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A Comparison of Freezing Injury in Oat and Rye: Two Cereals at the Extremes of Freezing Tolerance[1]

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A detailed analysis of cold acclimation of a winter rye (*Secale cereale* L. cv Puma), a winter oat (*Avena sativa* L. cv Kanota), and a spring oat cultivar (Ogle) revealed that freezing injury of leaves of nonacclimated seedlings occurred at −2°C in both the winter and spring cultivars of oat but did not occur in winter rye leaves until after freezing at −4°C. The maximum freezing tolerance was attained in all cultivars after 4 weeks of cold acclimation, and the temperature at which 50% electrolyte leakage occurred decreased to −8°C for spring oat, −10°C for winter oat, and −21°C for winter rye. In protoplasts isolated from leaves of nonacclimated spring oat, expansion-induced lysis was the predominant form of injury over the range of −2 to −4°C. At temperatures lower than −4°C, loss of osmotic responsiveness, which was associated with the formation of the hexagonal II phase in the plasma membrane and subtending lamellae, was the predominant form of injury. In protoplasts isolated from leaves of cold-acclimated oat, loss of osmotic responsiveness was the predominant form of injury at all injurious temperatures; however, the hexagonal II phase was not observed. Rather, injury was associated with the occurrence of localized deviations of the plasma membrane fracture plane to closely appressed lamellae, which we refer to as the "fracture-jump lesion." Although the freeze-induced lesions in the plasma membrane of protoplasts of spring oat were identical with those reported previously for protoplasts of winter rye, they occurred at significantly higher temperatures that correspond to the lethal freezing temperature.

Susceptibility to low temperatures and freezing is a primary determinant of the geographical distribution and yield of many agricultural crops. Although it is well known that rye (*Secale cereale* L.), the most freezing tolerant, and oat (*Avena sativa* L.), the least freezing tolerant, represent the extremes in freezing tolerance of the winter cereals, there have been few studies to document the precise differences during cold acclimation. In winter rye, destabilization of the plasma membrane is a primary cause of freezing injury and is a consequence of the osmotic stresses and cell dehydration that occur during freezing (Steponkus, 1984). In rye, freezing results in several different lesions in the plasma membrane, the incidence of which depends on the magnitude of the osmotic stress, the extent of cell dehydration, and the stage of cold acclimation. However, the plasma membrane lesions that are responsible for freezing injury in oat have not been characterized.

In NA protoplasts of winter rye, freezing injury occurs at temperatures below −2.5°C. Over the temperature range of −2.5 to −5°C, the predominant form of freezing injury in NA protoplasts of rye is expansion-induced lysis (Dowgert and Steponkus, 1984; Gordon-Kamm and Steponkus, 1984a). Freeze-induced osmotic contraction results in endocytotic vesiculation of the plasma membrane, which is irreversible. During thawing of the suspending medium and osmotic expansion of the protoplasts, lysis occurs before the protoplasts regain their initial volume/surface area. However, at temperatures below −6°C, freeze-induced dehydration is more severe and injury is manifested as a loss of osmotic responsiveness of the protoplasts (Dowgert and Steponkus, 1984). In NA protoplasts of rye, loss of osmotic responsiveness is associated with the formation of aparticulate domains in the plasma membrane, aparticulate lamellae subtending the plasma membrane, and the H_{II} phase (Gordon-Kamm and Steponkus, 1984b).

In ACC protoplasts of winter rye, freeze-induced osmotic contraction results in the reversible formation of exocytotic extrusions of the plasma membrane (Gordon-Kamm and Steponkus, 1984c), and, as a result, expansion-induced lysis does not occur (Dowgert and Steponkus, 1984). Injury in ACC protoplasts of rye occurs at temperatures lower than −20°C and is also manifested as a loss of osmotic responsiveness, but the H_{II} phase does not occur regardless of the temperature to which the protoplast suspensions are frozen (Gordon-Kamm and Steponkus, 1984c). Rather, freeze-fracture EM studies have revealed that injury in ACC protoplasts of rye is associated with the occurrence of localized deviations of the plasma membrane fracture plane to closely appressed subtending lamellae that are either aparticulate or particle depleted; we refer to this ultrastructural manifestation of

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Abbreviations: ACC protoplasts, protoplasts isolated from leaves of cold-acclimated seedlings; EF fracture face, ectoplasmic fracture face; EL, electrolyte leakage; T_{EL50}, temperature at which 50% electrolyte leakage occurred; FDA, fluorescein diacetate; H_{II}, hexagonal II phase; IMP, intramembrane particles; LT_{50}, temperature at which 50% of the protoplasts are lethally injured; NA protoplasts, protoplasts isolated from leaves of nonacclimated seedlings; osm, osmolal; 18:2/18:2-PC, 1,2-dilinoleoylphosphatidylcholine; PF fracture face, protoplasmic fracture face.

injury as the "fracture-jump lesion" (Fujikawa and Steponkus, 1990). Freeze-induced formation of the H_{II} phase in nonacclimated rye and the fracture-jump lesion in cold-acclimated rye during freezing-induced dehydration are not unique to isolated protoplasts and are also observed in the leaves from which the protoplasts are isolated (Webb and Steponkus, 1993).

The differential cryobehavior of the plasma membrane of NA and ACC protoplasts of rye is, in large part, the result of alterations in the lipid composition of the plasma membrane during cold acclimation (Steponkus and Lynch, 1989; Steponkus et al., 1990; Steponkus and Webb, 1992). A decrease in the incidence of expansion-induced lysis in rye protoplasts is associated with increased proportions of unsaturated species of phosphatidylcholine in the plasma membrane during cold acclimation (Uemura and Steponkus, 1994) and can also be effected by artificial enrichment of the plasma membrane with either mono- or di-unsaturated species of phosphatidylcholine (Steponkus et al., 1988; Uemura and Steponkus, 1989). Furthermore, during freeze-induced osmotic contraction of large unilamellar vesicles composed of the plasma membrane lipids from nonacclimated rye leaves, the liposomes form daughter vesicles that are sequestered within the mother liposome, whereas extraliposomal extrusions are formed in those composed of the plasma membrane lipids isolated from cold-acclimated leaves (Steponkus and Lynch, 1989). In addition, 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). Subsequent studies have shown that the decrease in the propensity for freeze-induced formation of the H_{II} phase in the plasma membrane of rye protoplasts occurs during the first week of cold acclimation (Sugawara and Steponkus, 1990).

Recently, we have initiated studies to determine whether genotypic differences in freezing tolerance are associated with differences in the lipid composition of the plasma membrane. In the present study, for the first step to provide a basis for understanding the biochemical and biophysical factors that limit the freezing tolerance of oat and rye, the freezing tolerance of a spring and a winter cultivar of oat is determined and compared with that of winter rye. Specifically, the objectives of this study were (a) to provide a detailed analysis of the freezing tolerance of leaves of spring (cv Ogle) and winter (cv Kanota) cultivars of oat and a winter rye cultivar (cv Puma) at weekly intervals during cold acclimation, (b) to determine the forms of injury and ultrastructural alterations in the plasma membrane that are associated with freezing injury of oat protoplasts, and (c) to compare the incidence of the freeze-induced lesions in the plasma membrane of oat with those that occur in rye in relation to the differences in freezing tolerance in nonacclimated and cold-acclimated leaves.

MATERIALS AND METHODS

Plant Materials

Seeds of a spring oat (*Avena sativa* L. cv Ogle) and a winter oat (*Avena sativa* L. cv Kanota) and of winter rye (*Secale cereale* L. cv Puma) were germinated in moist vermiculite and grown in a controlled environment at 20/15°C day/night (16-h photoperiod) and irrigated with Hoagland nutrient solution. Nonacclimated plants were kept in this environment for 10 to 12 d. Seedlings (7-d-old plants) that were to be cold acclimated were first transferred to 13/7°C day/night (11.5-h photoperiod) for 1 week before being transferred to the cold acclimation regime of 2°C (10-h photoperiod) for 1 to 4 weeks.

Determination of Freezing Tolerance of Leaves

Freezing injury of leaves was assessed by electrolyte leakage (EL). The leaves (0.5 g fresh weight) were cut into small pieces and placed in test tubes. Samples were cooled to −2°C for 1 h, and then ice formation was achieved by introducing a small piece of ice into the test tubes. After incubation overnight at −2°C, the samples were cooled in steps of 1°C at 15-min intervals and kept for 2 h at the specified temperatures. After the samples were thawed at 4°C and then incubated with 10 mL of distilled water at room temperature for 2 h, the EL from the leaves (EL_{frozen}) was measured with a conductivity meter (model 32; Yellow Springs Instrument, Yellow Springs, OH). EL from unfrozen leaves ($EL_{unfrozen}$) and from leaves frozen in liquid nitrogen (EL_{LN}) were taken as 0 and 100% EL, respectively. The percentage of EL from frozen leaves at the specified temperatures was calculated by the following equation:

$$\% \; EL = \frac{EL_{frozen} - EL_{unfrozen}}{EL_{LN} - EL_{unfrozen}} \times 100 \qquad (1)$$

The results shown are the average and SD of several different experiments; two samples were used for a given experiment. If no SD values are visible in the figure, they are smaller than the size of the symbols.

Protoplast Isolation

Protoplasts were enzymically isolated from leaves that were first gently abraded with HCl-washed carborundum (Fisher) and then rinsed with tap water and an appropriate isotonic solution of sorbitol. The isotonic osmolality for the nonacclimated, 1-week-acclimated, and 4-week-acclimated leaves was determined by plasmolysis-deplasmolysis tests with sorbitol solutions. The isotonic osmolality was estimated to be 0.45 for leaves of nonacclimated seedlings, 0.53 for leaves of 1-week-acclimated seedlings, and 0.65 for leaves of 4-week-acclimated seedlings. The leaves were cut into small pieces and incubated in an enzyme solution consisting of 1.3% (w/v) cellulysin (Calbiochem), 0.4% (w/v) macerase (Calbiochem), 0.6% (w/v) potassium dextran sulfate (Calbiochem), and 10 mM Mes/KOH (pH 5.6) in an isotonic sorbitol solution. After the leaves were incubated at 28°C for 2 to 3 h in the dark with gentle shaking, the undigested leaf sections were removed through four layers of cheesecloth, and the filtrate, which contained the isolated protoplasts, was centrifuged at 50*g* for 10 min at 0°C. The pellet was suspended in an isotonic sorbitol solution containing 1 mM Mes/KOH (pH 5.6) and then washed twice by centrifugation as described above. The washed protoplasts were resuspended in the

isotonic sorbitol solution containing 1 mM Mes/KOH (pH 5.6) and kept on ice until used.

Determination of Survival of Protoplasts

An aliquot of the protoplast suspension (0.5 mL, 2×10^5 protoplasts) was placed in a glass test tube (10 × 105 mm) and cooled to −2°C for 15 min prior to ice nucleation by touching the suspension with a spatula precooled in liquid nitrogen. After an additional 15 min at −2°C, the samples were cooled to the specified temperatures at a rate of 0.8°C min^{-1}. After a 30-min period at the specified temperatures, the samples were thawed at room temperature and then kept on ice. In some experiments, a hypertonic-thaw treatment was used to minimize the osmotic expansion of the protoplasts during thawing of the suspending medium and to preclude expansion-induced lysis; this is referred to as a freeze/hypertonic-thaw treatment. In this treatment, the frozen samples were warmed at −2°C for 5 min prior to addition of a hypertonic solution of sorbitol that contained 1 mM Mes/KOH (pH 5.6) and that was precooled to −2°C. This procedure yielded a final osmolality of 1.08 after melting of the remaining ice. After the ice melted, the protoplast suspensions were kept on ice.

Protoplast survival was determined by staining with FDA at a final concentration of 0.001% (w/v) (Widholm, 1972). After the samples were incubated for 5 min at room temperature with FDA, the number of stained protoplasts was counted in a hemocytometer. The results shown (percentage of survival of unfrozen control) are the average and SD of at least three different experiments; three hemocytometer samples were counted for each treatment of a given experiment. If no SD values are visible in the figure, they are smaller than the size of the symbols.

Protoplast-Liposome Fusion

Liposomes were prepared by sonication of an aqueous suspension of lipid. The lipid, 2 μmol of 18:2/18:2-PC (Avanti Polar Lipids, Alabaster, AL), was first dissolved in chloroform and then placed in a glass test tube (10 × 105 mm). After the solvent was evaporated by a stream of nitrogen gas, an isotonic sorbitol solution (0.9 mL of either 0.45 osm for NA protoplasts, 0.53 osm for 1-week-ACC protoplasts, or 0.65 osm for 4-week-ACC protoplasts) containing 1 mM Mes/KOH (pH 5.6) was added over the dried lipid film, and the test tube was filled with nitrogen gas and capped. The suspension was then sonicated for 10 to 15 min at room temperature until the suspension appeared clear. Fusion of protoplasts with the liposomes was performed by the pH-induced fusion procedure described previously (Arvinte and Steponkus, 1988). Briefly, aliquots of the protoplasts (2×10^6 protoplasts) and the liposome suspension (2 μmol of lipid) were added to 5 mL of a fusion buffer containing 0.15 M NaCl, 20 mM NaOH/acetic acid buffer (pH 4.6), and sorbitol, which had a final osmolality of 0.45 for NA protoplasts, 0.53 for 1-week-ACC protoplasts, or 0.65 for 4-week-ACC protoplasts of oat. The reaction was carried out at 28°C for 3 min after which 5 mL of the isotonic sorbitol solution, which contained 10 mM Mes/KOH (pH 5.6), was added at 0°C to stop the reaction. The protoplast suspension was centrifuged at 50*g* for 10 min at 0°C and washed twice by resuspension and centrifugation to remove unfused liposomes. Protoplast-liposome fusion was verified by fluorescence microscopy of the samples prepared with liposomes containing *N*-rhodamine-phosphatidylethanolamine (Avanti Polar Lipids) at a final concentration of 1% (w/w).

Effect of Osmotic Dehydration on Protoplast Survival

An aliquot (0.25 mL, 2×10^5 protoplasts) of the suspension of protoplasts stained with FDA was placed in a glass test tube (10 × 105 mm), and a hypertonic sorbitol solution (0.25 mL) containing 1 mM Mes/KOH (pH 5.6) was added to achieve the specified osmolality. After a 30-min incubation at 0°C, the suspension was warmed to room temperature, and the number of protoplasts that remained stained with fluorescein was counted in a hemocytometer. Survival of protoplasts kept in an isotonic sorbitol solution for 30 min at 0°C was taken as 100% survival. The results shown are the average and SD of at least three different experiments; three hemocytometer samples were counted for each treatment of a given experiment. If no SD values are visible in the figure, they are smaller than the size of the symbols.

Freeze-Fracture EM

The EM studies were conducted with protoplasts isolated from the leaves of the spring oat cultivar (Ogle). Small aliquots (approximately 2 μL) of the spring oat protoplast pellet, which was obtained after centrifugation of the protoplast suspension at 50*g* for 10 min, were loaded onto a freeze-fracture sample holder and placed in a small well of a copper block, which was cooled by a circulating ethanol bath (Neslab ULT-80). 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 suspensions were frozen at various temperatures over the temperature range of −2 to −10°C for 30 min before cryofixation for freeze-fracture EM. All cooling rates were 0.8°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 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 then examined on a Philips EM300 electron microscope at 80 kV of accelerating voltage.

The freeze-fracture replicas were examined for the occurrence of the ultrastructural alterations; only protoplasts in which the plasma membrane was discernible were scored for the presence or absence of the ultrastructural alteration. That is, although each freeze-fracture replica contained many protoplasts, a fracture plane through the plasma membrane occurred in only a small percentage of the protoplasts. The

incidence of the ultrastructural alterations is reported as a percentage of the protoplasts in which both the plasma membrane and the ultrastructural alteration were discernible; protoplasts that did not contain a discernible region of the plasma membrane were not scored. The values represent the percentages obtained for a minimum of 20 to 30 protoplasts for each treatment (protoplasts isolated from leaves of non-acclimated and cold-acclimated seedlings and subjected to three freezing temperatures: −3, −5, and −10°C). To obtain this number of protoplasts in which the plasma membrane was discernible for each treatment, it was necessary to pool the results from two to three different freezing tests.

RESULTS

Freezing Tolerance of Leaves

When grown under nonacclimating conditions (20/15°C day/night, 16-h photoperiod), the freezing tolerance of leaves of spring oat (cv Ogle) was similar to that of winter oat (cv Kanota) but significantly less than that of winter rye (cv Puma) (Fig. 1). EL from leaves of both cultivars of oat first occurred after freezing to −2°C and increased to more than 80% at −4°C. In contrast, EL from leaves of winter rye did not occur until after freezing to −4°C and then increased progressively with decreasing freezing temperature over the range of −4 to −10°C. The T_{EL50} was approximately −3°C for both cultivars of oat and −6°C for rye. It should be noted that the T_{EL50} values, which are based on the extent of EL from the samples after a freeze/thaw treatment, are used only as a common reference point and do not necessarily reflect the true lethal temperature for 50% survival as is sometimes inferred.

During 4 weeks of cold acclimation at 2°C, there was a significant increase in the freezing tolerance of leaves of all of the cultivars; however, the rate at which the freezing tolerance increased and the maximum freezing tolerance that was attained differed among the three cultivars (Fig. 1). In all three cultivars, the largest increase in freezing tolerance occurred during the first week of cold acclimation, but the magnitude of the increase differed for the different cultivars. It was the least in spring oat and the greatest in winter rye; after the first week of cold acclimation, the T_{EL50} decreased by approximately 3°C in spring oat, 4.5°C in winter oat, and 6°C in winter rye. In addition, in leaves of seedlings acclimated for 1 week, the threshold temperature at which EL increased was different among the cultivars, i.e. −4°C in spring oat, −6°C in winter oat, and −10°C in winter rye. The maximum freezing tolerance in all three cultivars was attained after 4 weeks of cold acclimation at 2°C; at that time, the T_{EL50} was decreased by 5°C in spring oat (from −3 to −8°C), 7°C in winter oat (from −3 to −10°C), and 15°C in winter rye (from −6 to −21°C). Additional increases in freezing tolerance were not elicited after 5 weeks of cold acclimation (data not shown). Although there was only a small difference (2°C) in the maximum freezing tolerance of the spring and winter cultivars of oat (T_{EL50}, −8°C versus −10°C), the rate at which the freezing tolerance increased was different in spring and winter oat. The maximum freezing tolerance of spring oat attained after 4 weeks of cold acclimation (T_{EL50} of −8°C) was equivalent to the freezing tolerance of winter oat after 1 week of cold acclimation (T_{EL50} of −7.5°C). Similarly, the maximum freezing tolerance of winter oat attained after 4 weeks of cold acclimation (T_{EL50} of −10°C) was less than the freezing tolerance of winter rye after 1 week of cold acclimation (T_{EL50} of −12°C).

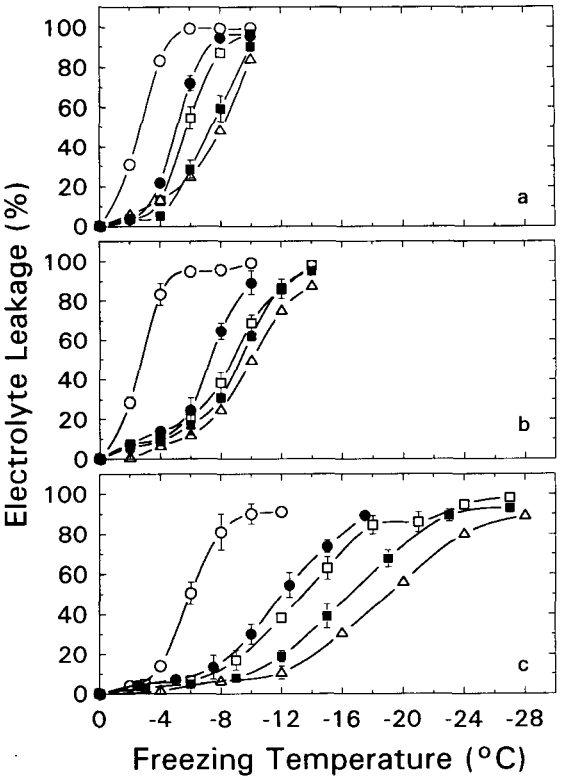


Figure 1. Freezing tolerance of leaves of spring oat (cv Ogle; a), winter oat (cv Kanota; b), and winter rye (cv Puma; c) during 4 weeks of cold acclimation as determined by measurement of EL from leaves after a freeze/thaw treatment. O, Nonacclimated leaves; ●, 1-week-acclimated leaves; □, 2-week-acclimated leaves; ■, 3-week-acclimated leaves; △, 4-week-acclimated leaves.

Forms of Freezing Injury of Oat Protoplasts

To determine what forms of freezing injury occur in the spring cultivar of oat, which is the least freezing tolerant of the cultivars examined, we investigated the manifestations of injury in protoplasts isolated from leaves. In suspensions of NA protoplasts frozen over the temperature range of −2 to −10°C, survival of protoplasts declined significantly after freezing to −2°C, and less than 10% survived at −6°C, with an LT_{50} of −4°C (Fig. 2). In contrast, more than 80% of ACC protoplasts survived freezing at temperatures over the range of 0 to −4°C, with an LT_{50} of −8°C (Fig. 2).

Next, to determine the temperature ranges over which expansion-induced lysis and loss of osmotic responsiveness occurred as a consequence of freeze-induced dehydration, survival of protoplasts after either a conventional freeze/thaw treatment or a freeze/hypertonic-thaw treatment was determined. In NA protoplasts, survival after the freeze/

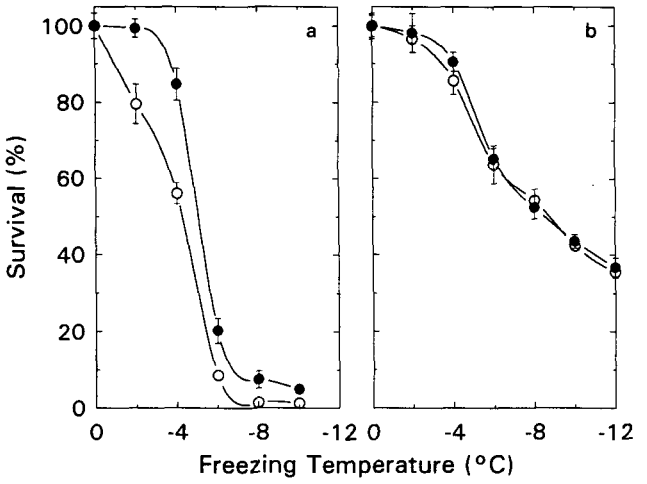


Figure 2. Survival of NA protoplasts (a) and ACC protoplasts (b) isolated from spring oat leaves after thawing in a hypertonic solution to preclude expansion-induced lysis. Survival was determined with FDA staining. O, Protoplasts after thawing in an isotonic solution (conventional freeze/thaw treatment); ●, protoplasts after thawing in a hypertonic solution (freeze/hypertonic-thaw treatment).

hypertonic-thaw treatment was greater than that after the conventional freeze/thaw treatment at temperatures over the range of −2 to −4°C (Fig. 2). However, there was little difference between the two treatments if the protoplast suspensions were frozen to temperatures of −6°C or below. In contrast, there was no difference between survival of ACC protoplasts subjected to either the conventional freeze/thaw treatment or the freeze/hypertonic-thaw treatment, regardless of the temperature at which protoplast suspensions were frozen (Fig. 2).

The decrease in survival of protoplasts after a conventional freeze/thaw treatment is the result of the combined incidence of expansion-induced lysis and loss of osmotic responsiveness. Because a freeze/hypertonic-thaw treatment limits osmotic expansion of the protoplasts and hence precludes the occurrence of expansion-induced lysis, the decrease in survival after the freeze/hypertonic-thaw treatment is attributable only to the loss of osmotic responsiveness. Consequently, the difference in survival after the conventional freeze/thaw treatment and the freeze/hypertonic-thaw treatment equals the incidence of injury resulting from expansion-induced lysis. Therefore, from the results in Figure 2, we conclude that expansion-induced lysis is the predominant form of freezing injury of NA protoplasts of oat at temperatures over the range of −2 to −4°C and that loss of osmotic responsiveness is the predominant form of the injury in both NA and ACC protoplasts of oat that are frozen to temperatures of −6°C or below.

In NA protoplasts of oat, fusion with liposomes composed of 18:2/18:2-PC resulted in a significant increase in survival over the temperature range of −2 to −4°C; however, there was no difference at −6°C or below (Fig. 3). Survival of NA protoplasts fused with liposomes composed of 18:2/18:2-PC was similar to that of NA protoplasts after a freeze/hypertonic-thaw treatment (Fig. 2), indicating that enrichment of the plasma membrane with 18:2/18:2-PC precludes expansion-induced lysis of NA protoplasts. In contrast, enrichment of the plasma membrane of ACC protoplasts with 18:2/18:2-PC did not elicit an increase in survival at any subzero temperature, which is consistent with the results that expansion-induced lysis does not occur in ACC protoplasts of oat at any injurious subzero temperatures (Fig. 2).

Although both the freeze/hypertonic-thaw treatment and enrichment of the plasma membrane with 18:2/18:2-PC effectively precluded the occurrence of expansion-induced lysis of NA protoplasts of oat, these two treatments affected the incidence of expansion-induced lysis in different ways. The freeze/hypertonic-thaw treatment of the protoplasts precluded expansion-induced lysis by reducing the extent of osmotic expansion, whereas enrichment of the plasma membrane with 18:2/18:2-PC precluded expansion-induced lysis by transforming the cryobehavior of the plasma membrane during freeze-induced osmotic contraction (i.e. formation of exocytotic extrusions) such that protoplasts are able to tolerate osmotic expansion during thawing (Steponkus et al., 1988).

Tolerance of Oat Protoplasts to Osmotic Dehydration

To determine the sensitivity of oat protoplasts to osmotic stress and cell dehydration, the survival of NA protoplasts was determined after exposure to hypertonic sorbitol solutions at 0°C without subsequent osmotic expansion. Survival of NA protoplasts remained at 100% after exposure to 1.03 osm but decreased from more than 90% at 1.61 osm to less than 10% at 3.23 osm (Fig. 4). The osmolality of the unfrozen portion of the suspending medium at −3 and −6°C is equivalent to 1.61 and 3.23, respectively; osmolality of the unfrozen portion of the solution = $-T/1.86$, in which T is the freezing temperature (°C). Therefore, NA protoplasts of oat

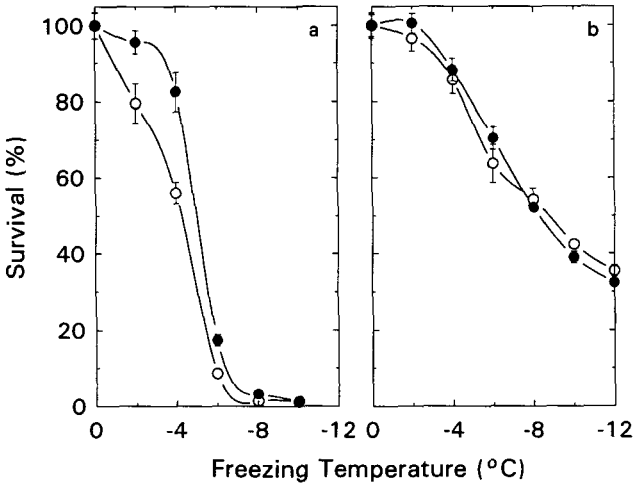


Figure 3. Effect of artificial enrichment of the plasma membrane with 18:2/18:2-PC on the survival of NA protoplasts (a) and ACC protoplasts (b) isolated from spring oat leaves; survival was determined with FDA staining. O, Control protoplasts (unfused but treated with the fusion buffer); ●, protoplasts fused with liposomes composed of 18:2/18:2-PC.

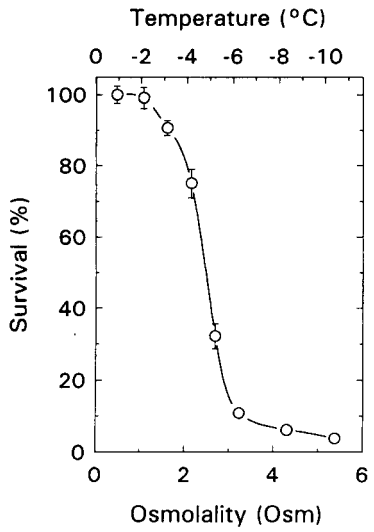


Figure 4. Survival of NA protoplasts isolated from spring oat leaves after exposure to a hypertonic sorbitol solution. Protoplasts, which were stained with FDA, were incubated in a hypertonic solution with the specified osmolality for 30 min at 0°C. Survival was determined by counting the number of FDA-stained protoplasts that remained after the osmotic treatment, i.e. without subsequent osmotic expansion. The temperature at which the equivalent osmotic stress is incurred in a partially frozen solution is given at the top of the figure.

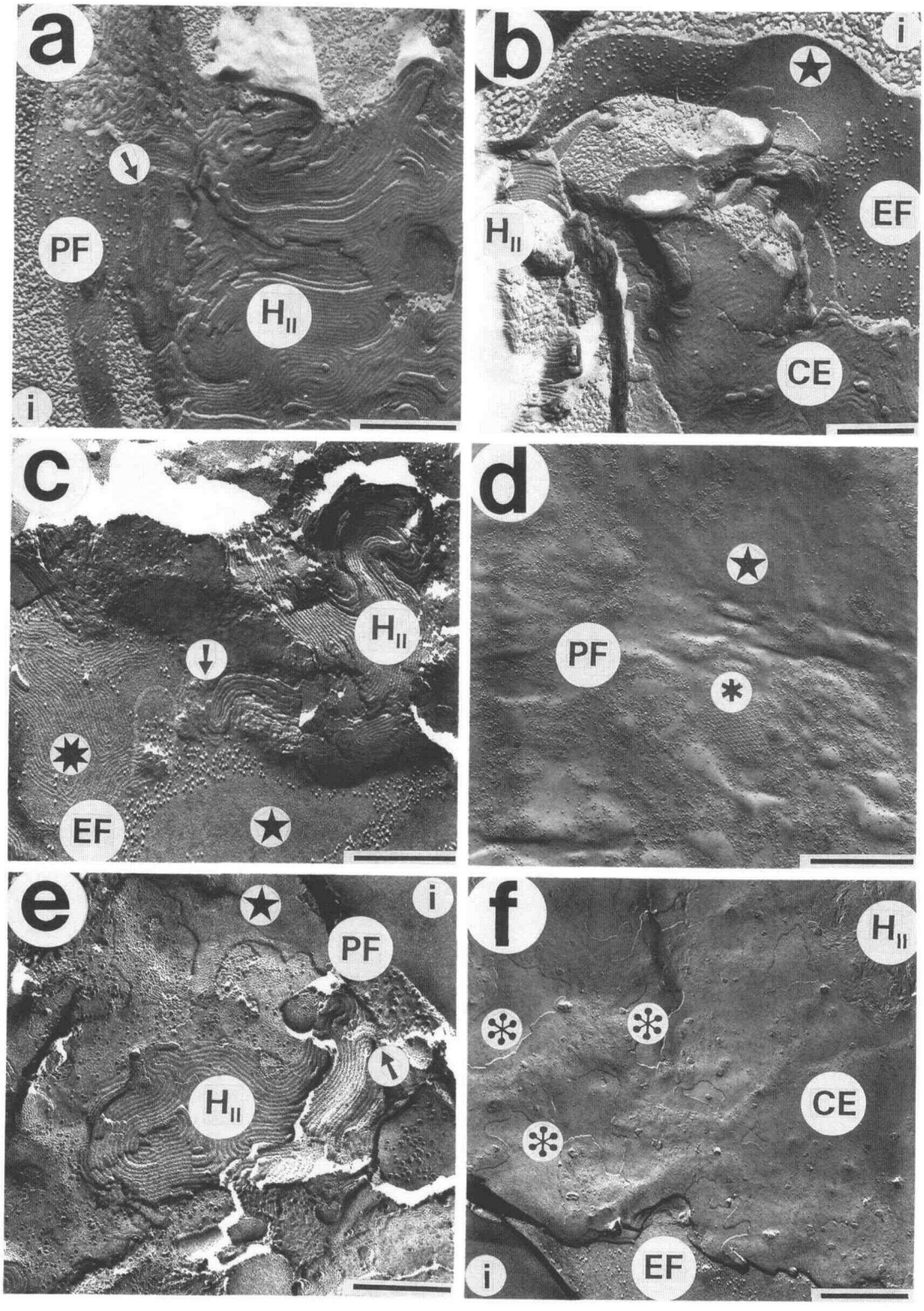
are injured by relatively mild osmotic stresses similar to those experienced during freezing between −3 and −6°C (Fig. 2).

Freeze-Induced Ultrastructural Alterations in Oat Protoplasts

To determine the membrane ultrastructural alterations that were associated with the loss of osmotic responsiveness, frozen suspensions of NA and ACC protoplasts of spring oat (cv Ogle) were examined by freeze-fracture EM. In NA protoplasts frozen at −2°C, aparticulate domains in the plasma membrane and aparticulate lamellae subtending the plasma membrane were observed. Aparticulate domains in the plasma membrane and aparticulate lamellae subtending the plasma membrane were observed in approximately 45% of the NA protoplasts frozen at −3°C and became more frequent with decreasing subzero temperature, e.g. aparticulate domains were observed in approximately 81% of the protoplasts frozen at −10°C (Fig. 5). At −3°C, the H_{II} phase was observed interior to the plasma membrane in both PF (Fig. 5a) and EF fracture faces (Fig. 5, b and c). Because the H_{II} phase is a nonlamellar phase and the liquid crystalline-to-H_{II} phase transition is an interbilayer event, the inverted cylindrical micelles of the H_{II} phase are expected to appear adjacent to, but not in, the plasma membrane per se. We observed that the outer membrane of the chloroplast envelope was the intracellular membrane most commonly associated with the H_{II} phase (Fig. 5, b and f). The participation of the plasma membrane in the formation of the H_{II} phase was confirmed by the observation that the cylindrical micelles of the H_{II} phase were physically continuous with the inner monolayer of the plasma membrane (Fig. 5, a and e, arrows). In NA protoplasts of oat frozen at −3°C, the H_{II} phase was observed in approximately 25% of the protoplasts. At temperatures below −4°C, the H_{II} phase was observed at significantly higher frequencies; the H_{II} phase was observed in 48% of the protoplasts frozen at −5°C (Fig. 5c) and in 71% of the protoplasts frozen at −10°C (Fig. 5, e and f) and was the predominant ultrastructural alteration observed at these temperatures.

Several other ultrastructural alterations were also observed in NA protoplasts of oat that were frozen to lethal temperatures. The regions of the plasma membrane EF fracture face that were closely associated with the H_{II} phase were frequently IMP depleted and occasionally had distinct undulations in the membrane (Fig. 5c). We interpret these undulations as arising from the contouring of the plasma membrane EF monolayer to the shape of the cylindrical micelles in adjacent domains of the H_{II} phase. This structural alteration suggests that the inner monolayer, but not the outer monolayer, of the plasma membrane participates in the formation of the H_{II} phase. This interpretation is consistent with the expectation that the lattice of the H_{II} phase will include an outermost lipid monolayer that is in the lamellar phase as a

Figure 5. (on facing page). Freeze-fracture electron micrographs of NA protoplasts of spring oat (cv Ogle) frozen at temperatures over the range of −3 to −10°C. a, PF fracture face of the plasma membrane of an NA protoplast frozen at −3°C. The H_{II} phase (H_{II}) was observed in close association with the plasma membrane. A region where the inverted cylindrical micelles of the H_{II} phase are continuous with the plasma membrane is indicated by an arrow. b, EF fracture face of the plasma membrane of an NA protoplast frozen at −3°C. The plasma membrane contains an aparticulate domain (★) and is closely appressed to the chloroplast envelope (CE) and the H_{II} phase. Note the loosely organized ripples in the chloroplast envelope. c, EF fracture face of the plasma membrane of an NA protoplast frozen at −5°C. The plasma membrane contains aparticulate domains (★) and is associated with the H_{II} phase. A region of the plasma membrane EF fracture face with well-defined undulations is indicated by an eight-point star. Close association of these undulations with the inverted micelles of the H_{II} phase is indicated by an arrow. d, PF fracture face of the plasma membrane of an NA protoplast frozen at −5°C. The plasma membrane contains frequent aparticulate domains (★) as well as paracrystalline arrays of IMPs (✱). e, PF fracture face of the plasma membrane of an NA protoplast frozen at −10°C. The H_{II} phase and aparticulate lamellae (★) are in close association with the plasma membrane. A region where the inverted cylindrical micelles are continuous with the inner monolayer of the plasma membrane (PF) is indicated by an arrow. f, EF fracture face of the plasma membrane of an NA protoplast frozen at −10°C. The plasma membrane is associated with an overlying and closely appressed chloroplast envelope (CE) that contains the fracture-jump lesion (✱) and a small domain of the H_{II} phase. i, Extracellular medium. Bars represent: a, 300 nm; b, 200 nm; c, 300 nm; d, 500 nm; e, 200 nm; f, 400 nm.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consequence of the unfavorable free energy of exposing the acyl chains of the lipids to the aqueous environment.

In addition, very loosely organized ripples were observed in the fracture faces of the chloroplast envelope (Fig. 5b). However, the ripples observed in the chloroplast envelope were readily distinguishable from the undulations observed in the plasma membrane EF fracture face (Fig. 5, cf. b and c). Paracrystalline arrays of IMP (Fig. 5d) were also observed on the plasma membrane PF fracture plane in NA protoplasts of oat. However, the frequency of the paracrystalline arrays did not increase significantly with decreasing temperatures below −3°C (approximately 10% of protoplasts frozen at −3, −5, and −10°C contained paracrystalline arrays).

The H_{II} phase was not observed in suspensions of ACC protoplasts of oat frozen over the range of −3 to −10°C. At −3°C, the plasma membrane had very few membrane ultrastructural alterations. The plasma membrane was exclusively in the bilayer configuration and appeared to have randomly distributed IMPs (Fig. 6, a and b), although no statistical analysis of the IMP distribution was performed. In some instances, paracrystalline arrays of IMP were observed (not shown), but these occurred at a low frequency (22 and 12% of protoplasts frozen at −3 and −10°C, respectively). At −5°C, the fracture-jump lesion was observed to occur in the plasma membrane and subtending lamellae of 33% of the examined protoplasts. We use the phrase fracture-jump lesion to refer to a localized region of 0.1 to 2.5 μm in diameter where the fracture plane has "jumped" from the plasma membrane to closely appressed subtending lamellae that are either aparticulate or have a greatly reduced IMP frequency. At −10°C, the fracture-jump lesion was the predominant membrane ultrastructural alteration in ACC protoplasts, occurring in 88% of the protoplasts, and was observed in the plasma membrane and subtending intracellular membranes (Fig. 6, c–e). The fracture-jump lesion was observed in both the EF (Fig. 6, c and d) and PF (Fig. 6e) fracture faces of the plasma membrane and was often closely associated with aparticulate domains within the plasma membrane (Fig. 6c). In addition, aggregation of IMP was observed in both the EF (Fig. 6d) and PF (Fig. 6f) fracture faces of the plasma membrane in 30 and 35% of the protoplasts frozen at −3 and −10°C, respectively.

Temporal Aspects of Freezing Injury

As previously reported for rye (Steponkus et al., 1990), the freeze-induced lesions associated with freezing injury of oat differ depending on the stage of cold acclimation; however, it remains to be resolved when these changes occur during cold acclimation of oat. To compare the temporal aspects of freezing injury in spring oat and winter rye, preliminary studies of 1-week-ACC protoplasts of spring oat were initiated.

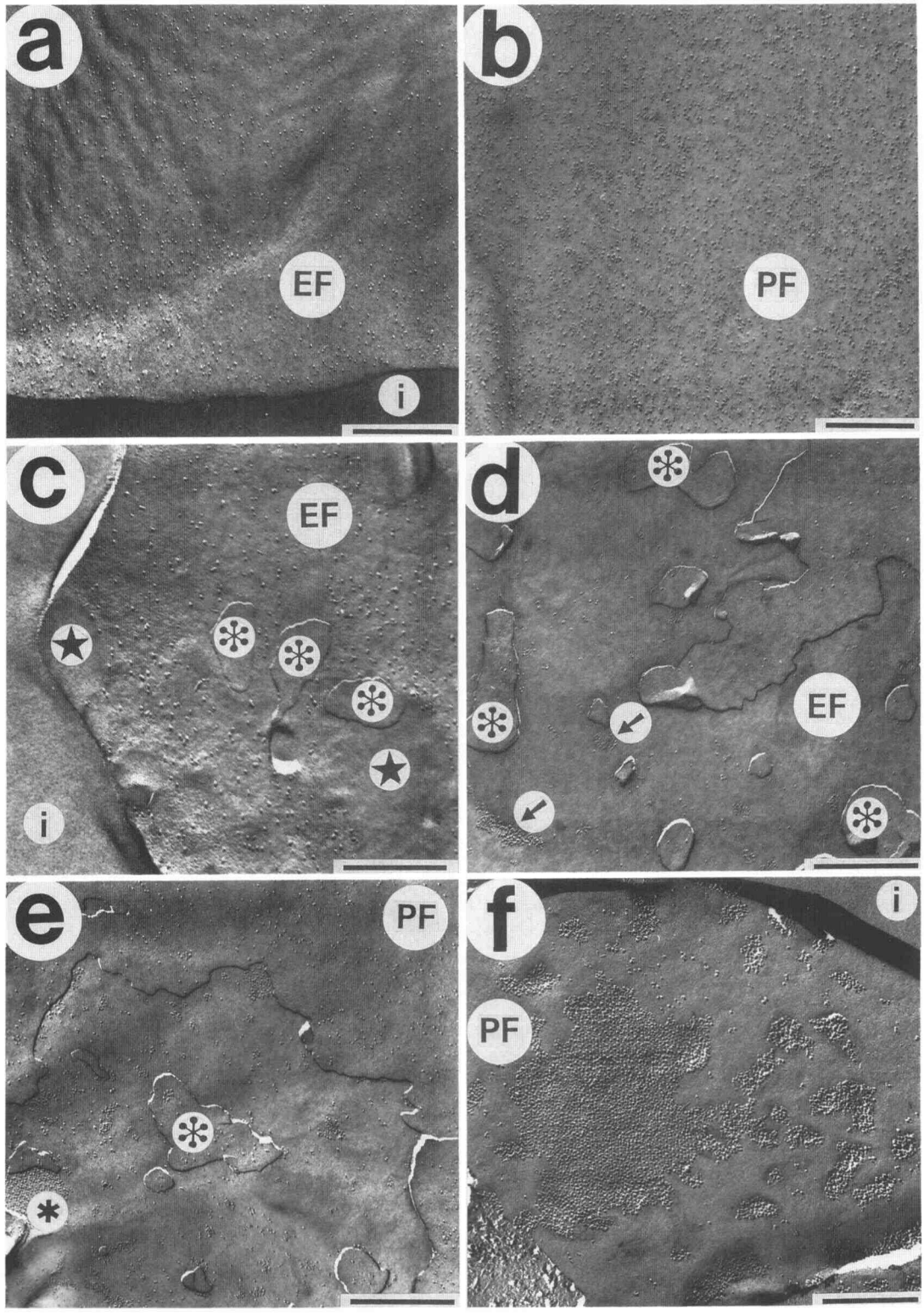
In 1-week-ACC protoplasts of oat, both expansion-induced lysis and loss of osmotic responsiveness were responsible for freezing injury (Fig. 7). After a conventional freeze/thaw treatment, more than 95% of the protoplasts survived at −2°C, but survival declined progressively over the range of −4 to −10°C, with an LT_{50} of −6°C. When survival was determined after a freeze/hypertonic-thaw treatment, survival was substantially higher than that after a conventional freeze/thaw treatment over the range of −4 to −6°C. The difference in survival between a conventional freeze/thaw treatment and a freeze/hypertonic-thaw treatment (i.e. the incidence of expansion-induced lysis) was 20% at −4°C and 25% at −6°C. However, there was little difference in survival between these two treatments at temperatures of −8°C or below. Thus, these results indicate that the incidence of expansion-induced lysis still accounted for a significant decrease in survival of protoplasts isolated from oat leaves after 1 week of cold acclimation.

In suspensions of 1-week-ACC protoplasts of oat frozen at −5°C, the H_{II} phase was not observed (data not shown). Instead, injury was associated with the occurrence of the fracture-jump lesion in the plasma membrane and chloroplast envelope (data not shown). The frequency of the fracture-jump lesion in both the plasma membrane and the chloroplast envelope of 1-week-ACC protoplasts at −5°C was greater than that which occurred in 4-week-ACC protoplasts at −5°C. In addition, aparticulate domains within the plasma membrane and aparticulate lamellae subtending the plasma membrane were observed in 1-week-ACC protoplasts frozen at −5°C.

DISCUSSION

This study demonstrates that the plasma membrane lesions associated with freezing injury of NA and ACC protoplasts of spring oat are identical with those previously reported for protoplasts (Gordon-Kamm and Steponkus, 1984b; Fujikawa and Steponkus, 1990) and leaves (Webb and Steponkus, 1993) of winter rye. In NA protoplasts of both spring oat and winter rye, expansion-induced lysis is the predominant form of injury at or above the LT_{50}. At lower temperatures, loss of

Figure 6. (on facing page). Freeze-fracture electron micrographs of ACC protoplasts of spring oat (cv Ogle) frozen at −3 and −10°C. a, EF fracture face of the plasma membrane of an ACC protoplast frozen at −3°C. The plasma membrane contains randomly distributed IMP. b, PF fracture face of the plasma membrane of an ACC protoplast frozen at −3°C. The plasma membrane contains randomly distributed IMP. c, EF fracture face of the plasma membrane of an ACC protoplast frozen at −10°C. The plasma membrane contains several fracture-jump lesions (✻) and aparticulate domains (★). d, EF fracture face of the plasma membrane of an ACC protoplast frozen at −10°C. The plasma membrane contains numerous fracture-jump lesions (✻) to subtending (interior) lamellae. The aggregation of IMP into small, nonparacrystalline, domains is indicated by arrows. e, PF fracture face of the plasma membrane of an ACC protoplast of oat frozen at −10°C. A large region of lamellae subtending the plasma membrane that contains several fracture-jump lesions (✻) is shown. Paracrystalline arrays of IMPs (✱) were also observed. f, PF fracture face of the plasma membrane of an ACC protoplast of oat frozen at −10°C, showing aggregation of IMP and large regions of particle-free lamellae. i, Extracellular medium. Bars represent: a, 400 nm; b, 300 nm; c, 300 nm; d, 400 nm; e, 400 nm; f, 300 nm.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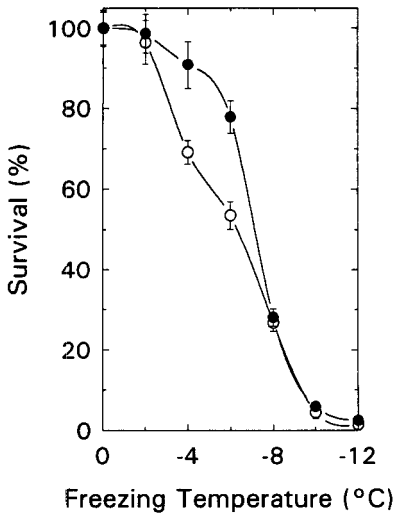

Figure 7. Survival of 1-week-ACC protoplasts isolated from spring oat (cv Ogle) leaves after thawing in a hypertonic solution to preclude expansion-induced lysis. Survival was determined by staining with FDA. O, Protoplasts after thawing in an isotonic solution (conventional freeze/thaw treatment); ●, protoplasts after thawing in a hypertonic solution (freeze/hypertonic-thaw treatment).

osmotic responsiveness is the predominant form of injury and is associated with the formation of the H_{II} phase. In ACC protoplasts of both oat and rye, loss of osmotic responsiveness is the predominant form of injury at all injurious temperatures and is associated with the fracture-jump lesion rather than the formation of the H_{II} phase. Nevertheless, the temperature range over which each of the plasma membrane lesions occurs is higher in oat than in rye.

Over the range of −2 to −4°C, expansion-induced lysis is the predominant form of freezing injury in NA protoplasts of spring oat. This form of injury is also observed in NA protoplasts of rye but occurred at somewhat lower temperatures, i.e. over the range of −2.5 to −6°C (Uemura and Steponkus, 1989). At −2°C, survival of NA protoplasts of oat decreased by 20% because of the occurrence of expansion-induced lysis (Fig. 2); whereas there was no decrease in survival of NA protoplasts of rye until after freezing to −2.5°C (Uemura and Steponkus, 1989; Steponkus et al., 1993). In NA protoplasts of rye, freeze-induced osmotic contraction results in endocytotic vesiculation of the plasma membrane, which decreases the surface area of the plasma membrane. Although endocytotic vesiculation per se is not injurious, sufficiently large area reductions (>25%) are irreversible and limit the expansion potential during subsequent thawing of the suspending medium (Dowgert and Steponkus, 1984). It is possible that the difference in the temperature range over which expansion-induced lysis occurs in NA protoplasts of oat and rye is attributable to differences in the potential of the protoplasts for osmotic expansion during thawing. However, it should be noted that the lower limit of the temperature range over which expansion-induced lysis occurs, which is higher in oat (−4°C) than in rye (−6°C), is delimited by the threshold temperature at which loss of osmotic responsiveness that is associated with freeze-induced formation of the H_{II} phase occurs. That is, osmotic expansion of the protoplasts during thawing of the suspending medium, which is required for manifestation of expansion-induced lysis, will be precluded if the protoplasts are osmotically unresponsive. This is discussed in greater detail in a subsequent section.

When frozen to temperatures of −4°C or below, loss of osmotic responsiveness is the predominant form of freezing injury in NA protoplasts of oat. This form of injury is also observed in NA protoplasts of rye (Gordon-Kamm and Steponkus, 1984b; Pihakaski and Steponkus, 1987). However, the first decrease in survival that is attributable to the loss of osmotic responsiveness occurs at a higher temperature in oat (−4°C) (Fig. 2) than in rye (−5°C) (Uemura and Steponkus, 1989). In NA protoplasts of both oat and rye, loss of osmotic responsiveness is associated with the formation of the H_{II} phase in the plasma membrane and subtending lamellae. In protoplasts of oat, the H_{II} phase is first observed at −3°C and occurs in approximately 50% of the protoplasts at −5°C; in protoplasts of rye, the H_{II} phase formation is first observed at −6°C (Pihakaski and Steponkus, 1987) and occurs in 50% of the protoplasts at −10°C (Gordon-Kamm and Steponkus, 1984a). Furthermore, in both spring oat and winter rye, the threshold temperature for the formation of the H_{II} phase corresponds to the temperature at which the loss of osmotic responsiveness is first observed, and the temperature at which the H_{II} phase is observed in approximately 50% of the protoplasts corresponds to the temperature at which 50% of protoplasts are injured.

The lower tolerance of NA protoplasts of oat to the osmotic stresses experienced during freezing is a manifestation of the differential propensity for freeze-induced formation of the H_{II} phase in NA protoplasts of oat and rye (threshold temperature of the freeze-induced formation of the H_{II} phase was −3 and −6°C, respectively). At −3°C, the osmolality of the unfrozen portion of the suspending medium is 1.6 (corresponding to an osmotic pressure of approximately 3.6 MPa); at −6°C, the unfrozen medium is 3.2 osm (7.2 MPa). Thus, the minimum osmotic pressure that is sufficient to induce formation of the H_{II} phase is lower in NA protoplasts of oat than in NA protoplasts of rye. This is consistent with the observation that the survival of NA protoplasts of rye declined after exposure to hypertonic solutions of 3 osm (M. Uemura and P.L. Steponkus, unpublished results), whereas survival of NA protoplasts of oat decreased after dehydration in a 2-osm solution (Fig. 4). These results indicate that NA protoplasts of oat are more sensitive to osmotic dehydration than NA protoplasts of rye.

There are both similarities and differences in the alterations in the cryostability of the plasma membrane during cold acclimation of oat and rye. As with 4-week-ACC protoplasts of rye (Gordon-Kamm and Steponkus, 1984b), freeze-induced formation of the H_{II} phase was not observed at any injurious temperature in 4-week-ACC protoplasts of oat. In protoplasts of rye, the propensity for the freeze-induced formation of the H_{II} phase is decreased during the first 7 to 10 d of cold acclimation (Sugawara and Steponkus, 1990). The decreased propensity for the freeze-induced formation of the H_{II} phase was also observed in protoplasts isolated

from oat leaves that were cold acclimated for 1 week; after freezing at −5°C, the H_{II} phase occurred in approximately 50% of NA protoplasts, but was not observed in 1-week-ACC protoplasts of oat. Thus, amelioration of the freeze-induced H_{II} phase formation occurs during the first week of cold acclimation in both oat and rye.

In contrast, alterations in the plasma membrane during cold acclimation that preclude the occurrence of expansion-induced lysis appear to occur more rapidly in rye than in oat. In rye, expansion-induced lysis is precluded during the first 7 to 10 d of cold acclimation (Uemura and Steponkus, 1989). However, in protoplasts of oat, the incidence of expansion-induced lysis of protoplasts still accounted for a significant decrease in survival (20% at −4°C and 25% at −6°C) after the first week of cold acclimation. Apparently the higher temperature range for the occurrence of expansion-induced lysis of NA protoplasts of oat (−2 to −4°C) in contrast to rye (−2.5 to −6°C) is the consequence of a higher threshold temperature for the occurrence of the freeze-induced formation of the H_{II} phase. This is suggested by the results with 1-week-ACC protoplasts of oat in which the incidence of expansion-induced lysis accounted for a 25% decrease in survival at −6°C; whereas there was only a small incidence of expansion-induced lysis (<10%) in NA protoplasts of oat at −6°C, and the freeze-induced formation of the H_{II} phase occurred in more than 50% of the protoplasts at these temperature ranges. Nevertheless, there was little occurrence of either expansion-induced lysis or the H_{II} phase in protoplasts of oat after 4 weeks of cold acclimation, indicating that spring oat undergoes the initial stage of cold acclimation during which the cryostability of the plasma membrane increases so as to preclude both expansion-induced lysis and the formation of the freeze-induced H_{II} phase.

Freezing injury of ACC protoplasts of oat is manifested as loss of osmotic responsiveness and is associated with the fracture-jump lesion as previously reported for ACC protoplasts of rye (Fujikawa and Steponkus, 1990) and leaves of cold-acclimated rye seedlings (Webb and Steponkus, 1993). In oat, however, the fracture-jump lesion occurs at higher temperatures, and the temperature range over which it occurs does not decrease substantially during cold acclimation, as observed in rye. That is, in 1-week-ACC protoplasts of rye, the fracture-jump lesion was first observed after freezing to −10°C and increased in frequency between −10 and −20°C; in 4-week-ACC protoplasts of rye, the fracture-jump lesion was first observed after freezing to −20°C and increased in frequency in suspensions that were frozen to temperatures between −20 and −40°C (Fujikawa and Steponkus, 1990). In contrast, the fracture-jump lesion occurred at a high frequency in 1-week-ACC protoplasts of oat frozen at −5°C and in 4-week-ACC protoplasts of oat at −10°C. Thus, during cold acclimation the magnitude of the decrease in the temperature range over which the fracture-jump lesion is the predominant lesion is much smaller in spring oat than in winter rye.

We have also observed an additional ultrastructural alteration in ACC protoplasts of oat—the aggregation of plasma membrane IMP (Fig. 6f)—that has not been observed in either ACC protoplasts or cold-acclimated leaves of rye. Neither the physical cause nor the possible injurious consequences of this aggregation is known; however, freeze-induced aggregation of plasma membrane IMP has been reported during freezing in the tertiary hyphae of mushroom (Fujikawa, 1988).

In summary, we have demonstrated that the plasma membrane lesions that limit the freezing tolerance of spring oat and winter rye—two species that differ widely in their freezing tolerance—are identical. However, the temperatures at which the freeze-induced lesions occur are significantly higher in spring oat than in winter rye, especially for the freeze-induced formation of the H_{II} phase in NA protoplasts and the fracture-jump lesion in ACC protoplasts. Given that previous studies have established that the differences in the cryostability of the plasma membrane of rye before and after cold acclimation are a result of alterations in the lipid composition of the plasma membrane that occur during cold acclimation (see a review by Steponkus et al., 1990), we now wish to determine whether the differences in the freezing tolerance of oat and rye at the temperatures at which the various freeze-induced lesions occur are also consequences of genotypic differences in the lipid composition of the plasma membrane. In a companion paper (Uemura and Steponkus, 1994), we present a detailed analysis of the lipid composition of the plasma membrane of oat and rye that documents that there is a vast difference in the lipid composition of the plasma membrane of oat and rye and suggests that these differences in plasma membrane lipid composition are, in part, responsible for the difference in the freezing tolerance of spring oat and winter rye.

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