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A leaf-specific gene stimulated by light during wheat acclimation to low temperature

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Abstract

We report here the identification and characterization of a new leaf-specific light-stimulated gene induced during cold acclimation of wheat. Sequence analysis revealed that the gene encodes a protein of 19 kDa with a pI of 8.8. This is a novel protein with a particular charge distribution. The C-terminal half has a high propensity to form an α-helix and contains all the acidic amino acids with a net negative charge of −7. On the other hand, the N-terminal half is rich in proline, lysine and arginine with a net positive charge of + 10. These properties are commonly found in several transcription factors. The protein is also rich in alanine (21%), is hydrophilic but not boiling soluble in contrast to other alanine-rich proteins. During low temperature exposure, the corresponding mRNA accumulates rapidly in the leaf and remains at a constant level in two tolerant cultivars used. However, in a less tolerant cultivar, the mRNA level declines despite maintaining the plants at 4 °C. Southern blot analysis indicates that the differential expression in the less tolerant genotype is not due to a different genomic organization or gene copy number. The mRNA was specifically localized in leaf tissues and increased several-fold during the greening at 4 °C. Furthermore, this gene is not induced in callus cultures acclimated in the absence or presence of light. This suggests that the full expression of this gene is dependent on organized leaf tissue. The expression of this gene was not affected by ABA, drought, heat shock, salinity, wounding or anaerobiosis, demonstrating that it is specifically induced by low temperature. The *Wcs19* mRNA is preferentially expressed in tolerant Gramineae species.

Introduction

Wheat is a temperate cereal that possesses the capacity to develop a high degree of freezing tolerance (FT) after a period of low-temperature growth. In general, winter cultivars, compared to spring ones, possess better protective mechanisms allowing them to optimize growth at low temperature while the induction of FT takes place. This difference is genetically programmed and provides winter cultivars with a competitive advantage to grow at low temperatures. This results in lengthening the growing season since the plant is able to capitalize on favourable conditions that may occur late in the season. Understanding the complexity of this genetic system and its regulation by environmental factors leading to the increase of FT is still a challenge. Development of FT in plants is a metabolically active process induced by low temperature and is associated with

altered gene expression [9, 33]. Several proteins and their corresponding mRNAs accumulate during cold acclimation. In certain cases, their accumulation was associated with the capacity of plant and tissues to develop FT [11, 24]. Some of these genes are specifically upregulated by low temperature [10, 19, 23, 36] while others are also induced by other factors such as ABA and water stress [14, 16, 18]. Sequence analysis of some of these genes has not revealed any information that improves our understanding of their function. Unlike heat shock, water or salinity stresses, FT is not associated with a universal response. The proteins that accumulate during cold acclimation were first believed to be species-specific but recent results indicate that they are family-specific [11, 36]. We have identified a wheat protein family which is upregulated specifically by low temperature and found it to be expressed only in freezing-tolerant Gramineae species [11]. The kinetics of accumulation and the abundance of these proteins during cold acclimation suggest a close relationship between the development of FT and the amount of these proteins [11]. However, their exact function in FT remains to be established. Since FT is a multigenic trait, the isolation of all the genes involved is required to understand the overall genetical and physiological bases regulating the process of cold acclimation and the induction of FT. In this report, we describe a novel gene regulated specifically by low temperature and associated with the leaf development. This gene, *Wcs19*, is preferentially expressed in green leaf tissues of tolerant Gramineae species and requires both light and low temperature for maximal induction. The possible functions of this gene during cold acclimation of wheat leaves are discussed.

Materials and methods

Plant material and growth conditions

In this study we used three 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, cv. Norstar, 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} −3 °C) rice (*Oriza sativa*, LT_{50} 4 °C), alfalfa (*Medicago falcata* cv. Anik, LT_{50} −12 °C), canola (*Brassica napus* cv. Jet neuf, LT_{50} −16 °C). FT was determined as reported previously [24] and expressed as the temperature required to kill 50% of the seedlings (LT_{50}).

Seeds 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) with a 15 h light period at an irradiance of 250 μmol m^{-2} s^{-1}. Control plants were maintained under the same conditions while cold acclimation was performed by subjecting the seedlings to acclimation conditions (6 °C/2 °C day/night, 10 h photoperiod). In the case of rice exposed to low temperature, the day/night cycle was of 10 °C/5 °C. For ABA treatment, 7-day old seedlings were watered daily for 4 days with nutrient solution containing 0.01 mM ABA. As expected, ABA-treated plants showed a reduced growth rate compared with the control, indicating that ABA elicited the proper physiological response. Water stress was induced by withholding water until plants became visibly wilted (4 days). Heat shock was performed by incubating seedlings at 40 °C for 3 h. This treatment was sufficient to induce typical heat shock proteins as described in our previous work [6]. Salt-stressed plants were grown for 18 h in solutions containing 500 mM NaCl. Anaerobic treatment was accomplished by submerging seedlings under water in an airtight container for 24 h. Wounding was performed by slicing leaves to 1 cm pieces and floating on water for 14 h. Etiolated seedlings were grown in complete darkness. Deacclimation was performed by returning cold-acclimated plants (36 days at 4 °C) to normal growth conditions for 1 and 5 days.

Preparation of calli

The calli used in this study were derived from winter wheat (*T. aestivum* L. cv. Fredrick). Plants

were grown under the same environmental conditions mentioned above, using a mixture of soil/peat/vermiculite (1:1:1; v/v/v) supplemented with a soluble fertilizer (N/P/K 20:20:20). Inflorescences were tagged at the onset of anthesis and the spikes were harvested 12 days after anthesis. The kernels were surface-sterilized as described earlier [4]. The embryos were aseptically isolated and cultured on Murashige and Skoog [21] (MS) medium supplemented with 30 g/l sucrose, 10 μM 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.8% Difco Bacto Agar (pH 5.7). The cultured plates were incubated at 24/20 °C with a 15 h photoperiod under low irradiance (100 μmol m^{-2} s^{-1}). The callus cultures were maintained by subculturing every 2 weeks on the same medium. Low temperature exposure of calli was done at 6/2 °C (day/night).

Construction and screening of the cDNA library

Poly(A)$^+$ RNA was isolated from cold-acclimated winter wheat Norstar [7]. A cDNA library was constructed in λZAPII (Stratagene) using *Eco* RI-*Not* I linkers from Pharmacia, and transformed into *Escherichia coli* strain XL-1 blue. The subtractor kit from Invitrogen was used to generate a cDNA-subtracted probe, prepared from poly(A)$^+$ RNA isolated from cold-acclimated and unacclimated winter wheat plants. The plaques showing an increased hybridization signal with the subtracted probe were selected and purified. The screening of the library and all the recombinant DNA techniques were performed as described [29].

Northern and Southern blot analyses

Poly(A)$^+$ RNA (4 μg) or total RNA (10 μg) samples were mixed with ethidium bromide before electrophoresis on formaldehyde agarose gels, as suggested [28]. This allowed visual evaluation of RNA quality and loads on gels. A clone previously isolated, p*2.1*, that did not display differential hybridization during cold acclimation was also used to verify the equal loading of RNA. After electrophoresis, RNA or DNA was transferred to nitrocellulose membranes (BAS-85, Schleicher & Schuell) in 20× SSC. The filters were air-dried and then baked for 1 h at 80 °C prior to hybridization with the ^{32}P-labelled p *Wcs19* insert [26]. Filters were washed at 65 °C with several buffer changes of decreasing SSC concentration (5 to 0.1×) and then autoradiographed on Kodak XRP films with intensifying screens (DuPont, Cronex Lightning Plus) at −80 °C.

Genomic DNA was prepared from shoots of 8-day old seedlings as previously described [27], and DNA samples (10 μg) were digested with appropriate restriction endonucleases prior to electrophoresis.

DNA sequence analysis

Plasmid DNA was prepared, and deletion subclones were generated using exonucleases III and VII, as described [37]. Plasmids were sequenced by the dideoxynucleotide chain-termination method [30] with the aid of T7 and Gene-ATAQ kits from Pharmacia. Sequence comparison was carried out with the Genetic Computer Group's Sequence Analysis Software package, version 6.0, with a Vax computer (Université de Montréal). The database was searched with the TFASTA program. The hydropathy profile was calculated according to Kyte and Doolittle [13], using a 6 amino acid window. Secondary structure predictions were made by the method of Garnier *et al.* [8].

In vitro *transcription/translation*

p*Wcs19* was linearized by digestion with *Hin*d III and the coding strand was transcribed *in vitro* with T3 RNA polymerase. The reaction volume of 20 μl contained 5 mM $MgCl_2$, 50 mM Tris-HCl pH 7.5, 10 mM DTT, 2 mM spermidine, 10 U of RNase inhibitor, 0.5 mM ATP, 0.5 mM GTP, 0.5 mM CTP, 0.5 mM UTP, 500 ng of linearized

plasmid and 40 U of T3 RNA polymerase (Pharmacia). Nucleic acids were extracted with phenol/chloroform/isoamyl alcohol (25:24:1) and precipitated with ethanol. The pellet was resuspended in TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA) and translated *in vitro* using a wheat germ extract from Promega in presence of ^{35}S-methionine. Translation products were fractionated on SDS-PAGE, dried and exposed to Kodak XOMAT-AR film.

Results

Northern and Southern analyses

The cDNA clone *Wcs19*, for wheat cold-specific gene, was identified using a subtracted probe to screen a cDNA library made from cold-acclimated wheat shoots of cv. Norstar. Based on reprobing the library with the purified insert, the representation was estimated to be 0.02%. The isolated clone hybridizes preferentially to an mRNA of 1.0 kb that accumulates upon exposure to low temperature. The kinetic studies using northern blot analysis (Fig. 1) show that the accumulation of *Wcs19* is very rapid, and remains at a constant level throughout the acclimation period in both freezing-tolerant cultivars, Fredrick (A) and Norstar (B). On the other hand, in the less tolerant cultivar Glenlea (C), the expression of this mRNA declines despite maintaining the plants at 4 °C. When the plants were deacclimated at 24 °C, the steady-state level of *Wcs19* transcripts declined rapidly.

Figure 2 shows that the *Wcs19* mRNA was not induced by water stress, exogenous ABA application or heat shock. Positive controls were used to demonstrate that the treatments elicited the typical molecular responses in addition to the physiological ones described in Materials and methods. Other treatments such as wounding, anaerobic and salt stresses did not show any effect on the expression of *Wcs19* (not shown). These results indicate that the accumulation of *Wcs19* is specifically induced by low temperature.

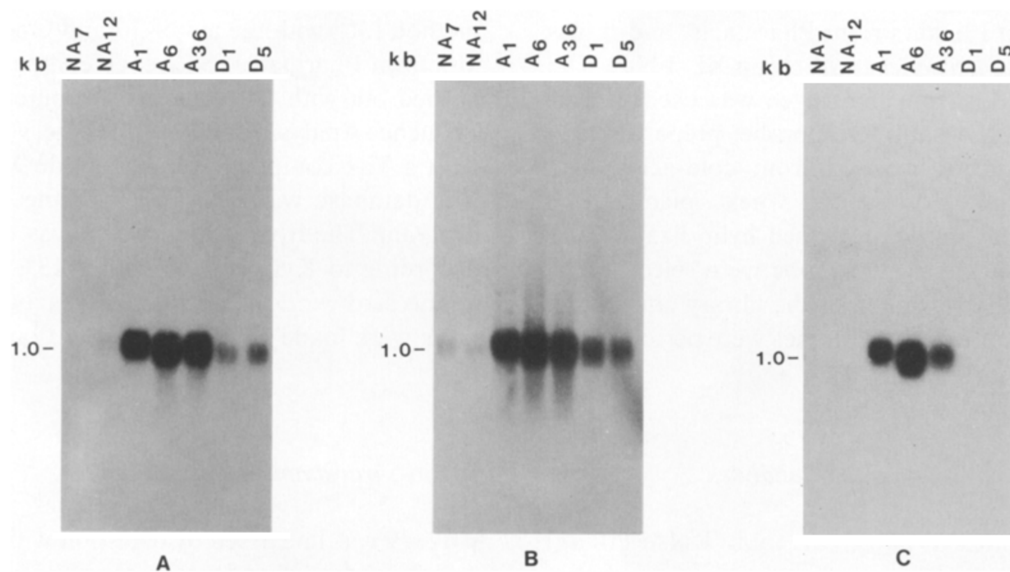

Fig. 1. Kinetic analysis of *Wcs19* mRNA expression during cold acclimation. Poly(A)$^+$ RNA (4 μg per lane) was separated and transferred to nitrocellulose membranes as described in Materials and methods and hybridized with ^{32}P-labelled cDNA insert from p*Wcs19*. NA_7 and NA_{12}, control plants (non-acclimated) grown for 7 and 12 days at 24 °C; A_1, A_6 and A_{36}, plants cold-acclimated for 1, 6 and 36 days; D_1 and D_5, cold-acclimated plants (36 days) were deacclimated for 1 and 5 days at 24 °C. Formaldehyde gels were visualized with ethidium bromide and the clone p*2.1* was used to control the equal loading and quality of RNA (not shown). A. Winter wheat Fredrick. B. Winter wheat Norstar. C. Spring wheat Glenlea.

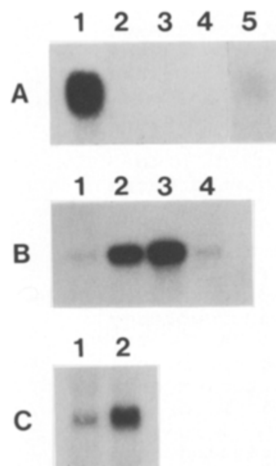

Fig. 2. Northern blot analysis of total RNA (10 μg per lane) from wheat plants (cv. Fredrick) exposed to different treatments. A. The filter was hybridized with *Wcs19*. 1, plants cold-acclimated for one day; 2, plants water-stressed for 4 days; 3, plants treated with 0.01 mM ABA for 4 days; 4, non-acclimated plants grown at 24 °C; 5, plants heat-shocked for 3 h at 40 °C. B. The filter was hybridized with *Wab1*, an ABA-responsive clone isolated from wheat in our laboratory. Lanes 1 to 4 as in A. C. The filter was hybridized with HSP70B cDNA (*Stress*Gen, Victoria, B.C., Canada). 1, plants grown at 24 °C; 2, plants heat-shocked for 3 h at 40 °C.

Southern analysis, shown in Fig. 3, did not reveal any differences in the restriction pattern between the three genotypes. This result suggests that the reduced expression of *Wcs19* in the less tolerant cultivar is not due to a detectable difference in the genomic organization of the gene or in the relative gene copy number since the bands intensities are comparable. At least three strong hybridizing fragments were detected after digestion with 12 enzymes indicating the presence of a putative multigene family.

Sequence analysis

The complete DNA sequence of *Wcs19* is shown in Fig. 4. A long open reading frame (ORF) was found in both DNA orientations. Northern blots were thus probed with labelled RNA produced by *in vitro* transcription of the p*Wcs19* insert using the T3 or T7 promoters and corresponding RNA polymerases. One of these orientations (T7) was complementary to the *Wcs19* mRNA and allowed us to identify the coding strand. The predicted polypeptide is 190 amino acids in length and has a calculated molecular mass of 19 kDa and a pI of 8.8. Search of the GenBank database revealed no homology with any protein. However, at the DNA level, a significant homology was found with a cold-regulated partial DNA sequence (p*T59*) from barley [3]. The sequence analysis indicates that the protein is alanine-rich (21%) and has a high content of glycine (8%), lysine (8%) and

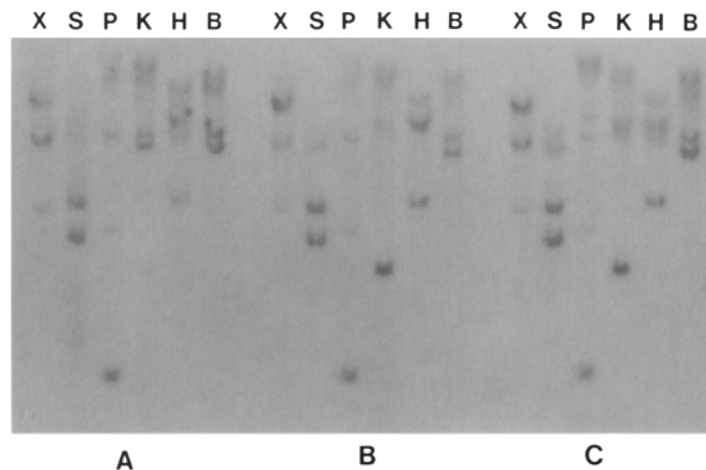

Fig. 3. Southern blot analysis of wheat genomic DNA. Wheat DNA from three genotypes was digested with 6 different restriction enzymes, separated by agarose gel electrophoresis, transferred to nitrocellulose and then probed with *Wcs19*. X, *Xba* I; S, *Sac* I; P, *Pst* I; K, *Kpn* I; H, *Hind* III; B, *Bam* HI. A, Glenlea; B, Norstar; C, Fredrick.

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  1    ttttttttgcgaccaaaatgaacaagtaaatttactccctcacaagc
 49    atatgcaaatatattccaccaagaatattagtcggtcctcgctatcaa
 97    ccacatctaaaaccatgtcaacgaatggaaacaacaccaccttaaaag
145    tatccacacgagaaggctccttatatttgtattaacagaagagcaaaa
193    agatatagctgtatgatttcagcgatccaaatccgcatggtgcagcga
(1)                M  I  S  A  I  Q  I  R  M  V  Q  R
                   α  α  α  α  α  α  α  α  α  α  α  α
241    tgcgcaagactaccatttccaatcggcacacatcctgtctccttccac
(13)   C  A  R  L [P] F [P] I  G  T  H [P] V  S  F  H
       α  ↲  ↲  ϖ  ↲  ϖ  β  β  ϖ  ϖ  ϖ  β  β  β  β  β
289    aacctaccctacccacccatccatcagcagtttttctatcgaccaatg
(29)   N  L [P] Y [P][P] T  H  Q  Q  F  F  Y  R [P] M
       ϖ  ϖ  ↲  ϖ  ϖ  ϖ  ϖ  ϖ  ϖ  β  β  β  β  ϖ  α  ↲
337    gcttcttcttccgtgctgctcggagcctcggccacggccgcgctcacc
(45)   A  S  S  S  V  L  L  G  A  S  A  T  A  A  L  T
       ↲  ↲  ↲  β  β  β  β  β  α  α  α  β  β  β  β  β
385    ggcacccccggcaggcaaggcccttccccggccttgcttcctcgccgct
(61)   G  T [P] A  G  K  A  L [P] R [P] C  F  L  A  A
       ϖ  ϖ  ϖ  ϖ  ϖ  ϖ  ϖ  ϖ  ϖ  ϖ  ↲  α  α  α  α  α
433    cgcccgcgcaccgtgagcggtggccgtctctgcctgcagaacgctcca
(77)   R [P] R  T  V  S  G  G  R  L  C  L  Q  N  A [P]
       ϖ  ↲  β  β  β  β  ↲  β  β  β  β  β  ↲  ↲  ϖ  ϖ
481    agggcgactccggcgtacaacgacgctgcggatgccaccgacaaggcc
(93)   R  A  T [P] A  Y  N (D) A  A (D) A  T (D) K  A
       ↲  ϖ  ϖ  ϖ  ϖ  ϖ  ϖ  α  α  α  α  α  α  α  α  α
529    atcgacggcgtgaagggggtggccgacgagttgaagaagggcgtggcg
(109)  I (D) G  V  K  G  V  A (D)(E) L  K  K  G  V  A
       α  α  α  α  α  α  α  α  α  α  α  α  α  α  α  α
577    gaggctgcggaggccgtctcgggcaacaccgagaaggccgcggaggaa
(125) (E) A  A (E) A  V  S  G  N  T (E) K  A  A (E)(E)
       α  α  α  α  α  α  α  α  α  α  α  α  α  α  α  α
625    gccggcaagggcgcgagcgaggtggacgcgaaggccaaggacttcggc
(141)  A  G  K  G  A  S (E) V (D) A  K  A  K (D) F  G
       α  α  α  α  α  α  α  α  α  α  α  α  α  α  α  α
673    gagcaggcgaagaaggcgacggaggaggcgtgggacggcgccaaggac
(157) (E) Q  A  K  K  A  T (E)(E) A  W (D) G  A  K (D)
       α  α  α  α  α  α  α  α  α  α  α  α  α  α  α  α
721    gccgcacagggcatcacggacaaagtcgccgccgcggccaaaaaggaa
(173)  A  A  Q  G  I  T (D) K  V  A  A  A  A  K  K (E)
       α  α  α  α  α  α  α  α  α  α  α  α  α  α  α  α
769    gctagctaagctaacactacgttgactagtccgatctgtatcgctcaa
(189)  A  S
       α  α
817    ttcattttccattgtaaggaatgcatatacgtatttcggtacaagaga
865    taagatagctgtatttattttctgtgatataggattaccgcactgtta
913    atgtcaaacgcaataaagaaaatgattttt
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Fig. 4. DNA sequence and deduced amino acid sequence of *Wcs19*. The coding strand has been determined using T3 and T7 RNA transcription of *Wcs19* in the Bluescript vector (Stratagene) and subsequent hybridization to RNA from acclimated plants. The longest ORF is shown here (570 nucleotides). The consensus polyadenylation signal is shown as a double underline; proline residues are boxed; acidic residues (D and E) are circled. On a third line, secondary structure predictions were made by the method of Garnier *et al.* [8]. Symbols are: α, α helix; β, β sheet; ↲, turns; ϖ, random coil. GenBank accession number L13437.

proline (7%). These four amino acids represent 44% of the polypeptide. The protein has a particular charge distribution. The acidic amino acids aspartate and glutamate (Fig. 4, circled) are localized towards the C-terminal half and thus give this region a net negative charge of −7. Furthermore, this region (from amino acid 100 to the end) has a high propensity to form an α-helix as observed for some transcription factors [25]. The N-terminal half is rich in proline (boxed), lysine and arginine, and has a net positive charge of + 10. The hydropathy profile (not shown) indicates that most of the protein is hydrophilic except between amino acids 42 and 59.

In vitro transcription/translation experiments with *Wcs19* were performed as described in Materials and methods. Figure 5 shows a specific translation product of 26 kDa (lane WCS19). The apparent molecular mass of 26 kDa is higher than the predicted mass of 19 kDa. This discrepancy

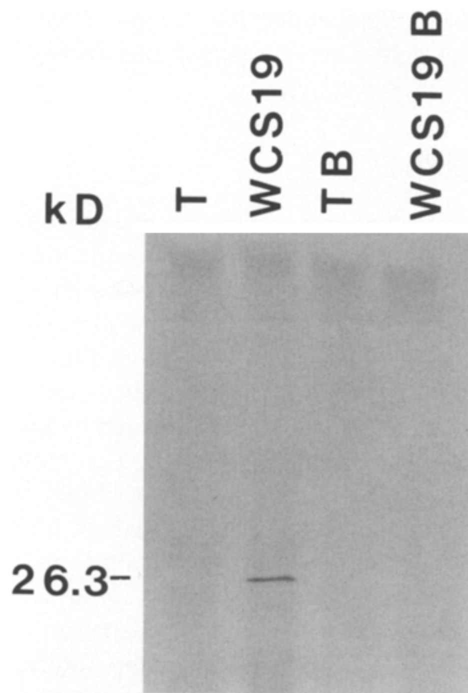

Fig. 5. In vitro transcription/translation of p*Wcs19*. The labelled translation products were separated on SDS-PAGE and visualized by autoradiography. T, translation products of the linearized vector alone; WCS19, translation products of the linearized p*Wcs19*; TB and WCS19B, the translation products were boiled for 10 min.

has already been observed for several other stress proteins [10, 35]. Since three other ATG start codons present in the 5′ region of the cDNA are followed by in-frame stop codons, we determined whether the longest ORF identified was able to encode the same protein. This ORF was subcloned using the polymerase chain reaction and the transcription/translation experiment was repeated with the subclone. The same translation product size was obtained indicating that the ATG at position 205 is the first one that can be used and that the stop codons identified before this ATG cannot be due to sequencing errors. The proper stop codon is found at position 775, and the consensus polyadenylation signal is found at position 924. The product of translation does not remain in solution after boiling as shown in lane WCS19B and is thus different from other alanine- and glycine-rich stress proteins cloned up to date [5, 10, 12, 14, 23].

Tissue and species specificity

Northern blot analyses of *Wcs19* mRNA expression in root, crown and leaf tissues of acclimated wheat seedlings are shown in Fig. 6. The results indicate that the expression of *Wcs19* is specific to leaf tissue. The expression was hardly detected in root and crown. To determine if the expression is strictly associated with the leaf structure, the expression of the gene in non-differentiated tissues such as wheat calli was compared to that of leaves. The results in Fig. 6, lane E, show that no accumulation of mRNA occurs during calli acclimation to low temperature. Furthermore, there was no detectable expression in other tissues such as the flower parts, developing or mature embryos (not shown). These results demonstrate that the *Wcs19* mRNA accumulation is leaf-specific and is not directly needed for the acquisition of FT in other tissues. The accumulation of *Wcs19* mRNA was found to be present in most cereal species that can cold-acclimate and suggests that it may play a crucial role in the leaf acclimation to low temperature. The highest level of accumulation was found in the most tolerant species, wheat and rye, compared to that of barley, a less tolerant species. Oat and rice did not show any accumu-

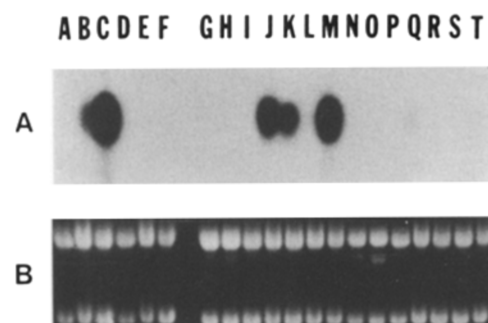

Fig. 6. Tissue and species specificity of *Wcs19* expression. Total RNA (10 μg per lane) was isolated from the different tissues. Plants and calli were cold-acclimated for 6 days. Panel A. Lanes A, B and C, root, crown and leaf tissues of cold-acclimated wheat (cv. Fredrick); lane D, non-acclimated wheat leaves; lanes E and F, cold-acclimated and non-acclimated wheat calli G to M, cold-acclimated species (G, *Brassica*; H, alfalfa; I, rice; J, rye; K, barley; L, oat; M, wheat). Lanes N to T correspond to the non-acclimated tissues of G to M respectively. Panel B. Ethidium bromide-stained gel.

lation of *Wcs19*. On the other hand, the two dicot tolerant plants examined, *Brassica* and alfalfa, did not show any induction. This indicates that the *Wcs19* is Gramineae-specific and that its expression is correlated to the capacity of each genotype to develop FT.

Light requirement

The association of *Wcs19* expression to the leaf tissue and its complete absence in the non-photosynthetic tissues drew our attention to the possibility that light may be required for expression. The results in Fig. 7 show that light is required for maximal mRNA accumulation since etiolated plants accumulated at least 4-fold less *Wcs19* transcripts. Another cold-regulated gene isolated in our laboratory (*Wcor410*) was not affected by the presence or absence of light (Fig. 7B). This result confirms the light stimulation of *Wcs19*. In addition, as for cold-acclimated callus cultures, albino seedlings lacking chloroplastic structures and chlorophyll (generated from some callus cultures) were not able to accumulate any detectable amount of *Wcs19* transcript in the presence of light and low temperature (not shown). These results indicate that the *Wcs19* expression is dependent on organized leaf tissue and that light acts as a stimulating factor.

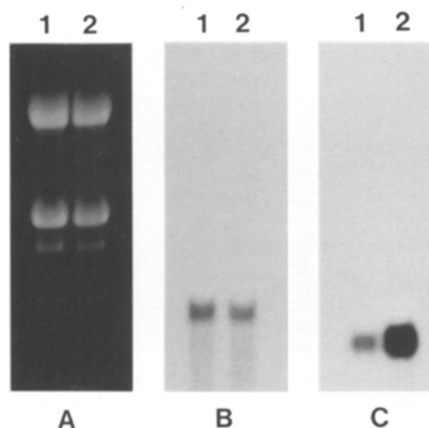

Fig. 7. Light requirement for the expression of *Wcs19*. Total RNA (10 μg per lane) was isolated from etiolated wheat seedlings (cv. Fredrick) cold-acclimated in the dark (lane 1) or in the presence of light (lane 2) for 4 days. A, ethidium bromide-stained gel; B, the filter was hybridized with *Wcor410*, a cold-regulated clone isolated in our laboratory; C, the filter was hybridized with *Wcs19*.

Discussion

Understanding the molecular genetic bases of cold acclimation in wheat requires the identification of genes involved in this complex mechanism. Towards this goal, we have identified and characterized several cDNA clones [10, 11, 23]. The cDNA clone *Wcs19* described here represents a novel gene which is regulated by low temperature and stimulated by light. This clone was identified by screening a cDNA library with a subtracted probe to reduce the high signal of abundant mRNAs and thus facilitate the isolation of new cDNAs. Northern analyses have shown that the mRNA accumulates only when the plants are exposed to low temperature. After 24 hours of exposure, the three genotypes accumulated mRNA to nearly their maximal level indicating that the accumulation is very rapid. The mRNA level remained constant thereafter in the two winter genotypes while it declined in the less tolerant spring genotype after 36 days of low-temperature exposure. This result suggests that a constant level of mRNA may be necessary to allow the accumulation of a sufficient amount of protein required for the development of FT. A similar result was found for other cDNAs cloned in our laboratory [10, 11, 23]. Southern analysis has not shown any differences in restriction patterns between the most tolerant and less tolerant genotypes. One might infer that the promoter structure is different and could not be detected by simple restriction analysis. This possibility is not likely since all genotypes accumulate the *Wcs19* mRNA to similar levels at early stages of cold acclimation. We do not yet understand the mechanism underlying this differential expression.

Our results indicate that *Wcs19* is expressed specifically in tolerant Gramineae species. There was no detectable expression in sensitive Gramineae such as rice or in any of the tolerant dicot species examined. However, this does not

exclude the possibility that proteins with a similar function are present in dicots. Moreover, this gene is not induced by ABA, drought, heat shock, salt, wounding, or anaerobic stresses indicating that the gene is induced specifically by low temperature.

Sequence comparison did not reveal any homology with other published genes or with other genes cloned in our laboratory. Furthermore, we have not found any of the repeated sequences observed in RAB [20], dehydrins [5], or LEA [1]. This suggests that *Wcs19* plays a distinct role in cold acclimation. Several structural properties of this protein are similar to those found in transcription factors. The C-terminal half of the protein contains all the acidic residues with a net charge of −7. In addition, an α-helical structure is predicted for the last 91 amino acids. It has been suggested that an α-helical structure might be an essential element of the activating region of several transcription factors [25]. Furthermore, the acidic character of the activating region is an important feature which allows transcription factors to interact with RNA polymerases and increase transcription rates [25]. It has been shown that the overall RNA polymerase activity increases markedly during cold acclimation [31]. This could contribute to increase the expression of several genes important for the development of FT.

Another important feature of transcription factors is the presence of a positively charged domain. This domain is essential for the interaction with DNA. In WCS19, the N-terminal half is rich in proline (14%) and contains basic residues (lysine and arginine) with a net charge of + 10. Those characteristics are found in transcription factors such as CPRF-1 [34], HBP1-a [32], and CTF/NF-1 [17]. However, detailed experiments are required to confirm this hypothesis.

Our results indicate that *Wcs19* expression is leaf-specific and not directly associated with FT in other plant tissues or callus cultures. It seems that its expression is dependent on leaf tissue organization and on the plant capacity to acclimate at low temperature. The role of leaf during cold acclimation is to provide the energy required for the development of FT. Photosynthesis is responsible for the production of this energy and it has been shown that tolerant cultivars have a higher photosynthetic capacity than the spring ones [2, 22]. This modulation of the photosynthetic apparatus by the tolerant plants to optimize growth at suboptimal conditions is correlated with an increased resistance to photoinhibition [22]. The close correlation of *Wcs19* gene expression with the capacity of leaves to develop FT suggests that this gene may play a role in this process.

The specific expression in the leaf indicates that cell or callus cultures mimic only in part the response of intact plants to low temperature stress. Our results emphasize that the leaf tissue response could be very important to the survival of the whole plant. It appears from these observations, and that of others [3, 15, 36], that we must distinguish between cellular responses to low temperature which may be ubiquitous to all cells from the tissue-specific reactions. This is an important consideration when one wants to improve FT at the whole plant level.

Based on the available properties of the WCS19 protein, we cannot yet assign it a definite role. Currently, we are in the process of expressing the protein in a bacterial system in order to produce a polyclonal antibody that will allow us to determine the exact cellular location of this protein. Furthermore, the production of sense and antisense *Wcs19* in transgenic plants is under way. This may help us to define more precisely the function of WCS19 during low temperature acclimation.

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