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Cloning, Characterization and Regulation of Expression of a Cold-Acclimation-Specific Gene, *cas18*, in a Freezing Tolerant Cultivar of Alfalfa

Lawrence A. Wolfraim

DEPARTMENT OF BIOLOGY
McGILL UNIVERSITY, MONTREAL,
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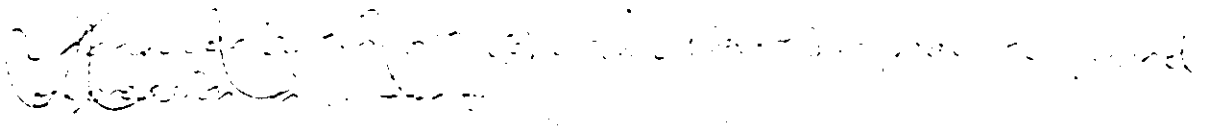

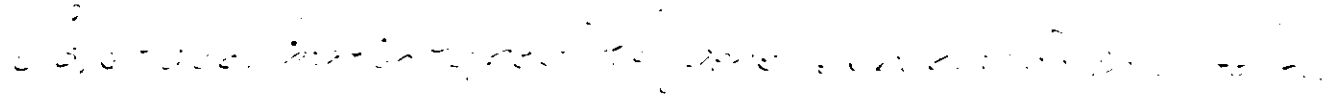

This thesis is dedicated to my mother and father, without whose love, support and encouragement this work would not have been possible.

Abstract

Cold-acclimation-specific (CAS) gene expression was examined by screening a cDNA library prepared from poly(A)$^+$ RNA of cold-acclimated seedlings of a freezing-tolerant variety of alfalfa (*Medicago falcata* cv Anik). Three distinct CAS cDNA clones, pSM784, pSM2201, and pSM2358 were isolated. The genes corresponding to all three clones are coordinately induced by cold. Expression of these genes is not triggered by other stress treatments such as heat shock, water stress, wounding, or treatment with exogenous ABA. A positive correlation was observed between the level of expression of each gene and the degree of freezing tolerance of four alfalfa cultivars.

A full-length cDNA clone for the most abundantly-expressed gene, *cas18* was isolated and sequenced. The deduced polypeptide, CAS18, is relatively small (167 amino acids), is highly hydrophillic, rich in glycine and threonine, and contains two distinctive repeat elements. It exhibits homology with members of the LEA/RAB/Dehydrin gene family - proteins which accumulate in response to water stress or abscisic acid (ABA). The *cas18* cDNA hybridizes to three transcripts of 1.6, 1.4 and 1.0 kb in cold acclimated seedlings and cell cultures. The clone described here, Acs784, corresponds to the 1.0 kb transcript.

Expression of this gene is 30-fold greater in cold-acclimated cells than in nonacclimated cells after one week of low temperature treatment. Return to room temperature (deacclimation) results in the rapid disappearance of the three

transcripts within just 5 hours. Studies of nuclear "run-on" transcription and transcript stability show that low temperature regulates the expression of *cas18* at both the transcriptional and post-transcriptional levels.

Résumé

L'expression de gènes spécifiques à l'acclimatisation au froid (CAS) a été étudiée par le criblage d'une banque d'ADN complémentaire (ADNc) préparée à partir d'ARN poly(A)$^+$ de grains acclimatés au froid d'une variété d'alfafa qui est tolérante à la congélation. Trois ADN complémentaires de CAS, pSM784, pSM2201 et pSM2358 ont été isolés. Les gènes correspondant à ces ADN complémentaires sont induits de façon coordonnée par le froid. L'expression de ces gènes n'est pas induite par d'autres traitements tels la chaleur, la sécheresse, les traitements physiques et chimiques comme l'ABA. Une correllation positive a aussi été observée entre le niveau d'expression de chaque gène et le degré de tolérance à la congélation de quatre alfafa cultivars.

Un ADNc contenant toute la région codante pour le gène qui est le plus exprimé, cds 784, a été isolé et séquencé. La séquence en acide aminé deduite de l'ADNc est relativement petite (167 acide amines), très hydrophilique, riche en glycine et thréonine et contient deux éléments répétitifs distinctifs. Il existe aussi une similarité de séquence avec les gènes membres de la famille LEA/RAB/Dehydrine qui codent pour des protéines, qui s'accumulent en réponse à la sécheresse et le ABA. L'ADNc Acs784 forme des hybrides spécifiques avec trois ARN messagers de 1.6, 1.4 et 1.0 kilobases tels qu'observé par analyse Northern d'ARN isolé de grains acclimatés au froid et en culture cellulaire. Le

clone décrit dans ce rapport, Acs784, correspond à l'ARN messager de 1.0 kilobase.

L'expression du gène *cas18*, est 30 fois plus élevée dans les cellules acclimatées au froid comparativement aux cellules qui ne sont pas acclimatées au froid après une semaine de traitement au froid. Les trois ARN messagers ne peuvent être détectés lorsque les cellules sont incubées à la température de la pièce pendant 5 heures. Des études de transcription nucléaire et de stabilie des ARN messagers ont démontré que le froid régularise l'expression de *cas18* au niveau de la transcription et post-transcription.

(Traduit par Stephane Lee et Dr. Lise Hébert)

Contributions to Original Knowledge

1. In collaboration with Dr. Mohapatra, I have described the molecular cloning of three Cold-Acclimation-Specific, (CAS), genes in alfalfa seedlings (Mohapatra et al., 1989). These genes are coordinately induced by low temperature, are not induced by other stress treatments, and their level of expression is positively correlated with varietal freezing tolerance of four alfalfa cultivars. This was the first description of cold acclimation-specific gene expression correlated with freezing tolerance.

2. I have isolated and sequenced a cDNA clone containing a full-length ORF, pAcs784, for the most abundantly expressed cold-inducible gene, *cas18*, of alfalfa (*Medicago falcata* cv. Anik) seedlings. This was achieved by screening a cDNA library which I constructed in λUni-ZAPII using poly(A)$^+$ RNA from cold acclimated seedlings. The probe used to screen this library was the partial length cDNA clone previously isolated, pSM784, (Mohapatra et al., 1989).

3. I have demonstrated that the predicted translation product of this gene, the polypeptide CAS18, has significant homology with proteins that accumulate in response to drought and ABA, collectively referred to as LEA/RAB/Dehydrin proteins.

4. I have developed a cell suspension culture system of the most freezing tolerant alfalfa cultivar identified in our previous studies, *Medicago falcata* cv. Anik. This system has proved very useful for detailed analyses of cold-inducible gene expression and signal transduction pathways.

5. I have demonstrated that *cas18* is expressed in cell suspension cultures in a manner which is qualitatively and quantitatively similar to the pattern of expression seen in seedlings. Expression is inducible and reversible: transcripts accumulate in response to low temperatures (5°C) and rapidly disappear upon return to nonacclimating temperatures (25°C).

6. I have shown that the expression of *cas18* is regulated at both the transcriptional and post-transcriptional levels.

Preface and Acknowledgements

This thesis is assembled in accordance with the regulations of the Faculty of Graduate Studies and Research. It consists of an Abstract, Résumé, Introduction, Literature Review (Chapter 1), Materials and Methods (Chapter 2), Results (Chapters 3 through 6), Discussion (Chapter 7) and References.

Part of this work has been published:

Mohapatra, S. S., Wolfraim, L., Poole, R. S., Dhindsa, R. S. 1989. Molecular cloning and relationship to freezing tolerance of cold-acclimation-specific genes of alfalfa. *Plant Physiol.* 89:375-380

Two additional manuscripts have been accepted for publication:

Dhindsa, R. S., Monroy, A. F., Wolfraim, L. A., Dong, G. 1992. Signal transduction and gene expression during cold acclimation of alfalfa. *In* Li, P. H., Christersson, L., eds, *Advances in Plant Cold Hardiness*, CRC Press, Roca Baton, Florida. pp 57-71

Wolfraim, L. A., Langis, R., Tyson, H., Dhindsa, R. S. 1992. cDNA sequence analysis and regulation of expression of a cold-acclimation-specific gene in alfalfa cell cultures. Plant Physiol. (in press)

All of the data presented in this thesis is the work of the author with the following exceptions:

1. The original pBR322 plasmid library was constructed by Dr. Shyam S. Mohapatra. The work described in the first publication (above) was conducted in collaboration with Dr. Mohapatra.

2. The freezing survival analysis of cell suspension cultures was performed in collaboration with Dr. Robert Langis.

I am indebted to my thesis supervisor, Dr. Rajinder Singh Dhindsa, for his advice, encouragement, enthusiasm and open-mindedness throughout the course of this work. I would also like to thank the individuals who served on my supervisory committee: Drs. Gregory Brown, Hugh Tyson, Jody Banks and Ronald Poole. I gratefully acknowledge the assistance and encouragement of all members of our lab: Drs. Antonio Monroy, Shyam Mohapatra and Robert Langis; Alison Taylor, Angie Rosenhauer, Sharon Forrest and G. Dong. I would like to acknowledge the encouragement and support of Guy Laverdure, Shelly Adamo, Liz DeBlois et al., Gillian R. Witchell, Mahipal Singh, Peter Cserjesi, the Copaholics and many other members of the Department of Biology. I would like to acknowledge the support of Canadian Pacific in providing me with a Canadian Pacific Fellowship in Biotechnology for four years of my Graduate studies.

Finally, I would like to express my appreciation to Catherine Liu for her assistance with word processing and Stephane Lee and Lise Hébert for their translation of the Abstract into French.

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List of Abbreviations

A	acclimated
ABA	abscisic acid
Acs	alfalfa cold-specific
Acs784	the cloned cDNA sequence removed from the pBluescript vector
bp	base pairs
cm	centimetre
CA	cold acclimated
CAS	cold acclimation-specific
cas18	the gene to which the cloned cDNA sequence (Acs784) corresponds
CAS18	the protein encoded by *cas18*
cDNA	complementary DNA
cpm	counts per minute
Ci	Curie
DA	deacclimated
Denhardt's	1X = 0.2% each of bovine serum albumin, Ficoll, and polyvinylpyrrolidone
DHN	dehydrin
DNA	deoxyribonucleic acid
DEPC	diethylpyrocarbonate
EDTA	ethylene diamine tetraacetic acid

h	hour
kb	kilobase
kDa	kilodaltons
LEA	late embryogenesis abundant
LT_{50}	Lethal Temperature of 50% survival
L	litre
mRNA	messenger RNA
m	metre
K_m	Michaelis-Menten constant
μCi	microcurie
μE	microeinstein
μg	microgram
μl	microlitre
mCi	milliCurie
ml	millilitre
mm	millimetre
mM	millimolar
min	minute
M	molar
NA	nonacclimated
ORF	open reading frame

pAcs784	the cloned cDNA sequence in the pBluescript vector
poly(A)$^+$	polyadenylated
RNA	ribonucleic acid
RAB	rice abscisic acid-inducible
s	second
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SSC	standard saline citrate
Tris-Cl	tris(hydroxymehyl)aminomethane hydrochloride
TBE	Tris borate EDTA
TCA	trichloroacetic acid
TTC	2,3,5-triphenyltetrazolium chloride
2D-PAGE	two-dimensional polyacrylamide gel elctrophoreis

INTRODUCTION

Low temperatures constitute one of the most significant constraints limiting the growth and reproduction of organisms. Freezing temperatures in particular represent a significant barrier to the survival of most species. The freezing of cells and tissues is almost always lethal. Nonetheless, a variety of different species are capable of inhabiting the coldest regions of the planet. Fish species that inhabit polar waters are able to resist freezing of their body fluids through the production of antifreeze proteins and glycoproteins (DeVries, 1983). Insects that inhabit cold climates where they may be exposed to subzero temperatures have similarly evolved mechanisms to survive freezing (Lee, 1989). Certain species of insects are able to supercool to as low as -60°C (Miller, 1982).

The most dramatic examples of freezing tolerance in higher eukaryotes are found in certain freezing-hardy plant species. When fully acclimated, some plant tissues can survive experimental freezing in liquid nitrogen (Sakai, 1960). Plants are generaly sessile and are limited in their ability to avoid environmental extremes. Certain annuals are able to avoid freezing temperatures by producing seed before the onset of winter. Seeds are resistant to environmental extremes of temperature. Perennial species have evolved mechanisms to survive overwintering and to resume active growth in the spring.

"Although the fact that plants freeze and are often killed by freezing is well known to everyone, the details of the process are known to but very few, as most textbooks give little attention to the subject."

K. M. Wiegand, 1906 (c.f., Steponkus, 1984)

Since Wiegand described the status of knowledge of freezing injury at the beginning of the twentieth century, thousands of articles on cold acclimation and freezing stress of plants have been published. Numerous attempts have been made to catalogue the many biochemical and physiological changes that occur during cold acclimation in hardy versus sensitive species, in the hope of identifying the factors responsible for increased freezing tolerance. As emphasized in a recent review (Guy, 1990), most of these attempts have failed to increase significantly our understanding of the underlying mechanisms of freezing tolerance.

In 1912, the first detailed studies of the genetics of freezing tolerance were conducted (Nilson-Ehle, 1912). However, it was not until 1970 that changes in gene expression were proposed to mediate the cold-inducible component of freezing tolerance (Weiser, 1970). Direct evidence for this proposal was obtained in 1985 when Guy and coworkers observed that low temperature resulted in rapid and persistant changes in translatable mRNAs in spinach (Guy et al., 1985).

The first report of the molecular cloning of genes which are induced by low temperature and which are correlated with varietal differences in freezing tolerance appeared in 1989 (Mohapatra et al., 1989). This publication forms part of this thesis. Since this first report, several other groups have published the cloning of cold inducible genes in a variety of other plant species.

The approach taken in the present study involved the construction of a recombinant cDNA library from RNA of cold acclimated tissue. Differential screening of such a library was performed using single-stranded cDNA probes prepared from RNA isolated from (1) cold acclimated and (2) nonacclimated plants. Clones which hybridized to probes prepared from cold acclimated material but not to those prepared from nonacclimated sources represent low-temperature-induced genes. Next, the level of expression of these genes, as determined by northern analysis, was compared in cultivars differing in their level of freezing tolerance. Freezing tolerance can be assessed physiologically by determining the freezing temperature which results in 50% survival (LT_{50}) after a simulated freeze/thaw cycle. Survival can be measured by visual inspection, solute leakage, or in the case of cell cultures by reduction of tetrazolium salts (eg.,TTC) and by a variety of vital dyes such as fluorescein diacetate. This approach has led to the identification of genes which are induced by low temperature and which are correlated with the development of freezing tolerance. Correlation of expression with the gradual onset and sudden loss of freezing

tolerance further strengthens the case for a positive role of these genes in freezing tolerance.

Sequence analysis of these genes permits the prediction of the protein product. Clues to potential function can be gleaned by searching for homologies shared by these genes and other published sequences in a computer databank. Finally, an investigation of the mechanisms involved in the regulation of these cold-inducible genes leads to a better understanding of the signal transduction pathways underlying freezing tolerance. Such studies would also enable the characterization of cold-inducible promoter, enhancer or other regulatory elements.

Alfalfa is an important forage crop for the cattle and dairy industries of Canada, particularly those of Québec (Genest and Winter, 1983). However, it suffers considerable loss during overwintering and certain high-yielding cultivars cannot be grown successfully in Québec because they are not sufficiently freezing tolerant. A understanding of the molecular genetic basis of freezing tolerance in lower-yielding cultivars which are relatively more freezing-resistant, could be of considerable economic benefit. With respect to freezing tolerance, there is a range of alfalfa genotypes available which forms a continuous spectrum of freezing resistance levels. Since the different cultivars have similar genetic backgrounds, it is reasonable to expect that introducing a gene which confers increased tolerance from a resistant cultivar into a sensitive cultivar should have

a high probability of success in increasing the level of tolerance in the sensitive plant. Another advantage of alfalfa as a system is the relative ease with which it can be transformed. Also, successful regeneration of alfalfa can be achieved. Accordingly, transgenic alfalfa plants harboring freezing tolerance genes could be created. In this way, the freezing tolerance of high-yielding but freezing-sensitive varieties could possibly be improved by the introduction into these plants of freezing-tolerance genes from resistant cultivars. Obviously, the cloning and characterization of genes which confer freezing resistance are a necessary prerequisites for any future attempts at increasing the level of freezing tolerance of high-yielding cultivars through applications of recombinant DNA technology.

The focus of this thesis is the identification, characterization and regulation of expression of a low-temperature-induced gene in alfalfa (*Medicago falcata* cv. Anik) and its possible functional significance to low-temperature-induced freezing tolerance. The first report of the cloning and characterization of cold-acclimation-specific genes which are not induced by other stress treatments and whose expression is positively correlated with the development of freezing tolerance and with varietal differences in levels of tolerance appeared in 1989 (Mohapatra et al., 1989). This work was carried out in collaboration with Dr. Shyam Mohapatra. Part of the data from this publication, representing my contribution, has been included in this thesis and constitutes Chapter 3. Initial

characterization of one clone, pAcs784, corresponding to one of these *c*old-*a*cclimation-*s*pecific (*cas*) genes, *cas18*, is presented. The development of a cell culture system is described along with an analysis of the kinetics of transcript accumulation during acclimation and deacclimation of these cultures. An examination of the regulation of expression of *cas18* at both the transcriptional and posttranscriptional levels is given. Finally, speculations as to the possible functional significance of the gene product of *cas18* are presented in light of homologies with other published sequences.

Chapter 1 provides a comprehensive review of the literature pertaining to low temperatures stress, specifically freezing stress. The chapter begins with an overview of the many environmental stresses faced by plants, of which low temperature is but one. Since freezing stress is intimately linked with drought stress, an overview of the latter has been included, together with a discussion of the role of the phytohormone abscisic acid (ABA). Cold acclimation, which results in increased freezing tolerance, is itself a chilling stress. Often, the stresses of freezing and chilling are confused with one another; therefore, a detailed overview of chilling stress is included. An understanding of the nature of freezing tolerance would be incomplete without a discussion of the events involved in a freeze/thaw cycle; hence, a discussion of these physiological and biochemical events and the possible injuries produced are provided. This is followed by a review of the current knowledge of the genetics of freezing

tolerance. Also included is a summary of the myriad biochemical alterations reported to occur during cold acclimation and which are correlated, in some cases, with the development of freezing tolerance. Finally, evidence for changes in gene expression is reviewed, concluding with the most recent studies describing the isolation and characterization of low-temperature-induced genes.

CHAPTER 1.

LITERATURE REVIEW

1.1. Variety of Environmental Stresses Faced by Plants

Plants are subjected to numerous environmental stresses. In what has become a classic treatise in the field of plant stress, Levitt (1980) delineates environmental stresses into two broad categories: (1) biotic (infection or competition by other organisms) and (2) physiochemical. Physiochemical stresses include the following: temperature (high or low); water (excess or deficit); radiation (infrared, visible, ultraviolet, ionizing); chemical (eg. salts, ions, gases, heavy metals, herbicides); and mechanical/electromagnetic. A stress on a biological system may be defined as any environmental factor capable of inducing a potentially injurious strain in a living organism (Levitt, 1980). The biological strain is the reaction of the organism to the imposed stress and may manifest itself either physically (eg. a change in dimension) or chemically (eg. a shift in metabolism). These various stresses may disrupt normal growth and reproduction or even result in severe injury or death to a plant. In the case of important crop species, injury results in a lowering of food quality and yield.

Many plant species have developed strategies for dealing with these various stress situations. Much work has been conducted in the areas of: (1) pathogen stress; (2) response to heavy metals; (3) oxidative stress; (4) anaerobic stress; (5) heat shock; (6) drought and water stress; and (7) low temperature stresses.

In response to infection by pathogens such as fungi, bacteria, or viruses, or to application of elicitor molecules (small glucans released from pathogen cell

walls), the plant synthesizes a set of pathogen-related (PR) proteins, and defense-related compounds such as phytoalexins which are involved in halting the advancement of the pathogen into healthy tissue (Matters and Scandalios, 1986). Plants grown in soils contaminated with high concentrations of heavy metals such as cadmium and copper, exhibit several metabolic dysfunctions. In response to heavy metals, resistant plants are capable of synthesizing low molecular weight, cysteine-rich, water soluble, metal-binding proteins (Jackson et al., 1985), analogous to the metallothionein proteins studied in animals (Webb, 1979). Oxidative stress refers to the increased intracellular accumulation of activated oxygen species such as superoxide, hydrogen peroxide or hydroxyl radical, which are potentially injurious to the plant cell. Anaerobic stress (anaerobiosis) occurs naturally in tissues of plant roots subjected to flooding. This stress has been shown to repress the normal pattern of protein synthesis and to induce the synthesis of a characteristic set of proteins collectively termed: major anaerobic proteins (ANPs)(Sachs et al, 1980). Synthesis of ANPs is biphasic with the synthesis of an early set of polypeptides, followed by the induction of a late set of polypeptides (Sachs and Ho, 1986).

High temperature is probably the best studied stress. The most extensively documented response to high temperatures is the universal heat shock response: a global change in the pattern of protein synthesis resulting from the induction of heat shock proteins (Hsp) and a concomitant repression of the normal pattern of

protein synthesis. Hsp are detected within 20 minutes of heat shock. Increases in the levels of transcripts for hsp are detected as early as 3 to 5 min after exposure to high temperatures - making this the most rapid temperature-inducible response known in plants (Key et al., 1981). In many organisms, the induction of hsp allows cells to establish increased thermotolerance (Scharf and Nover, 1982).

1.2. Drought, Desiccation, Water Deficit or Osmotic Stress.

Water deficit results in drought or water stress. As is the case with most stresses, two resistance strategies are employed by plants: avoidance or tolerance. Avoidance of osmotic stress is achieved through specialized adaptations involving root and shoot architecture and phenology (Paleg and Aspinal, 1981). Tolerance involves more subtle biochemical changes. The phytohormone abscisic acid (ABA) is involved in mediating various developmental and physiological events including responses to dehydration or osmotic stress. While even a mild water deficit is injurious to a plant, at other stages of its life cycle desiccation is a natural event and facilitates seed survival during a dormant period. Desiccated mature seeds are extremely resistant to many environmental stresses. ABA has been implicated in the control of many of the events during embryogenesis and seed maturation, including tolerance to desiccation (Kermode and Bewley, 1987)

and in fact, ABA levels peak shortly before the beginning of seed desiccation (King, 1976; Suzuki et al. 1981).

Plants are frequently subjected to periods where the availability of water is limited. Under conditions of water stress, growth of the plant is inhibited (Cleland, 1967; Gates, 1968) and a variety of morphological and biochemical changes occur (Hsiao, 1973). Water stress inhibits the incorporation of amino acids into proteins (Ben-Zioni et al., 1967; Nir et al., 1970) and leads to a decrease in protein content of tissues (Shah and Loomis, 1965; Stutte and Todd, 1969), and a decrease in polysome levels (Hsiao, 1970). Work conducted with oat coleoptiles using a double-labeling ratio technique showed that water stress causes a differential inhibition of protein synthesis with the synthesis of some proteins being affected more than that of others (Dhindsa and Cleland, 1975). Polysome loss during desiccation of the drought-tolerant moss *Tortula ruralis* was shown to precede increases in ribonuclease activity (Dhindsa and Bewley, 1976). The authors demonstrated that ribosome runoff from mRNA coupled with a failure to re-form an initiation complex was the primary cause of polysome loss during desiccation.

The drought-tolerant moss *Tortula ruralis* can be dried rapidly (less than 30 min), or slowly (8 to 10 h), to less than 20% of the original fresh weight (Dhindsa and Bewley, 1976). Upon rehydration, the moss regains nearly 99% of its original fresh weight in less than 90 s. The total RNA pool, and the

poly(A)$^-$ RNA subpool of *T. ruralis* are highly stable to desiccation, regardless of the speed of drying, and to rehydration. In contrast, the same RNA pools of the aquatic moss *C. filicinum* are stable to desiccation but are partially lost during the initial stages of rehydration (Oliver and Bewley, 1984a). In *T. ruralis*, the stable conserved total cellular RNA pool is utilized in protein synthesis during rehydration; this does not occur in the drought-intolerant moss, *C. filicinum*. In *T. ruralis* there is synthesis of new RNA during this rehydration which is rapidly processed and recruited into ribosomal subunits, ribosomes, and polysomes, the rate being more rapid in slowly dried than in rapidly dried moss (Oliver and Bewley, 1984a). RNA synthesized during the first hour of rehydration is preferentially recruited into the polysomal fraction. In *C. filicinum*, the ability to engage in new RNA and protein synthesis upon rehydration is lost. It appears therefore, that the ability of *T. ruralis* to utilize conserved mRNA for protein synthesis and its capacity for new mRNA synthesis upon rehydration is an important part of its capacity to survive desiccation.

Initially, poly(A)$^+$ RNA conserved in the dry moss *T. ruralis* is complexed with polysomes, but by 2 h of rehydration there is an overwhelming recruitment of newly synthesized poly(A)$^+$ RNA, at the expense of the conserved messages (Oliver and Bewly, 1984b). In rehydrated moss, there is synthesis *in vivo*, as determined by 2D-PAGE, of new polypeptides, termed rehydration proteins. However, protein patterns from *in vitro* translation of bulk RNA from hydrated,

desiccated, and rehydrated moss were qualitatively identical. This suggested that changes in protein synthesis *in vivo* as a result of desiccation were the result of control at the translational level.

Dehydration of plants has been shown to trigger a rise in endogenous ABA levels of up to 50-fold (Hensen and Quarrie, 1981) due to *de novo* biosynthesis of the phytohormone (Milborrow and Robinson, 1973; Zeevaart, 1980). A loss of cell turgor (Pierce and Raschke, 1980), or cell membrane perturbation associated with pressure potentials approaching zero are believed to be key factors in triggering ABA accumulation (Ackerson and Radin, 1983). Also required for ABA accumulation in dehydrated plants is transcription of nuclear genes since pretreatment of excised pea plants with transcriptional inhibitors prior to plant dehydration inhibits the synthesis of ABA (Guerrero and Mullet, 1986).

The ABA-deficient tomato mutant, *flacca* (*Lycopersicon esculentum* Mill. cv Ailsa Craig), does not synthesize ABA in response to drought stress (Neill and Horgan, 1985). This mutant has been used to distinguish polypeptides and *in vitro* translation products that are synthesized during drought stress in response to increased levels of ABA from those that are induced directly by altered water relations (Bray, 1988). This study demonstrated that many of the polypeptides and mRNAs synthesized during drought are regulated by changes in ABA concentration.

Reduction of turgor induced rapid changes in leaf translatable RNA in pea (*Pisum sativum*) (Guerrero and Mullet, 1988). Analyses using 2D-PAGE of radiolabeled translation products of poly(A)$^+$ RNA from wilted versus control leaves led to the identification of several messages that increased in wilted plants. Most of these did not accumulate in response to heat shock or exogenously applied ABA despite elevated endogenous ABA levels. Differential screening of a λgt10 cDNA library constructed using poly(A)$^+$ RNA from wilted shoots identified four clones corresponding to genes induced in wilted shoots. The sequences of three of these clones were reported (Guerrero et al., 1990). Clone 7a encoded a 289 amino acid protein with limited homology to soybean nodulin-26, was abundant in roots and induced in shoots by dehydration, heat shock and to a lesser extent by ABA. The protein is predicted to have six potential membrane spanning domains similar to proteins that form ion channels. Clone 15a encoded a 363 amino acid protein with high homology to cysteine proteases and was more abundant in roots than in shoots of control plants. Transcript levels were induced by dehydration but not by heat shock or drought. Clone 26g encoded a 508 amino acid protein with limited homology to several aldehyde dehydrogenases. Transcript levels were induced by dehydration of shoots but not of roots. ABA and heat shock failed to increase levels of this message. Rehydration of wilted shoots caused levels of mRNA hybridizing to cDNA 26g to decline to pre-stress levels within 2 h. Nuclear run-on experiments showed

that transcription of the three genes was induced within 30 min following reduction of turgor pressure. Together, these results indicate that plant cells respond to changes in cell turgor by rapidly increasing transcription of several genes.

Although dangerous at other phases of a plant's life cycle, desiccation stress is a normal part of seed formation. The desiccation of seeds is characterized by the accumulation of LEA proteins (Late Embryogenesis Abundant) which are not found abundantly in either other plant organs or in embryonic tissues of seeds of earlier stages of development (Dure et al., 1989). Levels of LEA proteins remain constant in resting seeds but disappear at the onset of imbibition and germination. First discovered in cotton (Galau et al., 1986), indications suggest that LEA proteins are widely distributed in higher plant species (Scherer and Potts, 1989). *Lea* genes may also be induced to high levels of expression in other tissues and at other times of ontogeny by ABA and/or desiccation or osmotic stress (Baker et al., 1988; Galau et al., 1986).

Approximately 18 *Lea* genes have been cloned from cotton (Galau et al., 1992). Homologs of the cotton *Lea* genes have been identified as *rab* (responsive to ABA) in rice seedlings exposed to ABA or salt (Mundy and Chua, 1988; Yamaguchi-Shinozaki et al., 1989) and maize embryos and calli exposed to ABA (Vilardell et al., 1990), *Dhn* (dehydrin) in maize and barley seedlings exposed to water stress (Close et al., 1989), pcD27-04 and pcC6-19 in dried leaves and

ABA-treated calli of the resurrection plant *Craterostigma planagineum* (Piatkowski et al., 1990), RSLEA2 in dry seeds of radish (Raynal et al., 1990), and TAS14 in tomato seedlings exposed to osmoticum or ABA (Godoy et al., 1990). Recent work has demonstrated a correlation between the expression of certain *lea* genes and the development of desiccation tolerance in embryos (Bartels et al, 1988).

ABA has also been demonstrated to play an important role in the responses of vegetative tissues to osmotic stress (Singh et al. 1987; Zeevaart and Creelman, 1988). Current models suggest the following chain of events. Water deficit results in dehydration which in turn leads to a loss of turgor pressure which is first perceived by the plasmalemma. This leads to an increase in cytosolic and apoplastic ABA levels due to *de novo* synthesis and/or the release of the hormone from internal stores where it is sequestered in organelles (Zeevaart and Creelman, 1988). This increase in free ABA is dependant on *de novo* transcription. Increased levels of ABA induce the expression of genes of the *lea/rab/dehydrin* family. The available evidence suggests that the rise in ABA levels and the expression of *lea/rab/dehydrin* genes lead to an increase in osmotic stress tolerance. For example, glycophyte plants and cells respond to high osmoticum by changes in the composition of cell wall proteins and polysaccharides (Iraki et al., 1989), by accumulating osmoprotectants such as proline (Rhodes et al., 1986) and by accumulating RAB proteins (Ramagopal, 1987; Singh et al., 1987). These

changes are maintained in cells and plants which are adapted to high salt (Bressan et al., 1987; Gulick and Dvorak, 1987).

Sequence analysis of several *lea* genes showed these proteins to be extremely hydrophilic and revealed the presence of a conserved 11 amino acid repeat, tandemly repeated, which probably exists as an amphiphilic alpha-helix (Dure et al, 1989). Likewise, the dehydrins are extremely hydrophillic. Dehydrins (DHNs) are glycine-rich and possess repeated units in a conserved linear order. These units are lysine-rich and occur twice: once at the carboxyl terminus; and again near the middle of the polypeptide, immediately adjacent to a series of consecutive serine residues. Both the repeating unit and the adjacent flanking stretch of serines are highly conserved with minimal variation among all the dehydrins studied thus far (Close et al. 1989). This structural organization is seen in pcC6-19 and pcC27-01 of *Craterostigma* (Piatkowski et al.,1990), RAB 21 from rice (Mundy and Chua, 1988) and dehydrins from barley and maize (M3) (Close et al., 1989), and in cotton LEA protein D11 (Baker et al., 1988). These conserved domains are thought to be functionally important in desiccation protection (Dure et al. 1989).

LEA proteins are believed to be desiccation protectants. It has been postulated that cellular proteins are stabilized during desiccation through interactions with LEA proteins - a model reminiscent of the arguments invoked for heat shock proteins (Pelham, 1986) and protein stabilization by proline

(Csonka, 1989). These proteins are characterized as being extremely hydrophillic and contain a conserved lysine-rich amino acid repeat at the carboxy terminus (Baker et al., 1989; Galau and Close 1992). Certain of the LEA proteins are predicted to form amphiphilic helices which are predicted to form intramolecular helical bundles. These would present a globular surface for the binding of ions and the formation of salt bridges. This would prevent crystallization due to increasing ion concentration caused by desiccation. Other LEA proteins which have an unusual abundance of glycyl residues which allow free rotation around the peptide bond are predicted to exist as random coils. Their preponderance of hydroxylated amino acids such as threonine could serve to solvate structural surfaces. These proteins, because they can span greater distances, would be superior to sugars and would be less likely to crystallize (Baker et al., 1988).

1.3. Chilling Stress.

Low temperature stress can be divided into two categories (Levitt, 1980): chilling stress, and freezing stress. Chilling stress occurs at temperatures from about 15°C down to 0°C; that is, at low, non-freezing temperatures. Freezing stress occurs at subzero temperatures and usually involves the formation of ice. Chilling temperatures are a significant constraint on the production of crops of tropical or subtropical origin as growers try to extend northward their range of cultivation.

The term "chilling injury" was first coined by Molisch (1896) in a series of early studies to describe the killing of a number of species of plants at low temperatures above the freezing point (Levitt, 1980). In most plant species, chilling injury is observed below the range 12 to 10°C. Some exceptional cases exist where an organism may be killed at freezing temperatures in the absence of freezing, where the cell is in the undercooled state (Levitt, 1980). However, for the vast majority of plants, chilling stress occurs at temperatures below about 12° to 10°C but above 0°C. In general, the severity of chilling injury increases with decreasing chilling temperatures (Lyons, 1973). Chilling injury occurs mainly in plants of tropical or subtropical origin although certain cells of plants from temperate climates may exhibit symptoms of injury (Lyons, 1973). For example, species such as cotton, cowpea, peanut, corn and rice exhibit symptoms of chilling injury when exposed to chilling temperatures (Sellschop and Salmon 1928). In wheat from temperate climates, foliar temperatures of 0° to 3°C constitute chilling temperatures producing sterility when pollen is in the stage of the first nuclear division (Toda, 1962). The chilling-sensitivity of some plants can be reduced if the plant is exposed to temperatures slightly above the chilling range (Apeland, 1966; Lutz, 1945; McColloch, 1962; Wheaton and Morris, 1967), a process referred to as acclimation or hardening to chilling temperatures. Acclimation to chilling temperatures is not common and should not be confused with the more frequent

phenomenon of acclimation to freezing temperatures. In fact, the terms acclimation and hardening are widely used only in the context of adaptation to freezing temperatures.

Chilling-injured plants initially give the appearance of water-soaked leaves or soft spots on fruits which undergo necrosis or are invaded by secondary pathogens resulting in tissue decay (Lyons, 1973). The water soaked appearance is the result of the loss of membrane semi-permeability and leakage of electrolytes into the apoplast. These symptoms may be immediate or delayed depending on the species and the severity of the stress (Markhart, 1986).

> "To every problem, however complicated, there is a single, elegant solution which one will discover if one looks hard enough. This solution will turn out to be wrong."
>
> Rothchilde's Rule (c.f., Steponkus, 1984)

Lyons and Raison (1970), in an attempt to unify the many observations on chilling injury, suggested that the primary event in chilling injury is due to phase transitions of the plasmalemma bulk lipids from a fluid-crystalline state to a less-fluid gel state. Such phase transitions are thought to cause an alteration in the activity of membrane-associated enzymes and enzyme systems which in turn could lead to major shifts in cell metabolism. Supporting evidence comes from

analyses of diverse physiological and biochemical processes using Arrhenius plots (for review, see Markhart, 1986; Graham and Patterson, 1982; Lyons, 1973). Such studies have shown discontinuities in Arrhenius plots at temperatures that cause chilling injury in chilling-sensitive plants; these discontinuities are not seen for chilling-resistant plants. Enzymes examined include several dehydrogenases (Duke et al, 1977), plasmalemma ATPase (Wright et al, 1982), and cytochrome oxidases (Maeshima et al, 1980).

A number of probes used to measure the freedom of movement of molecules within the membranes of cells have correlated the sharp decline in membrane fluidity with the temperatures that cause chilling injury in chilling-sensitive plants (Markhart, 1986). These investigations have led to a mechanistic interpretation of the critical temperature characteristic of chilling injury (Markhart, 1986; Lyons and Raison, 1970). This threshold or critical temperature was explained as the temperature of the phase transition of the most sensitive membrane.

However, recent evidence and review of the some of the original studies have forced a re-evaluation of the phase transition hypothesis. First, biochemical analysis of the fatty acid composition of membranes has revealed that the high levels of unsaturated fatty acids in most membranes would not be predicted to give a sharp phase transition of bulk lipid in the temperature range of chilling injury (Bishop et al, 1979). Corroborating these studies, are re-examinations of

many of the claimed discontinuities in Arrhenius plots. In some cases, a better interpretation of the data would be a gradual reordering of the lipid bilayer over a fairly wide temperature range, rather than the reported sharp transitions (McMurdo and Wilson, 1980).

Differential scanning calorimetry (DSC), perhaps one of the most theoretically sound techniques currently available for the investigation of physical properties of membranes (Markhart, 1986), has convincingly shown transitions in pure lipid vesicles (McKersie and Thompson, 1979). However, results obtained with isolated membrane vesicles or isolated membrane lipids are inconsistent (O'Neil and Leopold, 1982; Dalziel and Breidenbach, 1979).

Others (Grierson et al, 1982) have reported discontinuities in Arrhenius plots for the activities of soluble enzymes such as phosphoenol pyruvate carboxylase (PEPCase). Here, a break in the plots was observed at 14°C in corn leaves. This has led to questioning of whether membrane changes are indeed not secondary events of chilling injury.

Carter and Wick (1984) point to the cytoskeleton as a potential primary site of chilling injury. It has long been known that depolymerization of microtubules is enhanced when cells are exposed to chilling temperatures. The breakdown of the cytoskeleton would be expected to have myriad ramifications on cell metabolism and membrane function. In support of this hypothesis, Rikin and co-workers (Rikin et al 1979) have shown that treatment of cucumber cotyledons

with antimicrotubular drugs exacerbated chilling injury. Abscisic acid pretreatment protected these cells from the effects of drug treatment and chilling. In animal cells, it has been shown that depolymerization of microtubules leads to an increase in the motional freedom of molecular probes in the plasmalemma (Aszalos et al, 1985) - providing a link between microtubules and membrane fluidity.

The homeoviscous hypothesis predicts that in chilling-tolerant plants one should find a greater percentage of unsaturated fatty acids than in the lipids from chilling-sensitive plants and that as chilling-tolerant plants are acclimated to chilling temperatures, the degree of unsaturation should increase (Markhart, 1986). Many studies have been conducted along these lines (for reviews see Markhart, 1986). Plants of the same species grown under cool, acclimating temperatures have a greater degree of unsaturated fatty acids than warm-grown plants. However, the correlation between chilling tolerance and degree of unsaturation is not precise (Lyons and Asmundson, 1965; Uritani and Yamaki, 1969; Yamaki and Uritani, 1972). Therefore, it would appear that although the degree of unsaturation of membrane phospholipids is important in acclimation to growth at low temperatures, it is not the only factor responsible for low temperature tolerance.

The controversy surrounding much of the work aimed at demonstrating a correlation between lipid alterations and cold acclimation may arise from the use

of whole tissue or crude membrane fractions, rather than plasma membrane *per se*. The lipid composition of the plasma membrane is distinct from that of other plant membranes (Lynch and Steponkus, 1987). The most notable distinguishing features are the relatively high content of glucocerebrosides and the very high proportion of free sterols and sterol derivatives. A study of membrane lipid alterations, associated with cold acclimation, in highly enriched plasma membrane fractions of winter rye seedlings showed an increase in free sterol content, primarily β-sitosterol (Lynch and Steponkus, 1987). Bound sterols (steryl glucosides and acetylated steryl glucosides) decreased. Phospholipid content increased and substantial changes in the molecular species of phospholipids were observed during cold acclimation. Di-unsaturated molecular species of phosphatidylcholine and phosphatidylethanolamine increased during cold acclimation. Sterols are known to alter the phase transition temperature in isolated erythrocyte ghosts (Chapman, 1968) and artificial liposomes (McKersie and Thompson, 1979).

A recent study (Wada et al, 1990) in a cyanobacterium showed enhancement of chilling tolerance by genetic manipulation of fatty acid desaturase, an enzyme that introduces double bonds into fatty acids of membrane lipids. The introduction of a plant-type desaturase gene (desA) from a chilling-resistant cyanobacterium into a chilling-sensitive one, increased the tolerance of the recipient to low temperature. At the same time, the recipient cyanobacterium

acquired desaturase activity, and the phase-transition temperature of the plasma membrane was lowered. Since cyanobacteria are widely accepted as a model system for plant cells (Murata and Nishida, 1987; Murata, 1989), a similar mechanism of enhancement of chilling tolerance may operate in higher plants.

The phytohormone ABA has been linked to increased chilling tolerance in a number of species. Nutrient stress, water stress, salt stress, chilling-stress and the application of exogenous ABA have all been shown to raise endogenous levels of ABA and to increase tolerance to subsequent chilling treatments (Rikin et al, 1979). Studies conducted by Markhart and coworkers have demonstrated the protective effect of ABA during chilling (Markhart, 1984). Chilling-induced water stress caused by an increase in root resistance was lessened by ABA treatment. The effect of ABA was not due to stomatal closure but was instead a direct effect on the membrane that limits water flow through the root (Markhart et al, 1979a; Markhart et al 1979b). When water flux was measured as a function of temperature, Arrhenius plots showed a marked discontinuity at around 15°C. This discontinuity was eliminated if the roots were pretreated with ABA. Time course experiments suggested that the effect of ABA was too rapid to allow for alterations in protein synthesis. Markhart proposes that ABA may act directly on the membrane by intercalating between the membrane phospholipids in a manner similar to sterols; alternatively, ABA may act indirectly on the membrane by causing a change in metabolism that in turn leads to a change in the membrane

(Markhart, 1986). DSC scans indicate that ABA broadens the temperature range over which phase transitions occur (Markhart, 1986). Such data provide evidence of a direct role for ABA in changing the fluid properties of a lipid bilayer.

The process of cold acclimation to freezing temperatures requires that the plant withstand chilling. Therefore, superimposed upon the metabolic changes associated with increased freezing tolerance are those changes which are involved in adaptation to chilling temperatures.

1.4. Freezing Stress

Freezing injury is a major cause of crop loss (Parker, 1963). In Canada, yields of forage crops are often limited by winter injury and the annual loss due to low temperatures can be as high as 40% or more in certain regions of Québec (Villemont et al., 1971). The stresses of late spring and early autumn frosts, low midwinter minima, and rapid temperature fluctuations are responsible for various types of injury directly and indirectly associated with the freezing of water in plant tissues (Burke et al., 1976). The forms of injury incurred by different plants include (Burke et al., 1976): crown kill in winter cereals, biennials, and herbaceous perennials; sunscald on thin-barked trees; winter burn to evergreen foliage; blackheart and frost cracking in xylem of trees and shrubs; blossom kill; death of vegetative shoots in late maturing perennials; death of buds and bark in

plants that rapidly lose hardiness during transient warm periods in winter; and death of tender annuals.

The ability of temperate perennials to withstand freezing follows a seasonal rhythm (Levitt, 1956; 1972). Cold acclimation or hardening denotes the seasonal transition from a freezing-sensitive to a freezing-tolerant state, typical of hardy species. Traditionally, cold acclimation has been viewed as having two separate functions (Guy, 1990). First, acclimation results in a more universal adjustment of basic cellular functions to the biophysical constraints imposed by low temperatures. Second, acclimation leads to the induction of freezing tolerance. The first function differentiates chilling sensitive from chilling tolerant species. The second function separates chilling tolerant but freezing sensitive from chilling tolerant and freezing tolerant species. Although not directly involved in the development of freezing tolerance, the ability to adapt to chilling temperatures is certainly a prerequisite for the second function of cold acclimation; obviously, if a plant cannot undergo the metabolic adjustments necessary for proper function during extended exposures to low temperatures, it is unlikely to acquire freezing tolerance. Henceforth in this thesis, the term cold acclimation will be used in the more restrictive sense of changes directly linked to the development of increased freezing tolerance.

This process is normally triggered by environmental cues such as low nonfreezing temperatures and photoperiod (short days). Cold acclimation is

measured physiologically as an increase in freezing survival. Freezing survival is most often expressed in terms of the LT_{50} (the freezing temperature at which 50% survival is observed). Deacclimation or dehardening is the reverse process and results in the loss of freezing resistance.

In temperate woody perennials, photoperiod (short days) signals the end of the growing season and triggers the physiological and biochemical processes which lead to the onset of dormancy and an increase in freezing tolerance (Van Huystee et al., 1967). Subsequent to photoperiodic induction, development of full freezing tolerance potential occurs in response to low nonfreezing temperatures. The first phase, photoperiodic induction, is thought to involve a translocatable factor (Fuchigami et al., 1971), which at least in some cases appears to be sucrose (Levitt, 1980). The second phase, induced by low temperatures, does not involve a translocatable factor (Howell, 1969). Interestingly, in spite of the basic differences between the two stages of acclimation, either low temperature or short days can produce acclimation (ie., increased freezing tolerance) in the absence of the other inductive factor. Woody perennials exposed to low temperatures and long days will eventually acclimate fully. Similarly, plants exposed to short days and noninductive temperatures will acclimate, although not fully (Van Huystee et al., 1967). A third stage of acclimation is thought to occur in hardy woody species and is induced by prolonged exposures to very low temperatures (-30° to -50°C) (Tumanov and

Krastsev, 1959). This third stage results in a level of hardiness not commonly attained in nature (Weiser, 1970). Deacclimation occurs when temperatures return to warm, nonacclimating levels, freezing tolerance is rapidly lost, and active growth resumes.

For most other species, primarily herbaceous perennials, photoperiodic cues may play little or no role in initiating acclimation; only exposure to low nonfreezing temperatures is necessary (Fennel and Li, 1985). Studies conducted in the field with alfalfa (*Medicago media* Pers.) have demonstrated that acclimation continued even under snow cover, suggesting that photoperiod or even the presence of light, was unimportant to the attainment of full hardiness (Paquin and Pelletier, 1980). These studies clearly showed that cold hardening and dehardening were closely and linearly related to mean air and soil temperatures at plant level, with correlation coefficients very close to unity. Natural hardening began in autumn when air temperatures reached 5° to 6°C, coinciding with the first nocturnal frosts, and continued under snow cover to reach maximum levels between January and March. In March, alfalfa plants began to deharden when soil temperatures increased.

The differences in seasonal extremes of freezing tolerance are indeed dramatic. For example, when in the nonacclimated state during the active growth period of late spring, mulberry twigs can be severely injured or even killed by temperatures around -3°C. However, when fully acclimated by mid- or late-

winter, these same trees can survive experimental freezing temperatures of -196°C (Sakai, 1960). Poplar callus, when fully acclimated, is not injured by exposure to temperatures approaching absolute zero (-269°C) (Sakai and Sugawara, 1973).

Cold acclimation is thus an inducible and transient response. Time course studies have shown that the induction of freezing tolerance during cold acclimation is highly temperature- and species-dependant (Guy, 1990). Fennel and Li examined the rates of cold acclimation and deacclimation of freezing-sensitive and freezing-resistant spinach cultivars (Fennel and Li, 1985; Guy, 1990). Maximal freezing tolerance was attained within 2 to 3 weeks. Upon transfer to deacclimating conditions, freezing tolerance returned to nonacclimated levels within one week. In ivy, maximum tolerance was not reached until after 6 weeks; deacclimation was just as rapid as in spinach- requiring just one week (Steponkus and Lamphear, 1968; Guy, 1990). These findings illustrate another important, universally observed fact: The acquisition of freezing tolerance (ie., cold acclimation) is a slow, gradual process; in contrast, the loss of tolerance (deacclimation) is a much more rapid event.

As cogently emphasized in a recent review on freezing stress (Guy, 1990), cold acclimation, when compared to heat shock, is an exceedingly slow process. Whereas heat shock and the induction of thermotolerance are measured in minutes or hours, cold acclimation is measured in units of days or

weeks. Studies with certain herbaceous species such as spinach suggest that measurable increases in freezing tolerance can be observed in as little as one day (Guy, 1990).

Factors other than low temperature or photoperiod are capable of inducing freezing tolerance. In woody perennials, there is evidence for an effect due to endogenous rhythms. During the spring flush of growth, such plants cannot be made to acclimate fully regardless of the regimes of temperature and photoperiod provided in a controlled growth chamber (van Huystee et al., 1967).

Exogenous application of ABA to cell cultures, stem cultures, and seedlings at nonacclimating temperatures can cause a rapid induction of freezing tolerance (Chen et al., 1979; Chen et al; 1983; Chen and Gusta, 1983; Keith and McKersie, 1986; Orr et al., 1985; 1986; Reaney and Gusta, 1987). In many cases, the level of freezing tolerance achieved by ABA treatment was comparable to levels attained by low temperature treatment alone (Chen et al., 1979; Chen et al., 1983; Chen and Gusta, 1983; Keith and McKersie, 1986; Orr et al., 1986). However, in alfalfa seedlings, ABA treatment resulted in freezing tolerance levels which were only about 50% of those obtained after 2 weeks of low temperature treatment alone (Mohapatra et al., 1988). This finding is consistent with earlier studies (Waldman et al., 1975).

Limited desiccation increases freezing tolerance in the absence of low temperatures (Cloutier and Andrews, 1984; Cloutier and Siminovitch, 1982).

The level of freezing tolerance induced by 24 hours of desiccation at nonacclimating temperatures in epicotyls of several varieties of wheat and rye was comparable to the extent of hardiness reached after 4 weeks of cold acclimation at 2°C. Interestingly, tolerance was not correlated with water content in the desiccated plant but rather with the genetic capacity of the plant to harden (Guy, 1990). Studies with alfalfa (*Medicago media* Pers.) showed that a decrease in the the LT_{50} from -4° to -7°C was achieved if drought stress was applied to seedlings, grown at 20° to 25°C, by reducing the soil moisture from 100% to 30% of the soil water-holding capacity prior to freezing (Jung and Larson, 1972). Drought and low temperature stresses had additive effects on freezing tolerance.

Desiccation is known to induce accumulation of ABA in plant cells (Wright, 1977; Terry et al., 1988). Since low temperatures also lead to an increase on endogenous ABA levels in many species (Chen et al., 1983; Kacperska-Palacz, 1978; Chen and Gusta, 1983), it has been proposed that the increase in freezing tolerance by desiccation and low temperature may be mediated by ABA. Recent studies with ABA-deficient and ABA-insensitive mutants of *Arabidopsis thaliana* indicate that three separate but converging signal pathways may mediate low temperature-, water stress-, and ABA-inducible freezing tolerance (Nordin et al., 1991). An ABA-deficient mutant was unable tc cold acclimate. Moreover, freezing tolerance was not increased by low

temperature treatment, but addition of exogenous ABA to the growth medium restored freezing tolerance to wild type levels (Heino et al., 1990).

1.5. The Freezing Process

Several facts and assumptions require special emphasis in order to understand better the events involved in the freezing of plant tissues (Guy, 1990): (1) In an intact plant, the cells are not immersed in an aqueous solution but are surrounded by a water-saturated environment; (2) in many plant tissues, part of the internal extracellular volume is free air space which is usually saturated with water vapour; (3) the amount of osmotically-active extracellular water is small relative to the water inside living cells (Wenkert, 1980); (4) the apoplastic solution has a much lower solute concentration than the intracellular solution of the cell; (5) the colligative effect of higher solute concentration in cellular water results in a freezing-point depression (Levitt, 1980); (6) a functionally intact plasma membrane is an effective barrier against seeding of the intracellular solution by extracellular ice and against propagation of extracellular ice fronts; (7) the effectiveness of the plasma membrane as a barrier to ice is thought to be diminished for temperatures below about -10°C (Mazur, 1969); (8) liquid water can move freely in either direction across the plasma membrane, but solutes cannot; (9) the existence of intracellular heterogeneous ice nucleators is masked, minimized, or excluded (Burke et al., 1976; Sakai and Larcher, 1971; DeVries,

1983); (10) the type of freezing (intracellular versus extracellular) is determined largely by the rate of cooling (Mazur, 1963; Mazur, 1969; Orr et al., 1976).

Depression of the freezing point of an aqueous solution is known as supercooling (Burke et al., 1976). Theoretically, in the absence of ice nucleating substances, pure water can be supercooled down to about -47°C. At this temperature, known as the homogeneous nucleation point, ice forms spontaneously in the absence of nucleators (Chen et al., 1983; Kuczenski and Sueller, 1970). Some plant tissues in nature (flower buds, xylem ray parenchyma cells of deciduous forest species) do indeed supercool - to temperatures as low as about -40°C to -47°C (Burke et al., 1976; George et al., 1974). Intracellular freezing is a cataclysmic event, resulting in disruption of the integrity of the cell, and is generally regarded as a universally lethal event.

During a freeze/thaw cycle, both the cells and the extracellular spaces initially supercool. Ice first forms extracellularly, on internal surfaces of cell walls, in water-transporting elements, or on external surfaces (Jeffree et al., 1987). In nature, external ice such as hoar frost or soil ice can easily nucleate plants via entry sites such as stomates, lenticels, and wounds (Burke et al, 1976). Since the cell membrane acts as an effective barrier to seeding by extracellular ice, the cell remains unfrozen and supercooled. The cell contains a higher concentration of solutes and its contents remain supercooled; consequently, its aqueous vapour pressure is greater than that of the extracellular water and water

is drawn out of the cell to the extracellular spaces, where it freezes. This freezing-induced dehydration of the cell leads to an increase in the internal concentration of solutes. The chemical potential of ice is a direct function of subzero temperature (Guy, 1990). The decline in potential with temperature is predicted to be large,- about -1.16 MPa/°C (Rajashenkar et al., 1989). Therefore, the severity of the dehydration stress increases as the freezing temperature drops. Depending on the initial osmotic potential of the unfrozen cell, all osmotically-active water can be frozen out of a cell at some subzero temperature (Rajashenkar and Burke, 1982).

There are two ways in which the cell can achieve equilibrium between the intracellular and extracellular aqueous vapour pressures (Steponkus, 1984). If the cooling rate is slow ($\leq$ 3°C/h), the cell will dehydrate to the extent required to maintain equilibrium and intracellular freezing will not occur. Alternatively, if the cell membrane is not sufficiently permeable to liquid water or if the rate of cooling is too rapid ($\geq$3°C/h), the intracellular solution will not reach equilibrium but will continue to supercool until eventually equilibrium is achieved by internal freezing. Intracellular freezing will result from either homogeneous nucleation (at temperatures below about -40°C) or from heterogeneous nucleation promoted by foreign nucleating substances or seeding by extracellular ice (Steponkus, 1984). In nature, atmospheric cooling rates rarely exceed about 1°C/h (Steffen et al., 1989; Guy, 1990).

Viewed in this context, the ability to tolerate freezing-induced dehydration can be regarded as a freezing survival strategy. Indeed, it is a general observation (Burke et al., 1976) that hardier plants can survive with more of their water frozen than can less-hardy plants. Dehydration leads to numerous possibilities to account for injury, collectively referred to as "solution effects" (Steponkus, 1984). These include volumetric and area contraction, concentration of intra- and extracellular solutes, possible pH changes due to differential solubilities of various buffering compounds, eutectic crystallization, and possibly removal of the water of hydration of macromolecules.

The majority of studies indicate that intracellular ice formation is not caused by the presence of intracellular ice nucleators; rather, it is a consequence of seeding by external ice (Steponkus, 1984). Such seeding by extracellular ice is likely to be a consequence of mechanical breakdown of the cell membrane (Steponkus, 1984). Mazur (1969) has suggested that ice crystals with radii of curvature sufficiently small to penetrate membrane pores are only sufficiently stable at temperatures below about -10°C. This was proposed to explain the inability of the cell membrane to act as an effective barrier below -10°C. However, there is no direct experimental evidence to support this claim (Guy, 1990).

In a series of detailed and elegant studies, Steponkus and co-workers have examined the behaviour of plasma membranes of isolated protoplasts during

freeze/thaw cycles (Steponkus, 1984; Dowgert and Steponkus, 1984). Among the many manifestations of membrane injury observed was expansion-induced lysis. Isolated protoplasts shrink when dehydrated by freezing. Upon thawing, the cells rehydrate and expand. Elastic expansion is limited to only about 2 to 3%. Nonacclimated (NA) protoplasts from rye seedlings were observed to lyse before attaining their original surface areas. Apparently, membrane material is not conserved during sufficiently large contractions in surface area. Protoplasts from cold acclimated (CA) rye seedlings had a much greater ability to re-expand after a freeze/thaw cycle than did the NA protoplasts. In this case, membrane material is apparently conserved. Light and electron microscopic observations of these events showed that the membranes of protoplasts from CA and NA seedlings were different (Gordon-Kamm and Steponkus, 1984; Steponkus, 1984). Upon freezing to -5°C, NA protoplasts dehydrated and shrank, with the plasma membrane forming numerous endocytotic vesicles. Thus, membrane material had been deleted. When the protoplasts were subsequently thawed, the vesicular material was not reincorporated back into the membrane and expansion-induced lysis ensued due to intolerable osmotic pressures. Protoplasts from CA rye seedlings behaved differently. These protoplasts also dehydrated and shrank but rather than forming endocytotic vesicles, the plasma membrane formed exocytotic extrusions which were still continuous with the membrane. The critical difference here is that the extrusions remained in association with the plasma

membrane and were reincorporated into the plasma membrane during subsequent rehydration and expansion.

Endocytotic vesiculation requires breakage and subsequent fusion of the plasma membrane, which may predispose the NA protoplasts to seeding by extracellular ice (Steponkus, 1984). In contrast, exocytotic extrusion by CA protoplasts does not involve any breakage of the plasma membrane which would be expected to remain stable.

However, the situation is likely to be more complex in intact cells owing to the presence of a relatively rigid cell wall and the appropriateness of protoplasts as a model system has been called into question (Pearce and Williams, 1981; Johnson-Flanagan and Singh, 1986; Singh et al., 1987). For example, CA whole cells of *Brassica napus*, winter rye (Singh et al., 1987), and alfalfa (Johnson-Flanagan and Singh, 1986), instead of forming exocytotic extrusions, produce many more osmotically-active plasmalemma strands during plasmolysis than do NA cells. These strands appear to tether the protoplast to the cell wall during plasmolysis. Like the exocytotic extrusions of the protoplasts, these plasmalemma strands are reincorporated back into the expanding protoplast during deplasmolysis.

It is clear that the cell wall does indeed play a role in determining freezing tolerance. Investigations with potato cells and protoplasts revealed that the protoplasts were more freezing tolerant than were the intact cells from which the

protoplasts were generated (Tao et al., 1983) - a phenomenon first observed by Siminovitch using rye cells and protoplasts (Siminovitch et al., 1978). Tao and co-workers observed a difference as large as 10°C between the LT_{50} values of potato protoplasts and intact cells. The explanation for these differences is that contact between the cell wall and the protoplast during an extracellular freeze/thaw cycle provides an additional strain on the plasma membrane (Tao et al, 1983). Since the cell wall is relatively rigid, it cannot shrink and is deformed by the extracellular ice front. This forces it into contact with the protoplast resulting in an asymmetric stress on the protoplast.

At freezing temperatures below about -5°C, the secondary dehydration stress described above becomes the major factor (Steponkus, 1984). A loss of osmotic responsiveness is frequently observed (Steponkus, 1984; Steponkus and Lynch, 1989) in NA cells and in CA cells exposed to lethal freezing temperatures and involves: the formation of lateral phase separations; aparticulate lamellae; lamellar-to-hexagonal$_{II}$ phase transitions, and multilamellar vesicles (Gordon-Kamm and Steponkus, 1984; Pearce and Willison, 1985; Johnson-Flanagan and Singh, 1986; Singh et al., 1987). Similar membrane damage can be observed in NA protoplasts, in the absence of freezing, by subjecting them to equivalent degrees of dehydration induced by suspension in hypertonic solutions at 0°C; this indicates very clearly that injury is a direct result of dehydration and not of low temperature *per se*.

Another consequence of freezing of aqueous solutions which is often overlooked in cryobiology is the generation of electrical potentials caused by charge separation (Steponkus, 1984). Freezing of aqueous solutions results in an increase in the concentration of solutes in the unfrozen part of the solution, generating large, steady and transient potential differences at the ice water interface - a phenomenon referred to as the Workman-Reynolds effect (Workman and Reynolds, 1950). Large asymmetric ion concentrations across the plasma membrane may result in increased transmembrane potentials which go through a maximum as the freezing rate increases. Experimental studies of the spatial and temporal properties of the electric fields generated are lacking. However, it is known that electrical fields applied across biological and artificial membranes lead to electrical breakdown or membrane depolarization, increases in permeability to solutes and membrane fusion (Zimmermann, 1982; Zimmermann et al., 1981). Transmembrane potentials of 150 to 200 mV can be generated in the diffusion layer (Steponkus, 1984). The high field strength that occurs naturally in lipid protein membranes suggests that the field strength is just below the critical value for breakdown in many parts of the membrane (Steponkus, 1984). It is possible that small increases in the transmembrane potential could result in membrane breakdown, even if only transiently, allowing seeding of the protoplast by extracellular ice. Cold acclimation alters the electrical characteristics of the plasma membrane and increases the critical membrane potential that results in

electroporation of the plasma membrane (Steponkus et al., 1985). Accordingly, changes in lipid composition observed for the plasma membrane during cold acclimation may stabilize the membrane against mechanical breakdown (Steponkus et al., 1985).

What has emerged from studies of freezing injury is the realization that a freeze/thaw cycle involves myriad events of considerable complexity. Many studies have made the naive attempt to invoke a single mechanism to explain the various forms of injury. Instead, the freeze/thaw cycle is more accurately viewed as a sequential series of potentially lethal stress barriers, which are surmounted by individual facets of the cold acclimation process (Steponkus, 1984). A progressive increase in cold hardiness may then reflect the surmounting of these individual stress barriers as opposed to an increased tolerance to any one particular stress.

1.6. Genetics of Freezing Tolerance

Freezing tolerance involves a complex genetically-programmed, integrated process (Sutka and Veisz, 1988; Weiser, 1970). The genetics of overwintering or freezing tolerance has been extensively reviewed recently (Guy, 1990; Thomashow, 1991). Genetic studies of the inheritance of freezing tolerance have focused almost exclusively on cultivar and varietal trials in wheat and other cereals. The most common observation is that freezing tolerance is a complex,

quantitatively inherited trait involving several genes which are largely additive in their effects.

The first detailed studies were conducted by Nilson-Ehle in 1912. Crosses between two varieties of winter wheat, intermediate in their ability to harden, resulted in progeny whose frost tolerance was largely intermediate between that of the parental genotypes. Occasionally, progeny were found which were either more or less tolerant than either parental genotype; that is, the winter hardiness of some progeny segregated outside the parental boundaries - a phenomenon referred to as transgressive segregation. Transgressive segregation is consistent with the quantitative nature of the winterhardiness trait (Guy, 1990). Subsequent studies with winter and spring wheats confirmed these earliest findings (Hayes and Aamodt, 1927; Worzella, 1935; Quisenberry, 1931).

In the seventies, Gullord and coworkers demonstrated that the inheritance of freezing tolerance involved several partially dominant genes, mostly additive in their effect and that two different sets of genes were involved depending on the severity of the imposed freezing stress (Gullord 1975; Gullord et al., 1975). One set was associated with a "high intensity" freeze; the other set was involved in tolerance to the "low intensity" freeze.

Studies aimed at determining the direction of dominance (Puchkov and Zhirov, 1978; Schafer, 1923; Sutka, 1981, 1984) have been reviewed by Orlyuk (1985). A graphical analysis of the variance-covariance of freezing tolerance,

in progeny from a cross of two complete dialleles of winter wheat, suggested that frost sensitivity was partially dominant (Sutka, 1981, 1984). However, Gullord (1975), also working with winter wheat, found that freezing tolerance, not freezing sensitivity, was in the direction of dominance. These conflicting results may be attributed in large part to differences in freezing protocols, since different sets of genes are involved in low- versus high-intensity freezing stress. Indeed, more recent work by Sutka and Veisz (1988), has revealed that the genes on chromosome 5A of winter wheat which are implicated in freezing tolerance switch direction of dominance depending on the temperature at which freezing tolerance is measured. At higher freezing temperatures around -10°C (low stress), frost resistance was dominant; whereas, at more severe freezing temperatures of -14°C or lower (high intensity stress), frost sensitivity was dominant.

Inspection of the data for hexaploid wheat shows that 11 of the 21 chromosomes have effects on freezing tolerance (Thomashow, 1991). Chromosomes 5A and 5D have been implicated most frequently and appear to have the major effects on freezing tolerance. Variations in these results have been attributed to differences in the particular cultivars tested and to differences in the freezing protocols used.

Hardening conditions can influence the results. Roberts (1986) using 42 reciprocal chromosome substitution lines of 2 spring wheats, Cadet and Rescue,

presented evidence that chromosomes 2A, 5A, and 5B carried loci affecting freezing tolerance after hardening under conditions of 8-week day (6°C) and night (4°C) cycle. However, if plants were hardened in darkness for 7 weeks at 0.8°C followed by 8 weeks at 5°C, chromosomes 6A, 3B, 5B and 5D were found to affect freezing tolerance. Versz and Sutka (1989) used substitutions of chromosomes 5A, 5B, 5D, 4B and 7A from a highly freezing tolerant winter wheat into a relatively sensitive spring wheat. They found that the levels of freezing tolerance were positively correlated with the duration of the hardening period. Moreover, the different freezing tolerance genes had their effects at different times in the cold acclimation process.

Work with barley (Eunus et al., 1962; Rhode and Polham, 1960) and oats (Amirshashi and Patterson, 1956; Jenkins, 1969; Pfahler, 1966; Coffman, 1962; Finkner, 1966) have yielded results similar to those obtained for wheat. That is, genes involved in freezing tolerance were mostly additive in their effects, although transgressive segregation of the freezing hardiness character was also observed with F3 progenies that were more tolerant than the original parents in many crosses. Both dominant and recessive genes contributed to winter hardiness and the direction of dominance (towards freezing tolerance or freezing sensitivity) appeared to depend on the severity of the imposed freezing conditions.

Freezing survival encompasses two phenomena: the inheritance of the minimum survivable temperature and the timing of photoperiodic and low

temperature induction of freezing tolerance. The capacity of winter hardy plants to undergo cold acclimation is a significant component of winter hardiness. There exists a paucity of information concerning the genetics of this inducible component of freezing tolerance. Thus, while many studies have concentrated on differences in freezing tolerance between cultivars, very few have addressed the nature of seasonal differences within a single cultivar or isogenic line. The available data indicate that cold acclimation-induced freezing tolerance follows a dominant/recessive pattern of inheritance coupled to an additive dominance system (Gullord et al., 1975).

In view of the complexity of the freezing process and the multitude of stress barriers encountered by plants during a freezing event, it is not surprizing that the genetics of freezing tolerance should likewise prove to be very complex.

1.7. Metabolic Changes during Cold Acclimation

Attempts to understand the phenomenon of plant cold hardiness have taken two distinct approaches (Guy, 1990). The first approach (detailed above) has been to describe the mechanisms underlying a freeze/thaw cycle which lead to injury or death of the cell. The goal here has been to determine the site of lethal injury. An underlying assumption with this approach is that once the mechanisms of injury are well understood, it should be relatively easy to determine the biochemical and physiological alterations required to confer increased freezing

tolerance. Due to the very complex nature of freezing injury in plants, the mechanisms involved have proved to be extraordinarily complex. The second major approach has been to catalog and understand the biochemical and physiological changes which occur during cold acclimation. One major problem with correlative studies of this type is the confounding result of low temperature effects not related to the development of freezing tolerance. Field studies are further complicated by the physiological changes associated with dormancy, quiescence, autumnal colour change, and senescence (Johnson-Flanagan and Singh, 1988). The various biochemical and physiological changes that occur during cold acclimation have been extensively reviewed (Johnson-Flanagan and Singh, 1988; Guy, 1990; Thomashow, 1991).

Low molecular weight cryoprotectants

In many plants, exposure to low nonfreezing temperatures results in the accumulation of low-molecular-weight cryoprotective compounds (Guy, 1990). These are all compatible solutes and include disaccharide and trisaccharide sugars (Yelenoski and Guy, 1977; Steponkus and Lanphear, 1968); sorbitol (Sakai and Yoshida, 1968); glycinebetaine (Sakai and Yoshida, 1968); proline (Yelenosky, 1979; Kushad and Yelenoski, 1987) and polyamines (Kushad and Yeleoski, 1987). These compounds may function as cryoprotectants through an ability to maintain the ordered vicinal water around proteins by minimizing protein-solvent

interactions. In this way, subunit association and native conformations may be maintained at low water activities (Yancey et al., 1982; Guy, 1990). In addition, many of these substances may act to stabilize membranes through interactions with the polar head groups of phospholipids; others may form hydrophobic associations with the membrane (Anchordoguy et al., 1987; Guy, 1990).

Sucrose is the most commonly occurring cryoprotective carbohydrate (Guy, 1990). Levels of sucrose can increase as much as 10-fold during exposure to low temperature (Salerno and Pontis, 1989). Generally, there exists a correlation between the concentration of sucrose and freezing survival (Sakai and Yoshida, 1968). In fact, induction of freezing tolerance by low temperature is lost if sugar accumulation is blocked (Guy et al., 1980; Steponkus and Lanphear, 1968). Proline may act in a manner similar to that of sucrose and may have a membrane stabilizing effect (Hellergren and Li, 1981). Others suggest that proline may act as a nontoxic intracellular solute which protects cells against denaturation caused by freezing-induced dehydration (Withers and King, 1979). Alternatively, proline may act as an osmoticum at high concentrations (Stewart and Lee, 1974) or as a protective agent for enzymes and cellular structures (Schobert and Tschesche, 1978).

Although increases in sugar content do protect the cell from freezing injury to a limited extent, sugar contents are rarely high enough to account fully for the levels of freezing resistance often observed. Sugars and other conventional

cryoprotective agents act mainly on a colligative basis by preventing in a nonspecific manner the accumulation to damaging levels of potentially membrane-toxic compounds during freezing-induced dehydration (Volger and Heber, 1975).

Membrane lipids

The plasma membrane plays a central role in cellular behaviour during a freeze/thaw cycle and rupture or changes in its semipermeable properties is a primary cause of freezing injury (Steponkus, 1984). The biochemical and physical restructuring of cell membranes during cold acclimation is a well-documented phenomenon (Guy 1990; Thomashow, 1991; Steponkus, 1984; Steponkus and Lynch, 1989). Uemura and Yoshida (1984) and Yoshida and Uemura (1984) reported significant increases in phospholipid-to-protein ratios during cold acclimation of rye seedlings and orchard grass, respectively. Lynch and Steponkus (1987) found during cold acclimation of rye seedlings increases in both free sterols and phospholipids and decreases in steryl glucosides, acylated steryl glucosides and glucocerebrosides. Levels of diunsaturated molecular species of phosphatidylcholine and phosphatidylethanolamine doubled in cold-acclimated cells. Decrease in glucocerebroside and increases in free sterol and phospholipid would be expected to increase membrane fluidity by preventing the formation of separate phases or domains within the membrane. Given that cell dehydration is the principal stress that occurs during freezing of cells (Steponkus,

1984), and that dehydration-induced lamellar-to-hexagonal$_{II}$ phase transitions are associated with destabilization of the plasma membrane (Gordon-Kamm and Steponkus, 1984), the causal relationship between lipid composition and the cryobehaviour of the plasmamembrane is best viewed from the perspective of lyotropic phase transitions such as dehydration-induced liquid crystalline to gel and lamellar to hexagonal$_{II}$ phase transitions (Lynch and Steponkus, 1987).

The functional significance of alterations in membrane composition has been demonstrated. Steponkus and coworkers were able to increase the freezing tolerance of nonacclimated rye protoplasts by elevating the levels of monounsaturated and diunsaturated molecular species of phosphatidylcholine (Steponkus et al., 1988). Such modified nonacclimated protoplasts, instead of forming endocytotic vesicles, formed the typical extracellular extrusions seen in CA protoplasts, following moderate dehydration. However, enrichment of mono- and diunsaturated molecular species of phosphatidylcholine did not result in freezing tolerance at the more severe stress temperature of -10°C - the point where dehydration is sufficiently severe to cause lateral phase separations, lamellar-to-hexagonal$_{II}$ phase transitions, and osmotic unresponsiveness (Thomashow, 1991). Therefore, development of tolerance to freezing temperatures below -10°C must involve other processes, possibly including multiple alterations to the plasma membrane.

DNA

There is no agreement on changes in DNA levels during cold acclimation (Johnson-Flanagan and Singh, 1988). This is due to conflicting findings with different plant species and to wide variations in DNA levels during acclimation which suggests that any trends in the data were not statistically significant. Most studies indicate that DNA levels remain constant (Li and Weiser, 1967; Gusta and Weiser, 1972; Siminovitch, 1963).

RNA

In general, total RNA increases during cold acclimation and precedes the period of increased freezing tolerance. In potato (Chen and Li, 1980) and wheat (Sarhan and Chevrier, 1985) the increase in total RNA content was positively correlated with the low temperature induction of freezing tolerance. The activities of chromatin-bound RNA polymerases I and II increase during acclimation (Sarhan and Chevrier, 1985). Similar studies with alfalfa also show an increase in RNA content during cold acclimation (Mohapatra et al., 1987a). In alfalfa, increases in poly(A)$^+$ and poly(A)$^-$ RNA during cold acclimation were reported (Mohapatra et al., 1987a,b). A marked increase in RNA content was observed after only 2 days in the less resistant cultivar (*Medicago sativa* cv. Saranac). Two days were also sufficient for maximal hardiness. In the more freezing-resistant cultivar (*Medicago falcata* cv. Anik) a more gradual increase

in RNA content was observed which closely paralleled the more gradual increase in freezing tolerance. Drought stress, which also increases freezing tolerance, was correlated with an increase in RNA content in red osier dogwood (*Cornus stolonifera*) after an initial decline in RNA content during the first 3 days of increased tolerance. Most studies report an increase in ribosomal RNA (rRNA) (Johnson-Flanagan and Singh, 1988) and indeed the rate of rRNA synthesis increased at low temperatures in freezing-resistant winter wheat but not in freezing-sensitive spring wheat (Sarhan and D'Aoust, 1975; Paldi and Devay, 1977). These studies also found a strong correlation between the LT_{50} of wheat cultivars and the rate of rRNA synthesis at low temperatures. In nontolerant cultivars there was an accumulation of rRNA precursors, suggesting a possible inhibition of the ribosome maturation process. Further studies with winter wheat demonstrated the existence of different rRNA size classes and cistron numbers (Palvi and Devay, 1977), indicating the presence of different ribosome populations. One population was synthesized at low temperatures and degraded at higher temperatures; the other population was synthesized at higher temperatures and was stable at these temperatures. Studies of rRNA-DNA hybridization demonstrated higher cistron numbers in winter wheat, the activity of which varied with temperature (Palvi and Devay, 1983).

Soluble RNA (sRNA), which comprises messenger RNA (mRNA) and transfer RNA (tRNA), and rRNA increased during cold acclimation of apple (Li

and Weiser, 1969). However, only rRNA declined during deacclimation. Consistent with this are reports that RNA polymerase I was more active than RNA polymerase II during acclimation of winter wheat (Sarhan and Chevrier, 1985), suggesting more rapid synthesis of rRNA than of mRNA. It has been proposed that the increase in rRNA may be responsible for the increased freezing tolerance observed in these studies (Li and Weiser, 1969; Sarhan and Chevrier, 1975). That *de novo* RNA synthesis is indeed involved in the acclimation process is clear from studies using the transcriptional inhibitor, actinomycin D. In winter wheat, actinomycin D caused a 70% inhibition of RNA synthesis and an incomplete inhibition of freezing tolerance (Zvereva and Trunova, 1985). This explanation is probably too simplistic. It is more likely that the increase in rRNA synthesis is a general response to low temperature stress, facilitating more rapid protein synthesis. Increasing the amount of enzyme may be a means to overcome the inhibitory effect of low temperature on the specific activity of most enzymes. This may represent a general metabolic adaptation to low temperatures, rather than a specific event involved in increased freezing tolerance.

Increased RNA content may also result from the documented decrease in RNase activity (Gusta and Weiser, 1972; Brown and Bixby, 1973; Ho and Brown, 1964). Sarhan and Chevrier (1985) showed that low temperature exposure of spring and winter wheat resulted in a decrease in activity of

chromatin-bound RNase, and suggested that this is a response to low temperature rather than a change specifically associated with increased freezing tolerance.

Ribosomes and polysomes

Studies conducted with wheat seedlings showed phenotypical temperature adaptation (nongenetic temperature adaptation) of protein synthesis (Weidner et al., 1982). The optimum temperature of [^{14}C] leucine incorporation into total protein varies as a function of growing temperature (ie., preadaptation treatment). For 10 day-old plants kept for 2 d at 4°C, 20°C, or 36°C, the protein synthesis optima were at 27, 31, and 35°C, respectively. The temperature coefficient μ, calculated from Arrhenius plots of *in vivo* protein synthesis rates shifted to progressively higher values with increasing preadaptation temperature. Specific differences in the yield of ribosomes, in polyribosomal profiles, and in the apparent activation energy of protein synthesis were seen depending on temperature pretreatment (Fehling and Weidner, 1986). Heat leads to a dramatic increase in translational efficiency but yields only minor changes in the ribosome and polysome levels. Cold, however, causes a significant increase in the polysomal level and the ribosomal quantity.

Quantitative changes in ribosomes and polysome size affect the rate of protein synthesis (Johnson-Flanagan and Singh, 1988). During cold acclimation, there is a doubling in ribosome concentration in black locust seedlings (Bixby and

Brown, 1975). In potato, polysome size and *in vitro* translation rates increased during low temperature acclimation (Brown and Li, 1981). This increase was more pronounced in the tolerant cultivars than in the sensitive ones. Rapid increases in polysome size may reflect either increased initiation (Davies and Larkins, 1980) or decreased termination (Fehling and Weidner, 1986) or both.

Bixby and Brown (1975) reported differences in ribosomal proteins when cold-hardened black locust seedlings were compared to nonhardy ones. These changes were associated with increased thermal stability, during acclimation. Differences in three acidic ribosomal polypeptides between cold acclimated and nonacclimated winter rye have been reported (Laroche and Hopkins, 1987). These polypeptides may be initiating factors since they are released from ribosomes by 0.6 M KCl. Modest changes in isoelectric point and relative molecular weight were observed for these peptides. It has been suggested that such small changes may result from differences in phosphorylation (Johnson-Flanagan and Singh, 1988). Differences in ribosomal polypeptides or base pairing of rRNA may act to increase ribosome stability. Indeed, higher *in vitro* translation rates observed at low temperatures in polysomes isolated from freezing-tolerant cells support this idea (Johnson-Flanagan and Singh, 1987; 1988). All of these findings, taken together, suggest that the protein-synthesizing machinery is well integrated to facilitate continued protein synthesis during cold acclimation (Johnson-Flanagan and Singh, 1988).

Protein

Increase in soluble protein content during acclimation is a nearly universal observation. A statistical analysis of the linear regression of freezing tolerance and soluble protein content in potato yielded a correlation coefficient greater than 0.97 (Chen and Li, 1980). Studies with alfalfa seedlings showed a similar trend (Mohapatra et al., 1987a,b). This increase is due, at least in part, to increased rates of incorporation (ie., synthesis) during the later part of the cold acclimation period, after an initial decline in synthesis at the beginning of the cold aclimation period (Siminovitch et al., 1968; Guy et al., 1985; Mohapatra et al., 1987a,b). Protein synthesis inhibitors block the low-temperature induction of freezing tolerance in winter wheat, if applied during the first week of acclimation (Zvereva and Trunova, 1985). After 7 days, cycloheximide addition had no effect on the development of freezing tolerance. A similar finding was reported for potato-stem cultures (Chen et al., 1983). These data lend credence to the idea that *de novo* protein synthesis during cold acclimation is causally related to the development of freezing tolerance.

Qualitative changes in protein composition

Subtle changes in protein populations rather than gross changes in total protein level may be more directly linked to freezing tolerance. The general strategy has been to compare the polypeptide profiles of CA versus NA plant

material and to correlate any changes with increased freezing tolerance. Numerous reports have clearly shown that plants exposed to low temperatures synthesize a new set of polypeptides (reviewed by Guy, 1990; Thomashow, 1991). *In vivo* radiolabelling followed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) or two-dimensional gel electrophoresis (2D-PAGE) has shown that cold acclimation is associated with both the appearance of new polypeptides and increases and/or decreases in others. These experiments suggest that many of the alterations involve changes in polypeptide synthesis. However, relatively long labeling periods (6 to 24 h) were required for plants labelled at low temperatures (Thomashow, 1991). Therefore, it is possible that these changes may reflect posttranslational events such as protein turnover. In alfalfa (*Medicago falcata* cv. Anik), Mohapatra et al. (1987b, 1988b) reported the appearance of 11 new polypeptides after 2 days of cold acclimation, ranging in size from about 92 kDa to 13 kDa. Two polypeptides of 43 and 90 kDa increased during cold acclimation. Another polypeptide (molecular mass of 11 kDa) appeared after 8 days of CA. After 8 days, no new polypeptides were detected, although the proteins detected within 2 and 8 days of CA continued to increase. These changes preceeded and were correlated with the induction of freezing tolerance. Several proteins declined during acclimation (Mohapatra et al., 1988). A polypeptide of about 200 kDa disappears during cold acclimation. Changes in four membrane polypeptides (molecular mass of 76, 45, 37 and 26 kDa) were

also seen. Other studies documenting changes in protein profiles during acclimation include reports on *Arabidopsis thaliana* (Kurkela et al., 1988; Gilmour et al., 1988), *Bromus inermis* cell suspension cultures (Robertson et al., 1987, 1988), *Citrus sinenis* (Guy et al., 1988), *Dactylis glomerata* (Yoshida and Uemura, 1984), *Medicago sativa* (Robertson and Gusta, 1985; Mohapatra et al., 1987a), wheat (Perras and Sarhan, 1989; Cloutier, 1983, 1984), *Spinacia oleracea* (Guy and Haskell, 1987), *Zea mays* (Yacoob and Filion, 1986), and *Brassica napus* (Meza-Basso et al., 1986).

Several trends are clear. Unlike heat shock, the synthesis of housekeeping proteins continues at low temperature. The polypeptide profiles of CA and NA plant material are largely similar; changes in protein synthesis during cold acclimation are relatively modest. There does not appear to be a uniform appearance or decline of common proteins in the different systems studied. This again is in sharp contrast to the situation seen for heat shock. Time-course studies indicate that the accumulation of these low-temperature-induced polypeptides can be as rapid as 1 h (Marimoli et al., 1986) or as slow as 8 d (Mohapatra et al., 1987b). The induction of new polypeptides by low temperature is therefore very slow when compared to the minutes or hours required for the appearance of hsp. Synthesis of many low temperature-specific proteins is continuous throughout the period of cold acclimation (Guy and Haskell, 1987; Guy et al., 1985; Mohapatra et al.,1987a, b); that of others is

transient (Cattivelli and Bartels, 1989; Tseng and Li, 1987). Comparison of the polypeptides synthesized in response to low temperature versus heat shock suggests that the two responses are dissimilar, eliciting the synthesis of different sets of proteins (Guy et al., 1985; Ougham, 1987; Yacoob and Filion, 1986). More sensitive immunological studies, where polyclonal antisera raised against corn heat shock proteins were reacted against proteins from cold-shocked corn, showed an increase in polypeptides of 18 kDa and 70 kDa in size (Yacoob and Filion, 1987). This is supported by studies with chilled soybean where there is an increase in the transcript levels of the 28 kDa hsp (Kuznetsov et al., 1987). In spinach, there is an increase in a protein sharing sequence homology with the hsp70 (Guy, 1990). In the study with corn, the authors believe that the increased synthesis of the 70 kDa hsp may be a wound response rather than a low temperature response (Yacoob and Filion, 1987). Although heat shock and cold acclimation may involve the synthesis of common subset of stress proteins, the two responses are otherwise markedly different. Freezing stress and heat shock are fundamentally different stresses that probably involve dissimilar adaptive responses each requiring the synthesis of a unique set of stress proteins.

A few studies have correlated changes in protein synthesis with the induction and loss of freezing tolerance during acclimation and deacclimation, respectively. In spinach and alfalfa, the synthesis of cold acclimation-inducible proteins was abolished upon deacclimation (Guy and Haskell, 1987; Mohapatra

et al., 1987a,b). Perras and Sarhan (1989) reported a positive correlation between the level of accumulation of certain cold-inducible proteins and the degree of freezing tolerance in wheat cultivars, indicating that these proteins may be causally linked to the development of freezing tolerance.

To date, very little information exists concerning the possible functions of these low temperature induced polypeptides detected by *in vivo* radiolabelling studies (Guy, 1990). Some of these proteins may be involved in ameliorating freezing-induced drought stress. Cloutier (1983) found that freezing tolerance in winter rye induced by either desiccation or cold acclimation was correlated with an increase in a protein of 46 kDa. However, the changes in protein profiles resulting from these two treatments were generally dissimilar.

ABA in the absence of low temperature can induce maximal freezing tolerance in some species. Robertson and Gusta (1985) reported the enrichment of a 42 kDa protein and reduced amounts of four other extracellular polypeptides in both ABA- and low temperature-treated cell suspension cultures of *Medicago sativa*. A 190 kDa glycoprotein was absent in ABA and cold treated cultures. There was an apparent decrease in the electrophoretic mobility of the major extracellular polypeptides in ABA and low-temperature treatments, indicating an increase in molecular masses of 2 to 3 kDa. This increase in apparent molecular mass suggests possible modifications of extracellular proteins during cold acclimation, the nature of which is not yet understood. Mohapatra et al. (1988a)

detected two membrane polypeptides (molecular masses of 42 and 120 kDa) which were induced by both low temperature and ABA treatment in a freezing tolerant cultivar of alfalfa, *Medicago falcata* cv. Anik. Proteins common to ABA and cold treatments have been reported for cell suspension cultures of *Brassica napus* (Johnson-Flanagan and Singh, 1987) and *Bromus inermis* (Robertson et al., 1987). It is tempting to speculate that common proteins induced by cold and ABA may play a role in tolerance to freezing-induced dehydration.

As early as 1975, evidence existed for the synthesis of cryoprotective proteins during cold acclimation (Volger and Heber, 1975). Leaves of frost-resistant spinach were found to contain a number of low molecular mass (10 to 20 kDa) soluble proteins capable of protecting thylakoid membranes against freezing damage. These were found to be heat-stable and to contain a high proportion of polar amino acids. These cryoprotective proteins were found to be 1,000 times more effective on a molar basis than other low molecular weight cryoprotective agents such as sucrose, glycerol or dimethylsulfoxide; therefore, their mode of action was likely noncolligative.

Enzyme variation

There are numerous reports describing changes in activity, freeze stability, and isozymic variation of enzymes during cold acclimation (Guy, 1990; Thomashow, 1991). In one of the earliest studies, McCown and coworkers used

electrophoretic techniques to separate enzymes from acclimated and nonacclimated tissues of four widely unrelated woody species (McCown et al., 1969). Peroxidase activity increased. In three of the species, several peroxidase isozymes were unique to the acclimated tissues.

A series of comprehensive studies was conducted by Krasnuk and colleagues using alfalfa (*Medicago sativa* L.) (Krasnuk et al., 1975, 1976a, 1976b). Increased activity was observed during winter of a number of dehydrogenases involved with respiratory pathways, including glucose-6-phosphate dehydrogenase, lactate, and isocitrate dehydrogenase. These increases in enzyme activity paralleled increases in soluble protein content during winter, suggesting that the increased activity may be due in large part to elevated levels of these enzymes. Also, new isozymic variants were found of ATPases, esterases, acid phosphatases, leucine aminopeptidases, peroxidases, and some dehydrogenases. In many cases, freeze stability increased during winter.

In spinach, a study of glutathione reductase activity revealed in cold acclimated tissue the appearance of additional isozymic variants, increased activity, enhanced freeze/thaw stability, and altered kinetic behaviour (Guy and Carter, 1984). Based on a comparison of K_m values for substrates, the enzyme from hardened tissues was better suited to function at lower temperatures. The best-documented change in enzyme activity during cold acclimation is for ribulose bisphosphate carboxylase/oxygenase (Rubisco). Early *in vitro* studies of this

enzyme from winter rye showed an increased stability of catalytic activity to denaturants and freeze/thawing in acclimated plants (Huner and Macdowell, 1979) and evidence for a stable *in vivo* conformational change during low temperature exposure resulting in altered K_m values for CO_2 with respect to temperature (Huner and Macdowell, 1978).

It is not clear whether these changes during cold acclimation in enzyme activity or isozymic variants are directly linked to the development of freezing tolerance. These changes may instead represent a more universal adjustment of basic cellular metabolism to the biophysical constraints imposed by low temperature, increasing the overall fitness of the plant for survival in cold environments.

1.8. Evidence for Changes in Gene Expression

In vitro translation studies

As early as 1970, Weiser first suggested that changes in gene expression may mediate the process of cold acclimation (Weiser, 1970). Direct evidence was not obtained until 15 years later, when Guy and coworkers observed that low temperatures resulted in rapid and stable changes in translatable mRNAs in spinach (Guy et al., 1985). Subsequent studies have confirmed this finding in spinach (Guy and Haskell, 1988), barley (Cattivelli and Bartels, 1989; Hughes and Pearce, 1988), *Arabidopsis thaliana* (Kurkela et al., 1988; Gilmour et al.,

1988; Lin et al., 1990), *Brassica napus* seedlings (Meza-Basso et al., 1986) and cell suspension cultures (Johnson-Flanagan and Singh, 1987), alfalfa (*Medicago falcata* cv. Anik) (Mohapatra et al., 1987b), tomato (Schaffer and Fischer, 1988) and potato (Tseng and Li, 1987). Some of the *in vitro* translation products had similar masses and/or electrophoretic mobilities as cold-inducible polypeptides detected by *in vivo* labeling.

In alfalfa (*Medicago falcata* cv. Anik), four polypeptides, 90, 38, 36 and 48 kDa, detected by *in vivo* labeling, had similar electrophoretic mobilities to *in vitro* translation products (Mohapatra et al., 1987b, 1988b). Within hours (Cattivelli and Bartels, 1989; Gilmour et al., 1988) to one day (Guy and Haskell, 1988) of low temperature exposure, new mRNAs begin to appear. This provides strong, but not conclusive, evidence for altered gene expression since selective translation can occur. For example, the mRNA for CAT-2 of maize is present in the leaves of dark-grown seedlings but it is rendered translatable only after exposure to white light (Skadsen and Scandalios, 1987); this differential translation was not only observed *in vivo*, it was also found to occur *in vitro*.

Molecular cloning of low-temperature-induced genes

The most recent approach to understanding the molecular genetic basis of low-temperature-induced gene expression and freezing tolerance has been to screen cDNA libraries and identify cDNA clones which are low-temperature-specific. The first report of low temperature-induced genes appeared in 1988 (Mohapatra et al., 1988c; Schaffer and Fischer, 1988).

Working with cold acclimated seedlings of alfalfa, Mohapatra and coworkers described the isolation of a low temperature-induced gene (pSM1409) whose expression is correlated with differences in varietal freezing tolerance (Mohapatra et al., 1988c). Expression of this gene is induced by water stress and treatment with exogenous ABA, in addition to cold.

In tomato, two cDNA clones were identified, one of which, C14, encodes a polypeptide that is homologous to the plant thiol proteases actinidin and papain and to the animal thiol protease cathepsin H. However, the level of expression of both genes, as detected by northern hybridization analysis, was not correlated with the level of chilling tolerance: both genes were expressed in both the chilling-tolerant and the chilling-sensitive fruit, suggesting that induction of expression of these two genes is not responsible for the differential tolerance of the two lines.

Mohapatra et al. (1989) were the first to report the cloning of three cold-acclimation-specific (cas) genes in alfalfa seedlings, whose expression was

positively correlated to differences in varietal freezing tolerance (Mohapatra et al., 1988c; 1989). A part of this report is included in this thesis. Since the publication of this work, several other groups have succeeded in isolating and characterizing cDNA clones which are induced during cold acclimation in plant species.

One group working with *Arabidopsis thaliana* reported the cloning and characterization of two cold- and ABA-inducible genes, *kin1* and *kin2* (Kurkela and Frank, 1990; Kurkela et al., 1992). *Kin1* codes for a polypeptide of 6.5 kDa, rich in alanine, glycine and lysine. This clone belongs to a family of at least two genes and its expression was increased 20-fold in cold- or ABA- or water stress-treated plants. Sequence analysis showed limited similarities, at the amino acid level only, to type I fish antifreeze proteins (AFPs) (Pickett et al., 1984). KIN2 is similar to KIN1 in size and composition except that KIN2 responds strongly to drought and salinity stresses and has a detectable constitutive level of message. Recently, another group working independently has cloned a virtually identical gene, *cor6.6* (pHH29), which encodes a polypeptide of 6.6 kDa (Gilmour et al., 1992). This gene, *cor6.6*, differs from *kin1* by 0.1 kDa. Both may function as antifreeze or thermal hysteresis proteins (Gilmour et al., 1992). Hajela et al. (1990) have isolated cDNA clones by differential screening of an *Arabidopsis thaliana* cDNA library constructed from poly(A)$^+$ RNA isolated from CA plants. Initially, 25 clones were identified which fell into four different

groups, represented by pHH7.2, pHH28, pHH29 and pHH67, and which hybridize to transcripts of sizes 1.4, 2.5, 0.6 and 0.7 kb, respectively. Levels of transcripts increased 10-fold or more in CA plants. All 4 *cor* transcripts increased dramatically within 4 hours, continued to accumulate until 12 h, and then reached a plateau after 12 h. Transcripts were present at these levels for at least 14 days (the longest time point tested). Deacclimation resulted in rapid decreases in the levels of all 4 mRNAs; messages were undetectable within 4 to 8 h. Levels of *cor* transcripts increased in response to low temperature, exogenous ABA, and water stress.

Three genes appeared to be regulated at the posttranscriptional level (pHH7.2, pHH28, pHH29), while the third (pHH67) was regulated at the transcriptional level. Clone pHH7.2 (*Cor47*) encodes a polypeptide of 47 kDa which is boiling stable and detects a related gene in wheat (Lin et al., 1990). Recently published analysis of a partial-length cDNA clone (Gilmour et al., 1992) showed the predicted polypeptide product to share sequence homology with the Group II class of LEA proteins, including RAB16A from rice (Mundy and Chua, 1988), several barley and maize dehydrins (Close et al., 1989), LEA protein D11 from cotton (Dure et al., 1989), and a tomato salt-induced polypeptide (Godoy et al., 1990). A serine cluster and a lysine-rich sequence, sequence motifs found in Group II LEA proteins, were also found in COR47.

The authors speculate that COR47 may function to protect the plant against freezing-induced dehydration.

Certain freeze-avoiding fish (Davies and Hew, 1990) and freezing tolerant insects (Zachariassen, 1985) synthesize antifreeze or thermal hysteresis proteins during cold acclimation. In the case of marine teleosts, the freezing point of seawater (-1.9°C) is well below the freezing point of the body fluid of the fish (-0.8°C). This small difference is sufficient to permit freezing because supercooling cannot occur in the presence of ice (Scholander et al., 1957). However, the body fluids of such fishes will not freeze until the temperature is lowered below -2°C (DeVries and Lin, 1977). About 40 to 50% of the freezing point depression is due to the presence of antifreeze peptides and glycopeptides.

Four distict macromolecular antifreezes have been isolated and characterized from various marine fishes (Davies and Hew, 1990). These include the glycoprotein antifreezes (AFGP) (molecular masses of 2.5 - 33 kDa), which consist of a repeating tripeptide $(Ala\text{-}Ala\text{-}Thr)_n$ with a dissacharide attached to the threonyl residues, and three antifreeze protein (AFP) types.

Type I AFP of right eye flounders and sculpins have been the most extensively studied. This is the only type for which detailed structure/function relationships have been proposed based on x-ray chrystallography studies. The two major AFPs from winter flounder are each 37 amino acids in length and contain three 11-amino acid tandem repeats of the sequence $Thr\text{-}X_2\text{-}Asx\text{-}X_7$,

where X is usually alanine or some other amino acid compatible for α-helix formation. The tandem repeat acts to generate a helix with amphiphilic properties, stabilized by dipolar interactions and intrachain salt bridges. Many of the AF(G)P are synthesized as precursors which are eventually processed into the mature protein.

All of the AFP appear to lower the freezing point of a solution beyond the value predicted from the colligative effect alone. However, the melting point is not altered in such a noncolligative manner; such a difference between freezing and melting points is termed thermal hysteresis. On a molal basis, these AFP depress the freezing point 200 to 300 times more than expected on the basis of colligative relationships.

Microscopic observation of ice crystal growth has shown that the presence of AFP not only lowers the freezing point of the solution but also alters the growth habits and growth rates of ice (Scholander and Maggert, 1971). All of the antifreeze peptides and glycopeptides adsorb to ice crystals and act as a barrier to the advancing front of water molecules which are joining the ice lattice at the step during crystal growth. Inhibition of ice crystal growth results from a inability of the ice front to over-grow the adsorbed antifreeze molecules or pass in between them. The amphiphilicity of the α-helix, with its alignment of hydrophilic side chains, provides the hydrogen bonds needed for interaction with the ice lattice, while the hydrophobic side chains serve to impede the growth of

the ice nuclei. Anomolously low freezing points of water in tissues have been explained on the basis of increases in surface area free energy resulting from a high ratio of surface area to volume. Adsorption of antifreezes to ice crystals leads to an increase in surface free energy of the ice crystal and depression of the freezing point (DeVries, 1983; Raymond, 1976).

AFP can also lower the average spontaneous freezing temperature of leaves which are infiltrated with a solution of the protein (Cutler et al., 1989). For example, cotyledons of *Brassica napus* showed a freezing point depression of 1.8°C. Expression of the *afa-3* antifreeze gene from winter flounder has been achieved recently in transgenic plants (Hightower et al., 1991). In these studies ice recrystallization inhibition was detected in trangenic tissue. However, freezing point depression is limited to about 1.2°C. The significance of AFP is, therefore, likely to be important only in situations where a difference of one or two degrees is crucial, such as with certain tender spring crops, such as *Brassica napus* (canola), which are subject to damage from late spring or early fall frosts. Similarly, Florida citrus crops are occassionally devastated by winter cold spells; here, a depression in the freezing point of the plant tissue of only a degree or two could separate a good yield from economic catastrophe (Cutler et al., 1989). It is unlikely that AFP or their equivalents in plants would affect overwintering.

Independent evidence for cryoprotective proteins comes from studies with spinach and cabbage (Hincha et al., 1990). This group has succeeded in partially

purifying protein fractions, from cold acclimated, frost hardy plants, which protect isolated thylakoids of non-hardy leaves against mechanical membrane rupture during an *in vitro* freeze/thaw cycle. As these proteins are boiling-stable, at least some of the genes coding for low molecular-weight proteins cloned in *Arabidopsis* may be analogous to these spinach cryoprotective proteins. Confirmation of these speculations must necessarily await purification and sequencing of the cryoprotective proteins from spinach and cabbage. Recently, Lin and Thomashow (1992) published the sequence of a cDNA for a gene, *cor15*, whose protein product is a hydrophilic, boiling-stable polypeptide having an N-terminal amino acid sequence that closely resembles transit peptides that target proteins to the stromal compartment of chloroplasts. Immunoblot analysis using antiserum raised against COR15 detected the mature polypeptide, COR15m, in the soluble fraction of chlorplasts. Although COR15 shares many properties with the cryoprotective proteins described by Heber and coworkers (Volger and Heber, 1975), it remains to be established whether COR15 or COR15m has any cryoprotective properties *in vitro* or *in vivo*. Boiling stability and low molecular weight are also properties of the LEA/RAB/Dehydrin family of proteins. These cryoprotective proteins may therefore be members of this family and as such, protect cells against damage due to freezing-induced dehydration.

The cryoprotective proteins described for frost-hardy leaves of cabbage and spinach (Hincha et al., 1990; Volger and Heber, 1975) have a different

mechanism of protection. Rather than achieving freezing avoidance, these proteins protect isolated thylakoid membranes in the presence of frozen water. This protective effect is based, at least in part, on a decrease in solute permeability of the membranes which confers protection against osmotic stresses that lead to rupture of the membrane during subsequent thawing. In addition, a reduced suceptibility to a rapid component of mechanical freeze-thaw damage, related to expansion-induced lysis after thawing appears to be involved. To date, these cryoprotective proteins have not yet been purified or sequenced.

Also working with *Arabidopsis thaliana*, Nordin et al. (1991) have recently cloned a low-temperature-induced gene, *lti40*, which is induced by cold, ABA and water stress but not by heat shock. Low-temperature induction of *lti40* mRNA was not mediated by ABA, as demonstrated by normal induction of the *lti40* transcripts in both ABA-deficient and ABA-insensitive mutants and after treatment with the ABA biosynthesis inhibitor fluridone. However, the effects of low temperature and exogenous ABA were not cumulative, indicating that the two pathways converged. In addition, induction of *lti40* expression by ABA or water stress was abolished in the ABA-insensitive mutant *abi-1*, suggesting that this mutation defines a component in the ABA response pathway and that induction by water stress may be mediated, at least in part, by ABA. Taken together, the data lead one to conclude that three separate but converging signal pathways regulate expression of the *lti40* gene. Subsequently, work by another

group with similar mutants of the same plant supports such a conclusion (Gilmour and Thomashow, 1991).

Cattivelli and Bartels (1990) reported the cloning of five cold-regulated genes in barley. The transcripts detected by these clones accumulated to different levels during cold treatment and several of these genes were expressed in a tissue-specific manner. Homologous cold-induced transcripts were detected for wheat and rye. Expression of these genes was not induced by heat shock treatment. The hybrid-release translation product of one of the clones correlated with a polypeptide of an apparent molecular mass of 22 kDa, found only for *in vitro* translation products of RNA from cold acclimated leaves. DNA sequence analysis of two of the clones revealed a longest open reading frame which contains an arginine-rich basic domain. A computer search revealed no significant homologies between these cold-induced sequences and published DNA or protein sequences entered in the Genbank and EMBL gene data bank.

Dunn and coworkers (Dunn et al., 1990, 1991) have reported the cloning and characterization of two low temperature induced genes in barley. Clone BLT14 was induced to high levels in response to low temperature treatment, hybridized to a single transcript of 514 bp and included an open reading frame encoding a polypeptide of 88 amino acids. The gene was estimated to be present at one copy per haploid genome and appears to be localized on barley chromosome 2. A computer search of a sequence databank failed to reveal any

significant homologies to any published sequences. Therefore, the function of this particular gene is unknown. A second gene represented by the cDNA clone BLT4, was similarly induced after 3 days of low temperature treatment in shoot meristematic tissue. This gene was shown to reside on chromosome 3 and has sequence homology with genes in wheat and oats. However, expression did not correlate with frost hardiness in doubled haploid lines. Its expression was also induced by drought stress. Some homology was found with the probable α-amylase/protease inhibitor PAPi from barley (Svenson et al., 1986).

In summary, several groups working with a variety of different plant species have succeeded in identifying genes which are cold-inducible and whose expression is correlated with low temperature-induced freezing tolerance. These studies, therefore, support Weiser's earlier prediction that changes in gene expression mediate the cold-inducible component of freezing tolerance (Weiser, 1970).

CHAPTER 2.

MATERIALS AND METHODS

2.1. Plant Material

Two-week old alfalfa seedlings (*Medicago falcata* c.v. Anik), were used throughout this work. For analyses of varietal freezing tolerance, seedlings of the following alfalfa cultivars were also used: *Medicago media* c.v. Iroquois, *Medicago media* c.v. Algonquin, and *Medicago sativa* c.v. Trek. Seeds were surface-sterilized for 20 minutes in tap water containing 1% sodium hypochlorite and two drops of 10% Tween 20. The seeds were then rinsed extensively with tap water for several hours. Seeds were planted in vermiculite soaked in 1X Hoagland's solution and autoclaved for twenty minutes. Germination was carried out in darkness at 25°C. Once the seedlings had broken the surface of the vermiculite, the trays were uncovered and grown at 25°C/20°C (day/night) with a 16 hour photoperiod and a light intensity of 250 μE $m^{-2}s^{-1}$. Seedlings were watered every two days with tap water. For all experiments, two-week old seedlings were used.

2.2. Cell Suspension Cultures

For the initiation of callus cultures, a more rigorous sterilization regime was needed. Seeds of the freezing-tolerant cultivar, *Medicago falcata* c.v. Anik, were surface-sterilized with sterile (autoclaved) water containing 1.5% sodium hypochlorite plus two drops of 10% Tween 20. This and all subsequent

manipulations were carried out in a laminar flow hood. Seeds were then rinsed four times with sterile water for 1 h each wash. During this time the solution containing the seeds was stirred continuously using a magnetic stirrer. Seeds were individually pressed onto the surface of sterile solidified 1X Hoagland's medium (1.5% agar) in a 125 ml Erlenmeyer flask, stoppered with a foam plug. The seeds were allowed to germinate under continuous light at 25°C for two to three weeks.

The hypocotyls of individual seedlings were cut into segments approximately 1 cm in length. These segments were then sterilized in 1.5% sodium hypochlorite containing two drops of 10% Tween 20 for 5 min, rinsed extensively (about five times) in sterile water, placed for 15 seconds into a solution of 70% ethanol and rinsed several times in sterile water. These hypocotyl explants were then pressed onto the surface of solid B_5h medium (Atanassov and Brown, 1984) in a plastic petri dish. Callus production was then allowed to proceed under darkness for about one month. Failure to sterilize the seedlings themselves resulted in the growth of a microbial contaminant around the hypocotyl segments.

Cell suspension cultures were initiated by transferring a segment of 'friable' callus tissue approximately 1 cm^3 in volume to 50 ml of liquid B_5h medium in a 125 ml Erlenmeyer flask. The flask was shaken on an orbital shaker (New Brunswick) at 150 rpm under continuous light at 25°C.

Subculturing was carried out once per month by transferring 10 ml of the shaken culture to a new flask containing 40 ml of fresh media. After four months, large 500 ml suspension cultures were established by subculturing 100 ml of the cultures into 400 ml of fresh media in a 1 L flask. Thereafter, subculturing was carried out once every three weeks.

2.3. Growth Curves

The growth of the cell suspension cultures was followed by measuring fresh and dry weights. Aliquots of 15 ml from a 500 ml culture were removed and collected by vacuum filtration onto a pre-weighed Whatman 2 filter paper disc fitted into a Buchner funnel. The fresh weight of the sample was measured using an electronic Mettler balance, after correcting for the wet weight of the filter alone. Since the duration of the vacuum filtration greatly affected water content and hence the weight of the filter and sample, these times were standardized throughout. The filters were then placed in a drying oven set at 60°C and incubated overnight. After about 15 hours, the filters were weighed and the dry weight determined by subtracting the dry weight of the filter from the combined weight of the filter plus the sample. A growth curve was also constructed for a culture which had been kept at 25°C for 10 days and then subsequently transferred to cold (5°C/2°C, day/night, 12 hour photoperiod) to assess the effect of cold temperature on growth.

2.4. Cold Temperature Acclimation and Deacclimation

Seedlings which had been grown for two weeks at 25°C/20°C (day/night) under a 16-hour photoperiod in a growth chamber (Conviron) were transferred to a second growth chamber kept at 5°C/2°C (day/night) under a 12 hour photoperiod. Acclimation was carried out for periods ranging from 1 to 14 days.

Cell suspension cultures, grown under a 16 hour photoperiod and at 25°C/20°C (day/night) were transferred during either log phase (10 days after subculture) or early stationary phase (21 days) to a second growth chamber kept at 5°C/2°C (day/night) and a 12 hour photoperiod. Cultures were acclimated for periods ranging from 2.5 h to 14 days. Deacclimation was carried out by returning to the previous growth chamber (25°C/20°C, 16 hour photoperiod), cultures which had been incubated for 7 days at the colder temperatures. Deacclimation was carried out for periods ranging from 30 min. to 2 days.

2.5. Freezing Survival Analyses

Seedlings used in freezing survival tests were transferred to a temperature controlled freezer set at 5°C. The temperature was gradually lowered at a rate of approximately 2.5°C/h to final freezing temperatures ranging from -5°C to -20°C. Seedlings were kept at this final freezing temperature for a total of 3 hours. The temperature was then increased at a rate of approximately 2.5°C/h to 4°C. Thereafter, seedlings were transferred to a cold cabinet (4°C) overnight

before being returned to 25°C. Seedlings were finally transferred to a growth chamber set at 25°C/20°C (day/night, 16 hour photoperiod), for 3 days. Plants were classified as living or dead by visual inspection. Dead seedlings were easily distinguishable by their discoloured, flaccid appearance. Survival was determined by maintenance of turgidity and resumption of growth. Physically counting the number of individual living seedlings after freezing and dividing this quantity by the number of seedlings before freezing, gave an estimate of the percentage of survivors. Sample sizes typically ranged from 120 to 200 seedlings.

Freezing survival analyses were also conducted for cell suspension cultures of alfalfa. Cell suspension cultures, 10 days after subculture, were acclimated for various time intervals. One millilitre aliquots were removed from each culture and transferred to small glass test tubes. The cells were allowed to settle to the bottom of the tube for several minutes before the media was removed by aspiration. The cells were then rinsed with sterile deionized distilled water (sddH_2O), allowed to settle, and finally resuspended in 2 ml of sddH_2O. The tubes containing the cells were left to equilibrate on ice for 30 min. A programmable freezing bath (Neslabs, Portsmouth) containing polyethylene glycol as a coolant, was used to freeze the cells. The tubes were placed at -1.5°C for 15 min. Ice crystal nucleation was achieved by placing against the side of the tube a metal probe cooled with liquid nitrogen. The temperature of the bath was then decreased at a rate of 3°C/h. Tubes were removed when the desired

temperature was reached and placed immediately on ice until all samples had been removed. The bath was then adjusted to 5°C and tubes were then incubated at this temperature for a further 90 min.

Viability was assessed by a modification of the 2,3,5-triphenyltetrazolium chloride (TTC) reduction assay (Towill and Mazur, 1974; Steponkus and Lanphear, 1967). Water was removed and 3 ml of TTC (0.8% in 50 mM potassium phosphate buffer, pH 7.4) were added. The tubes were then incubated in the dark, without shaking, at room temperature for 18 h. To extract the reduced red formazan dye, the supernatant was removed and the cells resuspended in 5 ml of 95% ethanol. The tubes were capped and incubated in the dark at room temperature for about 1 day. The 5 ml were removed to another tube, and the pellet resuspended in an additional 4 ml of 95% ethanol and incubated for another 1 day. The 4 ml were removed and pooled with the previous 5 ml. The amount of dye extracted was measured spectrophotometrically by transferring about 3 ml to a plastic cuvette and measuring the absorbance of the solution at 485 nm using an LKB spectrophotometer. A solution of 95% ethanol served as a reference blank. The absorbance readings were corrected for small differences in sample size by measuring the dry weight of the pellet from each sample. Cells which had not been frozen served as a control. Percent survival was expressed as the absorbance reading for the frozen cells divided by that for the unfrozen cells.

2.6. Administration of Other Stress Treatments

Seedlings were subjected to various other stress treatments: water stress, heat shock, wounding, and treatment with ABA. Water stress was administered by placing seedlings in polyethylene glycol-6000, yielding a water potential of -15 bars for 12 h. Heat shock was delivered by exposing seedlings to 42°C for 3 h; wounding was achieved by cutting the seedlings into small (1 cm) segments and placing them in water at 25°C for 3 h; and ABA was used at 75 μM with the roots of the seedlings placed in the solution for a period of 12 h.

2.7. Extraction of Total RNA

All glassware used in the isolation of RNA, or for the storage of stock solutions for work with RNA, were baked at 400°C for five hours to inactivate any contaminating ribonucleases (RNase). All aqueous solutions used in the extraction of RNA, with the exception of the 4 M guanidinium buffer and any Tris buffers, were treated with diethylpyrocarbonate (DEPC), 0.1% final concentration, overnight with stirring. All such solutions were then autoclaved for 30 min to inactivate the DEPC.

Three different methods were used to extract total RNA from plant material. A modification of the guanidinium isothiocyanate/cesium chloride method (Chirgwin et al., 1979) was used to extract the total RNA later to be used

in the construction of the cDNA library. Two-week old seedlings of *Medicago falcata* c.v. Anik which had been cold acclimated for 7 days, were gently removed from the vermiculite trays, washed, and blotted dry with paper towels: this, and all subsequent manipulations were carried out at 4°C. Thirty five grams (fresh weight) of seedlings were frozen in liquid nitrogen and ground to a fine powder using a mortar and pestle pre-cooled with liquid nitrogen. The powder was immediately transferred to a pre-cooled 250 ml centrifuge bottle. Five volumes (175 ml) of 4 M guanidinium isothiocyanate solution were added. After the resultant slurry had thawed (about 10 min), it was split into several 50 ml conical tubes and homogenized for 5 min using a Polytron homogenizer set at medium speed. The sample was centrifuged twice at 12,000 r.p.m. for 15 min each time, using a Sorvall SS-34 rotor, to remove any debris. Approximately 28 ml of the homogenate was carefully layered over a 9.5 ml cushion of 5.7 M cesium chloride in 0.1 M EDTA, pH 7.5, in a Beckman SW28 ultracentrifuge tube. The tubes were centrifuged at 27,000 rpm for 26 hours.

After ultracentrifugation, the supernatant was carefully removed by aspiration, leaving about a one-half inch layer of the CsCl solution in the tube. The walls of the tube were washed with 4 M guanidinium solution to denature any ribonuclease adhering to the walls of the tube. The CsCl was removed and the RNA pellet was rinsed with 70% ethanol and dried. The pellet was finally resuspended in 1 ml of DEPC-treated HEPES buffer (0.1 M HEPES pH 7.5, 25

mM EDTA, 1% SDS), extracted once with a 4:1 mixture of chloroform:1-butanol to remove any traces of RNase, and precipitated with 0.1 volume of RNase-free 3 M sodium acetate (pH 5.2) and 2.5 volumes of absolute ethanol at -20°C overnight. The RNA was collected as a pellet after centrifugation at 12,000 g for 20 min, washed twice with 70% ethanol, lyophilized and finally redissolved in 1 ml of DEPC-treated ddH_2O. RNA prepared in this way was stored at -85°C until needed.

For most routine extractions of total RNA, the single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction was employed (Chomczynski and Sacchi, 1986). The only modification was the use of 5 volumes of extraction buffer, rather than the 10 volumes described in the original paper.

Much of the initial work with seelings involved extraction of total RNA using a modified hot phenol-SDS method and is described elsewhere (Mohapatra et al., 1989).

2.8. Extraction of Total Nucleic Acids

Experiments designed to examine the kinetics of uptake and incorporation of [^{3}H]uridine into cells, in the presence and absence of the transcriptional inhibitor cordycepin, involved the use of a cold phenol-chloroform extraction

protocol carried out at 4°C. This method was rapid and quantitative. Two volumes of cold (4°C) phenol, equilibrated with 0.2 M Tris-Cl pH 8.0, were added to cells in a mortar chilled to 4°C and ground thoroughly (30 s) using a chilled pestle. All manipulations were carried out at 4°C. Four volumes of phenol were then added and the sample ground further for about 1 min. The resultant slurry was transferred to a 15 ml conical tube. The mortar and pestle were rinsed with 2 volumes of phenol and the wash was transferred to the conical tube. The mortar and pestle were finally rinsed with 8 volumes of Nucleic Acid Extraction Buffer (50 mM Tris-Cl pH 8.0, 10 mM EDTA, 1% sodium sarkosyl, 5% 2-mercaptoethanol) which was transferred to the conical tube.

The tube was capped, the phases were mixed thoroughly for about 30 s and the tube was placed on ice for 5 min. The phases were separated by centrifugation in a swinging bucket bench-top centrifuge at 8,000 rpm for 10 min at 4°C. The aqueous phase was removed to a new tube and processed further as described in section 2.20.

2.9. Quantification of Nucleic Acids

Nucleic acids were quantified spectrophotometrically by measuring the absorbance of a solution at 260 nm, in 1 ml or 0.5 ml quartz (U.V. transparent) cuvettes with a pathlength of 1 cm using an LKB spectrophotometer. A solution with an absorbance of 1.0 at a wavelength of 260 nm was assumed to have a

concentration of 50 μg/ml and 40 μg/ml for DNA and RNA, respectively (Maniatis et al, 1982). This relationship was found to be linear over the range 0.050 to 0.800 absorbance units for solutions of RNA. For synthetic oligonucleotides, a solution having an optical density of 1.0 was assumed to represent a concentration of 33 μg/ml.

2.10. Selection of Polyadenylated RNA

Polyadenylated RNA (poly(A)$^+$ RNA) was separated from bulk total RNA by conventional affinity chromatography on oligo(dT)-cellulose (Aviv and Leder, 1972) as described by Sambrook et al., (1989)

2.11. Size Fractionation of RNA

Total or poly (A)$^+$ RNA was analyzed by electrophoresis in 1.5% agarose gels containing 0.66 M formaldehyde as a denaturant in 1X MOPS/EDTA ([3-(N-morpholino) propanesulfonic acid] buffer (Lehrach et al., 1977), as modified by Fourney et al., (1989). RNA was subsequently immobilized onto nitrocellulose membranes by a modified capillary blotting procedure (Fourney et al 1989). The only modification was the inclusion of 0.5 μg/ml ethidium bromide in the gel itself rather than in the sample. These 'northern blots' were placed in a U.V. Statalinker (Stratagene) to fix the RNA to membrane, according to the

manufacturer's instructions, before use in nucleic acid hybridization experiments.

2.12. Nucleic Acid Hybridization

Northern blots were prehybridized for several hours to overnight in a solution of 6X SSC, 1% SDS, 5X Denhardt's solution, and 100 μg/ml denatured calf thymus DNA in heat sealable heavy duty hybridization bags (Kapak Corp.) immersed in a water bath set at 65°C (1X SSC is 0.15 M NaCl, 0.015 M sodium citrate; 50X Denhardt's solution is 100 mg Ficoll 400/L, 100 mg/L polyvinylpyrrolidone, 100 mg/L Bovine Serum Albumin Fraction V) . After prehybridization, 500 ng of heat denatured nick translated probe were added to the hybridization bag and the bag resealed and incubated overnight (12 to 18 h) at 65°C with reciprocal shaking.

Northern blots were washed twice, 15 min each at room temperature in a solution of 2X SSC, 0.1% SDS; followed by a second 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 X SSC, 0.1% SDS, at 65°C. All washes were performed on an orbital shaker or in a reciprocal shaking water bath. Blots were wrapped in Saran Wrap or placed in a thin heat sealable plastic bag and used for autoradiography with Kodak O-Mat XAR film with or without intensifying screens, as described by Maniatis et al., (1982).

2.13 Labelling of Probes with ^{32}P

Probes used in hybridization experiments were either purified inserts from recombinant plasmids or synthetic oligonucleotides. Recombinant plasmids were digested with the appropriate restriction endonucleases to release the cloned inserts. Inserts were separated from the vectors by size fractionation on 1% nondenaturing agarose gels in 1X Tris-Borate-EDTA (TBE), 0.5 μg/ml ethidium bromide, in 1X TBE buffer. The inserts were recovered by electrophoresis onto strips of DEAE cellulose membrane (Schleicher and Schuell) according to the manufacturer's instructions (Winber and Hammarskjold, 1980). Five hundred ng of insert were labelled to high specific activity with [α^{32}P]dCTP (ICN, 3000 Ci/mmole) by nick translation (Rigby et al.,1977) using a BRL nick translation kit according to the instructions provided by the manufacturer. Unincorporated nucleotides were removed by spun column chromatography through Sephadex G50 equilibrated in Tris-Cl/EDTA (pH 8.0) as described by Maniatis et al., (1982). Specific activity was determined by liquid scintillation spectrometry (Brinkman liquid scintillation counter) using the ^{32}P channel.

Synthetic oligonucleotides were end-labelled with [γ^{32}P]ATP (ICN, 300 Ci/mmole), using T4 polynucleotide kinase (Pharmacia), according to the supplier's instructions. The oligonucleotides were purified by spun column chromatography as described above. Specific activity was determined by liquid scintillation counting.

2.14 Construction of Recombinant cDNA Library in Lambda Uni-Zap

The clone originally identified in seedlings, pSM784, was a partial length clone as judged by a comparison of the size of the clone with the sizes of the transcripts to which it hybridizes. Therefore, an attempt was made to clone a full-length version of pSM784.

Five μg of poly(A)$^+$ RNA were prepared, after two rounds of selection by oligo(dT)-cellulose affinity chromatography, from total RNA of 7 d cold acclimated seedlings of *Medicago falcata* cv. Anik. This was used as the starting material for construction of a cDNA library in the lambda phage vector Uni-Zap XR using a kit supplied by the manufacturer (Stratagene). This vector system was chosen for several reasons. First, it combines the high efficiency of lambda library construction and the convenience of a plasmid system with blue/white colour selection; inserts can be recovered by an '*in vivo* excision' process in the form of the pBluescript phagemid, obviating subcloning the insert into a plasmid. This system allows for unidirectional cloning, a feature which greatly facilitates subsequent characterization of inserts and reduces the frequency of nonrecombinant (background) phage. The vector can accommodate large DNA inserts from 0-10 kb in length. Finally, many of the convenient features of the pBluescript plasmid system can be exploited, including: rescue of single-stranded DNA probes for DNA sequencing or site-directed mutagenesis; generation of

unidirectional deletions using exonuclease III and mung bean nuclease; the generation of transcripts from the T3 and T7 promoters flanking the cloning site to be used as probes in Southern and Northern blotting; and the expression of fusion proteins by using the upstream lacZ promoter, to be used for western analysis, generation of antibodies or protein purification.

The instructions provided by the manufacturer were followed throughout except that methylmercuric hydroxide was used to relax secondary structure of the source mRNA (Krug and Berger, 1987) and thus facilitate first strand synthesis. Briefly, first strand synthesis was primed using a fifty-base oligonucleotide consisting of a Xho I restriction enzyme recognition sequence and a 3' 18-base poly (dT) sequence. Second strand synthesis was achieved by replacement synthesis using RNaseH and DNA polymerase I. Separately-labelled first and second strand cDNA reactions were analyzed by alkaline agarose gel electrophoresis (McDonell et al., 1977) in order to analyze the size of the products. After blunting the double-stranded cDNA ends, dephosphorylated Eco RI adaptors were ligated to the ends. The ligated adaptors were then phosphorylated, digested with Xho I and the digested products size-separated on a Sephacryl S-400 column. The eluate from five rounds of centrifugation were analyzed by agarose gel electrophoresis and autoradiography. Those fractions containing a majority of products above about 400 bp were pooled and ligated into prepared Uni-Zap vector arms (Eco RI, Xho I digested and

dephosphorylated). The ligation products were then packaged and plated. This primary library was then titred. Titering the library involved making serial dilutions of the phage stock, infecting bateria with these dilutions and plating. In this way, the concentration of phage particles in the library was determined. Results were expressed as the number of plaque forming units (pfu) per microlitre of library stock.

From the primary library, 200,000 plaques, at a density of 40,000 plaque forming units (pfu) per large (150 mm diameter) plate, were screened using nick translated insert from pSM784, a partial length *cas* cDNA clone, and duplicate plaque lifts from each plate. Two rounds of plaque purification were performed to isolate individual clones.

The "*in vivo* excision" protocol supplied by the manufacturer was used in order to recover the inserts as pBluescript phagemids. In order to identify homologs of SM784, the 3' ends of the inserts were sequenced and compared to the 3' end of SM784.

2.15 Determination of Kinetics of Transcript Accumulation

Ten μg of total RNA from cell suspension cultures were size fractionated through denaturing (formaldehyde) agarose gels. RNA was prepared from nonacclimated cell cultures, 10 d after subculture; from cultures acclimated for 2.5, 5.0 hours, 1, 2, 4, 7 and 14 days; and from cultures cold acclimated for 7

days and subsequently deacclimated for 2.5, 5.0 hours, 1, 2 days. The RNA was blotted onto a nylon membrane (Genescreen Plus, DuPont) and probed with inserts from (1) pAcs784 and (2) p2.1 (see below). These inserts had been uniformly labelled to high specific activity by nick translation. The blot was first probed with Acs784. The filter was stripped of the first probe, according to the instructions provided, and reprobed with the insert from a constitutive clone, p2.1. The plasmid p2.1, was isolated from winter wheat under a collaborative research programme between our laboratory and that of Fathy Sarhan at U. Q. A. M. It represents a gene which is induced to equal levels in nonacclimated and cold acclimated winter wheat (Houde et al., 1992). It also has been shown to accumulate to equal levels in seedlings and cell suspension cultures of alfalfa (Dhindsa et al., 1992; Monroy et al., 1992). It therefore served as an internal constitutive control for all of the subsequent work with alfalfa described here.

The levels of transcripts in each sample were quantified by scanning densitometry of autoradiographs using an LKB Gel Scanner. At least three different exposures were scanned in order to verify that the signals were within the linear response range of the film. A beam width of 4000 microns was used throughout.

2.16 Isolation of Nuclei

Nuclei were isolated according to Lawton and Lamb (1987) with modifications (Luthe and Quatrano, 1980; Watson and Thompson, 1988). Thirty grams (fresh weight) of cells were frozen in liquid nitrogen and ground to a fine powder with a mortar and pestle cooled with liquid nitrogen. The powder was transferred to a pre-cooled bottle and 10 volumes (300 ml) of Buffer A were added. Buffer A consisted of 0.44 M sucrose, 25 mM Tris-Cl pH 7.6, 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 50 g/L Dextran T40, 25 g/L Ficoll 400, 5 g/L Triton X-100, 2 mM spermidine HCl, and 2 mM PMSF (phenylmethylsulfonyl fluoride). The resultant slurry was allowed to thaw on ice for 10 min. This and all subsequent manipulations were carried out at 4°C. The slurry was homogenized at low speed for 30 s using a Polytron homogenizer. The homogenate was then filtered twice through two layers of Miracloth with gentle suction, and then successively through a layer of Miracloth overlaying a 60 micron and finally a 40 micron nylon mesh, without suction.

Nuclei were collected by centrifugation at 600 g for 10 min using an SS-34 fixed angle rotor. Nuclei were then resuspended in 30 ml of Buffer A using a soft brush. Fifteen ml of nuclei were layered on top of a 25 ml cushion of 30% Percoll (Pharmacia) in 0.44 M sucrose, 25 mM Tris-Cl pH 7.5, 10 mM $MgCl_2$. The resultant nuclear pellet was resuspended in 10 ml of Buffer A and layered onto 20 ml of 30% Percoll containing the above buffer and salts. After a 10 min

centrifugation at 600 g, the pellet was washed once in 20 ml of Buffer A minus spermidine HCl, twice in Nuclear Resuspension Buffer (NRB: 50 mM Tris-Cl pH 7.8), 5 mM $MgCl_2$ 20 mM 2-mercaptoethanol, 20% (v/v) glycerol), and finally resuspended in NRB with 50% glycerol. The nuclei were aliquoted into 1.5 ml centrifuge tubes and immediately frozen in liquid nitrogen. Nuclei were stored at -85°C until used in nuclear run-on transcription assays. Nuclei stored in this way are stable for several months.

2.17 Quantification of DNA Concentration in Isolated Nuclei

The DNA content of isolated nuclei was estimated by a modification of the Burton diphenylamine method. (Giles and Meyers, 1965; Burton, 1956). This is a colorimetric assay for the presence of the deoxyribose moiety of DNA in a tissue sample, in the presence of interfering substances. A standard curve was constructed using calf thymus DNA as a standard. The absorbance readings ($O.D._{595-700}$) for nuclei from all three samples (NA, CA and DA) fell within the range of the standard curve, and the DNA concentrations were read by interpolation of the corresponding values from the graph.

2.18 *In vitro* Transcription in Isolated Nuclei

Nuclei, equivalent to 30 μg of DNA, were resuspended in a total volume of 200 μl of transcription mixture on ice. Transcription mixture consisted of 100 mM $(NH_4)_2SO_4$, 30 mM Tris-Cl, pH 8.0 at 30°C, 7 mM $MgCl_2$, 50 mM KCl, 500 μM each of CTP, GTP, ATP (Pharmacia), 100 μM phosphocreatine (Sigma), 20 μg/ml creatin phosphokinase (Sigma), 3 mM 2-mercaptoethanol, 0.5 mCi [α^{32}P]UTP (3000 Ci/mmole, ICN), and 120 U RNA Guard (Pharmacia). *In vitro* transcription was performed essentially as described previously (Walling et al., 1986; DeLisle and Crouch, 1989). Nuclei were incubated at 30°C for 30 min.

RNA was isolated as described by Marzluff and Huang (1984). Reactions were terminated by diluting nuclei in 10 volumes of 1% SDS, 10 mM EDTA pH 7.0. After shaking vigorously for 30 s, one-tenth volume of 2 M sodium acetate, pH 4.0 was added, the solution shaken well, and an equal volume of water-saturated phenol:chloroform (2:1) was added and the mixture incubated at 55°C for 5 min, followed by chilling on ice for 5 min. This mixture was shaken thoroughly for 30s. The phases were separated after centrifugation at 10,000 g for 20 min at 4°C. The upper aqueous phase containing the RNA was transferred to a fresh tube. Fifty μg of E. coli tRNA was added as carrier for subsequent precipitations. An equal volume of isopropanol was added and the RNA was allowed to precipitate at -20°C overnight. The RNA was recovered as a pellet

after centrifugation at 10,000 g for 20 min at 4°C, washed twice with ice-cold 70% ethanol and dried using a SpeedVac (Savant, Toronto). The RNA pellet was finally redissolved in 100 μl of RNA Resuspension Buffer (0.3 M NaCl, 0.1% SDS, 1 mM EDTA, 10 mM Tris-Cl, pH 7.5).

Unincorporated nucleotides were removed after spun column chromatography through Sephadex G-50 equilibrated with RNA Resuspension Buffer. The RNA recovered in the void volume was precipitated with 0.1 volume of 3 M sodium acetate pH 5.2, and 2.5 volumes of 100% ethanol for at least 2 hours. After centrifugation to collect the RNA, the pellet was washed twice with 70% ethanol, lyophilized, and finally resuspended in 40 μl of DEPC-treated sddH_2O. The specific activity of the RNA samples was determined by removing a small aliquot (2.5 μl) and determining the amount of radioactivity incorporated into TCA precipitable material (Maniatis et al., 1982). Duplicate filters containing TCA precipitable material were subjected to liquid scintillation spectrometry in order to quantify the amount of radioactivity.

2.19 Hybridization of RNA Transcribed In Vitro to cDNA Immobilized onto Filters

Plasmids used included pAcs784, pUb (a ubiquitin probe from sea urchin, p2.1 (a constitutive control), and pBS(KS^+) (pBluescript vector alone). The ubiquitin probe was kindly provided by Dr. Bruce Brandhorst. Plasmids were

linearized by digestion with the appropriate restriction endonuclease (Eco RI for pAcs784, pBS(KS$^+$) and pUb, Pst I for p2.1). After phenol-chloroform and ethanol precipitation, the linearized plasmid DNA was denatured with a solution containing NaOH and NaCl at final concentrations of 0.2 M and 2.0 M, respectively. The DNA was then boiled for 2 min, followed by quick cooling on ice. Ethidium bromide was then added to a final concentration of 0.5 μg/ml in order to visualize the DNA. Using a commercially available slot blot apparatus (BRL), 5 μg of each plasmid in a volume of 50 μl, were applied with suction to separate wells. Following application of the DNA sample, each well was washed with 50 μl of 2X SSC. The nitrocellulose filter was cross-linked with U.V. and allowed to dry.

Filters were prehybridized for at least 1 h at 52°C in prehybridization buffer (final volume 0.5 ml): 0.25 ml deionized formamide, 0.125 ml 20 X SSC, 5 μl 10% SDS, 5 μl 0.1 M EDTA pH 7.5, 5 μl 1.0 M Tris-Cl pH 8.0 at 52°C, 10 μl 0.5 μg/ml poly(A), 40 μl of 50 X Denhardt's solution, 5 μl of 1 mg/ml denatured E. coli DNA, and 55 μl sddH_2O. After prehybridization, the bag was opened, the fluid removed and replaced with 0.5 ml of hybridization buffer, and then resealed. Hybridization buffer was identical to prehybridization buffer except that instead of adding the 55 μl of sddH_2O, 55 μl of RNA and sddH_2O were included. Each filter was hybridized to 30 x 10^6 cpm of RNA transcribed from nuclei of either NA, CA or DA cells. Cold acclimated cultures were

acclimated for 7 d. Deacclimated cultures were acclimated 7 d before being deacclimated for 2.5 h. Hybridization was carried out at 52°C in a shaking water bath for 72 h.

After hybridization, the filters were washed essentially as described elsewhere (Marzluff and Huang, 1984). Each filter was washed four times in 20 ml of 5X SSC, 0.1 % SDS, 1 mM EDTA, 10 mM Tris-Cl pH 7.5, with agitation at 52°C for 30 min each wash. Filters were then washed once with 20 ml of 0.1X SSC at 62°C for 30 min, followed by one wash in 50 ml of 10 μg/ml RNase A in 0.3 M NaCl at room temperature for 10 min. Finally, each filter was washed with 50 ml of 0.3 M NaCl at 25°C for 10 min. The filters were prepared for autoradiography as described earlier. The hybridization signals were quantified by scanning densitometry as outlined above. The amount of nonspecific or background hybridization was estimated by the amount of hybridization to the pBluescript vector alone. The amount of hybridization to each clone, for each filter, was therefore corrected by subtraction of this background signal.

2.20 Kinetics of Uptake and Incorporation of [^{3}H]Uridine in CA Cells In The Absence or Presence of the Transcriptional Inhibitor Cordycepin

One approach to measuring the half life of a transcript is to measure its rate of decay in the absence of transcription. Incubation of cells in the presence of the

transcriptional inhibitor, cordycepin, was used in order to measure the half life of *cas18* transcripts. In order to measure the inhibition of transcription by cordycepin, it was first necessary to measure transcription in the presence of radioactive tracer. Since the cells were labeled under conditions of low temperature, a lag period for both uptake and incorporation of tracer was expected. Incubtion in the absence of cordycepin was thus used in order to determine the extent of this lag period, the period beyond which uptake and incorporaion exhibited linear kinetics, and the point beyond which the kinetics of uptake and incorporation of tracer ceased to be linear.

To this end, cell suspension cultures were cold acclimated for 7 days. From a 500 ml culture, 100 ml (16 g fresh weight) of cells were removed to a sterile 250 ml flask prechilled on ice. All work was conducted at 4°C. Uridine dissolved in water, was added to the culture to a final concentration of 100 μM. Immediately, 500 μCi of [^{3}H]Uridine (ICN, 600 Ci/mmole) were added. A 5 ml aliquot was immediately removed (time zero) after thoroughly shaking the flask. Thereafter, aliquots were removed at various time interval, up to 10 h. The cells were collected onto filter paper, washed twice with 50 ml of cold water and weighed. A sample weighing 250 mg (fresh weight) was quickly transferred to a chilled mortar. Total nucleic acids were extracted as described above.

One hundred μl of the aqueous phase was spotted onto a Whatman GF/A glass fibre filter. The filter was placed into a scintillation vial and 5 ml of

scintillation cocktail were added. The amount of radioactivity was measured by liquid scintillation spectrometry. This provided an estimate of the total cpm. Only a negligible fraction of the unincorporated label partitioned with the phenol phase and this method gave a fairly good estimation of the total cpm in the tissue and was useful in comparing the relative levels of label in different samples. The amount of label incorporated into TCA precipitable material was estimated by removing a 1.5 ml aliquot of the aqueous phase and precipitating it on ice in the presence of TCA and E. coli tRNA carrier at final concentrations of 10% and 5 μg/ml, respectively.

The effect of cordycepin upon the uptake and incorporation of label in CA cells at 4°C was determined. In this series of experiments, it was important to determine how quickly cordycepin acts to abolish completely transcription. From the previous experiment it was determined that uptake and incorporation of tracer were linear from 2h to 10h. Therefore, in this second series of experiments, the cells were incubated for 2 h in the presence of labelled and unlabelled uridine, as above. This was done in order to achieve a detectble rate of transciption. This original culture was split equally into 3 different flasks. To one flask was added cordycepin to a final concentration of 200 μg/ml from a 5 mg/ml stock solution. To the second flask, cordycepin was added to a final concentration of 20 μg/ml. Nothing was added to the third flask of cells. Total and TCA precipitable cpm were determined for various time points as above. In this way,

the effects of two different concentrations of cordycepin on the kinetics of uptake and incorporation of tracer were examined. These experiments allowed the determination of the speed with which cordycepin acted to inhibit completely transcription at low temperature and of the efficacy of low (20 μg/ml) versus high (200 μg/ml) concentrations of the inhibitor. The time point at which cordycepin was found to abolish transcription completely was used as the zero time point in the experiments detailed below (section 2.21).

2.21 Analysis of Transcript Stability in the Presence and Absence of Cordycepin

Cell suspension cultures (500 ml) were cold acclimated for 7 days. From one culture, 125 ml were transferred to a new sterile, pre-cooled 500 ml flask. This and all subsequent procedures were carried out under aseptic conditions. Cordycepin (filter sterilized) was added to this flask to a final concentration of 200 μg/ml. The flask was incubated at 4°C for 8 hours to allow the inhibitor enough time to inhibit transcription completely. After this 8 hour period, an aliquot was removed (zero time point) and the contents of the flask were split into two equal halves (about 60 ml each) in two separate flasks. One flask was kept at 4°C. The other flask was transferred to 25°C for deacclimation. Aliquots were removed from each flask after 12, 24, 48, 60 and 84 hours. Another culture of about 60 ml, similarly cold acclimated, and to which no cordycepin had

been added, was transferred to 25°C for deacclimation. From this last flask, samples were removed after 30, 60 minutes, 2.5, 5.0, and 12 hours.

Total RNA was extracted by the single-step method of RNA extraction by acid guanidinium thiocyanate-phenol-chloroform extraction detailed above. Northern analysis was also carried out as above. The blots were probed with inserts from (1) pAcs784, (2) p2.1, and (3) pUb. Levels of the different transcripts were quantified by scanning densitometry.

2.22 DNA Sequencing

Inserts from recombinant plasmids were sequenced using the enzyme Sequenase and a commercially available kit (U.S. Biochemical Corp., Cleveland). Both strands of a version of SM784, named Acs784 (Alfalfa cold-specifc), which includes the entire coding and 3'-noncoding regions, was sequenced using the double-stranded sequencing protocol provided by the manufacturer, and [^{35}S]dATP (Amersham). The sequencing reactions themselves are a modification of the dideoxy-chain termination method (Sanger et al., 1977). Sequencing reaction products were resolved by denaturing acrylamide gel electrophoresis through 6% polyacrylamide containing 7 M urea in 1X TBE. The electrode buffer was 1X TBE. Sequencing gels were not routinely fixed in acetic acid/methanol prior to being dried down since this step was found to have little or no effect on the resolution of the gel as seen in the autoradiograph. The

sequencing apparatus used was from BRL (model S2). The gels were poured 2 to 24 hours in advance. A bottom spacer was fitted between the glass plates and the entire assembly clamped using small 'bull-dog' clamps. To prevent leakage, the sides and bottom of the gel assembly were carefully sealed with molten agarose. In addition, three large clamps were placed over the middle and extreme ends of the gel assembly directly over the sharkstooth comb. Once placed in the electrophoresis unit with buffer in the bottom chamber, air bubbles at the bottom of the plates were removed using a large syringe with a bent needle. Before placing the electrophoresis buffer in the top chamber, molten agarose was applied to the inner glass plates next to the rubber gasket; this prevented any leaking of buffer from the top to the bottom chamber which can lead to "frowning" artifacts involving unequal rates of migration of samples through the gel. Both strands of Acs784 were sequenced completely, using a combination of synthetic oligonucleotides and by sequencing smaller subcloned fragments.

2.23 Sequence Analysis

Analysis of clone Acs784 was carried out using the departmental PC/Gene facility. Analysis of the nucleic acid sequence to determine the longest open reading frame was performed using the method developed by J. W. Fickett (Fickett, 1982). Computation of the hydropathy index for the longest open

reading frame was carried out according to the method of Kyte and Doolittle (1982). A search for any putative membrane spanning domains was conducted using the method of Klein, et al. (1985). Calculation of pH properties of the putative translation product was carried out using the Chargepro programme of PC/Gene. Protein secondary structure was predicted using the GOR method for predicting secondary structures in proteins (Garnier and Robson, 1989).

A computer search of the GenBank database was carried out for both the nucleotide sequence and the amino acid sequence of the longest open reading frame for Acs784 using the FASTA programme developed by Pearson and Lipman (1988) on January 16 and January 24, respectively, 1992.

A multiple alignment of the amino acid sequence of Acs784 with 9 dehydrin proteins was carried out using a Clustal V alignment of similar sequences program (Higgins and Sharp, 1988).

CHAPTER 3.

COLD ACCLIMATION SPECIFIC (CAS) GENE EXPRESSION IN ALFALFA SEEDLINGS

3.1. Introduction

The ability of a plant to cold acclimate to freezing temperatures is best measured physiologically by subjecting the plant to a freezing test. A comparison of the survival values for nonacclimated plants versus plants cold acclimated for various periods of time provides an evaluation of the effect of prior low temperature exposure on the development of freezing tolerance. In this chapter, evidence is provided which shows that seedlings of *Medicago falcata* cv. Anik are able to undergo cold acclimation. A description of the initial characterization of cold-acclimation-specific cDNA clones in alfalfa seedlings is presented. This work has been previously published (Mohapatra et al., 1989). This was the first report of the cloning and initial characterization of CAS genes in plants.

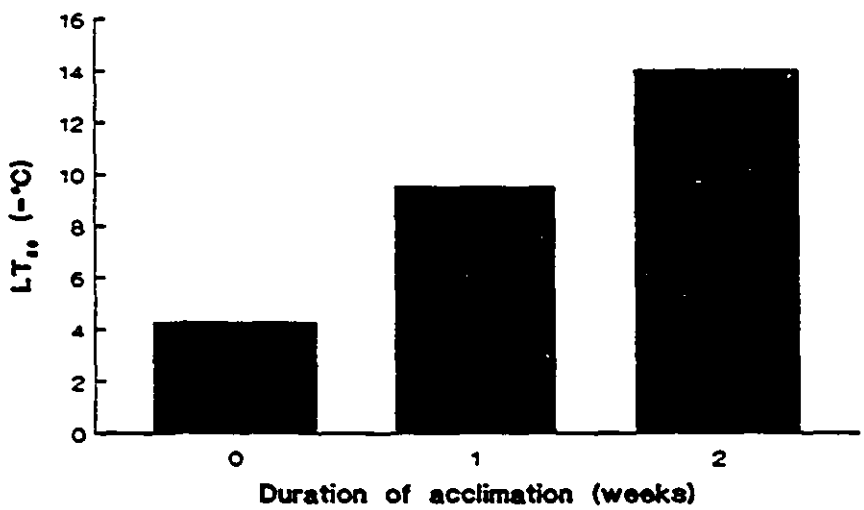
3.2. Results of Freezing Studies

Seedlings of the most freezing tolerant cultivar of alfalfa, *Medicago falcata* cv. Anik, were cold acclimated for various time periods and subjected to a controlled freezing test (Figure 1). The LT_{50} value represents the freezing temperature at which 50% of the seedlings survive upon subsequent thawing. This parameter is a useful and widely used indicator of freezing tolerance. Nonacclimated seedlings exhibited an LT_{50} of -4.25°C.

Seedlings acclimated for 1 week showed a lower LT_{50} of -9.5°C. Seedlings which had been acclimated for 2 weeks, exhibited an LT_{50} of -14.0°C,

a drop of 9.75°C from that of nonacclimated material. These results provide evidence for a progressive increase in freezing survival as the duration of prior low temperature exposure is increased.

Figure 1. Increase in freezing tolerance with duration of low temperature exosure of seedlings of *Medicago falcata* cv. Anik. Approximately 150 - 200 two-week old seedlings were cold acclimated for 0, 1 or 2 weeks and then subjected to a laboratory freezing test. The freezing temperature at which 50% survival was obtained (LT_{50}) was used as an index of freezing tolerance. Survival was determined by visual inspection of seedlings.


16
14
12
10
8
6
4
2
0
LT_{50} (-°C)
0
1
2
Duration of acclimation (weeks)

3.3. Kinetics of Accumulation of CAS Transcripts in Seedlings

A recombinant cDNA library was constructed in the plasmid vector pBR322 using poly(A)$^+$ RNA from seedlings which had been cold acclimated for 14 days. Differential colony hybridization was performed on duplicate nitrocellulose replica filters using radiolabelled single-stranded DNA prepared from (1) cold acclimated seedlings and (2) nonacclimated seedlings (Mohapatra et al., 1989). In this way, clones induced by cold treatment were identified. Three cDNA clones which do not cross hybridize were characterized (Table I and Figure 2). The most abundant cDNA clone (pSM784) was a partial length clone of 684 bp. This clone hybridized to three transcripts of 1.6, 1.4, and 1.0 kb. The sizes of 1.4, 1.0 and 0.4 kb, published previously (Mohapatra et al., 1989), were later found to be incorrect. The correct sizes are 1.6, 1.4 and 1.0 kb. A Genomic Southern blot analysis indicated that as many as 6 bands, ranging in size from 4 to 11 kb, hybridized to the probe. Clone pSM2358 was half as abundant as pSM784, was 861 bp in size, hybridized to two transcripts of 1.2 and 0.9 kb, and recognized 7 distinct bands on a genomic Southern blot. The third clone, pSM2201, was about 720 bp in length, was only half as abundant as pSM2358, hybridized to a single transcript of 0.9 kb, and identified 2 bands on a genomic Southern. Clones pSM784 and pSM2358 may therefore be encoded by several genes which constitute a small gene multigene families, since each clone

hybridizes to several fragments in a genomic Southern analysis. Alternatively, there may exist in each clone, repetitive sequence elements which are present in several unrelated genes. By contrast, clone pSM2201 hybridizes to a single transcript and identifies only two bands in a genomic Southern blot. In this latter case, there may exist a single gene which contains an EcoR1 restriction site outside of the cloned DNA region, or two genes which encode a single transcript or two transcripts of nearly identical size. Similar restriction patterns were obtained when genomic DNA was digested with BamH1.

The induction patterns of all three cDNA clones suggest that the genes corresponding to all three clones are coordinately induced by cold treatment (Figure 3). Accumulation is more rapid during the first 2 days, with transcript levels reaching more than 60% of the final levels. Thereafter, accumulation is more gradual until 7 days when levels reach a maximum. After 7 days the levels of transcripts reach a plateau. These levels are maintained for at least 10 weeks, the longest cold acclimation time point sampled (data not shown). There exists, therefore, a correlation in time between the induction of these CAS genes and the subsequent development of freezing tolerance.

Figure 2. Northern hybridization analysis of CAS transcripts in alfalfa seedlings. 1 μg of poly(A)$^+$ from nonacclimated (NA) or acclimated (A) seedlings was separated by denaturing agarose gel electrophoresis, blotted onto nitrocellulose membranes and hybridized with nick-translated cDNA inserts of clones mentioned under each set of lanes. Clone pSM355 is slightly downregulated during acclimation and was used to reprobe the filter originally hybridized with SM2201; this verified that RNA in the NA lanes was intact.

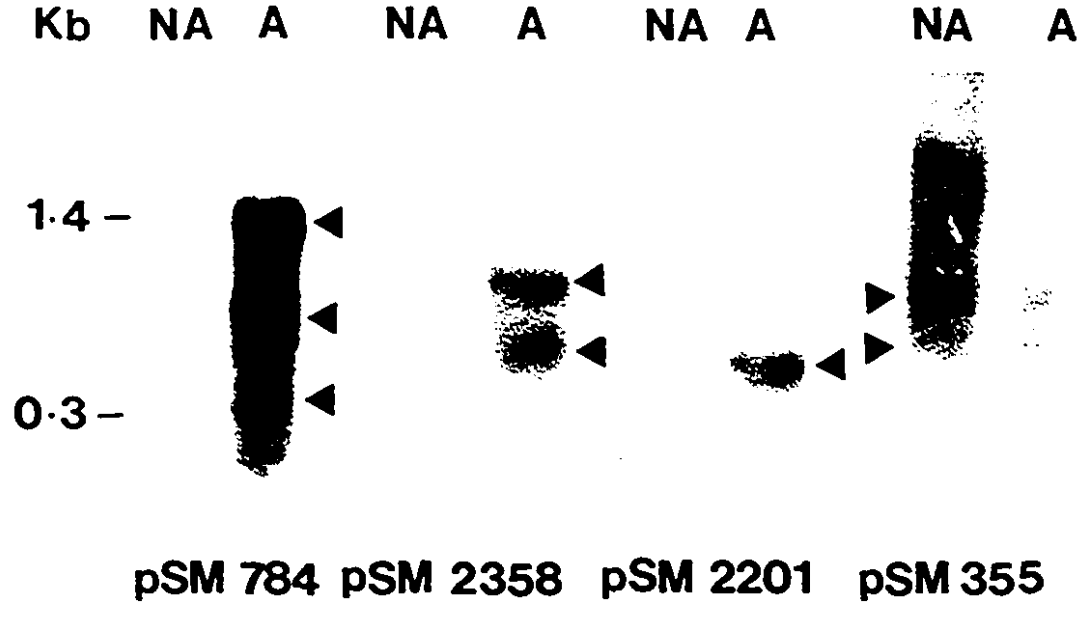
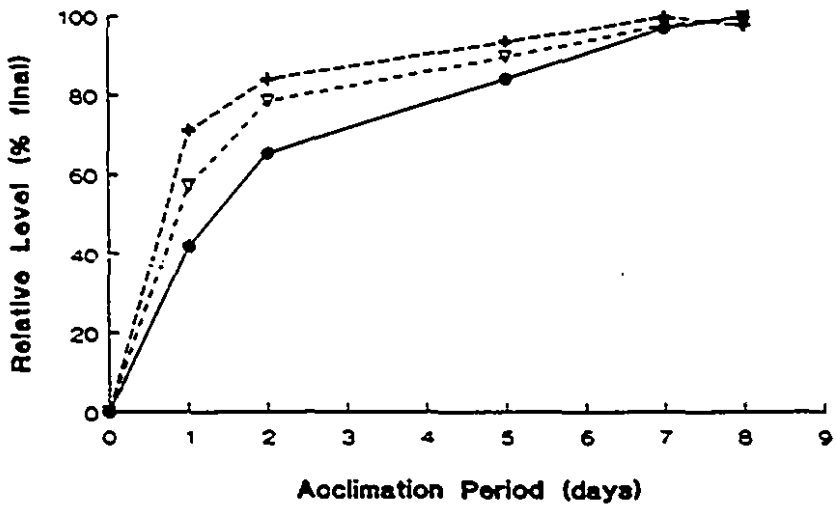

Kb
NA
A
NA
A
NA
A
NA
A
1·4 –
0·3 –
pSM 784
pSM 2358
pSM 2201
pSM 355

Table. I Characterization of Alfalfa CAS cDNA Clones

Clone	Insert size (kb)	Transcript size (kb)	Genomic EcoRI fragments (sizes in kb)
pSM784	684	1.6, 1.4, 1.0	6 (4-11)
pSM2358	681	1.2, 0.9	7 (4.8-12)
pSM2201	720	0.9	2 (5.5, 6.1)

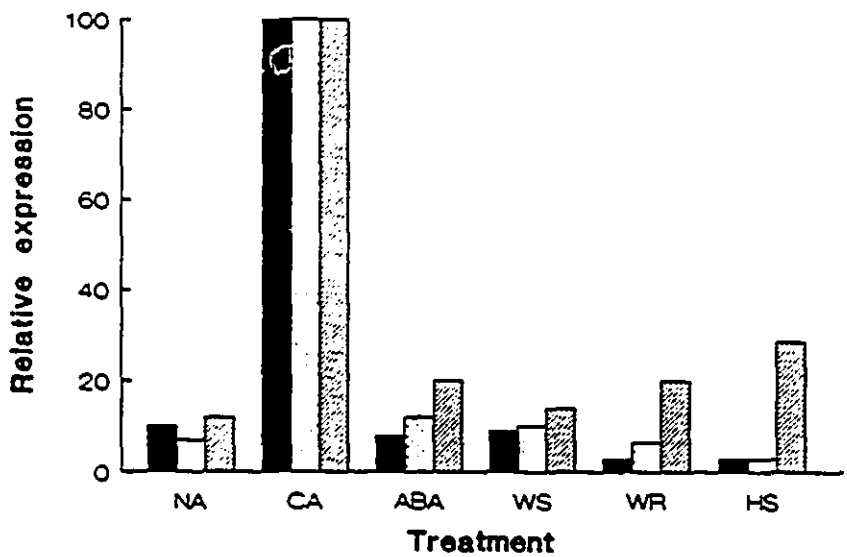
Figure 3. Time-course of accumulation during cold acclimation of CAS transcripts corresponding to clones pSM784, pSM2358 and pSM2201. Ten μg of total RNA isolated from seedlings at 0, 1, 2, 5, 7, and 8 d of acclimation was dot-blotted in triplicate onto nitrocellulose filters and hybridized to the nick-translated cDNA inserts. The hybridization of each dot was quantified by liquid scintillation spectrometry. The values are means of three replicates (SE $\leq$ 10% of the mean). (+), clone pSM2201; (▽), pSM2358; (●), clone pSM784.


Relative Level (% final)
100
80
60
40
20
0
0
1
2
3
4
5
6
7
8
9
Acclimation Period (days)

3.4. Specificity of CAS Gene Expression

Although these CAS genes could be induced by low temperature treatment, the possibility remained that the accumulation of these CAS transcripts represented a general response which could be achieved by a variety of different stresses. To investigate this possibility, seedlings of alfalfa were subjected to a variety of different stress treatments: low temperature, treatment with abscisic acid (ABA), wounding, water stress and heat shock. The phytohormone ABA was chosen since it is known to be involved in plant responses to water stress and low temperature. RNA was extracted from seedlings for each of the treatments and the relative level of expression of each clone was determined (Figure 4). Northern hybridization analysis and RNA dot blot hybridization studies revealed that all three clones failed to be induced to high levels by the other stress treatments; relative levels were comparable to those seen for nonacclimated seedlings. Clone pSM2201 was induced to low levels by ABA, wounding and heat shock, attaining levels no greater than about 25% of those observed for cold acclimated seedlings. From these studies it can be concluded that all three clones are specifically induced in response to low temperature treatment and are not induced by other stress treatments.

Figure 4. Determination of specificity of expression of CAS transcripts. Total RNA was isolated from seedlings subjected to the following treatments: nonacclimating temperatures (NA), cold acclimating temperatures (CA), exogenously applied abscisic acid (ABA), water stress (WS) wounding stress (WR), and heat shock (HS). Ten μg of total RNA from each were dot-blotted in triplicate onto nitrocellulose filters and hybridized to nick-translated cDNA inserts of clones: pSM784, solid bar; pSM2358, stippled bar; or pSM2201, hatched bar. Hybridization to each dot was quantified by liquid scintillation spectrometry. Values are means of three replicates (SE $\leq$ 10%). The hybridization values for each treatment are expressed relative to the level observed in cold acclimated seedlings which is set at 100%.


Relative expression
100
80
60
40
20
0
NA
CA
ABA
WS
WR
HS
Treatment

3.5. Correlation of CAS Gene Expression with Freezing Tolerance

If there exists any causal relationship between the expression of these CAS genes and the development of freezing tolerance, then one might reasonably expect to find a correlation between the relative level of expression of these genes and the degree of freezing tolerance of different varieties of alfalfa. To this end, several cultivars of alfalfa were cold acclimated for 2, 8 and 15 days. Freezing studies were used to determine the LT_{50} values for the different cultivars after two weeks of cold acclimation. Relative expression for each clone was determined by RNA dot blot analysis, using RNA samples from 2, 8 and 15 day CA seedlings.

It is clear from these studies (Table II) that a strong positive correlation exists between the relative level of expression of each clone and the degree of freezing tolerance for each cultivar. The correlation coefficients for all clones are highly significant at the 0.05% confidence level ($r > 0.96$). The expression of a fourth clone, pSM355, which is not cold inducible, shows no significant correlation with varietal freezing tolerance ($r = -0.055$).

Table II. Expression of CAS genes and Freezing Tolerance of Four Alfalfa Cultivars. Total RNA was isolated from various cultivars after 2, 8, and 15 days of cold acclimation as well as from nonacclimated seedlings and spotted in triplicate onto nitrocellulose filters and hybridized with nick-translated cDNA inserts from clones pSM784, pSM2358 pSM2201 and pSM355. Quantification of the amount of radioactivity hybridized to each dot was achieved by liquid scintillation spectrometry. The values indicate the sum total of hybridization (means of three replicates, SE did not exceed 10% of the mean) to RNA from 2, 8 and 15 d- acclimated seedlings. Hybridization to RNA of nonacclimated seedlings was similar in all genotypes and constituted only 3 to 4% of the acclimated Anik control. Values represent radioactivity hybridized to the filter for each clone (cpm x 10^{-1}). Number in parentheses indicate expression of the corresponding sequences as a percentage of that in Anik.

Alfalfa cultivar (Species)	pSM355	pSM784	pSM2358	pSM2201	LT_{50} (°C)
Anik (*M. falcata*)	5.1 (100)	49.2 (100)	8.2 (100)	33.4 (100)	14.6
Iroquois (*M. media*)	5.2 (102)	16.6 (33)	3.4 (41)	17.7 (53)	11.8
Algonquin (*M. media*)	5.5 (100)	12.1 (24)	3.0 (36)	14.4 (43)	11.5
Trek (*M. sativa*)	4.9 (96)	5.4 (10)	1.3 (15)	7.6 (23)	9.7
r^a	-0.055	0.968	0.987	0.993	-

[a] The correlation coefficient (r) for each clone indicates the correlation between the level of expression of the clones sequence and the LT_{50} in different cultivars.

CHAPTER 4.

CHARACTERIZATION OF *cas18* AND HOMOLOGIES WITH OTHER KNOWN GENES

4.1. Introduction

In order to isolate a of SM784 containing a full-length open reading frame, a new cDNA library was constructed in the phage vector, lambda (Uni)-Zap, using a unidirectional cloning procedure (for details, see Materials and Methods). A clone containing the entire coding region, from the initiator methionine to the stop codon, was isolated and sequenced (clone Acs784). The putative translation product for the longest open reading frame was analyzed. In this chapter, the nucleotide sequence of the clone as well as the amino acid sequence of the translation product are presented. In an effort to glean some useful information concerning the possible function of this clone, both the DNA and amino acid sequences were analyzed for homologies with other known genes.

4.2. Isolation and Characterization of Acs784

Approximately 200,000 plaque forming units (PFU) were screened using the insert of the partial length clone, (pSM784), as a probe. From the initial screening of duplicate filters, 137 double positives were identified. Since non-recombinant plaques should represent between 1 and 10% of the total PFU, this gives a relative abundance for SM784 of about 0.07%. Forty of these positives were randomly selected for further analysis. After two additional rounds of plaque purification, 35 true positives remained. One of these was homologous to SM784 over the sequenced 3'region and was selected for further study.

The sequence of a clone (Acs784) containing a full-length open reading frame is depicted in Figure 5. The clone is 1019 base pairs in length with a G+C content of 33.5%. The initiator methionine (beginning at nucleotide 246) and stop codon (nucleotide 747) are included. The full 3'-noncoding region is represented as well as a portion of the 5'-noncoding sequence. There are four in-frame stop codons upstream of the first ATG of the longest open reading frame, suggesting that this upstream region is indeed noncoding. In the other two reading frames there are 6 stop codons in this region. Three polyadenylation consensus sequences are present in the 3' noncoding region (underlined in Figure 5), suggestive of multiple sites of polyadenylation. One striking difference between the original clone (SM784) and the new full-length ORF clone (Acs784), is the presence of an extra 100 bp at the 3'end of the latter, beginning immediately before the poly(A) tail in pSM784. In several other clones, heterogeneity in the length of the 3' region was observed. It is conceivable, therefore, that the different size classes of transcript observed on northern blots, may arise, at least in part, from the use of different sites of polyadenylation.

The three transcripts which hybridize to the insert from pAcs784, are 1.6, 1.4, and 1.0 kb in size. The size of the insert is about 1.0 kb, suggesting that the 1.0 kb transcript has been cloned.

Figure 5. Nucleotide and derived amino acid sequences for clone Acs784. Polydenylation consensus sequences and dehydrin-like repeats are underlined once, Gly-Thr repeats are double-underlined.

CGGCACGAG

(-236) GAAAGCTACAAAAAAGAAGAATTAATATTGTTGTGGAGCCACAATTTTATTTGTTATAT

(-177) TTGTTAATTTTTGGAATTTTTAGTTTTTCTTTTTTATGAGAAAACCATTTATGATTATT

(-118) AGTTTTTGTGTTAAGGATAATTTATGGCATTTTATATTTTTAATGTTATAGTACTTGCA

(-59) TCTACATTAAACTTGTTCATTTTTTACATTAGAAAATTGTGAAGAAAAATAAAATAACA

(1) ATG TCT CAA TAT CAT GGA GAA AAT AGA GGA GTT GTG GAC AAG ATC
(1) Met Ser Gln Tyr His Gly Glu Asn Arg Gly Val Val Asp Lys Ile

(46) AAG GAG AAG ATT CCT GTG GCA CTG GAA CTG GAA CTG GAA CAT GGA
(16) Lys Glu Lys Ile Pro Val Ala Leu Glu Leu Glu Leu Glu His Gly

(91) ACA GGA ACT GGA ACA GGT CAT GGA ACA ACT GGT TAT GGG TCA TGG
(31) Thr Gly Thr Gly Thr Gly His Gly Thr Thr Gly Tyr Gly Ser Trp

(136) AAC AAC TGT GCT AGT GTA GTG CTG GTC ATG GTC ATC AAC AAC ATG
(46) Asn Asn Cys Ala Ser Val Val Leu Val Met Val Ile Asn Asn Met

(181) GAG AGA AAT AGA GGA GCT GTG GAC AAG ATT AAG GAG AAG ATT CCT
(61) Glu Arg Asn Arg Gly Ala Val Asp Lys Ile Lys Glu Lys Ile Pro

(226) GGT ACT GAA CAA AAT GTT TAT GGG ACA GGA ACG GGA ACT GGA ACT
(76) Gly Thr Glu Gln Asn Val Tyr Gly Thr Gly Thr Gly Thr Gly Thr

(271) GGA ACT GGA CAT GGG ACA GGA ACA GGA ACT GGA CAT GGA CAT GGA
(91) Gly Thr Gly His Gly Thr Gly Thr Gly Thr Gly His Gly His Gly

(316) ACA ACT ACT GGG TAT GGA AGC ACT GGA CAA GAG TAT GGA AAA GAG
(106) Thr Thr Thr Gly Tyr Gly Ser Thr Gly Gln Glu Tyr Gly Lys Glu

(361) GGT CAT CAT GGA CAT GAT GAG CAA CAC CTT GGT GAG AAA AAA GGG
(121) Gly His His Gly His Asp Glu Gln His Leu Gly Glu Lys Lys Gly

(406) ATT ATG GAA AAA ATT AAG GAG AAG ATT CCT GGT ACT GGA TCA TGT
(136) Ile Met Glu Lys Ile Lys Glu Lys Ile Pro Gly Thr Gly Ser Cys

(451) ACT GGA CAT GGA CAA ACT AAA CCA TAT TGT GTG TAT ATG GAT GCA
(151) Thr Gly His Gly Gln Thr Lys Pro Tyr Cys Val Tyr Met Asp Ala

(496) TGC ATT TGA TATGATCAATCAGTAGAATAAATATGTGTGTATTGGTGTATTTAAGT
(166) Cys Ile *

(552) GTATTTTAAACTTCTGTTTTTGAGTAAGTTGGTTCATATGTAACATATCTTATCACCTC

(611) ATGTATCTATATGTGGTGTATAATGATGGATACATAGATGAATAAAATATGTATGTTGA

(670) ATTTTAGTGTAGGGGCATATGTAAAGTTACTTTGAAATAGAAGTTTCTTTCATTGAAAA

(729) AGTAATAAAAGTTATAAGTATTTGAAGTAAAAAAAAAAAAAAAAA

4.3. Analysis of Putative Translation Product of Longest ORF

Detection of protein coding regions by the method of Fickett (Fickett, 1982), revealed a longest continuous open reading frame of 167 amino acids. The protein is largely hydrophilic (Figure 6), contains no significant membrane spanning domains (hence it is a soluble or peripheral protein) and is rich in glycine (20.9%) and threonine (12.5%). The amino acid composition of the protein is given in Table III. The isoelectric point (pI) of the protein was predicted to be 6.64.

Two distinctive repeats are evident: a $(Gly\text{-}Thr)_n$ motif which is present 3 times throughout the sequence; and a second repeat, (V/M)(D/E)KIKEK(I/L)P, which appears three times. In the case of the second repeat, there is an alternation between two amino acids at positions 1, 2, and 8; these all represent conservative amino acid substitutions.

4.4. Predicted Secondary Structure

The predicted secondary structure for CAS18 is depicted in Figure 7. The protein is predicted to exist largely as extended β-sheet and α-helix with small regions of turns (loops) and coiled conformation. The first 4 residues starting from the amino terminus comprise a region which exists mainly as an extended β sheet. This is followed by a turn and a region of α-helix extending from residue 8 to 29, interrupted by a coiled region at residue 9. A region of extended β-sheet is predicted to exist from residue 30 to residue 57, interrupted once by a small turn and α-helical region between residues 47 and 50. The middle of the protein is predicted to exist as a α-helix, followed by a series of turns and coiled conformation and a large stretch of extended β-sheet. From residue 115 to 142, the protein forms an α-helix. This is followed by a series of turns and coils from 142 to 145, extended β-sheet from 146 to 151, coiled conformation from 152 to 157, extended β-sheet from 158 to 162. The carboxy terminus exists as an α-helix.

Figure 6. Hydropathy plot of translation product for *cas18*. An interval of 9 amino acids was used to construct the plot.

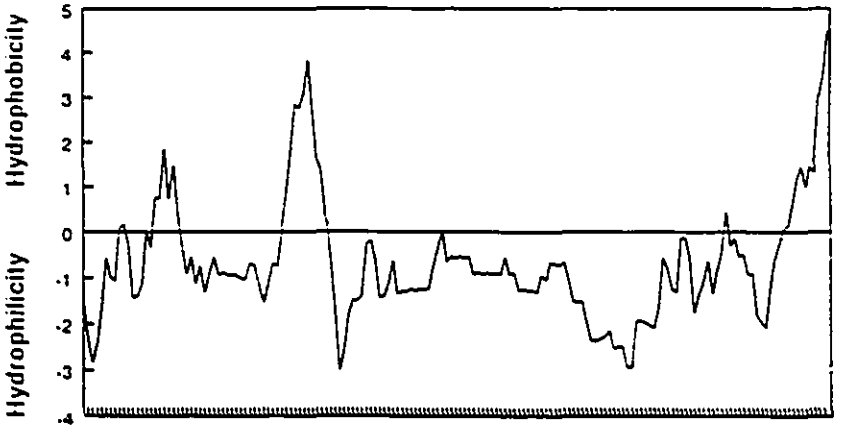
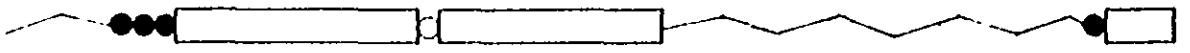

Hydrophobicity
Hydrophilicity
5
4
3
2
1
0
-1
-2
-3
-4

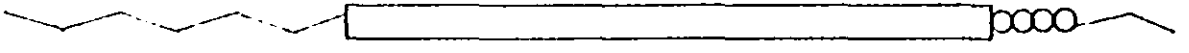
Figure 7. Predicted secondary structure of translation product of longest open reading frame for *cas18*. Open circle represents coiled conformation; wavy line shows extended β-sheet conformation; open rectangles depict helical regions, and filled circles represent turns.

MSQYHGENRGVVDKIKEKIPVALELELEHGTGTGTGHGTTGYGSWNNCAS 50



VVLVMVINNMERNRGAVDKIKEKIPGTEQNVYGTGTGTGTGTGHGTGTGT 100

GHGHGTTTGYGSTGQEYGKEGHHGHDEQHLGEKKGIMEKIKEKIPGTGSC 150



TGHGQTKPYCVYMDACI 167

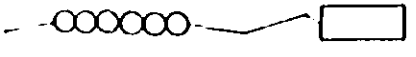


Table III. Amino acid composition of translation product of longest open reading frame for the clone Acs784.

Amino acid	Mole percentage	Amino acid	Mole percentage
Ala (A)	2.3	Leu (L)	2.9
Arg (R)	1.7	Lys (K)	7.7
Asn (N)	4.1	Met (M)	2.9
Asp (D)	2.3	Phe (F)	0
Cys (C)	2.3	Pro (P)	2.3
Glu (E)	2.9	Ser (S)	2.9
Gln (Q)	8.3	Thr (T)	12.3
Gly (G)	20.9	Trp (W)	0.5
His (H)	6.5	Tyr (Y)	4.1
Ile (I)	0.3	Val (V)	5.9

4.5. Homologies with Other Genes

The GenBank library was searched for protein and DNA sequences sharing homology with *cas18*. The results of the data search are summarized in Tables IV and V. At the amino acid level, CAS18 exhibits homology to several water-stress, drought-inducible or ABA-inducible proteins. CAS18 exhibits 49.4% homology with dehydrin DHN3 from barley in a 87 amino acid overlap; 31.3% homology in a 147 amino acid overlap with DHN4 of barley; and 52.9% identity with RAB21 (rice ABA-inducible) over a stretch of 85 amino acids (Figure 8). There is also an identity of 42.0% over an overlap of 50 amino acids with a late embryogenesis abundant (LEA) protein (Figure 9). There exists a 50.0% identity over an overlap of 66 amino acids between CAS18 and the period clock protein (PER) from *Drosophila*. The period clock proteins from mouse and *Acetabularia* show identities of 34.1% over a 126 amino acid overlap, and 42.7% identity over 82 amino acids, respectively (Figure 10).

At the nucleic acid level, there is also homology between *cas18* and other genes (Table V). *Cas18* exhibits 57.1% identity in a 168 nucleotide overlap with an ABA responsive gene from wheat (Figure 11), and 56.5% identity over 398 nucleotides with an ABA-inducible gene from tomato (Figure 12).

An alignment of the protein sequences for 8 dehydrins, RAB21 (RAB16A) and CAS18 (Figure 13), reveals the existence of two conserved boxes (Table V). One striking difference between CAS18 and the dehydrins is the universal

presence of a stretch of 7 to 8 consecutive serine residues in the RAB and dehydrin proteins, which is absent in CAS18. The first conserved box has the sequence: RkkG(i/m/l)k(D/E)KIKEK(L/I)PG (The lower case letters represent residues conserved in dehydrins but not present in CAS18). The second box has a similar sequence: GEKKG(I/F/V/M)(M/V)(D/E)KIKEK(I/L)PG.

Figure 8. Regions of homology shared by the translation product of Acs784 (CAS18) and Dehydrins or RAB proteins. Colons (:) represent identical amino acid residues; periods (.) show amino acid similarities; gaps represent spaces inserted to give optimal alignment of the two sequences; X demarks the boundaries of the region of overlap.

a)

```
DH3SHORVU  DEHYDRIN DHN3 (B17) (GENE NAME: DHN3).
initn= 150   init1= 150   opt= 159       49.4% identity in 87 aa overlap

             40        50        60        70        80        90
ACS784  GTGTGHGTTGYGSWNNCASVVLVMVINNMERNRGAVDKIKEKIPGTEQNVYGTGTGTGTG
                                          X..:  :::::::.:.. .   :: :: .
DH3SHO  TREEHKAGGILQRSGSSSSSSSEDDGMGGRRKKGLKDKIKEKLPGGHGDQQQTG-GTYGQ
             50        60        70        80        90        100

            100       110       120       130       140
ACS784  TGHGTGTGTGHGHGTTTG--YGSTGQEYGKEGHHGHDEQHLGEKKGIMEKIKEKIPGTGS
         :::.. :::::  .:.:: :: :.. :.:: .:.... :::::::::::::::X
DH3SHO  HGHTGMTGTGEHGATATGGTYGQQGHT-GMTGTGAHGTDGTGEKKGIMDKIKEKLPGQH
            110       120       130       140       150       160

     150       160
ACS784  CTGHGQTKPYCVYMDACI
```

b)

```
DH4SHORVU  DEHYDRIN DHN4 (B18) (GENE NAME: DHN4).
initn= 136   init1=  91   opt= 145       31.3% identity in 147 aa overlap

                                        10        20        30
ACS784                              MSQYHGENRGVVDKIKEKIPVALELELEHGTGTGTGH
                                    ..:. .::::::.: . . . ..... : :.
DH4SHO  DEHQTGRGILHRSGSSSSSSSEDDGMGGRRKKGIKEKIKEKLPGGHGDQQHNAGTYGYGQ
          50        60        70        80        90        100

          40        50        60        70        80        90
ACS784  GTTGYGSWNNCASVVLVMVINNMERNRGA-VDKIKEKIPGTEQNVYGTGTGTGTGHGT
         .:: .. ..  .      ...:. . :.  .. .. ..::...  .....:: .  ::..
DH4SHO  QGTGMAGTGGTYGQQGHTGMTGMGATDGTYGQQGHTGMAGTGAHGTAATGGTYGQQGHTG
        110       120       130       140       150       160

          100       110           120       130       140
ACS784  GTGTGHGHGTTTGYGSTGQE-------YGKEGHHGHDEQHLGEKKGIMEKIKEKIPGTGS
         ::::  :::...:: :..        .X..: .:... . ::::::::::::::X
DH4SHO  MTGTGM-HGTGGTYGQQGHTGMTGTGMHGTGGTYGQHGTDTGEKKGIMDKIKEKLPGQH
        170       180       190       200       210       220

     150       160
ACS784  CTGHGQTKPYCVYMDACI
```

c)

```
DH21SORYSA WATER-STRESS INDUCIBLE PROTEIN RAB21 (GENE
initn= 129   init1= 129   opt= 172       52.9% identity in 85 aa overlap

             40        50        60        70        80        90
ACS784  GTGTGHGTTGYGSWNNCASVVLVMVINNMERNRGAVDKIKEKIPGTEQNVYGTGTGTGTG
                                          :..:  .::::::.::....    . : v:~
DH21SO  MREEHKTGGVLQRSGSSSSSSSEDDGMGGRRKKGIKEKIKEKLPGGNKGEQQHAMG-GTG
       50        60        70        80        90        100

            100       110       120       130       140       150
ACS784  TGHGTGTGTGHGHGTTTGYGSTGQEYGKEGHHGHDEQHLGEKKGIMEKIKEKIPGTGSCT
         :: ::::::: ..:  :.: :: . :..: :: .. . :::::::.::::::X
DH21SO  TGTGTGTGTGGAYG-QQGHG-TGMTTGTTGAHGTTTTDTGEKKGIMDKIKEKLPGQH
       110       120       130       140       150       160

            160
ACS784  GHGQTKPYCVYMDACI
```

Figure 9. Region of homology shared between CAS18 and a LEA protein from rapeseed. Colons show identical amino acids; periods denote similar residues; X denotes boundary of overlap. (Baker et al., 1988).

```
DHLESPAPSA LATE EMBRYOGENESIS ABUNDANT (LEA) PROTEIN.
initn= 108   init1=  69   opt=  83       42.0% identity in 50 aa overlap

         70        80        90       100       110       120
ACS784 VDKIKEKIPGTEQNVYGTGTGTGTGTGHGTGTGTGHGHGTTTGYGSTGQEYGKEGHHLHD
                                        :.:. ... :.:. ..:::. .... . .
DHLESR SGSSSSSSSEDDGQGGRRKKSIKDKIKDKLGGGKHHDEQTPTTATTTGPTTTTTTTGAAA
     100       110       120       130       140       150

        130       140       150       160
ACS784 EQHLGEKKGIMEKIKEKIPGTGSCTGHGQTKPYCVYMDACI
       .::  X::::.:::::::.:X
DHLESR DQH-HEKKGTLEKIKEKLPGHHNHHP
     160       170       180
```

Figure 10. Homology shared by CAS18 and clock proteins from (a) mouse, (b) *Acetabularia*; (c) *Drosophila* (Shin et al., 1985).

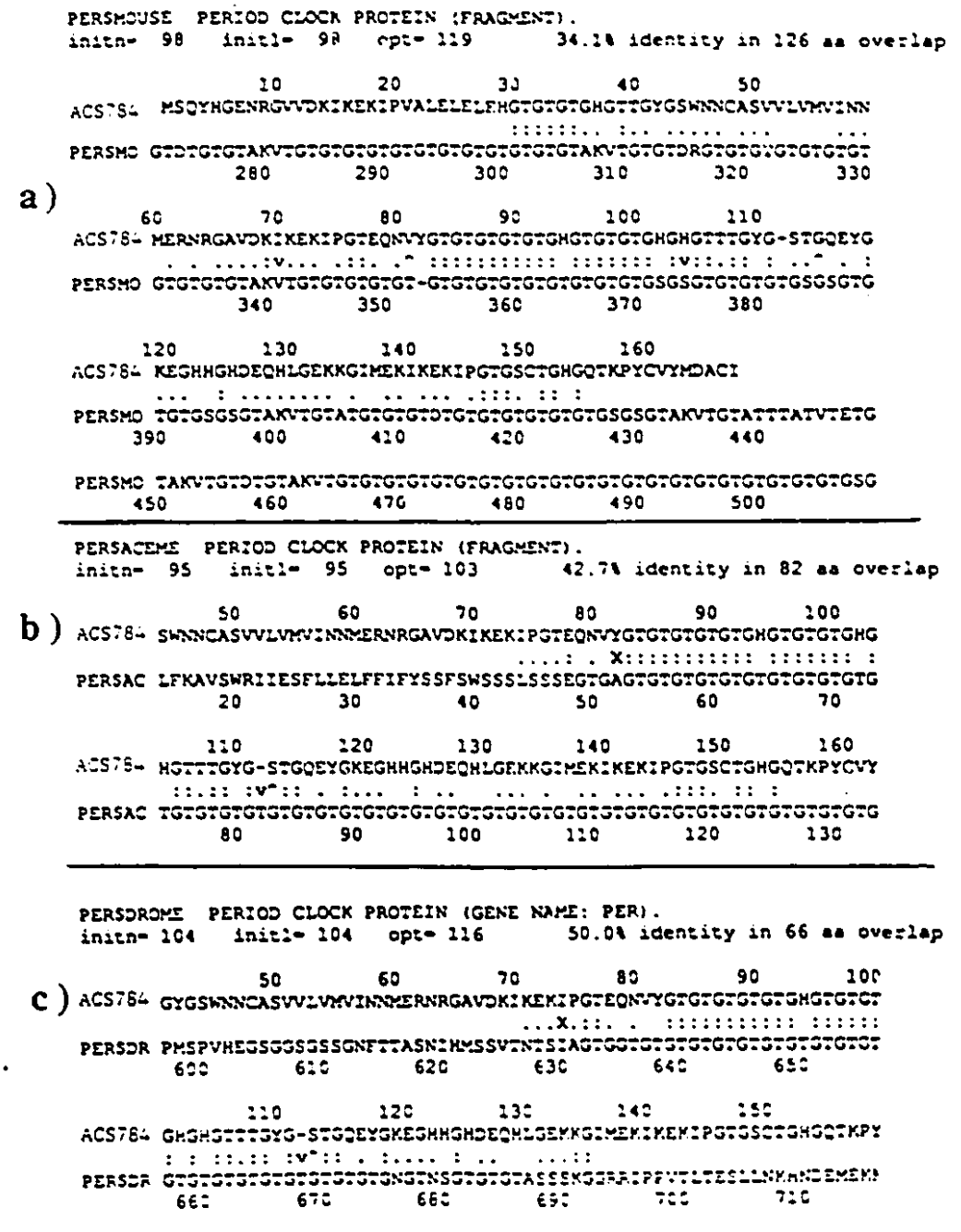

a)
PERSMOUSE PERIOD CLOCK PROTEIN (FRAGMENT).
initn= 98 init1= 98 opt= 119 34.1% identity in 126 aa overlap
b)
PERSACEME PERIOD CLOCK PROTEIN (FRAGMENT).
initn= 95 init1= 95 opt= 103 42.7% identity in 82 aa overlap
c)
PERSDROME PERIOD CLOCK PROTEIN (GENE NAME: PER).
initn= 104 init1= 104 opt= 116 50.0% identity in 66 aa overlap

Figure 11. Region of shared homology between *cas18* and a wheat *Rab* gene (Yamaguchi-Shinozaki et al., 1989).

```
WHTRAB     T.aestivum L. mRNA for an ABA responsive gene
initn= 240   init1= 113   opt= 128       57.1% identity in 168 nt overlap

       490       500       510       520       530       540
ACS784 TTATGGGACAGGAACGGGAACTGGAACTGGAACTGGACATGG-GACAGGAACAGGAACTG
                                          : : : : :::: ::: :: ::::: ::::
WHTRAB AGAAGCTCCCCGGTGGCCACAAGGACAACCAGCAGCATATGGCGACGGGGACAGGGACTG
             280       290       300       310       320       330

        550       560       570       580       590       600
ACS784 GACATGGACATGGAACAACTACTGGGTATGGAAGCACTGGACAAGAGTATGG-AAAAGAG
       ::   :   : ::  :    ::::::  :::  :  :        :: : ::  : : ::
WHTRAB GAGGAGCCTACGGGCCGGGAACTGGGACTGGTGGAGCCTACGGGCAGCAAGGCCACACAG
             340       350       360       370       380       390

         610       620       630       640       650       660
ACS784 GGTCATCATGGACATGATGAGCA-ACACCTTGGTGAGAAAAAAGGGATTATGGAAAAAAT
       :      :::: :       :::    :   :: X:::: :: ::::: ::::: :: ::
WHTRAB G-----AATGGCCGGCGCCGGCACTGGCACGGGCGAGAAGAAGGGGATCATGGACAAGAT
                  400       410       420       430       440

         670       680       690       700       710       720
ACS784 TAAGGAGAAGATTCCTGGTACTGGATCATGTACTGGACATGGACAAACTAAACCATATTG
        ::::::::: : :: :X
WHTRAB CAAGGAGAAGCTGCCGGGACAGCACTGAGCCACCGGTCCGGCTGGCTGCTTCCTTGTAGC
        450       460       470       480       490       500
```

Figure 12. Region of homology shared by *cas18* and an ABA-inducible gene from tomato, *Tas14* (Godoy et al., 1990).

```
TOMTAS14   Lycopersicon esculentum TAS14 mRNA inducibl
initn= 204   initl= 128   opt= 174        56.5% identity in 398 nt overlap

           400       410       420       430       440       450
ACS784 AGTGTAGTGCTGGTCATGGTCATCAACAACATGGAGAGAAATAGAGGAG-CTGTGGACAA
                                          :::: : : : : : :::: : ::::
TOMTAS ATGATGGGGGGCACGGGTACTGGCGGTATGATGGGGGGCACTGGTGGAGAATATGGA---
        150       160       170       180       190       200

           460       470       480       490       500       510
ACS784 GATTAAGGAGAAGATTCCTGGTACTGAACAAAATGTTTATGGGACAGGAACGGGAAC-TG
       : : : :  : : : ::::::: : ::  :::     ::: ::  : : :   : :
TOMTAS -ACTCAAGGCATGGGTACTGGTACTCATCACCATG----AGGGGCAACAGCAGCTTCGTC
           210       220       230           240       250

             520       530       540       550       560
ACS784 GAACTG-GAACTGGACATGGGACAGGAACAGGAACTGGACATGGACATGGAACAACTACT
       :: : :  : ::  :  : :    :::  : ::  :::: : ::     . : ::  :
TOMTAS GATCCGACAGCTCTAGCTCG--TCGGAGGATGA--TGGAGAAGGTGGGAGGAGAAAGA-A
     260       270         280         290       300       310

    570           580       590       600         610
ACS784 GGGTATG-----GAAG-CACTGGACAAGA-GTATGGAAAA---GAGGGTCATCATGGACA
       :::: ::     ::::  : :::: :::: :  :::: ::   :: ::: :  :::::::
TOMTAS GGGTTTGAAGGAGAAGATAATGGAGAAGATGCCTGGACAACATGAAGGTGAGTATGGACA
          320       330       340       350       360       370

    620       630       640       650       660       670
ACS784 TGATGAGCAACACCTTGGTGAGAAAAAAGGGATTATGGAAAAAATTAAGGAGAAGATTCC
         : :::::   ::  X:::: ::::: :: ::::: ::::: ::::: ::::: ::
TOMTAS -----AACAACA-GGTGAAGAGAAGAAAGGAATGATGGACAAAATCAAGGACAAGATCCC
               380       390       400       410       420

    680       690       700       710       720       730
ACS784 TGGTACTGGATCATGTACTGGACATGGACAAACTAAACCATATTGTGTGTATATGGA---
       ::: X :: ::  :: ::           : ::  : : :::  : :: :::   :
TOMTAS TGGGA-TGCAT--TGAACACCTTTGTTTTCATCTCCATCTTATCTTATGAATAAATAAGG
       430           440       450       460       470       480

          740       750           760       770       780
ACS784 ---TGCATGCATTTGATATGAT----CAATCAGTAG-AATAAATATGTGTGTATTGGTGT
         ::: :: :::  :: : ::     ::: ::::: :::   ::::   ::: :  : :
TOMTAS TAGTGCTTG-ATTCTATTTCATGCACTAATTAGTAGTAATCGTTATGCCAGTAATTATCT
          490       500       510       520       530       540

      790       800       810       820       830       840
ACS784 ATTTAAGTGTATTTTAAACTTCTGTTTTTGAGTAAGTTGGTTCATATGTAACATATCTTA
       : ::: ::
TOMTAS AATTACGTACACTCTTGTGTTTAAAGTCGTGTAAAGTGTGCTGACGCTATATACATGTGT
          550       560       570       580       590       600
```

Table IV. Homologies between *cas18* and other genes at the amino acid level.

Protein (Ascension #)	Percent identity	Overlap (amino acids)	Organism	Reference
DHN3	49.4	87	barley	Close et al., 1988
DHN4	31.3	147	barley	Close et al., 1988
RAB21	52.9	85	rice	Mundy and Chua, 1988
LEA	42.0	50	rape	Dure et al., 1989
PER	50.0	66	*Drosophila*	Li-Weber et al., 1987
(PER$MOUSE)	34.1	126	mouse	Shin et al., 1985
(PER$ACEME)	42.7	82	*Acetabularia*	Li-Weber et al., 1987

Table V. Homologies between *cas18* and other genes at the nucleic acid level.

Gene	Percent identity	Overlap (bp)	Organism	Reference
rab21	57.1	168	wheat	Yamaguchi-Shinozaki et al.,1989
tas14	56.5	398	tomato	Godoy et al., 1990

Figure 13. Multiple alignment of several dehydrins, RAB16A (RAB21) and CAS18. The two regions highly conserved between Cas18, dehydrins and RAB proteins are boxed.

CLUSTAL V multiple sequence alignment

CHAPTER 5.

KINETICS OF ACCUMULATION OF *cas18* TRANSCRIPTS IN CELL SUSPENSION CULTURES

5.1 Introduction

The insert from the previously isolated partial-length cDNA clone, pSM784, was used to probe a cDNA library constructed in Lambda-Zap, prepared from poly(A)$^+$ RNA from cold acclimated seedlings. In this way, a full-length ORF copy of the clone was isolated. This new full-length ORF cDNA clone, pAcs784, was used in all subsequent experiments on the kinetics of expression and regulation of the corresponding gene, *cas18*, in cell suspension cultures. The cell culture system offers several advantages over seedlings for such studies. Cell cultures consist essentially of undifferentiated, rapidly growing cells. They, therefore, represent a more homogeneous mass of tissue, devoid of the many differentiated cell types found in intact seedlings. Consequently, cell cultures respond more uniformly to experimental manipulations. Parameters such as temperature, hormonal and nutritional status can be more easily and accurately controlled with cell cultures. Finally, suspension cultures are ideal for studies involving uptake of radiollabeled precursors or inhibitors.

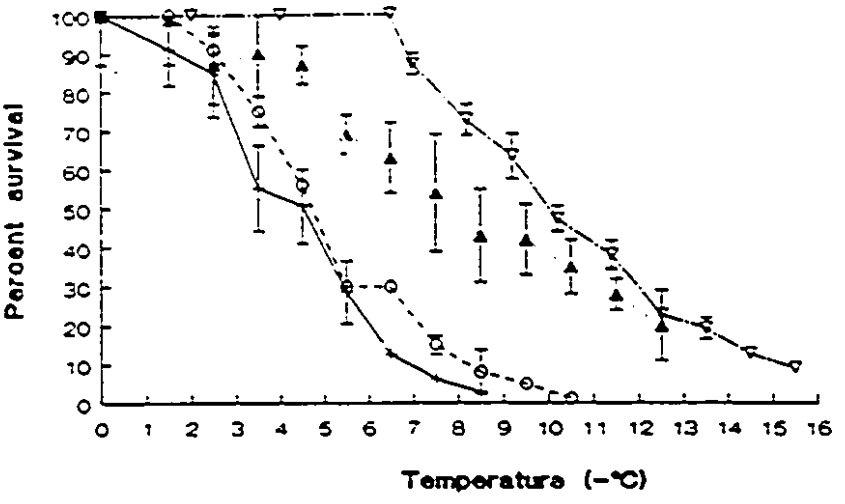
In this chapter, evidence is presented that cell suspension cultures are able to cold acclimate in a manner qualitatively similar to that of seedlings. The kinetics of accumulation of *cas18* in cell suspension cultures, during cold acclimation and deacclimation are examined. As well, the effect of the growth stage of the culture on transcript accumulation is investigated.

5.2. Freezing Survival Studies

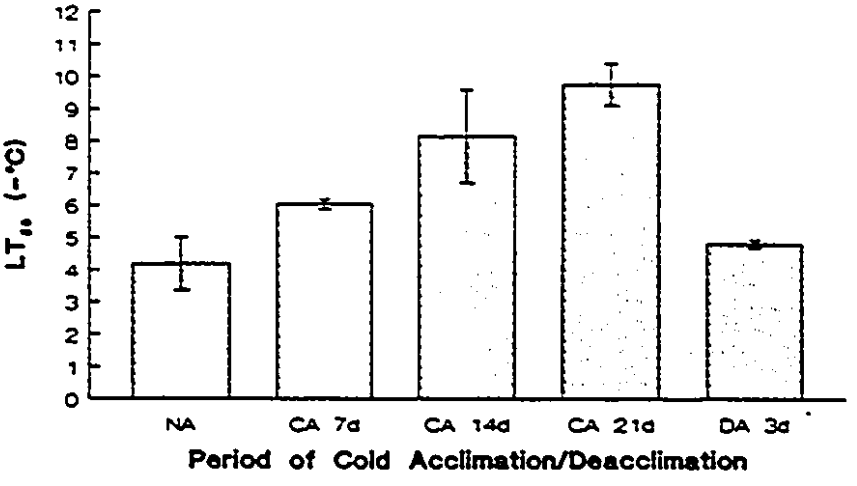
Cell suspension cultures of *Medicago falcata* c.v. Anik were evaluated for their ability to cold acclimate to freezing temperatures (Figure 14). Cell cultures were transferred 10 days after subculture to low temperatures for various periods of time as indicated in the figure legend. Nonacclimated cell cultures had an LT_{50} value of -4.18 ±0.82°C. As cultures were acclimated for 1, 2 and 3 weeks, the freezing survival curves shifted progressively to the right (toward increasingly more negative values). The LT_{50} values interpolated from the graph were -6.03 ± 0.15°C, -8.14 ± 1.44°C and -9.74 ± 0.65°C for cultures acclimated for 1, 2 and 3 weeks, respectively. Cultures acclimated for 3 weeks and then deacclimated for 3 days gave a curve virtually indistinguishable from that obtained with nonacclimated cultures; deacclimated cultures had an LT_{50} value of -4.79 ± 0.12°C. Cell suspension cultures do not exhibit the same degree of freezing tolerance as do seedlings (Figure 14C). Whereas seedlings acclimated for 2 weeks exhibited an LT50 of -14°C, cell cultures after 3 weeks of acclimation showed an LT_{50} value of only -9.74°C. Although the absolute level of freezing tolerance achieved by cell cultures and seedlings differs, cell cultures are nonetheless able to cold acclimate and, therefore, mimic qualitatively, the physiological response observed with seedlings. The ability to cold acclimate to freezing temperatures is, therefore, a property not just of intact plants but of individual cells as well.

Figure 14. Freezing survival of cell suspension cultures as a function of length of prior cold acclimation period. A. Freezing survival curves for cultures cold acclimated for (+) 0 days; (▲) 2 weeks; (▽) 3 weeks; (o) acclimated for 3 weeks, deacclimated 3 days. B. LT_{50} as a function of acclimation/deacclimation. Values were interpolated from curves in (A). C. Comparison of LT_{50} values of cultures (hatched bars) versus seedlings (solid bars).

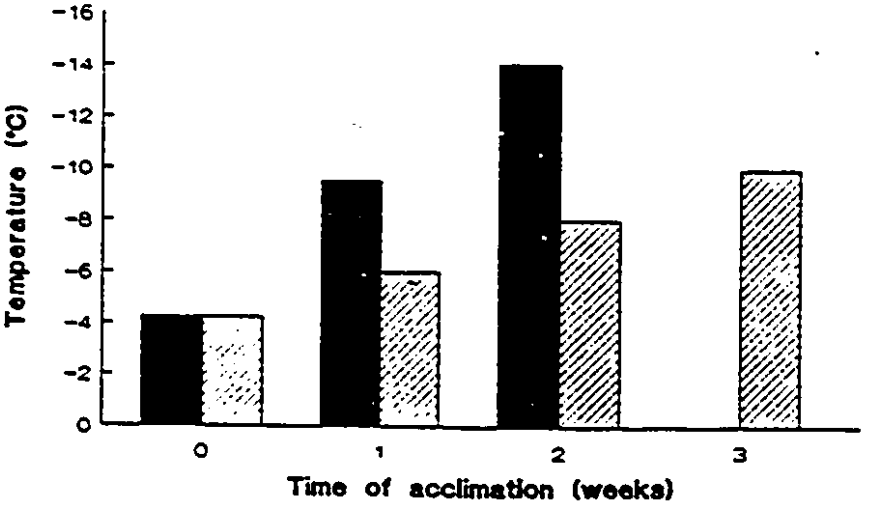
a)


Percent survival
Temperature (-°C)

b)


LT50 (-°C)
NA
CA 7d
CA 14d
CA 21d
DA 3d
Period of Cold Acclimation/Deacclimation

c)


Temperature (°C)
Time of acclimation (weeks)

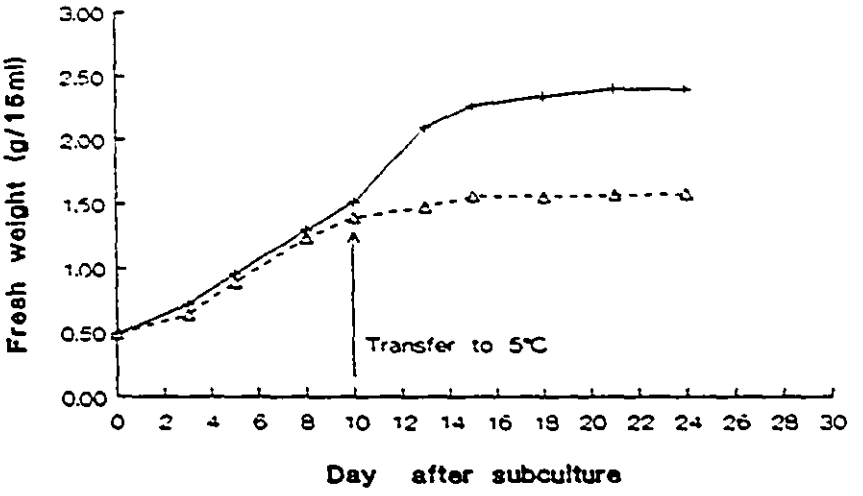
5.3. Effect of Growth Stage of Culture on Expression

Growth curves for the alfalfa cell suspension culture are shown in Figure 15. Cultures incubated at 25°C show no detectable lag phase. Exponential growth continues until about 14 days after subculture. After 14 days, the cultures begin to enter stationary phase. Cultures transferred during log phase to low temperatures (5°C) show a cessation of growth as measured by fresh weight (Figure 15A). The increase in dry weight (Figure 15B) continues but at a much reduced rate compared to the culture grown at 25°C.

Cell cultures were incubated at 25°C for 10 days (logarithmic or exponential phase) or 21 days (stationary phase) before being transferred to low temperatures. Cold acclimation was then carried out for one week. Analysis of the relative level of accumulation of *cas18* transcripts revealed that cultures transferred to cold while in stationary phase of the growth cycle showed 26% of the level measured for seedlings after 7 days of cold acclimation (Figure 16). Cultures cold acclimated while still in the logarithmic phase of the growth exhibited higher levels of transcript accumulation: After 7 days of acclimation, levels were about 80% of those found in seedlings acclimated for 1 week. Thus, for maximum induction of *cas18*, cultures must be cold acclimated while still in the logarithmic phase of the growth curve.

Figure 15. Growth curves for cell suspension cultures grown at 25°C (+), or grown at 25°C for 10 days followed by transfer to 5°C. A. Fresh weight. B. Dry weight. Equal volumes of culture were used throughout.

A


Fresh weight (g/15ml)
3.00
2.50
2.00
1.50
1.00
0.50
0.00
Transfer to 5℃
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
Day after subculture

B

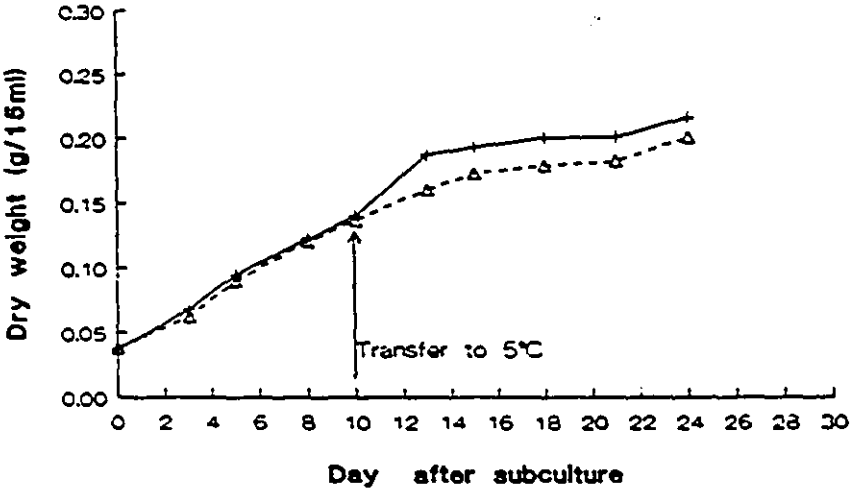
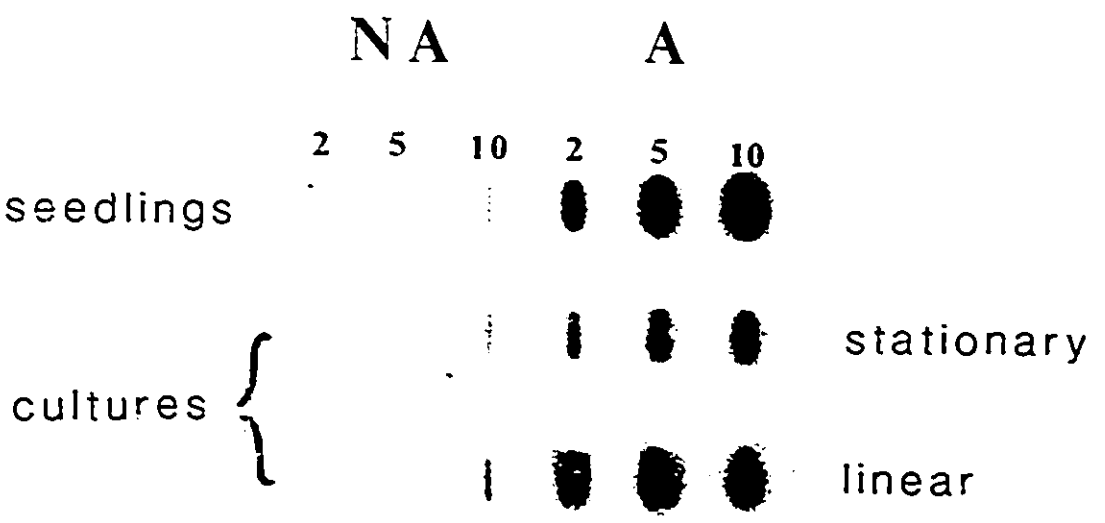

Dry weight (g/15ml)
0.30
0.25
0.20
0.15
0.10
0.05
0.00
Transfer to 5℃
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30
Day after subculture

Figure 16. Levels of *cas18* transcripts in seedlings and in cell cultures at stationary phase or logarithmic phase of the growth cycle. NA, RNA from nonacclimated material; A, RNA from cold acclimated sources. The numbers at the top of the slot blot indicate the amount of RNA immobilized to the filter in micrograms. The blots were probed with nick-translated insert from pAcs784.


NA
A
2
5
10
2
5
10
seedlings
cultures
stationary
linear

5.4. Kinetics of Accumulation of Transcripts

The time-course of accumulation of transcripts hybridizing to the cloned sequence Acs784 was studied (Figure 17). Cell suspension cultures were cold acclimated for various intervals as indicated in the Figure legend. Three transcripts are visible by 1 day of cold acclimation (Figure 17A). Levels continue to increase until 7 days, reaching levels 30-fold higher than those observed for nonacclimated cells. The level of expression remains essentially constant after 7 days until at least 14 days. Although not visible by northern analysis, slot blot quantification clearly shows that increased levels of expression are detectable as early as 5 hours (Figure 17B). In addition, the kinetics of transcript disappearance during deacclimation was examined. The levels of message increase slowly during incubation at low temperature (Figures 18, 19). Levels increase 1.5- and 3.0 -fold by 2.5 and 5.0 hours, respectively. By 1 day, three transcripts are clearly visible; these have sizes of 1.6, 1.4 and 1.0 kb. The most abundant size class is the 1.6 kb message, followed by the 1.4 kb transcript. The least abundant mRNA is the 1.0 kb band. By one day, expression is about 7-fold higher than in nonacclimated cells. By 2 days, levels increase to approximately 13-fold, and by 4 and 7 days expression increases to 22- and 31-fold, respectively (Figure 18). Upon deacclimation, levels of transcripts decline rapidly. Within 2.5 hours expression falls from 31-fold to 12-fold; by 5.0 hours levels drop to 1.8-fold; and by 1 day, levels return to those seen for nonacclimated cells. In

order to correct for loading artifacts, the same blot was probed with a constitutive control clone (Figure 18B). This clone has been shown previously to be expressed to equal levels in nonacclimated, cold acclimated and deacclimated seedlings and cells of alfalfa. Two transcripts hybridize to this probe: a minor band of 2.0 kb; and a highly abundant major band of 1.4 kb. The levels of expression have therefore been normalized relative to the expression of this control clone. Figure 18C is a photograph of the ethidium bromide-stained gel from which the blots were made. The kinetics of transcript accumulation have been summarized graphically in Figure 19. It is clear that the accumulation of transcripts is gradual during cold acclimation; whereas, upon deacclimation, decline in transcript levels is sudden and dramatic.

Figure 17. Time course of accumulation of *cas18* transcripts during cold acclimation. A. 15μg of total RNA from cell cultures cold acclimated for 0, 5 h, 1, 2, 4, 7, and 14 d was size fractionated on a 1.5% agarose-formaldehyde gel and blotted onto nitrocellulose. Blots were probed with nick-translated insert from pAcs784. B. Slot blot analysis. Total RNA from cells aclimated for 0, 5, 24 h was immobilized onto nitrocellulose and probed with ^{32}P-labelled insert from clone pAcs784.


a)
0
5h
1d
2d
4d
7d
14d
b)
0
5h
24h
µg
2 5 10 2 5 10 2 5 10

Figure 18. Expression of *cas18* is reversible upon deacclimation. Total RNA was extracted from cells acclimated for 0, 2.5, 5.0 h, 1, 2, 4, 7 d, and from cells acclimated for 7 days followed by deacclimation at 25°C for 2.5, 5.0 h, 1, 2 d. RNA was fractionated through a 1.5% agaose-formaldehyde gel and blotted onto Genescreen Plus nylon filters. After probing with Acs784, the probe was stripped off the blot according to the instructions of the manufacturer (DuPont), and reprobed with insert from p2.1. A. Blot probed with insert from pAcs784. B. Blot probed with insert from a constitutively expressed clone, p2.1. C. Photograph of gel stained with ethidium bromide, used for the transfer.

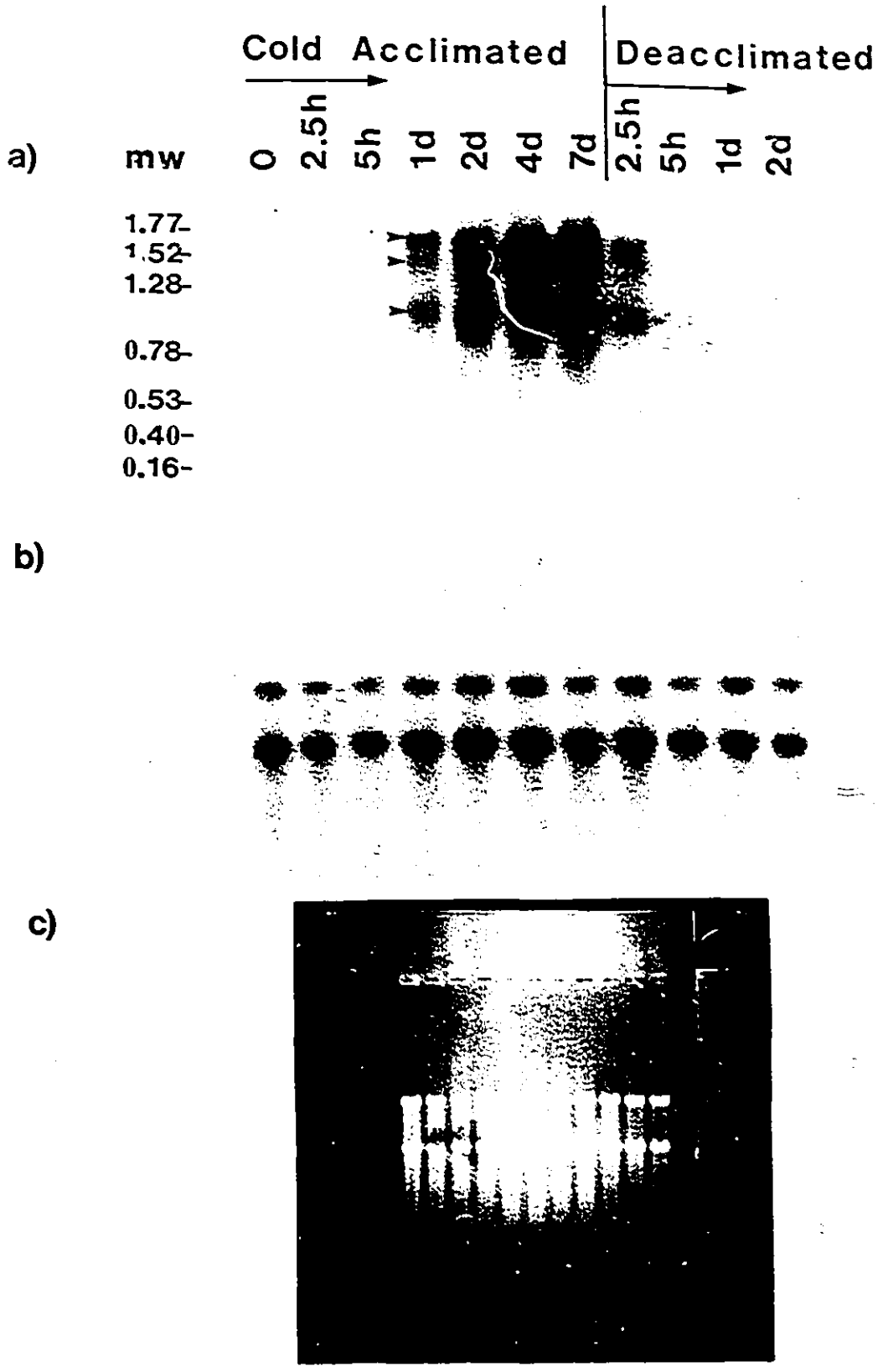
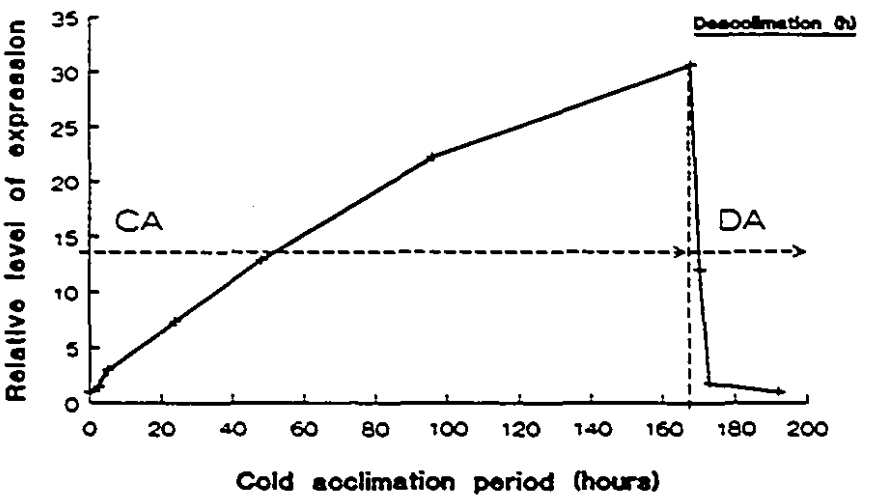

Cold Acclimated
Deacclimated
a)
mw
0
2.5h
5h
1d
2d
4d
7d
2.5h
5h
1d
2d
1.77
1.52
1.28
0.78
0.53
0.40
0.16
b)
c)

Figure 19. Summary of kinetics of transcript accumulation in cells during acclimation and deacclimation. The data presented here were obtained by scanning densitometry of the autoradiograms shown in Figure 18.


Deacclimation (h)
Relative level of expression
35
30
25
20
15
10
5
0
CA
DA
0
20
40
60
80
100
120
140
160
180
200
Cold acclimation period (hours)

CHAPTER 6.

REGULATION OF CAS GENE EXPRESSION

6.1. Introduction

Analysis of the accumulation of transcripts during cold acclimation and deacclimation indicates that there is approximately a 31-fold level of induction by low temperature. In this chapter, the underlying mechanisms for this accumulation are investigated. Specifically, regulation of expression at the transcriptional and post-transcriptional levels is examined.

Nuclear "run-on" analyses of *in vitro* transcription levels were used to examine the contribution of transcriptional regulation. Here, nuclei were isolated from nonacclimated, cold acclimated or deacclimated cells and then used in an *in vitro* transcription reaction. Under the conditions used, there is little or no re-initiation of transcription as measured by capping (Chappell and Hahlbrock, 1986). The fraction of total *in vitro* or *in vivo* labelled RNA complementary to various specific DNA segments is similar (Darnell, 1982). Therefore, the transcriptional profile of isolated nuclei faithfully reflects that of intact cells at the time of isolation of the nuclei.

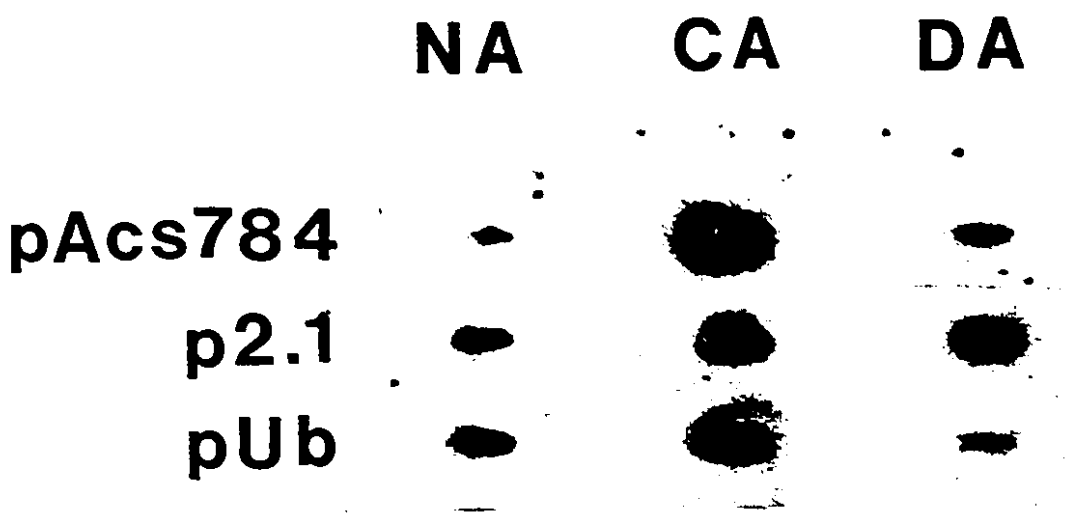
Post-transcriptional regulation can occur at many levels. These include: stability of the RNA within the nucleus; differences in the rate at which processed transcripts are exported from the nucleus to the cytoplasm; differences in rates of processing of the heterogeneous nuclear (hn) RNA; and, most commonly, stability of the cytoplasmic mRNA as measured by differences in the half life of the transcript.

Post-transcriptional regulation was examined by comparing the stability of transcripts in cold versus deacclimating temperatures in the presence of the transcriptional inhibitor, cordycepin. Cordycepin (2'-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). When used in high concentrations, cordycepin has been shown to completely inhibit plant RNA synthesis with no detectable effect on uptake of tracer (Delseny et al., 1975).

6.2. Results of Nuclear "Run-on" Transcription Studies

Results of the nuclear run-on studies are given in Figure 20. In nuclei from nonacclimated cells, the level of transcription of *cas18* is very low. Nuclei from cold acclimated cells show an increase in levels of transcription of approximately 8.7-fold over levels observed for nonacclimated nuclei. Nuclei from deacclimated cells exhibit much reduced levels of transcription of *cas18*; transcription drops to levels about 2.7-fold higher than those of nonacclimated cells after only 2.5 hours of deacclimation. Ubiquitin has previously been shown to be induced about 2.5-fold by low temperatures (Monroy et al., 1993). Levels of transcription for ubiquitin increase 1.2-fold over nonacclimated cells during low temperatures but decline to about 1.8-fold lower than cold acclimated cells after 2.5 hours of deacclimation.

Figure 20. Nuclear run-on transcription assays. Nuclei were isolated from nonacclimated (NA) cells, cells cold acclimated for 7 days, and from cells cold acclimated for 7 days and then deacclimated for 2.5 h. Autoradiograms from nuclear run-on assays. Five μg of linearized denatured plasmid were immobilized onto nitrocellulose filters and probed with *in vitro* radiolabeled RNA from nuclei. Plasmids used were: pAcs784, p2.1, pUb (ubiquitin probe from sea urchin). Scanning densitometry was performed to quantify signals on the autoradiogram. Hybridization to vector sequences alone, pBluescript, were subtracted from all signals in order to correct for nonspecific hybridization. Hybridization and washing conditions are described in section 2.19 of Materials and Methods.


NA
CA
DA
pAcs784
p2.1
pUb

Levels of transcription for the constitutive control clone, p2.1, are similar for all three treatments. Levels increase slightly during cold acclimation about over nonacclimated cells. As a result of deacclimation, the level of transcription remains essentially unaltered (1.1-fold decrease compared with CA).

Studies of the kinetics of accumulation demonstrated a 31-fold increase in the levels of *cas18* transcipts by 7 days of cold treatment of cells. After 7 days, levels reach a steady state. However, nuclear "run-on" analyses show only a 8.7-fold increase in the level of transcription during cold acclimation. Clearly, there must exist a significant contribution at the post-transcriptional level in order to account for the large induction of *cas18* during cold acclimation and the very rapid disappearance of these same transcripts during deacclimation.

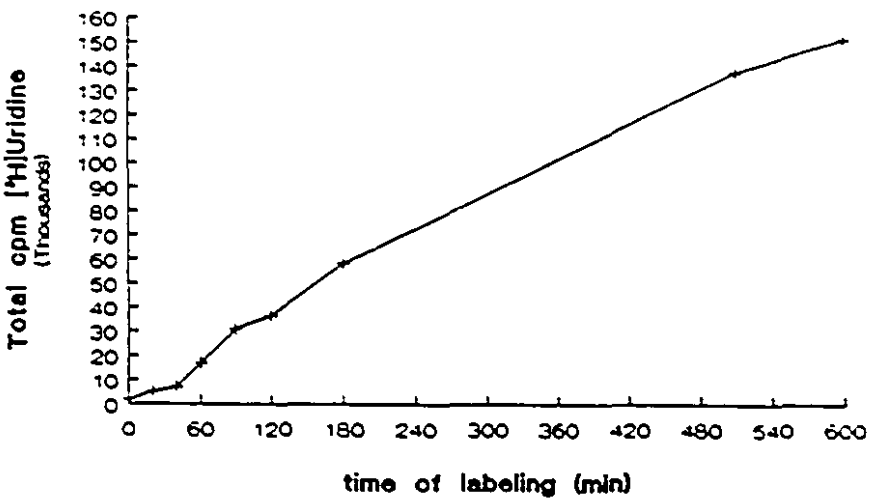
6.3. Studies of Transcript Stability

The major site of post-transcriptional regulation in eukaryotes is at the level of mRNA stability. Accordingly, an analysis was undertaken of the rate of decay of *cas18* transcripts in the presence of cordycepin. The rates of decay of *cas18* transcripts at 5°C and 25°C (deacclimating conditions) were compared. In order to evaluate inhibition of transcription, it is necessary to be able to follow transcription *in vivo* in the absence of inhibitors. To this end, transcription was monitored *in vivo*, for cold acclimated cells at 5°C, by following the incorporation of labelled uridine, [^{3}H]uridine, into TCA precipitable material. It

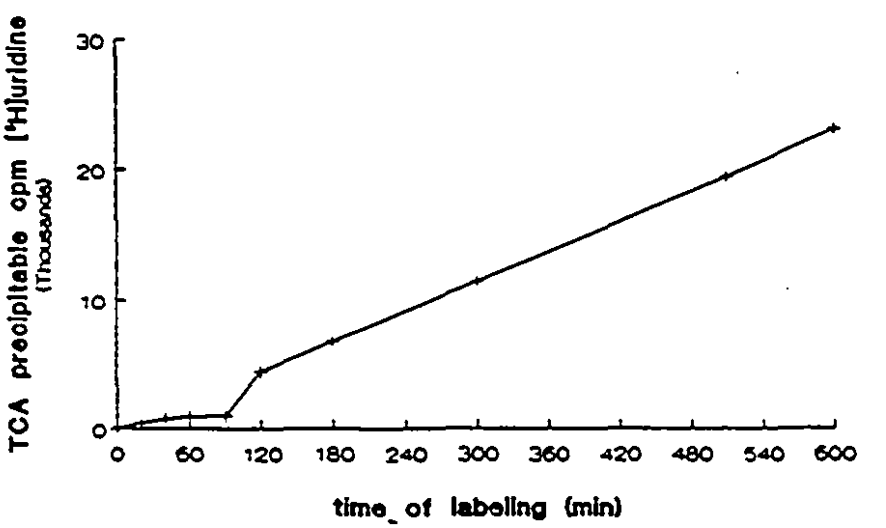
was also important to know the kinetics of uptake of labelled uridine, in order to determine that cordycepin-inhibited transcription was genuinely due to reduced transcription and not a secondary effect of reduced uptake. The results of these preliminary studies are presented in Figure 21. In the absence of inhibitors, uptake of uridine is linear from 120 min to 600 min, after an initial lag of about 30 min (Figure 21A). Uptake continues to exhibit linear kinetics after 24 hours of incubation (results not shown). Incorporation of labelled uridine into TCA precipitable material is linear from 120 min to about 600 min, after an initial lag of about 90 min (Figure 21B). After 600 min, incorporation levels off (results not shown).

Figure 21. Kinetics of uptake and incorporation of [^{3}H]uridine in cell suspension cultures at 25°C. Uptake was measured by determining the total cpm in samples at various time points after addition of label. Incorporation was determined by counting the radioactivity in TCA precipitable material. A uptake. B. incorporation.

a)


Total cpm [³H]Uridine
(Thousands)
160
150
140
130
120
110
100
90
80
70
60
50
40
30
20
10
0
0
60
120
180
240
300
360
420
480
540
600
time of labeling (min)

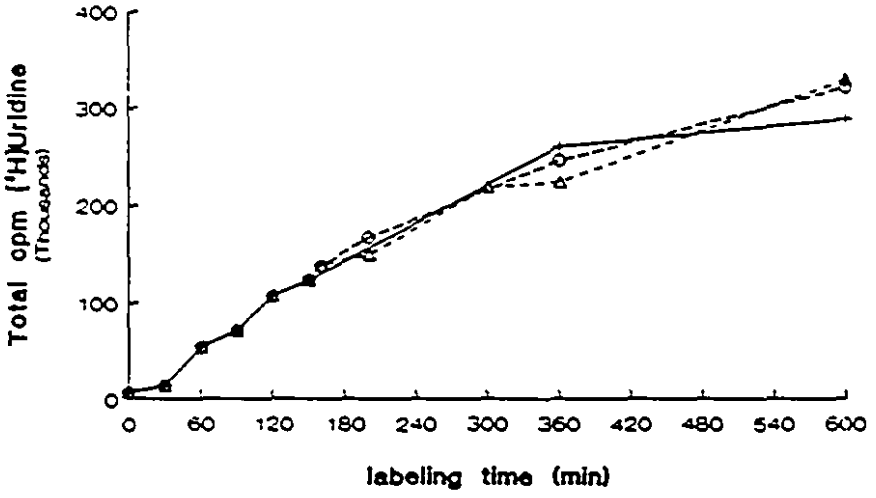
b)


TCA precipitable cpm [³H]uridine
(Thousands)
30
20
10
0
0
60
120
180
240
300
360
420
480
540
600
time of labeling (min)

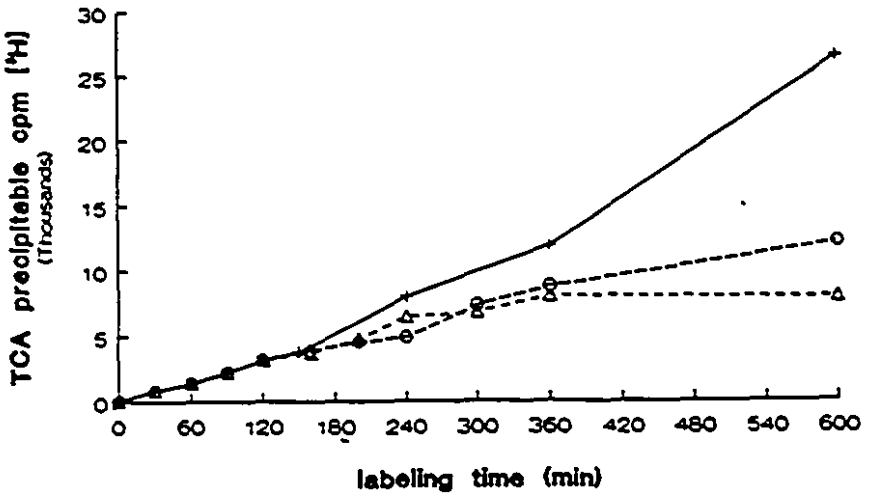
Next, the effect of cordycepin was studied (Figure 22). Cordycepin did not inhibit uptake of label, even at the highest concentration used, 200 μg/ml (Figure 22A). There was essentially no difference between the control (no inhibitor) and cordycepin-treated cells. Results were very different for the study of incorporation (Figure 22B). Whereas incorporation continued to increase linearly for the culture with no inhibitor, cells incubated in the presence of cordycepin exhibited a marked reduction in the rate of transcription after 2 h. Transcription was completely inhibited in the culture containing the higher concentration of cordycepin (200 μg/ml) after 8 hours.

Figure 22. Effect of cordycepin on the kinetics of (A) uptake and (B) incorporation of [^{3}H]uridine. (+) no cordycepin; (○) 20 ug/ml cordycepin; (△) 200 ug/ml cordycepin. A single culture was incubated at 25°C for 2 h. After 2 h, the culture was split into 3 equal parts in 3 new flasks and cordycepin was added to two of the flasks.

a)


Total cpm [³H]Uridine
(Thousands)
400
300
200
100
0
0
60
120
180
240
300
360
420
480
540
600
labeling time (min)

b)


TCA precipitable cpm [³H]
(Thousands)
30
25
20
15
10
5
0
0
60
120
180
240
300
360
420
480
540
600
labeling time (min)

The kinetics of decay of *cas18* transcripts in the presence of cordycepin was investigated in the following manner. Cell suspension cultures which had been cold acclimated for 7 days were incubated in the presence of 200 μg/ml cordycepin for 8 hours at 5°C. After 8 hours, the culture was split: half of the culture was incubated at 5°C as before; the other half was transferred to 25°C for deacclimation. An identical culture, to which no cordycepin was added, was deacclimated at 25°C.

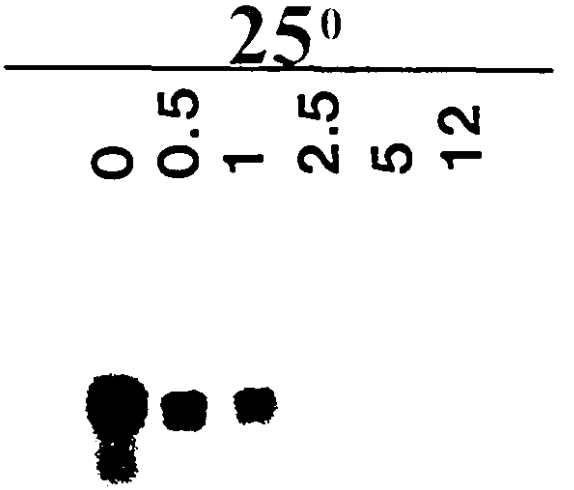
Deacclimation in the absence of inhibitor (Figure 23A) results in a very rapid decay of *cas18* transcripts. Within 2.5 hours, virtually all of the message disappears. Levels of the constitutive clone, remain essentially constant (Figure 23B). Deacclimation in the presence of cordycepin shows that the rate of decay of *cas18* transcripts is slower than in the absence of inhibitor (Figure 23C). The *cas18* transcripts are still detectable after 12 hours, but have almost completely disappeared after 24 hours.

In contrast, *cas18* transcripts are much more stable at 5°C (Figure 23C). Decay is slow over the entire period; transcripts are still visible even after 84 hours. Levels of *cas18* in the absence of cordycepin remain essentially constant throughout this period. The rate of decay of p2.1 transcripts during deacclimation is not affected by cordycpin (Figure 23D). Levels of p2.1 begin to decline only after about 84 hours. These are the expected findings for the control clone since

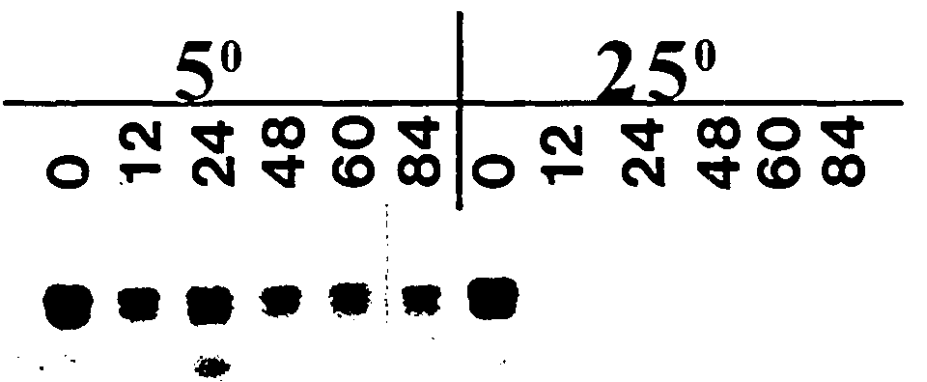
the steady state levels and the levels of transcription are virtually identical in cold acclimated and deacclimated cells.

Figure 23. 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, 12 h; total RNA was size fractionated on a denaturing agarose gel, blotted, and probed with Acs784; B. cold acclimated cells incubated in the presence of cordycepin (200 μg/ml) at 5°C for 8h and then transferred to either 5°C or 25°C for 0, 12, 24, 48, 60, 84 h; blot probed with the cloned sequence, Acs784. C. same samples as in (B) but blot probed with insert from p2.1. Decline in p2.1 transcipts at 25°C is not affected by cordycepin (data not shown).

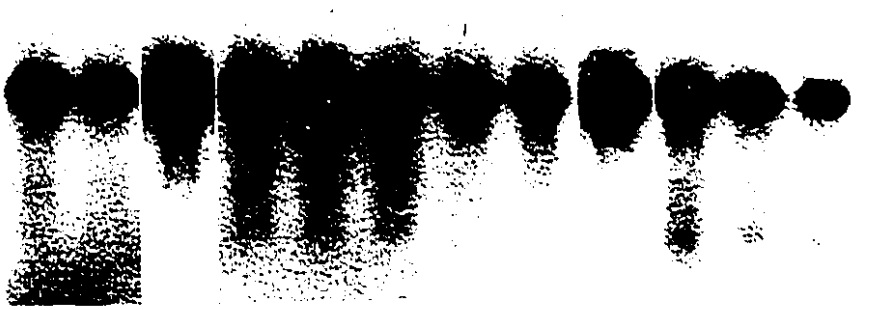
A



B



C

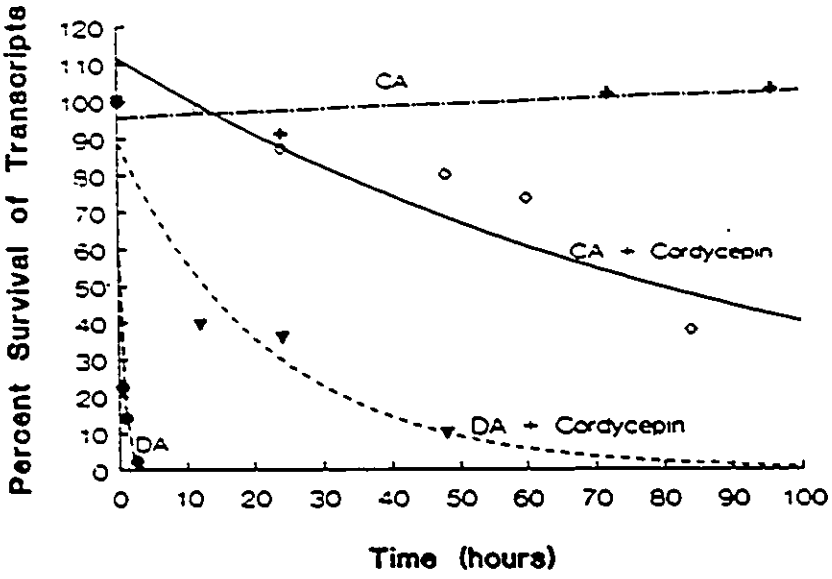


Quantitative analysis of these results was achieved by scanning densitometry. Since p2.1 is highly stable and does not show significant decay over the time period of these experiments, it constitutes a reasonable control. Decay curves were constructed using the densitometry data (Figure 24A). The approximate half life of *cas18* messages at 5°C in the presence of cordycepin was 80 hours. In the absence of inhibitor, levels of *cas18* transcripts do not decay, but rather, remain essentially constant until at least 14 days at 5°C. In the presence of cordycepin, the half life of *cas18* messages is about 12 hours at 25°C, under deacclimating conditions. In the absence of cordycepin, the transcripts are extremely unstable at 25°C, with a half life of about only 20 min (Figures 24B).

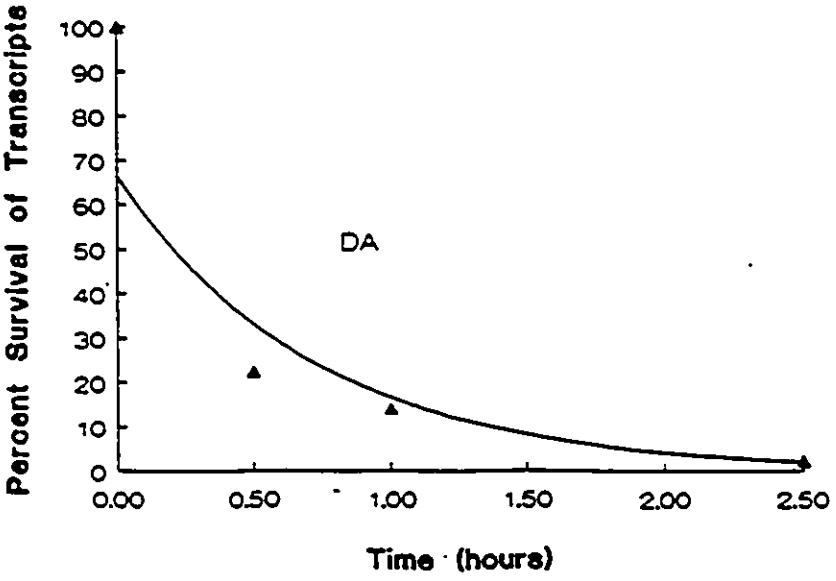
Two things are clear from these findings. First, cordycepin stabilizes *cas18* transcripts during deacclimation. Second, the transcripts are much more stable at 5°C than at 25°C.

Figure 24. Results of scanning densitometry analysis of decay of *cas18* transcripts in the absence or presence of cordycepin at 5°C or 25°C. (+) CA in absence of cordycepin; (◇) CA in presence of cordycepin; (●) DA in absence of cordycepin; (▾) DA in presence of cordycepin. a) Decay curves. b) Enlargement with expanded time scale of curve for cells DA in absence of cordycepin (curve DA in a)

a)


Percent Survival of Transcripts
120
110
100
90
80
70
60
50
40
30
20
10
0
CA
CA + Cordycepin
DA + Cordycepin
DA
0
10
20
30
40
50
60
70
80
90
100
Time (hours)

b)


Percent Survival of Transcripts
100
90
80
70
60
50
40
30
20
10
0
DA
0.00
0.50
1.00
1.50
2.00
2.50
Time (hours)

CHAPTER 7. GENERAL DISCUSSION

Our understanding of the molecular genetic basis of freezing tolerance in plants has increased greatly during the past five years (Guy, 1990). The first report of the molecular cloning of low-temperature-specific genes correlated with varietal differences in freezing tolerance appeared in 1989 (Mohapatra et al., 1989) and forms part of this thesis. Subsequent to this report, several other groups have cloned genes which are expressed to high levels in response to low temperature exposure (Cattivelli and Bartels, 1990; Dunn et al., 1990, 1991; Kurkela and Franck, 1990; Hajela et al., 1990; Gilmour and Thomashow, 1991; Gilmour et al., 1992; Houde et al., 1992). Some of these genes are also induced by ABA or drought (Hajela et al., 1990; Gilmour and Thomashow, 1991; Kurkela and Frank, 1990).

The three low-temperature-induced genes cloned in alfalfa seedlings, pSM784, pSM2358, and pSM2201 (Chapter 3) are not expressed in response to other stress treatments; expression is triggered only by low temperatures. Treatment with ABA, which is only about half as effective as low temperatures in inducing freezing tolerance in *Medicago falcata* cv. Anik (Mohapatra et al., 1988c), does not induce expression of *cas* genes. These *cas* genes are coordinately induced, suggesting a common regulatory mechanism for their accumulation.

Accumulation of these *cas* transcripts precedes the physiologically-measured development of increased freezing tolerance; their kinetics of

accumulation is therefore temporally consistent with a role in freezing tolerance. Significantly, the expression of these *cas* genes shows a high positive correlation with the degree of freezing tolerance of several cultivars ($r > 0.96$).

A detailed analysis was conducted for the cDNA clone pAcs784 - a full-length ORF version of the previously reported cDNA clone, pSM784 (Mohapatra et al., 1989; Wolfraim and Dhindsa, 1990) corresponding to the most-abundantly expressed *cas* gene, *cas18*. The expression of *cas18* is abundant in both seedlings and cell suspension cultures and it closely parallels the progress of cold acclimation. The cDNA characterized in this study hybridizes to three transcripts and the sequence reported here corresponds to the 1.0 kb message.

This study shows that while cold acclimation is a relatively slow process, deacclimation is very rapid. This suggests that *cas18* transcript stability is an important factor in the regulation of expression of this gene. The results of *in vitro* nuclear "run-on" experiments shows that transcription increases by nearly 9-fold with cold acclimation. During deacclimation, the estimated half life of *cas18* transcripts is less than 30 min but in the presence of cordycepin, which completely blocks transcription *in vivo*, the half life increases to nearly 12 hours - a 24-fold increase. The half life of the transcripts at low temperature in the presence of cordycepin is greater than 100 h; therefore, *cas18* messages are at least 8 times more stable at low temperatures. Since transcript levels remain essentially constant during cold acclimation, their decay in the presence of

cordycepin indirectly corresponds to the rate of *in vivo* transcript synthesis necessary to maintain steady state levels. Accordingly, there should be a level of *in vivo* transcription at least 8 times higher in cold acclimated cells; this predicted value is in agreement with the directly measured *in vitro* rates in isolated nuclei of approximately 9-fold. Thus, there is increased transcript synthesis in cold acclimated cells and an increased stability of the transcript at low temperatures.

The cordycepin-induced stability of the *cas18* transcripts during deacclimation shows that rapid decay during deacclimation is not merely a simple effect of higher temperatures *per se*. Probably, there is a requirement for *de novo* transcription of a factor(s) which is involved in the rapid decay of the *cas18* transcripts. Collectively, these studies indicate that both transcriptional and posttranscriptional mechanisms are involved in regulating the low-temperature induced expression of *cas18*.

This study constitutes the first attempt to examine specifically the role of transcript stability in low-temperature-induced gene expression. A previous study of this phenomenon in *Arabidopsis thaliana* used nuclear run-on transcription assays (Hajela et al., 1990). The authors concluded that of the four clones isolated, one was regulated at the transcriptional level; negative results were interpreted as evidence for posttranscriptional regulation for the other three clones. In this and other studies, a discrepancy between the apparent transcription rates and the steady state transcript levels has been interpreted as evidence for

regulation at the posttranscriptional level. However, discrepancies of this nature can also be attributed to technical difficulties in measuring the actual *in vivo* transcription rates by *in vitro* run-on studies using isolated nuclei (Fritz et al., 1991). Direct measurements of transcript half lives were not reported in this earlier study (Hajela et al., 1990).

Cas18 shares extensive homologies, at both the nucleotide and amino acid levels, with members of the Group 2 LEA/RAB/Dehydrin gene family, but unlike members of this gene family, *cas18* is not induced by either ABA or drought (Mohapatra et al., 1989; Wolfraim and Dhindsa, 1990; Wolfraim et al., 1992). Its putative translation product, CAS18, is relatively small (about 18 kDa) and is extremely hydrophilic. It is rich in glycine (21%) and threonine (12.5%), and in this respect it resembles the wheat gene *Wcs120* recently reported by this lab (Houde et al., 1992). CAS18 possesses, in common with members of the Group 2 LEA/RAB/dehydrin, two lysine-rich boxes of conserved amino acids. A multiple serine motif, present in members of the Group 2 LEA/RAB/Dehydrin family, is absent in CAS18. Recently, a low-temperature- and ABA -inducible gene from *Arabidopsis thaliana*, with homologies with Group 2 LEA/RAB/Dehydrin proteins was reported (Gilmour et al., 1992). Analysis of the predicted translation product of a partial length cDNA clone, pHH7.2 (*Cor47*), revealed the existence of the same lysine-rich motif conserved in Group 2 LEA/RAB/Dehydrins, CAS18, and WCS120: GEKKGKIKEKLPG. The region

of homology of COR47 with several LEA proteins was more extensive than was the case for CAS18. COR47 contains a lysine-rich motif of 21 residues, repeated 3 times, and which is virtually identical over 18 residues to the motif found in Group 2 LEA/RAB/Dehydrin proteins. In addition, the serine-rich motif found in Group 2 LEA/RAB/Dehydrins, but which is absent in CAS18 and WCS120, is present in COR47. *Cor47* transcripts, unlike *lea* mRNA, did not accumulate in siliques or mature seeds.

The expression during cold acclimation of a gene whose protein product has homology with members of the Group 2 LEA/RAB/Dehydrin family of proteins is potentially significant. This significance lies in the fact that during a freezing event plant cells become severely dehydrated. Therefore, tolerance to dehydration is necessary if a plant is to survive freezing. It is reasonable to hypothesize that drought and freezing tolerance might involve similar genetic and physiological mechanisms. This idea is supported by the observation that drought stress can induce increased freezing tolerance in cereals (Siminovitch and Cloutier, 1983) and in cabbage (Cox and Levitt, 1976). Several functions for LEA proteins have been proposed based largely on predicted secondary structure analysis (Baker et al., 1988). LEA proteins may function in the solvation of cytosolic structures.

The structural integrity of proteins and membranes is determined by the dielectric constant of water (Tanford, 1978; Creighton, 1983). The removal of

water from the cell during dehydration results in the loss or drastic modification of this essential driving force for structural organization. Sucrose and other disaccharides have been suggested to substitute for water in maintaining these dielectric constraints during times of water deficit. However, these sugars will eventually crystallize as the water content decreases. Trisaccharides such as raffinose, abundant in most seeds, inhibit sucrose crystallization; this is considered important in seed survival (Caffrey et al., 1988). Dehydrins and LEA proteins have a high content of glycine and hydroxylated amino acids (eg., threonine). Glycyl residues allow free rotation around the peptide bond suggesting that these proteins have no thermodynamically preferred structure in aqueous solution; instead, they may exist as amorphous random coils. Hydroxyl groups on these proteins could act to solvate structural surfaces. In this respect, they would be superior to sugars because they can span greater distances and are less likely to crystallize (Baker et al., 1988).

CAS18 similarly is very hydrophilic and has an unusually high preponderance of glycine and of the hydroxylated amino acid threonine. The predicted secondary structure shows the existence of large stretches of extended β-sheet and α-helix. It is possible that CAS18 serves to solvate cell structures in much the same way as LEA proteins during dessication.

Another consequence of desiccation is the increase in ionic strength of the cytosol. Crystallization would seem inevitable but likely disastrous for the

integrity of cytosolic structures. An alternative to crystallization would be the formation of salt bridges with amino acid residues of highly-charged proteins. Certain LEA proteins might play such a role (Baker et al., 1988). The amphiphilic helices found in their structures are likely to form intramolecular helical bundles which would present a globular surface for the binding of ions. In the case of CAS18, there is a significant content of α-helical regions, relatively large contents of the charged amino acids histidine (6.5%) and lysine (7.7%) and smaller contents of glutamic acid (2.9%) and aspartic acid (2.3%). Similar formation of salt bridges is conceivable.

Only *Rab16A* is known to be induced by cold as well as by drought and ABA (Hahn and Walbot, 1989). Despite the lack of common inducers for Group 2 Lea/Rab/Dehydrin and *cas* genes, these homologies raise important questions about the evolution of stress-induced genes and the possible functional significance of the conserved sequence domains.

To date, no member of the Group 2 Lea/Rab/Dehydrin family has been cloned in alfalfa. Therefore, the possibility remains that *cas18* may be a drought-tolerance gene which is regulated differently; that is, it is induced by cold and not by ABA or drought. *cas18* may share a common ancestor with *Rab16A* but has lost, during the course of evolution, the effective cis- and/or trans-regulating elements responsive to ABA and drought and retained or acquired the capacity to respond to low temperatures. Indeed, loss of regulation of a member of the *rab*

gene family provides an example. The rice haploid genome contains four *rab* genes tandemly arrayed in a locus of some 30kbp in length which are believed to have arisen relatively recently by gene duplication to form a small multigene family (Yamaguchi-Shinozaki et al., 1989). Like *cas18* the four *rab16* genes (*rab16A, B, C, D*) are coordinately regulated in most tissues by ABA. However, gene *D* is not expressed at detectable levels in mature seeds and is therefore not a *lea* gene in the strictest sense. This indicates that gene *D* may lack developmentally regulated promoter element(s) capable of controlling gene expression in seeds.

Could CAS18 have antifreeze properties? No homology with AFP was detected during the computer database search. Type I AFP have an extremely high content of alanine (>60%), are rich in threonine (about 16%) and possess tandem repeats of an 11-amino acid sequence (Thr-X_2-Asx-X_7), where X is usually alanine or some other amino acid that favours α-helix formation (Davies and Hew, 1990). Other glycopeptides have the repeating unit $(Ala\text{-}Ala\text{-}Thr)_n$, with a dissacharide moiety attached to the threonyl residues. Threonine residues can hydrogen-bond with water. AFP exist as α-helices. In this conformation, the polar side chains are located on one side of the helix, and threonine is separated from aspartic acid residues by a distatnce of 4.5 Å - the same distance that separates adjacent oxygen atoms in the prism faces of hexagonal ice. This lattice match between the polar residues and the oxygens in the ice lattice is important

to the function of these AFP. A similar conformation does not appear to exist for Cas18, which is predicted to exist largely in a extended β-sheet and with significant regions of α-helix. Also, the spacing of threonyl residues is different, being separated by one and not two amino acids. However, AFP from different antarctic and arctic fishes are known to be structurally very different and yet possess antifreeze properties (Davies and Hew, 1990). Also, there is a strong possibility that additional antifreeze types will be discovered in fish. At present, detailed knowledge is only available for type I AFP. Antifreeze proteins have also been identified in insects (Ramsay, 1964) where they are referred to as thermal hysteresis proteins (THP). The depression of freezing point is more dramatic in these terrestrial insects than is the case for marine fishes; hemolymph temperatures can be lowered by as much as 8 - 10°C (Duman and Horwath, 1983). Unfortunately, these proteins have not been well-enough characterized, due in large part to the difficulty in obtaining sufficient material (Davies and Hew, 1990). Accordingly, the failure to observe any significant homology between CAS18 and AFP should not be construed as strong evidence against CAS18 having antifreeze properties; too few AFP or THP have yet been characterized to justify such a conclusion. The simplest way to test whether or not CAS18 has antifreeze properties would be to look for its ability to produce thermal hysteresis in aqueous solutions.

Since Cas18 is relatively small (molecular mass of 17.6 kDa), very hydrophilic and rich in glycine, it is similar to the cryoprotective proteins decribed by Volger and Heber, (1975). Unfortunately, sequence data is not yet available for these proteins. A direct test for cryprotective activity of Cas18, using isolated thylakoids, could be used to examine this possibility. It is not known whether CAS18 is boiling-stable (ie., remains soluble after boiling), although, given its high degree of hydrophilicity and homology with Group 2 LEA/RAB/Dehydrins, this appears likely.

One intriguing feature of CAS18 is the presence of Gly-Thr repeats, a characteristic of clock proteins (Li-Weber et al., 1987; Shin et al., 1985). No other low-temperature-inducible gene yet identified, with the exception of the cloned sequence Acs2358 (Dong and Dhindsa, in preparation), appears to exhibit this peculiar feature. While the significance of this homology with clock proteins is not clear, it has been reported that an endogenous rhythm of increased chilling resistance interacts with cold acclimation to chilling (McMillan and Rikin, 1990). Also, it has recently been reported that low temperature alters the transcriptional regulation of the circadian expression of nuclear genes encoding chloroplast proteins (Martino-Catt and Ort, 1992). It is possible that following cold shock, which a plant must necessarily experience at the start of cold acclimation, a readjustment of the endogenous rhythm may be required for the development of freezing tolerance and that new isoforms of proteins related to such rhythms

appear during cold acclimation. As proposed by Guy (1990) in a recent review, many of the responses of temperate perennials to low temperature, at the mRNA level, may parallel that found in winter flounder, where antifreeze protein mRNA synthesis is preferentially regulated at low temperatures subsequent only to priming by endogenous clock mechanisms (Price et al., 1986). Indeed, it is well known that in hardy woody species, endogenous rhythms influence the photoperiod- and cold-induced development of freezing tolerance (Weiser, 1970). It is not known whether similar endogenous rhythms operate in herbaceous perennials.

A similar Gly-Thr repeat is found in a dehydrin protein from rice, RAB16A (previously referred to as RAB21) (Mundy and Chua, 1988; Yamaguchi-Shinozaki et al., 1989) in which there are 9 isolated Gly-Thr pairs and a tract of 6 contiguous Gly-Thr pairs.

Is *cas18* involved in the development of increased freezing tolerance? There is no direct genetic evidence for a causal relationship between the expression of *cas18* and the development of freezing tolerance during cold acclimation. Notwithstanding, there are several lines of strong correlative evidence for a positive role of this gene in the cold-induced development of freezing tolerance. First, the accumulation of *cas18* transcripts, in both seedlings and cell cultures, parallels the gradual development and rapid loss of freezing tolerance during cold acclimation and deacclimation, respectively. Second, the

extremely hydrophilic nature of its putative protein product, CAS18, with similarities to members of the LEA/RAB/Dehydrin family of proteins, suggests that it may play a protective role against injury from freezing-induced dehydration - an important and often decisive factor in survival of a freezing stress. Third, 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. Fourth, in a recent study of low temperature signal transduction (Dhindsa et al., 1992; Monroy et al., 1992), it was shown that cold-induced phosphorylation of specific proteins is required for the development of freezing tolerance. When this phosphorylation is prevented by specific inhibitors, not only does a cold-induced increase in freezing tolerance fail to occur but the transcription of *cas18* is substantially reduced in alfalfa cell cultures. Taken together, these findings strongly support a causal role for *cas18* in the development of cold-induced freezing tolerance.

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