Plant Cell Physiol. 35(2): 175–182 (1994)

Cold-Induced Alterations in Plasma Membrane Proteins That are Specifically Related to the Development of Freezing Tolerance in Cold-Hardy Winter Wheat[1]

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The objective of this study was to identify plasma membrane proteins that are specifically induced by cold acclimation in wheat (*Triticum aestivum* L.). Two cultivars with a marked difference in the genetic ability to cold-acclimate, namely, spring wheat (cv. Chinese Spring) and winter wheat (cv. Norstar), were used as the experimental material. After four weeks of growth in a cold chamber, the freezing tolerance in the shoots of winter wheat increased to −18°C, whereas it increased only to −8°C in the shoots of spring wheat. In the case of roots from both cultivars, freezing tolerance increased only slightly after the growth in the cold environment. Cold acclimation induced remarkable changes in the electrophoretic patterns of plasma membrane proteins which depended on both the cultivar and the tissue examined. Levels of polypeptides with molecular masses from 22 to 31 kDa decreased in both the root and shoot plasma membranes from both cultivars. Among these polypeptides, levels of those of 28 and 26 kDa decreased abruptly after one week of cold acclimation. By contrast, levels of polypeptides of 89, 83, 52, 23, 18 and 17 kDa increased specifically in the shoots of winter wheat. The increases in the levels of the 23-, 18- and 17-kDa polypeptides were proportional to the development of freezing tolerance. Freeze-fracture electron microscopy of plasma membranes from shoot cells revealed that the number of intramembrane particles on the fracture faces decreased markedly in winter wheat after cold acclimation, but to a lesser extent in spring wheat. These results suggest that the plasma membranes might undergo molecular reorganization during cold acclimation.

Key words: Freezing tolerance — Intramembrane particle — Membrane ultrastructure — Plasma membrane proteins — Winter-hardy wheat.

Research into the mechanism of freezing injury in plants has led to the generally accepted concept that the irreversible dysfunction of plasma membranes is the primary cause of freezing injury (Levitt 1972, Steponkus 1984, Yoshida and Uemura 1990). Biochemical studies (Uemura and Yoshida 1986) and electron microscopic studies (Fujikawa and Miura 1986, Gordon-Kamm and Steponkus 1984, Fujikawa 1987) have revealed that the structure of the plasma membrane is irreversibly altered by lethal freezing. Perennial and biennial plants growing in cold climates increase their freezing tolerance from late autumn to early winter as environmental temperatures decrease. Therefore, it seems likely that the plasma membrane per se may be structurally reorganized during cold acclimation to mitigate the freezing-induced damage to the membrane, which may be mediated by changes in the molecular components of the membrane. Biochemical analysis of plasma membranes has shown that cold acclimation involves marked changes in protein composition concomitant with a relative increase in phospholipid content per mg of protein in a wide variety of plant species (Yoshida 1984b, Yoshida and Uemura 1984, Uemura and Yoshida 1984, Ishikawa and Yoshida 1985). The changes in protein composition of the plasma membrane during cold acclimation are, however,

Abbreviations: MOPS, 3-(*N*-morpholino)propanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; DTT, dithiothreitol; IMP, intramembrane particle; PF, protoplasmic fracture face; EF, exoplasmic fracture face; TCA, trichloroacetic acid; BSA, bovine serum albumin.

[1] Contribution no. 3709 from the Institute of Low Temperature Science, Hokkaido University.

very dependent on the genetic backgrounds of the plants examined. Therefore, it is difficult to identify those proteins that are specifically related to the development of freezing tolerance.

In the present study, to obtain more insight into the role of changes in protein content of the plasma membrane during the development of freezing tolerance, we chose to examine two wheat cultivars, namely, a spring and a winter wheat, that differ in their genetic ability to harden. The changes associated with proteins in the plasma membranes were compared between these cultivars during cold acclimation in an attempt to identify those polypeptides that are specifically associated with the development of freezing tolerance in winter-hardy wheat.

Materials and Methods

Plant materials—Two cultivars of wheat (*Triticum aestivum* L. cv. Chinese Spring and cv. Norstar) were used as the experimental material. Seeds were surface-sterilized with 70% (v/v) ethanol and germinated in a growth chamber on wet vermiculite in a plastic tray with small holes in the bottom (35 × 25 cm, 5 cm depth). The plastic tray was placed in a plastic box (36 × 26 cm, 18 cm depth) which was filled with tap water to the base of the vermiculite layer. After two weeks of growth at 18°C (15-h light period) and 15°C (9-h dark period), seedlings had three to four leaves and their roots had emerged through the vermiculite layer into the tap water. At this stage, the tap water was replaced by Hoagland's nutrient solution (1/3 strength) and the roots were aerated by use of an air pump. The light intensity was around 20,000 lux at seedling level.

Cold acclimation—After two weeks of growth at 18/15°C, as described above, the temperature was changed to 4°C (12-h light period) and 2°C (12-h dark period) for cold acclimation. Shoots and roots were sampled separately at weekly intervals during the cold acclimation and were used for the isolation of plasma membranes.

Evaluation of freezing tolerance—Freezing tolerance of the seedlings was evaluated by an ion-leakage test, as described elsewhere (Uemura and Yoshida 1984). One gram of fresh tissue was cut into small pieces which were rinsed with distilled water and blotted. They were transferred to a test tube and frozen at −3°C with ice seeding for 2 h and then they were further cooled at the rate of 2.4°C h^{-1} to −30°C. The tissue samples were removed at designated temperatures separated by 2.5°C intervals and thawed at 4°C in air overnight. After thawing, five ml of distilled water were added into the each test tube which was then incubated at 26°C for 3 h in the dark with a gentle shaking prior to the measurement of the electric conductivity of the resultant solution. Values from tissues frozen to −80°C and from tissues that had been kept at 4°C were taken as having relative conductivity of the 100% and 0% conductivity, respectively. Freezing tolerance was expressed as the freezing temperature that caused 50% ion leakage from tissues.

Freeze-etch electron microscopy—The middle part of laminae from both acclimated and non-acclimated seedlings were cut into small pieces (1 × 2 mm), fixed with 2% (w/v) glutaraldehyde in 25 mM sodium phosphate buffer solution (pH 7.0) for 6 h at 4°C, and cryoprotected by exposure to 30% (w/v) glycerol in the same buffer solution overnight at 4°C. The chemical fixation by glutaraldehyde and following cryoprotectant treatment by glycerol were needed for fixing IMPs against fracturing and for keeping smooth fracture surfaces, respectively. The specimens were mounted on freeze-fracture holders, frozen by abrupt immersion into liquid nitrogen slash (Fujikawa 1991) and stored in liquid nitrogen. The freeze-replicas were made using a freeze-etching apparatus (JFD-7000; JEOL CO., LTD) and observed with a electron microscope (JEM 1200EX; JEOL CO., LTD).

Isolation of plasma membranes—Preparation of crude microsomal membrane fractions and isolation of plasma membranes were carried out in the same way as reported previously for seedlings of winter rye (Uemura and Yoshida 1983), with the exception that 5 mM EGTA and 10 mM KF were included in the homogenization medium, and 6.0% (w/w) polymers were used in the phase-partitioning system instead of 5.6% (w/w). After phase partitioning, the upper phase, which was enriched for plasma membranes, was diluted with 0.5 M sorbitol in a buffer that contained 5 mM MOPS-KOH (pH 7.3), 1 mM EDTA, 0.1 mM PMSF, 10 mM KCl and 2 mM DTT, then the suspension was centrifuged at 156,000 × *g* for 20 min to sediment plasma membrane vesicles. The plasma membrane vesicles obtained in this way were resuspended in the same buffer at an appropriate concentration of protein, usually 1 mg ml^{-1}, and stored at −80°C until use.

Solubilization of plasma membrane proteins with neutral detergent—Plasma membrane proteins were solubilized in a buffered solution of Triton X-100. An aliquot of plasma membranes (200 μg of protein) was centrifuged at 156,000 × *g* for 20 min and the membrane pellet was resuspended in 300 μl of 1% (w/v) Triton X-100 solution that contained 10 mM Tris-HCl (pH 7.3), 2 mM DTT and 0.1 mM PMSF. After incubation at 0°C for 1 h with stirring, the membrane mixture was centrifuged at 156,000 × *g* for 20 min. The supernatant (detergent-soluble) and the pellet (detergent-insoluble) fractions were separately analyzed by one-dimensional SDS-polyacrylamide gel electrophoresis.

One-dimensional SDS-polyacrylamide gel electrophoresis—For analysis of total membrane proteins, plasma membrane samples equivalent to 200 μg of protein were solubilized in SDS-solubilization buffer that contained 2% (w/v) SDS, 62.5 mM Tris-HCl (pH 6.8), 100 mM DTT, 15% (w/v) sucrose and 1 mM PMSF at room temperature.

For electrophoretic analyses of the detergent-soluble and detergent-insoluble protein fractions, the final concentration of SDS and Triton X-100 were adjusted to 10% (w/v) and 0.05% (w/v), respectively, in each fraction. The solubilized membrane samples were heated at 70°C for 15 min prior to electrophoresis. The discontinuous SDS-buffer system of Laemmli (1970) was used for the SDS-slab gel electrophoresis. A 9–14% polyacrylamide gradient gel was prepared as described by Ames (1974). Electrophoresis was performed at a constant current of 15 mA. After electrophoresis, gels were fixed overnight in a 10% (w/v) solution of TCA and rinsed thoroughly with distilled water with several changes. Protein bands were visualized by a silver-staining method (Wray et al. 1981).

Determination of protein concentration—Protein concentration was determined by the Lowry's method (Lowry et al. 1951) with precipitation by TCA and with BSA as the standard.

Results

Development of freezing tolerance—Changes in the freezing tolerance of the two wheat cultivars during cold acclimation are presented in Figure 1. The increase of freezing tolerance of shoots differed markedly between the two cultivars. In winter wheat, the freezing tolerance increased considerably from −5°C at the beginning to −18°C at the end of the cold-acclimation treatment (4 weeks), whereas in spring wheat the freezing tolerance increased only from −4°C to −8°C within the first two weeks and no further increase was observed thereafter. In roots, by contract, the change in freezing tolerance was very slight in both cultivars with tolerance increasing from −3°C to about −5°C after 4 weeks of cold acclimation. Thus, there was a marked difference in the development of freezing tolerance not only between the two cultivars but also between different tissues.

Changes in electrophoretic patterns of plasma membrane proteins—The patterns after SDS-polyacrylamide gel electrophoresis of plasma membrane proteins isolated

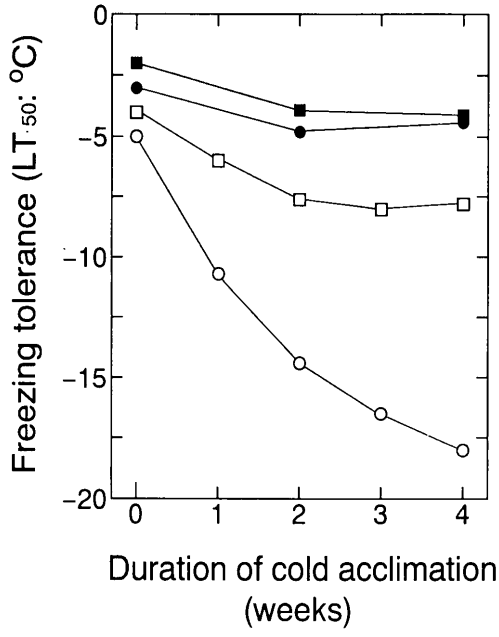

Fig. 1 Increases in freezing tolerance as a function of the duration of the cold-hardening period. During cold acclimation, shoot and root tissues were collected at one-week and two-week intervals, respectively, and then they were subjected to the freezing test as described in Materials and Methods. The data were averages of two experiments. Spring wheat, cv. Chinese Spring, (□, ■); winter wheat, cv. Norstar, (○, ●). Open and solid symbols refer to shoot and root tissues, respectively.

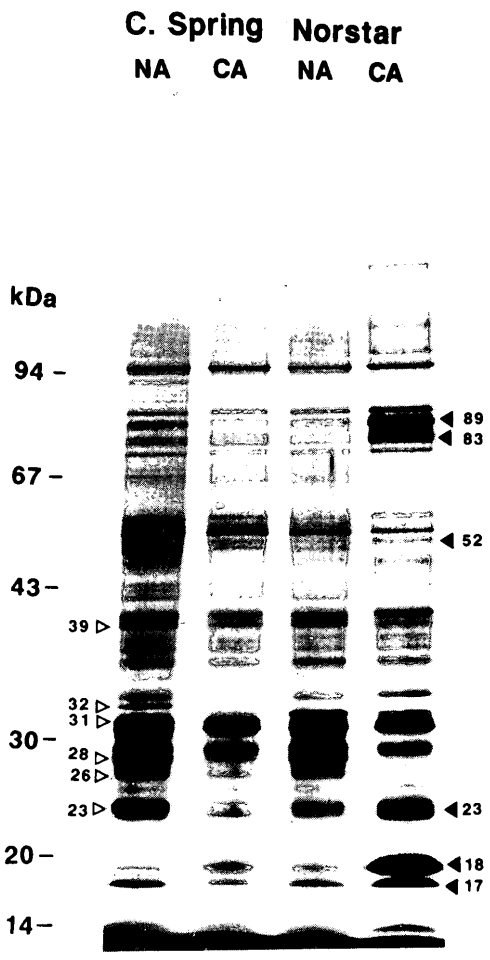

Fig. 2 Electrophoretic patterns of plasma membrane proteins from shoot tissues before (NA) and after cold acclimation for four weeks (CA). Plasma membrane proteins were solubilized in the SDS-buffer system and analyzed on SDS-polyacrylamide gradient gels (9–14%). Five μg of protein were applied to each lane. Arrowheads indicate polypeptides for which the intensity of staining increased (◀) or decreased (▷) after cold acclimation. C. Spring, spring wheat; Norstar, winter wheat.

from shoots are presented in Figure 2. The electrophoretic patterns of proteins solubilized from plasma membranes prepared from shoots of non-acclimated seedlings were quite similar for both cultivars, with the exception for a polypeptide of 32 kDa which was apparently specific to the shoots of spring wheat. Polypeptides of 31, 28, 26 and 23 kDa were the major polypeptides in the plasma membranes before cold acclimation from both cultivars. After cold acclimation for 4 weeks, the polypeptide composition of plasma membranes changed markedly in both cultivars. The polypeptides of 39, 28 and 26 kDa decreased in relative amount in both cultivars and the 23-kDa polypeptide decreased in relative amount in spring wheat. Levels of polypeptides of 89, 83, 52, 23, 18 and 17 kDa increased significantly only in winter wheat, although the level of an 18-kDa polypeptide increased very slightly in spring wheat.

Changes in polypeptide patterns of roots during cold acclimation are presented in Figure 3. In plasma membranes before cold acclimation, there were no great qualitative differences between plasma membranes from shoots (Fig. 2) and roots, but quantitative differences were noted for each polypeptide. The 28-, 26- and 23-kDa polypeptides were the major polypeptides in the plasma membranes before cold acclimation from both cultivars. After cold acclimation for 4 weeks, the levels of 28- and 26-kDa polypeptides showed a remarkable decrease in both cultivars. The level of the 23-kDa polypeptide decreased in roots of both cultivars (Fig. 3), whereas it increased in shoots of winter wheat (Fig. 2). The polypeptides of 89 and 83 kDa in roots showed no changes in levels in either cultivar even after 4 weeks of cold treatment, although their levels increased significantly in shoots of winter wheat (Figs. 2 and 3). The 52- and 18-kDa polypeptides showed slight increases in roots of winter wheat. The 17-kDa polypeptide in plasma membranes of roots decreased in both cultivars after cold acclimation. Thus, there appeared to be a marked difference between the electrophoretic patterns of plasma membrane proteins from shoots and roots after cold acclimation.

The time courses of the changes in polypeptides of shoot plasma membrane in winter wheat with relatively low molecular masses from 17 to 28 kDa are presented in Figure 4. The levels of the 28- and 26-kDa polypeptides decreased abruptly during the first week of cold acclimation. The extents of increases in levels of the 23-, 18- and 17-kDa polypeptides in winter wheat were dependent on the dura-

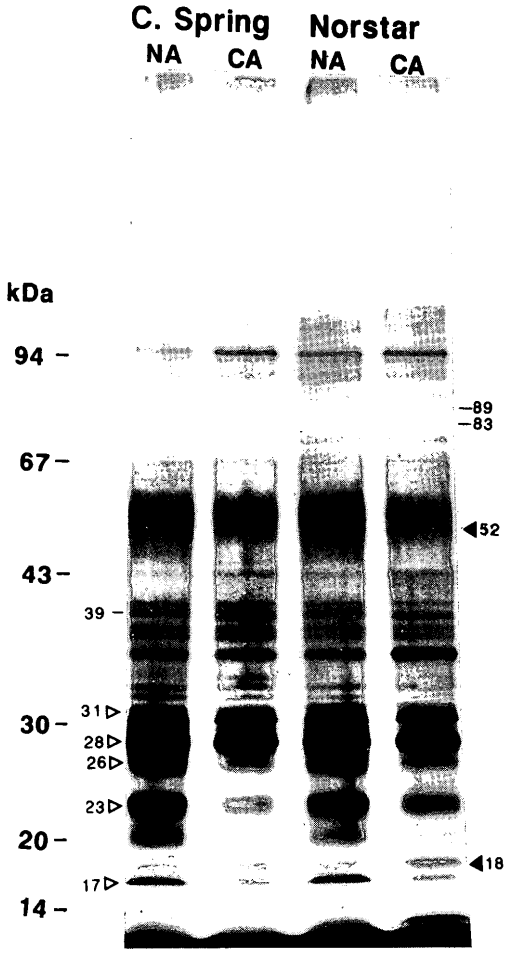

Fig. 3 Electrophoretic patterns of plasma membrane proteins from root tissues before (NA) and after cold acclimation for four weeks (CA). Plasma membrane proteins were solubilized in the SDS-buffer system and analyzed on SDS-polyacrylamide gradient gels (9–14%). Five μg of protein were applied to each lane. Symbols indicate polypeptides for which the intensity of staining increased (◀), unchanged (—) or decreased (▷) after cold acclimation. C. Spring, spring wheat; Norstar, winter wheat.

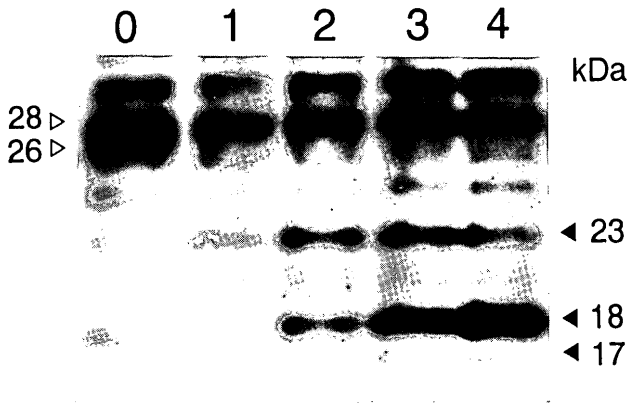

Fig. 4 Changes in intensities of staining of plasma membrane polypeptides from shoot tissues of winter wheat (cv. Norstar) as a function of the duration of the cold-acclimation period. Electrophoretic patterns of polypeptides with molecular masses from 17 to 35 kDa are shown. SDS-solubilized membrane protein (6 μg) was analyzed on SDS-polyacrylamide gels (12%). Arrowheads indicate polypeptides for which the intensity of staining increased (◀) or decreased (▷) during cold acclimation. Lane numbers indicate duration of cold acclimation (weeks).

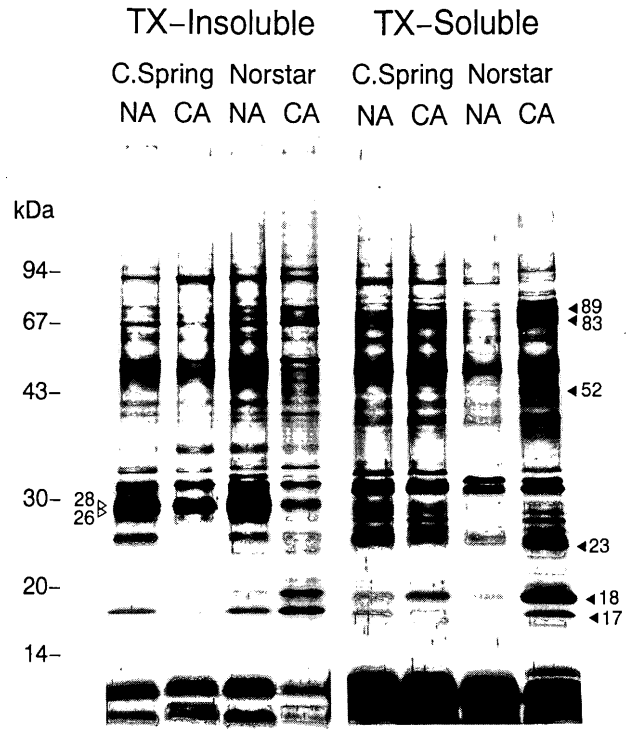

Fig. 5 SDS-polyacrylamide gel electrophorograms of plasma membrane proteins that were soluble and insoluble, respectively, in Triton X-100 (TX). Plasma membranes from shoot tissues before (NA) and after cold acclimation for four weeks (CA) were solubilized in 1% (w/v) Triton X-100. After centrifugation, soluble (right) and insoluble (left) protein fractions were analyzed by electrophoresis on an SDS-polyacrylamide gradient gel (9–14%). Arrowheads indicate polypeptides for which the intensity of staining increased (◀) or decreased (▷) after cold acclimation. C. Spring, spring wheat; Norstar, winter wheat.

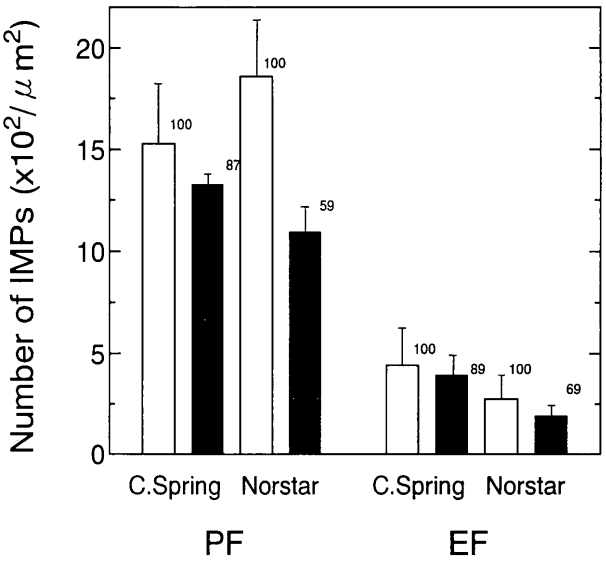

Fig. 7 Changes in numbers of intramembrane particles (IMPs) on plasma membrane fracture faces of leaf cells after cold acclimation for four weeks. Open bars, before cold acclimation; solid bars, after cold acclimation. Numerals on the top of the bars indicate relative numbers of IMPs as percentages. PF, protoplasmic fracture face; EF, exoplasmic fracture face. C. Spring, spring wheat; Norstar, winter wheat.

tion of cold acclimation and were proportionally related to the increase in freezing tolerance.

The electrophoretic patterns of Triton X-100-soluble and -insoluble proteins are presented in Figure 5. Of special interest is the distinct difference in terms of solubility between the two groups of plasma membrane proteins, namely, the polypeptides that were more or less abundant after cold acclimation. Polypeptides of 89, 83, 52, 23, and 18

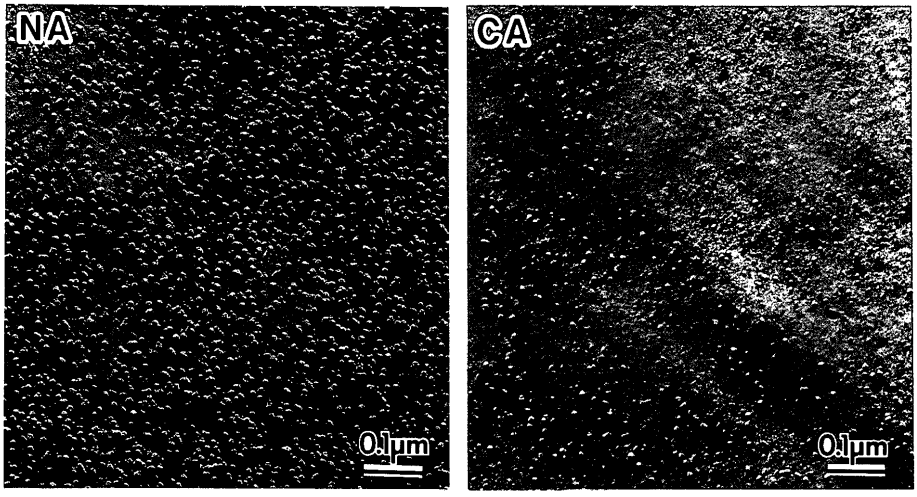

Fig. 6 Freeze-etch electron micrographs showing structural changes in the plasma membrane of leaf cells of winter wheat (cv. Norstar) before (NA) and after cold acclimation for four weeks (CA). Experimental details are given in Materials and Methods.

kDa were mostly recovered in the detergent-soluble fraction, but polypeptides of 28 and 26 kDa were mostly recovered in the detergent-insoluble fraction.

Changes in numbers of intramembrane particles in plasma membranes—To gain some insight into the relationship between the cold-induced changes in protein composition and alterations in plasma membrane structure after cold acclimation, freeze-fracture electron microscopy was used to examine intact leaf tissues. Figure 6 shows electron micrographs of the protoplasmic fracture face (PF) of winter wheat plasma membranes before (NA) and after (CA) cold acclimation. The number of intramembrane particles (IMPs) decreased significantly after cold acclimation. As shown in Figure 7, the percent decrease in the number of IMPs on the protoplasmic (PF) and exoplasmic (EF) fracture faces after cold acclimation were more marked in winter wheat than in spring wheat. In the apical parts of root tissues from winter wheat, by contrast, the decrease in the number of IMPs after cold acclimation was only marginal (data not shown). Thus, the percent decrease in the number of IMPs in the plasma membrane seems to be correlated with the development of freezing tolerance in wheat.

Discussion

When plants freeze under natural conditions, the rate of cooling is sufficiently low that extracellular ice can form outside cells in the tissues. As a result, cells experience severe dehydration and mechanical deformation, namely, cell shrinkage, the extent of which depends on the freezing temperature. Under deep-freezing condition, not only intracellular bulk water, but also the water molecules oriented on the surface of macromolecules and the polar heads of lipids in cellular membranes are removed. Biochemical studies (Uemura and Yoshida 1986) and morphological observations of the freezing and thawing of cells under the cryomicroscope (Steponkus et al. 1983, Dowgert and Steponkus 1984) and the electron microscope (Gordon-Kamm and Steponkus 1984, Fujikawa and Miura 1986, Fujikawa 1987) have led to the widely accepted concept that injury to cells is the direct result of structural and functional damage to plasma membranes. Although the cause of freezing-induced structural alterations in plasma membranes has not been fully characterized at the molecular level, a thermotropic (Yoshida 1984a) and/or a lyotropic (Webb and Steponkus 1990) phase transition of membrane lipids, as well as conformational changes in membrane proteins caused by the freezing-induced dehydration (Yoshida 1984a), and also the fusion of cellular membranes by mechanical contact (Fujikawa and Miura 1986, Fujikawa 1987) have all been considered.

During cold acclimation, the structure of the plasma membrane may be reorganized in such a manner that it is stabilized against freezing-induced structural damage. Hitherto, an increase in levels of highly unsaturated phospholipids (Lynch and Steponkus 1987), changes in the protein composition, and an increase in the relative content of lipids (Yoshida and Uemura 1984, Uemura and Yoshida 1984, Yoshida 1984b, Ishikawa and Yoshida 1985) have been reported to occur in plasma membranes during cold acclimation of various plant species.

According to studies by Steponkus's group, the increase in levels of unsaturated phospholipids during the early stages of cold acclimation in seedlings of winter rye is closely related to the initially induced, moderate increase in freezing tolerance and, furthermore, to the transformation of the cryobehavior of the isolated protoplasts from endocytotic vesiculation to the exocytotic extrusion of protoplast surfaces (Steponkus et al. 1983, Dowgert and Steponkus 1984). This conclusion was directly supported by the artificial incorporation of highly unsaturated phospholipids into protoplast surfaces isolated from non-acclimated cells (Steponkus et al. 1988). In their experiments (Lynch and Steponkus 1987) and our experiments (Uemura and Yoshida 1984) with the same plant material, the unsaturation of phospholipids is completed during the early stages of cold acclimation (within 10 days) at a time when freezing tolerance increases to around −8°C. Upon further prolonged cold acclimation for up to 4 weeks, freezing tolerance increases below −20°C without further changes in the unsaturation of phospholipids. Therefore, it seems difficult to explain the total process of cold acclimation in terms of the increase of unsaturated phospholipids per se. Changes in some other lipids such as glucocerebroside and sterols, and/or membrane proteins so reported here may be responsible for the completion of the development of freezing tolerance.

We reported previously that the protein composition of plasma membranes changed significantly during cold acclimation in various plant species, such as orchard grass (Yoshida and Uemura 1984), winter rye (Uemura and Yoshida 1984), Jerusalem artichoke (Yoshida and Uemura 1990) and mulberry (Yoshida 1984b). Levels of several proteins appeared to increase or decrease after cold acclimation. In Jerusalem artichoke tubers (Yoshida and Uemura 1990), the changes in the protein composition were apparently correlated with the increase of freezing tolerance. Most of the accumulated proteins were soluble in a neutral detergent, whereas the proteins whose levels decreased were not soluble, suggesting a marked difference in their properties (Bordier 1981). However, not all of the changes in the plasma membrane proteins induced by cold acclimation may necessarily be responsible for the increase in freezing tolerance but, perhaps, some of these changes may be involved in the adjustment of the physical and functional properties of the membranes to the chilling environment and also in the growth retardation caused by such an environment.

In the present study, we attempted to identify the plasma membrane proteins whose levels change specifically with the increase in freezing tolerance that occurs during cold acclimation. We compared the cold-induced changes in plasma membrane proteins between two wheat cultivars (Figs. 2 and 3) that have a marked difference in the genetic ability to harden (Fig. 1). A comparison was also made between shoots and roots, which are quite different in terms of the development of freezing tolerance (Figs. 1–3). The plasma membrane proteins whose levels changed during cold acclimation were categorized into two groups: those whose levels decreased dramatically during the early process of cold acclimation regardless of cultivar or tissue (polypeptides of 31, 28 and 26 kDa); and those whose levels increased proportionally to the increase in freezing tolerance only in shoots of cold-hardy winter wheat (polypeptides of 89, 83, 52, 23, 18 and 17 kDa) (Figs. 2–4). The abrupt decrease in levels of the former polypeptides in the early process of cold acclimation seems to be involved in the functional adjustment of the membranes to the chilling environment and/or to the moderate increase in freezing tolerance. By contrast, the gradual increase in levels of the latter polypeptides in shoots of winter wheat seems to be related to further increase in freezing tolerance up to −18°C, although their functional roles have not been clarified. It is noticeable, however, that these two groups of proteins show a marked difference in the solubility in the neutral detergent, Triton X-100 (Fig. 5). From the results, it is assumed that the properties and/or the assembly to the membrane are different between these protein groups. Further detailed studies are needed to clarify the properties, in particular, the physiological functions of those proteins.

Intramembrane particles (IMPs) on fracture faces of membranes are believed to be integral membrane proteins. In cambial cells of willow tree (Parish 1974) and callus from Jerusalem artichoke tubers (Sugawara and Sakai 1978), it was reported that the size of the population of IMPs in the plasma membrane varied significantly during cold acclimation and deacclimation. Upon cold acclimation the size of the population of IMPs decreased significantly and while the reverse occurred upon deacclimation. Nearly the same results were obtained with the wheat cultivars in the present study. The percent decrease in the number of IMPs after 4 weeks of cold acclimation was more marked in the plasma membranes from winter wheat than in those from spring wheat, and the percent decrease in the number was oppositely related to the freezing tolerance (Figs. 6 and 7). The percent decrease in the number of IMPs per specific membrane area of plasma membranes may be interpreted as (a) a result of the reduction in levels of integrated proteins with a concomitant increase in lipids, (b) a result of the vertical movement of the integral membrane proteins, or (c) a result of the dissociation of large protein complexes into small proteins. However, it has not been clarified as yet which are the real cause. At the moment, it is difficult to compare directly the changes in numbers of IMPs with the changes in the electrophoretic patterns of the plasma membrane proteins. However, the remarkable changes in the protein composition and in numbers of IMPs, which are intimately related to the increase in freezing tolerance in winter wheat, lead us to hypothesize that plasma membranes undergo a molecular reorganization during cold acclimation to cope with the freezing-induced destabilization of membrane structure and function. Further experiments are under way in our laboratory focusing on the biochemical processes responsible for the protein-associated changes, in particular, the remarkable increases in the levels of polypeptides with low molecular mass and their functional roles.

This work was supported in part by a Grants from the Ministry of Education, Science and Culture of Japan (no. 01480009). The authors gratefully acknowledge to Dr. Y. Amano of Hokkaido Prefectural Kitami Agricultural Experimental Station for his kind supply of the wheat seeds.

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(Received June 28, 1993; Accepted November 25, 1993)