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Effect of Cold Acclimation on the Incidence of Two Forms of Freezing Injury in Protoplasts Isolated from Rye Leaves[1]

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ABSTRACT

The freezing tolerance and incidence of two forms of freezing injury (expansion-induced lysis and loss of osmotic responsiveness) were determined for protoplasts isolated from rye leaves (*Secale cereale* L. cv Puma) at various times during cold acclimation. During the first 4 weeks of the cold acclimation period, the LT_{50} (*i.e.* the minimum temperature at which 50% of the protoplasts survived) decreased from −5°C to −25°C. In protoplasts isolated from nonacclimated leaves (NA protoplasts), expansion-induced lysis (EIL) was the predominant form of injury at the LT_{50}. However, after only 1 week of cold acclimation, the incidence of EIL was reduced to less than 10% at any subzero temperature; and loss of osmotic responsiveness was the predominant form of injury, regardless of the freezing temperature. Fusion of either NA protoplasts or protoplasts isolated from leaves of seedlings cold acclimated for 1 week (1-week ACC protoplasts) with liposomes of dilinoleoylphosphatidylcholine also decreased the incidence of EIL to less than 10%. Fusion of protoplasts with dilinoleoylphosphatidylcholine diminished the incidence of loss of osmotic responsiveness, but only in NA protoplasts or 1-week ACC protoplasts that were frozen to temperatures over the range of -5 to -10°C. These results suggest that the cold acclimation process, which results in a quantitative increase in freezing resistance, involves several different qualitative changes in the cryobehavior of the plasma membrane.

Cold acclimation of rye seedlings is induced by exposure to temperatures of 2 to 5°C, with the maximum freezing tolerance achieved in 4 to 6 weeks. For example, the freezing tolerance of the crowns of *Secale cereale* L. cv Puma increases from approximately −5°C to −25°C after 4 weeks of cold acclimation (14). This difference in freezing tolerance is also observed in protoplasts isolated from both epicotyls (4) and leaves (5) of nonacclimated and fully acclimated seedlings. In the case of isolated mesophyll protoplasts, destabilization of the plasma membrane is a primary cause of freezing injury and is a consequence of freeze-induced osmotic stresses and dehydration (10, 11). However, the mechanism of injury in protoplasts isolated from leaves of nonacclimated seedlings (NA[3] protoplasts) is different from that responsible for injury in protoplasts isolated from leaves of cold-acclimated seedlings (ACC protoplasts).

In NA protoplasts, freeze-induced dehydration results in two different forms of injury—the incidence of which depends on the extent of dehydration (which is determined by the minimum temperature to which the protoplast suspension is frozen) (11). When frozen to temperatures over the range of 0 to -5°C, injury is a consequence of osmotic excursions incurred during the freeze/thaw cycle. Freeze-induced osmotic contraction results in endocytotic vesiculation of the plasma membrane and the surface area of the plasma membrane is reduced (5, 7). Sufficiently large area reductions are irreversible and the protoplasts lyse during osmotic expansion following thawing of the suspending medium—before regaining their initial size. This form of injury is referred to as expansion-induced lysis (EIL). When frozen to lower temperatures (*e.g.* −10°C), dehydration is more severe and injury is manifested as a complete loss of osmotic responsiveness (LOR) (5). This form of injury is a consequence of dehydration-induced alterations in the ultrastructure of the plasma membrane that include the formation of large aparticulate domains and lamellar-to-hexagonal$_{II}$ phase transitions (6).

Following cold acclimation the behavior of the plasma membrane during a freeze/thaw cycle is altered such that freeze-induced osmotic contraction results in the reversible formation of exocytotic extrusions of the plasma membrane (8) and, as a result, EIL occurs at only a very low frequency in ACC protoplasts (5). Further, although injury in ACC protoplasts at the LT_{50}(-5°C) is manifested as a loss of osmotic responsiveness, dehydration-induced lamellar-to-hexagonal$_{II}$ phase transitions are not observed at any subzero temperature—even at -35°C (6).

These differences in the cryobehavior of the plasma membrane of NA and ACC protoplasts are the result of alterations in the lipid composition of the plasma membrane following cold acclimation (11). This is evidenced by the fact that liposomes prepared from plasma membrane lipid extracts of nonacclimated and cold-acclimated rye leaves (referred to as NA liposomes and ACC liposomes, respectively) also exhibit the differential behavior observed in the plasma membrane of NA and ACC protoplasts. For example, NA liposomes

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[3] Abbreviations: NA protoplasts, protoplasts isolated from nonacclimated leaves; *x*-week ACC protoplasts, protoplasts isolated from leaves cold acclimated for *x* weeks; LT_{50}, the temperature at which 50% of protoplasts are injured; EIL, expansion-induced lysis; LOR, loss of osmotic responsiveness; PC, phosphatidylcholine; osm, osmolal; DL_2PC, dilinoleoylphosphatidylcholine.

undergo endocytotic vesiculation during freeze-induced osmotic contraction, whereas ACC liposomes form exocytotic extrusions (12). And, severe dehydration results in lamellar-to-hexagonal$_{II}$ phase transitions in NA liposomes but not in ACC liposomes (3).

Detailed analyses of the lipid composition of the plasma membrane fractions isolated from nonacclimated and cold-acclimated rye leaves revealed that the proportion of virtually every lipid species is altered following cold acclimation (9). However, among the more than 100 different species identified to date, there is no single lipid species that is unique to the plasma membrane of either nonacclimated or cold-acclimated leaves. Therefore, the differential cryobehavior of the plasma membrane following cold acclimation apparently is a consequence of altered lipid-lipid interactions that result from differences in the proportions of the various lipid species.

Subsequent 'membrane engineering' studies to establish the specific lipid alterations responsible for the differential cryobehavior of the plasma membrane have involved a protoplast × liposome fusion technique (1) to selectively modify the lipid composition of the plasma membrane. Enrichment of the plasma membrane with either mono- or diunsaturated species of phosphatidylcholine transforms the cryobehavior of the plasma membrane such that exocytotic extrusions (rather than endocytotic vesicles) are formed during osmotic contraction, and EIL does not occur during osmotic excursions (13).

Collectively, these studies, which contrast the behavior of protoplasts isolated from nonacclimated rye leaves to that of protoplasts isolated from leaves of plants at maximum hardiness, indicate that the quantitative increase in freezing tolerance is a consequence of different qualitative changes in the cryobehavior of the plasma membrane. However, the temporal aspects of these changes are not known. The objectives of this study were to quantify the progressive increase in freezing tolerance of rye protoplasts and the incidence of the two forms of injury (EIL and LOR) during the cold acclimation process so as to determine when the incidence of EIL first reached a minimum. Further, we wished to determine if artificial enrichment of the plasma membrane with DL_2PC, which precludes EIL in NA protoplasts (13), elicits any additional increase in freezing tolerance in protoplasts isolated from leaves at intermediate stages of cold acclimation—when EIL is precluded because of the natural acclimation process.

MATERIALS AND METHODS

Plant Materials

Seeds of *Secale cereale* L. cv Puma were germinated in moist vermiculite and grown in a controlled environment at a 20°C-day and 15°C-night (16-h photoperiod). Nonacclimated plants remained in this environment for 10 to 14 d. Cold acclimation was achieved by exposing 1-week-old plants to 13°C-day and 7°C-night (11.5-h photoperiod) for 1 week, which is designated as the pre-acclimation stage, and then to 2°C (10-h photoperiod) for 1 to 6 weeks.

Protoplast Isolation

Changes in the osmotic potential of cells during cold acclimation were determined by plasmolysis-deplasmolysis tests with sorbitol solutions. The osmotic potentials were estimated to be 0.53 osm for nonacclimated leaves and 0.66, 0.78, 0.90, and 1.03 osm for leaves acclimated for 1, 2, 3, and 4 weeks, respectively (data not shown). For protoplast isolation, the leaves were gently abraded with acid-washed carborundum 500 (Fisher Co.) and washed in the appropriate isotonic sorbitol solution. The leaves were cut into small pieces (0.5 to 1 mm in length) and incubated in an isolation medium consisting of 1.3% (w/v) cellulysin (Calbiochem), 0.4% (w/v) macerase (Calbiochem), 0.6% (w/v) potassium dextran sulfate and 10 mM Mes/KOH (pH 5.6) in an isotonic sorbitol solution. The digestion was performed at 28°C for 2 to 4 h, with the longer times required for cold-acclimated leaves. After incubation, the digested materials were passed through four layers of cheesecloth and centrifuged at 50*g* for 10 min at 0°C. The pellets were suspended in an isotonic sorbitol solution including 1 mM Mes/KOH (pH 5.6) and then washed and purified by centrifugation as above. The purified protoplasts were resuspended in the isotonic sorbitol solution and kept on ice until use.

Determination of Freezing Tolerance

An aliquot of the protoplast suspension (0.5 mL, 1 to 2 × 10^5 protoplasts) was placed in a small test tube (15 × 100 mm) and cooled to −2.5 or −3°C, depending on the osmolality of the solution, for 10 min prior to inoculation of ice in the suspension. After ice inoculation, the samples were maintained at the above temperature for 15 min before cooling at a rate of approximately 0.8°C/min to the desired temperatures. Following a 30-min period at the specified temperatures, the samples were first thawed in air at room temperature and then kept on ice. For the treatments in which the osmotic expansion of the protoplasts was limited during thawing of the suspending medium (referred to as a freeze/hypertonic thaw), the frozen samples were warmed to −2.5 or −3°C for 5 min prior to addition of a hypertonic sorbitol solution precooled to the same temperature. The tonicity of the hypertonic solution was varied to yield a final osmolality that was twice that of the isotonic solution. Following melting of the suspending medium the samples were kept on ice.

Protoplast survival was determined by staining with fluorescein diacetate (15). Fluorescein diacetate was dissolved in acetone (0.5% w/v) and added to the protoplast solution at a final concentration of 0.001% (w/v). After incubation for 5 min at room temperature, the number of surviving protoplasts was counted in a hemocytometer. Typically, in the unfrozen controls, 200 to 400 protoplasts were counted in each of the hemocytometer fields, with three such samples taken for each treatment for a given experiment. Survival of the frozen samples was expressed as a percent of the unfrozen control. The results shown are the average and standard deviations of at least three different experiments. If no standard deviations are shown, they were smaller than the size of symbols in the figures.

Protoplast-Liposome Fusion

Liposomes were prepared by sonication of aqueous solutions of DL_2PC (Avanti Polar Lipid Inc.). The lipid (2 mg) was first dissolved in chloroform and then placed in a small glass tube (15 × 100 mm). Following evaporation of the chloroform by a stream of nitrogen gas, an isotonic sorbitol solution (0.9 mL) was added over the dried lipid film. 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 method described by Arvinte and Steponkus (1). Aliquots of the protoplasts (3 x 10^6 protoplasts) and the liposome suspensions were added to 5 mL of the fusion buffer, which consisted of 0.15 M NaCl, 20 mM Na-acetate buffer (pH 4.6) and sorbitol. The final osmolality of the solution was adjusted so that it was isotonic to the protoplast sample. The reaction was carried out at 28°C for 3 min after which 5 mL of the isotonic sorbitol solution, including 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 more by resuspension and centrifugation. Protoplast-liposome fusion was verified by fluorescence microscopy of samples prepared with liposomes containing rhodamine-labeled phosphatidylethanolamine (Avanti Polar Lipid Inc.) at a final concentration of 1% (w/w).

RESULTS AND DISCUSSION

During the first 4 weeks of the cold acclimation period at 2°C, the freezing tolerance of the isolated protoplasts, (*i.e.* the LT_{50}) decreased from approximately −5°C to −26°C, with the LT_{50} values for the 1-, 2-, and 3-week ACC protoplasts being −13°C, −18°C, and −21°C, respectively (Fig. 1). Maintaining the seedlings at 2°C for an additional 2 weeks neither increased nor decreased the LT_{50}.

Although it is useful to express the freezing tolerance as an LT_{50} for simple quantitative comparisons, qualitative differences in the survival pattern (% survival *versus* temperature) are obscured. Analysis of the survival pattern is especially important if one considers the possibility that the quantitative increase in freezing tolerance is the collective effect of several different qualitative changes rather than a simple quantitative change in a single factor. Examination of Figure 2 illustrates this point. The survival of NA protoplasts decreased linearly as a function of the freezing temperature over the range of 0 to −10°C such that approximately 50% of the protoplasts survived at −5°C and <10% survived at −10°C. After 1 week of cold acclimation, survival also decreased linearly over the range of 0 to −10°C, albeit to a lesser extent. As a result, >80% of the 1-week ACC protoplasts survived -5°C, approximately 60% survived −10°C, and 10% survived −30°C. Following 2 or 3 weeks of acclimation, the survival pattern was both quantitatively and qualitatively different. While there were quantitative increases in survival at all the freezing temperatures, survival at −5°C was 100%, which, as will be elaborated later, can be considered as a qualitative change. Similarly, after 4 weeks of acclimation, proportional increases in survival were apparent at all temperatures, with 100% survival at −10°C.

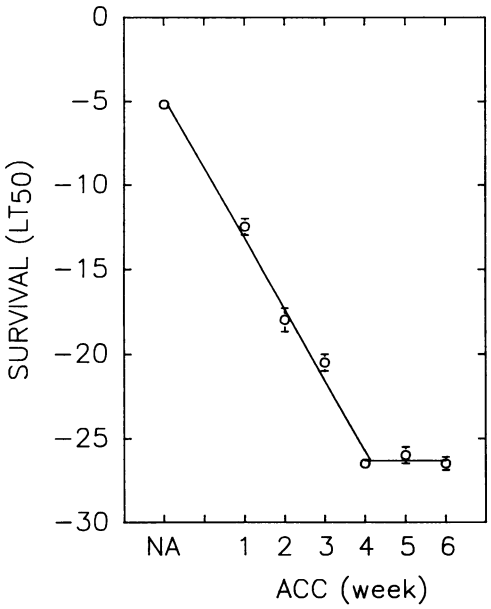

Figure 1. The LT_{50} of protoplasts isolated from nonacclimated rye leaves and those exposed to 2°C for various periods of time. Single regression line of the data is given.

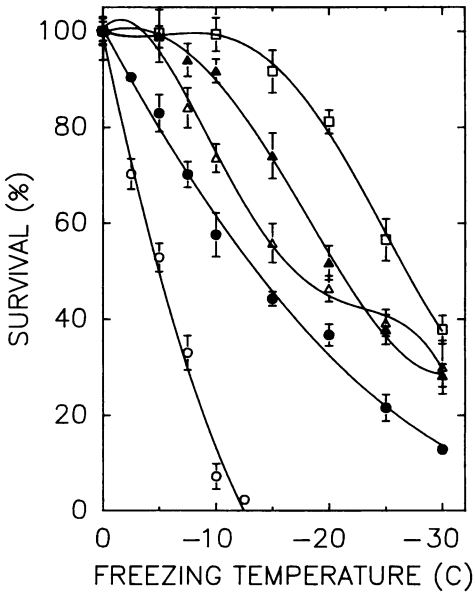

Figure 2. Development of freezing tolerance of isolated rye protoplasts during cold acclimation. The survival of protoplasts suspended in an isotonic sorbitol solution was determined by staining with fluorescein diacetate following a freeze/thaw cycle to the specific temperatures. Polynomial regression curves of the data are given. NA protoplasts (○); 1 -week-ACC protoplasts (●) 2-week-ACC protoplasts (△); 3-week-ACC protoplasts (▲) 4-week-ACC protoplasts (□). Error bars represent the standard deviation of the mean of at least three different experiments.

From our previous studies (10), we know that, in NA protoplasts, injury over the range of 0 to −5°C is a consequence of EIL; whereas at lower temperatures, injury is manifested as LOR and is a consequence of lamellar-to-

hexagonal$_{II}$ phase transitions in the plasma membrane. Further, in 4-week ACC protoplasts, EIL occurs at only a low frequency and LOR is not associated with lamellar-to-hexagonal$_{II}$ phase transitions. Therefore, as a working hypothesis to consider the survival patterns at intermediate stages of cold acclimation, we submit that there are three cardinal temperature ranges to consider in relation to freezing injury of rye protoplasts: 0 to −5°C, −5 to −10°C, and −10 to −30°C. The large increase in survival at −5°C after 1 wk of cold acclimation and 100% survival at −5°C after 2 weeks of cold acclimation reflect alterations in the plasma membrane that mitigate EIL. The large increase in survival at −10°C after 1 week of cold acclimation and, to some extent, after 2, 3, and 4 weeks reflect changes in the plasma membrane that preclude LOR that is associated with lamellar-to-hexagonal$_{II}$ phase transitions. The proportional increases in survival over the range of −10 to −30°C represent modifications that preclude LOR that is characteristic of fully acclimated protoplasts, but the cause of which is unknown at this time.

To test this hypothesis experimentally, the incidence of EIL and LOR was estimated for protoplasts at weekly intervals during a 4-week acclimation period. For this, protoplast suspensions isolated from leaves at the different stages of cold acclimation were divided into two samples. One sample was subjected to the conventional freeze/thaw treatment; the other was subjected to a freeze/hypertonic thaw treatment (see "Materials and Methods") so that the volumetric expansion following thawing of the suspending medium was limited and EIL was precluded. Thus, survival of the first group reflected injury resulting from both EIL and LOR; whereas, survival of the second group, which was higher, reflected injury that resulted from only LOR. The difference in survival between the two treatments reflected the incidence of EIL. The results of these studies are presented graphically in Figure 3, which shows the survival measurements, and in Table I, which presents the calculated incidence of the two forms of injury. The difference in survival between the two treatments (*i.e.* conventional freeze/thaw *versus* freeze/hypertonic thaw), which reflects the incidence of EIL, was greatest in NA protoplasts, with the greatest difference in protoplasts frozen to either −2.5°C or −5°C. At subsequent stages of acclimation, the difference in survival between the two treatments diminished considerably. For example, in the 2-week ACC protoplasts, there was no difference over the range of 0 to −5°C, although there was a small difference over the range of −10 to −15°C in both 1- and 2-week ACC protoplasts. No difference in survival between the two treatments was observed in 3- or 4-week ACC protoplasts at any temperature.

These results suggest that the membrane alterations responsible for the decreased sensitivity to osmotic excursions (*i.e.* the formation of exocytotic extrusions rather than endocytotic vesicles during osmotic contraction) occur primarily during the first week of cold acclimation and are completed by the second week. Previously, we have presented evidence that artificial enrichment of the plasma membrane of NA rye protoplasts with DL_2PC alters the cryobehavior of the plasma membrane so that osmotic contraction results in the formation of exocytotic extrusions in a manner analogous to natural cold acclimation (13) and that the increase in diunsaturated species of PC occurs during the first week of the cold acclimation period (2). Taken together, these results suggest that an increase in survival resulting from artificial enrichment of the plasma membrane with DL_2PC would be manifested only in NA protoplasts or those isolated from rye leaves in the first week of cold acclimation because after this time enrichment with diunsaturated PC species has already occurred naturally (2). If this is so, then one would expect (a) the survival patterns of protoplasts fused with DL_2PC to be similar to the survival patterns of unfused protoplasts subjected to the freeze/hypertonic thaw treatment and (b) no difference in the survival patterns of fused protoplasts subjected to either a conventional freeze/thaw treatment or a freeze/hypertonic thaw treatment.

Figure 4 shows the survival patterns of protoplasts fused with DL_2PC compared with the unfused controls, both of which were subjected to a conventional freeze/thaw treatment. Enrichment of the plasma membrane with DL_2PC resulted in the largest increase in survival in NA protoplasts, less in 1- or 2-week ACC protoplasts, and had no effect in 3- or 4-week ACC protoplasts. These results are consistent with the hypothesis presented above. Further, regardless of the stage of cold acclimation, there was essentially no difference

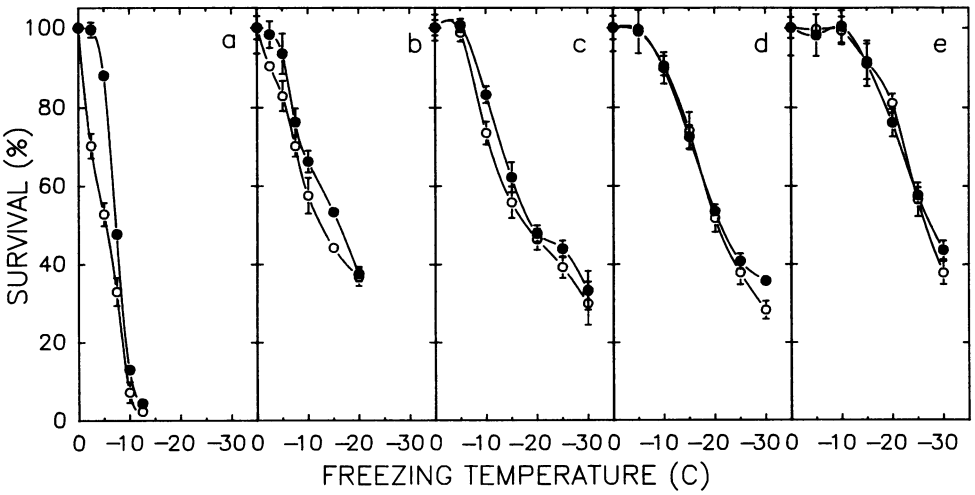

Figure 3. Effect of a freeze/thaw cycle (○) or a freeze/hypertonic thaw (●) on the survival of control protoplasts. Protoplasts were isolated from rye leaves that were either non-acclimated (a) or acclimated for 1 week (b), 2 weeks (c), 3 weeks (d), or 4 weeks (e). Error bars represent the standard deviation of the mean of at least three different experiments. If the error bars are not visible, they are smaller than the size of the symbol (*i.e.* <2%).

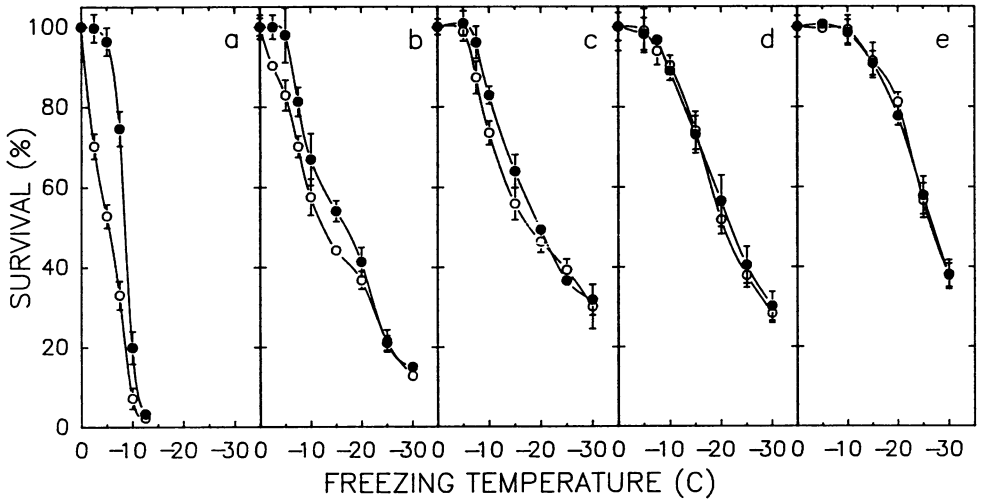

Figure 4. Effect of fusion with DL_2PC liposomes on the freezing tolerance of protoplasts. Protoplasts were isolated from rye leaves that were either nonacclimated (a) or acclimated for 1 week (b), 2 weeks (c), 3 weeks (d), or 4 weeks (e). Freezing tolerance of control protoplasts (○) or protoplasts fused with DL_2PC liposomes (●) is given in all panels. Error bars represent the standard deviation of the mean of at least three different experiments. If the error bars are not visible, they are smaller than the size of the symbol (*i.e.* <2%).

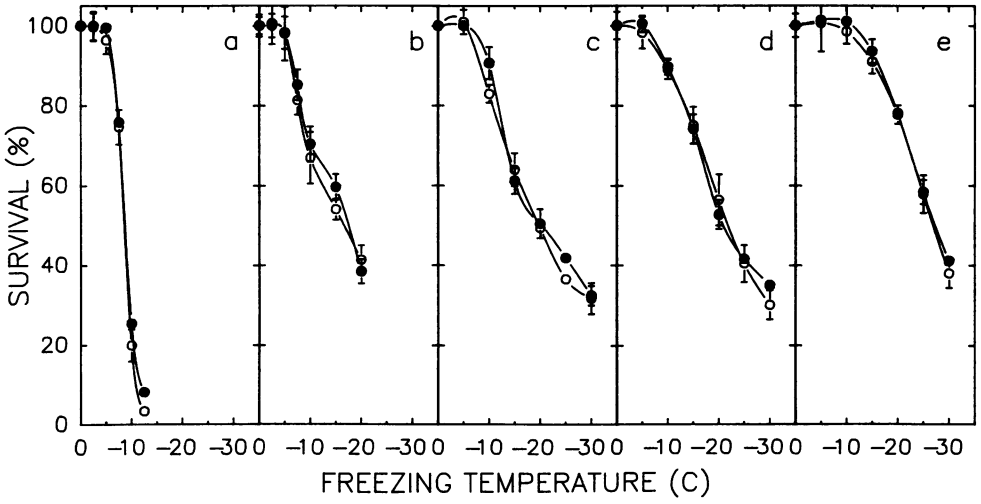

Figure 5. Effect of a freeze/thaw (○) or a freeze/hypertonic thaw cycle (●) on the survival of protoplasts fused with DL_2PC liposomes. Protoplasts were isolated from rye leaves that were either nonacclimated (a) or acclimated for 1 week (b), 2 weeks (c), 3 weeks (d), or 4 weeks (e). Error bars represent the standard deviation of the mean of at least three different experiments. If the error bars are not visible, they are smaller than the size of the symbol (*i.e.* <2%).

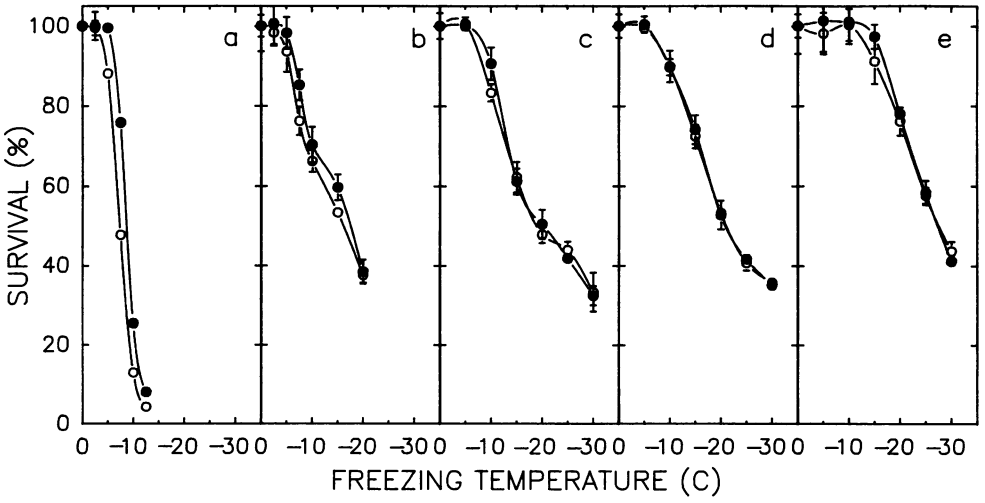

Figure 6. Effect of fusion with DL_2PC liposomes on the survival of protoplasts following a freeze/hypertonic thaw cycle. Protoplasts were isolated from rye leaves that were either nonacclimated (a) or acclimated for 1 week (b), 2 weeks (c), 3 weeks (d), or 4 weeks (e). Survival of control protoplasts (○) or protoplasts fused with DL_2PC (●) is given in all panels. Error bars represent the standard deviation of the mean of at least three different experiments. If the error bars are not visible, they are smaller than the size of the symbol (*i.e.* <2%).

in the survival patterns of the fused protoplasts subjected to either a conventional freeze/thaw cycle or a freeze/hypertonic thaw treatment (Fig. 5), which is also predicted by the hypothesis.

Thus, it appears that the initial increase in freezing tolerance that occurs within the first week of cold acclimation is a consequence of alterations in the plasma membrane that preclude EIL and that these alterations (the formation of exocytotic extrusions rather than endocytotic vesicles during osmotic contraction) are a consequence of increases in the diunsaturated species of PC. The question arises as to whether the effects of enrichment with DL_2PC are limited to this

Table I. *Calculated Incidence of EIL and LOR in Rye Protoplasts after Various Periods of Cold Acclimation*

The incidence of EIL was calculated as the difference in survival of protoplasts subjected to a conventional freeze/thaw cycle and those subjected to a freeze/hypertonic thaw treatment. The incidence of LOR was calculated as the decrease in survival of protoplasts subjected to the freeze/hypertonic thaw treatment. The numbers preceding and following the slash represent the incidence (%) of EIL and LOR, respectively. Control protoplasts (*C*) are shown in the left column, and protoplasts fused with DL_2PC (*F*) are shown in the right column.

Freezing Temperature	Protoplasts Isolated from Rye Leaves									
	Non-acclimated		Acclimated for							
			1 week		2 weeks		3 weeks		4 weeks	
	(*C*)	(*F*)	(*C*)	(*F*)	(*C*)	(*F*)	(*C*)	(*F*)	(*C*)	(*F*)
°C					%					
0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0	0/0
−2.5	29/1	0/0	8/2	0/0	ND[a]	ND	ND	ND	ND	ND
−5	35/12	3/1	11/7	0/2	1/0	0/0	1/1	2/0	0/2	0/0
−7.5	15/52	1/24	6/24	4/15	ND	ND	ND	ND	ND	ND
−10	6/87	6/75	9/34	3/30	10/17	8/9	1/10	1/10	1/0	2/0
−12.5	2/96	5/92	ND	ND	ND	ND	ND	ND	ND	ND
−15	ND	ND	9/47	6/40	7/38	0/39	0/28	0/26	0/9	3/7
−20	ND	ND	1/63	3/62	2/52	1/50	2/47	0/47	0/24	0/22
−25	ND	ND	ND	ND	5/56	5/58	3/59	1/58	1/42	0/42
−30	ND	ND	ND	ND	3/67	1/68	7/64	5/65	6/56	3/59

[a] Not determined.

transformation in the behavior of the plasma membrane. Qualitatively, the results presented in Figure 5 suggest that this is the case. However, a direct comparison of the survival patterns of fused *versus* nonfused protoplasts subjected to the freeze/hypertonic thaw treatment (Fig. 6) reveals that there is an additional effect of enrichment with DL_2PC in NA protoplasts that cannot be ascribed to a mitigation of EIL. Although the survival pattern of the fused protoplasts appears only slightly displaced to lower temperatures, there is a significant difference in survival at any given subzero temperature over the range of −5 to −10°C (*i.e.* compare the % survival between the treatments at −5°C, −7.5°C, or −10°C), with the maximum difference at −7.5°C. This difference is a consequence of a decrease in the incidence of LOR (Table I). The decreased incidence of LOR at −7.5°C (from 52% to 24%) in NA protoplasts fused with DL_2PC liposomes was comparable to that resulting from 1 week of cold acclimation (Table I). However, in protoplasts frozen to −10°C, the effect of cold acclimation for 1 week was much greater than that of fusion with DL_2PC (*i.e.* the incidence of LOR was reduced from 87% to 34% after 1 week of cold acclimation, but fusion of NA protoplasts with DL_2PC only decreased the incidence of LOR from 87% to 75%, Table I). Further, enrichment of the plasma membrane with DL_2PC had little, if any, effect on LOR in protoplasts isolated from leaves of plants that were cold acclimated for more than 1 week. Presumably, enrichment of the plasma membrane with DL_2PC also has an effect on the incidence of LOR that is associated with lamellar-to-hexagonal$_{II}$ phase transitions in the plasma membrane of NA protoplasts but not on the incidence of LOR that occurs in protoplasts isolated from leaves acclimated for more than 1 week because enrichment of the plasma membrane with diunsaturated PC species has occurred naturally during the first week of cold acclimation (2).

In conclusion, these results demonstrate that the quantitative increase in freezing tolerance of rye protoplasts during cold acclimation (*i.e.* a decrease in the LT_{50} from −5°C to −25°C) is the result of several different qualitative changes in the cryostability of the plasma membrane during freeze-induced dehydration. The first change to occur is a transformation in the cryobehavior of plasma membrane during freeze-induced osmotic contraction (*i.e.* from endocytotic vesiculation to endocytotic extrusion), which minimizes the incidence of EIL and is the result of alterations in the phospholipid composition of the plasma membrane. Studies are currently in progress to determine if at the same time, the incidence of LOR associated with lamellar-to-hexagonal$_{II}$ phase transitions in the plasma membrane is also reduced by the enrichment of diunsaturated PC species in the plasma membrane.

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