Differential Responses to Heat Stress in Activities and Isozymes of Four Antioxidant Enzymes for Two Cultivars of Kentucky Bluegrass Contrasting in Heat Tolerance

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ABSTRACT. Understanding antioxidant mechanisms for heat stress is important for improving heat tolerance in cool-season plant species. The objective of this study was to identify antioxidant enzymes associated with cultivar variations in heat tolerance in Kentucky bluegrass (*Poa pratensis*) by comparing heat responses of activity and isoforms of antioxidant enzymes in two cultivars contrasting in heat tolerance. Plants of heat-tolerant 'Eagleton' and heat-sensitive 'Brilliant' were exposed to 20 °C (control) or 40 °C (heat stress) for 28 days in growth chambers. Chlorophyll (Chl) a content remained unchanged and Chl b content increased in 'Eagleton', while both of them decreased in 'Brilliant', and by 28 days, 'Eagleton' had significantly higher Chl a and b content than 'Brilliant'. The activities of superoxide dismutase (SOD) were significantly higher in 'Eagleton' than in 'Brilliant' by 28 days of heat stress. An isozyme SOD2 was induced early during heat stress in 'Eagleton', while isozyme SOD3 degraded, to a lesser extent in 'Eagleton' than in 'Brilliant'. Catalase (CAT) activity significantly increased in 'Brilliant' but remained constant in 'Eagleton', and 'Brilliant' had a significantly higher CAT activity and isozyme CAT1 than 'Eagleton' during heat stress. Significant increases in ascorbate peroxidase (APX) activities occurred under heat stress, to a greater extent in 'Eagleton', whereas isozymes did not exhibit difference between cultivars. Guaiacol-peroxidase (POD) activity declined during heat stress in both cultivars. The intensity of POD isozymes in 'Brilliant' remained constant, while 'Eagleton' showed a transient increase in POD1 at 7 days of heat stress. Our results indicated that antioxidant defense mechanisms for heat tolerance in Kentucky bluegrass could be mainly associated with changes in activity and forms of isozymes of SOD for O₂ scavenging and APX activity for H₂O₂ scavenging under heat stress.

The optimum temperatures for cool-season grass growth are between 15 and 24 °C (Beard, 1973). High temperatures for prolonged periods during summer in transitional and warm climatic regions often cause turf quality decline, which is characterized by leaf senescence. Leaf chlorophyll (Chl) content is a good indicator of leaf senescence, and both forms of Chl a and Chl b are components of peripheral antenna complexes and complement each other in absorbing sunlight for photosynthesis (Streitweiser and Heathcock, 1976). Alleviating heat-induced leaf senescence is critical in maintaining high visual quality of cool-season species during periods of high temperatures.

Abiotic stresses, including heat stress, cause the production of reactive oxygen species (ROS), resulting in oxidative damage that has been associated with stress-induced leaf senescence (Foyer et al., 1994; Gong et al., 1997; Scandalios, 1992; Zhang and Kirkham, 1996). Oxidative stress can cause lipid peroxidation and, consequently, membrane injury, protein degradation, and enzyme inactivation (Meriga et al., 2004; Sairam et al., 2000). Intracellular membrane damage due to ROS can cause pigment breakdown (Scandalios, 1993). Multiple antioxidant

enzyme systems are involved in the enzymatic scavenging of ROS. Superoxide dismutase (SOD) is the enzyme for O₂ scavenging in the first step of ROS metabolism (Elstner, 1982). Its activity determines the concentrations of O₂ and H₂O₂, the two Haber-Weiss action substrates, and it is therefore likely to be central in the defense mechanism (Bowler et al., 1992). Hydrogen peroxide is scavenged by catalase (CAT) and peroxidases, which convert hydrogen peroxide to water and molecular oxygen (Bowler et al., 1992). Ascorbate peroxidase (APX) is characterized by its high degree of specificity for ascorbate as the electron donor and by its physiological role in the scavenging of hydrogen peroxide (Asada, 1992; Asada et al., 1993). Guaiacol peroxidases (POD) are characterized by their broad specificity, with guaiacol and pyrogallol as electron donors in assays of their activities (Amako et al., 1994).

Heat stress induces oxidative stress and alters antioxidant enzyme activities in many plant species (Foyer et al., 1994; Gong et al., 1997), including turfgrass species (Jiang and Huang, 2001a, 2001b; Larkindale and Huang, 2004; Liu and Huang, 2000). Decreases, increases, and unchanged activities of antioxidant enzymes were reported, depending on specific enzymes, stress duration, the level of temperatures, and plant species (Almeselmani et al., 2006; Chaitanya et al., 2002; Dat et al., 1998; Foyer et al., 1997; Sairam et al., 2000). Plant species and cultivars with superior heat tolerance generally have been associated with maintaining higher antioxidant enzyme activities

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(Liu and Huang, 2000; Scandalios, 1993). Several studies in cool-season turfgrass species investigated changes in antioxidant enzymes with heat stress (Jiang and Huang, 2001b; Liu and Huang, 2000; Wang et al., 2003). Plants have multiple isozyme forms of antioxidant enzymes, which may be differentially responsive to developmental events and environmental factors (Lee and An, 2005; Mori et al., 1992; Scandalios, 1993). For example, wheat (*Triticum aestivum*) leaves had three isoforms of Mn-SOD, one isoform of Fe-SOD and Cu/Zn-SOD, and the response of enzyme isoforms to drought stress varied between two cultivars differing in drought tolerance (Zhang et al., 2004). Multiple isoforms of CAT are found in many plant species and are differentially regulated by developmental events and environmental factors (Mori et al., 1992). Four CAT isoforms were identified in *Bruguiera parviflora* in response to salt stress, among which the prominent CAT-2 isoform level was reduced more than other isoforms, suggesting differential down-regulation of CAT isoforms by NaCl (Parida et al., 2004). This study concluded that the differential changes in the level of isoforms may be useful markers for identifying salt tolerance. Despite previous research on antioxidant enzymes in relation to heat tolerance, specific antioxidant enzymes, particularly the isozyme forms associated with heat tolerance in cool-season turfgrasses, have not yet been documented.

Kentucky bluegrass is a cool-season turfgrass widely used in temperate zones. Turf quality decline associated with leaf senescence is a major concern in Kentucky bluegrass lawn and golf turf management. There exists a wide range of genetic variability in summer stress tolerance in this grass species (Bonos and Murphy, 1999). Understanding genetic variations in responses to antioxidant enzyme activities and isozyme forms to heat stress may shed light on antioxidant mechanisms for heat tolerance in cool-season grass species. The objective of this study was to identify antioxidant enzymes associated with heat tolerance in Kentucky bluegrass by comparing responses of enzyme activity and isozymes to heat stress in two cultivars contrasting in heat tolerance.

Materials and Methods

PLANT MATERIALS AND GROWTH CONDITIONS. The heat-tolerant Kentucky bluegrass cultivar Eagleton and the heat-sensitive cultivar Brilliant (He and Huang, 2007) were examined. Sod plugs of 'Brilliant' and 'Eagleton' were collected from 3-year-old field plots at Horticulture Farm II, Rutgers University, North Brunswick, NJ, and were transferred into PVC pots (20 cm in diameter and 25 cm in height with holes at the bottom for drainage) filled with a mixture (1:3, v/v) of sand and soil (fine-loamy, mixed mesic Typic Hapludult). Plants were maintained in a greenhouse for 60 d and were then moved into growth chambers. The greenhouse had an average day/night temperature of 21/14 °C, photosynthetically active radiation (*PAR*) of 680 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$, and a 12-h photoperiod. The chambers were set at 20/15 °C (day/night temperature), 75% of relative humidity, a 14-h photoperiod, and 500 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ *PAR*. Plants were fertilized once per week with 40 mL of full-strength Hoagland's solution (Hoagland and Arnon, 1950) and were cut twice a week to keep the height at ≈ 10 cm. For all treatments, pots were watered three times per week until drainage occurred at the bottom of the pot at each irrigation.

HEAT STRESS TREATMENTS. Plants were allowed to acclimate to growth chamber conditions for 14 d before temperature

treatments were imposed. All plants were cut to the canopy height of 10 cm 2 d before heat stress treatments were imposed to start with a uniform turf canopy. Eight pots of plants (four pots for each cultivar as four replicates) were maintained at 20/15 °C (day/night) as the optimum growth temperature control, while another set of eight pots of plants (four pots for each cultivar as four replicates) were exposed to 40/35 °C (day/night), which imposed heat stress. Plants were watered twice per day to maintain soil water content at the field capacity (27%). Other environmental conditions and fertility were the same as during the growing period described above.

Plants were treated to heat stress for 28 d. Leaf samples were collected every 7 d, which was on 0 d (control at 20/15 °C), and 7, 14, 21, and 28 d of the heat stress treatment. Fresh leaf tissues were collected from different areas of the turf canopy in each pot at each sampling date to measure content of Chl a, Chl b, salt-soluble protein, and tissue dry weight. Leaf samples for enzyme analysis were frozen in liquid nitrogen and kept at -70 °C until use.

LEAF SENESCENCE MEASUREMENTS. The content of Chl a and Chl b was measured to evaluate the level of leaf senescence during heat stress. Chlorophyll was extracted by soaking 0.1 g of leaves in 10 mL of dimethyl sulfoxide for 72 h (Hiscox and Israelstam, 1979). Absorbance of chlorophyll extracts was measured at 663 and 645 nm with a spectrophotometer (Genesys 2; Spectronic Instruments, Rochester, NY). Content of Chl a and Chl b was calculated using the formula of Arnon (1949).

ENZYME EXTRACTION. Frozen leaves (0.5 g) were ground in liquid N₂ and extracted in 2 mL of 150 mmol PBS (pH 7.0) kept in ice for SOD, CAT, and POD extraction, and in PBS (pH 7.0) containing 5 mmol ascorbate and 1 mmol EDTA (Mittler and Zilinskas, 1993) for APX in an ice-water bath. The homogenate was centrifuged twice at 16,000 g_n at 4 °C for 30 min. Supernatant was stored at 4 °C before measurement and in an ice-water bath during measurement.

ENZYME ACTIVITY ASSAYS. Superoxide dismutase activity was determined by measuring its ability to inhibit the photochemical reduction of nitroblue tetrazolium chloride (NBT), according to the method of Giannopolitis and Ries (1977) with slight modifications. The reaction solution (3 mL) contained 37 mmol PBS (pH 7.8), 4×10^{-3} mmol riboflavin (7,8-dimethyl-10-ribitylisoalloxazine), 13 mmol methionine [2-amino-4-(methylthio)-butyric acid], 1×10^{-4} mmol EDTA, 0.08 mmol NBT, and 50 μL of enzyme extract. Reaction solution was irradiated under incandescent lights at 1000 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ for 40 min. Absorbance of reaction solution at 560 nm was determined with the spectrophotometer as described above. One unit of enzyme activity was defined as the amount of the enzyme bringing 50% inhibition of the photochemical reduction of NBT.

Catalase activity was assayed by measuring the rate of decomposition of H₂O₂ at 240 nm in 3 mL of reaction mixture consisting of 50 mmol PBS [pH 7.4 (He et al., 2001)] and 15 mmol H₂O₂ (modified method of Aebi, 1984). Absorbance of reaction solution at 240 nm was determined every 10 s six times with a spectrophotometer (Genesys 2). One unit of enzyme was defined as the decrease in absorbance by 1 at 240 nm as H₂O₂ was decomposed.

Ascorbate peroxidase activity was measured by monitoring the decrease in absorbance at 290 nm as ascorbate was oxidized in reaction solution, as described by Nakano and Asada (1981) with slight modification. Twenty-five microliters of enzyme extract was added to 3 mL of reaction solution containing

0.1 mmol HAC-NaAC buffer [pH 5.8 (He et al., 2001)], 1×10^{-4} mmol EDTA, 8.3×10^{-2} mmol H_2O_2 , and 0.17 mmol ascorbate. Absorbance of reaction solution at 290 nm was determined every 10 s six times with the spectrophotometer as described above. One unit of enzyme was defined as the decrease in absorbance by 1 at 290 nm as ascorbate was oxidized.

Guaiacol peroxidase activity was determined by monitoring the increase in absorbance at 470 nm as guaiacol was oxidized, according to the method of Chance and Machly (1955) with slight modification. The assay mixture contained 0.07% HAC-NaAC buffer [pH 5.0 (He et al., 2001)], 0.08% guaiacol, 0.03% H_2O_2 , and 25 μL of enzyme extract. The total reaction mixture was 3.0 mL. Absorbance of reaction solution at 470 nm was determined every 10 s six times with the spectrophotometer, as described above. One unit of enzyme was defined as the increase in absorbance by 1 at 470 nm as guaiacol was oxidized.

ELECTROPHORESIS FOR ISOZYME ASSAYS. Samples were subjected to discontinuous polyacrylamide gel electrophoresis under nondenaturing, nonreducing conditions as described by Laemmli (1970) with some modifications. SOD was detected on 10.8% acrylamide gels, and GPX, CAT, and APX were detected on 7.2% gels. Protein in 66 μg for SOD and CAT and 68 μg for APX and GPX were loaded on each gel. Electrophoresis was carried out using a Mini-Protean system (Bio-Rad Laboratories, Hercules, CA) at 20 °C for 5 to 6 h in 0.04 mol Tris (hydroxymethyl) aminomethane-glycine buffer (pH 8.3) for SOD, GPX, CAT, and in the same buffer with addition of 2 mmol ascorbate for APX.

ISOZYMES VISUALIZATION. The SOD isozymes were detected using the NBT staining method according to Beauchamp and Fridovich (1973) with modification. Briefly, the gels were soaked in 100 mL of staining solution containing 2.45×10^{-3} M NBT for 20 min in the dark, and were then soaked in 100 mL of solution containing 5.6×10^{-5} M VB_2 , 2.8×10^{-2} N,N,N',N'-tetramethylethylenediamine (TEMED), and 3.6×10^{-2} PBS (pH 7.8) for 15 min in the dark, and finally soaked in a solution containing 1×10^{-4} EDTA and 5×10^{-2} M PBS (pH 7.8). The gels were exposed to incandescent lights at $1000 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ for 40 min or until the transparent bands (SOD isozymes) were visible.

The CAT isozyme staining was performed according to Woodbury et al. (1971) with modifications. After gel electrophoresis, the gel was washed in three changes of distilled water for a total of about 6 min to remove the buffer from the gel surface where staining occurred. The gel was then soaked in 0.3% H_2O_2 for 15 min at 20 °C in the dark with light agitation, and then in the stain mixture containing 1% (w/v) FeCl_3 and 1% (w/v) $\text{K}_3\text{Fe}(\text{CN})_6$ in distilled water under 20 °C for 10 min or until the light yellow bands (CAT isoenzymes) were visible.

All the steps of detection of APX isozymes in the gels were performed according to the procedure described in Mittler and Zilinskas (1993). After the electrophoretic separation, the gels were equilibrated with 0.1 mmol sodium acetate buffer (pH 5.8) and 2 mmol ascorbate for 30 min; the equilibration buffer was changed three times every 10 min. The gels were then incubated for 20 min in 0.1 mmol sodium acetate buffer containing 4 mmol ascorbate and 2 mmol H_2O_2 . H_2O_2 was added to this solution immediately before the incubation of the gel. The gels were subsequently washed with 0.1 mmol sodium acetate buffer for 1 min and submerged in a solution of 0.1 mmol sodium acetate buffer, 28 mmol TEMED, and 2.45 mmol NBT with gentle agitation. The gels were exposed to incandescent

lights at $1000 \mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ for 10 min. The APX activity isozymes were observed as an achromatic band.

Guaiacol peroxidase isozymes were detected using the benzidine acetate staining method modified from Ros Barceló et al. (1987). The gel was incubated in a staining solution containing 0.074% ascorbate, 20% benzidine solution [2.2% benzidine in 18% (v/v) acetic acid], and 0.12% H_2O_2 for 3 min. The gels were subsequently destained in 7% (v/v) acetic acid for several seconds until the bands were visible in dark brown.

In all gels, bromophenol blue was used as the marker for estimating the relative mobility (R_f) values of isozymes (Bernardi et al., 2001), which indicates the mobility of enzyme bands relative to the mobility of the bromophenol blue front. The isozymes were defined or numbered by their R_f in order from the cathode to the anode.

EXPERIMENTAL DESIGN AND STATISTICAL ANALYSIS. Treatments were arranged in a randomized complete block design with four replicates (four pots of each treatment). For enzyme activity measurement, each extraction sample (replicate) was measured three times (three subsamples per replicate). The mean of the three subsamples was used to represent a single replicate in the analysis of variance. Effects of control and high temperature and cultivar variations under the temperature treatments were analyzed with analysis of variance using Excel 2000 (Microsoft, Redmond, WA) (Levine et al., 2001), and mean separations were performed with the Fisher's protected least significance difference test at $P = 0.05$ (Steel and Torrie, 1980). Cultivars did not differ in any of the parameters measured under control temperature conditions and exhibited no significant changes over temperature treatment duration. Therefore, only the data concerning cultivar variations under heat stress and changes in each parameter with heat stress duration are presented and discussed.

Results

CHLOROPHYLL A AND CHLOROPHYLL B CONTENT. At the initiation of heat stress or before heat stress (0 d), 'Brilliant' had a significantly higher Chl a content than 'Eagleton' (Fig. 1A). The content of Chl a was constant during heat stress in 'Eagleton'; it was significantly higher in 'Brilliant' than in 'Midnight' from 0 to 14 d of heat stress, but decreased significantly after 14 d of heat stress in 'Brilliant'. By 28 d of heat stress, Chl a content was significantly higher in 'Eagleton' than in 'Brilliant'.

Chl b content increased significantly at 14 d and the elevated level was sustained at 28 d of heat stress in 'Eagleton', but decreased significantly by 28 d of heat stress in 'Brilliant' (Fig. 1B). By the 28 d of heat stress, 'Eagleton' had significantly higher Chl b content than 'Brilliant'.

SUPEROXIDE DISMUTASE ACTIVITY AND ISOZYME PROFILES. Before heat stress (0 d), SOD activities did not differ between 'Eagleton' and 'Brilliant' (Fig. 2). During heat stress, SOD activity remained unchanged in 'Eagleton', but decreased significantly at 21 and 28 d of heat stress. At 28 d of heat stress, SOD activity was significantly higher in 'Eagleton' than in 'Brilliant'.

Both cultivars had five SOD isozymes with R_f of 0.19, 0.50, 0.82, 0.85, and 0.89 before heat stress (0 d) (Fig. 3). SOD2 with R_f 0.36 was induced in 'Eagleton' at 7 d of heat stress and in 'Brilliant' at 14 and 21 d. The intensity of SOD3 with R_f 0.5 decreased in 'Brilliant' at 28 d of heat stress, but was maintained in 'Eagleton'. Other isozymes did not change during heat stress in either cultivar.

Fig. 1. Changes in chlorophyll a content (A) and chlorophyll b content (B) of 'Brilliant' and 'Eagleton' kentucky bluegrass during heat stress. Vertical bars at the bottom represent LSD values at $P = 0.05$ for comparisons between cultivars at a given day of treatment. The LSD values ($P = 0.05$) for comparisons of chlorophyll a and chlorophyll b over time in both cultivars were 0.082 and 0.025, respectively.

CHANGES IN CATALASE ACTIVITY AND ISOZYME PROFILES. In 'Brilliant', CAT activity increased significantly at 7 d, the elevated activity was sustained at 21 d, and it then decreased to its control level by 28 d of heat stress. The activity of CAT did not change significantly from 0 to 28 d of heat stress (Fig. 4). The activity of CAT did not differ between the two cultivars before heat stress, but was significantly higher in 'Brilliant' than in 'Eagleton' under heat stress.

No CAT isozymes were present at 0 d of heat stress for either cultivar (Fig. 5). Two isozymes (Rf of 0.15 and 0.21) were detected under heat stress in 'Brilliant' at 7, 14, and 21 d of heat stress. Only one isozyme, CAT2 (Rf of 0.21), was detected in 'Eagleton' during heat stress.

CHANGES IN ASCORBATE PEROXIDASE ACTIVITY AND ISOZYME PROFILES. No significant differences in APX activities were observed between the two cultivars before heat stress (0 d) (Fig. 6). The activity of APX increased significantly in 'Eagleton' during 28 d of heat stress. It increased up to 14 d and then decreased afterward in 'Brilliant'. The activity of APX was significantly higher in 'Eagleton' than in 'Brilliant' at 28 d of heat stress, but not at 7 to 21 d.

Fig. 2. Changes in superoxide dismutase (SOD) activities of 'Brilliant' and 'Eagleton' kentucky bluegrass during heat stress. Vertical bars at the bottom represents LSD values at $P = 0.05$ for comparisons between cultivars at a given day of treatment. The LSD value ($P = 0.05$) for comparisons over time of SOD activity in both cultivars was 19.45.

Fig. 3. Changes in superoxide dismutase (SOD) isoforms of 'Brilliant' (A) and 'Eagleton' (B) kentucky bluegrass during heat stress. Equal amounts (90 μ g) of protein were loaded in each lane: lane 1 = 20 $^{\circ}$ C (0 d of heat stress), lane 2 = 40 $^{\circ}$ C for 7 d, lane 3 = 40 $^{\circ}$ C for 14 d, lane 4 = 40 $^{\circ}$ C for 21 d, lane 5 = 40 $^{\circ}$ C for 28 d; Rf = relative mobility (i.e., relative electrophoretic mobility with respect to the bromophenol blue band).

Both cultivars had three APX isozymes (Fig. 7). The changes in APX isozymes during heat stress were similar in both cultivars, with increasing intensity of APX2 (Rf of 0.58) and APX3 (Rf of 0.75), but APX1 remained unchanged (Rf of 0.16).

CHANGES IN GUAICOL PEROXIDASE ACTIVITY AND ISOZYME PROFILES. Guaiacol peroxidase activity declined during heat stress in both cultivars (Fig. 8). POD activity did not differ significantly between 'Brilliant' and 'Eagleton' before and during heat stress.

Eight POD isozymes were detected before and during heat stress in both cultivars (Fig. 9). The intensity of POD isoenzymes in 'Brilliant' remained unchanged, while the intensity of three isozymes, POD1 (Rf 0.06), POD2 (Rf 0.13), and POD3 (Rf 0.21) increased at 7 d of heat stress and was stronger in 'Eagleton' than in 'Brilliant', but then decreased at 14, 21, and 28 d of heat stress.

Fig. 4. Changes in catalase (CAT) activities of 'Brilliant' and 'Eagleton' Kentucky bluegrass during heat stress. Vertical bars at the bottom represents LSD values at $P = 0.05$ for comparisons between cultivars at a given day of treatment. The LSD value ($P = 0.05$) for comparisons over time of CAT activity in both cultivars was 6.11.

Fig. 5. Changes in catalase (CAT) isoforms of 'Brilliant' (A) and 'Eagleton' (B) Kentucky bluegrass during heat stress. Equal amounts (90 μ g) of protein were loaded in each lane: lane 1 = 20 $^{\circ}$ C (0 d of heat stress), lane 2 = 40 $^{\circ}$ C for 7 d, lane 3 = 40 $^{\circ}$ C for 14 d, lane 4 = 40 $^{\circ}$ C for 21 d, lane 5 = 40 $^{\circ}$ C for 28 d; Rf = relative mobility (i.e., relative electrophoretic mobility with respect to the bromophenol blue band).

Discussion

Leaf Chl content is an important factor determining photosynthetic capacity. Various studies have shown that heat stress leads to reductions in the content of Chl a and Chl b in cool-season turfgrasses, contributing to leaf senescence and photosynthesis inhibition under heat stress (He and Huang, 2007; Jiang and Huang, 2001a; Liu and Huang, 2000; Zhang et al., 2010). In this study, Chl a and Chl b content decreased significantly in 'Brilliant', but remained constant or even increased (Chl b) in 'Eagleton' under heat stress; in addition, Chl a and Chl b content were significantly higher in 'Eagleton' than in 'Brilliant' following prolonged periods of heat stress (28 d); to a greater degree for Chl b. It was previously reported that Chl b-less mutants of rice (*Oryza sativa*) were more susceptible to heat stress than wild type, indicating the importance of maintaining Chl b synthesis for heat tolerance (Lin et al., 2005). Our results suggested that 'Eagleton' was better able to maintain leaf chlorophyll synthesis and sustain less degradation, thus it had better heat tolerance compared with 'Brilliant'. These results are consistent with a previous study reporting that 'Eagleton' had superior heat tolerance than 'Brilliant' (He and

Fig. 6. Changes in ascorbate peroxidase (APX) activities of 'Brilliant' and 'Eagleton' Kentucky bluegrass during heat stress. Vertical bars at the bottom represents LSD values at $P = 0.05$ for comparisons between cultivars at a given day of treatment. The LSD value ($P = 0.05$) for comparisons over time of APX activity in both cultivars was 8.0.

Fig. 7. Changes in ascorbate peroxidase (APX) isoforms of 'Brilliant' (A) and 'Eagleton' (B) Kentucky bluegrass during heat stress. Equal amounts (90 μ g) of protein were loaded in each lane: lane 1 = 20 $^{\circ}$ C (0 d of heat stress), lane 2 = 40 $^{\circ}$ C for 7 d, lane 3 = 40 $^{\circ}$ C for 14 d, lane 4 = 40 $^{\circ}$ C for 21 d, lane 5 = 40 $^{\circ}$ C for 28 d; Rf = relative mobility (i.e., relative electrophoretic mobility with respect to the bromophenol blue band).

Huang, 2007). The cultivar variations in heat-induced leaf senescence, as shown in this study and in overall heat tolerance in Kentucky bluegrass, as demonstrated by differential physiological responses of the two cultivars reported previously (He and Huang, 2007), could be associated with differential changes in antioxidant defense systems in response to heat stress.

The two cultivars of Kentucky bluegrass exhibited different responses of SOD activities and isoenzymes to heat stress. In heat-tolerant 'Eagleton', constant SOD activity was observed during the 28 d of heat stress, whereas that SOD activity decreased in heat-sensitive 'Brilliant'. Unchanged SOD activity was also detected between heated (42 $^{\circ}$ C for 24 h) and unheated rice seedlings (Sato et al., 2001). The unaltered SOD

Fig. 8. Changes in guaiacol peroxidase (POD) activities 'Brilliant' and 'Eagleton' Kentucky bluegrass during heat stress. Vertical bars at the bottom represents LSD values at $P = 0.05$ for comparisons between cultivars at a given day of treatment. The LSD value ($P = 0.05$) for comparisons over time of POD activity in both cultivars was 91.4.

Fig. 9. Changes in guaiacol peroxidase (POD) isoforms of 'Brilliant' (A) and 'Eagleton' (B) Kentucky bluegrass during heat stress. Equal amounts (90 μ g) of protein were loaded in each lane: lane 1 = 20 °C (0 d of heat stress), lane 2 = 40 °C for 7 d, lane 3 = 40 °C for 14 d, lane 4 = 40 °C for 21 d, lane 5 = 40 °C for 28 d; Rf = relative mobility (i.e., relative electrophoretic mobility with respect to the bromophenol blue band).

activity might reflect that $O_2^{\cdot -}$ production in the heat-tolerant cultivar was not enhanced as much as in the heat-susceptible cultivar or that heat-tolerant plants were better able to maintain active SOD under heat stress for detoxifying $O_2^{\cdot -}$. Increased SOD activity was observed in the leaves of grape (*Vitis vinifera*) cultivars resistant to lime-induced chlorosis compared with less resistant cultivars (Ostrovskaya et al., 2009). The increased SOD activity was considered a protective mechanism against the formation of superoxide (Ostrovskaya et al., 2009). The maximal steady-state levels of the appropriate SODs might be required at all times to provide adequate protection, as the $O_2^{\cdot -}$ and the products of its peroxidation are highly and immediately toxic to the cell (Scandalios, 1993). The decreased SOD activities in 'Brilliant' may result in less protection of the leaves from attack by the increase of $O_2^{\cdot -}$ production and thus more severe heat damages in leaves.

Environmental stresses may result in high protein turnover, resulting in the requirement for new SOD enzyme synthesis to maintain SOD levels sufficient for effective protection (Scandalios, 1993). A previous study in *Catharanthus roseus* (Elkhoui et al., 2005) found that the increase in SOD activity observed in salt-treated cells was associated with increasing amounts of three of five isozymes. In the current study, Kentucky bluegrass cultivars exhibited five SOD isozymes. Heat-induced SOD2 (Rf 0.36) was detected in both cultivars, but occurred earlier (7 d) in 'Eagleton' than in 'Brilliant' (14 and 21 d), which could be involved in scavenging ROS produced during early phase of heat stress. The SOD2 isozymes with Rf 0.36 have been found in the cytoplasm in pea (*Pisum sativum* cv. Alaska) leaves (Foster and Edwards, 1980). The SOD3 (Rf 0.50), similar to the reported SOD isozyme with Rf 0.52 located in chloroplast in wheat (*Triticum aestivum* cv. Argee) leaves (Foster and Edwards, 1980), decreased only in 'Brilliant'. The SOD of Rf 0.52 in wheat chloroplast was the predominant SOD, contributing about 80% of the total SOD isozymes (Foster and Edwards, 1980). Localization of this enzyme in chloroplasts is consistent with a requirement for a more H_2O_2 -resistant enzyme at a site where generation of H_2O_2 occurs (Foster and Edwards, 1980). The reduction of SOD3 (Rf 0.50) in 'Brilliant', corresponding to the reduction of SOD activity at 28 d of heat stress, may account for its high sensitivity to heat stress, while the maintenance of this isozyme in 'Eagleton' could be related to the maintenance of higher SOD activity even after 28 d of heat stress.

Catalase is the major H_2O_2 -scavenging enzyme in all aerobic organisms. Reports on the effects of stress on CAT activities vary based on experiment and plant species or cultivars. Decreases in CAT activity has been reported in plants exposed to short-term heat shock in various species (Dat et al., 1998; Foyer et al., 1997; Sato et al., 2001) and during long-term heat stress in perennial grass species (Jiang and Huang, 2001b). In contrast, some studies have reported increases in CAT activity in response to short-term heat stress followed by decreases after prolonged periods of heat treatment (He et al., 2005). Increasing CAT activity may be beneficial to detoxifying H_2O_2 induced by heat stress, and also may be a response to the increase in H_2O_2 production under stress (Dat et al., 1998; Foyer et al., 1997). In the present study, CAT activity increased during 21 d of heat stress and then decreased at 28 d in 'Brilliant', while it was relatively unchanged in 'Eagleton' during 28 d of heat stress. Our results suggest that higher CAT activity under heat stress in Kentucky bluegrass may reflect increases in H_2O_2 production associated with heat injury. An increase in CAT activity with heat stress was also observed in other plant species such as wheat (Almeselmani et al., 2006; Sairam et al., 2000).

Multiple isozymes of CAT were found in various plant species that were responsive to environmental factors (Lee and An, 2005). Water stress activates CAT isozymes in drought-induced oxidative stress events in wheat (Zhang et al., 2004). A transient increase in CAT isozymes was also observed in NaCl-treated barley (*Hordeum vulgare*) root and shoot (Kim et al., 2005) and *C. roseus* (Elkhoui et al., 2005). In the present study, heat stress induced CAT2 with Rf 0.21 in both cultivars and CAT 1 with Rf 0.15 at 7, 14, and 21 d of heat stress only in 'Brilliant'. More and stronger CAT isozymes detected in 'Brilliant' during heat stress coincided with the higher CAT activity in 'Brilliant' compared with 'Eagleton'. The higher

CAT activities and stronger intensity of CAT isozymes in 'Brilliant' indicated that CAT metabolism may play important role in antioxidant defense in the heat-sensitive cultivar of kentucky bluegrass in response to heat stress.

Peroxidases are a variety of enzymes that catalyze the breakdown of H_2O_2 with the concomitant dependent oxidation of a wide variety of substrates. In plants, APX uses ascorbate as the specific electron donor in this enzymatic reaction (Teixeira et al., 2006). Its essential role in the scavenging of H_2O_2 in chloroplasts, where CAT is absent, has been well established (Asada and Takahashi, 1987). Some studies in annual crops have reported increases in the activities of APX during heat stress (Almeselmani et al., 2006; Chaitanya et al., 2002; Sairam et al., 2000), and this increase is more pronounced in heat-tolerant cultivars, such winter wheat (Dash and Mohanty, 2002; Sairam et al., 2000). The APX activity in leaves of creeping bentgrass increased with temperatures from optimum (20 °C) to moderate levels (26 °C), but declined to the lowest level at 30 °C (Xu and Huang, 2004). Results from the current study showed that APX activity increased during heat stress in 'Eagleton' and was significantly higher than that in 'Brilliant' by 28 d. The higher APX activity might enhance the H_2O_2 -scavenging system in chloroplasts and impair accumulation of H_2O_2 , resulting in less heat-induced leaf senescence. The reduction of H_2O_2 by the ascorbate-glutathione cycle is an extremely efficient reaction that dissipates energy and aids in the adjustment of ATP:NADPH ratios with increasing temperatures (Chaitanya et al., 2002). Plants have multiple APX isozymes. In this study, three APX isozymes were detected in kentucky bluegrass in response to heat stress. APX 2 with Rf 0.58 was enhanced by heat stress in both cultivars, which corresponded to the increase in APX activity. However, the presence of this isozyme in kentucky bluegrass may not be related to the cultivar differences in heat tolerance, as APX isozymes exhibited the same responses in both cultivars differing heat tolerance.

Peroxidases participate in a number of physiological processes, such as plant development and organogenesis (O'Neil and Scot, 1987; Schneider and Wightman, 1974). The POD activities in leaves and roots of heat-stressed plants increased to levels significantly above their respective controls for two cultivars of bentgrass at 28 d of heat stress (35/25 °C, day/night) (Liu and Huang, 2000). Results from former studies suggest that increases in POD activity are related to an accelerated production of ROS in some plant species (Okuda et al., 1991); however, the elevated activities of POD did not appear to have relieved maize (*Zea mays*) plants from excessive generation of ROS (Kumar et al., 2008). In the current study, a decline in POD activity was observed during heat stress, and, to a similar extent, in both cultivars during the 28-d heat treatment. Decreases in POD activity during heat stress were also observed in other species, such as wheat (Almeselmani et al., 2006). Heat-induced decline in POD activities in both cultivars suggested that POD could be sensitive to high temperatures, and the enzyme activity was not related to heat tolerance in kentucky bluegrass. Although peroxidases are catalytically very active, they exhibit very little specificity for substrates and exist in various isozyme forms (Gaspar et al., 1991; Siegel, 1993; Welinder, 1992). The expression of specific isozymes has been found responsive to development events such as senescence (Abeles et al., 1988) and external stress, such as wounding and pathogens (Albert and Anderson, 1987). In this study, eight

POD enzymes were detected at normal temperature and heat stress, suggesting that all of these isozymes were constitutively present in kentucky bluegrass. Three isozymes, POD1 (Rf 0.06), POD2 (Rf 0.13), and POD3 (Rf 0.21), showed differential responses to heat stress between the two cultivars, which exhibited a transient increase at 7 d of heat stress in 'Eagleton' but remained unchanged in 'Brilliant'. Certain POD isozymes use the phenolic compounds and H_2O_2 to initiate the biosynthesis of several secondary metabolites required for plant growth, development, and differentiation (Gaspar et al., 1991). The transient increase in POD isozyme intensity may reflect transient H_2O_2 production during the early phase of heat stress and/or changes in the production of secondary metabolites for stress defense.

In summary, our results demonstrate that antioxidant enzymes and their isozyme forms responded differently to heat stress in two cultivars of kentucky bluegrass. Superior heat tolerance in 'Eagleton', as indicated by lesser heat-induced leaf senescence and other physiological changes (He and Huang, 2007), could be associated with antioxidant protection through increasing activities and maintaining isozymes of SOD for $O_2^{\cdot -}$ scavenging and increasing APX activities for H_2O_2 scavenging. Thus, a tolerant genotype preferably should have greater expression of most of the antioxidant enzymes, such as SOD and Halliwell-Asada Pathway enzymes, such as APX. The lack of cultivar differences in CAT and POD activities or their isozymes could not account for the cultivar variation in heat tolerance in kentucky bluegrass. Manipulation of those antioxidant enzymes that exhibited differential responses to heat stress for cultivars differing in heat tolerance may lead to improvement in heat tolerance. The direction involvement, cellular location, characteristics of these enzymes, and the isoforms related to heat tolerance in perennial grasses are largely unknown, and deserve further investigation.

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