

Bermudagrass Freezing Tolerance Associated with Absciscic Acid Metabolism and Dehydrin Expression during Cold Acclimation

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ABSTRACT. Recent advances in bermudagrass [*Cynodon dactylon* (L.) Pers. var. *dactylon*] breeding and cultural management practices have enabled its use as a sports surface in U.S. Department of Agriculture cold hardiness zones 5 and 6. Use of these more cold-hardy bermudagrass cultivars further into transition- and cool-season zones increases the probability of freezing injury and increases the need for an improved understanding of physiological responses to chilling and freezing temperatures. Absciscic acid (ABA) has been shown to increase during cold acclimation (CA) and play a role in dehydration tolerance. This study investigated changes in ABA metabolism and dehydrin expression during CA and their association with freezing tolerance in four bermudagrass cultivars. Two cold-tolerant ('Patriot' and 'Riviera') and two relatively cold-sensitive ('Tifway' and 'Princess') cultivars were either subjected to CA at 8 °C day/4 °C night with a light intensity of 250 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ over a 10-h photoperiod for 21 days or maintained at 28 °C day/24 °C night over a 12-h photoperiod. In a separate study, exogenous ABA at 0, 50, 100, and 150 μM was applied to 'Patriot' bermudagrass without CA. ABA content in leaf and stolon tissues increased substantially during the first week of CA and remained relatively stable thereafter. 'Patriot' and 'Riviera' had greater ABA content and less stolon electrolyte leakage (EL) relative to 'Tifway' and 'Princess'. Expression of a 25 kDa dehydrin protein increased during CA in all four cultivars. A significant correlation was found between ABA content and freezing tolerance. Exogenously applying ABA to 'Patriot' at 50, 100, and 150 μM significantly increased endogenous ABA content and the 25 kDa dehydrin expression and reduced stolon EL. The results suggest that alteration of ABA metabolism during CA is closely associated with freezing tolerance. Selection and use of cultivars with substantial accumulation of ABA and certain dehydrins during CA or in response to exogenous ABA could improve bermudagrass persistence in transition zone climates.

Common bermudagrass is widely distributed throughout the world between latitudes 45°N and 45°S (Anderson et al., 1993; Harlan and de Wet, 1969), and both common and triploid hybrid [*Cynodon* \times *magennisii* Hurcombe (= *C. dactylon* \times *C. transvaalensis* Burt-Davy)] are used in turf systems (McCarty and Miller, 2002). Bermudagrasses grown in the transition zone of the United States are subject to freeze damage (Anderson et al., 2003; Fry, 1990; Taliaferro et al., 2004) and periodic severe winterkill (Anderson et al., 1997; Hiscock, 1996; Munshaw, 2004; Zhang et al., 2006).

Bermudagrasses undergo cold acclimation (CA), which is induced by a combination of reduced photoperiod and temperatures of less than 10 °C in the autumn. Cold-acclimated cultivars can develop metabolic defenses rapidly to cope with freezing stress (Zhang et al., 2006). Increases in certain sugars or amino acids, synthesis of novel proteins, and increases in the degree of unsaturation of membrane lipid fatty acids, antioxidant capacity, and certain hormones are some of the most important metabolic defenses against freezing stress (Cyril et al., 2002; Hughes and Dunn, 1996; Kalberer et al., 2006; Karpinski et al., 2002; Lee and Chen, 1993; Munshaw et al., 2006; Perras and Sarhan, 1989; Zhang et al., 2006; Zhang and Ervin, 2008).

Absciscic acid (ABA) plays an important role in low temperature response and is correlated with enhanced freezing tolerance in some plant species (Lee and Chen, 1993). It has

been reported that endogenous ABA increases during cold acclimation and that application of ABA may induce freezing tolerance in several plant species (Lee and Chen, 1993). A mutant of *Arabidopsis* [*Arabidopsis thaliana* (L.) Heynh.] deficient in ABA was unable to cold-acclimate unless treated with exogenous ABA (Heino et al., 1990). Exogenous ABA has been shown to induce dehydrin gene expression (Han and Kermode, 1996).

Dehydrin proteins (late embryogenesis abundant D11 family) are members of a family of proteins that range in size from 9 to 200 kDa. They have been shown to be expressed after plant exposure to environmental stimuli with a dehydrative component, including low temperature, drought, salinity, and developmental stages such as seed and pollen maturation (Close, 1997). Dehydrins have been postulated to stabilize cell structures against dehydration (Close, 1997; Danyluk et al., 1998). In several plant species, dehydrins have been shown to possess *in vitro* cryoprotective activity and *in vivo* antifreeze activity (Wisniewski et al., 1999). Dehydrins have also been suggested to function as possible osmoregulators (Nylander et al., 2001) or as radical scavengers (Hara et al., 2003). Several researchers have reported that dehydrin accumulation is correlated with freezing tolerance in some plant species (Marian et al., 2003; Zhu et al., 2000). Danyluk et al. (1998) noted that the WCOR410 dehydrin protein accumulated near the plasma membrane during CA of wheat (*Triticum aestivum* L.) and suggested that this accumulation protected integrity of the plasma membrane when plants were subjected to stress. Patton

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et al. (2007) found that dehydrin polypeptides of 23 and 25 kDa increased during CA and that the 23 kDa dehydrin polypeptide was associated with freezing tolerance of zoysiagrass (*Zoysia* Willd.).

The electrolyte leakage technique is commonly used to assess the level of cell injury caused by low temperatures and to test the relative freezing tolerance of turfgrasses (Anderson et al., 1988, 2002; Cardona et al., 1997; Fry et al., 1991; Miller and Dickens, 1996; Shashikumar and Nus, 1993; Zhang et al., 2006). The concept of lethal temperature for 50% loss of electrolytes (LT50) has been used as a measure of cold hardness and is defined as the predicted test temperature resulting in 50% or greater loss of total electrolytes (Shashikumar and Nus, 1993). When interpreting these results, it has been assumed that an electrolyte leakage (EL) of 50% or more is lethal (Fry et al., 1993). Predicted LT50 based on EL and glasshouse regrowth evaluations have been reported to be in close agreement when bermudagrass was tested (Anderson et al., 1988; Miller and Dickens, 1996; Zhang et al., 2006).

Bermudagrass cultivars vary largely in freezing tolerance (Anderson et al., 2003; Taliaferro et al., 2004). 'Patriot' ($2n = 4x = 36$ chromosomes) and 'Tifway' ($2n = 3x = 27$ chromosomes) are vegetatively propagated hybrid cultivars, whereas 'Riviera' and 'Princess' are common seeded cultivars ($2n = 4x = 36$ chromosomes). 'Patriot' and 'Riviera' were top performers among all cultivars in the 2003 to 2006 National Turfgrass Evaluation Program (NTEP) bermudagrass test and have excellent cold tolerance and high turf quality ratings (NTEP, 2008; Taliaferro et al., 2004). 'Tifway' and 'Princess' are widely used cultivars with highly rated visual quality but a medium level of cold tolerance. Enhancement of cultivar freezing tolerance as a means of reducing risk of winterkill has been a major goal of many bermudagrass improvement programs (Anderson et al., 2003). However, there are few reports on the physiological mechanisms of cultivar variation in freezing tolerance. Investigations concerning the physiological bases of cultivar differences in freezing tolerance would provide valuable selection information for turfgrass breeders and practitioners, especially in the transition zone. The objectives of this study were: 1) to examine changes in the levels of endogenous ABA and dehydrin proteins during CA and to investigate relationships of ABA and dehydrins with freezing tolerance; and 2) to examine if exogenous ABA affects endogenous ABA level and dehydrin expression associated with freezing tolerance in bermudagrass.

Materials and Methods

TURFGRASS CULTURE, COLD ACCLIMATION, AND ABSCISIC ACID TREATMENT. Four bermudagrass cultivars, 'Patriot' and 'Riviera' (cold-tolerant) and 'Tifway' and 'Princess' (relatively cold-sensitive), were used for this study. Mature bermudagrass plugs (10 cm diameter \times 5 cm deep) were taken from field plots at the Virginia Tech Turfgrass Research Center, Blacksburg, VA, on 2 Aug. 2007 and grown in pots (12.5 cm diameter \times 12.0 cm deep) filled with medium-textured sand and having three holes at the bottom to allow drainage. The grass was grown in a temperature-controlled glasshouse at 25 °C day/23 °C night with natural sunlight plus artificial light (Ruud Lighting, Racine, WI) [average $\approx 380 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ photosynthetically active radiation (PAR), a 12-h photoperiod]. Nitrogen was applied at 45 kg·ha⁻¹ with a 20N-8.8P-16.6K soluble fertilizer

containing micronutrients 2 d after the plugs were transplanted and every 4 weeks thereafter.

Twelve weeks after transplanting, the four cultivars were subjected to two treatments: CA and noncold acclimation (NA). Four pots from each cultivar were transferred to a growth chamber and subjected to CA at 8/4 °C (day/night) with a PAR intensity of $250 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ over a 10-h photoperiod for a period of 21 d. For NA, one pot from each cultivar was maintained at a normal temperature (28 °C day/24 °C night) in another growth chamber with the same PAR intensity and 12-h photoperiod.

To investigate effects of exogenous ABA application, ABA solutions at 0, 50, 100, and 150 μM , containing 0.01% (v/v) Tween 20 (Sigma, St. Louis), were sprayed uniformly to the canopy of 'Patriot' bermudagrass at a dosage of 10 mL per pot on 23 Oct. and again on 24 Oct. 2007. The treated grass was transferred to a growth chamber and maintained at a normal temperature (28 °C day/24 °C night) with the same PAR intensity as described previously. Only one cultivar was used in the exogenous ABA treatment trial because of space limitations in the growth chamber. 'Patriot' was selected because it is one of the most important cultivars used in Virginia and more genetically uniform relative to the seeded cultivars. The grass was mowed to 1.25 cm weekly until CA was initiated or ABA treatment. The grass plugs were watered once per week but not fertilized during the 3-week period in the growth chambers.

SAMPLE COLLECTION. At 0, 7, 14, and 21 d after CA initiation, leaf and stolon samples were collected for analysis of EL, ABA content, and dehydrin expression. The EL was measured immediately after sampling and the samples for ABA and dehydrin analysis were immediately frozen with liquid N and stored at -80 °C.

ABSCISIC ACID ASSAY. Leaf or stolon tissue (100 mg) was ground to a powder using a mortar and a pestle in liquid nitrogen. The sample was extracted in 2 mL methanol containing 0.01% butylated hydroxytoluene overnight. The sample was centrifuged at 2000 g_n for 15 min and the supernatant was collected and dried in a stream of air.

The ABA sample was purified using a C₁₈ Sep-Pack column (Waters, Milford, MA). The C₁₈ Sep-Pack column was flushed with 1 mL 100% methanol and then 1 mL 10% methanol. The ABA sample was dissolved in 10% methanol and loaded into the column. The container was washed with 1 mL 10% methanol two times and the column flushed. The ABA was eluted with 1 mL 80% methanol and dried in a stream of air. The sample was dissolved in 100 μL methanol and diluted with tris-buffered saline (TBS) buffer.

Indirect enzyme-linked immunosorbent assay was used for ABA estimation according to Walker-Simmons et al. (2000) with some modifications. Briefly, wells of a 96-unit plate were coated with 200 μL per well ABA-C4-bovine serum albumin (BSA) conjugate (1:250 dilution) and incubated overnight at 4 °C in the dark. The plate was emptied and washed three times with TBS buffer containing 0.05% Tween 20 (v/v) and 0.1% BSA (w/v). The reaction was blocked with 200 μL TBS containing 0.05% Tween 20 and 1% BSA (25 °C; 15 min). After the plate was washed three times with TBS containing 0.05% Tween 20 (v/v) and 0.1% BSA (w/v), 200 μL anti-ABA monoclonal antibody (ABA antibody; Agdia, Elkhart, IN) + standard or ABA antibody + sample was added to each well. ABA antibody + standard and ABA antibody + sample

mixtures were made by incubating the mixtures (1:1 ratio) overnight at 4 °C in the dark. The plate was incubated 2.5 h at 25 °C in the dark, emptied, and washed three times.

To each well, 200 μ L antimouse IgG alkaline phosphatase (1:1000 dilution) solution (Sigma) was added, and the plate was incubated 2 h at 25 °C in the dark. After three washes with TBS containing 0.05% Tween 20 (v/v) and 0.1% BSA (w/v), 200 μ L *p*-nitrophenyl phosphate disodium salt (1 mg·mL⁻¹ in 0.05 M NaHCO₃, pH 9.6) were added to each well and incubated in the dark for \approx 1 h. The color reaction was determined by measuring absorbance at 405 nm with an enzyme immunoassay microplate reader (Opsys MR; Thermo Labsystems, Chantilly, VA). The ABA concentration was calculated on the basis of a prepared standard curve and expressed as ng·g⁻¹ dry weight.

DEHYDRIN EXPRESSION ANALYSIS. Stolon tissues were used for dehydrin expression analysis because they are closely associated with winter survival of bermudagrass and also used in evaluating freezing tolerance based on stolon EL in this study. Stolons (250 mg) were grounded using liquid nitrogen and transferred to 2-mL microcentrifuge tubes. Then the powder was mixed with 1.5 mL precooled extraction buffer [50 mM Tris-HCl pH 6.8, 1 mM ethylenediaminetetraacetic acid, 1% SDS, and 0.5% polyvinylpyrrolidone, plus the protease inhibitor phenylmethylsulfonyl fluoride (1 mM; Sigma)]. The mixture was kept on ice for 5 min before centrifugation. The extracts were centrifuged at 16,000 g_n at 4 °C for 15 min, and the supernatant was transferred to new microcentrifuge tubes and kept on ice. Next, 20 μ L of fourfold diluted aliquot of extract was used for a protein concentration assay using bicinchoninic acid reagent (Sigma). The reaction mixture was read at 562 nm after 30-min incubation at 37 °C with BSA as the standard.

An aliquot of 100 μ L supernatant mixed with 100 μ L of 2 \times SDS buffer [0.125 mol·L⁻¹ Tris/HCl, pH 6.8, 20% (v/v) glycerol, 0.01% (v/v) bromophenol blue, 200 mM dithiothreitol, and 4% (w/v) SDS] was used for Sodium dodecylsulfate polyacrylamide gel electrophoresis analysis (Laemmli, 1970) after heating in a boiling water bath for 3 min. Each lane was loaded with 50 μ g protein with the proteins separated in 1.0-mm-thick gels (15% separating gel, 5% stacking gel) at constant volts of 200 using Bio-Rad Mini-Protean III (Bio-Rad Laboratories, Hercules, CA). The separated proteins was transferred for 1 h at constant volts of 100 and blotted onto 0.45- μ m nitrocellulose membrane (Bio-Rad Laboratories). After blotting, the membrane was blocked with 3% BSA in TBS (25 mM Tris-HCl, 150 mM NaCl, pH 7.5) for 1.5 h at room temperature. After a brief rinse with TBS, the membrane was incubated in TBS with a dehydrin polyclonal antibody raised from rabbit (Assay Designs, Ann Arbor, MI) at a dilution of 1:250 for 1.5 h. After that the membrane was rinsed in TBS containing 0.5% Tween 20 (TBS-T) four times and then placed for 1 h in a solution of goat antirabbit IgG (dilution 1:17,500) conjugated to alkaline phosphatase (Sigma). The membrane was rinsed in TBS-T four times and then developed using nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Sigma). Immunoblotting was conducted for three replications and the representative data are presented here.

ELECTROLYTE LEAKAGE. At the end of CA, the soil was washed from the plants and the roots were removed. Turf plugs from acclimated and nonacclimated treatments were then divided into five subsamples. Each subsample was wrapped in a wet paper towel and subjected to a series of freezing temperatures. A freezing chamber was programmed to cool

to -2, -4, -6, -8, and -10 °C with a 2° decline every 2 h at which time a subsample was removed for analysis. After thawing overnight at 4 °C, stolons (100 mg) from each subsample were rinsed with distilled water and transferred into a 50-mL centrifuge tube and deionized distilled water (20 mL) was added. The samples were placed in a shaker for 12 h at 25 °C. After an initial electrical conductivity (EC₁) reading using a conductivity meter, the samples were autoclaved at 120 °C for 30 min. After cooling, a second EC (EC₂) reading was taken. The EL (%) is expressed as (EC₁/EC₂) \times 100. The EL was also measured for the samples collected at 0, 7, 14, and 21 d after CA initiation or ABA treatment.

DETERMINATION OF LETHAL TEMPERATURE FOR 50% LOSS OF ELECTROLYTES. Freezing tolerance was evaluated based on predicted LT50 from EL measurements in this study. Predicted LT50 and glasshouse regrowth evaluation after freezing treatment of bermudagrasses have been shown to be in close agreement (Anderson et al., 1988; Miller and Dickens, 1996). The response curve between test temperature and EL was fitted to a sigmoidal response using Sigmaplot (version 10; Systat Software, San Jose, CA). Response curves were developed for each treatment and each LT50 was determined from the inflection point of the curve based on the sigmoidal response equation (Anderson et al., 1988; Cardona et al., 1997; Zhang et al., 2006).

EXPERIMENTAL DESIGN AND DATA ANALYSIS. In each of the two experiments (cultivars under CA and ABA treatment), a randomized complete block design was used with four replications. The cultivars without CA were sampled for analysis of dehydrin expression only. The data were analyzed using an analysis of variance and mean separations were performed using a Fisher's protected least significant difference test. Correlations between LT50 and ABA level in each experiment were analyzed (SAS version 8e for Windows; SAS Institute, Cary, NC). Regression between LT50 and ABA level was also analyzed using Sigmaplot (version 10). Because the sample sizes for the freezing tests were small, no attempt was made to examine growth recovery from nodes after freezing.

Results

CULTIVAR DIFFERENCES IN ABSCISIC ACID CONTENT. ABA content in leaves and stolons increased substantially during the first 7 d of CA and remained relatively stable thereafter (Fig. 1). Significant differences in ABA content were found among cultivars. When averaged over the four cultivars, leaf ABA content was increased by 3.2-fold from day 0 through day 7 (Fig. 1A). 'Patriot' and 'Riviera' had greater leaf ABA content relative to 'Tifway' and 'Princess' when measured at 21 d of CA. Similar patterns were observed in stolon ABA content of the four cultivars (Fig. 1B). Stolon ABA content in 'Patriot', 'Riviera', 'Tifway', and 'Princess' was increased by 4.5-fold, 3.5-fold, 2.9-fold, and 2.6-fold, respectively, from day 0 through day 7 of CA. When measured at 21 d of CA, stolon ABA content was 39.6 ng·g⁻¹ for 'Patriot', 35.1 ng·g⁻¹ for 'Riviera', 27.2 ng·g⁻¹ for 'Tifway', and 13.0 ng·g⁻¹ for 'Princess', respectively. 'Patriot' and 'Riviera' had greater ABA content in leaf and stolon tissues relative to 'Tifway' and 'Princess' during CA.

CULTIVAR DIFFERENCES IN DEHYDRIN EXPRESSION. Dehydrin with a size of 40 kDa was found in all cultivars, regardless of CA. Cold acclimation induced expression of a 25 kDa dehydrin.

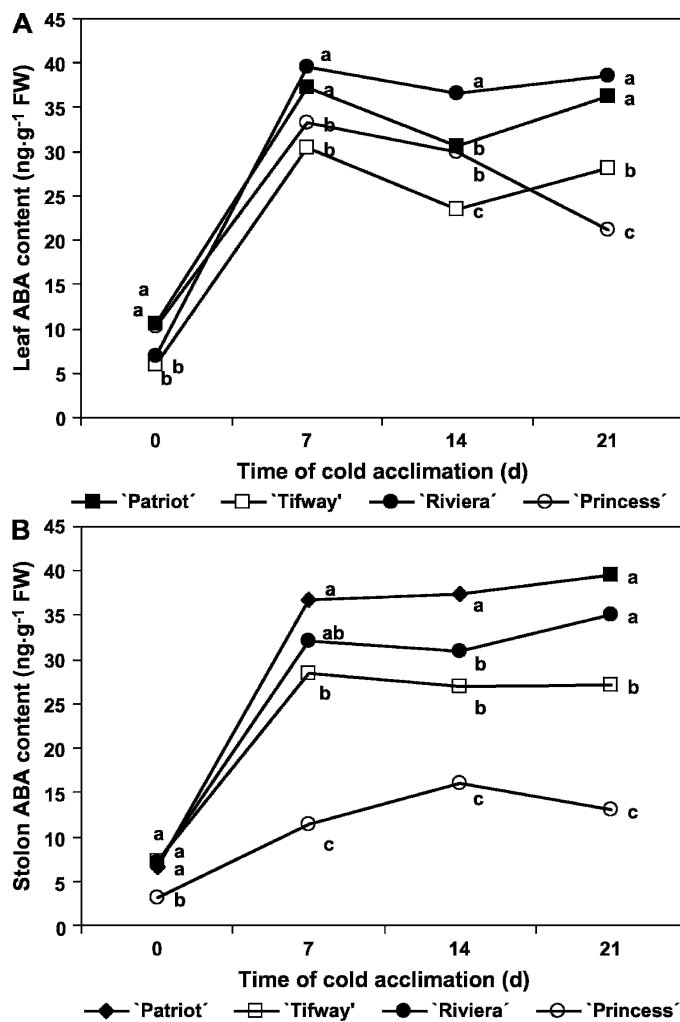


Fig. 1. Abscissic acid (ABA) content in (A) leaf and (B) stolon tissues of four bermudagrass cultivars (Patriot, Tifway, Riviera, and Princess) during cold acclimation (8 °C day/4 °C night); ABA values marked with same letters on each sampling date are not significantly different at $P \leq 0.05$.

However, there was no cultivar difference in abundance of this dehydrin (Fig. 2A).

EXOGENOUS ABSICISIC ACID IMPACTS ON DEHYDRIN EXPRESSION. Dehydrin with a size of 40 kDa was found in all treatments. Applying ABA at various concentrations to nonacclimated 'Patriot' induced expression of a 25 kDa dehydrin (Fig. 2B). The abundance of the 25 kDa dehydrin was greater when the grass was treated with ABA at 50 μM relative to 100 μM and 150 μM .

CULTIVAR DIFFERENCES IN ELECTROLYTE LEAKAGE AND LETHAL TEMPERATURE FOR 50% LOSS OF ELECTROLYTES. Leaf EL increased during CA, especially from 14 d through 21 d of CA (Fig. 3). 'Patriot' and 'Riviera' had less EL relative to 'Tifway' when measured at 14 and 21 d of CA. 'Princess' had less EL relative to 'Tifway' at 21 d of CA.

Stolon EL increased as temperature decreased from -2 to -10 °C (Fig. 4). 'Patriot' had less EL relative to 'Princess' at temperatures of -4, -8, and -10 °C. 'Riviera' had less EL relative to 'Princess' at -6 and -8 °C. 'Patriot' had a lower average LT50 relative to 'Tifway' and 'Princess' (Fig. 5).

RELATIONSHIP BETWEEN LETHAL TEMPERATURE FOR 50% LOSS OF ELECTROLYTES AND ABSICISIC ACID CONTENT. Linear regres-

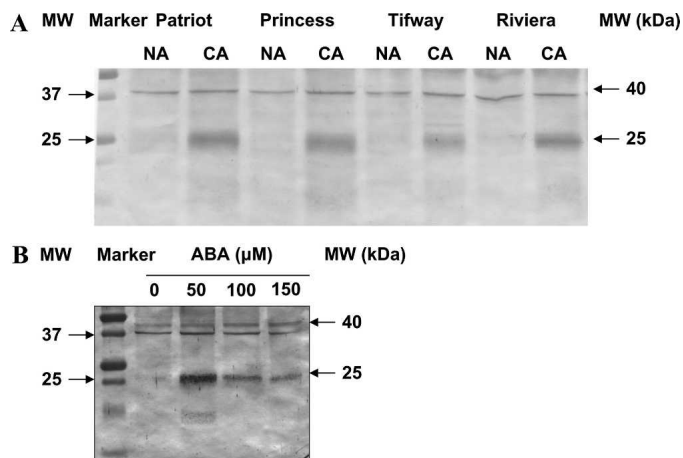


Fig. 2. Dehydrin expression in stolon tissues of four bermudagrass cultivars (Patriot, Tifway, Riviera, and Princess). Each of the cultivars was grown either under normal temperatures [not cold-acclimated (NA), 28 °C day/24 °C night] or subjected to cold acclimation (CA, 8 °C day/4 °C night) for 21 d (A). Dehydrin expression in stolon tissues in NA 'Patriot' bermudagrass treated with ABA at 0 (ABA0), 50 μM (ABA50), 100 μM (ABA100), and 150 μM (ABA150) and grown under normal temperatures (28 °C day/24 °C night) for 21 d (B). Molecular weight (MW) markers represent dehydrins sized 25 and 37 kDa (A and B, far left).

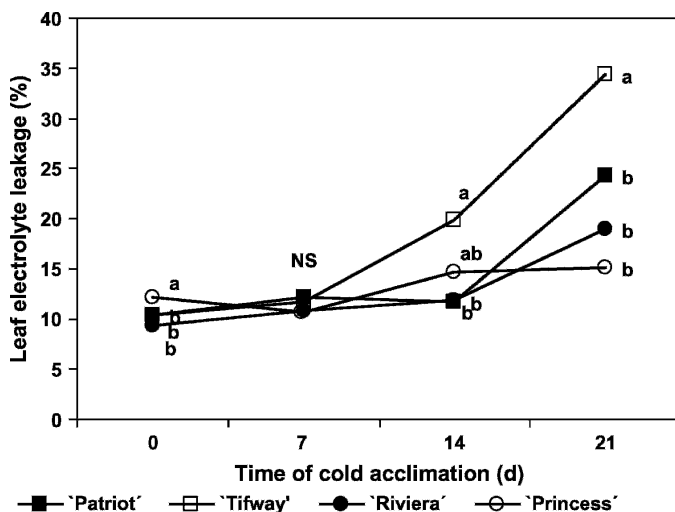


Fig. 3. Leaf electrolyte leakage (EL) of four bermudagrass cultivars (Patriot, Tifway, Riviera, and Princess) during cold acclimation (8 °C day/4 °C night). EL values marked with same letters on each sampling date are not significantly different at $P \leq 0.05$; NS = nonsignificant at the sampling date.

sions of leaf and stolon ABA content with LT50 were found (Fig. 6). Spearman correlation coefficients of LT50 with leaf ($r = 0.686^{**}$) and stolon ($r = 0.752^{**}$) ABA content were statistically significant at a 1% probability level.

EXOGENOUS ABSICISIC ACID IMPACTS ON ENDOGENOUS ABSICISIC ACID CONTENT. ABA treatment at 150 μM consistently increased leaf and stolon ABA content in 'Patriot' bermudagrass (Fig. 7). Applying ABA at 100 μM also increased stolon ABA content. ABA treatment at 50 μM increased leaf and stolon ABA content when measured 21 d after application. Applying ABA at 50, 100, and 150 μM increased stolon ABA content by 2.1-fold, 2.4-fold, and 2.9-fold, respectively, when measured 21 d after treatment.

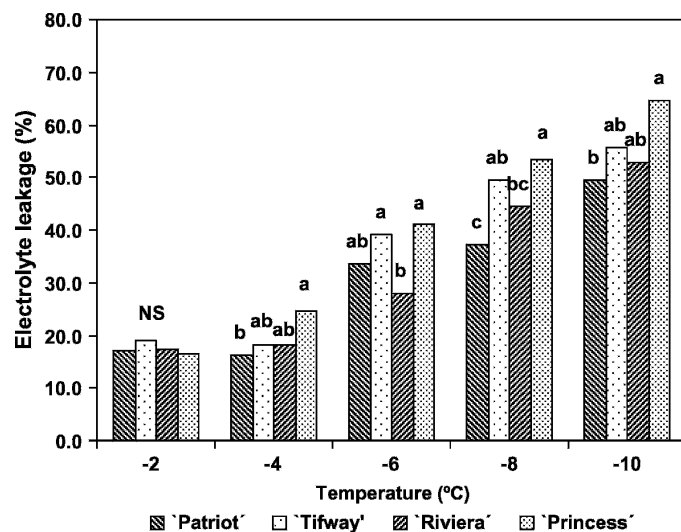


Fig. 4. Stolon electrolyte leakage (EL) of four bermudagrass cultivars (Patriot, Tifway, Riviera, and Princess) after 21 d of cold acclimation (8 °C day/4 °C night) and under freezing temperatures. EL values marked with same letters on each temperature are not significantly different at $P \leq 0.05$; NS = nonsignificant at the specific temperature.

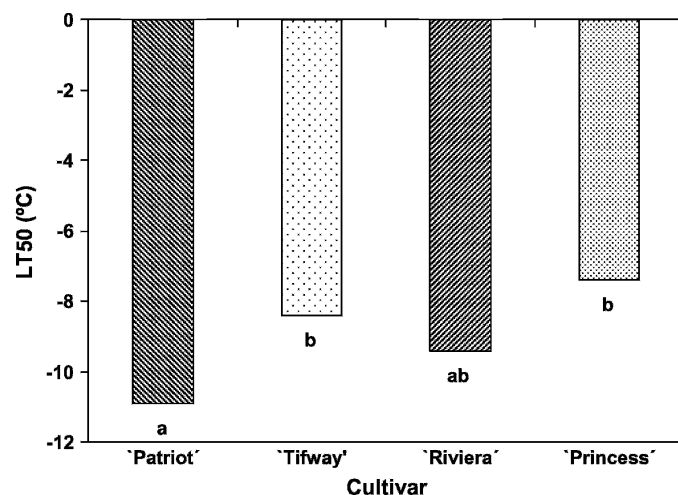


Fig. 5. Lethal temperature for 50% loss of electrolytes (LT50) of four bermudagrass cultivars (Patriot, Tifway, Riviera, and Princess) after 21 d of cold acclimation (CA, 8 °C day/4 °C night). LT50 values marked with same letters on each sampling date are not significantly different at $P \leq 0.05$.

EXOGENOUS ABSICISIC ACID IMPACTS ON ELECTROLYTE LEAKAGE AND LETHAL TEMPERATURE FOR 50% LOSS OF ELECTROLYTES. Exogenous ABA at 100 and 150 μM reduced leaf EL at 7 d after treatment (Fig. 8). Applying ABA at 100 μM reduced stolon EL at temperatures of -4 and -8 °C. Exogenous ABA at 150 μM also reduced stolon EL at -6 and -10 °C (Fig. 9). All ABA treatments significantly improved freezing tolerance of 'Patriot' bermudagrass in terms of average LT50 values (Fig. 10).

Positive linear regression of stolon ABA content with LT50 was found (Fig. 11). Spearman correlation coefficients of LT50 with leaf ($r = 0.504^*$) and stolon ($r = 0.812^{**}$) ABA content were statistically significant at 5% and 1% probability levels, respectively.

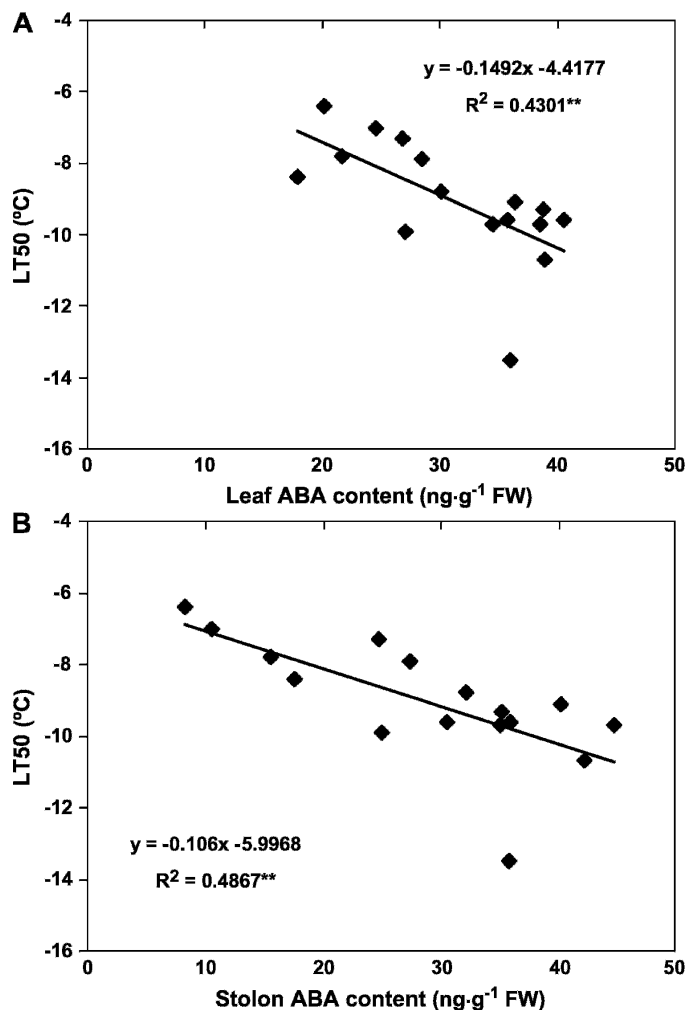


Fig. 6. Relationship of LT50 with abscisic acid (ABA) content in (A) leaf and (B) stolon tissues of four bermudagrass cultivars (Patriot, Tifway, Riviera, and Princess) after 21 d of cold acclimation (8 °C day/4 °C night); **significance of the linear regression at $P = 0.01$.

Discussion

The results of this study indicated that ABA content in leaf and stolon tissues increased substantially during CA. 'Patriot' and 'Riviera' (cold-tolerant) had significantly greater ABA content when compared with 'Tifway' and 'Princess' (relatively cold-sensitive). A significant correlation was found between ABA content and LT50. The results are in agreement with previous studies with other plant species (Lee and Chen, 1993; Thomashow, 1999). Several lines of evidence suggest that ABA is involved in CA of plants (Thomashow, 1999). ABA content has been shown to increase transiently in both herbaceous and woody plants exposed to low temperature (Lee and Chen, 1993; Li et al., 1997). Exogenous application of ABA at nonstress temperatures leads to increased chilling and freezing tolerance (Chen and Gusta, 1983; Lang et al., 1989; Li et al., 2003; Thomashow, 1999), de novo protein synthesis, and induction of cold-responsive genes (De Los Reyes et al., 2001; Gatschet et al., 1996). In addition, both ABA-insensitive (*abi*) and ABA (*aba*)-deficient mutants have been shown to have impaired CA (Gilmour and Thomashow, 1991; Heino et al.,

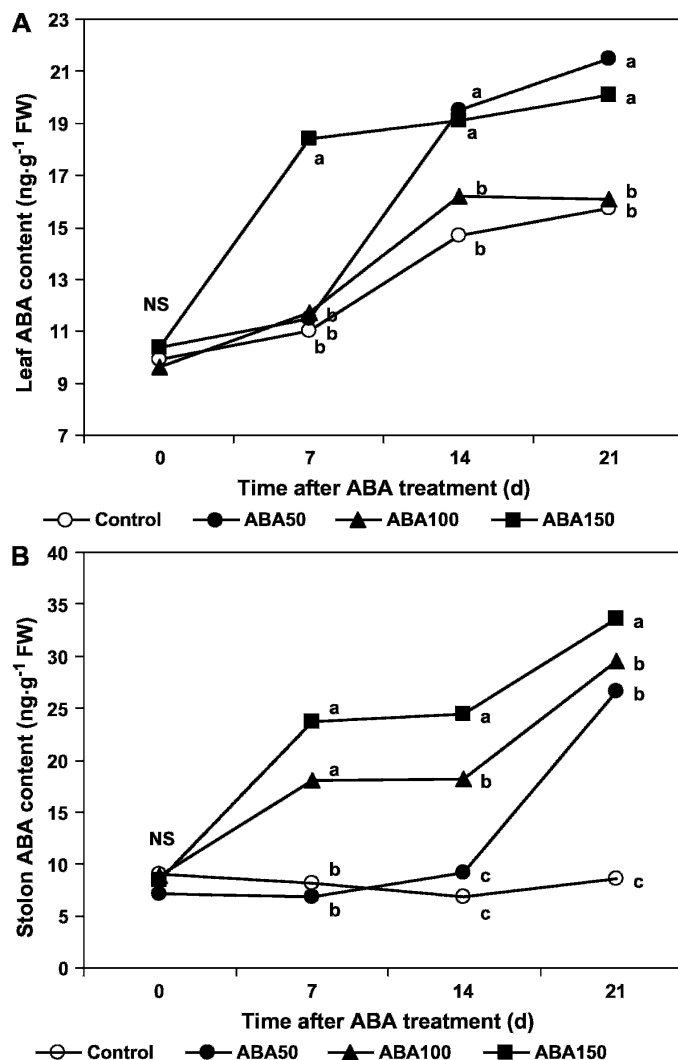


Fig. 7. Abscisic acid (ABA) content in (A) leaf and (B) stolon tissues of 'Patriot' bermudagrass grown under normal temperatures (28 °C day/24 °C night) as influenced by exogenous ABA. The ABA was applied to the canopy at concentrations of 0 (Control), 50 μ M (ABA50), 100 μ M (ABA100), and 150 μ M (ABA150). ABA values marked with same letters on each sampling date are not significantly different at $P \leq 0.05$; NS = nonsignificant at the specific sampling date.

1990; Thomashow, 1999). Furthermore, it has been shown that application of ABA can suppress the impaired CA phenotype in the *aba* mutant (Heino et al., 1990; Thomashow, 1999). This suggests that changes in ABA metabolism during CA or in response to exogenous ABA may contribute to freezing tolerance improvement.

Cold acclimation induced expression of the 25 kDa dehydrin, but no cultivar differences were found in terms of abundance of this dehydrin. Exogenous ABA induced expression of this dehydrin in 'Patriot' bermudagrass. This suggests that expression of the 25 kDa dehydrin may be associated with CA or exogenous ABA treatment. Lim et al. (1999) first studied the role of dehydrins in rhododendron (*Rhododendron* L.) cold hardiness and found levels of a 25 kDa dehydrin were closely associated with differences in leaf freezing tolerance. They suggest that this dehydrin could serve as a biochemical marker to distinguish between superhardy and less hardy rhododendron

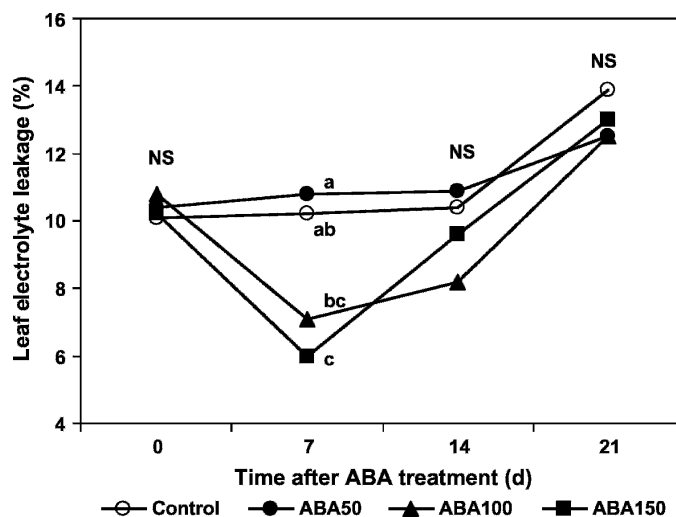


Fig. 8. Leaf electrolyte leakage (EL) of 'Patriot' bermudagrass grown under normal temperatures (28 °C day/24 °C night) as influenced by exogenous abscisic acid (ABA). The ABA was applied to the canopy at concentrations of 0 (Control), 50 μ M (ABA50), 100 μ M (ABA100), and 150 μ M (ABA150). EL values marked with same letters on each sampling date are not significantly different at $P \leq 0.05$; NS = nonsignificant at the specific sampling dates.

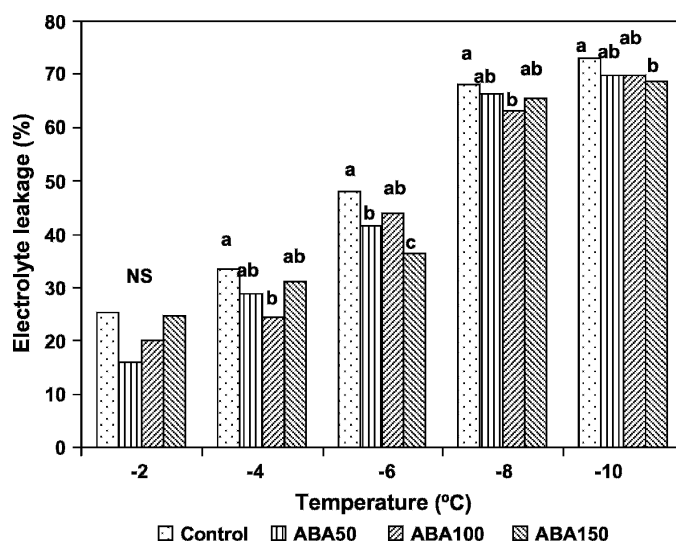


Fig. 9. Stolon electrolyte leakage (EL) of 'Patriot' bermudagrass under normal temperatures (28 °C day/24 °C night) as influenced by exogenous abscisic acid (ABA). The ABA was applied to the canopy at concentrations of 0 (Control), 50 μ M (ABA50), 100 μ M (ABA100), and 150 μ M (ABA150). EL values marked with same letters on each sampling date are not significantly different at $P \leq 0.05$; NS = nonsignificant at the specific temperature.

genotypes. Marian et al. (2003) examined the accumulation pattern of dehydrins in non- versus cold-acclimated leaves of 21 species comprising two divergent groups of rhododendron and found a close association ($R^2 = 0.95$) between relative changes in leaf freezing tolerance and 25 kDa dehydrin accumulation. Patton et al. (2007) reported that dehydrin polypeptides (23 and 25 kDa) increased during CA and abundance of the 23 kDa dehydrin polypeptide was positively associated with genetic variation in zoysiagrass freezing tolerance, but the 25 kDa dehydrin was not. The results of this study suggest that exogenous ABA or CA may induce

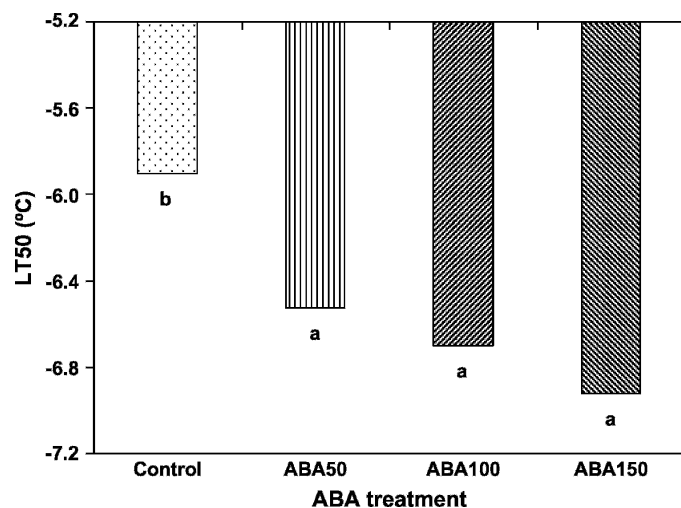


Fig. 10. Lethal temperature for 50% loss of electrolytes (LT50) of 'Patriot' bermudagrass grown under normal temperatures (28 °C day/24 °C night) as influenced by exogenous abscisic acid (ABA). The ABA was applied to the canopy at concentrations of 0 (Control), 50 μM (ABA50), 100 μM (ABA100), and 150 μM (ABA150). LT50 values marked with same letters on each sampling date are not significantly different at $P \leq 0.05$.

expression of the 25 kDa dehydrin in bermudagrass, and this dehydrin may contribute to freezing tolerance in bermudagrass but may not be a major factor causing the cultivar differences in freezing tolerance. Exogenous ABA or CA may induce other defense mechanisms [such as increases in expression of many cold-regulated genes (including other dehydrins), endogenous ABA, cellular pools of carbohydrates and amino acids, antioxidant activity, and so on], thus improving freezing tolerance (Kalberer et al., 2006; Thomashow, 1999; Zhang et al., 2006; Zhang and Ervin, 2008).

The results of our study indicated that exogenous ABA promoted endogenous ABA content in leaf and stolon tissues and dehydrin expression in stolon tissues and increased freezing tolerance in nonacclimated 'Patriot' bermudagrass. Stolon ABA content was positively correlated to freezing tolerance. The 25 kDa dehydrin in stolon tissues of 'Patriot' was induced by either CA or ABA treatment. To our knowledge, this is the first report on effects of CA and exogenous ABA on endogenous ABA and dehydrin expression associated with freezing tolerance in selected bermudagrass cultivars differing in freezing tolerance. It has been documented that plant freezing tolerance is associated with ABA (Lee and Chen, 1993; Thomashow, 1999) and certain dehydrins (Danyluk et al., 1998; Marian et al., 2003; Patton et al., 2007). These studies, however, addressed either ABA or dehydrins only. The results of our study indicated that both ABA and the 25 kDa dehydrin may be induced by CA or exogenous ABA, but ABA may be more closely associated with freezing tolerance of the bermudagrass cultivars relative to the 25 kDa dehydrin. In seedlings of alfalfa (*Medicago* L.), tolerance to freezing at -10 °C was greatly improved by previous cold exposure (4 °C) or by treatment with exogenous ABA without cold exposure. These treatments caused changes in the pattern of newly synthesized proteins. Some of the newly synthesized proteins induced by cold appear to be the same as those induced by ABA (Taiz and Zeiger, 2006). The results of this study suggest that applying ABA to 'Patriot' bermudagrass may mimic CA in inducing

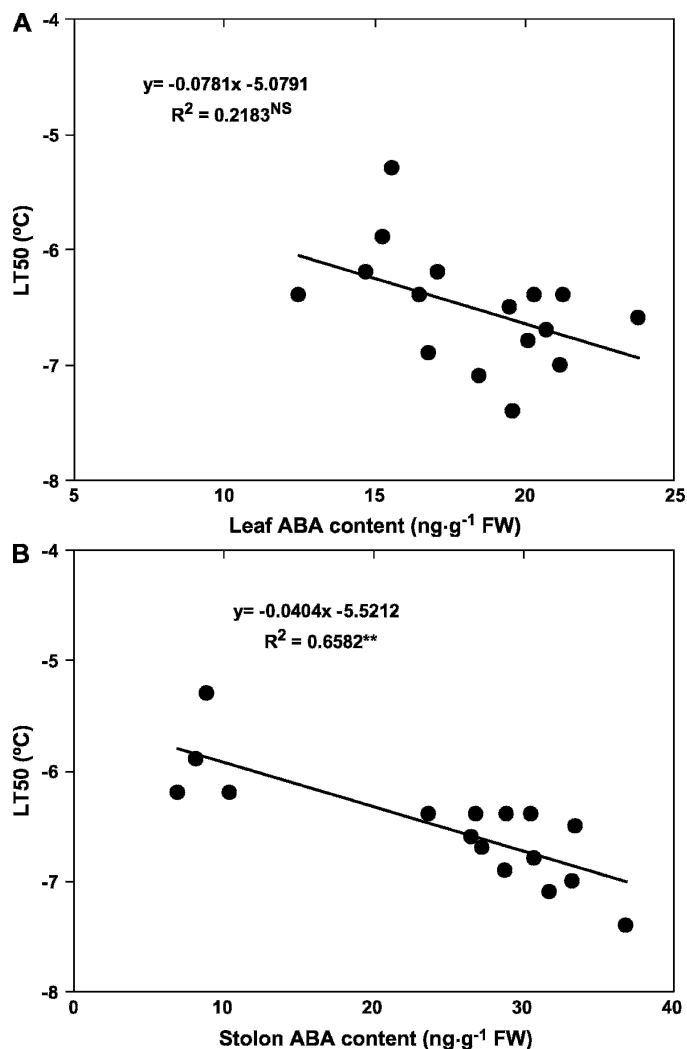


Fig. 11. Relationship of LT50 with abscisic acid (ABA) content in (A) leaf and (B) stolon tissues of exogenous ABA-treated 'Patriot' bermudagrass grown under normal temperatures (28 °C day/24 °C night); **significance of the linear regression at $P = 0.01$, NS = nonsignificant at $P = 0.05$.

plant defense responses and improving freezing tolerance. The complete underlying mechanisms of improved freezing tolerance remain unclear. There is evidence showing that exogenous ABA may signal initiation of a metabolic transition, increasing metabolic defenses and freezing tolerance (Thomashow, 1999). Exogenous ABA may trigger an accumulation of signaling molecules [such as reactive oxygen species (ROS), endogenous ABA; Xiong et al., 2002]. These molecules may signal plant defense responses such as an increase in expression of cold-regulated genes (including dehydrins), antioxidants, nonstructural carbohydrates, and amino acids and alteration of cell membrane composition (De Los Reyes et al., 2001; Kalberer et al., 2006; Thomashow, 1999; Xiong et al., 2002; Zhang and Ervin, 2008; Zhou et al., 2005).

The results of this study indicated that leaf EL increased during CA. This suggests that CA may cause mild injury to cell membrane and an increase in EL possibly resulting from ROS accumulation in leaf tissues. The ROS may signal initiation of plant metabolic defense responses (such as increase in ABA, dehydrins, and antioxidants), which may improve integrity of

cell membrane and reduce EL and LT50 (Zhang and Ervin, 2008). During CA, cold-tolerant cultivars may develop greater defense responses to suppress ROS toxicity and have lower EL and LT50 when compared with cold-sensitive cultivars. The cultivars' difference in EL and LT50 may be associated with their genetic background and capacity of metabolic defense responses (such as ABA, dehydrins, antioxidants, membrane lipids, and fatty acid constituents) during CA (Cyril et al., 2002; Zhang and Ervin, 2008). Our results showed that exogenous ABA reduced EL and LT50 of nonacclimated 'Patriot'. It is possible that exogenous ABA may induce plant metabolic defense responses directly and indirectly (through ROS) and improve integrity of cell membrane and freezing tolerance.

In summary, rapid increase in ABA content in leaf and stolon tissues during CA was positively associated with freezing tolerance. Bermudagrass cultivars with more ABA accumulation during CA may have better tolerance to freezing stress when compared with those with less ABA accumulation. ABA may be an important component in the signaling network for inducing freezing tolerance. Cold acclimation may induce expression of certain dehydrins such as the 25 kDa dehydrin. Exogenous ABA at proper dosages (50 to 150 μM) may induce endogenous ABA and other defense responses similar to those occurring during CA, improving bermudagrass freezing tolerance. Selection and use of cultivars with rapid accumulation of ABA and certain dehydrins during CA or in response to exogenous ABA could improve bermudagrass persistence in transition zone climates.

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