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A New Cold-Induced Alfalfa Gene Is Associated with Enhanced Hardening at Subzero Temperature[1]

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When alfalfa (*Medicago sativa* L. cv Apica) plants grown at room temperature are transferred to 2°C, the temperature at which 50% of the plants fail to survive (LT_{50}) decreases from −6 to −14°C during the first 2 weeks but then increases to −9°C during the subsequent 2 weeks. However, when plants are kept for 2 weeks at 2°C and then transferred to −2°C for another two weeks, the LT_{50} declines to −16°C. These changes in freezing tolerance are paralleled by changes in transcript levels of *cas15* (cold acclimation-specific gene encoding a 14.5-kD protein), a cold-induced gene. Cold-activation of *cas15* occurs even when protein synthesis is inhibited by more than 90%, suggesting that cold-initiated events up to and including the accumulation of *cas15* transcripts depend on preexisting gene products. *cas15* shows little homology to any known gene at the nucleotide or amino acid level. The deduced polypeptide (CAS15) of 14.5 kD contains four repeats of a decapeptide motif and possesses a bipartite sequence domain at the carboxy terminus with homology to the reported nuclear-targeting signal sequences. Although the relative amount of *cas15* DNA as a fraction of the total genomic DNA is similar in cultivars with different degrees of freezing tolerance, its organization in the genome is different. The possible role of *cas15* in the development of cold-induced freezing tolerance is discussed.

When exposed to low but nonfreezing temperatures (cold acclimation), many plants develop tolerance to subsequent freezing temperatures (Levitt, 1980; Guy, 1990). Plants capable of cold acclimation respond to the low-temperature signal by accumulation of transcripts of specific genes. During the past 3 years, several genes associated with cold acclimation have been cloned and characterized from several plants including alfalfa (Mohapatra et al., 1988, 1989; Wolfraim et al., 1993), barley (Cattivelli and Bartels, 1990; Dunn et al., 1991), *Arabidopsis* (Kurkela and Franck, 1990; Gilmour et al., 1992), and wheat (Houde et al., 1992). In some plants, such as alfalfa and wheat, the level of cold-induced transcript accumulation shows a strong positive correlation with the degree of cold-induced freezing tolerance in different cultivars (Mohapatra et al., 1989; Houde et al., 1992).

Sequence analysis of cold-induced genes has not revealed a definite clue to their function, although some sequence features such as Lys-rich repeat motifs, which they share with genes induced by drought and ABA (Baker et al., 1988; Close et al., 1989), have been noted. The development of freezing tolerance as a multigenic trait involves the action of many genes (Levitt, 1980; Guy, 1990). Furthermore, structural and metabolic alterations that accompany the process of cold acclimation suggest that the genes involved in the process are of a diverse nature. Thus, changes that occur in the composition and properties of plasma membrane (Steponkus, 1984; Steponkus et al., 1988; Hugly et al., 1990) are expected to involve lipid synthesis and desaturation enzymes.

Because dehydration is an important component of freezing injury (Levitt, 1980; Steponkus, 1984), cold-induced genes coding for dehydrin-like proteins have been characterized (Houde et al., 1992; Wolfraim et al., 1993). Similarly, cold-induced genes coding for proteins with homology to the fish antifreeze proteins have been described (Kurkela and Franck, 1990; Orr et al., 1992). The presence of such antifreeze-like proteins (Griffith et al., 1992) is proposed to inhibit ice crystal growth and thus protect the protoplast from ice seeding. It appears to be reasonable to assume that, like other complex and multifaceted biological processes, cold acclimation involves communication between cytoplasm and the nucleus. However, no cold-induced gene potentially involved in such a communication appears to have been described.

Here we report the characterization of a cDNA, apparently with a full-length open reading frame, that possesses sequence features distinct from those of previously characterized cold-induced genes and encodes a putative nuclear protein. Transcript accumulation of this gene (*cas15*) parallels the changes in freezing tolerance developed at 2 or −2°C. The possible physiological function of *cas15* is discussed.

MATERIALS AND METHODS

Plant Material

Seedlings of *Medicago sativa* L. cv Apica were grown at 21°C day/17°C night with a 16-h photoperiod and a PPFD

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Abbreviations: DA, deacclimation; LT_{50}, temperature at which 50% of the plants fail to survive; NTS, nuclear-targeting signal.

of 350 μmol m^{-2} s^{-1}. Seedlings of *M. sativa* ssp. *falcata* cv Anik were grown as previously described (Mohapatra et al., 1989). Etiolated seedlings of various cultivars used for extraction of genomic DNA were grown on moist filter paper at 25°C for 7 d and then transferred to 4°C for 2 d.

Cell Cultures

Experiments to determine whether de novo protein synthesis was required for cold-induced accumulation of *cas15* transcripts were conducted on cell-suspension cultures, which, compared to intact plants, are more amenable to uniform chemical treatments. These cell cultures have been derived from hypocotyl tissue of *M. sativa* spp. *falcata* cv Anik. The procedures for developing, maintaining, and using these cell cultures in studies of cold acclimation have been described elsewhere (Wolfraim et al., 1993). It has been shown that the genes that are specifically induced by low temperature in alfalfa seedlings (Mohapatra et al., 1989) are also expressed in cell cultures (Wolfraim et al., 1993). These cell cultures have been used previously with success in studies involving treatment with cordycepin to inhibit transcription for studies of transcript stability (Wolfraim et al., 1993). Rapidly growing cultures in the log phase of their growth were used in these experiments.

Cold Acclimation and Measurement of Freezing Tolerance

Five-week-old plants were cold acclimated in a growth chamber at 2°C with an 8-h photoperiod and light intensity of 150 μmol m^{-2} s^{-1} for 2 weeks. Half of the plants were then transferred to a freezer at −2°C for an additional 2 weeks to simulate natural hardening conditions that are known to promote freezing tolerance in frozen soil (Olien, 1984). The other half of the plants was maintained at 2°C for 2 additional weeks. Freezing tests were performed, and the LT_{50} was determined after 3 weeks of regrowth at 20°C as described by Paquin and Pelletier (1987).

Extraction of mRNA and Construction of cDNA Libraries

mRNA from cold-acclimated seedlings of a freezing-tolerant cultivar, *M. sativa* spp. *falcata* cv Anik, was used to construct a cDNA library in the bacteriophage vector λ-Uni-Zap XR (Stratagene, San Diego, CA) using the manufacturer's protocols (Wolfraim et al., 1993). This cDNA library was screened using the previously isolated partial-length cDNA clone pSM2201 (Mohapatra et al., 1989), radiolabeled to a high specific activity by nick translation (Sambrook et al., 1989). A full-length cDNA clone, pAcs2201, was isolated and characterized in this study. Independently, mRNA extracted from the crown tissue of cold-acclimated plants of another freezing-tolerant alfalfa cultivar, *M. sativa* cv Apica, was used to construct a cDNA library in the bacteriophage λgt10 using the cDNA synthesis and cloning kit from Amersham Corp. (Arlington Heights, IL). This cDNA library was screened by differential hybridization using, as probes, radiolabeled single-stranded cDNAs synthesized against mRNAs from nonacclimated or cold-acclimated crown tissue. A full-length cDNA clone pMSACIB was isolated and characterized. Both cDNA clones, pAcs2201 and pMSACIB, isolated from the two cultivars were found to be homologous, corresponding to the gene named *cas15* (cold acclimation-specific gene coding for a 14.5-kD protein).

Northern Hybridization Analysis

Total RNA was prepared as described by De Vries et al. (1988). Total RNA (10 μg) was denatured in formaldehyde, separated on a 1% agarose-formaldehyde gel, transferred by vacuum to Hybond-N membranes (Amersham), and hybridized at 68°C in 2× standard Na citrate and 0.25% BLOTTO (Sambrook et al., 1989). The gel-purified cDNA insert (500 ng), radiolabeled to a high specific activity by nick translation, was used as a probe. Blots were hybridized at 42°C in 50% formamide and washed at 55°C in 0.1× SSPE (18 mM NaCl, 10 mM Na phosphate [pH 7.7], 1 mM EDTA) and 0.1% SDS as described by Sambrook et al. (1989). Blots were then exposed, without intensifying screens, to Kodak O-Mat XAR film for autoradiography.

Genomic Southern Analysis

Genomic DNA was extracted from etiolated seedlings by the cetyltrimethylammonium bromide procedure as described by Rogers and Bendich (1988). Each DNA sample (10 μg) was digested separately with each of the indicated restriction endonucleases, separated on a 0.8% agarose gel, and transferred to a Biotrans nylon membrane (ICN). For slot blots, 1 to 8 μg of each sample was applied to the membrane under vacuum following the manufacturer's protocols. Hybridization and posthybridization washes of the blots were performed as described above for northern blots (Sambrook et al., 1989).

DNA Sequence Determination and Analysis

cDNA fragments were cloned into M13 mp18 and M13 mp19 bacteriophages and sequenced by the dideoxynucleotide chain termination method (Sanger et al., 1977), using a T7 DNA polymerase sequencing kit (Pharmacia). The complete sequence of both strands was determined. A computer search of data bases was carried out with the BLAST (Altschul et al., 1990) and T-FASTA (Pearson and Lipman, 1988) programs. Protein sequence analysis was aided by the SEQAID II, version 3.81, program (D. Rhodes and D. Roufa, Kansas State University, Manhattan).

RESULTS

Sequence Analysis of pAcs2201 and pMSACIB cDNAs (*cas15* Gene)

A full-length cDNA clone, pAcs2201, was isolated from freezing-tolerant *M. sativa* L. spp. *falcata* cv Anik. A homologous cDNA, pMSACIB, was independently isolated from the equally freezing-tolerant *M. sativa* L. cv Apica. Because the coding regions of the two cDNAs have been found to be almost identical, only one of them is shown here. The nucleotide sequence of MSACIB, the larger of the two cDNAs, and the deduced polypeptide sequence are shown in Figure

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                                                CATTTAGAAACAAATAGAAATACAAT    26

ATGGCAGGAATCATGAACAAGATTGGTGATGCCCTTCACGGAGGAGGAGACAAGAAAGAGGGAGAGCACA    96
M  A  G  I  M  N  K  I  G  D  A  L  H  G  G  G  D  K  K  E  G  E  H  K    24

AAGGAGAACAACATGGGCATGTAGGAGGAGAACATCATGGTGAGTACAAAGGAGAACAACATGGATTTGT   166
   G  E  Q  H  G  H  V  G  G  E  H  H  G  E  Y  K  G  E  Q  H  G  F  V    47

TGGAGGACATGCTGGTGACCACAAAGGAGAACAACATGGTTTTGTAGGAGGACATGGGGGTGATTACAAA   236
   G  G  H  A  G  D  H  K  G  E  Q  H  G  F  V  G  G  H  G  G  D  Y  K    70

GGAGAGCAACATGGATTTGGTCATGGAGACCACAAGGAGGGATACCATGGGGAAGAGCACAAGGAGGGAT   306
   G  E  Q  H  G  F  G  H  G  D  H  K  E  G  Y  H  G  E  E  H  K  E  G  F    94

TTGCCGACAAGATCAAGGACAAAATTCATGGTGAAGGTGCAGATGGTGAAAAGAAAAAGAAGAAGGAGAA   376
   A  D  K  I  K  D  K  I  H  G  E  G  A  D  G  E  K  K  K  K  K  E  K    117

GAAGAAACATGGAGAGGGTCATGAACATGGCCATGATAGCAGCAGCAGTGACAGTGATTAGATCTTAATT   446
 K  K  H  G  E  G  H  E  H  G  H  D  S  S  S  S  D  S  D  *              136

TCACTGCTTCATCATGTTGAGAGATGTATGGAGTCATATGCGACGGCAATCTCCTATGTTTTAATTTCTT   516
GTTGTAATATCTACGTATGTGTGTGGAAAGAAATACGTGTTCGTAGTTGGTAATTCGAATGCTTGTAAAA   586
ATAATGTGCCGGTCAGATTGGTTTGTTAAACCCCTACTGCTTTGTTGATGTTTGGCTGTATATTATCGTA   656
AAGAAATTATAAGGATTTCTATTAGTAGTAAGTATGAGTTATTACTATTGAGCTGCTTATTCAAAAAAAA   726
AAAAAAAAAA                                                               736
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Figure 1. Nucleotide sequence and the deduced amino acid sequence of pMSACIB cDNA from cv Apica (*cas15B* gene). The four repeats of the decapeptide motif are underlined. The 47-bp segment just before the poly(A) tail, which is present here in pMSACIB but absent in pAcs2201 (cv Anik), is double underlined. The nucleotide G shown at position 293 in pMSACIB is C in pAcs2201 (cv Anik). Consequently, the amino acid E at position 89 is replaced by D in pAcs2201 from cv Anik.

1. The cDNA is 736 bp long and contains a single open reading frame, starting at nucleotide 27 and ending with the stop codon at nucleotide 437. It can code for a polypeptide consisting of 136 amino acid residues. The 5′ untranslated region contains an in-frame stop codon at nucleotides 15 to 17. Nucleotides immediately before the start codon correspond to the sequences frequently found in dicotyledonous plants (Cavener and Ray, 1991). The coding region of the sequence of Acs2201 cDNA from cv Anik is almost identical with that of MSACIB from cv Apica shown in Figure 1. The only difference between the two sequences is that the nucleotide at position 293 is G in MSACIB but C in Acs2201. In the 3′ untranslated region, although there are several single mismatches, replacements, or insertions, an important difference is that the 47 nucleotides, 672 to 718, present just before the poly(A) tail in MSACIB (double underlined in Fig. 1) are absent in Acs2201. Because the two cDNAs are not completely identical, the corresponding *cas15* gene is named *cas15A* in Anik and *cas15B* in Apica.

The search in the GenBank sequence data base did not reveal a significant homology at the nucleotide or the amino acid level between *cas15* and previously reported genes. The deduced polypeptide (CAS15) contains 136 amino acid residues in both cultivars with the only difference being that the amino acid at position 89 is Glu (E) in MSACIB and Asp (D) in Acs2201. The predicted molecular mass of the CAS15A polypeptide (cv Anik) is 14,530 and that of CAS15B (cv Apica) is 14,544. The calculated isoelectric point is 6.72 in both cases. The CAS15 polypeptide contains a decapeptide motif repeated four times at regular intervals with the following consensus sequence: Lys-Gly-Glu-Gln-His-Gly-Phe(His)-Val(Gly)-Gly(His)-Gly (underlined in Fig. 1). CAS15 is rich in Gly (25.7%). Other abundant amino acids are His (15.4%), Lys (14.7%), and Glu (11.8%). Together, these four amino acids account for 67.6% of the polypeptide. Five amino acids, Cys, Pro, Arg, Thr, and Trp, are absent from the sequence. CAS15 is predicted to be hydrophilic and to be a soluble or peripheral protein (Fig. 2). The predicted secondary structure (Fig. 2B) is predominantly α-helical in conformation. Thus, more than 65% of the protein molecule is predicted to be α-helical in conformation, whereas only about 18% is in extended β-sheet conformation, and 12% is in the form of turns or loops.

A search of protein sequence data bases revealed a weak homology with nuclear proteins. We identified a putative bipartite motif of basic residues, K or R, characteristic of NTS sequences, near the carboxy terminus of CAS15. The alignment of the putative NTS sequence of CAS15 with those of six others from various organisms, TGA1a from tobacco (Katagiri et al., 1989), VirE2 from *Agrobacterium* (Citovsky et al., 1992), Top1 from *Arabidopsis* (Kieber et al., 1992), O2 from maize (Varagona et al., 1992), and nucleoplasmin and N1 from *Xenopus* (Robbins et al., 1991), is shown in Figure 3. The basic amino acid residues (K or R) at the two termini of the signal sequences, either known to be essential, such as in VirE2, nucleoplasmin, and O2, or predicted to be essential to the function of the signal sequence, are in bold uppercase, and the nonessential residues are in lowercase. It can be seen that, with respect to the essential basic amino acid residues

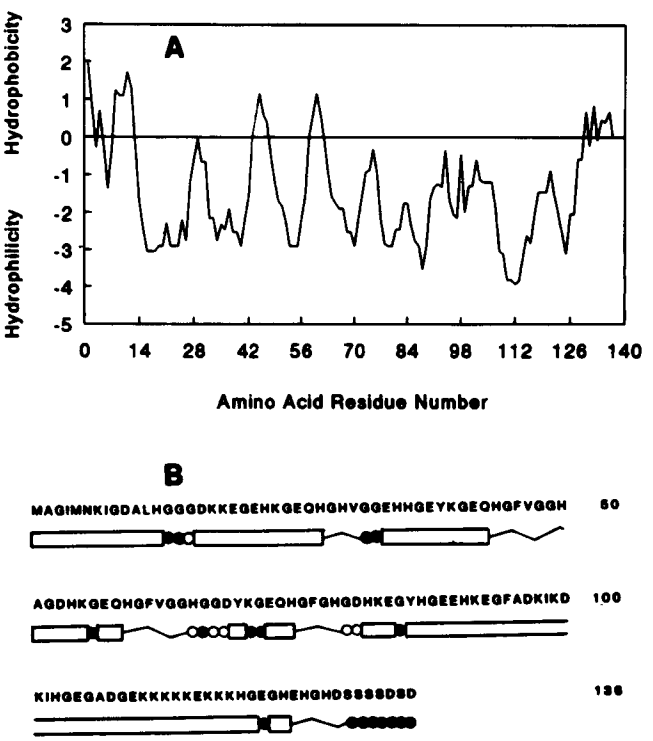

Figure 2. A, Hydropathy profile of CAS15 protein generated according to procedure of Kyte and Doolittle (1982) using a 9-amino acid averaging window. B, Predicted secondary structure of CAS18 protein. Letters indicate amino acid sequence in one-letter codes. Predicted secondary structure is shown under the amino acid sequence. Rectangle, α-Helical conformation; wavy line, extended β-sheet conformation; open circles, random coil; filled circles, turn or loop.

Protein	Organism	AA#	NTS Sequence
CAS15	Alfalfa	97	KiKdkihegadge------KKKKKek
TGA1a	Tobacco	134	KiKlegvnaniskcssl--KRKKsse
VirE2 NSE2	*Agrobacterium*	296	KtKygsdtei----------KlKsKsgi
Top1	*Arabidopsis*	821	KgKpplegsdg--------KKiRsle
O2	Maize	237	RKesnresa----------RRsRyRK
Nucleoplasmin	*Xenopus*	155	KRpaatkkagga------KKKKldn
N1	*Xenopus*	534	KRkteeesplkd-------KdaKKsk

Figure 3. The alignment of the putative NTS sequence present in CAS15 protein with six other NTS sequences. Sources of the reported NTS sequences are as follows (in parentheses): TGA1a (Katagiri et al., 1989), VirE2 (Citovsky et al., 1992), Top1 (Kieber et al., 1992), O2 (Varagona et al., 1992), Nucleoplasmin and N1 (Robbins et al., 1991). AA#, The amino acid residue number at the start of the bipartite motif in the respective NTS sequence.

located at the two termini, the putative CAS15 NTS sequence is quite similar to other NTS sequences.

Low-Temperature Induction of *cas15* Gene Expression

Both cloned cDNAs, pAcs2201 and pMSACIB, hybridized to a single transcript of about 0.85 kb in northern gel-blot hybridization. The transcript size and number are in agreement with those identified earlier with a partial-length clone (Mohapatra et al., 1989).

Cold-induced accumulation of *cas15* transcripts and their disappearance on DA (return of plants to ambient temperature) are shown in Figure 4A. The transcript level is hardly detectable in the absence of cold acclimation. However, it increases rapidly with cold acclimation and reaches a maximum after about 96 h. The transcript level appears to be highest at 168 h (7 d) but appears to decrease slightly by 336 h (14 d). When cold-acclimated plants are returned to room temperature for DA, the level of *cas15* transcripts rapidly declines and is barely detectable after 24 h.

The nature and relationship with freezing tolerance of the decline in *cas15* transcripts on prolonged cold acclimation at 2°C has been further explored. Thus, the transcript level in different plant parts after acclimation at 2°C for 2 or 4 weeks, or at 2°C for 2 weeks followed by another 2 weeks at −2°C, has been determined. The results obtained are shown in Figure 4B. The changes in LT_{50} for the entire plant with these various cold-acclimation treatments are also shown in the lower part of Figure 4B. When plants are kept at 2°C for 2 weeks (CA1), *cas15* transcripts increase in all three parts of the plant, leaf, crown, and root. Concomitantly, the LT_{50} declines from −6°C in the nonacclimated plants to −14°C. When plants are kept at 2°C for 4 weeks (CA2), the transcript level is much lower compared with those present at 2 weeks of cold acclimation at 2°C. The corresponding values for LT_{50} are −9 and −14°C. When plants are cold acclimated for 2 weeks at 2°C and for another 2 weeks at −2°C, the transcript level is higher in all plant parts than when plants are acclimated at 2°C (CA1) and the LT_{50} declines to the lowest value, −16°C (maximum freezing tolerance). The transcript level is highest in the crown and lowest in the leaf. The relative increase in *cas15* transcript level when plants are transferred from 2 to −2°C is particularly noteworthy in the root, where the level is considerably more than in the leaf and is about equal to that in the crown.

To determine whether synthesis of new proteins is required for the cold induction of *cas15* transcript accumulation, we used cell cultures of cv Anik, which have been used successfully before for similar purposes (Wolfraim et al., 1993). Cycloheximide was added at 150 $\mu g\ mL^{-1}$ to the cell cultures at room temperature, causing a greater than 90% inhibition of protein synthesis within 2 h of its application. Therefore, 2 h after the addition of cycloheximide, cultures were transferred to 2°C for cold acclimation. After 3, 6, and 9 h of exposure to 2°C in the absence or presence of cycloheximide, the level of *cas15* transcripts was determined by northern hybridization. The results are shown in Figure 5. It can be seen that accumulation of *cas15* transcripts occurs during cold acclimation, whereas the majority of protein synthesis is inhibited. Furthermore, the level of *cas15* transcripts is higher in cycloheximide-treated than in untreated control cells exposed to low temperature for the same duration. Therefore, it is concluded that cold-induced accumulation of *cas15* transcripts does not require de novo protein synthesis.

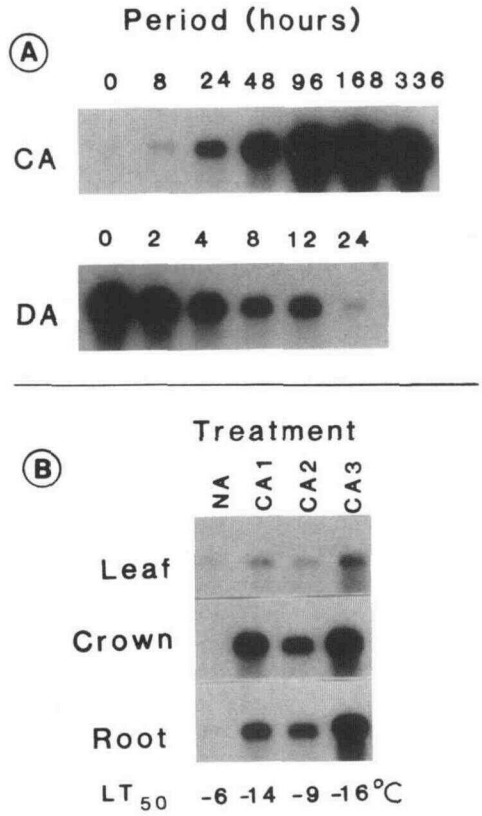

Figure 4. Cold acclimation-induced expression of *cas15* gene in *M. sativa* cv Apica. A, Level of *cas15* transcripts in crown tissue during cold acclimation (CA) and subsequent DA. Cold acclimation was at 2°C for times (h) indicated. DA of 2-week cold-acclimated seedlings was carried out by returning plants to 20°C for times indicated. B, Accumulation of *cas15* transcripts in leaves, crowns, and roots either without cold acclimation (NA) or with cold acclimation for 2 weeks at 2°C (CA1), 4 weeks at 2°C (CA2), or 2 weeks at 2°C and then 2 weeks at −2°C (CA3). The values for LT_{50}, in°C, achieved with NA, CA1, CA2, and CA3 are given at the bottom of the figure.

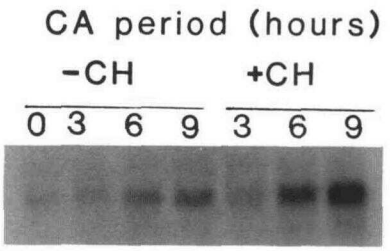

Figure 5. Cold-induced accumulation of *cas15* transcripts in the absence (−CH) or presence (+CH) of 150 μg mL^{-1} cycloheximide added 2 h before the start of cold acclimation. Transcript levels at 0 (nonacclimated control), 3, 6, and 9 h of cold acclimation were determined. CA, Cold acclimation.

Relative Genomic Content and Organization of *cas15* DNA in Cultivars with Different Degrees of Freezing Tolerance

Cultivars of alfalfa differ in their capacity for cold acclimation-induced development of freezing tolerance (Mohapatra et al., 1989). We have investigated two of the possible reasons for differential freezing tolerance of various cultivars: (a) gene dosage may be lower in less tolerant than in more tolerant cultivars and (b) gene organization may be different in different cultivars, resulting in defective or less efficient genes in less tolerant cultivars. To answer these questions, we have determined the relative amount of *cas15* DNA as a fraction of total genomic DNA in three cultivars that differ in their capacity to develop cold-induced freezing tolerance. We have also examined the differences in restriction fragment-length polymorphism in two cultivars that belong to the same species but differ in freezing tolerance.

Figure 6A shows LT_{50} values, cold-induced transcript levels, and relative genomic contents of *cas15* DNA in three alfalfa cultivars. It can be seen that the relative amount of *cas15* DNA in the genome appears to be the same in the three cultivars, although they differ in cold-induced freezing tolerance and *cas15* transcript levels. The genomic organization of *cas15* DNA in *M. sativa* cv Trek and cv Apica, which differ in their freezing tolerance (LT_{50} of −9 and −14.5°C, respectively), was then investigated. The results of genomic Southern analysis using three different restriction enzymes are shown in Figure 6B. The number and size of the DNA fragments hybridizing to the pAcs2201 probe, generated with each enzyme used, are different in the two cultivars. It may, therefore, be concluded that the organization of *cas15* DNA in the genome is different in the two cultivars of *M. sativa* examined.

DISCUSSION

The present study shows that the accumulation of *cas15* transcripts is rapidly induced during cold acclimation and does not require de novo protein synthesis. The level of *cas15* transcripts correlates with the degree of freezing tolerance developed during cold acclimation at 2 or −2°C. Two features of the cDNA sequence suggest that the open reading frame is full length: (a) an in-frame stop codon is present before the initial Met and (b) the nucleotides immediately before the start codon correspond to the sequence frequently found in dicotyledonous plants (Cavener and Ray, 1991).

The location and arrangement of the basic amino acids in the putative bipartite motif at the carboxy terminus of CAS15 show high homology with the essential basic amino acids of other NTS sequences. Three of the NTS sequences shown in Figure 3, VirE2 from *Agrobacterium* (Citovsky et al., 1992), nucleoplasmin from *Xenopus* (Robbins et al., 1991), and O2 from maize (Varagona et al., 1992), have been demonstrated to be functional by deletion analysis or point mutations. The spacer region between the two termini of the NTS sequences is known to be variable in length and may consist of up to 22 residues without loss of NTS function (Robbins et al., 1991). Thus, CAS15 is likely to be a nuclear-targeted protein. It is pertinent to point out that only about 4% of the cellular proteins possess such bipartite motifs of basic residues (Dingwall and Laskey, 1991). Because the bipartite NTS sequences are rich in basic amino acid residues (Arg or Lys), the presence of Lys residues in the case of CAS15 gives rise to an apparent homology between the region of the putative NTS sequence and the LEA/dehydrin proteins. However, the two conserved Lys-rich domains, characteristic of the LEA/dehydrin proteins, are absent in CAS15. Outside the putative NTS sequence of the CAS15, there is little homology to the LEA/

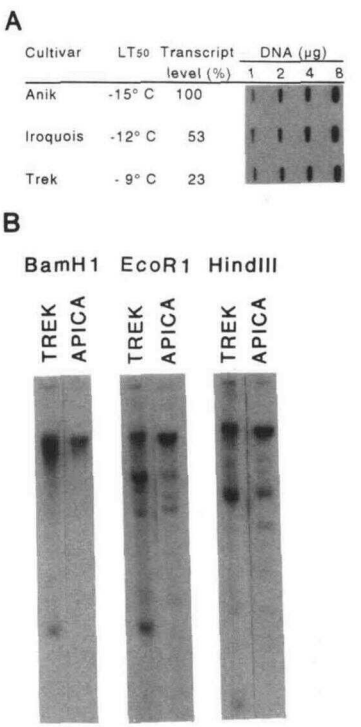

Figure 6. Genomic analysis of *cas15* DNA in different cultivars. A, The relationship between cold-induced freezing tolerance (LT_{50}), transcript level, and relative genomic content of *cas15* DNA in three different cultivars of alfalfa differing in freezing tolerance. B, Genomic organization of *cas15* DNA is different in two *M. sativa* cultivars, Apica (LT_{50} of −14.5°C) and Trek (LT_{50} of −9°C). DNA samples (10 μg) were digested separately with different enzymes indicated above and subjected to southern blot analysis with the radiolabeled pAcs2201 insert.

dehydrin proteins. Although the computer programs developed for predicting secondary structure have limited accuracy, it is interesting that the predicted secondary structure of CAS15 is different from that of CAS18, a dehydrin-related protein product of the cold-induced gene *cas18* (Wolfraim et al., 1993). Whereas CAS15 is predominantly α-helical (about 65%), CAS18 contains regions of α-helical and extended β-sheet conformations in equal proportions, together constituting nearly 90% of the protein molecule. The similarity of the predicted secondary structure of CAS15 to that of other known nuclear proteins has not been examined. However, the diverse nature of functional interactions of nuclear proteins is likely to be associated with a corresponding diversity in secondary structure.

Although experimental induction of cold acclimation is generally carried out at nonfreezing low temperatures alone, the additional promotive effects of exposure to subzero temperatures on freezing tolerance have been reported. (Olien, 1984). The appearance of new proteins in *M. sativa* L. cv Apica crown tissue when plants are transferred from 2 to −2°C has also been reported (Castonguay et al., 1993). The present study provides correlative evidence for a molecular genetic basis for these effects of subzero temperatures on freezing tolerance and protein synthesis. The underlying mechanisms of this enhanced transcript accumulation at subzero temperatures are presently unclear, but increased promoter activity and/or transcript stability may be contributing factors. It is interesting that the cold-induced level of *cas15* transcripts is the highest in the crowns, the overwintering part, and lowest in the leaves that do not survive winter. There is a particularly remarkable increase in *cas15* transcript level in roots when plants are transferred from 2 to −2°C. As underground plant parts, roots are protected from atmospheric temperature changes and probably respond only when ambient temperature declines further.

Different cultivars of alfalfa vary in their capacity to develop cold-induced freezing tolerance, and the cold-induced level of transcripts of cold acclimation-specific genes shows a high positive correlation with the degree of freezing tolerance (Dhindsa and Mohapatra, 1988; Mohapatra et al., 1989). It has been suggested that the low level of cold induction of these genes in sensitive varieties is not due to their absence but due to their defective regulation or structure. The present study shows that the relative amount of *cas15* DNA as a fraction of total genomic DNA is the same in three cultivars that vary in their freezing tolerance. Thus, gene dosage is unlikely to be the underlying cause of the differential freezing tolerance of these cultivars.

The genomic organization of the *cas15* DNA is different in cultivars with different degrees of freezing tolerance (*M. sativa* cv Apica and cv Trek in Fig. 6B). This situation is in marked contrast to that in wheat, in which the size and number of fragments that are generated with several different restriction enzymes and hybridized to the cDNAs of two cold-induced genes, *Wcs120* (Houde et al., 1992) and *Wcs200* (Oullet et al., 1993), are similar in freezing-tolerant and freezing-sensitive cultivars. It is tempting to speculate that altered genomic organization of the *cas15* DNA in the relatively sensitive cultivars may have rendered this gene defective or less efficient in its induction by cold. If the altered genomic organization of *cas15* DNA in the relatively freezing-sensitive cultivars is responsible for less efficient cold induction of this gene, the mechanisms underlying this effect are unclear but worth investigating.

A significant observation of the present study is that *cas15* induction by cold can occur while the majority of protein synthesis is inhibited. Furthermore, it can be seen (Fig. 5) that the cold-induced transcript level is considerably higher in cycloheximide-treated cells than in untreated controls. These results suggest that (a) processes involved in low-temperature signal perception and transduction, up to and including the accumulation of *cas15* transcripts, can take place without requiring de novo protein synthesis and (b) transcript stability may be an important factor in determining the transcript abundance. The time allowed for transcript accumulation after cycloheximide treatment was selected to be long enough to obtain a detectable transcript level but as short as possible to avoid secondary effects of inhibition of protein synthesis.

If CAS15 indeed turns out to be a nuclear protein, as this study suggests, what is its possible functional significance? There is a large diversity of nuclear proteins performing different functions, including gene regulation and maintenance of nuclear structure and function. Thus, CAS15 may be a regulatory protein acting either directly through its interaction with DNA or indirectly through protein-protein interactions. However, comparison of CAS15 sequence with those of other proteins reported in the data bases revealed a weak homology to several nuclear proteins but not to any DNA-binding domain. Alternatively, CAS15 may contribute to the stabilization of nuclear structure and/or function as a part of the process of cold acclimation. Although the elucidation of the precise function of *cas15* gene must await a detailed and prolonged study, evidence from two different sources suggests that *cas15* plays an important role in cold-induced development of freezing tolerance: (a) accumulation of *cas15* transcripts is rapid and positively correlated with the development of freezing tolerance and (b) when Ca^{2+}-mediated protein phosphorylation during cold acclimation is inhibited, not only does freezing tolerance not develop but the expression of *cas15* is sharply reduced (Dhindsa et al., 1993; Monroy et al., 1993). Studies of the precise intracellular localization of CAS15 and elucidation of its function during cold acclimation will now be possible with the characterization of this cDNA.

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