Bermudagrass Drought Tolerance Associated with Dehydrin Protein Expression during Drought Stress

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ABSTRACT. Drought stress is a major limiting factor for warm-season turfgrass growth during the summer in the U.S. transition zone. Genotypic variation in drought resistance exists among bermudagrasses (*Cynodon* sp.), but the mechanisms of drought resistance are poorly understood. Our objectives were to investigate physiological changes in three bermudagrass cultivars under a well-watered condition and drought stress, to determine expression differences in soluble protein and dehydrin of the three cultivars under well-watered and drought stress conditions, and to identify the association between dehydrin proteins and drought tolerance. Grasses included a high drought-resistant cultivar, Celebration, a low drought-resistant cultivar, Premier, and a newly released cultivar, Latitude 36. In both well-watered and drought treatments, 'Latitude 36' had the highest visual quality and lower or medium electrolyte leakage among three cultivars. In the drought treatment, 16- and 23-kDa dehydrin proteins were observed in 'Latitude 36' but not in 'Celebration' or 'Premier'. Our results indicate that the 16- and 23-kDa dehydrin expressions could be associated with drought tolerance and contribute to drought tolerance in bermudagrass.

Drought is a major limiting factor for sustainable turfgrass management in the United States. Climate prediction models indicate that the world may experience increased global temperatures, decreased precipitation, and an increase in the occurrence and persistence of drought periods over the next century. A growing challenge facing the turfgrass industry is limited availability of water for irrigation (Snow, 2001). Thus, understanding the mechanisms of drought resistance is increasingly important for turfgrass breeders and managers.

Dehydrins are the late embryogenesis abundant (LEA) D 11 family of hydrophilic proteins with a wide range of molecular masses from 9 to 200 kDa (Close, 1996). They accumulate in plants in response to environmental influence with a dehydration component such as drought, salinity, and low temperature (Beck et al., 2007; Close, 1997). These proteins have been postulated to stabilize macromolecules or cellular structure and help to maintain the integrity of cell membranes against dehydration (Beck et al., 2007; Bray, 1997; Campbell and Close, 1997; Close, 1997). Most work with dehydrins in turfgrass has been completed in cold acclimation and freezing tolerance (Gatschet et al., 1994; Patton et al., 2007; Zhang et al., 2008,

2011). There are also studies reporting that dehydrins are present in turfgrass when drought-stressed (Hu et al., 2010; Jiang and Huang, 2002; Pan et al., 2013; Voltaire, 2002). Very few data exist in turfgrass that support the popular hypothesis that dehydrin accumulation is positively correlated with dehydration tolerance. Hu et al. (2010) reported that accumulation of 31- and 40-kDa dehydrins may contribute to drought tolerance in bermudagrass through investigating four hybrid bermudagrasses (*Cynodon dactylon* × *Cynodon transvaalensis*) and four common bermudagrasses (*C. dactylon*).

Bermudagrass, the most important warm-season turfgrass grown in the southern portion of the United States, is regarded as a drought-resistant species (McCarty and Miller, 2002) with genotypic variation in drought resistance. Its drought resistance mechanisms include drought avoidance and drought tolerance. Some bermudagrasses have growth features such as deeper root systems, thicker cuticles, and smaller stomatal openings, which can improve their drought avoidance (Carrow, 1995, 1996; Qian et al., 1997). Others can sustain biochemical and physiological processes during internal water deficits (Hu et al., 2010; Huang et al., 1997). Different drought resistance mechanisms among bermudagrass cultivars may exhibit different patterns of dehydrin expression in response to drought stress. Three cultivars of bermudagrass selected for this study were two common bermudagrass cultivars, Celebration and Premier, and a clonally propagated hybrid cultivar developed by Oklahoma State University, Latitude 36. Several researchers have reported that 'Celebration' is a top-performing drought-resistant cultivar with a deep root system, whereas 'Premier' has high visual quality but

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is a drought-sensitive cultivar (Baldwin et al., 2006; Poudel, 2010; Steinke et al., 2009). ‘Latitude 36’, tested as OKC1119, was an excellent performer among all cultivars in the 2007 to 2011 National Turfgrass Evaluation Program (NTEP) bermudagrass test and had high turf quality rating (NTEP, 2013). However, there are few reports on the drought resistance mechanisms associated with dehydrin protein expression during drought stress. Investigations concerning the physiological bases of cultivar differences in drought resistance would provide valuable selection information for turfgrass breeders and managers. The objectives of this study were to investigate physiological changes in three bermudagrass cultivars under well-watered conditions and drought stress, to determine expression differences in soluble protein and dehydrin of three cultivars under well-watered and drought stress conditions, and to identify the association between dehydrin proteins and drought tolerance.

Materials and Methods

PLANT PREPARATION, MAINTENANCE, TREATMENTS, EXPERIMENTAL DESIGN, AND STATISTICAL ANALYSIS. Thirty sod plugs of ‘Celebration’, ‘Premier’, and ‘Latitude 36’ were collected on 19 Mar. 2010 from established swards at the Oklahoma State University Turfgrass Research Center in Stillwater (lat. 36°63′56″ N, long. 97°3′29″ W) and each cultivar was planted in 10 lysimeters (10 cm diameter × 45 cm deep). Lysimeters were filled with a mixture of sand and silt loam topsoil (1:1 v:v) and were maintained in a greenhouse for 3 months. The particles of sand and topsoil were less than 1 mm in diameter. Average day/night air temperature was 30/20 °C. The supplemental light with incandescent lamps was included for 14 h·d⁻¹ and average photosynthetically active radiation (*PAR*) was 1000 μmol·m⁻²·s⁻¹ in a quantum light meter (3415F; Spectrum Technologies, Plainfield, IL) during the daylight period. Grasses were clipped weekly at 5.0 cm and watered twice with fertilizer water at 0.25 g·L⁻¹ of 20N–8.7P–16.6K water-soluble fertilizer (Jack’s Professional 20-20-20; J.R. Peters, Allentown, PA).

After 3 months in the greenhouse, lysimeters were transferred to a walk-in growth chamber and acclimated for 3 weeks at optimal growth temperatures (30/25 °C, 14-h light/10-h darkness) with *PAR* at 600 μmol·m⁻²·s⁻¹ during the daylight period. Turfgrasses were mowed once per week at 5.0 cm and irrigated every 3 d. To maintain well-watered conditions, 100% of water lost through evapotranspiration (ET) during the previous 3 d was replaced; 100% ET was determined gravimetrically with the lysimeters (Bremer, 2003). Using this method, lysimeters were irrigated, allowed to drain until free drainage ceased, sealed, and weighed. Lysimeters were weighed again after 3 d and the water loss was attributed to ET. Turfgrasses were fertilized every 6 d with a solution supplying 16 kg·ha⁻¹ nitrogen (20N–8.7P–16.6K).

On 11 July 2010, ‘Celebration’, ‘Premier’, and ‘Latitude 36’ were exposed to well-watered and drought treatments in the walk-in growth chamber. The lysimeters under the well-watered condition continued to be irrigated by 100% ET replacement every 3 d and drought treatment was conducted by completely withholding irrigation.

A randomized complete block design with five replications (i.e., five blocks) was used. Totally there were 30 lysimeters with three cultivars, two treatments, and five replications. Thirty lysimeters were used to collect for volumetric soil water content (θ_v), visual quality, and electrolyte leakage. These data were

analyzed using the MIXED procedure of SAS (Version 9.1; SAS Institute, Cary, NC) and differences between means were separated by the SAS PDIFF option ($P = 0.05$). Eighteen lysimeters (three replications) were used to extract protein.

MEASUREMENTS OF VOLUMETRIC SOIL WATER CONTENT, VISUAL QUALITY, AND LEAF ELECTROLYTE LEAKAGE. Volumetric soil water content at 0- to 20-cm profile from each lysimeter was measured on 12 July 2010 [0 d of treatment (0 DOT)] and 20 July 2010 (8 DOT) using time domain reflectometry (HydroSense system; Campbell Scientific, Logan, UT).

Turf visual quality was rated on a scale of 1 to 9 (1 = poorest quality, 6 = minimally acceptable, and 9 = highest quality) according to color, texture, density, and uniformity (Emmons, 2000). Quality ratings were recorded on 0 and 8 DOT by the same individual.

The drought damage severity of turfgrass cell membrane was estimated by measuring leaf electrolyte leakage. Fifteen young fully expanded living leaves approximately similar age were collected from each lysimeter on 0 and 8 DOT. These young fully expanded leaves from each lysimeter were rinsed three times with distilled deionized water and placed in a test tube filled with 20 mL distilled deionized water. Test tubes were shaken on a shaker table at 120 rpm for 24 h to dissolve the electrolytes leaking from cells resulting from the drought stress. The solution conductivity (*C*₁) was measured with a portable conductivity meter (Accumet AP85; Fisher Scientific, Pittsburgh, PA). Then the leaf tissues were killed in an autoclave at 140 °C for 20 min to destroy the cell membranes, shaken for 24 h at room temperature to extract all the electrolytes from the cells, and the conductivity (*C*₂) was measured again. The percentage electrolyte leakage was calculated as $C_1/C_2 \times 100$ and its lower percentage indicates greater resistance to drought stress.

PROTEIN EXTRACTION. Total soluble protein was extracted according to the procedure of Damerval et al. (1986) with minor modifications. Briefly, fully expanded leaves (0.15 g fresh weight) were collected from each lysimeter on 0 and 8 DOT and grounded in a mortar and pestle with liquid nitrogen to a fine powder. The powder was mixed with 1.0 mL 10% w/v trichloroacetic acid and 0.07% v/v 2-mercaptoethanol in cold (–20 °C) acetone in 1.5-mL microcentrifuge tube. The mixture was incubated overnight at –20 °C and vortexed four times during the incubating process. Samples were then centrifuged at 16,000 *g*_n at 4 °C for 15 min and the supernatant was removed by decanting. Acetone containing 0.07% v/v 2-mercaptoethanol (1 mL at –20 °C) was used to wash the pellet and remove pigments and lipids until the pellet was colorless. The pellet was dried in a Speed Vac (Thermo Electron, Waltham, MA) for 15 min and re-suspended in 1-mL rehydration buffer (8 M urea, 2% 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate, and 50 mM dithiothreitol). Proteins were extracted in a water-bath sonicator (FS60; Fisher Scientific) for 30 min and centrifuged at 16,000 *g*_n at 4 °C for 25 min. The supernatant containing predominantly soluble protein was transferred to a new microcentrifuge. Next, 25-μL aliquots of supernatant were removed for protein quantification. Buffer-soluble protein was estimated using the Bradford procedure (Bradford, 1976). Buffer-soluble protein concentrations were determined using bovine serum albumin as a standard and absorbance was read at 595 nm in a spectrophotometer (Novaspec Plus; Amersham Bio-Sciences, Piscataway, NJ). The remaining supernatant was retained for sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting.

SDS-PAGE AND IMMUNOBLOTTING. An aliquot of 50 μL supernatant mixed with 50 μL Laemmli sample buffer (0.0625 $\text{mol}\cdot\text{L}^{-1}$ Tris-HCl, pH 6.8, 20% glycerol, 2% SDS, 0.01% bromophenol blue, 5% β -mercaptoethanol) was used for SDS-PAGE analysis (Laemmli, 1970) after heating 3 to 5 min at 100 $^{\circ}\text{C}$ in a sealed screw-cap microcentrifuge tube. Lanes were loaded with 20 μg sample protein, and proteins were separated in 1.0-mm-thick gels containing 5% stacking gel and 12% running gel with the Mini-Protean Tetra Cell (Bio-Rad Laboratories, Hercules, CA). The gel was stained for 1 h with Bio-Safe Coomassie G-250 stain (Bio-Rad Laboratories).

For immunoblotting, SDS unstained gel containing 20 μg separated proteins was transferred onto an Immun-Blot polyvinylidene difluoride membrane (0.2 μm ; Bio-Rad Laboratories) with an electrophoresis transfer unit Mini-Protean Tetra Cell (Bio-Rad Laboratories) in transfer buffer (25 mM Tris base, 192 mM glycine, 10% v/v methanol, pH 8.3) at constant volts of 100 for 1 h. The gel membrane was blocked with blocking buffer [5% dry skim milk with 0.05% Tween 20 (Bio-Rad Laboratories) in tert-butyl dimethylsilyl (TBS) (20 mM TrisHCl, 150 mM NaCl, pH 7.5)] at 4 $^{\circ}\text{C}$ overnight. Then the membrane was probed with 1:250 dilution of rabbit antidehydrin polyclonal antibody (PLA-100; Enzo Life Sciences International, Plymouth Meeting, PA) for 1 h. After being washed three times by TTBS (0.05% Tween 20 in TBS), goat antirabbit AP secondary antibody (Bio-Rad Laboratories) was diluted in TTBS (dilution 1:3000) to incubate the membranes for 1 h. After three washes in TTBS and one wash in TBS, a representative blotting image was detected by gently shaking the membrane immersed in color development solution [mixture of 0.2 mL AP color reagent A, 0.2 mL color reagent B, and 19.6 mL 1 $\times$ color development buffer (Bio-Rad Laboratories)] at room temperature on a shaker table at 30 rpm (Midwest Scientific, St. Louis, MO). The SDS-PAGE and immunoblotting were conducted for three replications and the representative data are presented here.

Results and Discussion

RESPONSES OF VOLUMETRIC SOIL WATER CONTENT, VISUAL QUALITY, AND LEAF ELECTROLYTE LEAKAGE TO DROUGHT STRESS. Volumetric soil water content at 0- to 20-cm profile was higher in ‘Premier’ than in ‘Latitude 36’ under well-watered treatment on 0 DOT, although absolute differences in θ_v at 0- to 20-cm profile among the three cultivars were markedly small (i.e., θ_v at 0 to 20 cm ranged from 30.0% to 31.8%) (Fig. 1A). Under the drought treatment and well-watered treatment on 8 DOT, there were no differences in θ_v at 0- to 20-cm profile among three cultivars (i.e., three bermudagrass cultivars received the same level of drought stress). On 8 DOT, the drought treatment reduced θ_v at 0- to 20-cm profile to 7.6% to 9.0% (Fig. 1A–B).

In well-watered conditions, visual quality was highest in ‘Latitude 36’ and lower, but similar, between ‘Celebration’ and ‘Premier’ (Fig. 2A). Lower visual quality in ‘Celebration’

was primarily the result of its coarse leaf texture. Higher turf quality in ‘Latitude 36’ than in ‘Premier’ was also reported in the 2007 to 2011 NTEP bermudagrass test (NTEP, 2013). Drought stress reduced visual quality among three cultivars. On 8 DOT, the drought stress reduced visual quality 10.0% in ‘Celebration’, 15.6% in ‘Latitude 36’, and 19.0% in ‘Premier’. However, visual quality was higher in ‘Latitude 36’ than in ‘Celebration’ and ‘Premier’ mainly because of its fine leaf texture (Fig. 2B).

On 0 DOT in well-watered and drought conditions, living leaf electrolyte leakage (EL) was higher in ‘Premier’ than in ‘Celebration’, although absolute differences in EL among the three cultivars were markedly small (i.e., EL ranged from 2.0% to 3.3% at 0 DOT in well-watered and from 2.4% to 3.5% at 0 DOT in drought conditions) and there was no difference between ‘Celebration’ and ‘Latitude 36’ (Fig. 3A–B). After exposure to drought, the EL in ‘Celebration’, ‘Latitude 36’, and ‘Premier’ were 3.5%, 4.7%, and 6.2%, respectively (Fig. 3B). Electrolyte leakage was lowest in ‘Celebration’ and highest in ‘Premier’ indicating less damage to cellular membranes in ‘Celebration’ and more damage to cellular membranes in ‘Premier’. These data showed that ‘Celebration’ has greater drought resistance likely because ‘Celebration’ may use its deeper root system to absorb deeper soil water in the lysimeters, thus avoiding drought stress. Steinke et al. (2009) reported that the mean canopy temperature was lower in ‘Celebration’ than in ‘Premier’ on several measurement dates during the 60-d drought in a 2-year field study. Lower canopy temperatures in

Fig. 1. Soil water content at 0- to 20-cm depth of bermudagrass ‘Celebration’, ‘Latitude 36’, and ‘Premier’ under well-watered (A) and drought (B) conditions. Means with the same letters on a given day after treatment initiation (day of treatment) were not significantly different ($P = 0.05$).

Fig. 2. Visual quality rated on a scale of 1 to 9 (1 = poorest and 9 = highest) of bermudagrass ‘Celebration’, ‘Latitude 36’, and ‘Premier’ under well-watered (A) and drought conditions (B). Means with the same letters on a given day after treatment initiation (day of treatment) were not significantly different ($P = 0.05$).

‘Celebration’ may enhance heat dissipation through greater evapotranspiration and allowing it to maintain leaf turgor longer in the drought situation.

PROTEIN CHANGES. The SDS-PAGE analysis of buffer-soluble protein from leaves indicated that all polypeptides maintained stable bands in response to drought stress. There were similar SDS-PAGE profiles of buffer-soluble protein from leaves of ‘Celebration’, ‘Latitude 36’, and ‘Premier’ under well-watered and drought conditions (Fig. 4).

Immunoblots indicated that dehydrin polypeptides of ≈ 16 and 23 kDa were detected in ‘Latitude 36’ under the drought treatment at 8 DOT but no dehydrin proteins were observed in ‘Celebration’ and ‘Premier’ under the drought condition and no dehydrin polypeptides were detected in three cultivars under the well-watered condition (Fig. 5). Drought-induced dehydrin polypeptides have been observed in many turfgrass studies (Hu et al., 2010; Jiang and Huang, 2002; Volaire, 2002). Jiang and Huang have found that 23- and 27-kDa dehydrin polypeptides

were observed at 10 d in drought stress and were more pronounced in the drought-stressed tall fescue (*Festuca arundinacea*) without abscisic acid. Our results in ‘Latitude 36’ also indicated that accumulation of 16 and 23 kDa was responsive to drought stress. Dehydrin polypeptides were not detected in ‘Premier’ under drought stress, although it suffered more drought damage than ‘Latitude 36’ and ‘Celebration’ has been reported that ‘Premier’ is a drought-sensitive bermudagrass cultivar (Poudel, 2010; Steinke et al., 2009). However, dehydrin polypeptides were also not present in the drought-resistant cultivar Celebration under the drought treatment. It is mainly because of its different drought resistance mechanisms. Poudel (2010) has reported that ‘Celebration’ was a top drought-resistant cultivar with a deep root system among 23 bermudagrass genotypes. In our study, lowest electrolyte leakage in ‘Celebration’ among three cultivars under drought treatment also confirmed less drought damage to cellular membranes in ‘Celebration’. Under the condition at 8 DOT, the three bermudagrass cultivars

received the same level of drought stress in the 0- to 20-cm profile, and our lysimeters were 45 cm deep. ‘Celebration’ may use its deep root system to absorb deeper soil water in the lysimeters and avoided drought stress. Our results also supported the popular hypothesis that dehydrin accumulation is positively correlated with drought tolerance (Beck et al., 2007; Close, 1997; Close et al., 1993; Hu et al., 2010; Labhili et al., 1995; Lopez et al., 2003).

Our results suggest that dehydrin polypeptides of 16 and 23 kDa play a role in drought tolerance of bermudagrass. The function of 16 kDa for drought tolerance in our study is consistent with other findings in wheat [*Triticum durum* (Labhili et al., 1995)] and olive [*Olea europaea* (Tripepi et al., 2011)]. Lopez et al. (2003) found that expression of a 24-kDa dehydrin was observed in some drought-tolerant wheat (*Triticum aestivum*) cultivars after 4 d of drought stress and positively associated with drought tolerance in wheat. As far as the function of 23 kDa was concerned, several researchers reported that the 23-kDa dehydrin polypeptides were associated with chilling or freezing tolerance (Kang et al., 2005; Patton et al., 2007). Gatschet et al. (1994) have reported that accumulation of low-molecular-weight (20 to 26 kDa) cold-regulated proteins similar to dehydrins was positively correlated to freeze tolerance in bermudagrass. Cold and drought affect similarly the water relations of plant at the cellular or whole plant levels (Beck et al., 2007). The molecular weight of dehydrin proteins varies from 9 to 200 kDa (Close, 1996). The wide range of molecular weights in dehydrin proteins is caused by the repeat number of K segments, which is a 15 amino acid consensus sequence (EKKGIMDKIKEKPLG). The K segment resembles lipid-binding class A₂ amphipathic α -helical proteins and stabilizes the cell membrane to tolerate drought

Fig. 3. Living leaf electrolyte leakage of bermudagrass ‘Celebration’, ‘Latitude 36’, and ‘Premier’ under well-watered (A) and drought conditions (B). Means with the same letters on a given day after treatment initiation (day of treatment) were not significantly different ($P = 0.05$).

Fig. 4. Dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) profiles of buffer-soluble protein from leaves of bermudagrass ‘Celebration’, ‘Latitude 36’, and ‘Premier’ under well-watered and drought conditions. Lane 1, molecular marker; lane 2, ‘Celebration’ under well-watered condition; lane 3, ‘Latitude 36’ under well-watered condition; lane 4, ‘Premier’ under well-watered condition; lane 5, ‘Celebration’ under 8 d drought stress [drought on 8 d of treatment (DOT)]; lane 6, ‘Latitude 36’ under 8 d drought stress; lane 7, ‘Premier’ under 8 d drought stress. Equal amounts of buffer-soluble protein (20 μ g) were loaded into each lane.

Fig. 5. Immunoblots of buffer-soluble protein from leaves of bermudagrass 'Celebration', 'Latitude 36', and 'Premier' under well-watered and drought conditions and probed with 1:250 dilution of rabbit antidehydrin polyclonal antibody. Lane 1, molecular marker; lane 2, 'Celebration' under well-watered condition; lane 3, 'Latitude 36' under well-watered condition; lane 4, 'Premier' under well-watered condition; lane 5, 'Celebration' under 8 d drought stress [drought on 8 d of treatment (DOT)]; lane 6, 'Latitude 36' under 8 d drought stress; lane 7, 'Premier' under 8 d drought stress. All lanes were loaded with 20 µg buffer-soluble protein.

stress (Close, 1997; Davidson et al., 1998; Koag, et al., 2003). The contribution of dehydrins to drought tolerance is achieved by considering dehydrins as membrane stabilizers and involving alleviation of cell damage during drought stress although the precise mechanisms is still to be explored (Bartels and Sunkar, 2005; Koag et al., 2003). Moreover, Goyal et al. (2005) provided the first evidence of antiaggregation activity of *LEA* proteins resulting from water stress. Larsson et al. (2006) reported that drought stress changed membrane phospholipid composition by enhancing phosphatidylserine decarboxylase activity in root cells of oat (*Avena sativa*). Our previous study of membrane lipid composition and drought tolerance of the same three bermudagrass cultivars indicated that lower monogalactosyldiacylglycerol and higher phospholipid contents contributed to drought tolerance in bermudagrass (Su et al., 2013). Therefore, more research is needed to identify the association between dehydrin proteins and drought tolerance to decide whether these dehydrin proteins could be used as practical protein markers for drought tolerance.

Conclusions

Our data indicate that 'Latitude 36' had the highest visual quality and lower or medium EL among three cultivars in both well-watered and drought treatments. In the drought treatment, 16- and 23-kDa dehydrin proteins were observed in 'Latitude 36'. Our results indicate that the 16- and 23-kDa dehydrin expressions could be associated with drought tolerance and contribute to drought tolerance in bermudagrass.

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