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

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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 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.

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 μE m⁻² 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 LT₅₀ 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.

Abbreviations: LT₅₀, 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 methylmercurichloride 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-32P]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× 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× SSC, 1% SDS, 5X Denhardt’s solution, and 100 μg mL⁻¹ of denatured calf thymus DNA in a shaking water bath at 65°C. Then, 500 ng of the heat-denatured, nick-translation 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× 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× 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 μm.

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 casl8 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 μg of DNA were resuspended in 200 μl of transcription mixture: 100 mM (NH₄)₂SO₄, 30 mM Tris-Cl, pH 8.0, 7 mM MgCl₂, 50 mM KCl, 500 μM each of CTP, GTP, ATP, 100 μM phosphocreatine, 20 μg/mL of creatine phosphokinase, 3 mM β-mercaptoethanol, 0.5 mCi [α-32P]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 (Dhirinda 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 [3H]uridine and on its incorporation into TCA-precipitable material. These experiments showed that cordycepin at 200 μg mL⁻¹ had little effect on the uptake of [3H]uridine but inhibited in vivo transcription completely within 8 h at 5°C. Thus, an 8-h treatment with 200 μg 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 μg 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 casl8 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 LT₅₀) with increasing duration
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 to be an appropriate system for molecular studies 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, 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

<table>
<thead>
<tr>
<th>Days Cold Acclimated</th>
<th>Days Deacclimated</th>
<th>LT90°C</th>
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<tr>
<td>0</td>
<td>0</td>
<td>-4.18 ± 0.82</td>
</tr>
<tr>
<td>7</td>
<td>0</td>
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<tr>
<td>14</td>
<td>0</td>
<td>-8.14 ± 1.44</td>
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<tr>
<td>21</td>
<td>0</td>
<td>-9.74 ± 0.65</td>
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<tr>
<td>21</td>
<td>3</td>
<td>-4.79 ± 0.12</td>
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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.

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.

Characteristic of a Cold-Specific cDNA of Alfalfa

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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 manner. 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 nonacclimated, cold-acclimated, and deacclimated cell cultures
Characterization of a Cold-Specific cDNA of Alfalfa

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.

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.
The densitometry data, and the approximate half-lives of 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 obtained 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 correlative 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.

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