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A molecular marker to select for freezing tolerance in Gramineae

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Summary. We isolated, and expressed in *Escherichia coli*, a gene (*Wcs120*) that is strongly induced during cold acclimation of wheat. The gene product was purified and used to produce antibodies. Immunoblotting experiments with the anti-WCS120 antibody identified several cold-induced proteins named FTMs for Freezing Tolerance Markers since they are associated with the development of freezing tolerance. This protein family was found to be coordinately regulated specifically by low temperature, highly hydrophilic, stable to boiling, and to have a pI above 6.5. The accumulation kinetics during the acclimation period indicated a positive correlation with the capacity of each genotype to develop freezing tolerance. Accumulation of the proteins was higher in the freezing-tolerant genotype than in the less tolerant one. In addition, their accumulation was more pronounced in the crown and leaf tissues compared with roots, confirming a relationship to the capacity of the different tissues to develop freezing tolerance. Analysis of different species (eight monocots and four dicots) indicated that this protein family is specific for freezing-tolerant cereals. The antibody did not cross-react with any of the non-cereal species examined. The anti-FTMs antibody represents a potential tool for breeders to select for freezing tolerance traits in the Gramineae.

Key words: Cold acclimation – Molecular marker – Antibody – Freezing tolerance – Gramineae

Introduction

Cold acclimation of hardy plants involves the coordinated action of many enzymes and proteins that are required to increase freezing tolerance (Levitt 1980; Sakai and Larcher 1987; Steponkus and Lynch 1989; Guy 1990). These physiological and biochemical changes are regulated by low temperature at the gene expression level (Guy 1990). However, the mechanism by which low temperature regulates the expression of this poly genic trait is not well understood. Increasing efforts have been directed toward the identification of genes that are regulated by low temperature. From the few publications available, no similarity has emerged between the genes so far identified, even within the same species. Recently, we have identified several cDNA clones that were induced during cold acclimation and correlated with the capacity of the plants to develop freezing tolerance (Houde et al. 1991; Houde et al. 1992). One of these genes, *Wcs120*, encodes a major protein that was detected in vivo (Perras and Sarhan 1990, 1989) and in vitro (Danyluk and Sarhan 1990) during cold acclimation. The protein is stable to boiling and has an apparent molecular weight of 50 kDa with a pI of 7.3. The cDNA was introduced into the vector pET11a, which allowed the production of a large quantity of the intact protein in *Escherichia coli*. The protein was purified and was used to prepare antibodies for immunocharacterization studies. Using the anti-WCS120 antibody, we identified a protein family sharing a common antigenicity. This protein family is coordinately regulated by low temperature, and is boiling stable, hydrophilic, and Gramineae specific. The use of the anti-WCS120 antibody as a selection tool for freezing tolerance in graminaceous crops is discussed.

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Materials and methods

Plant material and treatments. Seeds were germinated in moist Vermiculite for 5 days in the dark and 2 days under artificial light (25°/20° C, day/night with a 15 h photoperiod). Cold acclimation was performed by subjecting the seedlings to a day/night temperature cycle of 6°/2° C for different periods of time as specified for each experiment. In the case of rice and corn, the day/night cycle was of 10°/5° C. Freezing tolerance was determined as reported previously (Perras and Sarhan 1989) and expressed as the temperature required to kill

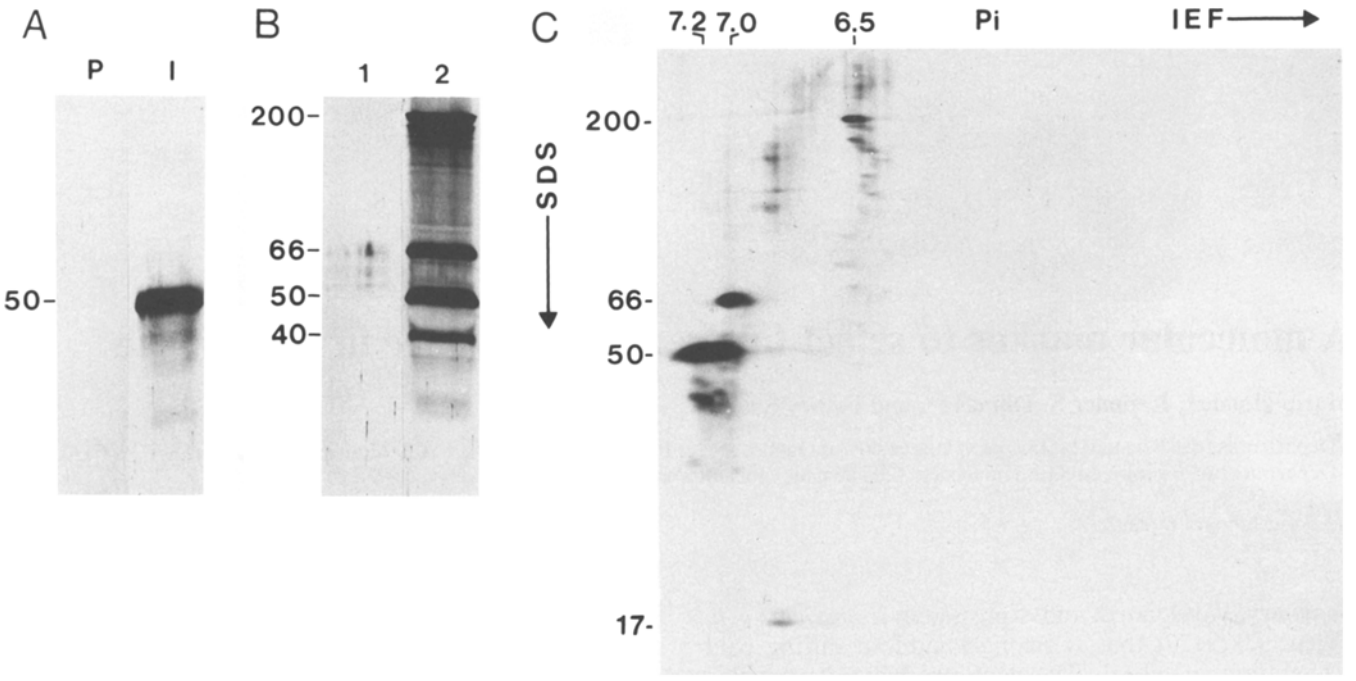

Fig. 1A–C. Immunoblot characterization of anti-WCS120 antibody. **A** Purified WCS120 protein used for immunization. P, preimmune serum, I, affinity purified immune serum. **B** Lane 1, soluble proteins from non-acclimated wheat (cv. Fredrick) seedlings; lane 2, 36 day cold-acclimated wheat seedlings. **C** Proteins were isolated from winter wheat cv. Fredrick cold-acclimated for 36 days, and separated on a 2D gel. After transfer to nitrocellulose, the filter was incubated with the anti-WCS120 antibody and processed. Several proteins are revealed in the basic portion of the gel

50% of the seedlings (LT_{50}). Salt-stressed plants were treated with 75 mM NaCl in water for 1 day, then with 150 mM NaCl for 1 day, and finally with 200 mM NaCl for 1 day. Water-stressed plants were obtained by withholding water for 4 days by which time they were visibly wilted. Abscisic acid (ABA)-treated plants were watered daily for 4 days with nutrient solution containing 10^{-5} M ABA. Heat shock treatment was administered for 3 h at 40° C.

In this study we used two wheat genotypes: spring wheat (*Triticum aestivum* L. cv. Glenlea, LT_{50} −8° C), and winter wheat (*T. aestivum* L. cv. Fredrick, LT_{50} −16° C), couch-grass (*Agropyron repens* L., LT_{50} −19° C), winter rye (*Secale cereale* L. cv. Musketeer, LT_{50} −21° C), barley (*Hordeum vulgare* L. cv. Winchester, LT_{50} −7° C), oat (*Avena sativa* L. cv. Laurent, LT_{50} −6° C), Timothy grass (*Phleum pratense* L., LT_{50} −6° C), corn (*Zea mays*, LT_{50} 4° C), rice (*Oriza sativa*, LT_{50} 4° C), canola (*Brassica napus* cv. Jet neuf, LT_{50} −16° C), mint (*Mentha canadensis*, LT_{50} −20° C), petunia (*Petunia hybrida*, LT_{50} −20° C), and alfalfa (*Medicago falcata* cv. Anik, LT_{50} −12° C).

Antibody production and purification. Antibodies against WCS120 were generated using antigen synthesized in *E. coli* (Houde et al. 1992). The purified protein was used to generate antibodies in a New Zealand rabbit. Preimmune serum was taken from the rabbit before the first immunization and immune serum was taken 10 days after the second and subsequent injections.

For antibody purification, the purified WCS120 protein was coupled to Affi-gel 10 (Bio Rad) at 3 mg/ml of bed resin in 0.1 M HEPES buffer, pH 7.5 containing 80 mM $CaCl_2$. 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 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 dialyzed against PBS and lyophilized.

Protein extraction, separation and immunoblot analysis. Soluble proteins were extracted from different tissues by grinding in a precooled mortar with TRIS buffer [0.1 M TRIS-HCl, pH 9.5 containing 1 mM phenylmethylsulfonyl fluoride (PMSF)]. The extract was immediately centrifuged for 5 min at 12000 *g* and the supernatant was adjusted to final buffer concentration with 5× SDS electrophoresis sample buffer (Laemmli 1970). Samples were separated by electrophoresis on 10% polyacrylamide-SDS gels (SDS-PAGE) or on two-dimensional (2D) gels as described (Danyluk and Sarhan 1990).

Total soluble proteins separated by SDS-PAGE were electrophoretically transferred to nitrocellulose (BAS-85, Schleicher and Schuell). After blocking with powdered milk (2%) in PBS containing 0.2% Tween-20 (Blotto), the blot was incubated with a 1:1000 dilution of the

purified WCS120 antibody. After washing with PBS-Tween, the proteins recognized by the primary antibody were revealed with alkaline phosphatase coupled to anti-rabbit IgG as secondary antibody. The complex was revealed by incubating in 100 mM TRIS, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$, 0.2 mg/ml nitroblue tetrazolium, and 0.2 mg/ml of 5-bromo-4-chloro-3-indolyl phosphate.

Northern blot analysis. RNA isolation and Northern blot analysis were as previously described (Houde et al. 1992) except that washing stringency was lowered to 5 × SSC at 55° C.

Results

Specificity of anti-WCS120 antibody

The purified WCS120 protein produced in the bacterial system and proteins extracted from control and cold acclimated wheat seedlings were separated by SDS-PAGE and transfered to nitrocellulose. Preimmune serum did not react with the WCS120 protein while the purified anti-WCS120 antibody recognized the 50 kDa protein expressed in *E. coli* and that synthesized in cold acclimated seedlings (Fig. 1). No proteins were recognized in the control non-acclimated plant. The anti-WCS120 antibody also recognized several other proteins from cold acclimated seedlings. Analysis by 2D gel electrophoresis (Fig. 1C) revealed that they were neutral to slightly basic (from pI 6.5 to 7.3) and correspond to proteins previously identified during cold acclimation by in vivo labeling of proteins and by in vitro translation of mRNA isolated from cold acclimated plants (Danyluk and Sarhan 1990).

Northern blot analysis of Wcs120 *related mRNAs*

The anti-WCS120 antibody identified at least four protein groups on 1D and 2D gels. In order to examine whether this homology was apparent at the mRNA level, we probed a Northern blot with the p*Wcs120* insert and washed it at low stringency (55° C, 5 × SSC). Five mRNA species, ranging in size from 0.8 to 5 kb, were detected (Fig. 2). This low stringency washing condition allowed us to detect three new mRNA species that were not seen at higher stringency (Houde et al. 1992). These mRNAs may encode the different proteins recognized by the anti-WCS120 antibody. We have recently isolated several anti-WCS120 positive clones. One of these has a cDNA insert size of 4.7 kb and encodes the 200 kDa protein (Ouellet et al. 1991). We thus believe that the proteins revealed by the anti-WCS120 antibody are encoded by different genes.

Accumulation kinetics of freezing tolerance markers (FTMs) during cold-acclimation

The anti-WCS120 antibody was reacted against soluble proteins isolated from cold-acclimated seedlings. Equal amounts of proteins separated by gel electrophoresis were used in assays of any given cultivar. Since the cultivar Glenlea is relatively freezing sensitive, it is likely to synthesize less FTMs and, therefore, 50% more proteins were loaded to increase the chance of immunodetection (compare Fig. 3A and 3C). Results in Fig. 3B show that proteins from non-acclimated seedlings react very weakly to this antibody. When the seedlings were transferred to 4° C, FTMs accumulated rapidly. A large amount of these proteins was seen after 6 days of cold acclimation in both Fredrick (more tolerant) and Glenlea (less tolerant) cultivars. The accumulation of these FTMs was more pronounced and began earlier in Fredrick (Fig. 3B) and the level was maintained or continued to increase slightly during the 36 days of cold treatment. On the other hand, these FTMs had reached their maximum level in Glenlea after 15 days of cold acclimation and they then declined despite the seedlings being maintained at 4° C. When the plants were returned to normal

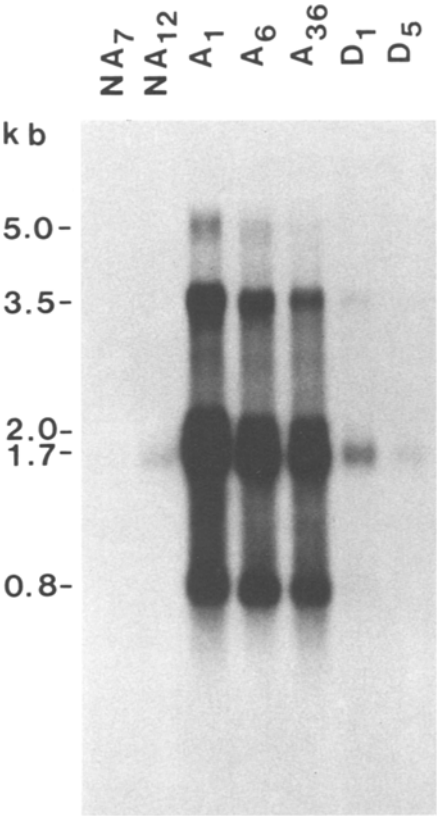

Fig. 2. Northern blot analysis of winter wheat cv. Fredrick mRNA after cold acclimation and deacclimation. Poly $(A)^+$ RNAs (4 µg) isolated from non-acclimated, cold-acclimated, and deacclimated wheat plants were separated by agarose gel electrophoresis in the presence of formaldehyde and then transfered to a nitrocellulose membrane. The blots were probed with ^{32}P-labeled cDNA insert from clone p*Wcs120* and bands were visualized by autoradiography. One major band of 1.7 kb and four minor bands varying in size from 0.8 to 5 kb were detected. NA_7 and NA_{12} non-acclimated 7 and 12 day plants; A_1, A_6, and A_{36}, cold-acclimated for 1, 6 and 36 days; D_1 and D_5, deacclimated for 1 and 5 days. The plants used in the deacclimation experiment had been cold-acclimated for 36 days

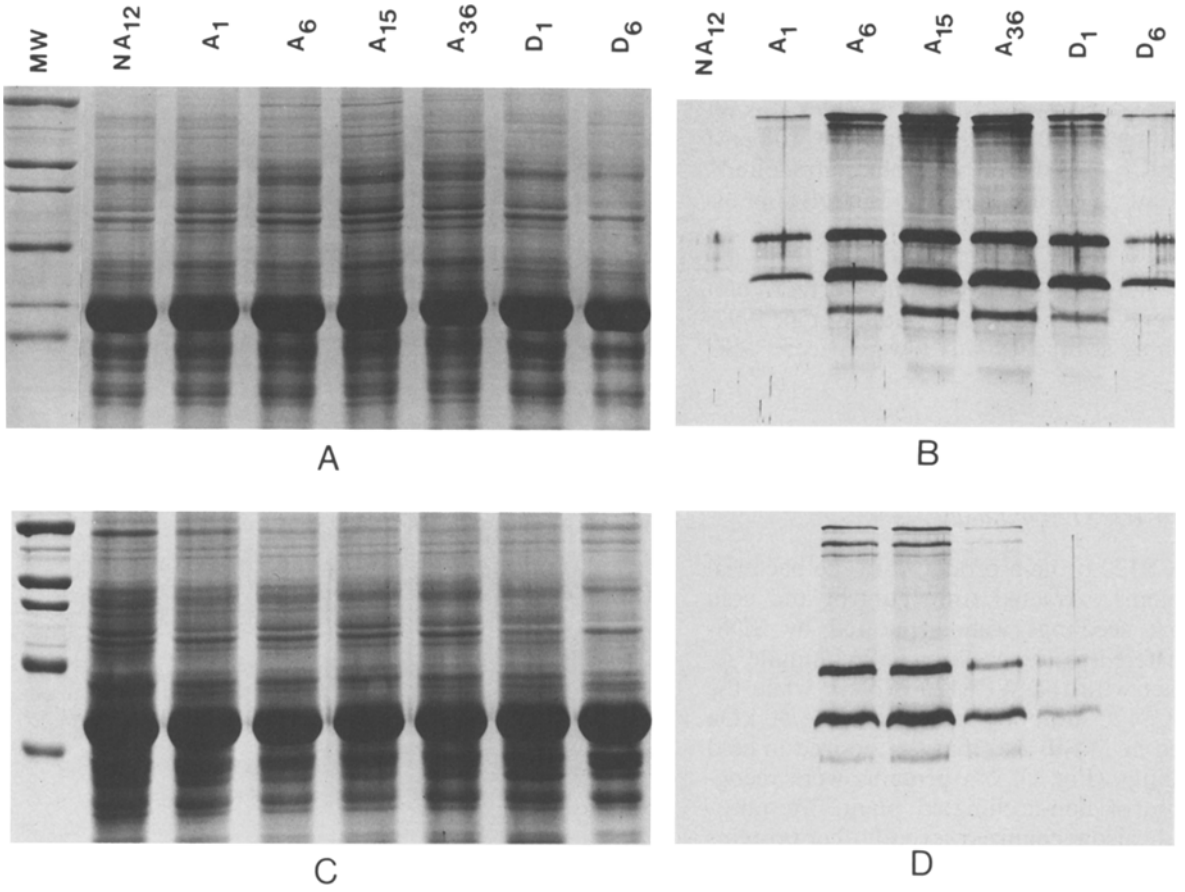

Fig. 3A–D. Accumulation kinetics of freezing tolerance markers (FTMs) in *Triticum aestivum* L. cv. Fredrick and cv. Glenlea. **A** Coomassie Blue-stained gel of cv. Fredrick. **B** Parallel gel transferred to nitrocellulose and probed with the purified anti-WCS120 antibody. **C** Coomassie Blue-stained gel of cv. Glenlea; 50% more proteins were loaded compared with **A**. **D** Parallel gel transferred to nitrocellulose and probed with the purified anti-WCS120 antibody. NA_{12} non-acclimated 12 day old plants; A_1, A_6, A_{15} and A_{36}, cold-acclimated for 1, 6, 15 and 36 days; D_1, and D_6, deacclimated for 1 and 6 days. The plants used in the deacclimation experiment had been cold-acclimated for 36 days. High molecular weight markers (Bio-Rad) are shown on the left side

growth conditions, the FTMs declined rapidly and reached near control level after 6 days of deacclimation for Fredrick and after only 1 day for Glenlea. These results indicate that the accumulation of FTMs is correlated with the capacity of each genotype to develop freezing tolerance during the cold acclimation period.

Expression of FTMs in different tissues and during other stresses and ABA treatments

On comparing the relative abundances of these proteins in leaves, crown, and roots, we concluded that FTMs accumulate to higher levels in those parts of the plant with the highest freezing tolerance. Figure 4A shows that the leaves and meristematic crown (more tolerant) contain more FTMs than the basal region of the crown and roots (less tolerant).

FTMs are specifically induced by low temperature (Fig. 4B). These proteins are slightly induced by water stress and ABA, while they are not induced by heat shock or salt stress. Interestingly, the antibodies recognized proteins of lower molecular weight (14 to 21 kDa) that are strongly induced by water stress and ABA. These proteins presumably belong to the RAB and/or dehydrin families since proteins of that molecular weight range have been shown to be induced during water stress and ABA treatments (Vilardell et al. 1990; Yamaguchi-Shinozaki et al. 1989; Close et al. 1989). Furthermore, the WCS120 sequence shares homology with these two protein families through two repeated motifs (see Houde et al. 1992). In order to verify that dehydrins are recognized by our antibody, barley seedlings were subjected to water stress and the soluble proteins were extracted and reacted with the anti-WCS120 antibody. The results in Fig. 4B show that the low molecular weight proteins strongly induced during water stress were not detected in cold-treated barley. Moreover, a protein of ca. 72 kDa was strongly induced by cold treatment while it was only slightly induced by water stress. This indicates that proteins strongly induced by cold treatment are induced either poorly or not at all by water stress.

Species specificity of FTMs

In order to determine whether proteins similar to WCS120 accumulate during cold acclimation in other

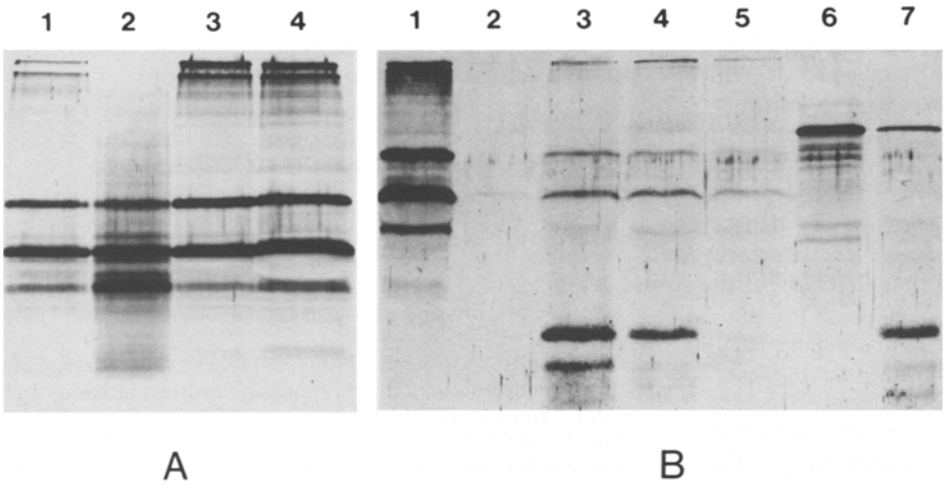

Fig. 4A and B. Immunoblot analysis of FTMs isolated from different tissues and from plants grown in different conditions. **A** Proteins isolated from different plant tissues (cold acclimated cv. Fredrick) and probed with the purified anti-WCS120 antibody. Lane 1, root; lane 2, basal region of the crown; lane 3, meristematic crown; lane 4, leaf. **B** Effect of different growth conditions on FTMs isolated from shoot tissue. Lane 1, *Triticum aestivum* L. cv. Fredrick cold-acclimated for 36 days; lane 2, salt-treated cv. Fredrick; lane 3, water-stressed cv. Fredrick; lane 4, abscisic acid-treated cv. Fredrick; lane 5, heat-shocked cv. Fredrick; lane 6, *Hordeum vulgare* L. cv. Winchester cold-acclimated for 36 days; lane 7, water-stressed cv. Winchester

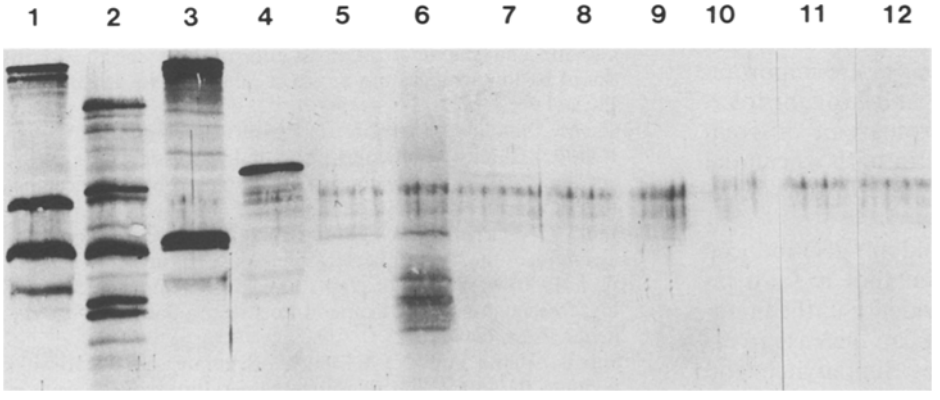

Fig. 5. Immunoblot analysis of soluble proteins isolated from different cold-acclimated species. Lane 1, *Triticum aestivum* L. cv. Fredrick; lane 2, *Agropyron repens* L.; lane 3, *Secale cereale* L. cv. Musketeer; lane 4, *Hordeum vulgare* L. cv. Winchester; lane 5, *Avena sativa* L. cv. Laurent; lane 6, *Phleum pratense* L.; lane 7, *Zea mays*; lane 8, *Oryza sativa*; lane 9, *Brassica napus* L.; lane 10, *Mentha canadensis*; lane 11, *Petunia hybrida*; lane 12, *Medicago falcata* cv. Anik

species, we examined a number of tolerant and less tolerant cereals as well as four tolerant dicot species. Figure 5 shows that proteins from tolerant cereals (lanes 1–3) reacted most strongly with the anti-WCS120 antibody. Less tolerant cereals (lanes 4–6) reacted less strongly. It appears that the correlation between LT_{50} and FTMs may not be perfect for all graminaceous species, as shown in the case of oat (lane 5). The antibody did not recognize any protein in the sensitive cereal varieties (lanes 7 and 8) or within the tolerant dicotyledonous group (lanes 9–12). This suggests that these FTMs are not associated with cold acclimation in these species or that the proteins have evolved in a way that does not allow cross-reactivity with the antibody.

Discussion

We have produced and purified a polyclonal antibody against a major protein induced during cold acclimation of wheat. The corresponding cDNA was shown to hybridize with at least five different mRNA species, which have the potential to encode all the proteins recognized by the anti-WCS120 antibody. The accumulation kinetics of these coordinately expressed proteins are positively correlated with the time of development and the degree of freezing tolerance. Hence, we refer to this protein family as Freezing Tolerance Markers. The winter wheat variety Fredrick accumulates more of these FTMs and continues accumulating them throughout the cold acclimation period. In contrast, the spring variety Glenlea accumulates less FTMs and their level starts to decline early during the acclimation period correlating with the sharp reduction in mRNA seen in our previous study (Houde et al. 1992). Moreover, FTMs accumulated in greater amounts in tissues with higher capacity to develop freezing tolerance. This observation is consistent with our earlier observation, which showed a preferential synthesis of the 200 kDa protein (a member of this protein family) at the shoot level compared with the roots (Perras and Sarhan 1989).

We have also demonstrated that the antibody recognizes several proteins in other cold-acclimated cereals. The amount of cross-reactive material was much higher

in the freezing-tolerant species. These results confirm the positive correlation between the induction of these proteins and the capacity of plant or tissues to develop freezing tolerance. Furthermore, the accumulation of this protein family did not appear to be associated with the plant developmental stage since it was induced at low temperature in calli, sprouts, and germinating seedlings at different growth stages (not shown). The expression of FTMs was induced to the same level in dark- or light-grown seedlings, suggesting that this is light independent.

No cross-reactivity could be found in freezing-tolerant dicotyledonous species suggesting that freezing tolerance in monocots and dicots involves different proteins. However, these proteins may have similar properties (such as boiling stability or high hydrophilicity). The presence of proteins of variable molecular weight having a similar antigenicity is novel and suggests that their function is determined through common small repeated elements within their structure. Such repeats do exist within the 50 kDa (Houde et al. 1992) and 200 kDa proteins (Ouellet et al. 1991). Similar motifs have been found in the dehydrin (Close et al. 1989; Baker et al. 1988) and RAB (Yamaguchi-Shinozaki et al. 1989; Vilardell et al. 1990) families. These results suggest that the number of repeats within the protein molecule and the amount of these proteins synthesized are important factors in the acquisition of freezing and drought tolerance. However, the induction of proteins of different sizes during cold and drought stress (in both wheat and barley) suggests that they probably have distinct functions with some complementarity.

Some of the FTMs induced by cold are also induced slightly by water stress and ABA but they are not induced by salt stress or heat shock. Evidence in the literature indicates that ABA and drought can increase freezing tolerance (Cox and Levitt 1976; Siminovitch and Cloutier 1982; Gagné et al. 1989). However, in the case of the intact wheat plant, the freezing tolerance conferred by ABA is $-8°$ C ($-4°$ C for the non-acclimated control) compared with $-16°$ C after cold acclimation (Gagné et al. 1989; Perras and Sarhan 1989). This may be due to the partial induction of FTMs and other cold-regulated genes necessary for the acquisition of freezing tolerance. This assumption requires a detailed study to determine the exact role that ABA and drought may play during cold-acclimation. It is however interesting to observe that small molecular weight proteins recognized by the anti-WCS120 antibodies are present in high concentration during water stress and ABA treatment. This suggests that low temperature induction of freezing tolerance involves pathways distinct from that induced by ABA and water stress. A similar conclusion has been reached in studies, with *Arabidopsis thaliana* (Nordin et al. 1991).

The characterization of FTMs should help us to understand their role in the acquisition of freezing tolerance. However, the antibody represents a very important and easily accessible tool to identify cereal cultivars with a superior capacity for cold acclimation. This potential can be assessed rapidly with proteins extracted from as little as 50 mg of plant tissues. This provides breeders with a simple and economic method of selection for potential freezing tolerance of new cereal crops.

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