Mol Gen Genet (1998) 257: 157–166

ORIGINAL PAPER

A. Vazquez-Tello · F. Ouellet · F. Sarhan

Low temperature-stimulated phosphorylation regulates the binding of nuclear factors to the promoter of *Wcs120*, a cold-specific gene in wheat

Received: 9 June 1997 / Accepted: 18 August 1997

Abstract The *Wcs120* gene encodes a highly abundant protein which appears to play an important role during cold acclimation of wheat. To understand the regulatory mechanism controlling its expression at low temperature, the promoter region has been characterized. Electrophoretic mobility shift assays using short promoter fragments revealed the presence in nuclear extracts from non-acclimated (NA) plants of multiple DNA-binding proteins which interact with several elements. In contrast, no DNA-binding activity was observed in the nuclear extracts from cold-acclimated (CA) plants. In vitro dephosphorylation of these CA nuclear extracts with alkaline phosphatase restored the binding activity. Moreover, okadaic acid (a potent phosphatase inhibitor) markedly stimulated the in vivo accumulation of the WCS120 family of proteins. This suggests that protein phosphatases PP1 and/or PP2A negatively regulate the expression of the *Wcs120* gene. In addition, both Ca^{2+}-dependent and Ca^{2+}-independent kinase activities were found to be significantly higher in the CA nuclear extracts. Western analysis using antibodies directed against protein kinase C (PKC) isoforms showed that a PKCγ homolog (84 kDa) is selectively translocated into the nucleus in response to low temperature. Taken together, our results suggest that, in vivo, the expression of the *Wcs120* gene may be regulated by nuclear factors whose binding activity is modulated by a phosphorylation/dephosphorylation mechanism.

Key words Cold · Phosphorylation · Promoter · Transcription · Wheat

Communicated by H. Saedler

A. Vazquez-Tello · F. Ouellet · F. Sarhan (✉)
Département des Sciences biologiques,
Université du Québec à Montréal,
C.P. 8888, Succursale Centre-Ville,
Montréal, Québec, Canada H3C 3P8
Fax: +1-514-987-4647;
e-mail: sarhan.fathey@uqam.ca

Introduction

During exposure of plants to low temperature, many physiological and biochemical changes occur, leading to the development of freezing tolerance (FT). The survival of tolerant plants at freezing temperatures depends on the timely modulation of specific sets of genes; the accumulation of both mRNAs and protein products of such genes correlates with the development of FT (Guy 1990; Thomashow 1990). A cryoprotective function has been proposed for several low temperature-responsive genes (Kurkela and Franck 1990; Houde et al. 1995; Sieg et al. 1996). However, the exact role that they play in the cells during cold acclimation is still unknown.

The molecular mechanisms governing gene expression at low temperature are not understood. Recent reports suggest a role for calcium as second messenger in the early events following exposure to chilling temperatures (Monroy and Dhindsa 1995; Knight et al. 1996). The Ca^{2+} signal is likely to be transduced by a series of phosphorylation events which may involve Ca^{2+}-dependent protein kinases. However, there is little information regarding the downstream signaling components leading to the activation of specific sets of genes. A major difficulty lies in the fact that many low temperature-responsive genes are also induced by other stimuli, such as drought, abscisic acid (ABA) and salinity. It is still unclear, however, how such different stimuli converge to induce the same gene. For example, it has been reported that low temperature and ABA regulate gene expression through separate transduction pathways (Nordin et al. 1991; Dallaire et al. 1994). An alternative explanation is that several *cis*-acting elements are present in the promoter regions of these genes that respond to multiple factors

Few *cis*-acting elements responsive to low temperature have been identified so far. In the case of the *cor15a* gene of *Arabidopsis thaliana*, Baker et al. (1994) suggested a potential role for G-box-like elements in ABA and drought responsiveness. However, it is

unclear whether these elements also play a role in low-temperature responsiveness. The DR1 core motif (TACCGACAT) in the promoter of the *A. thaliana rd29A* gene is a *cis*-element implicated in the response to dehydration, high salt and low temperature (Yamaguchi-Shinozaki and Shinozaki 1994). A similar low-temperature regulatory element (TGGCCGAC), found in the promoter of the *Brassica napus BN115* gene, contains the pentamer CCGAC motif, which confers low-temperature responsiveness (White et al. 1994b; Jiang et al. 1996). This motif or similar variants is also present in the promoters of *cor15a* (Baker et al. 1994), *rd29A* (Yamaguchi-Shinozaki and Shinozaki 1994), *lti78* and *lti65* genes in Arabidopsis (Nordin et al. 1993) and the *blt4.6* and *blt4.9* genes of barley (White et al. 1994a).

In previous work, we have characterized several low temperature-responsive cDNA clones from wheat. Among these, the *Wcs120* gene is specifically regulated by low temperature (Houde et al. 1992). The encoded 50-kDa protein is the major member of the WCS120 protein family (Houde et al. 1995). Southern analysis indicated that the gene copy number and gene organization are identical in both freezing-tolerant and -sensitive wheat cultivars. On the other hand, the accumulation of both *Wcs120* mRNA and protein was shown to correlate closely with the differential capacity of wheat cultivars to develop FT (Limin et al. 1995). Homologs of *Wcs120* and other cold-regulated genes are present in chilling-sensitive Gramineae species, such as rice and corn, but they are not induced by low temperature (Danyluk et al. 1994). Therefore the inability of these species to develop FT could be due to the absence of signal transduction components or to inefficient *cis*/*trans*-acting factors.

In our efforts to identify the nuclear events that regulate the cold-specific expression of the *Wcs120* gene, we have characterized its promoter region to gain insight into both the *cis*-elements and the nuclear factors that recognize these sequences. Our analyses revealed the presence of DNA-binding proteins in nuclear extracts from non-acclimated plants that interact with elements in this promoter. These factors are inactivated during the exposure of plants to low temperature and can be reactivated by in vitro dephosphorylation. A hypothetical model describing the regulation of the *Wcs120* gene is discussed.

Materials and methods

Plant material and growth conditions

Winter wheat (*Triticum aestivum* L. cv Fredrick) was grown in a controlled environment. Seeds were germinated for 5 days at 24/20° C (day/night) in a mixture of vermiculite, soil and peat. Non-acclimated seedlings were kept at 24° C for 10 days, while those treated for cold acclimation were transferred to 4/2° C (day/night) for 20 days. Leaves were collected at the end of the indicated growth period and immediately used for preparation of nuclei.

Identification of the *Wcs120* promoter

The *Wcs120* cDNA (Houde et al. 1992) was used as a probe to isolate the corresponding genomic clone from a wheat genomic library (Clontech). The promoter region of the gene, identified by Southern analysis, was subcloned into pBluescript vector (Stratagene) and the nucleotide sequence was determined using the T7 sequencing kit (Pharmacia). Primer extension analysis was performed following the procedure of Ausubel et al. (1992). Transient expression experiments were performed by microprojectile bombardment of wheat leaves, using a construct bearing a transcriptional fusion of the full-length promoter to the luciferase reporter gene. Co-transformation with a vector bearing the glucuronidase gene (GUS) driven by the ubiquitin promoter (pAHC27; Christensen and Quail 1996) allowed us to standardize for the inherently variable transformation efficiency. Luciferase activity was determined using the Luciferase Assay System (Promega) and β-glucuronidase activity was determined according to Jefferson et al. (1987).

Isolation of nuclei and preparation of nuclear protein extracts

Nuclei were isolated and purified from leaves using a modification of the procedure described by Nagao et al. (1981). All manipulations were carried out at 4° C. Briefly, 50 g of leaves were ground in a Waring blender in 200 ml of homogenization buffer [1 × basal buffer (25 mM MES pH 6.0, 10 mM $MgCl_2$, 1 mM $CaCl_2$, 20 mM KCl, 25 mM NaCl, 0.1% β-mercaptoethanol), 40% glycerol, 0.6 M sucrose]. After filtration through four layers of cheese cloth, the filtrate was centrifuged for 20 min at 2000 × *g*. The pellets were gently resuspended in 50 ml of 'W' buffer (1 × basal buffer, 25% glycerol, 0.5 M sucrose, 0.01% Triton X-100) and recentrifuged. The resulting pellets were resuspended in 10 ml of 'G' buffer (1 × basal buffer, 0.5 M sucrose, 0.001% Triton X-100) and loaded on two discontinuous Percoll gradients (80%, 50%, 35%, 22.5% and 15% Percoll, prepared in 'G' buffer) and centrifuged for 30 min at 6000 × *g*. The nuclei at the 80/50% Percoll interphase were collected, washed with two volumes of 'G' buffer and centrifuged for 20 min at 5000 × *g*. The purified nuclei were gently and thoroughly disrupted with a pestle in 6 ml of lysis buffer (20 mM HEPES-KOH pH 7.6, 420 mM NaCl, 20% glycerol, 0.5 mM DTT, 0.2 mM EDTA, 0.5 mM PMSF, 15 µg/ml leupeptin) (Allen et al. 1989) and centrifuged for 30 min at 100 000 × *g*. The proteins in the supernatant were precipitated overnight on ice with the addition of solid ammonium sulfate (80% saturation). After centrifugation for 30 min at 100 000 × *g*, the nuclear proteins were resuspended in 500 µl of dialysis buffer (20 mM HEPES-KOH pH 7.8, 50 mM KCl, 20% glycerol, 0.5 mM DTT, 0.2 mM EDTA, 0.5 mM PMSF, 15 µg/ml leupeptin) (Allen et al. 1989), and dialyzed against the same buffer for 5 h. These nuclear extracts (8–10 mg/ml protein) were stored frozen in small aliquots at −80° C.

DNA probes and electrophoretic mobility shift assay (EMSA)

Oligonucleotides were synthesized with a Gene Assembler Plus DNA synthesizer (Pharmacia LKB) and purified on polyacrylamide-urea gels. The DNA primers were used to amplify overlapping fragments of the *Wcs120* promoter: for fragment 120.1, the primers CAGCCCTCTTTATGGGCTAGTCG and TGTGTACTTGCCTTGGTAGTGTGA were used for upstream and downstream annealing, respectively; fragment 120.2, CCTAGTCGGCAGCCACCTGC and CGACTAGCCCATAAAGAGGGCTG; fragment 120.3, ATGCCGACACTTTGGATCTT and GCAGGTGGCTGCCGACTAGG; fragment 120.4, GCACTTCTCAGAATCCTACT and AAGATCCAAAGTGTCGGCAT; fragment 120.5, TTTGCATCCGCGCGGGTATACGT and TGAGAAGTGCTTCGGTAGACC; fragment 120.6, TTGGGACGGCGGCGATCGGCCA and ACGTATACCCGCGCGGATGCAAA. The PCR-amplified fragments were subcloned into pBlue-

script (Stratagene). Plasmids were isolated and the nucleotide sequences confirmed by dideoxy sequencing using the T7 Sequencing kit (Pharmacia). For the EMSA and Southwestern assays, the promoter fragments were radiolabelled by including 2.5 μCi each of [α-^{32}P]dCTP and [α-^{32}P]TTP in the PCR reaction, and then purified on agarose gels. Protein-DNA binding reactions were performed essentially as described by Harter et al. (1994). The 20-μl mixture, containing 5 to 15 μg of nuclear proteins and 3 μg of double-stranded poly(dI-dC) as nonspecific competitor (Pharmacia), was preincubated for 10 min at 4° C. The DNA probe (50 000 cpm) was added and the mixture incubated for 20 min at 4° C. Competition experiments were performed under identical conditions by including unlabelled competitor fragments in the binding reaction prior to the addition of probe. For the dephosphorylation and phosphorylation treatments, the nuclear extracts were preincubated at 28° C for 30 min with 1 unit of immobilized alkaline phosphatase (Sigma), or with 5 mM ATP and 2 mM sodium metavanadate (a phosphatase inhibitor) before the addition of the probes. The DNA-protein complexes were resolved on a 4% polyacrylamide gel prepared in TCE buffer (10 mM Tris-HCl pH 7.9, 3 mM sodium citrate, 1 mM EDTA) containing 8% glycerol, after a 1 h prerun at 180 V. Electrophoresis was carried out for 3 h at the same voltage in TCE buffer at 4° C (Ausubel et al. 1992). Gels were dried and exposed to X-ray films at −80° C.

Southwestern assays

Nuclear proteins (15 μg) from NA and CA plants were either phosphorylated or dephosphorylated as for the EMSA assays, separated by SDS-PAGE, and then blotted onto nitrocellulose membranes. For protein renaturation, the membranes were submerged in the binding buffer described by Harter et al. (1994) containing 6 M urea for 10 min and transferred sequentially to fresh binding buffer with decreasing concentrations of urea (3, 1.5 and 0.75 M) for 10 min each, followed by two final rinses in binding buffer alone. The membranes were blocked in binding buffer containing 5% non-fat dry milk for 30 min, briefly rinsed twice with binding buffer containing 0.25% non-fat dry milk, and incubated overnight at 4° C without shaking in buffer (200 μl/cm^2) containing 10 μg/ml sheared herring sperm DNA and radiolabelled probe (>10^6 cpm/ml). After three 5-min washes in cold binding buffer containing 0.25% non-fat milk, the membranes were exposed to X-ray film at −80° C.

Determination of endogenous kinase activity

To reveal qualitative changes in the phosphorylation pattern following cold treatment, the nuclear extracts from both NA and CA plants (10 μg protein) were incubated for 5 to 40 min at 30° C in binding buffer (Harter et al. 1994) containing 1 mM $MgCl_2$, 50 μCi [γ-^{32}P]ATP and 1 mM $CaCl_2$. At specified intervals, reactions were stopped by adding one volume of 2 × SDS-sample buffer and the proteins were separated by SDS-PAGE. Gels were dried, exposed to X-ray film and the signal intensity of individual bands was determined by quantitative densitometry. To quantify the Ca^{2+}-dependent and Ca^{2+}-independent nuclear kinase activities accurately, the incorporation of ^{32}P into histone III-S, casein or the PKC-specific substrate peptide MARCKS (Biomol; Blackshear 1993) was assessed as described elsewhere (Kitano et al. 1986). Histone III-S and casein are suitable substrates for phosphorylation assays, particularly when dealing with a crude mixture of uncharacterized protein kinases.

In vivo okadaic acid treatment and Western analyses

Wheat shoots were cut and immediately incubated in water or in the presence of the phosphatase inhibitor okadaic acid (OA) at 10, 100 or 1000 nM. The plants were left at room temperature for 4 h to allow translocation of the inhibitor, then transferred to 4° C for 24 h, and the total soluble proteins were extracted as described (Limin et al. 1995). For Western analysis, proteins from total soluble extracts (10 μg) were separated by SDS-PAGE, electroblotted onto nitrocellulose membranes and probed with the anti-WCS120 antibody following procedures described previously (Houde et al. 1995). The signal intensity of individual protein bands on the X-ray films was quantified with a Personal Densitometer SI (Molecular Dynamics) using ImageQuaNT version 4.2 software. The results are presented as the integrated intensity of all the pixels in each band, excluding the background. Similarly, the detection of PKC protein was done by Western analysis of total soluble extracts and nuclear extracts (10 μg proteins) from NA and CA plants, using individual mammalian (rabbit) polyclonal antibodies specific to isoforms α, β, γ, δ, ε, and ζ of PKC (Boehringer Mannheim).

Results

Characterization of the *Wcs120* promoter region

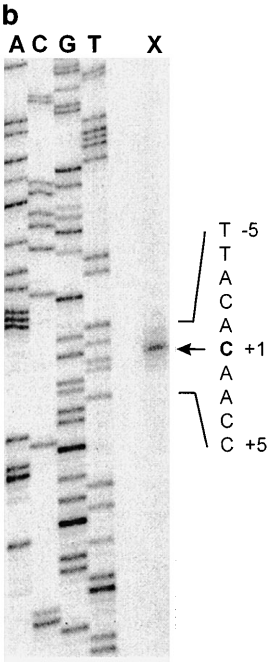
The 5′ region of the *Wcs120* gene, comprising 942 bp of sequence upstream from the ATG translation start site, was sequenced (Fig. 1a). Primer extension analysis revealed a transcription initiation start site 82 nucleotides upstream of the ATG codon (Fig. 1b). This nucleotide was assigned the position +1, and all the elements discussed in the text are numbered accordingly. The sequence analysis (Fig. 1a) showed a TATA box-like motif at position −34 and several repeated motifs were also found. The element CACCTGC is repeated three times, while the CANNTG consensus motif is repeated eight times. The core CANNTG motif is also present in the promoter region of many genes that are regulated by a variety of environmental and physiological stimuli, including light-regulated and ABA-responsive genes (Guiltinan et al. 1990; Williams et al. 1992). This core sequence was identified as the preferred binding site for the common plant regulatory factors (CPRFs), the G-box binding factors (GBFs) belonging to the basic leucine zipper (bZIP) class of proteins, and for the b-HLH (basic helix-loop-helix) family of transcription factors proteins which play a key role in cell progression and developmental gene regulation (Davis et al. 1990; Weisshaar et al. 1991; Anthony-Cahill et al. 1992; Harter et al. 1994; Kusano et al. 1995). Another element, CACTCAC, is repeated twice and has been identified as the recognition site for GCN4 and Zeste factors, which play a direct role in enhancing gene transcription in yeast and Drosophila, respectively (Thireos et al. 1984; Chen et al. 1992). Interestingly, the pentamer CCGAC, which has been reported to be essential for the low-temperature responsiveness of the *BN115* gene from *B. napus* (Jiang et al. 1996) is repeated twice in the *Wcs120* promoter. Several other repeated elements are present but do not show significant homology to known motifs: CGTCGG, repeated five times; GGGTATA, twice; and ACTACCA, twice. There is also a major direct repeat, found between −810 and −703, which contains two 52-bp elements separated from each other by 4 bp. The core motif ACGTCC present in this repeat was reported to be recognized by

Fig. 1a, b Promoter of the low-temperature-responsive *Wcs120* wheat gene. **a** Nucleotide sequence of the promoter region. The region corresponding to the mRNA is shown in *lower case letters* up to the translation initiation codon ATG. The TATA box is located at −34 bp. The repeated motifs and putative *cis*-elements are *underlined* and discussed in the text.
b Determination of the mRNA transcription start site. Lanes A, C, G, T contain sequencing reactions performed using the primer used for extension analysis. Lane X, extension products. The longest extension product is indicated with an *arrow* and was assigned the position +1. The corresponding transcription start site is shown in *bold* in the nucleotide sequence of the coding strand. Genbank Accession Number: AFO 31235

a

```
-860 AAACCACGGG TTTTTGGCCG GATCCGTGGC GGGGGACGAC
-820 AACGCGGTCA GTCGCGGCAG AGGCGGCGTC GGACATCGGG
-780 CCGTTCACGT CCGCGGTGTC GGACGGGGAC GGTGAGATGC
-740 GGTGTCGAAC GTCGGGCCGT TCACGTCCGC GTCGTCGGAC
-700 GGGCACGGTG AGATGCGGCG TCGGGCGGGG TTGGGACGGC
-660 GGCGATCGGC CAGTTGGAAA AATGGAACGG GAGGAGCATG
-620 ATCGCCGGGC GGGCGAGAAG ATCATGCAAC TGCCTCTTTT
-580 TTCCCGTACA CGGGCGATGC CTTTTTTTTT GCATCCGCGC
-540 GGGTATACGT CGTCGGACCT GTATGTACAA TAGAAGGTGG
-500 GTATATCGTT TCCTTCATAT GGCCATTCTG CCCTTCTACA
-460 TTTTGTTGGG GGTCTACCGA AGCACTTCTC AGAATCCTAC
-420 TGTATAAAAT TATTTCGAAT CAAAGCCCTA AGCCTCTCGT
-380 ATGCTTCTTC TAGTTACTCT CATAGTCTCA TTGTCGTTAC
-340 ATGCCGACAC TTTGGATCTT CCATCCTCTT AAGCAAACAA
-300 TACTACCATT TTTGCAAGAG AAAAGAATCA TCTTCTTCCC
-260 GGACAAGGAC GAATGAGCTG GGACGTGGCG ACCCGGACGC
-220 GCCACTGGCT TCAGAGGCCC GGCCCCCCTA GTCGGCAGCC
-180 ACCTGCCGAC CACTGATGCG ACCACACGTA GCTCCCAGCC
-140 GCGGCGATTC GTCCATCTGA CCAGCCCTCT TTATGGGCTA
-100 GTCGGCACTC ACCTGCCCAT CCACTCACGA GCGCGCACGT
 -60 CGTGGTTCGT ATACCCTCCA ACGGCCTATA AATACTGCGT
 -20 CGCGCTGCAT ATGCTTTACA caaccacctg cttcacacta
 +21 ccaaggcaag tacacagcag caatacgtag tagatttccc
 +61 gagtgaggag ctcagcgcaa gatg
```

b



mlip15, a bZIP protein induced by low temperature in maize (Kusano et al. 1995). This observation opens up the possibility that a bZIP protein may be involved in *Wcs120* gene regulation. Sequence comparison between the *Wcs120* promoter and those of several genes regulated by either low temperature, drought, salinity or ABA did not reveal significant homologous regions (Baker et al. 1994; Ouellet et al. 1994; White et al. 1994b; Yamaguchi-Shinozaki and Shinozaki 1994), except in the case of the barley homolog *Dhn5* (Close et al. 1995).

To confirm that the *Wcs120* promoter is indeed responsive to low temperature, transient expression experiments were performed by microprojectile bombardment of wheat leaves using a construct bearing the full-length promoter fused to the luciferase reporter gene. The results showed that luciferase activity was on average eightfold higher in the transformed leaves exposed to 4° C compared to the leaves maintained at 25° C. In contrast, the luciferase activity driven by the ubiquitin promoter did not increase at low temperature. The results of a more detailed analysis of transient expression, using different deletion constructs of the promoter, will be published elsewhere.

Nuclear proteins from non-acclimated plants interact with sequences of the *Wcs120* promoter

In order to investigate the interactions of nuclear DNA-binding factors with elements in the *Wcs120* promoter, six overlapping fragments (100–160 bp) spanning approximately 700 bp of the promoter region (Fig. 2) were amplified by PCR and used as probes in gel retardation experiments. DNA-protein complexes with different relative mobilities were detected when the six fragments

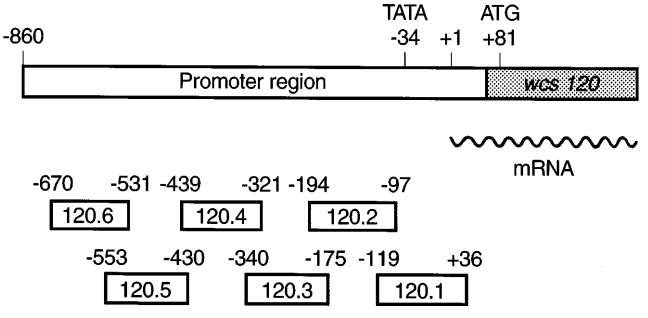


Fig. 2 Schematic representation of the *Wcs120* promoter region. The map shows the relative position of the six overlapping promoter fragments used as probes in EMSA and Southwestern experiments. Positions of the different elements are indicated relative to the transcription initiation start site (+1)

were incubated with nuclear extracts from NA plants (Fig. 3, NA, lanes " − "). These differences in mobility suggest that the binding proteins are of different sizes and/or that the DNA-protein complexes have different conformations. In spite of the presence of similar motifs (putative *cis* elements) in the different promoter fragments, competition experiments performed with non-labelled fragments indicated that the nuclear proteins bind each fragment in a specific manner. The differences in the relative mobility of the complexes and the specificity of the interactions indicate that the promoter region binds several and distinct nuclear proteins at normal growth temperature. In contrast, no detectable complexes were formed when the same probes were incubated with nuclear extracts from CA plants (Fig. 3, CA, lanes " − "). This lack of DNA-binding activity in the CA nuclear extracts may result either from the absence, or from the in vivo inactivation of the nuclear DNA-binding factors during the acclimation of wheat plants at 4° C.

In vitro dephosphorylation restores DNA-binding activity of proteins in CA extracts

To discover the basis of the differences in pattern of DNA-protein interactions between NA and CA nuclear extracts, we performed experiments to test whether the binding factors are modulated by phosphorylation or dephosphorylation. The results in Fig. 3 (NA, lanes AP and ATP) show that, in the NA extracts, the DNA-binding activities for all probes except 120.2 were not significantly affected by alkaline phosphatase (AP) nor ATP treatments. The ATP treatment partially inhibited binding to fragment 120.2, observed as an accumulation of unbound probe (Fig. 3b, NA, lane ATP). In contrast, when the CA extracts were dephosphorylated with AP, most of the DNA-binding activities were restored (Fig. 3, CA, lanes AP). These reactivated nuclear factors produced complexes with mobilities similar to, or slightly different from those observed in untreated NA extracts. In the case of the CA extracts treated with ATP and the phosphatase inhibitor Na-metavanadate, a partial restoration of binding was observed. This partial restoration could be the result of the phosphorylation of the nuclear factors by the active endogenous kinases, or of the incomplete inhibition of unknown phosphatases, or both. These results indicate that the DNA-binding factors in the nuclear extracts from NA plants are in a dephosphorylated state and probably interact in vivo with elements in this promoter at normal growth temperature. The inability of ATP to stimulate phosphorylation (and inactivate the DNA-binding factors) in the NA extracts may be due to the absence and/or inactivation in vivo of a particular protein kinase(s) in the nucleus at normal growth temperature. Furthermore, we suggest that the preferential binding of these factors at normal temperature may result in the transcriptional repression of the *Wcs120* promoter.

To determine whether the DNA-binding factors that are regulated by protein kinases and phosphatases are homodimeric or heterodimeric in nature, Southwestern analyses were performed. The results in Fig. 4 show that at least five distinct DNA-binding proteins in the NA extracts interact with elements present in fragment 120.4. Their binding capacity was not significantly modified by phosphorylation or dephosphorylation, as found in EMSA experiments (Fig. 4, NA, lanes AP and ATP). In the CA extracts, only two out of the five proteins could bind to the DNA probe, indicating that they may be unrelated to low-temperature regulation since proteins of similar molecular weight from both NA and CA extracts also bind to the other promoter fragments. Therefore, at least three DNA-binding proteins were presumably inactivated in vivo during cold acclimation. The binding capacity in the CA extracts was not restored by in vitro AP pretreatment (Fig. 4, CA, lane AP). This is in contrast with the EMSA results (Fig. 3d), which clearly showed that dephosphorylation can restore the DNA-binding activity. However, we cannot rule out the possibility that under the conditions used, the factors may not have been renatured to their active conformation. Since the Southwestern technique allows the detection of the primary DNA ligands of homodimeric but not heterodimeric proteins, it is possible that the phosphorylation target is a regulatory subunit(s)

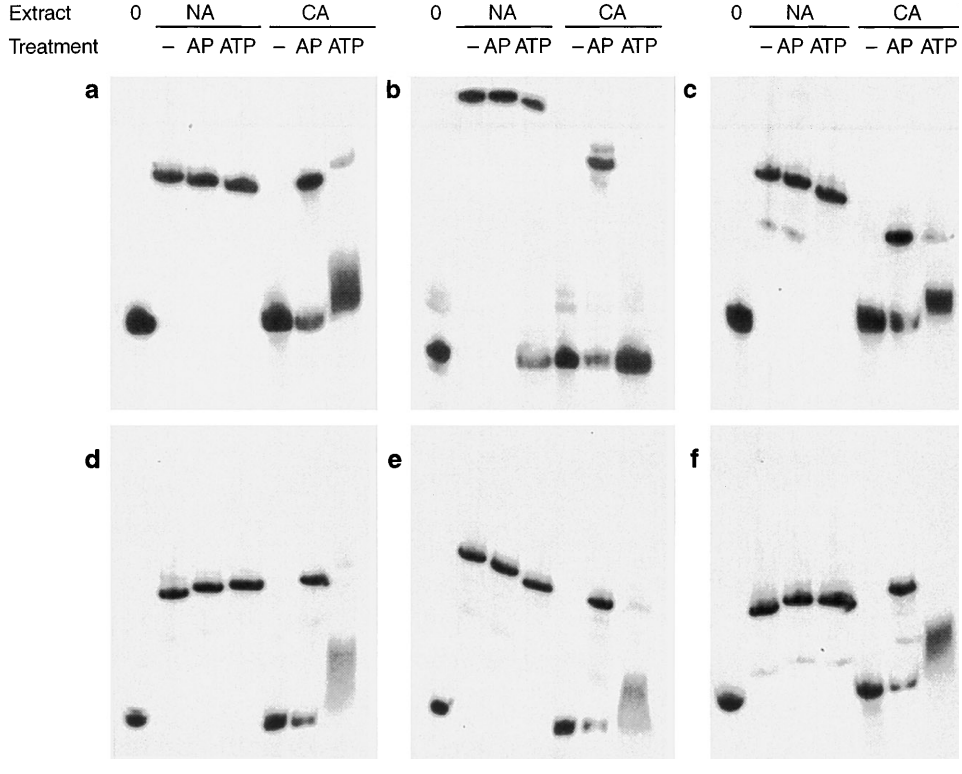


Fig. 3a–f Electrophoretic mobility shift assays showing the binding activity of *Wcs120* promoter fragments with nuclear factors from non-acclimated (NA) and cold-acclimated (CA) extracts. The nuclear extracts (15 μg of protein) were pre-incubated in binding buffer alone (–), with immobilized alkaline phosphatase (AP) or with ATP and the phosphatase inhibitor sodium metavanadate (ATP). The ^{32}P-labelled probes were added and the complexes were resolved on Tris-citrate-EDTA native polyacrylamide gels. Free probe (0). The positions of the probes with respect to the promoter region are indicated in Fig. 2. **a** Probe 120.1. **b** Probe 120.2. **c** Probe 120.3. **d** Probe 120.4. **e** Probe 120.5. **f** Probe 120.6

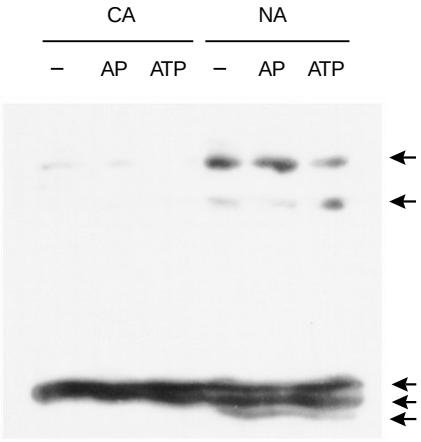


Fig. 4 Southwestern analysis showing the binding of nuclear proteins from non-acclimated (NA) and cold-acclimated (CA) plants to probe 120.4. The nuclear extracts (15 μg of protein) were pre-incubated in binding buffer (–), with alkaline phosphatase (AP) or with ATP and the phosphatase inhibitor sodium metavanadate (ATP). The proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. The proteins were then renatured by successive washes in decreasing concentrations of urea and incubated with the labelled probe. The membranes were washed and exposed to X-ray film

associated with the DNA-binding subunits. Taken together, these data, though intriguing, suggest that the factors regulated by protein kinases and phosphatases revealed by EMSA may be heterodimeric proteins. It is well established that many DNA-binding proteins of the bHLH and plant bZIP classes of proteins selectively form DNA-binding heterodimers (Armstrong et al. 1992; Davis et al. 1990).

Nuclear kinase activity is stimulated by cold acclimation

In order to find an explanation for the differences in phosphorylation state of the nuclear factors in the NA and CA extracts, endogenous kinase activity was determined. The results in Fig. 5a show that protein kinase activity was significantly higher in the nuclear CA extracts than in the NA extracts, with a major difference being noted in the phosphorylation of a 24-kDa protein. Densitometric quantification showed that incorporation of ^{32}P was sixfold higher in CA extracts compared to NA extracts. The possible implication of Ca^{2+}-dependent and Ca^{2+}-independent protein kinases in the phosphorylation of nuclear proteins was determined.

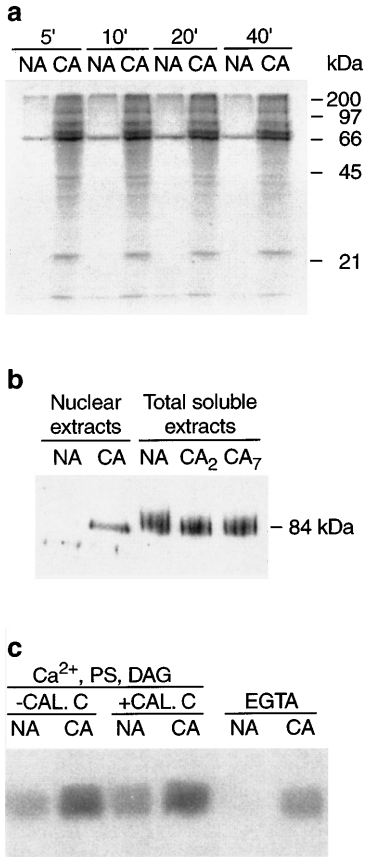


Fig. 5a–c Analysis of endogenous kinases in nuclear extracts from non-acclimated (NA) and cold-acclimated (CA) wheat plants. **a** In vitro phosphorylation of nuclear proteins. Equal amounts of nuclear proteins (10 μg) were incubated with [γ-^{32}P]ATP and separated by SDS-PAGE. The gel was dried and exposed to an X-ray film. **b** Immunodetection of a PKCγ homolog in nuclear and soluble extracts from non-acclimated (NA) and cold-acclimated (CA) plants. Equal amounts of proteins (10 μg) were separated by SDS-PAGE, blotted onto nitrocellulose membranes and probed with the anti-PKCγ antibody. CA2 and CA7 indicate soluble extracts from plants cold-acclimated for 2 and 7 days, respectively. **c** PKC activity was measured in the nuclear extracts with the peptide MARCKS as the PKC-specific substrate and containing the indicated chemicals. Calphostin C (CAL.C) and EGTA concentrations are 0.1 μM and 0.5 mM, respectively. Aliquots of each reaction were separated by SDS-PAGE and visualized by autoradiography. PS, phosphatidylserine; DAG, diacylglycerol

The results in Table 1 show that the amount of ^{32}P incorporated into histone by the action of both Ca^{2+}-independent and Ca^{2+}-dependent kinase activities was significantly higher in the CA extracts compared to the

Table 1 Ca^{2+}-independent and Ca^{2+}-dependent protein kinase activities in nuclear extracts from non-acclimated (NA) and cold-acclimated (CA) plants

Protein substrate	Ca^{2+}-independent[a]		Ca^{2+}-dependent[a]	
	NA	CA	NA	CA
Histone-III-S	16034 ± 4703*	29929 ± 2660*	1236 ± 1618*	10233 ± 3865*
Casein	15186 ± 1223	17235 ± 758	14127 ± 1255*	24494 ± 1331*

[a] Values are expressed as the amount of ^{32}P incorporated in cpm. Each datum represents the mean (± S.E.) from three replicates. This experiment was repeated three times with similar results each time
* Significant at $P < 0.05$, Student's t-test

NA extracts. A similar increase in the incorporation of ^{32}P into casein catalyzed by Ca^{2+}-dependent kinase activity was observed in the CA extracts relative to the NA extracts (Table 1). The overall stimulation of kinase activity in the CA extracts supports our hypothesis that the DNA-binding factors are inactivated by phosphorylation in vivo.

Cold acclimation induces the accumulation of a PKCγ-like protein in the nucleus

The increase in kinase activity in the nuclear CA extracts could result from the activation and/or the translocation of cytoplasmic kinases into the nucleus when the plants are exposed to low temperature. To test this hypothesis, antibodies directed against six mammalian isoforms of protein kinase C (PKC) were used to detect wheat PKC homologs. A signal was obtained only with the anti-PKCγ antibody, which cross-reacted with an 84-kDa protein (Fig. 5b). While this protein was equally abundant in the total protein extracts prepared from both NA and CA plants, its relative abundance in the nuclear CA extract was 22-fold higher than in the nuclear NA extract. To confirm this observation, the PKC-like activity was assayed in the nuclear extracts using the PKC-specific substrate peptide MARCKS (Blackshear 1993). The results in Fig. 5c show that the CA extracts phosphorylate the substrate at higher levels than the NA extracts. The protein kinase involved is stimulated by Ca^{2+}, PS and DAG, thus suggesting the presence of a PKC-like activity in the CA extracts. The addition of 0.5 mM EGTA resulted in only partial inhibition of the phosphorylation of MARCKS. This incomplete inhibition could be due to the insufficient chelation of Ca^{2+} by EGTA under the standard experimental conditions used. The addition of Calphostin C at 0.1 μM, a concentration at which mammalian PKC activity is inhibited (Lee and Yang 1996), did not significantly inhibit the MARCKS phosphorylation. However, complete inhibition of phosphorylation, as determined by ^{32}P incorporation and scintillation counting, was obtained when 0.5 μM Calphostin C was added to the reaction. Together, these results indicate that during cold acclimation of wheat plants, a putative PKCγ homolog is selectively translocated from the cytosol into the nucleus. While this observation correlates with the increased Ca^{2+}-dependent kinase activity in the CA extracts, it remains to be determined whether a PKCγ homolog is involved in the phosphorylation and inactivation of the nuclear transcription factors that interact with the *Wcs120* promoter.

Okadaic acid stimulates the accumulation of the WCS120 family of proteins in vivo

To determine the role of protein phosphatases in the expression of the *Wcs120* gene, wheat seedlings were incubated at 4° C in the presence of okadaic acid (a potent and specific inhibitor of protein phosphatases PP1 and PP2A) (Smith and Walker 1996). Soluble proteins were extracted and the accumulation of the WCS120 family of proteins was analyzed by quantitative densitometry of Western blots. The results showed a significant increase in the levels of the 50-, 66- and 200-kDa proteins at 4° C (Fig. 6a, lane 2) compared to 25° C (Fig. 6a, lane 1). When the plants were exposed to low temperature in the presence of different concentrations of OA (Fig. 6a, lanes 3, 4 and 5), accumulation of these proteins was significantly stimulated. At the highest concentration tested (1 μM), the densitometric analysis revealed a 2.2-fold increase in amounts of the 50- and 66-kDa proteins with respect to the plants exposed to low temperature without inhibitor (Fig. 6a, lane 2 and Fig. 6c). The stimulated accumulation of the 200-kDa protein was even more striking, showing a 9-fold increase (Fig. 6c). These experiments suggest that, in vivo, the accumulation of the WCS120 protein family at low temperature is negatively regulated by PP1 and/or PP2A phosphatases, which antagonize the effects of protein kinases on the state of phosphorylation of the putative repressors, and hence modulate their DNA-binding activity.

Discussion

Our work provides evidence that several factors present in nuclear extracts from non-acclimated wheat plants interact with elements found in the promoter of the cold-inducible *Wcs120* gene. Based on these results, we postulate that the regulation of *Wcs120* gene expression in response to low temperature involves specific interactions of multiple nuclear factors. The preferential DNA-binding activity present in nuclear extracts from non-acclimated plants (24° C) is thought to repress gene transcription. Conversely, when the plants are exposed to low temperature (4° C) for cold acclimation, the nuclear factors are inactivated, allowing transcription to take place. This temperature-dependent pattern of transcriptional activation/repression suggests the participation of nuclear proteins whose DNA-binding activity may be modulated by post-translational modifications such as phosphorylation (Clark and Docherty 1993; Hunter and Karin 1992).

The mobility shift assays performed with different promoter fragments showed that only proteins from the NA extracts produced DNA-protein complexes. In vitro dephosphorylation of the CA nuclear extracts restored the DNA-binding activity, suggesting that during cold acclimation of wheat in vivo, these factors are probably inactivated by phosphorylation. Indeed, both Ca^{2+}-dependent and Ca^{2+}-independent kinase activities were significantly higher in CA extracts than in NA extracts. This low-temperature-stimulated kinase activity in the CA nuclear extracts may contribute to the phosphory-

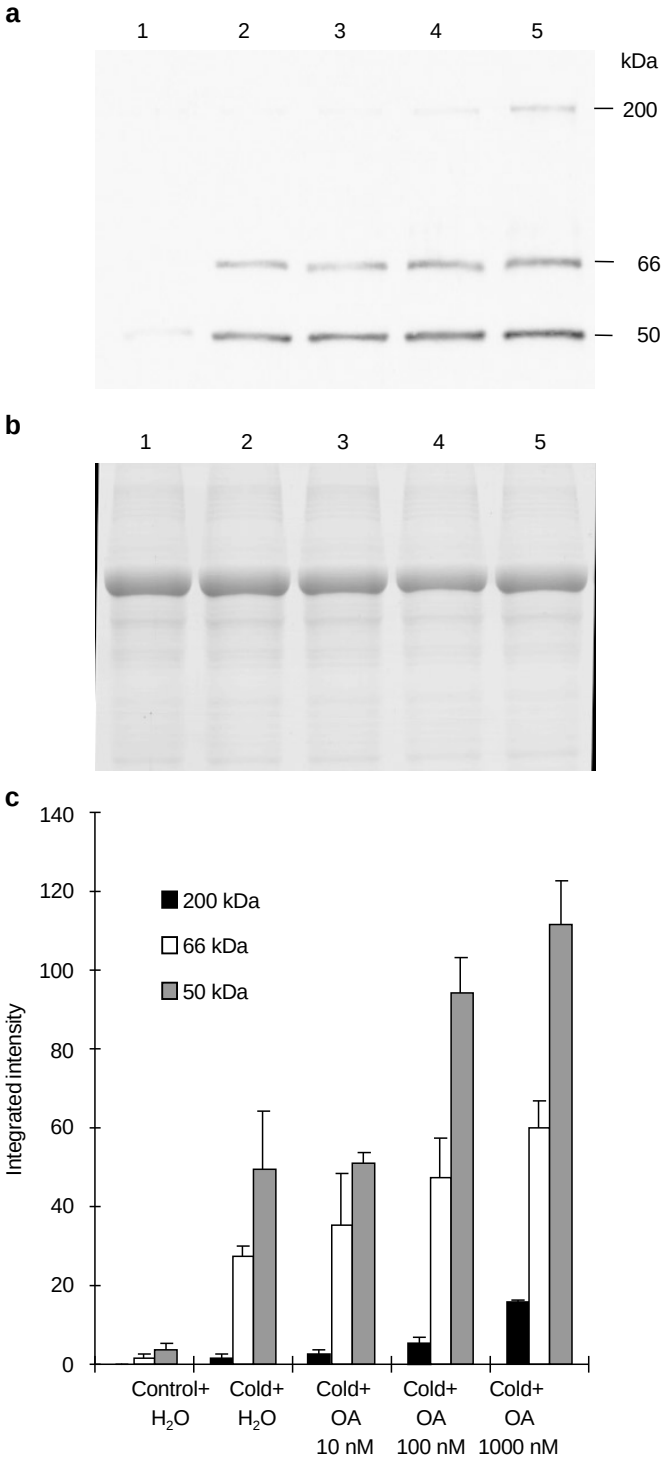


Fig. 6a–c Effect of in vivo okadaic acid treatment on the accumulation of the cold-inducible WCS120 family of proteins. Wheat seedlings were incubated at low temperature without or with different concentrations of okadaic acid (OA), and soluble proteins were extracted and separated by SDS-PAGE. **a** Immunodetection of the WCS120 protein family in the soluble extracts. Following the treatment, the proteins were analyzed by Western blotting using the anti-WCS120 antibody. **b** SDS-PA gel stained with Coomassie blue showing equal protein loading. **c** Densitometric quantification of the individual protein bands of 50, 66 and 200 kDa on the immunoblot shown in **a**. The 50-kDa protein is encoded by the *Wcs120* gene. Values represent averages and standard deviations of the integrated intensity of the pixels for each protein band, excluding the background, from at least three independent experiments

lation of the putative repressors. In support of these observations, several studies have demonstrated that phosphorylation can negatively affect the binding of nuclear factors to DNA (Datta and Cashmore 1989; Hunter and Karin 1992; Tjaden and Coruzzi 1994). It has been reported that Ca^{2+}-dependent protein kinases may play a role in signal transduction during the early events following exposure to low temperature (Monroy and Dhindsa 1995; Knight et al. 1996). Our analysis of the pattern of protein phosphorylation in both nuclear extracts suggests the participation of both Ca^{2+}-independent and Ca^{2+}-dependent protein kinases, which possibly became activated and/or were translocated into the nucleus in response to low temperature. However, the identity of the nuclear kinases that are involved in the inactivation of the putative repressors at low temperature remains to be determined. In this work, we determined that a PKCγ homolog is present in similar abundance in the cytosol of both NA and CA plants but is selectively translocated into the nucleus in response to low temperature. Similarly, it was reported that PKCγ may be activated and targeted to the nucleus in mammalian T-cells treated with the tumor-inducer phorbol ester (Avraham et al. 1994). Consistent with this observation, the CA nuclear extracts phosphorylated the PKC-specific substrate MARCKS at higher levels than the NA extracts in the presence of the activators Ca^{2+}, PS and DAG. However, relatively high concentrations of Calphostin C were required to inhibit this PKC-like activity, which is in agreement with the observations reported by Subramaniam et al. (1997). Presumably the plant PKC is less sensitive to Calphostin C than is the mammalian PKC. Indirect evidence for the existence of a PKC homolog in plants has been published recently (Nanmori et al. 1994; Xing et al. 1996; Subramaniam et al. 1997).

The phosphorylation status of proteins is governed by the complementary activities of protein kinases and phosphatases (Hunter and Karin 1992). Therefore, we expected that the in vivo inactivation of protein phosphatase activity would shift the equilibrium state of the putative repressors towards the phosphorylated state because protein kinase activity would then predominate. This shift in phosphorylation would decrease the DNA-binding capacity of the repressors and hence inactivate them, as our EMSA experiments have suggested. The overall effect on gene regulation is expected to result in increased accumulation of the 50-kDa WCS120 protein. Our in vivo experiments with okadaic acid, which specifically inhibits phosphatases PP1 and PP2A (Smith and Walker 1996), support the data obtained from the EMSA experiments, and suggest that PP1 and/or PP2A may act as negative regulators of *Wcs120* gene expression.

The presence of multiple and distinct *cis*- and *trans*-acting elements suggests a complex mechanism of transcriptional regulation of the *Wcs120* gene, such as those reported for light-regulated promoters (Schindler and Cashmore 1990; Weisshaar et al. 1991). In the case of the

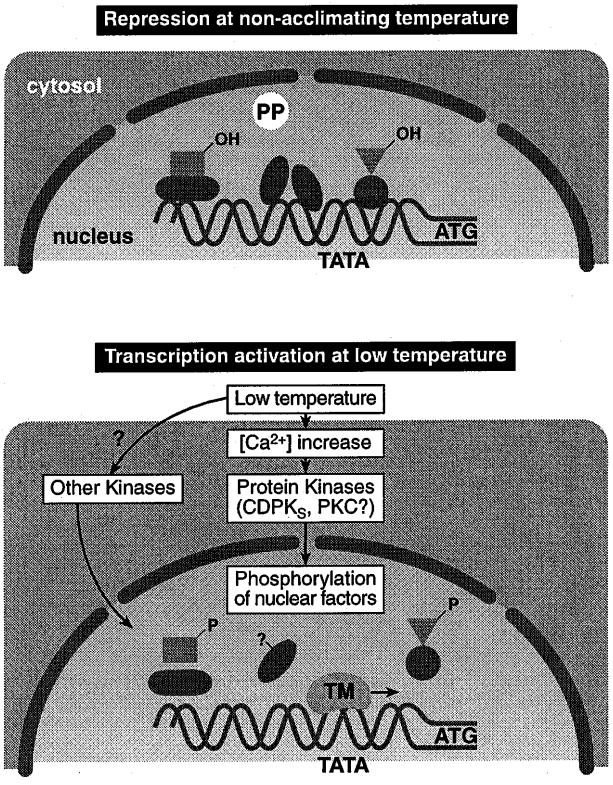


Fig. 7 Hypothetical model describing how temperature shifts regulate the expression of low-temperature-responsive genes

CAB gene, Schindler and Cashmore (1990) characterized five nuclear proteins involved in its light-mediated regulation. However, the authors observed that the DNA-binding factors were equally active in extracts from both dark- and light-adapted tobacco plants, thus preventing the establishment of a physiological relationship between in vitro DNA-binding activity and in vivo gene regulation. Similarly, the nuclear factors which interact with the DR1 motif (TACCGACAT) present in the promoter of the *rd29A* gene are active in extracts prepared from both high-salt-stressed and control plants (Yamaguchi-Shinozaki and Shinozaki 1994). In contrast, our data provide strong evidence that the interaction of the nuclear repressors with the promoter of the low-temperature-responsive *Wcs120* gene is regulated by a temperature shift. Although we suggest that repressor factors bind specifically to their cognate elements, we cannot rule out the possibility that positive transcription factors may also act as repressors (Davis et al. 1990; Sakamoto et al. 1996). These factors may act as repressors by binding near, or overlapping with, the binding site of the RNA polymerase complex, thus interfering with the assembly of the transcriptional machinery (Clark and Docherty 1993; McBryant et al. 1995). Based on the data presented in this report, we propose in Fig. 7 a working model that describes the possible regulation of gene expression by low temperature. Under non-acclimated conditions, protein phosphatases such as PP1 and/or PP2A maintain the putative repressors in a dephosphorylated state; they can thus bind actively to the *Wcs120* promoter. The lower nuclear kinase activity at 25° C (compared to 4° C) suggests that some protein kinases are downregulated or absent from the nucleus. The repressor factors may interfere with the assembly of the general transcriptional machinery perhaps by binding near or at the site of the TATA box, resulting in the repression of *Wcs120* expression. When the plant is exposed to low temperature, it is suggested that the signal is sensed and transduced into an increase in cytosolic Ca^{2+}, leading to a series of phosphorylation events mediated by Ca^{2+}-dependent protein kinases. Our evidence indicates that a cytosolic PKCγ homolog is selectively translocated into the nucleus during cold acclimation. The low temperature signal also up-regulates nuclear Ca^{2+}-independent kinases. We postulate that these low temperature-activated protein kinases phosphorylate, and hence inactivate, the repressor proteins, which are released from the promoter region. However, other post-translational modifications leading to the inactivation of DNA-binding factors cannot be ruled out. This derepression would allow the transcriptional machinery to assemble, thus activating gene transcription. Cloning and functional characterization of the transcription factors, PKC and the protein phosphatases regulating the expression of *Wcs120* will help us to understand this complex mechanism of regulation.

Acknowledgements We are grateful to Claude Daniel for his help in the figures' preparation. This work was supported by a "Natural Sciences and Engineering Research Council of Canada" research grant (A7199) to F. Sarhan. F. Ouellet was supported by a Ph.D. scholarship from the "Fonds pour la Formation de Chercheurs et l'Aide à la recherche, Gouvernement du Québec".

References

Allen RD, Bernier F, Lessard PA, Beachy RN (1989) Nuclear factors interact with a soybean b-conglycinin enhancer. Plant Cell 1:623–631

Anthony-Cahill SJ, Benfield PA, Fairman R, Wasserman, ZR, Brenner SL, Stafford WF, Altenbach C, Hubbell WL, Degrado WF (1992) Molecular characterization of helix-loop-helix peptides. Science 255:979–983

Armstrong GA, Weisshaar B, Hahlbrock K (1992) Homodimeric and heterodimeric leucine zipper proteins and nuclear factors from parsley recognize diverse promoter elements with ACGT cores. Plant Cell 4:525–537

Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (1992) Short protocols in molecular biology (2nd edn). Greene Publishing Associates and John Wiley and Sons, New York

Avraham H, Erdos GW, Gazitt Y (1994) Alpha, gamma, delta, epsilon and zeta isoforms in AGF T-cells: nodification during PMA-induced differentiation. Inter J Oncology 5:237–241

Baker SS, Wilhelm KS, Thomashow MF (1994) The 5′ region of *Arabidopsis thaliana cor15a* has *cis*-acting elements that confer cold-, drought- and ABA-regulated gene expression. Plant Mol Biol 24:701–713

Blackshear PJ (1993) The MARCKS family of cellular protein kinase C substrates. J Biol Chem 268:1501–1504

Chen JD, Chang CS, Pirrotta V (1992) Conserved DNA binding and self-association domains of the Drosophila zeste protein. Mol Cell Biol 12:598–608

Christensen AH, Quail PH (1996) Ubiquitin promoter-based vectors for high level expression of selectable and/or screenable marker genes in monocotyledonous plants. Transgenic Res 5:213–218

Clark AR, Docherty K (1993) Negative regulation of transcription in eukaryotes. Biochem J 296:521–541

Close TJ, Meyer NC, Radik J (1995) Nucleotide sequence of a gene encoding a 58.5 kDa barley dehydrin that lacks a serine tract. Plant Physiol 107:289–290

Dallaire S, Houde M, Gagné Y, Saini HS, Boileau S, Chevrier N, Sarhan F (1994) ABA and low temperature induce freezing tolerance via distinct regulatory pathways in wheat. Plant Cell Physiol 35:1–9

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

Datta N, Cashmore AR (1989) Binding of a pea nuclear protein to promoters of certain photoregulated genes is modulated by phosphorylation. Plant Cell 1:1069–1077

Davis RL, Cheng PF, Lassar AB, Weintraub H (1990) The MyoD binding domain contains a recognition code for muscle-specific gene activation. Cell 60:733–746

Guiltinan MJ, Marcotte WR, Quatrano RS (1990) A plant leucine zipper protein that recognizes an abscisic acid responsive element. Science 250:267–271

Guy CL (1990) Cold acclimation and freezing stress tolerance: role of protein metabolism. Annu. Rev. Plant Physiol. Plant Mol Biol 41:187–223

Harter K, Kircher S, Frohnmeyer H, Krentz M, Nagy F, Schafer E (1994) Light-regulated modification and nuclear translocation of cytosolic G-box binding factors in parsley. Plant Cell 6:545–559

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

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

Hunter T, Karin M (1992) The regulation of transcription by phosphorylation. Cell 70:375–387

Jefferson RA, Kavanagh TA, Bevan MW (1987) GUS fusions: β-glucuronidase as a sensitive and versatile gene fusion marker in higher plants. EMBO J 6:3901–3907

Jiang C, Iu B, Singh J (1996) Requirement of a CCGAC cis-acting element for cold induction of the *BN115* gene from winter *Brassica napus*. Plant Mol Biol 30:679–684

Kitano T, Go M, Kikkawa U, Nishizuka Y (1986) Assay and purification of protein kinase C. Methods Enzymol 124:349–352

Knight H, Trewavas AJ, Knight MR (1996) Cold calcium signaling in Arabidopsis involves two cellular pools and a change in calcium signature after acclimation. Plant Cell 8:489–503

Kurkela S, Franck M (1990) Cloning and characterization of a cold- and ABA-inducible *Arabidopsis* gene. Plant Mol Biol 15:137–144

Kusano T, Berberich T, Harada M, Suzuki N, Sugawara K (1995) A maize DNA-binding factor with a bZIP motif is induced by low temperature. Mol Gen Genet 248:507–517

Lee SC, Yang SD (1996) Calphostin C induces tyrosine dephosphorylation/inactivation of protein kinase FA/GSK-3α in a pathway independent of tumor promoter phorbol ester-mediated down-regulation of protein kinase C. J Cell Biochem 60:121–129

Limin AE, Houde M, Chauvin LP, Fowler DB, Sarhan F (1995) Expression of the cold-induced wheat gene *Wcs120* and its homologs in related species and interspecific combinations. Genome 38:1023–1031

McBryant SJ, Kassavetis GA, Gottesfeld JM (1995) Repression of vertebrate RNA polymerase III transcription by DNA-binding proteins located upstream from the transcription start site. J Mol Biol 250:315–326

Monroy AF, Dhindsa RS (1995) Low-temperature signal transduction: induction of cold acclimation-specific genes of alfalfa by calcium at 25°C. Plant Cell 7: 321–331

Nagao RT, Shah DM, Eckenrode VK, Meagher RB (1981) Multigene family of actin related sequences isolated from a soybean genomic library. DNA 1:1–9

Nanmori T, Taguchi W, Kinugasa M, Oji Y, Sahara S, Fukami Y, Kikkawa U (1994) Purification and characterization of protein kinase C from a higher plant, *Brassica campestris* L. Biochem Biophys Res Comm 203:311–318

Nordin K, Heino P, Palva T (1991) Separate signal pathways regulate the expression of a low-temperature-induced gene in *Arabidopsis thaliana* (L.) Heynh. Plant Mol Biol 16:1061–1071

Nordin K, Vahala T, Palva ET (1993) Differential expression of two related, low-temperature-induced genes in *Arabidopsis thaliana*. Plant Mol Biol 21:641–653

Ouellet F, Houde M, Sarhan F (1994) Characterization of the promoter regions of two cold-induced genes from wheat. Meeting abstract, Annual Meeting of the Canadian Federation of Biological Societies, Montréal, Canada

Sakamoto A, Omirruleh S, Nakayama T, Iwabuchi M (1996) A zinc-finger-type transcription factor WZF-1 that binds to a novel *cis*-acting element of histone gene promoters represses its own promoter. Plant Cell Physiol 37:557–562

Schindler U, Cashmore AR (1990) Photoregulated gene expression may involve ubiquitous DNA binding proteins. EMBO J 9:3415–3427

Sieg F, Schröder W, Schmitt JM, Hincha DK (1996) Purification and characterization of a cryoprotective protein (cryoprotein) from the leaves of cold-acclimated cabbage. Plant Physiol 111:215–221

Smith RD, Walker JC (1996) Plant protein phosphatases. Annu Rev Plant Physiol Plant Mol Biol 47:101–125

Subramaniam R, Despres C, Brisson N (1997) A functional homolog of mammalian protein kinase C participates in the elicitor-induced defense response in potato. Plant Cell 9:653–664

Thireos G, Penn MD, Greer H (1984) 5′ untranslated sequences are required for the translational control of a yeast regulatory gene. Proc Natl Acad Sci USA 81:5096–5100

Thomashow MF (1990) Molecular genetics of cold acclimation in higher plants. Adv Genet 28:99–131

Tjaden G, Coruzzi GM (1994) A novel AT-rich DNA binding domain protein that combines an HMG 1-like DNA binding domain with a putative transcription domain. Plant Cell 6:107–118

Weisshaar B, Armstrong GA, Block A, Da Costa e Silva O, Hahlbrock K (1991) Light-inducible and constitutively expressed DNA-binding proteins recognizing a plant promoter element with functional relevance in light responsiveness. EMBO J 10:1777–1786

White AJ, Dunn MA, Brown K, Hughes MA (1994a) Comparative analysis of genomic sequence and expression of a lipid transfer protein gene family in winter barley. J Exp Bot 45:1885–1892

White TC, Simmonds D, Donaldson P, Singh J (1994b) Regulation of *BN115*, a low-temperature-responsive gene from winter *Brassica napus*. Plant Physiol 106:917–928

Williams ME, Foster R, Chua NH (1992) Sequences flanking the hexameric G-box core CACGTG affect the specificity of protein binding. Plant Cell 4:485–496

Xing T, Higgins VJ, Blumwald E (1996) Regulation of plant defense response to fungal pathogens: two types of protein kinases in the reversible phosphorylation of the host plasma membrane H^+-ATPase. Plant Cell 8:555–564

Yamaguchi-Shinozaki K, Shinozaki K (1994) A novel cis-acting element in an Arabidopsis gene is involved in responsiveness to drought, low-temperature or high-salt stress. Plant Cell 6:251–264