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Identification and characterization of a low temperature regulated gene encoding an actin-binding protein from wheat

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Abstract A cDNA corresponding to a putative actin-binding protein was cloned from a cold-acclimated wheat cDNA library. The cDNA, designated *Wcor719*, encodes a polypeptide of 142 amino acids with a calculated molecular mass of 15.8 kDa and a pI of 4.27. The protein has the two conserved domains identified as actin and phosphatidylinositol 4,5-biphosphate (PIP_2) binding sites found in members of the cofilin family. Northern analyses revealed that *Wcor719* transcript accumulation is rapid and strongly up-regulated by low temperature. This accumulation was greater in the tolerant winter wheat and rye species compared to the less tolerant ones. The rapidity of transcript induction and the significant homology with actin-binding proteins (ABP) from different organisms suggest that the product of this gene might be involved in the dynamic reorganization of the cytoskeleton during low temperature acclimation. It may also serve as a key factor in the signal transduction pathway during cold acclimation.

Key words: Cold acclimation; Gramineae; Cytoskeleton; Actin-binding protein; cDNA

1. Introduction

Cells of freezing tolerant plants have the ability to modify their cell wall and plasma membrane to protect them from freezing injury. These modifications are associated with a reduction in cell water content, increase in intracellular solutes, reduction in cell volume, and an increase in plant erectness [1]. It has been reported that low temperature causes microtubule depolymerization in winter rye root tips [2]. The level of depolymerization was related to the degree of freezing tolerance, suggesting that microtubule depolymerization is important for achieving maximal freezing tolerance. These adaptive adjustments are genetically programmed and induced by low temperature during the acclimation period. However, the molecular basis and the role of low temperature in regulating these modifications are still unknown. In the course of our studies on the molecular genetics of cold acclimation in wheat, we have identified a new cDNA clone, *Wcor719*, which is up-regulated by low temperature. Sequence analysis revealed that *Wcor719* encodes a protein with significant homology with ABP. This group of proteins is involved in the regulation of different cellular processes such as cytokinesis, cell locomotion, cytoplasmic streaming and actin assembly in the membrane cytoskeleton, and recently in signal transduction events [3–7]. The high expression of a gene with these characteristics suggests a possible function in regulating the structure of actin filaments during cold acclimation. This reorganization of the cytoskeleton may be important to preserve the structural integrity of the cell during freezing stress. In this report, we describe the molecular features of this gene and discuss its putative function in *Gramineae* species during cold acclimation.

2. Materials and methods

2.1. Plant material and growth conditions

In this study we used: two spring wheat genotypes (*Triticum aestivum* L. cv Glenlea, LT_{50} (lethal temperature that kills 50% of the seedlings) −8°C; and cv Concorde, LT_{50} −8°C), 4 winter wheat genotypes (*T. aestivum* L. cv Monopole, LT_{50} −15°C; cv Absolvent, LT_{50} −16°C; cv Fredrick, LT_{50} −16°C; and cv Norstar, LT_{50} −19°C), winter rye (*Secale cereale* L. cv Musketeer, LT_{50} −21°C), oat (*Avena sativa* L. cv Laurent, LT_{50} −6°C), barley (*Hordeum vulgare* L. cv Winchester, LT_{50} −7°C), rice (*Oryza sativa*, LT_{50} 4°C) and corn (*Zea mays*, LT_{50} 4°C).

Plants were germinated in moist sterilized vermiculite for 5 days in the dark and 2 days under artificial light at 25°C/20°C (day/night). Based on dry weight, control plants were maintained under the same conditions while cold acclimation was performed by subjecting the germinated seedlings to 6°C/2°C (day/night). Based on dry weight, control (7 and 12 days), and cold-acclimated (1 and 36 days) plants were at comparable physiological ages. Deacclimation was performed by transferring 36 days cold-acclimated plants to 25°C/20°C (day/night). Heat shock was performed by incubating seedlings at 40°C for 1 and 3 h. Wounding stress was induced by cutting the seedlings into 1 cm segments and placing them in water at 20°C for 3 and 14 h. Salt-stressed plants were obtained by incubating seedlings for 18 h with a nutrient solution containing 300 or 500 mM NaCl. Water stress was induced by removing seedlings from vermiculite and leaving them at 20°C without water for different time periods, after which the relative water content (RWC) was evaluated. ABA-treated plants were obtained by transferring seedlings for 18 h 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. In the case of rice and corn, control seedlings were maintained at 29°C/26°C (day/night) while low temperature exposure was performed by subjecting the seedlings for 24 h to temperature regimes described in the figure legends.

2.2. Cloning and molecular analysis

The p*Wcor719* clone was isolated from a Lambda Zap II library constructed from poly(A)$^+$ RNA isolated from cold-acclimated winter wheat. (T. *aestivum* L. cv Norstar). Differential screening of this library was done with cDNA probes synthesized from poly(A)$^+$ RNA isolated from control and cold-acclimated winter wheat. The *Wcor719* clone, which hybridized preferentially with the cold-acclimated probe, was purified and excised as a pBluescript vector as described (Stratagene). DNA sequence was determined on both strands using the T7 DNA sequencing kit (Pharmacia). Analysis and sequence comparisons were carried out with the Genetic Computer Group's sequence analysis software package, version 6.0. RNA and DNA gel blot analysis were performed as described previously [8].

3. Results and discussion

Differential screening of a wheat cDNA library from cold-

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A

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1   TTTCAATCCAGCTACCAGTTCCCGTCTCAGCATCAGCACACGGATTTCTCCTCGGGCCAA
61  CCTCCGGATCCATCAACAATGGCGAACTCTGTCTCCGGGGTGGCGGTGAATGAGGAGTGC
                  M  A  N  S  V  G  S  V  A  V  N  E  E  C
121 GTGAAGGTGTTCCAGGAGCTGCGGGCGGAGCGCAAGCACCGGTTCGTGGTGTACAAGATG
    V  K  V  F  Q  E  L  R  A  E  R  K  H  R  F  V  V  Y  K  M
181 GACGACGACGCGCAGCAGGTGGTGGTGGACAAGGTGGGCGCCCTCGACGCCACCTTCGAC
    D  D  D  A  Q  Q  V  V  V  D  K  V  G  A  L  D  A  T  F  D
241 GACCTGGCGGCGGCGATGCCCGCCGACGACTGCCGCTACGCGGTGTACGACCTGGACTTC
    D  L  A  A  A  M  P  A  D  D  C  R  Y  A  V  Y  D  L  D  F
301 GTGTCCGAGGACTCGGCGGGGGACACGCCGCGGAGCAAGATCTTCTTCATCCACTGGTCG
    V  S  E  D  S  A  G  D  T  P  R  S  K  I  F  F  I  H  W  S
361 CCGGAGTCTGCGGACGCGAGGAACAAGATGCTGTATGCCAGCTCCACGGAGGGGCTCAAG
    P  E  S  A  D  A  R  N  K  M  L  Y  A  S  S  T  E  G  L  K
421 AAGGAGCTGGACGGCGTGCAGATCGACGTGCAGGCCACCGACGCCAGCGAGCTCACCCTC
    K  E  L  D  G  V  Q  I  D  V  Q  A  T  D  A  S  E  L  T  L
481 AACATCCTCAAGGACTACACCACCTAATTAAGCTAATCCATCCCGTACGCGCGCACGATC
    N  I  L  K  D  Y  T  T
541 GGTCGGTGCCTGGATCCAAATAATGTTTTTCCATGTGTAGTGGTTTGTGAATGATTGGGT
601 TTTCCTTTTTACTTCCTCGAGATTGATAATTGAGTGCTTGATCTATTC
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B

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                1                                                  50
WCOR719         MANSVSGVAVNEECVKVFQELR.........AERKHRFVVYKMDDDAQQV
LMP131A         MANSSSGMAVDDECKLKFMELK.........AKRNFRFIVFKIEEKVQQV
BMP1            .........EDNCKLKFLELK.........KRIFRFIIFRI..DGQQV
Actophorin      ....MSGIAVSDDCVQKFNELKL........GHQHRYVTFKMNASNTEV
Cofilin         ...MASGVAVSDGVIKVFNDMKVRKSSTPEEVKKRKKAVLFCLSEDKKNI
COF1            ..MSRSGVAVADESLTAFNDLKL.........GKKYKFILFGLNDAKTEI
Consensus       ---s-SGVAV-Decv-kFnELK----------kRkfRfVvFkM-dd-qqV

                51                                                 100
WCOR719         VVDK.....VGALDATFDD....LAAAMPADDCRYAVYDLDFVSEDSAGD
LMP131A         TVER.....LGQPNESYDD....FTECLPPNECRYAVFDFDFVTDE...N
BMP1            VVEK.....LGNPQETYDD....FTASLPADECRYAVFDFDFTTNE...N
Actophorin      VVEH.....VGGPNATYED....FKSQLPERDCRYAIFDYEFQVDGG...
Cofilin         ILEEGKEILVGDVGQTVDDPYATFVKMLPDKDCRYALYDATYETKES...
COF1            VVKE......TSTDPSYDA....FLEKLPENDCLYAIYDFEY..EINGNE
Consensus       vVek-----VG-pn-TyDd----F---LPenDCrYAVyDfdF-tee----

                101                                                150
WCOR719         TPRSKIFFIHWSPESADARNKMLYASSTEGLKKELDGVQIDVQATDASEL
LMP131A         CQKSKIFFISWSPDTSRVRSKMLYASTKDRFKRELDGIQVELQATDPSEM
BMP1            CQKSKIFFIAWSPDSSRVRMKMVYASSKDRFKRELDGIQVELQATDPSEM
Actophorin      .QRNKITFILWAPDSAPIKSKMMYTSTKDSIKKKLVGIQVEVQATDAAEI
Cofilin         .KKEDLVFIFWAPESAPLKSKMIYASSKDAIKKKLTGIKHELQANCYEE.
COF1            GKRSKIVFFTWSPDTAPVRSKMVYASSKDALRRALNGVSTDVQGTDFSEV
Consensus       -qRskIfFI-WsPDSapVRsKMLYASSkD-LKKeLdGIqvEVQAtd-sEM

                151                        179
WCOR719         TLNILKDYTT*.................
LMP131A         SMDIIKARAF*.................
BMP1            SFDIIKSRAL*.................
Actophorin      SEDAVSERAKKDVK*.............
Cofilin         ....VKDRCTLAEKLGGSAVISLEGKPL*
COF1            SYDSVLERVSRGAGSH*...........
Consensus       S-diVkdra--------------------
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Fig. 1. (A) Nucleotide sequence and deduced amino acid sequence of *Wcor719*. The longest ORF is shown here (428 nucleotides). The predicted polypeptide is 142 amino acids in length, with a calculated molecular mass of 15.8 kDa and a pI of 4.27. The underlined regions represent the putative actin and PIP_2 binding sites. GenBank accession no. U58278. (B) Amino acid sequence alignment of WCOR719 with members of the cofilin family from *L. longiflorum* LMP131A, *B. napus* BMP1, *Acanthamoeba* actophorin, human cofilin and yeast COF1. Alignment was obtained using the Gap and Pretty programs of the Genetics Computer Group software package. Dots indicate gaps to maximize alignment. In the consensus line, identical and homologous amino acid residues in all six proteins are shown in capitals while identical and homologous amino acid residues in at least three proteins are shown in lowercase letters.

acclimated plants was used to isolate low temperature responsive clones. Northern analyses revealed that the relative amount of several transcripts increases during cold acclimation. DNA sequence analysis revealed that one of these cDNAs, *Wcor719*, encodes a protein with significant homology with a group of proteins involved in the regulation of the actin cytoskeleton. The nucleotide sequence of *Wcor719* and its deduced protein are shown in Fig. 1. The longest ORF is 428 nucleotides long and encodes a protein of 142 amino acids, with a molecular mass of 15.8 kDa and a pI of 4.27. A comparison of the WCOR719 protein with the Genbank database indicates that this protein is closely related to ABP of the cofilin family from several organisms. Sequence analysis also revealed that WCOR719 contains the two regions putatively identified as actin and PIP_2 binding sites found in these proteins (Fig. 1A). Using the GAP program for alignment, the percent identity/similarity of *L. longiflorum* actin-depolymerizing factor (ADF) [9], *B. napus* ADF [9], *Acanthamoeba* actophorin [10], human cofilin [11], and yeast cofilin [12] were 47.8/74.2, 48.8/77.1, 40.7/68.1, 35.8/62.5 and 37.6/65.9, respectively (Fig. 1B).

RNA gel blot analysis of *Wcor719* indicated that the cDNA hybridized preferentially with a transcript of 0.8 kb that is strongly up-regulated by low temperature. The kinetic studies show that the *Wcor719* transcript accumulates rapidly and then decreases gradually during the acclimation period in the three genotypes (Fig. 2). The RNA signal was detectable after 3 h of exposure to low temperature (Fig. 3A). However, the more freezing tolerant genotypes, Fredrick and Norstar, maintained a higher level of *Wcor719* mRNA at the end of the cold acclimation period (36 days) when compared to the less freezing tolerant genotype Glenlea. When the plants are deacclimated at 24°C, the transcript declines to the non-acclimated control level. Futhermore, expression during the cold acclimation period is not tissue-specific since the accumulation of transcripts was detected in the leaves, crown and roots.

To determine whether the *Wcor719* mRNA accumulation is specifically regulated by low temperature, plants were subjected to different treatments. Northern blot analysis (Fig. 3B) indicates that extensive water stress (RWC 57–44%) induces a very low level of transcript accumulation compared to a one day low temperature exposure. Exogenously applied ABA, heat shock, salinity and wounding did not induce its accumulation. Fig. 4A shows that the accumulation of *Wcor719* mRNA during cold acclimation is higher in winter wheat and rye, which are the most freezing tolerant species. We could not detect any accumulation of the transcript in the freezing sensitive species rice and corn under the four temperature regimes used (Fig. 4B). Southern analysis (Fig. 5) shows that *Wcor719* is present in all monocot species examined, including rice and corn. Northern and Southern analyses of two dicot tolerant plants, alfalfa and rapeseed, did not reveal any signal, even at low stringency and long exposure times (results not shown). However, since dicots have a lower GC content, similar genes may not be detectable using our probe. These results suggest that *Wcor719* is gramineae-specific and that its expression is correlated with the capacity of each species to develop freezing tolerance. The inability of the *Wcor719* gene to respond to low temperature in sensitive species may be explained by the absence of a low temperature responsive *cis*-element or of the appropriate transcription factor interacting with this element. The presence of freezing tolerance-associated genes in both sensitive and tolerant gramineae species with specific expression in tolerant ones is important since it may offer the possibility of activating the expression of these genes to improve cold tolerance in the sensitive species.

The significant homology of WCOR719 to proteins belonging to the cofilin family and the presence of actin-binding regions suggest that WCOR719 is an actin-binding protein. The rapid induction of the *Wcor719* transcript during cold acclimation may be related to its putative function in the dynamic reorganization of the actin cytoskeleton. It is possible that low temperature stress modulates the organization of the cytoskeleton by modifying the polymerization state of actin molecules. It has been shown in rat fibroblasts cells

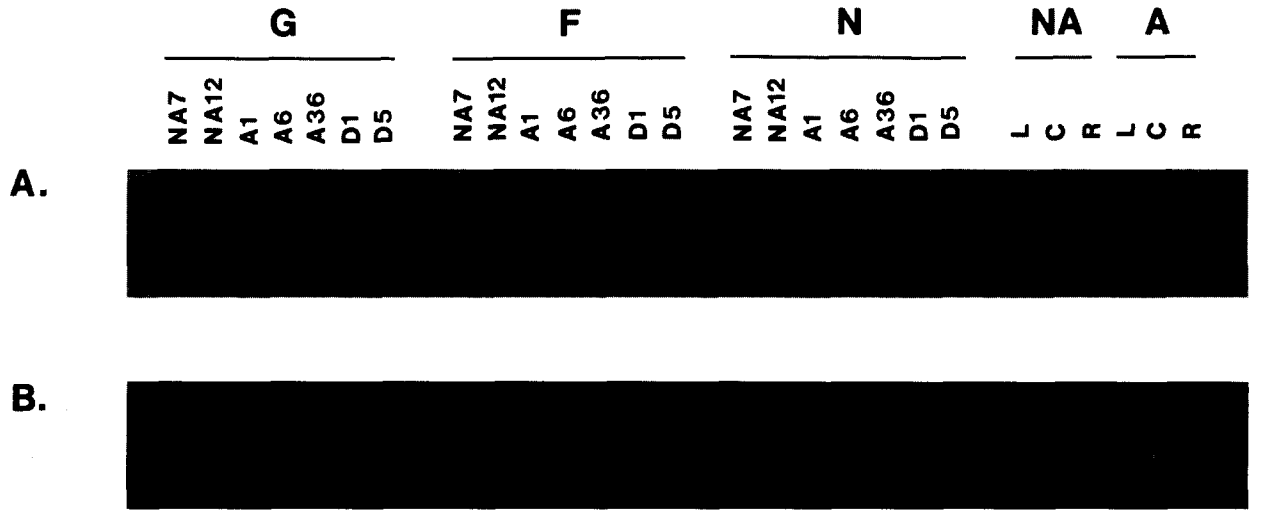

Fig. 2. Kinetic analysis and tissue specificity of mRNA expression during cold acclimation. (A) Total RNA (7.5 μg) extracted from the shoots of wheat genotypes Glenlea (G), Fredrick (F) and Norstar (N) was separated on a formaldehyde agarose gel, transferred to nitrocellulose and hybridized with a ^{32}P-labeled cDNA insert from p*Wcor719*. NA7 and NA12, control plants (non-acclimated) grown for 7 and 12 days; A1, A6 and A36, plants cold-acclimated for 1, 6 and 36 days; D1 and D5, cold-acclimated plants (36 days) were deacclimated for 1 and 5 days. Tissue specificity was determined in the genotype Fredrick for leaves (L), crown (C) and roots (R); NA, non-acclimated control plants grown for 12 days; A, cold-acclimated for 36 days. (B) Ethidium bromide-stained 28S ribosomal band (included to show RNA loads).

that dephosphorylation of cofilin accompanies its heat shock-induced nuclear accumulation [13]. This results in the formation of actin/cofilin rods, whose functions are unknown. Although the actual role of cofilin's phosphorylation state and the mechanisms controlling it remain unclear, it has been suggested that dephosphorylation is important for its activity. The dephosphorylated form of the chicken actin-depolymerizing factor, a cofilin-like protein, was able to bind and depolymerize actin, while the phosphorylated form was not [14]. If this is true for WCOR719 identified in our work, then low temperature may activate it by dephosphorylation and thus increase the depolymerization of actin filaments. In the budding yeast *Saccharomyces cerevisiae*, disruption of the COF1 gene encoding a cofilin-like protein caused cell lethality while expression of a mammalian cofilin rescued yeast cells from this lethality [12,15]. These results indicate that cofilin is

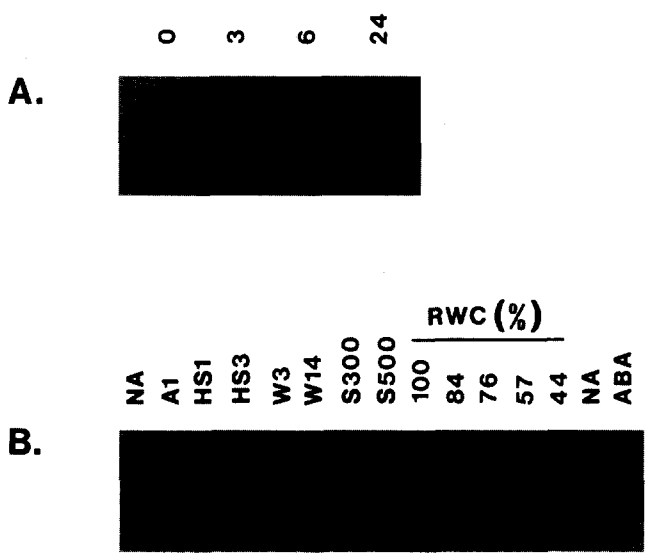

Fig. 3. (A) *Wcor719* expression in wheat shoots (cv Fredrick) exposed to short periods of cold treatment. 0, control plants (non-acclimated); 3, 6 and 24, plants cold-acclimated for 3, 6 and 24 h. (B) *Wcor719* expression in wheat shoots (cv Fredrick) exposed to different treatments. NA, control plants (non-acclimated); A1, plants cold-acclimated for 1 day; HS1 and HS3, plants heat-shocked at 4°C for 1 and 3 h; W3 and W14, 3 and 14 h after wounding plants; S300 and S500, plants salt-stressed for 18 h with 300 or 500 mM NaCl; RWC (%), relative water content after water stress; ABA, plants treated with 10^{-4} M ABA.

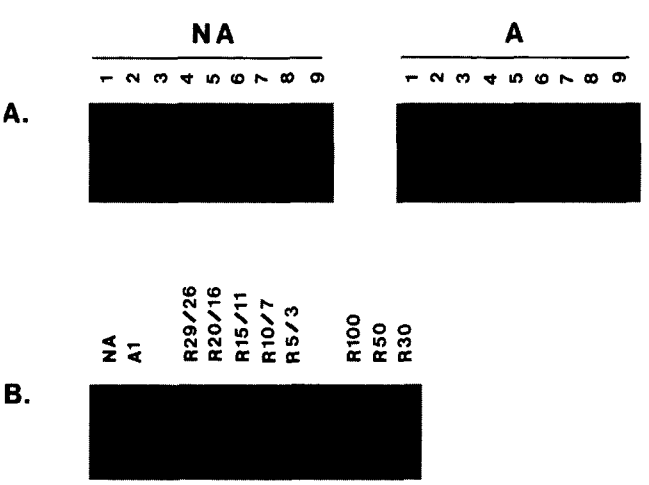

Fig. 4. (A) *Wcor719* mRNA expression in several plant species. NA, control plants (non-acclimated) grown for 12 days; A, plants cold-acclimated for 36 days; Lanes 1–6, wheat genotypes Glenlea, Concorde, Monopole, Absolvent, Fredrick and Norstar; 7, oat; 8, barley; 9, rye. (B) *Wcor719* mRNA expression in rice exposed to cold and water stress. Similar results were obtained with corn (results not shown). NA, control Fredrick wheat plants (non-acclimated); A1, Fredrick wheat cold-acclimated for 1 day; R29/26, control rice plants grown under a day/night temperature of 29°C/26°C; R20/16, R15/11, R10/7 and R5/3, rice plants were transferred for 24 h to the corresponding day/night temperatures. R100, control rice plants at 100% of relative water content; R50 and R30, rice at 50 and 30% of relative water content after water stress.

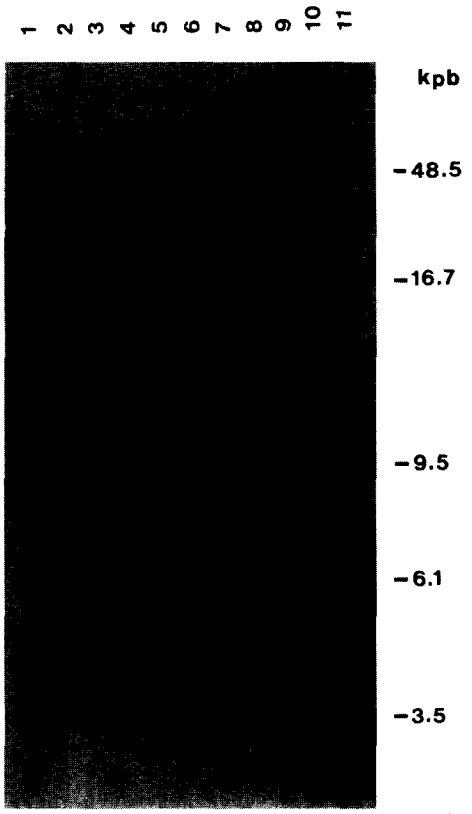

Fig. 5. Southern blot analysis of DNA from several plant species. Plant DNA (1.8 μg) was digested with *Xba*I, separated by field inversion agarose gel electrophoresis (Bio-Rad), transferred to nitrocellulose and then probed with *Wcor719*. Lanes: 1–6, wheat genotypes Glenlea, Concorde, Monopole, Absolvent, Fredrick and Norstar; 7, oat; 8, barley; 9, rye; 10, rice; 11, corn.

essential for cell survival and may be implicated in cell protection against stress. The function of cofilin appears to be conserved among eukaryotes from yeast to mammals and probably higher plants. The high level of *Wcor719* induction early during the acclimation period may suggest a similar protective function against low temperature stress. The higher accumulation of an ABP in the tolerant species may be related to their capacity to modulate the intracellular actin structure needed to develop higher freezing tolerance. It has been reported that low temperature causes microtubule depolymerization in winter rye root tips [2]. The level of depolymerization was related to the degree of freezing tolerance. It appears to be necessary for rye root-tip microtubules to depolymerize to threshold levels in order to achieve their maximal freezing tolerance. Therefore, depolymerization of microtubules and actin filaments might be mechanisms required to maintain a more fluid plasma membrane during the cold acclimation process.

Recent reports have shown a possible involvement of the cofilin protein family in a signal transduction pathway. Cofilin was found to inhibit phospholipase C activity by protecting PIP_2 from hydrolysis, suggesting that the cytoskeleton participates in signal transduction processes in a cofilin-dependent manner [4]. A similar result was obtained with another actin binding protein, profilin, which was found to bind PIP_2 and inhibit phospholipase C in bean leaf plasma membrane [16]. In *C. communis*, disruption of actin organization impairs the ability of the stomata to respond to environmental cues. Microfilaments in guard cells, presumably through actin-binding proteins, may regulate intracellular Ca^{2+} which in turn modulates stomatal opening and several other physiological processes in the cell [17]. Considering these informations, it is possible that the actin-binding protein WCOR719 may have multiple functions. Detailed studies are in progress to elucidate the role of WCOR719 in plants during cold acclimation.

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