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Immunolocalization of freezing-tolerance-associated proteins in the cytoplasm and nucleoplasm of wheat crown tissues

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Summary

A protein family associated with the development of freezing tolerance in wheat has been identified. This protein family is Gramineae-specific and coordinately regulated by low temperature. Antibodies directed against the 50 kDa (WCS120) protein recognize at least 5 members of this family. Using these antibodies, the cellular content and location of this protein family was determined in cold-acclimated wheat seedlings. Western analyses of subcellular fractions indicated the presence of all members of the family in the cytosolic and purified nuclear fractions. These proteins accumulated to 0.9% of soluble proteins after 21 days of cold acclimation in winter wheat. This represents a cellular concentration of 1.34 μM. Immunohistochemical localization showed that these proteins are highly expressed in the vascular transition zone. No detectable expression was found in mature xylem, in the shoot apical meristem or lateral root primordia. This differential tissue expression suggests that the sensitive cells near the regions where water tends to freeze first require a higher amount of these proteins. This observation is consistent with the fact that regrowth after freezing stress is highly dependent on the viability of this region of the crown. Electron microscopy analysis using immunogold labelling showed that these proteins are present in the cytoplasm and in the nucleoplasm. They are not found in cell walls or other organelles. *In vitro* cryoprotective assays indicated that the WCS120 protein (PD_{50} of 10 μg ml^{-1} or 0.2 μM) are as effective as BSA and sucrose (at 250 mM) against freezing denaturation of lactate dehydrogenase. These results suggest that this protein family may be involved in a general mechanism of protection in the soluble fraction of the cell. Their presence in the nucleoplasm may also suggest a possible protective function of the transcriptional machinery. The high hydrophilicity, the abundance and stability of these proteins to boiling suggest that they may provide a particular micro-environment needed for cell survival in the sensitive vascular transition zone during freezing stress.

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Introduction

Plants avoid freezing damage by using two strategies: freezing avoidance (supercooling) and freezing tolerance (restriction of ice crystal formation to the intercellular spaces). Calorimetric measurements have shown that water freezes in several stages during the lowering of the external temperature (Franks, 1985). The major exotherm represents the freezing of extracellular water whereas the minor exotherm corresponds to the freezing of water in the living cell and is usually associated with plant death. The freezing of intercellular water increases water migration to the growing ice crystals causing the loss of vital intracellular water from the protoplasm (Guy, 1990). The degree of cellular dehydration increases with decreasing temperature to a point where dehydration and/or ice crystal formation is sufficient to kill the cells. In wheat, ice nucleation begins near the leaf surface and the first signs of ice formation appear below the epidermis at −2.4°C (Pearce and Ashworth, 1992). In rye, a similar result was observed at a slightly lower temperature and ice formation was more extensive in both sub-epidermal and perivascular tissues (Pearce, 1988). Cells bordering these regions are thus more likely to be affected by dehydration and must be better prepared to tolerate this stress.

We have identified several low-temperature responsive cDNAs from winter wheat (Chauvin *et al.*, 1994, 1993; Danyluk *et al.*, 1994; Houde *et al.*, 1992a; Ouellet *et al.*, 1993). Several of these genes encode proteins belonging to the same family and were found to be highly expressed in the hardy varieties during cold acclimation (Chauvin *et al.*, 1994; Houde *et al.*, 1992a; Ouellet *et al.*, 1993). Antibodies directed against the 50 kDa protein, encoded by the *Wcs120* gene, allowed us to detect several cross-reactive proteins which are highly abundant and coordinately regulated during cold acclimation. Their accumulation was specific to gramineae species and shown to be closely correlated with the development of freezing tolerance of each species (Houde *et al.*, 1992b). Although their precise function is still unknown, their hydrophilicity, stability upon boiling, high amount of charged amino acids, and structural similarity with the late embryogenesis abundant (LEA) group of proteins suggest that they may be associated with the protection of cells against desiccation caused by

freezing of intercellular water. It is known that winter survival is dependent on the capacity of the crown meristematic tissue to survive winter. Our previous results showed that the crown tissues, particularly the basal region, accumulate a large amount of these proteins during cold acclimation (Houde *et al.*, 1992b). Thus, we have focused our efforts to localize these freezing-tolerance-associated proteins in the crown region of cold-acclimated wheat seedlings. Using immunocytochemical methods, we have found that the highest level of expression of these proteins is found in the vascular regions with lower levels of expression in the parenchyma and epidermal cells. No expression was detected in the meristems or in mature xylem. Immunogold labelling indicated that the WCS120 protein family is located in both the nucleus and cytoplasm of cold-acclimated tissues. The significance of these observations in relation to the possible function of these proteins in freezing tolerance (FT) is discussed.

Results

Characterization of the anti-WCS120 antibody

The purified 50 kDa protein produced in *Escherichia coli* was previously shown to cross-react strongly with the anti-WCS120 serum (Houde *et al.*, 1992b). This protein was used to purify the polyclonal antibody by affinity chromatography. Immunoblot analysis using purified antibodies showed that they cross-react with a family of five proteins (40, 50, 66, 180, 200 kDa) induced during cold acclimation of wheat (Figure 1, lane 4). To test for the specificity of the reaction, the purified 50 kDa protein produced in the bacteria was added as a competitor when the immunoblot was incubated with the anti-WCS120 antibody. The results in Figure 1 (lanes 5–8) show that an incubation with 1 μg ml^{-1} of WCS120 was sufficient to completely block the cross-reaction with all members in the family. When a low concentration of competing WCS120 protein was added (lanes 6 and 7), the cross-reaction with the five members of the family was reduced in a similar manner suggesting that they are closely related. Sequence analysis of the WCS120 protein revealed the presence of two repeated domains, a glycine-rich domain repeated 11 times and a lysine-rich domain repeated six times (Houde *et al.*, 1992a). The WCS66 protein is 94% homologous at the protein level with WCS120 and contains 14 glycine-rich domains and seven lysine-rich domains (Chauvin *et al.*, 1994). Partial amino acid sequence and Southern analysis of the *Wcs200* cDNA using probes specific to these two domains have shown that the lysine-rich and glycine-rich domains are also present several times within the protein (Ouellet *et al.*, 1993). These results are summarized in Table 1. The strong consensus between the repeated domains of the different

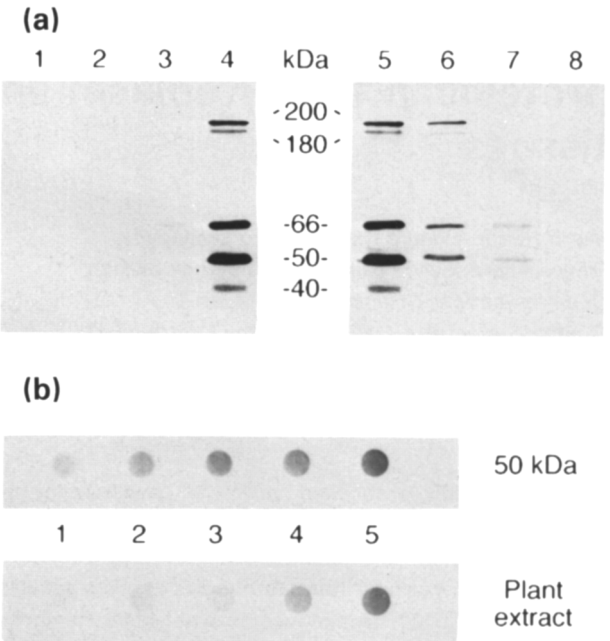


Figure 1. Specificity of the anti-WCS120 antibody and quantitation of immunoreactive proteins.
(a) Western analysis of soluble proteins from non-acclimated (lanes 1 and 3) and cold-acclimated (lanes 2 and 4) wheat seedlings. Proteins were separated by SDS–PAGE, transferred to nitrocellulose and incubated with pre-immune serum (lanes 1 and 2) and with the anti-WCS120 antibody (lanes 3 and 4). Competition of the antiserum by the addition of varying concentrations of purified WCS120 protein is shown in lanes 5–8, where 0 ng, 10 ng, 100 ng and 1 μg ml^{-1} were added, respectively.
(b) Dot blot quantitation of WCS120-related proteins in the soluble extract of cold-acclimated wheat seedlings. Different dilutions (dot 1, 60 nl; 2, 80 nl; 3, 100 nl; 4, 200 nl; 5, 500 nl; diluted to 250 μl) of boiling soluble proteins were blotted on to a nitrocellulose membrane along with several concentrations of the purified WCS120 protein as internal standard (dot 1, 4 ng; 2, 6 ng; 3, 8 ng; 4, 10 ng; and 5, 20 ng of purified WCS120 protein). The membrane was then processed for immunoblot analysis using the anti-WCS120 antibody. The quantity of immunoreactive proteins was determined through scanning densitometry and by comparison with purified protein standards as described in Experimental procedures.

proteins may explain the cross-reactivity of the anti-WCS120 antibody.

Densitometry quantitation using the 50 kDa protein produced in recombinant bacteria as standard allowed us to evaluate the absolute and relative abundance of the 40, 50, 66, 180, and 200 kDa proteins (Figure 1b, Table 2). Among the five members of the family, the 50 and 66 kDa proteins are present in higher amounts compared with the other three (64% of the total). Using dot blot quantitation, we estimated that after 21 days of cold acclimation, winter wheat seedlings (cv. Fredrick) had accumulated a total of 72 μg g^{-1} fresh weight of these proteins. This represents 0.9% of the soluble proteins and a cellular concentration of 1.34 μM (Table 2). Considering that only specific cells express the WCS120 protein family, the actual concentration should be much higher in these cells.

Table 1. Conserved lysine-rich and glycine-rich repeated domains in three members of the WCS120 protein family

cDNA clone	Lysine-rich repeat	Glycine-rich repeat
WCS120[a] and *WCS66*[b] (consensus)	KGVMENIKEKLPGGHGDHQQ	TGGTYGQQGHTGTT
WCS200[c] tryptic digests		
12 kDa	KGVKENINDKLPDGRGsdHQTt	Several copies of the glycine-rich repeat as determined by Southern analysis[c]
16 kDa	KGVmENINdklpxgxs	
24 kDa	KGVMENINDKLSGGHGDHHQT	

[a]From Houde *et al.* (1992a); [b]from Chauvin *et al.* (1994); [c]from Ouellet *et al.* (1993).

Table 2. General characteristics of the different members of the WCS120 protein family

Protein	MW[a] (kDa)	Isoelectric point[a]	Abundance[b]		Cellular concentration[b]	
			% total family	% soluble proteins	(μg g^{-1} fresh weight)	(μM (85% water content))
WCS200	200	6.5	17.8	0.16	12.8	0.075
WCS180	180	6.5	2.8	0.025	2.0	0.013
WCS66	66	7.1	34.0	0.31	24.5	0.44
WCS120	50	7.2	34.6	0.31	24.9	0.58
WCS40	40	7.2	10.8	0.10	7.8	0.23
Total			100	0.905	72	1.34

[a]As determined by two-dimensional electrophoresis (Houde *et al.*, 1992b).
[b]Values represent the mean of a triplicate determination from at least three separate experiments. Standard error did not exceed 10%.

Cryoprotection of lactate dehydrogenase (LDH)

A standard *in vitro* cryoprotective assay was used to evaluate the ability of the WCS120 protein to protect LDH against freezing inactivation. Two other substances recognized as being effective cryoprotectants and protein stabilizers were used for comparison (BSA and sucrose). We have determined a PD_{50} of 10 μg ml^{-1} for WCS120 compared with PD_{50} values of 10 and 500 μg ml^{-1} for BSA and RNase A, respectively. In order to obtain an equivalent protection of LDH, a concentration of 250 mM sucrose was needed. On a molar basis, the WCS120 protein (PD_{50} of 0.2 μM) was thus much more efficient as a cryoprotective agent than sucrose. Similar results were reported for the COR85 protein (low temperature-induced protein with properties similar to WCS120) from spinach where an estimated PD_{50} of 15 μg ml^{-1} was obtained (Kazuoka and Oeda, 1994). This result suggests that the WCS120 protein family may provide a similar protection of freezing-sensitive molecules *in vivo*.

Subcellular fractionation

Biochemical fractionation and Western analyses indicated the presence of the five members of the protein family in purified nuclei (Figure 2, lane 4). The 180 kDa protein becomes more visible with a longer revelation time. We have observed that increasing the homogenization time of the tissues resulted in breaking the nuclear envelope and the loss of the WCS120 protein family from nuclei (result not shown). This indicates that these proteins are not strongly attached to the chromatin and are present in the nucleoplasm. Furthermore, immunoblot analysis of the other cell organelles showed that these proteins are not found in intact chloroplasts or any other cellular fractions, except the soluble (100 000 ***g*** supernatant) and nuclear fractions. Western analysis of boiled and non-boiled extracts gave similar results (not shown). However, the use of boiled extracts improved the quality of migration on SDS–PAGE since the presence of a high DNA concentration caused some distortion.

Immunolocalization of the WCS120 protein family

Since the crown meristematic tissue and, most importantly, the vascular transition zone are of greatest importance for the regrowth of wheat seedlings after harsh winter conditions (Tanino and McKersie, 1984), we have focused our efforts to localize this protein family in the crown tissues.

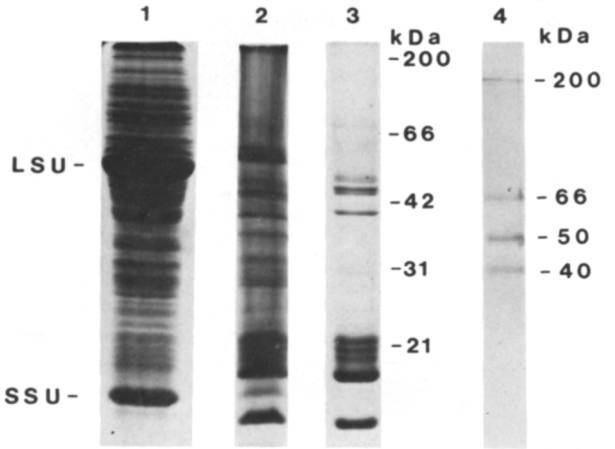


Figure 2. Immunoblot analysis of the WCS120 protein family in purified nuclei.
Different fractions were separated on a 12.5% SDS–PAGE and stained with Coomassie brilliant blue (lanes 1–3). Lane 1, crude extract after filtration through four layers of gauze; lane 2, crude nuclei preparation containing thylakoids (note the absence of stromal proteins such as LSU and SSU of rubisco); lane 3, purified nuclei. In lane 4, the boiling soluble proteins from purified nuclei were separated on a 10% SDS–PAGE, transferred to a nitrocellulose membrane, and processed for immunoblotting using the anti-WCS120 antibody.

Transverse and longitudinal sections of wheat seedlings were incubated with the purified anti-WCS120 antibody. The protein family was not detected in control non-acclimated specimens (Figures 3a and 4a) while in cold-acclimated specimens, the strongest reaction was found in the vascular bundles and bordering parenchyma cells, with weaker expression in epidermal and nearby parenchyma cells (Figures 3b, c and 4b).

The expression of the WCS120 protein family is associated with differentiating leaf traces in the apical region of the seedling as shown in Figures 3(b) and (c). A detailed view of a leaf trace is shown in Figure 3(d) (non-acclimated), and (e) and (f) (cold-acclimated). The section in Figure 3(f) is the same as in Figure 3(e), counterstained to clearly identify the nuclei. The antibody bound strongly to nuclei in the leaf traces, suggesting a significant presence of the WCS120 protein family in this organelle.

In the more differentiated (basal) part of the seedling, there was no apparent reaction in the xylem region of the vascular bundles of the stele, despite the presence of numerous nuclei-containing cells. However, cells of the phloem region (companion cells, and/or sieve tubes, and/or parenchyma cells) and parenchyma cells bordering the xylem region reacted with the antibody (Figure 4e and f). A weaker reaction was obtained in parenchyma cells near tracheary elements running horizontally in the stele. Although the reaction is variable in intensity in different cells, the WCS120 protein family is found in both the nuclei and the cytoplasm. Control experiments using pre-immune serum (Figure 4g) or competition with an excess of purified WCS120 protein (Figure 4h) did not show any reaction. The omission of the primary antibody gave a similar result (not shown).

The WCS120 protein family is not expressed in the shoot apical meristem (Figure 3b and c) nor in lateral root primordia (Figure 4b and d) suggesting that other mechanisms are involved in protecting meristems. The detailed view of a developing lateral root (Figure 4d) shows that in parenchyma cells of the crown cortex, both the nuclei and cytoplasm reacted with the antibody while no reaction occurred within the small meristematic cells of the root primordium.

The ultrastructural localization of the WCS120 protein family was performed on sections prepared from non-acclimated and cold-acclimated crown tissues by using the protein A-gold technique. On tissue sections of non-acclimated cells (Figure 5a and c show representative micrographs), the gold labelling was practically absent. However, in cold-acclimated cells (Figure 5b and d), the labelling density using the anti-WCS120 antibodies was significantly increased in both the nucleus (10.5 $\pm$ 0.7 particles per μm^2, SEM (standard error of the mean), $n=6$) and the cytoplasm (10.8 $\pm$ 1.6 particles per μm^2, SEM, $n=6$) as compared with control cells where gold particles were seldomly found. Some cells did not react significantly above background and these are likely found in parenchyma or meristematic regions that did not react with the anti-WCS120 antibodies (Figures 3 and 4). In cells that were reacting positively, the labelling was particularly intense in the soluble part of the nuclei and cytoplasm (Figure 5b and d). Mitochondria were found to contain few gold particles. However, biochemical fractionation did not reveal the presence of any member of this protein family in the mitochondrial fraction. The cell wall region and cytoplasmic vacuoles were essentially free of labelling. In the nucleus, the areas containing dispersed chromatin were strongly labelled while the nucleolus and areas containing dense chromatin showed little labelling. It is important to note, however, that the analysis of several sections showed that there was no reaction in any cells from non-acclimated tissues. In control experiments, the use of pre-immune serum, omission of the primary antibody or addition of purified WCS120 proteins to the incubation mixture reduced the labelling to the non-acclimated level.

Discussion

We have isolated several genes associated with the development of freezing tolerance in wheat. Several of the highly expressed genes belong to the WCS120 protein family. This protein family was found to be coordinately regulated by low temperature, Gramineae-specific, highly hydrophilic, stable to boiling and have a pI above 6.5 (Chauvin *et al.*, 1994; Houde *et al.*, 1992a, 1992b; Ouellet *et al.*, 1993). They have homologous sequences and share

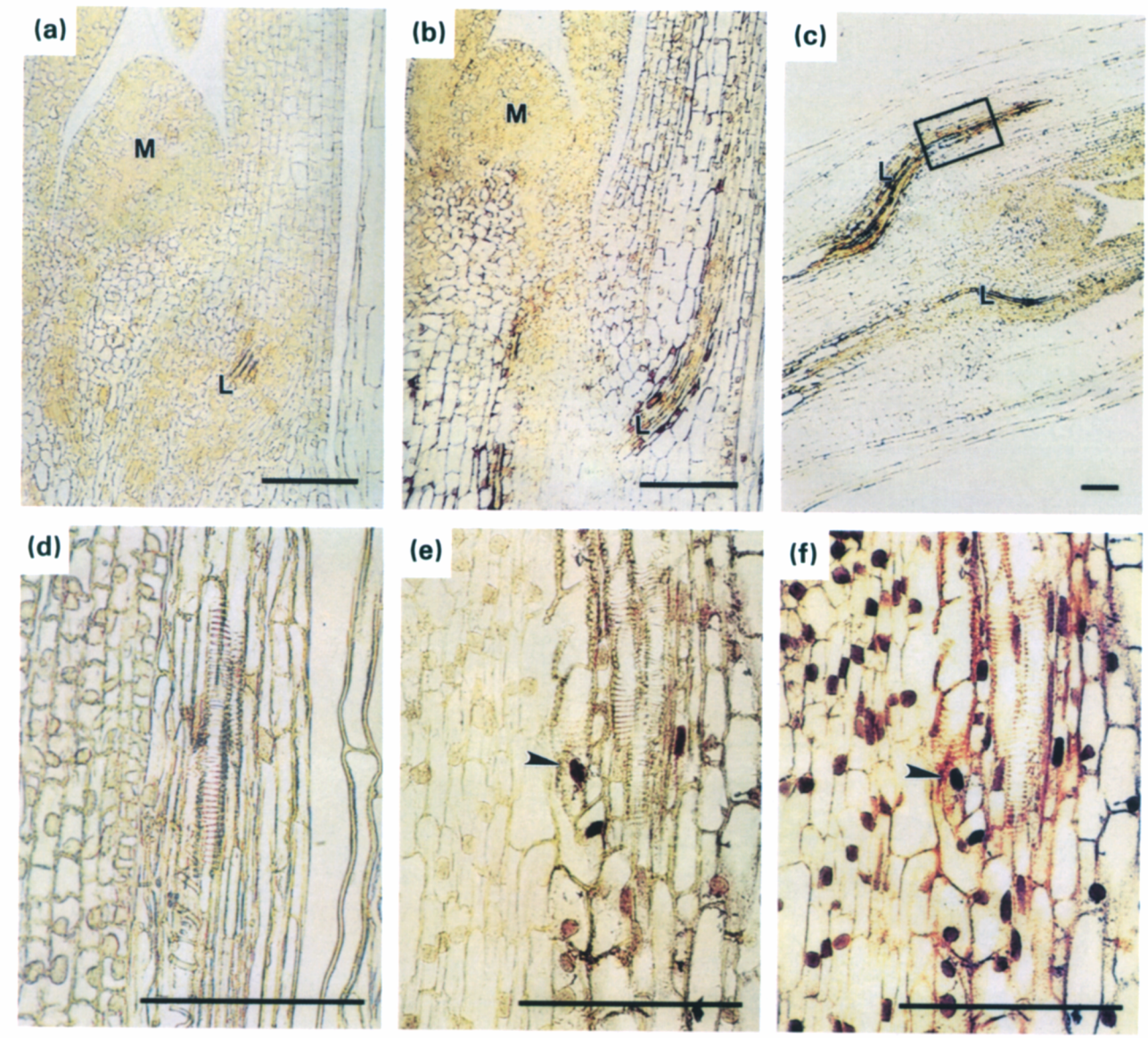
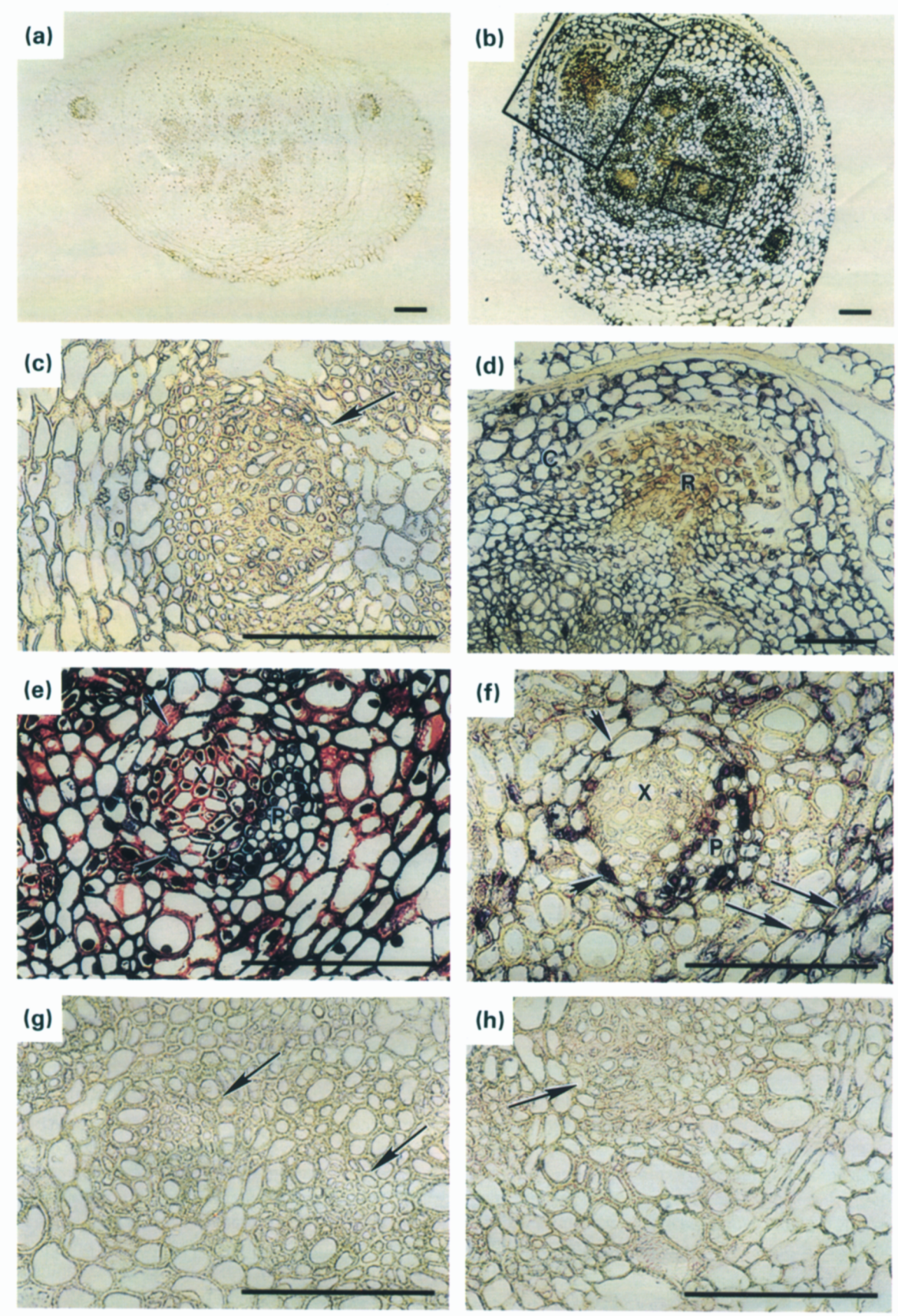


Figure 3. Immunocytochemical detection of the WCS120 protein family in longitudinal sections of a young seedling apical region using purified anti-WCS120 antibody.
(a) Non-acclimated seedling.
(b and c) Cold-acclimated seedlings. Note the strong reaction in the leaf traces.
(d) Detailed view of a leaf trace and surrounding parenchyma in a non-acclimated seedling.
(e and f) Detailed view of a leaf trace and surrounding parenchyma in a cold-acclimated seedling. Enlarged region of boxed area in (c). In (f), the section was counterstained to enhance nuclei. Arrowhead points to a labelled nucleus in a tracheary element.
Bar equals 125 μm. L, leaf trace; M, apical meristem.

a similar antigenicity but differ in their molecular weights and relative abundance. The most abundant are the 50 and 66 kDa proteins. The role of each member and the significance of their relative expression during cold acclimation are still unknown. Our results indicate that this protein family is highly expressed in the vascular transition zone with lower expression in epidermal cells and nearby parenchyma cells.

During freezing stress, intercellular ice crystal formation increases in the subepidermal cell layers causing the epidermal cells to collapse faster than mesophyll cells (Pearce and Ashworth, 1992). Furthermore, Pearce (1988) has shown that ice crystal formation also occurs in vascular bundles. Since ice formation creates a water vapour deficit that increases water migration from the nearest cells, dehydration of the protoplasm should be much stronger in cells adjacent to extracellular ice. The presence of the 50 kDa protein family in the vicinity of the regions where water freezing is more likely to occur indicates that these proteins may alleviate the dehydration stress associated


(a)
(b)
(c)
(d)
C
R
(e)
X
P
(f)
X
P
(g)
(h)

with freezing and thus result in increasing tolerance to freezing. The expression of these proteins in the epidermal and nearby parenchyma cells is consistent with a greater need for protection against dehydration.

Tanino and McKersie (1984) have shown that the vascular transition zone in cold-acclimated winter wheat plants was more sensitive to freezing stress than the apical meristem and that regrowth of the crown was primarily dependent on the viability of this region. The high level of expression of the WCS120 protein family observed in vascular tissues

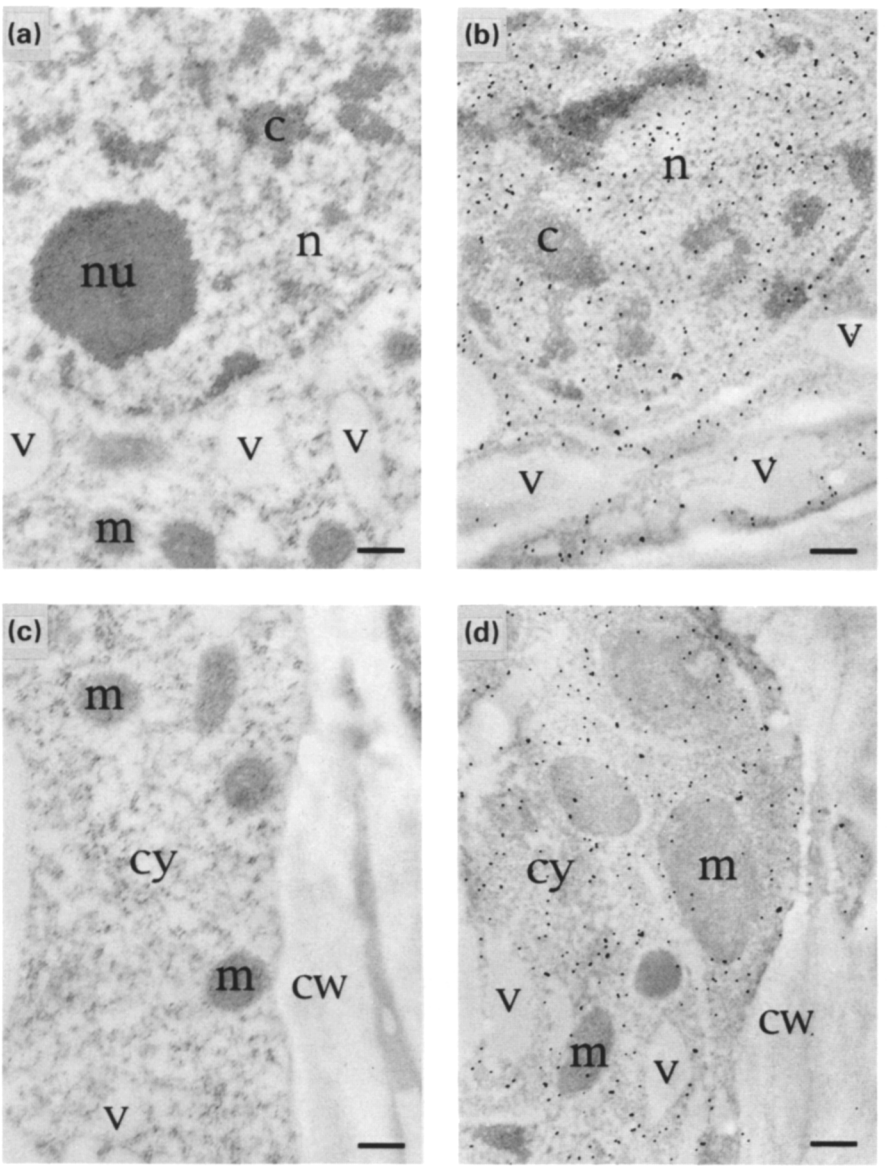


Figure 5. Electron micrographs of control non-acclimated (a and c), and cold-acclimated (b and d) crown transverse sections from wheat seedlings.
The sections were incubated with the anti-WCS120 antibody and immunoreactive sites were revealed using the protein A–gold complex.
Bar equals 0.5 μm. C, dense chromatin; Cy, cytoplasm; CW, cell wall; m, mitochondria; n, nucleus; nu, nucleolus; V, vacuole.

Figure 4. Immunocytochemical detection of the WCS120 protein family in crown transverse sections using purified anti-WCS120 antibody.
(a) Non-acclimated seedling.
(b) Cold-acclimated seedling.
(c) Detailed view of a vascular bundle (arrow) in a non-acclimated seedling.
(d) Lateral root primordium from a cold-acclimated seedling. Enlarged region of large boxed area in (b).
(e and f) Detailed view of a vascular bundle from a cold-acclimated seedling. Enlarged region of small boxed area in (b). In (e), the section was counterstained to enhance nuclei. Arrowheads point to reacting parenchyma cells bordering the fascicular xylem region. Arrows point to reacting parenchyma cells near tracheary elements running horizontally in the stele.
(g) Detailed view of vascular bundles (arrows) from a cold-acclimated seedling incubated with pre-immune serum.
(h) Detailed view of a vascular bundle from a cold-acclimated seedling incubated with immune serum in the presence of 1 mg ml^{-1} of purified WCS120 protein.
Bar equals 125 μm. C, crown cortex; P, phloem; R, lateral root primordium; X, xylem.

suggests that these proteins may also play a role in maintaining or improving metabolite transport at low temperature. The superior performance and growth characteristics observed in freezing-tolerant cultivars supports this possibility. Interestingly, the presence of this protein family in the companion cells would coincide with the localization of sucrose synthase in these cells and its greater activity during wheat cold acclimation (Crespi *et al.*, 1991; Guy *et al.*, 1992; Nolte and Koch, 1993). This enzyme is involved in the regulation of sucrose-starch interconversion and could thus be a key enzyme in the accumulation of sucrose during cold acclimation (Guy, 1990; Guy *et al.*, 1992; Perras and Sarhan, 1984). Although the physiological significance of sucrose synthase in transport tissues is unclear, it may provide the necessary supply of UDP-glucose to repair minor injuries. These precursors may allow callose formation which could reduce the size-exclusion limit of sieve-plate pores in sieve tubes and lead to rapid sealing of sieve tubes in the case of severe injury (Nolte and Koch, 1993). A parallel can be made with cryptobiotic organisms where dehydrins are induced concomitantly with sucrose phosphate synthase and sucrose synthase leading to sucrose accumulation (Bartels *et al.*, 1993).

Subcellular localization of the WCS120 protein family using light microscopy indicated that this protein family is present both in the cytoplasm and nucleus. Similarly, an ABA-induced protein (Goday *et al.*, 1994) and a drought-induced protein (Close *et al.*, 1993) were localized in the nucleus and cytoplasm of several cell types of the embryo. Electron microscopy using immunogold localization and biochemical fractionation have confirmed that these proteins are present both in the cytoplasm and the nucleoplasm. They were not found in cell walls, organelles or in dense chromatin. Their absence in cell walls is significant since several glycine-rich proteins were localized in cell walls of vascular tissues and are believed to be involved in lignification (Showalter, 1993). This indicates that the antibody raised against the WCS120 protein family (which contains glycine-rich repeats) reacts specifically with this family. The presence of these proteins in both cytoplasm and nucleoplasm suggests that they are involved in a general protective role. The high amount found in the nucleus of some cells (see Figure 4f) indicates that they may be important for the protection of the transcriptional machinery. It also raises the question of how these proteins are targeted to the nucleus. Sequence analysis did not reveal the presence of a known nuclear signal peptide. The WCS120 protein family is also devoid of the serine-rich cluster found in Rab proteins which is believed to be involved in nuclear import. However, the basic nature of the lysine-rich repeat which was found in all members of this family may play a role in nuclear import. Furthermore, computer analysis showed that the glycine-rich repeat has weak homology with several viral nuclear proteins. It is also possible, as suggested by Goday *et al.* (1994), that the WCS120 proteins are co-transported to the nucleus through protein–protein interactions.

The predicted high hydrophylicity of the WCS120 protein family may indicate that these proteins could provide a micro-environment that reduces protein denaturation resulting from the loss of polar water molecules (Jaenicke, 1991) during severe dehydration. Cryoprotection assays indicated that the WCS120 protein protects lactate dehydrogenase against denaturation upon freezing. The PD_{50} is 10 μg ml^{-1} and is comparable to that of bovine serum albumin or 250 mM sucrose. A similar result was also found by Kazuoka and Oeda (1994) using COR85 from cold-acclimated spinach. This protein has a PD_{50} of 15 μg ml^{-1} and is homologous to WCS120. The cryoprotective effect of these proteins may be related to their abundance during cold acclimation. An accumulation of 72 μg g^{-1} fresh weight (1.34 μM considering a water content of 85%) after 21 days of cold acclimation is very significant and is well above the PD_{50} of 10 μg ml^{-1} (0.2 μM) calculated using the *in vitro* assay. On a molar basis, the WCS120 protein used at 0.2 μM has the same efficiency as 250 mM sucrose in protecting LDH. Considering the fact that the WCS120 protein family is expressed only in specific cells, the concentration of these proteins is much higher than 1.34 μM in these cells. It is possible that these proteins surround vital cellular proteins and protect them from unfolding or aggregating during freezing or dehydration. A similar explanation was given for the cryoprotective role of BSA in protecting enzyme preparations during storage at low temperature (Tamiya *et al.*, 1985).

Our results indicate that the WCS120 proteins are not present in the meristems. Thus these proteins are not involved in their protection against freezing. However, the meristems may accumulate high solute concentrations such as sucrose or proline to help lowering the freezing point of intracellular water and increase their resistance. The freezing tolerance of winter wheat appears to be limited by the tolerance of the vascular transition zone. The presence of the WCS120 protein family in these tissues may play an important role for the survival of this critical region needed for regrowth. Further physicochemical studies of these proteins and their expression in transgenic plants are under way to determine more precisely the function of the WCS120 protein family in freezing tolerance.

Experimental procedures

Plant material and growth conditions

Winter wheat (*Triticum aestivum* L. cv. Fredrick) was germinated in moist vermiculite for 5 days in the dark and 2 days under artificial light. Cool white fluorescent and incandescent lights were

combined to provide an irradiance of 300 μmol m^{-2} sec^{-1}. The temperature was maintained at 24° ± 1°C (15 h photoperiod) during the day and 20° ± 1°C during the night. The relative humidity was maintained at 70 ± 5%. Seedlings were watered daily with a nutrient solution (20:20:20, N:P:K). At the end of this period, control plants were maintained under the same conditions of light and temperature for 6 more days. Cold acclimation was performed by subjecting germinated seedlings to a temperature of 6° ± 1°C during the day (10 h photoperiod) and 2° ± 1°C during the night. Control non-acclimated and cold-acclimated seedlings were compared at similar growth stages (Cadieux *et al.*, 1988).

Protein purification and antibody production.

The ORF encoded by the gene *Wcs120* was expressed in *Escherichia coli* using the pET11a expression vector, and the protein was purified as previously described (Houde *et al.*, 1992a). For antibody purification, the purified WCS120 protein was coupled to Affi-gel 10 (Bio-Rad) at 3 mg ml^{-1} of bed resin in 0.1 M Hepes buffer, pH 7.5 containing 80 mM of $CaCl_2$. The coupling was performed at 4°C overnight. Free sites were saturated with 0.2 M ethanolamine for 1 h. The coupled resin was washed with phosphate-buffered saline (PBS) containing 0.1% NP-40 and the immune serum was incubated for 1 h with the beads. After washing with PBS, the bound antibodies were eluted with 0.3 M glycine, pH 2.0 and immediately neutralized with Tris base. The purified antibodies were then dialysed and lyophilized before reconstitution in half of the original volume.

Quantitation of WCS120 proteins

Soluble proteins were extracted from fresh plant tissues by grinding in 100 mM Tris-HCl buffer (pH 8.5) containing 1 mM phenylmethylsulphonyl fluoride. The 100 000 ***g*** supernatant was used for protein analyses. A fraction of this supernatant was boiled for 10 min and boiling soluble proteins were recovered after centrifugation at 12 000 ***g*** for 10 min. Protein extracts (boiled and non-boiled) were separated on a 10% SDS–PAGE and proteins were electrophoretically transferred to nitrocellulose according to our previously described procedures (Houde *et al.*, 1992b). The protein bands reacting with the anti-WCS120 antibody (used at 1:10 000 dilution) were revealed using the ECL chemiluminescence detection kit (Amersham). The secondary antibody (Goat anti-rabbit IgG peroxidase-coupled, Jackson Immunoresearch Inc.) was used at 1:25 000 dilution. In order to quantitate the amount of WCS120 proteins by Western blotting, several concentrations of purified WCS120 (from bacteria) and of plant extracts were loaded on the same gel and transferred to nitrocellulose. Results were identical when the same proportions of extract from boiled or non-boiled supernatant were used indicating that there is no loss of WCS120 proteins upon boiling. This method was used to estimate the relative abundance of each member of the WCS120 protein family.

To quantitate more accurately the total amount of WCS120-related proteins in plant extracts, different dilutions (in 100 mM Tris-HCl pH 9.5 to ensure a net negative charge of the WCS120 proteins having pIs less than 7.2, see Table 2) of the boiled extract were dot blotted on a nitrocellulose membrane along with different concentrations of the purified WCS120 protein as standard. This is to eliminate possible variability in reagents and handling. The use of a boiled extract reduced the risk of saturating the nitrocellulose filter and allowed us to load several concentrations of proteins within the linear range of sensitivity of the assay. The dot blot procedure gave results that are less variable than Western blotting quantitation. The internal standards were used to construct a standard curve by densitometry and allowed an accurate estimation of the amount of WCS120 proteins in the plant extract. Quantitation of the total amount of WCS120 by either Western or dot blotting gave the same results but dot blotting was faster and easier to standardize.

The molar concentration of WCS120 proteins was determined from the total immunoreactive proteins per gram fresh weight considering a water content of 85% as determined for 21 days cold-acclimated wheat plants. The molecular weight of each member of the WCS120 family was used to estimate its molar concentration as presented in Table 2. The total amount of soluble proteins was determined from the trichloroacetic acid-insoluble fraction of the non-boiled 100 000 ***g*** supernatant using the Bio-Rad protein assay kit.

Subcellular fractionation

Intact chloroplasts were isolated using a buffer containing the appropriate osmotic concentration as previously described for cold-acclimated wheat tissues (Cadieux *et al.*, 1988). Isolation of wheat nuclei and purification on discontinuous Percoll gradient were conducted according to Nagao *et al.* (1981) with minor modifications. Ethidium bromide was omitted from the homogenization buffer and a blender was used to homogenize the tissues (4 × 5 sec bursts). The concentration of the bottom layer of Percoll gradient was 80% instead of 50%. Microscopic observation after staining with propidium iodide or ethidium bromide indicated that most nuclei band at the 50–80% Percoll interface. The nuclei were intact and devoid of major contaminants. To confirm the presence of the WCS120 protein family, a fraction of the nuclei was used directly for immunoblotting while another fraction was boiled for 10 min, centrifuged and the boiling soluble proteins in the supernatant were analysed by SDS–PAGE and immunoblotting as described above.

Lactate dehydrogenase cryoprotection assay

The assay and PD_{50} calculation were essentially performed as described by Kazuoka and Oeda (1994) except that siliconized tubes were used to avoid the loss of enzyme activity due to adsorption on the tube wall. PD_{50} was calculated as the concentration of added substance required to give 50% residual activity after freezing and thawing.

Immunocytochemistry and light microscopy

Cold-acclimated and control wheat crown meristematic tissues from at least three seedlings were fixed *in situ* using a mixture of 1% glutaraldehyde and 4% paraformaldehyde in 0.1 M PBS, pH 7.2, for 2 h at room temperature, followed by at least 48 h at 4°C. Dehydration was performed in a graded series of cold ethanol, and the tissue was embedded in paraffin.

The immunocytochemical reaction used to localize the 50 kDa protein was performed on 7 μm thick tissue sections prepared from cold-acclimated and control crown tissues that were pre-incubated with 1% gelatin in PBS for 5 min in a moist chamber at room temperature. The embedded sections were incubated with the anti-WCS120 affinity-purified antibody diluted 1:250 in 1% gelatin-PBS for 1 h at 25°C. After 3 × 10 min washes with

PBS-0.1% Tween 20, the sections were incubated in 1% gelatin-PBS for 5 min and allowed to react with the alkaline phosphatase coupled anti-rabbit IgG (Jackson Immunoresearch Laboratories Inc.). The complex was revealed by incubating in 100 mM Tris, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$, 0.2 mg ml^{-1} nitroblue tetrazolium, and 0.2 mg ml^{-1} of 5-bromo-4-chloro-3-indolyl phosphate.

Some sections were counterstained in the following manner. Sections were incubated in 1% tannic acid for 10 min, washed with water for 5 min, and transferred to 3% aqueous ferric chloride for 5 min. After another 5 min wash, the previous staining steps were repeated once to increase the colour. Finally, sections were stained with 1% safranin in 95% ethanol (diluted fresh 1:2 with water) for 10 min. After dehydration through 95% and 100% ethanol, and three changes of xylene, sections were mounted with Permount.

Immunogold labelling and electron microscopy

Tissues were fixed and dehydrated as for light microscopy. Embedding was carried out at low temperature in Lowicryl K4M (Lachapelle and Bendayan, 1991; Lachapelle *et al.*, 1992). Tissue sections were deposited on electron microscope grids and pre-incubated with 1% gelatin in PBS for 30 min. The grid was then deposited for 16 h at 4°C on to a drop of anti-50 kDa affinity-purified antibody at a 1:100 dilution. After 3×10 min washes, the protein A–gold complex (15 nm gold particles) was reacted for 30 min at room temperature (Lachapelle *et al.*, 1993; Wan *et al.*, 1993). The optimal dilution for the protein A–gold complex was found to be 1:10 (OD_{525}=0.5) in 0.01 M PBS-PEG (molecular weight 20 000; 0.2 mg ml^{-1}). The preparations were then washed with PBS-Tween, distilled water, and then dried. Sections were contrasted with uranyl acetate prior to examination under the electron microscope.

In both light and electron microscopy, the specificity of the labelling was assessed by the following control experiments: tissue sections were incubated with (i) the primary antibody to which the 50 kDa protein was previously added (1 mg ml^{-1}) followed by the protein A–gold complex; (ii) rabbit pre-immune serum followed by the protein A–gold complex; (iii) the protein A–gold complex, omitting the primary antibody step.

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