

**BIOCHEMICAL AND MOLECULAR ANALYSIS OF COLD
ACCLIMATION IN BERMUDAGRASS (*Cynodon spp.*)**

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EXECUTIVE SUMMARY

Disruption of cellular membrane integrity, as a result of low temperature-induced water removal, is a primary cause of cold injury. The lipid bilayer (Fig 1) provides the necessary environment for proper functioning of membrane proteins. Membrane lipid "fluidity" is thought to be one of the prerequisites for unimpaired survival at low temperature. Membrane fluidity is affected by the degree of unsaturation (i.e., number of double bonds) in the constituent fatty acid (FA) side-chains of membrane lipids. These double bonds induce "kinks" in the molecules (Fig. 2), thus resisting molecular compaction (Fig. 3), and maintaining fluidity. Therefore, increase in FA unsaturation reduces the temperature at which membranes undergo damaging, dehydration induced, phase transitions from a flexible to more rigid, gel-like, state.

'Midiron' and 'U3' bermudagrass were exposed to conditions that induce cold acclimation, and crown tissue from rhizomes was harvested for total membrane lipid isolation. Fatty acid analysis of total lipids revealed a significant increase in tri-unsaturated species (i.e., linolenic acid; C18:3), over shorter and saturated species, and an overall increase in the double-bond index. These changes were more pronounced in 'Midiron' (relatively cold tolerant) than in 'U3' (relatively cold sensitive) bermudagrass, during cold acclimation. Preliminary biochemical analysis of the isolated membrane lipids identified neutral lipids, glycolipids and phospholipids. At least four species of neutral lipids were detected, two of which were in abundance. Three glycolipids species were detected, in equimolar amounts. Phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidyl glycerol (PG) and phosphatidylserine (PS) were the four phospholipids detected. Their relative abundance is $PC \gg PE = PG = PS$. Exact species determination, quantification, and FA and intermolecular positional analysis during cold acclimation in the two cultivars is currently under investigation.

Differential display is used to identify genes expressed in bermudagrass during cold acclimation. Comparisons of "displays" from 0, 12, 24, 48 and 72 hrs post-low temperature exposure to that from non-acclimating tissue over the same time period, allows for the identification of genes differentially expressed between treatments, timepoints and/or cultivars. The cDNA clones obtained represent both "RAPD"-type fragments, those with 10-mer/complimentary sequences on the ends, as well as gene fragments derived from amplifications utilizing both the anchor (e.g., T₁₁AG or T₁₁AC) and a specific 10-mer. The latter have an easily recognizable poly(A) addition signal, just 5' of the anchor primer sequence. The clones range in size from 300 to 450 bp. All were chosen as "up-regulated" genes from differential display gels. Searching DNA and protein databases with translations of these sequences failed to show significant homology to any previously cloned gene or protein/peptide sequence. This is to be expected for at least two reasons: (i) since the clones are biased toward the 3'-untranslated end of a gene transcript, protein databases or those containing gene sequences from genomic or random-primed cDNA libraries may not have sequence data for this region of any of their clones; (ii) since, to date, few genes whose expression is regulated during cold acclimation have been cloned from any organism, such genes would be under-represented in current databases (i.e., we are cloning new, undiscovered genes).

Expenditure Summary:

Salaries: (graduate student) \$2,500, (hourly student) \$2,750; Fringe Benefits: \$50; Supplies: \$6,415; Total = \$11,715

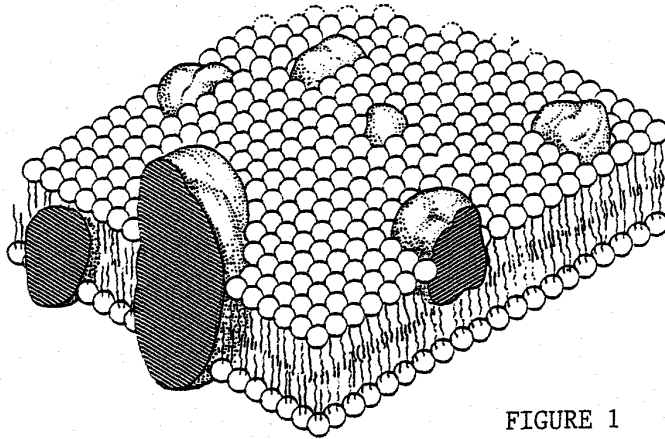


FIGURE 1

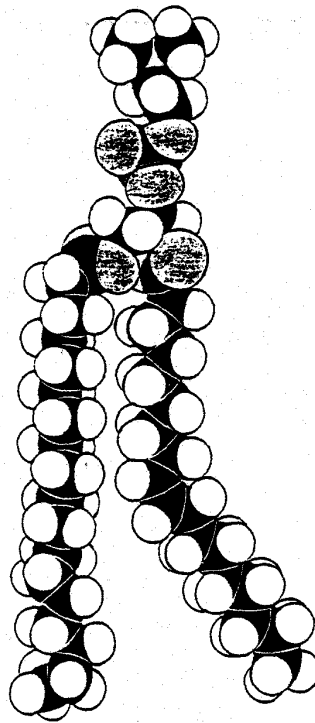
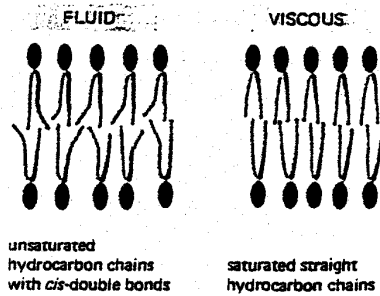


FIGURE 2



Double bonds in unsaturated hydrocarbon chains increase the fluidity of a phospholipid bilayer by making it more difficult to pack the chains together.

FIGURE 3

ANNUAL REPORT (11/1/95 to 10/31/96)

INVESTIGATOR: Wm. Vance Baird (33% of research program)

INSTITUTION: Clemson University, Horticulture Department

C. U. PROPOSAL No: R 0375-96-0295

SPONSOR: United States Golf Association, Green Section Research

PROJECT: Biochemical and Molecular Analyses of Cold Acclimation in Bermudagrass [*Cynodon* spp.]

OBJECTIVES:

- (1) Characterize membrane-specific changes in fatty acid and lipid composition during cold-acclimation.
- (2) Characterize the expression of genes involved in fatty acid and lipid biosynthesis during cold acclimation, using heterologous gene probes.
- (3) Clone fatty acid biosynthesis genes (e.g., desaturases) expressed in bermudagrass in response to low temperature.

The primary goal of this project is to gain a more complete understanding of the process of cold acclimation in bermudagrass, by characterizing the changes in membrane composition and gene expression that accompany exposure to low temperature. Previous experience has shown crowns and stolon nodes to be biologically and physiologically appropriate tissue for the proposed studies, confirmed the utility of 'Midiron' and 'U3' germplasm in these analyses, and provided insight as to the important enzymes regulating fatty acid and lipid composition of cellular membranes.

INTRODUCTION

Environmental stress occurs in many forms such as low temperature or water deficit. The physical and biochemical changes, which occur in chilling sensitive plants exposed to reduced temperature, together with the subsequent expression of low temperature stress symptoms, are collectively referred to as cold or chilling injury. The physiological changes include alterations in cytoplasmic streaming, enzyme activity, respiration, photosynthesis as well as effects on membrane permeability, structure and composition. Which if any of these changes is responsible for the primary low temperature-induced injury remains uncertain.

It is clear, however, that disruption of cellular membrane integrity, as a result of low temperature-induced water removal, is a primary cause of cold injury. The membrane lipid bilayer (Fig. 1) provides the necessary environment for proper functioning of proteins and enzymes associated with a particular membrane system. Maintenance of

a fluid state for membrane lipids is thought to be one of the prerequisites for unimpaired survival at low temperature.

Physical properties such as flexibility and molecular motions of membrane diacylglycerols (e.g., polar phosphoglycerides), depend, to a large extent, on the degree of unsaturation (i.e., number of double bonds) in their fatty acid (FA) side chains. Double bonds produce bends or kinks (Fig. 2), that collectively enable membranes to resist damage at low temperature. Damage results from the detrimental effects of molecular compaction, partitioning and the formation of flexibility-limiting molecular contacts between adjacent lipid molecules (Fig. 3). Thus, changes in FA unsaturation can affect biophysical properties like the temperature at which membranes undergo dehydration induced phase transitions (i.e., flexible/fluid ---> rigid/gel).

Early work in woody plant species, was the first to propose that cold acclimation and low temperature survival involve fundamental changes in the physiology of the plant at the level of gene expression. This concept was extended in studies of cold acclimation in spinach. Here, researchers obtained very high correlation coefficients between levels of expression of cold-induced genes and the degree of freezing tolerance in acclimated alfalfa. Since then, several workers have documented a causal relationship between exposure of plants to low temperature and increases in gene expression or induction of previously unexpressed genes. Whether these genes are biologically significant in conferring tolerance to the effects of low temperature, e.g., code for enzymes involved in lipid or FA metabolism, and whether they have homologs in bermudagrass or other turf species remains unknown at this time. One notable exception is the recent identification of a category b-II chitinase, with presumptive anti-fungal and/or cryo-protective properties, expressed in crowns of bermudagrass in response to cold.

A better understanding of the nature and basis for tolerance to low temperature and the process of cold acclimation in bermudagrass will be very useful for programs focused on germplasm improvement, whether through breeding or biotechnology.

Analysis of Membrane Lipid Composition During Cold Acclimation

(Mr. Suresh Samala and Ms. Jenith Cyril; Doctoral students)

Physiological studies have correlated changes in the composition of membrane lipids with survival of exposures to low and subsequently to lethal temperatures. Expanding on our earlier results with bermudagrass (e.g., identifying specific alterations in the fatty acid composition of polar membrane lipids), we are now investigating the composition of membrane lipids, and their specific fatty acid components, in the rhizome crowns of 'Midiron' and 'U3'. Samples were harvested at 0, 7, 14, 21, and 28 days of cold treatment, and processed for the isolation of total lipids.

Total lipids are broadly classified into three groups. These are the phospholipids (including phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylglycerol, phosphatidylinositol, phosphatidic acid); the glycolipids (including monogalactosyldiglycerides, digalactosyldiglycerides, sterylglucosides, acylated sterylglucoside and cerebrosides); and the neutral lipids (including sterols and

triacylglycerols). Total lipids from bermudagrass crowns were fractionated into these three major groups, and then further separated into their constituent classes by thin layer chromatography. Individual lipids within each class are identified by co-chromatography with commercially available authentic standards, and the use of specific spray detection reagents to reveal their presence and relative abundance on the thin layer chromatography plates.

Among the phospholipids group we detected the presence of four lipid classes. These were, in order of relative abundance phosphatidylcholine >> phosphatidylethanolamine = phosphatidylserine = phosphatidylglycerol. Not surprisingly, our preliminary evidence indicates that these phospholipid components are the same for both 'Midiron' and 'U3'. Among the glycolipids isolated from the two bermudagrass cultivars we detected the presence of only three lipid classes. Their specific identification is not yet complete, but preliminary analysis indicates the presence of sterylglucosides, acylated sterylglucoside and cerebrosides. These three glycolipids were detected in relatively equimolar amounts. Neutral lipids were by far the least abundant class of the three groups of membrane lipids isolated from membranes. Because of the low abundance, identification of individual lipid species within this class is of low priority at this time.

We want to direct future efforts at (1) fatty acid species analysis of each phospholipid class; (2) quantification of the three different groups of lipids; (3) intramolecular positions analysis (e.g., *sn-1* and *sn-2*) of the fatty acyl moieties; (4) molecular species of cerebrosides (glycolipids) will be separated by HPLC using a C18 reverse phase column and their identification will be accomplished using the mass spectrum obtained by GC-Mass Spectrometer analysis; (5) analysis of free sterols species will be accomplished by GC using an HP-1 capillary column; (6) fatty acid analysis of other bermudagrass cultivars (e.g., Tifton 96, Tifgreen, Brookings?) and/or other turfgrass species that show variation in cold hardiness (e.g., seashore paspalum, *Paspalum vaginatum* Swartz.). Overall, these chemical analyses will be performed on material from the five time points during the experimentally induced cold acclimation process, and compared/contrasted over time and cultivar.

Differential Gene Expression During Cold Acclimation

(Mr. John Wells, Research Technician)

Work continued using differential display (DD-RT-PCR) to screen for changes in genes expression (i.e., up or down regulation) in response to low temperatures during cold acclimation. Titration of polymerase chain reaction (PCR) amplification parameters (e.g., primers, MgCl₂, Taq polymerase, dNTP and oligonucleotide concentrations) was performed to optimize cDNA synthesis, the amplification procedure, as well as removal of amplified DNA from the gel matrix and subsequent re-amplification. For example, we have concentrated on two anchor primers, but are screening each with the "standard" 25 10-mer primers to determine if any pattern for predicting effective combinations arises. From this round, a half dozen bands representing putative differentially expressed mRNA's from primer pairs (Anchor:10-mer); T₁₁AG:1, T₁₁AG:20 and T₁₁AC:22 were cloned into the pNoTAT/7 cloning vector.

Further optimization included the use of multiple lane loading to increase template concentration. This reduced the need for two-fold amplification of expressed RNAs (as cDNAs), a possible mitigating factor in cloning exclusively "RAPD-type" fragments (see below). Use of anchor-primers with a variable base in the penultimate position (i.e., T₁₂VN; where V is an equimolar mixture of G, A, and C, and N is any one of G, A, T, or C) is being employed to more rapidly screen all possible anchor-primer choices. To date anchor-primers T₁₂VG and T₁₂VC have been the most successful when paired with the various 10-mer downstream primers, consistent with the unique anchor-primers used initially. Over 70 variable anchor:10-mer primer pairs have been screened on total RNA isolated from 0 hr, 12 hr, 48 hr (and some at 72 hr and 7 day as well) cold acclimating bermudagrass crowns. Of these, nearly two dozen have exhibited differential gene expression, displaying one to three "polymorphic" bands.

DNA sequencing and nucleotide sequence analysis of the original cloned differential display products (ranging from 300 to 450 nucleotides in length), using an Applied Biosystems, Inc. automated sequencer and GeneWorks software, identified sequencing primer sites, vector restriction sites, and 10-mer differential display primer sites in all clones. However, the 3' anchor-primer sites were "missing" from many of the clones. For example, two of the clones, amplified from different primer pairs, were identified as essentially identical. In these and two other cases, complementary sequence of the 10-mer primer was found. This indicates that the clones were likely to be "RAPD" fragments, resulting from amplification by 10-mer primers only. This was confirmed by re-amplification of either the original isolated fragments, or their plasmid clone, using only 10-mer primers without combination with an anchor-primer. To recognize and/or avoid such products, characterization of PCR amplification products now includes the use of 10-mer primers only in re-amplification 'control' reactions.

Additional clones have revealed the expected 10-mer and anchor primer on opposite ends of the DNA fragment. In addition, in these clones the poly(A) addition signal (e.g., AAUAAA) can be identified just upstream of the anchor primer. The sequences obtained from all these clones were computer translated, in three frames, to convert from nucleotide to amino acid (~peptide/protein) sequence. These sequence data were used to search protein/gene data bases. However, to date none of the clones show significant homology to any previously cloned genes or sequenced proteins. This is not to surprising, and is actually somewhat expected for at least two reasons. First, in light of the length of the clones compared to typical length of 3'-untranslated regions, protein data bases or those containing genes from genomic or random-primed cDNA libraries may not have sequence data for this region of any of their clones. Second, and more exciting, it is a fact that, to date, few genes whose expression is regulated by cold acclimation have been cloned from any organism, and therefore would not exist in present data bases.

To confirm the differential expression of these clones Northern and "reverse" Northern hybridizations are being performed. This will give us information about the full-length nature of the gene transcripts from which our clones arose, thus providing insight to the development of improved cloning methods. Finally, these analyses will give us a measure of the relative change in gene expression between clones and between cultivars. Poly(A)-enriched RNA fraction was selected, by oligo(dT) chromatography, from isolations of total RNA prepared from the cultivars, treatments, tissues and collection times mentioned above. This RNA was size fractionated and transferred to

membranes for hybridization with each clone. Alternatively, we will fractionate a complete set of clones, including a dilution series, and transfer them to a membrane(s), for subsequent hybridization with radiolabeled RNA as the probe.