

cDNA Sequence, Expression, and Transcript Stability of a Cold Acclimation-Specific Gene, *cas18*, of Alfalfa (*Medicago falcata*) Cells¹

Lawrence A. Wolfrain, Robert Langis, Hugh Tyson, and Rajinder S. Dhindsa*

Department of Biology, McGill University, 1205 Avenue Docteur Penfield, Montreal, Quebec H3A 1B1, Canada

The nucleotide sequence of a full-length cDNA, the deduced amino acid sequence, and the regulation of expression of a cold acclimation-specific gene, *cas18*, in cell suspension cultures of a freezing-tolerant cultivar of alfalfa (*Medicago falcata* cv Anik) have been determined. The deduced polypeptide, CAS18, is relatively small (17.6 kD), is highly hydrophilic, is rich in glycine and threonine, and contains two distinctive repeat elements. It exhibits homology with members of the LEA/RAB/dehydrin family of proteins, which accumulate in response to abscisic acid (ABA) or water stress. It is intriguing that *cas18* is induced by neither ABA nor water stress. The *cas18* cDNA hybridizes to three transcripts of 1.6, 1.4, and 1.0 kb, and the cDNA characterized here corresponds to the 1.0-kb transcript. The expression of this gene is about 30-fold greater in cold-acclimated cells than in nonacclimated cells. Although the accumulation of transcripts during cold acclimation is relatively slow, their disappearance during deacclimation is dramatically rapid, becoming undetectable in less than 5 h. Studies of nuclear run-on transcription show that cold acclimation enhances the transcription of this gene nearly 9-fold. The stability of *cas18*-detectable transcripts during deacclimation is considerably increased if transcription is inhibited with cordycepin. It therefore appears that low temperature regulates the expression of *cas18* at both the transcriptional and posttranscriptional levels.

During the past 5 years, cold-induced genes have been cloned from several plants, including alfalfa (*Medicago falcata*) (Mohapatra et al., 1988, 1989), *Arabidopsis* (Hajela et al., 1990; Kurkela and Franck, 1990; Gilmour et al., 1992), barley (Cattivelli and Bartels, 1990; Dunn et al., 1991), maize (Christie et al., 1991), tomato (Schaffer and Fischer, 1988), and winter wheat (Houde et al., 1992). We have previously reported (Mohapatra et al., 1989) the isolation of three cDNA clones (pSM784, pSM2201, pSM2358) from a pBR322 library constructed with mRNA of cold-acclimated seedlings of a freezing-tolerant cultivar of alfalfa that are specifically expressed in response to low temperature. Their level of expression shows a high positive correlation with the degree of

freezing tolerance developed in different cultivars of alfalfa in response to low temperature.

In this paper, we report the characterization of a full-length cDNA clone, pAcs784 (alfalfa cold-specific), isolated from a cDNA library constructed in λ Uni-Zap XR by using pSM784 as probe. We show that the corresponding *cas* (cold acclimation-specific) gene, *cas18*, is expressed abundantly in cell-suspension cultures and is regulated at both the transcriptional and posttranscriptional levels. The physiological significance of *cas18* is discussed.

MATERIALS AND METHODS

Plant Material

Seedlings of a freezing-tolerant alfalfa cultivar (*Medicago falcata* cv Anik) were grown for 2 weeks under sterile conditions. Callus cultures were initiated by transferring 1-cm hypocotyl segments to solid B₅h medium (Atanassov and Brown, 1984). After 3 weeks, a portion of friable callus was transferred to liquid B₅h medium on an orbital shaker to establish cell-suspension cultures, which were subcultured every 3 weeks. Cell-suspension cultures were grown at 25/20°C (day/night) under a 16-h photoperiod and a light intensity of 250 $\mu\text{E m}^{-2} \text{s}^{-1}$.

Cold Acclimation and Deacclimation

Cell-suspension cultures in log phase of growth were cold acclimated at 5/2°C (day/night) under a 12-h photoperiod. Cold acclimation was assessed by measuring the freezing survival of the cells at several freezing temperatures by the TTC reduction assay (Towill and Mazur, 1974) and then determining the LT_{50} values. Cells that had not been frozen served as a control in the TTC reduction assay. Deacclimation was administered by returning the cold-acclimated cells to 25°C.

RNA Extraction and cDNA Cloning

Total RNA was extracted by the guanidinium isothiocyanate/cesium chloride method, and poly(A)⁺ RNA was fractionated by affinity chromatography on oligo(dT)-cellulose

¹ Work supported, in part, by Natural Sciences and Engineering Research Council of Canada grant A2724, and by Fonds pour la Formation de Chercheurs et l'Aide à la Recherche (Quebec) grant AS-2740 to R.S.D. L.A.W. was supported by a Canadian Pacific Biotechnology Fellowship.

* Corresponding author; fax 1-514-398-5069.

Abbreviations: LT_{50} , freezing temperature at which 50% of the cells survived; TTC, 2,3,5-triphenyltetrazolium chloride.

(Maniatis et al., 1982). RNA to be used for northern hybridization was extracted by the single-step method (Chomczynski and Sacchi, 1987) except that 5 volumes of the extraction buffer were used. RNA extracted from seedlings cold acclimated for 7 d served as the source material for cDNA cloning. A cDNA library was constructed using the vector λ Uni-Zap XR (Stratagene), and the manufacturer's protocols were followed except that treatment with methylmercurichydroxide was used to relax RNA secondary structure prior to synthesis of the first-strand cDNA. The primary library was screened using the previously isolated (Mohapatra et al., 1989), cold acclimation-specific, partial-length cDNA clone pSM784, labeled to high specific activity with [α - 32 P]dCTP by nick translation (Maniatis et al., 1982).

Northern Blot Analysis

Total RNA was size-fractionated by electrophoresis in 1.5% agarose gels containing 0.66 M formaldehyde as denaturant in 1 \times Mops/EDTA buffer (Fourney et al., 1988), transferred to nitrocellulose or nylon (GeneScreen Plus, Dupont, Wilmington, DE) membranes, and fixed by cross-linking in a U.V. Stratalinker (Stratagene). Northern blots were prehybridized overnight in a solution of 6 \times SSC, 1% SDS, 5 \times Denhardt's solution, and 100 μ g mL $^{-1}$ of denatured calf thymus DNA in a shaking water bath at 65°C. Then, 500 ng of the heat-denatured, nick-translated probe, pAcs784, were added to the hybridization bag and hybridization was carried out overnight at 65°C. Blots were washed twice, 15 min each time, at room temperature in a solution of 2 \times SSC, 0.1% SDS; this was followed by a wash in the same buffer at 65°C for 30 min. Finally, the blots were washed twice for a total of 1 h in a solution of 0.2 \times SSC, 0.1% SDS at 65°C. Blots were exposed to Kodak O-Mat XAR film for autoradiography as described (Maniatis et al., 1982), without intensifying screens. Several different exposure times were used to ensure that the signals were within the linear response range of the film. Autoradiographs were analyzed by scanning densitometry using an LKB (Piscataway, NJ) gel scanner and a beam width of 4000 μ m.

Subcloning and Sequencing

Inserts were recovered as pBluescript phagemids and DNA sequences were determined in both directions using Sequenase Version 2.0 (United States Biochemical Corp.) and synthetic oligonucleotides. DNA and amino acid sequences were analyzed using PC Gene software (Intelligenetics, Mountain View, CA). Amino acids regarded as functionally equivalent are: Ala, Gly; Thr, Ser, His; Arg, Lys; Leu, Ile, Val, Met; Tyr, Phe, Trp; Glu, Asp; and Asn, Gln.

A computer search of the GenBank data base was carried out for the nucleotide sequence of the coding strand and the amino acid sequence using the FASTA program (Pearson and Lipman, 1988). Multiple alignment was achieved using the Clustal V alignment package (Higgins and Sharp, 1988), and relationships among amino acid sequences were established through optimum alignments (Tyson, 1992).

The hydropathic index and the predicted secondary structure of the putative protein product of the *cas18* gene were

obtained with the Sequaid program (D. Rhodes and D. Roufa, Kansas State University, Manhattan).

Transcription in Isolated Nuclei

Nuclei were isolated according to Watson and Thompson (1988), and the DNA content was estimated according to Giles and Meyers (1965). Nuclei equivalent to 30 μ g of DNA were resuspended in 200 μ L of transcription mixture: 100 mM (NH $_4$) $_2$ SO $_4$, 30 mM Tris-Cl, pH 8.0, 7 mM MgCl $_2$, 50 mM KCl, 500 μ M each of CTP, GTP, ATP, 100 μ M phosphocreatine, 20 μ g/mL of creatine phosphokinase, 3 mM β -mercaptoethanol, 0.5 mCi [α - 32 P]UTP (3000 Ci mmol $^{-1}$, ICN), and 120 units of RNA Guard (Pharmacia). In vitro transcription was performed as described (DeLisle and Crouch, 1989).

RNA transcribed in vitro from isolated nuclei was hybridized to cDNA clones immobilized onto nitrocellulose filters. The clones used were: (a) pAcs784, the cold acclimation-specific clone under study; (b) pUb, a ubiquitin cDNA clone from sea urchin that is constitutively expressed in alfalfa, and (c) p2.1, an unidentified cDNA clone from wheat that is constitutively expressed in wheat (Houde et al., 1992) and alfalfa (Dhindsa et al., 1992; A.F. Monroy, F. Sarhan, and R.S. Dhindsa, unpublished data). Filters were washed and subjected to autoradiography. Quantitation of signals was obtained by scanning densitometry as described above for northern analysis. Background hybridization to the cloning vector, pBluescript, was subtracted.

Determination of Transcript Decay

Decay of transcript levels was examined following blockage of in vivo transcription with cordycepin. Cordycepin (3'-deoxyadenosine) is a specific inhibitor of transcriptional elongation and has been used successfully in plant systems to analyze the half-lives of various transcripts (Fritz et al., 1991). Preliminary experiments were conducted on the effects of different concentrations of cordycepin on the uptake of [3 H]-uridine and on its incorporation into TCA-precipitable material. These experiments showed that cordycepin at 200 μ g mL $^{-1}$ had little effect on the uptake of [3 H]uridine but inhibited in vivo transcription completely within 8 h at 5°C. Thus, an 8-h treatment with 200 μ g mL $^{-1}$ of cordycepin was used in further experiments reported here.

Cell cultures, cold acclimated for 7 d, were divided into two lots; cordycepin was added to one of these to 200 μ g mL $^{-1}$. Both lots of cultures were maintained for a further 8 h at 4°C and then each lot was subdivided into two parts. One part was maintained at low temperature and the other was transferred to 25°C. The decline, with time, in *cas18* transcripts occurring at 4 or 25°C in the absence or presence of cordycepin was determined by northern hybridization as described above.

RESULTS

Cold Acclimation of Cell-Suspension Cultures

Cold acclimation-induced development of freezing tolerance is shown in Table I. There is a progressive increase in freezing tolerance (decreased *LT* $_{50}$) with increasing duration

Table 1. Comparison of freezing survival values for cell-suspension cultures cold acclimated and/or deacclimated for various time periods

Cell-suspension cultures of alfalfa were cold acclimated at 5/2°C (day/night) for 0, 7, 14, and 21 d. An identical culture was cold acclimated for 21 d and then deacclimated at 25/20°C (day/night) for 3 d. Survival was estimated by the TTC method.

Days Cold Acclimated	Days Deacclimated	LT ₅₀ °C
0	0	-4.18 ± 0.82
7	0	-6.03 ± 0.15
14	0	-8.14 ± 1.44
21	0	-9.74 ± 0.65
21	3	-4.79 ± 0.12

of cold acclimation. These results are in agreement with previous reports on cold acclimation of alfalfa cell-suspension cultures (Orr et al., 1985). Deacclimation results in a decline in freezing tolerance levels to those seen for nonacclimated cultures. Thus, cell cultures used by us in this investigation appear to be an appropriate system for molecular studies of cold acclimation.

Isolation and Sequence Analysis of pAcs784 (cas18)

A total of 200,000 plaque-forming units from the cDNA library were screened using pSM784 as probe, and 138 positives were isolated. From the latter, 40 were selected at random. Several of these were sequenced and those that appeared truncated or to have double inserts were rejected. Finally, the clone pAcs784 was selected for further characterization. The nucleotide sequence of the coding strand of the cloned Acs784 cDNA and the deduced amino acid sequence are shown in Figure 1. This cDNA is 1019 bp in length, and the longest open reading frame, including the stop codon, starts at nucleotide 246 and ends at nucleotide 749. The 5' untranslated region contains an in-frame stop codon starting at nucleotide 215. The longest open reading frame can encode a polypeptide (CAS18) containing 167 amino acids and with a predicted molecular mass of 17,572.5 D and an isoelectric point of 6.64. CAS18 contains two types of repeat elements, a nonapeptide with the sequence Met(Val)-Asp(Glu)-Lys-Ile-Lys-Glu-Lys-Ile-Pro (underlined in Fig. 1), and a (Gly-Thr)_n repeat with n = 3 to 5 (double underlined in Fig. 1). Each of these repeat elements occurs three times in the CAS18 sequence. CAS18 is relatively rich in Gly (21%) and Thr (12.5%).

The predicted hydrophathy profile and the conformational features of CAS18 are shown in Figure 2. The polypeptide is largely hydrophilic (Fig. 2, upper) and is predicted to be a peripheral or soluble protein. It is predicted to be more than 45% in extended β-sheet and more than 42% in α-helical conformation (Fig. 2, lower). The rest consists of turns (loops) and random coil.

A search of the GenBank database was conducted for both the DNA and protein sequences. At the nucleotide level, cas18 shows maximum homology, 56% in an overlap of 398

bases, to the *Tas14* gene of tomato, which is induced by drought, salinity, and ABA (Godoy et al., 1990), and to *rab21* (also called *rab16A* or *dh21*), a gene from rice that is induced by drought and ABA (Mundy and Chua, 1988), about 50% in an overlap of 150 bases. At the amino acid level, CAS18 shows maximum homology to the LEA/RAB/dehydrin family of proteins, greatest with dehydrins and lowest with LEA proteins. A multiple alignment of CAS18 with nine members of the LEA/RAB/dehydrin family of proteins (Fig. 3) shows the existence of two boxes of highly conserved regions of amino acid sequences. The first conserved box is located between amino acids 62 and 76 and the second between 131 and 147 in CAS18. The absence of multiple Ser residues in CAS18 is noteworthy.

Kinetics of Transcript Accumulation

The time-course of accumulation of *cas18*-detectable transcripts during cold acclimation and of their disappearance during deacclimation, as determined by RNA gel blot analysis, is shown in Figure 4. It can be seen from Figure 4A that pAcs784 probe binds to three transcripts of 1.6, 1.4, and 1.0

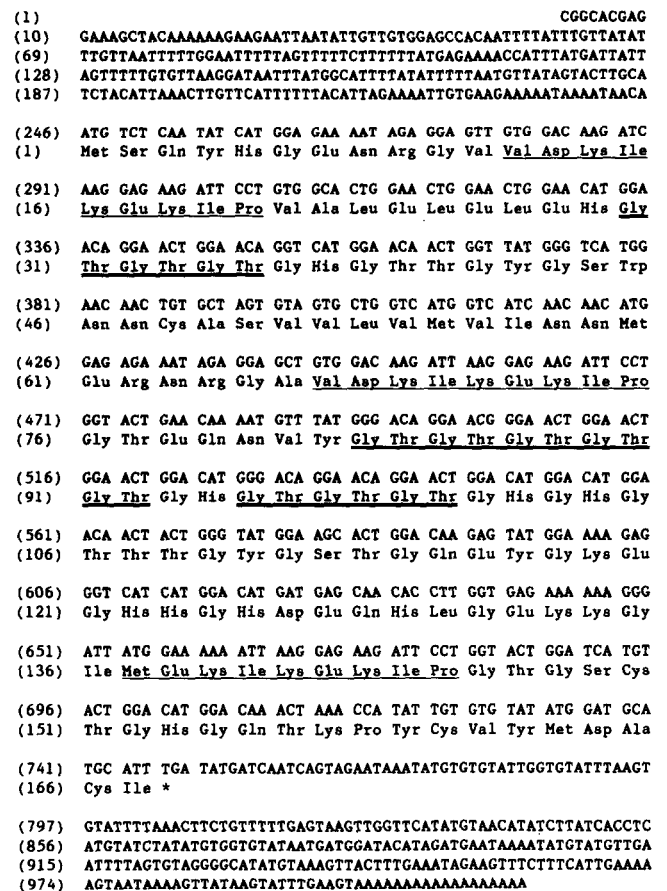


Figure 1. Nucleotide and deduced amino acid sequence of cloned Acs784 cDNA. Numbers in parentheses indicate nucleotide number (upper number in pairs) and amino acid number (lower number in pairs). Two repeat elements are indicated, one by single underlining and the other by double underlining.

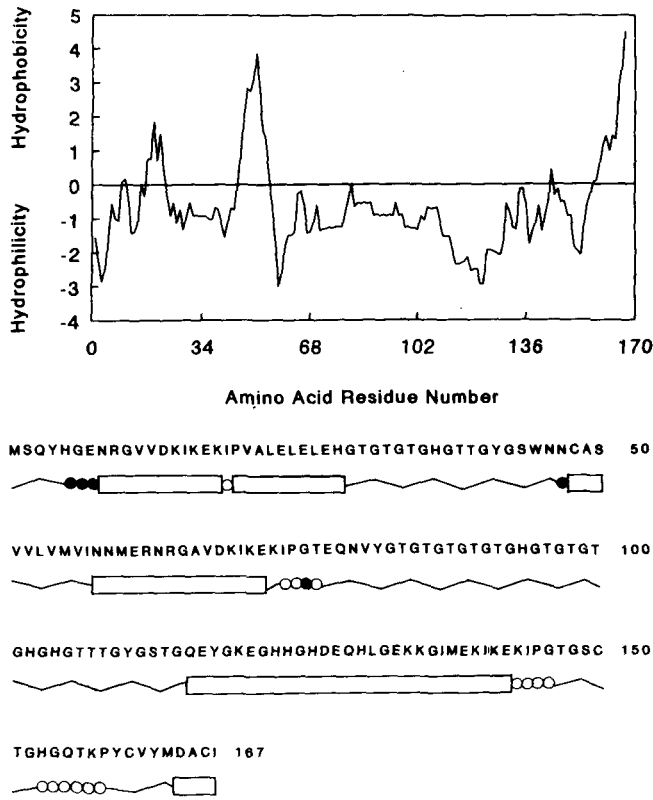


Figure 2. Upper, The hydrophobicity profile of CAS18 generated according to Kyte and Doolittle (1982) using a 9-amino acid window. Lower, Predicted secondary structure of CAS18 protein. Letters indicate amino acid sequence in single-letter code. Predicted secondary structure is shown under the amino acid sequence. Rectangle, α -Helical conformation; wavy line, extended β -sheet conformation; open circle, random coil; closed circle, turn.

kb. There is no detectable hybridization in nonacclimated cells (0 cold acclimation). With cold acclimation, *cas18* transcripts are undetectable up to 5 h but become easily detectable by 24 h and reach maximum levels in 7 d of cold acclimation. When cells cold-acclimated for 7 d are deacclimated, there is a rapid decline in the level of transcripts, which become barely detectable by 5 h.

The results presented in Figure 4A show that the three *cas18*-detectable transcripts are expressed in a coordinate manner. The levels of transcripts corresponding to the cDNA clone p2.1, which is constitutively expressed in wheat (Houde et al., 1992) and alfalfa (Dhindsa et al., 1992) and is used here as a control, do not change much during cold acclimation or deacclimation (Fig. 4B). The quantitative results obtained by densitometric scanning of the autoradiograph are shown in Figure 4C. The accumulation of *cas18*-detectable transcripts reaches half maximal levels after about 65 h of cold acclimation. The maximum levels of the transcripts are more than 30-fold greater in cold-acclimated than in nonacclimated cells. During deacclimation, transcript levels decline rapidly. It may, therefore, be concluded that the *cas18* cDNA characterized here hybridizes to three transcripts, which accumulate in response to low temperature in a coordinate man-

ner. Furthermore, their accumulation during cold acclimation is slow, whereas their disappearance during deacclimation is rapid.

Transcriptional and Posttranscriptional Regulation

Nuclear run-on transcription in isolated nuclei from non-acclimated, cold-acclimated, and deacclimated cell cultures

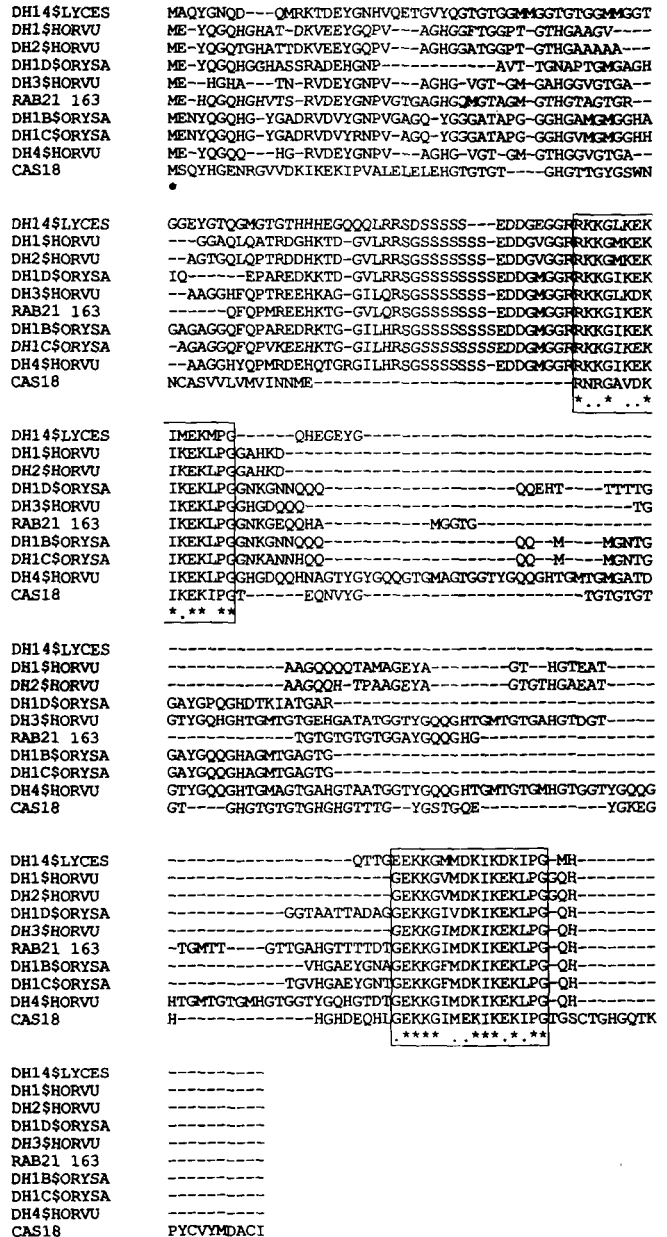


Figure 3. A comparison of CAS18 and members of the dehydrin/LEA/RAB family of proteins. A Clustal V alignment of similar sequences was used to locate regions highly conserved between nine dehydrins and CAS18. Asterisk (*) denotes identical residue. Dot under the column indicates one mismatch in that column. Conserved domains are boxed. Sources of these sequences are: DH14 (also called TAS14), Godoy et al. (1990); DH1-DH4, Close et al. (1989); DH1B-D, Yamaguchi-Shinozaki et al. (1989); RAB21, Mundy and Chua (1988).

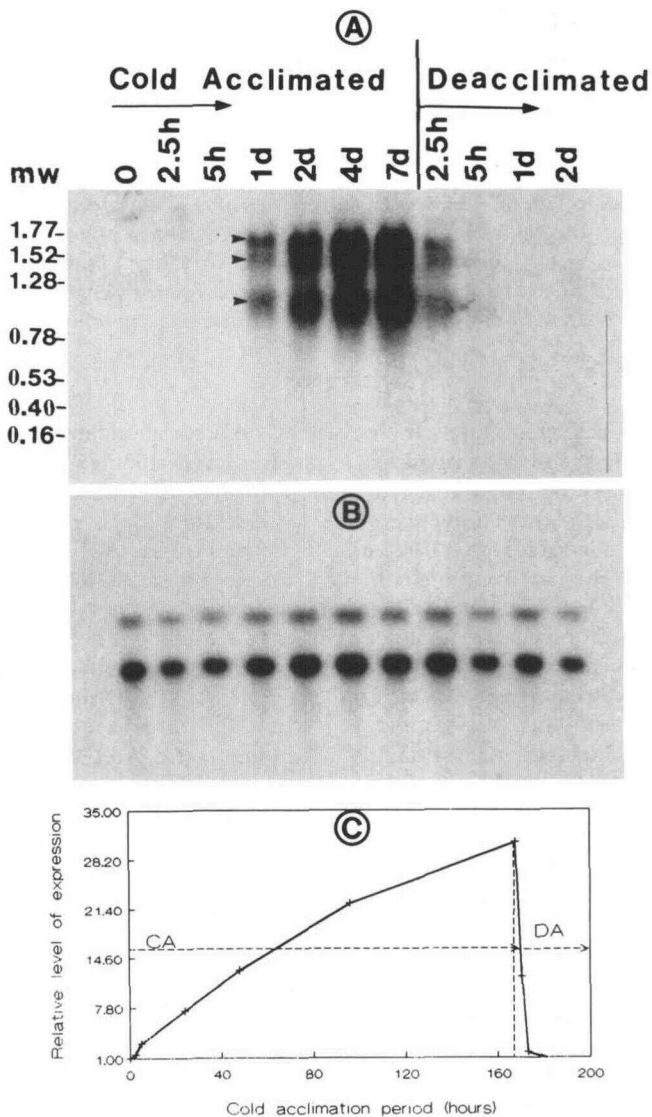


Figure 4. Northern blot analysis of kinetics of accumulation of *cas18* transcripts during cold acclimation of cell-suspension cultures of *M. falcata* cv Anik. Lanes 1–7, Cultures incubated at 5/2°C (day/night) for 0, 2.5, or 5.0 h, or 1, 2, 4, or 7 d; lanes 8–11, cultures incubated for 7 d at 5/2°C (day/night) followed by incubation at 25/20°C (day/night) for 2.5 or 5.0 h or 1 or 2 d. A, Blot probed with pAcs784; B, blot probed with p2.1; C, line graph summary of scanning densitometry of blots, showing relative levels of *cas18* transcripts.

was used to determine the relative contribution of transcriptional regulation in the accumulation of *cas18* transcripts. Three independent experiments yielded similar results, and data from one of them are presented here. The results of scanning densitometry of slot-blot hybridization, after subtracting the background hybridization to the cloning vector, pBluescript, are shown in Figure 5. In nuclei from nonacclimated cells, the level of transcripts for pAcs784 (*cas18*) is very low. The level in the case of nuclei from cold-acclimated cells is nearly 9-fold greater than that in nonacclimated cells. The level in nuclei from deacclimated cells is much reduced. Hybridization to the transcripts corresponding to the clones

used as controls, pUb and p2.1, does not differ much among nuclei from nonacclimated, cold-acclimated, and deacclimated cells.

Because the cold acclimation-induced 9-fold increase in nuclear run-on transcription of the *cas18* genes is not sufficient to account for the observed 30-fold increase in transcript levels in vivo, it may be concluded that there is a substantial regulation of the transcript abundance at the posttranscriptional level. Thus, the stability of *cas18*-detectable transcripts was examined by monitoring the decline in their level by gel-blot analysis under conditions where transcription had been almost completely inhibited with cordycepin. The decay of these transcripts in the absence or presence of cordycepin in cold-acclimated cells either maintained at 5°C or transferred to 25°C for deacclimation is shown in Figure 6. The two larger transcript bands are not well-separated due to the shorter time allowed for electrophoresis. However, the results pertaining to transcript decay, the objective of this experiment, are clear. Deacclimation in the absence of cordycepin (Fig. 6A) results in a very rapid decay of *cas18*-detectable transcripts. Within 2.5 h, transcripts have virtually disappeared. Deacclimation in the presence of cordycepin leads to a slower rate of decay for the transcripts, which are detectable even after 12 h (Fig. 6B, right). In the cold-acclimated cells maintained at 5°C in the presence of cordycepin (Fig. 6B, left), the level of the transcripts is easily detectable even after 84 h. Cordycepin had little effect on the rate of decay of transcripts corresponding to the constitutively expressed clone p2.1 at 25°C during the time of experimentation (data not shown), suggesting a much lower rate of turnover of these transcripts compared with that of the *cas18*-detectable transcripts.

The quantitation, by densitometer scanning, of the hybridization data on the decay of the *cas18*-detectable transcripts is shown in Figure 6C. Three different times of exposure to the film were used to make sure that the response was in the

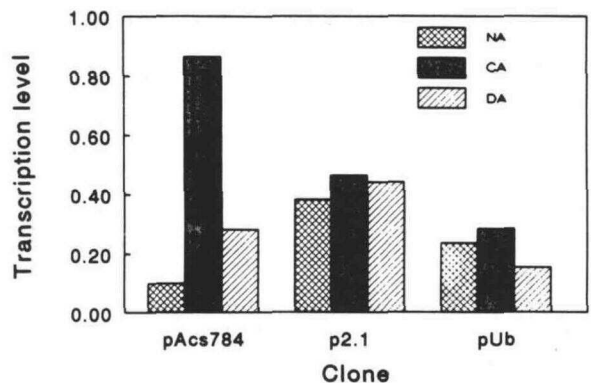


Figure 5. Level of run-on transcription of *cas18* in nuclei isolated from cell-suspension cultures. Clones analyzed were pAcs784, p2.1, and pUb (ubiquitin). NA, Nuclei isolated from nonacclimated cells; CA, nuclei from cells cold acclimated for 7 d; DA, nuclei from cells cold acclimated for 7 d and deacclimated for 2.5 h. Results presented are from scanning densitometry. Signal due to background hybridization to the cloning vector, pBluescript, has been subtracted.

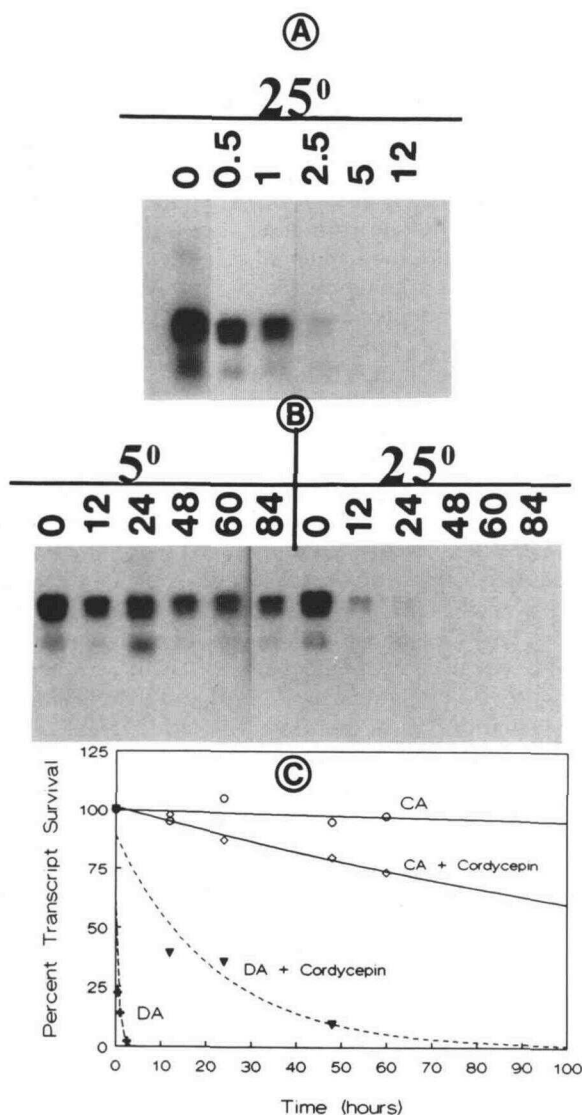


Figure 6. Northern hybridization analysis of the decay of *cas18* transcripts in the absence or presence of cordycepin. A, Cold-acclimated cells incubated at 25°C to deacclimate for 0, 0.5, 1.0, 2.5, 5.0, or 12 h; total RNA gel blot was probed with pAcs784. B, Cold-acclimated cells incubated in the presence of cordycepin (200 μ g/mL) at 5°C for 8 h and then transferred to either 5°C (left of vertical line) or 25°C (right of vertical line) for 0, 12, 24, 48, 60, or 84 h; total RNA gel blot was probed with pAcs784. C, Results of scanning densitometry analysis of decay of *cas18* transcripts in the presence or absence of cordycepin at 5 or 25°C. CA, Cold-acclimated cells; CA + cordycepin, cordycepin added to cold-acclimated cells kept at 5°C; DA, deacclimation in the absence of cordycepin; DA + cordycepin, deacclimation in the presence of cordycepin. Exponential curves were fitted to the data points.

linear range of the film. Decay curves were constructed using the densitometry data, and the approximate half-lives of transcripts were estimated. It can be seen by extrapolation of the curve CA + cordycepin in Figure 6C that the approximate half-life of the *cas18*-detectable transcripts at 5°C in the presence of cordycepin was greater than 100 h. In the cold-

acclimated cells, once the level of transcripts increases to a maximum it remains more or less constant (hybridization data not shown). When cold-acclimated cells are transferred to 25°C, the *cas18*-detectable transcripts become extremely unstable, with an estimated half-life of less than 30 min (determined from an expansion of the time scale of the curve DA in Fig. 6C). However, in the presence of cordycepin, the half-life of the transcripts at 25°C increases to about 12 h (curve DA + cordycepin, Fig. 6C). Therefore, it may be concluded that transcription is required for the rapid decay of the *cas18*-detectable transcripts during deacclimation.

DISCUSSION

The present study shows that *cas18* is abundantly expressed in cell-suspension cultures and its accumulation parallels the progress of cold acclimation. The cDNA characterized in this study hybridizes to three transcripts, and the sequence reported here corresponds to the 1-kb transcript.

The putative protein product (CAS18) of *cas18* is relatively small, about 17,572.5 D in molecular mass, and is extremely hydrophilic. The presence of in-frame stop codons before the initial Met indicates that the open reading frame is full length. In its compositional bias for Gly (21%), CAS18 is similar to most other stress proteins. *cas18* cDNA shares extensive homologies with a family of drought- and ABA-induced proteins collectively referred to as LEA/RAB/dehydrin proteins (Baker et al., 1988; Close et al., 1989; Galau and Close, 1992; Galau et al., 1992). There are about 20 different sequences reported for this family. The greatest homology is seen with RAB21 (also called DH21), a dehydrin from rice (Mundy and Chua, 1988), but unlike dehydrin, *rab*, and *lea* genes, *cas18* is not induced by either ABA or drought (Mohapatra et al., 1989). Because no members of the *lea/rab/dehydrin* gene family have been cloned for alfalfa, the possibility remains that *cas18* is indeed a member of this gene family that happens to be regulated differently; it is regulated by low temperature and not by ABA or drought. It is interesting that *rab21* is also induced by low temperature (Christie et al., 1991). One intriguing feature of CAS18 is the presence of Gly-Thr repeats, a characteristic of clock proteins (Shin et al., 1985; Li-Weber et al., 1987). Two such Gly-Thr repeats are also present in RAB21 (Fig. 3) (Mundy and Chua, 1988). Although the significance of this homology with clock proteins is not clear, it has been reported that a circadian rhythm of transcriptional activity is interrupted by low temperature (Martino-Catt and Ort, 1992). It is possible that following cold shock, which a plant must experience at the start of cold acclimation, a readjustment of the endogenous rhythm may be required for the development of freezing tolerance.

The results of the present study show that *cas18* is regulated at both the transcriptional and posttranscriptional levels. The dramatically rapid decline in the level of *cas18*-detectable transcripts during deacclimation suggests an important role of transcript stability in determining the transcript abundance. Furthermore, the cold acclimation-induced 9-fold increase seen in nuclear run-on transcription is not sufficient to account for the 30-fold increase in the in vivo transcript abundance. Thus, kinetics of decay of the *cas18*-detectable transcripts have been examined with minimum interference

due to transcript synthesis by using cordycepin to inhibit *in vivo* transcription. Cordycepin has been successfully used in determining half-lives of transcripts (Fritz et al., 1991). During deacclimation, the half-life of *cas18*-detectable transcripts is less than 30 min (Fig. 6C), but in the presence of cordycepin the half-life of the transcripts increases to nearly 12 h. The half-life of the transcript at low temperature in the presence of cordycepin is more than 100 h (Fig. 6C). A limitation of the data presented in Figures 5 and 6C is that the transcript levels shown represent the composite total of all three *cas18*-detectable transcripts. Thus, the results of experiments on nuclear run-on transcription and on cordycepin-induced transcript stability may not necessarily apply to individual transcripts considered separately. However, the data in Figure 3 show the coordinate manner in which the three individual transcript populations are expressed. Therefore, their expression in nuclear run-on transcription and their behavior in stability experiments are likely to be similar. Because the transcript level remains constant during cold acclimation (curve CA, Fig. 6C), the rate of decay in the presence of cordycepin is likely to correspond to the rate of *in vivo* synthesis of the *cas18*-detectable transcripts to maintain the steady-state level. The cordycepin-induced stability of the transcripts during deacclimation shows that rapid decay upon deacclimation is not a simple effect of high temperature. It appears that there may be a factor(s) that is transcription-dependent and that is involved in the rapid decay of the transcripts. Thus, the present study shows that *cas18* is regulated at both the transcriptional and posttranscriptional levels.

What is the possible functional significance of *cas18*? There is no direct evidence for a causal relationship between *cas18* expression and the development of freezing tolerance during cold acclimation. The similarities between CAS18 and members of the LEA/RAB/dehydrin family of proteins lead us to speculate that CAS18 may play a role in protecting the cell from freezing-induced dehydration. CAS18 may function to solvate cytosolic structures by virtue of its relatively high content of Gly and Thr. Hydroxyl groups of the Thr residues may serve to solvate structural surfaces in a manner similar to that proposed for LEA proteins (Baker et al., 1988). Indeed, it is the ability to withstand this secondary dehydration stress that in many cases determines the freezing tolerance of a plant (Levitt, 1980). There are several lines of strong relative evidence for a causal involvement of *cas18* in the cold-induced development of freezing tolerance: (a) the level of its transcripts shows temporal relationship with the development of freezing tolerance during cold acclimation; (b) in several cultivars of alfalfa differing in freezing tolerance, the level of expression of *cas18* shows a high positive correlation with the degree of freezing tolerance (Mohapatra et al., 1989); and (c) in a recent study of low-temperature signal transduction (Dhindsa et al., 1992; A.F. Monroy, F. Sarhan, and R.S. Dhindsa, unpublished results), we observed that cold-induced phosphorylation of specific proteins is required for the development of freezing tolerance. When this phosphorylation is inhibited by specific inhibitors, not only does cold acclimation not occur, but the transcription of *cas18* is substantially reduced. Thus, there are strong indications that

cas18 is causally involved in the development of cold-induced freezing tolerance.

ACKNOWLEDGMENT

The ubiquitin cDNA clone, pUb, was kindly provided by Dr. Bruce Brandhorst of Simon Fraser University.

Received September 23, 1992; accepted December 23, 1992.

Copyright Clearance Center: 0032-0889/93/101/1275/08.

The EMBL/GenBank/DBJ accession number for the sequence reported in the article is L07516.

LITERATURE CITED

- Atanassov A, Brown DCW (1984) Plant regeneration from suspension culture and mesophyll protoplasts of *Medicago sativa* L. *Plant Cell Tissue Organ Cult* 3: 149-162
- Baker J, Steele C, Dure L (1988) Sequence and characterization of 6 Lea protein and their genes from cotton. *Plant Mol Biol* 11: 277-291
- Cattivelli L, Bartels D (1990) Molecular cloning and characterization of cold-regulated genes in barley. *Plant Physiol* 93: 1504-1510
- Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162: 156-159
- Christie PJ, Hahn M, Walbot V (1991) Low-temperature accumulation of alcohol dehydrogenase-1 mRNA and protein activity in maize and rice seedlings. *Plant Physiol* 95: 699-706
- Close TJ, Kortt AA, Chandler PM (1989) A cDNA-based comparison of dehydration-induced proteins (dehydrins) in barley and corn. *Plant Mol Biol* 13: 95-108
- DeLisle AJ, Crouch ML (1989) Seed storage protein transcription and mRNA levels in *Brassica napus* during development and in response to exogenous abscisic acid. *Plant Physiol* 91: 617-623
- Dhindsa RS, Monroy A, Wolfrain LA, Dong G (1992) Signal transduction and gene expression during cold acclimation of alfalfa. In PH Li, L Christersson, eds, *Advances in Plant Cold Hardiness*. CRC Press, Boca Raton, FL, pp 57-71
- Dunn MA, Hughes MA, Zhang L, Pearce RS, Quigley AS, Jack PL (1991) Nucleotide sequence and molecular analysis of the low temperature induced cereal gene, BLT4. *Mol Gen Genet* 229: 389-394
- Fritz CC, Herget T, Wolter FP, Schell J (1991) Reduced steady-state levels of *rbcS* mRNA in plants kept in the dark are due to differential degradation. *Proc Natl Acad Sci USA* 88: 4458-4462
- Fourney RM, Miyakoshi J, Day RS, Paterson MC (1988) Northern blotting: efficient RNA staining and transfer. *Focus* 10: 5-7
- Galau GA, Close TJ (1992) Sequences of the cotton group 2 LEA/RAB/dehydrin proteins encoded by *Lea3* cDNAs. *Plant Physiol* 98: 1523-1525
- Galau GA, Wang HY-C, Hughes WD (1992) Cotton *Lea4* (D19) and *LeaA2* (D132) group 1 *Lea* genes encoding water stress-related proteins containing a 20-amino acid motif. *Plant Physiol* 99: 783-788
- Giles KW, Myers A (1965) An improved diphenylamine method for the estimation of deoxyribonucleic acid. *Nature* 206: 93
- Gilmour SJ, Artus NN, Thomashow MF (1992) cDNA sequence analysis and expression of two cold-regulated genes of *Arabidopsis thaliana*. *Plant Mol Biol* 18: 13-21
- Godoy JA, Pardo JM, Pintor-Toro JA (1990) A tomato cDNA inducible by salt stress and abscisic acid: nucleotide sequence and expression pattern. *Plant Mol Biol* 15: 695-705
- Hajela RK, Horvath DP, Gilmour SJ, Thomashow MF (1990) Molecular cloning and expression of *cor* (cold-regulated) genes in *Arabidopsis thaliana*. *Plant Physiol* 93: 1246-1252
- Higgins DG, Sharp PM (1988) CLUSTAL: a package for performing multiple sequence alignments on a microcomputer. *Gene* 73: 237-244
- Houde M, Danyluk J, Laliberté J-F, 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
- Kurkela S, Franck M** (1990) Cloning and characterization of a cold- and ABA-inducible *Arabidopsis* gene. *Plant Mol Biol* **15**: 137–144
- Kyte J, Doolittle RF** (1982) A simple method for displaying the hydropathic character of a protein. *J Mol Biol* **157**: 105–132
- Levitt J** (1980) *Responses of Plants to Environmental Stresses*. Vol I. Chilling, Freezing, and High Temperature Stresses. Academic Press, New York
- Li-Weber M, de Groot EJ, Schweiger HG** (1987) Sequence homology to the *Drosophila Per* locus in higher plant nuclear DNA and in *Acetabularia* chloroplast DNA. *Mol Gen Genet* **209**: 1–7
- Maniatis T, Fritsch EF, Sambrook J** (1982) *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
- Martino-Catt S, Ort DR** (1992) Low temperature interrupts circadian regulation of transcriptional activity in chilling sensitive plants. *Proc Natl Acad Sci USA* **89**: 3731–3735
- Mohapatra SS, Poole RJ, Dhindsa RS** (1988) Abscisic acid-regulated gene expression in relation to freezing tolerance in alfalfa. *Plant Physiol* **87**: 468–473
- Mohapatra SS, Wolfrain L, Poole RJ, Dhindsa RS** (1989) Molecular cloning and relationship to freezing tolerance of cold-acclimation-specific genes of alfalfa. *Plant Physiol* **89**: 375–380
- Mundy J, Chua N-H** (1988) Abscisic acid and water stress induce the expression of a novel rice gene. *EMBO J* **7**: 2279–2286
- Orr W, Singh J, Brown DCW** (1985) Induction of freezing tolerance in alfalfa cell suspension cultures. *Plant Cell Rep* **4**: 15–18
- Pearson WR, Lipman DJ** (1988) Improved tools for biological sequence comparison. *Proc Natl Acad Sci USA* **85**: 2444–2448
- Schaffer MA, Fischer RL** (1988) Analysis of mRNAs that accumulate in response to low temperature identifies a thiol protease gene in tomato. *Plant Physiol* **87**: 431–436
- Shin HS, Bargiello TA, Clark BT, Jackson FR, Young MW** (1985) An unusual coding sequence from *Drosophila* clock gene is conserved in vertebrates. *Nature* **317**: 445–448
- Towill LE, Mazur P** (1974) Studies on the reduction of 2,3,5-triphenyltetrazolium chloride as a viability assay for plant tissue cultures. *Can J Bot* **53**: 1097–1102
- Tyson H** (1992) Relationships, derived from optimum alignments, among amino acid sequences of plant peroxidases. *Can J Bot* **70**: 543–556
- Watson JC, Thompson WF** (1988) Purification and restriction endonuclease analysis of plant nuclear DNA. In A Weissbach, H Weissbach, eds, *Methods in Plant Molecular Biology*. Academic Press, New York, pp 57–75
- Yamaguchi-Shinozaki K, Mundy J, Chua N-H** (1989) Four tightly linked *rab* genes are differentially expressed in rice. *Plant Mol Biol* **14**: 29–39