EXECUTIVE SUMMARY

PROJECT:	Low temperature and drought regulated gene expression in bermudagrass
INVESTIGATOR(S):	Wm. Vance Baird and Landon C. Miller
INSTITUTION:	Clemson University (Horticulture Department)
SPONSOR:	United States Golf Association, Green Section Research

'Midiron' and 'U3' bermudagrass were exposed to conditions known to induce cold acclimation [e.g., 8/3°C (d/n) temperature, 10 hr photoperiod, 250 μmol/m^2/s photosynthetic photon flux density] for one or four week time periods. Tissues (leaves, crowns and roots) were harvested and total lipids isolated by organic extraction. The polar fraction was purified by thin layer chromatography. The fatty acids (FA) were released from the polar lipids by saponification, and then converted to FA-methyl esters. Separation and identification of individual FAs within the mixture was accomplished by gas chromatography, with the aid of an automated, computer-based identification system. Different organs of the same plant responded differentially to low temperature. Crowns showed the most dramatic and sustained increase in total FA content. Overall, significantly greater than 95% of the polar FA content was accounted for by four FA species: palmitic acid (16:0), stearic acid (18:0), linoleic acid (18:2) and linolenic acid (18:3). 'Midiron' (relatively cold- tolerant) responded more rapidly and to a greater extent than did 'U3' (relatively cold-sensitive) for the changes in FA composition documented in this study. This was illustrated by the nearly four-fold increase of unsaturated:saturated FA ratio for "Midiron' over. 'U3', and by the significant difference between the double bond index of the two genotypes, at the end of the cold acclimation treatment. Our results point to specific desaturase enzymes (e.g., ω-3 and Δ-12) as being of primary importance in controlling membrane lipid-FA composition in response to low temperature, and ultimately in avoiding the winter damage suffered by bermudagrass along its northern zone of adaptation.

Differential display / RNA profiling has been used to identify genes expressed in bermudagrass in response to low temperature (i.e., during the process of cold acclimation). This procedure uses total or poly(A)+ RNA as a substrate for the synthesis of cDNA molecules (i.e., partial gene clones) expressed in the particular organ, developmental stage or treatment from which the RNA was isolated. Additionally, this method uses a set of "random" anchor and 10-mer oligonucleotide primers to initiate clone synthesis. By comparing gel "displays" from 0, 24 and 48 hrs post-low temperature exposure (as above) to that from control non-acclimating tissue over the same time frame, four sequences putatively expressed in response to low temperature were identified. These have been subcloned for further analysis and characterization; such as DNA sequence determination and use as probes on Northern blots to confirm their differential, cold-specific expression. In addition to continuing this avenue of investigation, we are designing degenerate (sequence variable) oligonucleotide primers to conserved sequence regions of fatty acid biosynthesis genes for use in differential display experiments and in reverse transcriptase-polymerase chain reaction (RT-PCR) analysis.

FINAL REPORT

INVESTIGATOR(S):	Wm. Vance Baird and Landon C. Miller
INSTITUTION:	Clemson University
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SPONSOR:	United States Golf Association, Green Section Research
PROJECT:	Low temperature and drought regulated gene expression in bermudagrass

Four cultivars of bermudagrass (Midiron, U3, Tifgreen and Tifway) were chosen as experimental material in screens for the presence of low-temperature and/or drought induced gene sequences in the bermudagrass nuclear genome. Southern blot membranes containing restriction endonuclease (*Eco*RI, *Hin*dIII, and *Pst* I) digested total genomic DNA from each cultivar were prepared. Preparation of genomic DNA was at first problematic, but is now a routine operation. Twelve plasmid sub-clones of genomic or cDNA clones, expressed during exposure to low temperatures or water deficit in other plant systems (i.e., Arabidopsis, barley, maize, spinach, tobacco, tomato and wheat), were used as heterologous gene probes. Because of the heterologous nature of the probes, molecular hybridizations were performed at moderate to low stringency in hopes of detecting related sequences in the bermudagrass genome, while avoiding spurious cross-hybridization signals.

Four categories of hybridization signals were observed -- based on homology between the heterologous gene clones and sequences in the nuclear genome of the four bermudagrass cultivars. One class detected homologous gene sequences (more than one fragment), and the overall banding patterns showed variation between the four bermudagrass cultivars (i.e., restriction fragment length polymorphisms = RFLPs). These probes may be useful in comparisons between germplasm (e.g., exotic accessions, hybrids, segregating populations, etc.) for the genetic variation associated with their potential response to low temperatures and/or drought conditions. The best example of a clone in this class is a cold regulated gene sequence (from *Arabidopsis*), which hybridizes to between 5 and 7 fragments (i.e., gene family) in the bermudagrass genome depending upon the cultivar and restriction enzyme used.

Similarly, a second class detected homologous sequences in the bermudagrass genome, but showed little or no variation between the four cultivars. Some of the members in this class detected ten or more homologous sequences in the bermudagrass genome, occasionally revealing minor hybridization pattern differences between the genotypes. These gene clones represented cold regulated sequences from *Arabidopsis* and wheat. One member, a drought-induced gene sequence from tobacco, detected only a single fragment and it was monomorphic (no variation) in all four genotypes.

The third class (with only a single member, a sequence from *Arabidopsis* whose transcript accumulates in response to low temperature) was interesting in that it showed a very strong hybridization signal to only a single sequence in a single cultivar (U3).

The biological significance of this finding is unknown. It may be the result of cross-hybridization to an unrelated but highly abundant sequence or, more interestingly, it may be related to a unique physiological capability of the 'U3' genotype.

A fourth group of clones showed no significant homology to nuclear DNA sequences in bermudagrass, even at low hybridization stringency. Surprisingly, four of these clones (three from tomato and one from corn) are induced by drought/dehydration or by the exogenous application of the plant hormone abscisic acid (ABA). These heterologous gene clones were deemed uninformative, and will not be used in future analyses.

Heterologous gene clones from the first and third class have been used as probes on Northern blots -- a moderately sensitive measure of gene expression (for steady-state transcript levels, rather than actual transcription rates). The membranes contain 2 ug of poly(A)$^+$ and 20 ug of poly(A)$^-$ RNA from cold acclimated (12, 24, 48 and 72 hr) and control (0 and 72 hr) 'U3' plants, along with molecular size standards and hybridization "control" sequences. To date we have not detected any significant expression using one clone from either class. This may be due no expression of sequences "homologous" to these "stress" gene probes under our conditions of cold acclimation, or to very low levels of expression -- and therefore the use of more sensitive methods [e.g., reverse transcriptase-polymerase chain reaction (RT-PCR) or RNA protection assays] may be necessary. However, the latter explanation would be surprising due to the strong hybridization signals detected on Southern blots. Another explanation is that because the Northern blots used lacked 'Midiron' RNA, the stress gene clones would not be detected as their expression in response to low temperature is unique to the more cold-tolerant 'Midiron' cultivar. This is a more likely explanation, but a little surprising for the single class-three clone that had homology to 'U3' sequences only. Finally, it could be that these genes are expressed, not in the early stages of cold acclimation, but rather are present (accumulate to detectable levels) only after longer exposures to cold acclimating conditions. Probings of Northern blots is continuing, and will include RNA from both 'Midiron' and 'U3", as well as RNA from later stages of cold treatment (e.g., 2, 3 and 4 weeks) of these two cultivars.

The principle target of chilling and freezing injuries is the cellular membranes, and a primary component of these membranes is the lipid bilayer. We have investigated temporal and quantitative changes in fundamental membrane lipid components, the fatty acids, that occur during cold acclimation in bermudagrass. This type of information, although representing physiological parameters considered central to surviving extended exposures to low temperature, is currently unknown for many plants. This is especially true among the numerous turfgrass species and their cultivars. As mentioned above, we have restricted our investigations to 'Midiron' and 'U3'.

Lipid extraction and fatty acid purification procedures were optimized for leaf, root and crown tissue. The plants were grown essentially as reported by Anderson *et al.* (HortSci. 28:955, 1993), where cold acclimation conditions included an 8/2 °C day/night temperature, 10 hr photoperiod and 250 μmol/m^2 /s PPFD. This was accomplished in controlled environment chambers. Total lipids were extracted from whole organ homogenates by sequential organic extraction (chloroform). Polar lipids were isolated by the development of thin layer chromatography plates, spotted with total lipid extracts, in a non-polar solvent. Fatty acids were released from the polar lipids by saponification, and converted to volatile fatty acid methyl esters for analysis by gas chromatography

(GC) and mass spectroscopy. Individual fatty acid species were identified by their retention times, using an automated computer-based microbial identification system (aerobic database). Fatty acids not present in the GC database were identified by analysis on the mass spectrometer.

Our findings showed that different organs of the same genotype responded differentially to low temperature. Crowns showed the largest sustained increase in polar fatty acid content (ug per mg of total lipid) over the four week experiment, especially when contrasted to leaf tissues. Even the response in roots was attenuated as compared to crowns. This trend was the same for both 'Midiron' and 'U3', although the magnitude of the changes for 'U3' was not as great as for 'Midiron'. Overall, significantly greater than 95% of the total polar fatty acid content was accounted for by four fatty acid species: palmitic acid (16:0 =16 carbon chain-length and no double bonds), stearic acid (18:0), linoleic acid (18:2) and linolenic acid (18:3). 'Midiron' (relatively cold- tolerant) responded more rapidly and to a greater extent than did 'U3' (relatively cold-sensitive) for the changes in fatty acid composition documented in our study. This was illustrated by the four-fold increase of unsaturated:saturated fatty acid ratio for "Midiron' over 'U3', and by the significant difference between the double bond index (DBI) of the two genotypes by the end of the first week of cold treatment. Although the DBI was essentially identical for the two genotypes at the start of the experiments, the observed increase in DBI during cold acclimation was initiated earlier, increased more rapidly and increased to a greater degree in 'Midiron' than it did in 'U3'.

The alterations in membrane fatty acid content documented in our results (e.g., increased levels of 18:3, and decreased levels of 16:0 and 18:0) are consistent with a number of other studies, which have correlated increases in membrane lipid fatty acid unsaturation with enhanced cold tolerance, and emphasize the importance of fatty acid unsaturation and its role in membrane fluidity -- thus protecting these membranes from undergoing damaging phase transitions at higher temperatures. In addition, our results, especially those observed changes in 18:3, point to specific desaturase (e.g., ω-3 and Δ-12) and transferase-type (e.g., 3-keto-acyl-ACP synthase II) enzymes as being of primary importance in regulating membrane lipid/fatty acid composition in response to low temperature, and ultimately in avoiding the winter damage suffered by bermudagrass along its northern zone of adaptation. Specifically, the increase documented for 18:3 is a consequence of the activity of a linoleic acid (ω-3) desaturase. Because this increase occurred in response to low temperature, the gene for this (iso)enzyme may be temperature regulated, especially so in 'Midiron'. Our findings point to the need for more detailed and in-depth investigations of the lipids and their constituent fatty acids from specific membrane enriched fractions of bermudagrass. In addition, molecular studies of the genes (families) for the lipid and FA biosynthesis enzymes is now possible because of the availability of heterologous probes for these genes from various higher plant species.

Differential display / RNA profiling has been used to identify genes expressed in bermudagrass in response to low temperature, during cold acclimation. This procedure used total or poly(A)$^+$ RNA as a substrate for the synthesis of cDNA molecules (i.e., partial gene clones) from transcripts expressed in the particular organ, at the particular developmental stage, in the particular treatment from which the RNA was isolated. Additionally, this method uses a set of "random" anchor and 10-mer oligonucleotide primers to initiate clone synthesis. By comparing gel-based "displays" from 0, 24 and 48 hrs post-low temperature exposure (as above) to displays from control non-

acclimating tissue over the same time frame, four sequences putatively expressed in response to low temperature were identified. They ranged from 300 to 450 base pairs in size. These fragments have been subcloned for further characterization: such as DNA sequence determination, as well as for use as probes on Northern blots to confirm their differential, cold-specific induction of expression.

In addition, a related methodology, RT-PCR, also has been employed in a collaboration with Drs. Mark Gatschet and Charles Taliaferro (Oklahoma State University, Stillwater) to aid in their search for a chitinase-like gene characterized by amino-terminal sequencing of a protein visualized by 2-D poly acrylamide gel electrophoresis of four-week cold acclimated 'Midiron' plants. We have used their N-terminal amino acid sequence, unfortunately all from regions containing amino acids with multiple codons, to design degenerate (variable sequence) oligonucleotides for use as primers in the RT-PCR experiments. We have used these in experiments with total RNA from 48 and 72 hour cold acclimated plants, but have only observed a single weak signal in the size-class expected. Therefore, we will use RNA from 4-week cold acclimated plants in future experiments, as well as using poly(A)$^+$ enriched fractions.

Infrastructure/New Resources: We were again fortunate to leverage funds from the SC Agric. Expert. Station. One expenditure was for the purchase of an image acquisition and analysis system. This computer-based system runs *pdi, inc.* (Protein-DNA Identification, Inc.) Diversity One analytical software. The system includes a Sharp high-resolution, color, wet/dry, transmitted/reflected light scanner, and a thermally cooled CDC camera, with filters for white or UV light, connected to a thermal printer for hard copy images. We use this equipment to document our gel-based results, store our database for future analysis and comparative studies, and quantify the amounts of individual products in particular reactions. However, it is available for general image acquisition to all members of the "Turf Group" for use in their programs.

The other grant of funds was for the purchase of three additional controlled environmental chambers (Conviron, model E-15) and the upgrade of a fourth, for the collective use of the turf group, through the Enhancement of Research and Extension in Turf Program. Two other E-15 chambers, purchased ~2 years ago with SC bond money, are in place at the Musser Research Farm of Clemson University and have been the workhorses of this project. The three new chambers were purchased in June, 1995, and have been installed in the Newman Bldg. adjacent to the Horticulture Department (Poole Agric. Center). This brings to a total of six the number of growth chambers available within the Horticulture Department. These chambers will greatly expand our ability to set-up and run experiments designed to evaluate bermudagrass cultivars during cold acclimation, under controlled, highly reproducible conditions.

Changes in Polar Lipid Fatty Acid Composition during Cold Acclimation in 'Midiron' and 'U3' Bermudagrass

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Abbreviations: FA = fatty acid(s)

ABSTRACT

'Midiron' and 'U3' bermudagrass were exposed to conditions known to induce cold acclimation [e.g., 8/3 °C (d/n) temperature, 10 hr photoperiod, 250 μmol/m^2/s photosynthetic photon flux density] for one and four-week periods. Tissue (leaves, crowns or roots) was harvested and stored frozen until processed. Lipids were isolated by organic extraction, and the polar lipid fraction was purified by thin layer chromatography. The fatty acids (FA) were released from the polar lipids by saponification, and then converted to methyl esters. Separation and identification of individual FA within the mixture was accomplished by gas chromatography, with the aid of an automated, computer-based identification system. Different organs of the same plant responded differentially to low temperature. Crowns showed the most dramatic increase in total FA content, and were the focus of this study. Overall, significantly greater than 95% of the polar FA content was accounted for by four acyl species: palmitic acid (16:0), stearic acid (18:0), linoleic acid (18:2) and linolenic acid (18:3). 'Midiron' (relatively cold- tolerant) responded more rapidly and to a greater extent than did 'U3' (relatively cold-sensitive) for the changes documented in this study. This was illustrated by the four-fold increase in the ratio of unsaturated:saturated FA for "Midiron' over 'U3', and by the significant difference between the double bond index of the two genotypes. These results point to specific desaturase enzymes (e.g., ω-3 and Δ-12) as being of primary importance in controlling membrane lipid/FA composition in response to low temperature, and ultimately in avoiding the winter damage suffered by bermudagrass along its northern zone of adaptation.

Environmental stress occurs in many forms such as low temperature, water deficit, soil salinity, etc. Plants of tropical and sub-tropical origin undergo sharp reductions in growth rate and development at temperatures below about 15°C. The changes, both physical and biochemical, which occur in chilling sensitive plants exposed to reduced temperature, together with the subsequent expression of low temperature stress symptoms, is referred to as cold or chilling injury. The physiological changes include alterations in cytoplasmic streaming, enzyme activity, respiration, photosynthesis and effects on membrane permeability, structure and composition (Graham and Patterson, 1982; Guy, 1990; Huner, 1988; Lyons, 1973). Which, if any of these changes is responsible for the primary low temperature-induced injury remains uncertain.

A principle target of chilling injury is the cell membranes; lipid bilayers with their peripheral and integral proteins. The major membrane lipids are diacylglycerides with amphipathic properties. These complex glycerolipids have a polar head-group (hydrophilic) attached at one of the primary hydroxyl groups of the glycerol back bone (i.e. the *sn*-3 carbon), while the remaining two hydroxyl groups are esterified to long chain FA (hydrophobic) (Ohlrogge and Browse, 1995).

The membrane lipid bilayer provides the necessary environment for proper functioning of proteins and enzymes associated with the 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 glycerolipids depend to a large extent on the degree of unsaturation in the attached FA (Chapman, 1975; Quinn et al., 1985; Wada et al., 1994),. To a first approximation, the greater the degree of unsaturation the lower the melting (= freezing) point. Thus, changes in FA unsaturation can affect biophysical properties such as the temperature at which membranes undergo dehydration induced phase transitions (Lyons, 1973) (and see discussion in Palta et al., 1993)

Numerous biochemical and physiological studies have demonstrated that changes in membrane lipid and FA composition are effected by exposure to low temperatures (Browse and Somerville, 1991; Uemura et al., 1995; Wolter et al., 1992). For example, in winter rye the level of di-unsaturated lipid species in plasma membranes increased after 7 to 10 days of cold acclimation (Uemura and Steponkus, 1994) . Conversely, mutants of *Arabidopsis*, deficient in plastid or endoplasmic reticulum localized FA desaturase activity contained markedly reduced levels of trienoic FA and showed increased sensitivity to chilling injury (Browse et al., 1989; Hugly and Somerville, 1992; Kunst et al., 1989; Miquel et al., 1993).

An better understanding of the nature and basis for tolerance to low temperature and the process of cold acclimation in bermudagrass would be very helpful for programs focused on germplasm improvement through breeding or biotechnology. In the experiments presented here, the first to characterize specific temporal and biochemical changes in membrane components of bermudagrass, we investigated the effect of low temperature on the FA composition of total polar lipids during cold acclimation. Our findings demonstrate that plants exposed to low temperature show a decrease in the proportion of saturated FA and an increase in a longer chain unsaturated FA. Furthermore, the rapidity and magnitude of these changes differ between genotypes as well as between organs of the same genotype.

MATERIALS AND METHODS

Plant Material

'Midiron' (relatively cold- tolerant) and 'U3' (relatively cold-sensitive) bermudagrass were kindly provided by Dr. C. M. Taliaferro (Dept. of Agronomy, Oklahoma State University, Stillwater) and grown in Rootrainers (Hummert International, St. Louis, MO) containing soilless growth medium (3-B, Fafard Inc., Anderson SC). Plants were propagated vegitatively and maintained in the greenhouse until used for experimentation. The plants were irrigated daily using an automated system and fertilized with liquid 20-10-20 (N-P-K) once every two weeks at the recommended rate.

Temperature Treatment

Environmental conditions (cold acclimation) known to induce cold hardiness in bermudagrass have been previously determined experimentally (Anderson et al., 1993). We mimicked those conditions in the treatments reported here. All experimental treatments were conducted in controlled environment growth chambers (model E-15; Conviron, Ashville, NC). Pre-conditioning of the experimental plants and growth of control (check) plants were at 28/22°C (day/night) with a 10 hr photoperiod at 250 umol/m^2/s PPFD. Cold acclimation (Anderson et al., 1988; Anderson et al., 1993) was achieved by growing the experimental plants at 8/3°C (d/n) under light and day-length parameters identical to those above. Air and soil temperature were monitored during the treatment periods. Plants were watered once each day, but were not fertilized, during the acclimation treatment period.

Experimental Design and Sampling

Experiments were conducted for one week and three weeks time periods. Each experiment was repeated twice. Samples, 1 gm fresh weight, of plant material (leaves, crowns or roots) were harvested, washed and dried, weighed, frozen in liquid N_2 and stored at -80°C until processed. For both cultivars a minimum of three samples (1 gm)

were collected per time point per treatment, and each sample was divided into two replicates at the start of processing.

Total Lipid Extraction

Frozen tissue samples were ground to a fine powder in liquid nitrogen in a precooled mortar and pestle. Each sample of powdered tissue (1 gm) was divided equally and transferred into two test tubes for extraction of total lipids. These replicates were processed simultaneously. Three ml of extraction buffer (chloroform:methanol:water at 1:2:0.8; v/v/v) were added to each tube and the tubes were incubated at room temperature for 20 min. One ml of 1% (w/v) sodium chloride and 3 ml of chloroform were added, mixed and the tube centrifuged at 3000 x g for 5 min to separate the phases. The lower chloroform layer, containing the lipid fraction, was transferred to a new test tube. The upper aqueous layer was re-extracted two times, as above. The three extractions were combined, and dried at 45°C under a stream of nitrogen. Each dried lipid sample was resuspended in 250 ul of chloroform and stored at 4°C over night prior to processing.

Polar Lipid Purification and Fatty Acid Isolation

Each resuspended lipid sample was loaded on a 20 x 20 cm thin layer chromatographic (TLC) plates, at 1.5 cm from the bottom, in a band about 2 cm long. Polar lipids were separated from non-polar lipids by developing each TLC plate in a non-polar solvent (hexane:ethyl either:acetic acid at 80:20:1) for 60 to 90 min until the solvent front reached at least 16 cm from the TLC plate bottom. The silica at the TLC origin was scraped into a 5 ml plastic syringe fitted with a 0.2 um Acrodisc nylon filters (Gelman Sciences Inc., Ann Arbor, MI). The polar lipids were eluted from the syringe with 5 ml of chloroform:methanol (2:1; v/v). Prior to this elution, 50 ng of nonadecanoic acid (19:0) was added to each lipid sample as an internal control and quantitation standard. This standard was washed out of the syringe with 3 ml ethyl ether. These

samples (polar lipids and FA standard) were dried at 45°C under a stream of nitrogen as before.

Saponification reagent (1 ml of a 15% sodium hydroxide in 50% aqueous methanol) was added to each test tube and the tubes were heated at 100°C for 30 min followed by immediate cooling to room temperature. This digests the mixture of complex lipids to release their FA components. These free FA were then converted to FA-methyl esters by adding 2 ml of acidified methanol (6N hydrochloric acid:methanol at 54:46; v/v), heating at 80°C for 10 min, and then immediately cooling the tubes to room temperature. FA-methyl esters were purified from the acidic aqueous phase by organic extraction with 1.25 ml of hexane:methyl-tert butyl ether (1:1, v/v). The aqueous phase (lower) is discarded and residual contaminants present in the organic phase were removed by adding 3 ml of mild base solution (1.5% aqueous sodium hydroxide solution; w/v) and mixing for 5 minutes. The upper organic phase was transferred to a sealed vial for subsequent analysis of FA.

Fatty Acid Quantification

The mixture of purified FA were analyzed on a gas chromatograph (GC) (5890 Heweltt-Packard, Avondale, PA) and mass spectrometer (5971A Hewlett-Packard, Avondale, PA). Samples (2 ul) were analyzed under aerobic conditions by GC on a 25 m x 0.2 mm Ultra 2 column and quantified with flame-ionization detection. The chromatograph was programmed for an initial temperature of 170 °C, followed by a 5 °C/min ramp to 270 °C and secondary ramp of 30°C/min to 310 °C. The final temperature was maintained for 2 min. The aerobic library (version 3.8) of the Microbial ID system, version 4 (MIDI, Inc., Newark, DE), was used to identify individual FA peaks based on their retention time. Octadecatrienoic acid (18:3) could not be unambiguously identified by this GC method. Therefore, samples were also analyzed on a mass spectrometer.

Weights of individual FA were calculated by comparing their peak areas to that of the 19:0 internal standard and its known quantity. Total FA weight is the sum of weights of individual FA. Amounts of FA, either individually or in combination were expressed as weight percent (mean ± s.d.) , relative to the total.

RESULTS AND DISCUSSION

Initial studies exposing bermudagrass plants to experimental conditions known to induce cold acclimation showed that the leaves, crowns and roots responded differentially with respect to changes in the amount of total polar FA (Fig. 1). Overall, the crowns showed the largest sustained increase in FA content over the four week experiment, especially when contrasted to leaf tissues. Even the roots, which showed an initial increase similar to those observed in crowns, leveled off in their response after approximately 10 days. These differences in organ-specific changes were essentially the same for both 'Midiron' and 'U3', although the absolute amounts and magnitude of change were not identical. This is not entirely unexpected since comparative studies have shown similar trends of altered lipid composition in response to low temperature between different genotypes or cultivars of the same species or even between closely related species (Lemieux et al., 1990; Palta et al., 1993). Similarly it is not surprising that different organs of the same plant (e.g.., roots and crowns) show variation in absolute amounts of FA as such static differences have been found in other plant species (Rochester et al., 1987; Sandstrom and Cleland, 1989).

This differential response is consistent with the growth and reproductive biology of warm season turfgrass, which typically perennate as crowns and stolons, and initiate post-winter regrowth from these subterranean organs. For these reasons we chose to focus our subsequent analyses on crown tissue.

Characterization of the FA composition of bermudagrass polar lipids identified hexadecanoic acid (palmitic acid; 16:0), octadecanoic acid (stearic acid; 18:0), octadecadienoic acid (linoleic acid; 18:2) and octadecatrienoic acid (linolenic acid; 18:3) as the four most abundant components. This was out of a total of approximately 10 species identified. The four major components accounted for greater than 95% of the total FA isolated from any organ at any time-point during the experiments (Fig. 2). The remaining five or six secondary components individually accounted for significantly less

than 1% of the total, and were inconsistent in their appearance throughout the experiment. This is likely a combined function of their minor role in bermudagrass membrane lipids and our use of whole tissue homogenates rather than purified membrane preparations (Palta et al., 1993). Again, these FA identifications and proportional relationships were found in both genotypes.

Identification and quantification of FA during the cold acclimation process revealed significant changes in the proportions of the individual components (e.g., 16:0, 18:0, 18:2 and 18:3) in the crowns of both genotypes examined. For example, the level of 16:0 in 'Midiron' steadily decreased in the first seven days of cold treatment, while for 'U3' there was no significant change in 16:0 over that same time frame (Fig. 3a). Because the observed decrease in 'Midiron' membrane C16:0 was not observed to level off, and to determine if a similar decrease might be initiated in 'U3' with longer exposures to low temperature, a second series of analyses was performed. This was accomplished over a three week low temperature treatment. Again, a similar trend was observed as before for both genotypes in the first seven days (Fig. 3b). However by the end of the first week no further decrease in proportion of 16:0 was observed in 'Midiron'. As before 'U3' failed to show any significant change in 16:0 content during the entire treatment period.

The other major saturated FA (18:0) identified in this study represented only about 4% of the total polar lipid FA. 'Midiron' plants showed a decrease in 18:0 levels during cold acclimation. However, unlike changes observed for 16:0, this decrease in 18:0 for 'Midiron' was gradual but sustained for the entire cold treatment period. The change in 18:0 for 'U3' was minimal. It decreased very little, essentially remaining the same during the entire treatment period. Figure 4 illustrates these changes in C18:0 for 'Midiron' and 'U3'.

Overall, the amount of 18:2 in polar lipids of 'Midiron' and 'U3' decreased during cold acclimation (Fig. 5). Interestingly, 'Midiron' showed a more dramatic and rapid

change than did 'U3'. However, there was no significant net change in the amount of 18:2 within the first week of the experiment for either genotype. For 'Midiron' this was due to an initial increase followed by a return to original levels, while in 'U3' 18:2 levels remained relatively constant. This pattern was even more apparent in our seven day experiments, where samples were collected each day (data not shown). Once initiated, the decrease in 18:2 was quite pronounced for 'Midiron' and reached its lowest value by 12 to 14 days of cold treatment. In 'U3' the decrease was more gradual, and never reached the minimal level attained in 'Midiron'.

In general, levels of the 18:3 long chain polyunsaturated FA increased noticeably in polar lipid fractions during cold acclimation (Fig. 6). In both genotypes, prior to this increase, a short lag phase was observed. After this stasis period levels of 18:3 increased, at least gradually. For 'Midiron' the increase in 18:3 was first observed after four or five days exposure to low temperature, whereas the increase in C18:3 for 'U3' was not observed until day 8 or 9. Also, 'Midiron' attained a more than two-fold greater level of 18:3, and did so more rapidly, than 'U3'. Interestingly, this pattern of change in relative amounts of 18:3 is almost inverse of that observed for 18:2, and both of these lag behind the more rapidly initiated decrease shown for 16:0.

Figures 7a and 7b summarize observed changes in the proportions of all four major membrane FA for 'Midiron' and 'U3', respectively. The relative amounts of each component are shown for four time points (one week intervals) over a three week treatment period. The FA composition of 'Midiron' and 'U3' are nearly identical at the initiation of the low temperature treatment. However by 21 days of exposure they are quite different from one another. In general, increase in the 18:3 component is mimicked by decreases in one or more of the other FA. Decreases are most notable in C18:2 and C16:0. It can be seen that 'Midiron' alters its polar lipid FA composition more rapidly and to a greater extent than does 'U3'.

The ratio of the amount of unsaturated:saturated FA species [i.e., (18:2 + 18:3):(16:0 + 18:0)] is a convenient method to compare and evaluate changes in FA composition. Both bermudagrass genotypes showed an increase in this ratio following cold acclimation; from 2.65 at the start of the experiment to 2.71 (2.3%) for 'U3' and to 2.94 (10.5%) for 'Midiron'. The increase for 'Midiron' was four times that observed for 'U3'. Similarly, Palta et al. (1993) found a two-fold increase in the ratio of unsaturated:saturated FA after cold acclimation of a freezing tolerant (20.5%) and a freezing susceptible (12.2%) species of *Solanum*. Interestingly, for both genotypes of bermudagrass the increase was entirely due to 18:3, while that in *S. commersonii* (acclimating) was due to 18:2 and that in *S. tuberosum* (non-acclimating) was due to 18:3 (Palta et al., 1993).

The exact role of any individual unsaturated FA species in affecting cold tolerance is not clear. Results from studies using transgenic tobacco showed that increased levels of trienoic FA correlated with enhanced cold tolerance (Kodama et al., 1994). Our study is consistent with their results, and indicates that increased levels of 18:3 may be important for bermudagrass in acclimating to low temperatures and avoiding subsequent freezing damage. However, an earlier study of two *Solanum* spp. contradicts this conclusion (Palta et al., 1993). There the authors found that one of the changes unique to the acclimating species was an increase in 18:2 and a decrease 18:3. In addition, they provide an argument for their hypothesis based upon phase transition temperatures of various mono- and di-unsaturated phosphatidylcholine lipids. It must also be kept in mind that the presence and importance of any particular membrane lipid, with its associated FA moieties, may have a plant species specific component as well a cryoprotective function (Cronan and Roughan, 1987).

Double bond index (DBI) is another parameter for comparing changes in FA composition between treatments and between genotypes. In the first week of cold acclimation there is no real difference between 'Midiron' and 'U3' with respect to DBI.

However after that time the DBI of 'Midiron' diverged from that of 'U3'. 'Midiron' showed a significant increase in DBI after seven days and then leveled off by the second week, while 'U3' did not show any significant change in DBI throughout the treatment period (Fig. 8).

The changes in FA composition observed for bermudagrass during cold acclimation represent a complex interplay between the composition at the start of cold treatment and the activity of specific acyltransferase and desaturase enzymes (Browse and Somerville, 1991). The increase documented for 18:3 is a consequence of the activity of a linoleic acid (ω-3) desaturase. Because this increase occurred in response to low temperature, the gene for this (iso)enzyme may be temperature regulated, especially so in 'Midiron'. This situation has been identified in *Arabidopsis thaliana* where a chloroplast localized ω-3 desaturase (encoded by the *fad8* gene) was shown to be induced at low temperature (Gibson et al., 1994; McConn et al., 1994).

These findings all point to the need for more detailed and in-depth investigations of the lipids and their constituent FA from specific membrane enriched fractions of bermudagrass. In addition, molecular studies of the genes (families) for the lipid and FA biosynthetic enzymes is now possible because of the availability of heterologous probes for these genes from various higher plant species.

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REFERENCES

Anderson, J.A., M.P. Kenna, and C.M. Taliaferro. 1988. Cold hardiness of 'Midiron' and 'Tifgreen' bermudagrass. HortScience 23:748-750.

Anderson, J.F., C.M. Taliaferro, and D.L.Martin. 1993. Evaluating freeze tolerance of bermudagrass in a controlled environment. HortScience 28:955.

Browse, J., L. Kunst, S. Anderson, S. Hugly, and C. Somerville. 1989. A mutant of *Arabidopsis* deficient in the chloroplast 16:1/18:1 desaturase. Plant Physiol. 90:522-529.

Browse, J. and C. Somerville. 1991. Glycerolipid synthesis: biochemistry and regulation. Annu. Rev. Plant Physiol. Plant Mol. Biol. 42:467-506.

Chapman, D. 1975. Phase transition and fluidity characteristics of lipids and cell membrane. Q. Rev. Biophys 8:185-235.

Cronan, J.E., Jr. and P.G. Roughan. 1987. Fatty acid specificity and selectivity of the chloroplast sn-glycerol 3-phosphate acyltransferase of the chilling sensitive plant, *Amaranthus lividus*. Plant Physiol. 83:676-680.

Gibson, S., V. Arondel, K. Iba, and C. Somerville. 1994. Cloning of a temperature-regulated gene encoding a chloroplast ω-3 desaturase from *Arabidopsis thaliana*. Plant Physiol. 106:1615-1621.

Graham, D. and D.B. Patterson. 1982. Responses of plants to low, nonfreezing temperatures: proteins, metabolism and acclimation. Annual Review of Plant Physiology. 33:347-372.

Guy, C. 1990. Cold acclimation and freezing stress tolerance: role of protein metabolism. Annual Review of Plant Physiology and Plant Molecular Biology 41:187-223.

Hugly, S. and C. Somerville. 1992. A role for membrane lipid polyunsaturation in chloroplast biogenesis at low temperature. Plant Physiol. 99:197-202.

Huner, N.P.A. 1988. Low-temperature alterations in photosynthetic membranes. CRC Crit. Rev. Plant Sci. 7:257-278.

Kodama, H.T., G. Hamada, G. Horiguchi, M. Nishimura, and K. Iba. 1994. Genetic enhancement of cold tolerance by expression of a gene for chloroplast W-3 fatty acid desaturase in transgenic tobacco. Plant Physiol. 105:601-605.

Kunst, L., J. Browse, and C. Somerville. 1989. A mutant of *Arabidopsis* deficient in desaturation of palmitic acid in leaf lipids. Plant Physiol. 90:943-947.

Lemieux, B., M. Miquel, M. Somerville, and J. Browse. 1990. Mutants of *Arabidosis* with alterations in seed lipid fatty acid composition. Theor. Appl. Genet. 80:234-240.

Lyons, J.M. 1973. Chilling injury in plants. Annual Review of Plant Physiology 24:445-466.

McConn, M., S. Hugly, J. Browse, and C. Somerville. 1994. A mutation at the *fad8* locus of *Arabidopsis* identifies a second chloroplast ω-3 desaturase. Plant Physiol. 106:1609-1614.

Miquel, M.D., H. James, H. Dooner, and J. Browse. 1993. Arabidopsis requires polyunsaturated lipids for low temperature survival. Proc. Natl. Acad. Sci., U.S.A. 90:6208-6212.

Ohlrogge, J. and J. Browse. 1995. Lipid biosynthesis. Plant Cell 7:957-970.

Palta, J.W., B.D. Whitaker, and L.S. Weiss. 1993. Plasma membrane lipids associated with genetic variability in freezing tolerance and cold acclimation of Solanum species. Plant Physiol 103:793-803.

Quinn, P.J., F. Joo, and L. Vigh. 1985. The role of unsaturated lipids in membrane structure and stability. Prog. Biophys. Mol. Biol. 53::71-103.

Rochester, C.P., P. Kjellbom, and C. Larsson. 1987. Lipid composition of plasma membrane from barley leaves and roots, spinach leaves and cauliflower inflorescences. Plant Physiol. 71:257-263.

Sandstrom, R.P. and R.E. Cleland. 1989. Comparison of lipid composition of oat root and coleoptile plasma membranes: lack of short-term change in response to auxin. Plant Physiol. 90:1207-1213.

Uemura, M., R.A. Joseph, and P.L. Steponkus. 1995. Cold acclimation of *Arabidopsis thaliana*. Plant Physiol. 109:15-30.

Uemura, M. and P.L. Steponkus. 1994. A contrast of the plasma membrane lipid composition of oat and rye leaves in relation to freezing tolerance. Plant Physiol. 104:479-496.

Wada, H., Z. Gombos, and N. Murata. 1994. Contribution of membrane lipids to the ability of the photosynthetic machinery to tolerate temperature stress. Proc. Natl. Acad. Sci. 91:4273-4277.

Wolter, F.P., R. Schmidt, and E. Heinz. 1992. Chilling sensitivity of *Arabidopsis thaliana* with genetically engineered membrane lipids. EMBO J. 11:4685-4692.

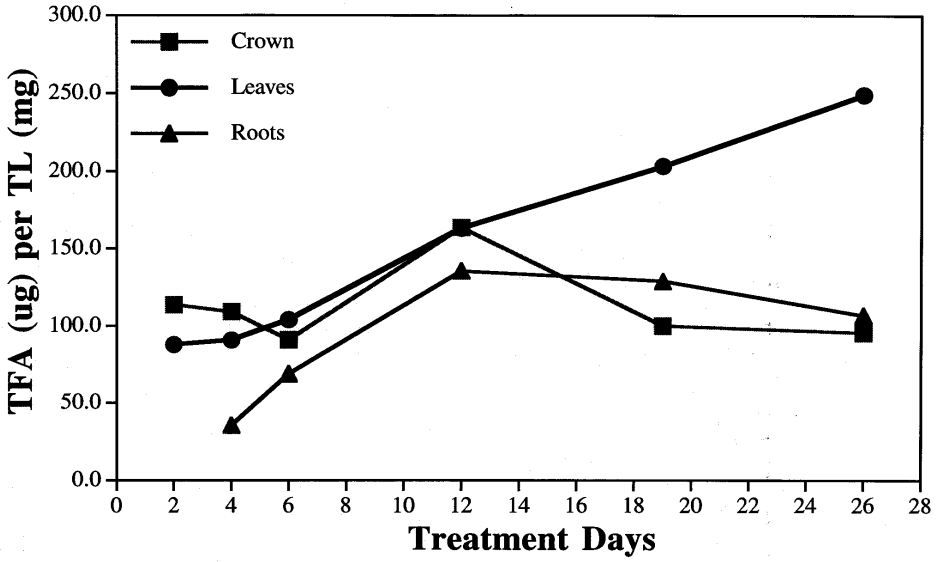

Fig .1. Change in lipid composition as a response to low temperature. Alterations of total membrane fatty acids (ug) were quantified with respect to total lipids (mg). This anlysis was made for leaves, crowns and roots of 'Midiron' bermudagrass. The low temperature was carried over the four week period in a controlled environment chamber. TFA = total fatty acids, TL = total lipids

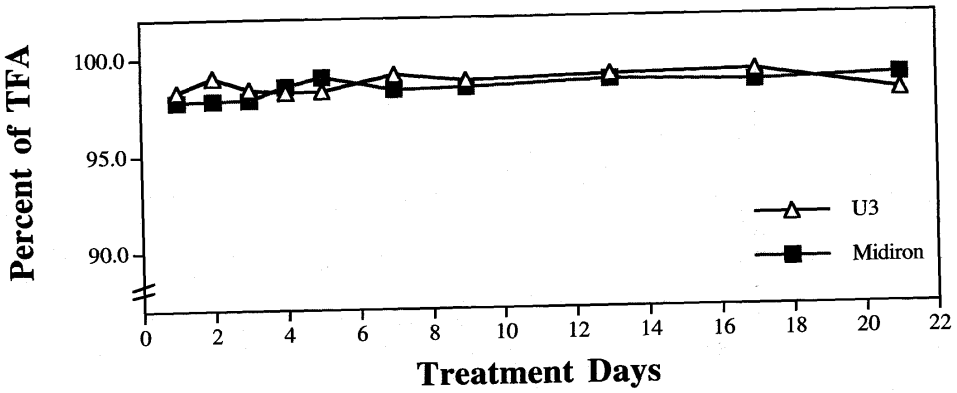

Fig. 2. Change in cumulative amounts of four major membrane fatty acids in response to low temperature. The membrane fatty acids composition of 'Midiron' and 'U3' bermudagrass crowns was characterized over the three week low temperature treatment period. The major fatty acids were hexadecanoic acid (C16:0), octadecanoic acid (C18:0), octadecadienoic acid (C18:2) and octadecatrienoic acid (C18:3). The values represent the mean sum of all four components, and are displayed as a percentage of total fatty acids (TFA) isolated.

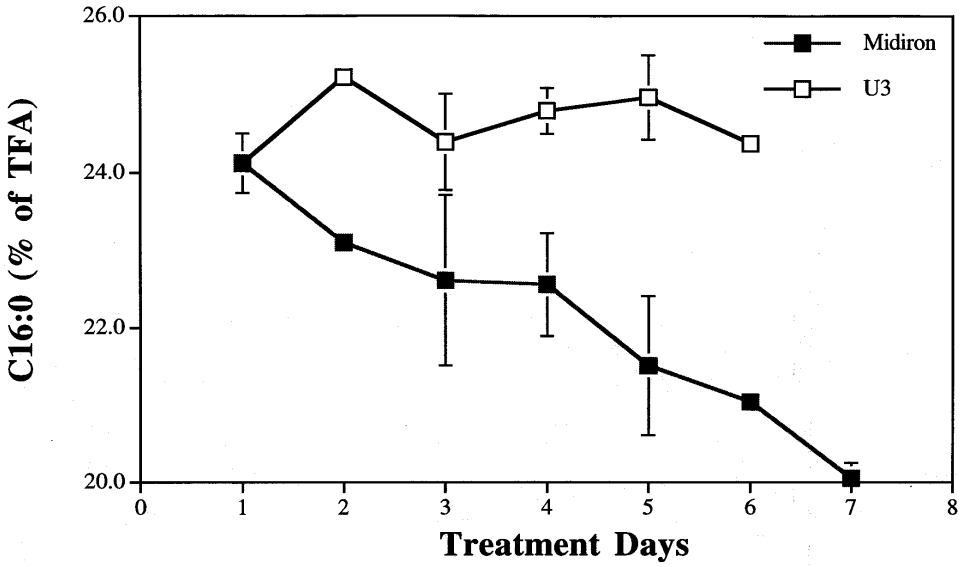

Fig. 3a. Changes in hexadecanoic acid (C16:0) in responce to temperature. The C16:0 component of the fatty acid fraction of 'Midiron' and 'U3' bermudagrass crowns was characterized at specific time points over the seven day low temperature treatment period. The amount of C16:0 was quantified, and displayed as percentage of total fatty acids (TFA) isolated. Error bars represent one standard deviation from the mean.

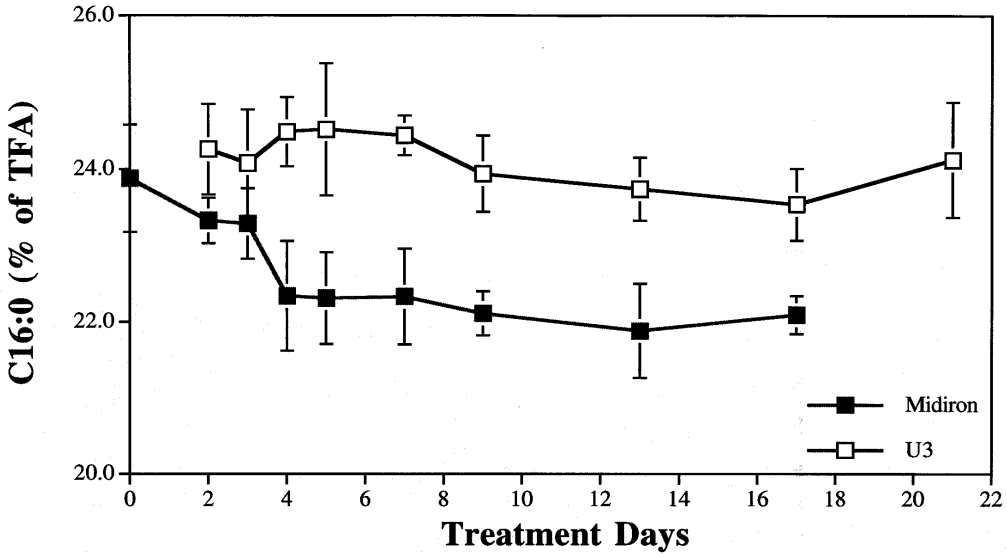

Fig. 3b. Change in hexadecanoic acid (C16:0) in response to low temperature. The C16:0 component of the fatty acid fraction of 'Midiron' and 'U3' bermudagrass crowns was characterized at specific time points over three week treatment period. The amount of C16:0 was quantified, and is displayed as a percentage of total fatty acids (TFA) isolated. Error bars represent one standard deviation from the mean.

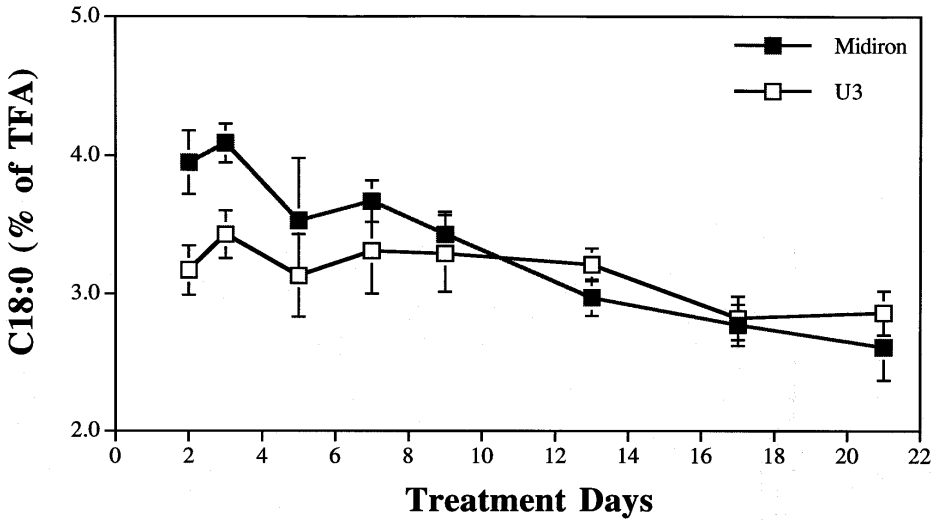

Fig. 4. Changes in octadecanoic acid (C18:0) in response to low temperature. The C18:0 component of the fatty acid fraction of 'Midiron' and 'U3' bermudagrass crowns was characterized at specific times over the three week treatment period. The amount of C18:0 was quantified, and is displayed as a percentage of total fatty acids (TFA). Error bars represent one standard deviation from the mean.

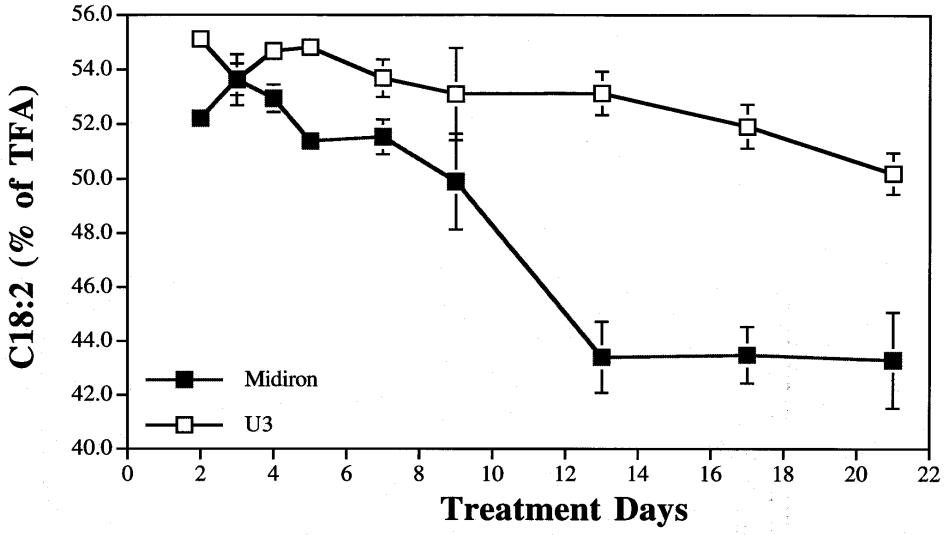

Fig. 5. Changes in octadecadienoic acid (C18:2) in response to low temperature. The C18:2 component of the fatty acid fraction of 'Midiron' and 'U3' bermudagrass crowns was characterized at specific times over the three week treatment period. The amount of C18:2 was quantified, and is displayed as a percentage of total fatty acid (TFA). Error bars represent one standard deviation from the mean.

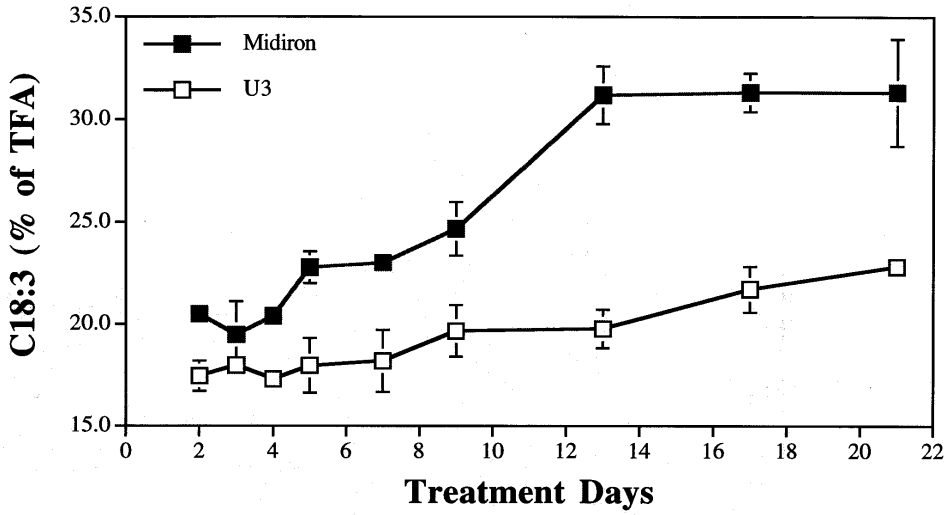

Fig. 6. Change in octadecatrienoic acid (C18:3) in response to low temperature. The C18:3 component of the fatty acid fraction of 'Midiron' and 'U3' bermudagrass crowns was characterized at various time points over the three week treatment period. The amount of C18:3 was quantified, and displayed as a percentage of total fatty acids (TFA). Error bars represent one standard deviation from the mean.

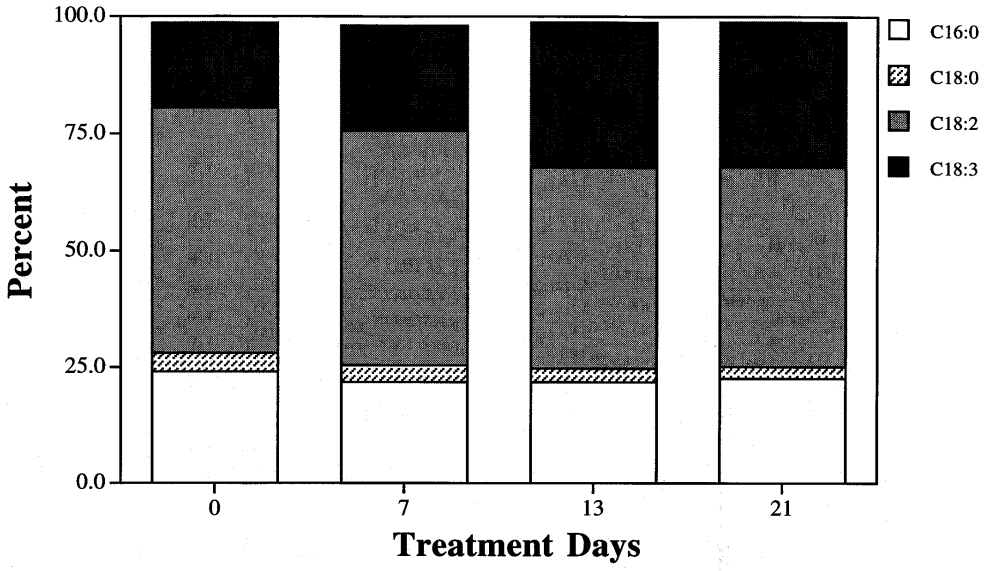

Fig. 7a. Changes in the relative amounts of the four major membrane fatty acids in response to low temperature. Fatty acids isolated from 'Midiron' bermudagrass crowns at various times over a three week exposure to low temperature were quantified as described in the Materials and Methods. Four representative time points were chosen to illustrate the changes in fatty acid composition. The fatty acid species identified were hexadecanoic acid (C16:0), octadecanoic acid (C18:0), octadecadienoic acid (C18:2), octadecatrienoic acid (C18:3).

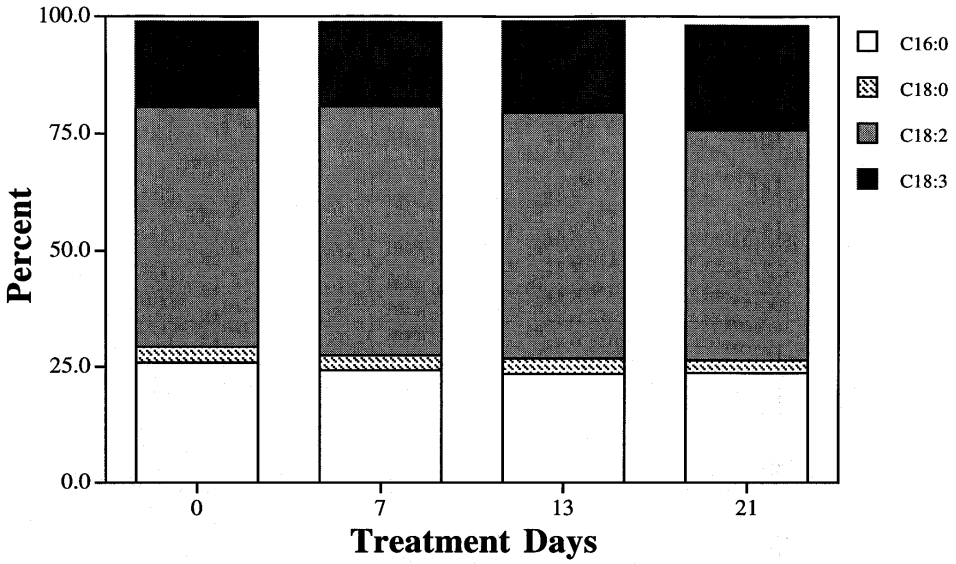

Fig. 7b. Changes in the relative amounts of the four major membrane fatty acids in response to low temperature. Fatty acids isolated from 'U3' bermudagrass crowns at various times over a three week exposure to low temperature were quantified as described in materials and methods. Four representative time points were chosen to illustrate the changes in fatty acid composition. The fatty acid species identified were hexadecanoic acid (C16:0), octadecanoic acid (C18:0), octadecadienoic acid (C18:2) and octadecatrienoic acid (C18:3).

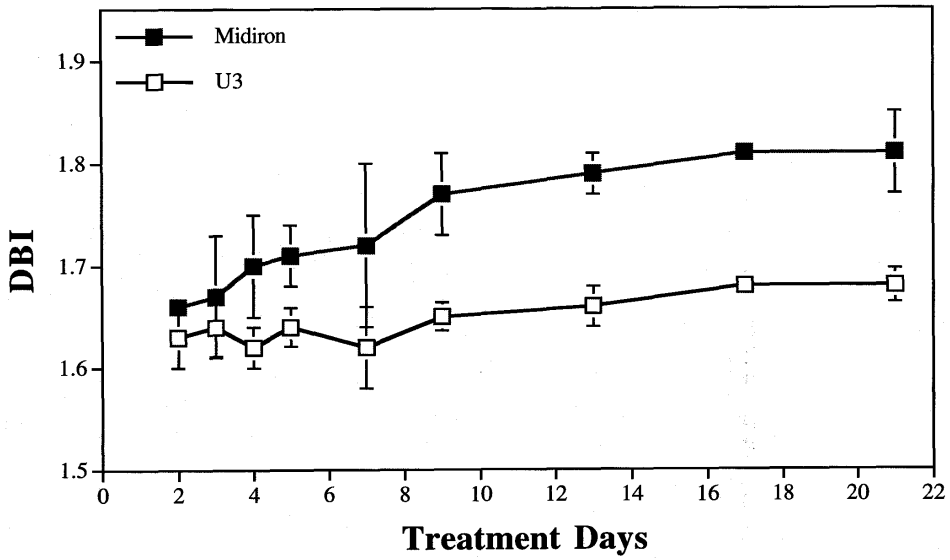

Fig. 8. Changes in the double bond index (DBI) in response to temperature. The sum of products divided by 100, of the relative percent of each unsaturated fatty acids times the number of double bonds in that fatty acid component gives the DBI. DBI was calculated for various time points during cold acclimation for both 'Midiron' and 'U3'.