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

For the Period

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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.

Experiments were initiated to determine the low temperature tolerance (LT_{50}) of turf bermudagrasses using laboratory based methodology. The LT_{50} values will be determined sequentially for selected bermudagrasses in each of four groups. The groups are: 1) vegetatively-propagated fairway types, 2) seeded fairway types, 3) vegetatively-propagated putting green types, and 4) experimental fairway breeding lines. Experiments with the vegetatively-propagated fairway types are underway.

Substantial progress has been made toward the goal of isolating and characterizing cold regulated *(Cor)* genes. A *Cynodon* genomic library was constructed from Midiron ,C. dactylon x C. 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 indeed chitinase genes, which we designate as *CynCht-1, CynCht-2* and *CynCht-3*. We expect other of the clones to also be chitinase genes.

Northern blot analyses indicated chitinase gene expression in Midiron, Uganda, and MSU turf bermudagrasses to be strongly affected by acclimation temperatures (4-8 °C). Substantial increases (75-100%) in gene activity in crown and root tissues occurred after 24 hours cold acclimation. Increases of gene activity in crown and root tissues were proportional to the LT_{50}'s and ploidy levels of the three cultivars. Cold acclimation for 28 days caused an approximate three-fold increase in chitinase gene activity in leaves of Midiron and Uganda, but had little effect on MSU. Leaves of MSU remained relatively green during the 28 day acclimation, while those of Midiron and Uganda strongly senesced. Different *Cor* gene regulatory mechanisms may be involved in leaf and crown/root 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. 1998). 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; Hiscock, 1996; Schaffer, 1994). 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, 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

The cold hardiness evaluation research, led by stress physiologist Jeffrey Anderson and as outlined in the research proposal project, has been implemented. Cold hardiness evaluations are 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 first two plantings have been established and the first set is undergoing acclimation treatment prior to determination of LT_{50}s (lethal temperature for 50% of plants) using a freeze chamber. Cold hardiness evaluations of fairway bermudagrasses will be completed during winter of 1998/1999.

The second set of bermudagrasses comprises seeded varieties from the last NTEP trial: Jackpot, Mirage, OKS 91-11, and Guymon (standard). These experiments will be conducted during late winter/early spring of 1998-1999. The third series of plants represent bermudagrasses used for putting greens: Floradwarf, Champions, Tifeagle, MS Supreme, Miniverde, Tifdwarf, and Tifgreen (standard). These experiments will be conducted during summer 1999.

The final set of cold hardiness determinations will examine advanced selections from the OSU breeding program in fall/early winter 1999. Plans call for repeating experiments for each use type on 3 dates. We will generate LT_{50} values for each genotype on each date, and determine significant differences in cold hardiness within each group by multiple comparisons. Results will be reported in journal and popular press articles.

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 expression 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 be able to dissect the molecular basis of this biological phenomenon in *Cynodon* through the use of recombinant DNA techniques. We expect to discover novel genes that have potential use for genetic improvement of the freezing tolerance not only of bermudagrasses but of other turfgrass species as well.

For the last 6 years we have examined 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 function in conferring 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 Midiron (very freeze tolerant) than in Tifgreen (moderately freeze tolerant) (Gatschet et al., 1996). A similar situation was recently documented by the Hon et al. (1995) and Antikainen et al. (1997) in 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, *C. dactylon* X *C.*

transvaalensis, (2) isolation and sequencing of bermudagrass chitinase genes, and (3) analysis of temporal and spatial expression patterns of chitinase genes in three cold acclimating bermudagrass cultivars.

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 1A). 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. Cloning and Sequencing of Cynodon Chitinase Genes.

Screening the Library for Chitinase Genes. Eight hundred thousand clones from the library were plated at a density of 50,000 pfu in sixteen 150 mm petri dishes by infecting XL1-Blue MRA host cells grown on NZY-agar overnight at 37°C. The plates were incubated at 4°C for at least 4 hours to harden the media and terminate further growth of the plaques. Duplicate plaque lifts were prepared from each of the plates using Hybond N+ nylon membrane (Amersham Life Science, Arlington Heights, IL) following the manufacturer's instruction.

To screen the library for chitinase genes, we used a 300-bp cDNA clone representing the putative conserved region of the bermudagrass chitinase genes. This clone was isolated, sequenced and kindly provided to us by Mr. Stephen MacMaugh from the University of Sydney, Australia. The cDNA probe was random primed labeled with ^{32}P-dCTP using the RediPrime Labeling Kit (Amersham Life Science) following the manufacturer's instruction. Hybridization with the plaque lifts was allowed overnight at 65°C following

the procedure of Sambrook et al. (1989). The filters were washed twice for 10 minutes with 2x SSC + 0.1 % SDS at room temperature and twice for 15 minutes with 0.25X SSC at 65°C before they were subjected to autoradiography. A total of fourteen positive plaques were identified after the primary screening. Autoradiogram showing one of the fourteen primary positive clones is shown in Figure 1B (top). Individual clones were isolated from the plates and then subjected to secondary and tertiary screenings. Nine out of the fourteen primary positives appeared in the secondary and tertiary screenings. The results of the tertiary screen is shown in Figure 1B (bottom). Representative plaques from the tertiary plates were isolated from individual groups and designated as putative *Cynodon* chitinase genomic clones.

Subcloning and Sequence Analysis. DNA from these clones was isolated using the Stratagene Lambda DNA isolation kit following the manufacturer's instructions. The clone containing the longest insert (20kb) based on *Not*I digestion was selected (Cyn4-5.6) and then digested with *Sal*I. The *Sal*I fragments were directly subcloned into the *Sal*I region of the pBluescript-SK (Stratagene Inc. La Jolla, CA) generating the first set of sublibrary. The *Sal*I sublibrary was first screened with the radiolabeled 300-bp chitinase partial cDNA probe. One clone from the *Sal*I sublibrary (designated as Sal456-1) showed strong hybridization with the partial cDNA probe. The 900bp insert from this clone was sequenced (Figure 2) at the Recombinant DNA Core Resource Facility (Figure 2). Analysis of sequence homology with known genes through Blast Search Algorithms indicated very high homology with a number of known plant chitinase genes (data not shown). Sequence alignment data indicated that this 900 bp clone contained a stretch of at least 600 bp that is highly conserved among many plant chitinase genes (Hamel et al., 1997) as shown in region 1600-2200 in Figure 4A. We concluded that this clone represents the partial genomic sequence of a Midiron chitinase gene, and therefore we decided to use this as the probe to look for longer subclones containing full-length chitinase genes.

A second sublibrary was constructed by digesting Cyn4-5.6 with *StuI* generating larger fragment sizes than the *Sal*I sublibrary. The ends of the Cyn4-5.6 digests were blunt end-ligated with *Xho*I adapters and then cloned into the *Xho*I site of pBluescript-SK. This sublibrary was screened using Sal456-1 as probe. One subclone containing an insert of about 3.5-kb was identified based on the result of Southern blot analysis (data not shown). This clone was designated as Stu456-1. Three more StuI sublibraries (from clones Cyn1-1.3, Cyn8-1.7 and Cyn3-1.9) were constructed in pBluescript-SK and similarly screened with the Sal456-1 probe. Fragments of approximately the same size (3.5-kb) were identified by Southern blot analysis from each of the three *Stu*I sublibraries designated as Stu113-1, Stu817-1 and Stu319-1 (Figure 3).

Series of unidirectional deletions were constructed on each of the four putative chitinase clones using the Erase-A-Base Kit (Promega, Madison, WI) following the manufacturer's instruction. Overlapping deletion clones were sequenced at the Recombinant DNA-Core Resource Facility. So far we have generated about 1.8-2.8 kb of sequence information on the four clones subjected to unidirectional deletion. Sequencing of the second strand and the remaining parts are in progress.

Fragment sequences were arrayed using MacVector computer programs. Arrayed sequences were aligned among each other to determine the extent of sequence diversity among the four putative bermudagrass chitinase genes. Sequence alignment among these four putative chitinase genes indicate that Stu113-1 and Stu456-1 were identical clones, thus designated as *CynCht-1*. Stu817-1 and Stu319-1 exhibit slight sequence divergence relative to *CynCht-1* and to each other and therefore were designated as *CynCht-2* and *CynCht-3*, respectively (Figure 4B). In all cases, sequence differences were found flanking the highly conserved region. Such highly conserved regions are found in many plant chitinase genes (Figure 4C). We then concluded that these three clones represent potentially related chitinase genes, which are members of the chitinase gene family in *Cynodon*. Southern blot analysis of Midiron genomic DNA using the conserved region of the chitinase genes as probe shows multiple bands supporting the result on multiple chitinase genes in bermudagrass genome (Figure 5). Further analysis of the sequence alignment of *CynCht-1* with other known plant chitinase genes (mostly from monocot species) indicated very high homology at the conserved region and moderate to low homology in the flanking regions. The same trend was found in all of the sequence alignments (Figure 4C) with most of the base mismatches and insertions occurring at the regions flanking the conserved sequence. Highest sequence homologies were observed with the *O. sativa Cht-1, H. vulgare CHI26* and *T. aestivum chi* gene. The same trend was observed in the case of *CynCht-2* and *CynCht-3* (data not shown).

3) Characterization of Chitinase Gene Expression in *Cynodon*

Pathogenesis-related (PR) proteins which include chitinase, glucanase and thaumatin have been implicated with freezing tolerance in a number of plant species including bermudagrass (Antikainen and Griffith, 1997; Gatschet et al., 1996; Hon et al., 1995; Hincha et al., 1997). In a study on winter rye (Hon et al., 1995), it was demonstrated that cold acclimation induced the synthesis of these PR-proteins which accumulated in the apoplastic sap. The previous results of Gatschet et al. (1996) in bermudagrass led to a hypothesis that there must be an increase in the expression of chitinase genes in the crown tissues in response to cold-acclimating conditions. To test this hypothesis we conducted northern blot experiments to study the induction of expression of bermudagrass chitinase genes during the cold acclimation process. We included three cultivars for this experiment. The first is Midiron, a triploid interspecific hybrid (*C. dactylon* x *C. transvaalensis*) which is also one of the most freezing tolerant bermudagrass cultivars with an LT_{50} value of $-10^{\circ}C$ (Anderson et al., 1993). The second cultivar, Accession A12195, is a tetraploid *C. dactylon* collected on the campus of Michigan State University in 1996. This cultivar, which we call 'MSU' has an LT_{50} value slightly lower than $-10^{\circ}C$ and a slightly greater level of freezing tolerance than Midiron (data not presented). The third cultivar is the diploid *C. transvaalensis* cultivar Uganda. Based on LT_{50} determinations, this cultivar has freeze tolerance comparable to that of Tifgreen ($LT_{50}= -8^{\circ}C$). Furthermore, Uganda is one of the putative parents of the triploid hybrid Midiron; hence we decided to include this in our analysis. These three cultivars represent a wide range of freezing tolerance that is available in Cynodon germplasm.

Vegetatively propagated shoots of each cultivar were grown in pots at greenhouse condition for 3 wks. The plants were then transferred and kept for another 2 wks in a controlled chamber at 28°C day/24°C night. One fourth of the plants were kept in the 28°C/24°C chamber for 28 d serving as the control or non-acclimated plants. Leaf, crown and root tissues were harvested from the control plants after 28 d in the chamber. The remaining three fourths of the plants were transferred for cold acclimation in a cold chamber at 8°C day/2°C night. Leaf, crown and root tissues were harvested from each sample following 2 and 28 d of cold acclimation. The remaining plants, which have been acclimated for 28 d, were transferred and kept for additional 2 d at the 28°Cday/24°Cnight chamber for deacclimation. Similarly, leaf, crown and root tissues were harvested from the deacclimated plants.

Total RNA was isolated from each sample with Guanidine-Hydrochloride by a method modified from Logemann et al. (1987). Equal amounts (20 ug) of total RNA samples from each treatment and tissue were separated in 1.2% formaldehyde-agarose gel using the Northern Max Kit (Ambion, Austin, TX). RNAs were blotted on Hybond N nylon membrane by the downward transfer method for two hours. Filters were hybridized with random primed–labeled (^{32}P) Sal456-1 probe (Rediprime Kit, Amersham Life Sciences, and IL). Hybridization and washing of the filters were performed using the Northern Max Kit according to the manufacturer's instruction. The filters were autoradiographed for three d at –80°C.

Expression in the Crowns and Roots. The northern blot analyses indicated significant increases in the level of expression of chitinase genes in the crowns following cold acclimation and a corresponding decrease after deacclimation in the case of Midiron (Figure 6). The highest increase (100%) in chitinase gene expression was observed after 2 d of cold acclimation. After 28 d of cold acclimation, the magnitude of increase in the level of chitinase gene expression started to decrease to about 80%. This was possibly caused by the sudden change in temperature after 2 d in the cold chamber. This sudden change might have caused a very rapid response resulting in the higher level of gene expression. As acclimation proceeded to 28 d, the expression became more stable. This may also be related to the fact that no significant increase in freezing tolerance is usually observed beyond 28 d of cold acclimation. It is therefore highly possible that the maximum level of cold-induced chitinase gene expression occurs between 2-28 d of cold acclimation. Deacclimation resulted in a decrease in the level of expression of chitinase genes to a level that approximates the level before cold acclimation. This pattern of changes in the chitinase gene expression clearly supports our initial findings (Gatschet et al., 1996) that low temperature causes an increased level of synthesis of chitinase in bermudagrass crowns. Whether these chitinases exhibit antifreeze activity remains to be determined using biochemical assays for ice crystal binding ability of the protein products of these genes.

The chitinase gene expression during cold acclimation and deacclimation in MSU and Uganda follows the same general trend as that observed in Midiron. The surprising difference is that the level of chitinase mRNAs before cold acclimation is highest in MSU and lowest in Uganda when compared with Midiron (Figure 6). MSU had 100% and 85%

mRNAs increases after 2 d and 28 d of cold acclimation, respectively. Uganda had increases of about 75% after 2 d and 50% after 28 d. These results suggest a possible relationship between the level of chitinase gene expression and ploidy level (or chitinase gene copy number) and level of cold hardiness. As noted earlier, tetraploid MSU is the most cold hardy of the three cultivars, followed by triploid Midiron and diploid Uganda. We intend to investigate these possible relationships in future experiments by comparing the chitinase gene copy number between the three cultivars and also include comparisons with a non-hardy tetraploid and diploid cultivars. The root tissues exhibit significantly lower levels of chitinase mRNA compared to the crown tissues. However, the same general trend of chitinase gene expression was observed in the roots of all three cultivars studied (data not shown).

Expression in the Leaves. Northern blot analyses indicate differential expression of chitinase genes in the leaves. However, the pattern of expression is very distinct from the pattern observed in the crowns and roots. Furthermore, differential expression was observed only with Midiron and Uganda and not with MSU (Figure 7). In contrast to the crowns and roots, the initial level (before cold acclimation) of chitinase mRNAs in the leaves was relatively the same for all three cultivars. Two d of cold acclimation did not cause any significant increase in chitinase gene expression in Midiron and Uganda. However, a drastic increase in chitinase gene expression was observed after 28 d (about 200% in Midiron and 180% in Uganda). Deacclimation did not cause a decrease in the level of chitinase mRNA in the leaves of Midiron but a decrease was observed in the leaves of Uganda. No visible increase in the level of chitinase gene expression was observed in the leaves of MSU as indicated by the low levels of mRNAs across the different treatments. This pattern of expression seems to be an induction event, which is independent to the induction of chitinase gene expression observed in the crowns and roots. Furthermore, it appears that the expression of chitinase genes in the crowns and roots is controlled by a mechanism entirely different and independent from the mechanism in the leaves. Both Midiron and Uganda exhibited very high levels of leaf chlorosis and senescence after the 28 d of cold acclimation. Deacclimation caused further senescence in Midiron to the point that the younger leaves appeared very pale green and the older leaves were yellow. Uganda leaves were totally wilted after deacclimation. In stark contrast, MSU leaves did not exhibit the same level of chlorosis and senescence after cold acclimation and deacclimation. The leaves retained much of their green color throughout the duration of the experiment with very low levels of chlorosis in older leaves compared to the non-acclimated or control plants. This suggests a correlation between chitinase gene expression in the leaves and some physiological events related to leaf senescence. As pathogenesis-related proteins, chitinases are induced primarily during pathogen invasion or insect attack as a primary response to wounding. One possibility is that low temperature causes senescence, which further induces modification of cell wall materials in the leaves, an event where the chitinases are involved. This could be an event similar to wounding that occurs during pathogen invasion and insect feeding. A possible explanation of these results is that the induction of chitinase gene expression in the leaves is not related at all to cold acclimation, but may instead be an event triggered by leaf senescence. In contras to this, the induction of expression in the crowns and roots is consequential with the cold treatment as evidenced

by the pattern of expression indicated by the northern blot results (Figure 6). Thus, this could very well be a response directly related to cold acclimation and possibly controlled by a mechanism specific to the crowns (and roots) that may be involved in protecting the meristem from being damaged during freezing periods. As earlier stated, demonstration of the antifreeze activity of the crown and root chitinases (from apoplastic extracts of crown and root tissues after cold acclimation) should provide clarification on these intriguing observations.

Research in Progress

Studies in other plant species (Antikainen and Griffith, 1997) suggest that the involvement of PR-proteins with cold acclimation and freezing tolerance could be a mechanism specific to cold acclimating monocot species. Most of the cold-regulated (*Cor*) genes that have been isolated in dicot species are also induced by abscisic acid (ABA) and drought or desiccation stress (Wang et al., 1995). An important question is whether chitinase gene expression can also be induced by other external factors that mimics the effect of cold acclimation like the exogenous application of ABA and drought? Or is it involved only with cold stress, in addition to being a defense mechanism against pathogen attack? The current line of thought is that PR-proteins are primarily devoted to a specific function (defense against pathogen and insects), and that they have been co-opted to a second function as antifreeze proteins in recent evolutionary periods due to some structural attributes favorable for ice crystal-binding function (Worral et al., 1998). Chitinases are involved with cell wall modifications during pathogen attack and cold stress. It is therefore logical that proteins like chitinases be recruited for a second function of being antifreeze factors since freezing in plants occurs in the apoplasts, or the space between cell walls of adjacent cells. The observation that most freezing tolerant bermudagrass cultivars (including Midiron) are also resistant to spring dead spot caused by the fungus *Ophiosphaerella herpotricha* favors the hypothesis that chitinase gene induction may be specific only to pathogen and cold stress and not to drought. We are currently seeking possible answers to these questions. We are investigating the expression of chitinase genes in response to drought stress, exogenous application of ABA and their effect on the cold hardiness of Midiron, MSU and Uganda. Future research will include more extensive search for other genes that may have some roles in mechanisms that govern freezing tolerance in *Cynodon*.

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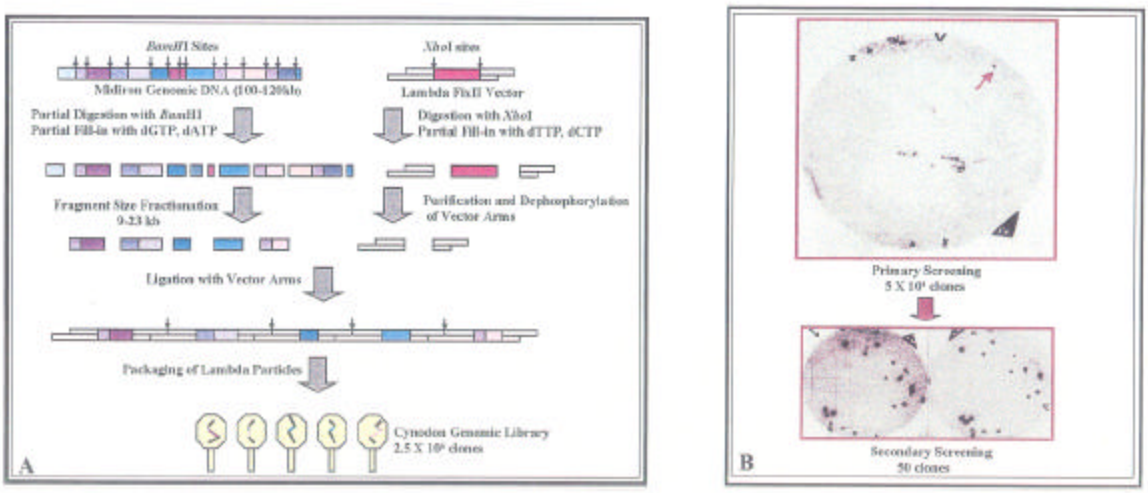

Figure 1. Construction and screening of *Cynodon* genomic library. (A) Midiron total genomic DNA was isolated from leaf tissues. High molecular weight DNA was partially digested with *Bam*HI and then ligated with Lambda FixII Vector generating a primary library of at least 2.5 X 10^6 plaque forming units (pfu). (B) Eight hundred thousand pfu from the primary library were plated on XL1-Blue MRA (P2) host cells. A single plaque is shown (arrow) hybridizing with radiolabeled partial chitinase gene probe in the primary screen (top, duplicate filter is not shown). The positive plaque from the primary screen was further purified through secondary and tertiary screens (bottom) until a single plaque was obtained which was used for subcloning and sequencing.

GTCGACGTTGAAAATCTGGCAGTACCGTCGGTAAAACCCGATGCGGTTGTTCACGCGCG
CGTCGTCCGTGCGGTTGCACTCGAGGCCGCCGTTGACGATGTTGGTGACGAGCCCGAAC
CCGGGCATCCGGTTCCCCGCAACATCGGTGGCGGTGGGTCGGTACTCCCCGACCATGAC
CTCGTGGCACGACGGCTTGGGTCTCCGCGGCGTCATCCACAACCAGAGCGCCGTCCGGA
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CCTGCCCCGCCGGCCCGTAATTGAAGTTCCACGAAAGCTGGATGGGGCCCCGGCCGTG
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CCGGGCTGATCTCCTCCTTGTACACAAGCCCCACAAGTACGGGCCGTCCGGAACCGTCC
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TTTAAAAAAATCATTGTTTGATCATCTGTCTACTACGTGCCTCCTACGCTACCTGTGGTC
TCGTGGGAGATTTGCGCGAAGAAGGCGGCGAGCTCGCGCTTGCGGGTGGCAAGGTCGC
CGGTGCCGGCGAACTTGGGGAAGGCGCGGGCGGCCTGGATGAAGGACGAGTAGGTGTA
GAACCCCTTTGCCGGGCAGGCCGGGTCGTCCTTGTGCAGGAACAACGAGTTGTACAGCC
GCTCGCTCACGAGCGCCTCCACGGAGCTGCACTTCCCGCCGGGGCCGTACCACCGCGCC
TCCGCGAAGAAGCCGCCGGAAGTGACCAGGGAAGCAACAGCCGTGACGGCGACAATAA
TGCGTCGGAATATGCCATC

Figure 2. Nucleotide sequence of Sal456-1 clone. This clone represents a fragment of the *Cynodon* chitinase genes containing a sequence conserved among plant chitinase genes and among the *Cynodon* chitinase genes isolated. This clone was used as probe for the subcloning of *CynCht-1*, *CynCht-2* and *CynCht-3*, as well as for Southern and northern blot analyses.

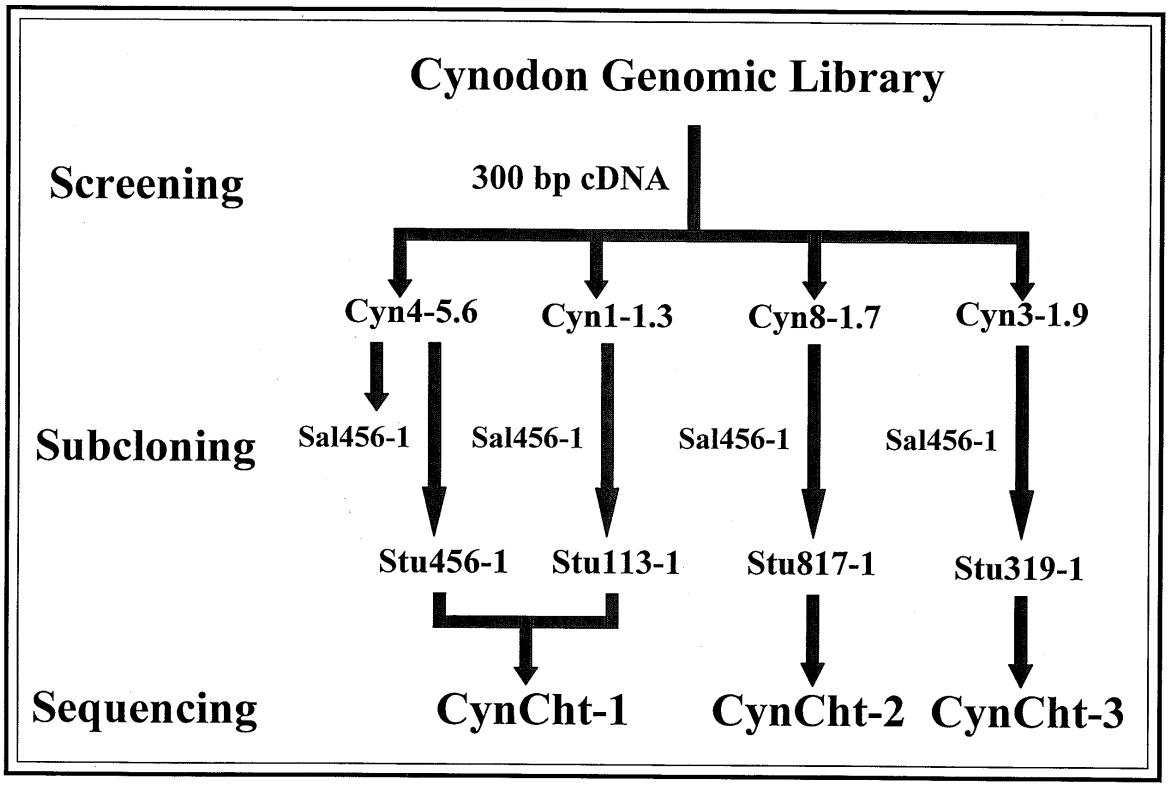

Figure 3. Isolation of the chitinase genomic clones from the library. The primary library was screened for chitinase genes using the 300bp cDNA probe. Nine positives were isolated after the tertiary screening. Four of these positive clones were subcloned using Sal456-2 as probe and then sequenced resulting in the identification of *CynCht-1, CynCht-2* and *CynCht-3*.

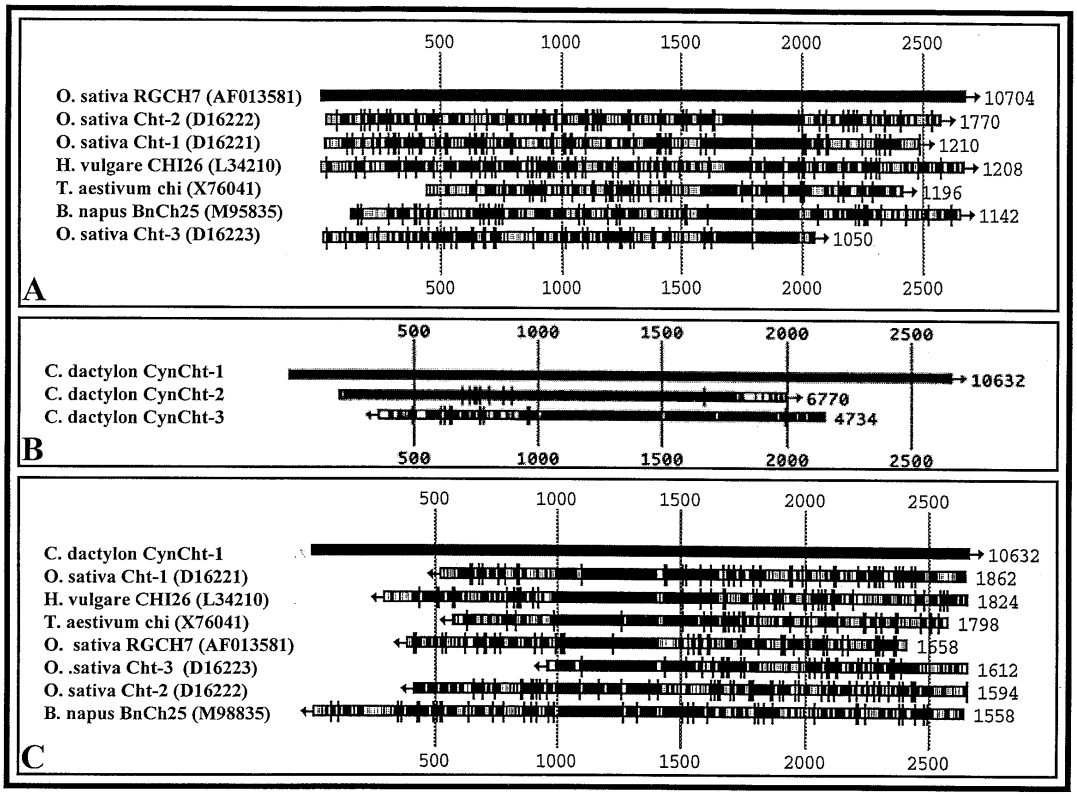

Figure 4. Analysis of nucleotide sequence homology among plant chitinase genes. Solid black bars represent sequence match, white bars represent sequence mismatch and black lines represent regions of base insertions. (A) Sequence alignment among chitinase genes from different plant species. Highly conserved sequence is shown in region 1600-2200. (B) Sequence alignment among *Cynodon* chitinase genes. *CynCht-3* is in reverse orientation relative to *CynCht-1* and *CynCht-2*. (C) Alignment of bermudagrass *CynCht-1* (cloned in reverse orientation) with other plant chitinase genes. Conserved sequence is shown in region 1000 to 1600.

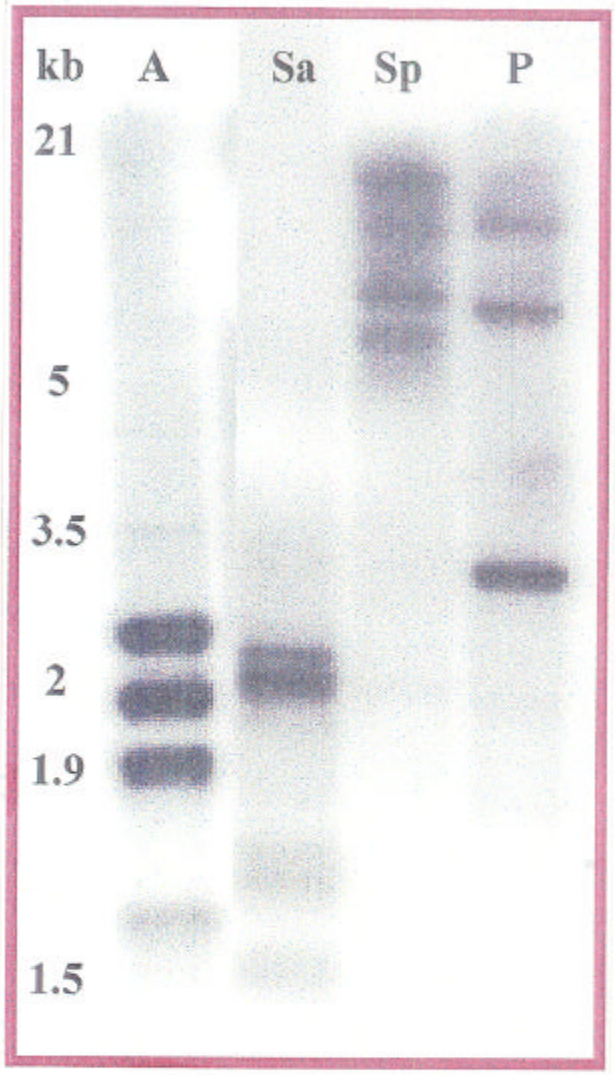

Figure 5. Southern blot anlaysis of Midiron total genomic DNA using Sal456-1 probe. Multiple bands indicate multiple chitinase genes in the Midiron genome. A=*Ava*I; Sa=*Sau*3AI; Sp=*Spe*I; P=*Pst*I

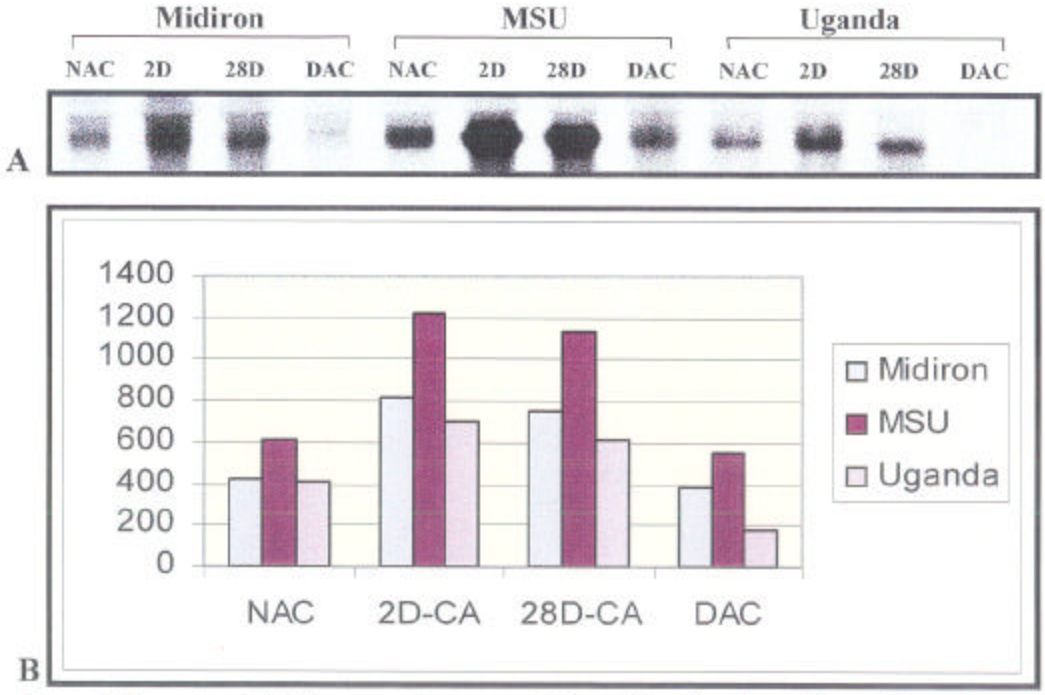

Figure 6. Expression of chitinase gene in crowns of three cold acclimating cultivars of bermudagrass. (A) Northern blot of crown tissue total RNA (20 ug) probed with radiolabeled chitinase gene. NAC=Non-acclimated; 2D=2 days cold acclimation; 28D=28 days cold acclimation; DAC=Deacclimated. (B) Densitometer scan of northern blot bands showing increase in the level of chitinase mRNAs during cold acclimation and decrease during deacclimation.

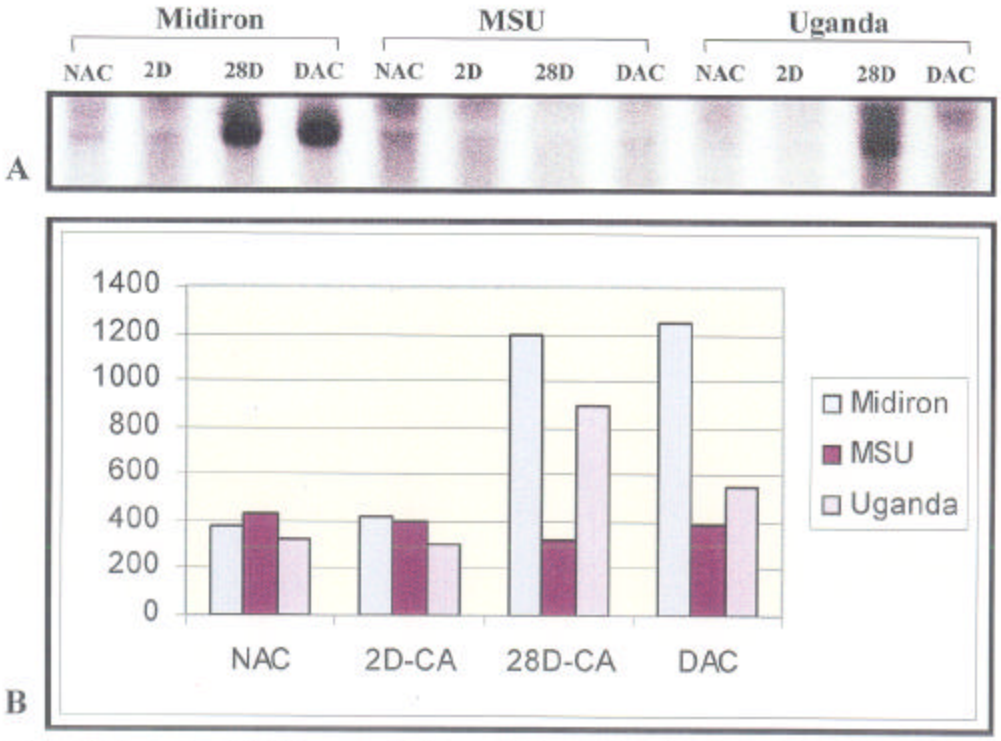

Figure 7. Expression of chitinase gene in leaves of three cold acclimating cultivars of bermudgrass. (A) Northern blot of leaf tissue total RNA (20 ug) probed with radiolabeled chitinase gene. NAC=Non-acclimated; 2D=2 days cold acclimation; 28D=28 days cold acclimation; DAC=Deacclimated. (B) Densitometer scan of northern blot bands showing the pattern of changes in the level of chitinase mRNAs in the leaves during cold acclimation and deacclimation.