Protein Changes in Response to Heat Stress in Acclimated and Nonacclimated Creeping Bentgrass

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ABSTRACT. The acclimation of plants to moderately high temperature plays an important role in inducing plant tolerance to subsequent lethal high temperatures. This study was performed to investigate the effects of heat acclimation and sudden heat stress on protein synthesis and degradation in creeping bentgrass (*Agrostis palustris* Huds.). Plants of the cultivar Penncross were subjected to two temperature regimes in growth chambers: 1) heat acclimation—plants were exposed to a gradual increase in temperatures from 20 to 25, 30, and 35 °C for 7 days at each temperature level before being exposed to 40 °C for 28 days; and 2) sudden heat stress (nonacclimation)—plants were directly exposed to 40 °C for 28 days from 20 °C without acclimation through the gradual increase in temperatures. Heat acclimation increased plant tolerance to subsequent heat stress, as demonstrated by lower electrolyte leakage (relative EL) in leaves of heat-acclimated plants compared to nonacclimated plants at 40 °C. Heat acclimation induced expression of some heat shock proteins (HSPs), 57 and 54 kDa, detected in a salt-soluble form (cystoplasmic proteins), which were not present in unacclimated plants under heat stress. However, HSPs of 23, 36, and 66 kDa were induced by both sudden and gradual exposure to heat stress. In general, total protein content decreased under both heat acclimation and sudden heat stress. Cystoplasmic proteins was more sensitive to increasing temperatures, with a significant decline initiated at 25 °C, while sodium dodecyl sulphate (SDS)-soluble (membrane) protein content did not decrease significantly until temperature was elevated to 30 °C. The results demonstrated that both a gradual increase in temperature and sudden heat stress caused protein degradation and also induced expression of newly synthesized HSPs. Our results suggested that the induction of new HSPs during heat acclimation might be associated with the enhanced thermotolerance of creeping bentgrass, although direct correlation of these two factors is yet to be determined. This study also indicated that protein degradation could be associated with heat injury during either gradual increases in temperature or sudden heat stress.

High temperature stress is a major environmental factor limiting plant growth in tropical and subtropical areas (Radin et al., 1994). High temperature inhibits photosynthesis, limits carbohydrate accumulation, damages cell membranes and leads to cell death (Liu and Huang, 2000). Plants have the ability to acquire thermotolerance by exposure to a gradual increase in temperature (heat acclimation) (Hong et al., 2003; Vierling, 1991), which often occurs in the natural environment. Understanding mechanisms of heat acclimation will facilitate the development of new strategies for crop improvement under high temperature stress.

Cellular membranes are among the most sensitive components of a plant cell to heat stress (Raison et al., 1980). Heat stress may disrupt membranes and leads to the leakage of organic and inorganic solutes (electrolytes) from the cell (Levitt 1980). Electrolyte leakage of cells is widely used to evaluate membrane thermostability (Marcum et al., 1998). The ability of maintaining high membrane thermostability under heat stress is positively correlated to whole-plant tolerance to heat stress (Howarth et al., 1997; Marcum et al., 1998).

Plants produce a family of proteins called heat shock proteins (HSPs) in response to either rapid heat shock or a gradual increase in temperature (Al-Niemi and Stout, 2002; Nguyen et al., 1994; Vierling, 1991). Based on their molecular weight, the major HSPs synthesized by plants belong to five classes: HSP100, HSP90, HSP70, HSP60 (kDa), and small HSPs (≈17 to 30 kDa) (Vierling, 1991). Proteins in plant cells are also classified into different groups based on their solubility properties

and localization in cells (Loponen et al., 2004). Salt- and water-soluble proteins (simplified as salt-soluble protein) are usually localized in cytoplasm and called cystoplasmic protein. Sodium dodecyl sulphate (SDS) is a good solvent for membrane proteins (Ames and Nikaido, 1976; Hurkman and Tanaka, 1986), and SDS-soluble proteins are mainly considered as cell membrane proteins. In recent investigations, acquired thermotolerance of plants has been associated with the presence of HSPs in several plant species (Burke, 2001; Hong and Vierling, 2000; Malik et al., 1999; Queitsch et al., 2000). All major classes of HSPs are proposed to act as molecular chaperones, functioning by binding to substrate proteins that are in unstable, nonnative structural states (Gething, 1997). By virtue of this property, the different HSPs/chaperones are able to aid in a variety of cellular processes that involve assisted protein folding, including rescue of misfolded or aggregated proteins. This latter activity is presumed to explain their important role in heat stress, a condition that often leads to protein denaturation (Vierling, 1991).

Creeping bentgrass is a commonly used cool-season turfgrass grown in temperate regions of the world. Many cool-season turfgrasses are often subjected to heat stress during summer in warm climatic regions, exhibiting decline in turf quality and growth. A previous study showed that heat acclimation improved the ability of creeping bentgrass to survive subsequent exposure to potentially lethal temperatures (Larkindale and Huang, 2004). The induction of HSPs has been associated with enhanced heat tolerance in other perennial grass species (Al-Niemi and Stout, 2002). We hypothesized that increased thermotolerance of creeping bentgrass induced by heat acclimation could be related to expression of certain new proteins, and heat injury is associated with protein degradation. The objective of this study was to examine changes in protein content and expression during heat acclimation and sudden heat stress in creeping bentgrass.

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Materials and Methods

PLANT MATERIALS AND GROWTH CONDITIONS. Sod plugs of 'Penncross' creeping bentgrass were collected from 3-year-old field plots at the Rutgers Univ. Horticulture Farm II, North Brunswick, N.J., in May 2002. They were transferred into polyvinyl chloride (PVC) tubes (10 cm diameter and 60 cm long) filled with washed sand. Plants were maintained in a greenhouse for 1 month and then moved into a growth chamber at 20 °C day/16 °C night temperature, 75% relative humidity, 14-h photoperiod, and 400 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ photosynthetically active radiation (PAR). Plants were fertilized once per week with 40 mL Hoagland's solution (Hoagland and Arnon, 1950) and cut twice per week to keep the height at ≈ 5 cm.

HEAT ACCLIMATION AND SUDDEN HEAT STRESS TREATMENTS. For heat acclimation treatment, eight tubes of plants were maintained at 20, 25, 30, and 35 °C (day/night temperature) for 7 d at each temperature before exposed to 40 °C for 28 d. For sudden heat stress (nonacclimation), eight tubes of plants were exposed only to 20 °C for 7 d before exposure to 40 °C for 28 d. Four growth chambers were used for each temperature at each 7-d period and during the 28 d of sudden heat stress. During sudden heat stress or heat acclimation, leaves were sampled once a week. Fresh samples were used to measure EL and frozen samples were kept at -70 °C for protein analysis. During the temperature treatments, growth chambers were at 75% relative humidity, 14-h photoperiod, and 400 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ PAR. Plants were watered twice a day, fertilized once per week with 40 mL Hoagland's solution (Hoagland and Arnon, 1950), and cut twice per week to keep the height at ≈ 5 cm.

MEASUREMENT OF ELECTROLYTE LEAKAGE. Cell membrane stability was estimated by measuring electrolyte leakage (EL) following the method described by Liu and Huang (2000). Approximately 0.2 g of fresh leaves were randomly sampled from each of four pots in each treatment and cut into 1-cm segments. Leaves were rinsed twice with de-ionized water and placed into test tubes containing 40 mL deionized water. Test tubes were then placed on a shaker for 24 h at 22 °C. Electrical conductivity (EC) of the solution containing fresh leaves (EC_0) was measured with a conductivity meter (YSI model 32; Yellow Springs Instrument Co., Yellow Springs, Ohio) at 22 °C. All leaves in each test tube were then killed at 100 °C for 1 h in a water bath and left at room temperature for 2–4 h until cooled to 22 °C. EC of the solution containing killed leaves (EC_{100}) was measured with the conductivity meter described above. Relative conductivity, or relative EL (%), was calculated as $(\text{EC}_0 / \text{EC}_{100}) \times 100$.

EXTRACTION OF PROTEINS AND QUANTIFICATION. Extraction of proteins from shoots was performed following the method described by Shimoni et al. (1997). Frozen leaves (0.5 g fresh weight) were ground in liquid N_2 to fine powder. The powder was extracted in 3 mL of buffer containing 0.10 M Tris-HCl (pH 7.6) and 0.15 M NaCl on ice bath (around 4 °C) and then centrifuged twice at 16,000 g_n at 4 °C for 30 min. The resulting supernatant, containing the salt-soluble proteins, was kept for further analysis. The pellet was subsequently resuspended in 3 mL of the buffer containing 2% (w/v) SDS, 0.10 M Tris-HCl (pH 7.6), and 0.15 M NaCl. Following extraction for 1 h at 4 °C with agitation, the suspension was centrifuged as described above and the supernatant, termed "SDS-soluble protein," was kept. Protein content of the supernatant was determined by the method of Bradford (1976). Briefly, 100 μL of protein extraction (diluted five times) was mixed with 3 mL of coomassie G-250

reagent (0.01% coomassie brilliant blue G, 4.75% ethanol, and 8.5% phosphoric acid), and the absorbance was measured at 595 nm between 5 and 30 min after reaction using a spectrophotometer (Spectronic Genesys 2; Spectronic Instruments, Rochester, N.Y.). Bovine serum albumin was used as a standard.

PROTEIN EXPRESSION. For protein expression sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was done according to the method of Laemmli (1970) with modifications. Salt-soluble protein extraction was dissolved in SDS-PAGE sample buffer containing 75 mM Tris-HCl (pH 6.8), 50% (w/v) sucrose, 10% (w/v) SDS, 20% (v/v) β -mercaptoethanol, and 1% bromophenol blue at the sample to buffer volume ratio of 4:1. Proteins were separated by discontinuous SDS-PAGE with an electrophoresis unit (PROTEIN II; Bio-Rad, La Jolla, Calif.) using a 6% stacking gel and 12% running gel. Gels were run at room temperature (20–22 °C) and were stained overnight with coomassie brilliant blue R. Proteins with known molecular weights (MW) were used as markers to identify MW of proteins in the samples in each gel. As the log (MW) and the migration rate (distance the band migrated from the top of the separating gel divided by the distance migrated by the tracking dye of bromophenol) is linearly related when protein MW is between 12–200 kDa (Viney and Fenton, 1998), the distance and migration rate were used to calculate the MW of protein bands in the samples based on the linear regression of the markers in the same gel. By comparing protein profiles between control and high-temperature-treated samples, the proteins that were present only in treated samples, but were not present in the control were defined as heat shock proteins.

EXPERIMENTAL DESIGN AND STATISTICAL ANALYSIS. Treatments were arranged in a randomized complete-block design with four replicates (four pots for each treatment). Protein content was measured for three sub-samples taken from each extraction sample (replicate). The mean of the three sub-samples was used to represent a single replicate in the analysis of variance. Data were analyzed with analysis of variance using Microsoft Excel 2000 (Microsoft Corp., Redmond, Wash.) (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).

Results

EFFECTS OF HEAT ACCLIMATION AND SUDDEN HEAT STRESS ON CELL MEMBRANE STABILITY. Cell membrane stability decreased, as demonstrated by the increase in relative EL, with an increase in temperature from 20 to 35 °C during heat acclimation. An increase of 32% in relative EL was observed at 25 °C compared with that at control temperature (20 °C) as shown in Fig. 1A. During sudden heat stress (40 °C), EL increased rapidly with the duration of treatment. The increase of 79% in relative EL was initially observed at 7 d of treatment compared with 0 d of treatment as shown in Fig. 1B. Plants exposed to increasing temperatures had significantly higher relative EL than those grown at 20 °C (Fig. 1C, 0 d) but had 33% and 57% lower relative EL than unacclimated plants when both were exposed to 40 °C for 14 and 28 d, respectively, as shown in Fig. 1C.

EFFECT OF HEAT ACCLIMATION AND SUDDEN HEAT STRESS ON PROTEIN CONTENT. Significant decreases in salt-soluble (Fig. 2) and SDS-soluble (Fig. 3) protein content were observed in plants exposed to gradually increasing temperatures from 20 to 35 °C and sudden heat stress. A decrease of 12% in salt-soluble protein occurred when the temperature was increased to 25 °C

Fig. 1. Electrolyte leakage in leaves of creeping bentgrass during heat acclimation (A), under sudden heat stress (40 °C) (B), and at 0, 14, and 28 d of sudden heat stress for heat- acclimated and unacclimated plants (C). Bars represent SE (n = 4) and different letters indicate a significant difference at $P = 0.05$.

(Fig. 2A) and a decrease of 25% in SDS-soluble protein content decreased when temperature was increased to 30 °C (Fig. 3A) as compared with the control at 20 °C. Both salt-soluble and SDS-soluble protein content decreased with duration of sudden heat stress treatment as shown in Fig. 2B and Fig 3B, respectively. The decline in SDS-soluble protein content at 7 d of sudden heat stress was 54% (Fig. 3B) and 8% in salt-soluble protein content (Fig. 2B) as compared to that of control at 20 °C, which showed the changes of SDS-soluble protein content was more pronounced than that of salt-soluble protein content at 7 d of heat treatment.

Fig. 2. Salt-soluble protein content [percent dry weight (DW)] in leaves of creeping bentgrass during heat acclimation (A) and under sudden heat stress (40 °C) (B). Bars represent SE (n = 4) and different letters indicate a significant difference at $P = 0.05$.

Plants exposed to increasing temperatures had significantly lower salt-soluble (Fig. 4A, 0 d) and SDS-soluble (Fig. 4B, 0 d) protein contents than plants maintained at 20 °C. However, no significant differences in salt-soluble (Fig. 4A) or SDS-soluble (Fig. 4B) protein content were observed between heat-acclimated and nonacclimated plants when both plants were exposed to heat stress (40 °C) for 14 or 28 d.

PROTEIN EXPRESSION DURING HEAT ACCLIMATION AND SUDDEN HEAT STRESS. Proteins that were induced under heat stress but were absent under normal temperature (20 °C in this study) are considered HSPs. Some heat shock proteins (HSPs) were

Fig. 3. Sodium dodecyl sulphate (SDS)-soluble protein content [percent dry weight (DW)] in leaves of creeping bentgrass during heat acclimation (A) and under sudden heat stress (40 °C) (B). Bars represent SE (n = 4) and different letters indicate a significant difference at $P = 0.05$.

Fig. 4. Comparisons of salt-soluble (A) and sodium dodecyl sulphate (SDS)-soluble (B) protein content [percent dry weight (DW)] between heat acclimated and unacclimated creeping bentgrass under heat stress (40 °C). Bars represent SE (n = 4) and different letters indicate a significant difference at $P = 0.05$.

detected in the treatment of sudden exposure of plants to 40 °C, which occurred between 7 and 28 d after exposure to the stress (Fig. 5). Three HSPs with molecular weights of 23, 36, and 66 kDa were detected in heat-stressed plants (lanes 2 through 5 in Fig. 5), whereas these proteins were absent in the control plants exposed to 20 °C (lanes 1 and 6 in Fig. 5). In addition to the three HSPs (23, 36, and 66 kDa) as shown in plants exposed directly to 40 °C, two additional HSPs, 54 and 57 kDa, were detected in heat-acclimated plants after the gradual increase in temperature from 20 to 35 °C at 14 d of 40 °C (lane 4 in Fig. 6). However, as heat stress was prolonged to 28 d, the acclimation induced HSPs disappeared (lane 2 in Fig. 6). These two HSPs were not detected in plants directly exposed to 40 °C for 14 d and 28 d (lanes 3 and 5 in Fig. 6).

Heat stress damages membrane systems and can lead to the leakage of electrolytes from plant cells (Levitt, 1980). The increase in relative EL indicates cell membrane damage had occurred. Cell membrane thermostability measured by the relative amount of EL from leaf segments exposed to a heat shock has been used to predict whole-plant thermotolerance for different turfgrass cultivars (Marcum, 1998). In this study, heat-acclimated plants had significantly lower EL than unacclimated plants when exposed to 40 °C for 14 and 28 d (Fig. 1C). This result indicated that membrane destabilization was lower in acclimated leaves

Discussion

Fig. 5. Salt-soluble protein profile for creeping bentgrass exposed to sudden heat stress. Lane 1 = 20 °C for 7 d, 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, lane 6 = 20 °C for 7 d. Equal amounts of protein (18 μ g) were loaded in each lane. Arrows indicate newly induced proteins under sudden heat stress. MW = molecular weight, Marker = standard protein with known MW.

than those in nonacclimated plants being exposed to heat stress, which may contribute to the enhanced creeping bentgrass tolerance to subsequent exposure as previously reported by Larkindale and Huang (2004).

Proteins are localized mainly in cellular membranes as insoluble form and cytoplasm in the soluble form. Membrane proteins have good solubility in SDS (Ames and Nikaido, 1976; Hurkman and Tanaka, 1986), and thus in the present study, SDS-soluble proteins are considered as membrane proteins and salt-soluble proteins are mainly cytoplasmic proteins. Synthesis of normal proteins in either soluble or membrane form usually decreases under heat stress conditions (Key et al., 1981). Both heat acclimation through gradual exposure to temperatures from 20 to 35 °C before exposure to 40 °C and sudden exposure of plants from 20 to 40 °C caused reduction in the content of both salt-soluble (Fig. 2) and SDS-soluble protein (Fig. 3) groups. A significant net decline in salt-soluble cytoplasmic protein content occurred at 25 °C (Fig. 2A), while a significant net decline in SDS-soluble membrane proteins was not detected until temperature was increased to 30 °C (Fig. 3A), suggesting that soluble cytoplasmic protein was more susceptible to a gradual increase in temperatures than membrane proteins. A decrease in soluble protein content with increasing temperatures has also been detected in other species (Zhou et al., 1995). In our study, the net decline in soluble and insoluble membrane protein content corresponded with the increase in EL under both heat acclimation and sudden heat stress conditions. It is reasonable to infer that the net decline in protein content could be associated with heat injury in shoots, as manifested by the increased EL.

The decrease in normal protein synthesis is often accompanied by the synthesis and accumulation of HSPs. High-molecular-mass HSPs (HSP70, HSP90, HSP100) are characterized by a high level of sequence similarity within the plant kingdom (Maestri et al., 2002). The low-molecular-mass HSPs are the most abundant proteins induced by heat stress in higher plants (Vierling, 1991). A study of the heat shock response in creeping bentgrass variants indicated that both selected heat-tolerant lines (SB) and nonselected (non-heat tolerant) lines (NSB) from a parent cultivar, 'Penncross', synthesized HSPs of 97, 83, 70, 40, 27, and 18 kDa in 1-cm leaf segments incubated at 40 °C for 1.5 h

Fig. 6. Comparison of salt-soluble protein profiles for creeping bentgrass during heat acclimation and sudden heat stress. Lane 1 = 20 °C for 7 d; lane 2 = 40 °C for 28 d after exposure for 7 d at each temperature (20, 25, 30, 35 °C); lane 3 = direct exposure to 40 °C for 28 d; lane 4 = 40 °C for 14 d after exposure for 7 d at each temperature (20, 25, 30, 35 °C); lane 5 = direct exposure to 40 °C for 14 d; lane 6 = 20 °C for 7 d. Equal amounts of protein (18 μ g) were loaded in each lane. Arrows indicate newly induced proteins. MW = molecular weight, Marker = standard protein with known MW.

(Park et al., 1996). In our study, we used the Penncross cultivar that is developed from the open pollination of three genotypes to investigate heat induction of HSPs in a genetically balanced population. HSPs of 66, 36, and 23 kDa were detected in leaves of 'Penncross' creeping bentgrass when the whole plant was exposed to 40 °C for 7 to 28 d. HSP66 is a member of the HSP60 family, which has been found to facilitate proper folding of other proteins (Vierling, 1991). HSP23 has also been found to accumulate abundantly at 40 °C in tomato (*Lycopersicon esculentum* Mill.) leaves, which served as molecular chaperons in an in vitro test (Liu and Shono, 1999). HSP36 has also been detected in tomato (Rose et al., 2003), but the function of this protein is unknown and warrants further study.

HSP induction has been correlated with acquired thermotolerance (Kimpel and Key, 1985; Krishnan et al., 1989; Lin et al., 1984; Lindquist, 1986). Park et al. (1996) reported that heat tolerant line "SB" of creeping bent grass synthesized two additional HSP25 polypeptides that did not accumulate in non-heat tolerant line "NSB." The production of these additional HSP25 isoforms was genetically correlated with improved thermotolerance. Creeping bentgrass plants with the additional HSP25 polypeptides recovered from heat stress faster and were able to resume typical levels of protein synthesis $\approx$ 2 h earlier than those without the new protein expression (Park et al., 1997). Park et al. (1996) also proved that these two additional HSP25 could only be extracted by phenol extraction procedure, which was developed for the efficient isolation of membrane-associated proteins (Hurkman and Tanaka, 1986). In the present study, HSP23, a member of small heat shock protein family, was detected in both heat acclimated plants and unacclimated plants, which indicated that HSP23 may be the result of a general response of plants to increasing temperatures, but not specifically involved in heat acclimation.

Heat acclimation induced two unique HSPs, 57 and 54 kDa (Fig. 5). These two proteins were not expressed in unacclimated creeping bentgrass plants. The functions of these HSPs are unknown in higher plants, except for limited studies in other organisms (Gerner et al., 2000). The 57-kDa cytoplasmic protein binds to the promoters of heat-shock genes and activates transcription during heat shock in human cells (Soncin et al.,

1997). HSP54 has been found to be correlated with the reduction of copper toxicity in sponge (*Crambe crambe* L.) (Agell et al., 2001). It is possible that the two additional polypeptides, HSP57 and HSP54, may contribute to acquired thermotolerance through acclimation since only acclimated plants that exhibited better heat tolerance had the expression of those proteins. Investigation of the functions of these unique HSPs in creeping bentgrass might provide insight into mechanisms of acquired thermotolerance in cool-season grasses.

In summary, heat acclimation enhanced creeping bentgrass tolerance to subsequent heat stress, as manifested by increased membrane thermostability. The exposure of plants to a gradual increase in temperature and sudden heat stress induced the expression of some HSPs, but different HSPs were synthesized in heat-acclimated plants compared to heat-stressed plants, suggesting that the unique HSPs expressed during heat acclimation may contribute to the enhanced thermotolerance due to heat acclimation. Gradual increases in temperature and direct exposure to heat stress also caused the degradation of normal proteins, which may be associated with heat injury in leaves.

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