

Leaf Senescence and Protein Metabolism in Creeping Bentgrass Exposed to Heat Stress and Treated with Cytokinins

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ADDITIONAL INDEX WORDS. *Agrostis stolonifera*, high temperature, chlorophyll, protease, photochemical efficiency

ABSTRACT. Heat stress induces leaf senescence and causes changes in protein metabolism. The objective of this study was to investigate effects of exogenous application of a synthetic form of cytokinin, zeatin riboside (ZR), on protein metabolism associated with leaf senescence under heat stress for a cool-season grass species. Creeping bentgrass (*Agrostis stolonifera* L.) (cv. Penncross) plants were exposed to optimum temperature control (20/15 °C, day/night) and heat stress (35/30 °C) in growth chambers. Before heat stress treatments, foliage was sprayed with 10 μmol ZR or water (untreated) for 3 days and then once per week during 35 days of heat stress. Leaf chlorophyll content, photochemical efficiency (F_v/F_m), and soluble protein content declined, whereas protease activity increased during heat stress. Treatments with ZR helped maintain higher leaf chlorophyll content, F_v/F_m , and soluble protein content under heat stress. Protease activity in ZR-treated plants was lower than that of untreated plants. Zeatin riboside-treated plants had less severe degradation of ribulose-1,5-bisphosphate carboxylase proteins than untreated plants exposed to heat stress. In addition, ZR treatment upregulated the expression of 32- and 57-kDa proteins under heat stress conditions. These results demonstrated that the exogenous application of ZR ameliorated the negative effects of heat stress, as manifested by suppression or delay of leaf senescence. Cytokinins may have helped to alleviate heat stress injury, probably by slowing down the action of protease and by induction or upregulation of heat-shock proteins.

Temperatures greater than the optimal growth temperature negatively affect plant growth and induce various physiological and metabolic changes, including premature leaf senescence. Leaf senescence is characterized by loss of chlorophyll and proteins (Richmond and Lang, 1957; Selivankina et al., 2001). Heat stress has also been reported to accelerate the process of protein degradation (Gulen and Eris, 2004; He et al., 2005; Jiang and Huang, 2002). Under conditions of high temperature, protein loss is accelerated as a result of increased protease activity and this, in turn, leads to accelerated leaf senescence (Al-Khatib and Paulsen, 1984; Ueda et al., 2000; Zavaleta-Mancera et al., 1999). Plant adaptation to high temperatures also involves induction of protein synthesis or altered protein functions (Teeri, 1980). A family of proteins, called heat-shock proteins (HSPs), are often induced when plants are exposed to elevated temperatures. Heat-shock proteins are divided into two major classes based on their molecular size, with high-molecular mass HSPs ranging from 60 to 110 kDa and low-molecular mass HSPs ranging between 15 and 30 kDa (Vierling, 1991). These proteins protect biomolecules and organelles in plant cells from stress damage by enhancing thermotolerance (Park et al., 1996; Sun et al., 2002).

Different approaches have been examined in alleviating heat stress injury, including exogenous application of plant hormones, such as cytokinins. Cytokinins regulate many aspects of growth and development, including leaf senescence (Binns,

1994; Hare and Van Staden, 1997). Cytokinins have the ability to retard senescence, and this may be the result of their influence on different metabolic processes. Senescent leaves showed a decline in cytokinin level, and exogenous application of cytokinin resulted in delayed senescence in various plant species (Badenoch-Jones et al., 1996; Liu and Huang, 2002; Nooden et al., 1979). It has also been reported that transgenic tobacco (*Nicotiana tabacum* L.) plants that overexpressed the isopentenyltransferase (*ipt*) gene controlling cytokinin synthesis showed increased cytokinin content, which resulted in delayed senescence (Gan and Amasino, 1995). Cytokinins prevent senescence and its accompanying chlorophyll breakdown by preventing proteolysis (Martin and Thimman, 1972) or by reducing the messenger RNA (mRNA) and protein level of proteases (Li et al., 2000). Cytokinins inhibited the loss of proteins and prevented the increase in proteolytic activity of senescing leaves in *Hordeum vulgare* L. (Peterson and Huffaker, 1975). The enhancement in protein content of *Tropaeolum majus* L. leaves treated with cytokinin has been attributed to the inhibition of protein degradation (Mizrahi et al., 1970). Cytokinins are able to counteract the deleterious effect of heat stress, probably by scavenging free radicals or by modulating the activity of various antioxidant enzymes (Caers et al., 1985; Dhindsa et al., 1982; Lesham, 1984; Liu and Huang, 2002; Synkova et al., 2006; Wang et al., 2003).

Creeping bentgrass is a widely used cool-season turfgrass species and there is often a considerable decline in turfgrass quality resulting from leaf senescence under conditions of high-temperature stress. Previous studies have demonstrated that exogenous application of cytokinins to the root zone enhanced shoot and root growth and delayed leaf senescence (Liu and Huang, 2002; Liu et al., 2002). Foliar application of cytokinin may delay leaf senescence or promote better heat tolerance in creeping bentgrass by affecting protein metabolism.

Received for publication 11 Oct. 2006. Accepted for publication 2 Feb. 2007. The authors thank Rutgers Center of Turfgrass for funding support of this research project.

Thanks also go to Dr. Jiang Tian, Steve McCann, Yan Xu, and Emily Merewitz for critical review of the manuscript.

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Understanding the relationship of cytokinins and heat-induced changes in protein metabolism may help elucidate their role in regulating heat tolerance. Therefore, the objective of this study was to determine the effects of an exogenous application of cytokinins on protein metabolism and leaf senescence for creeping bentgrass under heat stress.

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 University Horticulture Farm II (New Brunswick, NJ). They were transferred into PVC tubes (10 cm in diameter and 30 cm long) filled with washed sand. Plants were maintained in a greenhouse for 2 months and then moved into growth chambers. The growth chamber conditions were set at a temperature of 20/15 °C (day/night), 75% humidity, a 14-h photoperiod, and 400 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$ of photosynthetically active radiation. Plants were fertilized once per week with 100 mL half-strength Hoagland solution (Hoagland and Arnon, 1950) and cut once per week to the height of ≈ 5 cm. Plants were allowed to acclimate to growth chamber conditions for 14 d before treatments were imposed.

CYTOKININ AND TEMPERATURE TREATMENTS. Plants were pretreated daily by foliar spray with 50 mL of water (untreated) or 50 mL (this volume saturated the canopy until some dripping occurred) of 10 μmol zeatin riboside (ZR; ZR-treated) for 3 d before high-temperature treatment (heat stress) and on the day of heat stress initiation. Water and ZR (transzeatin riboside; Acros Organics, Fair Lawn, NJ) solution contained 0.05% Tween 20 (Sigma-Aldrich, St. Louis) and was adjusted to a pH of 6.7 to -6.9 with NaOH or HCl. After 3 d of pretreatment, both untreated and ZR-treated plants were then exposed to heat stress at 35/30 °C (day/night) for 35 d in the growth chamber. During heat stress treatment, plants were treated again with 50 mL of water or 10 μmol ZR once per week. Plants were watered with 200 mL water twice daily to prevent water deficit. The 10- μmol ZR concentration was used in this study because it has been found to be the most effective dose in alleviating heat injury in creeping bentgrass through root-zone injection (Liu and Huang, 2002). Plants grown under optimum temperature conditions at 20/15 °C (day/night) were treated with water and used as a control for comparison with the heat-stressed plants.

MEASUREMENTS. Two parameters, including leaf chlorophyll content and photochemical efficiency, were measured to evaluate leaf senescence and the severity of heat injury. All measurements were made on leaves at weekly intervals. Chlorophyll was extracted by soaking 0.1 g fresh leaves in 10 mL dimethyl sulfoxide for 72 h. The absorbance of the extract was estimated at 663 nm and 645 nm with a spectrophotometer (Spectronic Genesys 2; Spectronic Instruments, Rochester, NY). Chlorophyll content was calculated according to the formula of Arnon (1949).

Leaf photochemical efficiency was determined by measuring chlorophyll fluorescence, the ratio of variable fluorescence to maximal fluorescence (F_v/F_m), with a leaf photochemical efficiency analyzer (Fim 1500; ADC BioScientific, Hoddesdon, UK) according to the method described by Havaux (1992). When the leaf is kept in the dark, the amount of fluorescence is at the basal level (F_0); when the leaf is flashed with bright light, the fluorescence increases to a maximum (F_m). The difference between the maximum and minimum fluorescence is the

variable fluorescence (F_v) and the ratio of F_v/F_m is the photochemical efficiency of Photosystem II (PSII). Intact leaves were covered in specially designed leaf chambers and allowed to adapt in the dark for 30 min before measuring fluorescence. Measurement was made on three subsamples in each pot.

PROTEIN EXTRACTION AND QUANTIFICATION. Total soluble proteins were extracted from leaves according to the method of Shimoni et al. (1997). Leaf tissue (0.5 g fresh weight) was ground with liquid nitrogen to a fine powder. Protein was extracted in 3 mL buffer containing 0.10 mM Tris-HCl (pH, 7.6) and 0.15 M NaCl. Samples were then centrifuged twice at 16,000 g_n at 4 °C for 30 min and the resulting supernatant was collected. Protein content of leaves was quantified by the method of Bradford (1976) using bovine serum albumin as standard.

PROTEASE ACTIVITY MEASUREMENT. Extracts were assayed according to a modified method of Benbella and Paulsen (1998). Fresh shoots (1.0 g) were ground to a fine powder in liquid nitrogen and placed in 4 mL extraction medium (25 mM HEPES at a pH of 7.5, 1 mM ethylenediamine tetraacetic acid, and 4 mM dithiothreitol). The homogenized samples were centrifuged twice at 16,000 g_n at 4 °C for 30 min. Crude protease activity was assayed by adding 0.1-mL aliquots of enzyme extract to 1.5 mL 200 mM sodium citrate-citric acid buffer (pH, 5.2) and 0.4 mL 1% (w/v) azocasein. The reference tube contained 0.1 mL extraction medium instead of enzyme extract. The tubes were incubated for 2 h at 37 °C. The reaction was stopped by adding 1 mL 15% (w/v) trichloroacetic acid. Both sample and reference tubes were then incubated at 4 °C for 30 min. The resulting precipitate was removed by centrifugation twice at 4000 g_n for 5 min. The absorbance of clear supernatant was read at 340 nm in a spectrophotometer (Spectronic Genesys 2). The absorbance of reference was subtracted from the sample. One unit of enzyme activity was defined as the changes of absorbance by 0.1.

PROTEIN EXPRESSION. Samples from 21-d heat-stressed leaves were analyzed for changes in protein expression by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (1970), with slight modifications. Samples were solubilized 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. The ratio of sample to buffer volume was 4:1. Proteins were separated by discontinuous SDS-PAGE with an electrophoresis unit (Protein II; Bio-Rad, La Jolla, CA) using a 6% stacking gel and 12% separating gel. Gels were stained overnight with Coomassie brilliant blue R-250. Gels for SDS-PAGE were run on three samples (replicates) for each treatment. The gel of one representative replicate is presented.

EXPERIMENTAL DESIGN AND STATISTICAL ANALYSIS. Cytokinin and temperature treatments were arranged in a randomized, complete block design, each with three replicates. High-temperature treatment was repeated simultaneously in three growth chambers. Three tubes of plants for each treatment were placed inside each chamber as three subsamples. The mean of the three subsamples was used to represent a single replicate in the analysis of variance. Sampling was done at an interval of 7 d for the entire duration of experiment, which lasted for 35 d. Data were analyzed by analysis of variance according to the general linear model procedures of SAS (SAS Institute, Cary,

NC). Differences between untreated plants, ZR treatment, and nonstressed controls were separated by the LSD test at a 0.05 *P* level.

Results

LEAF CHLOROPHYLL CONTENT. Leaf chlorophyll content of both ZR-treated and untreated plants decreased significantly after 7 d of heat stress (35 °C) compared with that at 20 °C (Fig. 1). The decrease in chlorophyll content at 7 d was 13% in heat-stressed plants treated with ZR and 20% in untreated plants. By 35 d of heat stress, chlorophyll content decreased by 39% in ZR-treated plants and 51% in untreated plants compared with nonstressed control plants grown at 20 °C. During heat stress, ZR-treated plants had significantly higher chlorophyll content than untreated plants.

LEAF PHOTOCHEMICAL EFFICIENCY. Photochemical efficiency (F_v/F_m) decreased significantly with heat stress, beginning at 7 d for untreated plants and 14 d for ZR-treated plants (Fig. 2).

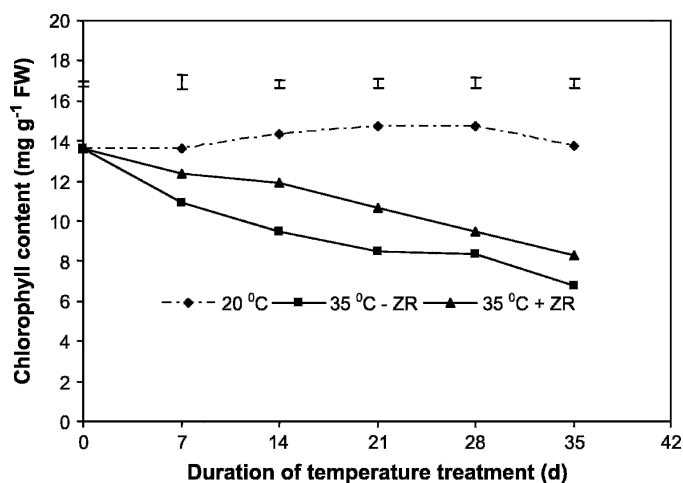


Fig. 1. Effect of zeatin riboside (ZR) on chlorophyll content of creeping bentgrass exposed to heat stress. Vertical bars are LSD values ($P \leq 0.05$) for treatment comparisons at a given day of treatment. FW, fresh weight.

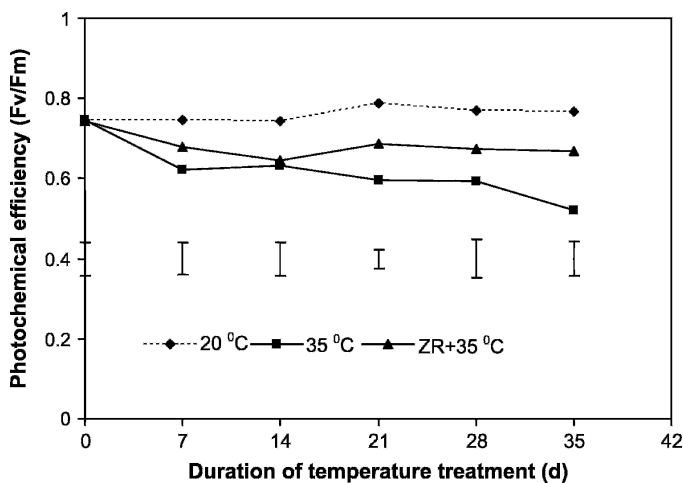


Fig. 2. Effect of zeatin riboside (ZR) on leaf photochemical efficiency of creeping bentgrass exposed to heat stress. Vertical bars are LSD values ($P \leq 0.05$) for treatment comparisons at a given day of treatment.

The decline in F_v/F_m at 7 d was 17% in untreated plants and 9% in plants treated with ZR, compared with nonstressed control plants. By 35 d, the decline in F_v/F_m was 32% in untreated plants and 13% in ZR-treated plants. Zeatin riboside-treated plants maintained a significantly higher F_v/F_m than untreated plants at 21 and 35 d of heat stress.

SOLUBLE PROTEIN CONTENT. Soluble protein content was significantly lower than control plants, beginning at 7 d for untreated plants and 14 d for ZR-treated plants (Fig. 3). Zeatin riboside-treated plants had significantly higher protein content than untreated plants at 21 d of heat stress. The difference in protein content between the treatments diminished at 28 and 35 d.

PROTEASE ACTIVITY. Protease activity of both ZR-treated and untreated plants increased during heat stress compared with nonstressed control plants (Fig. 4). Zeatin riboside-treated plants had significantly lower protease activity than untreated plants at 21 and 35 d of heat stress. Protease activity at 21 d of heat stress was 15% higher in ZR-treated plants and 32% higher in untreated plants compared with nonstressed plants. Toward

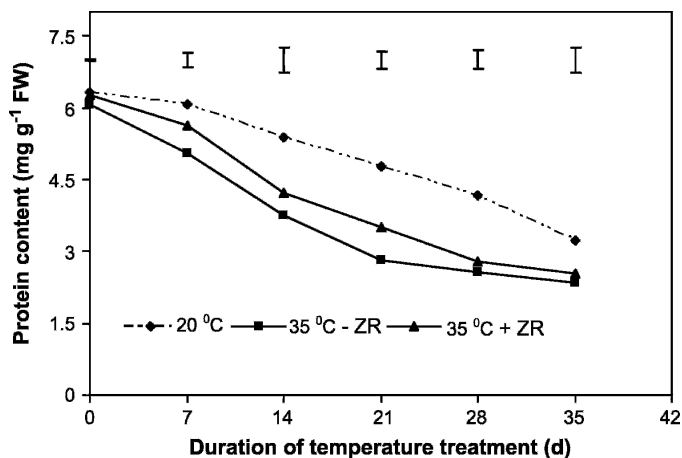


Fig. 3. Effect of zeatin riboside (ZR) on soluble protein content in leaves of creeping bentgrass exposed to heat stress. Vertical bars are LSD values ($P \leq 0.05$) for treatment comparisons at a given day of treatment. FW, fresh weight.

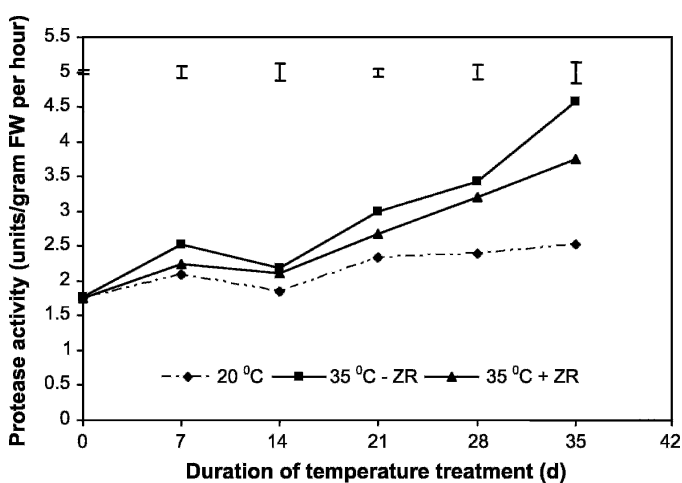


Fig. 4. Effect of zeatin riboside (ZR) on protease activity in leaves of creeping bentgrass exposed to heat stress. Vertical bars are LSD values ($P \leq 0.05$) for treatment comparisons at a given day of treatment. FW, fresh weight.

the end of the treatment period (day 35), protease activity was 81% higher in untreated plants and 48% higher in plants treated with ZR compared with plants grown at 20 °C.

PROTEIN CHANGES. Leaf protein SDS-PAGE showed effects of heat stress and ZR application on protein expression at 21 d (Fig. 5). Heat stress caused degradation of the large subunit of Ribulose-1,5-bisphosphate (RuBP)-carboxylase proteins (55 kDa), and also increased the intensity of small-molecular weight proteins at 23 kDa. Exogenous application of ZR in heat-stressed plants upregulated several proteins, including 23, 32, and 57 kDa. The 57-kDa proteins were present only in ZR-treated plants under heat stress. Zeatin riboside-treated plants had less severe degradation of RuBP-carboxylase proteins (55 kDa) than untreated plants exposed to heat stress.

Discussion

Heat stress at 35 °C induced leaf senescence in creeping bentgrass, as indicated by the decline in chlorophyll content and photochemical efficiency. Results from this study confirmed results from previous studies with creeping bentgrass (Fu and Huang, 2003; Liu and Huang, 2000). Heat-induced senescence has also been reported in other plant species (Crafts-Brandner and Salvucci, 2002; Yamasaki et al., 2002). Foliar application of the synthetic cytokinin ZR helped maintain higher chlorophyll concentration and photochemical efficiency for a longer duration of heat stress, suggesting that cytokinin delayed and suppressed leaf senescence induced by heat stress. Exogenous application of ZR into the root zone also suppressed the decrease in chlorophyll content and photochemical efficiency when roots were exposed to high soil temperatures (Liu and Huang, 2000). The effects of ZR on leaf senescence through foliar or root-zone application may be a result of increased chlorophyll synthesis, reduced chlorophyll degradation, or a

combination of both these factors (Fletcher et al., 1973; Genkov et al., 1997).

The alleviation of heat-induced senescence by cytokinins may be associated with alteration in protein degradation. It has been reported that chlorophyll loss is linked to protein degradation (Hashimoto et al., 1989). Our results showed that total soluble protein content was greatly reduced by heat stress. This is consistent with earlier reports that showed a decline in total soluble protein under conditions of heat stress in other plant species (Chaitanya et al., 2001; Key et al., 1981). It has been suggested that the progressive decline in protein content of leaves may be the result of a general decrease in synthesis or the result of enhanced proteolytic degradation (Hieng et al., 2004; Zavaleta-Mancera et al., 1999). Decrease in protein content was accompanied by increased activity of serine proteases in senescent leaves of *Phaseolus vulgaris* L. (Huffaker, 1990). In our experiment, heat-stressed plants treated with ZR maintained higher total soluble protein content than untreated plants. Heat-stressed plants also showed higher levels of protease activity, and treatment with ZR resulted in lower protease activity. These results suggested that ZR may have delayed or suppressed heat-induced leaf senescence of creeping bentgrass by inhibiting proteolytic activities. Cytokinins may delay senescence by reducing the mRNA and protein levels of proteases (Jiang et al., 1999; Li et al., 2000). Cytokinins may also inhibit the activation of proteases during senescence. It has been reported that treatment with protease inhibitors resulted in delayed senescence in other plant species (Park and Doorn, 2005; Wang et al., 2004). The expression of most senescence-associated genes was repressed by cytokinins (Hajouj et al., 2000; Weaver et al., 1998).

Tolerance to heat stress involves mechanisms that protect biomembranes, organelles, and molecules that enable them to maintain their functions, and HSPs play an important role in these mechanisms (Vishwanathan and Khanna-Chopra, 1996). Our study showed the synthesis of small HSPs of 23 and 32 kDa was upregulated under heat stress, particularly when plants were treated with ZR, which may contribute to heat stress adaptation. The accumulation of 23-kDa proteins has also been reported in tomato (*Lycopersicon esculentum* L.) plants exposed to heat stress (Liu and Shono, 1999). The synthesis of 32-kDa D1 protein was observed in *P. vulgaris* leaves during senescence (Roberts et al., 1987), which may be a result of preferential sequestration of ribosomes by psb A transcript (Droillard et al., 1992). Both 23- and 32-kDa proteins are located in PSII, and are involved in the light-driven oxidation of water in photosynthesis (Seidler, 1996). Our results suggest that the upregulation of 23- and 32-kDa proteins in PSII by application of ZR could contribute to increased photochemical efficiency, as indicated by a higher F_v/F_m ratio in ZR-treated than untreated plants. It has been previously reported that increases in synthesis of small HSPs is positively correlated with whole-plant tolerance to heat stress (Park et al., 1996). Heat-stressed plants treated with ZR showed the accumulation of 57-kDa proteins. The accumulation of a 57-kDa protein, which is considered to be a subunit of 130-kDa cytokinin-binding protein complex (CBP 130) has been reported in the leaves of *N. sylvestris* Speg. and Comes (Mitsui et al., 1993). It is possible that these ZR-induced polypeptides may contribute toward thermotolerance in plants treated with ZR.

In conclusion, foliar application of cytokinin ZR delayed heat-induced leaf senescence or promoted heat tolerance of

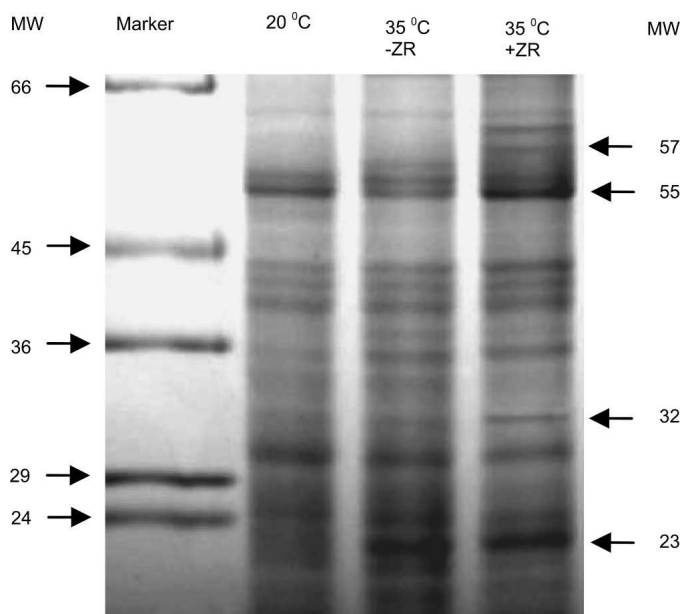


Fig. 5. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis profile of soluble protein for creeping bentgrass exposed to heat stress for 21 d and treated with zeatin riboside (ZR). Equal amounts of protein were loaded in each lane. MW, molecular weight in kilodaltons.

creeping bentgrass, as manifested by maintaining higher chlorophyll content and photochemical efficiency. Cytokinins may have helped to keep plants from heat stress injury by induction of HSPs that protect cellular functions or slow the action of proteases that cause breakdown of proteins. Any means that promote cytokinin synthesis would be beneficial for maintaining stay-green leaves and improving heat tolerance of cool-season turfgrass.

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