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Abscisic acid (ABA) increases the freezing tolerance of bromegrass (*Bromus inermis* Leyss cv Manchar) suspension-cultured cells from an LT_{50} of -7°C to -30°C after 5 d of treatment. Associated with increased freezing tolerance was the induction or increased abundance of 22 polypeptides and 29 translatable RNAs. Five days of +4°C treatment neither hardened the cells to a level of hardiness nor induced as many polypeptide changes as with ABA treatment. There were however, some translation products that had the same pI and MW as with ABA treatment. Many of the ABA responsive translatable RNAs persisted well after ABA was excluded from the medium during which hardiness remained relatively high.

Differential screening of a cDNA library constructed from poly(A^+) RNA of ABA hardened cells yielded 16 cDNAs. RNA gel blot analysis indicates that 9 RNAs were expressed only with ABA treatment, 7 RNA had increased steady state levels and 1 was transiently expressed. Five of the 7 increased level RNAs

were also cold responsive. Nine of the RNAs were responsive with 10 μM ABA or greater, which was also the minimum concentration for inducing hardiness. Preliminary sequencing data identified cDNAs with high homology to aldose reductase, phosphoglucomutase, dehydrin, germin, cathepsin D and embryo globulin.

The cDNA putatively identified as aldose reductase was fully sequenced and found to share 92% nucleotide similarity to the coding region of barley NADPH-dependent aldose reductase. mRNA accumulation was detected 3 h after ABA treatment and reached the highest level after 5 d when cells were hardiest. Cold treatment caused a transient increase in the mRNA. PEG 8000 increased hardiness slightly but there was no RNA accumulation. $GA_{4,7}$ in combination with ABA lowered hardiness and mRNA accumulation relative to ABA alone. Increased aldose reductase activity was detected after 5 d ABA treatment but cellular sorbitol levels did not increase. Aldose reductase expression during ABA induced hardening in bromegrass cells is probably not directly responsible for increasing hardiness but may be involved in the adjustment of metabolic processes during cold acclimation.

Bromegrass cell cultures treated with 75 μM (+)-ABA or (-)-ABA induced similar levels of hardiness and the accumulation of the 16 mRNAs between the 2 treatments were also similar. Cells treated with the ABA analog (+)-2',3'-dihydro ABA were more hardy and also accumulated more mRNA than the (-) enantiomer. Nine of the mRNAs most strongly reflected this difference in hardness.

Molecular Biology of Abscisic Acid Induced Freezing Tolerance in Bromegrass Cell Suspension Cultures

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Typed by Stephen Lee for Stephen P. Lee

To my mother,

Kay W. Lee

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Molecular Biology of Abscisic Acid Induced Freezing Tolerance in Bromegrass Cell Suspension Cultures

1.0 Introduction

Low temperature stress poses a major environmental constraint on natural plant distribution and is a major limiting factor in agricultural production. As more crops are cultivated north of their native distribution, the greater the chance of frost damage will be. Lethal freezing of economically important species occurs annually with estimated losses of $1.5 billion in the USA and $14 billion worldwide. Increasing the freezing tolerance of certain major crop species may be difficult as some are at their limit of genetic potential for winter hardiness and therefore only small gains in freezing tolerance will be realized via conventional breeding technology. Before we can address the issue of major crop improvements directly, our fundamental understanding into the mechanisms of freezing tolerance must be greatly expanded.

The sequence of events leading to an increase in freezing tolerance under low temperature conditions in winter hardy plant species is called cold acclimation and, as many studies have shown, this is a metabolically intensive process. Associated with acclimation are quantitative shifts in carbohydrate, lipid, protein

and nucleic acid metabolism. Within each class of compounds are changes in the distribution of the various molecular species as well as the accumulation of other compounds not normally abundant in the cell of a non-acclimated plant. Of particular interest are those molecules which exert control over the change in metabolism (i.e. the nucleic acids and proteins).

In the early seventies, Weiser (207) had suggested that cold acclimation includes a requirement for changes in gene expression. This may be required for the adjustment to metabolism at low temperatures and for hardening. At the time, this was difficult to demonstrate due to the limited resolution of available procedures. With the advent of high resolution electrophoretic techniques, researchers have been able to resolve literally hundreds of changes in polypeptide species and upon development and adaptation of molecular biology techniques to plant research, compositional changes in the translatable RNA population were also observed. There are indeed extensive changes to the pattern of protein synthesis and translatable RNA during cold acclimation (75).

The question is then which of these proteins or RNAs are responsible for the increase in freezing tolerance? A common approach is to build a cDNA library from RNA of a hardened plant and select cDNA clones correspond to RNAs that are differentially expressed during acclimation. These cDNAs can then be used to analyze patterns of transcript accumulation, identify corresponding genomic DNA sequences for gene regulation studies, determine if similar cDNA sequences with known functions exist and produce transgenic plants expressing the gene product and possibly altering the phenotype. The cDNA cloning approach

is rapidly becoming the tool of choice for those interested in the genetic regulation of cold hardiness. Cold inducible RNAs expressed during cold acclimation in alfalfa (140, 143), *Arabidopsis* (76, 112, 150) and barley (18) have been cloned.

It has been suggested that during cold acclimation, endogenous levels of the phytohormone, abscisic acid (ABA), increases and thus modulates freezing tolerance. There are several lines of evidence to support this proposal: (1) Many hardy plant species show an increase in endogenous ABA during cold acclimation (23, 73), (2) exogenous application of ABA at room temperature can also increase freezing tolerance in whole plants (23, 117) and cell culture systems (25, 105), (3) ABA induced freezing tolerance occurs only in species which have the capacity to cold acclimate (25) and (4) both low temperature and ABA induce expression of a common set of polypeptides and translatable RNAs (117, 200).

The evidence strongly suggests that ABA plays a major role in freezing tolerance, however, studies involving the cloning of ABA inducible genes related to hardiness have not been reported. The goal of this thesis is to identify and characterize the molecular changes associated with ABA induced freezing tolerance in bromegrass cell suspension cultures. Specifically, the thesis will deal with 4 objectives: (1) characterization of changes in gene expression during ABA induced freezing tolerance, (2) cloning of ABA-responsive mRNAs, and identification of a set of cDNAs most correlated with hardening, (3) analyze the expression of an aldose reductase-like gene during hardening and (4) determine the pattern of gene expression associated with the induction of freezing tolerance by ABA and its analogs.

Although there have been several studies that have characterized the changes in the pattern of protein synthesis and translatable RNAs during cold acclimation (75, 198), none of the plant systems used increase freezing tolerance by more than 10°C. Bromegrass cell suspension cultures on the other hand can acclimate from LT_{50} of -7°C to well below -30°C within 5 days of ABA treatment. This substantial increase in hardiness has two major advantages. First, because there is such a large difference in hardiness between the tender and hardy state, use of rapid viability assays such as TTC reduction (199) are more 'robust' and less sensitive to experimental error. Second, bromegrass suspension cultures are one of the most hardy monocot cultures available for cold hardiness studies and therefore may represent a good source of 'hardiness genes'. The bromegrass culture is also very fast growing and the characteristics of growth and cold acclimation are well established (25, 94, 196). As this study demonstrates (Chapter 3), the bromegrass cell suspension culture not only responds to ABA treatment in such a dramatic manner at the physiological level, but for the first time we also can observe the complexity of ABA induced gene expression during the induction of freezing tolerance.

The next step in characterizing the molecular changes observed at the translatable RNA level is to construct a cDNA library and screen for RNAs expressed during ABA induced hardening. cDNAs representing ABA responsive genes were isolated and used as probes for identifying specific molecular changes in response to a change in hardiness. Using a quick screening method, putative functions were assigned to some of the cDNAs. The cDNA cloning section of this

thesis (Chapter 4) represents the first time that genes with known functions could be linked to the hardening process in bromegrass cell cultures.

One of the cDNA clones identified contained a sequence with high homology to barley NADPH-dependent aldose reductase. In animal systems, this enzyme is responsible for the conversion of glucose to sorbitol. The expression of a gene involved in sugar accumulation is of great interest because it is well known that during the induction of freezing tolerance, sugars generally accumulate (122). The most common sugar to accumulate is sucrose, however the sugar alcohols, mannitol and sorbitol, have also been observed to increase in certain species (122). This thesis (Chapter 5) describes the characterization of aldose reductase mRNA expression in response to ABA induced freezing tolerance and of its enzyme activity.

Another method that is available to identify which of the 16 ABA-responsive cDNAs (see Chapter 4) are most correlated with ABA induced hardiness is to study their induction response with ABA analogs. Studies have shown that changing the structure of the ABA molecule, can alter its physiological effects (139) therefore it may be possible to identify analogs which induce a hardening response but not other physiological effects, characteristic of ABA that are not related to hardening. Consequently, this would facilitate the identification of ABA inducible genes required for the acclimation process. In this study (Chapter 6), expression of some of the 16 mRNAS were found to be strongly induced by (+)-2',3'-dihydro ABA but not by the (-) enantiomer. The level of freezing tolerance was also greater for the (+) form than the (-) form.

2.0 Literature Review

2.1 Introduction

Most temperate plant species increase freezing tolerance when exposed to progressively lower temperatures (137) but the ability to survive winter is also dependent on the rate of hardening and the ultimate level and stability of hardiness. Tolerance to freezing induced dehydration is the major survival strategy in most cold hardy plant species. The changes in water relations at the cellular level during freezing have been well documented by others (95, 55).

Weiser (159) first proposed a requirement for the alteration in gene expression during cold acclimation. Throughout the 1970's and early 1980's, considerable evidence suggested that gene expression was involved in cold acclimation. Direct evidence to support Weiser's (159) hypothesis was not available until the development of more recent molecular biological techniques. Direct evidence to demonstrate that low temperature alters gene expression was first reported by Guy et al. (59) and subsequently, rapid progress has been made in characterizing molecular changes associated with frost tolerance.

Currently, genes directly involved in freezing tolerance have not been identified, however, several candidate genes regulated by cold (111, 113, 60, 87) may be associated with hardening. It is not known what role these genes play in a plants' adaptation to freezing stress but it is certain that more than one gene will be required for the induction, and maintenance of freezing resistance. The complex multigenic nature of winter hardiness has been demonstrated in genetic studies with winter wheat (149).

A review of the current knowledge on the processes of cold acclimation and frost tolerance is beyond the scope of this thesis. For a thorough analysis of the physiological, biophysical and biochemical aspects of cold acclimation, the reader is directed to reviews by Franks (41), Sakai and Larcher (137), Levitt (95), Burke et al.(9), Steponkus (146), Graham and Patterson (50) Singh and Laroche (143), Johnson-Flanagan and Singh (78) and Weiser (159). The role of protein metabolism (55), molecular genetics (153), and growth regulators (127) in cold acclimation have also been reviewed recently.

Low temperature is not the only stimulus that can induce freezing tolerance in hardy plant species. Other factors that can induce freezing tolerance without low temperature conditioning include abscisic acid (ABA), desiccation, and short day photoperiod. It is currently not known whether the different factors share any a common pathway in inducing cold hardiness. Comparison of molecular alterations associated with cold acclimation induced by various factors may identify similar biochemical events that are required for increased tolerance to freezing stress. This paper will compare the molecular events associated with cold acclimation induced by low temperature, ABA, desiccation and short day photoperiod.

2.2 Low temperature induced freezing tolerance

The exposure of plants to low temperatures involves metabolic adjustments required for growth and adaptation to freezing stress. The issue of how these metabolic changes observed during acclimation increase hardiness has not yet been addressed. It is known that cold acclimation is a highly complex metabolic phenomenon, therefore likely to be a quantitative trait as indicated by conventional genetic studies. The inheritance of frost tolerance has been recently reviewed by Thomashow (153) and by Sakai and Larcher (137). The myriad of evidence for altered metabolism during cold acclimation and data from genetic studies suggest that frost tolerance involves the expression of a group of genes.

Early studies have shown qualitative changes in protein patterns during hardening (29, 38, 46). Altered soluble protein and isozyme patterns were able to roughly hint at qualitative changes. More recent studies have applied newer electrophoretic techniques (89, 117) to identify cold induced changes in protein synthesis (24, 59, 154, 155, 76, 109, 110, 140, 56, 47, 88, 91, 111, 112, 10, 58, 124, 92, 93) with greater resolution and consistency. Both *in vivo* and *in vitro* protein labeling methods have been used extensively to resolve these changes.

Even though many of the early reports on cold acclimation have dealt with woody plant species, virtually all of the current research on the molecular aspects of hardening revolves around herbaceous species. A summary of the molecular changes associated with cold acclimation for each species will be presented. The plant species that have been used for the molecular analysis of cold acclimation

are listed in Table 2.1.

2.2.1 Alfalfa

The process of hardening often involves the conversion of complex carbohydrates into lower molecular weight products (94). Isoforms of the hydrolases in cold tolerant and non-tolerant alfalfa cultivars were compared (86). Amylase isoform differences between hardened and control plants were small but some variation was observed. Esterase isoforms from both cultivars were present year round but some had higher activity or were more abundant in hardened samples. Isozymic pattern differences in acid phosphatase and leucine aminopeptidase show seasonal abundance changes, but all forms were present regardless of the time of year. The data in these studies were complicated by variations in the procedures used to store the enzymes but resulted in an interesting finding. When enzyme extracts from both summer and winter plants were frozen and then thawed, only the winter sample showed any catalytic activity therefore indicating that freeze tolerant isoforms occur in the winter. It is possible that cold acclimation entails synthesizing new isoforms or modification of existing enzymes to prevent freeze denaturation (86).

Synthesis of nucleic acids and proteins during hardening increases the requirement for reduced coenzymes and ATP (85). In a study similar to the isozyme analysis of the hydrolases (86), electrophoretic comparisons of the isozymes of glutamate, NAD-malate, NADP-malate, isocitrate, lactate, 6-phospho-

gluconate and glucose-6-phosphate dehydrogenases from hardened and non-hardened alfalfa cultivars were made. The isoforms of all these enzymes were present in non-hardy plants but the activity increased during hardening and decreased after deacclimation. Glutamate, NAD and NADP-malate dehydrogenase extracts retained catalytic activity after freezing from both hardy and control plants where as the other enzymes analyzed appear to be freeze tolerant in hardy but not control plants. Overall dehydrogenase activity appears to increase during cold acclimation and decreases during dehardening (85).

Gerloff et al. (46) examined soluble protein content and peroxidase activity in hardy and non-hardy alfalfa cultivars and determined that there was increased soluble protein content and peroxidase activity in all the cultivars after low temperature growth. Two peroxidase isozymes appeared in the hardy variety only. New peroxidase isoforms were not detected in hardened plants but it appears that the energy of activation of the existing isoforms were lower than in control plants. Krasnuk et al.(84) also reported increased total peroxidase activity in both frost tolerant and frost sensitive cultivars of alfalfa during cold acclimation.

The steady state polypeptide population changes during cold acclimation in alfalfa (*M. sativa* L. cv. Wisconsin 22C) cell suspension cultures (134). Proteins normally secreted into the liquid media in the control cultures were not detected in hardened cultures. This may reflect a change in membrane permeability during hardening.

The pattern of protein synthesis is also altered in cold acclimating alfalfa (*M. sativa* cvs 'Anik' and 'Saranac') seedlings (109,110). Ten novel or increased

steady state level polypeptides were identified and eleven polypeptides were synthesized during the first two days of acclimation. Maximum hardiness, however, required a considerably longer period of time to develop. The less hardy alfalfa cultivar 'Saranac' shares similar polypeptide differences but attained maximum hardiness much more quickly. It appears that cold induced biochemical changes observed in alfalfa are rapid but maximum hardiness development is a much slower process in the more hardy cultivar. Deacclimation was rapid for both cultivars as two days exposure of hardened plants to room temperature was sufficient to decrease hardiness to control levels and suppress cold induced polypeptide synthesis (109, 110).

Changes in the pattern of gene expression were observed in the alfalfa cultivars 'Anik' and 'Saranac' during cold acclimation (109,110). The *in vitro* translation products of two novel RNAs from 'Anik' appear to have the same molecular weights as the translation products of two novel RNAs from 'Saranac'.

The cold induced differential expression of RNAs observed in alfalfa was the basis for the differential screening of a cDNA library constructed from mRNA of cold acclimated plants (111, 113). The isolated cDNA clones hybridized only to RNA in cold acclimated plants from various alfalfa cultivars. One cDNA clone (pSM1409) from 'Anik' also hybridized to RNA from ABA or water stressed plants. The other clones were not water stress, heat shock, wound nor ABA inducible and thus appear to represent cold specific genes. Northern blot analysis indicates that some of the clones hybridize to more than 1 size of transcript from poly (A^+) RNA of cold acclimated plants (113). This suggests that

posttranscriptional modification of the RNAs produced different size transcripts or that the different RNAs represent members of a gene family.

2.2.2 *Arabidopsis*

The molecular aspects of cold acclimation have been most well studied in *Arabidopsis thaliana* (47, 88, 91, 60, 65, 96). Cold acclimation of *A. thaliana* involves novel protein synthesis (47, 88, 91). By *in vivo* labeling with ^{35}S-methionine, polypeptides synthesized during cold treatment were labelled and resolved by 1D or 2D-gel electrophoresis and identified by fluorography. Approximately eleven new polypeptides were synthesized and one disappeared during cold treatment (91). Some of the polypeptides apparently disappeared but with prolonged low temperature exposure reappeared. The actual hardening process was very rapid. Within one or two days of acclimation, hardiness increased and so did the expression of new polypeptides (47, 88).

Heino et al.(65) have questioned the role of these cold regulated polypeptides in frost tolerance. An ABA deficient *A. thaliana* mutant was determined to be incapable of cold acclimating but could harden when ABA was added to the medium. Even though the mutant did not harden under low temperature treatment, the altered pattern of protein synthesis was strikingly similar to that of the hardened wildtype. Virtually all of the cold induced novel polypeptides were present in the mutant. This indicates that these novel polypeptides do not have a direct role in conferring frost tolerance and may only

represent metabolic adjustments for adaptation to low non-freezing temperatures.

Current evidence favors the view that novel gene expression occurs during cold acclimation. Low temperature altered protein synthesis indicates a change in the population of *in vivo* translated RNAs. This was verified by *in vitro* translation studies which identified alterations in the pool of translatable poly (A^+) RNA (59, 106, 109, 47, 88, 10, 92, 93, 155, 33). The question of whether new genes are actually transcribed during cold hardening required the cloning of differentially expressed cold regulated mRNAS. Northern blot analysis using the cold regulated cDNAs as probes indicated a cold induced steady state accumulation of corresponding RNAs. Nuclear run-on experiments, at least in *Arabidopsis* (96), suggest that the regulation of cold induced RNAs is predominantly post-transcriptional or is a combination of transcriptional and post-transcriptional control. More cold regulated genes will need to be analyzed to determine if cold acclimation does transcriptionally activate new genes.

Low temperature induced changes in the translatable RNA population were observed in plants grown in both soil (47) and artificial media (88). There appears to be only 3 or 4 induced or increased level translatable RNAs after 1 day of low temperature treatment. No consistant decrease or disappearance of RNAs were observed. Callus cultures similarly treated also induced similar RNA species but a few RNAs clearly disappeared (47). There were several *in vitro* translation products showing similar molecular weight as the silver stained polypeptides indicating that these cold induced RNAs are probably *in vivo* translated resulting

in the observed *in vivo* protein synthesis changes (47, 88). Lin et al. (96) have demonstrated that 4 cold induced translation products are heat stable as they remain in solution after boiling.

Arabidopsis cDNA libraries have been constructed from 1 day (87) and 3 day (60) cold treated plants. A genomic DNA library was also made (87). From differential screening of a genomic library with single strand cDNA from mRNA of 1 day control and cold treated plants, a genomic clone was isolated. After subcloning the insert into a cloning vector, it was used to probe the 1 day cDNA library to isolate the corresponding cDNA clone (kin1) (87). The kin1 transcript is present within 6 hrs of cold treatment and remains for the duration of the treatment. When the plant is brought back to room temperature, the transcript disappears within 12 hrs. It is also induced by drought and ABA, but not by heat shock treatment. Southern blot analysis indicates the presence of 2 genes with high homology to the kin1 cDNA in the *Arabidopsis* genome.

Sequence analysis of the kin1 cDNA predicts a putative polypeptide of 66 amino acids in length. The putative protein is very hydrophilic and contains repetitive sequences. Comparison of the kin1 cDNA with published sequences indicates similarities to a number of antifreeze proteins from arctic flounder. This suggests that the polypeptide may function as an antifreeze protein to promote intracellular supercooling (87) or to minimize ice crystal formation.

Four cold regulated cDNAs were isolated by differential screening of a cDNA library from *Arabidopsis* plants hardened for 3 days (60). The cold

regulated (*cor)* genes are also present in single or low copy numbers per genome. Northern blot analysis with control, 3 day cold acclimated and 1 day deacclimated total RNA shows the presence of all 4 *cor*-related transcripts in 3 day acclimated plants only. The transcripts were rapidly induced by 1 to 4 hrs of cold treatment. *cor* transcripts are also induced by ABA and drought but not by heat shock treatment. Three of the *cor* genes are apparently regulated post-transcriptionally and the fourth regulated transcriptional and post-transcriptionally (60).

From the few cold regulated cDNAs that have been isolated, most of the DNA sequences do not appear to match with published sequences. This may be indicative of a requirement for unique protein function under an extremely low water potential generated by extracellular freezing. Evidence for this unique requirement was found in *Arabidopsis* in which the *kin1* gene, coding for an antifreeze-like protein, and one of the *cor* genes coding for a boil stable protein were identified.

2.2.3 Barley

Analysis of the cold shock response in barley (*Hordeum vulgare* L) plants indicated very little change in protein synthesis (101). Barley seedlings were exposed to low temperature (+6°C) for 4 hours in the presence of ^{35}S-methionine and the labeled polypeptides separated by 1D-SDS PAGE. Minor polypeptide differences between a winter and a spring cultivar were identified. In contrast to

the barley data, the heat shock response observed in soybean indicated attenuation of most housekeeping proteins (83). Whether the expression of these cold shock polypeptides are directly involved in cold acclimation is not known.

In cold acclimated barley plants, relatively few translatable RNA changes were observed when *in vitro* translation products were resolved by 1D PAGE (69). The most prominent band identifies an RNA coding for a 77 KDa polypeptide (present at either 2 or 50 days of low temperature growth). A cDNA library was constructed from mRNA of cold acclimated barley plants and differentially screened. A cDNA was selected from several clones isolated and was used for further analysis (36). Northern blot analysis identifies the presence of a homologous transcript in 3 cold acclimated winter and 1 spring barley cultivars. A polypeptide 88 amino acids long was predicted from the DNA sequence of the cDNA. The function of the polypeptide is not known as there was little sequence similarity with published sequences. Restriction fragment length polymorphism analysis with 5 different restriction enzymes shows 2 allelic forms in 19 cultivars tested.

Two other cultivars of barley were also analyzed for gene expression during cold acclimation. Three different types of responses were observed when 'Georgie' seedlings, a winter cultivar, were hardened for 4 days: (1) induction of novel translatable RNAs, (2) increased level of existing RNAs or (3) decrease level or disappearance of RNAs (10). A similar pattern of response was observed in the winter cultivar 'Onice' with the exception of a few minor differences. From these results, it was concluded that rapid gene expression occurs in response to

low temperature but 3-4 days of cold treatment were required for maximum expression of the cold induced RNAs (10). It is possible that 4 days of low temperature treatment is not sufficient to induce some of the genes that are more genotype spcific as they may require a longer low temperature exposure for induction.

Five cDNA clones were selected by differential screening of a cDNA library constructed from mRNA of cold acclimated 'Georgie' seedling shoots (11). These clones hybridize to mRNA predominantly from cold treated seedlings and within 8 hrs of deacclimation, cold induced transcripts hybridizing to the cDNA probes were not detectable. Some of the clones are only expressed in certain tissues after cold treatment and also hybridize weakly to mRNA from hardened wheat and rye. DNA sequences for 2 of the cDNA clones contain putative polypeptides with an extremely basic domain. No function has been assigned to the gene products yet, as these sequences contain very little similarity with other published sequences (11).

2.2.4 *Brassica*

It has been suggested that membrane lipid oxidation at low temperatures generates harmful peroxides (141). Peroxidases must therefore be active at low temperatures to remove these compounds. Qualitative and quantitative changes in peroxidase isozymes of cold acclimated plants have been reported (46, 102, 61, 5, 80, 84, 75). In hardened winter rape (*Brassica napus* L var. Oleifera cv.

Gorczanski), total peroxidase activity increased by ten fold over control plants (80). Cold hardened winter rape contained 2 low molecular weight soluble protein fractions with unusual properties (79). Amino acid analysis indicates a highly basic protein with very few proline and methionine residues when compared to control proteins from the same fraction.

B. napus cv. Jet Neuf seedlings expressed approximately fourteen novel or increased abundance polypeptides and decreased the expression of six others within 48 hrs of low temperature treatment (106). A similar change in protein synthesis was detected in as early as 6 hrs of cold treatment. Although freezing tolerance was not determined at the end of 6 hrs, it is improbable that hardiness would increase significantly during this time. Rapid changes in protein synthesis during the initial phases of hardening appears to be a common process in cold acclimation. *In vitro* translation products separated by 2D PAGE reveal the induction or increased abundance of some RNAs and a decrease in others but the majority of the RNA species were present after cold treatment. One RNA that did decrease coded for the small subunit of RUBP carboxylase/oxygenase.

2.2.5 Bromegrass

Cold acclimation of bromegrass (*Bromus inermis* Leyss cv. Manchar) cell cultures harden from -7 to -17°C after 5 days at +2 to +4°C (21, 92). After 5 days of low temperature treatment, 6 new SDS soluble polypeptides were expressed and the abundance of 1 other decreased. After 1 day, 1 polypeptide

disappeared but novel expression was not apparent (92). *in vivo* labeling with [^{14}C]-leucine for 86 hrs at +4°C indicated an induction of 3 new polypeptides and the reduction of 2 others (135). *In vitro* translation experiments revealed that after 5 days of +4°C hardening, the expression of 7 novel RNAs and the repression of 5 other RNAs were identified. It appears that a considerable increase in freezing tolerance was attained with relatively few polypeptide changes, even though the cells were not at maximum hardiness after 5 days of low temperature treatment. Further analysis of these cold induced polypeptides associated with hardening and deacclimation will more precisely determine their correlation with frost tolerance.

2.2.6 *Solanum*

Cold acclimation in potato requires protein synthesis. Chen et al.(20) demonstrated that cycloheximide treatment of *Solanum commersonii* stem cultures inhibited the development of cold hardiness. Low temperature hardening of *S. commersonii* induces the synthesis of 11 to 14 new polypeptides (154,155). After 1 day of deacclimation, the majority of the cold induced polypeptides disappeared and hardiness had correspondingly decreased. Some cold induced polypeptides, expressed in the early and middle phases of hardening, were not present at maximum hardiness (155). The transient expression of these polypeptides during

cold acclimation may reflect the involvement of a cascade of regulatory proteins required for the induction and maintenance of frost tolerance.

An increase in frost tolerance in *S. commersonii* stem cultures was associated with a low temperature induced expression of novel translatable RNAs (154, 155). Similar to the temporal pattern of cold induced protein synthesis, the changes in the RNA population over a period of 14 days at +5°C indicates a pattern of transient expression for a sub-population of the RNAs. After 1 day of deacclimation, hardiness was lost and concurrently many of the RNAs declined to control levels. The appearance of these RNAs with the development of frost tolerance and their disappearance after dehardening implies a direct role for gene expression during cold acclimation.

Suspension cultures of *S. commersonii* can also cold harden to the same level of hardiness as with whole plants (93). Treatment of cell cultures for 48h at 4°C altered the pattern of translatable RNA. Many of the cold induced RNAs were present by 12h treatment but not all of them were present after 48h suggesting a requirement for a number of genes in regulating frost tolerance.

2.2.7 Spinach

Guy et al. (59) provided direct evidence to support the hypothesis that cold acclimation of spinach (*Spinacia oleracea*) involves changes in gene expression. They determined that at least 6 mRNAs increased in abundance upon exposure to 5°C. A close correlation was found between hardiness and the expression of

certain cold acclimation proteins (CAPs) (160, 85 and 75 KDa) in spinach (59, 56, 57). Upon exposure to warm temperatures, hardened spinach plants deacclimated very rapidly and many of the cold induced polypeptides disappeared (56). Heat shock treatment did not induce any of the cold induced polypeptides nor was there any increase in hardiness. Conversely, there was no increase in tolerance to heat stress when cold acclimated plants were heat stressed.

Protein fractionation studies (58) indicated that spinach CAPs neither accumulated within chloroplasts nor mitochondria therefore they are likely to be cytoplasmic proteins. The CAPs were purified from 2-D gels and were micro sequenced to determine the partial amino acid composition. It appears that all three CAPs are rich in aspartic acid, glutamic acid and glycine.

2.2.8 Wheat

During cold acclimation in winter (Kharkov) and spring (Rescue) wheat, an increase in ferredoxin-NADP reductase synthesis was observed (130). However, the specific activity was higher in the more hardy winter wheat cultivar although the Km's for both cultivars were similar.

Invertase activity between hardened winter wheat (Kharkov - most hardy) and 2 spring wheats (Thatcher - intermediate hardiness and Rescue - frost sensitive) was compared. A substantially lower energy of activation was observed with invertase from hardened winter wheat than from the spring cultivars (131). In a subsequent experiment (132), three invertase isoforms were identified by gel

filtration chromatography. Upon hardening, the activity of isoforms I and II from winter wheat changed significantly with a shift in activity from form II to form I. The ratio of form I to form II activity from twelve wheat cultivars ranging in ability to harden were compared, and found to roughly vary with the hardiness potential of the cultivar (133). Because hardening increases form I and decreases form II invertase activity, it was concluded that one isoform replaces another during cold acclimation (133). Although such a detailed analysis has not been performed with other isozymes, it is likely that similar changes occur with other enzymes during cold acclimation.

RNA levels were compared between low temperature grown spring and winter wheat (139). Spring wheat does not harden appreciably relative to winter wheat. Both sRNA and rRNA increased markedly in acclimated winter wheat but not in acclimated spring wheat. The base composition of the acclimated winter wheat sRNAs also shifted towards a higher (G+C)/(A+ΨU+U) ratio which indicates the presence of new sRNA species associated with cold acclimation. A low temperature induced change of DNA-dependent RNA polymerase activity was observed in winter wheat (138). Chromatin DNA-dependent RNA polymerases isolated from both winter and spring cultivars indicates that polymerase activity was higher in the winter cultivar. The predominant increase in activity was contributed by RNA polymerase I. Since RNA polymerase I is responsible for rRNA synthesis, it would account for the increase in rRNA observed during cold acclimation. Increases in rRNA were confirmed in other cultivars of winter wheat by Devay and Paldi (35). They determined that increased rRNA was not from

increased expression of the rRNA genes, but was the result of winter wheat containing more rRNA cistrons than spring wheat (121).

A comparative analysis of the soluble protein changes between etiolated seedlings of winter wheat (*Triticum aestivum* cvs. Frederick and Norstar) and a spring wheat (*T. aestivum* cv. Glenlea) indicates that the majority of the polypeptides induced or increased by low temperature were common to all 3 cultivars (140). The major difference between the 3 cultivars was due to a higher abundance of a 200 KDa polypeptide in both winter wheats. It was concluded that the majority of the low temperature induced changes were not associated with hardening but reflected an adjustment to growth at low temperatures. *In vivo* protein analysis of root, crown and leaf tissues indicated a synthesis of at least 8 new polypeptides which were more abundant in the 2 winter cultivars (124). A 200 kDa cold induced polypeptide was found to be most abundant in all 3 tissues of the winter cultivars after acclimation, but was especially abundant in the aerial tissues. Since aerial organs are usually more hardy than root tissues, the differential expression of this polypeptide suggests a close correlation with increased frost tolerance (124).

From genetic studies, it is known that winter hardiness is a complex trait. In wheat, regulation of frost tolerance is associated with 11 of the 21 chromosomes and therefore likely to involve many genes (149). Evidence to suggest that gene expression occurs at the molecular level during cold acclimation of winter wheat was reported by Danyluk and Sarhan (33). They determined that 23 increased abundance mRNAs were differentially expressed in frost tolerant cultivars but not

all were present at the level of hardiness attained by the end of cold treatment. Some of the RNAs were transiently expressed while others remained throughout hardening. Upon deacclimation for 24 hrs, all of the cold regulated RNAs decreased to control levels. Cold treatment also decreased the expression of 14 RNAs in the frost tolerant cultivars of which 13 were in common with the cold sensitive cultivar (33). The cold regulated RNAs were shown to be actively translated and polysomes isolated from hardened plants had a higher *in vitro* translation activity than from control plants (125). The expression of cold regulated genes may be required to either maintain or increase the rate of protein synthesis required for hardening.

Lin et al. (96) reported that wheat has a *cor* gene related to *Arabidopsis cor* 47. A cDNA library of poly (A+) RNA isolated from cold acclimated winter wheat (cv. Winoka) was screened with *Arabidopsis cor* 47 cDNA probe and a positive clone, pWG1 was isolated. Northern blot analysis indicated that transcripts homologous to pWG1 were only presented in cold acclimated plants. Similar to the *cor* cDNAs from *Arabidopsis*, the pWG1 also encodes a boiling stable polypeptide.

2.3 Abscisic acid induced freezing tolerance

2.3.1. Involvement of ABA in cold acclimation

The first indication that a growth regulating substance modulated cold hardiness was observed in woody plants. It was thought that hardiness was the result of dormancy development in the late summer or early fall during which certain substances translocated to the bark would result in hardiness. Subsequent work by Irving and Lanphear (71,72) demonstrated that dormancy and cold hardiness development was independent of bud dormancy (71). It was suspected that the increased cold hardiness in *Acer negundo* may involve an ABA-like substance (72). Low temperature treatment resulted in high endogenous levels of an ABA-like substance. Chromatographic analysis of the ABA-like substance indicated a high degree of similarity with authentic ABA. Furthermore, plants treated with either the ABA-like substance or ABA increased hardiness (70).

Several lines of evidence from the current literature suggest that ABA is involved in plant adaptation to freezing stress: (1) endogenous levels of ABA were shown to increase during cold acclimation (108, 160, 19, 20, 90, 57), (2) application of ABA at room temperature can induce freezing tolerance in whole plants (70, 19, 20, 90, 111) and in cell cultures (21, 120, 82, 119, 92, 93, 118), (3) ABA induced freezing tolerance was only observed in those species which could cold harden and not in species which lacked the ability to cold acclimate (21) and (4) ABA has been shown to affect metabolic processes similar to those manifested by

cold treatment (20, 90, 150).

There is substantial evidence to indicate that endogenous ABA levels rise during cold acclimation. This was observed in both woody and herbaceous species. Under a long or short day photoperiod at +5°C, *A. negundo* plants increased hardiness as well as endogenous ABA levels (70). Irving (70) also demonstrated that hardiness development was more a function of increased ABA than a decreased gibberellin concentration because the application of GA to dormant and hardened plants broke dormancy but did not decrease hardiness as rapidly. An increase in ABA level was observed in the fall in sour cherry (*Prunus cerasus*) flower buds (107) but by mid-winter the increased ABA concentration decreased to basal levels.

Exposure of the frost tolerant potato *Solanum commersonii* to 2°C day/night lead to a large transient increase of ABA in the leaves (19, 20) during which frost hardiness and total soluble protein also increased. When the frost sensitive potato species *S. tuberosum* was subjected to the same treatment, there was no significant increase in frost tolerance, free ABA or total soluble protein level. Even though the ABA peak in *S. commersonii* was transient during acclimation, hardiness actually continued to increase well after the time of the ABA peak while still at low temperature. Application of the protein synthesis inhibitor cycloheximide to stem cultures of *S. commersonii* at the beginning of cold treatment prevented hardening. When the inhibitor was added after 5 days of low temperature treatment, hardiness induction was not inhibited. The induction of

freezing tolerance in *S. commersonii* is thus associated with a transient increase in free ABA and novel protein synthesis (20).

Analysis of the crown tissues from two acclimated winter wheat cultivars indicate a 3 fold higher ABA level in the hardier cultivar (160). Soluble protein accumulation paralleled hardiness for both cultivars. Cold acclimated winter wheat unlike sour cherry (107), potato (20), spinach (57) or *Arabidopsis* (M. Francks, personal communication) maintains a high ABA level throughout hardening. Cold acclimation nevertheless characteristically involves an increase in ABA concentration followed by an increase in frost tolerance.

A second line of evidence supporting ABA involvement in cold acclimation comes from the fact that application of ABA without low temperature treatment can induce a hardening response. Treatment of *A. negundo* seedlings with ABA under long day nonhardening conditions increased frost tolerance and under short day conditions enhanced hardiness (70). Chen et al.(20) not only demonstrated an increased endogenous ABA concentration in *S. commersonii* but that growing *S. commersonii* stem cultures in the presence of ABA at room temperture could also increase hardiness to the same extent as low temperature treatment. ABA induced hardening was also inhibited when cycloheximide was added at the beginning of ABA treatment. In two winter wheat cultivars, ABA treatment increased hardiness of both cultivars but the hardening effect was greater in the hardier cultivar (90). ABA treatment of both cultivars at low temperatures however could enhance the level of hardiness above ABA alone. There is also a

similar low temperature requirement in combination with ABA treatment to attain maximum hardiness in two alfalfa (*M. sativa*) cultivars (111). However, low temperature may be required to initiate and/or maintain a biophysical aspect of hardening.

Wild type *A. thaliana* is able to increase frost tolerance by low temperature (47, 88) or ABA treatment (91). An *A. thaliana* ABA deficient mutant was not able to cold acclimate but when ABA was added to the growth medium, freezing tolerance increased to a level similar to cold acclimated wild type plants (65). This is a strong indication that ABA plays a major role in the induction of cold hardiness.

In whole plants studies, ABA treatment by foliar sprays or soil drenching have not always been effective in increasing hardiness (39, 54). This may be due to ineffective ABA uptake, enzymatic breakdown or microbial degradation (21). To circumvent these technical difficulties, a number of workers have opted to use sterile *in vitro* cultured systems. Suspension cultures originating from mesocotyl tissue of bromegrass (*Bromus inermis* Leyss cv Manchar), winter wheat (*T. aestivum* L cv Norstar) and winter rye (*Secale cereale* L cv Cougar), capable of cold acclimation, grown in 75 μM ABA (21) were able to harden substantially at room temperature with four days of treatment. There was no further increase in hardiness when the same treatment was performed at +2°C. A similar effect was observed in lotus (*Lotus corniculatus* L.) callus culture originating from axillary buds (82). Freezing tolerance increased to the same level as field hardened plants

when calli were cultured at room temperture in media containing 10 μM ABA. There was no additional increase in hardiness when combined with low temperature.

Freezing tolerance was enhanced by ABA treatment in *Brassica napus* cv. Jet Neuf microspore-derived cell suspension culture (119) and in microspore derived embryos (118). Low temperature treatment alone was not as effective in increasing hardiness, although a higher level of hardiness was attained when ABA treated microspore embryos were followed by culturing at +2°C for 3 weeks. A combination of ABA and cold treatment was required for a maximum hardening response in alfalfa suspension culture derived from cotyledons (120). The necessity for ABA in combination with low temperature to induce ultimate hardiness in both alfalfa cultures (120) and whole plants (111) may indicate a requirement for cold conditioning in order to be ABA responsive or to maintain the cold hardiness.

The third line of evidence which indicates an integral role for ABA in inducing frost tolerance was reported by Chen and Gusta (21). They demonstrated that ABA treatment could only induce freezing tolerance in species which could cold acclimate and not in warm season species which are not capable of cold hardening. This suggests that the ability to cold acclimate is genetically regulated (21, 54). Genetic studies in winter wheat have already supported the notion of winter hardiness as a heritable trait (149) and thus the ability of ABA to trigger the hardening response lends further support to this phytohormone as a part of the cold acclimation response.

The fourth line of evidence involves the metabolic and physiological changes observed during ABA induced hardening. For example, cold acclimating alfalfa undergoes a morphological change by assuming a rosette growth habit (129). Under long day conditions, ABA treatment could also alter the growth form to a rosette habit and increase frost tolerance. As outlined earlier, cold acclimation is accompanied by gross changes in cellular metabolites. ABA induced hardening at room temperature similarly alters the abundance of certain cellular metabolites. Examples of these changes have been reported in wheat (90), bromegrass (150) and potato(20) studies.

Cold acclimation in potato can be inhibited by application of cycloheximide. Similarly, by preventing protein synthesis during ABA treatment, hardening does not occur. ABA, similar to cold treatment, can induce frost tolerance in winter wheat as well as increase proline and sugar levels (90). During cold acclimation, cellular water content decreases (13, 105, 14) and dry matter content increases (14, 148, 40, 103). These two responses were also observed in ABA hardened bromegrass suspension culture cells (152). Additionally, ultrastructural alterations were observed during cold acclimation in bromegrass cell cultures treated with ABA (151). Numerous examples of low temperature induced changes in protein synthesis have been reported. Similarly, ABA alters the pattern of protein synthesis during the induction of hardiness. Results of these studies will be presented in the following sections.

2.3.2 Gene expression induced by ABA or water stress

Currently, there is a paucity of data on the molecular changes during ABA induced cold acclimation. In contrast, considerably more is known about the inductive role of ABA in mediating biochemical responses during various developmental processes and other environmental stresses. Studies on ABA induced gene expression have focused primarily on events during embryo development, desiccation stress and more recently wounding.

Typical seed development involves embryo maturation followed by desiccation to produce a mature dormant seed. At a specific stage during embryogenesis, embryonic tissue ABA concentration rises and concurrently novel or increased levels of specific proteins are expressed. If premature embryos are excised from the seed, ABA levels do not rise, the novel proteins are not expressed and precocious germination results (32). By applying ABA to *in vitro* cultured premature embryos, precocious germination is prevented and the accumulation of storage or embryo proteins is maintained. ABA application to seed tissues of different species can also prevent precocious germination and or regulate the expression of various seed proteins including: storage proteins (8, 32), lectins (126), albumin (161), barley aleurone proteins (67) and other late embryogenesis abundant (LEA) or LEA-like proteins from cotton (37, 44, 43, 45), carrot (62), maize (48, 158) and *Brassica* (62). In each of the above examples, ABA inducible cDNA clones corresponding to the proteins were isolated. Northern blot analysis with the cDNA probe verified the presence of the

complementary RNA transcript after endogenous ABA levels increased or exogenous ABA application.

Further evidence for the involvement of ABA in regulating these proteins was demonstrated by fluridone treatment. ABA is thought to be a metabolite of carotenoid biosynthesis therefore fluridone, which interferes with carotenoid metabolism presumably inhibits ABA accumulation (165). In the presence of the inhibitor, endogenous ABA level did not increase, nor did ABA inducible protein (126) and RNA (8, 63) levels at the expected time during embryo growth. By supplementing the media containing fluridone with ABA, expression of the protein and mRNA was restored.

A unique physical property of some of these ABA inducible proteins was observed in barley aleurone cells (74). ABA treatment of the aleurone layers induced at least 25 new polypeptides. When the protein was extracted from ABA treated cells and boiled for 10 min, the majority of the proteins from the control cells precipitated, whereas most of the ABA induced polypeptides remained in solution. A similar effect was observed in dormant wheat grain tissues (128). Two ABA responsive proteins (called dehydrins) associated with dormant wheat embryonic axes also remained in solution when heated to 70°C. These heat stable proteins and the ABA inducible *cor* proteins of *Arabidopsis* (60) signify a common physical property for ABA responsive proteins and may therefore have similar functions.

Late in cotton embryo development, a set of proteins and associated RNA transcripts called LEA mRNAs are highly expressed (37). Many of the LEA

mRNAs are induced by treating excised embryos with ABA, however, storage protein accumulation is not regulated in this manner. DNA sequences of 6 of the LEA genes code for putative polypeptides that are unusually hydrophobic in nature. Two of the LEA polypeptides have a high proportion of glycine and hydroxylated amino acids. They do not seem to have a single thermodynamically preferred structure, and thus predicted to form amorphous random coils. The proteins could act as "solvation" molecules to substitute for the solvent characteristics of water. Two other LEA polypeptides have putative structures which could bind ionic species. As free water decreases under desiccation, ion concentrations can increase to the point of being cytotoxic. It has been suggested that these polypeptides can bind-up excess ions (1).

A cDNA of the gene for the wheat albumin, Em, was sequenced and like the LEA genes has a very hydrophilic amino acid composition and may exist as a random coil (98). The regulation of Em by ABA during embryo development was analyzed first by identifying the cis-acting elements of the Em gene required for ABA induction. A chimeric gene construct consisting of 650bp of the Em promotor region fused to the GUS reporter gene was required for ABA inducible expression in a rice transient assay (99). More detailed analysis revealed the presence of ABA responsive elements (ABREs) which when fused upstream of a 35s promotor-GUS chimeric gene, could increase GUS activity significantly over the basal level of the reporter gene alone. Thus the ABRE was able to confer ABA sensitivity to a constituitive promotor (100). A DNA binding protein was found to bind to an 8bp element within the ABRE. Mutations to the 8bp

sequence prevented the DNA binding protein from interacting with it and consequently ABA responsiveness decreased. The DNA binding protein appears to contain putative amino acid sequences characteristic of a class of transcription factors called leucine zippers (52).

The LEA proteins may play a desiccation protective role in programmed dehydration. Metabolic adjustments required for an embryo to tolerate drying may be a part of a more universal process in plants to withstand drought stress. Evidence for the involvement of ABA in modulating gene expression during a water deficit has been reported by many workers.

One of the most dramatic examples of desiccation tolerance in higher plants is seen in the African resurrection plant *Craterostigma plantagineum* Hochst. The mature leaves of this plant is able to withstand almost complete drying and upon rehydration can continue growth (42, 2). In response to drying, endogenous ABA concentration increases, the pattern of protein synthesis is altered and new translatable RNA are expressed (2). mRNAs corresponding to desiccation inducible cDNA clones were inducible by ABA treatment, and were repressed upon rehydration of the leaves. Other examples of desiccation altered gene expression have been observed in rice (114, 22), maize embryos (49), tomato (7, 28), barley (23), soybean (3, 31) and *Mesembryanthemum crystallinum* (4). Similar to *C. plantagineum,* drought specific mRNAs from rice (114, 22) and tomato (28) are ABA inducible without desiccation.

Exposure of plants to high salt conditions may elicit a similar biochemical response as desiccation because both types of stress subject the plant to a low

water potential environment. An altered pattern of gene expression has been reported in salt stressed *M. crystallinum* (4), *Distichlis spicata* (166), tobacco suspension culture cells (145) and rice (114, 22). The cDNAs corresponding to the RNAs induced by salt stress in *M. crystallinum* (4), tobacco cells (145) and rice (114,20) are also inducible by ABA treatment. Desiccation inducible RNAs from maize dry embryo (49) and rice (114, 22) are also salt inducible. It is not surprising that the rice and maize RNAs in addition to being desiccation and high salt inducible are ABA inducible as well.

Genetic evidence for the regulation of drought inducible genes by ABA was demonstrated with a mutant variety of tomato (*flacca*) that is only capable of synthesizing low levels of ABA (28). cDNAs corresponding to the RNA transcripts isolated from wilted wild type (*wt*) leaves could be induced in a non-wilted plant with ABA treatment. Wilted mutant leaves could not express these RNAs but were able to when treated with ABA. A higher endogenous ABA level in water stressed wt leaves was correlated with an accumulation of the wilt inducible RNAs. Cohen and Bray (28) concluded that drought stress induced changes in the endogenous ABA levels regulate the expression of these RNAs.

The regulation of the rice RAB21 (Responsive to ABA) gene was originally identified as water stress inducible, but it is also salt stress and ABA inducible (114). DNA sequencing of the Rab21 cDNA (subsequently renamed rab16 a-d) turns out to be a member of a small gene family consisting of 4 genes tandemly arrayed. The promotor region of the 4 genes contain a highly conserved sequence that is also found in cotton LEA genes (163) and the wheat Em gene (52, 100).

Gel retardation and DNase I footprint analysis indicate that some trans-acting factor(s) binds to this conserved sequence (163) and thus may be an ABRE (115).

A role for ABA during wound induction has been suggested (122). When the leaves of potato or tomato plants are wounded, such as by insect attack or mechanical damage, the proteinase inhibitors I and II (PI-I and PI-II) accumulate. These inhibitors are directed at decreasing the nutritional content of the plant by preventing protein digestion in the insect gut. It has been shown that even though wounding may be localized, PI-II accumulates throughout aerial portions of the plant and therefore is a systemic response (123). Previous work in water stressed maize seedling (64) and embryo (49) proteins indicated that they are also ABA and wound inducible. Pena-Cortes (122) tested whether PI-II was ABA responsive and if ABA was involved in the systemic induction of the wound response. The results were: (1) exogenous ABA treatment increased PI-II RNA without wounding, (2) ABA-deficient mutants of potato and tomato show no signs of PI-II wound inducibility but when the petiolar ends of the leaves were dipped in an ABA solution, PI-II RNA accumulated and (3) wounding increased endogenous ABA levels not only in the affected leaf but also in other leaves as well. The PI-II gene has been sequenced (81) and comparisons made by Guiltinan et al. (52) indicate the presence of the same ABRE found in Em and rab16 5' regulatory region.

Whether during embryo development, desiccation or wounding stress, endogenous ABA levels increase and a set of ABA inducible genes are expressed. Exogenous ABA application can also induce the same genes. These gene products

may perform different functions as they are components of different physiological responses to different stresses. However, the occurence of the LEAs and some rabs in embryos and other tissues during desiccation or ABA treatment may indicate a universal role for these proteins in desiccation tolerance. Since freezing stress can be considered a desiccation stress at low temperatures, similar proteins may be induced by ABA or cold acclimation treatment to increase freezing tolerance. In addition, ABA responsive homologous regulatory sequences were found to be in common with these genes. ABA may, therefore, play a central role in mediating a molecular response to developmental and environmental stimuli.

2.3.3 Gene expression during ABA induced acclimation

As mentioned above, ABA is thought to be involved in the adaptation of plants to freezing stress. In this section, we will concentrate on changes observed at the molecular level during ABA induced hardening.

Changes in protein synthesis during ABA induced hardening have been observed in alfalfa suspension culture (134), alfalfa seedlings (111, 112), *Arabidopsis* seedlings (91), *Brassica* suspension cultures (76, 77), bromegrass suspension culture (135, 92), potato stem (156) and suspension culture (93).

The electrophoretic pattern of soluble proteins change during ABA induced hardening in alfalfa (*M. sativa* cv Wisconsin 22C) suspension culture cells (134). ABA treated alfalfa (*M. falcata* cv 'Anik' and *M. Sativa* cv. 'Trek') seedlings also have an altered pattern of protein synthesis (111, 112). Some of the newly

synthesized polypeptides have molecular weights and pIs similar to those expressed in cold acclimated seedlings. It is difficult to determine if these novel polypeptides are associated with hardening from the results given.

ABA treatment of *Arabidopsis* seedlings for 3 to 4 days increases hardiness by 4°C (91). In comparison, low temperature hardened the plants much more slowly even though the ultimate hardiness was the same whether by ABA or low temperature. 2D-PAGE of *in vivo* labeled proteins reveal approximately 11 polypeptide species induced by low temperature and 15 by ABA. Eight of the polypeptides appear to be induced by either ABA or low temperature (91).

B. napus cv Jet Neuf suspension cells can harden to an LT_{50} of -20°C when cultured in ABA for 8 days (119) during which the pattern of protein synthesis changes (76, 77). One of the *in vivo* synthesized polypeptides was associated with an endoplasmic reticulum enriched membrane fraction.

Bromegrass suspension culture cells treated with ABA for 4 to 5 days increased hardiness to -30°C (21, 92). The change in protein synthesis throughout 86 h of ABA treatment indicated a sequential induction of new polypeptides during hardening (135). Two extracellular proteins were present only in the media of ABA or cold treated cultures (136). During the first day of treatment 7 new or increased level and 3 decreased level SDS soluble polypeptides were identified (92). After 5 days, 22 new polypeptides appeared. Cold treatment for the same period of time did not induce the same number of polypeptides nor the same level of hardiness as with ABA. Both treatments did not induce any polypeptides with similar molecular weights and pIs, although 1 polypeptide with the same apparent

molecular weight and pI did disappear from both treatments (92).

ABA increases the freezing tolerance of potato (*S. commersonii*) stem cultures by 5°C (156) and changes the pattern of *in vivo* synthesized polypeptides (156). Similar to cold treatment (155), the development of hardiness was characterized by transiently expressed polypeptides. Six of the ABA responsive polypeptides have the same estimated molecular weight and pI as six polypeptides induced by cold treatment and thus may be a part of the same hardening mechanism induced by cold or ABA treatment.

As expected, the pattern of gene expression is altered during ABA induced hardening (77, 111, 113, 60, 88, 92, 93, 156). In *Brassica* suspension culture cells, a translatable RNA induced by ABA was apparently induced by cold treatment as well (76). Translation products induced by both ABA and cold were identified in potato stem culture (156), potato suspension culture (93) and bromegrass (92).

A comprehensive analysis of the changes in gene expression during ABA induced hardening in bromegrass was reported by Lee et al. (92). In addition to assessing the translatable RNA changes during 1 or 5 days of cold or ABA treatment, RNA changes were monitored: (1) during the first 12 h of ABA treatment, (2) with various ABA concentrations and (3) during deacclimation (initiated by removing ABA from the media). The response to ABA at the molecular level was extremely rapid as the expression of 13 translatable RNAs were detected within 1 hr of treatment.

ABA induced freezing tolerance is dependent on the concentration of ABA

added to the media (21). The response of bromegrass cells to increasing ABA concentration (up to the level required for optimum hardiness) was an increasing number of new RNA species. From this data, one may speculate that since cold hardiness is quantitatively inherited therefore a multigenic trait, frost tolerance requires the expression of many genes. This is then reflected by the number of new RNAs expressed at the optimal ABA concentration required to attain maximum hardiness.

In many of the species used to study cold acclimation, the hardened plant deacclimates relatively quickly (59, 109, 110, 60, 87, 155). In contrast, bromegrass deacclimation when hardened by ABA, was considerably slower (127, 73, 92). Even when ABA hardened cells were cultured in ABA-free media for 7 days, hardiness was maintained well above the control level and correspondingly, many of the ABA induced RNAs continued to be expressed (92). A close association of these RNAs with hardening and deacclimation suggests that the mRNAs induced by ABA contributes to the induction and/or maintenance of frost tolerance.

The identification of ABA specific cDNAs directly associated with hardening has not been reported yet. However, one of the *cor* cDNAs from alfalfa (111) and all of the *cor* cDNAs isolated from *Arabidopsis* (60, 87) are ABA inducible. It is likely that many more of the *cor* genes isolated in the future will also be ABA inducible.

2.4 Desiccation induced freezing tolerance

An increase in endogeneous abscisic acid concentration in plants subjected to water stress (or desiccation) have been well documented (34). The elevated level of ABA is thought to mediate a number of physiological, morphological and molecular changes in responses to water stress. These changes are necessary for plants to survive during drought stress. Evidence indicates that ABA treatment also increases desiccation tolerance (6, 34). The increase in ABA level during plant dehydration requires nuclear gene transcription (51). The up-regulation of specific genes during drought stress have been reported in several plant species, (7, 49, 114, 23, 28) and many of these are regulated by ABA as well. It is likely that the products of these drought regulated genes may play important roles in the drought resistance of plants.

The ability of the cytoplasm to tolerate freezing induced desiccation is the key mechanism for hardy plant cells to survive freezing stress (95). It is not surprising that hardy plant species that are subjected to water stress at room temperature can increase freezing tolerance (97, 17, 16, 15, 157, 142, 27, 26). Desiccation, similar to low temperature stress, increases the endogenous levels of ABA (164, 6, 162, 51, 66). During cold acclimation, plant tissue water content decreases (105, 14) and desiccation tolerance increases (116).

In red-osier dogwood stems, Chen et al. (17) determined that water stress increased the frost hardiness from -3° to -11°C in 7 days. Freezing studies using nuclear magnetic resonance spectroscopy indicated that increased hardiness in

water-stressed plants resulted from both increased tolerance of freezing and an increased avoidance of freezing (16). During water-stress induced freezing tolerance, a decrease in protein, RNAs and starch and an increase in sugar content was observed after the first three days of water stress. Starch levels continuously declined while sugar levels increased continuously along with an intermittent rise in proteins and RNAs (151).

Exposure of wheat and rye seedlings to a dry atmosphere of 40% RH at room temperature (21°C) induced some degree of freezing tolerance in the plumules similar to those produced by cold conditioning for 4 weeks at 3°C (27). Both cold and desiccation treatments altered the protein patterns during the induction of freezing tolerance, however, they did not induce similar patterns changes (25). Protein bands increased upon hardening by both treatments and was correlated to increased freezing tolerance (24, 25).

The similarities in metabolic changes observed between low temperature and desiccation induced hardening suggests a similar mechanism of frost tolerance is involved. The similarity between low temperature and drought stress extends to the molecular level. Not only are the cold regulated genes from *Arabidopsis* ABA inducible but are also drought stress responsive (60, 87). Since both low temperature and desiccation elevate ABA levels, it is not known if ABA is involved in the induction of freezing tolerance via the same mechanism.

2.5 Short day photoperiod induced freezing tolerance

Short day (SD) photoperiod can induce the first stage of cold acclimation in woody plant species (159). It has been shown that dogwood plants grown under a short day (8 h) photoperiod at non-acclimating temperatures could increase freezing tolerance (104, 16, 18). The short day effect appears to be a classical example of a phytochrome mediated response (104).

Whether short day photoperiod induced freezing tolerance involves the alteration of gene expression is not known. Recently, a short day inducible gene coding for a 32 kD bark storage protein (BSP) from poplar (*Populus deltoides*) was cloned. The effect of short day (SD) photoperiod on the accumulation of the 32 kD protein of poplar was examined under controlled environment and natural growing conditions. SDS-PAGE and protein gel blot analysis indicates that the relative abundance of the 32 kD protein increase to 20% in 10 days, and to 50% in 17 days of SD exposure (30). Immunoprecipitation of *in vitro* translation products with anti-BSP serum suggests that SD protein accumulation is correlated with changes in the pool of translatable mRNA. A full length cDNA encoding for the poplar 32 KD BSP was isolated and its nucleotide sequence determined. The derived amino acid sequence indicates that poplar bark storage protein is a basic protein (estimated pI 8.0), and is rich in serine, leucine and lysine. Like the low temperature responsive genes identified, its function is currently unknown.

Table 2.1 Plant species used to study the molecular biology of cold acclimation. Transformation ability: '+' indicates species was successfully transformed, '?' indicates transformation was not reported for the species. *In vivo / In Vitro* labeling: '+' indicates changes in protein synthesis (via *in vivo* labeling) or gene expression (via *in vitro* labeling) occured during cold acclimation, '-' indicates procedure has not been reported. cDNA clone: '+' indicates cold and / or ABA responsive mRNAs have been cloned, '-' indicates cold reponsive genes not reported. Sequence reported: '+' indicates cDNA corresponding to a cold responsive gene(s) has been sequenced, '-' indicates no sequence data reported.

Plant Species	Maximum Cold Hardiness (LT_{50})	Transformation Ability	*In Vivo/ In Vitro* Labeling	cDNA Clone	Sequence Reported
Arabidopsis thaliana	-10°C	+	+/+	+	+*
Brassica napus	-20	+	+/+	-	-
Bromus inermis (cell culture)	-40	?	+/+	+	+
Hordeum vulgare	-15	?	-/+	+	+
Medicago spp.	<-10	+	+/+	+	-
Solanum commersonii	-11	+	+/+	+	+
Spinacia oleracea	-11	?	+/+	-	-
Triticum aestivum	-21	?	+/+	+	-

*: Genomic and cDNA sequences.

2.6 Conclusions and future directions

Low temperature, water stress, and short day photoperiod are environmental cues which can induce freezing tolerance. These signals may induce the accumulation of ABA and therefore provide an endogenous signal to trigger hardening. The application of molecular biology techniques to the study of cold acclimation has permitted us to demonstrate a link between heritable hardiness traits and gene expression. A high degree of similarity between the molecular events associated with each of the environmental stimuli and with exogenous ABA treatment can be discerned. For example, cold regulated cDNAs from alfalfa (111, 113) and *Arabidopsis* (60, 87) are ABA and drought inducible as well. In *Arabidopsis* one of the cDNA clones also contains a high degree of similarity to fish antifreeze protein sequences (87) and other clones code for boil stable proteins (60). These characteristics may provide an important function for a plant in avoiding or tolerating freezing stress. The availability of cloned genes signifies the beginning of an era in the search for molecular mechanisms of cold acclimation.

Currently we have not identified genes which contribute directly to hardiness. In the near future, it is imperative to identify and verify the low temperature induced genes which are directly involved in conferring frost tolerance. The mechanisms of frost tolerance will probably be identified by determining the function of the gene products of cold hardiness genes. Identification of mutant lines, *in vitro* mutation of putative hardiness genes, or

antisense technology will provide a more definitive correlation of a putative hardiness gene to frost tolerance. The cellular location of these proteins may also give some clues to their function.

The focus of future research in cold acclimation will likely be defined by the following questions. First, how does a plant perceive cold stimulus and translate it into a molecular response? Based on our knowledge of the physiology and biochemistry of hardening, it is possible that during the early stages of cold acclimation, ABA biosynthesis is initiated, which in turn activates transcription of genes required for increasing frost tolerance. Second, are there ABA receptors inside and/or outside a cell to receive the ABA signal? ABA binding proteins located on the apoplastic surface of *Vicia* guard cell plasma membrane suggest the presence of ABA receptors (68). ABA also modifies phospholipid blayer membrane permeability (147) thus may directly alter cellular response to cold stress. The prospects of answering the second question are promising as illustrated by recent reports on the regulation of ABA responsive genes (52, 115).

Finally, can we improve cold hardiness by transferring cold hardiness genes into less hardy varieties or species? Progress in the development of frost tolerant varieties has been very slow by conventional breeding methods. The reason for this is threefold. First, we know very little of the mechanism of cold acclimation and frost tolerance. Second, winter hardiness is most likely a quantitative trait and therefore many genes are involved in frost tolerance. A survey of the literature reporting the numerous physiological and biological changes associated with cold acclimation illustrates the complex multigenic nature of frost tolerance.

Third, most crop species have a narrow base of genetic variability for winter hardiness (53), consequently further breeding for cold hardiness will only realize small gains in freezing tolerance.

Once cold hardiness genes and their functions have been identified, their transformation into economically important crops to increase frost tolerance may be possible. Cold hardiness genes may also assist the breeder by providing RFLP maps of genuine cold hardiness genes and simplify the screening of germplasm for hardiness potential. Alternatively, we may be able to improve the quality and yield of a hardy genotype by combining desired agronomic traits with greater frost tolerance.

2.7 Literature cited

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3.0 Changes in the Translatable RNA Population During Abscisic Acid Induced Freezing Tolerance in Bromegrass Suspension Culture.

3.1 Abstract

Abscisic acid (ABA) has been shown to increase freezing tolerance of bromegrass *(Bromus inermis* Leyss cv Manchar) cell suspension cultures from a LT_{50} (the temperature at which 50% cells were killed) of -7 to -30°C in 5 days at 23°C. Our objective was to study the qualitative changes in the translatable RNA population during ABA induced frost tolerance. *In vitro* translation products of poly(A)$^+$ RNA isolated from bromegrass cells with or without 75μM ABA treatment for various periods of time were separated by 2D-PAGE and visualized by fluorography. SDS soluble proteins from the same treatments were also separated by 2D-PAGE. After 5 days treatment, at least 22 new or increased abundance SDS soluble polypeptides were observed. From fluorographs, 29 novel or increased abundance *in vitro* translation products could be detected. The pattern of changes between ABA induced SDS-soluble proteins and translation products from the 2D gels were similar. A time course study (0-7 days) showed that 17 of the 29 translation products were detected after 1 day ABA treatment, and at least 14 were present after 1 hour. Cold treatment (+4°C) induced fewer changes in the pool of translatable RNA than with ABA treatment. Three translation products induced by cold appear to share similar pI and MW to 3 of the ABA induced translation products. Majority of the ABA inducible translatable RNAs appeared at 10^{-5}M or higher which coincides with the induction of freezing tolerance. Many of these ABA inducible RNAs persisted 7 days after ABA was removed from the media and correspondingly the LT_{50} (-17°C) was still well above the control level (-7°C). The results suggest that ABA alters the pool of

translatable RNAs during induction of freezing tolerance in bromegrass suspension culture cells.

Abbreviations:

CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate; 2D-PAGE, 2-dimensional polyacrylamide gel electrophoresis; dH_2O, distilled water; IEF, isoelectric focusing; LT_{50}, temperature at which 50% cells were killed; 2-ME, 2-mercaptoethanol; pI, isoelectric point; PCV, packed cell volume; TTC, 2,3,5-triphenyl tetrazolium chloride.

3.2 Introduction

The adaptation of plants to low temperature stress involves changes in several metabolic pathways (14), including alterations in protein synthesis (8, 10, 17, 20, 26, 29). The appearance of novel polypeptides has been correlated with an increase in cold hardiness (8, 10). Furthermore, changes in the translatable RNA population during low temperature treatment were observed (9, 20, 21). These results suggest an important role for protein synthesis and gene expression during cold acclimation.

Several lines of evidence from the literature suggest that abscisic acid (ABA) is involved in the adaptation of plants to freezing stress: (1) endogenous levels of ABA were shown to increase during the process of cold acclimation (4); (2) exogenous application of ABA at room temperature can induce freezing tolerance in whole plants (4, 17, 21) and in cell cultures (5, 13); (3) ABA induced freezing tolerance was observed only in plant species which could be cold hardened, and not in species that lack the ability to cold acclimate (5) and; (4) similar to cold treatment, ABA has been shown to affect both the pattern of protein synthesis and gene expression during the induction of freezing tolerance (15, 21, 29). Novel polypeptides were observed during ABA induced cold hardening, and some of these polypeptides appear to be induced by low temperature treatment as well (17, 21, 26, 29).

Chen and Gusta (5) have shown that freezing tolerance in bromegrass cell suspension cultures can be induced by ABA at room temperature, thus bypassing the low temperature treatment required for hardening. The bromegrass system

furthermore has several desirable characteristics for examining ABA induced gene expression associated with freezing tolerance. These characteristics include: [1] a fast growing cell culture and established characteristics of culture growth and cold acclimation (5, 13, 27), [2] a homogenous system with minimum barriers to phytohormone uptake, [3] one of the most hardy monocot cultures available and is therefore a potential source of "cold hardiness genes", [4] a considerably hardier system than dicot systems (4, 8, 9, 15, 20) used to study cold hardiness, thus, may represent different regulatory mechanisms for cold acclimation (LT_{50} for ABA treated cells can be lower than -30°C, whereas in other systems the LT_{50}s are only -12 to -15°C), and [5] one of the few monocotyledonous systems where regeneration of cells and plants from protoplasts and cells respectively are possible (16).

This study presents evidence for SDS soluble polypeptide and translatable RNA population changes associated with ABA induced freezing resistance in bromegrass culture cells.

3.3 Materials and Methods

3.3.1 Plant material

Smooth bromegrass (*Bromus inermis* Leyss) cell suspension cultures (BG970) were obtained from Dr. K.N. Kao, Plant Biotechnology Institute, National Research Council, Canada. The cultures were maintained in 250 ml flasks containing 50 ml of Eriksson's liquid media (7) supplemented with 0.5 mg/l 2,4-D (5). Cultures were incubated at 23°C on a rotary shaker. Approximately 1.5 ml PCV (volume of cells after 1000 x g centrifugation for 5 min) of cells were transferred to fresh media weekly.

3.3.2 Treatments and freezing tests

Media from 5 day old cultures were decanted and the cells were transferred to fresh media either with or without 75μM (±) ABA (Calbiochem). The cultures were then incubated at 4°C or 23°C for various periods of time. After treatment, the cultures were harvested by filtering the cells through 2 layers of Kimwipe. A sample of the treated cells were immediately frozen in liquid nitrogen, and stored at -70°C. The remaining cells were washed with 250 ml dH_2O to remove remaining media and prepared for freezing tests.

The washed cells were placed into 10x75 mm culture tubes (0.2 ml PCV/tube), water aspirated off, and then placed into a Neslab low temperature bath set at -1°C. Ice crystals were then added to the cells to initiate freezing and

left overnight at -1°C before lowering the temperature. Cells were then cooled at 4°C/h, with samples removed at 2°C intervals and thawed at 4°C overnight. LT_{50} was determined by the TTC reduction assay (28).

3.3.3 Protein extraction

Cell samples were removed from -70°C storage, and ground to a fine powder in liquid nitrogen with a mortar and pestle. The powder was then transferred into a 50 ml polypropylene centrifuge tube and 3 volumes (w/v) of protein extraction buffer (50 mM Tris-HCl pH 8.0, 2% SDS, 5% 2-ME and 1 mM PMSF) was added and homogenized with a Tekmar Tissumizer set at 90 (4 x 30 s pulses). The slurry was centrifuged at 15,000 x g at +4°C for 30 min and the supernatant filtered through a glass wool plugged funnel into a 30 ml Corex tube. Proteins were then precipitated with 5 volumes (v/v) of cold acetone for 30 min at -20°C. After centrifuging at 9000xg at 4°C for 30 min, the supernatant was discarded and the protein pellet washed twice with cold acetone and air dried.

3.3.4 RNA isolation

Extraction of total RNA is essentially as described by Commere et al. (6). Frozen cells were ground to a fine powder in liquid nitrogen with a mortar and pestle and then transferred to a polypropylene tube containing three volumes (w/v) of RNA extraction buffer (50 mM Tris-HCl pH 8.0, 10 mM EDTA, 5M guanidine isothiocynate, 5% 2-ME and 2%(w/v) sarcosine) and homogenized with

the Tissumizer (set at 90) for 4 x 30 s pulses. After centrifugation at 15,000xg at 20°C for 30 min, the supernatant was collected and adjusted to 0.1 mg/ml CsCl with 5.7 M CsCl solution, containing 50 mM EDTA, and then layered onto a 4ml CsCl cushion in a 13.5 ml polyallomer Quick-seal tube (Beckman). The sample was centrifuged at 60,000 rpm (Ti70.1 rotor, Beckman) at 15°C for 4.5 h. The resultant RNA pellet was resuspended in 400 μl of resuspension buffer (10 mM Tris-Cl pH 7.5, 1 mM EDTA and 0.1% SDS). The RNA solution was heated at 65°C for 10 min, followed by centrifugation for 5 min at 10,000xg to remove any remaining particulate matter. The RNA was precipitated with 0.1 volume 3 M NaOAc and 2.2 volumes 100% ethanol at -20°C overnight. The precipitate was collected by centrifuging at 10,000xg at 4°C for 20 min and then washed 2 times with cold 70% ethanol. The pellet was then solubilized in resuspension buffer and reprecipitated 2 additional times and finally dissolved in dH_2O. RNA concentration was estimated by measuring 260 nm absorbance. The ratio of A_{260}/A_{280} ranged between 1.85 and 2.10. RNA samples were then stored at -70°C.

Poly(A)$^+$ RNA was purified twice by oligo(dT) cellulose chromatography using a Stratagene mRNA purification kit (La Jolla, CA) according to the instructions provided by the manufacturer. The oligo(dT) columns were regenerated by standard methods (18). The poly(A)$^+$ RNA was then precipitated with 0.1 volume (v/v) of 3 M NaOAc and 2.2 volumes (v/v) of ethanol and stored at -20°C overnight. After centrifuging at 9000xg at 4°C for 30 min, the RNA pellet was washed 2 times with cold 70% ethanol and dried in a Speed-Vac (Savant). The pellet was then resuspended in 10 μl H_2O, and then stored at -70°C.

3.3.5 *In Vitro* translation

Poly(A)$^+$ RNA was translated using a rabbit reticulocyte lysate *in vitro* translation system (NEN-Dupont) in the presence of 50 μCi [^{35}S]-methionine (specific activity 1149 Ci/mmol). Procedures for the *in vitro* translations followed the protocols described in the NEN-Dupont translation kit manual.

3.3.6 Electrophoresis

Proteins from either the SDS extracted cell samples or the *in vitro* translation products were separated by 2D-PAGE as described by O'Farrell (23) and modified by Holloway and Arundel (11) and BioRad (Bulletin #1144). The acetone precipitated protein pellet was resuspended in approximately 500 μl of lysis buffer (9.5 M urea, 3% (w/v) CHAPS (Sigma), 3% (w/v) ampholytes (1.5% pH 3-10 and 1.5% pH 5-7, BioRad) and 5% 2-ME). The protein solution was spun in a microfuge (10,000xg, 30 min) to remove any remaining particulate matter. A volume of protein solution equivalent to 200 μg protein was mixed with a 1/2 volume of glycerol and 0.5 part (w/v with respect to glycerol) solid urea and mixed until the urea dissolved. The mixture was loaded onto a 2.5 x 140 mm isoelectric focusing tube gel consisting of 9.5 M urea, 4% acrylamide, 3% CHAPS and 3%(w/v) ampholytes (same ratio as in the lysis buffer). Gels were focused using 2 M NaOH cathode and 0.015 M H_3PO_4 anode electrolytes for 15 hrs at 600 V followed by 1 h at 1000 V. Methods for the second dimension SDS-PAGE run were essentially those by O'Farrell (23) with stacking and running gel

concentrations at 4% and 12.5% respectively and electrophoresed at 20 mA/gel constant current. Slab gels were stained with Coomassie blue R-250 (Sigma). Quantification of proteins was based on a modified Lowry's method using BSA as the standard (25).

The procedure for separating the labeled *in vitro* translation products is similar to the above procedure for SDS soluble proteins except for the following modification. To 400,000 cpm of TCA precipitable proteins, one volume of 2x lysis buffer (1x urea), 1/2 volume glycerol, and 0.75 part (w/v of sample) solid urea were added. The labeled slab gels were fixed in 50% methanol, 10% acetic acid for 30 min, followed by a 20 min dH_2O wash and then soaked in 10 volumes (v/v) of 1 M sodium salicylate (Sigma) for 25 min (3). After drying between 2 layers of cellulose acetate, the gel was exposed to X-ray film (Kodak XAR-5) for 36 hr at -70°C. The pH gradient of the first dimension gel was determined with a blank IEF gel. [^{14}C]-labeled molecular weight markers (BRL) were applied at the second dimension to estimate the molecular weight (M_r) of each polypeptide spot.

All of the above procedures were replicated at least 2 times. To facilitate analysis of figures containing electrophoretic data, induced or increased abundance polypeptide spots are indicated by circles and polypeptide spots which have decreased in abundance or disappeared are indicated by triangles. ABA or cold induced changes are assigned a sequential number or letter respectively.

3.4 Results

3.4.1 Cold hardiness

The effect of ABA treatment on bromegrass freezing tolerance (LT_{50}) is presented in Fig. 3.1. Cells cultured in media containing 75 μM ABA at 23°C for 5 days decreased the LT_{50} from -7°C to -30°C. During the same period of time, the LT_{50} of cells transferred to media without ABA decreased only by 2 degrees to -9°C. These results are in agreement with those reported by Chen and Gusta (5). After 5 days of ABA treatment, the cells were washed with 5 volumes of fresh media and then transferred to fresh ABA-free media for a further 7 days of growth. Control cells were also similarly treated. The effect of ABA removal on cold hardiness was then determined (Fig. 3.1). Washing did not immediately change the hardiness of the control or ABA treated cells. Seven days after the removal of exogenously applied ABA from the medium, the LT_{50} of cells increased from -30°C to -17°C.

A comparison of the effects between ABA and low temperature (+4°C) treatment on the freezing tolerance of bromegrass cells were also made in a separate experiment. Table 3.1 summarizes the results of hardiness levels after 1 or 5 days treatment. One day of ABA treatment lowered the LT_{50} by approximately 7°C and by 5 days in ABA, the LT_{50} decreased from -6.5°C to -30.1°C. When cells were grown at +4°C, the LT_{50} decreased only to -15°C after 5 days. A combination of ABA at +4°C for 5 days resulted in an intermediate level of hardiness at -24.8°C.

3.4.2 Alterations in the polypeptide population

Changes in the polypeptide population are shown in Fig. 3.2. A summary of the relative molecular masses (M_r) and isoelectric points of polypeptides that changed in abundance is given in Table 3.2. One day of ABA treatment, induced or increased the abundance of 7 polypeptides as visualized by Coomassie blue staining. The abundance of 3 low molecular mass polypeptides decreased after 1 day of ABA treatment. One day at +4°C resulted in the disappearance of a 60 kDa, pI 6.33 polypeptide but no new polypeptides were observed. Five days of ABA treatment induced the expression or increased the abundance of 22 polypeptides and attenuated expression of 8 other polypeptides. Approximately two-thirds of these novel polypeptides occur towards the basic end; ranging from pH 6.67 to 7.10 with M_r of 52 to 21 kDa. Incubation at +4°C for 5 days induced 6 new polypeptides and decreased the abundance of 2 others.

Neither ABA nor low temperature treatment induced expression of similar SDS extractable polypeptides. However, the expression of a 12 kDa, pI 6.75 polypeptide (D2=D8, Table 3.2) was attenuated by either treatment.

3.4.3 Alterations in the translatable mRNA population

ABA and to a certain extent +4°C treatment altered the SDS soluble polypeptide population in the bromegrass system (Fig. 3.2). To determine whether qualitative changes also occurred with the translatable RNA population, *in vitro* translations of the poly(A)$^+$ RNA fraction from each of the treatments were

performed. The 2D fluorographs of the *in vitro* translated products are presented in Fig. 3.3 and the respective M_r and pI's are summarized in Table 3.3. One day of ABA treatment induced or increased the expression of at least 17 translatable mRNA species. The translation products were distributed in a pattern similar to the polypeptide profile of the same treatment (Fig. 3.2). The majority of the changes occur around 18 to 55 kDa, pI 6.5 to 7.4. Only 1 translation product decreased after 1 day ABA treatment. At +4°C (1 day), 3 new translatable mRNAs were observed and 2 large M_r translation products (94 kDa, pI 6.33 and 58 kDa, pI 6.65) disappeared.

There were at least 29 new or increased abundance translation products observed after 5 days of ABA treatment (Fig. 3.3), which is an increase of 12 more translatable RNA relative to 1 day ABA treatment. Of the 12 new translatable RNAs, 6 of these have translation products with lower M_r, ranging from 19 to 13 kDa (pI 6.65 to 6.74). Although the 2D gel profile of the 29 ABA induced translation products do not correspond exactly with the 22 or so ABA induced SDS soluble polypeptides a similar pattern of change is evident (cf. Fig. 3.2 and Fig. 3.3 ABA treatments). Little change in the translatable RNA population was detected between 5 and 7 days of ABA treatment (data not shown).

After 5 days at +4°C, 7 new translatable mRNAs were detected, and in addition to the 2 translation products that decreased after 1 day treatment, 3 more decreased in abundance (Fig. 3.3). Overall the degree of change in the translatable RNA population by cold treatment was considerably less than with ABA treatment. There were, however, some similarities between the two after 5

days treatment. The 3 translation products with M_r of 19 (C22, see Table 3.3), 13 (C26) and 19 (C23) kDa and pIs of 6.65, 6.74, and 6.18 respectively, induced by 5 day ABA treatment appear to be the same ones as CE (see Table 3.3), CF, and CK from 5 day +4°C treatment. The two translation products that disappeared from both 5 day ABA and +4°C had M_rs of 27 (D1 = DE) and 50 (D2 = DD) kDa and pIs of 6.68 and 6.79 respectively.

Since 1 day of ABA treatment already induced 17 novel translatable RNAs, a short time course was performed to determine when they appeared. Fig. 3.4 shows the translation products after 0, 1, 8 and 12 h of 75 μM ABA treatment. Within 1 h after ABA addition, at least 13 novel translatable RNAs were detected. By 8 h, 24 translatable RNAs were observed, as well as the disappearance of polypeptide D1 (see Table 3.3). The polypeptide pattern at 12 h, consisting of 25 new polypeptides relative to the control, has more changes than after 1 day of ABA treatment as some of the novel polypeptides present after 1 or 8 h disappeared by 24 h. Polypeptides 15 and 16 are expressed intensely within 1 h and up to 1 day treatment but disappear after 5 days. Polypeptide 14 on the other hand is strongly expressed by 1 h, however the signal attenuates considerably by 8 h but persists even after 5 days.

It has been shown that ABA induced freezing tolerance is dependent upon the concentration of ABA applied (5). After 1 day of ABA treatment at concentrations of 0, 1, 10 or 75 μM, changes in the pattern of translatable products were determined as illustrated in Fig. 3.5 . At 1 μM, only 1 new polypeptide was observed, but at 10μM, 15 polypeptides were present as well as the disappearance of polypeptide D1 (see Table 3.3). At 75 μM, which was

previously shown to be the optimal concentration for inducing maximal frost hardiness (5), polypeptides 14 and 16 appeared. At 100 μM, there were no new translation products detected, but rather attenuated some that occurred at lower concentrations (data not shown). Thus, changes in the pool of translatable RNAs also appears to be dependent upon ABA concentration.

It has been reported that the removal of ABA from bromegrass cell suspension cultures results in a decrease in hardiness (13). However, even after allowing for a period of deacclimation (ABA removal), the LT_{50} was still above the control LT_{50}. This was confirmed by this study as illustrated in Fig. 3.1. The growth rate of bromegrass cell cultures in the presence of ABA is significantly reduced relative to cultures grown without ABA (27). This was also observed with the ABA treated cultures used for this experiment (data not shown), but the growth rate increased (based on pack cell volume) after ABA was removed. By the end of 7 days deacclimation, the PCV had almost doubled relative to pre-washed ABA treated cells while hardiness remained high. Changes in the pool of translatable RNAs were also observed at 0, 2, 4 and 7 days after ABA removal. Only the translation products at 0 and 7 days deacclimation are shown in Fig. 3.6 since changes at 2 and 4 days were minor. As mentioned earlier, deacclimation was initiated by washing the cells with fresh media containing no ABA. Immediately after washing, ABA and control cells were frozen in liquid nitrogen and subsequently poly(A)$^+$ RNA was *in vitro* translated. Washing apparently attenuated the expression of some ABA induced translatable RNAs. There are fewer ABA induced translation products immediately after washing (Fig. 3.6, 0 day deacclimation) than just before washing (Fig. 3.2, 5 day ABA). Washing however,

did not affect the LT_{50} (-30°C). After 7 days deacclimation, only approximately 5 translatable mRNAs disappeared. Thirteen of the ABA induced translation products were still present after 7 days and correspondingly the LT_{50} (-17°C) was still well above the pre-ABA treatment level (-7°C).

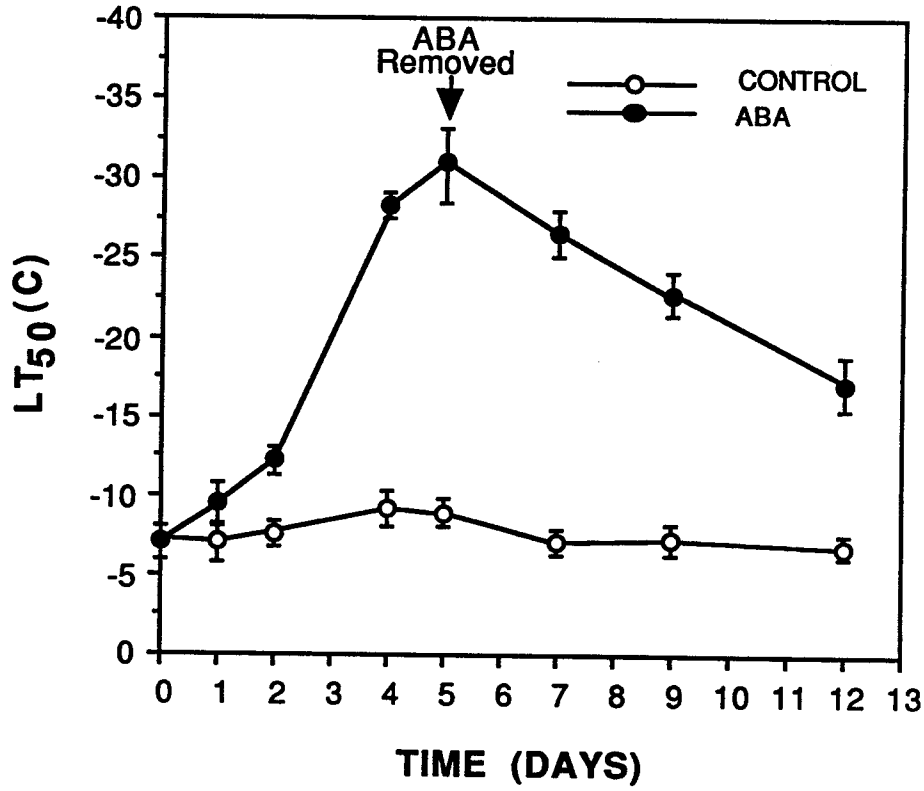

Figure 3.1 Time course of ABA-induced cold hardiness. For the ABA removal experiment, cells were washed with fresh ABA-free media after 5 days treatment and cultured in ABA-free media for up to 7 days. Arrow head indicates the removal of ABA from culture medium. Vertical bars represent ± SE (n=3).

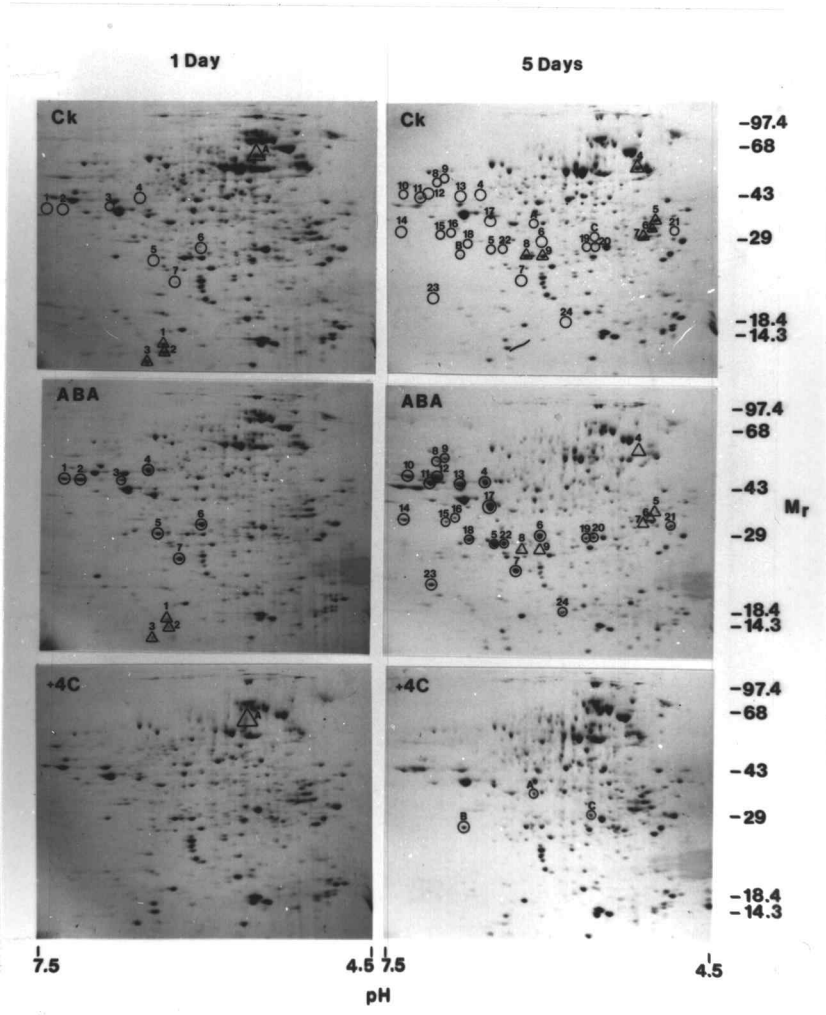

Figure 3.2 2D-PAGE of SDS soluble polypeptides extracted from bromegrass cells after 1 or 5 days ABA (75μM) or cold (+4^{o}C) treatment. Open circle in the controls (Ck) indicate the induction or increased abundance of the polypeptides after treatment. Open triangle in the controls indicate the disappearance of the polypeptide after treatment. Numbers indicate ABA treatment and letters indicate cold treatment.

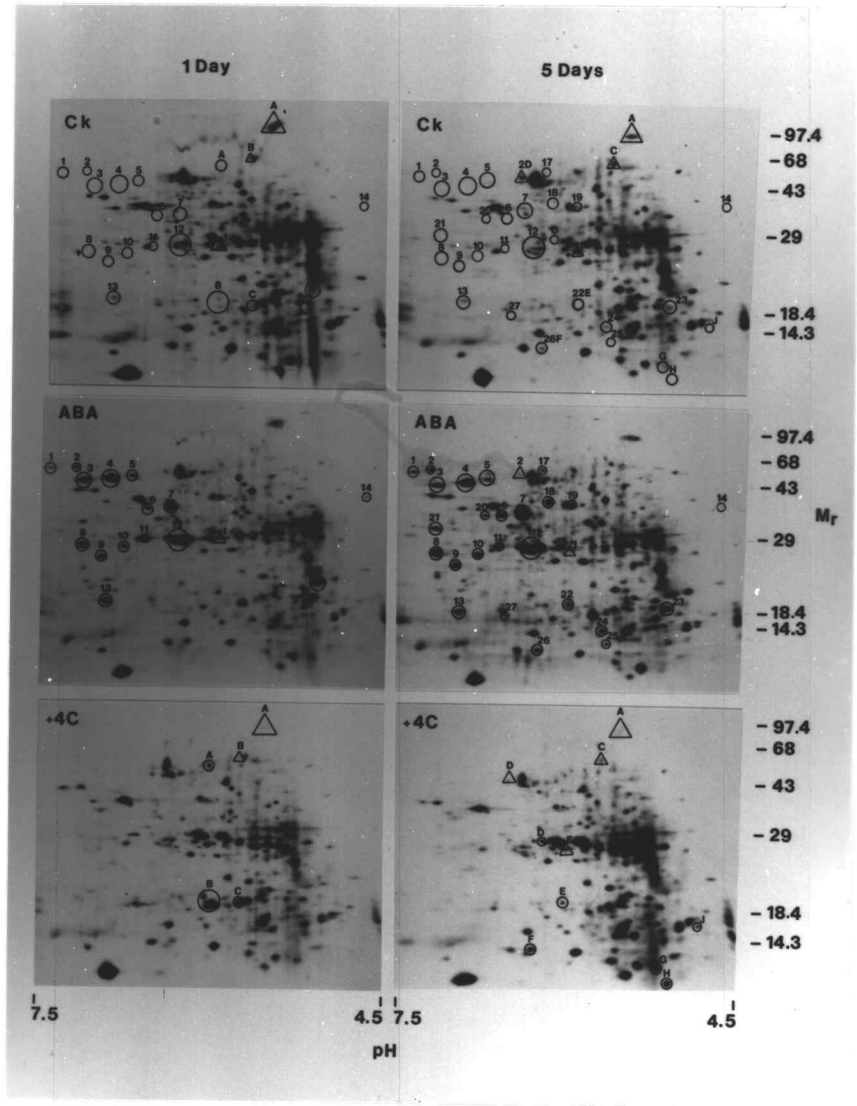

Figure 3.3 Fluorographs of 2D-PAGE gels from *in vitro* translation products of poly $(A)^+$ mRNA from bromegrass cells after 1 or 5 days ABA (75 μM) or cold ($+4^oC$) treatment. Open circles in the controls (Ck) indicate the induction or increased abundance of the translatable mRNA after treatment. Open triangles in the controls indicate the disappearance of the mRNA after treatment. Numbers indicate ABA treatment and letters indicate cold treatment.

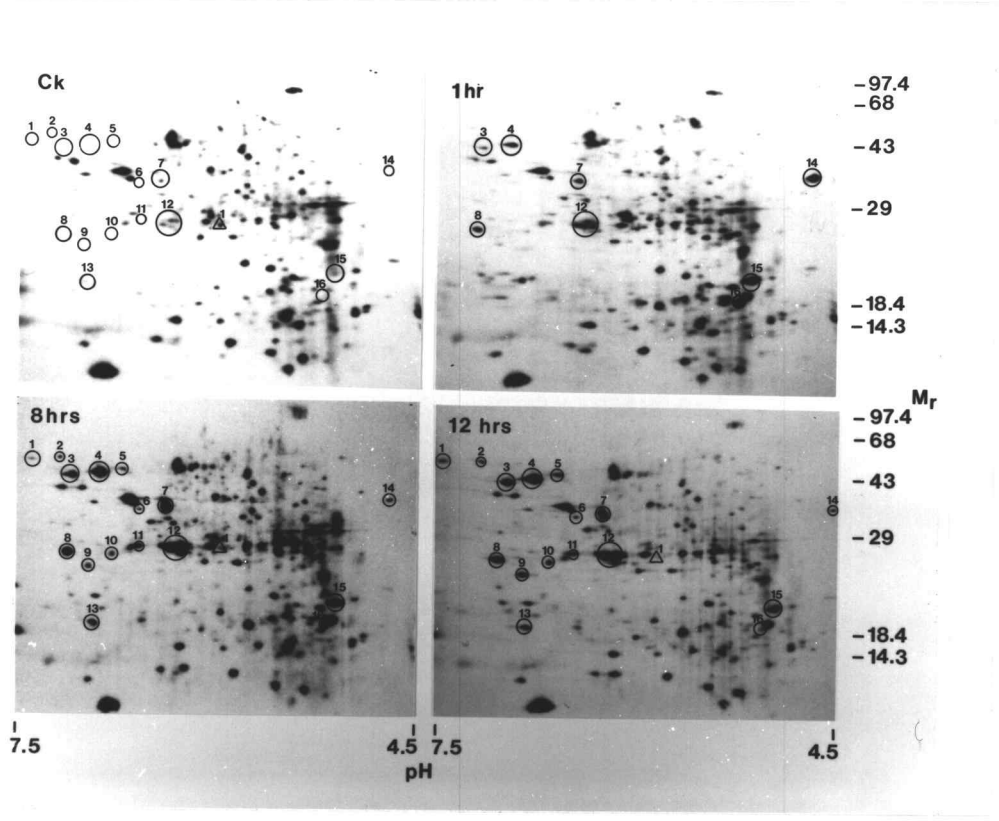

Fig. 3.4 Fluorographs of the translation products after 0, 1, 8 or 12 hours of 75 μM ABA treatment. Open circles in the control (Ck) indicated the induction or increased abundance of translatable RNA after the specified treatment time. Open triangle indicates disappearance. Numbers in common with those in Fig. 3.3 (1 day ABA) refer to the same peptides.

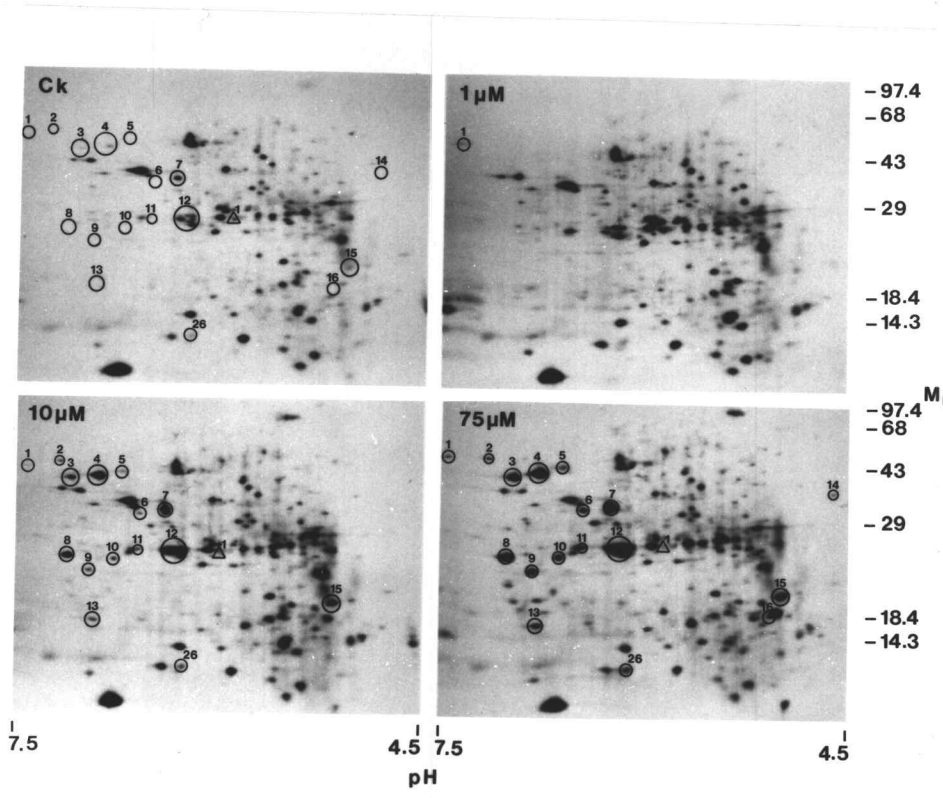

Fig. 3.5 Fluorographs of the translation products after 1 day of 0, 1, 10, or 75 μM ABA treatment. Open circles in the control (Ck) indicated the induction or increased abundance of the translatable mRNA after treatment with the specified concentration and open triangle means disappearance. Numbers in common with those in Fig. 3.3 (1 day ABA) refer to the same peptides.

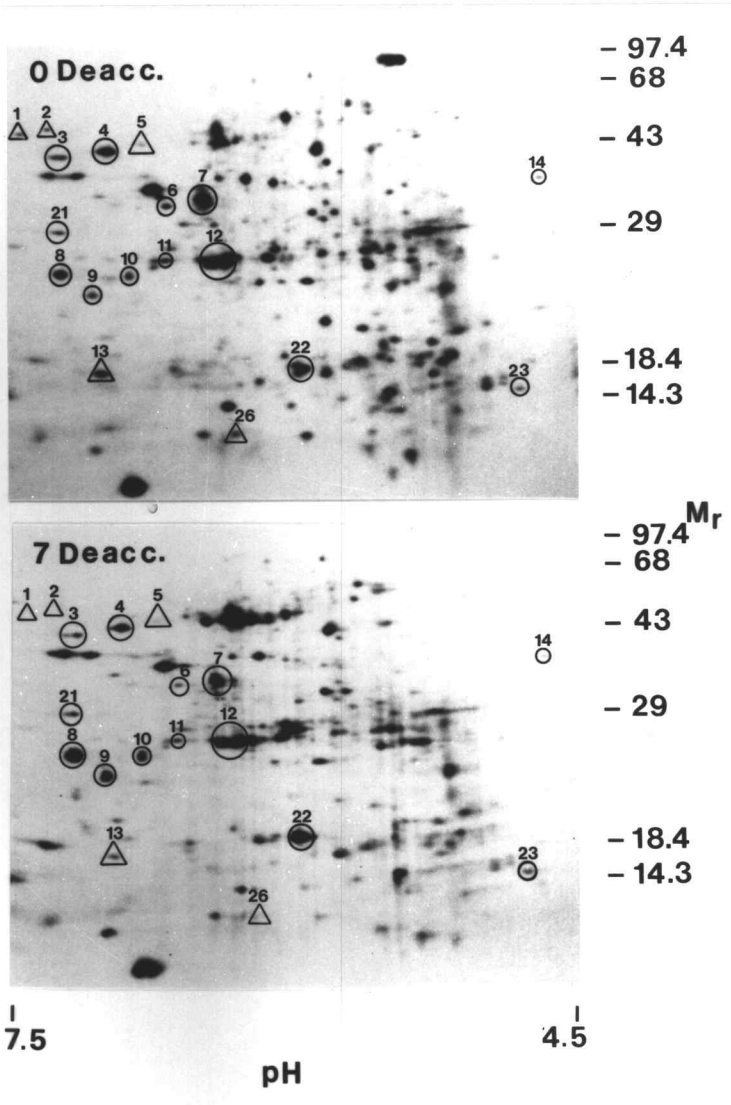

Fig. 3.6 Fluorographs of the translation products after 0 or 7 days deacclimation. Bromegrass cells were washed with fresh ABA-free media after 5 days ABA treatment and then mRNA isolated immediately (0 Deacc.) or 7 days later (7 Deacc.). Open circles indicate the persistence of ABA induced translatable mRNA after ABA removal and open triangles denote the disappearance of the mRNA 7 days after washing. Numbers in common with those in Fig. 3.3 (5 day ABA) refer to the same peptides.

Table 3.1 Effect of low temperature, ABA or both on the frost tolerance of bromegrass cell suspension cultures after 1 or 5 days treatment. Values are means ± SE (n=3).

	LT_{50}(C)		
	Days of Treatment		
Treatments	0 Day	1 Day	5 Day
Control	6.5±0.8	-6.8±0.4	-7.7±0.6
ABA	---	-13.2±1.9	-30.1±2.3
+4°C	---	-7.1±0.5	-15.3±1.8
ABA, +4°C	---	-6.9±1.1	-24.8±2.0

Table 3.2 SDS extractable polypeptides which have increased or decreased in abundance after ABA or cold treatment.

ID[a]	M_r (kDa)	pI	Time[b] (Day)	Treatment	ID[a]	M_r (kDa)	pI	Time[b] (Day)	Treatment
C1	43	7.10	1	ABA	C23	21	6.92	5	ABA
C2	42	7.00	1	ABA	C24	18	6.58	5	ABA
C3	41	6.80	1	ABA	C25	46	6.76	5	ABA
C4	46	6.77	1, 5	ABA	D1	15	6.78	1	ABA
C5	28	6.76	1, 5	ABA	D2	12	6.75	1, 5	ABA
C6	30	6.67	1, 5	ABA	D3	11	6.77	1	ABA
C7	24	6.72	1, 5	ABA	D4	58	6.21	5	ABA
C8	51	6.91	5	ABA	D5	37	6.10	5	ABA
C9	52	6.89	5	ABA	D6	33	6.13	5	ABA
C10	46	7.07	5	ABA	D7	32	6.15	5	ABA
C11	44	6.95	5	ABA	D8	28	6.71	5	ABA
C12	45	6.91	5	ABA	D9	28	6.65	5	ABA
C13	44	6.83	5	ABA	D10	19	5.35	5	ABA
C14	33	7.09	5	ABA	D11	16	5.68	5	ABA
C15	32	6.86	5	ABA	CA	33	6.68	5	+4C
C16	33	6.84	5	ABA	CB	27	6.82	5	+4C
C17	36	6.77	5	ABA	CC	30	6.33	5	+4C
C18	28	6.81	5	ABA	CD	59	6.40	5	+4C
C19	29	6.43	5	ABA	CE	58	6.33	5	+4C
C20	29	6.33	5	ABA	CF	59	6.31	5	+4C
C21	31	5.83	5	ABA	DA	60	6.33	1	+4C
C22	27	6.74	5	ABA	DB	12	6.75	5	+4C

[a] ID's corresponding to Figure 2. The first character "C" or "D" refers to circles (increased abundance) or triangles (decreased abundance) respectively in the figure.

[b] Increased or decreased abundance after 1, 5 or both 1 and 5 days of treatment.

Table 3.3 Polypeptides of *in vitro* translated RNAs which have increased or decreased in abundance after ABA or cold treatment.

ID[a]	M_r (kDa)	pI	Time[b] (Day)	Treatment	ID[a]	M_r (kDa)	pI	Time[b] (Day)	Treatment
C1	47	7.40	1, 5	ABA	C26**	13	6.74	5	ABA
C2	55	7.20	1, 5	ABA	C27	18	6.82	5	ABA
C3	43	7.17	1, 5	ABA	C28	22	7.12	5	ABA
C4	44	6.97	1, 5	ABA	C29	20	6.75	5	ABA
C5	44	6.84	1, 5	ABA	C30	17	6.60	5	ABA
C6	34	6.82	1, 5	ABA	C31	12	6.75	5	ABA
C7	35	6.77	1, 5	ABA	D1	27	6.68	1, 5	ABA
C8	26	7.13	1, 5	ABA	D2	50	6.79	5	ABA
C9	24	6.97	1, 5	ABA	CA	55	6.65	1	+4C
C10	26	6.87	1, 5	ABA	CB	18	6.63	1	+4C
C11	27	6.82	1, 5	ABA	CC	19	6.46	1	+4C
C12	28	6.75	1, 5	ABA	CD	28	6.78	5	+4C
C13	18	6.95	1, 5	ABA	CE*	19	6.65	5	+4C
C14	38	5.25	1, 5	ABA	CF**	13	6.74	5	+4C
C15	21	6.15	1	ABA	CG	12	6.19	5	+4C
C16	18	6.21	1	ABA	CH	11	6.09	5	+4C
C17	55	6.74	5	ABA	CJ	16	5.50	5	+4C
C18	38	6.72	5	ABA	CK***	19	6.18	5	+4C
C19	37	6.65	5	ABA	DA	94	6.33	1, 5	+4C
C20	34	6.84	5	ABA	DB	58	6.65	1	+4C
C21	30	7.15	5	ABA	DC	60	6.46	5	+4C
C22*[c]	19	6.65	5	ABA	DD	50	6.79	5	+4C
C23***	19	6.18	5	ABA	DE	27	6.68	5	+4C
C24	15	6.46	5	ABA	DF	21	5.80	5	+4C
C25	14	6.39	5	ABA					

[a] ID's corresponding to Figure 3. The first character "C" or "D" refers to circles (increased abundance) or triangles (decreased abundance) respectively in the figure.

[b] Increased or decreased abundance after 1, 5 or both 1 and 5 days of treatment.

[c] Three translation products induced by ABA and +4°C appear to be identical. They are: C22=CE; C26=CF; and C23=CK.

3.5 Discussion

It is well known that metabolic changes during cold acclimation are evident at both the protein and RNA level (4, 8, 9, 10, 14, 17, 20, 24, 26, 29). By [^{14}C]-leucine *in vivo* labeling, Robertson, et al. (26) have shown that ABA altered the pattern of protein synthesis in bromegrass cell cultures. The results presented in this study further demonstrate that the ABA-induced changes in the SDS-extractable polypeptide and translatable RNA population are associated with increased freezing tolerance.

An increase in the number of SDS-extractable polypeptides and translatable RNA were observed when cells were at their maximum hardiness. For example, there were 3 times more SDS-extractable polypeptides induced after 5 days in ABA (LT_{50}=-30.1°C) than after 1 day treatment (LT_{50}=-13.2°C). Furthermore, the LT_{50} of cells treated at +4°C for 5 days was about -15°C, with only 6 new polypeptides visualized. Similar changes were also observed in the pool of translatable RNA. Five days of ABA treatment resulted in more ABA induced translatable RNAs than with 1 day treatment. The cells in 75μM ABA were much hardier than the cells treated with 10μM ABA (5), and the former treatment resulted in the presence of many more translatable RNA species than the latter (Fig. 3.5). These alterations in the translatable RNA population may reflect changes in the stability or translatability of the RNA or may be the result of novel gene expression. In either case, maximum hardiness appears to be related to ABA treatments which induced the greatest number of polypeptides or translatable

RNAs.

Cold treatment induced considerably fewer polypeptide and translatable RNAs than ABA treatment. The most probable reason for the difference is that cells grown at +4°C have a much lower rate of metabolism, and therefore changes in the protein and RNA pool are expected to be slower. A longer period of cold treatment may increase the level of hardiness similar to ABA treatment and possibly accumulate more polypeptide species. Secondly, it has been shown that in *Solanum commersonii*, protein synthesis is required for cold acclimation and that elevated cold hardiness is related to increased endogenous ABA levels (4). This suggests that the induction of freezing tolerance by cold treatment is slower than by exogenous ABA application. There was nevertheless an increase in hardiness with both 5 day ABA and +4°C treatment and an apparent similarity of 3 novel translation products between 5 day ABA and +4°C treatment. Further characterization of these three RNA species and the encoding polypeptides may provide evidence for the involvement of ABA in the response of plants to cold acclimation.

Some researchers have shown that exogenous ABA treatment induces rapid gene expression (12,22). Our results indicate that a considerable number of changes in the pool of translatable RNA occurred within 1 hour of ABA treatment. This rapid response is not exclusive to ABA alone, but has also been observed with auxin (19) and cytokinin (1) induced genes. The ABA specific "rapidly inducible RNAs" from bromegrass may not be directly responsible for cold acclimation, since increased hardiness was not detected until after 1 day of

treatment. However, it is possible that a time lag occurs between molecular change and the resulting physiological response. It is also possible that some of the ABA induced RNAs may be components of other ABA mediated responses.

Given that there may be a temporal lag between gene expression and phenotypic expression, changes in the translatable RNA population were monitored during deacclimation. Certain RNAs which change in abundance during dehardening may give us a clue to which RNAs are related to hardiness. After 7 days of deacclimation, the LT_{50} was still well above -7°C and only 5 ABA induced translatable mRNAs disappeared relative to immediately after washing. The maintenance of a high level of freezing tolerance after ABA removal suggests that: (1) ABA was not thoroughly removed, therefore sufficient ABA remained to maintain a high level of hardiness; or (2) ABA may play a major role in inducing cold hardening mechanisms, but may perform a lesser role in maintaining hardiness. Two examples of the delay in physiological response after ABA levels peaked were observed in potato and maize. Firstly, in potato plants, endogenous ABA levels peaked during the early stages of hardening at +2°C, but hardiness continued to increase after the peak (4). The second example involves subjecting intact maize plants to water stress. In response to a water deficit, endogenous ABA levels increased over 20 fold resulting in stomata closure. After rewatering, ABA concentration dropped rapidly, but the stomata remained closed for a considerably longer period of time (2). These examples suggest that a similar mechanism of response can also be elicited by exogenous ABA application.

Two novel features were observed during deacclimation. First, it was

noticed that there was a rapid change in the translatable RNA population as a result of washing with fresh ABA-free media but there was no immediate change in hardiness. It is possible these translatable RNA changes are due to: (1) loss of induction of certain highly ABA- dependent translatable RNAs or (2) washing itself alters the pattern of translatable RNAs. Since the LT_{50} did not change immediately after washing, the translation products of these RNAs probably do not contribute directly to frost resistance. Another possibility is that the novel translation products are stable for sometime and thus, even in the absence of ABA, hardiness is maintained. Secondly, it was noticed that even though ABA treatment attenuated cell culture growth, removal of the hormone reversed the process, but hardiness did not decrease to non-acclimated levels. Apparently hardiness above the pretreatment level was maintained even though cell division and growth had resumed.

In summary, ABA induced cold acclimation in bromegrass cell suspension cultures altered the pattern of protein synthesis and the population of translatable RNAs. Associated with an increase in ABA induced cold hardiness is the induction or increased abundance of 22 novel polypeptides and at least 29 translatable RNA species after 5 days treatment. Cold hardiness was not induced to as great an extent by low temperature treatment when compared to ABA treatment, and fewer alterations to the pattern of SDS extractable polypeptides and *in vitro* translation products were observed. ABA rapidly changes the pool of translatable RNAs, but cold hardiness develops much later. The majority of these translatable RNAs, however, persisted for at least 7 days after ABA removal

where hardiness remained high.

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4.0 Molecular Cloning of Abscisic Acid Responsive mRNAs Expressed During the Induction of Freezing Tolerance in Bromegrass Suspension Culture.

4.1 Abstract

The freezing tolerance of bromegrass (*Bromus inermis* Leyss) suspension culture cells can be increased by abscisic acid (ABA) treatment at room temperature. Induction and maintenance of freezing tolerance by ABA, like cold treatment, is accompanied by the expression of novel polypeptides and translatable RNAs. The objective of this study was to isolate ABA responsive cDNA clones from ABA hardened bromegrass culture cells and to characterize the pattern of transcript expression during the development of freezing tolerance. Among the 16 ABA responsive cDNA clones isolated: nine were expressed only with ABA treatment; seven showed increased transcript level; and one was transiently expressed. Five of the seven increased level transcripts were also cold responsive. Deacclimation of ABA hardened cells, by transferring the culture to ABA-free media, was a relatively slow process. All of the novel transcripts persisted (albeit at lower levels) during this time. Not only is a minimum of 10μM ABA required for the induction of freezing tolerance but also for significant transcript accumulation of nine of the ABA responsive genes. Preliminary sequence data from the cDNAs have identified several clones with high homology to genes associated with sugar metabolism, desiccation tolerance and protease activity. The expression of RNAs corresponding to the cDNAs identified in this study are closely associated with the induction and maintenance of freezing tolerance in bromegrass and thus may represent genes required for tolerance to freezing stress.

Abbreviations: LT_{50}, temperature at which 50% of the cells were killed; TTC, 2,3,5-triphenyltetrazolium chloride, EST, expressed sequence tag.

4.2 Introduction

Many temperate plant species increase freezing tolerance when exposed to low non-freezing temperatures. The acclimation process involves numerous metabolic changes including an accumulation of sugars, increase in RNA levels and the requirement for protein synthesis (22). Not only is there a quantitative increase in protein and RNA levels, but the population of protein and translatable RNA species is also altered during cold acclimation (12, 36, 21). Cold inducible cDNAs corresponding to RNAs expressed during cold acclimation in alfalfa (23, 24), *Arabidopsis* (14, 18, 26) and barley (4) have been isolated. Currently, the roles of these cold inducible gene products have yet to be identified.

It has been proposed that cold induced biosynthesis of the phytohormone abscisic acid ultimately leads to an increase in freezing tolerance (7). There are three lines of evidence to support this proposal: (1) Many plant species respond to low acclimating temperatures by increasing endogenous ABA levels (7), (2) exogenous application of ABA to either whole plants (7, 20) or cell cultures (5, 16) at room temperature also increases freezing tolerance. and (3) both low temperature and ABA treatment induce expression of a common set of polypeptides and translatable RNAs (20, 38, 21).

We have previously shown that bromegrass cell cultures increased freezing tolerance by 23°C (LT_{50} from -7°C to -30°C) after four to five days of ABA treatment (5, 21). Associated with the rapid increase in hardiness were changes in the population of polypeptides (29) and translatable RNA's (21). ABA

treatment induced a greater level of hardiness and expression of more novel polypeptides and RNAs than did cold treatment for the same period of time (21). Deacclimation of ABA hardened bromegrass cells is a relatively slow process (21, 28) compared to other cold hardy species (13, 14, 38) and correspondingly, the disappearance of many of the novel polypeptides and RNAs is slow (21). The hardening response of bromegrass by ABA treatment is concentration dependent (4) and this is also reflected at the molecular level where expression of many of the translatable RNA's are also ABA dose dependent (21).

The objective of this study was to isolate and characterize ABA responsive cDNA's associated with increased freezing tolerance. Differential screening of a cDNA library constructed from Poly(A)$^+$ RNA of ABA treated bromegrass cells has resulted in the identification of 16 ABA responsive cDNA clones. Using the expressed sequence tag (EST) method of Adams et al. (1), we have identified several cDNA clones with high homology to genes involved with sugar metabolism, desiccation tolerance and protease activity.

4.3 Materials and Methods

4.3.1 Plant material and treatments

Cell suspension cultures of smooth bromegrass (*Bromus inermis* Leyss) were maintained and subcultured as described by Chen and Gusta (5). ABA treatments consisted of transferring five day old cells into fresh media containing 0.5 mg l^{-1} 2,4-D with or without 75μM ABA and incubating at 23°C on an orbital shaker for various times. For the ABA removal experiment (deacclimation), ABA treated cells were thoroughly washed with ABA-free media and then transferred into fresh ABA-free media. Cells were cold treated by incubation at +4°C on a shaker for one or five days (21). Freezing tests were essentially those of Lee et al. (21) and the TTC reduction assay (37) was used to determine the LT_{50}'s.

4.3.2 RNA isolation

Both total and poly(A)$^+$ RNA isolation have been detailed elsewhere (21). Briefly, frozen cells were grounded in liquid nitrogen and total RNA extracted by the guanidine isothiocynate / CsCl ultracentrifugation method. Poly(A)$^+$ RNA was isolated from total RNA by the push column method (21).

4.3.3 cDNA library construction and differential screening

Poly(A)$^+$ RNA (5 μg) from bromegrass cells treated with 75μM ABA for five days was used for the construction of a cDNA library. Double stranded cDNA was synthesized by the linker-primer / RNase H method of Gubler and Hoffman (11) and cloned into the EcoRI / XhoI site of the Lambda ZapII vector (Stratagene, LaJolla, CA). Primary recombinant clones were *in vitro* packaged with Gigapack II (Stratagene) and subjected to one round of amplification using *Escherichia coli* PLK-F' as the host strain.

Recombinant phage were plated at a density of 5 x 10^4 PFU / 132mm plate with the *E. coli* host strain XL1-Blue (Stratagene) and duplicate plaque lifts were made on nitrocellulose filters (Hybond-C, Amersham, Arlington Heights, IL.). First strand ^{32}P-dCTP labeled cDNA probes were made from poly(A)$^+$ RNA (30) of control or ABA treated cells, and then hybridized to the plaque lifts. Prehybridization and hybridization conditions were 6 x SSC (1 x SSC = 0.15M NaCl, 0.015M NaCitrate), 0.06 x Blotto (1 x = 5% (w/v) non-fat dried milk, 0.02% NaN_3) and 10 μg/ul poly(A)RNA at 65°C. Membranes were washed with 2 x SSC, 0.1% SDS three times at 65°C for 20 min each followed by a 30 min 1 x SSC, 0.1% SDS wash and then exposed to X-ray film (Kodak XAR-5) with one intensifier screen for 36 h.

Plaques which differentially hybridized to the probe from ABA treated cells were selected and subjected to one round of plaque purification. Positive clones were hybridized to each other to remove the ones in common. The phagemid

containing the cDNA was rescued by the *in vivo* excision method according to the manufactures directions (Stratagene). A total of 4.5 x 10^5 recombinant plaques were screened for ABA responsive genes.

4.3.4 RNA slot blot analysis

Total RNA (20μg) from ABA or cold treated bromegrass cells was slot blotted (30) onto nitrocellulose membrane (BioRad) and prehybridized, hybridized and washed under the same conditions as for the plaque lifts. Each of the cDNA probes were ^{32}P-dCTP labeled by the random priming method and hybridized to the blotted membrane. Autoradiographs of the membranes were scanned with a densitometer (model 1650, BioRad) to determine the relative steady state transcript levels.

4.3.5 RNA gel blot analysis

Total RNA from treated bromegrass cells was electrophoresed on a 1.3% agarose - formaldehyde gel (30) and blotted onto a nylon membrane (Zetaprobe, BioRad) according to the manufactures directions. Each blot was probed with one of the unique cDNAs isolated. Prehybridization and hybridization was carried out in 0.25M Na_2HPO_4, pH 7.2, 1mM EDTA, 7% SDS at 65°C. Washes were conducted in 5% SDS, 20mM Na_2HPO_4, 1mM EDTA at 65° for 30 min followed by two more washes in 1% SDS, 20mM Na_2HPO_4, 1mM EDTA at 65°C. The

membranes were then exposed to X-ray film with one intensifier screen for 2 to 24 h.

4.3.6 Cross hybridization with cDNAs from other plant species

Each of the unique bromegrass cDNAs were dot blotted on to a nylon membrane and probed with a cold or ABA responsive cDNA from other species using the same prehybridization and hybridization conditions as for RNA gel blot analysis. Filters were first washed under low stringency conditions (2 x SSC, 0.1% SDS) at 65°C and exposed to X-ray film and then washed under high stringency (0.2 x SSC, 0.1%SDS) at 65°C and re-exposed to X-ray film.

4.3.7 Generation of expressed sequence tags

Phagemids containing the bromegrass cDNAs were purified from overnight cultures by the plasmid column purification method (Stratagene). Approximately 1.5μg of phagemid in 6 μl of H_2O was then used for automated dideoxynucleotide chain-termination (31) sequencing using the Applied Biosystems, Model 373A DNA sequencer. The sequencing strategy is based on the method of Adams et al. (1) where approximately 350-400 bp of the 5' end is sequenced, thus providing enough information for the preliminary identification of similar sequences from GenBank and EMBL databases.

4.4 Results

4.4.1 Freezing tolerance

The freezing tolerance of bromegrass cells after various treatments are expressed as LT_{50}s and are summarized in Table 4.1. 75μM of ABA treatment increased the freezing tolerance from -6.5°C to -30.8°C in five days. Cold treatment for the same period of time only increased hardiness by 8.8°C to -15.3°C. Removal of ABA from the media did not affect hardiness immediately (data not shown), but seven days after washing, the LT_{50} decreased from -30.8°C to -17.1°C. ABA induced freezing tolerance is a dose dependent response and at 10μM or greater an increase in hardiness was detected after one day of treatment.

4.4.2 Isolation of ABA responsive cDNA clones

Poly(A)$^+$ RNA from bromegrass treated with ABA for five days was used for the construction of a cDNA library. Approximately 4.5 x 10^5 recombinant phages were differentially screened for ABA responsive clones using first strand cDNA probes synthesized from poly(A)$^+$ RNA of ABA-treated or control cells. Of the 100 cDNA clones isolated, cross hybridization of the clones with each other resulted in 16 unique clones.

4.4.3 RNA gel and slot blot analysis

To verify the ABA inducibility of the genes corresponding to these cDNAs, RNA slot blot analyses of total RNA from control and ABA treated cells (75μM, 1,2 or 5 day treatment) were performed (data not shown). All 16 cDNA clones were identified as ABA responsive.

Additional analysis was performed by RNA gel blot of total RNA from zero to five days ABA treatment, as well as RNA from cells that were deacclimated for seven days after five days of ABA treatment, using the ABA responsive cDNAs as probes. Three patterns of transcript accumulation were observed (data not shown). RNA gel blot examples of each pattern are illustrated in Figure 4.1. Clone A60 is an example of transcripts which are expressed predominantly during ABA treatment. The transcripts accumulate to high levels after one day ABA treatment and continued to increase after two or five days treatment. Immediately after washing the residual ABA from the cells and culturing in fresh ABA-free media, the transcript level decreased (Fig. 4.1, -ABA, 5d) with no noticeable decrease in hardiness (data not shown). Seven days of deacclimation reduced the transcript level. Clone A8 is an example of transcripts which are expressed in control cells but are present at elevated levels during ABA treatment. Increased transcript levels were detected by one day ABA treatment and continued to increase after two and five days treatment, but a low level of the transcript was also present in the controls. Very little A8 RNA remained immediately after ABA removal and after seven days deacclimation. For clone

A25, transcript accumulation during ABA treatment was transient. The RNA level was elevated after one day treatment but decreased after two and five days treatment and was expressed at very low levels after seven days of deacclimation. Clone A89 transcript level, was minimally affected by ABA treatment.

It is well known that many cold inducible genes are ABA responsive (14, 18, 23, 26). RNA gel blot analysis with RNA from cells treated for one or five days at +4°C was performed to determine if any of the bromegrass ABA responsive clones were cold responsive. Probes of the A25, A34, A56, A85 and A12 cDNAs have detected corresponding RNAs that are both ABA and cold responsive (Fig. 4.2).

As noted in Table 4.1, the level of frost tolerance attained with bromegrass culture cells is dependent on the concentration of ABA applied. The dose dependent response is also reflected at the translatable RNA (21) and in this study, at the steady state RNA level. Examples of ABA concentration responsive clones are shown in Figure 4.3. At 0.1μM ABA, little or no change in transcript level was detected for most of the clones, but with ABA concentrations greater than 1μM, significantly stronger hybridization signals were observed. Clone A56 appeared to be highly ABA responsive as a strong signal was detected at 0.1μM and by 1μM or greater, there was no further increase in the signal.

Table 4.2 summarizes the response of the ABA inducible genes to the various treatments. Fourteen of the 16 clones have an ABA dose dependent response. There were five clones that appeared to be cold responsive after five days cold treatment. Clone A25 had increased transcript levels during ABA

treatment and was cold responsive, but both ABA and cold induced gene expression were transient.

4.4.4 Cross-hybridization analysis

Cold responsive cDNAs from other species were used as probes to determine if they hybridized to any of the bromegrass ABA responsive cDNAs. All of the bromegrass cDNAs were dot blotted onto nylon membrane and were probed with barley cDNAs pA029, pT59, pA086 and pAF93 (4), *Arabidopsis* cDNAs pHH28, pHH29, pHH67 (14) and Lti18 (E. T. Palva, personal communication) and potato cDNAs (B. Zhu, personal communication) cDNAs. Under low stringency washing conditions (2 x SSC, 0.1% SDS, 65°C), *Arabidopsis* clones pHH29 and Lti18 hybridized to bromegrass clones A86 and A55 respectively. There was no hybridization signal detected after high stringency (0.2 x SSC, 0.1% SDS, 65°C) washes. Barley clone pAF93 also hybridized to bromegrass clone A55 under low stringency only. None of the potato ABA responsive clones hybridized to any of the bromegrass clones.

4.4.5 Similarity of expressed sequence tags to other genes

Expressed sequence tags (1) were determined for each of the bromegrass cDNAs and used to search for sequence similarities in both GenBank and EMBL databases using the Intelligenetics suite (Mountain View, CA). The searches

resulted in the identification of six bromegrass ESTs with high (>50%) homology to reported sequences (Table 4.3). Of interest are clones A61, A55, A56, A85, A86 with nucleic acid match to barley NADPH dependent aldose reductase (2), barley dehydrin (8), wheat germin (19), human cathepsin D (10) and barley embryo globulin (Heck et al., unpublished data) respectively and clone A72 with an amino acid match to rabbit phosphoglucomutase (27).

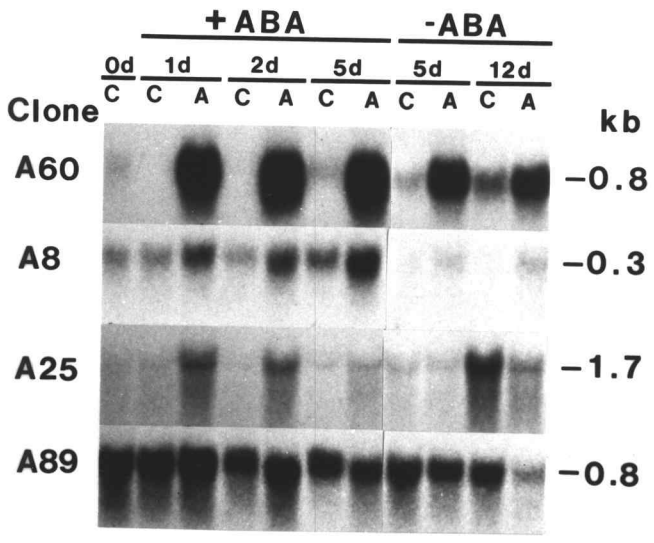

Figure 4.1 RNA gel blot of steady state transcript levels during ABA induced acclimation (+ ABA) and deacclimation (- ABA) treatment. Twenty μg of total RNA was separated on a 1.3% agarose formaldehyde gel, blotted onto nylon membrane and probed with a labeled ABA responsive cDNA. Three ABA responsive and an ABA non-responsive pattern are shown.

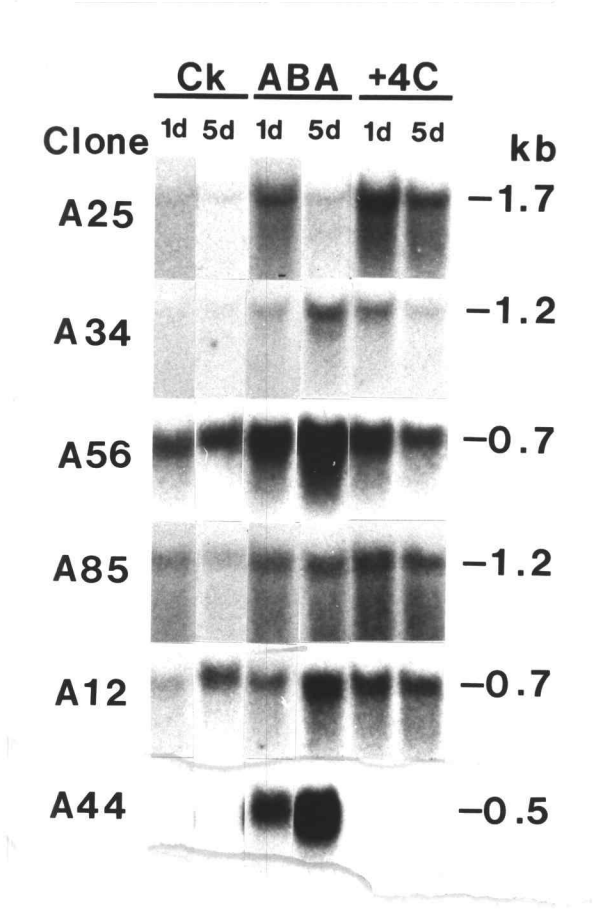

Figure 4.2 RNA gel blot of steady state transcript levels from ABA induced RNAs that are cold inducible. Total RNA (20μg) was separated on a 1.3% agarose-formaldehyde gel, blotted onto nylon membrane and hybridized with a labeled ABA responsive cDNA clone. Clone A44 is an example of an ABA responsive gene that is not inducible after five days cold treatment.

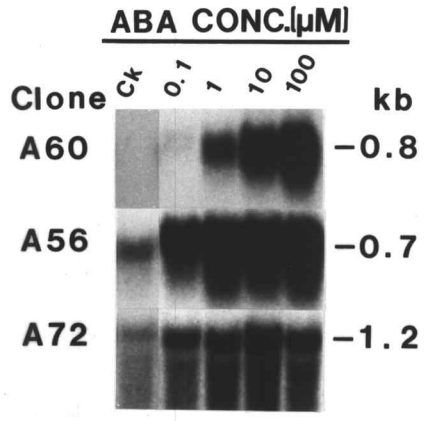

Figure 4.3 Effect of ABA concentration on steady state ABA responsive transcript levels. Total RNA (20μg) from bromegrass cells treated with various concentrations of ABA for one day were separated on a 1.3% agarose-formaldehyde gel, blotted onto nylon membrane and hybridized with labeled ABA responsive cDNA clones. Two ABA dose responsive (A60 and A56) and a non-dose responsive (A72) transcript pattern are illustrated.

Table 4.1 Freezing tolerance of bromegrass culture cells after ABA or low temperature treatment. Bromegrass cells were treated with various concentrations of ABA at 23°C or without ABA at +4°C. LT_{50}'s were assessed by the TTC reduction assay as described in Materials and methods.

Treatment		LT_{50} (°C)					
		Days of treatment					
ABA Conc.(μM)	Temp (°C)	0	1	2	4	5	12[a]
0	23	-6.5	-6.8	-7.5	-9.2	-7.7	-6.8
0	4	-	-7.1	-	-	-15.3	-
0.1	23	-	-6.5	-	-	-	-
1	23	-	-6.4	-	-	-	-
10	23	-	-7.1	-	-	-	-
75	23	-	-9.4	-12.2	-	-30.8	-17.1
100	23	-	-8.5	-	-	-	-

[a] ABA was removed on day five and cultured for seven more days in ABA-free medium.

Table 4.2 Accumulation pattern of the bromegrass ABA-responsive mRNAs in response to various treatments. Both RNA slot blot and gel blot analyses were used to determine the treatment response.

		Treatment Response				
		ABA			+4°C	ABA Conc.[a]
Clone	Transcript Size (kb)	Novel	Increased	Transient		
A55	0.6	X				X
A86	1.0	X				X
A60	0.8	X				X
A6	0.5	X				X
A44	0.5	X				X
A61	1.1	X				X
A36	1.5	X				X
A80	1.8	X				X
A64	1.4	X				X
A56	0.7		X		X	X
A34	1.2		X		X	
A85	1.2		X		X	
A72	1.2		X			
A12	0.7		X		X	
A8	0.3		X			
A25	1.7		X	X	X	
A89	0.8					

[a] ABA concentration responsive.

Table 4.3 Bromegrass ESTs with high nucleic acid or amino acid similarity to genes from other organisms. ESTs of the 16 bromegrass cDNAs were used to search both GenBank and EMBL databases. Identification refers to the name of the sequence matching to the EST; %NA and %AA indicates the percent nucleic acid or amino acid match respectively; Number and Reference refers to the GenBank accession number (unless otherwise noted) and corresponding literature citation of the matching sequence respectively.

EST	Identification	Organism	%NA	%AA	Number	Reference
A55	Dehydrin	Barley	82	-	X15287	8
A56	Germin	Wheat	55	-	M63224	19
A61	Aldose reductase	Barley	89	-	X57526	2
A72	Phosphoglucomutase	Rabbit	[a]	69	A01175[b]	27
A85	Cathepsin D	Human	53	-	M11233	10
A86	Embryo globulin	Barley	76	-	M64372	[c]

[a] The nucleic acid sequence was not available for this gene.

[b] Accession number from the PIR database.

[c] Heck et al., unpublished data

4.5 Discussion

Numerous reports have shown that novel proteins and translatable RNAs are expressed during cold acclimation (12, 36). Recently, cold acclimation specific mRNAs have been cloned (for review see 36) and the expression of transcripts corresponding to these cDNAs were highly correlated with hardiness. In potato, however, certain translatable RNAs were expressed transiently during hardening (38, 39) suggesting that the development of frost tolerance requires a cascading pattern of gene expression. In this study, we have identified 16 cDNA clones from an ABA treated bromegrass cDNA library. Transcript accumulation corresponding to these cDNAs can be categorized into three distinct patterns. The first pattern includes transcripts that are detectable predominantly under treatment conditions. This type of response is similar to cold responsive genes identified in other species (4, 14, 18, 23, 24, 29). There are two additional patterns in which transcripts are present in the controls but are up-regulated by ABA or cold treatment. In the second pattern, transcript levels remain elevated during hardening and in the third pattern the increased transcript signal is transient.

While not all ABA inducible genes may be involved in the hardening process, expression of some of these genes are likely required for the development of frost tolerance. These gene products may be responsible for the numerous novel physiological and biochemical changes detected during hardening (22). A decrease in steady state transcript levels, corresponding to the ABA inducible

cDNAs during dehardening, further suggests that some of those genes are associated with freezing tolerance. Although deacclimation was apparent as indicated by dehardening and RNA level attenuation, the process was still much slower relative to *Arabidopsis* (14, 18) at the transcript level, and spinach (13), alfalfa (25) and potato (38) at the translatable RNA level. The difference in response time may reflect a difference between monocots and dicots or the fact that bromegrass is much hardier than the other species. The persistence of ABA inducible transcripts nevertheless, may be required for maintaining an elevated level of hardiness.

Many translatable RNA changes in bromegrass (21) and potato (38, 39) were observed during the first few hours of ABA or cold treatment but were not present in later treatments. Transient gene expression during hardening indicates that the gene product is only required temporarily. Transient expressed gene such as clone A25 may not directly confer tolerance to freezing but may be components of a cascade pathway ultimately leading to some physiological response. More these type of clones would certainly have been isolated if the bromegrass cDNA library were made from RNA of one or two days ABA treatment.

It has been demonstrated that the degree of ABA induced frost tolerance is dependent on the concentration of ABA applied (5, 7, 39). A minimum of 10μM ABA is required to induce a hardening response during which many of the ABA inducible-translatable RNAs are expressed (21). Steady state transcript levels of the ABA inducible genes identified in this study do not accumulate

substantially until 10μM ABA or greater. This is consistent with the concentration of ABA required to induce hardening and therefore these genes are ideal candidates for further analysis.

Expressed sequence tags (ESTs), consisting of approximately 400 bp of the cDNA 5' end, were obtained for all 16 cDNA clones and compared to sequences in both GenBank and EMBL data bases. EST analysis of the cDNA clones corresponding to ABA responsive genes from bromegrass have provided some intriguing results. Clones A61, A55, A56, A85, A86 and A72 have high homology to a barley NADPH dependent aldose reductase (2), barley dehydrin (8), wheat germin (19), human cathepsin D (10), barley embryo globulin (Heck et al., unpublished data) and rabbit phosphoglucomutase (27) respectively.

Aldose reductase (EC 1.1.1.21) is considered an important enzyme in animals which catalyzes the stereospecific reduction of glucose to sorbitol (30). Bartels et al. (2) have cloned an ABA inducible aldose reductase related protein that is expressed during the desiccation phase of embryogenesis in barley. The association of this gene with sugar metabolism during desiccation in barley and with ABA induced freezing tolerance in bromegrass is of significance. First, it is known that sugars accumulate during cold acclimation in many species (22) including bromegrass (35). Second, cryoprotection studies have demonstrated that sorbitol is one of the most effective cryoprotectants (6). It has been suggested that there is a requirement for the upregulation of genes dealing with sugar metabolism during cold acclimation (12). Evidence for this was demonstrated in winter wheat where sucrose synthase polypeptide and poly(A)$^+$ RNA levels

increased with low temperature treatment (9). This study also suggests that a phosphoglucomutase gene, which codes for an enzyme of the glycolysis / gluconeogenesis pathway, has a higher level of expression during ABA induced hardening. The upregulation of aldose reductase and phosphoglucomutase in bromegrass and sucrose synthase in wheat therefore indicates the occurrence of osmotic adjustment during hardening.

Expression of a dehydrin-like gene in bromegrass during ABA induced freezing tolerance suggests that hardening involves increased tolerance to dehydration stress. Desiccation of barley seedlings induces the expression of dehydrin (8) and like many of the Rab (Responsive to ABA) genes (for review see 34), it is also induced by ABA treatment. Rabs are expressed concurrently with increased tolerance to water stress, however, the function of these genes are not known. Freezing stress is considered a form of desiccation stress since extracellular freezing causes cellular water to move outside the cell (22). It has been demonstrated that ABA treated bromegrass suspension culture cells not only have greater tolerance to freezing stress but also to salt stress (28) and therefore likely greater resistance to desiccation stress.

Another gene associated with desiccation stress that is expressed during ABA induced hardening in bromegrass is wheat germin. Wheat germin is expressed in partially hydrated (60% water) germinating embryos (19). The function of this protein is not known, however, a polypeptide from barley that is specifically salt stress inducible (15) has high sequence similarity to it. Germin also has significant amino acid similarity to *Physarum polycephalum* spherulins.

P. polycephalum spherulins are expressed if plasmodia are subjected to environmental stresses including osmotic and temperature extremes (19). Germin-like genes including bromegrass clone A56 may thus represent members of a class of genes associated with desiccation stress resistance.

A thiol protease from tomato fruit was expressed during low temperature treatment (32). The tomato cDNA shares regions of homology with other plant proteases and to the animal protease cathepsin H. The bromegrass A85 clone is not only ABA responsive , but like the tomato thiol protease gene, is also cold responsive. Schaffer and Fischer (32) have suggested that low temperatures may denature certain polypeptides thus requiring proteases to degrade the denatured polypeptides. Cold or ABA induced protease expression may therefore play an important role in supporting cellular metabolism during low temperature growth.

Although the identities of aldose reductase, dehydrin, germin and phosphoglucomutase-like RNAs expressed in hardening bromegrass remain to be confirmed, the expression of these type of genes may be indicative of a requirement for tolerance to desiccation stress manifested by freezing temperatures. It is certainly possible that expression of these genes are required for the accumulation of osmolytes and tolerance to extracellular freezing-induced dehydration stress. From the results presented here and by others, we postulate that acclimation to freezing requires the expression of genes involved in the tolerance to desiccation stress. While desiccation resistance may not be the only mechanism involved in cold hardiness, the association of these types of genes with hardiness helps to explain many of the physiological changes related to hardening

induced osmotic adjustments. Work is in progress to characterize these genes. Clone A86, on the other hand, shares high homology with barley embryo globulin (Heck et al., unpublished data) and with a class of maize embryo proteins (3). Since the maize globulins most likely function as storage proteins (3), clone A86 probably has a similar role and therefore not related to cold acclimation.

In summary, we have isolated 16 cDNAs from $4.5x10^5$ recombinant clones of a bromegrass cDNA library representing genes expressed during ABA induced freezing tolerance. Three distinct patterns of gene expression have been identified. Expressed sequence tag analysis indicates that bromegrass cDNAs A61, A72, A55, and A56 have a high sequence similarity to the barley aldose reductase, rabbit phosphoglucomutase, barley dehydrin, wheat germin genes respectively. The association of genes involved with sugar metabolism and osmotic stress during ABA induced hardening suggests that tolerance to dehydration stress may be required for the induction of freezing tolerance in plant cells.

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5.0 Expression of an Aldose Reductase-like Gene During ABA Induced Frost Tolerance in Bromegrass Suspension Culture

5.1 Abstract

Abscisic acid (ABA) has been shown to increase freezing tolerance (from LT_{50} -7 to -30°C) of bromegrass cell suspension cultures at 23°C and elicits many metabolic changes similar to those observed during cold acclimation. We have previously isolated 16 cDNA clones of ABA responsive genes from AN ABA treated bromegrass library. In this study, we characterized one of the cDNAs, putatively identified as a NADPH-dependent aldose reductase. Nucleotide sequencing of the cDNA (pBGA61) strongly suggests its identity as aldose reductase based on a 92% nucleotide and 91% predicted amino acid similarity to barley aldose reductase. RNA gel blot analysis revealed that the accumulation of pBGA61-related mRNA was detected within 3 h of 75 μM ABA treatment, and the highest levels were observed after 5 days treatment when the cells were at highest level of hardiness. Cold acclimation (+4°C) for 10 days also induced the accumulation of the mRNA, but after 20 or 30 days at +4°C, the mRNA signal had disappeared while the level of freezing tolerance remained elevated. Bromegrass cells treated with 4% PEG 8000 for 5 days increased hardiness slightly but there was no detectable pBGA61-related mRNA accumulation. Treatment with a combination of $GA_{4,7}$ (75μM) and ABA (75 μM) resulted in lower hardiness and steady state RNA level than with ABA alone. A significant increase in aldose reductase activity was detected in bromegrass cells after ABA treatment, but cellular sorbitol levels did not increase. It is unlikely that the expression of aldose reductase during ABA induced hardening in bromegrass cells is directly

responsible for the increase in freezing tolerance but is possibly involved in the adjustment of metabolic processes during cold acclimation.

5.2 Introduction

Cold acclimation in plants is a metabolically complex process that includes the re-adjustment of cellular processes to maintain functions at low temperatures and an increase in freezing tolerance (10). In many plant species which have the ability to cold acclimate, exogenous application of ABA at room temperature can also increase freezing tolerance (6). In bromegrass cell suspension cultures, ABA can elicit responses similar to the physiological and biochemical changes observed in cold acclimating plants (35). These changes include the accumulation of sugars (35), altered expression of polypeptide (29, 21) and translatable RNA (21) patterns. Specific RNAs which accumulate under cold or ABA treatment have recently been cloned from alfalfa (23), *Arabidopsis* (12, 19, 26), barley (4), and bromegrass (20).

ABA responsive mRNAs from ABA hardened bromegrass cell suspension cultures have been identified (21) and cloned (20). Differential screening of a cDNA library from RNA of ABA hardened cells yielded 16 unique cDNAs. Expressed sequence tag screening (1) of the bromegrass cDNAs indentified a clone with sequence similarity to barley NADPH-dependent aldose reductase (2). This gene is responsible for the sterospecific reduction of glucose to sorbitol (32), thus expression of aldose reductase during ABA induced hardening may indicate a change in sugar metabolism. The association of this gene with frost tolerance provides evidence for ABA induced changes in the regulation of sugar metabolism

during hardening.

In this study, the complete DNA sequence of the bromegrass cDNA clone pBGA61 has been determined. It shares very high homology with barley NADPH-dependent aldose reductase (2) and to a lesser degree with human lens aldose reductase (25). RNA gel blot analysis using pBGA61 as the probe indicates that RNA accumulation is strongly associated with the development of ABA induced frost tolerance and is ABA concentration dependent. Both cold and $GA_{4,7}$ treatment also appear to modulate steady state levels of the RNA. A specific protein fraction from ABA hardened bromegrass cells has been determined to contain aldose reductase-like activity but there was no detectable increase in sorbitol concentration.

5.3 Materials and methods

5.3.1 Plant material and treatments

Smooth bromegrass (*Bromus inermis* Leyss cv Manchar) cell suspension cultures were subcultured and grown in media containing 0.5 mg l^{-1} 2,4-D as described by Chen and Gusta (6). Five day old cells were transferred to fresh media containing various concentrations of (±)-ABA, PEG 8000, NaCl or $GA_{4,7}$. Cold treatment consisted of growing cell cultures at +4°C on an orbital shaker for various periods of time. ABA treated cells were deacclimated by washing cells with ABA-free media and then further culturing in ABA-free media. A sample of the cells from each treatment was collected to determine the level of freezing tolerance (21) and the remaining portion immediately frozen in liquid nitrogen and stored at -70°C for RNA or protein extraction.

5.3.2 DNA sequencing

A set of nested deletions for each strand of the pBGA61 cDNA (20) was made according to the procedure of Henikoff (13). Purified plasmids for each deletion were then dideoxynucleotide chain-termination sequenced (31) with the Applied Biosystems DNA sequencer (Model 373A). The cDNA sequence was then used to search the GenBank and EMBL databases for homologous sequences.

5.3.3 RNA gel blot analysis

Total RNA was isolated by the guanidine isothiocyanate / CsCl ultracentrifugation method as described by Lee et al. (21). RNA concentration was determined spectrophotometrically.

Total RNA from bromegrass cells was electrophoresed in a 1.3% agarose - formaldehyde gel for 2 h (30) and then blotted onto a nylon membrane (Zetaprobe, BioRad) according to the suppliers directions. Prehybridization and hybridization was carried out in 0.25M Na_2HPO_4, pH 7.2, 7% SDS at 65°C. The probe used for the hybridizations were made by agarose gel purifying an Xba I / Kpn I restriction digest released cDNA insert from pBGA61 and then labeled with ^{32}P-dCTP by the random priming method (30). The membranes were washed for 5 min in 2 x SSC, 0.1% SDS at room temperature, two times in 2 x SSC, 0.1% SDS for 20 min, two times in 0.2 x SSC, 0.1 x SDS for 20 min and then autoradiographed with one intensifier screen.

5.3.4 DNA gel blot analysis

Bromegrass DNA was isolated by grinding approximately 4 g of cells in liquid nitrogen to a fine powder and transferred to a 50 ml centrifuge tube containing 20 ml of homogenization buffer (100mM Tris-HCl pH 9.0, 100mM NaCl, 10mM $MgCl_2$, 0.5M sucrose, 0.1% 2-mercaptoethanol and 0.4% diethyldithiocarbamic acid) and homogenized for 30 s (Tekmar Tissumizer set at 50). The homogenate

was centrifuged at 7500 x g, +4°C for 3.5 min and the pellet resuspended in 2 ml of homogenization buffer. At room temperature, 4 ml of lysis buffer (100mM Tris-HCl pH 8.3, 100mM NaCl, 50mM EDTA, 1.5% SDS, 15% phenol) was added, gently mixed and incubated at 55°C for 5 min with occasional mixing, then 1.5ml of 5M KOAc added, mixed and then incubated on ice for 5 min. The mixture was centrifuged at 2000 x g, +4°C for 15 min, the supernatant collected and 0.5 ml of 10 M NH_4OAc and 2.5 ml of chloroform : isoamyl alcohol (24:1) was added to the supernatant and thoroughly mixed. After centrifuging at 2000 x g, +4°C for 15 min, the aqueous phase was collected and the DNA precipitated with 7 ml of isopropyl alcohol. The DNA was pelleted, washed with cold 70% ethanol and then redissolved in 0.9 ml TE (10 mM Tris-HCL pH 8.0, 1mM EDTA). The DNA was further purified by precipitating-out the impurities with 0.44ml 10 M NH_4OAc, centrifuged for 2 min at 10000 x g and the DNA precipitated with 1.5 ml isopropyl alcohol. The DNA pellet was washed with 70% ethanol, dried, dissolved in 0.5ml TE and then heated to 65°C for 5 min. DNA concentration was determined spectrophotometrically.

The bromegrass DNA was digested with BamH I, Kpn I, Xba I or EcoO109 I, electrophoresed on a 0.8% agarose gel (30), and blotted onto a nylon membrane (Zetaprobe) according to suppliers instructions. Membranes were prehybridized and hybridized under the same conditions as for the RNA gel blots using the pBGA61 cDNA as the probe and then washed under low stringency (2x SSC, 0.1% SDS) two times at 65°C for 15 min and then exposed to X-ray film for 5 d. The membranes were then washed under high stringency (0.2x SSC, 0.1% SDS) at 65°C

for 15 min and re-exposed to X-ray film for 5 days.

5.3.5 Enzyme purification and sorbitol determination

Bromegrass cells from -70°C storage were used for the partial purification of aldose reductase-like enzymes. Samples from five day control and ABA treatments were used for the extraction procedure.

Approximately five g of cells were ground to a fine powder in liquid nitrogen, transferred into a 50 ml centrifuge tube and 20 ml of extraction buffer (20 mM potassium phosphate buffer, pH 7.5, 0.5 mM EDTA, 5 mM 2-mercaptoethanol) (2) containing 1 mM PMSF added and homogenized (five 15 sec pulses at a setting of 90). The homogenate was centrifuged at 12,000 x g for 30 min at +2°C and the supernatant filtered through glass wool into a clean centrifuge tube. Solid ammonium sulfate was added to 40% saturation and centrifuged at 12,000 x g for 30 min at +2°C. The supernantant was then transfered to a fresh tube. Additional ammonium sulfate was added to the supernatant in 10% increments to reach 50, 60, 70, 80 and 100 % saturation. Each protein fraction was dissolved in 0.75 ml of extraction buffer and dialyzed against the same buffer without PMSF.

Aldose reductase activity was determined spectrophotometrically by monitoring the oxidation of NADPH in the presence of the substrate DL-glyceraldehyde (2). 100 μl of extract containing 100 μg protein was added to 900 μl of assay buffer (100 mM potassium phosphate buffer, pH 6.9, 50 mM glyceraldehyde, 0.15 mM NADPH) to start the reaction and the decrease in absorbance was then monitored

at 340 nm, 23°C for 5 min. One unit of activity is defined as the amount of enzyme catalyzing the oxidation of 1 μmole of NADPH per minute with the above assay conditions. Specific activity is defined as units per mg of protein. Protein concentration was measured by a modified Lowery's method using bovine serum albumin as the standard (27).

Soluble sugars were extracted from bromegrass cells (3 g FW) by grinding with liquid nitrogen to a fine powder in a mortar and transferred to a tube containing 15 ml of water. The mixture was heated to 80°C for 30 min with mixing, then cooled to room temperature and water added to a final volume of 25 ml. After centrufugation (25,000 x g, 15°C, 30 min), the supernatant was filtered through Whatman #1 filter paper and stored at +4°C.

Sorbitol concentration of the supernatant was determined by the coupled enzyme assay method (Boehringer Mannheim, Indianapolis, IN). The enzyme, sorbitol dehydrogenase, was used to catalize the oxidation of sorbitol with NAD to fructose and NADH and the activity monitored by the change in absorbance at 340 nm.

5.4 Results

5.4.1 Nucleotide and amino acid sequence

The cDNA clone, pBGA61, was isolated by differential screening a cDNA library constructed from RNA of ABA hardened bromegrass cells (previously labeled as A61, 20). Both strands of the cDNA were sequenced. The coding strand along with the predicted amino acid sequence is listed in Figure 5.1. The longest open reading frame, starting at base 79 and ending at base 1071, specifies a polypeptide of 36,922 Da. Based on the percentage of molecular weight, the most abundant amino acids are lysine (11.5%), glutamic acid (9.1%), leucine (8.9%), valine (6.2%), aspartic acid (6.2%) and alanine (4.7%). There does not appear to be any bias for any specific amino acid.

Preliminary sequencing of pBGA61 (20) indicated a very high level of homology to barley NADPH-dependent aldose reductase (2). A search made with the complete pBGA61 cDNA sequence has confirmed its identity as that of NADPH-dependent aldose reductase. The identification is based on a coding region nucleotide and amino acid similarity of 92% and 91% respectively, with respect to the putative coding region of barley clone pG22-69 (2). Nucleotide similarity of the 3' untranslated region between the two clones was considerably lower (ca. 50%). Figure 5.2 compares the putative amino acid sequences of pBGA61 with pG22-69 and human lens aldose reductase (25). The amino acid match between pBGA61 and pG22-69 is high but neither are as high with the

human lens cDNA. As Bartels et al. (2) have noted though, pG22-69 (and pBGA61) share regions of significant similarity to human lens aldose reductase, including a site reported to be required for aldehyde and aldose reductase activty (3).

5.4.2 mRNA accumulation and development of freezing tolerance

The development of ABA induced freezing tolerance in bromegrass cells is illustrated in Figure 5.3. A significant level of hardiness was not apparent until after 24 h of 75 μM ABA treatment and maximal hardiness was reached after 5 d of treatment. When the bromegrass culture was transfered to ABA-free medium, hardiness decreased but remained high for three days and then declined to an LT_{50} of -17°C after four additional days of culturing. This is in close agreement with previously reported LT_{50} values for ABA treated bromegrass cultures (21). A close correlation of the accumulation of pBGA61-related mRNA with a high level of frost tolerance was observed. RNA gel blot analysis indicates that the RNA accumulates to detectable levels after only 3 h of ABA treatment and continues to increase with time (Figure 5.3, inset). However, high levels of the transcript was mostly associated with a high degree of hardiness, as observed after 5 d of ABA treatment. A considerably high level persisted after 3 d of deacclimation. Very little transcript was detected 7 d after ABA was excluded from the medium.

Induction of freezing tolerance in bromegrass cell cultures by ABA is also a

dose dependent response (6, 20). At ABA concentrations of 10 μM or higher, an increase in freezing tolerance was observed (24h treatment). Figure 5.4 shows this dose dependent increase in hardiness. The accumulation of pBGA61-related mRNA was also dose dependent as a minimum of 10 μM ABA was required for a detectable hybridization signal (Figure 5.4, inset).

Bromegrass cell suspension cultures can also be hardened by growth at low temperatures (6, 16, 21). In this study, bromegrass cultures were incubated on a rotary shaker for 10, 20 or 40 days at +4°C. Freezing tolerance of the cells increased from an LT_{50} of -7.5°C to -13.6°C after 10 d but after 20 d (LT_{50} -17°C) or 30 d (LT_{50} -20.2°C) the increase was less dramatic (Figure 5.5). The pack cell volume of the cold treated cells, unlike with ABA treatment, did not change very much. A pBGA61-related mRNA signal was detected after 10 d but was not present after 20 or 30 d (Figure 5.5, inset). We previously determined that 5 days incubation at +4°C did not induce the expression of the transcript to detectable levels (20). Under low temperature growth, pBGA61-related mRNA rapidly accumulated between 5 and 10 d but the effect was transient as the mRNA signal was not present after 20 or 30 d.

It has been demonstrated that desiccation stress by witholding water (7) or artificially by PEG treatment (39), can increase freezing tolerance. At the RNA level, Bartels et al. (2) have isolated a barley cDNA which identified a RNA that was temporally associated with programmed embryo desiccation. In this study, bromegrass suspension culture cells were osmotically stressed by the addition of PEG 8000 to the culture medium to determine the effect on freezing tolerance

and the expression of pBGA61-related mRNA. There was an increase in hardiness with 4% PEG treatment only (Figure 5.6). The 9% PEG treated cells were less hardy than the control and cells after 16% PEG treatment did not survive. PEG 8000 treatment did not induce the accumulation of pBGA61-related mRNA at any of the test concentrations.

Bromegrass cells were cultured in NaCl to determine if a salt/osmotic stress could induce hardiness and increase pBGA61-related mRNA levels. NaCl concentrations ranging from 25mM to 250mM were added to the cell culture and shaken at room temperature for 5 d. Bromegrass cells appeared to be very sensitive to NaCl as the lowest concentration (25mM) was sufficient to kill the culture. There was no RNA hybridization signal when probed with pBGA61 (Figure 5.6).

Reaney et al. (28) have reported that adding GA_4, GA_7, or GA_9 could reduce the hardening effect of ABA induced freezing tolerance in bromegrass cell suspension culture significantly. In this experiment, cells were treated with 75 μM each of $GA_{4,7}$ and ABA for 5 d at 23°C. There was a small decrease in hardiness with $GA_{4,7}$ + ABA treatment relative to ABA alone (Figure 5.6). Correspondingly, pBGA61-related mRNA signal strength was attenuated (Figure 5.6, inset).

5.4.3 DNA gel blot analysis

Figure 5.7 illustrates the hybridization pattern of the pBA61 probe to bromegrass DNA. The pBGA61 cDNA probe indicates that the aldose reductase-like gene is probably respresented by a small multi-gene family consisting of approximately 3-5 copies of related genes.

5.4.4 ABA induced aldose reductase activity and sorbitol level

Since the nucleotide and amino acid sequence of pBGA61 was very similar to the barley cDNA, pG22-69, and the accumulation of pBGA61 mRNA closely followed the development of ABA induced hardiness, aldose reductase activity was assayed in hardening bromegrass cells. Preliminary assays were made with cells after 5 d control or ABA treatment. The level of aldose reductase activity in the crude protein extract, with glyceraldehyde as the substrate, was only marginally higher in the ABA cells (data not shown). The greatest difference in activity between control and ABA treated cells was after ammonium sulfate fractionation. The 80% fraction of ABA treated cells was at least two times higher than the control. Aldose reductase-like activity for both one and five days ABA treatment is illustrated in Figure 5.8. After one day of ABA treatment, there was a small increase in activity over the control in the 80% fraction but after 5 days treatment, there was a substantial increase in enzyme activity. When glucose was used as the

substrate, the difference in activity between treatment and control became insignificant. There was also very little activity when no substrate was added to the assay buffer (data not shown).

An increase in aldose reductase-like activity lead us to suspect that there may also be a corresponding increase in cellular sorbitol levels. Using the coupled enzyme assay method, we were not able to detect any significant change in sorbitol concentrations after 1 or 5 days of ABA treatment.

```
                                                                      1
                                                                      CGGCACG
AGGACCACCAGACTAGGAAGGAGAAGGCGTCTGAAGAGAGAAGAGAGGTTAGAGATTCAGAAGGTGCGATC
                                                                     78
                                  105                                 
ATG GCG AGC GCC AAG GCG ATG ATG GGG CAG GAG AGG CAG GAT CAC TTT GTT CTG
MET Ala Ser Ala Lys Ala MET MET Gly Gln Glu Arg Gln Asp His Phe Val Leu
                                  159                                 
AAG AGC GGG CAT GCC ATC CCA GCC GTC GGA TTG GGC ACC TGG AGA GCT GGC TCC
Lys Ser Gly His Ala Ile Pro Ala Val Gly Leu Gly Thr Trp Arg Ala Gly Ser
                                  213                                 
GAT ACT GCT CAC TCC GTT CAG ACG GCC ATC ACC GAG GCC GGG TAC AGG CAT GTG
Asp Thr Ala His Ser Val Gln Thr Ala Ile Thr Glu Ala Gly Tyr Arg His Val
                                  267                                 
GAC ACA GCT GCT GAA TAC GGA GTA GAA AAG GAG GTC GGC AAA GGG CTT AAA GCT
Asp Thr Ala Ala Glu Tyr Gly Val Glu Lys Glu Val Gly Lys Gly Leu Lys Ala
                                  321                                 
GCA ATG GAA GCT GGG ATC GAC AGG AAA GAT TTG TTT GTG ACG TCA AAA TTA TGG
Ala MET Glu Ala Gly Ile Asp Arg Lys Asp Leu Phe Val Thr Ser Lys Leu Trp
                                  375                                 
TGC ACA GAC TTG GTT CCT GAC AGG GTG CGG CCA GCG TTA GAG AAG ACG CTC AAG
Cys Thr Asp Leu Val Pro Asp Arg Val Arg Pro Ala Leu Glu Lys Thr Leu Lys
                                  429                                 
GAT CTA CAG TTG GAC TAC CTC GAT CTT TAC CTT ATC CAC TGG CCG TTC CGG CTG
Asp Leu Gln Leu Asp Tyr Leu Asp Leu Tyr Leu Ile His Trp Pro Phe Arg Leu
                                  483                                 
AAA GAT GGG GCA CAC AAG CCA CCG GAA GCA GGG GAG GTG CTG GAA TTC GAC ATG
Lys Asp Gly Ala His Lys Pro Pro Glu Ala Gly Glu Val Leu Glu Phe Asp MET
                                  537                                 
GAG GGG GTG TGG AAG GAA ATG GAG AAC CTT GTC AAG GAT GGA CTG GTC AAG GAC
Glu Gly Val Trp Lys Glu MET Glu Asn Leu Val Lys Asp Gly Leu Val Lys Asp
                                  591                                 
ATC GGT GTC TGC AAC TAC ACA GTG ACA AAG CTC AAC CGG CTG CTG CAA TCT GCA
Ile Gly Val Cys Asn Tyr Thr Val Thr Lys Leu Asn Arg Leu Leu Gln Ser Ala
                                  645                                 
AAG ATT NCG CCG GCC GTA TGC CAG ATG GAA ATG CAC CCT GGT TGG AAG AAC GAC
Lys Ile     Pro Ala Val Cys Gln MET Glu MET His Pro Gly Trp Lys Asn Asp
                                  699                                 
AAG ATT CTC GAG GCC TGC AAG AAG CAC GGA ATC CAT GCC ACC GCT TAC TCC CCA
Lys Ile Leu Glu Ala Cys Lys Lys His Gly Ile His Ala Thr Ala Tyr Ser Pro
                                  753                                 
TTG TGT TCT TCA GAG AAG AAC CTT GCT CAT GAC CCG GTT GTT GAA AAG GTG GCC
Leu Cys Ser Ser Glu Lys Asn Leu Ala His Asp Pro Val Val Glu Lys Val Ala
                                  807                                 
AAC AAG CTG AAC AAG ACC CCG GGG CAG GTG CTC ATC AAG TGG GCT CTG CAG AGG
Asn Lys Leu Asn Lys Thr Pro Gly Gln Val Leu Ile Lys Trp Ala Leu Gln Arg
                                  861                                 
GGG ACG ATC GTG ATC CCC AAA TCG AGC AAA GAC GAG AGG WTC AAG GAG AAC ATC
Gly Thr Ile Val Ile Pro Lys Ser Ser Lys Asp Glu Arg     Lys Glu Asn Ile
                                  915                                 
CAG GTG TTC GGA TGG GAG ATC CCT GAA GAG GAC TTC CAG GTC CTG TGC AGC ATC
Gln Val Phe Gly Trp Glu Ile Pro Glu Glu Asp Phe Gln Val Leu Cys Ser Ile
                                  969                                 
AAA GAT GWG AAG CGT GTG CTG ACC GGG GAG GAG CTC TTC GTG AAC AAG ACC CAC
Lys Asp     Lys Arg Val Leu Thr Gly Glu Glu Leu Phe Val Asn Lys Thr His
                                 1023                                 
GGG CCG TAC AAG AGT GCA TCC GAG GTC TGG GAT AAC GAG AAC TWG AAG NNC TNN
Gly Pro Tyr Lys Ser Ala Ser Glu Val Trp Asp Asn Glu Asn     Lys
                                 1071                                 
TCG CCA GCA GGC ACC GTC TAG GACGACGACTCGAATAATGGAGGCTGAAGAAGTGAAGAAGGAA
Ser Pro Ala Gly Thr Val  .

GATGGTACTCCTGCATCAATTCAGACTCTGCCTGATGCTCTGTAAACAAACGCTGTGTGCAATGAATAAAT
                                 1222
ATGGCTTCGTCGTGCTCCAAAAAAAAAAAAAAAAAAA
```

Figure 5.1. Nucleotide and predicted amino acid sequence of the pBGA61 cDNA insert. The longest open reading frame starts at 79 and ends at 1071.

```
 BGA61    1 MASAKAMMGQERQDHFVLKSGHAIPAVGLGTW  RAGSDTAHSVQTAITEAGYRHVDTAAE
G22-69    1 ------T---GE----------M--------  ----------R----------------
 HUMAR    1 ---            RLL-NN-AK--IL-----KSPP-QV- EA-KV-- DV----I-C-HV

 BGA61   60 YGVEKEVG KGLKAAMEAGIDRKDLFVTSKLWCTDLQPDRVRPALEKTLKDLQLDYLDLYL
G22-69   60 -------- ----------------------I---I-A-E-------N---------I---H
 HUMAR   49 -QN-N---VAIQEKLR-QVVK-EE--IV------YHEKGL-KG-CQ---S--K--------

 BGA61  120 IHWPFRLKDG AHKP PEAGEVLEFD  MEGVWKEMENLVKDGLVKDIG  VCN YTV TK
G22-69  120 ---------- --M- ----------  ----------------------  --- --- --
 HUMAR  110 ----TGF-P-KEFF-LD-S-N-VPS-TNILDT-AA--E--DE----A--ISNF-HLQ-EMI

 BGA61  173 LNRLLQSAKIXPAVCQMEMHPGWKNDKILEACKKHGIHATAYSPLCSSEKNLA  HDP VV
G22-69  173 -----R----P----------------F---------V------G-------  --- --
 HUMAR  171 --  KPGL-YK---N-I-C--YLTGE-LIGY-QSK--V---------PDRPW-KPE--SLL

 BGA61  231 E    K VANKLNKTPGQVLIKWALQRGTIVIPKSSKDERXKENIQVFGWEIPEEDFQVLC
G22-69  231 E    - ----------------------S----------I---------------K---
 HUMAR  230 -DPRI-AI-A-H---TA----RFPM--NLV-----VTP--IA--FK--DF-LSSQ-MTT-L

 BGA61  287 SIKDXKRVLTGEELFVNKTHGPYKSASEVWD nENxkxxspagtvX
G22-69  287 ----E----------------R--AD---H --           -
 HUMAR  291 -YNRNW--   CA- LSC-     -HK-YPFHE-          F-
```

Figure 5.2 Comparison of the bromegrass cDNA (BGA61) putative amino acid sequence with barley (G22-69, 2) and human lens (HUMAR, 25) putative aldose reductase sequences. A dash (-) indicates an amino acid match with BGA61. Gaps were introduced to optimize sequence alignment.

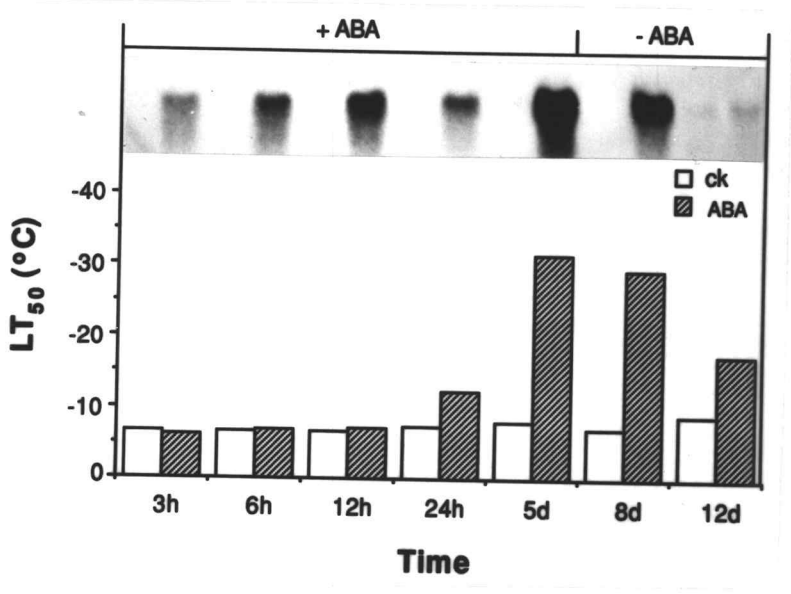

Figure 5.3 Development of ABA induced freezing tolerance. Bromegrass suspension cultures were treated with 75 μM ABA for various times and the LT_{50} determined. The inset shows a RNA gel blot of pBGA61-related mRNA levels for the corresponding treatment times. Twenty μg of total RNA was applied to each lane of the gel. '+ABA' refers to the inclusion of ABA in the medium and '-ABA' refers to the exclusion of ABA from the medium after 5 days of ABA treatment.

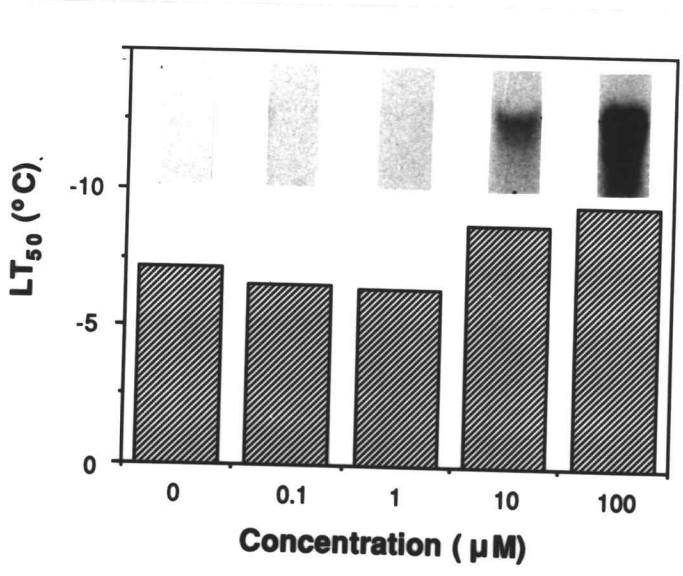

Figure 5.4 Dosage response of ABA with respect to the level of freezing tolerance and the induction of pBGA61-related mRNA accumulation. Bromegrass suspension cultures were treated with various concentrations of ABA for 1 day and the LT_{50} determined. Twenty μg of total RNA was used for the RNA gel blot analysis (inset).

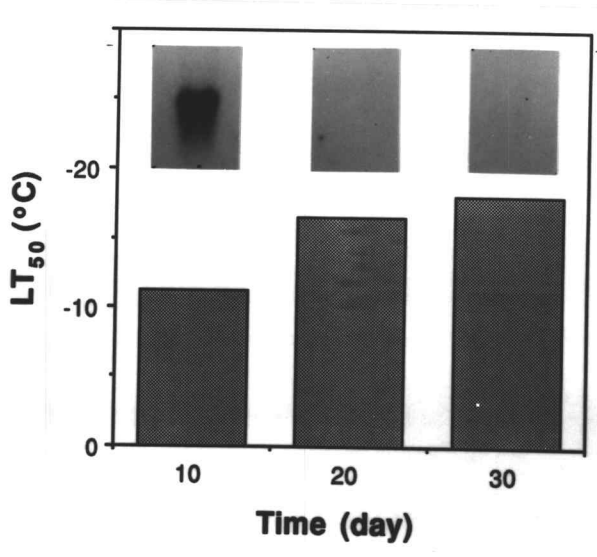

Figure 5.5 Induction of freezing tolerance by low temperature treatment and accumulation of pBGA61-related mRNA. Bromegrass suspension cultures were incubated on a rotary shaker at +4°C for various times. The RNA gel blot for the treatments are placed above the corresponding incubation times.

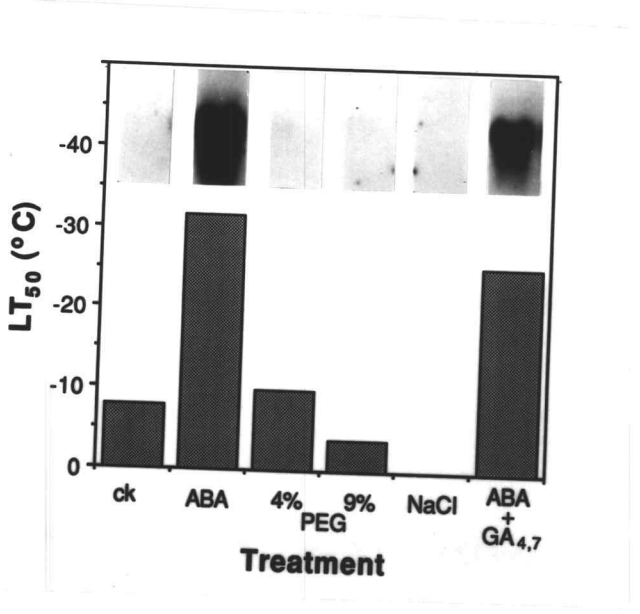

Figure 5.6 Freezing tolerance and pBGA61-related RNA accumulation after ABA, PEG, NaCl or ABA+$GA_{4,7}$ treatment. LT_{50} values and RNA gel blot hybridization signals are based on 5 days treatment in media containing 75 μM ABA, 4% or 9% PEG 8000, 25 mM NaCl or 75 μM ABA + 75 μM $GA_{4,7}$. Twenty μg of total RNA was used for RNA gel blot analysis.

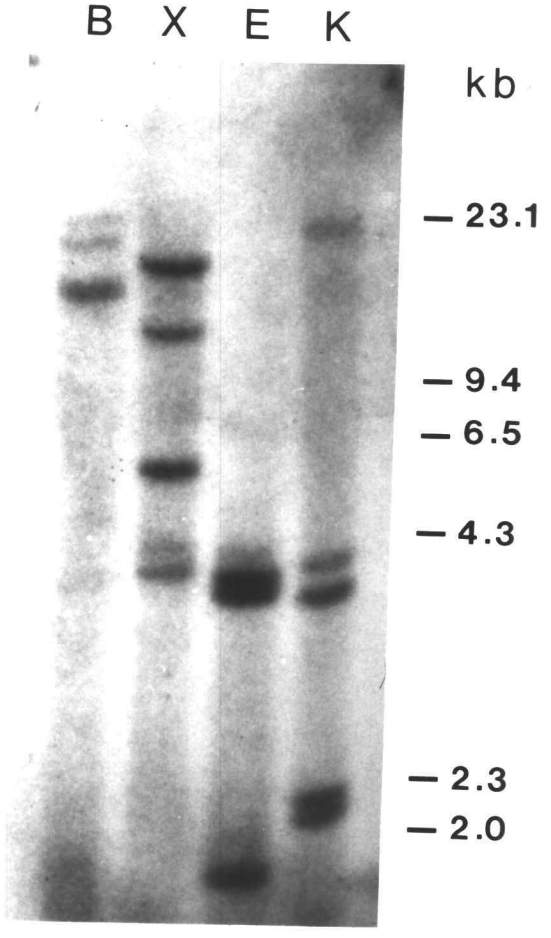

Figure 5.7 DNA gel blot of bromegrass genomic DNA. Forty μg of DNA was digested with BamH I (B), Xba I (X), EcoO109 I (E) or Kpn I (K), electrophoresed in an 0.8% agarose gel, blotted and probed with pBGA61. Hind III digested λ DNA markers are included on the right.

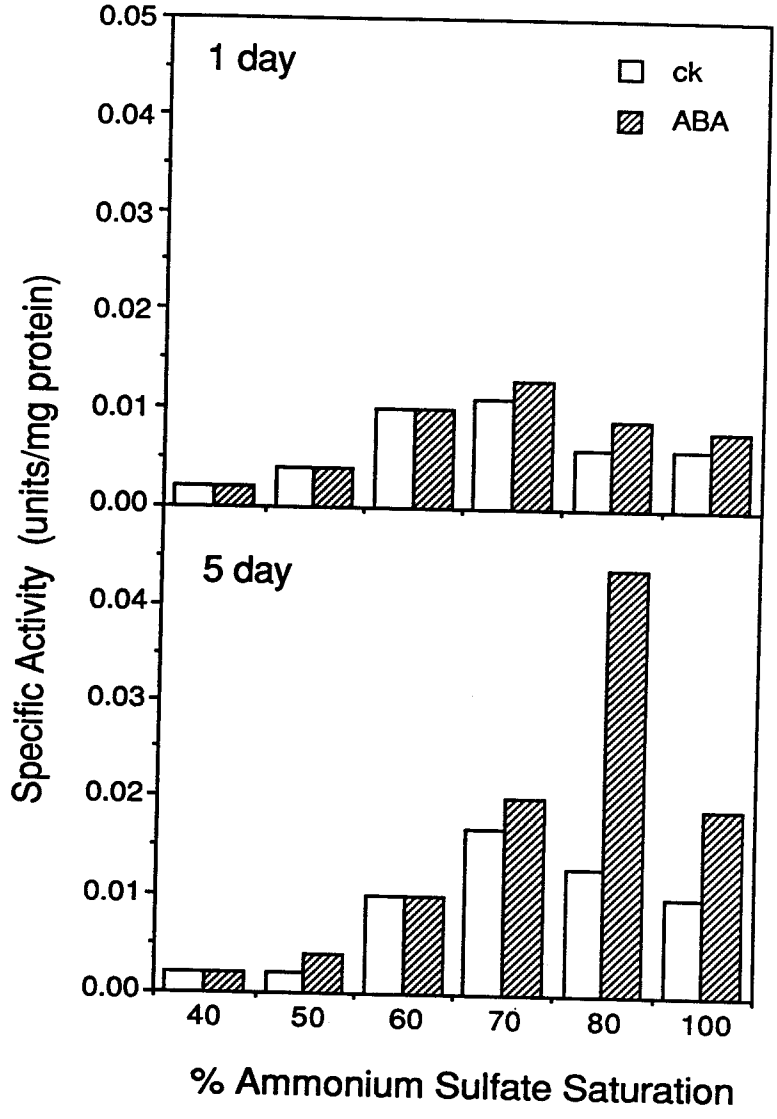

Figure 5.8 Comparison of NADPH-dependent aldose reductase-like activity between 1 and 5 days of ABA treatment. Water soluble proteins from bromegrass suspension culture cells were ammonium sulfate fractionated and each fraction assayed for enzyme activity. Aldose reductase activity was determined by monitoring the oxidation of NADPH in the presence of DL-glyceraldehyde. One unit of activity is the amount of enzyme catalyzing 1 μmole of NADPH per minute.

5.5 Discussion

There is a large body of evidence which supports the proposal that sugar metabolism is altered during the process of cold acclimation (32). Most of these studies are based on the observation that total sugar or specific sugars accumulate during hardening. Relatively little data is available on the genetic regulation of these sugars, although with the application of molecular biology techniques to these types of studies, this will soon no longer be the case. Currently two research groups have identified changes in the pattern of sucrose synthase gene expression in cold acclimating wheat (8, 24). They had determined that both sucrose synthase protein and poly(A^+) RNA levels increased in response to cold temperatures. In this study, we demonstrate that the expression of an NADPH-dependent aldose reductase-like gene is strongly associated with a high level of ABA-induced freezing tolerance in bromegrass suspension culture.

The bromegrass cDNA, pBGA61, contains essentially the same putative amino acid sequence as the barley cDNA, pG22-69, which codes for an aldose reductase-related protein (2). By virtue of the high sequence similarity, pBGA61, also contains the same regions of amino acid identity with the animal monomeric aldose reductase cDNAs. We conclude that the gene represented by pBGA61 most likely codes for an aldose reductase-related protein.

Accumulation of pBGA61-related mRNA is associated with a high level of ABA induced freezing tolerance. A weak transcript signal was present throughout the early stages of hardening but the signal was the strongest when the cells

displayed a rapid increase in hardiness (ie. LT_{50} increase from -13°C to -30°C). The strong hybridization signal was present only during early deacclimation while hardiness remained high.

Bromegrass suspension-cultured cells likely require many genes for the induction and maintainance of freezing tolerance. Classical genetic studies have linked the requirement of numerous genes to the winter hardiness trait in cereal crops. Molecular genetic studies also indicate that a complex pattern of gene expression is associated with freezing tolerance induction (36). The development and maintainance of freezing tolerance therefore likely includes the sequential (transient or contiguous) expression of genes was well as the constituitive expression of other genes. Evidence for transient or contiguous gene expression was observed in cold and ABA induced acclimation in potato (38, 37) and in ABA hardened bromegrass (21, 20). There are numerous examples of genes which are constituitively expressed during hardening (11, 12, 20). The expression of transcript hybridizing to pBGA61 cDNA probe appears to correspond to a stage where a high level of freezing tolerance is maintained.

Bromegrass pBGA61-related mRNA accumulates in response to low temperature although unlike ABA treatment, the elevated RNA level was transient. The level of freezing tolerance also increased significantly after 1 week of cold treatment but considerably less than after 2 or 4 weeks and did not reach the level of hardiness attained by ABA. It is well known that endogenous ABA concentrations can increase sharply in response to low temperatures (5). Even though endogenous ABA was not quantified in this study, it is possible that the

expression of pBGA61-related mRNA may reflect the variations in the endogenous level ABA levels during hardening. We surmise that ABA levels increased in response to low temperature, but then decreased as reflected by the transient increase in pBGA61-related mRNA and a slower rate of cold acclimation.

When equilibrum freezing of plant tissues occur, the individual cells are subjected to desiccation stress as a result of the negative water potential generated by extracellular ice formation (22). It has been suggested that tolerance to freezing requires a mechanism to deal with the osmotic stress imposed by the loss of cellular water (14). Bartels et al. (2) proposed that ABA inducible expression of aldose reductase, leading to sorbitol accumulation, may play an important role in protecting maturing barley embryo from osmotic stress encountered during programmed dessication. The PEG experiment indicated that bromegrass cells under a moderate osmotic stress (4% PEG) had increased freezing tolerance, but there was no indication of pBGA61-related mRNA accumulation. It is likely that the small increase in hardiness was simply due to a decrease in cellular water content and not the result of metabolic adjustment to increase freezing tolerance. The removal of cellular water appears to be a small component of the mechanism of ABA induced freezing resistance in bromegrass suspension cultures.

The association of pBGA61-related mRNA expression with freezing tolerance was further strengthened by the results of the GA experiment. GA has been shown to reverse or attenuate the effects of ABA treatment; most notably during seed development (15). In a previous experiment, bromegrass suspension cultures

treated with ABA and GA were considerably less hardy than with ABA alone (28). The difference in hardiness between the two treatments was not as large in this experiment, however, the effect was still obvious. The level of hardiness did not decrease to the extent as previously reported (28), but the difference may be due to the use of a commercial preparation of $GA_{4,7}$ rather than applying the purified forms of GA_4 and GA_7. A decrease in hardiness was mirrored by a weaker pBGA61-related mRNA signal as well. The antagonistic interaction of GA with pBGA61-related gene expression and hardiness suggests that the aldose reductase gene plays a role in freezing resistance. We recognize that GA can modulate the expression of other ABA inducible genes which may also be involved in hardening (data not shown).

There are two pieces of evidence to support the hypothesis that sorbitol metabolism changes during ABA induced freezing tolerance in bromegrass suspension-cultured cells. First, as mentioned above, pBGA61-related mRNA, encoded by a NADPH-dependent aldose reductase-like gene, accumulates to high levels only in very hardy bromegrass cells. Second, there is considerably greater aldose reductase-like activity present in ABA treated cell cultures. It is possible that enzymes other than aldose reductase, in the 80% ammonium sulfate fraction, are responsible for the oxidation of NADPH when glyceraldehyde is used as the substrate. Even if aldose reductase was not a contributing factor to the enzyme activity detected, we can still be certain that ABA treatment has induced the expression of certain enzymes which are involved in aldose metabolism.

In many overwintering plant species, sugar accumulation is a common

occurence in the fall. Specific sugars which been found to increase during cold acclimation include glucose, fructose, sucrose and raffinose (22). In some species, the polyhydric alcohols, sorbitol and mannitol, can account for up to 40% of the total sugar content (22). The results of this study, however, did not reveal any difference in sorbitol concentration between the control and ABA hardened bromegrass cells. Sorbitol itself is probably not a major factor in conferring freezing tolerance but rather may be an intermediate compound for some other process. Kuo et al. (18) have identified what is possibly a sorbitol pathway in which sorbitol may be an intermediary metabolite involved in the inter-conversion of glucose and fructose. They suggested that since excessive fructose inhibits fructokinase activity, and therefore glycolysis / gluconeogenesis, the alternative sorbitol pathway could fulfill the requirement for lower fructose concentration by facilitating the conversion of fructose to glucose via sorbitol. In acclimating bromegrass cells and in other species, the presence of a sorbitol pathway may help to explain a number of phenomena associated with hardening: (1) Sucrose accumulation, as observed in many species (22) would be enhanced by the conversion of glucose to fructose (via sorbitol) resulting in more fructose available for sucrose synthesis and (2) A sorbitol pathway would permit an alternate route for the metabolism of glucose to triose phosphates (17) thus bypassing some of the major regulatory points of glycolysis leading to the 3 carbon compounds. This is significant because low temperatures have been shown to inactivate certain key enzymes of glucose metabolism (10). Phosphofructokinase is a major regulatory enzyme in glycolysis / gluconeogenesis, that happens to be chilling sensitive (9).

Guy (10) has suggested that some of these chilling sensitive enzymes may be upregulated to offset the effects of low temperatures. A sorbitol pathway, similar to one present in animal systems (17), could also maintain the continuity between glucose and the production of other metabolites further down the glycolytic pathway and therefore provide energy and other substates to support the acclimation process. For example, it has been demonstrated that plasma membrane augmentation (33) and increase in lipid bodies (35) occurs in plant cells during hardening. The induction of aldose reductase and therefore the sorbitol pathway would provide an alternative metabolic pathway to generate sufficient energy to maintain cellular function under low temperatures which are inhibitory to glycolysis. The excess fructose could be channeled into sucrose synthesis via sucrose synthase. This mechanism could help to explain the sucrose accumulation commonly observed in cold acclimating plants. In addition, the alternative pathway could maintain the flow of carbon from glucose to glycerol to support increased *de novo* lipid biosynthesis.

5.6 Conclusion

We had previously suggested that the bromegrass cDNA, pBGA61, encoded an NADPH-dependent aldose reductase (20). The complete sequencing of the cDNA now confirms this based on a 92% nucleotide similarity to the barley NADPH-dependent aldose reductase cDNA (2). Expression of pBGA61-related mRNA is predominantly associated with a high level of ABA-induced freezing tolerance in bromegrass suspension-cultured cells. Freezing tolerance developed much more slowly with cold treatment and did not reach the level of hardiness attained with ABA treatment within the experimental timeframe. Accumulation of the pBGA61-related mRNA was transient for the duration of cold treatment. PEG treatment did not increase freezing tolerance substantially nor induce the accumulation of pBGA61-related mRNA . $GA_{4,7}$ treatment was able to lower ABA induced freezing tolerance and correspondingly decreased the pBGA61-related steady state mRNA level. Aldose reductase-related activity was much more prominant only after a high level of ABA-induced hardiness was attained, however there was no increase in the sorbitol level. Taken together, these results support the hypothesis that a change in sorbitol metabolism is associated with a high level of ABA-induced freezing tolerance and that sorbitol itself does not directly contribute to an increase in freezing tolerance.

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6.0 Gene Expression Associated with the Induction of Freezing Tolerance in Bromegrass Cell Cultures by Abscisic Acid and 2',3'-dihydroabscisic Acid

6.1 Abstract

The freezing tolerance of bromegrass cell suspension cultures has been shown to increase rapidly with ABA treatment at room temperature and is associated with the upregulation of a group of polypeptides and translatable RNAs. In a previous study, 16 cDNAs of ABA responsive genes were isolated from an ABA hardened bromegrass cDNA library. Structure-activity relationship studies with ABA analogs indicated that certain isomers of ABA were able to induce a hardening response comparable to that of ABA. This study was initiated to test the hypothesis that ABA analogs which induce a weaker hardening response than ABA will also have a different pattern of mRNA accumulation corresponding to the 16 cDNAs. The goal is to identify ABA-responsive genes that are most associated with the induction of freezing tolerance. Bromegrass cell cultures were treated with 75 μM of the (+) or (-) enantiomers of ABA or the analog 2',3'-dihydro ABA for 5 days and the LT_{50} determined. (+)-ABA induced a level of freezing tolerance (LT_{50} -19°C) similar to (-)-ABA treatment (LT_{50} -18°C), and there was little difference in the accumulation of the 16 mRNAs between the 2 enantiomers. Bromegrass cells treated with (+)-2',3'-dihydro ABA, however induced a higher level of hardiness and a different pattern of RNA accumulation than treatment with (-)-2',3'-dihydro ABA. Cells that were treated with the (-)-enantiomer were less hardy (LT_{50} -12.7°C) than the (+)-enantiomer (LT_{50} -22°C). Most of the 16 mRNAs were weakly induced by the (-) form compared to the (+) form. Nine of the mRNAs showed a much stronger hybridization signal with (+)-

2',3'-dihydro ABA treatment and thus are candidates for further analysis. This study has demonstrated that (-)-2',3'-dihydro ABA is less effective in promoting a hardening response than the (+)-enantiomer and correspondingly, the expression of many of the 16 mRNAs was lower. Nine of the mRNAs were found to reflect this difference in hardiness.

6.2 Introduction

Several lines of evidence suggests that abscisic acid (ABA) plays an important role in plant cold acclimation. During acclimation, endogenous ABA levels frequently increase (3, 8). It is also well established that exogenous ABA treatment at warm temperatures enhances freezing tolerance in whole plants (3, 13) or cell culture systems (4, 10, 14). Associated with the induction of freezing tolerance by ABA or cold treatment is the upregulation of a group of polypeptides and translatable RNAs (13, 25, 14). Recently, cDNAs corresponding to cold responsive genes of alfalfa (19) and *Arabidopsis* (9, 11, 20) have been isolated and were found to be ABA responsive as well.

ABA analogs have been used to study the effect of structural changes in the ABA molecule on biological activity (18, 27). It has been shown that the effect of ABA treatment on seed embryo development (26), transpiration (2) and cold acclimation (23, 21) is altered by changes in the structure of ABA. Walker-Simmons et al. (26) have generated a series of systematic permutations to the ABA molecule to determine which structures elicite an immunological response similar to ABA itself. By studying the stereo specific requirements for monoclonal antibody recognition of ABA, the authors (26) intend to design ABA specific receptor probes and to analyze the changes in physiological response induced by the various ABA analogs.

A promising area of cold hardiness research is the use of ABA analogs to understand the mechanisms of ABA induced freezing tolerance (21). Robertson

et al. (23) observed that the ABA analog, (±)-2',3'-dihydroabscisic acid, was effective in inducing freezing tolerance in bromegrass cell cultures (23) and a subsequent study (21) had identified the (+) enantiomer as more active than the (-) enantiomer. A group of proteins modulated by racemic ABA was also responsive to racemic (±)-2',3'-dihydroabscisic acid (23). Since it is known that different ABA analogs produce different physiological effects (18) and ABA induced freezing tolerance at least in part is due to altered gene expresion (14, 15), it may be possible to identify analogs which induce a specific group of ABA responsive genes that are important for the acclimation process.

We have previously shown that the addition of racemic ABA to bromegrass cell suspension cultures increases the level of freezing tolerance (4) and alters the pattern of protein synthesis (22, 14) and translatable RNAs (14). Bromegrass cDNAs, isolated from a cDNA library made from RNA of ABA hardened cell cultures, have enabled us to identify the accumulation of mRNA closely related with the development and maintainance of freezing tolerance (16). Preliminary sequencing data of the cDNAs indicate high nucleotide and amino acid homologies to genes in other organisms involved in sugar metabolism, dehydration stress and protease activity (16).

In this study, the objective was to test whether ABA analogs that are less effective in increasing freezing tolerance induce the expression of fewer ABA responsive genes or induce lower levels of the mRNAs corresponding to ABA responsive genes from bromegrass (16) than ABA treatment. We demonstrate that the ABA analog (+)-2',3' dihydro ABA induces a higher level of freezing

tolerance in bromegrass suspension-cultured cells than does the (-) enatiomer and correspondingly a greater accumulation of RNA was detected for most of the cDNA clones. Some of the clones revealed considerably greater RNA accumulation with (+)-analog than the (-)-analog.

6.3 Materials and Methods

6.3.1 Plant material and treatments

In addition to the bromegrass cell suspension culture (designated BG1) used in our previous studies (14, 16), another bromegrass suspension-cultured cell line (BG2) was used for this study. Preliminary experiments had indicated that BG1 was hardier than BG2 after ABA treatment (data not shown) thus we were interested in knowing whether this difference in hardiness was reflected at the RNA level. Both cell lines were subcultured and maintained as previously described (4). ABA treatments consisted of transferring five day old cells to media containing 0.5 mg l^{-1} 2,4-D with 75 μM commercial (±)-ABA (Calbiochem, San Diego, CA), (+)-ABA (**1**), (-)-ABA (**2**), (±)-ABA (**1+2**, in equal molar proportions), the ABA analog (+)-2',3' dihydro ABA (**3**) or (-)-2',3'-dihydro ABA (**4**) ABA (12). The chemical structures of these compounds are illustrated in Figure 6.1 and will be referred to by their assigned numbers. The cultures were treated at 23°C on an orbital shaker for five days. After treatment cells were harvested and either frozen immediately in LN_2 and stored at -70°C or prepared for freezing tests (14). The treatments were repeated three times.

6.3.2 RNA isolation and gel blot analysis

Total RNA was isolated from frozen bromegrass cells by the guanidine isothiocynate / CsCl method as described elsewhere (14). Twenty μg of total RNA was eletrophoresed on a 1.3% agarose - formaldehyde gel for 2 h (24). Blotting and hybridization conditions have been detailed previously (16). The probes used for hybridization were ^{32}P - labeled cDNA inserts from bromegrass cDNA clones previously isolated (16). Clone pBGA89, which does not preferentially hybridize to RNA from ABA treatments (16), was used to ensure equal loading in all the lanes.

6.4 Results

6.4.1 Induction of freezing tolerance

The level of freezing tolerance induced by the various treatments are illustrated in Figure 6.2. After five days of ABA treatment, the BG1 cell line hardened to an LT_{50} of -28°C and the BG2 cell line to -23°C, thus both cultures respond to ABA but the degree of hardiness attained was different.

Within the BG2 culture, commercial racemic (±)-ABA treated cells (LT_{50} -23°C) were hardier than with **1+2** (LT_{50} -18°C) by 5°C but were not much different compared to treatment with **1** or **3**. There was a clear difference in freezing tolerance between cells treated with **3** and **4**, with **3** (+ form) being 8.5°C hardier. Compound **1** (+ ABA) induced a level of hardiness similar to compound **2** (- ABA).

6.4.2 RNA accumulation

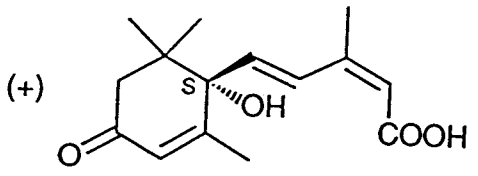
The accumulation of RNA hybridizing to the cDNA probes of ABA responsive genes from bromegrass (16) in response to the various ABA analog treatments are shown in Figure 6.3. Both cell lines, BG1 and BG2, exhibited a similar pattern of RNA accumulation after commercial (±)-ABA treatment. Steady state levels of RNA corresponding to each of the clones were considerably higher than the controls. The hybridization signal of pBGA80 and pBGA86

probes to BG1 RNA, however, was significantly stronger than with BG2 RNA. In contrast, pBGA56 and pBGA85 probes detected a much stonger signal in the BG2 cell line than the BG1 cell line.

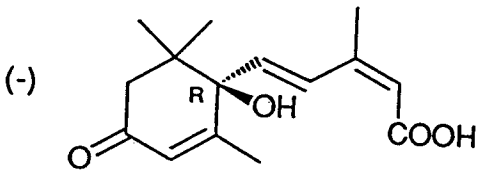
With the exception of clones pBGA6, pBGA72 and pBGA80, accumulation of the corresponding RNAs was greater with commercial (±)-ABA than with **1+2** mixture (Figure 6.3). This was clearly evident with clone pBGA55.

Many of the cDNA probe hybridization signals indicated that both **1** and **2** induced similar levels of RNA accumulation (Figure 6.3). Only clones pBGA80 and pBGA86 clearly show a stronger hybridization signal to RNA from cells treated with compound **1**. The signal was stronger with compound **2** treatment for clones pBGA25, pBGA55 and pBGA85.

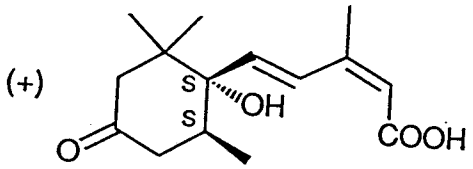
A majority of the probes show either no hybridization to RNA or a very weak signal at best with compound **4**. Only pBGA8, pBGA34, and pBGA85-related mRNA levels were either similar or greater than treatment with compound **3**. Compound **3** also appears to be as effective as **1** or **1+2** at inducing the accumulation of the RNAs corresponding to the respective cDNA clones. The clones that have a substantially stronger signal with **3** treatment than with the (-)-enantiomer include pBGA6, pBGA36, pBGA44, pBGA55, pBGA60, pBGA61, pBGA64, pBGA80 and pBGA86.



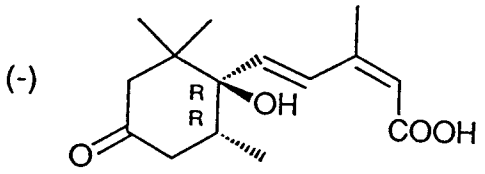
(1) (+)-S-abscisic acid



(2) (-)-R-abscisic acid



(3) (+)-(4S,5S)-dihydro ABA



(4) (-)-(4R,5R)-dihydro ABA

Figure 6.1 Chemical structures of enantiomers of ABA and 2',3'-dihydro ABA. Structures (1), (2), (3) and (4) are identified as (+)-ABA, (-)-ABA, (+)-2',3'-dihydro ABA and (-)-2',3'-dihydro ABA respectively in the text.

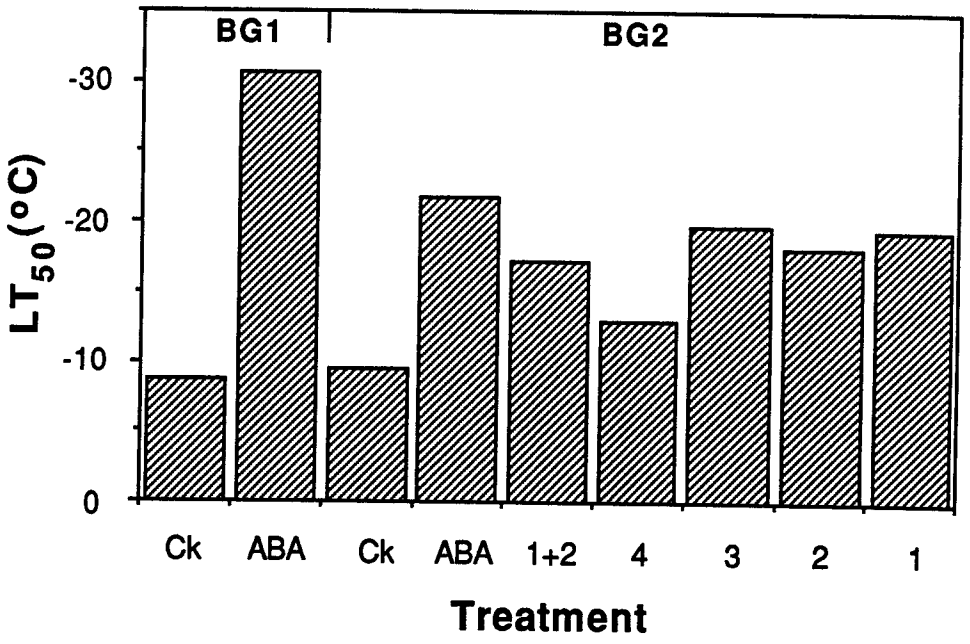


Figure 6.2 Induction of freezing tolerance by ABA and 2',3'-dihydro ABA. Two bromegrass cell lines, BG1 and BG2, were compared for their response to commercial racemic ABA. BG2 was used to compare the difference in hardiness of the compounds (+)-ABA (**1**), (-)-ABA (**2**), (+)-2',3'-dihydro ABA (**3**) and (-)-2',3'-dihydro ABA (**4**). **1+2** is an equal molar mix of **1** and **2**. All treatments were conducted with a 75 μM concentration.

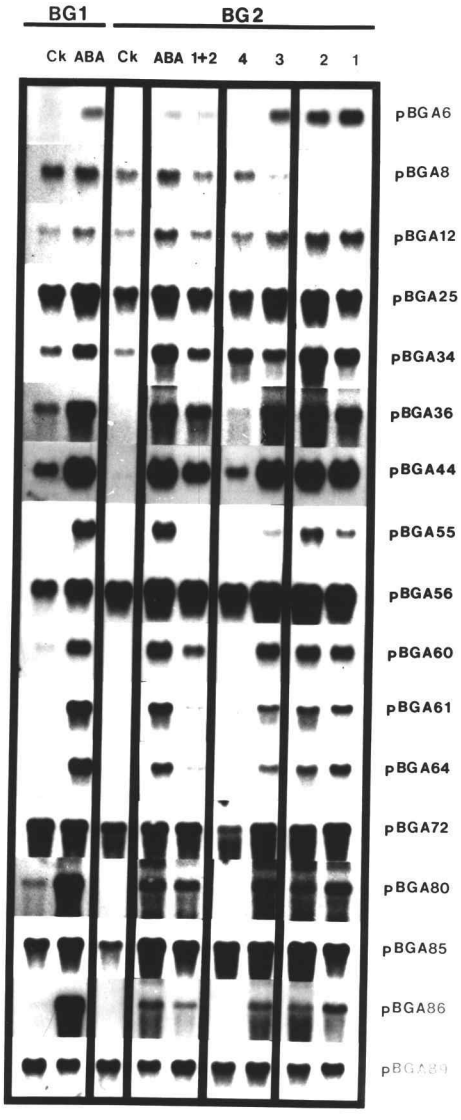


Figure 6.3 Accumulation of steady state RNA in response to ABA or 2',3'-dihydro ABA. Each row corresponds to a labeled probe from previously isolated cDNAs (16). Two bromegrass cell lines, BG1 and BG2, were compared for their response to commercial racemic ABA. BG2 was used to compare the difference in RNA accumulation with the compounds (+)-ABA (1), (-)-ABA (2), (+)-2',3'-dihydro ABA (3) and (-)-2',3'-dihydro ABA (4). 1+2 is an equal molar mix of 1 and 2. Twenty μg of total RNA was loaded into each lane of an agarose-formaldehyde gel.

6.5 Discussion

As was previously demonstrated (23), and confirmed in this study, structural differences in the ABA molecule results in a different level of freezing tolerance in bromegrass suspension-cultured cells. The (+)-enantiomer of 2',3'-dihydro ABA (3) induced a greater degree of hardiness than the (-)-enantiomer. Although the overall pattern of hardiness induction between the (-) and (+) form of 2',3'-dihydro ABA was the same in this and a previous study (23), we did not attain the same level of hardiness as was reported. This study also did not show any difference in hardiness after (+) or (-)-ABA treament. These differences may be due to the difference in treatment times (5 days for this study and 7 days for the previous study), the use of different freezing protocols, or different tissue culturing procedures.

Conceivably, the difference in hardening response after ABA treatment between BG1 and BG2 may be due to somaclonal variation. A common concern of suspension-cultured cells is the possibility of inadvertently selecting for somaclonal variants after many cycles of subculturing (17). Both BG1 and BG2, which originated from the same culture (6), retained the ability to harden with commercial racemic (±)-ABA treatment. Bromegrass cell lines derived from callus tissue of individual seeds also retained the ability to harden with ABA treatment (Lee et al. unpublished data). The pattern of BG2 RNA accumulation corresponding to each of the BG1 cDNA clones (16) was also similar to the pattern of BG1 RNA accumulation (Fig. 6.3). It can be concluded that even

though BG2 was less hardy than BG1, both cell lines still responded to ABA induce freezing tolerance in a similar manner and therefore it appears that whatever mutations that occured during culturing did not affect ABA induced hardening.

Commercial (±)-ABA treated cells were more hardy and expression of RNAs corresponding to the cDNAs were greater than after **1+2** treatment. Robertson et al. (21) however, reported that both treatments resulted in similar levels of hardiness. They also observed that (+)-ABA induced a greater level of hardiness than (-)-ABA in bromegrass cells but again this study did not reveal any difference in hardiness between the two enantiomers, thus it is possible that this difference in response to ABA was due to variations in cultural, treatment or freezing methodology. Despite these differences, the results of this study have consistently demonstrated that a higher level of freezing tolerance is associated with higher levels of the cDNA-related RNAs.

The (+)-form of 2',3'-dihydro ABA (**3**) induced a stronger hardening and RNA accumulation response than did the (-)-enantiomer (**4**). Robertson et al. (20) had observed that racemic 2',3'-dihydro ABA increased the freezing tolerance of bromegrass suspension-cultured cells by 22°C and induced the same set of proteins as (R,S)-ABA treatment. While there is no data available on which of the bromegrass cDNA-related-mRNAs (16) expressed are represented at the protein level, our results suggest that compounds **1**, **2** and **3** induce a similar pattern of gene expression. It would therefore appear that removing the ring double bond from ABA neither changes the effect of the molecule on hardiness

induction nor on expression of the cDNA-related RNAs.

The observation that **3** elicits a considerably stronger hardening and molecular response in bromegrass than **4**, affords us the opportunity to identify specific bromegrass cDNAs which are most closely associated with hardening. Clones pBGA6, pBGA36, pBGA44, pBGA55, pBGA60, pBGA61, pBGA64, pBGA80 and pBGA86 identify patterns of RNA accumulation concurrent with the induction of freezing tolerance. Clones pBGA55 and pBG61 have previously been associated to the dehydrin (5) and aldose reductase (1) genes of barley respectively (16).

This study has demonstrated that both (+)-ABA (**1**) and (-)-ABA (**2**) are equally effective in inducing freezing tolerance and the expression of the 16 BG1-cDNA-related RNAs. The (+)-enantiomer of 2',3'-dihydro ABA (**3**) induced a hardening and gene expression response pattern similar to (**1**) and (**2**) however treatment with the (-)-enantiomer did not induce as strong an effect. It appears that a higher level of freezing tolerance is associated with a greater accumulation of specific RNAs. Removing the ring double bond from (+)-ABA (**1**) to give (**3**) does not significantly affect hardiness induction nor BG1 cDNA-related RNA accumulation but when the double bond is removed from (-)-ABA (**2**) resulting in compound (**4**), a considerably weaker response at both the physiological and the molecular level was observed. Via the use of two bromegrass cell lines differing in hardiness and the ABA analog 2',3'-dihydro ABA, it was possible to identify mRNAs corresponding to the bromegrass cDNAs most strongly associated with freezing tolerance and are thus candidates for further analyses.

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