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Induced expression of the class II chitinase gene during cold acclimation and dehydration of bermudagrass (*Cynodon* sp.)

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Abstract Bermudagrass cultivars vary greatly in their ability to survive freezing temperatures as a result of a differential ability to cold acclimate (CA) at temperatures slightly above 0°C. Little information exists on the genetic and physiological mechanisms associated with the cold acclimation process in bermudagrass. Experiments were conducted to study the changes in chitinase gene expression during cold acclimation of freeze-tolerant bermudagrass cultivars. A chitinase gene (*CynCHT1*) was isolated from 'Midiron' bermudagrass. Because the hydrophilic protein putatively encoded by the gene lacked an N-terminal cysteine-rich domain and a hydrophobic C-terminal extension, it was classified a class II chitinase. The expression patterns of this and related chitinase genes in response to CA, drought, and ABA were investigated in freeze-tolerant 'MSU' (LT_{50}=−11°C), Midiron (LT_{50}=−10°C) and 'Uganda' (LT_{50}=−8°C) bermudagrasses. Northern-blot analysis indicated expression in the crown tissues induced by CA at 8°C/2°C day/night temperature cycles. Induction of gene expression was evident in tissues sampled at 2 and 28 days after initiating CA. Expression after 2-days de-acclimation at 28°C/24°C was similar to control levels. Significantly higher levels of CA-induced chitinase gene expression were observed in MSU and Midiron, compared to Uganda. Similar expression patterns were observed among the cultivars in responses to drought and ABA. These results suggest that chitinases have important roles in bermudagrass response to low temperature and dehydration stresses.

Keywords Bermudagrass (*Cynodon* sp.), Class II chitinase – Cold acclimation, Freeze tolerance, Gene expression

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Introduction

Bermudagrass is widely distributed in nature and of economic importance in cultivated pastures and turf. It is distributed most abundantly in tropical and subtropical regions, but is sparsely distributed at latitudes approaching 50°N-S (Harlan and de Wet 1969). Consequently, bermudagrass ecotypes vary greatly in their response to climatic and edaphic conditions. Bermudagrass plants are polymorphic, perennial, sod-forming, and have the C_4 photosynthetic pathway. In temperate climates bermudagrass is dormant during winter months and regenerates via crown buds, rhizomes, and in some cases stolon buds. Many bermudagrass cultivars used in the USA have only low to moderate freeze tolerance, and freeze injury to such cultivars occurs intermittently in regions subject to low freezing temperatures (Anderson et al. 1997). Enhancement of freeze tolerance in bermudagrass would facilitate its use in colder regions, primarily by lessening risk of winter injury.

Subjecting bermudagrass to cold acclimation (CA) elicits the production of specific chitinase enzymes (Gatschet et al. 1994, 1996). Chitin, a linear polymer consisting of N-acetyl-D-glucosamine (GlcNAc) units, is a ubiquitous molecule in nature, being an integral component of insect exoskeletons, crustacean shells and fungal cell walls. Organisms that contain chitin also synthesize chitinases (E.C.3.2.1.14), the enzymes that hydrolyze the polymer. Interestingly, most plants synthesize chitinases although they do not contain chitin. Potential endogenous substrates for plant chitinases were un-

known until the discovery that GlcNAc is also present in the secondary cell walls of higher plants in the form of glycolipids (Benhamou and Asselin 1989). Although the precise function of chitinases in plant metabolism is not fully understood, evidence suggests important roles in plant development and morphogenesis, specifically in flower formation and leaf abscission (Gomez-Lim et al. 1987; Neale et al. 1990; Harikrishna et al. 1996). The fungal cell wall-degrading activity is another important function of plant chitinases; hence research has focused on the role of chitinases as pathogenesis-related (PR) proteins (Graham and Sticklen 1994). Most chitinases are actively synthesized in response to wounding, fungal, bacterial and viral infections (Legrand et al. 1987; Hedrick et al. 1988; Majeau et al. 1990; Chang et al. 1995) and ethylene (Boller et al. 1983; Roby et al. 1986; Flach et al. 1992; Shinshi et al. 1995).

Multiple genes encode chitinases. The products of these genes constitute structurally distinct enzyme classes with different spatial and temporal regulatory patterns (Meins et al. 1992; Collinge et al. 1993). Like other redundant genes, the complex regulation of chitinase genes suggests that their functions extend beyond being PR proteins (Pickett and Meeks-Wagner 1995). Studies have shown that chitinase gene expression is induced by abiotic factors such as ozone (Ernst et al. 1992) and osmotic stress (Chen et al. 1994). Chitinases, along with other PR proteins (β-1, 3-glucanase and thaumatin), may function as antifreeze proteins (AFP) due to their ability to retard extracellular ice formation during freezing of plant tissues. In winter rye, these proteins were found to accumulate in leaf apoplastic sap following CA, an observation consistent with the extracellular location of known AFPs (Hon et al. 1995; Antikainen et al. 1996; Griffith et al. 1997).

Gatschet et al. (1996) demonstrated increased chitinase levels in the crowns of freeze-tolerant bermudagrass cultivars following 2 and 28 days CA. This study was conducted to provide information on changes in bermudagrass chitinase gene expression in response to CA and dehydration. We report here the molecular cloning of a member of the class II chitinase gene family and the expression patterns of chitinase genes in response to CA, drought stress and exogenous application of abscisic acid (ABA).

Materials and methods

Construction and screening of a genomic library

Genomic DNA (>80 kb) was isolated from leaf tissues of Midiron (*Cynodon dactylon*×*Cynodon transvaalensis*) by the CTAB method (Wagner et al. 1987). The genomic library was constructed in Lambda Fix II (Stratagene) using *Bam*HI-digested bermudagrass DNA (10–20 kb). The primary library consisted of about 2.2×10^6 recombinant plaques. A 350-bp bermudagrass chitinase genomic fragment produced by PCR was used to screen plaque-lifts from 8×10^5 pfu (McMaugh and Lyon 1997). The probe was labeled with α^{32}P-dCTP by random priming (Amersham Life Science). Hybridizing plaques were identified and amplified by standard procedures (Sambrook et al. 1989).

Subcloning and sequencing of GCyn clones

Two clones (GCyn113 and GCyn456) with the longest insert sizes were identified by *Not*I digestion of the recombinant phage DNA. These fragments were further digested with *Sal*I and *Stu*I. The digests were blotted onto a Hybond N^+ nylon membrane and hybridized with the same probe originally used in the library screening. A probe-reactive fragment (0.9 kb) from the *Sal*I-digests was subcloned into the *Sal*I site of pBluescript-SK(+) and sequenced using T3 and T7 primers. The 0.9-kb *Sal*I fragment was used as a probe to identify larger subclones from *Stu*I digests of GCyn113 and GCyn456. The probe-reactive *Stu*I fragment (3.5 kb) was subcloned into pBluescript-SK(+) with an *Xho*I adaptor. The 3.5-kb *Stu*I fragment was serially deleted from both ends with exonuclease III using the Erase-A-Base Kit (Promega). Overlapping deletion clones were sequenced from both directions with the T3 and T7 primers using an automated DNA sequencer (373 DNA Sequencer, Applied Biosystems) at the Recombinant DNA/Protein Resource Facility, Oklahoma State University. The DNA sequences were analyzed with the GENSCAN computer program (Burge and Karlin 1997). Analysis of the 5′-flanking region of the gene was performed with the Promoter Prediction by Neural Network (Reese et al. 1996). The encoded amino-acid sequence was analyzed with ExPASy Proteomics Tools (Wilkins et al. 1998). The chitinase nucleotide and amino-acid sequence comparisons with the NCBI sequences database and their alignment to selected plant chitinase sequences were performed by the BLAST and ClustalW programs (Altschul et al.1990; Thompson et al.1994), respectively.

Plant materials and stress treatments

Midiron, MSU, and Uganda bermudagrasses were used for both Southern- and Northern-blot analyses. Midiron (2n=3*x*=27 chromosomes) and Uganda, *C. transvaalensis*, (2n=2*x*=18 chromosomes) are commercial cultivars (Alderson and Sharp 1995; Hanson 1972). MSU (*C. dactylon* var. *dactylon*; 2n=4*x*=36 chromosomes) was originally collected from the campus of Michigan State University, East Lansing, Mich. The clonal plants were obtained from the *Cynodon* germplasm collection maintained by the Department of Plant and Soil Sciences, Oklahoma State University.

Mature phytomers from each cultivar were transplanted into each of 40 14-cm diameter pots and maintained in a greenhouse (22–37°C) for at least 3 weeks. The plants were transferred to a controlled environment chamber maintained at 28°C day/24°C night and 10/14 h light/dark. The plants were allowed to equilibrate under these conditions for 3 weeks before CA. The CA treatments were initiated by placing 30 pots in a controlled environment chamber maintained at 8°C day/2°C night and a photosynthetic photon flux of about 300 $\mu mol \cdot m^{-2} \cdot s^{-1}$ at 10/14 h light/dark (Anderson et al. 1993). Ten pots each were used for 2 and 28 days CA. The remaining plants (ten pots) were de-acclimated (DAC) for 2 days at 28°/24°C immediately after 28 days of CA. A control set (ten pots) was maintained in the growth chamber at 28°C day/24°C night and 10/14 h light/dark cycles during the CA period.

Drought stress was induced by withholding water for ten consecutive days, while the control plants were watered daily to field capacity. The relative water content (RWC) of both the control and drought-stressed plants was determined using the formula: RWC=(Fresh Weight-Dry Weight)/(Turgid Weight-Dry Weight)×100 (Baker et al. 1994).

The minimum ABA concentration (100 μM) that caused a significant increase in freeze tolerance was determined from a preliminary study using Midiron (data not shown). The ABA solution (100 μM) was applied to ten potted plants of each cultivar grown in a growth chamber at 28°/24°C. Plants were sprayed with 0.1% (v/v) Tween-20 surfactant 10-min prior to ABA application. The ABA solutions were applied to the same plants by both foliar spray and direct watering of the pots at 12-h intervals for 5 con-

secutive days. The control plants maintained in the same chamber received only Tween-20 and water.

RNA isolation and Northern hybridization

Total RNA was isolated from 4 g of bermudagrass tissues with 8 M guanidine hydrochloride (pH 7.0), following a procedure modified from Logemann et al. (1987). Modifications included a precipitation step with 4 M LiCl after guanidine hydrochloride extraction. Equal amounts of total RNA samples (18 µg) were electrophoresed on 1% formaldehyde-agarose gels using reagents from the NorthernMax kit (Ambion). The resolved RNA was transferred for 2 h onto a Hybond N^+ nylon membrane (Amersham Life Science) using a Turboblotter (Schleicher and Schuell) and fixed by UV cross-linking. Two kinds of DNA probes were labeled with α^{32}P-dCTP by random priming as described above. Probe P1 corresponds exclusively to the coding region while probe P2 corresponds to a small part of the C-terminal coding region and the 3′UTR regions of CynCHT1. Hybridization and post-hybridization washings were performed at 42°C using the NorthernMax kit. The hybridized filters were autoradiographed for 48 h at −80°C. The Northern blot signals were quantified using a GS-700 Imaging Densitometer (BioRad).

Southern hybridization

*Xho*I-digested genomic DNA samples (2 µg) were electrophoresed in 1% TAE-agarose gels and transferred on Hybond N^+ nylon membrane (Amersham Life Science) by alkali blotting (0.4 M NaOH). Pre-hybridization and hybridization with labeled probes were both performed overnight at 65°C in 5× SSPE, 2.5× Denhardt's solution, 0.1% SDS and 0.5 mg ml^{-1} of sonicated salmon sperm DNA. Post-hybridization washings were performed with 2× SSC, 0.1% SDS at room temperature, and 0.25× SSC, 0.1% SDS at 65°C. The hybridized filters were autoradiographed for 2–3 days at −80°C.

Results

Isolation of chitinase genomic clones

Two probe-reactive genomic library clones (GCyn113, GCyn456) with the strongest signals were chosen for further analysis. Digestion with *Not*I revealed that both clones contained inserts of about 20 kb. Two 0.9-kb fragments (one from each clone) were identified from *Sal*I-digested insert DNA by hybridization with the 360-bp probe. Analysis of the nucleotide sequences of these probe-reactive fragments indicated that they were identical and corresponded to a large part of the coding region of a chitinase gene. The open reading frames (ORF) contained in the two *Sal*I fragments were identical. The predicted amino-acid sequences of the ORFs were very similar (at least 50% identical) to those of the catalytic regions of a number of known plant chitinase genes.

To subclone the full-length gene from the 20 kb-inserts of GCyn113 and GCyn456, the *Sal*I fragment from GCyn113 was used as a probe (P1) to search for specific restriction fragments that contain the full-length chitinase gene. *Stu*I digestion of the both GCyn 113 and GCyn456 inserts generated 3.5-kb fragments that hybridized to probe P1. The nucleotide sequences of these fragments indicated that they contained both the coding and the flanking regions of the gene. Identical sequences confirmed that these clones contained the same gene, consistent with the partial sequences of the *Sal*I fragments. The gene was designated as *CynCHT1*.

Structure of *CynCHT1*

The complete *CynCHT1* nucleotide sequence and the deduced amino-acid sequence of the protein encoded are shown in Fig. 1. The predicted transcription start site is located 88 nucleotides upstream to the first AUG codon in the ORF. Computer analysis indicated that the coding region is divided into three exons (126, 100 and 524 bp) by two small introns (94 and 103 bp). The boundaries of the two introns were predicted based on consensus sequences for splice donor and acceptor sites in eukaryotes as defined by the motifs C/GT ... AG/G and G/GT ... AG/G, respectively. Three potential polyadenylation signals occur at the 3′ end located at 65, 288 and 361 nucleotides downstream from the UAG termination codon (Fig. 1). This predicted structure of *CynCHT1* is consistent with the lengths of hybridizing mRNA (1.1 kb) detected in Northern blots.

The translation initiation codon was assumed to be the first AUG codon immediately downstream from the putative transcription start site. This codon is imbedded within a sequence that conforms to the consensus for the optimal context of eukaryotic translation initiation as defined by the motif GCC(**G or A**)CC*AUG***G** (Kozak 1991). The two most important positions in this motif, the purine at position −3 and the last G at position +4, are conserved. The putative 27-kDa preprotein product of *CynCHT1* (designated as preCHT1) is comprised of 249 amino-acid residues and is slightly basic (pI approximately 7.52).

The putative TATA-signal was located at position –25. Two GC-rich motifs were also located upstream of the TATA-box (Fig. 1). The first motif, defined by the sequence GGCCGGCCGCCCTTG (position –100), is very similar to the GGCC-box that was also found upstream of the wound-inducible chitinase and glucanase genes from rice (Zhu and Lamb 1991; Simmons et al. 1992; Nishizawa et al. 1993). The second motif, defined by the sequence GCCCGGCGCGA GC GCG (position –125), is somewhat similar at four nucleotide positions to the ethylene responsive element (GAGCCGCC) in tobacco class I chitinase genes (Shinshi et al. 1995). The significance (if any) of these two motifs to chitinase gene regulation remains to be determined.

Amino-acid sequence analysis

The predicted primary structure of the preCHT1 consists of a signal peptide connected to the catalytic domain by a short hypervariable region (Fig. 2). The predicted mature polypeptide is comprised of 227 amino-acid residues with a calculated molecular weight of 25 kDa, and

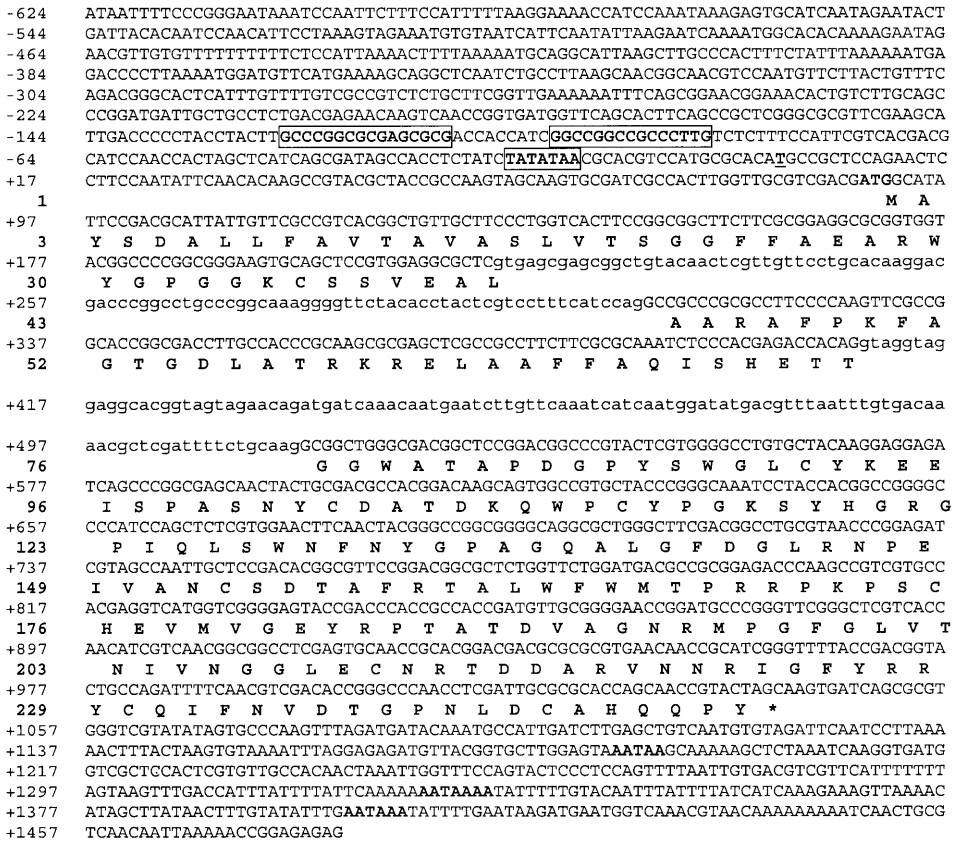

Fig. 1 Nucleotide sequence of *CynCHT1* (Acc. 105425) and the deduced amino-acid sequence of the encoded protein (preCHT1). The nucleotide positions and amino-acid residues are *numbered* at the left. Introns are represented by *lower case letters*. The sequences inside the box represent the GC-rich 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*

a pI of 8.10. The protein is hydrophilic with a Grand Average Hydropathicity (GRAVY) value of –0.493. Alignment of the amino-acid sequence of the CHT1 mature polypeptide suggested homology with a number of known plant chitinase gene products. The homologous region was localized in the catalytic domain of the protein (Fig. 2). This region aligned with both class I and II chitinases, although the level of shared identity was slightly higher with class II proteins. Class II chitinases from peanut (*Arachis hypogea*, S65069), strawberry (*Fragaria×ananassa*, AF147091), tomato (*Lycopersicon esculentum*, S69184) and potato (*Solanum tuberosum*, X67693) averaged 66% sequence similarity. Class I chitinases from alfalfa (*Medicago sativa*, U83591), wheat (*Triticum aestivum*, X76041) and rice (*Oryza sativa*, X87109) averaged 59% sequence similarity. Despite the sequence correspondence in the catalytic region, CHT1 differs from class I chitinases in the absence of both an N-terminal cysteine-rich domain and a hydrophobic C-terminal extension (Fig. 2) which are present in vacuolar chitinases (Bednarek and Raikhel 1991; Chrispeels and Raikhel 1992) but not in class II chitinases. Hence, CHT1 is probably a class II chitinase and an extracellular protein like most class II chitinases.

Cynodon chitinase gene family

The predicted restriction sites in *CynCHT1* were examined to identify a restriction enzyme that does not cut within the region spanned by probe P1 (Fig. 3A). A unique *Xho*I site was located near the 3′ end of the probe region. *Xho*I-digested genomic DNAs from the three bermudagrass cultivars were probed by Southern blotting and hybridization to determine the number of gene copies in the *Cynodon* genomes. Southern analysis revealed three major (strong) bands and several minor (faint) bands of Midiron that hybridized to probe P1 (Fig. 3B). The major bands were 3.5, 1.7 and 0.9 kb in length. The same three major bands were also detected in *Xho*I-digested MSU DNA while only one common major band (1.7 kb) was observed between Uganda and the other two cultivars and even this was of slightly slower mobility. Relative to the banding pattern in Midiron and MSU, polymorphic restriction fragments of 2.8 and 1.2 kb were observed in Uganda (Fig. 3B). These results suggest at least three members of the bermudagrass class II chitinase gene family per diploid genome. The minor bands probably represent cross hybridization with chitinase genes belonging to a different family.

Chitinase gene expression during CA

Both P1 and P2 probes were used to determine the level of chitinase gene expression. Transcripts of chitinase genes were not detected by Northern-blot analysis using probe P1 in the control crown tissues of Uganda (Fig. 4A). Very low levels of transcripts were detected in Midiron and MSU. However, chitinase mRNA levels

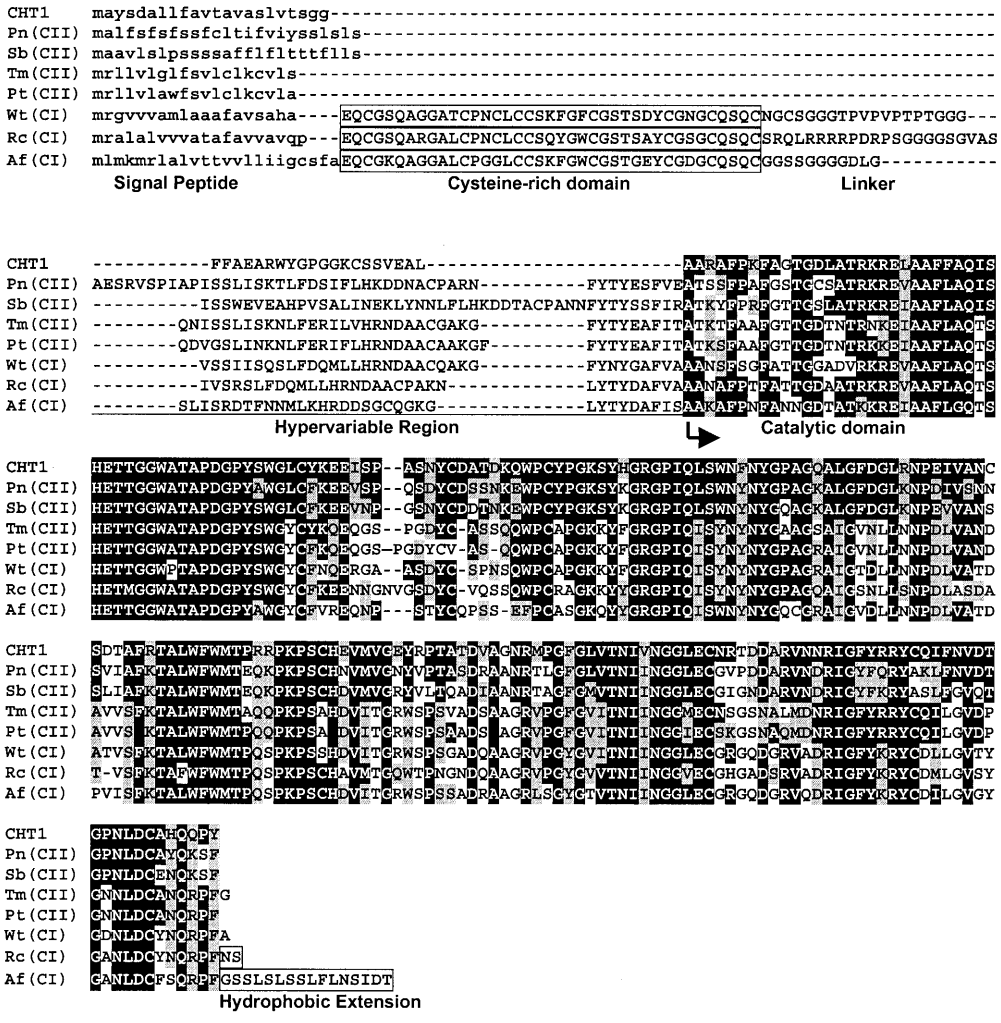

Fig. 2 Structural analysis and amino-acid sequence alignment of the CHT1 catalytic domain with class I and II chitinases from other plant species. *Pn*(*CII*): peanut, class II=S65069; *Sb*(*CII*): strawberry, class II=AF147091; *Tm*(*CII*): tomato, class II=S69184; *Wt*(*CI*): wheat, class I=X76041; *Rc*(*CI*): rice, class I=X87109; Af(CI): alfalfa, class I=U83591. The putative signal peptides are in *lower case letters*. The structural domains of class I, absent in class II, are also indicated. The *shaded residues* represent homologous regions in the catalytic domain

significantly increased following 2 and 28 days CA, as indicated by mRNA bands of about 1.1 kb (Fig. 4A and B). The general trend of induction of chitinase mRNA in response to CA, and of decay after DAC were similar in all three cultivars. However, the magnitude of low temperature induction was clearly distinguishable among the cultivars. The highest and lowest induction levels occurred in MSU and Uganda, respectively. After 2 days CA, the transcript levels in Midiron, MSU and Uganda increased approximately 5-, 6- and 3-fold, respectively, from the basal levels. This pattern clearly indicated that plants responded rapidly to CA by inducing the expression of chitinase genes. The transcript levels in Midiron, MSU and Uganda increased approximately 4-, 5- and

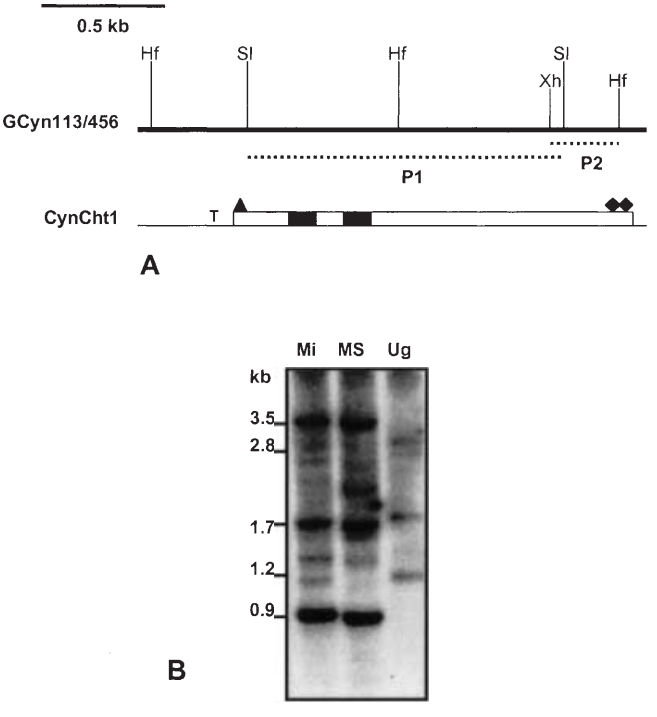

Fig. 3 **A** Structure of *CynCHT1*. The sequenced region is represented by *open bars*. The location of probes P1 (*Sal*I fragment) and P2 (3′ UT) are shown by the *dotted lines*. Introns are represented by *black bars*. ▲=transcription initiation site, ◆=poly-A signals, *Hf*=*Hinf*I, *Sl*=*Sal*I, *Xh*=*Xho*I **B** Southern blot of *Xho*I-digested bermudagrass total genomic DNA hybridized with probe P1. The fragments corresponding to each of the chitinase gene copies identified in Midiron, and the corresponding homologues in MSU and Uganda, are indicated

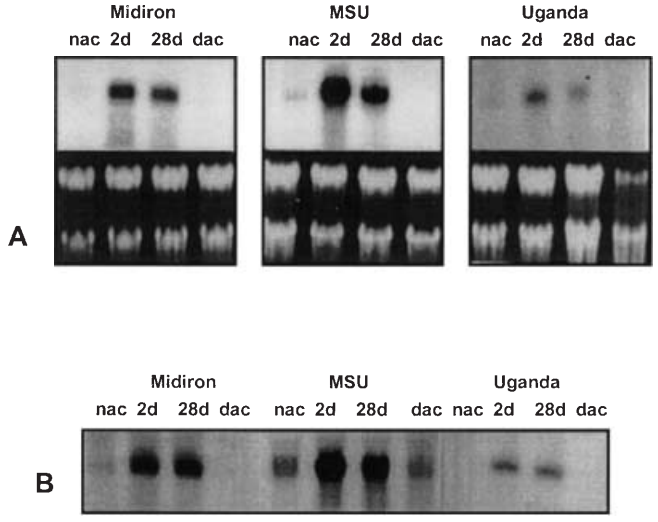

Fig. 4 Northern-blot analysis of chitinase gene expression in bermudagrass crowns during cold acclimation. Equal amounts of total RNA loaded in gel are shown by rRNA bands. RNA blots of non-acclimated (*nac*), 2-days cold acclimated (*2d*), 28-days cold acclimated (*28d*) and de-acclimated (*dac*) samples were hybridized with probes P1 (*A*) and P2 (*B*)

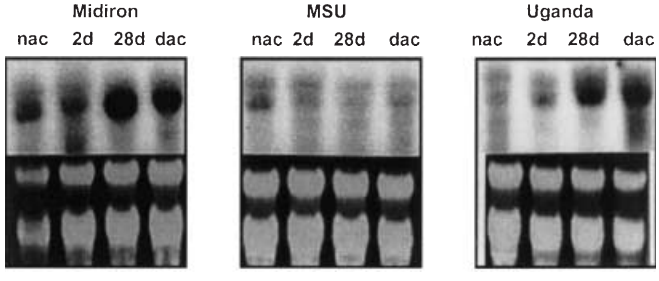

Fig. 5 Northern-blot analysis of chitinase gene expression in bermudagrass leaves during cold acclimation. Equal amounts of total RNA loaded in the gel are shown by rRNA bands. RNA blots of non-acclimated (*nac*), 2-day cold acclimated (*2d*), 28-day cold acclimated (*28d*) and deacclimated (*dac*) samples were hybridized with probe P1

2.7-fold, respectively, at 28 days CA compared with non-acclimated tissues (Fig. 4A). The similarity in levels at 2 and 28 days suggests that a steady state level of chitinase mRNA is achieved during CA. A similar trend of transcript accumulation during CA was observed using probe P2 (Fig. 4B).

Very low levels of chitinase mRNA were found in root tissues of Midiron and MSU after 2 and 28 days CA. There was no detectable chitinase transcript in Uganda roots (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 as detected by probe P1 (Fig. 5). After 2 days CA, transcript accumulation in Midiron and Uganda had increased approximately 3- and 2.5-fold, respectively, relative to base levels. Increased *chitinase* transcript accumulation occurred in Midiron and Uganda, but not in MSU, after 28 days CA. Unlike

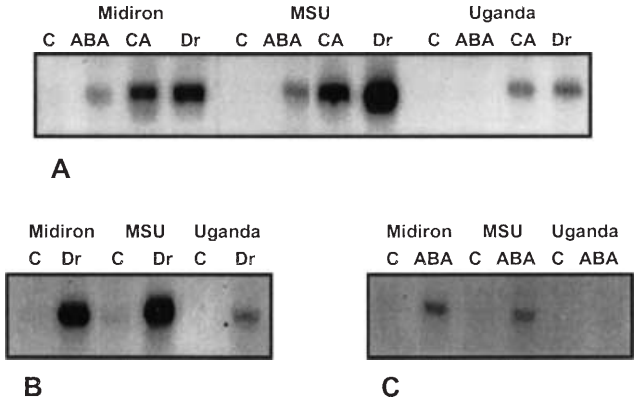

Fig. 6 A Stress-induced expression of *CynCHT1* in bermudagrass cultivars. Equal amounts of crown tissue total RNA from control or non-stressed (*c*), cold-acclimated (*ca*), drought-stressed (*dr*) and ABA-treated (ab) plants were blotted onto a nylon membrane and hybridized with probe P1. **B** Chitinase gene expression in crown tissues of drought-stressed plants. RNA blots were hybridized with probe P2. **C** Chitinase gene expression in crown tissues of ABA-treated plants. RNA blots were hybridized with probe P2

in crowns, the transcript levels in the leaves of Midiron and Uganda remained elevated after DAC, and approximately equivalent to those in 28 days CA-leaf tissues. No transcripts were detected in the leaves of MSU.

Effect of drought stress and ABA on chitinase gene expression

Plants were subjected to drought stress to determine the effect of desiccation on chitinase gene expression in the bermudagrass cultivars. The RWC's of the three cultivars were >75% and <25% before and after the drought stress, respectively. All plants showed clear symptoms of extreme desiccation following the 10-days dehydration. Drought stress significantly induced chitinase mRNA levels in the crowns of all three cultivars as indicated by Northern hybridization with probe P1 (Fig. 6A). Similar to the observations with CA-plants, the highest level of drought-induced chitinase mRNA was in MSU and the lowest in Uganda. A similar trend of drought-induced transcript accumulation was detected by probe P2 (Fig. 6B). Drought treatments did not induce chitinase gene expression in the root tissues of any cultivar (data not shown).

Results from initial studies (data not shown) indicated that 100 µM of ABA caused a moderate but significant increase in the cold hardiness of Midiron bermudagrass (from LT_{50}=–6°C before CA to LT_{50}=–8°C after CA, data not shown). Accordingly, chitinase gene expression was investigated in Midiron, MSU and Uganda plants treated with 100 µM of ABA. Unlike CA and drought, 100 µM of ABA caused only moderate increases in chitinase transcript levels in the crown tissues of Midiron and MSU, but not in Uganda, as shown by Northern blotting using probe P1 for hybridization (Fig. 6A).

Moreover, unlike in CA and drought, ABA induced slightly higher levels of chitinase mRNA in Midiron compared to MSU (Fig. 6A). Similar trends in transcript accumulation were observed using probe P2 (Fig. 6C). The application of ABA did not induce chitinase transcript accumulation in the leaf and root tissues of any cultivar (data not shown).

Drought stress resulted in higher levels of chitinase mRNA in crown tissues than did CA or ABA (Fig. 6A). 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. Drought-induced transcript levels were higher than those observed in 2-day CA crown tissues, which were approximately 5-, 6- and 3.25-fold increased from the basal levels in Midiron, MSU and Uganda, respectively. The ABA-induced mRNA levels (at least at 100 μM of ABA) 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.

Discussion

Analysis of both the nucleotide and translated sequences of *CynCHT1* indicated similarity with the catalytic regions of both class I and II chitinases of other plant species. Although the sequences of the catalytic regions of class I and class II chitinases are highly conserved (Collinge et al. 1993; Tomohiro and Torikata 1995; Hamel et al. 1997), class I proteins were distinguished from class II proteins based on two structural elements (Fig. 2). The first element is the N-terminal cysteine-rich domain necessary for binding of the protein to chitin (Shinshi et al. 1988; Flach et al. 1992; Iseli et al. 1993; Beintema 1994). This element consists of 40 amino acids with eight cysteine residues located at highly conserved positions along the motif. This domain is linked to the main catalytic region by a short domain that is usually glycine- and proline-rich. The second element is a C-terminal extension comprised mostly of hydrophobic amino acids (Fig. 2). This domain is necessary for vacuolar localization of class I proteins (Neuhaus et al. 1991; Chrispeels and Raikhel 1992). CHT1 was categorized as class II based on the absence of those two structural features and the higher sequence identity shared with the catalytic regions of other class II chitinases.

Unlike most class II acidic chitinases, the mature cynCHT1 protein has a basic pI (approximately 8.10). However, this predicted value may not reflect the true pI of the mature protein since it can be affected by post-translational modifications including the attachment of other moieties to the protein structure (e.g., potential asparagine N-glycosylation sites at positions 152–155 and 212–215 of CHT1) (Graham and Sticklen 1994). The current convention for classifying plant chitinases is based on both the structural features and the pI, with classes I and II as basic and acidic proteins respectively (Shinshi et al. 1990; Beintema 1994). However, genes encoding acidic chitinases with N-terminal cysteine-rich domains have been identified in some plant species (Margis-Pinheiro et al. 1991; Araki et al. 1992). Accordingly, CHT1 is an example of a basic chitinase that can be structurally classified as a class II protein. This is in agreement with the proposal of Meins et al. (1992) and Nishizawa et al. (1993) for a classification of chitinases purely on the basis of structural characteristics rather than on both structure and pI.

The multiple banding patterns observed in the Southern blot with probe P1 suggest that *Cynodon* class II chitinases are encoded by a small family comprising at least three genes per diploid genome. This result is consistent with reports that chitinase genes in higher plants occur as families of three to four members (Wu et al. 1991; Meins et al. 1992; Hudspeth et al. 1996). The species-associated allelic patterns are consistent with genomic differences between *C. dactylon* and *C. transvaalensis* (Harlan et al. 1970). The presence of both *C. dactylon*- and *C. transvaalensis*-associated alleles confirms the heterozygosity of the triploid hybrid Midiron.

Northern hybridizations using probes P1 and P2 were performed to gauge the level of chitinase gene expression in the cold-acclimated bermudagrass crown. P1 spans 82% of the coding region including the highly conserved catalytic domain. P2 spans a short 205-bp region including 119 bp of translated and 86 bp of 3′ untranslated region. Neither P1 nor P2 should be regarded as gene specific towards cynCHT1.

The results of this study are consistent with those of Gatschet et al. (1996) indicating that chitinase proteins are actively synthesized in the crown tissues of freeze-tolerant bermudagrasses during CA. The level of chitinase gene expression in the crown tissues of the three cultivars appears to be related to the magnitude of cold hardiness. Previous studies indicated that MSU and Midiron are two of the most freeze-tolerant genotypes so far identified in *Cynodon* germplasm (Anderson et al. 1988, 1993). Based on these studies, CA for 28 days under laboratory conditions (8°C/2°C) resulted in LT_{50} values of −11°C and −10°C for MSU and Midiron, respectively. In contrast, Uganda exhibited moderate tolerance to freezing stress with an LT_{50} value of –8°C (Anderson et al. 1993; J. Anderson, unpublished). Accordingly, the low temperature-induced chitinase gene expression was found to be highest in MSU and lowest in Uganda. This pattern suggests potential differences in the upstream regulatory regions of the homologous genes from each cultivar. Two days of DAC was sufficient to downregulate chitinase gene expression in all the cultivars. This temporal regulation pattern is very similar to the expression patterns exhibited by known cold-regulated (COR) genes in higher plants (Thomashow 1990).

The CA-induced chitinase gene expression appears to be specific to the crown. The transcript levels in the crown peaked after 2 days of CA. This pattern indicates a rapid response of the plants to environmental signals inducing gene transcription. As the acclimation process

proceeded from 2 to 28 days, a slight reduction in transcript level occurred. This pattern reflects a balance between transcription and mRNA turnover and can be interpreted as an indication of the steady state mRNA levels during the acclimation process. These data support Gatschet et al's. (1994) finding that CA beyond 28 days caused no further increase in the cold-hardiness of Midiron and 'Tifgreen' bermudagrasses. Accumulation of PR proteins has been demonstrated in other cold-hardy monocot species including wheat (Houde et al. 1995) and winter rye (*Secale cereale*) (Antikainen et al. 1996). It can therefore be inferred that chitinase gene expression during CA in bermudagrass crowns may be one of the cellular responses involved in protecting the meristem or its surrounding protective tissues from the damaging effects of low temperature and dehydration.

Contrary to the pattern of gene expression in crown tissues, the expression in leaf tissues does not correlate with CA or with the freezing tolerance of the cultivars used in the experiments. Induction of gene expression only occurred in Midiron and Uganda. Furthermore, this pattern of chitinase gene expression could be under a regulatory control that is different from that in the crowns. Moreover, differences in the regulation of gene expression may also result in the expression of an entirely distinct set of chitinase genes in leaves compared with crown tissues. Chitinases have functions in various physiological processes and are induced by several factors including wounding and pathogen infection. Both Midiron and Uganda exhibited very high levels of chlorosis during the CA periods, which were aggravated during DAC. This condition was not pronounced in MSU. The pattern of chitinase gene expression in the leaves of Midiron and Uganda may not be directly related to low temperature per se, but could be associated with other physiological events in the leaves including the loss of chlorophyll (Gomez-Lim et al. 1987).

The physiological relevance of the CA-induced chitinase transcript accumulation in bermudagrass crowns is potentially related to recent findings regarding the accumulation of PR proteins, including chitinase, β-1,3-glucanase and thaumatin, in the apoplast of cold-acclimated monocot species (Hon et al. 1995; Antikainen and Griffith 1997). Studies have shown that these proteins possess the ability to bind to the surface of potential ice nuclei, thereby inhibiting ice-crystal growth in vitro. This phenomenon, observed predominately in overwintering monocot species, has led to a hypothesis that some isozymes of PR proteins exhibit biological activity as antifreeze agents (Antikainen and Griffith 1997; Griffith et al. 1997; Thomashow 1998; Yu and Griffith 1999). The ability of these PR proteins to act as antifreeze agents in vivo has not been demonstrated. Nevertheless, the activity they exhibited in vitro and their high level of accumulation during CA of cold-hardy species imply that they probably have significant specific roles in plant response to low-temperature stress. Similarly, the cultivar-associated and CA-induced expression of the chitinase genes probed here point to a possible role in the tolerance mechanism(s). However, further biochemical characterization is necessary to determine the physiological role of these chitinases during low-temperature stress. The potential role of one or more chitinase genes in the coordinated control of freeze tolerance and fungal resistance should be addressed in future research. This possibility is consistent with a report that most freeze-tolerant bermudagrass cultivars have higher resistance to spring dead spot disease caused by *Ophiosphaerella herpotricha* than freeze-susceptible cultivars (Baird et al. 1998). Similarly, Tronsmo et al. (1993), also reported that CA in some cereal species could increase not only the tolerance to freezing but also the resistance to fungal infection.

The involvement of some PR proteins in cellular responses to low-temperature stress is unclear. One hypothesis is that pathogen infection or exposure to low temperature each induces biochemical processes leading to the modification of plant cell walls (Griffith and Brown 1982; Collinge et al. 1993; Rajashekar and Lafta 1996). These events may cause the synthesis of a common set of molecules, which probably include some PR proteins. The enzymatic activity of plant chitinases is most likely involved both with fungal cell-wall degradation and with plant cell wall modification during stress periods induced by low temperature and drought. This hypothesis is supported by the occurrence of endogenous substrates for this enzyme in plant cells as GlcNAc units in glycolipids of secondary cell walls are probably present in tough tissues like the crowns of bermudagrass (Benhamou and Asselin 1989). Some PR proteins may have acquired multiple functions because they are spatially and temporally in-frame relative to both forms of stresses. The hydrophilicity of *CynCHT 1* is consistent with the hydrophilic property and extracellular location of known class II chitinases and other PR-proteins with putative antifreeze activity.

The biochemical and physiological bases of plant tolerance to freezing and drought are very similar, if not totally identical (Siminovich and Cloutier 1983). Hence, the drought- and ABA-induction of bermudagrass crown chitinases was expected. Further investigations are necessary to determine the link between tolerance to freezing, drought, and resistance to fungal infection. It is possible that both low temperature and drought contribute to increased susceptibility of plants to pathogen infection, and chitinase gene expression may just be a secondary response to such conditions (Simpson 1981).

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