the plasma membrane have undulating striations (pm-s), which are less ordered than the H_{II} phase; these regions have a low density of IMP and frequently intergrade between typical lamellar regions and domains of the H_{II} phase. Aparticle domains (apd) are also commonly observed in the plasma membrane. v, Vitreous layer of unfrozen suspending medium; i, ice. Magnification: 45,200 \times . Bar represents 300 nm. Arrow indicates direction of shadowing. C, PF fracture face of the plasma membrane (pm) containing large areas of the undulating striations (pm-s), the H_{II} phase, and aparticulate domains (apd). Magnification: 58,900 \times . Bar represents 500 nm. Arrow indicates direction of shadowing.

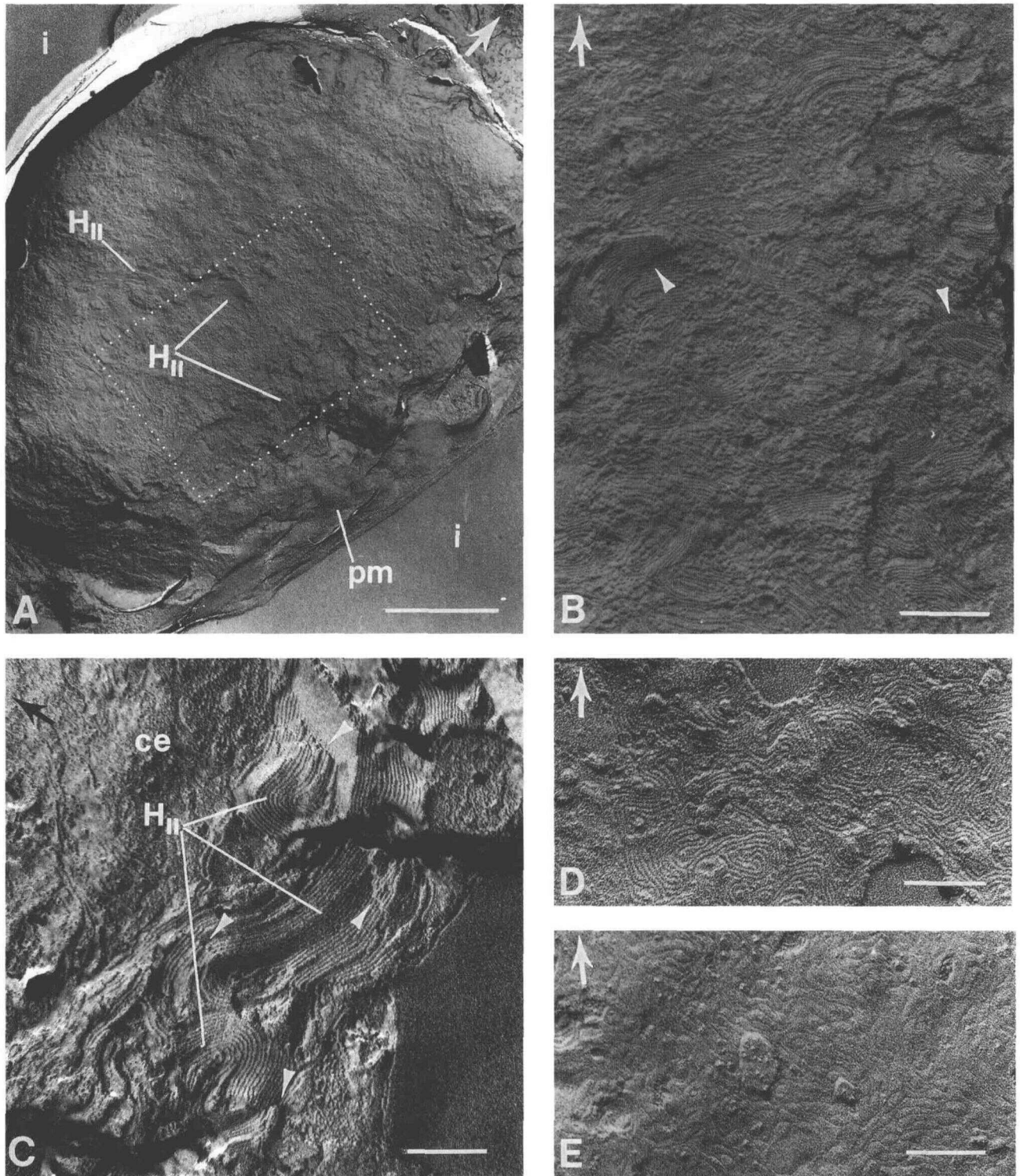


Figure 4. Freeze-fracture electron micrographs of chloroplast envelopes of protoxysts isolated from leaves of nonacclimated *A. thaliana* seedlings; protoxysts were frozen at -6°C , which was the highest temperature that resulted in less than 10% survival. A, Low magnification ($19,200\times$) micrograph illustrating the extensive formation of undulating striations over much of the chloroplast envelope and numerous domains of the H_{II} phase. The area in the dotted box is shown in higher magnification in Figure 4B. Bar represents $1\ \mu\text{m}$. Arrow indicates direction of shadowing. B, High magnification ($49,300\times$) of the region that is within the dotted box in A, showing more detail of the H_{II} phase including cross-fractures of the inverted cylindrical micelles (arrowheads) that constitute the H_{II} phase. Bar represents $300\ \text{nm}$. Arrow indicates direction of shadowing. C, High magnification ($67,800\times$) of a region of the H_{II} phase associated with the chloroplast envelope (ce). Bar represents $300\ \text{nm}$. Arrow indicates direction of shadowing. D, High magnification ($49,300\times$) of a region of the H_{II} phase. Bar represents $300\ \text{nm}$. Arrow indicates direction of shadowing. E, High magnification ($49,300\times$) of a region of the H_{II} phase. Bar represents $300\ \text{nm}$. Arrow indicates direction of shadowing.

versus roots in barley (Rochester et al., 1987) and coleoptiles versus roots in oat (Sandstrom and Cleland, 1989). The composition is also influenced by exposure to low temperatures (Lynch and Steponkus, 1987; Palta et al., 1993; Uemura and Steponkus, 1994) and drought (Norberg and Liljeborg, 1991).

Although there is a great diversity of molecular species within each lipid class, to our knowledge a molecular species analysis of the plasma membrane lipids has heretofore been reported only for rye and oat (Lynch and Steponkus, 1987; Cahoon and Lynch, 1991; Uemura and Steponkus, 1994). A comparison of the lipid composition of the plasma membrane of *A. thaliana* with that of rye and oat reveals that the molecular species of PC and PE are similar (Table IV) (Lynch and Steponkus, 1987; Uemura and Steponkus, 1994). The preponderant species are mono-unsaturated species, such as 16:0/18:2 and 16:0/18:3; di-unsaturated species, such as 18:1/18:3, 18:2/18:2, and 18:2/18:3, occur in smaller proportions. In contrast, there is a vast difference in the molecular species of CER. The majority (82 mol%) of the CER species in the plasma membrane of *A. thaliana* leaves contain 16:0h as the acyl moiety (Table V), but the proportion of 16:0h-containing species of CER is only 17.6 mol% in rye and 6.4 mol% in oat. Instead, 24:1h-containing species are predominant in the plasma membrane of rye (47.8 mol%) and oat (73.4 mol%) (Cahoon and Lynch, 1991; Uemura and Steponkus, 1994). It has been reported that the plasma membrane of oat root contains only one molecular species of CER, 24:1h-d18:2 (Norberg et al., 1991).

Effect of Cold Acclimation on Plasma Membrane Lipid Composition

An increase in the proportion of PL in the plasma membrane during cold acclimation is commonly observed in many plant species, including both herbaceous species, such as orchard grass leaves (Yoshida and Uemura, 1984), rye leaves (Uemura and Yoshida, 1984; Lynch and Steponkus, 1987; Uemura and Steponkus, 1994), oat leaves (Uemura and Steponkus, 1994), and Jerusalem artichoke tubers (Ishikawa and Yoshida, 1985), and woody species, such as mulberry bark cells (Yoshida, 1984). An increase in PL also occurs in the plasma membrane of *A. thaliana* leaves (Table II). Using two *Solanum* species that differ in their capacity for cold acclimation, Palta et al. (1993) recently reported that during growth at low temperatures (4°C during the day and 2°C at night) there is an increase in PL in the plasma membrane (expressed as $\mu\text{mol lipid/mg protein}$) of *Solanum commersonii*, which increases in freezing tolerance, but not in *Solanum tuberosum*, which does not increase in freezing tolerance.

In rye and oat, the increase in PL during cold acclimation is associated with an increase in the proportion of di-

unsaturated species of PC and PE and a corresponding decrease in mono-unsaturated species (expressed as mol% of total PC or PE) (Uemura and Steponkus, 1994). These changes also occur in *A. thaliana* (Table IV). Before cold acclimation, the majority of the PC species are mono-unsaturated in all three plant species. After cold acclimation, di-unsaturated species become predominant in rye, but mono-unsaturated species are still predominant in oat and *A. thaliana*. Thus, an increase in the proportion of di-unsaturated species of PC in the plasma membrane during cold acclimation occurs in all three species, but the extent of the increase is different. An increase in the proportion of di-unsaturated species of PE during cold acclimation is more pronounced in rye and *A. thaliana* than in oat; nevertheless, mono-unsaturated species of PE predominate in all three species both before and after cold acclimation.

The proportion of CER in the plasma membrane before and after cold acclimation varies among the plant species studied. In rye, the proportion of CER is intermediately high before cold acclimation and decreases progressively during cold acclimation (from 16.4 to 10.5 mol%); oat contains a high proportion of CER, and there is little change during cold acclimation (from 27.2 to 24.2 mol%) (Uemura and Steponkus, 1994); and potato (both *S. tuberosum* and *S. commersonii*) contains a low proportion of CER, which decreases only slightly during cold acclimation (from 6.5 to 5.0 mol% in *S. tuberosum* and from 6.1 to 4.9 mol% in *S. commersonii*) (Palta et al., 1993). In the present study, the proportion of CER in the plasma membrane of *A. thaliana* is low before cold acclimation and decreases during cold acclimation (from 7.3 to 4.3 mol%; Table II).

Although there are substantial differences in the proportion of free (FS) and glucosylated forms (SG and ASG) of sterols in the plasma membrane of plant species—FS are predominant in rye (38.1 mol% of total lipids), orchard grass (43.7 mol%), and *A. thaliana* (37.7 mol%); ASG are predominant in oat (27.3 mol%), *S. commersonii* (32.5 mol%), and *S. tuberosum* (31.9 mol%)—the changes in the relative proportions during cold acclimation are generally rather small (Uemura and Yoshida, 1984; Yoshida and Uemura, 1984; Palta et al., 1993; Uemura and Steponkus, 1994). In *A. thaliana*, there was a decrease in the proportion of FS in the plasma membrane during cold acclimation, but little change in the proportions of SG and ASG (Table II).

Effect of Lipid Composition on the Cryostability of the Plasma Membrane

Although the primary objective of the current study—to determine the effect of cold acclimation on the lipid composition of the plasma membrane of *A. thaliana* leaves—has been accomplished, our ultimate goal is to provide a mech-

Figure 4. (Continued from previous page.) Arrowhead, Region of cross-fracture of the inverted micelles. The fracture face of the envelope appears to be at a lower elevation than the region of the H_{II} phase; hence, the H_{II} phase involves the chloroplast envelope and membranes exterior to it (e.g. the plasma membrane, tonoplast, and ER). Bar represents 200 nm. Arrow indicates direction of shadowing. D, High magnification (67,800 \times) of undulating striations that are less ordered than the H_{II} phase and that may be an intermediate stage in the lamellar-to-H_{II} phase transition. Bar represents 200 nm. Arrow indicates direction of shadowing. E, High magnification (67,800 \times) of region showing less-ordered striations. Bar represents 200 nm. Arrow indicates direction of shadowing.

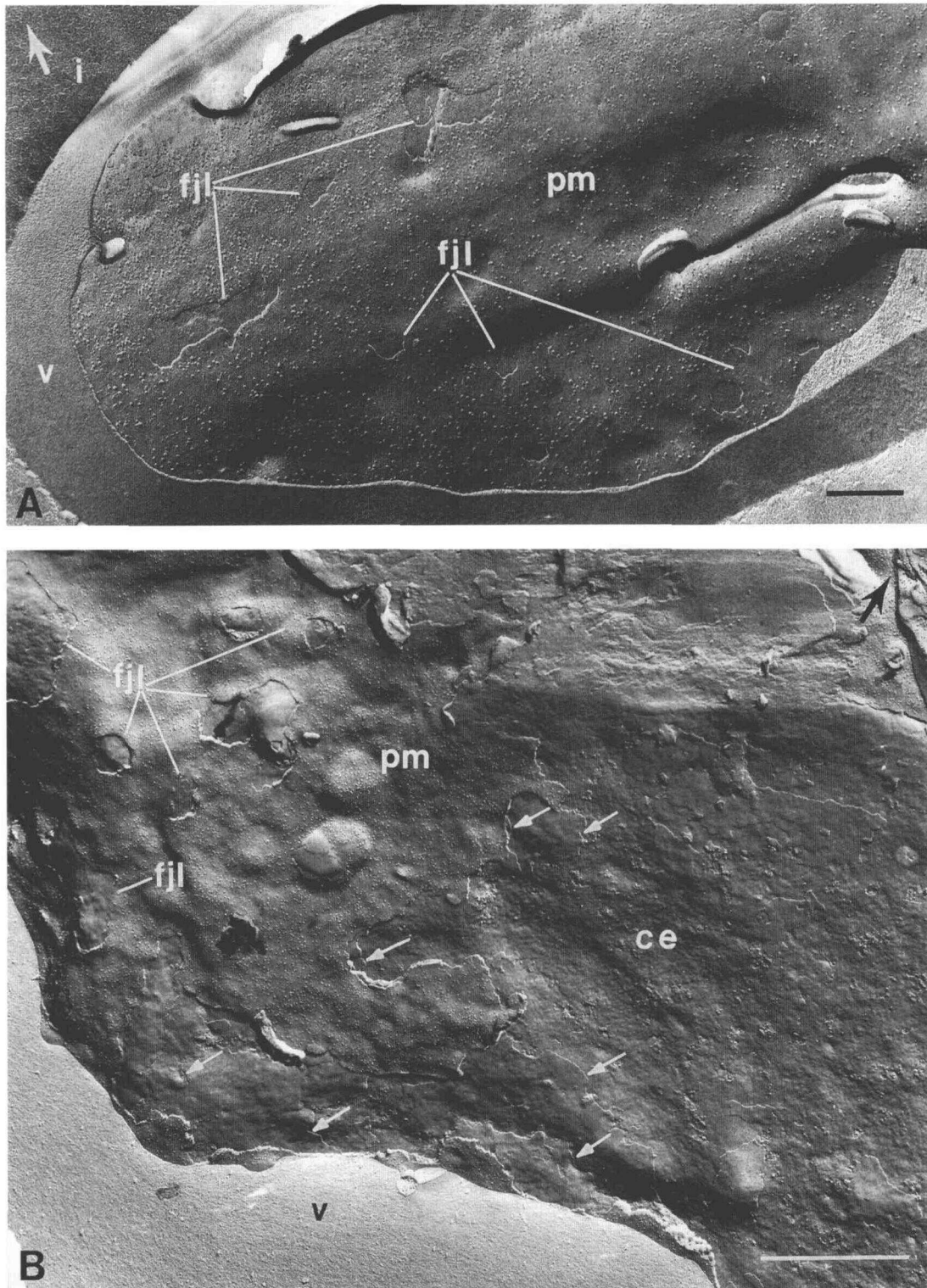


Figure 5. Freeze-fracture electron micrographs of protoplasts isolated from leaves of *A. thaliana* seedlings that were cold acclimated for 1 week; protoplasts were frozen at -16°C , which was the highest temperature that resulted in less than 10% survival. A, PF fracture face of the plasma membrane (pm) showing numerous fracture-jump lesions (fjl). i, Ice in the suspending medium; v, vitreous layer of unfrozen suspending medium. Magnification: $40,100\times$. Bar represents 300 nm. Arrow indicates direction of shadowing. B, Fracture-jump lesions (fjl) in regions of the PF fracture face of the plasma membrane (pm) (left side) that is overlying the chloroplast envelope (ce) (right side). At least three different fracture planes are apparent and may represent the plasma membrane and the outer and inner envelopes of the chloroplast; small arrows indicate the jumps in the fracture plane. v, Vitreous layer of unfrozen suspending medium. Magnification: $23,300\times$. Bar represents $1\ \mu\text{m}$. Arrow indicates direction of shadowing.

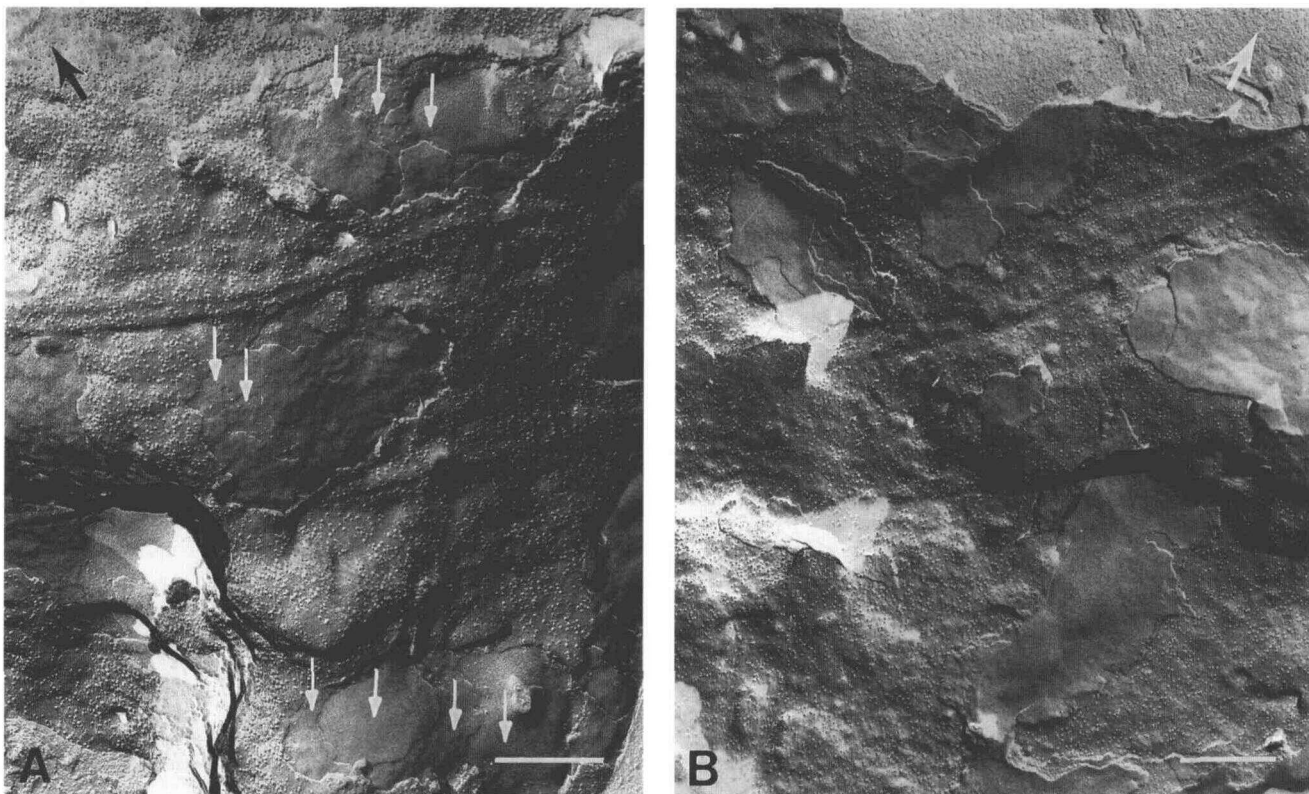


Figure 6. Freeze-fracture electron micrographs of protoplasts isolated from leaves of *A. thaliana* seedlings that were cold acclimated for 1 week; protoplasts were frozen at -16°C , which was the highest temperature that resulted in less than 10% survival. A, High magnification (35,500 \times) of the fracture-jump lesion showing several layers of lamellae (denoted by small arrows) below the primary fracture plane. Bar represents 300 nm. Arrow indicates direction of shadowing. B, High magnification (31,400 \times) of the fracture-jump lesion showing several layers of lamellae above the primary fracture plane, which is believed to be through the tonoplast. Bar represents 400 nm. Arrow indicates direction of shadowing.

anistic understanding of the influence of alterations in lipid composition during cold acclimation on the cryostability of the plasma membrane. This is best considered from the perspective of the specific freeze-induced lesions that limit the freezing tolerance of *A. thaliana* before and after cold acclimation.

Expansion-Induced Lysis

The incidence of expansion-induced lysis in *A. thaliana* was quite low (<10%) (Fig. 2) in comparison to that which occurs in rye (>40%) and oat (30%) (Steponkus et al., 1993). This difference appears to be the result of a difference in the behavior of the plasma membrane during osmotic contraction. During the microscopic determinations of survival of NA protoplasts of *A. thaliana* subjected to the freeze/hypertonic-thaw treatment, which involved maintaining the protoplasts in a hypertonic solution (1.08 osmolal sorbitol), we observed numerous exocytotic extrusions on the plasma membrane (data not shown). The extrusions appeared as long filamentous strands extending from the plasma membrane. The extrusions were not observed in protoplasts maintained in isotonic solutions. In preliminary cryomicroscopy studies of NA protoplasts isolated from *A. thaliana*, formation of the exocytotic extrusions occurred during freeze-induced osmotic contraction (data not shown).

The behavior of the plasma membrane of NA protoplasts of *A. thaliana* during osmotic contraction is quite different from that of NA protoplasts of rye. Whereas the plasma membrane of NA protoplasts of *A. thaliana* forms exocytotic extrusions during osmotic contraction, the plasma membrane of NA protoplasts of rye forms endocytotic vesicles (Dowgert and Steponkus, 1984). In NA protoplasts of rye, endocytotic vesiculation during osmotic contraction results in a reduction in the surface area of the plasma membrane, and sufficiently large area reductions are irreversible such that the protoplasts lyse before regaining their initial size; hence, this form of injury is referred to as expansion-induced lysis. In rye and oat, expansion-induced lysis is precluded after cold acclimation because of a transformation in the cryobehavior of the plasma membrane, that is, the plasma membrane of ACC protoplasts of rye and oat forms exocytotic extrusions during osmotic contraction and the surface area of the plasma membrane is conserved such that osmotic contraction is readily reversible. Thus, during osmotic contraction, the plasma membrane of NA protoplasts of *A. thaliana* behaves like that of ACC protoplasts of rye and oat.

The transformation in the behavior of the plasma membrane of rye and oat (formation of endocytotic vesicles before cold acclimation; formation of exocytotic extrusions

after cold acclimation) is a result of increases in the proportion of PC in the plasma membrane during cold acclimation (Steponkus et al., 1993). This transformation in the behavior of the plasma membrane and an increase in freezing tolerance can also be effected in NA protoplasts of rye (Steponkus et al., 1988) and oat (Webb et al., 1994) by artificial enrichment of the plasma membrane with mono- or di-unsaturated species of PC. Therefore, the formation of exocytotic extrusions in NA protoplasts of *A. thaliana* is consistent with the high proportion of PC present in the plasma membrane: 16.6 mol% of the total lipids in nonacclimated seedlings and 20.4 mol% in cold-acclimated seedlings (Table III). In fact, the proportion of PC in the plasma membrane of *A. thaliana* before cold acclimation exceeds that of rye (14.9 mol%) and oat (12.6 mol%) after cold acclimation (Uemura and Steponkus, 1994).

Freeze-Induced Formation of the H_{II} Phase

Freeze-induced formation of the H_{II} phase is an interbilayer event that occurs when the plasma membrane is brought into close apposition with various endomembranes as a consequence of freeze-induced dehydration. The H_{II} phase is a nonlamellar phase that is a three-dimensional array of inverted cylindrical micelles with water in the central core of each cylinder. Although the H_{II} phase does not occur within a given membrane per se, it is obvious that the plasma membrane participates in the formation of the H_{II} phase because in many instances (e.g. Fig. 3) we have observed that the plasma membrane is contiguous with the H_{II} domain. Most often, the chloroplast envelope (Fig. 4) and, less frequently, the tonoplast are also observed to participate in formation of the H_{II} phase; however, we have not observed the H_{II} phase in association with the chloroplast thylakoids.

Although the threshold temperature at which the H_{II} phase occurs in *A. thaliana* remains to be determined by freeze-fracture EM studies, it is inferred to be approximately -2 to -4°C , since at temperatures lower than -4°C , there is a steep decline in survival of leaves and protoplasts (Figs. 1 and 2) and expansion-induced lysis does not contribute significantly to the decreased survival at these temperatures (Fig. 2). The threshold temperature for freeze-induced formation of the H_{II} phase is -3°C in oat and -6°C in rye, with a high incidence (in 50% of the protoplasts) occurring in oat at -5°C (Webb et al., 1994) and at -10°C in rye (Gordon-Kamm and Steponkus, 1984a). Thus, *A. thaliana* appears to be intermediate between oat and rye in the temperature at which freeze-induced formation of the H_{II} phase occurs.

The higher threshold temperature in oat is attributed to the large proportion of ASG present in the plasma membrane (Uemura and Steponkus, 1994). Recently, we have shown that ASG are much more effective than FS in increasing the propensity for dehydration-induced formation of the H_{II} phase in mixtures of dioleoylphosphatidylcholine and dioleoylphosphatidylethanolamine (Webb et al., 1995). However, the proportions of FS and sterol derivatives in the plasma membrane of *A. thaliana* (Table II) are very similar to those in rye and unlike those in oat (Steponkus et al., 1993). The plasma mem-

brane of *A. thaliana* does, however, contain a higher proportion of PE (18.2 mol% of the total lipids, Table III) than does rye (12.4 mol%) (Uemura and Steponkus, 1994). Although PE is the lipid in the plasma membrane most prone to form the H_{II} phase, as will be discussed, it is unlikely that the propensity to form the H_{II} phase can be ascribed only to the proportion of PE. This is exemplified by the fact that the proportion of PE increases during cold acclimation (Table III) when the propensity for freeze-induced formation of the H_{II} phase is decreased.

The decreased propensity for freeze-induced formation of the H_{II} phase after cold acclimation is a consequence of alterations in membrane lipid composition (see reviews by Steponkus et al., 1990, 1993). For example, osmotic dehydration results in the formation of the H_{II} phase in liposomes prepared from the plasma membrane lipids of nonacclimated rye leaves but not in liposomes prepared from the plasma membrane lipids of cold-acclimated rye leaves (Cudd and Steponkus, 1988). In addition, membrane engineering studies in which the lipid composition of the plasma membrane was selectively altered have provided direct evidence that an increase in the proportion of di-unsaturated species of PC contributes to the decreased propensity for freeze-induced formation of the H_{II} phase (Sugawara and Steponkus, 1990). Thus, the increase in the proportion of PC (Table III), especially di-unsaturated species (Table IV), in the plasma membrane of *A. thaliana* after cold acclimation is expected to contribute to the decreased propensity for the freeze-induced formation of the H_{II} phase.

Nevertheless, it is likely that the decreased propensity for freeze-induced formation of the H_{II} phase after cold acclimation is influenced by the collective changes in the various lipid classes rather than in a specific lipid class or species. Freeze-induced formation of the H_{II} phase is an interbilayer phenomenon that occurs between bilayers when they are brought into close apposition during freeze-induced dehydration. The propensity of closely apposed membranes to undergo the lamellar-to- H_{II} phase transition is influenced by a complex interplay of factors that influence (a) the hydration characteristics of the membranes, which, in turn, will influence the spatial separation between bilayers at a given freezing temperature (osmotic pressure); (b) the propensity for dehydration-induced demixing of membrane components (lipid-protein and lipid-lipid demixing); and (c) the intrinsic curvature of the constituent monolayers (see Steponkus et al., 1993, for details).

An increase in bilayer hydration, which will result in a greater spatial separation between bilayers at a given osmotic pressure (freezing temperature), can be effected by either an increase in the proportion of highly hydrated lipid classes (e.g. PL) or a decrease in the proportion of poorly hydrated lipid classes (e.g. CER and FS), or by a combination of both. Changes in the proportions of highly hydrated species and poorly hydrated species will also influence the tendency for dehydration-induced lipid-lipid demixing and hence the tendency to form domains that are enriched in H_{II} -forming lipids (e.g. unsaturated species of PE) (Webb et al., 1992). A decrease in the intrinsic curvature of the constituent monolayers, which will decrease the propensity for formation of the H_{II}

phase, can be effected by an increase in the proportion of PC and/or a decrease in the proportion of CER and FS. Thus, during cold acclimation of *A. thaliana*, the increase in the proportion of PL and decreases in the proportion of CER and FS will collectively influence the propensity for freeze-induced formation of the H_{II} phase.

This complex interplay of different factors may also explain the apparent paradox that although cold acclimation decreases the propensity for freeze-induced formation of the H_{II} phase, it also results in an increase in the proportion of di-unsaturated species of PE, which are the species present in the plasma membrane that are most prone to undergo the lamellar-to-H_{II} phase transition. The increased content of PE will increase the hydration of the plasma membrane and thus maintain a greater spatial separation from the endomembranes during freeze-induced dehydration. This would diminish the possibility of dehydration-induced, lipid-lipid demixing as a prelude to the formation of PE-enriched domains that would participate in formation of the H_{II} phase.

Fracture-Jump Lesion

Although the cause of the fracture-jump lesion remains to be elucidated, we have previously suggested that it is the result of the formation of ILAs and fusion between the plasma membrane and various endomembranes when they are brought into close apposition during freeze-induced dehydration (Steponkus et al., 1993). When bilayers are subjected to extreme dehydration, removal of water from the lipid headgroups results in an increased lateral pressure in the acyl domain relative to that in the headgroup region, which results in a bending energy (Gruner 1989a, 1989b). At low water contents (<20%, w/w), the bending energy is minimized by formation of the H_{II} phase. However, the H_{II} phase does not form in ACC protoplasts; instead, membrane destabilization is manifested as the fracture-jump lesion. We believe that theoretical studies by Siegel (1986a, 1986b, 1986c, 1987) provide a possible explanation for this difference in behavior. Siegel has suggested that when lipid bilayers are brought into close apposition they may form either the H_{II} phase or ILA from a common structural intermediate, referred to as an IMI. Whether the IMI are converted to the H_{II} phase or ILA is thought to be dependent on the intrinsic curvature of the constituent monolayers: monolayers with a high intrinsic curvature form the H_{II} phase, whereas those with a low intrinsic curvature form ILA.

Thus, the differential behavior of NA and ACC protoplasts subjected to injurious levels of freeze-induced dehydration (i.e. formation of the H_{II} phase in NA protoplasts and the fracture-jump lesion in ACC protoplasts) is attributed to changes in membrane lipid composition during cold acclimation that increase membrane hydration characteristics and decrease the intrinsic curvature of the constituent monolayers. An increase in the proportion of PL and a decrease in the proportions of CER and FS during cold acclimation alter the desorption characteristics of the bilayer such that a greater amount of water is retained at a given osmotic pressure, which is a direct function of the freezing temperature. Therefore, with ACC protoplasts, a lower freezing temperature must be imposed to attain the

close apposition required for formation of the IMI. The lower temperature, however, is apparently below the lamellar-to-H_{II} phase transition temperature. Although the extent of dehydration is sufficiently great to result in the formation of IMI between the plasma membrane and various endomembranes, the decrease in the intrinsic curvature of the constituent monolayers favors formation of ILA and fusion of the plasma membrane with various endomembranes rather than formation of the H_{II} phase.

Although this working hypothesis provides an explanation to account for the differential behavior of NA and ACC protoplasts, further studies of *A. thaliana* are required to determine when during the cold acclimation process the decreased propensity for freeze-induced formation of the H_{II} phase occurs. Although this occurs during the 1st week of cold acclimation in rye and oat, which require 4 weeks to achieve maximum freezing tolerance (Webb et al., 1994), it is likely that the decreased propensity for freeze-induced formation of the H_{II} phase occurs more rapidly in *A. thaliana*, which attains maximum freezing tolerance in 1 week. Further studies are also required to determine if the threshold temperature for the fracture-jump lesion decreases with the stage of acclimation. In rye, it decreases from -10°C after 1 week of cold acclimation to -20°C after 4 weeks of cold acclimation; in oat, it decreases from -5 to -10°C at similar time intervals. Such studies, together with a temporal analysis of alterations in the lipid composition of the plasma membrane at daily intervals during the 1 week of cold acclimation required to attain maximum freezing tolerance in *A. thaliana*, will allow for a circumspect analysis of lipid changes that are associated with the decrease in the threshold temperature for the fracture-jump lesion.

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