The Plant Cell, Vol. 10, 623–638, April 1998 © 1998 American Society of Plant Physiologists

Accumulation of an Acidic Dehydrin in the Vicinity of the Plasma Membrane during Cold Acclimation of Wheat

Jean Danyluk,[a] André Perron,[a] Mario Houde,[a] Allen Limin,[b] Brian Fowler,[b] Nicole Benhamou,[c] and Fathey Sarhan[a,1]

[a]Département des Sciences Biologiques, Université du Québec à Montréal, C.P. 8888 Succursale Centre-ville, Montréal, Québec H3C 3P8, Canada
[b]Crop Development Centre, University of Saskatchewan, 51 Campus Drive, Saskatoon, Saskatchewan S7N 5A8, Canada
[c]Département de Phytologie, Université Laval, Pavillon C.E. Marchand, Ste-Foy, Québec G1K 7P4, Canada

Expression of the acidic dehydrin gene *wcor410* was found to be associated with the development of freezing tolerance in several Gramineae species. This gene is part of a family of three homologous members, *wcor410*, *wcor410b*, and *wcor410c*, that have been mapped to the long arms of the homologous group 6 chromosomes of hexaploid wheat. To gain insight into the function of this gene family, antibodies were raised against the WCOR410 protein and affinity purified to eliminate cross-reactivity with the WCS120 dehydrin-like protein of wheat. Protein gel blot analyses showed that the accumulation of WCOR410 proteins correlates well with the capacity of each cultivar to cold acclimate and develop freezing tolerance. Immunoelectron microscope analyses revealed that these proteins accumulate in the vicinity of the plasma membrane of cells in the sensitive vascular transition area where freeze-induced dehydration is likely to be more severe. Biochemical fractionation experiments indicated that WCOR410 is a peripheral protein and not an integral membrane protein. These results provide direct evidence that a subtype of the dehydrin family accumulates near the plasma membrane. The properties, abundance, and localization of these proteins suggest that they are involved in the cryoprotection of the plasma membrane against freezing or dehydration stress. We propose that WCOR410 plays a role in preventing the destabilization of the plasma membrane that occurs during dehydrative conditions.

INTRODUCTION

Low-temperature (LT) acclimation allows hardy plants to develop efficient tolerance mechanisms needed for winter survival. During this period, numerous physiological changes are induced, including increased levels of sugars, soluble proteins, proline, and organic acids, the appearance of new isoforms of proteins, and alteration in lipid membrane composition (Hughes and Dunn, 1990). Because these metabolic changes are presumed to be regulated at the gene expression level, efforts in recent years have focused on identifying LT-responsive genes. Several cold-induced genes and their products have been isolated and characterized in a number of dicotyledonous and monocotyledonous species (Hughes and Dunn, 1996). Some of these LT-responsive genes have also been reported to be responsive to the exogenous application of abscisic acid (ABA) and to abiotic stresses, such as drought and salinity.

Of the many LT-responsive genes characterized to date, several are predicted to encode proteins with the characteristics of the D-11 or dehydrin class of LEA (late embryogenesis–abundant) proteins (Hughes and Dunn, 1996). The induction of this class of protein has been observed in >100 independent studies on drought stress, cold acclimation, salinity stress, embryo development, and responses to ABA (Close, 1996). Dehydrin proteins have a wide size range, have no similarity with any enzymes or proteins of known function, and accumulate to high levels. They are largely hydrophilic proteins that contain different amounts of the K segment (lysine-rich repeat), the S segment (tract of Ser residues), and the Y segment (conserved N-terminal sequence). Using these segments, Close (1996) classified dehydrins into at least five distinct subtypes. In a recent study, we identified an LT-responsive dehydrin-like gene (*wcor410*) from wheat and found a positive correlation between the accumulation of *Wcor410* transcripts and the capacity of wheat genotypes to develop freezing tolerance (FT) (Danyluk et al., 1994). This gene encodes a protein of 28 kD (pI of 5.1) that is rich in the charged amino acids glutamate and lysine (30%) in addition to sharing certain characteristics with members of the D-11 family, such as three K segments and one S segment. Due to its acidic nature and the absence of the glycine-rich repeat present in the cold-regulated dehydrin

[1]To whom correspondence should be addressed. E-mail sarhan.fathey@uqam.ca; fax 514-987-4647.

WCS120 (Houde et al., 1992a), WCOR410 is thought to belong to a different subtype of the D-11 protein family, the so-called acidic dehydrin (Danyluk et al., 1994). These properties raised the possibility that this protein may perform a specific function during cold acclimation.

To gain insight into the function of this gene and its product in increasing FT of wheat, we have undertaken detailed studies to investigate the properties, expression, and subcellular localization of the WCOR410 protein during cold acclimation. In addition, genetic analyses were conducted to map this gene to particular chromosomes and to understand its regulation. Results of this study reveal that in hexaploid wheat, *wcor410* is part of a multigene family that encodes proteins accumulating to very high levels in the vicinity of the plasma membrane of cold-acclimated cells in the vascular transition area. The significance of this spatial localization in relation to the possible cryoprotective function of these proteins in FT is discussed.

RESULTS

Identification and Chromosomal Localization of the *wcor410* Family

During differential screening of the cold-acclimated wheat cDNA library, we identified and sequenced the termini of eight clones, revealing the presence of three cDNAs related to *Wcor410.* The complete DNA sequences of *Wcor410b* (GenBank accession number U73210) and *Wcor410c* (GenBank accession number U73211) were then determined. Sequence analysis indicated that *Wcor410b* and *Wcor410c* encode products that are almost identical to WCOR410 (97.7 and 97.3%, respectively). Gaps to maximize alignment between the corresponding proteins did not fall in regions containing the succession of serine residues, the three lysine-rich repeats, and the N-terminal sequence described for the first acidic dehydrin isolated from wheat (Danyluk et al., 1994).

The ditelocentric (DT) series of Chinese Spring (CS) wheat, in which one homologous pair of chromosome arms is missing in each line, was used to determine which chromosome arms carry the genes of this family. DNA gel blot analysis revealed that hexaploid wheat contains three strong hybridizing fragments corresponding to the three members of the *wcor410* family (Figure 1). With the use of the DT series, we were able to map the three genes to the long arms of the homologous group 6 chromosomes of all three genomes (A, B, and D) of hexaploid wheat. Related species carrying the A, D, or AB genomes were also examined using *Wcor410* as a probe. The results clearly confirm that each genome contains only one member of the *wcor410* gene family. SQ44 was the only one of the D genome species that produced a fragment of equal size to the one found in the hexaploid wheat CS, whereas in the three A genome species, the fragment size no longer corresponds to the hexaploid A genome. This polymorphism can also be seen at the level of the B genome between the hexaploids Norstar (NOR) and CS and among the five tetraploids (AB genome species).

Antibody Production and Biochemical Characterization of the WCOR410 Protein

To characterize the WCOR410 protein and determine its cellular localization and abundance during cold acclimation of wheat, the open reading frame of *Wcor410* was expressed as a histidine-tagged fusion product in *Escherichia coli*, and the WCOR410 protein was purified by affinity chromatography on a HisBind resin. Further purification was achieved by the separation of WCOR410 proteins on an SDS–polyacrylamide gel and electroelution. The analysis of the total amino

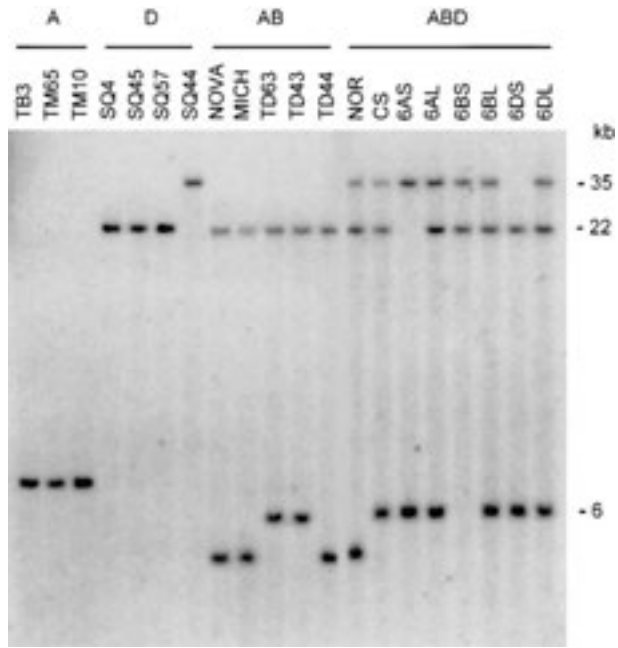

Figure 1. DNA Gel Blot Analysis of Wheat Genomic DNA Using *Wcor410* as a Probe.

DNA from related *Triticum* species carrying the A genome (TB3, TM65, and TM10), the D genome (SQ4, SQ45, SQ57, and SQ44), and the AB genome (NOVA, MICH, TD63, TD43, and TD44); wheat cultivars NOR and CS; and the group 6 chromosome DT lines of CS (6AS, 6AL, 6BS, 6BL, 6DS, and 6DL) were digested with EcoRV. The DNA quantity used for electrophoresis was adjusted according to the genome size: 0.6, 1.2, and 1.8 μg were used for the diploid, tetraploid, and hexaploid wheats, respectively. Numbers at right indicate estimated lengths of bands in kilobases.

acid composition of the recombinant protein indicated that it had the same composition as the one predicted from the *Wcor410* sequence. Separation on an SDS–polyacrylamide gel revealed that this purified protein has an apparent molecular mass of 55 kD, which is in contrast with the predicted one of 28 kD (Figure 2A). This discrepancy has also been observed with other plant stress proteins such as WCS120 (Houde et al., 1992a).

The immune serum raised against the purified WCOR410 protein cross-reacted partially with proteins of the WCS120 family (Figure 2B), probably due to the many copies of the lysine-rich repeat present in the members of this family. When antibodies recognizing the WCS120 proteins were removed, the resulting anti-WCOR410 antibody detected specifically the WCOR410 protein (Figure 2C). This anti-WCOR410 antibody (depleted of anti-WCS120 antibodies) was used for all further experiments. To confirm the specificity of this immunological probe, the anti-WCOR410 antibody was further purified with an affinity column containing pure WCOR410 protein. Immunoblot analysis with this affinity-purified anti-WCOR410 antibody showed identical results (Figure 2D). Longer exposures of Figures 2C and 2D did not reveal any cross-reaction with the purified WCS120 compared with a strong signal with the purified WCOR410. These experiments show that both antibodies detect specifically the WCOR410 protein and are free of any major nonspecific contaminants.

Preliminary protein gel blot analysis comparing the soluble protein extraction and the trichloroacetic acid–acetone extraction procedure for total proteins showed that the latter consistently resulted in higher recoveries of the WCOR410 protein. Furthermore, electrophoretic transfer to nitrocellulose using SDS in the transfer buffer was found to be variable. The omission of SDS and an increased methanol concentration in the transfer buffer improved transfer efficiency and apparently increased the affinity of the protein for the nitrocellulose membrane. Because these modifications produced consistent results, they were used routinely for all further protein gel blot analyses.

Accumulation Kinetics of the WCOR410 Protein Family and Abundance during Cold Acclimation

The anti-WCOR410 antibody was used to study the accumulation kinetics of the WCOR410 protein family during cold acclimation in the spring cultivar Glenlea and the winter cultivar Fredrick. When the seedlings were transferred to cold acclimating conditions, WCOR410 proteins accumulated rapidly, reaching a maximum level after 5 days, and then decreased gradually, despite plants being maintained at 4°C (Figure 3A). The timing of peak expression was similar to that of *Wcor410* transcripts (Danyluk et al., 1994) and corresponds to the period when cereal cold acclimation curves show the most rapid acclimation rates (Fowler et al., 1996). In addition, these results showed that during the whole period of cold acclimation, the tolerant cultivar Fredrick accumulated more of the WCOR410 proteins compared with the less tolerant Glenlea. After ~21 days of acclimation, cereal plants are very near their maximum FT (Fowler et al., 1996). At this time, Glenlea showed no accumulation compared with Fredrick (Figure 3A). This suggests that the poor FT of the spring compared with the winter cultivar is the result of an inability of the spring type to maintain LT tolerance genes in an upregulated state.

To quantitate the amount of WCOR410 proteins accumulated during cold acclimation, several concentrations of the recombinant WCOR410 protein (used as standards) and different dilutions of plant extracts were separated within the same gel, transferred to nitrocellulose, and probed with the anti-WCOR410 antibody. Using densitometry quantitation, we were able to estimate that after 5 days of cold acclimation, winter wheat Fredrick had accumulated WCOR410 proteins to 0.38 ± 0.08% of total proteins.

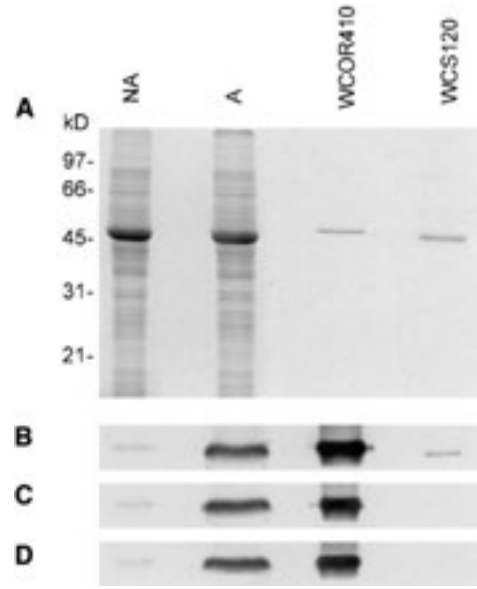

Figure 2. Antibody Specificity.

(A) Analysis of total proteins (5 μg) from nonacclimated (NA) and 2-week-old cold-acclimated wheat Fredrick (A) and 0.5 μg of purified WCOR410 and WCS120 proteins. The proteins were separated on a 10% SDS–polyacrylamide gel and visualized by staining with Coomassie Brilliant Blue R 250. The position of molecular mass markers in kilodaltons is indicated at left.

(B) Immunoblot analysis with the crude immune serum. Proteins separated on an SDS–polyacrylamide gel were transferred to a nitrocellulose membrane and probed with the immune serum.

(C) Immunoblot analysis with the anti-WCOR410 antibody (depleted of anti-WCS120 antibodies).

(D) Immunoblot analysis with the affinity-purified anti-WCOR410 antibody.

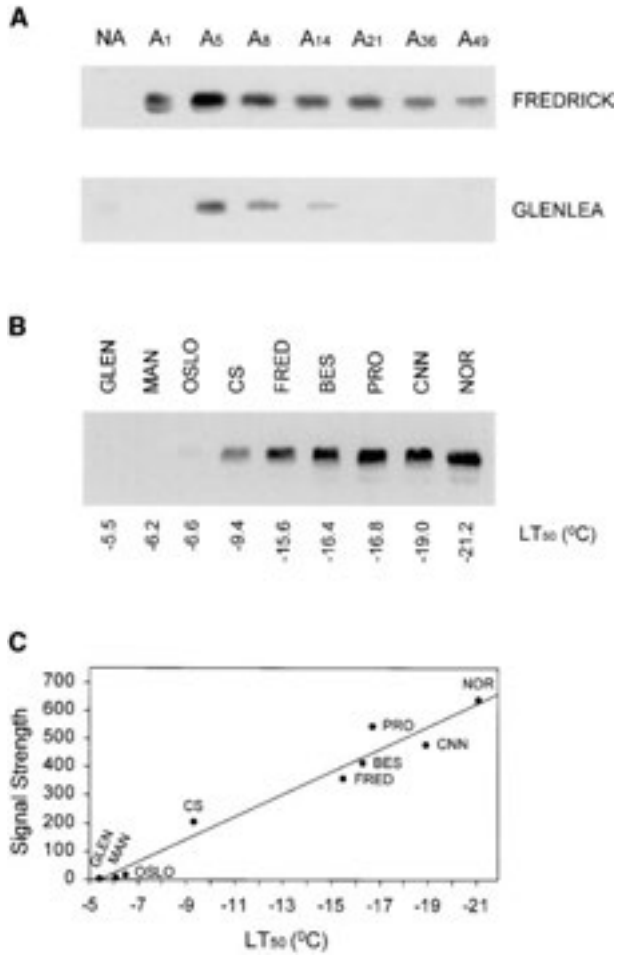

Figure 3. Accumulation of WCOR410 Proteins during Cold Acclimation of Wheat.

(A) Accumulation kinetics of WCOR410 in the cultivars Fredrick and Glenlea. Total proteins (5 μg) from aerial tissues were separated on an SDS–polyacrylamide gel, transferred to a nitrocellulose membrane, and probed with the anti-WCOR410 antibody. NA, nonacclimated; A1 to A49, cold acclimated for 1 to 49 days.

(B) WCOR410 accumulation in different wheat cultivars that were cold acclimated for 49 days. Immunoblot analysis was done with total proteins (5 μg) from aerial tissues of spring cultivars Glenlea (GLEN), Manitou (MAN), Oslo (OSLO), and Chinese Spring (CS) and of winter cultivars Fredrick (FRED), Besostoya (BES), Pioneer 2548 (PRO), Cheyenne (CNN), and Norstar (NOR).

(C) Graphical representation of the results shown in **(B)**. WCOR410 signal strength (expressed in arbitrary units) was plotted as a function of the cultivars LT_{50} (°C).

Accumulation of WCOR410 Proteins in Different Wheat Cultivars

To determine whether WCOR410 protein accumulation during cold acclimation could be used as an FT marker in wheat, we examined a number of cultivars differing in their acclimation capacity. The results shown in Figures 3B and 3C indicate that the accumulation of WCOR410 proteins correlates well with the capacity of each cultivar to cold acclimate and develop FT. This lends support to the concept that the anti-WCOR410 antibody may represent a useful tool for breeders to select for FT in cereals. We also evaluated the FT as measured by the temperature at which 50% of the plants are killed (LT_{50}) and WCOR410 protein accumulation for the chromosome substitution series in which the individual chromosomes of the more FT cultivar Cheyenne (CNN) are substituted in individual pairs for the corresponding homologous chromosome pair in the less FT CS cultivar. Chromosome 5A of CNN conferred a significant ($P < 0.05$) increase in FT over that of the recipient CS parent (Figure 4A). As expected, protein gel blot analysis showed that the more freezing-tolerant parent CNN accumulated a greater amount of the WCOR410 protein family than did the less tolerant CS parent (Figure 4B). Protein gel blot analysis of the CS/CNN substitution series (Figure 4B) showed that substitution of chromosome 5A of CNN for 5A of CS resulted in greater protein accumulation than was found in CS or any of the other substitution lines. Because the genes expressing WCOR410 proteins reside on the long arms of the group 6 chromosomes, regulation of the *Wcor410* gene family in this instance appears to lie on chromosome 5A.

We have shown previously that the accumulation of the WCS120 family of dehydrin proteins was also correlated with the capacity to develop FT in different Gramineae species (Houde et al., 1992b). The genes of the *wcs120* family have been mapped to the long arms of the homologous group 6 chromosomes, and the regulation of their expression seems also to lie on chromosome 5A (Limin et al., 1997). In both studies, no single chromosome substitution brought the FT or the accumulation of the WCOR410 and the WCS120 protein families in CS close to the CNN level, indicating that these traits are controlled by several genes. However, these results imply that a gene located on chromosome 5A is, at least in part, regulating the expression of both the *wcs120* and *wcor410* gene families during cold acclimation. This regulatory factor may be responsible for the differential expression of the WCOR410 family among the different cultivars, as shown in Figure 3. Detailed genetic analysis and molecular isolation of the regulator on chromosome 5A will help to confirm its biological significance in the development of FT. Knowledge gained from these studies will allow us to determine whether FT in cereals can be improved by modulating the expression level of this regulator in the less tolerant and sensitive Gramineae species.

Tissue Distribution and Specificity of Expression of WCOR410 Proteins

We compared the relative abundance of WCOR410 proteins in different tissues (Figure 5A) and found that their accumu-

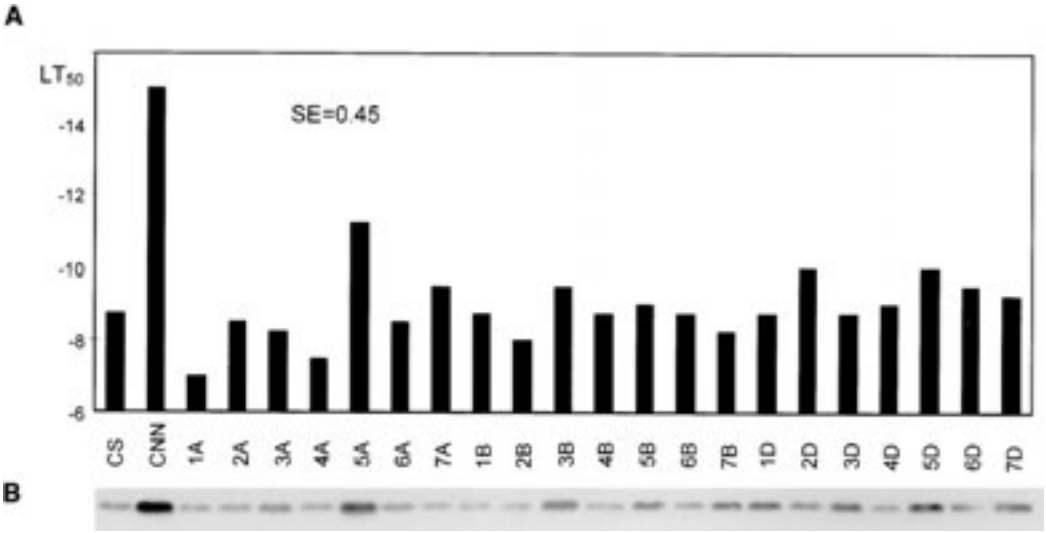

Figure 4. Freezing Tolerance and WCOR410 Protein Accumulation in the CS/CNN Chromosome Substitution Series.

(A) Freezing tolerance (LT_{50}) of 21-day-old cold-acclimated lines.
(B) Immunoblot analysis of total proteins (5 μg) from aerial tissues of cold-acclimated lines.

lation during cold acclimation showed a slight tissue specificity because it was detected at a higher level in the leaves than in the crown and roots. In addition, because evidence has accumulated indicating that water stress and ABA can increase FT to varying degrees (Guy, 1990; Thomashow, 1990), we analyzed WCOR410 protein accumulation in wheat plants subjected to different treatments. Figure 5B shows that severe water stress, polyethylene glycol–induced osmotic stress, and ABA treatment significantly induced the accumulation of WCOR410 proteins but to a lower level than did LT exposure in wheat. These results suggest that WCOR410 protein accumulation may be associated with the increased FT induced by these treatments. However, more in-depth correlative studies are required to confirm this assertion.

Immunolocalization of the WCOR410 Protein Family

When ultrathin sections of root, crown, stem, and leaf tissues from nonacclimated wheat (cv Fredrick) were incubated with the anti-WCOR410 antibody and with gold-conjugated antiserum to rabbit immunoglobulins, little or no deposition of gold particles was observed (Figures 6A, 7A, 8A, and 9A). However, sections from cold-acclimated wheat plants revealed a heavy deposition of gold particles over the plasma membrane lining the electron-opaque cell wall, whereas no labeling was detected in the cell cytoplasm, the nucleus, the organelles, and the vacuoles (Figures 6B, 6C, 7C, 8D, and 9B). Quantitative evaluation of labeling near the plasma membrane revealed that cold-acclimated tissues were densely

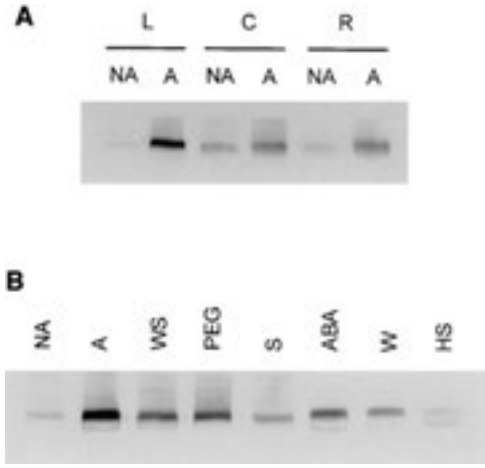

Figure 5. WCOR410 Tissue Distribution and Specificity of Expression.

(A) Immunoblot analysis of total proteins (5 μg) present in different tissues of the cultivar Fredrick grown under acclimating conditions. NA, nonacclimated; A, cold acclimated for 2 weeks; L, leaf (sheath and blade); C, crown; R, root.
(B) Immunoblot analysis of total proteins (5 μg) from aerial tissues of the cultivar Fredrick exposed to different treatments. NA, nonacclimated; A, cold acclimated for 2 weeks; WS, 20 hr after water stress; PEG, 48 hr after transferring to a solution of 40% (w/v) polyethylene glycol; S, salt stressed for 24 hr with 0.5 M NaCl; ABA, treatment with 10^{-4} M ABA for 18 hr; W, 18 hr after wounding stress; HS, heat shocked for 3 hr.

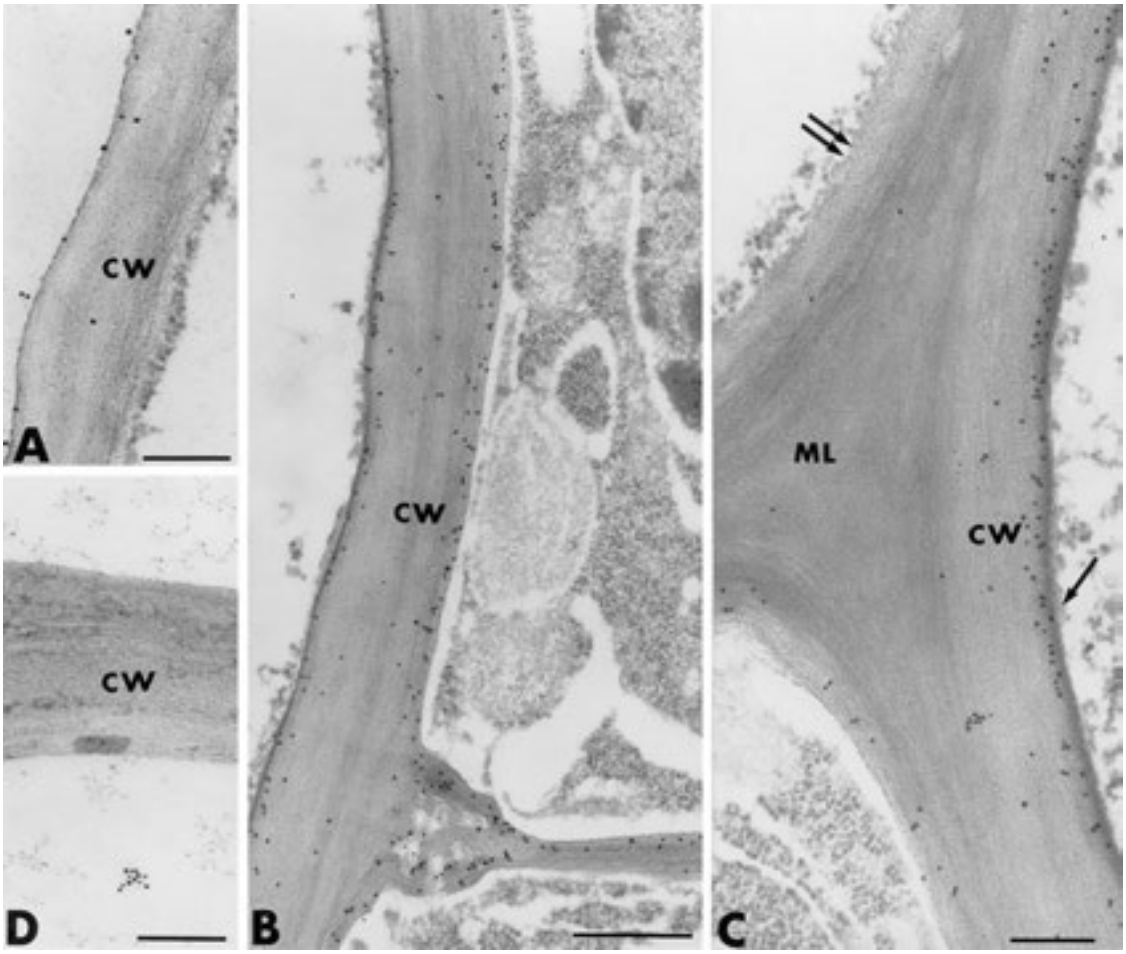

Figure 6. Electron Microscopy of Transverse Root Sections from Wheat Seedlings Incubated with the Anti-WCOR410 Antibody.

(A) Nonacclimated root. Bar = 0.25 μm.
(B) Cold-acclimated root. Bar = 0.5 μm.
(C) Cold-acclimated root. Bar = 0.25 μm.
(D) Cold-acclimated root (primary antibody omitted). Bar = 0.5 μm.
CW, cell wall; ML, middle lamella; the single arrow in **(C)** indicates the dense labeling on the plasma membrane; the double arrow indicates the absence of labeling in a neighboring cell.

labeled, ranging from 22 to 48 gold particles per μm^2 (Table 1) compared with nonacclimated tissues, which ranged from 1 to 3 gold particles per μm^2 (n = 10). In cold-acclimated tissues, labeling near the plasma membrane represented 53 to 75% of total gold particles counted (Table 1). The dense distribution of the labeling around the plasma membrane makes it very difficult to determine whether WCOR410 proteins are present on the external or internal side or on both sides of the membrane. In addition, this quantitative evaluation revealed that labeling near the plasma membrane was stronger in crown and stem tissues than in root and leaf tissues (Table 1). These results differ slightly from those presented in Figure 5A and may reflect a loss of immunoreactivity in some tissues during processing for electron microscopy.

A close examination of the tissue sections revealed that labeling was not only associated with the plasma membrane but was also present at a lower level in the matrix between adjacent cell walls (Figure 7B), in the amorphous material formed in some intercellular spaces (Figure 7D), and over the fibrillar network at the junction with cell walls (Figures 8B and 8C). This suggests that the WCOR410 protein must be transported via the membrane and exported outside of the cell to be present in these intercellular spaces. However, sequence analysis of the WCOR410 protein did not reveal any

known signal peptide that would be responsible for this translocation. An interesting feature of antibody reaction was the strong deposition of gold particles in some cells and the near absence of labeling in the neighboring ones (Figures 6C, 7C, and 8D). This differential distribution of gold particles was consistently observed from one section to another and may reflect our qualitative evaluation of labeling intensity that revealed that antibody binding sites were predominantly associated with the vascular transition area.

To confirm the specificity of labeling obtained with the anti-WCOR410 antibody, we conducted similar experiments with the affinity-purified anti-WCOR410 antibody. Figures 10A and 10B clearly show a significant accumulation of gold particles along the plasma membrane and confirm the results presented in Figures 6 to 9. This demonstrates the specificity and the strict affinity of both antibodies for the WCOR410 protein. All control tests, including the omission of primary antibody (Figure 6D), the use of preimmune serum (Figures 7E and 8E), and preincubation of the primary antibody with the WCOR410 protein before section labeling (Figure 10C), yielded negative results (0 to 2 gold particles per μm^2; $n = 10$). These results clearly show that the WCOR410 protein family is preferentially associated with the plasma membrane and may accumulate to a significant level on the external side of the membrane.

Subcellular Fractionation

Biochemical fractionation and protein gel blot analysis were performed to examine the association of WCOR410 with the plasma membrane in cold-acclimated wheat leaves. The results in Figure 11A show that on a protein basis, WCOR410 is more abundant in the upper phase fraction, which is enriched in plasma membranes, than in the crude microsomal pellet. This suggests that WCOR410 is preferentially associated with the plasma membrane fraction. However, evaluation of the total quantity of WCOR410 proteins in different fractions revealed that >70% of the protein was localized in the 156,000*g* supernatant fraction. The quantity of these proteins in the soluble fraction did not decrease after a 2-hr centrifugation at 200,000*g*, indicating that in this fraction, WCOR410 was not associated with vesicles (results not shown). In addition, WCOR410 proteins in the soluble fraction were found to be vulnerable to proteinase K digestion (Figure 11B). This indicated that the interaction between WCOR410 and the plasma membrane was labile under the extraction conditions used and suggested a peripheral localization for these proteins.

To examine in more detail WCOR410 localization within vesicles, the crude microsomal fraction was subjected to sodium carbonate treatment and proteinase K digestion. Our observations show that sodium carbonate treatment, which converts closed vesicles into open membrane sheets, caused the majority of microsomal WCOR410 proteins to become localized in the soluble fraction (results not shown). In addition, WCOR410 proteins in the microsomal fraction were found to be resistant to proteinase K digestion, unless 1% Triton X-100 was included to solubilize membranes (Figure 11B). When the microsomal pellet was resuspended in a low-osmoticum buffer containing proteinase K to burst vesicles and release their content, a lower quantity of WCOR410 proteins was protected from proteinase K digestion (Figure 11B). The behavior of the WCOR410 protein under these experimental conditions indicates that it is not an integral membrane protein but a peripheral protein.

DISCUSSION

The results presented here provide evidence that the accumulation of the WCOR410 protein family during cold acclimation is correlated with the differential capacity of wheat cultivars to develop FT. The cold-regulated *wcor410* genes were also found to be upregulated extensively by water stress, polyethylene glycol, and ABA and to a lesser extent by salt and wounding. Previous studies have shown that water stress and ABA can increase FT to varying degrees (Guy, 1990; Thomashow, 1990). Therefore, the accumulation of dehydrins, such as WCOR410, is consistent with a possible function in the protection of the cell against dehydrative conditions that result from these treatments.

During freezing stress, intercellular ice crystal formation is initiated in both the subepidermal and perivascular tissues (Pearce, 1988; Pearce and Ashworth, 1992). Understandably, cells bordering these regions are more likely to be affected by dehydration and higher ionic stress that result from water migration to the growing extracellular ice crystal. Thus, it is reasonable to assume that plant tolerance to abiotic stresses relies primarily on the ability to elaborate rapidly a defensive line for protecting the vascular area against dehydration. In line with this concept, Tanino and McKersie (1984) have shown that the vascular transition area in cold-acclimated winter wheat plants is more sensitive to freezing stress than is the apical meristem and that regrowth of the crown is primarily dependent on the viability of this region.

As for the induced WCS120 dehydrin protein family in wheat (Houde et al., 1995), WCOR410 proteins were found to accumulate preferentially in the vascular transition area. This observation supports the idea that massive deposition of the newly formed WCOR410 proteins in the perivascular region is directly related to the protection of the underlying tissues. It appears that the cultivar-dependent accumulation of two highly abundant LT-induced protein families, WCS120 and WCOR410, is crucial in protecting this highly sensitive region of the plant. Elucidating the signals that regulate the synthesis of these protein families in specific cell types warrants further investigation. This will help in producing transgenic plants expressing these proteins in tissues that need them most.

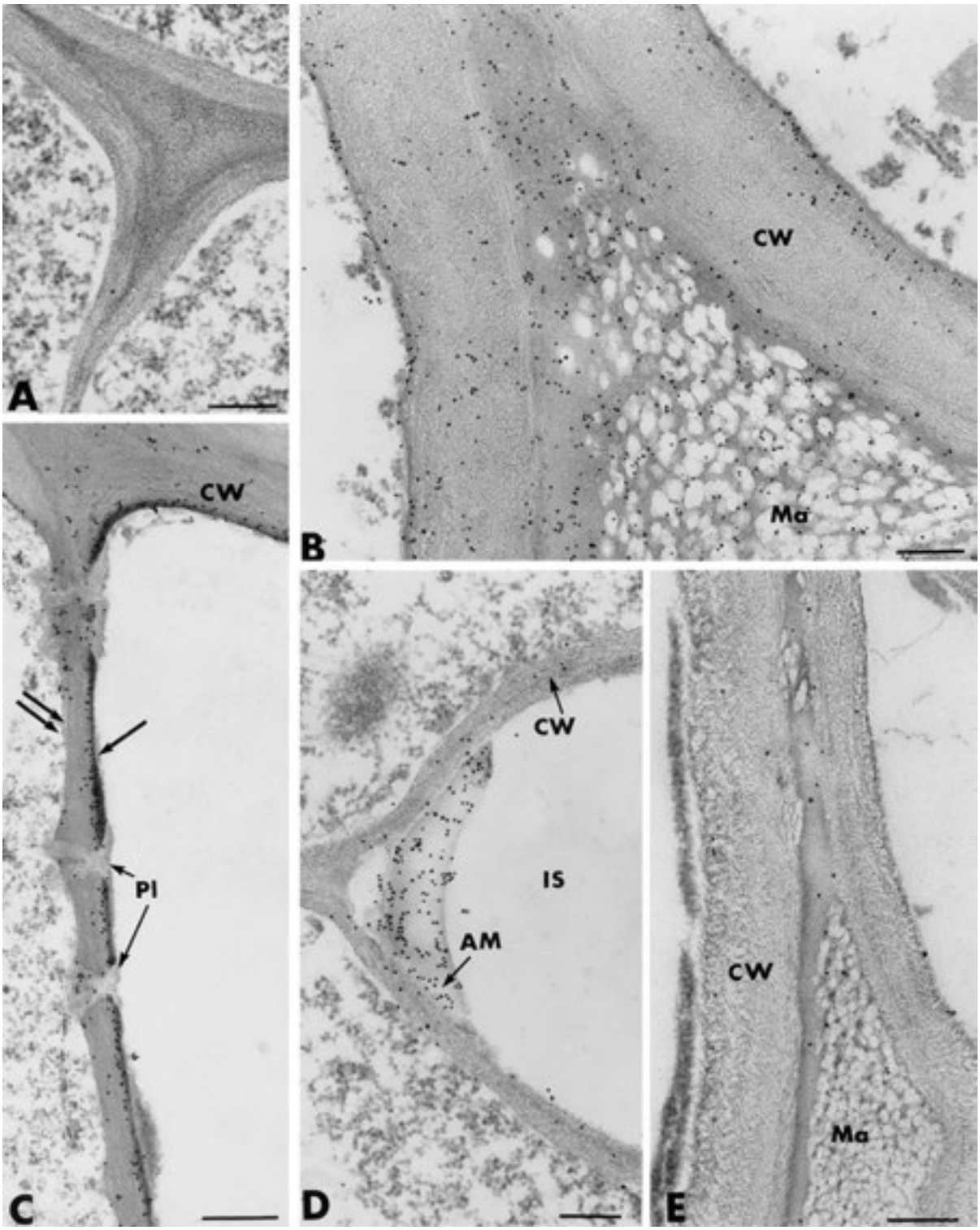

Figure 7. Electron Microscopy of Transverse Crown Sections from Wheat Seedlings Incubated with the Anti-WCOR410 Antibody.

(A) Nonacclimated crown. Bar = 0.25 μm.
(B) Cold-acclimated crown. Bar = 0.25 μm.

Immunoelectron microscopic observations indicate that WCOR410 proteins are concentrated mainly in the vicinity of the plasma membrane, with a lower accumulation in the amorphous material and the fibrillar network. These results provide direct evidence that a subtype of the dehydrin family accumulates near the plasma membrane. Localization work with other dehydrins has established that they can be present in the nucleus or cytoplasm (reviewed in Close, 1996). Our results confirm, at least in part, our previous hypothesis that dehydrin subtypes can accomplish their function in different compartments (Danyluk et al., 1994). WCOR410 proteins represent ~0.38% of total cellular proteins in the cold-acclimated tolerant cultivar Fredrick. If one considers that these proteins are present in specific cell types and concentrated at or near the plasma membrane, then their concentration in the vicinity of the membrane is much greater. The WCOR410 protein is largely hydrophilic because only 17% of residues are the hydrophobic amino acids Ile, Leu, Met, and Val. A distinguishing feature of this protein is its high content of charged residues (43% of total) and hydroxylated residues (12% of total). This protein's hydrophilicity combined with its abundance suggest a cryoprotective function during freezing stress.

It is not known how a protein such as WCOR410 interacts with the surface of the plasma membrane. In water, the surfaces of macromolecules remain well separated due to the entropic force of the ordered water molecules at the surface (Israelachvili and Wennerström, 1996). In biological systems such as ours, the hydration layer must be modified to bring the two systems into contact with each other. It has been proposed that dehydrating the surfaces is required to initiate these phenomena (Israelachvili and Wennerström, 1996).

During freezing or dehydration stress, water migration to the extracellular space leaves the paramural space between the plasma membrane and cell wall in a dehydrated condition, with the cell having increased osmotic pressure. This may result in the withdrawal of the ordered water molecules surrounding both the membrane surface and the WCOR410 proteins. The removal of water could initiate contacts between WCOR410 and membrane lipids. The dynamic association thus would be strongly influenced by water activity. This may explain why during biochemical fractionation the majority of WCOR410 proteins are found in the soluble fraction and not tightly associated with the plasma membrane. This type of reversible association can be advantageous for the cell because interaction under normal growth conditions may cause a steric hindrance or interference with vital metabolic processes involving the plasma membrane. However, WCOR410 proteins that accumulate during cold acclimation would be available to fulfill their cryoprotective function upon occurrence of freezing temperatures. In support of this concept, Ceccardi et al. (1994) have shown that maize G50 dehydrin is capable of substantial hydrophobic interaction in vitro. This led Close (1996) to suggest that a possible function of the amphipathic α helix within the K segment may be its hydrophobic interaction with partially denatured proteins or membranes.

From the present ultrastructural observations, it is difficult to determine accurately whether WCOR410 proteins are present on both sides of the plasma membrane and by which mechanism they may be exported outside of the cell. Some evidence, such as the presence of these proteins in intercellular spaces and in vesicles that may have an excretory role, indicates that they may accumulate to a significant level on the external side of the plasma membrane. Furthermore, the use of a buffer of an osmolarity similar to that of the cell in the fractionation experiment did not permit a quantitative recovery of WCOR410 proteins with membranes, suggesting that these proteins are soluble but are trapped in the paramural space. The association with the exterior membrane face would be of key importance to stabilize this side of the membrane bilayer. The exact mechanism by which such a protein may cryoprotect the plasma membrane is not known, but several possibilities can be proposed on the basis of the protein's composition and localization.

First, the features of acidic dehydrins suggest that they may either function in water retention or directly replace water for the "solvation" of the membrane. Because WCOR410 proteins are predicted to be largely α helical, it is reasonable to assume that their water binding capacity would be inferior to glycine-containing dehydrins that exist mostly in random coils. In the coil conformation, the dipolar peptide bonds are not involved in protein secondary structure and so are available for binding other polar molecules. However, at the moment, we cannot rule out the possibility that during dehydrative conditions, WCOR410 may exist in a conformation other than the one it has in a purified form in aqueous buffers. During dehydration, loss of water from membranes can alter their transition temperature from the liquid crystalline to gel phase and lead to irreversible damage (Crowe et al., 1993). However, if sugars are added before dehydration begins, the physical properties of dry membranes are altered

Figure 7. (continued).

(C) Cold-acclimated crown. Bar = 0.5 μm.

(D) Cold-acclimated crown. Bar = 0.25 μm.

(E) Cold-acclimated crown incubated with the preimmune serum. Bar = 0.25 μm.

AM, amorphous material; CW, cell wall; IS, intercellular space; Ma, matrix; Pl, plasmodesmata; the single arrow in **(C)** indicates the dense labeling on the plasma membrane; the double arrow indicates the absence of labeling in a neighboring cell.

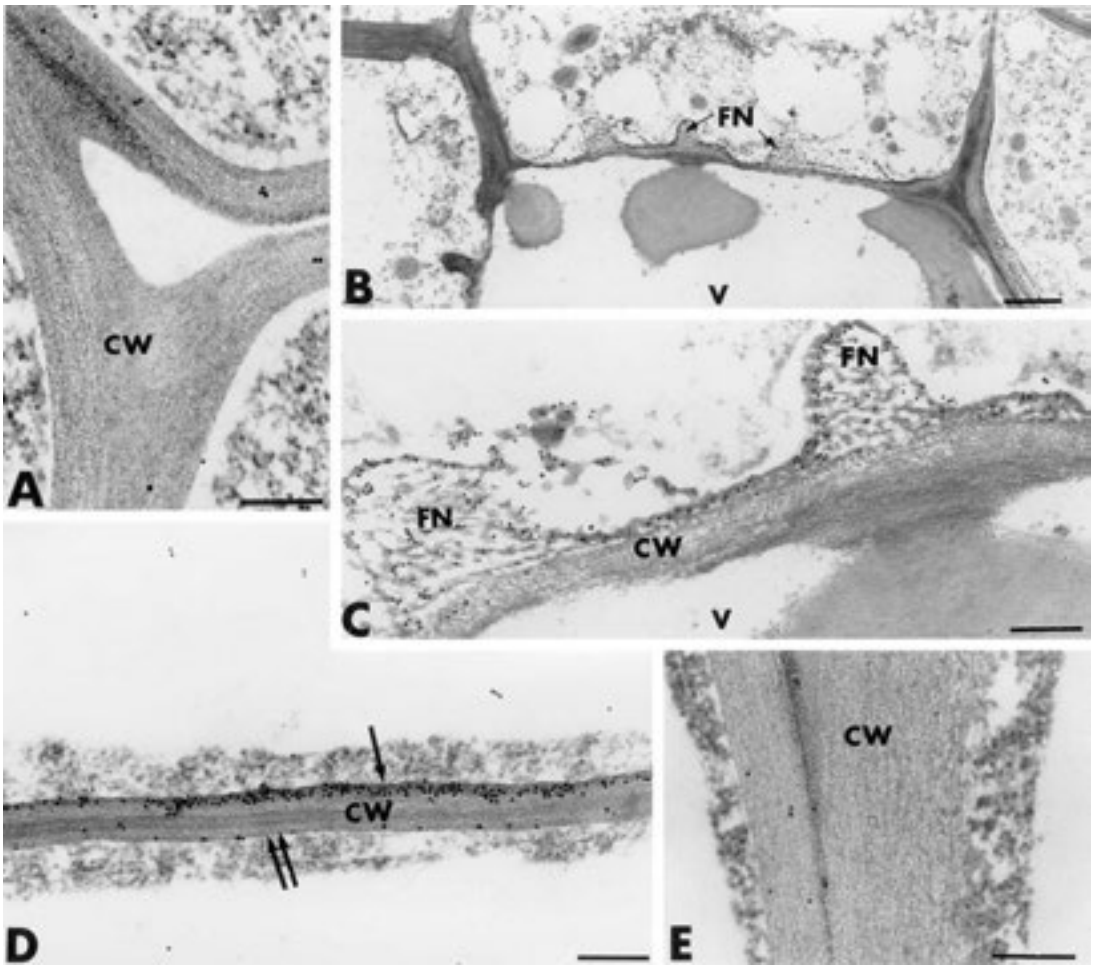

Figure 8. Electron Microscopy of Transverse Stem Sections from Wheat Seedlings Incubated with the Anti-WCOR410 Antibody.

(A) Nonacclimated stem. Bar = 0.25 μm.
(B) Cold-acclimated stem. Bar = 1 μm.
(C) Cold-acclimated stem. Bar = 0.25 μm.
(D) Cold-acclimated stem. Bar = 0.25 μm.
(E) Cold-acclimated stem incubated with the preimmune serum. Bar = 0.25 μm.
CW, cell wall; FN, fibrillar network; V, xylem vessel; the single arrow in **(D)** indicates the dense labeling on the plasma membrane; the double arrow indicates the absence of labeling in a neighboring cell.

and resemble those of fully hydrated biomolecules. This stabilization is achieved through hydrogen bonding between hydroxy groups on the sugar and polar residues in phospholipids. It seems that the hydrophilic nature of the WCOR410 protein is well suited to replace water and stabilize membranes through polar interactions. A detailed investigation is required to determine WCOR410 hydration characteristics and its propensity to interact with liposomes and alter their physical properties. This approach was recently used for two cold-regulated polypeptides from Arabidopsis (Webb et al., 1996).

Second, WCOR410 proteins may be involved in reducing the incidence of the dehydration-induced membrane alterations that lead to loss of osmotic responsiveness after a freeze–thaw cycle. During freeze-induced dehydration, membrane bilayers are brought close to each other. In these regions, several changes can be induced, such as demixing of membrane components that differ in hydration characteristics, followed by the formation of a nonbilayer structure known as the hexagonal II phase and fusion of bilayers (reviewed in Steponkus and Lynch, 1989; Steponkus et al., 1993). These ultrastructural changes affect membrane bilayer integrity

and lead to the freezing injury that is characterized by the loss of osmotic responsiveness during rehydration. Therefore, if WCOR410 proteins can prevent the interaction between membrane bilayers during dehydration and/or the dehydration-induced demixing of lipids, they may diminish the incidence of the lamellar to hexagonal II phase transition and the fusion of bilayers. Because WCOR410 protein accumulation was observed only in specific cell types, it seems reasonable to assume that their abundant association with the plasma membrane of these cells can cause a steric hindrance to the interaction between membrane bilayers. In addition, proteins such as WCOR410 contain a high content of acidic, basic, and hydroxylated amino acids that may interact with membrane lipids, such as the different phospholipids, cerebrosides, and sterols. If one WCOR410 was able to bind to several lipid species, this could be the basis for preventing lipid demixing that occurs during dehydration. This hypothesis is plausible because proteins known as annexins were shown to bind to acidic and neutral phospholipids in a calcium-dependent manner (Raynal and Pollard, 1994) and affect the lateral mobility of the phospholipids (Swairjo and Seaton, 1994). Further investigations are required, such as using the in vitro approach described by Webb et al. (1996).

Finally, WCOR410 proteins may function in counteracting the irreversibly damaging effects of the increasing ionic concentration during dehydration. Under nonstressful conditions, the high content of charged residues in WCOR410 (43%) may form predominantly intramolecular interactions, whereas in a high-salt environment, these may be replaced by the formation of salt bridges with ions. Thus, WCOR410 proteins may prevent the precipitation or crystallization of ions that are approaching the limits of their solubilities. In addition, because the interbilayer repulsion due to electrostatic forces is decreased by charge screening from the higher ionic concentration, this can lead to interaction between bilayers and the induction of the changes in membrane ultrastructure enumerated above. Ion binding by the WCOR410 protein may alleviate this interaction. A function in sequestering phosphate ions has been proposed for the conserved 11-mer repeated motif that is present in members

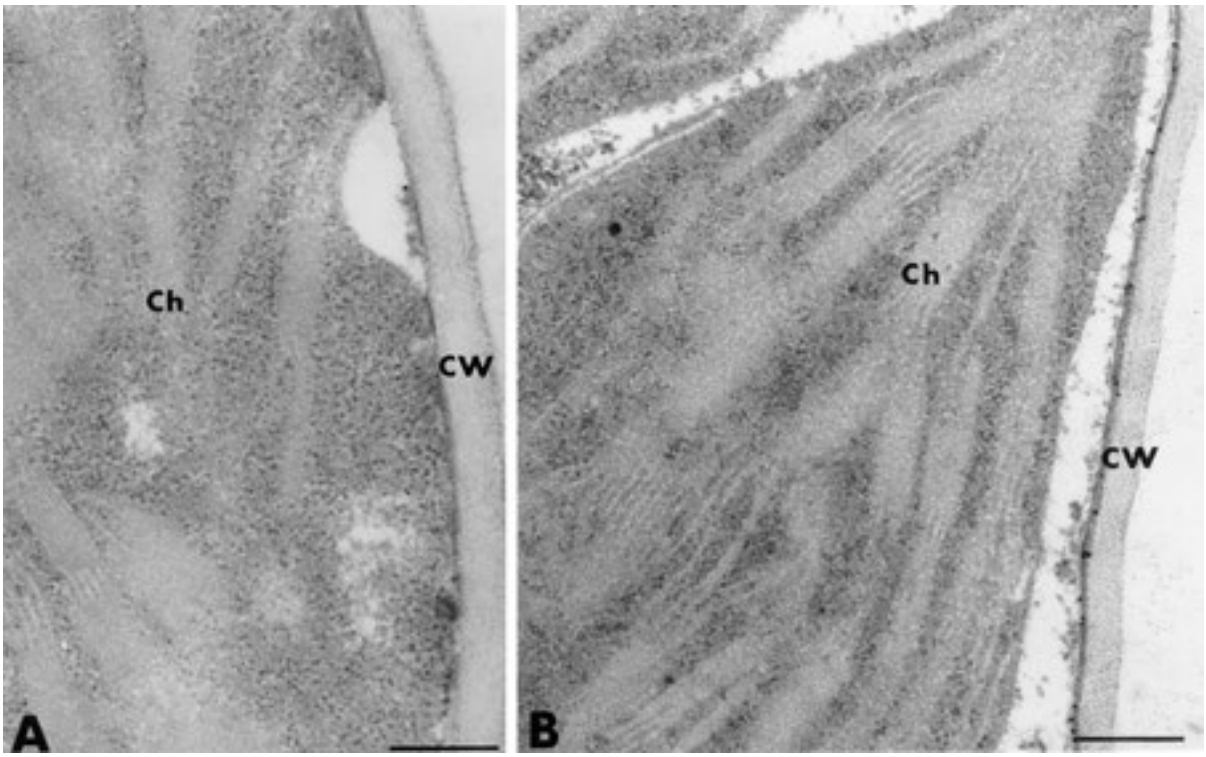

Figure 9. Electron Microscopy of Transverse Leaf Sections from Wheat Seedlings Incubated with the Anti-WCOR410 Antibody.

(A) Nonacclimated leaf. Bar = 0.25 μm.
(B) Cold-acclimated leaf. Bar = 0.25 μm.
Ch, chloroplast; CW, cell wall.

Table 1. Density of the Immunogold Labeling Obtained with the Anti-WCOR410 Antibody over Root, Crown, Stem, and Leaf Tissues of Cold-Acclimated Wheat Plants

	Gold Particles per $\mu m^2 \pm SD$[a]		
Plant Organ	Plasma Membrane	Intercellular Space	Wall Junction
Root	35.6 ± 5.4	6.5 ± 3.2	5.4 ± 2.0
Crown	48.2 ± 6.3	25.8 ± 4.3	16.3 ± 5.2
Stem	42.8 ± 9.5	8.4 ± 5.5	10.5 ± 2.4
Leaf	22.5 ± 6.4	3.4 ± 1.3	4.3 ± 1.5

[a] Densities were determined by counting the number of gold particles over specified areas on 25 micrographs for each organ.

of the D-7 family of LEA proteins (Dure, 1993). Acidic dehydrins do not contain this motif or any other motif that would show a strict conservation and be repeated through the whole protein. However, the content of charged residues in WCOR410 is equivalent to the percentage ranging from 33 to 43% in the 11-mer repeat domains of these proteins (Dure, 1993). If WCOR410 proteins can be induced to crystallize, studies using x-ray diffraction may show pockets for binding specific ions.

In summary, our results show that during cold acclimation, the acidic dehydrin WCOR410 accumulates to high levels in the vicinity of the plasma membrane of cells in the vascular transition area. The abundance and subcellular localization of members of this protein family suggest that they are involved in the cryoprotection of the plasma membrane during freeze-induced dehydration.

METHODS

Plant Material, Growth Conditions, and Stress Conditions

Wheat (*Triticum aestivum*) cultivars Glenlea and Fredrick were germinated in water-saturated vermiculite for 7 days. Cool-white fluorescent and incandescent lighting were combined to provide an irradiance of 250 μmol m^{-2} sec^{-1}. The temperature was maintained at 20 ± 1°C with a 15-hr photoperiod under a relative humidity of 70 ± 5%. At the end of this period, control plants were maintained under the same conditions of light and temperature. Cold acclimation was performed by subjecting germinated seedlings to a temperature of 4 ± 1°C with a 12-hr photoperiod for different periods of time as specified for each experiment. Seedlings were watered daily with a nutrient solution (20:20:20; N:P:K). Growth conditions and LT_{50} (temperature at which 50% of the plants are killed) determinations for the cultivars and the Chinese Spring/Cheyenne (CS/CNN) substitution series were the same as those described by Limin and Fowler (1988). Experimental design was a randomized complete block design with four replicates for the CS/CNN series and five replicates for the cultivars. After 49 days of cold acclimation, the LT_{50} values were as follows: spring cultivars Glenlea, −5.5°C; Manitou, −6.2°C; Oslo, −6.6°C; CS, −9.4°C; and winter cultivars Fredrick, −15.6°C; Besostoya, −16.4°C; Pioneer 2548, −16.8°C; CNN, −19.0°C; Norstar (NOR), −21.2°C.

Heat shock was performed by incubating seedlings at 40°C for 3 hr. Wounding stress was induced by cutting the seedlings into 1-cm segments and placing them in water at 20°C for 18 hr. Salt-stressed plants were obtained by incubating seedlings for 24 hr in a nutrient solution containing 500 mM NaCl. Water stress was induced by removing seedlings from vermiculite and leaving them at 20°C without water for 20 hr. Abscisic acid (ABA)–treated plants were obtained by transferring seedlings for 18 hr to a nutrient solution containing 10^{-4} M ABA and concomitantly applying a foliar spray containing 10^{-4} M ABA in 0.02% (v/v) Tween 20. Polyethylene glycol–induced osmotic stress was performed by removing seedlings from vermiculite and transferring them to a solution containing 40% (w/v) of polyethylene glycol (average molecular weight of 8000) for 48 hr.

Genetic Analyses

The identification of the first *Wcor410* sequence (GenBank accession number L29152) has been described previously (Danyluk et al., 1994). Further screening of the cDNA library has allowed us to isolate two other members of the *Wcor410* family. The complete DNA sequence of *Wcor410b* and *Wcor410c* was determined on both strands, as described previously (Houde et al., 1992a). To map these genes to a specific chromosome, we used several diploid and tetraploid wheats and the ditelocentric (DT) series of wheat cultivar CS CSDT (provided by the U.S. Department of Agriculture E.R. Sears collection, Aberdeen, ID). In this series, all chromosomes are present, except that in each line, one chromosome pair is represented by only the telocentric chromosomes of one arm. The presence of the long or short arm of the chromosome is indicated by L or S, respectively. Isolation of genomic DNA and DNA gel blot analysis was as described previously (Limin et al., 1997). To study the regulation of WCOR410 accumulation, we used the CS/CNN wheat chromosome substitution series of 21 lines, in which an individual pair of homologous chromosomes of CS is replaced by the corresponding homologous chromosome pair of the more tolerant winter wheat CNN (kindly provided by R. Morris, University of Nebraska, Lincoln).

Production and Purification of WCOR410 Antibodies

The open reading frame of *Wcor410* was subcloned in the pET22 vector (Novagen, Madison, WI) and expressed as a histidine-tagged fusion product in *Escherichia coli.* The WCOR410 protein was purified by affinity chromatography on a HisBind resin (Novagen) and then separated on a 10% SDS–polyacrylamide gel. The expressed protein was excised and electroeluted for 3 hr. Immune serum from rabbits injected with the WCOR410 protein was found to cross-react partially with the WCS120 protein family. To eliminate cross-reacting antibodies, we prepared an affinity column containing WCS120 proteins (produced in a bacterial system) by coupling these proteins with Affi-gel-10 beads (Bio-Rad), as described previously (Houde et al., 1995). The flowthrough containing the anti-WCOR410 antibodies (depleted of anti-WCS120 antibodies) was used for all further experiments unless stated otherwise. To confirm the specificity of this antibody preparation, the anti-WCOR410 antibody was further purified with an affinity column containing pure WCOR410 protein. Coupling

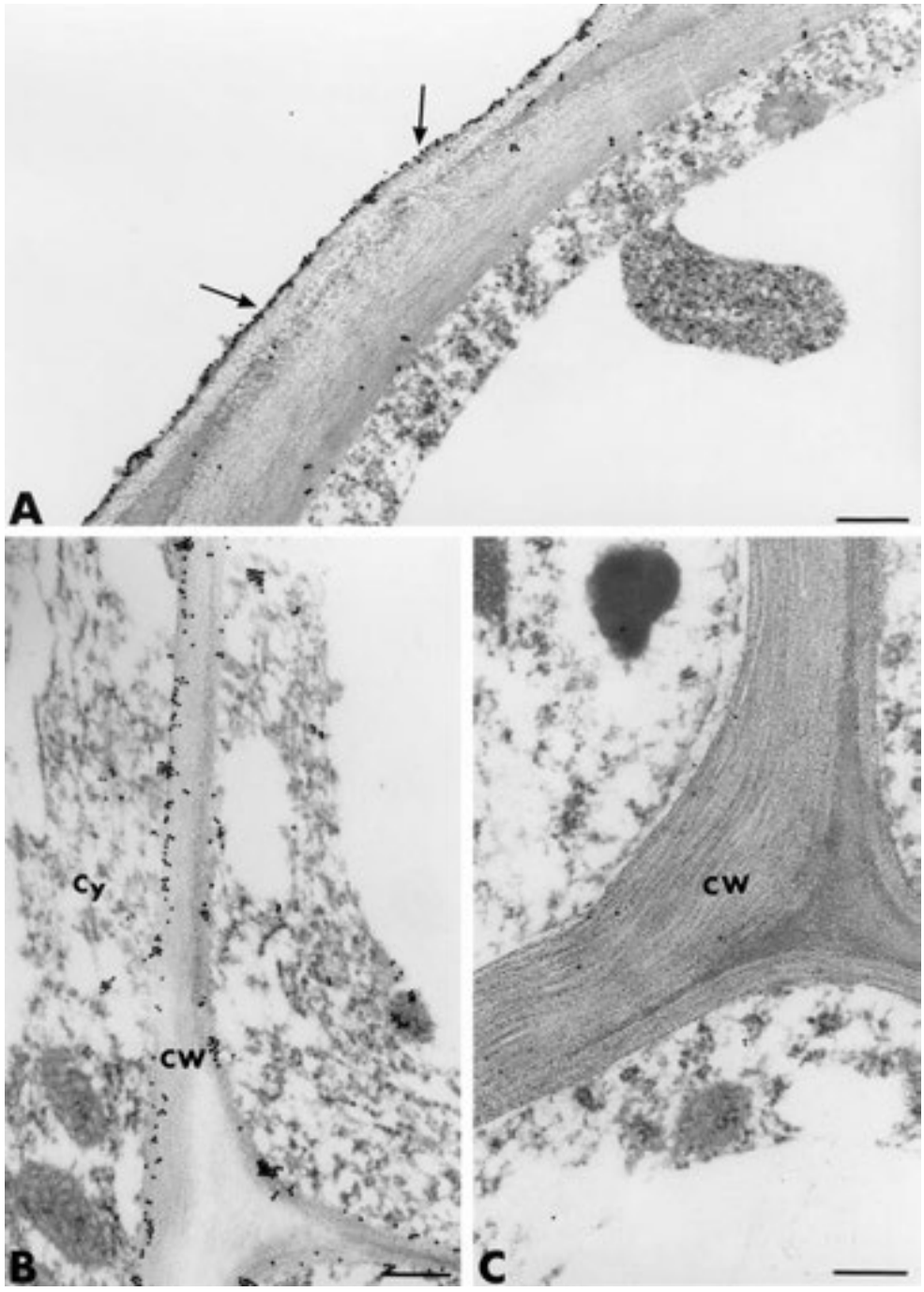

Figure 10. Electron Microscopy of Transverse Crown Sections from Wheat Seedlings Incubated with the Affinity-Purified Anti-WCOR410 Antibody.

(A) Cold-acclimated crown. Bar = 0.25 μm.
(B) Cold-acclimated crown. Bar = 0.25 μm.
(C) Cold-acclimated crown incubated with the affinity-purified antibody to which the WCOR410 protein was previously added. Bar = 0.25 μm.
CW, cell wall; Cy, cytosol; the arrows in **(A)** indicate the dense labeling on the plasma membrane.

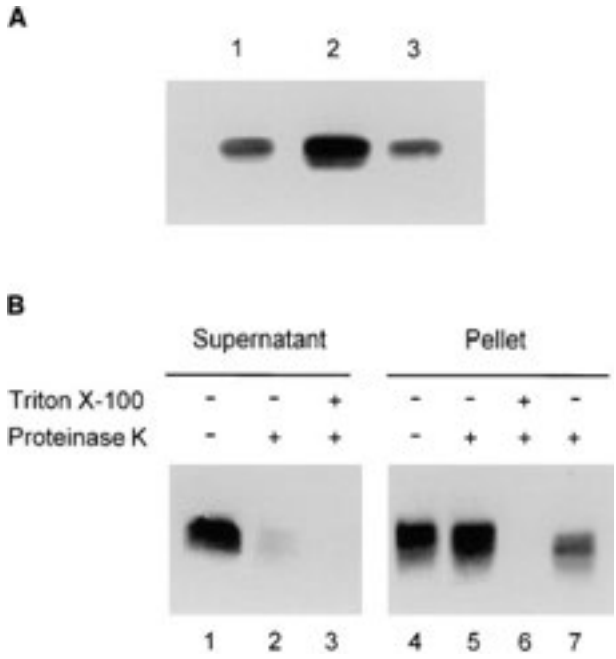

Figure 11. Biochemical Fractionation of WCOR410 Proteins and Their Susceptibility to Protease.

(A) Immunoblot analysis of total proteins (10 μg) from cellular fractions prepared from aerial tissues of cultivar Fredrick that were cold acclimated for 2 weeks. Lane 1 contains the 156,000*g* crude microsomal pellet; lane 2, membrane vesicles from upper phase; and lane 3, membrane vesicles from lower phase.
(B) Proteinase K digestion of the 156,000*g* supernatant (lanes 1 to 3) and the crude microsomal pellet (lanes 4 to 7). Microsomal pellets were resuspended in 0.25 M sucrose and 10 mM KH_2PO_4, pH 7.8 (lanes 4 to 6), or in 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA (lane 7). (+), treated with proteinase K or Triton X-100; (−), untreated.

at a protein concentration of 0.5 mg/mL and purification of the antibodies were as described previously (Houde et al., 1995).

Protein Extraction and Immunoblot Analysis

Total proteins were extracted from frozen plant tissue, according to Crosatti et al. (1995). Proteins, solubilized in sample buffer (60 mM Tris-HCl, pH 6.8, 10% [w/v] glycerol, and 2% [w/v] SDS), were quantitated using the Bio-Rad *Dc* Protein Assay. Equal amounts of protein were separated on a 10% SDS–polyacrylamide gel and transferred electrophoretically for 1 hr to a 0.45-μm nitrocellulose membrane (Hybond-C; Amersham) without SDS in the transfer buffer. The membranes were blocked in a 4% (w/v) solution of reconstituted skimmed milk powder prepared in PBS containing 0.2% (v/v) Tween 20 and then probed with the anti-WCOR410 antibody at a 1:10,000 dilution for 1 hr. After washing with PBS–Tween 20, the proteins recognized by the primary antibody were revealed with a horseradish peroxidase–coupled anti-rabbit IgG (Jackson Immunoresearch Laboratories, West Grove, PA) at a 1:20,000 dilution. The complexes were visualized using the enhanced chemiluminescent detection system (Amersham) and X-OMAT-RP film (Eastman Kodak, Rochester, NY).

To estimate the abundance of WCOR410 proteins in wheat tissue, we separated different amounts of the recombinant WCOR410 protein (quantitated with the Bio-Rad *Dc* Protein Assay and used as standards) on an SDS–polyacrylamide gel along with total leaf proteins from cultivar Fredrick and then transferred to a nitrocellulose membrane. Immunoblot analysis was as described above. Levels of WCOR410 proteins were then quantitated by densitometry, using a personal densitometer SI and the ImageQuaNT 4.2 software (Molecular Dynamics, Sunnyvale, CA).

Tissue Processing for Electron Microscopy

Root, crown, stem, and leaf samples from cold-acclimated (21 days) and nonacclimated wheat cultivar Fredrick were cut into 1-mm pieces with a razor blade and immediately transferred to 3% (v/v) glutaraldehyde buffered with 0.1 M sodium cacodylate buffer, pH 7.2, for 2 hr at room temperature and then overnight at 4°C. Samples were dehydrated in a graded ethanol series and embedded in Epon 812 (J.B. EM Services Inc., Pointe-Claire, Canada). Ultrathin sections (60 to 90 nm) were collected on Formvar-coated nickel grids and either stained with uranyl acetate and lead citrate or processed for gold labeling. Approximately 15 sections per sample were examined under an electron microscope (model 1200 EX; JEOL, Tokyo, Japan) operating at an accelerating voltage of 80 kV.

Immunogold Labeling

The anti-WCOR410 antibodies were used for studying the subcellular localization of the WCOR410 protein in different organs. Ultrathin sections of root, crown, stem, or leaf samples were first incubated on a drop of 0.01 M PBS, pH 7.4, containing 0.2% (w/v) ovalbumin (PBS–ovalbumin) for 5 min at room temperature. Sections were then transferred to a drop of normal goat serum diluted 1:10 in PBS–ovalbumin for 1 hr at room temperature and incubated on the primary antibody at a 1:100 dilution in PBS–ovalbumin for 2 hr at 37°C. In experiments using the affinity-purified anti-WCOR410 antibody, the primary antibody was incubated at a 1:10 dilution in PBS–ovalbumin. After washing with Tris-HCl, pH 8.2, containing 0.5 M NaCl and 1% (w/v) BSA, sections were incubated on a drop of colloidal gold (15 nm)–conjugated goat antiserum to rabbit immunoglobulins diluted 1:10 in the rinsing buffer for 30 min at room temperature. Grids carrying sections were finally washed with PBS, pH 7.4, thoroughly rinsed with distilled water, and counterstained with uranyl acetate and lead citrate. The specificity of the labeling was assessed by the following control tests: (1) incubation with preimmune serum instead of primary antiserum; (2) incubation with gold-conjugated antiserum to rabbit immunoglobulins only, the primary antibody step being omitted; and (3) incubation with the primary antibody, which was previously adsorbed overnight at 4°C with an excess of the corresponding antigen (WCOR410 proteins).

Quantification of Labeling

The density of labeling obtained with the anti-WCOR410 antibody over specified areas in root, crown, stem, and leaf tissues of cold-acclimated wheat plants was determined by counting the number of

gold particles per square micron. Area determinations were conducted by the point-counting method established by Weibel (1969) using negatives of electron micrographs projected on a lattice. The amount of labeling over specified wall areas (Sa) was estimated by counting the number of gold particles (Ni) on a photographic enlargement. Density of labeling (Ns) was calculated as follows: Ns = Ni/Sa, where Ns represents the number of gold particles per unit surface.

Biochemical Fractionation and Isolation of Plasma Membranes

Wheat leaves were ground in a mortar with the extraction medium described by Uemura and Yoshida (1983), except that BSA was omitted. The homogenate was squeezed through two layers of Miracloth (Calbiochem) and then subjected to differential centrifugations successively at 1500*g* for 7 min, 14,000*g* for 15 min, and 156,000*g* for 25 min. The crude microsomal pellet was washed once (Uemura and Yoshida, 1983) and partitioned in an aqueous two-phase polymer system (Zhou et al., 1994). After phase partitioning, the upper phase enriched in plasma membranes and the lower phase were diluted with 0.5 M sorbitol in a buffer containing 5 mM Mops-KOH, pH 7.3, 1 mM EDTA, 0.1 mM phenylmethylsulfonyl fluoride, 10 mM KCl, and 2 mM DTT. The suspension was centrifuged at 156,000*g* for 25 min to sediment membrane vesicles. The resulting membrane pellets were resuspended in the same buffer. Enrichment of plasma membrane vesicles was determined by measuring the activity of the vanadate-sensitive ATPase as the marker enzyme (Uemura et al., 1995). Proteins from the different fractions were extracted by the phenol extraction procedure described by Hurkman and Tanaka (1986). Protein estimation, electrophoretic separation, and protein gel blot analysis using the anti-WCOR410 antibody were as described above.

Treatments with Sodium Carbonate and Proteinase K

The microsomal fraction was treated with sodium carbonate, as described by Fujiki et al. (1982). WCOR410 proteins in the resulting supernatant and membrane pellet were detected by protein gel blot analysis using the anti-WCOR410 antibody. For the protease treatments, the crude microsomal pellets were resuspended in 0.25 M sucrose and 10 mM KH_2PO_4, pH 7.8, or in 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA (low-osmoticum buffer). The 156,000*g* supernatant and the microsomal pellets were adjusted to 200 μg/mL proteinase K and 1% Triton X-100, as indicated. After incubation for 1 hr on ice, proteinase activity was blocked by the addition of phenylmethylsulfonyl fluoride to a final concentration of 2 mM. Samples were separated on an SDS–polyacrylamide gel, transferred to nitrocellulose, and immunoblotted with the anti-WCOR410 antibody.

ACKNOWLEDGMENTS

This work was supported by a Natural Sciences and Engineering Research Council of Canada (NSERC) research grant (No. A7199) to F.S. The genetic analysis was supported by an NSERC strategic grant (No. STR0149258) to F.S. and B.F.

Received November 12, 1997; accepted January 27, 1998.

REFERENCES

Ceccardi, T.L., Meyer, N.C., and Close, T.J. (1994). Purification of a maize dehydrin. Protein Expression Purif. **5,** 266–269.

Close, T.J. (1996). Dehydrins: Emergence of a biochemical role of a family of plant dehydration proteins. Physiol. Plant. **97,** 795–803.

Crosatti, C., Soncini, C., Stanca, A.M., and Cattivelli, L. (1995). The accumulation of a cold-regulated chloroplastic protein is light-dependent. Planta **196,** 458–463.

Crowe, J.H., Crowe, L.M., Leslie, S.B., and Fisk, E. (1993). Mechanisms of stabilization of dry biomolecules in anhydrobiotic organisms. In Plant Responses to Cellular Dehydration during Environmental Stress, T.J. Close and E.A. Bray, eds (Rockville, MD: American Society of Plant Physiologists), pp. 11–20.

Danyluk, J., Houde, M., Rassart, É., and Sarhan, F. (1994). Differential expression of a gene encoding an acidic dehydrin in chilling sensitive and freezing tolerant Gramineae species. FEBS Lett. **344,** 20–24.

Dure III, L. (1993). A repeating 11-mer amino acid motif and plant desiccation. Plant J. **3,** 363–369.

Fowler, D.B., Limin, A.E., Wang, S.-Y., and Ward, R.W. (1996). Relationship between low-temperature tolerance and vernalization response in wheat and rye. Can. J. Plant Sci. **76,** 32–42.

Fujiki, Y., Hubbard, A.L., Fowler, S., and Lazarow, P.B. (1982). Isolation of intracellular membranes by means of sodium carbonate treatment: Application to endoplasmic reticulum. J. Cell Biol. **93,** 97–102.

Guy, C.L. (1990). Cold acclimation and freezing stress tolerance: Role of protein metabolism. Annu. Rev. Plant Physiol. Plant Mol. Biol. **41,** 187–223.

Houde, M., Danyluk, J., Laliberté, J.-F., Rassart, É., Dhindsa, R.S., and Sarhan, F. (1992a). Cloning, characterization, and expression of a cDNA encoding a 50-kilodalton protein specifically induced by cold acclimation in wheat. Plant Physiol. **99,** 1381–1387.

Houde, M., Dhindsa, R.S., and Sarhan, F. (1992b). A molecular marker to select for freezing tolerance in Gramineae. Mol. Gen. Genet. **234,** 43–48.

Houde, M., Daniel, C., Lachapelle, M., Allard, F., Laliberté, S., and Sarhan, F. (1995). Immunolocalization of freezing-tolerance–associated proteins in the cytoplasm and nucleoplasm of wheat crown tissues. Plant J. **8,** 583–593.

Hughes, M.A., and Dunn, M.A. (1990). The effect of temperature on plant growth and development. Biotechnol. Genet. Eng. Rev. **8,** 161–188.

Hughes, M.A., and Dunn, M.A. (1996). The molecular biology of plant acclimation to low temperature. J. Exp. Bot. **47,** 291–305.

Hurkman, W.J., and Tanaka, C.K. (1986). Solubilization of plant membrane proteins for analysis by two-dimensional gel electrophoresis. Plant Physiol. **81,** 802–806.

Israelachvili, J., and Wennerström, H. (1996). Role of hydration and water structure in biological and colloidal interactions. Nature **379,** 219–225.

Limin, A.E., and Fowler, D.B. (1988). Cold hardiness expression in interspecific hybrids and amphiploids of the Triticeae. Genome **30,** 361–365.

Limin, A.E., Danyluk, J., Chauvin, L.-P., Fowler, D.B., and Sarhan, F. (1997). Chromosome mapping of low-temperature induced *Wcs120* family genes and regulation of cold-tolerance expression in wheat. Mol. Gen. Genet. **253,** 720–727.

Pearce, R.S. (1988). Extracellular ice and cell shape in frost-stressed cereal leaves: A low temperature scanning-electron-microscopy study. Planta **175,** 313–324.

Pearce, R.S., and Ashworth, E.N. (1992). Cell shape and localization of ice in leaves of overwintering wheat during frost stress in the field. Planta **188,** 324–331.

Raynal, P., and Pollard, H.B. (1994). Annexins: The problem of assessing the biological role for a gene family of multifunctional calcium- and phospholipid-binding proteins. Biochim. Biophys. Acta **1197,** 63–93.

Steponkus, P.L., and Lynch, D.V. (1989). Freeze/thaw–induced destabilization of the plasma membrane and the effects of cold acclimation. J. Bioenerg. Biomembr. **21,** 21–41.

Steponkus, P.L., Uemura, M., and Webb, M.S. (1993). Membrane destabilization during freeze-induced dehydration. In Plant Responses to Cellular Dehydration during Environmental Stress, T.J. Close and E.A. Bray, eds (Rockville, MD: American Society of Plant Physiologists), pp. 37–47.

Swairjo, M.A., and Seaton, B.A. (1994). Annexin structure and membrane interactions: A molecular perspective. Annu. Rev. Biophys. Biomol. Struct. **23,** 193–213.

Tanino, K.K., and McKersie, B.D. (1984). Injury within the crown of winter wheat seedlings after freezing and icing stress. Can. J. Bot. **63,** 432–436.

Thomashow, M.F. (1990). Molecular genetics of cold acclimation in higher plants. Adv. Genet. **28,** 99–131.

Uemura, M., and Yoshida, S. (1983). Isolation and identification of plasma membrane from light-grown winter rye seedlings (*Secale cereale* L. cv Puma). Plant Physiol. **73,** 586–597.

Uemura, M., Raymond, A.J., and Steponkus, P.L. (1995). Cold acclimation of *Arabidopsis thaliana*: Effect on plasma membrane lipid composition and freeze-induced lesions. Plant Physiol. **109,** 15–30.

Webb, M.S., Gilmour, S.J., Thomashow, M.F., and Steponkus, P.L. (1996). Effects of COR6.6 and COR15am polypeptides encoded by *COR* (Cold-Regulated) genes of *Arabidopsis thaliana* on dehydration-induced phase transitions of phospholipid membranes. Plant Physiol. **111,** 301–312.

Weibel, E.R. (1969). Stereological principles for morphometry in electron microscope cytology. Int. Rev. Cytol. **26,** 235–244.

Zhou, B.L., Arakawa, K., Fujikawa, S., and Yoshida, S. (1994). Cold-induced alterations in plasma membrane proteins that are specifically related to the development of freezing tolerance in cold-hardy winter wheat. Plant Cell Physiol. **35,** 175–182.