

Freeze-Induced Membrane Ultrastructural Alterations in Rye (*Secale cereale*) Leaves¹

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Freezing injury in protoplasts isolated from leaves of nonacclimated rye (*Secale cereale* cv Puma) is associated with the formation of the inverted hexagonal (H_{II}) phase. However, in protoplasts from cold-acclimated rye, injury is associated with the occurrence of localized deviations in the fracture plane, a lesion referred to as the "fracture-jump lesion." To establish that these ultrastructural consequences of freezing are not unique to protoplasts, we have examined the manifestations of freezing injury in leaves of nonacclimated and cold-acclimated rye by freeze-fracture electron microscopy. At -10°C, injury in nonacclimated leaves was manifested by the appearance of aparticulate domains in the plasma membrane, aparticulate lamellae subtending the plasma membrane, and by the frequent occurrence of the H_{II} phase. The H_{II} phase was not observed in leaves of cold-acclimated rye frozen to -35°C. Rather, injury was associated with the occurrence of the fracture-jump lesion between the plasma membrane and closely appressed cytoplasmic membranes. Studies of the time dependence of H_{II} phase formation in nonacclimated leaves indicated that freeze-induced dehydration requires longer times in leaves than in isolated protoplasts. These results demonstrate that the freeze-induced formation of the H_{II} phase in nonacclimated rye and the fracture-jump lesion in cold-acclimated rye are not unique to protoplasts but also occur in the leaves from which the protoplasts are isolated.

Winter rye (*Secale cereale*) is the most freezing tolerant of winter cereals. Although leaves of nonacclimated rye are lethally injured by freezing at temperatures below -4°C, cold acclimation triggers numerous cellular and metabolic alterations that collectively defer injury to temperatures ≤ -25°C. In protoplasts isolated from the leaves of both NA protoplasts and ACC protoplasts rye seedlings, the plasma membrane is a primary site of damage due to freezing (Steponkus, 1984). In NA protoplasts at -10°C and in ACC protoplasts at ≤ -25°C, injury due to freezing is associated with the formation of lesions in the plasma membrane that result in loss of osmotic responsiveness of the protoplast during subsequent thawing (Steponkus, 1984).

In NA protoplasts, freezing at -10°C results in the formation of aparticulate domains in the plasma membrane, aparticulate lamellae subtending the plasma membrane, and the H_{II} phase in association with the plasma membrane and

subtending lamellae (Gordon-Kamm and Steponkus, 1984). These ultrastructural manifestations of freezing injury have been demonstrated to be consequences of freeze-induced dehydration rather than low temperature per se (Gordon-Kamm and Steponkus, 1984). In contrast, the H_{II} phase is not observed in freeze-injured ACC protoplasts. Rather, freezing to ≤ -25°C is associated with the formation of localized deviations of the plasma membrane fracture plane, as observed by freeze-fracture EM, to aparticulate or particle-depleted lamellae that are in close apposition with the plasma membrane, a phenomenon referred to as the "fracture-jump lesion" (Fujikawa and Steponkus, 1990).

Previously, Pearce and Willison (1985a) observed that leaf bases and laminae of warm-grown wheat frozen to damaging temperatures between -2 and -8°C had aparticulate domains in the plasma membrane, but they did not observe the H_{II} phase. However, in their study the tissue samples were cryofixed by quenching when they had reached the desired subzero temperature (Pearce and Willison, 1985b). In leaves, cellular dehydration due to the formation of extracellular ice occurs largely by vapor phase equilibration (Allan et al., 1990). As a result, it is likely that there is a significant time dependence for cellular dehydration and the development of freezing injury. We were concerned, therefore, that insufficient time had been allowed for cellular dehydration to occur in the study of Pearce and Willison (1985a) and hence their failure to observe the H_{II} phase.

To determine whether or not freeze-induced lamellar-to-H_{II} phase transitions are unique to isolated NA protoplasts, we have examined frozen leaves of nonacclimated rye by freeze-fracture EM. Similarly, to establish whether the fracture-jump lesion is unique to isolated ACC protoplasts, we have also examined frozen leaves of cold-acclimated rye seedlings. To overcome potential kinetic limitations on the formation of the H_{II} phase and the fracture-jump lesion, we have examined leaf sections after both short (1 h) and long (8 h) exposures to injurious temperatures.

MATERIALS AND METHODS

Plant Growth and Freezing Treatment

Seedlings of winter rye (*Secale cereale* cv Puma) were germinated and grown as described previously (Uemura and

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Steponkus, 1989). Briefly, nonacclimated plants were grown in a 16-h photoperiod at 20/15°C (day/night) for 10 to 14 d. Cold acclimation was achieved by transferring 7-d-old plants to a 11.5-h photoperiod at 13/7°C (day/night) for 1 week and then to a 10-h photoperiod at 2/2°C (day/night) for an additional 4 weeks. Sections of leaves approximately 2 mm² were floated on a 1.5- μ L droplet of a 0.1% solution of bacterial ice-nucleating protein (Kodak Snomax) placed on a freeze-fracture specimen carrier. Each sample was placed in a small well of a copper block that was cooled by a circulating ethanol bath (Neslab ULT-80). Sample temperature was monitored by a thermocouple placed in an identical position in an adjacent well of the copper block. Temperatures described here are those determined with this thermocouple. Excised leaf sections were cooled to -3°C for 15 min, and then ice nucleation was effected by touching the leaves with tweezers cooled in liquid nitrogen. Except where noted, the leaves were equilibrated for 16 h at -3°C to effect cellular dehydration at a noninjurious temperature. After this equilibration period, the leaves were either quenched in liquid propane supercooled with liquid nitrogen or further freeze dehydrated at either -10°C (nonacclimated leaves) or -35°C (cold-acclimated leaves) before cryofixation in liquid propane in preparation for freeze-fracture EM. All cooling rates were 0.8°C min⁻¹.

Freeze-Fracture EM

Samples were quenched for freeze-fracture EM by plunging into liquid propane supercooled by liquid nitrogen. Samples were fractured on a Balzers 360 freeze-fracture device at -102°C and $<2 \cdot 10^{-6}$ 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 for 3 d with 100% H₂SO₄ and then examined in a Philips EM300 electron microscope.

Two criteria were used to determine the presence of the H_{II} phase and to distinguish the H_{II} phase from closely appressed lamellae. First, we required that the lipid array consist of nonlamellar lipid structures that appear as inverted cylindrical micelles, which are characteristic of the H_{II} phase. Furthermore, the inverted cylindrical micelles were required to have transverse cross-sections of the cylinders that revealed "hexagonal packing" of the cylinders. Second, we required that the lipid array be fractured in such a way as to reveal periodicity in two dimensions normal to the long axis of the putative H_{II} phase cylinders. Because the H_{II} phase is a two-dimensional lipid mesophase, whereas the lamellar phase is a one-dimensional mesophase (Seddon, 1990), the H_{II} phase will show a regular periodic arrangement in two dimensions normal to the cylinder long axis. Such periodicity cannot be present in closely appressed lamellae. By requiring the presence of both the transverse cross-sections of the H_{II} phase cylindrical micelles and two-dimensional periodicity, we are confident that tightly appressed lamellar structures have not been mistaken for the H_{II} phase.

RESULTS

Nonacclimated Rye Leaves

In previous work with isolated protoplasts, freeze-induced dehydration for 30 min at -10°C was sufficient for the

formation of the H_{II} phase (Gordon-Kamm and Steponkus, 1984). However, in preliminary experiments with leaves of nonacclimated rye seedlings frozen using this protocol (i.e. without preequilibration at -3°C), there were few alterations in membrane ultrastructure. All membranes were present in the lamellar configuration and the IMP appeared to be randomly distributed, although no statistical analysis was performed. Furthermore, most cells in nonacclimated rye leaves frozen for 30 min at -10°C without an extended equilibration at -3°C were not significantly dehydrated, as indicated by the occurrence of large intermembrane distances between the intracellular membranes as well as the presence of turgid organelles (Fig. 1). Other cells were more dehydrated. In these cells, domains of the plasma membrane either were aparticle or had obvious decreases in the frequency of IMP (data not shown). These results indicate that freezing for 30 min at -10°C, which is sufficient to cause dehydration and injury in isolated protoplasts, was not sufficient for uniform cellular dehydration in leaf sections. Therefore, to effect significant cellular dehydration at a noninjurious temperature, further experiments were performed using an overnight (16 h) equilibration at -3°C before freeze-dehydration at the injurious temperature. It should be emphasized that this time/temperature protocol is similar to that routinely used to determine the freezing tolerance of rye leaves (M.S. Webb, M. Uemura, and P.L. Steponkus, unpublished data).

Cells in nonacclimated rye leaves frozen for 16 h at -3°C were significantly dehydrated. However, these cells had few membrane ultrastructural alterations as a consequence of freezing for 16 h at -3°C. The plasma membrane and endomembranes were predominantly in the bilayer configuration, and membrane IMP were randomly dispersed (Fig. 2a); however, in some instances small regions of plasma membrane were observed with either a reduced density of IMP or with IMP-free domains (Fig. 2a). Aggregates of inverted cylindrical micelles, characteristic of the H_{II} phase, also occurred in these freeze-dehydrated cells (Fig. 2b). Finally, localized deviations of the plasma membrane fracture plane to closely appressed lamellae that were either aparticle or IMP depleted, i.e. the fracture-jump lesion, were also observed in nonacclimated rye leaves frozen for 16 h at -3°C (Fig. 2c). The frequency of these ultrastructural alterations (aparticle domains, the fracture-jump lesion, and the H_{II} phase) was low after freezing for 16 h at -3°C (6, 18, and 6% of examined cells, respectively).

In nonacclimated rye leaves freeze-dehydrated for an additional 8 h at -10°C, nonlamellar aggregates occurred at a high frequency. It should be noted that the H_{II} phase is a nonlamellar phase and H_{II} phase formation is an interbilayer event. As a consequence, inverted cylindrical micelles that are formed by the plasma membrane and subtending membranes/lamellae that are brought into close apposition during freeze-induced dehydration will appear to be adjacent to, but not "in," the plasma membrane per se. The H_{II} phase was observed directly adjacent to the cell wall (Fig. 3a) and occurred in close association with both the PF (data not shown) and EF (Fig. 3b) faces of the plasma membrane. The H_{II} phase was identified by the presence of both transverse cross-sections of the inverted cylindrical micelle aggregates and by periodicity in two dimensions normal to the long axis

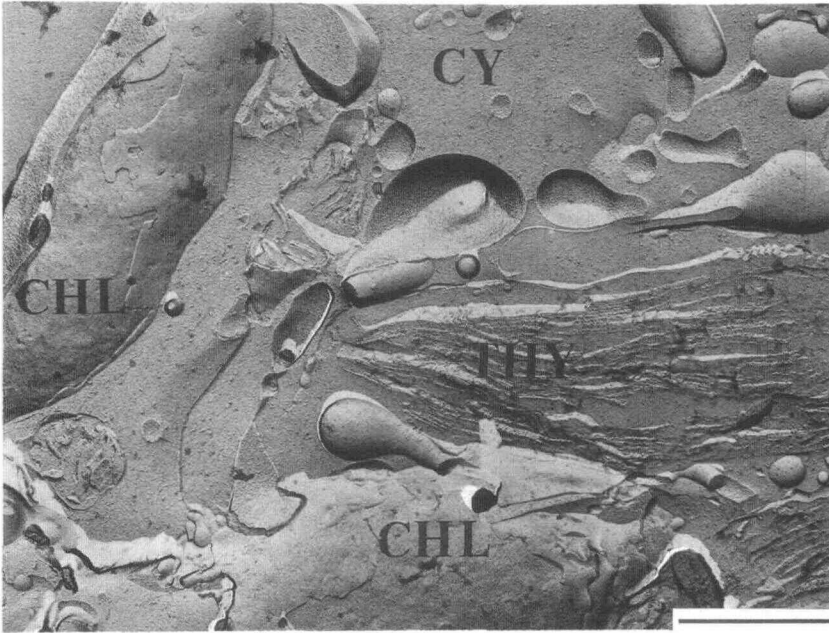


Figure 1. Freeze-fracture electron micrograph of a leaf of nonacclimated rye frozen at -10°C for 30 min. A low-magnification view of a cell showing large spatial separations between membranes and organelles. CY, Cytoplasm; CHL, chloroplast; THY, thylakoids. Bar represents $1\ \mu\text{m}$.

of the H_{II} phase cylinders (Figs. 2b and 3a). Our observations clearly indicate that the H_{II} phase occurs immediately adjacent to the cell wall and in close association with the plasma membrane. It is likely that the plasma membrane undergoes the formation of the H_{II} phase in conjunction with closely appressed intracellular membranes. The membrane most often observed to be closely appressed to the plasma membrane during freeze-induced dehydration was the outer membrane of the chloroplast envelope. The H_{II} phase observed in leaves after freezing at -10°C occurred in very large domains and was the predominant ultrastructural alteration in these cells. Nonetheless, freezing injury at -10°C in nonacclimated rye leaves was also associated with the occurrence of aparticle domains in the plasma membrane (data not shown) and lamellae subtending the plasma membrane that either were aparticle or had a low frequency of IMP (Fig. 3c). These lamellae were visible in regions where the fracture plane of the plasma membrane deviated to the subtending lamellae, i.e. the fracture-jump lesion. A detailed description of the fracture-jump lesion obtained from observations of frozen ACC protoplasts, first described by Fujikawa and Steponkus (1990), has been published (Steponkus and Webb, 1992). Briefly, the fracture-jump lesion consists of a localized region of 0.1 to $2.5\ \mu\text{m}$ in diameter in which the fracture plane in the plasma membrane has "jumped" to lamellae of the endomembranes that are in close apposition to the plasma membrane. The frequency of the fracture-jump lesion did not increase as a consequence of freezing at -10°C but occurred in 18% of the examined cells. However, the incidence of both aparticle domains within the plasma membrane and the H_{II} phase were significantly increased by freezing at -10°C (30 and 88% of examined cells, respectively).

To ensure that the formation of the H_{II} phase was not a consequence of the extended (8 h) equilibration at -10°C , the occurrence of the H_{II} phase in excised sections of nonac-

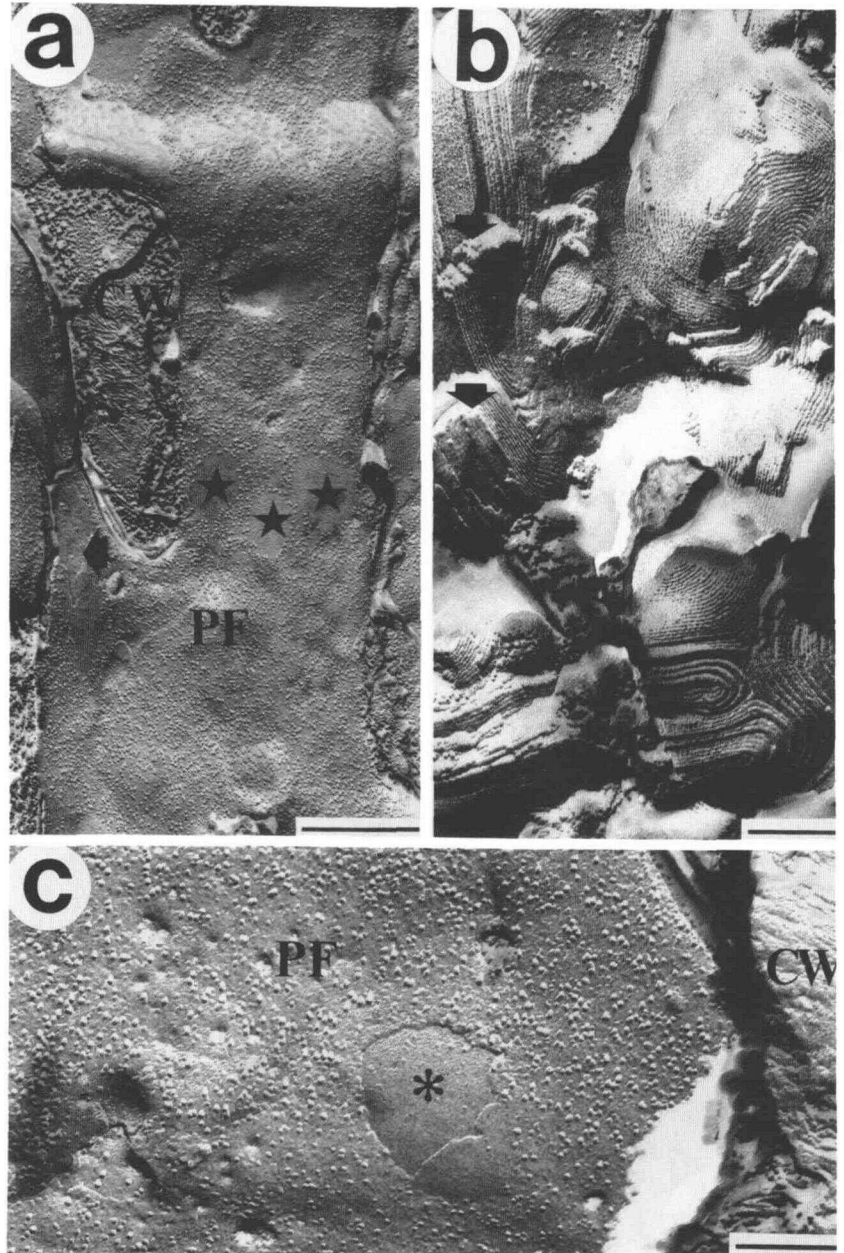
climated rye leaves was determined in leaves after either 30 min or 2 h at -10°C after overnight equilibration at -3°C . After 30 min at -10°C , the frequency of the H_{II} phase was very low. After 2 h at -10°C , cells were severely dehydrated (Fig. 4a, compare with Fig. 1a). The H_{II} phase was observed frequently in cells frozen for 2 h at -10°C . The H_{II} phase (Fig. 4b) occurred in close proximity to the cell wall and plasma membrane (data not shown) and in the intracellular membranes (Fig. 4c). Therefore, the H_{II} phase occurred at a high frequency using a time/temperature protocol very similar to that used to assess the survival of leaf sections to subzero temperatures (M.S. Webb, M. Uemura, and P.L. Steponkus, unpublished data).

Cold-Acclimated Rye Leaves

Excised sections of cold-acclimated rye leaves frozen for 16 h at -3°C were significantly dehydrated. Frequently the plasma membrane was observed to be in close apposition with three or more lamellae of the endomembranes (Fig. 5a). These closely appressed lamellae were often aparticle or had a greatly reduced IMP density (Fig. 5a). The plasma membrane and intracellular membranes were exclusively in the lamellar organization, and in most cells, plasma membrane IMP appeared to be randomly distributed (Fig. 5b). However, in 17% of the examined cells, regions of plasma membrane that either were aparticle or had obvious decreases in the frequency of IMP were observed (Fig. 5c). In addition, the fracture-jump lesion was also observed in the plasma membranes of cold-acclimated rye leaves after 16 h at -3°C , albeit at a low frequency—11% of examined cells (Fig. 5c). The H_{II} phase or other nonlamellar structures were not observed in acclimated rye leaves at this temperature.

Leaves of cold-acclimated rye that were freeze-dehydrated for 8 h at -35°C after equilibration for 16 h at -3°C had a number of membrane ultrastructural alterations. The plasma

Figure 2. Freeze-fracture electron micrographs of leaves of nonacclimated rye frozen at -3°C for 16 h: a, PF face of the plasma membrane with aparticulate domains (\star) and apparently randomly distributed IMP. Closely appressed aparticulate lamellae underlying the plasma membrane are present (arrow). b, Region of H_{II} phase in the intracellular membranes. The fracture plane reveals transverse cross-sections of the inverted cylindrical micelles (arrows). c, PF face of the plasma membrane showing randomly distributed IMP and a region of the fracture-jump lesion (\ast) that reveals aparticulate lamellae subtending the plasma membrane. CW, Cell wall. Bars represent 500 nm (a) and 200 nm (b and c).



membrane was in close apposition with aparticulate intracellular membranes subtending the plasma membrane (Fig. 6a). In regions where the plasma membrane appeared closely appressed to subtending lamellae, a reduction in the frequency of IMP was observed (Fig. 6a). The fracture-jump lesion occurred frequently in the plasma membrane (75% of examined cells) and was observed in both the PF (Fig. 6, b and d) and EF (Fig. 6c) faces of the plasma membrane. In some instances, the fracture-jump lesion included several aparticulate lamellae (Fig. 6c). In addition, the plasma membrane was observed to contain both paracrystalline arrays of IMP (Fig. 6d inset) and localized aparticulate domains (Fig. 6d). The H_{II} phase was not observed in acclimated rye leaves frozen at -35°C .

DISCUSSION

Previous studies have demonstrated that freeze-induced injury in NA protoplasts of winter rye is associated with the formation of aparticulate domains in the plasma membrane and aparticulate lamellae subtending the plasma membrane and with the lamellar-to- H_{II} phase transition resulting from the interaction of the plasma membrane and underlying lamellae (Gordon-Kamm and Steponkus, 1984). In contrast, the H_{II} phase is not observed in ACC protoplasts; rather, freezing injury is associated with a phenomenon referred to as the fracture-jump lesion (Fujikawa and Steponkus, 1990). This ultrastructural alteration appears in freeze-fracture electron micrographs as a localized deviation of the fracture plane

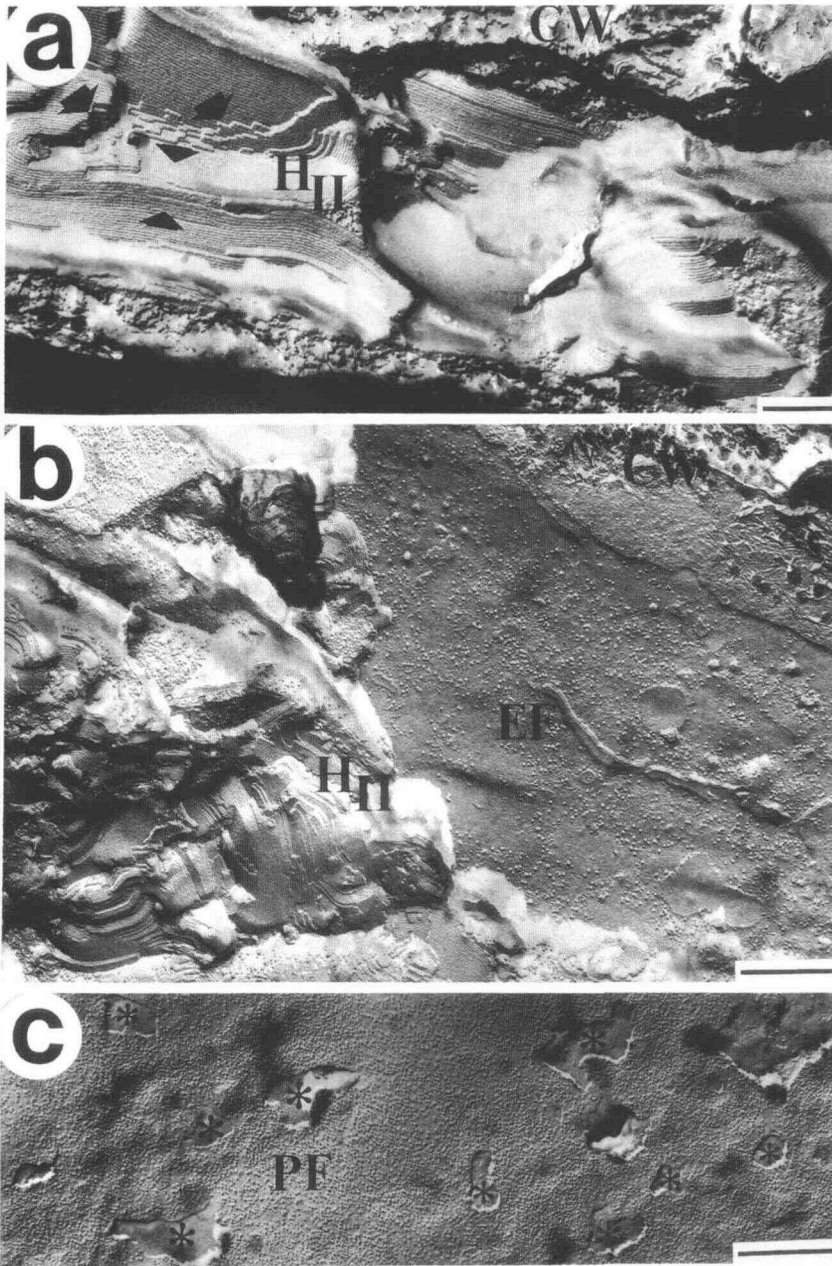


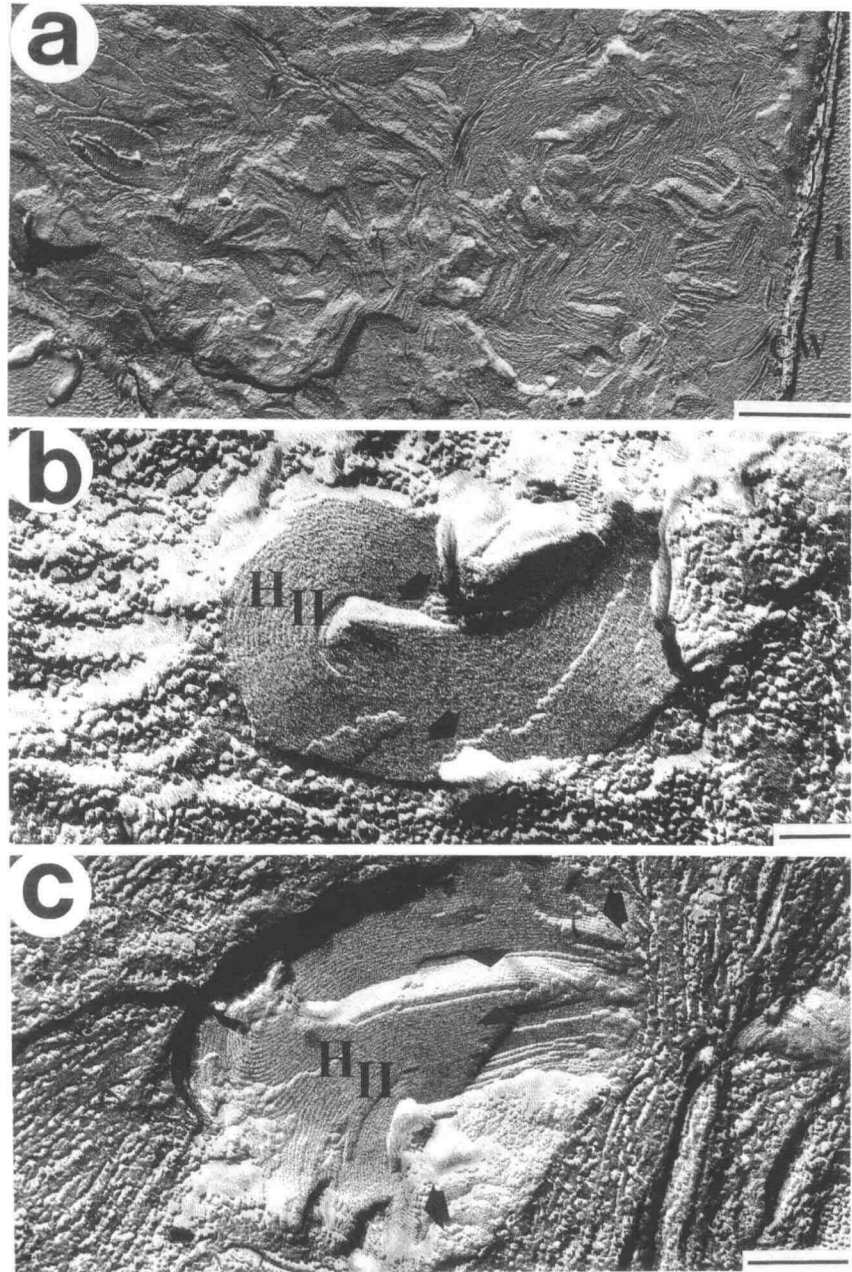
Figure 3. Freeze-fracture electron micrographs of leaves of nonacclimated rye that were first frozen at -3°C for 16 h and then at -10°C for 8 h: a, Region of the H_{II} phase in close association with the cell wall (CW). The fracture plane reveals transverse cross-sections of the inverted cylindrical micelles (arrows) and periodicity in two dimensions normal to the long axes of the cylinders (arrowheads). b, The H_{II} phase interior to the EF face of the plasma membrane. c, PF face of the plasma membrane in which the fracture-jump lesion (*) occurred in conjunction with lamellae subtending the plasma membrane that were aparticulate or IMP depleted. Bars represent 200 nm (a), 300 nm (b), and 400 nm (c).

from the plasma membrane to subtending lamellae that either are aparticulate or are obviously particle depleted. The physical cause of the fracture-jump lesion is not known. However, two possible causes are under consideration: (a) the freeze-induced interdigitation of lipids in the plasma membrane (Steponkus and Webb, 1992) and (b) freeze-induced fusion of the plasma membrane with closely appressed lamellae from the endomembranes.

The present study was initiated to determine if the membrane ultrastructural alterations described previously are unique to isolated protoplasts or whether they also occur in freeze-dehydrated leaves. The results clearly demonstrate that the freeze-induced ultrastructural alterations observed in both NA and ACC protoplasts also occur during freezing

of the leaves from which the protoplasts were isolated. Specifically, we observed that freezing injury in nonacclimated rye leaves is associated with the formation of aparticulate domains within the plasma membrane, aparticulate lamellae subtending the plasma membrane, and nonlamellar lipid structures, i.e. aggregates of inverted cylindrical micelles characteristic of the H_{II} phase (Figs. 2–4). Qualitatively, these findings are identical with those reported previously for isolated NA rye protoplasts (Gordon-Kamm and Steponkus, 1984). In addition, Fujikawa (1988) reported the freeze-induced formation of the H_{II} phase in tonoplasts in the tertiary hyphae of more than 20 species of edible mushrooms and in the tonoplast, nuclear envelope, chloroplast envelopes and ER of cortical parenchyma cells of mulberry trees. We

Figure 4. Freeze-fracture electron micrographs of leaves of nonacclimated rye frozen at -3°C for 16 h and then at -10°C for 2 h: a, Low-magnification view of a typical cell showing the severe dehydration of the cells at this temperature. CW, Cell wall; i, extracellular ice. b, The H_{II} phase occurring in membranes immediately adjacent to the plasma membrane and cell wall (not shown). Arrows indicate transverse cross-sections of the inverted cylindrical micelles. c, The H_{II} phase surrounded by various unidentified intracellular membranes. Arrows indicate transverse cross-sections of the inverted micelles, and arrowheads indicate periodicity in two dimensions normal to the cylinder long axis (arrowheads). Bars represent $1\ \mu\text{m}$ (a), $100\ \text{nm}$ (b), and $200\ \text{nm}$ (c).



have also determined that the fracture-jump lesion, which was first observed in ACC protoplasts (Fujikawa and Steponkus, 1990), is the predominant ultrastructural alteration in freeze-injured leaves of cold-acclimated rye (Figs. 5 and 6) and confirmed that the H_{II} phase does not occur in either cold-acclimated rye protoplasts (Gordon-Kamm and Steponkus, 1984) or leaves frozen to temperatures over the range of -3 to -35°C (Fig. 6).

The results show that the formation of the H_{II} phase in nonacclimated rye leaves requires a significantly longer equilibration time than that previously reported for NA protoplasts (Gordon-Kamm and Steponkus, 1984). Observations of frozen rye leaves by cryo-scanning EM have demonstrated that ice forms in discrete locations within the leaf and that cellular

dehydration probably occurs via vapor phase equilibration (Allan et al., 1990). Therefore, it is likely that the longer equilibration times at injurious temperatures required for the development of ultrastructural alterations in leaves compared to protoplasts are due to longer times required for cell dehydration by vapor phase equilibration. Although we have used long equilibration times at injurious temperatures to ensure that cellular dehydration occurs, it should be emphasized that significant amounts of the H_{II} phase are also observed after only 2 h at -10°C following overnight equilibration at -3°C (Fig. 4, b and c). The longer time requirement for cellular dehydration may explain the failure of Pearce and Willison (1985a) to observe the H_{II} phase in freeze-injured wheat leaf bases. In their studies, the tissues were cooled at

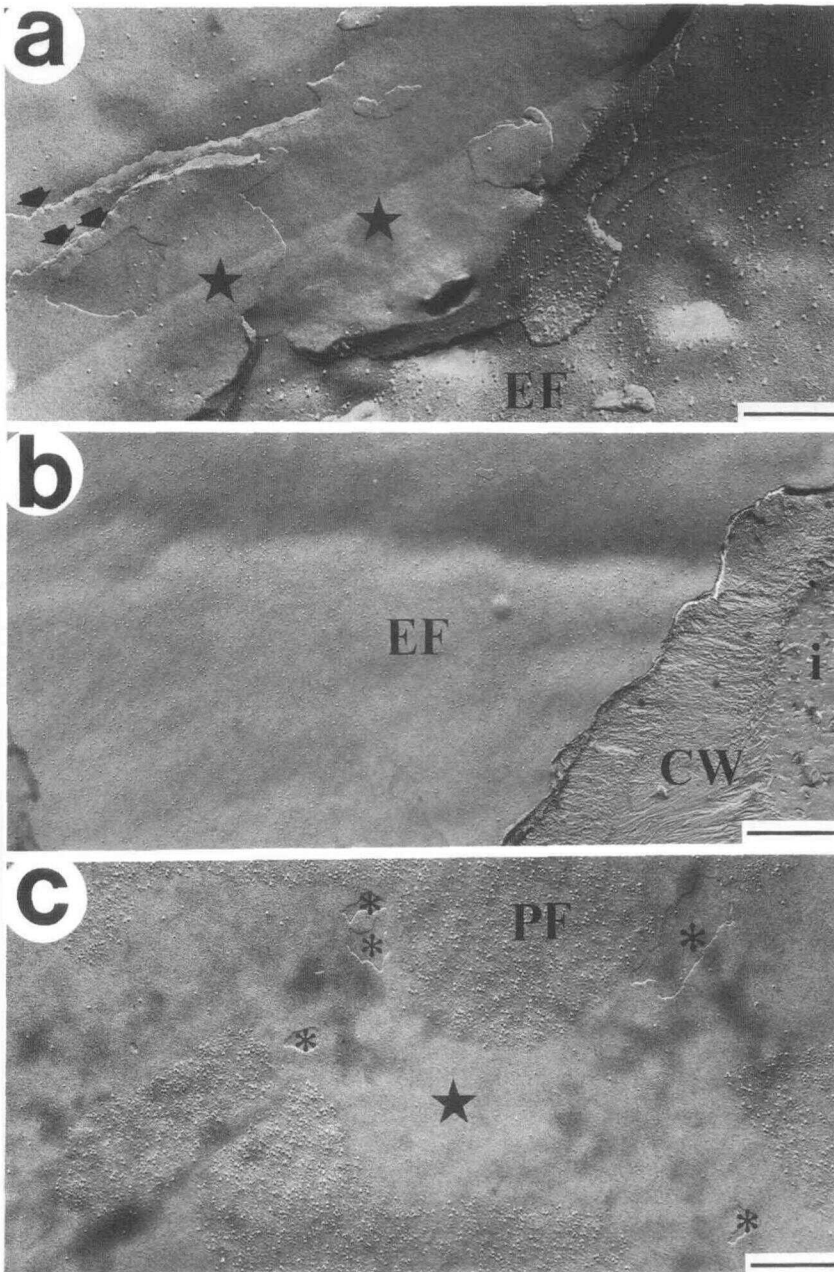


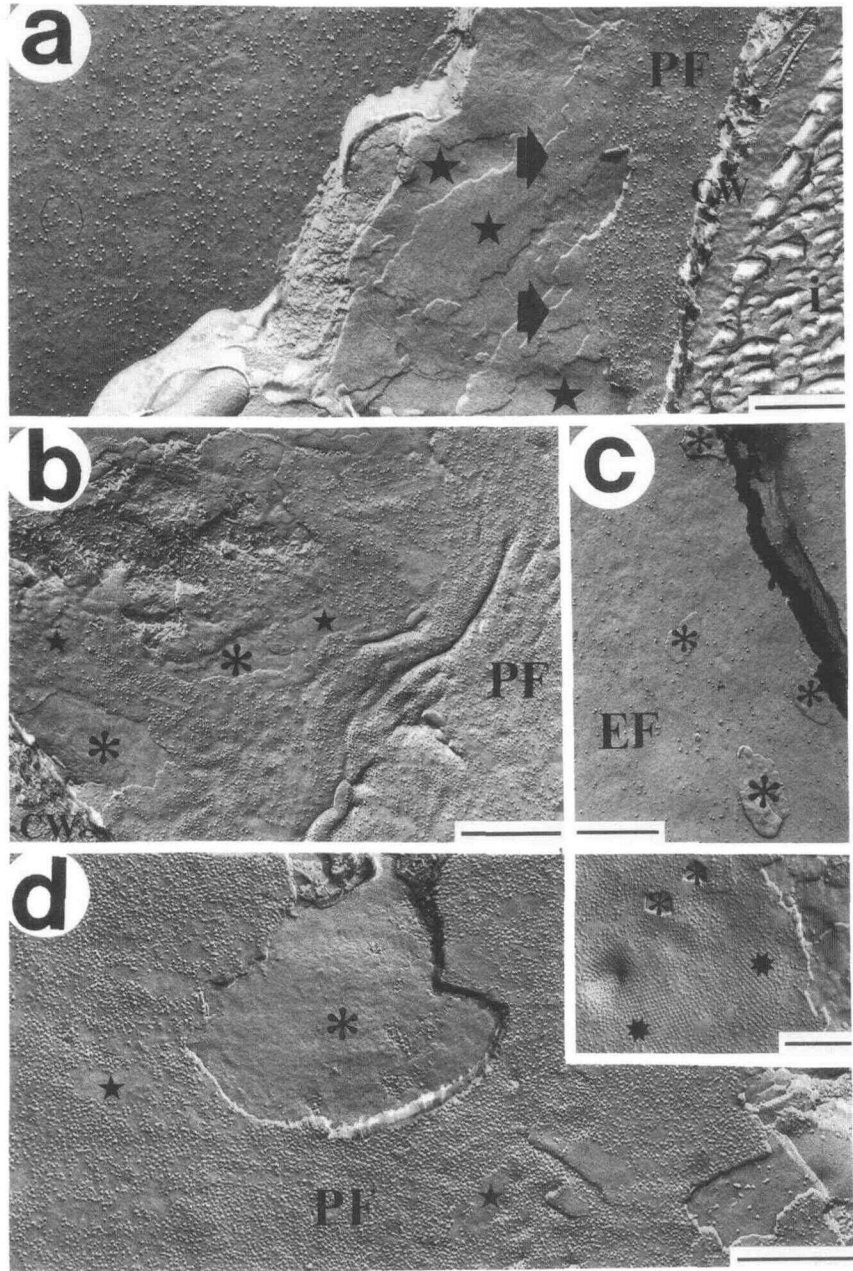
Figure 5. Freeze-fracture electron micrographs of leaves of cold-acclimated rye frozen at -3°C for 16 h: a, EF face of the plasma membrane with closely appressed "overlying" lamellae with both particulate and aparticle (\star) regions. Arrows indicate at least three layers of lamellae that were in close apposition to the plasma membrane. b, EF face of the plasma membrane showing apparently randomly distributed IMP. CW, Cell wall; i, extracellular ice. c, PF face of the plasma membrane showing a domain with a greatly reduced density of IMP (\star) and several regions of fracture-jump lesion (\ast) in conjunction with lamellae subtending the plasma membrane. Bars represent 300 nm (a), 500 nm (b), and 300 nm (c).

slow rates (7°C h^{-1}) to temperatures between -2 and -14°C , but were quenched upon achieving the desired temperature (Pearce and Willison, 1985a, 1985b). Therefore, these tissues were exposed to temperatures lower than -2°C for a maximum of only 2 h. Furthermore, because the warm-grown wheat leaves were frozen to a nadir temperature of -8°C , these tissues were exposed to temperatures lower than -2°C for <1 h and to injurious subzero temperatures for a significantly shorter time. In addition, in wheat grown in a 12/4 $^{\circ}\text{C}$ (day/night) temperature regimen, the H_{II} phase was not observed during freezing to temperatures as low as -14°C (Pearce and Willison, 1985a). The absence of the H_{II} phase in these partially cold-acclimated tissues is not unexpected because the occurrence of freeze-induced lamellar-to- H_{II}

phase transitions is ameliorated during the first 7 to 10 d of the cold acclimation period (Sugawara and Steponkus, 1990).

Although Pearce and Willison (1985a) did not observe the H_{II} phase, they did observe the extensive formation of aparticle domains within the plasma membrane of warm-grown wheat leaf bases, similar to those reported here. Because the formation of IMP-free domains is a likely consequence of cellular dehydration and is a prelude to membrane-membrane interactions that lead to the H_{II} phase (Steponkus and Webb, 1992), it is possible that their observation of only aparticle domains, but not the H_{II} phase, in frozen wheat leaves was a consequence of relatively short times at the injurious temperature. Aparticle (IMP-free) domains

Figure 6. Freeze-fracture electron micrographs of leaves of cold-acclimated rye frozen at -3°C for 16 h then at -35°C for 8 h: a, PF face of the plasma membrane with randomly distributed IMP and several layers of closely appressed aparticulate lamellae subtending the plasma membrane (\star). Note the reduction of IMP density in regions where the plasma membrane is in close apposition with the subtending aparticulate lamellae (arrows). CW, Cell wall; i, extracellular ice. b, PF face of the plasma membrane with regions of fracture-jump lesion (\ast) in conjunction with aparticulate lamellae subtending the plasma membrane. Note that the regions of plasma membrane immediately peripheral to the fracture-jump lesion are either aparticulate or have a reduced density of IMP (\star). CW, Cell wall. c, EF face of the plasma membrane showing randomly dispersed IMP and regions of fracture-jump lesion (\ast). Note that the lower region of fracture-jump lesion contains two layers of apparently aparticulate lamellae. d, PF face of the plasma membrane with a large region of fracture-jump lesion (\ast) as well as several small aparticulate domains (\star). Inset, High-magnification view of paracrystalline arrays of IMP (\ast) that were occasionally observed in the vicinity of the fracture-jump lesion (\ast). Bars represent 300 nm (a), 400 nm (b), 300 nm (c and d), and 200 nm (d and inset).



are, therefore, kinetic intermediates to the formation of the H_{II} phase during freeze-induced injury (Steponkus and Webb, 1992).

In summary, results presented here demonstrate the occurrence of the H_{II} phase in nonacclimated rye leaves frozen at -10°C and of the fracture-jump lesion in cold-acclimated rye frozen at -35°C . Qualitatively, these ultrastructural consequences of freezing injury in leaves are identical with those reported previously for protoplasts isolated from leaves of both nonacclimated and cold-acclimated rye seedlings (Gordon-Kamm and Steponkus, 1984; Fujikawa and Steponkus, 1990). In addition, these data confirm the validity of using rye protoplasts as a model system for studying freeze-induced alterations in membrane ultrastructure.

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