Genotypic Variation in Fatty Acid Composition and Unsaturation Levels in Bermudagrass Associated with Leaf Dehydration Tolerance

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ADDITIONAL INDEX WORDS. *Cynodon*, drought stress, turfgrass, water stress

ABSTRACT. Fatty acid metabolism may be involved in plant adaptation to drought stress. The objective of this study was to identify saturated and unsaturated fatty acids associated with leaf dehydration tolerance by comparing fatty acid composition and unsaturation levels at equivalent leaf water status of two bermudagrass genotypes contrasting in drought resistance. A drought-resistant hybrid bermudagrass (*Cynodon dactylon* × *C. transvaalensis*) genotype ('Tifway') and a drought-sensitive bermudagrass (*C. dactylon*) genotype ('C299') were maintained under well-watered (control) or water-withheld (drought) conditions. Drought treatment was imposed until soil water content decreased to 5% or leaf relative water content (RWC) dropped to 28% to 29%. 'Tifway' maintained higher RWC and lower electrolyte leakage (EL) at 5 and 10 days of drought stress. Leaves of 'Tifway' maintained lower EL when RWC of both genotypes declined to the same level of water deficit (28% to 29%) by the end of drought periods. The degree of fatty acid unsaturation, expressed as the double bond index, decreased in both genotypes during drought stress, which was mainly associated with the decline in linoleic (C18:2) and linolenic acids (C18:3) and an increase in palmitic (C16:0) and stearic acids (C18:0). A lipid composition characterized by a greater amount of unsaturated fatty acids was detected in 'Tifway' relative to 'C299' exposed to the same level of water deficit, mainly as a result of a greater content of C18:2 and a lower content of C16:0 and C18:0. Our results suggest that the ability to maintain a greater composition of unsaturated fatty acids in membrane lipids may contribute to superior leaf dehydration tolerance in bermudagrass.

Drought stress is one of the most detrimental abiotic stresses of plant growth and production, causing water deficit and various cellular and biochemical changes (Nilsen and Orcutt, 1996). Damage to cellular membranes is caused by leaf desiccation; therefore, maintenance of cellular membrane integrity and stability is vital for plant adaptation to drought stress (Anh et al., 1985; Monteiro De Paula et al., 1990). The level of cell membrane stability during drought stress depends largely on the composition and content of fatty acids in the lipid bilayers of the membrane (Gigon et al., 2004; Yordanov et al., 2000).

Total lipid content in leaves generally exhibits a decline in response to drought stress in various plant species (Gigon et al., 2004; Monteiro de Paula et al., 1993; Pham-Thi et al., 1985). Fatty acids in the lipid complex are classified into unsaturated and saturated fatty acid based on the extent of double bond formation. Change in fatty acid composition based on saturation levels within the drought response varies by plant species. In shoots of safflower (*Carthamus tinctorius*), the proportion of linolenic and linoleic acid [C18:2 (number of carbon:number of double bonds)] decreased with drought stress (Hamrouni et al.,

2001). A significant reduction in the composition of C18:3 and the disappearance of palmitoleic acid (C16:1) were reported in sage (*Salvia officinalis*) leaves under drought stress (Bettaieb et al., 2009). In *Boea hygrosopica*, a resurrection plant with leaves that can be fully rehydrated within hours of rewatering after complete desiccation, unsaturated fatty acid composition increased under drought stress (Navari-Izzo et al., 1995). An increase in fatty acid unsaturation level with drought stress was also reported in *Arabidopsis thaliana* (Gigon et al., 2004). The unsaturation level of polar lipids decreased in sensitive plants, whereas it remained unchanged or even increased in resistant cultivars under drought stress conditions (Monteiro De Paula et al., 1990; Repellin et al., 1997). Current literature suggests that specific adjustments in the fatty acid composition and unsaturation level of lipids under drought stress could help plants maintain membrane integrity in different plant species (Navari-Izzo et al., 1993; Toumi et al., 2008).

Leaf tolerance to dehydration is critically important for perennial turfgrass species cultivated for their aesthetic appearance. For turfgrass species, the ability to maintain a high level of unsaturation of fatty acids has been positively associated with leaf recovery from drought damage upon rewatering as demonstrated in kentucky bluegrass [*Poa pratensis* (Xu et al., 2010)]. The relationship of composition and saturation level for specific fatty acids with leaf dehydration tolerance at a given internal leaf water deficit is not clear, although changes in fatty acid composition and saturation or unsaturation levels with drought stress are well documented in a variety of plants

Received for publication 7 Oct. 2010. Accepted for publication 9 Nov. 2010. We thank Shanghai Jiao Tong University and Rutgers University for joint support of this research project. We also thank the Analytic Testing Center at Shanghai Jiao Tong University for help with the GC-MS analysis of fatty acids. The critical review of the article by Dr. Chenping Xu and Emily Merewitz at Rutgers University is appreciated.

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species, as discussed previously. The comparison of composition of individual fatty acids and saturation levels at the same level of internal water deficit for genotypes contrasting in drought resistance allows for the identification of fatty acids associated with leaf dehydration tolerance. Therefore, the objectives of this study were to examine fatty acid changes in response to drought stress and to compare composition and saturation levels of fatty acids in leaves subjected to the same level of water deficit for two bermudagrass genotypes contrasting in drought resistance. The ultimate goal was to identify specific fatty acids associated with leaf dehydration tolerance, which could be used as biochemical markers to select for drought-resistant germplasm. A drought-resistant hybrid bermudagrass genotype, 'Tifway', and a drought-sensitive common bermudagrass genotype, C299 (Hu et al., 2010a, b), were examined in this study.

Materials and Methods

PLANT MATERIALS AND GROWING CONDITIONS. Sod of two bermudagrass genotypes, hybrid bermudagrass (*Cynodon dactylon* × *C. transvaalensis*) 'Tifway' (drought-resistant) and common bermudagrass (*C. dactylon*) 'C299' (drought-susceptible), was collected from a research farm at Shanghai Jiao Tong University, Shanghai, China, and planted in 20-cm-diameter plastic pots in July 2009. The pots with holes at the bottom were filled with a mixture (1:3 v/v) of sand and loamy soil (fine-loamy, mixed mesic Typic Hapludult). The plants were maintained for 35 d in growth chambers (HP1500 GS-B; Wuhan Ruihua Instrument and Equipment Co., Wuhan, China) at 30/25 °C (day/night), 75% relative humidity, 600 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ photosynthetically active radiation, and a 14-h photoperiod. During this time, the plants were clipped once weekly to maintain canopy height at ≈ 6 cm, watered daily, and fertilized with controlled-release fertilizers (15N–6.5P–8.3K) at a total nitrogen amount of 50 $\text{kg}\cdot\text{ha}^{-1}$.

DROUGHT TREATMENT AND EXPERIMENTAL DESIGN. Plants of each species were subjected to two treatments: well-watered control plants, which were watered three times per week to maintain soil water content at pot capacity [25% (the amount of water remaining in a pot after an irrigation and visible drainage has stopped)] and drought-stressed plants by withholding irrigation until soil water content declined to 5%. Soil water content was measured using a time domain reflectometer (TDR200; Spectrum Technologies, Plainfield, IL). Each treatment had four pots as four replicates. Drought treatments and cultivars were arranged as a randomized split-plot design with drought as the main plot and cultivars as the subplots.

PHYSIOLOGICAL ANALYSIS. Cultivar variation in drought tolerance for each species was evaluated by measuring cell membrane stability and RWC at 0, 5, 10, and 15 d of drought stress. Measurements were made on the two youngest fully expanded leaves from multiple plants in each pot.

Leaf RWC was determined with 10 to 15 first and second fully expanded leaves per pot using the method described in Barrs and Weatherley (1962). Leaves were clipped and weighed [fresh weight (FW)] and then placed into small petri dishes filled with water. They were soaked in water for ≈ 24 h at 23 °C and then weighed immediately after excess moisture was removed with paper towels [turgid weight (TW)]. The leaves were then dried in an oven at 80 °C for 48 h to determine dry weight (DW). Leaf RWC was calculated as $(\text{FW} - \text{DW}) / (\text{TW} - \text{DW}) \times 100$.

Cell membrane stability was estimated by measuring EL from leaf tissues (Blum and Ebercon, 1981), the increase of which is generally considered as an index of membrane damage or deterioration (Simon, 1974). Samples of fresh whole leaves (0.2 g) were weighed, rinsed with deionized water, dried with paper towels, cut into ≈ 1 -cm pieces, and immersed in a 50-mL centrifuge tube with 20 mL of deionized water. The conductivity of the solution (C_{initial}) was measured after the leaves were shaken for 24 h using a conductivity meter (DDS-11C; Analytical Equipment Co., Tianjin, China). Leaves then were killed by an automatic autoclave at 121 °C for 30 min. The conductivity of killed tissues (C_{max}) was measured after samples were cooled down to room temperature. Relative EL (%) was calculated as the percentage of C_{initial} over C_{max} .

FATTY ACID EXTRACTION AND COMPOSITION ANALYSIS. Fatty acid analysis was carried out for leaf samples collected from well-watered plants when RWC in both genotypes was $\approx 95\%$ and at 10 d of drought stress for 'C299' and at 13 d for 'Tifway' when RWC of leaves in both genotypes declined to an equivalent level of 28% to 29%. The comparison of fatty acid content under the same level of water deficit enables the identification of major fatty acids contributing to leaf dehydration tolerance. Fatty acid content was analyzed using the method described in Browse et al. (1986) and Larkindale and Huang (2004). Briefly, leaves (0.05 g FW) were acidified with 1 N H_2SO_4 and fatty acids were methylated by heating at 80 °C for 90 min. A solution containing 0.9% NaCl and 200 μL of heptadecanoic acid (C17:0 as internal standard) was added to the sample and then vortexed. After centrifuging for 5 min at 250 g_n , 1 μL of the extract was subjected to gas mass spectrometry analysis. Fatty acids were separated and identified with a gas chromatography mass spectrometer [GC-MS (Auto-System XL GC and TurboMass MS; Perkin-Elmer, Waltham, MA). A 60-mHP-5MS capillary column with an i.d. of 0.25 mm was used (Agilent Technologies, Santa Clara, CA). The GC-MS was programmed to begin at 70 °C for 2 min followed by a 10-min ramp of temperatures until 270 °C at a flow rate of 1 $\text{mL}\cdot\text{min}^{-1}$. The samples were quantified against the internal standard (100 μg heptadecanoic acid) and the composition of each fatty acid in the sample was expressed as a percentage or proportion of the total fatty acids present in the sample. The unsaturation level of all fatty acids was estimated using a double bond index (DBI), which was calculated using the following equation: $\text{DBI} = 0 \times [(16:0) + (18:0)] + 1 \times [(16:1) + (18:1)] + 2 \times [(16:2) + (18:2)] + 3 \times (18:3)$ (Larkindale and Huang, 2004). Parentheses indicate the percentage of the total lipid content, which was made up by each lipid species.

STATISTICAL ANALYSIS. The significance of treatment and cultivar effects was determined using the analysis of variance according to the general linear procedure of SAS (Version 9.1; SAS Institute, Cary, NC) at $P = 0.05$. SES of means were calculated for four replicates per treatment or cultivar. In addition, correlation analysis between DBI and RWC and between DBI and EL was performed to determine the relationship between level of fatty acid unsaturation with drought tolerance characterized by RWC and EL.

Results and Discussion

PHYSIOLOGICAL CHARACTERIZATION OF CULTIVAR VARIATION DURING DROUGHT. Leaf RWC is an indicator of the internal water status, which is widely used for the evaluation of leaf

hydration state or internal water deficit level during dehydration (Rachmilevitch et al., 2006). Leaf RWC (Fig. 1A) remained $\approx 95\%$ under well-watered conditions in both genotypes. During drought stress, RWC declined rapidly in both genotypes, and 'Tifway' had significantly higher RWC than 'C299' at 5 and 10 d of treatment, indicating that 'C299' leaves lost water more quickly than 'Tifway' during drought stress. Plants were unwatered up to 10 d for 'C299', but for 13 d for 'Tifway' as a result of slower water loss until leaves of both genotypes were completely desiccated and RWC reached the same level in both genotypes (29% in 'Tifway' and 28% in 'C299').

Many biochemical factors are associated with dehydration tolerance of leaves, and the maintenance of cell membrane integrity or stability plays critical roles in leaf dehydration tolerance (Blum and Ebercon, 1981; Nilsen and Orcutt, 1996). The EL has been widely used as an indicator of estimating cell membrane stability (Blum and Ebercon, 1981; Rachmilevitch et al., 2006). Electrolyte leakage (Fig. 1B) increased during drought stress, to 69% for 'Tifway' and 86% for 'C299' by the end of treatment (10 d for 'C299' and 13 d for 'Tifway'), and these EL values were significantly different between the two genotypes. Leaf EL of 'C299' was also significantly higher than that of 'Tifway' at 10 d of drought stress. The increase of EL is positively associated with cellular membrane damage or deterioration (Simon, 1974).

The physiological analysis of both RWC and EL demonstrated that 'C299' was more sensitive to drought stress than 'Tifway', which is in agreement with previous reports in Hu et al. (2010a, b) and Zhao et al. (2010). Leaves of 'Tifway'

maintained lower EL (67%) at the same level of water deficit (indicated by RWC) by the end of the drought period compared with 'C299' (85%), suggesting leaves of 'Tifway' had superior dehydration tolerance.

GENOTYPIC VARIATION AND THE RESPONSE OF SATURATED FATTY ACID COMPOSITION TO DROUGHT. The alteration of fatty acid composition in membrane lipids is critical for plant adaptation to drought stress (Liljenberg, 1992; Navari-Izzo et al., 1993; Yordanov et al., 2000). In the present study, leaf samples were collected for fatty acid analysis at 10 d of drought stress for 'C299' and at 13 d of drought stress for 'Tifway', when plants suffered the same level of water deficit or RWC dropped to the same level (28% or 29%) in both genotypes. The comparison of fatty acids at the same level of water deficit for both genotypes enables the identification of fatty acids associated with leaf dehydration tolerance.

Three saturated fatty acids (SFAs) [palmitic acid (C16:0), stearic acid (18:0), and eicosanoic acid (C20:0)] were detected in leaves of both bermudagrass genotypes (Fig. 2). The C16:0 and C18:0 fatty acids are mainly found in plasma and organelle membranes (Millar et al., 2000). The C16:0 content was 12.6% in 'Tifway' and 16.6% in 'C299' under well-watered conditions; it increased significantly under drought stress, to 18.8% in 'Tifway' and 24.1% in 'C299' (Fig. 2A). The C16:0 content was significantly higher in 'C299' than in 'Tifway' under drought stress. Our results were similar to results reported in a cool-season turfgrass species, kentucky bluegrass; C16:0 accounted for 13% under well-watered conditions and 17% at 15 d of drought stress in a drought-sensitive cultivar (Midnight) and 15% and 20% in a drought-sensitive cultivar (Brilliant) in well-watered and drought-stressed plants, respectively (Xu et al., 2010). The C18:0 accounted for 2.3% in 'Tifway' and 3.5% in 'C299' under well-watered conditions and increased to 3.4% in 'Tifway' and 5.0% in 'C299' under drought stress (Fig. 2B). The C18:0 in 'C299' was significantly higher than in 'Tifway' under both well-watered and drought stress. The C18:0 also increased during drought stress to a greater extent in the drought-tolerant compared with the drought-sensitive cultivar in kentucky bluegrass, but C18:0 accounted for only $\approx 0.6\%$ of total fatty acids in that species (Xu et al., 2010). Increase in the levels of C16:0 and C18:0 may increase membrane rigidity as a result of the linearity of fatty acid arrangement in the membrane phospholipid phase (Cyril et al., 2002; Lehninger, 1977). Leaves of drought-resistant 'Tifway' could maintain more flexible membranes during dehydration associated with the lower levels of C16:0 and C18:0.

The C20:0 is a component of the long-chain fatty acid complex in the epicuticular wax present on the leaf surface (Rhee et al., 1998). The C20:0 was present in a very low amount relative to C16:0 and C18:0, which was only 0.6% in both genotypes under well-watered conditions; the percentage of C20:0 exposed to drought stress increased to 1.4% in 'Tifway' and to 1.8% in 'C299' (Fig. 2C), which was significantly higher in 'C299' than in 'Tifway'. However, C20:0 was not detected in well-watered or drought-stressed kentucky bluegrass in a previous study (Xu et al., 2010). Epicuticular wax accumulation on leaves has been associated with reduction in transpiration and improved drought tolerance in other grass species such as tall fescue [*Festuca arundinacea* (Fu and Huang, 2004)] and barley [*Hordeum vulgare* (González and Ayerbe, 2010)]. The increase in percent C20:0 under drought stress could reflect an adaptive response to drought stress in bermudagrass, which may reduce

Fig. 1. Changes in leaf relative water content (A) and electrolyte leakage (B) during drought stress in drought-resistant hybrid bermudagrass 'Tifway' and drought-sensitive common bermudagrass 'C299'. Vertical bars indicate least significant difference values ($P = 0.05$) for comparison across drought treatments and genotypes at a given day of treatment.

Fig. 2. Genotypic variations in saturated fatty acid (SFA) composition under well-watered and drought stress conditions with the same level of leaf relative water content in drought-resistant hybrid bermudagrass ‘Tifway’ and drought-sensitive common bermudagrass ‘C299’. Columns marked with the same letters are not significantly different based on least significant difference test ($P = 0.05$) comparing across drought treatments and two genotypes.

water loss from the leaf surface and protect leaves from desiccation.

The total SFA composition was 15.5% in ‘Tifway’ and 20.6% in ‘C299’ under well-watered conditions; it significantly increased under drought stress in both genotypes. ‘C299’ (30.9% SFA) had significantly higher SFA than ‘Tifway’ (23.6% SFA) (Fig. 2D). The higher total SFA was mainly the result of the higher C16:0, because C16:0 was the most abundant among the three SFAs detected in bermudagrass. These results suggested that lower membrane SFA composition, particularly C16:0, could be related to the better leaf dehydration tolerance in ‘Tifway’.

GENOTYPIC VARIATIONS AND DROUGHT RESPONSES AS RELATED TO UNSATURATED FATTY ACID COMPOSITION. Three unsaturated fatty acids (UFAs), primarily membrane lipid components [palmitoleic acid (C16:1), linoleic acid (C18:2), and linolenic acid (C18:3)], were detected in both bermudagrass genotypes (Fig. 3). Unlike SFA, UFA are loosely arranged in the lipid bilayers of cellular membranes as a result of the non-linearity of the fatty acid chains introduced by the presence of double bonds (Lehninger, 1977). The positive association of UFA content and membrane fluidity within lipid membranes is important for proper cellular metabolism and function under various environmental stresses (Hoekstra et al., 2001; Losa and Muratab, 2004).

The C16:1 accounted for 1.1% of total fatty acid content in ‘Tifway’ and 2.0% in ‘C299’ under well-watered conditions (Fig. 3A). The C16:1 content in both genotypes did not change in response to drought stress but was significantly higher in ‘C299’ than that in ‘Tifway’ under well-watered or drought conditions. The C18:2 (Fig. 3B) accounted for 26.3% of total fatty acid content in ‘Tifway’ and 15.2% in ‘C299’ under well-watered conditions. The C18:2 content was not significantly different between the control and drought-stressed plants for

‘Tifway’ but was significantly decreased under drought stress in ‘C299’. The C18:3 (Fig. 3C) accounted for 57.0% of total fatty acid content in ‘Tifway’ and 62.1% in ‘C299’ under well-watered conditions. The percentages of C18:3 in both genotypes significantly decreased under drought stress, to 50.0% in ‘Tifway’ and 54.5% in ‘C299’. ‘Tifway’ had lower C18:3 composition than ‘C299’ under both well-watered and drought conditions.

The composition of all detected UFA was 84.5% in ‘Tifway’ and 79.4% in ‘C299’ under well-watered conditions (Fig. 3D). The total composition of UFA decreased with drought stress but was significantly higher in ‘Tifway’ than in ‘C299’. Similar to total UFA composition, the DBI (Fig. 4) of fatty acids decreased under drought stress for both genotypes and ‘Tifway’ had significantly higher DBI than ‘C299’. Comparison of the composition of individual UFA and the combined UFA composition and DBI between the two genotypes suggested that the maintenance of higher total UFA composition, particularly C18:2, could contribute to the superior leaf dehydration tolerance in ‘Tifway’.

RELATIONSHIPS OF UNSATURATION LEVEL OF FATTY ACIDS AND LEAF WATER STATUS AND MEMBRANE STABILITY. Linear regression analysis between RWC and DBI was made to estimate the relationship of cellular water deficit and fatty acid unsaturation levels. DBI and RWC were positively correlated with coefficients of 0.908 and 0.944, respectively, for ‘Tifway’ and ‘C299’ (Fig. 5A). The DBI decreased as RWC decreased in both genotypes. The DBI value in ‘Tifway’ was higher than in ‘C299’ at the same level of internal water deficit (RWC below 40%). A negative correlation was found between DBI and EL with correlation coefficients of 0.925 and 0.960 for ‘Tifway’ and ‘C299’, respectively (Fig. 5B). Leaf EL decreased as DBI increased for both genotypes, and ‘Tifway’ had higher DBI value than ‘C299’ at the same level of EL. The EL in ‘C299’

Fig. 3. Genotypic variations in unsaturated fatty acid (UFA) composition under well-watered and drought stress conditions with the same level of leaf relative water content in drought-resistant hybrid bermudagrass 'Tifway' and drought-sensitive common bermudagrass 'C299'. Columns marked with the same letters are not significantly different based on least significant difference test ($P = 0.05$) comparing across drought treatments and two genotypes.

Fig. 4. Genotypic variations in unsaturation level of all fatty acids, expressed as double bond index (DBI), under well-watered and drought stress conditions with the same level of leaf relative water content in drought-resistant hybrid bermudagrass 'Tifway' and drought-sensitive common bermudagrass 'C299'. Columns marked with the same letters are not significantly different based on least significant difference test ($P = 0.05$) comparing between well-watered control and drought treatment and between the two genotypes.

changed more rapidly in DBI than in 'Tifway' with the linear slope being -2.904 (percent EL per unit change of DBI) for 'C299' and -2.089 for 'Tifway'. Similar relationships of DBI with RWC and with EL were found in kentucky bluegrass exposed to drought stress and rewatering (Xu et al., 2010).

In summary, drought-resistant 'Tifway' leaves were able to maintain higher cellular membrane stability (lower EL) than drought-sensitive 'C299' at the same level of water deficit. Our results suggested a positive relationship of unsaturation level (indicated by DBI) and composition of unsaturated fatty acids with leaf dehydration tolerance in bermudagrass. Such a relationship for drought tolerance has not been previously reported in turfgrass species, although it has already been established in the case of cold tolerance (Cyril et al., 2002). The higher level of unsaturation of fatty acids in 'Tifway' compared

Fig. 5. Relationships between leaf relative water content (RWC) and double bond index (DBI) and between electrolyte leakage (EL) and DBI in drought-resistant hybrid bermudagrass 'Tifway' and drought-sensitive common bermudagrass 'C299'. Correlation coefficients were significant at $P = 0.05$.

with 'C299' was mainly the result of the higher composition of C18:2 and the lower composition of C16:0 and C18:0. The combined results of genotypic variations in physiological performance and fatty acid composition suggested that C18:2,

C16:0, and C18:0 could be major fatty acids involved in regulating membrane stability, protecting leaves from dehydration in bermudagrass. These fatty acids have potential for use as biomarkers to select for drought-tolerant turfgrass genotypes.

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