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Bermudagrass Cold Hardiness: Characterization of Plants for Freeze Tolerance and Characterization of Low Temperature-Induced Genes

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Executive Summary

Injury to bermudagrass turf caused by freezing temperatures during winter is a persistent problem over much of its geographic area of use in the USA. This research seeks to reduce risk of freeze injury to bermudagrass grown in temperate regions. The research focuses on accurately assessing the freeze tolerance of bermudagrass cultivars, isolating genes responsible for enhanced freeze tolerance, and enhancing knowledge of the fundamental mechanisms associated with cold hardiness. Specific objectives are to: 1) quantify cold-hardiness of advanced breeding lines, recently released varieties, and established standard varieties and 2) isolate and characterize cold regulated (*Cor)* genes responsible for conferring freeze tolerance.

The low temperature tolerance (LT_{50}) of 11 turf bermudagrasses was evaluated. LT_{50} values (°C) for clonal varieties were: GN-1 =-5.8, Baby = -6.1, Tifway = -6.6, Tifton 94, Quickstand = -8.0, and Midlawn = -8.4. LT_{50} values for seeded varieties were: Arizona Common = -5.6, Mirage = -6.1, Jackpot = -6.3, Guymon = -7.4, and OKS 91-11 = -7.6. These evaluations will continue with selected varieties from: 1) vegetatively-propagated fairway types, 2) seeded fairway types, 3) vegetatively-propagated putting green types, and 4) experimental fairway breeding lines.

Cold-regulated (*Cor)* bermudagrass genes were identified. A *Cynodon* genomic library was constructed from Midiron (*Cynodon dactylon* X *Cynodon transvaalensis*) turf bermudagrass. Screening the library using a 300-bp cDNA bermudagrass clone provided by Mr. Stephen McMaugh from the University of Sydney, Australia, identified nine putative chitinase genes. Sequencing and homology studies completed for three of the clones provided strong evidence that they are chitinase genes, which we designated as *CynCht-1, CynCht-2* and *CynCht-3*. The nucleotide sequences of CynCht-1 and CynCht-2 are in the European Molecular Biology Laboratory (EMBL),Genbank and DNA DataBase of Japan (DDBJ) databases as accession numbers AF105425 and AF105426, respectively.

The expression of bermudagrass chitinases during cold acclimation (CA), drought, and exogenous application of abscisic acid were determined. Expression was investigated in three cultivars that differ in cold hardiness (MSU > Midiron > Uganda). Northern blot analysis indicated that chitinase gene expression is regulated by cold acclimation (CA) at 8°C/2°C day/night temperature cycles. The level of chitinase gene expression varied positively with cold hardiness. The MSU cultivar exhibited the highest level of low temperature-induced accumulation of chitinase mRNA. The low temperature-induced transcript accumulation was reversed by deacclimation at ambient temperatures immediately following the acclimation period. Drought stress induced the expression of the chitinase genes in the three cultivars at levels significantly higher than the levels induced by CA. Exogenous application of abscisic acid also induced chitinase gene expression but only in Midiron and MSU. The ABA-induced expression occurred at much lower levels compared to those resulting from CA and drought. Midiron exhibited a slightly higher level of ABA-induced chitinase transcript accumulation than MSU. CA-, drought- and ABA-induced gene expressions were specific to the crown tissues.

Introduction

Bermudagrass, *Cynodon* sp., is one of the most important turf species in the southern USA and throughout much of the world. Injury due to freezing temperatures during winter is a persistent problem throughout much of the geographic area of use of the species in the USA (Anderson, et al. 1997). For example, extensive winter injury was experienced in the winters of 1977- 78, 1978-79, 1989-90, 1993-94 and 1995-96, with many areas requiring re-establishment (Anderson et al., 1997; Gatschet et al., 1994; O'Brien, 1994; O' Brien, 1996). Bermudagrass winter injury is unsightly, it disrupts turfgrass use during repair, and it is costly. The economic loss from even a few thousand acres of bermudagrass winterkill can be in the millions of dollars (Anderson et al., 1997). Reducing the risk of freeze injury to bermudagrass grown in temperate regions can be accomplished by a combination of actions. These include: 1) identifying and using best adapted varieties and 2) following management practices that mitigate freeze injury, and 3) developing more cold tolerant varieties. Our research focuses on accurately assessing freeze tolerance of bermudagrasses and identifying genes involved in cold tolerance. Both are fundamental to the breeding improvement of turf bermudagrasses for freeze tolerance.

Cold Hardiness Evaluations

Bermudagrasses grown in the transition zone between warm- and cool-season grasses are subject to winter kill. Bermudagrass germplasm improvement programs have identified improved winter survival as a priority. Breeding programs require a rapid, reproducible means to quantitatively evaluate cold hardiness. Although test winters probably supply the best indication of winter survivability, their occurrence is unpredictable and not reproducible. Therefore, our objective is to quantify cold hardiness of advanced lines, recently released varieties, and established standards using laboratory-based methodology. Standardized, quantitative information on tissue cold tolerance is vital to scientists to track their progress in developing new varieties. Cold tolerance is also one of the most important pieces of information for turf managers selecting bermudagrasses for the transition zone.

Cold hardiness evaluations have been divided into four groups, three based on intended use and the fourth comprising advanced selections from the OSU breeding program. The vegetatively propagated fairway types include Baby, Midlawn (standard), Tifway, GN-1, Tifton 94, and Quickstand Common. The second set of bermudagrasses comprises seeded varieties from the last NTEP trial: Jackpot, Mirage, OKS 91-11, Guymon, and Arizona Common (standard). The third series of plants represent bermudagrasses used for putting greens: Floradwarf, Champions, Tifeagle, MS Supreme, Miniverde, Tifdwarf, and Tifgreen (standard). The final set of cold hardiness determinations will examine advanced selections from the OSU breeding program including OKS 95-1 and 18-4. Experiments with fairway and seeded bermudas have been completed. Putting green varieties are currently being evaluated and studies with advanced selections are planned for late 2000. Plans call for repeating experiments for each use type on three dates.

All plants were clonally propagated in cone-tainers except for the seeded group. After plants were established at 28/24 C day/night temperatures, they were acclimated at 8/2 C day/night temperatures for 4 weeks. The 10-hour photoperiod had a light intensity of 400 $E \cdot m^{-2} \cdot s^{-1}$. Tmid values (midpoint of survival vs temperature response curve) for each genotype were determined as previously described (Anderson et al., 1993). Significant differences in Tmid means from the seeded study were determined following ANOVA. Since one of the replications of the fairway study became infested with insects, it was discarded and mean separation could not be performed.

Fairway Bermudas

Genotype	Tmid (°C)
GN-1	-5.8
Baby	-6.1
Tifway	-6.6
Tifton 94	-7.4
Quickstand	-8.0
Midlawn	-8.4

Seeded Bermudas

Genotype	Tmid (°C)
Arizona Common	-5.6 a
Mirage	-6.1 ab
Jackpot	-6.3 abc
Guymon	-7.4 bc
OKS 91-11	-7.6 c

Although data from the fairway study should be interpreted with caution, it appears that GN-1 and Baby were the least hardy with Tmids around -6 C. Freeze tolerance increased from Tifway (-6.6 C), Tifton 94 (-7.4 C), Quickstand (-8.0 C) to Midlawn (-8.4 C). If these values represent true winter survival capacity, genotypes such as GN-1 and Baby will be at greater risk of freeze damage than Quickstand and Midlawn.

Among the bermudagrasses propagated from seed, Arizona Common (-5.6 C) was significantly less cold hardy than Guymon (-7.4 C) and OKS 91-11 (-7.6 C). Mirage (-6.1 C) and Jackpot (-6.3 C) were not significantly hardier than Arizona Common. Although we have not previously examined this combination of genotypes, Tmids of several genotypes were substantially lower (greater hardiness) in previous reports (Anderson and Taliaferro, 1999) where plants were propagated clonally. Although we did not compare seed vs clonal propagation, it is possible that the Tmids of our recently seeded materials reflect the frequent field observation of increased susceptibility to winter injury the first season after establishment.

Isolation and characterization of genes induced during cold acclimation in *Cynodon* sp.

The recent success of recombinant DNA technology in many aspects of crop improvement demonstrates its potential as a tool to further enhance or complement plant breeding efforts towards the development of more cold hardy turf bermudagrass cultivars. One way by which this goal can be accomplished is through the discovery of genes whose expressions contribute either directly or indirectly to increased survival of turfgrasses following periods of freezing stress. Some bermudagrass cultivars are capable of surviving under conditions of freezing temperatures by their ability to cold acclimate at temperatures slightly above 0°C before the occurrence of freezing conditions, a process known as cold acclimation or hardening. The main goal of this research project is to dissect the molecular basis of this biological phenomenon in *Cynodon* through the use of recombinant DNA techniques. Efforts in this area will lead to the discovery of novel genes that may have potential use for genetic improvement of the freezing tolerance not only of bermudagrasses but of other turfgrass species as well.

For the last seven years we have probed the molecular basis of cold acclimation and freezing tolerance in *Cynodon*. One of our earlier findings was the possible involvement of pathogenesis-related (PR) chitinase proteins that may also confer freezing tolerance in bermudagrass crown tissues. We have evidence from two-dimensional protein electrophoretic studies showing that some chitinases are synthesized in larger amounts in response to cold acclimation in the freezing tolerant cultivar Midiron than in the moderately freeze tolerant Tifgreen bermudagrass (Gatschet et al., 1996). A similar situation was recently documented by the Hon et al. (1995) and Antikainen et al. (1997) on winter rye, and by Hincha et al. (1997) in spinach. The results of these studies pointed to the possible secondary roles of PR proteins as antifreeze factors. It is now hypothesized that the biochemical basis for the involvement of PR proteins in freezing tolerance is by virtue of their structural ability to bind to growing extracellular ice crystals, thereby preventing further crystallization, a situation analogous to the mode of action of the AFPs originally isolated from polar fishes (Davies and Hew, 1990). Although still speculative, this possibility is very attractive due to the widespread occurrence of this phenomenon not only in winter rye but also in other overwintering cereal species. This leads to a further hypothesis that this may be an adaptive response specific to monocots (Antikainen et al., 1997). This also points to a possible pleiotropic nature of some PR-protein genes that occur as members of multigene families.

In line with the findings discussed above, we initiated a project with the aim of isolating chitinase genes from Midiron. Our major goal is to characterize members of the chitinase gene families in *Cynodon*. Expected outputs from this project include cloning and sequencing of chitinase genes and analysis of their temporal and spatial expression patterns in relation to cold acclimation and freezing. With this project we also expect to verify the results of initial protein studies by characterizing expression at the gene level. Our accomplishments so far on this project can be summarized into three parts, (1) construction of a *Cynodon* genomic library from Midiron (*Cynodon dactylon* X

Cynodon transvaalensis), (2) isolation and sequencing of bermudagrass chitinase genes, and (3) characterization of chitinase expression in three cold acclimating bermudagrass cultivars during CA, drought stress ad exogenous abscissic acid application

1. Construction of *Cynodon* genomic library.

High molecular weight genomic DNA was isolated from Midiron leaf tissues by the CTAB method. DNA molecules with an average size between 100-120 kb was successfully isolated using this methodology. The high molecular weight genomic DNA was partially digested with *Bam*HI. Digestion products between 9-23 kb in length was fractionated by gel electrophoresis and purified by ethanol precipitation. To avoid self-ligation of the inserts, the cohesive ends of the *Bam*HI digested genomic DNA was partially filled-in with dATP and dGTP by Klenow DNA polymerase enzyme (Promega, Madison, WI). *Xho*I pre-digested Lambda FixII vectors (Stratagene Inc., La Jolla, CA) was also partially filled in with dTTP and dCTP to make its cohesive ends compatible with the ends of the partially filled-in insert DNA. Ligation of the bermudagrass insert DNA with the Lambda FixII vectors was performed with T4 DNA ligase (Stratagene, La Jolla, CA) following the manufacturer's instruction. The ligation product was packaged using Stratagene Gigapack III packaging extracts following the recommended procedures (Figure 1). After packaging, the resulting primary library was titered by infecting XL1-Blue MRA (P2) host cells. The cultures were grown in NZY broth at 37°C overnight or until plaques are visible on the surface of the bacterial mass. An average of 2.5×10^6 plaque forming units (pfu) was obtained per packaging reaction. This size of a library is considered high enough to give at least 90% probability of successfully isolating a particular gene sequence present as a single copy in the genome. Quality analyses of random clones from the library indicate insert size between 9-20 kb. The primary library was subjected to a single round of amplification and is kept in cold storage for other applications in the future.

2. Isolation and Sequencing of Cynodon Chitinase Genes.

<u>Isolation of Chitinase Genomic Clones</u>.

Approximately 8×10^5 clones from the Lambda Fix II library consisting of 2.178×10^6 recombinant phages were screened with the 360 bp bermudagrass chitinase partial cDNA probe. Three clones that produced the strongest hybridization signals were identified (GCyn113, GCyn319, GCyn456) after the tertiary screening (Figure 2A). *Not*I digestion of purified lambda DNA revealed that these clones contained insert sizes ranging from 18-21 kb. The two clones that contained the longest inserts (GCyn113, GCyn456) were digested with several restriction enzymes. The digests were subjected to Southern blot analysis using the same 360 bp probe used in the library screening. *Sal*I fragments, which are 0.9 kb in length from both clones, hybridized to the probe (Figure 2B) and were subcloned into the *Sal*I site of pBluescript-SK and sequenced from both ends. The nucleotide sequences of these two fragments were identical and contained a single open reading frame (ORF). The ORFs contained in these clones were homologous (>50%)

with the conserved region of plant chitinase genes. This result confirmed that the clones isolated from the library indeed contained chitinase genes (data not shown).

To isolate the full-length chitinase genes, the *Sal*I fragment from GCyn113 was used as a probe to search for specific restriction fragments that were at least 2.0 kb. *Stu*I digestion generated probe-reactive fragments (Figure 2C) which were long enough to contain full length genes based on the average length of known chitinase genes in higher plants (2.5 kb) (Hamel et al., 1997). The *Stu*I fragments were subcloned in the *Xho*I site of pBluescript-SK and were subjected to unidirectional deletion (Figure 2D). The fragments were sequenced from both ends. Sequence alignments among the three clones indicated that GCyn113 and GCyn456 contained the same gene and was designated as *CynCht1*. The gene in clone GCyn319 was designated as *CynCht2*.

Structure of Bermudagrass Chitinase Genes

The three original clones (GCyn113, GCyn319, GCyn456) were further digested with other restriction enzymes. The digests were blotted on Hybond N^+ nylon membrane and hybridized with radiolabeled 0.9 kb probe isolated from *Sal*I digest of Cyn113 (Figure 2C). Larger fragments from *Stu*I digests were isolated from all four clones, blunt end-ligated to *Xho*I/*Not*I adapters (Stratagene, La Jolla, CA) and then cloned into the *Xho*I site of the pBluescipt plasmid. Unidirectional deletion series for both ends of the insert were prepared for each *Stu*I subclone using the Erase-A-Base Deletion Kit (Promega, Madison, WI) (Figure 2D). The overlapping deletion series from the three clones were sequenced at both strands by the dideoxy-termination method through an automated DNA sequencer at the Recombinant DNA/Protein Resource Facility, Oklahoma State University. The fragment sequences were analyzed using MacVector computer softwares. The open reading frames in the genomic sequences were analyzed and mapped using the GENSCAN computer program, Massachusetts Institute of Technology (Burge and Karlin, 1998; Burge and Karlin, 1997) while the promoter regions were analyzed using the Promoter Prediction by Neural Network (Reese et al., 1996). All genomic sequences were compared among themselves and aligned with known plant chitinase genes through the blast search algorithms (blastN, blastP, blastX) from the National Center for Biotechnology Information (NCBI), Bethesda, MD. The amino acid sequence of the protein encoded by each gene was analyzed by ExPASy Proteomics Tools (ExPASy Molecular Biology, Swiss Institute of Bioinformatics).

Nucleotide sequence analysis revealed complete coding sequences for both *CynCht1* and *CynCht2* genes. The transcription initiation sites were predicted at the same location within the two genes, 26 nucleotides downstream to the TATA-signal (Figures 3 and 4). The accuracy of the predicted origin of transcription should however be verified experimentally either by cDNA cloning or primer extension analysis.

The coding region of *CynCht1* (Figure 3) is divided into three exons of 126, 100 and 524 bp by two small introns of 94 and 103 bp. Computer analysis predicted the location of introns based on consensus sequences for splice donor and acceptor sites in eukaryotes. The two introns are defined by motifs C/GT...AG/G and G/GT...AG/G, respectively.

The gene encodes a 27 kDa preprotein comprised of 249 amino acid residues. The preprotein is slightly basic based on the calculated pI of 7.52. The predicted transcription start site is located 88 nucleotides upstream to the first AUG codon in the ORF. The coding region of this gene is terminated by a UAG nonsense codon. Three potential polyadenylation signals occur at the 3' end of the gene. The first is a partial signal (AATAA) which occurred 65 nucleotides downstream to the UAG termination codon. The second and third are full polyadenylation signals that occur 288 and 361 nucleotides downstream to the UAG termination codon.

Similarly, the coding region of *CynCht2* (Figure 4) is interrupted by two introns, which are 93 and 107 bp in length. The respective intervening sequences are also defined by C/GT...AG/G and G/GT...AG/G motifs for eukaryotic splice sites. Three exons which are 126, 100 and 530 bp comprised the whole coding sequence. The 27 kDa preprotein encoded by this gene comprised of 251 amino acid residues and has a calculated pI of 8.53. The protein sequence is terminated by UAG codon, which is immediately followed by the heptanucleotide consensus polyadenylation signal (AATAAA) 26 nucleotides downstream. A second potential polyadenylation signal also occur 160 nucleotides downstream to the UAG codon.

The amino acid sequences of the proteins encoded by the two genes were aligned (Figure 5). The sequence alignment revealed that CHT1 and CHT2 proteins are almost identical (96% identical and 97% similar). The homologous regions are located within the span delineated by the *Sal*I fragment identified from the original GCyn113 and GCyn456 subclones. The slight differences between the two proteins are due to few amino acid substitutions located at aa-16 of the signal peptide, and aa-97, aa-109, aa-115, aa-186 and aa-188 located within the catalytic region of the polypeptide. Furthermore, the C-termini of the two proteins are quite distinct from one another. The differences are illustrated as follows:

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                          C  A  H  Q  Q  P  Y  *
CynCht1   5'.....GATTGCGCGCACCAGCAACCGTACTAG..... 3'
CynCht2   5'.....GAT-GCGCACACCATCAACCGTATTAGCAAGTGA.....3'
                          A  H  T  I  N  R  I  S  K  *
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The differences are defined by aa-243 to 249 of CHT1 and aa-243 to 251 of CHT2, and can be explained by several events. These events include the occurrence of a single T-deletion between nt 1014 and 1015 of *CynCht2* relative to *CynCht1* (Figure 5). This deletion in *CynCht2* caused a frameshift and bypassed the first UAG termination codon that was in-frame in *CynCht1*. This frameshift mutation resulted in the extension of the coding region of *CynCht2* to two additional codons downstream where it is terminated by the second in-frame nonsense codon UGA. This event is the major cause of the differences observed in the C-termini of CHT1 and CHT2. Additionally, three nucleotide substitutions are present at the 3' end of the coding region of *CynCht2* relative to *CynCht1*. Although the sequence of this region of *CynCht2* had been confirmed, it is difficult to determine the exact cause of this frameshift. To prove that this is really a biological event requires the isolation and sequencing of the cDNAs corresponding to both *CynCht1* and *CynCht2*. Information on the cDNA sequences should also confirm the

predicted location of polyadenylation sites and essentially the 3' end of the genes. The sequences between the gene and the cDNA should be compared to confirm this observation.

Promoter Regions of Bermudagrass Chitinase Genes

CynCht1 had 649 bp sequenced 5' flanking region (-1 to –649) while *CynCht2* had only 227 bp (-1 to –227). The putative TATA-signals are located at position –25 in both genes (Figures 3 and 4). In addition to the TATA box, two GC-rich motifs were found upstream to the TATA-box (Figures 3 and 4). The first motif (GGCCGGCCGCCCTTG) is very similar to the GGCC-box which was also found in rice chitinase and □-1,3-glucanase genes (Nishizawa et al., 1993; Simmons et al., 1992; Zhu and Lamb, 1991). The second GC-rich motif is defined by the sequence GCCCGGCGCGAGCGCG (43%C, 50%G) located 25 nt upstream to the first GGCC-box.

The promoter regions of the bermudagrass chitinase genes are almost identical (Figure 6). The sequence differences between *CynCht1* (region -148 to +1) and *CynCht2* (region -140 to +1) were detected as two short deletions in *CynCht2* and two nucleotide substitutions located at the region upstream to the TATA-box. It appears that significant sequence variations in 5' flanking sequences of the two genes are located starting at position –141 of *CynCht1* and position –133 of *CynCht2*. However, the sequence information for *CynCht2* is not complete to allow accurate determination of the extent of sequence variation within these regions of the two genes. Definite comparison of these regions of the two genes require further sequencing of the 5' end of *CynCht2*.

The results of nucleotide sequence alignments in the 3'ends (C-terminus + 3' UT + 3'-flanking sequences) showed that the two genes diverged considerably in these regions. The sequences diverged starting at the region downstream from the translation termination codon (Figure 7). The location and number of the potential polyadenylation signals also varied between the bermudagrass chitinase genes. This region will be useful for future development of gene specific probes.

3) Characterization of Chitinase Gene Expression in *Cynodon*

Phytomers (root + crown + stem unit) of cultivars Midiron. Uganda and MSU were transplanted into 40 medium sized pots containing a mixture of fine sand, peat moss and vermiculite (2:3:2) and maintained in a greenhouse (22-37°C). The plants were watered as needed and supplied weekly with dilute fertilizer solution (No-Stain Formula Peter's Professional Plant Food) at a concentration of 240 mg/liter. After three weeks, the plants were transferred to a controlled environment chamber maintained at 28°C day/24°C night and 10/14 hours light/dark cycles. The plants were allowed to equilibrate at this condition for three weeks before initiating the CA treatments. The CA treatments were initiated by placing 30 pots in a controlled environment chamber maintained at 8°C day/2°C night cycles and photosynthetic photon flux of about 300 umol*m^{-2}*sec^{-1} at 10 hours photoperiod (Anderson et al., 1993). Ten plants were used each for 2 and 28 days of CA. The remaining 10 plants were deacclimated (DAC) immediately after 28 days of CA by

transferring to the ambient condition for additional 2 days. A control set consisting of ten pots were maintained in the growth chamber at 28°Cday/24°C night and 10/14 hours light/dark cycles for the duration of the CA treatments.

Immediately after the CA treatments, leaf, crown and root tissues were excised from each plant at appropriate temperature conditions. Tissues were excised from the acclimated plants inside the acclimation chamber while the control tissues were excised at ambient temperature conditions. The excised crown and root tissues were quickly and thoroughly washed with iced-distilled water and then rinsed with phosphate buffered saline with gentle shaking for 5-10 minutes at 4°C. The leaf tissues were washed thoroughly with iced-distilled water immediately after cutting from the plants. The tissues were blot-dried with tissue paper and then immediately frozen in liquid nitrogen prior to storage at –80°C.

For the drought studies, plants were grown in pots and under control chamber conditions as previously described. The drought stress condition was induced by withholding water for 10 consecutive days. The control plants were watered every day to full capacity. The relative water contents of both the control and drought stressed plants were determined using the formula: RWC=(Fresh Weight-Dry Weight)/(Turgid Weight-Dry Weight) X 100 (Baker et al., 1994). Only the crown and root tissues were harvested from the drought-stressed plants because the leaves were severely dehydrated and not suitable for RNA isolation. The harvested tissues were processed and stored as in the CA samples.

The minimum ABA concentration that effected CA was established using Midiron as experimental plant. This cultivar was used for this experiment because it is the genotype used as a standard in determining cold hardiness (LT_{50}) in bermudagrass. The ABA solutions (25, 50, 100 uM) were administered to 10 potted plants of each cultivar. Plants were grown in a growth chamber as previously described and ABA treatments were administered in the growth chamber. The plants were sprayed with 0.1% Tween-20 surfactant, 10-15 minutes prior to ABA application. The ABA solutions were applied to the same plants by both foliar spray and direct watering to the pots. The ABA treatment was repeated at 12-hour intervals for 5 consecutive days. The control plants were maintained in the same chamber. Leaf, crown and root tissues were harvested from control and treated plants 4 hours after the last ABA application. The excised tissues were processed and stored using the same procedures described for the CA samples.

Total RNA was isolated from the bermudagrass sample tissues following a procedure modified from Logemann et al. (1987). Samples of frozen tissue (2.5g) were ground in liquid nitrogen. The total RNA was extracted from the powdered tissues with 8M guanidine hydrochloride (pH 7.0) and purified by precipitation with 4M LiCl and absolute ethanol. The residual DNA in the total RNA samples was removed by DNase I treatment (Gibco-BRL, Grand Island, NY) at 37°C for 1 hour. The concentration and quality of the total RNA samples were determined by spectrophotometric analysis. The A_{260}/A_{280} ratio ranged between 1.6-1.8 in crown and root samples and between 1.7-2.0 in leaf samples. The integrity of the RNA fragments was further confirmed by running in 1% formaldehyde-agarose gels and staining with EtBr, following the electrophoretic

procedures of Sambrook et al. (1989). To verify the estimated concentrations, aliquots from each sample were serially diluted and compared with known concentration standards in EtBr-stained formaldehyde-agarose gels. The intensities of ribosomal RNA bands were compared.

Equal amounts of total RNA samples (18ug) were electrophoresed at 65 volts for 2.5 hours on formaldehyde-agarose gels using the NorthernMax kit (Ambion, Austin, TX). Duplicate gels were prepared for each set of samples. One of the duplicate gels was stained with EtBr to verify equal loading of samples in each of the lanes. The molecular weight plot was constructed based on RNA ladder (RNA Millenium Markers, Ambion, Austin, TX). This plot was used to estimate the size of the mRNAs that hybridized to the probe in the northern blots. The other duplicate gel was blotted for 2 hours on Hybond N nylon membrane (Amersham Life Science, Arlington Heights, IL) by the downward transfer method using a turbo blotter set-up. The RNA was cross-linked to the membrane by exposure to UV for 1 minute.

Three probes were used to detect the chitinase transcripts in northern blots (Figure 2). One was the 0.9 kb *SalI* fragment isolated from *CynCht1* and covers the highly conserved regions of the two genes (middle portion of the ORF). The other two probes were isolated from 3' end of both *CynCht1* and *CynCht2*. The double stranded DNA probes were labeled with □^{32}P-dCTP to about 1 X 10^7 cpm/ul by random-priming method using the RediPrime labeling kit (Amersham Life Science). Hybridization was performed overnight at 42°C using NorthernMax hybridization buffer. The filters were washed twice for 15 minutes with NorthernMax low stringency wash solution at room temperature with gentle shaking. The final washes were performed twice for 15 minutes at 42°C using the NorthernMax high stringency wash solution. The filters were autoradiographed for 48 hours at –80°C.

The semi-quantitative northern blot was optimized by determining the amount of RNA sample within the linear range of the autoradiography film. This was performed by titrating the samples in concentration gradient that covers a wide range of known amounts of total RNA. The trial blots were hybridized with the labeled probe and exposed for 24, 48 and 72 hours at –80°C. The signals obtained from the autoradiograms were quantified by scanning densitometry to generate a standard concentration curve. The curve indicated that concentrations between 10-22 ug of total RNA samples were moderately to highly detectable on autoradiograms and were within the linear range of the film when exposed for not more than 48 hours.

Expression of Chitinase Genes During CA

There was no detectable transcription of the chitinase genes in the control crown tissues of either Midiron or Uganda, as indicated by northern blot analysis using probe P1 (Figure 8). There was a very low basal level of transcription in MSU. However, chitinase gene expression was significantly increased following 2 and 28 days of CA, which was indicated by mRNA bands of about 1.0 kb (Figure 8). This size of the mRNA is consistent with the length of the transcript predicted through the analysis of the sequence

of the individual chitinase genes based on the location of the polyadenylation signals. The general trend of upregulated chitinase expression during CA periods, and the downregulated expression after DAC were similar for the three cultivars. However, the magnitude of induction was clearly distinguishable among the cultivars. The highest and lowest low temperature-dependent induction levels occurred in MSU and Uganda, respectively. After 2 days of CA, the transcript levels of Midiron, MSU and Uganda increased approximately 5-, 6- and 3-fold, respectively, from the base levels (Figure 8). This pattern clearly indicated that plants responded rapidly to CA by inducing the expression of chitinase genes. The transcript levels of Midiron, MSU and Uganda increased approximately 4-, 5- and 2.7-fold, respectively at 28 days of CA (Figure 8). The slight decrease in the transcript levels indicate the rate of mRNA turnover during CA process.

There were no detectable differences on pattern of transcript accumulation detected by individual probes P1, P2 and P3 (Figures 8 and 9). The specific gene responsible for the observed pattern of expression cannot be determined since none of the three probes is specific to a particular member of the gene family. Development of gene specific probes will be necessary in order to determine if both genes are responsible for the observed expression patterns.

Very low expression levels of the chitinase genes were found in root tissues of Midiron and MSU after 2 and 28 days CA. There was no indication of transcription in Uganda (data not shown).

The patterns of transcript accumulation in the leaves of all three cultivars following CA and DAC were quite distinct from the expression patterns observed in the crown. Increased transcript accumulation occurred in Midiron and Uganda but not in MSU only after 28 days of CA (Figure 10). After 2 days of CA, transcript accumulation in Midiron and Uganda had increased approximately 3- and 2.5- fold, respectively, relative to base levels. Unlike in the crowns, the transcript levels in the leaves of Midiron and Uganda remained elevated even after DAC (Figure 10). The transcript levels were estimated to be approximately the same as the transcript levels in 28 days CA leaf tissues. No detectable induction of gene expression was observed in the leaves of MSU as indicated by very low transcript levels across the different treatments (Figure 10).

Expression of Chitinase Genes During Drought Stress and Exogenous ABA application

The relative water contents of the three cultivars were >75% and <25% before and after the drought stress, respectively. Plants of each cultivar showed clear symptoms of extreme desiccation following the 10-day drought stress period. Drought stress significantly induced chitinase gene expression in the crowns of all three bermudagrasss cultivars as indicated by the northern blot analysis. The general pattern of transcript accumulation was the same using probes P1, P2 and P3. (Figures 11 and 14). Similar to the CA plants, the highest level of drought-induced chitinase gene expression was

observed in MSU and the lowest in Uganda. Drought treatments did not induce gene expression in the root tissues of any cultivar (data not shown).

Midiron is the cultivar used as a standard in determining cold hardiness (LT_{50}) of bermudagrass cultivars. The application of 100 uM ABA at 12 hour intervals for five consecutive days increased the cold hardiness of Midiron from LT_{50}=-6°C to LT_{50}=-8°C (data not shown). Consequently, the expression of chitinase genes was investigated in Midiron, MSU and Uganda plants treated with 100 uM ABA.

The ABA treatments induced chitinase gene expressions in the crown tissues of Midiron and MSU, but not in Uganda. The level of induction was much lower compared to that caused by low temperature and drought. Moreover, unlike in CA and drought, exogenous application of ABA induced a slightly higher level of expression in Midiron than in MSU. Identical results were obtained using probes P1, P2 and P3 (Figures 12 and 14). In contrast, the application of ABA did not induce chitinase gene expression in the leaf (Figure 13) and root tissues of any cultivar (data not shown).

Drought stress resulted in higher levels of chitinase gene expression in crown tissues than did CA or ABA. In comparison to the control levels, drought stress caused 6-, 7.5- and 4-fold increases in the transcript levels in crown tissues of Midiron, MSU and Uganda, respectively (Figure 14). These transcript levels were significantly higher than the transcript accumulation observed in 2-day CA crown tissues, which were approximately 5-, 6- and 3.25 fold increases from the basal levels in Midiron, MSU and Uganda, respectively. The ABA-induced expression levels, although significant, were relatively low with only about 2.5 and 2-fold increases from the basal levels observed in the crowns of Midiron and MSU, respectively (Figure 14).

Research in Progress

It is now well established that some plant species synthesize proteins with antifreeze function during cold acclimation. Many of these proteins have been identified as pathogenesis-related (PR) proteins (Hon et al, 1995;Griffith et al, 1997; Yu and Griffith, 1999). The salient features of the two PR protein genes encoding chitinases (*CynCht1*, *CynCht2*) from freeze-tolerant cultivar 'Midiron' are consistent with the possibility that they may be involved in freeze-tolerance mechanisms. The data on the temporal and spatial expression patterns strongly indicated low temperature-induced expression. These results have significant implications especially with regard to the hypothesized function of PR proteins as antifreeze molecules. Despite this information, the direct involvement of the products of these genes in bermudagrass freeze-tolerance mechanisms needs to be further confirmed experimentally. A major question that needs to be answered concerns the magnitude of increase in cold hardiness and drought tolerance if a chitinase gene is overexpressed without cold acclimation. To address this question, we will overexpress the bermudagrass chitinase gene (CynCht-1) in a suitable *Arabidopsis* strain. Chitinase overproducing transgenic plants will be evaluated at the phenotypic, biochemical and genetic levels in order to determine the role of chitinases in freeze-tolerance mechanism/s.

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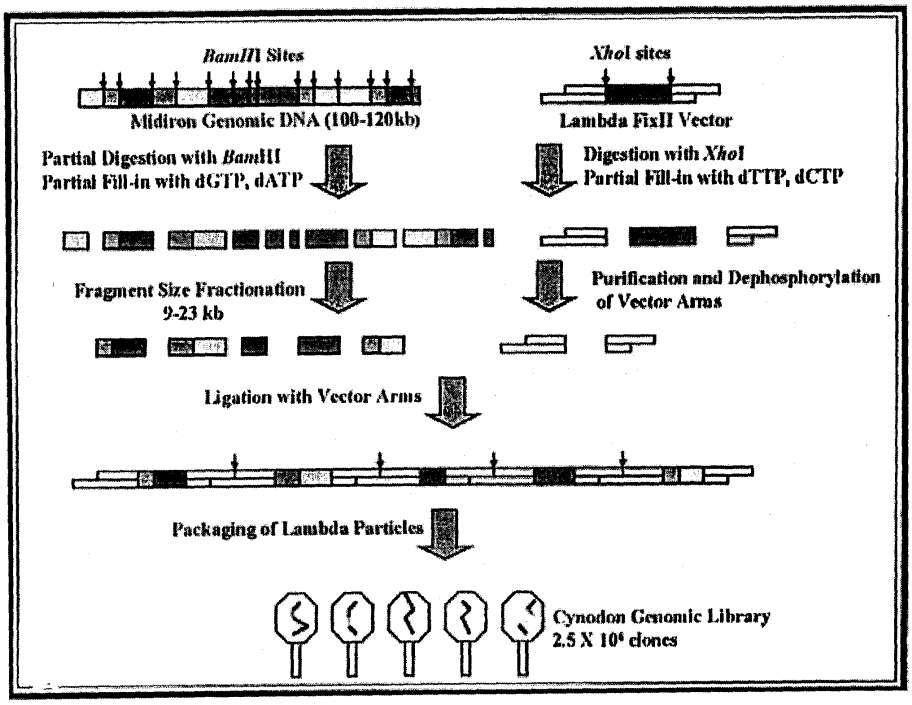


Figure 1. Construction of *Cynodon* genomic library. Midiron total genomic DNA was isolated from leaf tissues. High molecular weight DNA was partially digested with BamHI and then ligated with Lambda FixII Vector generating a primary library of at least 2.5 X 10^6 plaque forming units (pfu).

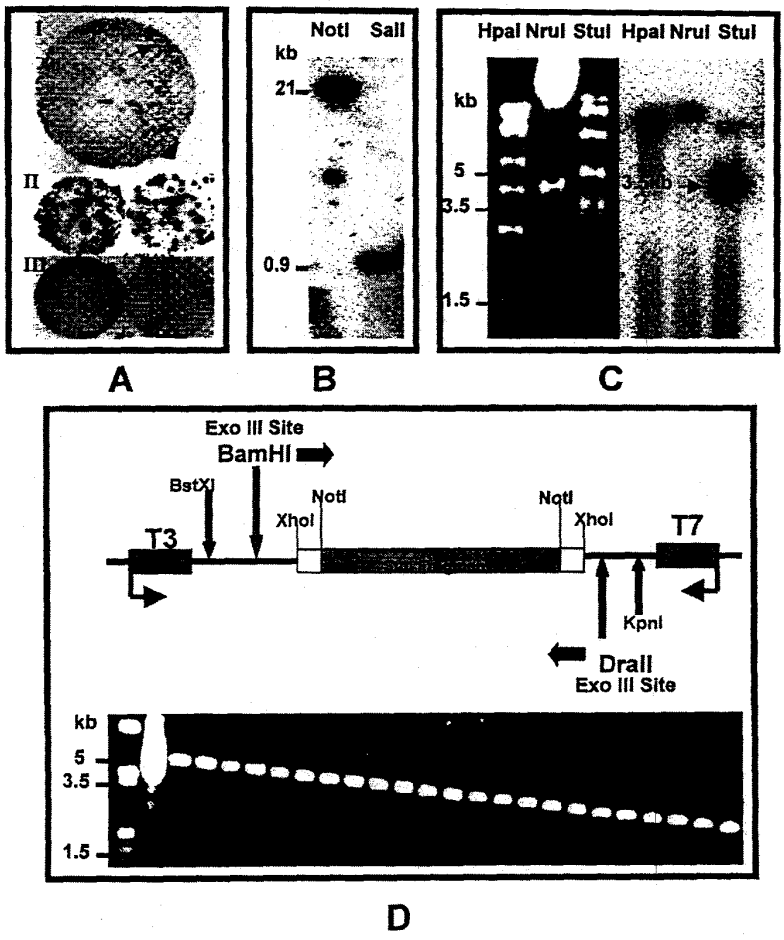


Figure 2. Cloning and sequencing of bermudagrass chitinase genes. (A) Screening of Lambda Fix II genomic library. Eight hundred thousand clones were screened with the 360bp chitinase partial cDNA probe at moderate stringency. Sample primary (I), secondary (II) and tertiary (III) screening filters are shown. (B) Identification and cloning of SalI probe. Southern blot shows the 21kb full-length insert (NotI) and the 0.9kb SalI fragment from GCyn113. (C) Identification of the fragment that contains the full length CynCht1 from GCyn113 clone. GCyn113 DNA was digested with StuI and hybridized with the SalI fragment. Southern blot shows the 3.5kb StuI fragment that contained the full length CynCht1 gene. (D) The 3.5kb StuI fragment from GCyn113 was purified and cloned in pBluescript-SK. Unidirectional deletion series from both ends of the pBluescript clone was constructed. Representative deletion clones were sampled at 1 minute intervals. Deletion clones are shown in the EtBr-stained gel as DNA fragments of decreasing size. The deletion clones were used to determined the full-length sequence of the genes.

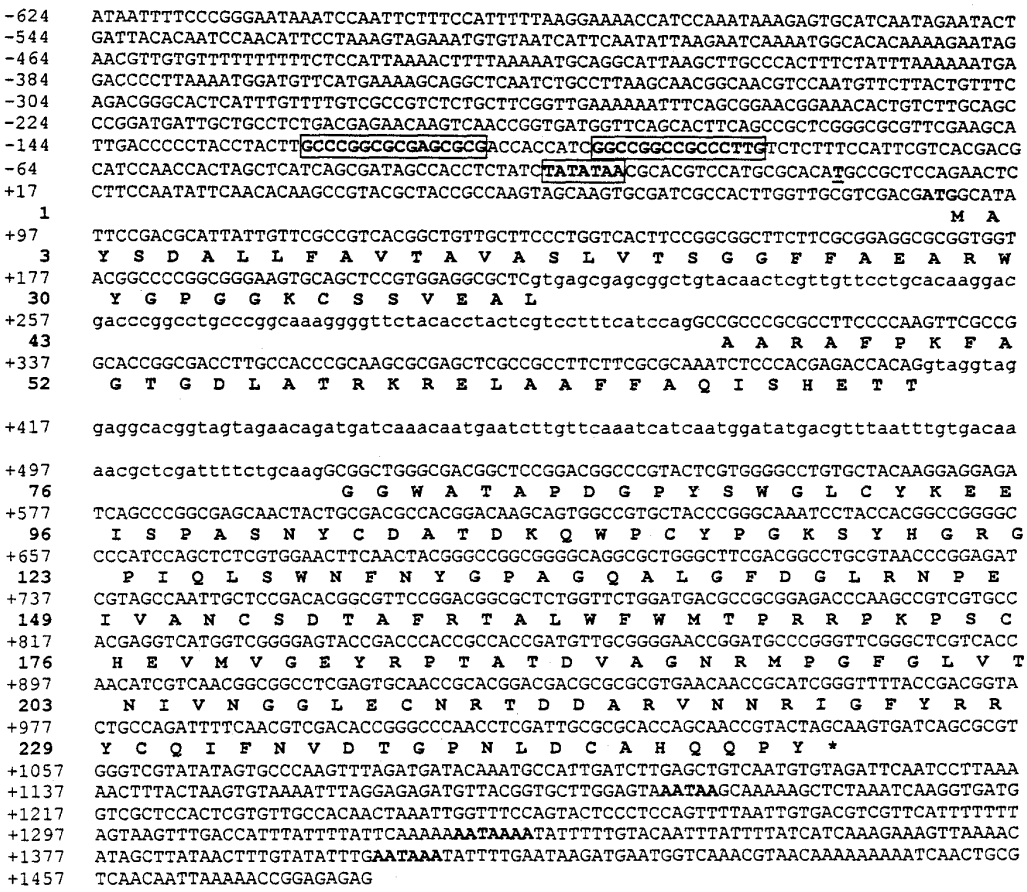


Figure 3. Nucleotide sequence of *CynCht1* and deduced amino acid sequence of the protein encoded by the gene (CHT1). The nucleotide positions and amino acid residues are numbered at the left. Introns are represented in lower case letters. The sequences inside the box represent the GC-rich motifs and TATA signal. The predicted transcription initiation site is underlined. The consensus sequences for polyadenylation signals found at the 3' flanking region of the gene are in bold letters. The nucleotide sequence of *CynCht1* appears in the EMBL, GenBank and DDBJ databases as accession number AF105425.

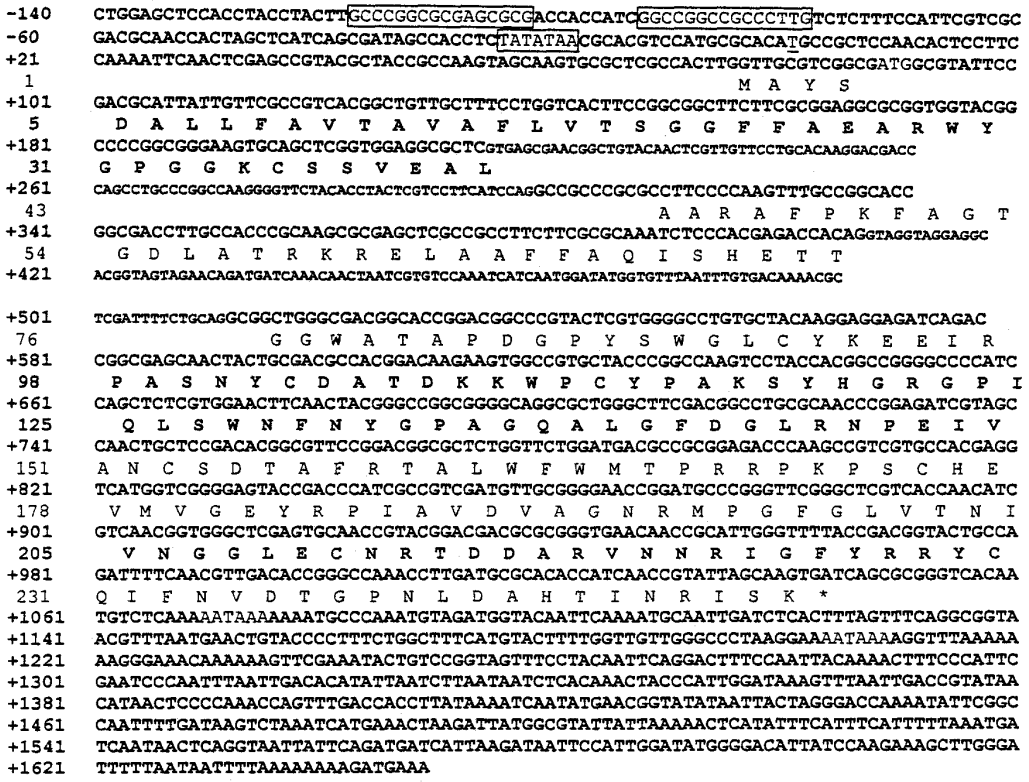


Figure 4. Nucleotide sequence of *CynCht2* and deduced amino acid sequence of the protein encoded by the gene (CHT2). The nucleotide positions and amino acid residues are numbered at the left. Introns are represented in lower case letters. The sequences inside the box represent the GC-rich motifs and TATA-signal. The predicted transcription initiation site is underlined. The consensus sequences for polyadenylation signals at the 3' flanking region of the gene are in bold letters. The nucleotide sequence of *CynCht2* appears in the EMBL, GenBank and DDBJ databases as accession number AF105426.

Figure 5. Amino acid sequence alignment of CHT1 and CHT2 with known class I and class II chitinases from other plant species. Pn(CII): Peanut, class II=*S65069*; Tm(CII): Tomato, class II=*S69184*; Af(CI): Alfalfa, class I= *U83591*. The putative signal peptides are in lower case letters. The highlighted sequences indicate the mismatched amino acids between *CynCht1* and *CynCht2*. The structural domains of class I chitinase not found in the class II chitinases are also indicated.

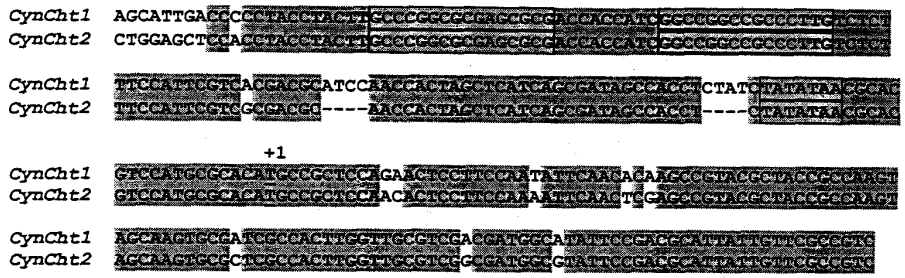


Figure 6. Analysis of the 5' flanking regions of bermudagrass chitinase genes (*CynCht1*:-148 to +121; *CynCht2*:-140 to +121). Sequences at promoter regions were aligned to determine the extent of sequence homology between the *CynCht1* and *CynCht2*. The predicted transcription initiation sites are underlined and in bold letters (+1). Sequences inside the box represent the GC-rich motifs and TATA-signal. Homologous sequences are shaded.

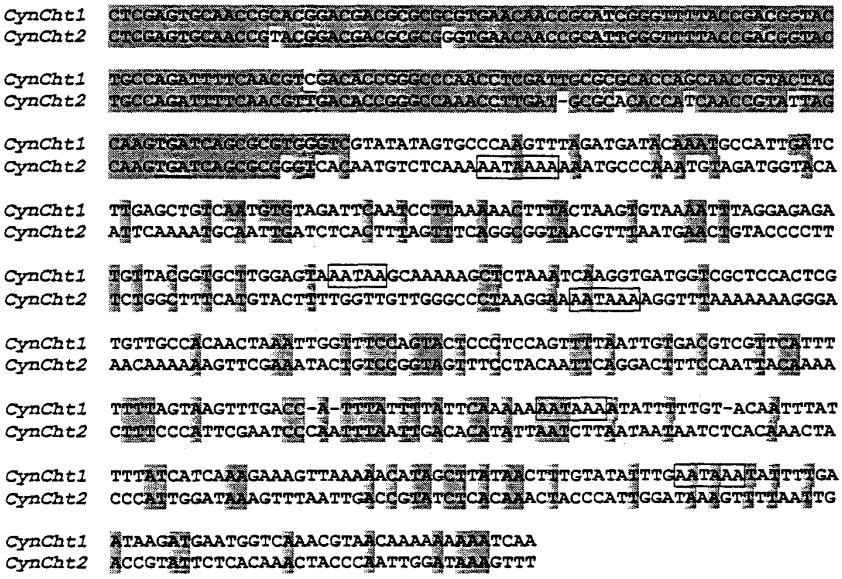
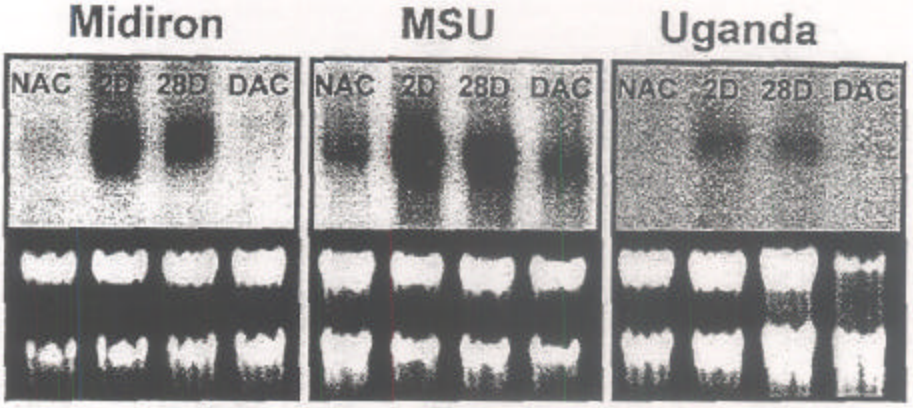


Figure 7. Analysis of the 3' flanking regions of bermudagrass chitinase genes (*CynCht1*:+914 and downstream; CynCht2:+913 and downstream). Sequences at the 3'UT regions were aligned to determine the extent of sequence homology between the *CynCht1* and *CynCht2*. The translation termination codons are underlined. The sequences inside a box represent the polyadenylation signals found at the end of the coding regions of the genes. The homologous sequences are shaded.



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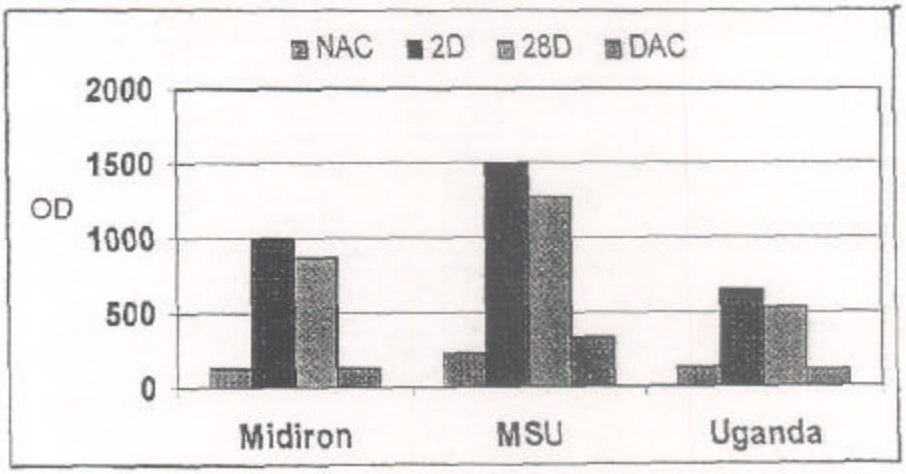


B

Figure 8. Northern blot analysis of chitinase gene expression in bermudagrass crowns during cold acclimation. (A) Equal amounts of crown tissue total RNA (18 ug) from non-acclimated (NAC), 2-days cold acclimated (2D), 28-days cold acclimated (28D) and deacclimated (DAC) were electrophoresed in formaldehyde-agarose gel (bottom), and then transferred to Hybond N nylon membrane. The filter was hybridized with probe P1 and autoradiographed for 48 hours (top). (B) Northern blot bands were analyzed by scanning densitometry to compare transcript levels between treatments.

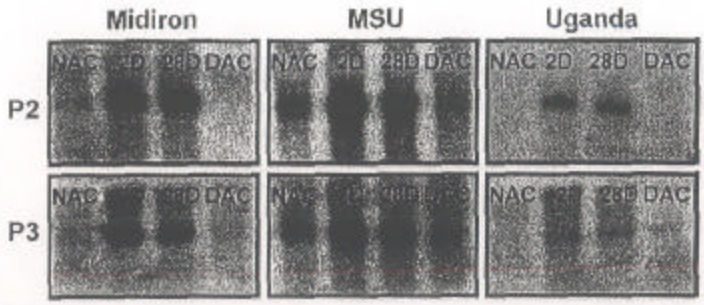
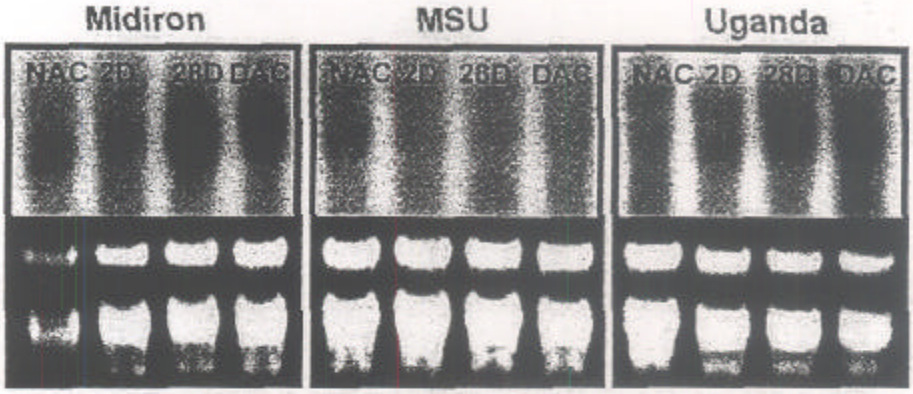


Figure 9. Northern blot analysis of chitinase gene expression in bermudagrass crowns during cold acclimation. Equal amounts of crown tissue total RNA (18 ug) from non-acclimated (NAC), 2-days cold acclimated (2D), 28-days cold acclimated (28D) and deacclimated (DAC) were electrophoresed in formaldehyde-agarose gel and transferred to Hybond N nylon membrane. The filter was hybridized with probes P2 and P3 and autoradiographed for 48 hours.



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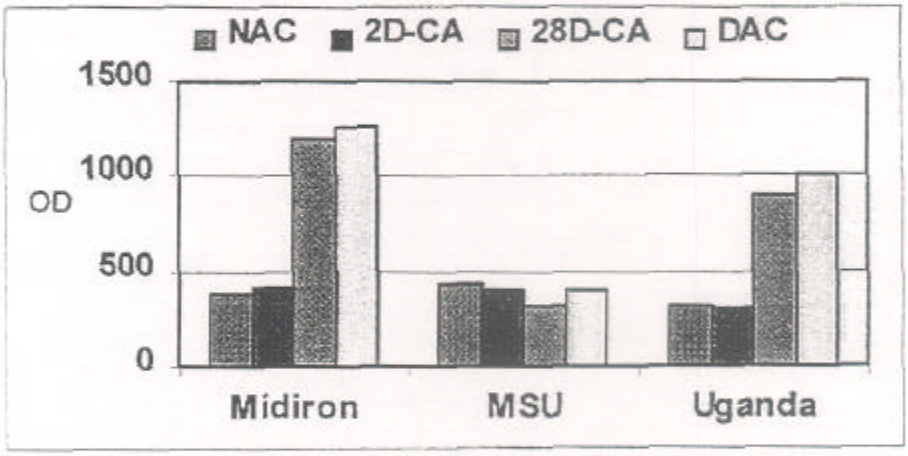

B

Figure 10. Northern blot analysis of chitinase gene expression in bermudagrass leaves during cold acclimation. (A) Equal amounts of leaf tissue total RNA (18 ug) from non-acclimated (NAC), 2-days cold acclimated (2D), 28-days cold acclimated (28D) and deacclimated (DAC) were electrophoresed in formaldehyde-agarose gel (bottom), and then transferred to Hybond N nylon membrane. The filter was hybridized with probe P1 and autoradiographed for 48 hours (top). (B) Northern blot bonds were analyzed by scanning densitometry to compare transcript levels between treatments.

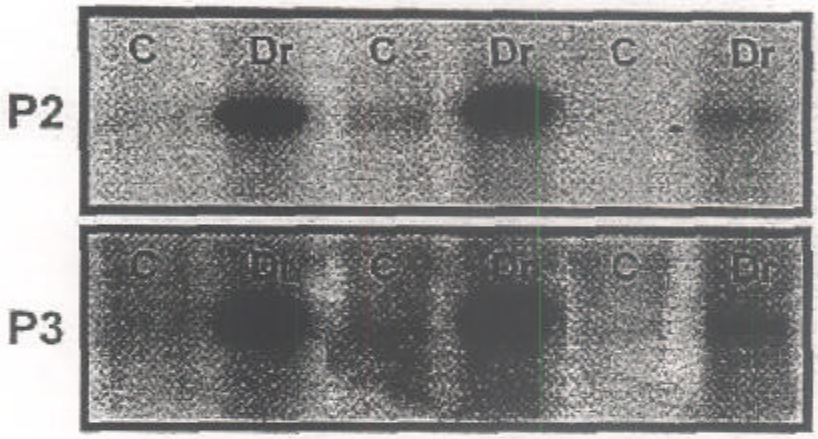

Figure 11. Northern blot analysis of chitinase gene expression in bermudagrass crowns during drought stress. Equal amounts of crown tissues total RNA (18 ug) from control or non-stressed (C) and drought-stressed (Dr) plants were electrophoresed in formaldehyde-agarose gel and then transferred to Hybond N nylon membrane. The filters were hybridized with probes P2 and P3 and autordiographed for 48 hours.

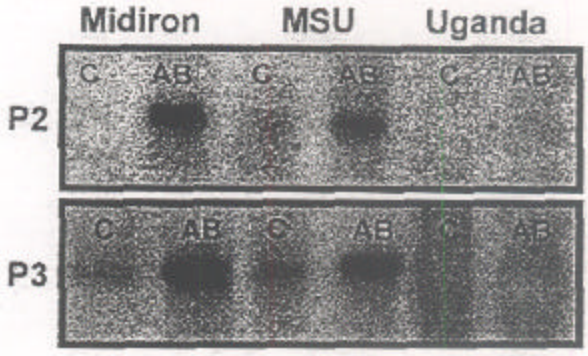

Figure 12. Northern blot analysis of chitinase gene expression in bermudagrass crowns treated with 100 uM ABA. Equal amounts of crown tissues total RNA (18 ug) from control (C) and ABA-treated plants (AB) plants were electrophoresed in formaldehyde-agarose gel and then transferred to Hybond N nylon membrane. The filter was hybridized with probes P2 and P3 and autoradiographed for 48 hours.

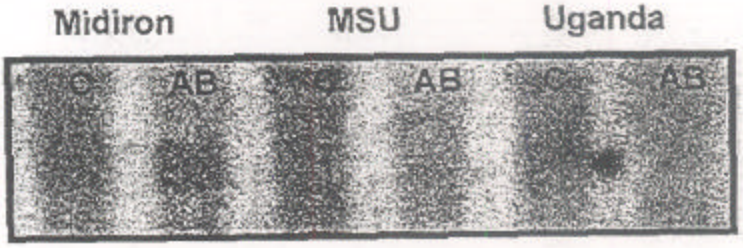

Figure 13. Northern blot analysis of chitinase gene expression in bermudagrass leaves treated with 100 uM ABA. Equal amounts of leaf tissuetotal RNA (18 ug) from control (C) and ABA-treated (AB) plants were electrophoresed in formaldehyde-agarose gel and then transferred to Hybond N nylon membrane. The filter was hybridized with probe P1 and autoradiographed for 48 hours.

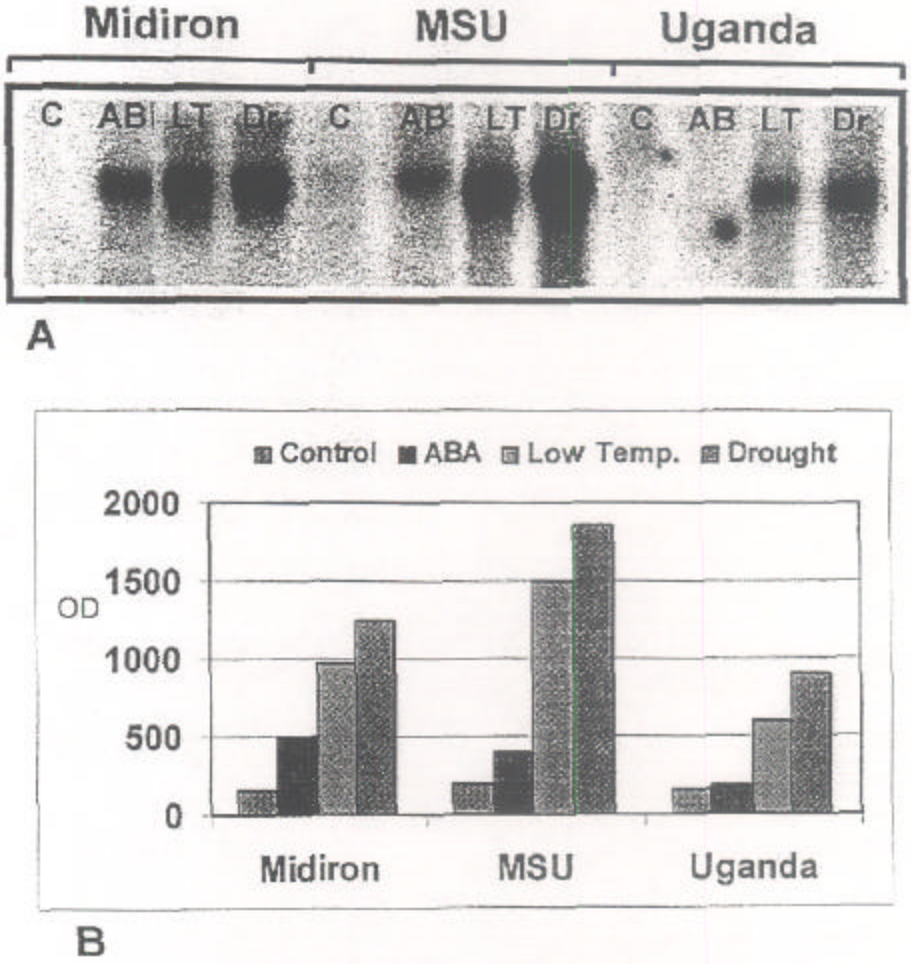

Figure 14. Comparison of chitinase gene expression in bermudagrass crowns during cold acclimation (LT), drought stress (Dr) and exogenous application of ABA (AB). (A) Equal amounts of crown tissue total RNA (18 ug) from control, cold-acclimated, drought-stressed and ABA-treated plants were electrophoresed in formaldehyde-agarose gel and then transferred to Hybond nylon membrane. The filter was hybridized with probe P1 and autoradiographed for 48 hours. (B) Northern blot bands were analyzed by scanning densitometry to compare transcript levels between treaments.