# Molecular Cloning of Abscisic Acid-Responsive mRNAs Expressed during the Induction of Freezing Tolerance in Bromegrass (*Bromus inermis* Leyss) Suspension Culture[1]

## Stephen P. Lee[2] and Tony H. H. Chen*

Department of Horticulture and Center for Gene Research and Biotechnology, Oregon State University, Corvallis, Oregon 97331

Abscisic acid (ABA) increases the freezing tolerance of bromegrass (*Bromus inermis* Leyss) cell-suspension cultures at 23°C and elicits many metabolic changes similar to those observed during cold acclimation. Induction and maintenance of freezing tolerance by ABA is accompanied by the expression of novel polypeptides and translatable RNAs. The objective of this study was to isolate and characterize ABA-responsive cDNAs associated with ABA-induced freezing tolerance in bromegrass cell cultures. Among the 16 ABA-responsive cDNA clones isolated, 9 were expressed only with ABA treatment, 7 showed increased transcript level, and 1 was transiently expressed. Cold responsiveness was determined in three clones with increased transcript levels and in the transiently expressed clone. Deacclimation of ABA-hardened cells was a relatively slow process, because all of the novel transcripts persisted for at least 7 d after cells were cultured in ABA-free medium. Preliminary sequencing of cDNAs has identified several clones that share high sequence homology with genes associated with sugar metabolism, osmotic stress, and protease activity. Clone pBGA61 was fully sequenced and tentatively identified as an NADPH-dependent aldose reductase. The predicted amino acid sequence of the coding region shared 92% similarity with that predicted for barley aldose reductase cDNA. It is proposed that expression of genes related to sugar metabolism and osmotic stress may be required for ABA-induced hardening.

Many temperate plant species develop greater freezing tolerance when exposed to low, nonfreezing temperatures. This process, cold acclimation, involves a variety of biochemical and physiological changes resulting in the induction of freezing tolerance (Levitt, 1980). Accumulating evidence indicates that changes in gene expression occur during cold acclimation (Guy, 1990; Thomashow, 1990). cDNAs corresponding to novel RNAs expressed during cold acclimation have been isolated from a number of plant species (Mohapatra et al., 1988, 1989; Cattivelli and Bartels, 1990; Hajela et al., 1990; Kurkela and Franck, 1990; Nordin et al., 1991; Houde et al., 1992; Luo et al., 1992).

In addition to low temperatures, exogenous application of ABA to either whole plants (Chen et al., 1983; Lang et al., 1989) or cell cultures (Chen and Gusta, 1983; Keith and McKersie, 1986) at room temperature also induces freezing tolerance. With bromegrass (*Bromus inermis*) cell cultures, it has been demonstrated that 4 to 5 d of ABA treatment increases freezing tolerance by 23°C, with a change in $LT_{50}$ from −7 to −30°C (Chen and Gusta, 1983; Lee et al., 1991). Associated with a rapid increase in hardiness were qualitative changes in the population of polypeptides (Robertson et al., 1987) and translatable RNAs (Lee et al., 1991). Deacclimation of ABA-hardened bromegrass cells is a relatively slow process (Reaney et al., 1989; Lee et al., 1991) compared to other cold-hardy species (Guy et al., 1985; Hajela et al., 1990; Tseng and Li, 1990). Correspondingly, the disappearance of novel polypeptides and RNAs was slow (Lee et al., 1991). It is not clear in this system whether freezing tolerance induced by ABA or by low temperature occurs via the same mechanism.

The objective of this study was to isolate and characterize ABA-responsive cDNAs associated with ABA-induced freezing tolerance. Differential screening of a cDNA library constructed from poly(A)+ RNA of ABA-treated bromegrass cells resulted in the identification of 16 ABA-responsive cDNA clones. Using the EST method of Adams et al. (1991), we have identified several cDNA clones with high nucleotide sequence homology with genes involved with sugar metabolism, desiccation tolerance, and protease activity. Clone pBGA61 was completely sequenced and was found to share a high degree of similarity to the barley NADPH-dependent aldose reductase cDNA (Bartels et al., 1991).

## MATERIALS AND METHODS

### Plant Material and Treatments

Cell-suspension cultures of smooth bromegrass (*Bromus inermis* Leyss) were maintained and subcultured as described by Chen and Gusta (1983). Five-day-old cells were treated with various concentrations of (±)-ABA in medium containing 0.5 mg L⁻¹ of 2,4-D and incubated at 23°C on an orbital shaker for various times. Deacclimation after ABA treatment was initiated by thoroughly washing cells with ABA-free medium and transferring them into fresh ABA-free medium (Lee et al., 1991). Cells were cold treated by incubating at 4°C for up to 5 d while shaking. A sample of the cells from

Abbreviations: EST, expressed sequence tag; $LT_{50}$, temperature at which 50% of the cells were killed; SSC, standard sodium citrate.

each treatment was collected to determine the level of freezing tolerance (Lee et al., 1991). The remaining portion was immediately frozen in liquid nitrogen and stored at −70°C. All treatments and freezing tests were repeated three times.

## RNA Isolation

As detailed by Lee et al. (1991), total RNA was extracted by the guanidine isothiocynate/cesium chloride ultracentrifugation method. Poly(A)$^+$ RNA was further separated by the push column method (Lee et al., 1992).

## cDNA Library Construction and Differential Screening

Poly(A)$^+$ RNA (5 $\mu$g) from bromegrass cells treated with 75 $\mu$M ABA for 5 d 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 (1983) and cloned into the EcoRI/XhoI site of the λZapII vector (Stratagene, La Jolla, CA). Primary recombinant clones were packaged in vitro with Gigapack II (Stratagene) and subjected to one round of amplification in Escherichia coli strain PLK-F'.

Recombinant phage were plated at a density of 5 × 10⁴ plaque-forming units/132-mm plate with E. coli strain XL1-Blue (Stratagene). 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 (Sambrook et al., 1989) of control or ABA-treated cells and then hybridized to plaque lifts. Prehybridization and hybridization conditions were 6× SSC (1× SSC = 0.15 M NaCl, 0.015 M Na citrate), 0.06× Blotto (1× = 5% [w/v] nonfat dried milk, 0.02% NaN₃), and 10 $\mu$g $\mu$L$^{-1}$ of poly(A)$^+$ RNA at 65°C. Membranes were washed three times in 2× SSC containing 0.1% SDS at 65°C for 20 min and once in 1× SSC, 0.1% SDS for 30 min and then exposed to x-ray film (Kodak XAR-5) with one intensifying screen for 36 h.

Plaques that differentially hybridized to the probe from ABA-treated cells were selected and purified. Positive clones were cross-hybridized to select unique clones. The phagemid containing the cDNA was rescued by the in vivo excision method according to the manufacturer's directions (Stratagene).

## Analysis of RNA

Total RNA (20 $\mu$g) from ABA- or cold-treated bromegrass cells was slot blotted (Sambrook et al., 1989) onto nitrocellulose membranes (Bio-Rad), prehybridized, hybridized, and washed using the same conditions described for plaque lifts. Each of the cDNA probes were [$^{32}$P]dCTP labeled by the random priming method and hybridized individually to the blotted membrane. Autoradiographs of membranes were scanned with a densitometer (model 1650; Bio-Rad) to determine relative steady-state transcript levels.

Total RNA from treated bromegrass cells was electrophoresed on a 1.3% agarose-formaldehyde gel (Sambrook et al., 1989). After electrophoresis, RNA was stained with ethidium bromide and visualized with UV light to ensure equal loading in each lane. RNA was blotted onto a nylon membrane

(Zetaprobe; Bio-Rad) according to the manufacturer's directions. Replicate blots were probed with each unique cDNA isolated. Prehybridization and hybridization were carried out in 0.25 M Na₂HPO₄ (pH 7.2), 1 mM EDTA, 7% SDS at 65°C. Washes were conducted in 5% SDS, 20 mM Na₂HPO₄, 1 mM EDTA at 65° for 30 min followed by two washes in 1% SDS, 20 mM Na₂HPO₄, 1 mM EDTA at 65°C. Membranes were then exposed to x-ray film with one intensifying screen for 2 to 24 h. A randomly selected clone from the bromegrass cDNA library identified a gene expressed at high levels in controls and minimally affected by ABA or cold treatment. As a probe, this clone was used to assure that approximately equal concentrations of RNA were loaded for each gel blot analysis.

## Generation of ESTs and DNA Sequencing

Phagemids containing bromegrass cDNAs were purified from overnight cultures by the plasmid column purification method (Stratagene). Approximately 1.5 $\mu$g of phagemid in 6 $\mu$L of H₂O was used for automated dideoxynucleotide chain termination (Sanger et al., 1977) sequencing with the Applied Biosystems model 373A DNA sequencer. The sequencing strategy was based on the method of Adams et al. (1991). Between 350 and 400 bases of the 5' end were sequenced, providing enough information for preliminary identification of similar sequences stored in GenBank and EMBL data bases.

A complete nucleotide sequence was obtained for one bromegrass cDNA by the nested deletion procedure of Heinikoff (1987). Each deletion construct was sequenced as described above. The complete cDNA sequence was used to search GenBank and EMBL data bases for similarity matches.

## RESULTS

### Freezing Tolerance

The frost hardiness of bromegrass cells after various treatments, expressed as $LT_{50}$ values, are summarized in Table I. ABA treatment (75 $\mu$M) for 1, 2, or 5 d increased freezing tolerance from −6.5 to −9.4, −12.2, or −30.8°C, respectively. After 5 d of ABA treatment, removal of ABA from the medium did not result in a noticeable decrease in freezing tolerance immediately. However, 7 d after ABA removal, the $LT_{50}$ decreased from −30.8 to −17.1°C. There was no increase in hardiness after 1 d of 0.1 (−6.5°C) or 1 $\mu$M (−6.4°C) ABA treatment, but ABA concentrations >10 $\mu$M increased hardiness (−7.1, −9.4, and −8.5°C for 10, 75, and 100 $\mu$M ABA, respectively). Cold treatment after 1 d did not increase hardiness, but after 5 d, hardiness increased to −15.3°C (Table I).

### Isolation of ABA-Responsive cDNA Clones

Poly(A)$^+$ RNA from bromegrass treated with 75 $\mu$M ABA for 5 d was used for the construction of a cDNA library. Approximately 4.5 × 10⁵ recombinant phage were differentially screened for ABA-responsive clones using first-strand cDNA probes synthesized from poly(A)$^+$ RNA of ABA-treated or control cells. Cross-hybridization detected 16 unique clones of the 100 cDNA clones isolated.

**Table I.** *Freezing tolerance of bromegrass culture cells after ABA or low-temperature treatment*

Bromegrass cells were incubated with 0 or 75 $\mu$M ABA at 23°C or without ABA at 4°C. $LT_{50}$ values were assessed by the 2,3,5-triphenyltetrazolium chloride reduction assay as described in "Materials and Methods." Values represent the means $\pm$ SE of three separate experiments.

| Treatment | | $LT_{50}$ | | | | | |
|---|---|---|---|---|---|---|---|
| ABA concentration | Temp | Days of treatment | | | | | |
| | | 0 | 1 | 2 | 4 | 5 | 12[a] |
| $\mu$M | °C | °C | | | | | |
| 0 | 23 | $-6.5 \pm 1.1$ | $-6.8 \pm 0.8$ | $-7.5 \pm 0.6$ | $-9.2 \pm 1.2$ | $-7.7 \pm 1.3$ | $-6.8 \pm 1.5$ |
| 0 | 4 | —[b] | $-7.1 \pm 1.6$ | — | — | $-15.3 \pm 2.8$ | — |
| 75 | 23 | — | $-9.4 \pm 0.9$ | $-12.2 \pm 1.2$ | — | $-30.8 \pm 2.1$ | $-17.1 \pm 3.2$ |

[a] ABA was removed on day 5 and cells were cultured for 7 more d in ABA-free medium. [b] —, No data.

## RNA Gel and Slot Blot Analysis

To verify ABA responsiveness of genes corresponding to these cDNAs, RNA slot blot analyses of total RNA from control and ABA-treated cells (75 $\mu$M; 1-, 2-, or 5-d treatment) were performed. All 16 cDNA clones were identified as ABA responsive (data not shown).

RNA gel blot analysis of total RNA after 0, 1, 2, or 5 d of ABA treatment and of RNA from deacclimated cells previously ABA treated for 5 d was performed using ABA-responsive cDNAs as probes. Three general patterns of ABA-induced gene expression were identified by this method and are illustrated in Figure 1. In the first pattern (pattern A; Fig. 1A), RNA of the corresponding clones accumulated to high levels after 1 d of ABA treatment and continued to increase with 2- or 5-d treatments. Immediately after residual ABA was washed from cells and the cells were cultured in ABA-free medium, the transcript level decreased (Fig. 1, 0d ABA Deacc.) with no noticeable decrease in hardiness (data not shown). Seven days of deacclimation (Fig. 1, 7d ABA Deacc.) reduced both the level of hardiness and the intensity of the hybridization signal. None of the transcripts in this group appeared to be cold responsive after 1 or 5 d of cold treatment. All of the pattern A genes required a minimum of 1 $\mu$M ABA to induce accumulation of steady-state RNA.

The second pattern of ABA-responsive gene expression (pattern B) includes transcripts that were expressed in control cells throughout 5 d of treatment but were present at elevated levels during ABA treatment (Fig. 1B). Transcript levels of the 0-d control (Fig. 1B, 0d ck) for pBGA12, pBGA56, pBGA72, and pBGA85 were greater than 1 d after transferring to fresh medium (1d ck). All pattern B gene transcript levels decreased to control levels after 7 d of deacclimation. Three of the transcripts (pBGA12, pBGA56, pBGA85) increased 2- to 3-fold in response to cold treatment (Fig. 1B, 1d +4C and 5d +4C). However, higher steady-state transcript levels were only transient, i.e. there is a higher level of transcript at 1 d than at 5 d. ABA, 0.1 $\mu$M, was sufficient to induce a detectable accumulation of transcript, suggesting a high sensitivity of these genes to ABA treatment.

A third pattern of ABA-responsive gene expression (pattern C) identified was similar to pattern B, but increased transcript accumulation was transient (Fig. 1C). Increased transcript corresponding to pBGA25 during ABA treatment was not

maintained throughout the treatment period. The transcript levels were elevated after 1 or 2 d but not after 5 d of treatment. Cold treatment increased transcript accumulation after 1 d but not after 5 d.

## Similarity of ESTs to Other Genes

ESTs were determined for each bromegrass cDNA, and searches for sequence similarities in both GenBank and EMBL data bases were performed using the Intelligenetics Suite (Intelligenetics, Mountain View, CA). Six bromegrass ESTs with high (>50%) homology to reported sequences were identified (Table II). Of interest are clones pBGA61, pBGA55, pBGA56, pBGA85, and pBGA86 with nucleic acid sequence matches to barley NADPH-dependent aldose reductase (Bartels et al., 1991), barley dehydrin (Close et al., 1989), wheat germin (Lane et al., 1991), human cathepsin D (Faust et al., 1985), and barley embryo globulin (G.R. Heck, unpublished data), respectively, and clone pBGA72 with amino acid sequence matches to rabbit phosphoglucomutase (Ray et al., 1983).

## Bromegrass Aldose Reductase Nucleotide and Amino Acid Sequence

To determine whether bromegrass ESTs correspond to predicted homologs of other genes, clone pBGA61 was selected for further analysis. The cDNA insert in pBGA61 was completely sequenced in both directions and determined to be a full-length copy (1192 bp) based on the length of the RNA (1.1 kb) to which it hybridized. The coding strand and predicted amino acid sequence are listed in Figure 2. The longest open reading frame starts at base 79, ends at base 1041, and specifies a polypeptide of 35,938 D, isoelectric point 6.28. Based on percentage of molecular mass, the most abundant amino acids are Lys (11.5%), Glu (9.1%), Leu (8.9%), Val (6.2%), Asp (6.2%), and Ala (4.7%). There does not appear to be a bias for any specific amino acid.

A search made with the complete pBGA61 cDNA sequence strongly suggests its identity as an NADPH-dependent aldose reductase (Bartels et al., 1991). This association is based on 92% similarity found in nucleotide-coding region and predicted amino acid sequence with respect to the putative coding region of the barley cDNA clone pG22-69 (Bartels et
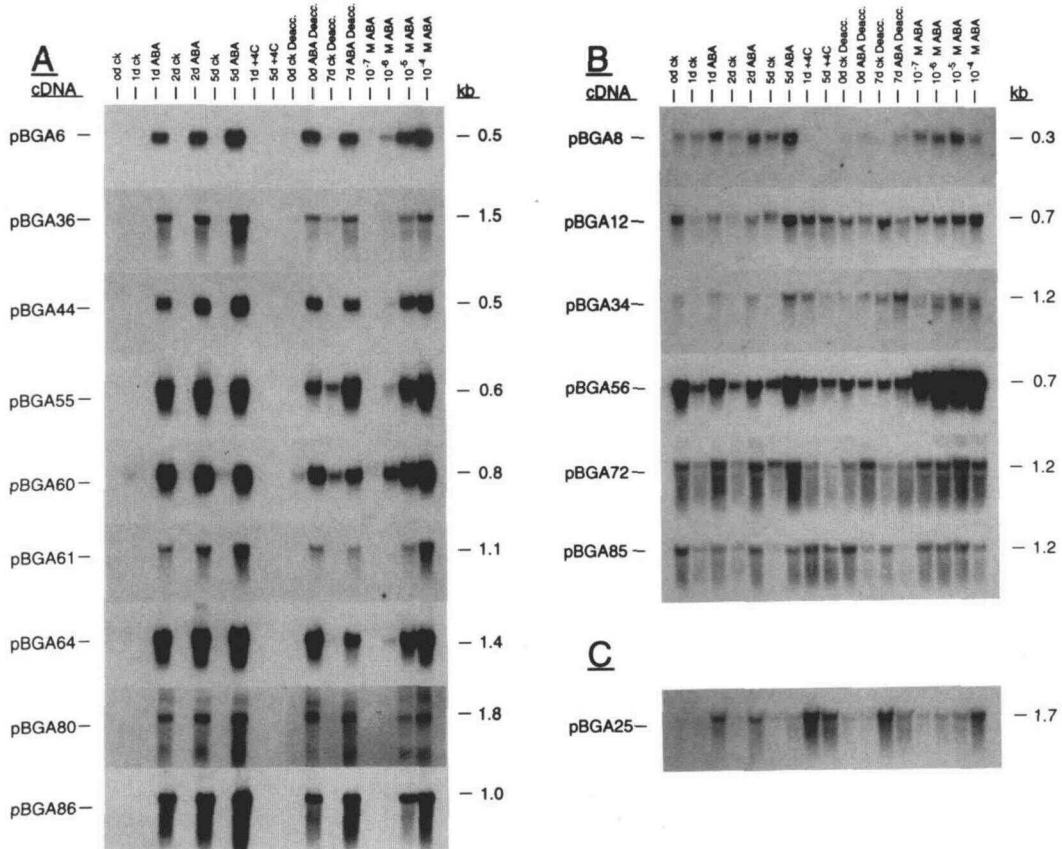
**Figure 1.** RNA gel blot of steady-state transcript levels after ABA or cold treatment. Bromegrass cells were treated for 0, 1, 2, or 5 d without (0d ck, 1d ck, 2d ck, 5d ck) or with 75 μM ABA (1d ABA, 2d ABA, 5d ABA). Five days after control or ABA treatment, cells were washed with ABA-free medium and immediately frozen for RNA extraction (0d ck Deacc., 0d ABA Deacc.) or cultured in fresh medium for 7 additional d (7d ck Deacc., 7d ABA Deacc.). Cultures were also treated with 0.1, 1.0, 10, or 100 μM ABA for 1 d, and the RNA was extracted for analysis. Twenty micrograms 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 transcript expression patterns are shown: A, Transcript detected only after ABA treatment; B, transcript present in controls but elevated after treatment; C, similar to pattern B but increased transcript level is transient.

**Table II.** *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 data bases. Identification refers to the name of the sequence matching the EST; Percentage NA and percentage AA indicate the percentage of nucleic acid or amino acid match, respectively; No. of bases is the number of contiguous nucleotides used for the sequence search; No. and Ref. refer to the GenBank accession number (unless otherwise noted) and corresponding literature citation of the matching sequence, respectively.

| EST | Identification | Organism | Percent NA | Percent AA | No. of Bases | No. | Ref. |
|---|---|---|---|---|---|---|---|
| pBGA55 | Dehydrin | Barley | 82 | 55 | 364 | X15287 | Close et al., 1989 |
| pBGA56 | Germin | Wheat | 55 | 45 | 408 | M63224 | Lang et al., 1989 |
| pBGA61 | Aldose reductase | Barley | 89 | 89 | 351 | X57526 | Bartels et al., 1991 |
| pBGA72 | Phosphoglucomutase | Rabbit | —[a] | 69 | 100[b] | A01175[c] | Ray et al., 1983 |
| pBGA85 | Cathepsin D | Human | 53 | 53 | 431 | M11233 | Faust et al., 1985 |
| pBGA86 | Embryo globulin | Barley | 76 | | 456 | M64372 | G.R. Heck, unpublished data |

[a] The nucleic acid sequence was not available for this gene. [b] Number of contiguous predicted amino acids used for the search. [c] Accession no. from the PIR data base.

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                                                                    CGGCACG   7
AGGACCACCAGACTAGGAAGGAGAAGGCGTCTGAAGAGAGAAGAGAGGTTAGAGATTCAGAAGGTGCGATC   78

ATG GCG AGC GCC AAG GCG ATG ATG GGG CAG GAG AGG CAG GAT CAC TTT GTT CTG  132
MET Ala Ser Ala Lys Ala Met Met Gly Gln Glu Arg Gln Asp His Phe Val Leu

AAG AGC GGG CAT GCC ATC CCA GCC GTC GGA TTG GGC ACC TGG AGA GCT GGC TCC  186
Lys Ser Gly His Ala Ile Pro Ala Val Gly Leu Gly Thr Trp Arg Ala Gly Ser

GAT ACT GCT CAC TCC GTT CAC ACG GCC ATC ACC GAG GCC GGG TAC AGG CAT GTG  240
Asp Thr Ala His Ser Val Gln Thr Ala Ile Thr Glu Ala Gly Tyr Arg His Val

GAC ACA GCT GCT GAA TAC GGA GTA GAA AAG GAG GTC GGC AAA GGG CTT AAA GCT  294
Asp Thr Ala Ala Glu Tyr Gly Val Glu Lys Glu Val Gly Lys Gly Leu Lys Ala

GCA ATG GAA GCT GGG ATC GAC AGG AAA GAT TTG TTT GTG ACG TCA AAA TTA TGG  348
Ala Met Glu Ala Gly Ile Asp Arg Lys Asp Leu Phe Val Thr Ser Lys Leu Trp

TGC ACA GAC TTG GTT CCT GAC AGG GTG CGG CCA GCG TTA GAG AAG ACG CTC AAG  402
Cys Thr Asp Leu Val Pro Asp Arg Val Arg Pro Ala Leu Glu Lys Thr Leu Lys

GAT CTA CAG TTG GAC TAC CTC GAT CTT TAC CTT ATC CAC TGG CCG TTC CGG CTG  456
Asp Leu Gln Leu Asp Tyr Leu Asp Leu Tyr Leu Ile His Trp Pro Phe Arg Leu

AAA GAT GGG GCA CAC AAG CCA CCG GAA GCA GGG GAG GTG CTG GAA TTC GAC ATG  510
Lys Asp Gly Ala His Lys Pro Pro Glu Ala Gly Glu Val Leu Glu Phe Asp Met

GAG GGG GTG TGG AAG GAA ATG GAG AAC CTT GTC AAG GAT GGA CTG GTC AAG GAC  564
Glu Gly Val Trp Lys Glu Met Glu Asn Leu Val Lys Asp Gly Leu Val Lys Asp

ATC GGT GTC TGC AAC TAC ACA GTG ACA AAG CTC AAC CGG CTG CTG CAA TCT GCA  618
Ile Gly Val Cys Asn Tyr Thr Val Thr Lys Leu Asn Arg Leu Leu Gln Ser Ala

AAG ATT GCG CCG GCC GTA TGC CAG ATG GAA ATG CAC CCT GGT TGG AAG AAC GAC  672
Lys Ile Ala Pro Ala Val Cys Gln Met Glu Met His Pro Gly Trp Lys Asn Asp

AAG ATT CTC GAG GCC TGC AAG AAG CAC GGA ATC CAT GCC ACC GCT TAC TCC CCA  726
Lys Ile Leu Glu Ala Cys Lys Lys His Gly Ile His Ala Thr Ala Tyr Ser Pro

TTG TGT TCT TCA GAG AAG AAC CTT GCT CAT GAC CCG GTT GTT GAA AAG GTG GCC  780
Leu Cys Ser Ser Glu Lys Asn Leu Ala His Asp Pro Val Val Glu Lys Val Ala

AAC AAG CTG AAC AAG ACC CCG GGG CAG GTG CTC ATC AAG TGG GCT CTG CAG AGG  834
Asn Lys Leu Asn Lys Thr Pro Gly Gln Val Leu Ile Lys Trp Ala Leu Gln Arg

GGG ACG ATC GTG ATC CCC AAA TCG AGC AAA GAC GAG AGG ATC AAG GAC AAC ATC  888
Gly Thr Ile Val Ile Pro Lys Ser Ser Lys Asp Glu Arg Ile Lys Glu Asn Ile

CAG GTG TTC GGA TGG GAG ATC CCT GAA GAG CAC TTC CAG GTC CTG TGC AGC ATC  942
Gln Val Phe Gly Trp Glu Ile Pro Glu Glu Asp Phe Gln Val Leu Cys Ser Ile

AAA GAT GAG AAG CGT GTG CTG ACC CGG GAG GAG CTC TTC GTG AAC AAG ACC CAC  996
Lys Asp Glu Lys Arg Val Leu Thr Arg Glu Glu Leu Phe Val Asn Lys Thr His

GGG CCG TAC AAG AGT GCA TCC GAG GTC TGG GAT AAC GAG AAC TAG GACGACGACTC  1052
Gly Pro Tyr Lys Ser Ala Ser Glu Val Trp Asp Asn Glu Asn  .

GAATAATGGAGGCTGAAGAAGTGAAGAAGCAAGATGGTACTCCTGGATCAATTCAGACTCTGCCTGATGCT  1123

CTGTAAACAAACGCTGTGTGCAATGAATAAATATGGCTTCGTCGTGCTCCAAAAAAAAAAAAAAAAAAAA  1192
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**Figure 2.** Nucleotide and predicted amino acid sequence of the pBGA61 cDNA insert. The longest open reading frame starts at base pair 79 and ends at 1041.

al., 1991). Nucleotide similarity of the 3'-untranslated region between the two clones was considerably lower (approximately 50%). The putative amino acid sequence of pBGA61 is compared to those of barley pG22-69 and human lens aldose reductase in Figure 3 (Nishimura et al., 1990). The amino acid match between pBGA61 and pG22-69 is high (92%), but neither is as high as with the human lens cDNA.

## DISCUSSION

Studies in the past have identified a host of metabolic changes associated with cold acclimation (for review, see Guy, 1990). These changes have been observed at the protein and translatable RNA levels, however, only a small number of these RNAs have been cloned. In this study, we have isolated 16 unique 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 (A) includes transcripts that were detected primarily after ABA treatment. This type of response is similar to that of the cold-responsive genes identified in other species (Robertson et al., 1987; Mohapatra et al., 1988, 1989; Cattivelli and Bartels, 1990; Hajela et al., 1990; Kurkela and Franck, 1990; Luo et al., 1992). With the second pattern (B) of gene expression, transcripts were detected in the controls but were up-regulated by ABA or cold treatment. In the third pattern (C), the increased transcript signal is transient.

Although not all ABA-inducible genes may be involved in the hardening process, expression of some of these genes could be required for development of frost tolerance and be responsible for the numerous physiological and biochemical changes detected during hardening (Levitt, 1980). A decrease in steady-state transcript levels, corresponding to the ABA-induced cDNAs during dehardening, further suggests that some of these genes could be associated with freezing tolerance. Although deacclimation was apparent, as indicated by dehardening and RNA level attenuation, the process was still much slower relative to those in *Arabidopsis* (Hajela et al., 1990; Kurkela and Franck, 1990) at the transcript level and spinach (Guy et al., 1985), alfalfa (Mohapatra et al., 1987; Luo et al., 1992), and potato (Tseng and Li, 1990) at the translatable RNA level. This difference in response time may reflect a difference between monocots and dicots or between cell cultures and whole plants, or it may occur because bromegrass is much hardier than the other species studied. Nevertheless, persistence of specific ABA-induced transcripts may be required for maintaining an elevated level of hardiness.

Previous reports have shown that certain translatable RNAs expressed in bromegrass (Lee et al., 1991) and potato (Tseng and Li, 1990, 1991) during the first few hours of ABA or cold treatment were not present in later treatments. Transient gene expression during hardening may indicate that the gene product represents an intermediary step in hardening. These types of genes, as represented by clone pBGA25, may not directly confer tolerance to freezing but may be components of a cascade pathway ultimately leading to a hardening response.

It was observed that some of the pattern B RNA gel blots revealed a higher transcript level immediately after transfer to fresh ABA-free medium (Fig. 1, 0d ck) than after 1 d of culture in the same medium (Fig. 1, 1d ck). After 1 d of culturing, however, the transcript level was much lower than the level determined after 1 d of ABA treatment. A stronger hybridization signal in the 0-d control is likely due to stress to the cells when they are transferred to fresh medium where the osmotic potential or pH may be different.

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pBGA61      MASAKAMMGQ ERQDHFVLKSGHA IPAVCLGTW  RAGSDT AHSVQTAITEAGYRHVDT   56
BLYALREDR   ------T---G-  ------------  M------  ------  ----R------------   56
HUMALRMB    ---       -  LL-NN-  -KM-IL-----KSPP-QV-E-  -KV-- DV----I-C   45

pBGA61      AAEYGVEKEVG   KGLKAAMEAGIDRKDLF VTSKLWCT     DLVPDRVRPALEKTL   107
BLYALREDR   -----------   ----------------- ------I---   N-A-E-------N--   107
HUMALRMB    -HV-QN-N---VAIQE-  -  R-QVVK-EE--I-  ------YHEKG-   -KG-CQ---   97

pBGA61      KDLQLDYLDLYLIHWP  FRLKDGAHK    P PEAGEVLEFD  MEGVWKEMENLVKD GL   159
BLYALREDR   -------I---H----  --------  M-  ----------  ----------------   159
HUMALRMB    S--K------------TG--  -P-  -EFP-LD-S-N-VPS-TNILDT-AA--E-- -E--   153

pBGA61      VKDIG  VCN YTV TKLN RLLQSAKIAPAVCQMEMHPGWKNDK ILEAC KKHGIHATA   213
BLYALREDR   -----  ----- --- ----R----P---------------- -F-- ----------V-   213
HUMALRMB    --A--ISNF-HLQ-EMI--KPG-   -YK---N-I-C--YLTQE-L- QY-QS- ---VV--   209

pBGA61      YSPLCSSEKNLA  HDP VVE    K VANKLNKTPGQVLIKWALQRGTIVIPKSSKDERI   266
BLYALREDR   ----G--------  ------- ---  ---------------------------S-------   266
HUMALRMB    ----G-PDRPW-KPE--SLL-DPRI-AI-A-H---TA----RFPM--NLV-----VTP---   270

pBGA61      KENIQVFGWEIPEEDFQVLCSIKDEKRV LTGEELFVNKT HGPYKSASEVWDNENX   321
BLYALREDR   ---------------------R------- ------------ ----R--AD---H---   321
HUMALRMB    A--FK--DF-LSSQ-MTT---YNRNW--CA-    - SC-S-KD- PFH-    -F-   317
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**Figure 3.** Comparison of the bromegrass cDNA (pBGA61) putative amino acid sequence with that of barley (BLYALREDR, Bartels et al., 1991) and human lens (HUMALRMB, Nishimura et al., 1990) putative aldose reductase sequences. A dash (-) indicates an amino acid match with pBGA61. Gaps were introduced to optimize sequence alignment.

Several of the ABA-responsive bromegrass genes appear to be cold responsive but only after 1 d of cold treatment. Because the accumulation of transcripts corresponding to many of these clones was sensitive to subculturing, perhaps a recovery period of 1 d at 4°C following transfer would have resulted in the identification of some false-positive signals. Therefore, only clones pBGA12, pBGA56, pBGA85, and pBGA25 are probable representatives of cold-responsive genes. However, the increase in transcript levels corresponding to these cDNAs was only 2- to 3-fold, which may be attributable to increased RNA stability rather than induced expression. Additional experiments are needed to confirm the cold responsiveness of these genes. Because freezing tolerance can be induced by either ABA or cold treatment, those ABA- and cold-responsive genes may have important roles in cold acclimation.

This study demonstrated that exogeneous ABA application alters the expression pattern of many ABA-responsive genes. The function of many of these genes remains unknown. Through RNA gel blot analysis, it has been possible to demonstrate that many of the ABA-responsive bromegrass genes are associated with development of freezing tolerance. By generating ESTs for the 16 bromegrass cDNAs, we were able to associate some of them to homologous nucleotide sequences of previously sequenced genes. As shown in Table I, clones share high homology to barley NADPH-dependent aldose reductase (Bartels et al., 1991), barley dehydrin (Close et al., 1989), wheat germin (Lane et al., 1991), human cathepsin D (Faust et al., 1985), barley embryo globulin (G.R. Heck, unpublished data), and rabbit phosphoglucomutase (Ray et al., 1983). Both the pBGA61 EST and the putative coding region of the pBGA61 cDNA insert share a high degree of predicted amino acid sequence similarity (89 and 92%, respectively) to barley aldose reductase. A similar strategy utilizing ESTs has been used to screen a potato cDNA library. Three cDNAs, putatively identified as osmotin-like genes, were fully sequenced and found to share a similar degree of homology to tobacco osmotin (B. Zhu, personal communication). Therefore, we recommend the EST method (Adams et al., 1991) as a useful tool for rapidly identifying genes that share homologous sequences to previously reported gene sequences.

Aldose reductase (EC 1.1.1.21) catalyzes the stereospecific reduction of Glc to sorbitol (Shiono et al., 1987). Bartels et al. (1991) 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, sugars accumulate during cold acclimation in many species (Levitt, 1980), including bromegrass (Tanino et al., 1990). Also, cryoprotection studies have demonstrated that sorbitol is one of the most effective cryoprotectants (Chen et al., 1984). It has been suggested that there is a requirement for the up-regulation of genes involved with sugar metabolism during cold acclimation (Guy, 1990). Evidence for this was demonstrated in winter wheat, where Suc synthase polypeptide and poly(A)$^+$ RNA levels increased with low-temperature treatment (Crespi et al., 1991). Results of this study also suggest that a phosphoglucomutase gene, encoding an enzyme of the glycolysis/gluconeogenesis pathway, has a higher level of expression during ABA-induced hardening. The up-regulation of aldose reductase and phosphoglucomutase in bromegrass and of Suc synthase in wheat may, therefore, indicate changes in the molecular regulation of sugar metabolism during hardening.

Expression of a dehydrin-like gene in bromegrass during ABA-induced freezing tolerance may suggest that hardening involves changes in tolerance to dehydration stress. Desiccation of barley seedlings induces the expression of dehydrin (Close et al., 1989) and like expression of many of the Rab (responsive to ABA) genes expression of dehydrin is also induced by ABA treatment (for review, see Skriver and Mundy, 1990). Rab genes are expressed concurrently with increased tolerance to water stress; however, the function of these genes is not known. Freezing stress is considered a form of desiccation stress, because extracellular freezing causes cellular water to move outside the cell (Levitt, 1980). It has been demonstrated that ABA-treated bromegrass suspension culture cells have greater tolerance to freezing stress and to salt stress (Reaney et al., 1989). It is likely that they also have greater resistance to desiccation stress.

A putative homolog (pBGA56) of the desiccation-related wheat germin gene (Lane et al., 1991) is expressed during ABA-induced hardening in bromegrass. Wheat germin is expressed in partially hydrated (60% water) germinating embryos (Lane et al., 1991). The function of this protein is not known; however, a polypeptide from barley that is specifically salt-stress inducible (Hurkman et al., 1991) has a high sequence similarity to it. Germin also has moderate amino acid similarity to spherulins of Physarum polycephalum, proteins expressed if plasmodia are subjected to environmental stresses, including osmotic and temperature extremes (Lane et al., 1991). Germin-like genes, including the bromegrass gene represented by clone pBGA56, may thus represent members of a class of genes associated with desiccation-stress resistance.

A low-temperature-inducible thiol protease cDNA has been cloned from tomato fruit (Schaffer and Fischer, 1988). The tomato cDNA shares regions of homology with other plant proteases and with the animal protease cathepsin H. The bromegrass pBGA85 clone is not only ABA responsive, but is also cold responsive, like the tomato thiol protease gene. Schaffer and Fischer (1988) have suggested that low temperatures may denature certain polypeptides, thus creating a requirement for proteases to degrade the denatured polypeptides. Cold- and ABA-induced protease expression may, therefore, play a more important role in supporting cellular metabolism during low-temperature growth than in developing hardiness.

Although the identities of aldose reductase, dehydrin, germin, and phosphoglucomutase-like RNAs expressed in hardening bromegrass remain to be confirmed, the expression of these types of genes may be indicative of a requirement for tolerance to desiccation stress manifested by freezing temperatures. Work is in progress to characterize these genes. It is certainly possible that expression of these genes is required for the accumulation of osmolytes and tolerance to extracellular freezing-induced dehydration stress.

Clone pBGA86, on the other hand, shares high homology

with barley embryo globulin (G.R. Heck, unpublished data) and with a class of maize embryo proteins (Belanger and Kriz, 1989). Because the maize globulins most likely function as storage proteins (Belanger and Kriz, 1989), clone pBGA86 probably has a similar role and is, therefore, not related to cold acclimation.

In summary, we have isolated 16 cDNAs from bromegrass cDNA library representing genes expressed during ABA-induced freezing tolerance. Three distinct patterns of gene expression have been identified. EST analysis indicates that some of the bromegrass cDNAs have high sequence similarity to barley aldose reductase, rabbit phosphoglucomutase, barley dehydrin, and wheat germin genes. The expression of genes putatively associated with sugar metabolism and osmotic stress may be a requirement for freezing tolerance in plant cells.

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