COLD ACCLIMATION AND CHITINASE GENE EXPRESSION IN BERMUDAGRASS (*CYNODON* SPP.)

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PREFACE

Substantial progress has been made towards the understanding of the molecular basis of low temperature stress tolerance in both prokaryotic and eukaryotic model systems. This wealth of information has furnished us with the fundamental knowledge necessary in order to establish a better strategy to investigate the processes of cold acclimation and freezing tolerance in more complex genomes, including most of the economically important plant species. These efforts not only enhance basic knowledge but also aid in the development of innovative approaches to increase freezing tolerance capabilities of crops and other plant species beneficial to human daily life.

Bermudagrass turfs (*Cynodon* spp.) provide a number of practical benefits for the improvement and preservation of the human environment. Unfortunately, these species are constantly subjected to winter injury over much of their geographic area of use in the southern United States and in other temperate countries. Information on the molecular events responsible for some bermudagrass cultivars withstanding freezing conditions was limited prior to this

investigation. Hence, this research was initiated to study the potential molecular mechanisms responsible for the ability of some bermudagrass cultivars to cold acclimate and develop tolerance to otherwise damaging temperature conditions. This goal was addressed in this particular investigation through the use of modern techniques of molecular genetics and recombinant DNA technology. These efforts led to the molecular cloning of specific pathogenesis-related (PR) protein genes potentially involved in tolerance mechanisms. Although still inconclusive, the results of the experiments presented in this manuscript are quite promising and present interesting hypothesis on the possible involvement of novel PR proteins with potential antifreeze mechanisms in *Cynodon*. I hope that this information will open an area of biochemical and molecular investigation on the occurrence of antifreeze proteins in *Cynodon*.

The humble contribution made by this investigation in an attempt to better understand the mechanisms responsible for freezing tolerance in *Cynodon* would not have been possible without the guidance and supervision of my

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I am very grateful for having good friends who made my stay in Stillwater more like home. Lastly, I thank my family for the encouragement and love despite our distance.

I dedicate this humble piece of work to my parents Anatalio and Lorieta, to my grandmother Maria, to my grandfather Jose, and to my mentor Dr. D.S. Brar, who all inspired me to pursue my dreams.

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CHAPTER I

INTRODUCTION

All living organisms are exposed to adverse environmental conditions during their life cycle. Because of this, survival is determined by the ability of the organism to adapt to such challenges. Animals are able to escape harsh environmental conditions mainly through their locomotive ability that allows them to move to a more suitable habitat. In contrast, plants are immobile and are unable to escape hostile environmental conditions. Therefore, in order to survive, plants should possess the ability to accommodate the harsh conditions that prevail within the environment. Plants having this ability have evolved novel mechanisms that provide protection against environmental extremes. Common environmental factors associated with plant stress include extremes in temperature, soil moisture and pH, and the presence of pathogenic organisms. Adaptive mechanisms are largely determined by the genetics, biochemistry and physiology of the organism and have significant impact on ecological distribution.

Among the environmental factors that challenge plants, the abiotic stress component, particularly drought and low

temperatures have more profound negative impact on global agricultural productivity (Weiser, 1970; Guy, 1990; Boyer, 1982). Accordingly, effort has been devoted to increase our understanding of the mechanisms by which plants respond and adapt to those conditions. The ultimate goal of these efforts is not only to enhance basic knowledge but also to develop innovative approaches to improve stress tolerance capabilities of economically important plant species.

The seasonal temperatures in temperate countries exhibit very pronounced fluctuation from ambient to subfreezing levels (Sakai and Larcher, 1987). Thus, freezing temperature is an important abiotic stress factor that affects plants in those regions. Freezing in plant tissues triggers various biochemical and physiological events that may result in cell injury. Injury is caused either by direct physical and mechanical effects of the growing intercellular ice to the cells or by freeze-induced cell dehydration. Some plant species obtain constitutive ability to protect themselves against these damaging changes by supercooling. This process maintains membrane lipids in the normal liquid crystalline phase at temperatures slightly below freezing point (Levitt, 1980). On the other hand, more recent evidence indicates that freezing tolerance in the majority of plant species is not

a constitutive process (reviewed by Thomashow, 1998 and Palva, 1994). Instead, freezing tolerance is an inducible process activated by exposure of plants to low but nonfreezing temperatures prior to the occurrence of freezing, a process known as cold acclimation (CA), or hardening.

Functionally, CA is a period of overall metabolic adjustment that prepares the plant for an impending freezing stress. It is accompanied by numerous biochemical and physiological changes that include increase in soluble protein and sugar contents, appearance of new isozymes, increase in the level of proline and organic acids and alteration in lipid composition (Steponkus and Lynch, 1989; Sakai and Larcher, 1987). These changes collectively function in mechanisms that allow the plants to adapt to abnormal conditions during CA and prevent the injuries caused by the freezing process to plant cells and tissues. As a whole, all of these processes are thought to be controlled by changes in gene expression during CA (Cativelli and Bartells, 1992; Weiser et al., 1970). Therefore, the ability of plants to cold acclimate is an important trait that must be considered in dealing with the problem of freezing damage to plants.

BERMUDAGRASS FREEZING TOLERANCE

The genus *Cynodon* consists of warm-season, perennial grass species which are widely used for turf, not only in tropical regions, but also in temperate countries including the United States (Taliaferro, 1995). Of particular interests are the cultivars belonging to *Cynodon dactylon* (2n=4x=36), *Cynodon transvaalensis* (2n=2x=18) and their triploid hybrids (2n=3x=27), also commonly known as bermudagrasses. The use of these species particularly as turf provides a number of benefits to human daily life. Turfgrasses are primarily used as grass cover established on a particular site to prevent soil erosion and maintain visibility. They not only add to the aesthetic beauty of the human environment, but they also contribute to the reduction of human health hazards by reducing dust, air pollution, glare, noise and surface temperatures. They also contribute to environmental preservation by preventing massive soil erosion, flooding and, by providing alternative environments for beneficial wildlife species. Turfgrasses possess features important to their use in human recreational facilities which include golf courses, athletic and other sport fields that requires resilient playing surfaces (Duble, 1996; Beard and Green, 1993). Because of these practical benefits, management of

turfgrasses has become an important concern in modern agriculture. One of the most persistent problems associated with the use of bermudagrass is its susceptibility to freezing stress during winter. Severe winter temperatures often produce very serious damage to freeze-susceptible bermudagrass cultivars on golf courses, house and public park lawns. Management of turf damage often involves considerable amount of human effort and financial resources. For example, Martin (1990), estimated the replacement value for bermudagrass turf in Oklahoma at around $1.3 billion in 1987. This figure was calculated from the product of turf establishment costs per acre and the total number of acres maintained for bermudagrass in that year.

Because of frequent freeze injury to bermudagrass, particularly in colder regions, it has been a major goal of bermudagrass improvement programs to combine superior turf quality with increased capabilities to withstand freezing conditions. Over the last several years, these efforts have resulted in modest but significant accomplishments. Traditional breeding for freezing tolerance in bermudagrass and plant species in general is slowed by the complex nature of this trait. The ability of plants to cold acclimate involves multiple genes, each of which

contributes either directly or indirectly to mechanisms of freezing tolerance (Thomashow, 1990). Combining classical plant breeding and biotechnological approaches has a great potential to speed the development of plant cultivars with enhanced freeze tolerance. However, the strategy assumes a basic understanding of the genetic basis of freeze tolerance and the underlying biochemical events that result in protection against freezing injury. Recombinant DNA technology offers possible ways to address this goal. A proven approach to elucidate a complex genetic mechanism is to isolate the genes and investigate their physiological function(s).

Anderson et al. (1993, 1988), demonstrated that freezing tolerance in 'Midiron' and 'Tifgreen' turf bermudagrass cultivars is enhanced following 28 days of CA at 8°C/2°C temperature cycles. Results from these studies indicated that electrolyte leakage was significantly reduced by CA and resulted in high levels of regrowth in cold acclimated plants after freezing. By determining the lethal temperature for 50% of the plants (LT_{50}), it was clearly indicated that CA increased the cold hardiness of 'Midiron' and 'Tifgreen' to as much as LT_{50}=-10°C and LT_{50}=-8°C, respectively. A prevailing hypothesis suggests that such increases in cold hardiness are due to changes in gene

expression. Hence, the identification of genes induced by CA will be a first step towards enhanced understanding of the molecular mechanisms involved in bermudagrass freezing tolerance.

MOLECULAR BASIS OF COLD ACCLIMATION AND FREEZING TOLERANCE IN HIGHER PLANTS

Freezing Stress

Ice formation in plant tissues can occur when the atmospheric and soil temperatures drop below the freezing point of water (0°C). However, the aqueous solutions within the plant tissues are capable of supercooling (Ashworth, 1990). Because of this, the temperature at which crystallization initiates is largely determined by both homologous and heterologous ice nucleating agents which are necessary to orient the water molecules into the crystalline structure of ice (Burke and Lindow, 1990; Ashworth, 1990). At relatively fast cooling rates, ice formation occurs within the intracellular compartments causing deleterious effects to the cell. However, cooling of the environment normally occurs at slow rates, and because of this ice formation is initiated external to the cell, primarily in the dilute apoplastic solution (Levitt,

1980). Hence, freezing injury is predominantly caused by ice crystallization in the intercellular spaces. Freeze-induced injuries to the cells are attributed to two major events. The first event involves direct physical and mechanical effects of the growing intercellular ice crystals on adjacent cells. During freezing, the growing crystals form adhesions between cell walls and membranes, causing mechanical rupture of structures that attach the cell wall and plasma membrane (Levitt, 1980). The second event involves prolonged exposure to 'solution effects' caused by the concentration of intercellular solutions (Steponkus, 1984). Intercellular ice formation is accompanied by decreased water potential outside of the cell, creating a chemical potential gradient. To balance this gradient, the unfrozen intracellular water moves out to the intercellular matrix, resulting in severe cell dehydration effect (Steponkus and Webb, 1992). This event may trigger several processes leading to physiological injuries. These include: 1) volume and area contraction, 2) concentration of both intracellular and intercellular solutes, 3) possible pH changes caused by different solubilities of buffering compounds, 4) eutectic crystallization and, 5) removal of water of hydration from macromolecules including membrane lipids and cellular

proteins (Steponkus, 1984). Thus, it appears that freezing tolerance is achieved through physiological adjustments related to these disturbances. Functionally, this is achieved by reducing the rate of intercellular ice formation. Other freeze tolerance mechanisms operate by repairing the physiological injuries mentioned. These mechanisms must be activated during the process of cold acclimation in order to achieve high levels of cold hardiness.

Metabolic Adjustments During Cold Acclimation

Heat stress in plants is normally accompanied by overall repression of protein synthesis. In contrast, CA leads to the induction of protein synthesis, causing a net overall increase in total soluble protein contents of the cell (Vierling, 1991; Sakai and Larcher, 1987). The changes in protein content are necessary both for optimal growth during CA periods and for the mechanisms involved with freezing tolerance. Moreover, the development of maximum freezing tolerance is determined by the ability of the plant to adjust its metabolism during the relatively abnormal growing conditions during the CA process. Protein synthesis is one of those adjustments. The results of many studies are in full agreement with this. It has been

demonstrated that the ability of wheat to cold acclimate is heavily impaired if protein synthesis is inhibited by cycloheximide (Trunova, 1982).

Different proteins or enzymes have different levels of sensitivity to low temperatures. Because of this, most enzymes occur in multiple forms or isozymes. The increase in overall protein content during CA can therefore be attributed to two events. CA in a number of plant species has been shown to cause increased synthesis of cold labile enzymes, or enzymes that are less stable or less active during periods of low temperature (Hughes and Dunn, 1996). A number of housekeeping enzymes like those involved in the respiratory pathway are cold labile. Increased synthesis of those enzymes contribute to overall metabolic adjustments and permit adaptation to the CA conditions (Guy, 1990). Furthermore, similar forms of adjustments also take place by increased synthesis of new isozymes that are more stable and have higher activities even at low temperature conditions. An example of this is the enzyme RUBISCO, a major component of the photosynthetic machinery. It was found that specific RUBISCO isozymes that are induced during CA have higher activity than those that are synthesized at normal conditions (Huner and MacDowall, 1979).

Other proteins that are synthesized in larger amounts during CA may be directly involved with freezing tolerance mechanisms. Examples of these proteins are those that exhibit cryoprotective and antifreeze or thermal hysteresis activities (Sieg et al., 1996; Worall et al., 1998). This aspect of freezing tolerance will be discussed in greater detail in the succeeding sections.

Cold Acclimation and Mechanisms of Freezing Tolerance

Antifreeze and Cryoprotective Proteins

The biochemical and physiological changes that occur during CA enable or result from metabolic adjustments. More importantly, some of these events are directly involved with mechanisms of tolerance to freezing stress. Different plant species have evolved diverse strategies that enable them to survive freezing conditions. Some plant species synthesize antifreeze proteins (AFP) which protect plant tissues against intercellular ice formation (Worall et al., 1998; Antikainen and Griffith, 1997; Griffith et al., 1997; Urrutia et al., 1992). During CA, AFPs are actively synthesized and secreted to the intercellular spaces in plant tissues. Unlike the AFPs of fishes and insects, plant AFPs have relatively limited thermal hysteresis activity. Instead, their major roles are to slow down the rate of ice

crystal formation and inhibit ice recrystallization, thereby preventing the potentially damaging effects of ice crystal formation (Worall et al., 1998; Urrutia et al. 1992; Davies and Hew, 1990). Biochemical studies on plant AFPs suggest that the basic structure is similar to animal AFPs. AFPs are structurally defined by unique repeating units of hydrophilic amino acid residues. The hydrophilic moieties are oriented towards the external surface allowing them to form hydrogen bonds with the prism phase of seeded ice nuclei. This event results in growth of ice crystals only in the basal plane. The continued binding of these proteins to the prism phase results in bipyramidal ice crystals, hence slower crystallization (Worall et al., 1998; Davies and Hew, 1990).

In a study conducted in overwintering monocot species including winter and spring rye, winter barley, spring oats and, winter and spring wheat, Griffith et al. (1997), demonstrated that AFPs indeed caused the formation of bipyramidal ice crystal structures. These crystal structures indicate interaction between the prism phase of ice nuclei and the AFP through the formation of hydrogen bonds with the hydrophilic surface of the protein. Some of these AFPs have already been identified as pathogenesis-related (PR) proteins such as chitinase, β-1,3-glucanase and

thaumatin. The potential roles of these AFPs in bermudagrass freezing tolerance mechanisms are discussed further in the results of this investigation (Chapters II and III).

Proteins with cryoprotective ability have also been isolated in several plant species. Sieg et al. (1996), purified a single cryoprotective protein from cold acclimated cabbage (*Brassica oleracea*), referred to as cryprotectin. This protein was found to be effective in protecting thylakoid membranes against freeze-induced dehydration damage. Interestingly, cryprotectin appears to be encoded by a COR gene since its temporal regulatory pattern is similar to the expression patterns of known COR genes. More recently, a class I β-1,3-glucanase from tobacco was shown to exhibit cyroprotective activity. Like the cabbage cryoprotectin, the tobacco β-1,3-glucanase exhibits cryoprotective properties and functions by protecting spinach thylakoid membranes against freeze-thaw injury in *in vitro* assay (Hincha et al., 1997).

Stabilization of Membranes and Macromolecules

The cell membranes and proteins are the major sites of freeze-induced injury to plant cells (Steponkus, 1984; Levitt, 1980). The plasma membrane is the principal barrier

between the cell cytoplasm and the intercellular environment, and hence plays a significant role in overall metabolic activities of the cell. Despite this, the cell membrane is highly vulnerable to dehydration-induced destabilization (Crowe et al., 1992). This destabilization results from changes in the configuration of lipid components that ultimately result in alteration of the semi-permeable properties or lysis of the plasma membrane.

Lysis of the plasma membrane is deleterious. This event is caused by osmotic excursions associated with the movement of bulk water from the cell cytoplasm during succeeding freeze-thaw cycles. Dowgert and Steponkus (1984), first demonstrated the mechanism of expansion-induced cell lysis by investigating the behavior of isolated rye protoplasts during freeze-induced dehydration cycles. At freezing temperatures, the intercellular water freezes first. This event results in lowering of water activity and increase in solute concentration in the unfrozen portion of the intercellular fluid. Consequently, the chemical and osmotic potentials of cells are changed, leading to rapid movement of intracellular fluid towards the intercellular compartments. This dehydration effect causes membrane shrinkage and cell contraction leading to irreversible reduction in cell volume. Thawing of the

intercellular ice causes the movement of water to be reversed towards the cell cytoplasm, hence effecting cell rehydration. Since the original cell volume had been reduced during the process of dehydration-induced membrane contraction, continuous influx of water towards the cytoplasmic side eventually leads to intolerable osmotic pressure and causes the cell to burst and die. However, it was further demonstrated that the process of dehydration-induced cell volume reduction could be reversed by cold acclimation. Protoplasts from acclimated plants exhibited greater potential to reexpand than the protoplasts from non-acclimated plants. During freezing, both the non-acclimated and acclimated protoplasts dehydrated. However, acclimated protoplasts formed exocytotic extrusions while non-acclimated protoplasts formed endocytic vesicles. After thawing, the exocytotic extrusions formed in the acclimated protoplasts are reincorporated into the plasma membrane allowing the cells to swell to their original size. In contrast, the vesicular materials formed by the non-acclimated protoplasts are not reincorporated, causing a reduction in cell volume. Apparently, expansion-induced cell lysis appears to be the predominant form of injury to non-acclimated plants, particularly at temperatures between -6°C to -10°C.

The CA-induced transformation of the plasma membrane during osmotic contraction is due to alterations in lipid composition. Specifically, the differential membrane cryobehavior is due to altered lipid-lipid interactions that result from increased proportions of unsaturated fatty acid species in the plasma membrane. These fatty acids include the diunsaturated species of phosphatidylcholine and phosphatidylethanolamine (Uemura and Steponkus, 1994; Palta et al., 1993; Miquel et al., 1993; Steponkus et al. 1990; Cheesebrough, 1989; Steponkus et al., 1988; Arvinte and Steponkus, 1988; Lynch and Steponkus, 1987). Furthermore, these changes are attributable to the increased level of the enzymes involved with fatty acid desaturation. Consequently, these events have been implicated as the major causes of increased levels of tolerance to freezing after cold acclimation in a number of plant species including *Arabidopsis thaliana* (Uemura et al., 1995; Kodama et al., 1995).

Freeze-induced cell injury can also be inflicted by the removal of water closely associated with the cell membrane. The removal of membrane-bound water molecules generally occurs under conditions that cause more severe dehydration than those conditions that caused expansion-induced lysis (-10°C and lower). In the absence of CA, more

severe dehydration can result in transition of membrane lipids from lamellar to hexagonal II (HII) phase. This damage occurs in regions where the plasma membrane is brought into close apposition with subtending endomembranes. In *Arabidopsis thaliana*, the decreased propensity for the occurrence of the HII phase during CA was also attributed to the alterations in membrane lipid compositions, particularly the increased proportions of highly hydrated lipid species such as phosphatidylcholine and decreased proportions of poorly hydrated lipid species such as cerebroside and phosphatidylserine. As dehydration becomes even more severe, the removal of water from the lipid headgroups results in an increased lateral pressure in the acyl domain relative to that in the headgroup region. This creates a bending energy that results in injury even in cold acclimated plants. Since the HII phase is precluded by CA, membranes can be damaged even during CA. Injury resulting from this condition is referred to as fructure jump lesion. This type of lesion is characterized by deviations of membrane fructure plane to subtending lamellae (Uemura et al., 1995; Uemura and Steponkus, 1994; Webb and Steponkus, 1993; Webb et al., 1994).

Enzymes may also be directly altered when freezing occurs in plant tissues. Low temperatures can cause

reversible protein denaturation, leading to unmasking of reactive SH-groups. Cell contraction due to freeze-induced dehydration decreases the distance between the denatured proteins within the contracted cytoplasm. Consequently, such an event can cause irreversible protein aggregation through the formation of intermolecular SS-interactions between the denatured protein molecules (Graham and Patterson, 1982). The exact mechanism by which this form of damage is repaired or prevented is not yet very clear. However, it is believed that some of the hydrophilic proteins synthesized both during drought and low temperature stress conditions may be involved not only in protecting the membranes but also the proteins during dehydration (Palva, 1994; Cativelli and Bartels, 1992; Lin et al., 1990). This hypothesis was supported by the finding that spinach seedlings synthesize 70kDa proteins during CA. Analysis of the sequence and immunological cross-reactivity of these proteins indicated that they belong to the family of 70kDa heat shock proteins. Interestingly, many of the members of this family of heat shock proteins have been classified as molecular chaperones. Therefore, it was inferred that these proteins may be involved with a mechanism that avoids aggregation of proteins and favors renaturation and correct re-folding of polypeptides to

restore their proper conformation and function after freezing (Neven et al., 1992).

Solute Accumulation

Another interesting physiological parameter observed during CA is the accumulation of substances such as sugar and carbohydrates (sucrose, fructosan), sugar alcohols, amino acids (proline, glycine betaine) and polyamines (Bartels and Nelson, 1994; Guy, 1990; Levitt, 1980). CA in spinach was demonstrated to result in increased activity of sucrose phosphate synthase. Moreover, the increase in enzyme activity led to increased concentration of sucrose and other sugars in cold acclimated plants (Guy et al., 1992). Similarly, Crespi et al. (1991), observed an induction of sucrose synthase gene expression during CA of wheat. Although the exact role of increased sugar concentration in CA has not been fully elucidated, it was proposed that such increases may be involved in osmoregulation and reduction of water potential inside the cell cytoplasm. This event is necessary to prevent freeze-induced dehydration of cellular components (Lang et al., 1994; Bartels and Nelson, 1994; Sakai and Larcher, 1987). Guy et al. (1992), also proposed that the increased sucrose concentration in spinach following CA reflects the possible

function of sucrose as cryoprotectant, perhaps through its ability to prevent membrane damage during freeze-induced cell dehydration.

The accumulation of osmolytes such as sugar alcohols, proline and glycine betaine has also been observed during CA in a number of plant species. Because these substances were originally implicated with increased drought and salinity tolerance, it was proposed that they possibly function in the same manner during freezing stress. The mechanism may involve osmoregulation and prevention of cell dehydration (Bartels and Nelson, 1994; Delauney and Verma, 1993; Anchordoguy et al., 1987).

COR Gene Expression

Numerous studies have demonstrated that the overall protein changes during CA can be accounted for by changes in translatable mRNA populations. Consequently, the development of freezing tolerance is associated with the expression of novel genes known as cold regulated (COR) genes (Hughes and Dunn, 1996, Palva, 1994; Thomashow, 1994; Thomashow, 1990). Several COR genes have been isolated and characterized in plants, majority of them from the model species *Arabidopsis thaliana* (Thomashow, 1998). An important common feature of these COR genes is that they

all encode hydrophilic and boiling stable proteins. The expression of these genes is highly induced by CA and a high level of expression is maintained over the duration of CA conditions. Interestingly, the expression of most, if not all of the COR genes is also induced by drought and abscisic acid (ABA)(Hughes and Dunn, 1996; Palva, 1994; Thomashow, 1994).

The COR genes of *Arabidopsis thaliana* comprise four distinct families, each one consisting of two members. These genes are physically linked in tandem order along the genome (Thomashow, 1998; Gilmour et al., 1992; Kurkela et al., 1988). Some COR genes encode proteins with known biochemical activities, and thus their physiological function in relation to CA and freezing tolerance can be deduced. Additionally, some of the COR genes encode unknown proteins. The functional roles of these COR genes in freezing tolerance can therefore be inferred only based on the structural features of the proteins they encode.

The *A. thaliana COR47* gene codes for a protein whose function has been determined (Gilmour et al., 1992). Based on analysis of sequence homology, this gene was designated to be a homologue of group II LEA protein which are also known as dehydrin D11 genes (Thomashow, 1998; Close, 1996; Dure et al., 1989). Furthermore, homologues of this gene

have also been identified in a number of species including barley (*Hordeum vulgare*), wheat (*Triticum aestivum*) and potato (*Solanum tuberosum*) (Thomashow, 1998). Because of their homology with group II LEA proteins, the products of this group of COR genes have been implicated in mechanisms that protect cells against freeze-induced dehydration.

Gilmour et al. (1992), identified an *A. thaliana* gene, *COR6.6*, to be responsible for the synthesis of a 6.6kDa basic protein that accumulates in response to low temperatures, ABA and water stress. Nucleic acid sequence analysis confirmed this gene to be highly homologous to the *kin1* gene which was earlier identified also in *Arabidopsis* by Kurkela and Franck (1990), and to *BN28* which was identified in oil seed rape (*Brassica napus*) by Orr et al. (1992). These genes are compositionally similar to genes that encode the fish alanine-rich antifreeze proteins. Hence, Hughes and Dunne (1996) proposed that both the *Arabidopsis* and *Brassica* genes may be involved either in cryoprotective or antifreeze mechanisms.

The *A. thaliana COR15* gene family comprised of *COR15a* and *COR15b* homologues, has been well characterized. Like the other COR genes, *COR15a* is also induced by drought and ABA (Thomashow, 1994; Wilhelm and Thomashow, 1993). Several homologues of these genes in other plant species have been

identified and include *BN115*, *BN26* and *BN19* from *Brassica napus* (Thomashow, 1998; Weretilnyk, 1993). The *COR15a* gene encodes highly hydrophilic and boiling stable 15kDa polypeptide. The mature polypeptide is rich in alanine, lysine, glutamic acid and asparagine, and is targeted to the stromal compartment of the chloroplast (Thomashow, 1998, Thomashow, 1994). Because of the lack of sequence homology with other genes of known biochemical activity, the physiological function of the protein encoded by this gene was unknown. It was later discovered that chloroplasts from transgenic plants overexpressing this gene exhibited 1°C to 2°C increases in freezing tolerance in *in vitro* condition compared to the chloroplasts isolated from non-transgenic plants. However, overexpression of this gene alone failed to result in maximum cold hardiness level that can be achieved with CA. This result indicated that *COR15a* expression could be involved in the prevention of some, but not all, membrane lesions that occur during freeze-induced dehydration. Hence, it was apparent that *COR15a* is involved in a mechanism that prevents damage to the chloroplast membranes (Artus et al., 1996).

The *A. thaliana COR78* gene is another example of a COR gene with no homology to other genes of known biochemical activity (Thomashow, 1998). This gene which was also

referred to as *lti78* has another homologue, the *lti65* (Nordin et al., 1993). Based on nucleotide sequence analysis, the genes were predicted to encode extremely hydrophilic proteins. Although both genes were responsive to low temperatures, the expression of *lti65* appeared to be more specific to drought and ABA. Accordingly, it was proposed that these two genes follow separate signal transduction pathways. It was also proposed that due to the high hydrophilicity of the proteins encoded by these genes, they may have significant roles in the prevention of freeze-induced dehydration damage to membranes and macromolecules during freezing stress.

Transcriptional Regulation of COR Gene Expression

It was recently discovered that the *COR15a* gene contains a cis-acting element involved with cold, drought and ABA regulated expression. This element is defined by the sequence TGG**CCGAC**, which was designated as 'C-repeat'. Interestingly, the core motif of this element (CCGAC) was also found in repeated copies in a number of COR genes including *COR15b*, *COR78*, *rab18*, *kin1* and *kin2* (Nordin et al., 1993; Lang and Palva, 1992; Kurkela and Franck, 1990). Even more interesting was the discovery that the core motif of this element existed in the promoter of almost all known

drought responsive genes. This core motif (CCGAC) was designated as a **D**rought **R**esponsive **E**lement (DRE). The motif was also confirmed as a cis-acting element responsible not only for water stress-inducible expression, but also for low temperature and salt stress-regulated expression of drought regulated genes (Yamaguchi-Shinozaki and Shinozaki, 1994). These findings led to a hypothesis that perhaps COR genes are coordinately regulated during CA and may be acting in concert in increasing freezing tolerance (Thomashow, 1998). This hypothesis is consistent with the polygenic and cumulative nature of the genes that control freezing tolerance (Thomashow, 1990). Another significant discovery was that of a cDNA that encodes a C-Repeat/DRE binding factor, designated as CBF1 (Stockinger et al., 1997). Analysis of the deduced amino acid sequence of the protein encoded by the CBF1 cDNA revealed the presence of both nuclear localization and acidic activation domains. The protein also contained a motif very similar to the AP2 domain responsible for the DNA binding activity of a number of transcription factors in *A. thaliana*. Based on structural features and results from expression studies, it was concluded that the CBF1 protein functioned as a transcriptional activator that recognizes and binds the DRE and regulates COR gene expression during both low

temperature and water stress conditions. It was later hypothesized that the CBF1 may be responsible for expressing the whole battery of COR genes that collectively provide different mechanisms involved in preventing freeze-induced damage to cells.

To test this hypothesis, Jaglo-Ottosen et al. (1998), attempted to express the entire battery of *Arabidopsis* COR genes by overexpressing CBF1 through the constitutive CamV35S promoter. They found that overexpression of the CBF1 induced the expression of COR genes and increased the freezing tolerance of transgenic *A. thaliana* plants without CA. Moreover, the constitutive expression of CBF1 abolished the requirement for CA for the expression of the COR genes. Thus, it was concluded that CBF1 regulates the response of plants to CA, which in turn mediates tolerance to freezing stress.

Signal Transduction During Cold Acclimation

The ability of plants to adapt to environmental stresses depends on intricate mechanisms that coordinate cellular events leading to exquisite sensitivity to physical and chemical changes in the environment. Recent investigations have focused on the mechanisms of detecting extracellular signals and how these signals are transduced

into appropriate intracellular responses (Chang and Stewart, 1998). Specifically, stress tolerant plants must have mechanisms that allow them to perceive and transduce low temperature signals during CA. These signals activate gene expression required for freezing tolerance mechanisms.

Different stimuli can trigger CA responses in plants. These include not only low temperatures, but also water stress and increased levels of ABA (Merlot and Giraudat, 1997; Shinozaki and Yamaguchi-Shinozaki, 1997; Giraudat, 1995; Palva, 1994). Like in the drought response, ABA was originally proposed to be the central mediator of low temperature response during CA, and that expression of low temperature- and drought-induced genes can be switched on by transient increases in cytoplasmic ABA concentration (Chen et al., 1983). Contrary to this, more recent experiments involving the use of ABA-defective and ABA-insensitive mutants of *A. thaliana* indicated that not all COR genes require increased ABA level for expression. Hence, it was proposed that there are ABA-dependent and independent signaling pathways involved in low temperature responses. Furthermore, Nordin et al. (1991), proposed three independent and parallel pathways involved in sensing the low temperature, drought and ABA stimuli. Each of these pathways leads to the expression of a specific set of COR

genes that results either in low temperature tolerance, drought tolerance or both. Neither simultaneous nor sequential applications of stimuli have a cumulative effect on stress tolerance. Hence, it was proposed that the parallel signaling pathways converge to produce a final response (Nordin et al., 1991; Gilmour and Thomashow, 1991).

Calcium has been recognized as a second messenger involved in a number of signaling mechanisms in higher plants (Bush, 1995; Shocklock et al., 1992; Gilroy et al., 1990). Transient increase in the level of calcium in the cell cytoplasm is one of the important physiological changes observed during CA (Minorsky, 1989). Definitive evidence for the role of calcium in low temperature signaling mechanisms has been obtained by Monroy and Dhindsa (1995), from physiological experiments on alfalfa (*Medicago sativa*). They observed that CA at 4°C increased the influx of extracellular $^{45}Ca^{2+}$ to as much as 15 times the basal level. Furthermore, the addition of calcium chelator (bis(O-aminophenoxy)ethane N,N,N',N'-tetraacetic acid or BAPTA), and calcium channel blockers (Lanthanum, Nitrendipine and Verapamil) inhibited the influx of extracellular $^{45}Ca^{2+}$. Consequently, this event also prevented CA. On the other hand, the addition of calcium ionophore or

calcium channel agonist to non-acclimated cells caused increased influx of extracellular $^{45}Ca^{2+}$ and caused transient induction of expression of the COR genes *cas15* and *cas18*.

Similarly, Tahtiharju et al. (1997) and Knight et al. (1996), demonstrated that CA in *A. thaliana* is accompanied by transient increases in cytoplasmic calcium levels. They also showed that induction of expression of *kin1* and *kin2* depends not only on the influx of extracellular Ca^{2+} but also on the release of Ca^{2+} from intracellular stores such as the vacuole.

Low temperature signal transduction is also associated with changes in protein phosphorylation (Monroy et al., 1993). Studies have shown that phosphorylation of preexisting proteins in alfalfa (*Medicago sativa*) can be inhibited by calcium channel blockers and antagonists of calmodulin and Ca^{2+}-dependent protein kinases. Similarly, Tahtiharju et al. (1997), showed that inhibitors of both calmodulin and calmodulin-dependent protein kinases prevented both CA and expression of *kin1* and *kin2* genes in *A. thaliana*.

A number of genes encoding calcium-dependent protein kinases have been isolated from several plant species. Several studies showed that mutation of these genes impair the plant's ability to respond to environmental stress

signals including low temperature, drought and salinity (Sheen, 1996; Jonak et al., 1996). These findings clearly indicate that protein phosphorylation is a key component of low temperature signaling mechanisms in plants. The elucidation of the exact cascade of events leading to the final response would enhance understanding of how tolerance mechanisms are activated.

OBJECTIVES OF THE STUDY

Previous studies indicated that CA in freezing tolerant bermudagrass cultivars is accompanied by increased synthesis of hydrophilic proteins. Analysis of the peptide sequences isolated from CA crown tissues indicated that these proteins are homologous to tobacco chitinases (Gatschet et al., 1996; Gatschet et al., 1994).

A current hypothesis is that PR-proteins, including chitinases, may have significant roles in freezing tolerance due to possible antifreeze activity of particular isozymes. This study was initiated to provide additional evidence regarding the possible roles of the PR-proteins in bermudagrass freeze tolerance. The major objectives of this study were to: 1) isolate and sequence some members of the bermudagrass chitinase gene family and, 2) characterize the temporal and spatial expression patterns of chitinase genes

in freezing tolerant bermudagrass cultivars. The expression of each gene in response to cold acclimation, drought stress and exogenous application of ABA were investigated in order to determine whether the genes are involved in freezing tolerance mechanisms.

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CHAPTER II

Molecular Cloning and Analysis of Sequence Variation Among Members of Bermudagrass Class II Chitinase Gene Family

ABSTRACT

A bermudagrass (*Cynodon* sp.) Lambda Fix II genomic library constructed from high molecular weight Midiron genomic DNA was screened for chitinase genes using a 360bp partial cDNA as probe. Three of the nine positive plaques (GCyn113, GCyn319 and GCyn456) were subcloned and sequenced. Sequence analysis revealed that the genes in GCyn113 and GCyn456 were identical and thus were designated as *CynCht1* (AF105425). The gene in GCyn319 was designated as *CynCht2* (AF105426). All three clones contained complete open reading frames. *CynCht1* and *CynCht2* exhibit high levels of sequence similarities at both the coding and flanking regions. Sequence alignment revealed that the two genes were homologous with the catalytic regions of known class I and class II chitinases of higher plants. However, all genes were classified as class II chitinases because of the lack of the structural domains (N-terminal cysteine-

rich domain and hydrophobic C-terminal extension) that distinguish class I from class II chitinase. Analysis of the deduced protein products indicated that both genes encode low molecular weight hydrophilic proteins (CHT1 and CHT2), which are targeted to the intercellular compartments. Southern blot analysis revealed that *CynCht1* and *CynCht2* are members of class II family composed of three genes. In addition to the TATA-signal, other interesting motifs were found at the promoter regions of the genes. These sequences include two GC-rich motifs located upstream to the TATA-box. It was hypothesized that these elements may be important for the induced expression of these chitinase genes.

Key Words: genomic library, *Cynodon*, clone, chitinase, gene family, Southern blot, promoter

INTRODUCTION

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. Accordingly, organisms that contain chitin also synthesize chitinase (E.C.3.2.1.14), the enzyme that hydrolyzes the polymer. Interestingly, most higher plants synthesize chitinases although they do not contain chitin. Potential endogenous substrates for plant chitinases were unknown until the discovery that GlcNAc is also present in secondary cell walls of higher plants in the form of glycolipids and not chitin (Benhamou and Asselin, 1989). Although the precise function of chitinases in plant metabolism is not quite fully understood, evidence suggests important roles of these enzymes in plant development and morphogenesis. These roles include flower formation and leaf abscission (Harikrishna et al., 1996; Neale et al., 1990; Gomez-Lim et al., 1987).

The fungal cell wall-degrading activity of plant chitinases is one of their important functions. Because of this, much research has been devoted on these proteins to explain their roles in mechanisms of defense against pathogen infection. Consequently, numerous plant chitinases

have been identified as pathogenesis-related (PR proteins) (Graham and Sticklen, 1994). In relation to their function as defense proteins, most chitinases are more actively synthesized in response to wounding of plant tissues, and following fungal and viral infections (Chang et al., 1995; Hedrick et al., 1988; Legrand et al., 1987). Furthermore, induction of chitinase gene expression following bacterial infection has been observed in a number of plant species and has been demonstrated to result in systemic resistance. This mechanism is associated with findings that some chitinase isozymes exhibit lysozymic activity, hence could be responsible for the observed resistance to bacterial infection (Majeau et al., 1990). Additionally, chitinase gene expression can also be induced by abiotic elicitors which include the hormone ethylene (Shinshi et al., 1995; Flach et al., 1992; Roby et al., 1986; Boller et al., 1983).

Plant chitinases are encoded by redundant nuclear genes. These gene copies occur in distinct classes, or families. The products of these multiple genes constitute different chitinase isozymes with distinct spatial and temporal regulatory patterns (Collinge et al., 1994; Meins et al., 1992). These isozymes display distinct structural features believed to be important to their functional

specificity. Because of this, plant chitinases have been categorized into four broad classes (Shinshi et al., 1990). Class I includes the basic proteins that contain an N-terminal domain important for chitin binding but not for catalytic and antifungal activities (Iseli et al., 1993; Shinshi et al., 1988). They also contain a characteristic peptide motif at the C-terminus that is important for targeting the protein to the vacuole (Neuhaus et al., 1991; Shinshi et al., 1988). Class II chitinases are acidic proteins and lack these two structural features, but exhibit high sequence similarity with class I proteins. Chitinases with no structural and sequence similarities with both class I and class II proteins are grouped under class III. Recently, an additional category, class IV has been proposed to include proteins with structural similarities and sequence differences with class I proteins (Collinge et al., 1994).

The occurrence of chitinases as multigene families has important biological implications. The occurrence of redundant gene copies seems to be a wasteful situation for any organism because of functional duplications that may not always be necessary for survival and adaptation. However, a proposed theory is that redundant genes, including members of multigene families, are favored during

evolution. It is reasoned that natural selection ensures that duplicated genes with mutually exclusive functions are selectively favored while the functions shared by the redundant copies are preserved (Pickett and Meeks-Wagner, 1995). This is thought to confer evolutionary advantage to the organism. Evidence supporting this theory includes observations that different isoforms of the protein exhibit distinct spatial and temporal regulatory patterns. The complexity of the regulation involved with multiple gene copies clearly suggests that the functions of these genes extend beyond being pathogenesis-related proteins. For example, numerous studies have shown the involvement of these genes in responses to abiotic stresses, such as mediating ozone (Ernst et al., 1992) and osmotic stress (Chen et al., 1994). Particularly interesting is the hypothesis that chitinases may have roles in freezing tolerance by virtue of their ability to retard the growth of intercellular ice. This suggests that some chitinases may have potential use in engineering plants for freezing tolerance (Thomashow et al., 1998; Antikainen et al., 1996; Hon et al., 1995).

Bermudagrass (*Cynodon* sp.) is an economically important warm-season grass species. It is most commonly used for turf in both tropical and subtropical regions of

the world. It is also commonly utilized as turf in colder regions including the southern part of the United States. Because of its presumed tropical origin, a major problem associated with the use of bermudagrass is its susceptibility to freezing temperatures. Accordingly, a major goal of many bermudagrass improvement programs is the development of cultivars that combine enhanced turf quality and tolerance to freezing temperatures. This goal can be best accomplished by combining classical plant breeding and biotechnological methods. The success of these approaches relies on the basic understanding of the genetics of freezing tolerance and the underlying molecular mechanisms that result in protection against freezing injury. Therefore, this research was undertaken to enhance understanding of the molecular basis of freezing tolerance in *Cynodon* through the discovery of genes involved with tolerance mechanisms. Specifically, the objective of this experiment was to isolate some members of bermudagrass chitinase gene families.

Gatschet et al. (1996), demonstrated an increased synthesis of some acidic chitinases in the crowns of freezing tolerant bermudagrass cultivars following acclimation at low, but non-freezing temperatures. This indicated possible specialized function of this group of

chitinase genes. These proteins may have the structural ability to bind extracellular ice thus, preventing crystallization (Hon et al., 1995). Because chitinase genes occur in multiple copies, it is necessary to identify the specific family or class of genes that are directly involved in this specialized function. The analysis of the regulation of expression of a particular chitinase gene, or gene family, is usually complicated by the occurrence of multiple gene copies producing very similar mRNAs. Therefore, isolation of individual members of the multigene families should facilitate detailed investigation and identification of specific genes of interest. This chapter describes the molecular cloning of two members of the class II chitinase gene family from the freezing tolerant cultivar 'Midiron'. The structural and sequence variations between the two genes are also discussed.

MATERIALS AND METHODS

Construction of genomic library

The genomic library was constructed from the cultivar 'Midiron' (*C. dactylon* X *C. transvaalensis*, 2n=3x=27). High molecular weight total genomic DNA (>80kb) was isolated from five grams of 'Midiron' leaf tissues with cetyltrimethylammonium bromide (CTAB) by a method modified from Wagner et al. (1987) (refer to Appendix A). Fifty micrograms of total genomic DNA was partially digested at 37°C with *BamH*I to generate fragments approximately between 9-23 kb. The *BamH*I ends of digested insert DNA was modified by partially filling-in the first two nucleotides of the *BamH*I site with 10mM each of dATP and dGTP using Klenow DNA polymerase (Gibco-BRL, Gaithersburg, MD). This partial fill-in step increased the efficiency of vector-insert ligation and prevented formation of chimeric clones that result from self-ligation of smaller insert DNA fragments. The partially filled-in insert DNA was purified by phenol-chloroform extraction and then precipitated with ethanol (absolute) and resuspended in 50 ul of TE buffer.

Five micrograms of *Xho*I-predigested Lambda Fix II vector arms (Stratagene, La Jolla, CA) was prepared by partially filling-in the first two nucleotides of the *Xho*I

site with 10mM each of dTTP and dCTP using Klenow DNA polymerase. The partially filled-in vector arms were purified by phenol-chloroform extraction and then precipitated with ethanol (absolute) and resuspended in 5 ul of TE buffer.

The primary library was constructed by ligating an equal molar ratio (1:1) of the compatible *BamHI*-digested insert and *Xho*I-digested Lambda Fix II vector DNA. One microgram each of the insert and vector DNA were ligated using 2 units of T4 DNA ligase and 10mM of ATP (Stratagene, La Jolla, CA) for 18 hours at 4°C.

The ligation mixture was purified by phenol-chloroform extraction. One microliter of the purified ligation product was packaged with Gigapack III Gold extracts (Stratagene, La Jolla, CA) at room temperature for 2 hours. Three independent packaging reactions were performed and the resulting products were pooled into one primary library. The packaged primary library was diluted with 500 ul of SM buffer (Stratagene) and the remaining debris was removed by chloroform extraction.

The primary library was titered by infecting XL1-Blue MRA host cells (Stratagene, La Jolla, CA) and plating on LB-agar topped with NZY-agarose. The recombinant plaques in the primary library was determined by titering on XL1-Blue

MRA (P2) host cells. Selection was provided by the *spi* (sensitivity to P2 inhibition) system of the Lambda Fix II vector. Recombinant plaques are *Red*$^-$/*Gam*$^-$ and therefore are insensitive to P2 inhibition and are able to grow on P2 lysogen of XL1-Blue MRA. The contaminating wild type plaques are *Red*$^+$/*Gam*$^+$ and therefore are sensitive to P2 inhibition and are unable to grow in XL1-Blue MRA (P2) strain.

Screening of genomic library

8 x 10^5 clones from the Lambda Fix II genomic library were plated on LB-NZY-agarose at a density of 40,000 plaques per plate (20 plates). The plaques were blotted in duplicate copies on Hybond N^+ nylon membrane (Amersham Life Science, Arlington Heights, IL). The library was screened using a previously isolated 360 bp *Cynodon* partial chitinase cDNA fragment (provided by S. McMaugh, University of Sydney, Australia) as probe. Fifty nanograms of probe were labeled with α^{32}P-dCTP by the random priming method using the RediPrime DNA Labeling Kit (Amersham Life Science, Arlington Heights, IL). The filters were hybridized overnight at 65°C in 5X SSPE, 5X Denhardt's solution, 0.5% (w/v) SDS and 500 ug sonicated salmon sperm DNA (Gibco-BRL, Grand Island, NY). The filters were washed

twice for 10 minutes at room temperature with 2X SSC and 0.1% SDS. A second higher stringency wash was performed twice with 0.5X SSC and 0.1% SDS at 65°C for 15 minutes each washing. The filters were used for autoradiography for 2-3 days at -80°C. A total of nine positive plaques were isolated after the tertiary screening. Of these plaques, the three containing the largest inserts based on *Not*I digestion (GCyn113, GCyn319, GCyn456) were selected for further analysis.

Subcloning and sequencing

The three positive plaques selected were amplified at high multiplicity in suspension cultures of XL1-Blue MRA following the method described by Sambrook et al. (1989). The lambda DNA was purified and precipitated from the liquid lysate by cetyltrimethylammonium bromide (CTAB) using the Lambda DNA purification kit (Stratagene, La Jolla, CA).

Two micrograms of DNA from clones containing the longest inserts of 20kb (GCyn113 and GCyn456) were digested with *Sal*I. The *Sal*I digests were blotted on Hybond N^+ nylon membrane and hybridized with random prime-labeled *Cynodon* partial chitinase cDNA probe that was originally used in the library screening. The hybridizing fragments (0.9 kb)

were purified from agarose gel with QiaExII Gel purification kit (Qiagen), and cloned into the *Sal*I site of pBluescript SK (Stratagene, La Jolla, CA). Both strands of the inserts were sequenced through the T3 and T7 promoter-primer sites of the pBluescript plasmid.

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 1C). 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 1D). 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).

Southern blot analysis

Southern blot analysis was performed to determine the chitinase gene copy number in the genome of bermudagrass and also to compare the number of gene copies between cultivars and species with different ploidy or chromosome numbers. The three bermudagrasses compared were 'Midiron' (*C. dacytlon* x *C. transvaalensis*, 2n=3x= 27), 'MSU'-A12195 (*C. dactylon*, 2n=4x= 36) and 'Uganda' (*C. transvaalensis*, 2n=2x= 18). These cultivars also cover a wide array of cold hardiness available in *Cynodon* germplasm (J. Anderson, unpublished).

DNA samples were digested with specific restriction enzymes (*Xho*I, *Eco*RI, *Pst*I and *Ava*I) at 37°C. Digested DNA samples were electrophoresed in 1% TAE-agarose gel at 75 volts for 3-4 hours. DNA was transferred to Hybond N^+ nylon membrane (Amersham Life Science, Arlington Heights, IL) by downward alkali (0.4M NaOH) blotting technique for 1.5 hours using a turbo-blotter set-up. The filters were hybridized with the *Sal*I fragment isolated from GCyn113 and labeled with α^{32}P-dCTP by random priming method using RediPrime Labeling kit (Amersham Life Science, Arlington Heights, IL). Hybridization was performed overnight at 65°C in 5X SSPE, 2.5% Denhardt's solution, 0.1% SDS and 0.5mg/ml sonicated salmon sperm DNA. The filters were washed twice for 10 minutes with 2X SSC + 0.1% SDS at room temperature, twice for 10 minutes with 1X SSC + 0.1% SDS at 65°C and then for 10 minutes with 0.25XSSC + 0.1%SDS at 65°C. Filters were autoradiographed for 2-3 days at -80°C.

RESULTS

Isolation of chitinase genomic clones

Approximately 8 x 10^5 clones from the Lambda Fix II library consisting of 2.178 x 10^6 recombinant phages (Table 1), 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 1A). *Not*I digestion of purified lambda DNA revealed that these clones contained insert sizes ranging from 18-21 kb (Figure 1B, Table 2). 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 1B) 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 1C, Table 2) 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 1D). 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

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 2 and 3). 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 2) 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 3) 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 4). 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:

```
                          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  *
```

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* (refer to diagram). 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.

Analysis of the 5′ and 3′ flanking regions

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 2 and 3). In addition to the TATA box, two GC-rich motifs were found upstream to the TATA-box (Figures 2 and 3). The first motif (GGCCGGCCGCCCTTG) is very similar to the GGCC-box which was also found in rice chitinase and β-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 5). 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 6). 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.

Cynodon chitinase gene family

The structure of the chitinase genes in the genome of Midiron is shown in Figure 7. The *Xho*I sites are located proximal to either or both the 5′ and 3′ ends of the region spanned by the 0.9 kb *Sal*I probe (P1). *CynCht1* has a unique *Xho*I site located 77 bp upstream from the 3′-*Sal*I site. In *CynCht2*, the conserved region spanned by the *Sal*I probe is flanked by unique *Xho*I sites that are about 880 base pairs apart. Southern blot analysis of *Xho*I-digested total genomic DNA (Figure 8) revealed three major or strong bands and several minor or faint bands in Midiron. These bands are 3.5, 1.7 and 0.9 kb in length. Since *Xho*I does not cut

in the middle of the region covered by the probe, this result suggests that the three fragments possibly correspond to distinct gene copies representing the members of the class II chitinase gene family in bermudagrass. The same three major bands were detected in *Xho*I-digested MSU total genomic DNA. Only one common major band (1.7 kb) was observed between Uganda and the other two bermudagrasses. Two polymorphic restriction fragments were observed in Uganda which are 2.8 and 1.2 kb in length. The minor bands observed in all three bermudagrasses represent cross hybridization with other genes possibly belonging to another class or family of chitinases.

Amino acid sequence homology

The primary structure of the preprotein encoded by the bermudagrass genes can be divided into two regions, the signal peptide and the catalytic domain. CHT1 and CHT2 have highly homologous N-termini with only a single amino acid substitution at the signal peptide (Figure 4). Computer analysis using ExPASy Proteomics Tools predicted that both proteins are cleaved at the glycine-phenylalanine junction, removing a signal peptide consisting of 22 amino acid residues. The signal peptides of CHT1 and CHT2 are 95% identical and are both hydrophobic as indicated by the

calculated Grand Average Hydropathicity (GRAVY) of 1.286 and 1.450, respectively (Kyte and Doolittle, 1982).

The CHT1 mature polypeptide is composed of 227 amino acid residues with a molecular weight of 25 kDa, and a calculated pI of 8.10. The CHT2 mature polypeptide consists of 229 amino acid residues and about 25.5 kDa. Computer calculation indicates that CHT2 is basic with a pI of 8.82. Both proteins are hydrophilic with GRAVY values of -0.493 and -0.465, respectively.

Alignment of the amino acid sequences of the mature polypeptides encoded by the two genes revealed significant homology with a number of known chitinases from higher plants (Table 3, Figure 4). The homologous regions are located at the catalytic domain of the two proteins defined by aa 43-243 and aligned quite well with the catalytic regions of both class I and class II chitinases. The sequence similarity is slightly higher for the class II than for the class I chitinases (Table 3). Apparently, these highly conserved regions of the two bermudagrass proteins correspond to the functional domain for catalytic activity of these chitinases (Flach et al., 1992; Beintema, 1994). The results of the sequence alignments also revealed that both bermudagrass chitinase genes are most closely related to class II chitinases from peanut (Kellmann et

al., 1996) and tomato (Harikrishna et al., 1996). No sequence homology was detected with known class III chitinase genes.

Computer analysis failed to detect any intracellular targeting signals in both genes. Most notable was the absence of the hydrophobic C-terminal extension that characterize chitinases which are targeted to the vacuole (Bednarek and Raikhel, 1991; Chrispeels and Raikhel, 1992). Thus, the mature proteins encoded by both bermudagrass genes are all predicted to be extracellular. This result is consistent with the extracellular location of known class II chitinases of higher plant species.

DISCUSSION

CynCht1 and *CynCht2* genes exhibit significant sequence homology with the catalytic regions of class I and class II chitinases, both at the nucleotide and amino acid sequence levels. However, despite the sequence similarity with class I chitinase, the overall structure of the proteins have indicated that the genes represent the members of class II gene family. The sequence of the catalytic regions of class I and class II chitinases are highly conserved (Hamel et al., 1997; Tomohiro and Torikata, 1995; Collinge et al., 1993). Class I chitinases can be distinguished from class II chitinases based on two important structural elements which are absent in mature class II proteins (Figure 4). The first element is the N-terminal cysteine-rich domain necessary for chitin binding of the protein (Flach et al., 1992; Iseli et al., 1993; Beintema, 1994). This element consists of about 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 composed mostly of hydrophobic amino acids (Figure 4). This domain is necessary for vacuolar localization of class I protein (Neuhaus et al., 1991; Chrispeels and Raikhel, 1992). It

was concluded that both bermudagrass genes are class II chitinases based on two characteristics. First is the absence of the structural features of class I chitinases and second, the higher sequence homology shared by these genes with the catalytic region of class II than class I chitinase.

Plant chitinases are small molecules with the mature proteins ranging from 25 to 40 kDa (Flach et al., 1994, Collinge et al., 1993). The bermudagrass genes isolated encode mature proteins within this size range. The signal peptides of known plant class II chitinases are generally comprised of 23 amino acid residues (Linthorst et al., 1990). Computer analysis predicted slightly shorter signal peptides for both CHT1 and CHT2, consisting of 22 amino acid residues. Both signal peptides consist of a hydrophobic core and a processing specificity domain that characterize all eukaryotic signal peptides (Von Heijne, 1983).

Class II chitinases are generally acidic proteins (Graham and Sticklen, 1994). Contrary to this information, the calculated pI of both CHT1 and CHT2 mature proteins indicated that they are basic chitinases. However, these predicted pI values may not reflect the true pIs of those proteins since the real values may still be affected by

various post-translational modifications including the attachment of other moieties to the protein structure. For example, two potential asparagine N-glycosylation sites (positions 152-155 and 212-215) were found in CHT1 mature protein. Likewise, the protein also has six potential N-myristoylation sites (positions 33-38, 76-81, 136-141, 199-204, 207-212 and 208-213), and three potential protein kinase-C phosphorylation sites (positions 58-60, 106-108, 167-169). Similar sites were also found in CHT2 (Table 4).

The current convention for classifying plant chitinases is based principally on both the structural features and the pI of the protein (Shinshi et al., 1990; Araki and Torikata, 1995; Beintema, 1994). Class I and class II chitinases are not only distinguished based on the presence of the two structural domains but also by being basic and acidic proteins, respectively. However, acidic chitinase genes with N-terminal cysteine-rich domains have been identified from different plant species (Margis-Pinheiro et al, 1991; Araki et al., 1992). Accordingly, the bermudagrass chitinase genes are examples of basic chitinase proteins, which can be structurally classified as class II proteins. Nishizawa et al. (1993), proposed that the classification scheme for chitinases should be modified and based only on their structural characteristics rather

than both structural features and pI. Furthermore, Meins et al. (1992), pointed out that the classification of chitinases and β-1,3-glucanases on the basis of pI is not a very robust and reliable criterion. The reasoning behind this is that most chitinases and β-1,3-glucanases have relatively small net charges at neutral pH. Because of this, small amino acid substitutions in regions of the proteins that are not highly conserved could possibly lead to the occurrence of both acidic and basic molecules within the same structural group.

In general, chitinase genes in higher plants are interrupted by intervening sequences (Meins et al., 1992). Some of the few exceptions to this are the *Chi1* gene from pea (Chang et al., 1995) and the rice chitinase genes *RCH10* (Zhu and Lamb, 1991), *RCG1* and *RCG3* (Nishizawa et al., 1993). The introns of *CynCht1* and *CynCht2* deduced by computer analysis are consistent with reports that most interrupted chitinase genes have two introns (Flach et al., 1992; Graham and Sticklen, 1994; Wu et al., 1997). Likewise, the peanut Chi2;1 gene to which the bermudagrass chitinase genes are most closely related also has two introns. All of the introns predicted for both bermudagrass genes are flanked by the splice motif for intron-exon junction and have AT content from 40-60% required for

processing of the premRNA (Goodall and Filipowicz, 1989). However, the proposed intron-exon boundary needs to be investigated experimentally to be able to confirm the computer-predicted results. This can be done either by S1 nuclease protection assay or by direct cloning and sequencing of corresponding cDNAs.

The translation initiation codons were identified as the first AUG codon immediately downstream from the putative transcription start sites. In both *CynCht1* and *CynCht2*, the translation initiation codons are embedded within a motif consistent with the consensus for optimal context of translation initiation in eukaryotes as defined by the GCC(**G or A**)CCAUG**G** (Kozak, 1991). The two most important positions in this motif, the purine (A or G) at position -3 and the last G at position +4 are particularly conserved in both chitinase genes.

In addition to the TATA-box, other potential cis-acting elements found in the sequenced region of the promoters of both *CynCht1* and *CynCht2* are two GC-rich motifs. The first motif defined by the sequence GGCCGGCCGCCCTTG is very similar to the GGCC-box (GGCCGGCYGCCCYAG) found at the promoter regions of several rice chitinase, *Cht-1*, *Cht-3* (Nishizawa et al., 1993), *RCH10* (Zhu and Lamb, 1991) and glucanase, *Gns1* (Simmons et

al., 1992) genes. This motif was found at positions -100 and -92 of *CynCht1* and *CynCht2*, respectively. Studies involving the rice genes having this element indicated that this GGCC-box is required for the induced expression of the chitinase and glucanase genes. It was proposed that this element is a potential transcription factor binding site and could be the regulatory control for the induced expression of chitinase genes particularly in response to both biotic and abiotic elicitors including ethylene and wounding. The 3′ region of the second GC-rich motif (GCCCGGCGC**GAGGCGCG**), which was found at positions -125 of *CynCht1* and -118 of *CynCht2*, is similar to the ethylene responsive element (GAGCCGCC), identified in tobacco class I chitinase genes (Shinshi et al., 1995). The possible roles of this motif in the expression of the bermudagrass chitinase genes should be investigated, particularly in relation to both biotic and abiotic stress factors.

The results of Southern blot analysis indicated that bermudagrass class II chitinases are encoded by a small gene family consisting of three members. This result is consistent with various reports that most chitinase genes in higher plants occur as families of 3-4 members (Meins et al., 1992; Wu et al., 1997; Hudspeth et al., 1996). The three major bands observed in the *Xho*I-digested Midiron

genomic DNA correspond to all the members of class II chitinase gene family in bermudagrass. Exactly the same number and size of major bands was also observed in MSU that had been digested with the same restriction enzyme. The *Sal*I probe used for the Southern blot also detected all three genes in Uganda although a different restriction fragment profile was observed. This species-specific allelic pattern (restriction fragment length polymorphism) suggests structural differences between the chitinase gene family members of *C. dactylon* and *C. transvaalensis*.

Midiron was derived from a polycross nursery. The female parent of Midiron is unknown, although it is believed that the actual female parent could be a cultivar closely related to the tetraploid MSU. Based on available information, Uganda is the putative pollen donor for the hybrid Midiron (R. Keen, personal communication). The RFLP patterns for the class II chitinase genes are consistent with these facts. Based on the *Xho*I restriction pattern, *CynCht1* could be represented either by the 1.7 kb or 3.5 kb fragments. The 1.7 kb fragment is common between the three bermudagrasses. The 3.5 kb fragment shared by Midiron and MSU is represented by the 2.8 kb polymorphic allele in Uganda, which is also present in the hybrid Midiron. This pattern is consistent with the heterozygosity of Midiron.

The shortest fragment which correspond to *CynCht2* is represented by 0.9 kb band in Midiron and MSU and 1.2 kb band in Uganda. Like in *CynCht1*, both the *C.dactylon*- and *C. transvaalensis*-specific alleles were detected in the triploid Midiron. These results suggest that the two chitinase genomic clones correspond to specific members of the gene family and not just allelic variants due to the heterozygosity of Midiron whose genome was used in the library. The RFLP patterns also suggest the possibility of distinct *dactylon* and *transvaalensis* genomes. This was also suggested by earlier works which showed the lack of pairing between the chromosomes of the tetraploid *C. dactylon* and diploid *C. transvaalensis* (Harlan et al., 1970). Most diploid plant genomes have three to four members for particular chitinase gene family. The number of class II chitinase gene family members is equal in both diploid and tetraploid bermudagrasses. Obviously, this number is not more than the number of genes observed in other diploid plant species (Van Buuren et al., 1992). Based on this fact, it appears that no additional unique chitinase gene copy has evolved as a result of polyploidy in *Cynodon*.

Like other redundant genes, multiple chitinase genes belonging to the same family are thought to have originated after the duplication followed by gene conversion. Gene

conversion results from base substitutions, deletions, insertions and rearrangements (Maeda and Smithies, 1986). The sequence differences observed both in the coding and flanking regions of the two bermudagrass chitinase genes exhibit some base substitutions as well as deletions and hence, consistent with this theory. Most interesting is the occurrence of a frameshift mutation that occurred due to single nucleotide deletion at the C-terminus of *CynCht2* relative to *CynCht1*. Whether this event is truly biological awaits further confirmation by the isolation and sequencing of cDNAs corresponding to *CynCht1* and *CynCht2*.

It is also interesting to prove if these redundant genes exhibit specialized functions. It was proposed that redundant genes which include multigene families are favored during evolution because they exhibit mutually exclusive in addition to the shared functions (Picket and Meeks-Wagner, 1995).

Biochemical studies in bermudagrass have indicated that chitinases may have potential roles in freezing tolerance (Gatschet et al., 1996), in addition to their original function as pathogenesis-related proteins. It is an interesting hypothesis that needs to be proven experimentally. However, our present study failed to detect significant matches between the two chitinase proteins

(CHT1 and CHT2) and the peptide sequences of the proteins earlier found by Gatschet et al. (1996), to be induced during cold acclimation in Midiron crown tissues.

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Table 1. Titer of the Lambda Fix II genomic library from Midiron total genomic DNA.

Sublibrary*	Titer (plaque forming units, pfu)		%Recombinant Phages
	XL1-Blue MRA	XL1-Blue MRA (P2)	
1	1.042×10^6	1.025×10^6	98.34
2	5.011×10^5	4.988×10^5	99.54
3	6.653×10^5	6.542×10^5	98.33
Pooled Primary Library =		2.178×10^6	Ave.= 98.74
Concentration = 1452 pfu/ul			

*Independent packaging reactions (insert:vector= 1ug:1ug)

Table 2. Positive plaques identified from the Lambda Fix II genomic library after tertiary screening with *Cynodon* chitinase partial cDNA probe.

Clone Number	Size of Fragment that Hybridized to the Probe (kb)		
	NotI*	***SalI****	***StuI****
GCyn113	21	0.9	3.5
GCyn122	15	nd	nd
GCyn135	15	0.9	2.5
GCyn319	18	nd	2.0
GCyn456	21	0.9	3.5
GCyn548	15	nd	nd
GCyn817	15	nd	2.5
GCyn829	10	nd	2.5
GCyn972	10	nd	nd

nd= not determined
*Probe: *Not*I=360bp cDNA; *Sal*I=360bp cDNA; *Stu*I=360bp cDNA and 0.9kb *Sal*I fragment from GCyn113

Table 3. Homology of the catalytic region of bermudagrass chitinases with some known class I and class II chitinases in higher plants.

Chitinase	% Identity	% Similarity
Cht1/Peanut (Class II)	73	87
Cht1/Tobacco (Class II)	62	89
Cht1/ Tomato (Class II)	60	76
Cht1/Class II (Average)	**65**	**84**
Cht1/Alfalfa (Class I)	59	74
Cht1/Pea (Class I)	59	74
Cht1/Potato (Class I)	57	73
Cht1/Class I (Average)	**58**	**73.6**
Cht2/Peanut (Class II)	70	84
Cht2/Tobacco (Class II)	60	77
Cht2/Tomato (Class II)	57	74
Cht2/Class II (Average)	**62**	**78**
Cht2/Alfalfa (Class I)	55	69
Cht2/Pea (Class I)	56	69
Cht2/ Potato (Class I)	53	69
Cht2/Class I (Average)	**54.6**	**69**

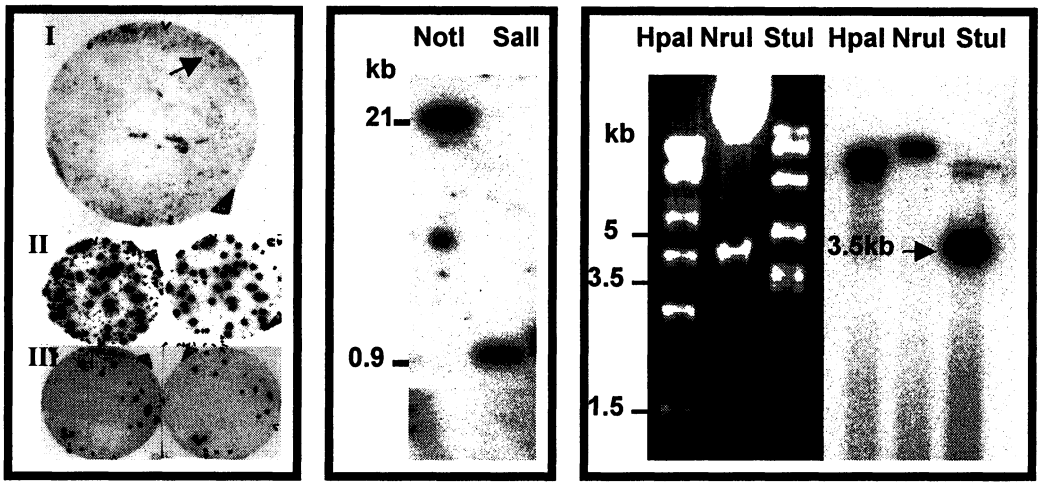
Accession Number: Peanut:*S65069* Tobacco:*AB008892*
Tomato:*S69184* Alfalfa:*U83591*
Pea:*S56694* Potato:*S43317*

Table 4. Predicted post-translational modification sites on CHT1 and CHT2 proteins.

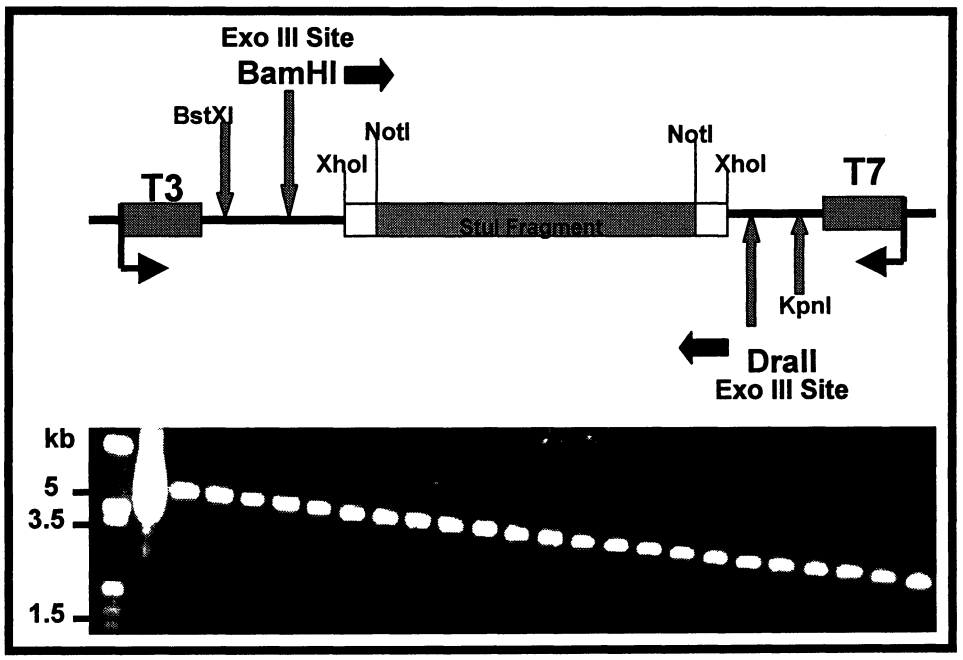
Chitinase	N-Glycn	N-Myristn	PKC-Phospn
CynCht1	152-155 (NCSD)	33-38 (GGKCSS)	58-60 (TRK)
	212-215 (NRTD)	76-81 (GGWATA)	106-108 (TDK)
		136-141 (CQALGF)	186-189 (TPR)
		199-204 (GLVTNI)	
		207-212 (GGLECN)	
		208-213 (GLECNR)	
CynCht2	183-186 (NCSD)	33-38 (GGKCSS)	44-46 (SER)
	243-246 (NRTD)	107-112 (GGWATA)	89-91 (TRK)
		167-172 (GQALGF)	137-139 (TDK)
		230-235 (GLVTNI)	198-200 (TPR)
		238-243 (GGLECN)	
		239-244 (GLECNR)	

N-Glycn: N-Glycosylation
N-Myristn: N-Myristoylation
PKC-Phospn: Protein kinase C phosphorylation

Figure 1. Cloning and sequencing of bermudagrass chitinase genes. (A) Screening of Lambda Fix II genomic library. Eight hundred thousand clones were screened with the 360 bp chitinase partial cDNA probe at moderate stringency. Sample primary (I), secondary (II) and tertiary (III) screening filters are shown. (B) Identification and cloning of the *Sal*I probe. Southern blot shows the 21kb full-length insert (*Not*I) and the 0.9kb *Sal*I fragment from GCyn113. (C) Identification of the fragment that contains the full length *CynCht1* from GCyn113 clone. GCyn113 DNA was digested with *Stu*I and hybridized with the *Sal*I fragment. Southern blot shows the 3.5 kb *Stu*I fragment that contained the full length *CynCht1* gene. (D) The 3.5kb *Stu*I 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 determine the full-length sequence of the genes.


I
II
III
NotI SalI
kb
21
0.9
HpaI NruI StuI HpaI NruI StuI
kb
5
3.5
1.5
3.5kb

A B C


Exo III Site
BamHI
BstXI
NotI
XhoI
NotI
XhoI
T3
T7
StuI Fragment
KpnI
DraII
Exo III Site
kb
5
3.5
1.5

D

Figure 2. 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 3. 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.

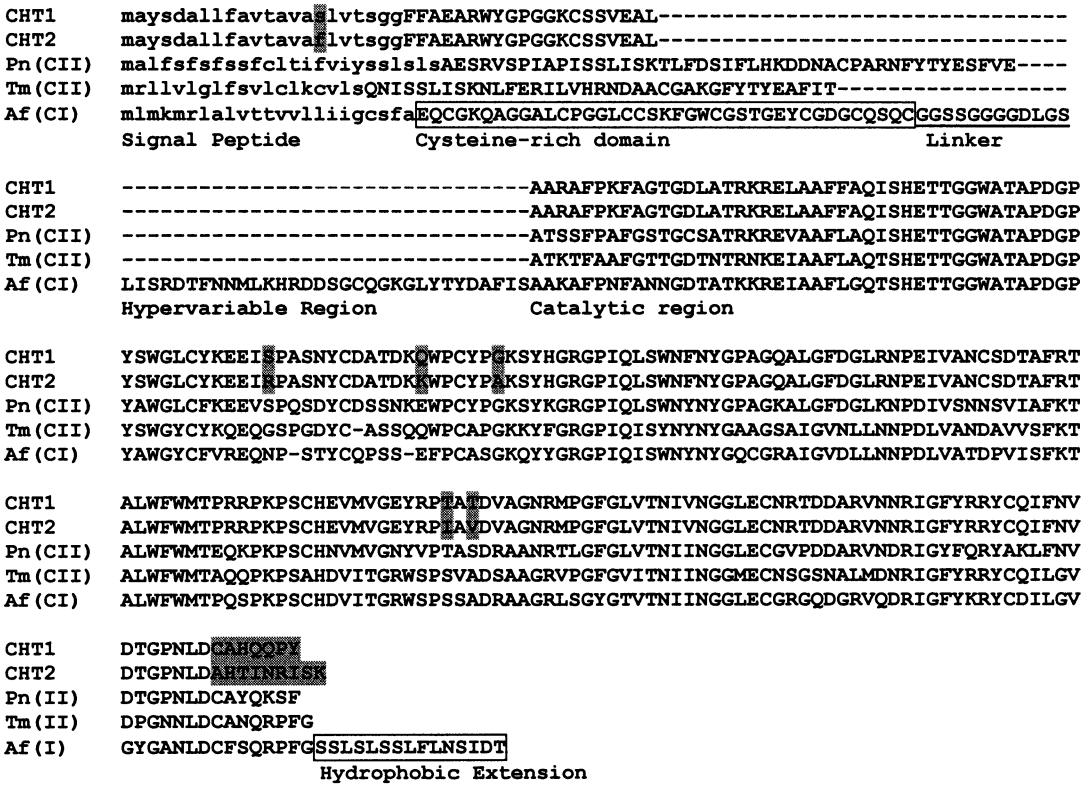


Figure 4. Amino acid sequence alignment of CHT1 and CHT2 with known class I and class II chitinases from other plant species. Pn(CII): Peanut, class II=*S65069;* Tm(CII): Tomato, class II=*S69184;* Af(CI): Alfalfa, class I= *U83591*. The putative signal peptides are in lower case letters. The highlighted 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.

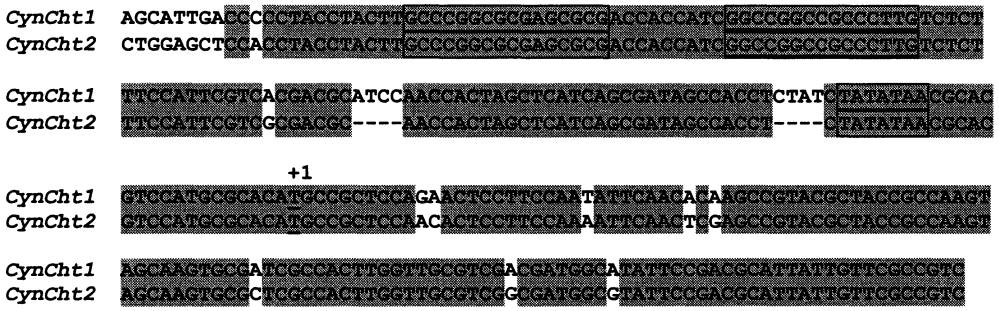


Figure 5. Analysis of the 5′ flanking regions of bermudagrass chitinase genes (*CynCht1*:-148 to +121; *CynCht2*:-140 to +121). Sequences at promoter regions were aligned to determine the extent of sequence homology between the *CynCht1* and *CynCht2*. The predicted transcription initiation sites are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

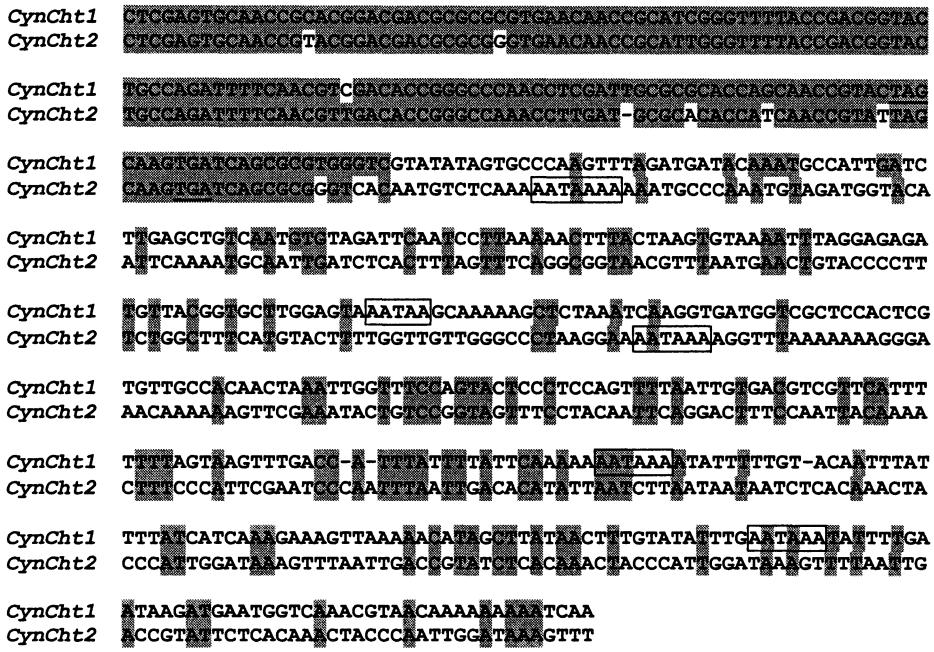


Figure 6. Analysis of the 3′ flanking regions of bermudagrass chitinase genes (*CynCht1*:+914 and downstream; CynCht2:+913 and downstream). Sequences at the 3′UT regions were aligned to determine the extent of sequence homology between the *CynCht1* and *CynCht2*. The translation termination codons are 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.

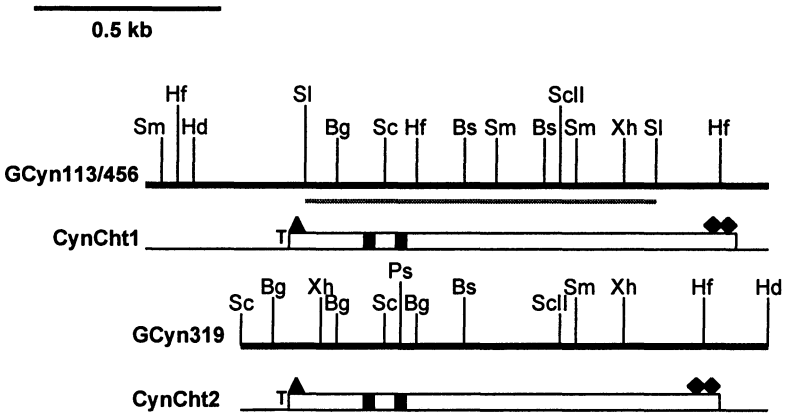


Figure 7. Structure of bermudagrass chitinase genomic clones. The sequenced regions are represented by open bars. The location of the 0.9 kb *Sal*I probe is indicated by the gray line. Introns are represented by black bars. The transcription initiation sites are indicated by ▲. The poly-A signals are indicated by ◆.
Bg= *Bgl*I, Bs= *Bsi*MI, Hd= *Hind*III, Hf= *Hinf*I, Ps= *Pst*I, ScI= *Sac*I, ScII=*Sac*II, Sl= *Sal*I, Sm= *Sma*I, Xh= *Xho*I

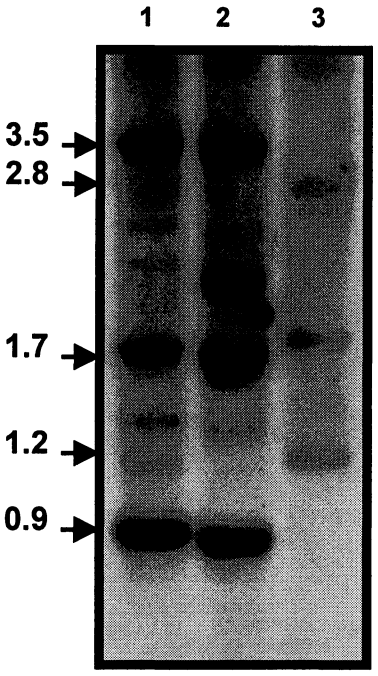


Figure 8. Southern blot analysis of bermudagrass class II chitinase gene family. Total genomic DNA (8 ug) isolated from leaves of bermudagrass cultivars Midiron (1), MSU (2) and Uganda (3) were digested with *Xho*I and probed with the labeled *Sal*I fragment that represents the conserved regions of the two genes. The arrows show the fragments corresponding to each of the three members of the class II chitinase gene family identified in Midiron, and the corresponding homologues in MSU and Uganda.

CHAPTER III

Expression of Bermudagrass Class II Chitinase Genes During Cold Acclimation of Freezing Tolerant Genotypes

ABSTRACT

Two members of bermudagrass class II chitinase gene family (*CynCht1* and *CynCht2*) were recently sequenced. This research was conducted to determine their expression during cold acclimation (CA), drought, and exogenous application of abscisic acid. 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 CA-induced expression of the genes was specific to the crown tissues. The level of chitinase gene expression varied positively with cold hardiness. The most cold hardy 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 each cultivar at a level significantly higher than those achieved 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. Both drought- and ABA-induced gene expressions were specific to the crown tissues. The salient features of the deduced protein products, as well as the temporal and spatial expression patterns of the genes are consistent with the theory that this class of chitinase may be involved with a mechanism of freezing tolerance in bermudagrass. The hypothesis regarding the possible antifreeze function of class II chitinases is discussed.

Key Words: cold acclimation, drought, abscisic acid, mRNA, gene expression, northern blot, antifreeze

INTRODUCTION

Bermudagrasses, *Cynodon* sp. are distributed worldwide between latitudes of approximately 45°N and 45°S. These perennial, sod-forming, warm-season grasses are economically important due to their wide use for forage, turf and soil stabilization. Bermudagrass plants generally have limited capacity to withstand freezing temperatures, though there is substantial genetic variation for this trait within the genus. Increasing the freeze tolerance of bermudagrass cultivars grown in regions subject to subfreezing temperatures is an ongoing research objective with important economic ramifications. It is generally agreed that this can best be done through the use of traditional breeding and biotechnology. The application of genetic engineering to this problem requires new knowledge regarding the genes involved in low temperature response and the genetic mechanisms involved in the process.

Previous studies on gene expression during CA in bermudagrass cultivars identified a cold regulated protein in crown tissues as a chitinase (Gatschet et al., 1996). This protein normally functions in other plant defense mechanisms, particularly against pathogens. As part of the current study, two genes encoding class II chitinases were

isolated from the freezing tolerant cultivar 'Midiron' (discussed in Chapter II). How these chitinases might function in defense mechanisms is another question of present concern.

Thermal hysteresis is an important mechanism of freezing tolerance in plants and animals (Levitt, 1980). Antifreeze proteins (AFP) with thermal hysteresis activity were first found in animal systems, particularly in fishes (Smaglik, 1998; Davies and Hew, 1990). These proteins which can lower the freezing point, but not the melting point, of water are thought to be the principal components in the main mechanism that imparts freeze tolerance to polar fishes and some overwintering insect species. The structures of these proteins have unique repeating units of hydrophilic residues or amino acids with sugar side chains. The mechanism of action involves hydrogen bonding of the hydrophilic moieties on the surface of the protein with the ice nuclei. In effect, it results in the inhibition or retardation of ice crystal growth.

That similar kinds of AFPs also occur in plants was long suspected but demonstrated only in 1992 by Urrutia et al. (1992). They confirmed that higher plants indeed synthesize AFPs that functioned in reducing the rate of ice crystal growth and recrystallization in the intercellular

spaces of plant tissues. More recently, structural analysis of an AFP isolated from carrots confirmed the earlier results of Urrutia et al. (1992) and defined plant AFPs as having the same structural features as fish AFPs (Worall et al., 1998).

Recently published plant AFP research details the discovery that some of the PR proteins, chitinase, β-1,3-glucanase and thaumatin, accumulate in the apoplastic sap of rye leaves during CA (Hon et al., 1995; Griffith et al., 1997; Antikainen et al., 1997). The results of *in vitro* assays on the biochemical activities of these cold-inducible PR proteins clearly suggested that they have potential ice nuclei-binding activity and probably function as AFPs.

The protein studies of Gatschet et al. (1996), along with the discovery of chitinase genes described in Chapter II, suggest that these products may function in bermudagrass freeze-tolerant mechanisms. This chapter describes research at determining the spatial and temporal expression patterns of three bermudagrass chitinase genes in response to cold acclimation, drought and abscisic acid (ABA).

MATERIALS AND METHODS

Plant materials and treatments

Three bermudagrass cultivars (genotypes), 'Midiron', 'MSU'-A12195 and 'Uganda', were used in the study. Midiron (*C. dactylon* X *C. transvaalensis*; 24=3x=27 chromosomes) and Uganda (*C. transvaalensis*; 2n=3x=18 chromosomes) are commercial cultivars. MSU-A12195 (*C. dactylon* var. dactylon; 2n=4x=36 chromosomes) was originally collected on the campus of Michigan State University, East Lansing, MI. Clonal plants of each cultivar were obtained for this study from the *Cynodon* germplasm collection maintained by the Department of Plant and Soil Sciences, Oklahoma State University.

Phytomers (root + crown + stem unit) of each cultivar 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.

RNA isolation

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.

Northern Hybridization

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 1). One was the 0.9 kb *Sal*I 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 α^{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.

RESULTS

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 2). 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 2). 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 2). 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 2). 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 2 and 3). 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 4). 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 4). 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 4).

Expression of chitinase genes during drought stress and exogenous application of ABA

The RWC's 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 5 and 8). 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 6 and 8). In contrast, the application of ABA did not induce chitinase gene expression in the leaf (Figure 7) 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 8). 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 8).

DISCUSSION

The results of this study clearly indicate that low temperatures during CA regulate chitinase gene expression in freeze-tolerant bermudagrass cultivars. Interestingly, the level of gene expression in the crown tissues of the three cultivars appears to be positively correlated with the level of cold hardiness exhibited by the individual cultivar. Previous studies indicated that MSU and Midiron are two of the most freezing tolerant genotypes so far identified in *Cynodon* germplasm (Anderson et al., 1993; Anderson et al., 1988; J. Anderson, unpublished). 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 was found to exhibit moderate tolerance to freezing stress with a LT_{50} value approximated to be within the same level as the cultivar Tifgreen (-8°C) (Anderson et al., 1993; J. Anderson, personal communication). Accordingly, the low temperature-induced chitinase gene expression was found to be highest in MSU and lowest in Uganda. Two days of DAC was sufficient to downregulate chitinase gene expression in every cultivar. This temporal regulation pattern is very similar to the expression patterns exhibited by known cold regulated (COR) genes in higher plants (Thomashow, 1990).

These observations provide good evidence that CA induces chitinase gene expression. It is but logical to assume that this expression produces gene products required by the mechanisms that determine freezing tolerance in bermudagrass.

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 temporal expression pattern indicates a rapid response of the plants to environmental signals inducing transcription of the chitinase genes. As the acclimation process proceeded from 2 to 28 days, a slight reduction in transcript level occurred. This pattern reflects a balance between mRNA synthesis and turnover, and can be interpreted as an indication of the steady-state mRNA levels during the acclimation process. This pattern seems to be related with the earlier findings that maximum freezing tolerance in bermudagrass is achieved after 28 days of CA Gatschet (1993). Additionally, it is interesting to investigate the exact timing at which mRNA steady state levels occur and how it correlates with cold hardiness. Consequently, the increased levels of chitinase mRNA were clearly due to low temperature response and were consistent with previous results which showed that CA caused an increased synthesis of chitinase in bermudagrass crowns

(Gatschet et al., 1996). Similarly, accumulation of COR proteins was demonstrated in other cold hardy monocot species including wheat and winter rye (Houde et al., 1995; Antikainen et al., 1996). It can therefore be inferred that chitinase gene expression during CA in bermudagrass crowns is an adaptive response involved with mechanisms that prevent or reduce the damaging effects of freezing temperatures to the meristem. This process implies important biological function, conditioning the ability of plants to regrow after exposure to freezing temperatures.

Contrary to the pattern of gene expression in crown tissues, the expression of the genes in leaf tissues appeared not to correlate with CA or with freezing tolerance of the cultivars used in the experiments. Induction of gene expression occurred only in Midiron and Uganda. This result suggests that this pattern of chitinase gene expression in the leaves is probably regulated in a different manner than that observed in the crown tissues. This regulatory control may be due to a mechanism quite different from the process responsible for the increased gene expression in the crown tissues. Chitinase proteins are involved with various physiological processes and are induced by several factors including physical wounding and pathogen infection. There is evidence supporting a

potential function of these proteins in leaf abscission process (Gomez-Lim et al., 1987). Both Midiron and Uganda exhibited very high levels of chlorosis and leaf abscission after about 15 days of CA, which was aggravated further during DAC. In stark contrast, chlorosis and leaf abscission were less severe in MSU throughout the duration of CA and DAC. Apparently, the pattern of expression of the genes in the leaves of Midiron and Uganda is associated with physiological events such as leaf chlorosis and abscission. Therefore, this event is not a direct response to low temperature and may not be associated with the development of freezing tolerance.

Based from the aligned sequences, the *CynCht1* and *ChyCht2* genes exhibit highly conserved 5′ flanking regions at least within 140 nucleotides upstream from the transcription start sites (refer to Chapter II). Located within the conserved regions of *CynCht1* and *CyCht2* are two GC-rich motifs. One of these motifs defined by the sequence GGCCGGCCGCCCTTG, is very similar to the GGCC-box (GGCCGGCYGCCCYAG) previously identified in several rice chitinase and β-1,3-glucanase genes (Nishizawa et al., 1993; Zhu and Lamb, 1991; Simmons et al., 1992). Previous studies have shown that the GGCC-box is essential for the induced expression some PR-protein genes. A hypothesis that

had been proposed suggest that this motif may be a cis-regulatory element that determines inducible expression of the chitinase and glucanase genes, particularly in response to wounding, salicylic acid, UV light, ethylene, cytokinin and various biotic elicitors. Based on this, it can also be inferred that the inducible expression of class II chitinase genes may also be due to this regulatory element. Consequently, the exact role of this GC-rich motif in the low temperature regulation of chitinase gene expression can be elucidated by experiments involving promoter fusion with suitable reporter genes.

As previously mentioned, early studies indicated that the expression of chitinase genes in plants is predominately induced by pathogen infection and wounding (Collinge et al., 1993; Meins et al., 1992). However, more recent biochemical and physiological studies suggest that CA in a number of cold hardy monocot species is accompanied by accumulation of PR proteins including chitinase, β-1,3-glucanase and thaumatin in the apoplast (Antikainen and Griffith, 1997; Gatschet et al., 1996; Hon et al., 1995). In vitro assays also indicated that these PR proteins possess the ability to bind to the surface of potential ice nuclei, thereby inhibiting ice crystal growth in the intercellular spaces. This phenomenon which was observed

predominantly in overwintering monocot species, has led to the theory proposing that some isozymes of PR-proteins exhibit biological activity as antifreeze agents (Thomashow, 1998; Antikainen and Griffith, 1997; Griffith et al., 1997). It was further speculated that the PR proteins accumulated during CA of cold hardy herbaceous plants could be potent inhibitors of ice crystal growth and recrystallization. These actions are in a manner analogous to the modes of action of AFPs of certain aquatic fishes and insects (Griffith et al., 1997; Urrutia et al., 1992). The exact contribution of the AFPs, particularly the PR-proteins involved in freezing tolerance of these plant species, is yet to be determined. Nevertheless, the activity exhibited by these proteins in *in vitro* assays and their high level of accumulation during CA of cold hardy species provides reasonable bases to assume that they have significant roles in freezing tolerance mechanisms. The results of Marentes et al. (1993), supported this hypothesis, which demonstrated that freezing tolerance of acclimated rye leaves are diminished upon the removal of the apoplastic proteins.

The potential antifreeze function of some PR-protein genes had been the subject of speculations, particularly with regard to how such a specialized function evolved in a

certain group of PR-protein genes. Pathogen infection and low temperature stress are both accompanied by biochemical processes that lead to modification of plant cell walls (Rajashekar and Lafta, 1996; Collinge et al., 1993; Griffith and Brown, 1982). This event may cause the synthesis of common set of molecules, which included the PR-proteins. The enzymatic activity of plant chitinases are likely to be involved not only with fungal cell wall degradation but also in plant cell wall modification during stress periods like low temperature. This hypothesis is supported by the occurrence of endogenous substrate for this enzyme in plant cells as N-acetyl-D-glucosamine units in glycolipids of secondary cell walls, present in tough tissues like the crown (Benhamou et al., 1989). A proposed theory is that during the evolution of plant responses to pathogen and low temperature stress, some of the PR-proteins may have acquired additional function as antifreeze proteins. A strong selective pressure on these molecules possibly caused some functional specialization to occur mainly because these proteins possess the structural attributes that made them suitable for such an additional function (Worall et al., 1998; Logsdon and Doolittle, 1997). Freezing injury is predominantly a result of ice crystallization in the intercellular spaces. Ice formation

results in cell dehydration, which is considered to be the primary cause of injury (Steponkus and Webb, 1992; Steponkus, 1984). Additionally, specific groups of PR-proteins that are functionally and temporally in frame relative to both pathogen infection and freezing stress may have been coincidentally recruited for both functions.

Chitinase proteins are encoded by multiple genes belonging to small groups, or families. It is also possible that functional specialization might have been favored during evolution mainly because of genetic redundancy. The theory proposed by Picket and Meeks-Wagner (1995), is relevant to this hypothesis. This theory demonstrated that genetic redundancy in eukaryotic organisms is associated with events that led to functional specialization. It can therefore be hypothesized that the low temperature-induced chitinase genes may have been products of such events and may have important functions in freezing tolerance.

The mature proteins encoded by the three bermudagrass genes are highly hydrophilic. Based on the absence of intracellular localization signals, computer analysis also predicted that all proteins are targeted to the intercellular matrix (discussed in Chapter II). This data is consistent with the extracellular location of class II chitinases in plant tissues. However, this needs to be

proven experimentally through immunological detection of the cellular distribution of the proteins. Theoretically, the characteristics of both bermudagrass proteins strongly suggest that they qualify for the second function as antifreeze proteins. Based on the expression patterns exhibited by these genes, they may also have significant roles in the coordinate expression of resistance to fungal infection and tolerance to freezing stress. This possibility is consistent with data indicating that most known freezing tolerant bermudagrass cultivars are also resistant to spring dead spot disease caused by the fungus *Ophiosphaerella herpotricha* (Baird et al., 1998). This is further supported by similar studies which indicated that CA in some cereal species can increase not only the tolerance to freezing but also the resistance to fungal infection (Tronsmo et al., 1993).

The biochemical and physiological bases of plant tolerance to freezing and drought are very similar if not totally identical (Siminovich and Cloutier, 1983). Because of this, the drought- and ABA-inducible expression patterns exhibited by the chitinase genes were not totally unexpected. Furthermore, because of the presumed warm season origin of bermudagrass, it is very tempting to speculate that the low temperature-regulated response of

chitinase gene expression may have been originally due to drought tolerance. However, there is insufficient basis to make this assumption because there is no experimental data regarding the drought tolerance of the three bermudagrass cultivars used in this experiment. Further investigations are necessary to determine the link between chitinase gene expression and tolerance to both freezing and drought. One possibility is that both low temperature and dehydration stress could contribute to increased susceptibility of plants to pathogen infection, and that the increased chitinase gene expression could just be a secondary response not necessarily directly associated with low temperature or drought tolerance. Simpson (1981), proposed that plants may have evolved protective mechanisms that protect them against opportunistic pathogens particularly during stressful periods such as drought. The high induction of expression of chitinase genes after 10 days of drought stress is quite consistent with this theory.

ABA has been implicated with plant responses to several environmental stresses including both drought and low temperature (Giraudat, 1995; Luo et al., 1993). A number of studies have demonstrated that ABA alone can replace CA for the development of freezing tolerance in some plant tissues (Robertson et al., 1987; Chen and Gusta,

1983). Contrary to this, the results obtained from the current investigation on bermudagrass indicated that exogenous application of ABA without CA caused only a moderate increase in the LT_{50} of Midiron from -6°C to -8°C. This increase in cold hardiness is significantly lower than that induced by CA of the same cultivar (-10°C). Previous studies in alfalfa (*Medicago sativa*) also demonstrated that the level of freezing tolerance resulting from exogenous application of ABA was much lower than the tolerance induced by CA (Mohapatra et al., 1988). The ABA-induced chitinase gene expression exhibited a pattern that was parallel to the results of the cold hardiness experiments. Based on these results, it appears that ABA is necessary but cannot totally substitute for CA in increasing the freezing tolerance of bermudagrass. Additionally, the efficacy of different ABA isomers (+ or -) to induce this physiological effect is another interesting aspect that needs to be investigated in future experiments.

Finally, the differential responses of the individual cultivars to all the stress factors used in the experiment also suggest that perhaps differential regulatory mechanisms are responsible for different levels of cold hardiness exhibited by those cultivars.

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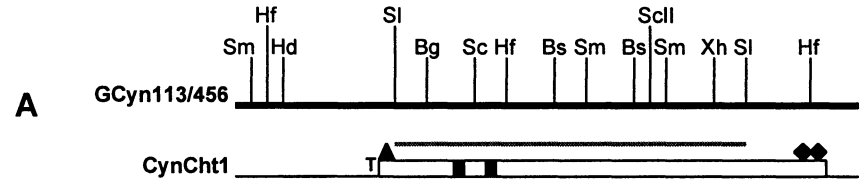

B

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CynCht1  ctcgagtgcaaccgcacggacgacgcgcgcgtgaacaaccgcatcgggttttaccgacggtac P2
CynCht2  ctcgagtgcaaccgtacggacgacgcgcgcggtgaacaaccgcattgggttttaccgacggtac P3

CynCht1  tgccagattttcaacgtcgacaccgggcccaacctcgattgcgcgcaccagcaaccgtactag P2
CynCht2  tgccagattttcaacgttgacaccgggccaaaccttgat-gcggcacaccatcaaccgtattag P3

CynCht1  caagtgatcagcgcgtgggtcgtatatagtgcccaagtttagatgatacaaatgccattgatc P2
CynCht2  caagtgatcagcgcgggtcacaatgtctcaaaaataaaaaaatgcccaaatgtagatggtaca P3

CynCht1  ttgagctgtcaatgtgtagattcAATCCTTAAAAACTTTACTAAGTGTAAAATTTAGGAGAGA P2
CynCht2  attcaaaatgcaattgatctcagtttagtttcgggggtaacgtttaatgaacgtaccccctt P3

CynCht1  TGTTACGGTGCTTGGAGTAAATAAGCAAAAAGCTCTAAATCAAGGTGATGGTCGCTCCACTCG
CynCht2  tctggcttcatgtactttggttgttgggccctaaggaaaataaaaggtttaaaaaaaggga P3

CynCht1  TGTTGCCACAACTAAATTGGTTCCAGTACTCCGTCCAGTTTTAATTGTGAGGTCGTTCATTT
CynCht2  aacaaaaagttcgaaatactgtccggtagtttcctacaattcaggactttccaattacaaaa P3

CynCht1  TTTTAGTAAGTTTGACC-G-TTTATTTTTTTTCAAAAAATAAAATATTTTTGT-ACAATTTAT
CynCht2  cttcccattcGAATCCCAATTTAATTGACACATTTAATCTTGATAATAATCTCACAAACTA P3
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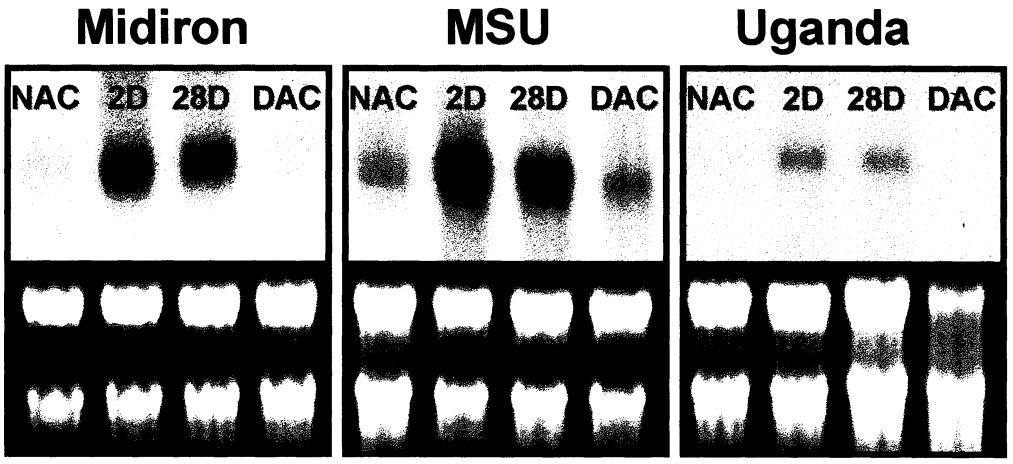
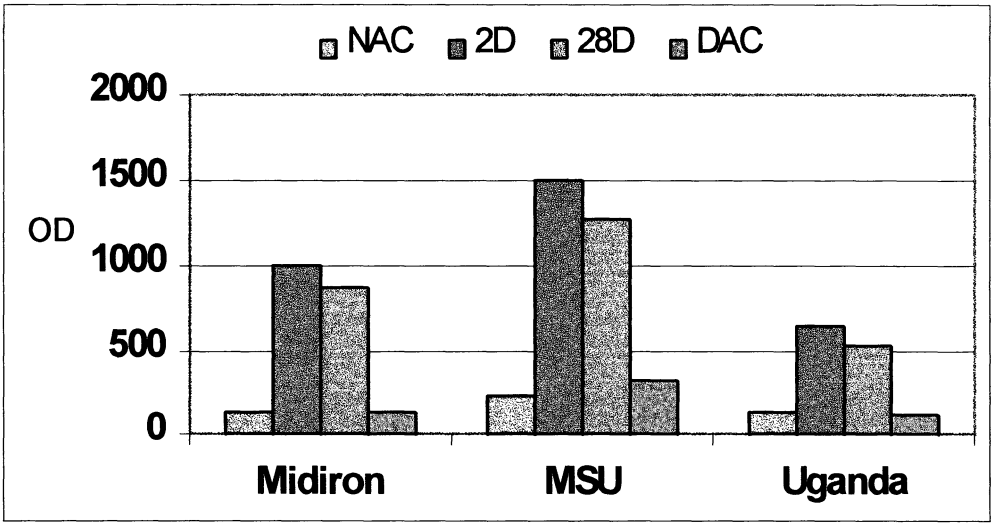
Figure 1. Probes used for northern blot analysis of chitinase gene expression.(A) Probe P1 (shown in gray line) covers about 90% of the coding regions (highly conserved) of *CynCht1* and *CynCht2*. (B) Probes P2 and P3 (shown in lower case letters) represent the region covering the C-terminus of the proteins and part of the 3′-UT regions of *CynCht1* and *CynCht2*, respectively.

Figure 2. 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.


Midiron
MSU
Uganda
NAC 2D 28D DAC
NAC 2D 28D DAC
NAC 2D 28D DAC

A


NAC
2D
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DAC
2000
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OD
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MSU
Uganda

B

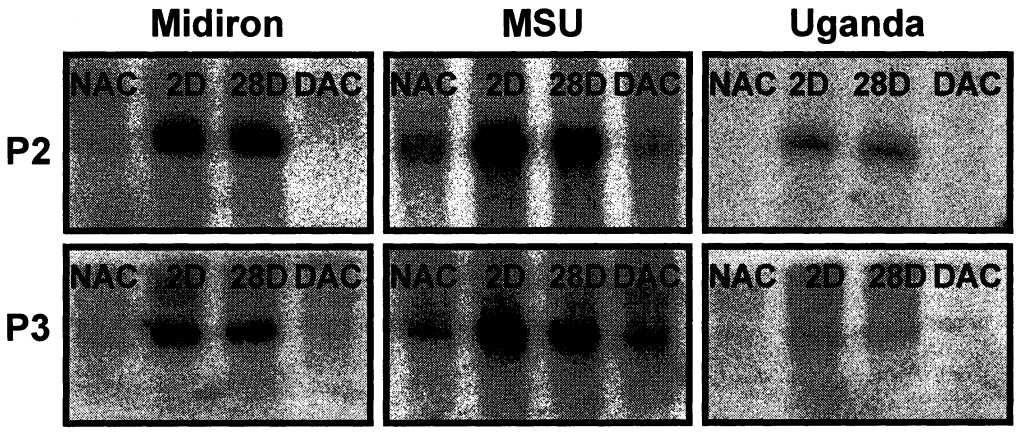
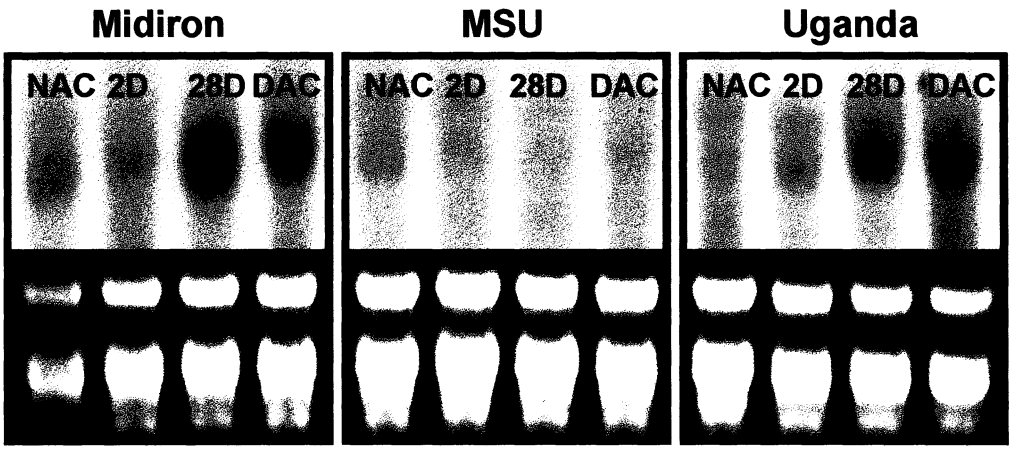


Figure 3. Northern blot analysis of chitinase gene expression in bermudagrass crowns during cold acclimation. Equal amounts of crown tissue total RNA (18 ug) from non-acclimated (NAC), 2-days cold acclimated (2D), 28-days cold acclimated (28D) and deacclimated (DAC) were electrophoresed in formaldehyde-agarose gel and transferred to Hybond N nylon membrane. The filter was hybridized with probes P2 and P3 and autoradiographed for 48 hours.

Figure 4. 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 bands were analyzed by scanning densitometry to compare transcript levels between treatments.


Midiron
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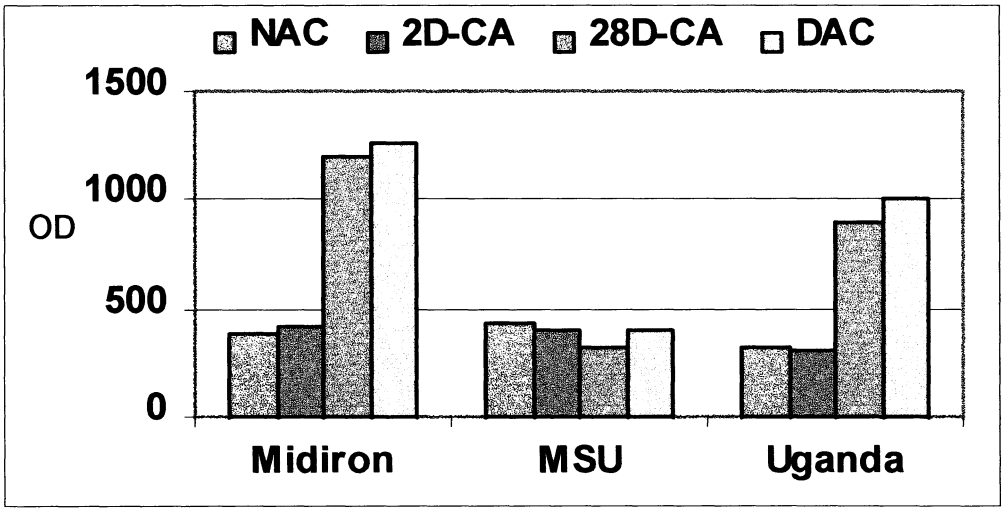
NAC 2D 28D DAC
NAC 2D 28D DAC
NAC 2D 28D DAC

A


NAC
2D-CA
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1500
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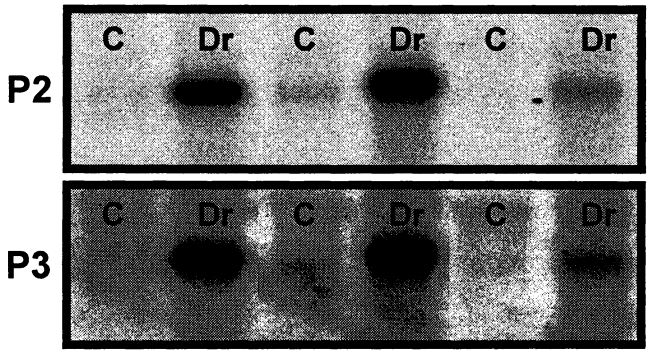


Figure 5. Northern blot analysis of chitinase gene expression in bermudagrass crowns during drought stress. Equal amounts of crown tissue total RNA (18 ug) from control or non-stressed (C) and drought-stressed (Dr) plants were electrophoresed in formaldehyde-agarose gel and then transferred to Hybond N nylon membrane. The filters were hybridized with probes P2 and P3 and autoradiographed for 48 hours.

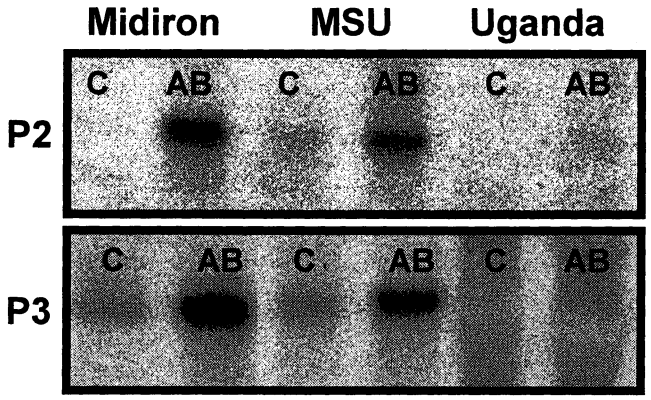


Figure 6. Northern blot analysis of chitinase gene expression in bermudagrass crowns treated with 100 uM ABA. Equal amounts of crown tissue total RNA (18 ug) from control (C) and ABA-treated (AB) plants were electrophoresed in formaldehyde-agarose gel and then transferred to Hybond N nylon membrane. The filter was hybridized with probes P2 and P3 and autoradiographed for 48 hours.

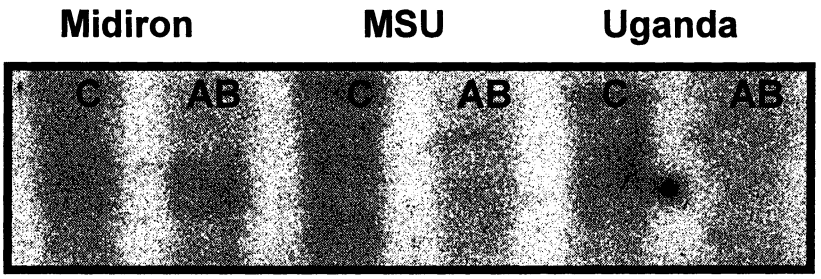
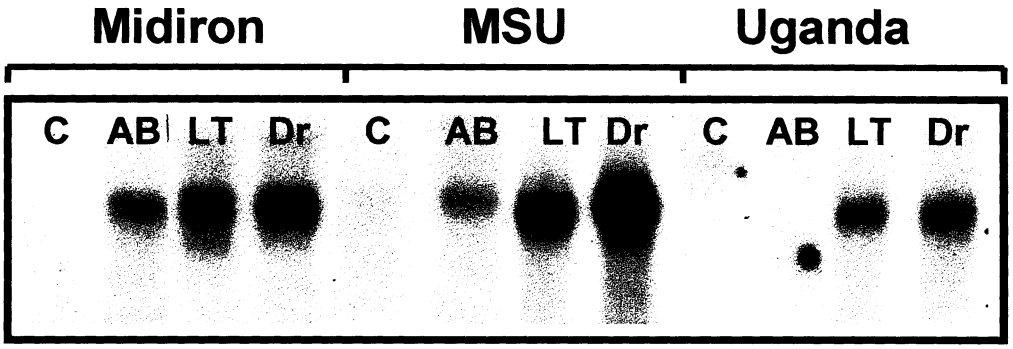


Figure 7. Northern blot analysis of chitinase gene expression in bermudagrass leaves treated with 100 uM ABA. Equal amounts of leaf tissue total RNA (18 ug) from control (C) and ABA-treated (AB) plants were electrophoresed in formaldehyde-agarose gel and then transferred to Hybond N nylon membrane. The filter was hybridized with probe P1 and autoradiographed for 48 hours.

Figure 8. 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, drought-stressed and ABA-treated 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. (B) Northern blot bands were analyzed by scanning densitometry to compare transcript levels between treatments.


Midiron
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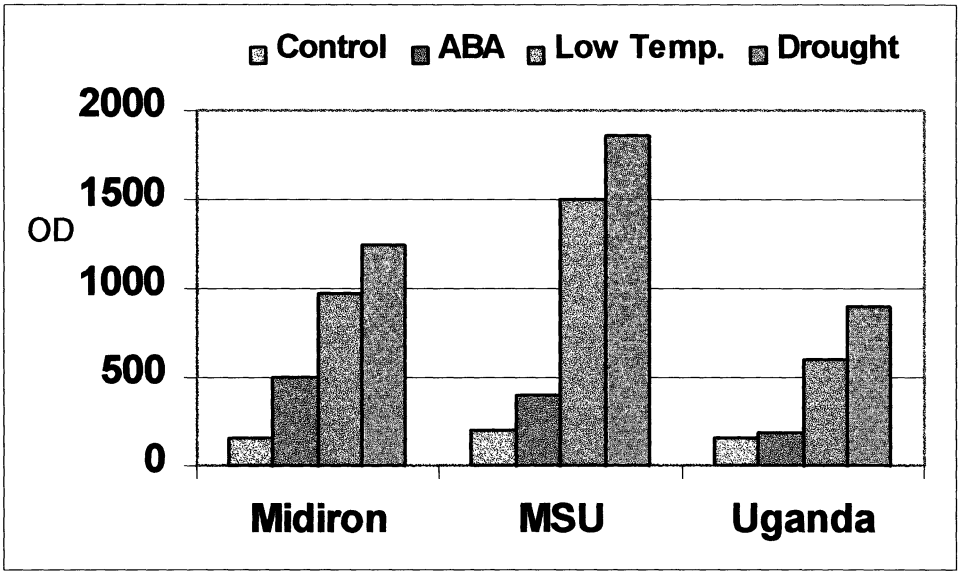
C AB LT Dr C AB LT Dr C AB LT Dr

A


Control
ABA
Low Temp.
Drought
2000
1500
1000
500
0
OD
Midiron
MSU
Uganda

B

CHAPTER IV

Conclusions: Current Status and Future Direction of Bermudagrass Freezing Tolerance Research

The ability of some bermudagrass cultivars to cold-acclimate is an important trait that can be explored towards the development of more cold hardy turfgrasses. The primary goal of this study was to enhance fundamental understanding of the molecular basis of CA and freezing tolerance in the genus *Cynodon*. This objective was addressed in this research through efforts to discover genes that are potentially involved with tolerance mechanisms.

The ability of some plant species to withstand freezing conditions is thought to be due to various mechanisms that either help to avoid, reduce or repair the damage(s) caused by freezing temperatures to the cells and tissues. How these mechanisms definitively work is a question yet to be answered. However, the potential general mechanisms that result in freezing tolerance have been identified to include preventing freeze-induced dehydration damage to cells and reducing direct physical damage caused by the accumulation of intercellular ice. Significant

progress in elucidating how these mechanisms work has been achieved in the past decade, the most notable of which was the discovery of a number of cold regulated (COR) genes, whose expression are coordinately regulated during CA. Although the exact physiological and biochemical functions of the proteins encoded by these genes are still not well understood, there is compelling evidence suggesting that they may be involved in preventing freeze-induced dehydration damage to cells, membranes and molecules (i.e proteins). More recently, an exciting discovery suggested that some plant species synthesize proteins with antifreeze function during cold acclimation. Many of these proteins have been identified as pathogenesis-related proteins. Consequently, this aspect of cold acclimation and freezing tolerance research became the main subject of this investigation mainly because of the following reasons. First, the previous studies of Gatschet et al. (1996), indicated that CA of the freeze-tolerant cultivars 'Midiron' and 'Tifgreen' caused an increased synthesis of specific chitinase proteins in the crowns. Second, Antikainen and Griffth (1997), suggested that the occurrence of the PR proteins with potential antifreeze function may be evolutionarily conserved among cold hardy

monocot species, which include the cereal and grass families.

In line with these facts, and with the major objective of this project, which was to discover genes potentially involved with tolerance mechanisms, two PR protein genes encoding chitinases (*CynCht1* and *CynCht2*) were isolated from the freeze-tolerant cultivar 'Midiron'. The salient features that characterize these genes are consistent with the possibility that they may be involved in freezing tolerance mechanisms in bermudagrass. The data on the temporal and spatial expression patterns strongly indicated low temperature-induced expression in the crown tissues. Accordingly, gene expression persists for as long as the CA conditions remain. Gene specific probes must be designed to be able to investigate and detect specific expression of individual chitinase genes. This experiment will allow investigation of whether the whole gene family or just specific members are responsible for this process.

The degree of induction of chitinase gene expression parallels the level of cold hardiness exhibited by the individual cultivar. The low temperature-induced expression is also specific to the crown, the plant organ that survives and regenerates after freezing. Analysis of the sequences of two chitinase genes indicated that they encode

low molecular weight proteins, which can be structurally classified as class II chitinases. These results have significant implications especially with regard to the hypothesized function of PR proteins as antifreeze molecules.

The proteins encoded by both genes possess the basic structural and spatial requirements for potential antifreeze proteins. However, the data provided by the current investigation are still insufficient to confirm this information. For example, the cellular location of these proteins is an important feature related to their predicted antifreeze function. Computer analysis suggests that the two proteins are hydrophilic and are localized in the intercellular compartments (apoplast) of plant tissues. The cellular location of the proteins encoded by these genes should be investigated experimentally through a combination of immunological and *in situ* hybridization techniques. Furthermore, the predicted sizes of the proteins should also be confirmed by antibody hybridization though western blot techniques.

Several questions must be answered in order to explain the physiological and/or biochemical roles of these proteins in freezing tolerance mechanisms of bermudagrass. These questions include, 1) How does the expression of

these genes respond to low temperature signals during CA? and, 2) Are these PR-proteins also antifreeze proteins?

To address the first question, it will be necessary to investigate the role of potential regulatory elements on the low temperature-dependent expression of these genes. Quite interesting is the presence of the GGCC-box in the promoter regions of *CynCht1* and *CynCht2*. This sequence motif was identified to be responsible for the stress-inducible expression of a number of similar genes in rice. Because of this function, it is logical to speculate that this GC-rich motif may be a regulatory element that also confers low-temperature induced expression. The elucidation of this potential regulatory role requires promoter-fusion studies. Deletion fragments of the chitinase gene promoter may be fused with suitable reporter gene like β-glucuronidase (GUS). The expression of these constructs must be tested by transforming a freezing susceptible bermudagrass cultivar and assaying for reporter gene activity in transgenic plants. The success of this approach will depend on the availability of a reliable genetic transformation system for bermudagrass. An alternative approach is to assay the promoter-reporter activity in a heterologous system like *Arabidopsis thaliana*. This experiment may also open new areas of investigation

including the possible involvement of known regulatory modules that determine low temperature-induced expression of other known COR genes like the DRE-CBF1 module in *Arabidopsis thaliana* (Stockinger et al., 1997; Jaglo-Ottosen et al., 1998).

The second question can be addressed through the use of both molecular genetic and biochemical approaches. The cDNA corresponding to the low-temperature inducible chitinase gene should be isolated and overexpressed in a heterologous system. For example, the cDNA may be fused with a strong constitutive promoter like CaMV35S and expressed in a freezing susceptible strain of *Arabidopsis thaliana*. The antifreeze activity of the proteins encoded by the transgene can be determined by *in vitro* assay for ice nuclei-binding activity using apoplastic fluids extracted from transgenic plants. The results from this biochemical assay should be correlated with changes in cold hardiness in the transgenic plants.

Because freezing tolerance is such a complicated trait, it may be necessary to investigate the different aspects of known low temperature responses to be able to enhance understanding of this biological process. This would require more extensive gene isolation and characterization. Candidate genes which can be investigated

are those involved with fatty acid unsaturation and other genes similar to the COR genes that have already been identified in the dicot systems. This research problem can also be approached on a global basis through the isolation and characterization of the whole battery of genes induced during CA. The DNA microarray technology provides a very powerful tool to address this goal.

The discovery that most plant COR genes contain cis-acting elements (DRE or C-repeat) that are subject to regulation by novel transcriptional activators has began to improve the potential approaches for genetic improvement of plant tolerance to freezing stress. The DRE-CBF1 module for low temperature-dependent regulation of COR genes in *Arabidopsis* is the best example. The potential of using this system to genetically improve freezing tolerance of economically important plant species is very promising because of the ability of the CBF1 gene to coordinately control the expression of a battery of genes required for maximum freezing tolerance, "*a multiple target with a single dart*" approach. There is no evidence to suggest that this system is totally exclusive to the dicot system. Therefore, it will be an interesting research to investigate whether a heterologous system exists in monocot species including *Cynodon*.

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Jaglo-Ottosen, K.R., S.J. Gilmour, D.G. Zarka, Schabenberger, O. and M.F. Thomashow. 1998. *Arabidopsis CBF1* overexpression induces COR genes and enhances freezing tolerance. Science 280:104-106.

Stockinger, E.J., S.J. Gilmour and M.F. Thomashow. 1997. *Arabidopsis thaliana CBF1* encodes an AP2 domain-containing transcriptional activator that binds to the C-repeat/DRE, a cis-acting DNA regulatory element that stimulates transcription in response to low temperature and water deficit. Proc. Natl. Acad. Sci. USA 94:1035-1040.

APPENDIX A

DNA Isolation Procedure (CTAB Method)

Modified from the protocol originally adapted for rice by B.G. de los Reyes, Japan Rice Genome Program/International Rice Research Institute, 1993)

Harvest 4-5 grams of bermudagrass leaves. Immediately put them at -80°C. The leaf tissues can be stored at this point for up to one month. Preheat 2X CTAB at 65°C water bath for at least 30 minutes.

Grind the leaf tissues in liquid nitrogen and then transfer the powdered tissues in 50 ml sterile conical tube. Add 20 ml of 2X CTAB to the powdered tissues. Mix well with a sterile spatula until homogenous. Incubate the mixture at 65°C for 15 minutes and then shake the mixture at 250 rpm at room temperature for 15 minutes. Add 20 ml of chloroform:isoamyl alcohol (24:1) to the mixture. Shake at 250 rpm for 15 minutes at room temperature and then centrifuge the mixture at 3000 rpm for 15 minutes.

Transfer the supernate in a fresh 50 ml sterile conical tube. Use a wide-mouthed pipet tip or sterile disposable aspirator to avoid shearing the DNA. Add 2 ml of 10% CTAB and one volume of chloroform:isoamyl alcohol (24:1). Shake or mix at 250 rpm for 10 minutes at room temperature and then centrifuge at 3000 rpm for 15 minutes.

Transfer the aqueous layer (uppermost layer) in fresh 50 ml sterile conical tube. Be sure not to disturb the interface to avoid pipeting out the protein layer. Use a wide-mouthed pipet tip or sterile disposable aspirator to avoid shearing the DNA. Add 20 ml of CTAB precipitation buffer.

Swirl the tubes gently until the DNA precipitates. Precipitation will be visible as the formation of white thread-like materials. Allow the mixture to stand at room temperature for 5-10 minutes. Pellet the DNA by centrifuging at 3000 rpm for 10 minutes. Decant the supernate and then air-dry the DNA pellet for at least 10 minutes. Add 5 ml of 1M NaCl to the pellet. Swirl the tube gently to allow the pellet to dissolve. Add 5 ul of 1ug/ml RNase to the DNA solution and incubate at 37°C for 10-15 minutes. If the DNA pellet is not completely dissolved, the tubes may be incubated at 65°C water bath for 15 minutes or until the DNA is completely in solution.

Add two volumes of 99% or absolute ethanol to reprecipitate the DNA. Swirl the tube gently to allow DNA precipitation. As in the previous step, precipitation will be visible as the formation of white thread-like materials. Hook the DNA either with a sterile toothpick or with a close-tipped pasteur pipet. Wash the DNA by immersing the

tip of the toothpick or pipet in a microfuge tube containing 70% ethanol. Do this step twice with fresh 70% ethanol each washing.

Air dry the DNA pellet for 5-10 minutes. Immerse the air-dried DNA pellet in a microfuge tube containing appropriate volume of sterile water. Allow the DNA threads to drop into solution. The tubes may be incubated at 65°C water bath for 10 minutes to hasten this process. Determine the DNA quantity by A_{260}/A_{280} reading. Check the DNA quality by running in 1% agarose gel for at least 40 minutes.

Solutions:

2X CTAB

CTAB, 2%	20 g
Tris-HCl (1M, pH 8)	100 ml (0.1M, pH 8)
EDTA (0.5M, pH 8)	40 ml (20 mM EDTA)
NaCl (1.4M)	81.8 g

Complete to 1 liter with distilled water. Autoclave for 15 minutes.

10% CTAB

CTAB	100 g
NaCl (0.7 M)	40.9 g

Complete volume to 1 liter with distilled water. Autoclave for 15 minutes.

CTAB precipitation buffer

CTAB, 1 %	1 g
Tris-HCl (1M, pH 8)	50 ml (50 mM)
EDTA (0.5M, pH 8)	20 ml (10 mM)

Complete the volume to 1 liter with distilled water. Autoclave for 15 minutes.

NaCl (1M)

NaCl (Molec. Biol. Grade)	58.44 g/ liter

Autoclave for 15 minutes.

Chloroform:Isoamyl alcohol (24:1)

Choloroform	24 ml
Isoamyl alcohol	1 ml

APPENDIX B

Construction of Genomic Library

Preparation of insert for Lambda Fix II genomic library

Partial digestion of genomic DNA. Prepare the following reaction mixture to digest 25 ug of genomic DNA with *Bam*HI (0.1 unit/ug DNA).

Genomic DNA sample (5 ug/ul)	5 ul
Reaction buffer (10x)	2.5 ul
*Bam*HI (0.5 unit/ul)	1 ul
Distilled water	16.5 ul
Total volume	20 ul

Prepare two reaction mixtures to digest 50 ug of genomic DNA. Incubate the reaction at 37°C for not more than 1.5 hours. Take 1 ul aliquot from each digestion reaction and run in 1X TAE-agarose gel to check the size range of the digestion product. The digestion product should show a smear ranging from about 8-25 kb in ethidium bromide-stained 0.9% agarose gel. If digestion is sufficient, purify the product by extracting with 0.5 volume of phenol-chloroform. Mix well and then centrifuge for 2 minutes at 12,000 rpm at 4°C. Transfer the aqueous layer into clean tubes. Add 0.1 volume of 3M Sodium acetate and two volumes of ethanol (95%) to precipitate

the DNA. Dissolve in 50 ul of 1x TE buffer.

Partial fill-in of insert DNA. Prepare the following reaction mixture to fill-in the first two nucleotides of the *Bam*HI-digested genomic DNA using 15 units of Klenow DNA Polymerase.

*Bam*HI-digested insert DNA (1 ug/ul)	50 ul
Fill-in buffer, React 2 (10x)	30 ul
dGTP (10mM)	5 ul
dATP (10mM)	5 ul
Klenow DNA Polymerase (4.3 units/ul)	3.5 ul
Distilled water to 300 ul final volume	206.5 ul

Incubate the reaction mixture at room temperature for 15 minutes. Add 33 ul of STE buffer (10x) and 100 ul of TE buffer (1x). Extract with 0.5 volume of phenol chloroform twice and then transfer the aqueous layer into clean tubes. Precipitate the DNA by adding 0.1 volume of sodium acetate (3M) and 2 volumes of ethanol (95%). Centrifuge at 12,000 rpm for 5 minutes at 4°C and then lyophilize the pellet. Resuspend the pellet in 25 ul of 1x TE buffer.

Ligating the insert DNA with Lambda Fix II vector arms

Note: The Lambda Fix II vector arms (Stratagene, Cat. # 248211) are pre-digested with XhoI and already separated from the stuffer fragment. The first two nucleotides of the XhoI sites are filled-in with dCTP and dTTP leaving 3'-CT-5' overhang and are ready to be ligated with the partially filled-in insert DNA.

Prepare the following ligation reaction.

Lambda Fix II vector arms (1 ug/ul)	1 ul
*Bam*HI digested insert DNA (1 ug/ul)	1 ul
Ligase buffer (10x)	0.5 ul
rATP (10 mM, pH 7.5)	0.5 ul
T4 DNA ligase (1 unit/ul)	2 ul
Total volume	5 ul

Incubate the ligation reaction at 4°C overnight. Prepare 3 ligation reactions to make a primary library from 3 ug of genomic DNA. Store at -20°C prior to packaging.

Packaging the primary library

Remove three tubes of Gigapack III Gold packaging extract from the -80°C freezer. Immediately place on dry ice to avoid sudden rise in temperature. Quickly thaw the extracts by holding the tubes between the your fingers

until the content begins to thaw. Immediately add 4 ul of the ligation product (0.8 ug) to the packaging extract. Mix the suspension very gently with a pipet tip. Avoid trapping air bubbles in the mixture as it will decrease the efficiency of packaging. Briefly spin the tubes to collect all the liquids at the bottom.

Incubate the reaction at room temperature for not more than 2 hours. Add 500 ul of SM buffer to the packaging mixture and then mix the contents by gentle pipeting. Add 20 ul of chloroform and mix the contents of the tube by gentle pipeting. Spin the tubes at 12,000 rpm for 1 minute at 4°C to pellet the debris. Transfer the library into clean tubes and then store at 4°C while preparing for titering. The library can be stored at this temperature for not more than one month.

Titering the primary library

Preparing the host bacteria. Prepare two LB-agar plates and then streak the XL1 Blue MRA and XL1-Blue MRA (P2) from the glycerol stock to separate plates. Grow the cultures by incubating overnight at 37°C.

Prepare LB broth supplemented with 0.2 % (w/v) maltose and 10 mM $MgSO_4$. Inoculate 50 ml of LB-maltose-$MgSO_4$ with a single colony of each host strain from the fresh plates.

Grow the cultures at 37°C with shaking (250 rpm) for 4-6 hours. Alternatively, the cultures may be grown at 30°C overnight with shaking at 200 rpm. These conditions allow the bacteria to grow up to a density of not more than OD_{600} = 1.0. Overgrowing the bacteria past OD_{600}= 1.0 will result in high number of non-viable cells which will decrease the titer of the library. Pellet the bacteria by centrifuging at 500g for 10 minutes at room temperature. Gently resuspend the cells in half the original volume (25 ml) using sterile 10mM $MgSO_4$. Dilute the cell suspension to OD_{600} = 0.5 using sterile 10mM $MgSO_4$.

Plating. Prepare ten LB-maltose (LB + 0.2% maltose + 10mM $MagSO_4$) plates in 80 mm petri dishes. Incubate the plates containing the media at 37°C at least 1 hour prior to plating for equilibration. Prepare NZY-agarose and equilibrate at 48°C at least 1 hour before plating. Prepare a serial dilution of the primary library (10^0, 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}). Mix 1 ul of the serially diluted packaged library sample with 200 ul of host bacteria, which had been diluted, to OD_{600}=0.5 with SM buffer (XL1-Blue MRA and XL1-Blue MRA (P2)). Add 3 ml of NZY-agarose (48°C), mix and pour immediately onto the LB-maltose plates. Allow the plates to

solidify for 10 minutes. Incubate the cultures for not more than 12 hours at 37°C.

Count the number of plaques per plate. Use the dilution factor to calculate the titer in plaque forming units (pfu) per milliliter. Both the recombinant and non-recombinant phages will grow in XL1-Blue MRA, but only the recombinant plaques will grow on XL1-Blue MRA (P2).

Amplification and storage of the library

Prepare the host strain as described in the titering steps. Combine 600 ul of host cells with library suspension containing 5 x 10^4 pfu. Mix with 7 ml of NZY-agarose (48°C). Pour the mixture on 150 mm LB-maltose plate, and allow to solidify for 10 minutes. Grow the phages at 37°C for not more than 12 hours.

Overlay the plates with 10 ml of SM buffer and incubate overnight at 4°C with gentle shaking to allow the phage particles to diffuse to the SM buffer. Harvest the bacteriophage suspension and pool into a clean 50 ml polypropylene tube. Add chloroform to a final concentration of 5% (v/v). Mix well and then incubate for 15 minutes at room temperature. Remove the cell debris from the phage suspension by centrifuging the mixture at 500xg for 10 minutes. Transfer the supernate into clean polypropylene

tube and add chloroform to a final concentration of 0.3% (v/v). Store aliquots of the amplified library in 7% (v/v) DMSO at -80°C.

Solutions

10X Fill-in buffer

60mM Tris-HCl (pH 7.5)

60mM NaCl

60mM $MgCl_2$

0.5% gelatin

10mM dithiothreitol (DTT)

10X Ligation buffer

500mM Tris-HCl (pH 7.5)

70mM $MgCl_2$

10mM Dithiothreitol

LB broth (per liter)

NaCl	10 g
Tryptone	10 g
Yeast Extract	5 g

Add deionized water to 500 ml. Adjust to pH 7.0 with 5N NaOH and then complete the volume to 1 liter. Autoclave

for 15 minutes. To prepare the LB-Agar, add agar to LB broth at a final concentration of 2% (w/v).

NZY broth (per liter)

NaCl	5 g
$MgSO_4$-$7H_2O$	2 g
Yeast Extract	5 g
NZ Amine	10 g

Add deionized water to 500 ml. Adjust to pH 7.5 with NaOH and then complete the volume to 1 liter. Autoclave for 15 minutes. To prepare the NZY-Agarose, add agarose to NZY broth to a final concentration of 1.5% (w/v).

SM buffer (per liter)

NaCl	5.8 g
$MgSO_4$-$7H_2O$	2 g
1M Tris-HCl (pH 7.5)	50 ml
2% (w/v) Gelatin	5 ml

Add deionized water to a final volume of 1 liter. Autoclave for 15 minutes.

10X STE buffer

1M NaCl

200mM Tris-HCl (pH 7.5)

100mM EDTA

TE buffer

10mM Tris-HCl (pH 7.5)

1mM EDTA

Appendix C

Screening the library

Plaque lifts. Plate the library on 150mm LB-Agar plates at a density of 5 x 10^4 pfu as described in the titering steps. Grow the phages at 37°C for not more than 12 hours. Do not allow the plaques to overlap. Chill the plates at 4°C for at least 4 hours to harden the media.

Place the first Hybond N^+ nylon membrane onto the plate. Mark the position of the membrane by pricking the media with a 15-gauge syringe containing black india ink. Allow the phage particles to transfer onto the membrane for 2 minutes. Remove the first membrane and place the second (duplicate) membrane and put the ink marks. Allow the phages to transfer to the membrane for 4-5 minutes.

Washing. After lifting, subject the membranes to denaturing conditions by immersing in solution containing 1.5M NaCl and 0.5M NaOH for 2 minutes. Neutralize the membranes by rinsing for 5 minutes in a solution containing 1.5M NaCl and 0.5M Tris-HCl (pH 8.0). Rinse the denatured membranes for 30 seconds in solution of 0.2M Tris-HCl (pH 7.5) and 2X SSC buffer.

Dry the membranes by blotting on clean Whatmann No.3 filter paper. Cross-link the membrane by baking at 80°C for not more than 1 hour.

Probe labeling. Dilute 25 ng of probe DNA in 45 ul sterile distilled water. Denature the double stranded DNA by boiling in water bath for 7 minutes. Immediately quench in ice bath. Transfer the denatured probe into a tube containing the labeling cocktail (dATP, dGTP, dTTP, Klenow DNA Polymerase, Klenow Buffer) (Rediprime Kit). Mix the content of the tube and then add 5 ul of α^{32}P-dCTP (5 uCi). Mix and incubate for 1 hour at room temperature. Remove unincorporated nucleotides using sephadex spin columns. Measure the radioactivity by scintillation counting. Denature the labeled probe in 100°C hot plate before hybridization.

Hybridization and washing. Prepare the prehybridization buffer as follows.

20X SSPE	12.5 ml
100X Denhardt's solution	2.5 ml
10% (w/v) SDS	2.5 ml

Complete the volume to 50 ml with sterile distilled water. Add 1 ml (1mg/ml) sonicated salmon sperm DNA. Preincubate

at 56°C before putting the membranes. Two pairs of duplicate membranes can be palced in hybridization reaction. Prehybridize for at least 1 hour at 56°C with gentle shaking. Hybridize overnight under the same conditions.

After 16 hours hybridization, perform the low stringency washes twice for 10 minutes in 2X SSC + 0.1% SDS at room temperature. Transfer the filters in 0.5X SSC + 0.1% SDS and wash twice with gentle shaking at 56°C for 10 minutes. Perform the final washing twice in 0.25X SSC with gentle shaking at 56°C for 10 minutes. Autoradiograph the filters for at least 48 hours at -80°C.

Solutions

20X SSPE

3.6M NaCl

0.2M Sodium phosphate

0.02M EDTA (pH 7.7)

20X SSC

3M NaCl

0.3M Sodium Citrate

Adjust to pH 8.0

100X Denhardt's solution

2% (w/v) bovine serum albumin (BSA)

2% (w/v) ficoll

2% (w/v) polyvinylpyrrolidone (PVP)

Appendix D

RNA isolation by Guanidine-HCl Method

Note: Adapted from the procedure of Logemann et al., 1997: Anal. Biochem. 163:16-20. All glasswares (i.e. corex centrifuge tubes) must be pre-baked at 200oC overnight

Homogenize 2.5 g of tissues in liquid nitrogen until powdered. Add two volumes of 8M Guanidine buffer for every 250 mg of tissues. Mix until thick slurry is produced. Transfer the mixture into clean 30 ml corex tubes. The mixture can be stored at -80°C at this point.

Incubate the slurry at room temperature for 5 minutes and then mix by vortexing at low speed. Centrifuge the extract at 10,000 rpm at 4°C for 15 minutes. Collect the supernate into clean corex tubes and then add one volume of phenol:chloroform:isoamyl alcohol. Mix by vortexing at medium speed and keep at room temperature for 5 minutes.

Centrifuge the mixture at 10,000 rpm for 10 minutes at 4°C. Transfer the aqueous layer into clean corex tubes. Add 1 volume of phenol:chloroform:isoamyl alcohol. Mix by vortexing at medium speed and then centrifuge at 10,000 rpm for 10 minutes at 4°C. Transfer the aqueous layer into clean corex tubes. Add one volume of absolute ethanol and 0.2 volume of 1M acetic acid. Swirl gently to precipitate the

total RNA. At this point, high molecular DNA and residual proteins will be left in solution.

Incubate the solution at -70°C for 1 hour. Centrifuge the mixture at 10,000 rpm for 5 minutes at 4°C. Discard the supernate and then wash the pellet twice with 70% ethanol. Air-dry the pellet for at 15 minutes. Dissolve the pellet in 5 ml DEPC-treated water. Add 2 units of RNase-free DNase I and then incubate for 1 hour at 37°C.

Reprecipitate the total RNA by adding 300 ul 4M LiCl and two volumes of absolute ethanol. Incubate the mixture for 30 minutes at -20°C. Centrifuge at 10,000 rpm for 10 minutes at 4°C to pellet the RNA. Discard the supernate and wash the RNA pellet twice with 70% ethanol. Dissolve in appropriate amount of DEPC-treated water or RNase-free TE buffer depending on the amount of the pellet (250-1000 ul). Determine the quantity and quality of total RNA by spectrophotometry and by analyzing fragment sizes in EtBr-stained formaldehyde-agarose gel.

Solutions

8M Guanidine buffer

Guanidine-hydrochloride	76.4 g
MES	0.44 g
EDTA-$2H_20$	0.76 g

Dissolve all the components in 50 ml nanopure water (not necessarily DEPC-treated) and then adjust to pH 7.0. Complete the volume to 100 ml with nanopure water. Add 180 ul 8M mercaptoethanol and then store at 4°C.

Phenol:chloroform:isoamyl alcohol

Liquid Phenol (in buffer, pH 4.3)	25 ml
Chloroform	24 ml
Isoamyl alcohol	1 ml

Store in amber bottle at -20°C.

1M Acetic acid

Glacial acetic acid	7.73 ml
DEPC-treated water	94.27 ml

4M Lithium chloride

Lithium chloride	16.95 g
DEPC-treated water	100 ml

VITA

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