PHYSIOLOGICAL, BIOCHEMICAL AND MOLECULAR FACTORS ASSOCIATED WITH HEAT TOLERANCE IN BENTGRASS (*AGROSTIS* SPP.)

by

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ABSTRACT OF THE DISSERTATION

Physiological, Biochemical and Molecular Factors Associated with Heat Tolerance in Bentgrass (*Agrostis* spp.)

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High temperature is a major factor limiting the growth of cool-season plant species during summer. Understanding mechanisms of plant tolerance to high temperature would help develop effective management practices and heat-tolerant cultivars through breeding or biotechnology. This dissertation research explored physiological, biochemical and molecular mechanisms for improving heat tolerance in two bentgrass species, creeping bentgrass (*Agrostis stolonifera* L.), a widely used cool-season grass species on golf course tees and putting greens, and thermal rough bentgrass (*Agrostis scabra* Willd.) adapted to geothermal areas in Yellowstone National Park.

The dissertation reports research in three main components. The first section compared differential heat-induced metabolism of hormones, proteins and metabolites between heat-sensitive creeping bentgrass and heat-tolerant *A. scabra*. Based on the findings that heat tolerance of bentgrass was associated with changes in stress-related hormone levels, the effects of foliar-applied hormone or hormone inhibitors on creeping

bentgrass to enhance heat tolerance were further studied. Results from both growth chamber and field studies confirmed the effectiveness of applying hormones or hormone-based plant growth regulators on alleviating heat injuries in creeping bentgrass. In the last part of the dissertation, a few transgenic creeping bentgrass lines with improved heat tolerance were characterized. These transgenic lines carry a gene (*ipt*) controlling cytokinin synthesis. Increased *ipt* gene expression and cytokinin levels were confirmed and changes in morphological and physiological traits of the plants were examined. Genome-wide protein responses to the addition of the gene and their association with heat tolerance were discussed. The results indicated that transformation with the *ipt* gene induced protein changes involved in multiple functional groups, mainly in energy, protein destination and storage, and disease/defense categories in both leaves and roots of creeping bentgrass, thus cytokinins may have regulatory roles in multiple metabolic pathways for heat tolerance. Taken together, these studies suggest summer performance of creeping bentgrass may be improved by properly applying hormone-based plant growth regulators or biostimulants, and incorporating molecular markers developed from heat- and/or hormone-responsive proteins and metabolites may facilitate selection of heat-tolerant creeping bentgrass cultivars.

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LITERATURE REVIEW

INTRODUCTION

Temperature rises beyond a threshold may cause irreversible damages to plant function and development or alteration of metabolism, resulting in reduction in growth and yield production (Porter, 2005). The temperature threshold is often defined as a value of daily mean temperature at which a detectable reduction in plant growth begins, usually 5-10 ^{o}C above ambient. According to a report of the EPA Global Warming site (2002), the mean global temperature will rise 1-4.5 ^{o}C in the next 50 years. Predicted global warming has become a serious threat for sustainable agriculture worldwide and imposed an increasing challenge to improve plant tolerance to temperature elevation.

The extent to which heat stress causes damage on plants is a complex issue. It depends on the intensity, duration and rate of increase in temperature, as well as other environmental conditions, such as when the high temperature occurs (during the day or the night) and where it occurs (in the air or the soil) (Sung et al., 2003; Weis and Berry, 1988). The destructive effects of heat stress on plant growth may directly result from increasing temperatures in tissues and organs, or indirectly from water deficits due to high evapotranspiration rates that increases exponentially with temperature elevation (Hall, 2001). Genetic variations exist among plant species and cultivars in their sensitivity to high temperatures. Based on the ranges of temperature and precipitation that plants adapt to, they are classified into warm-season and cool-season categories. Warm-season plants require higher temperature for germination and emergence. Cool-season

plants can tolerate light frost but perform poorly when growing in hot environments for extended periods.

Turfgrasses are plants that form a continuous ground cover that persists under regular mowing and traffic (Turgeon, 1999). Among the grass family Poaceae that is comprised of nearly 10000 species, less than 40 species are currently used as turfgrass. Millions of acres of turfgrasses function on home lawns, commercial landscapes, roadsides, parks, athletic fields and golf courses. According to the data of NTRI (2003), the U.S. turfgrass industry is a $40 billion annual business, which becomes part of the backbone of sustainable agriculture and contributes extensively to the world economy (Moser and Hoveland, 1996; Wang et al., 2001). There are five zones of turfgrass adaptation in the US, which are cool-humid, cool-arid, warm-humid, warm-arid and the transition zones. Cool-season turf species are typically grown in the cool-humid and cool-arid zones, and warm-season turf species are best adapted to the warm-arid and warm-humid regions. Additionally, cool-season and warm-season turf species have distinctive photosynthetic pathways, referred as C_3 and C_4 pathways, respectively. As C_3 photosynthesis is sensitive to temperatures above 30 ^{o}C and C_4 photosynthesis maximizes at higher temperatures (30-35 ^{o}C), the growth of warm-season turfgrasses thrives in summer while cool-season species shows a bimodal growth pattern. Specifically, the shoot growth of cool-season species reaches to its maximum in spring, slows or stops in summer, then resumes in fall, with an optimum temperature between 18 and 24 ^{o}C. The maximum root growth of cool-season species occurs at lower temperatures (10 and 18 ^{o}C), with slow and shallow rooting in summer. Summer decline in the quality and growth of cool-season species has become a major concern in turfgrass management, especially

when attempts are made to extend use of cool-season species into the transitional and warm climatic regions (Carrow, 1996; Fry and Huang, 2004).

Turfgrass may survive heat stress through heat avoidance or heat tolerance mechanisms (Levitt, 1972). Heat avoidance is the ability of plants to maintain internal temperatures below lethal stress levels, including transpirational cooling, leaf orientation, reflection of solar radiation, leaf shading of tissues that are sensitive to sunburn, and extensive rooting. The association of maintained transpirational cooling and root extension with better summer stress performance of major cool-season turfgrass species such as Kentucky bluegrass (*Poa pratensis*) has been reported (Bonos and Murphy, 1999; Lehman and Engelke, 1993). However, heat-avoiding turfgrass cultivars that thrive in the low humidity may lack heat resistance in humid areas due to reduced cooling effects of transpiration (Beard, 1997). Heat tolerance is the ability of plants to survive high internal tissue temperatures, and heat-tolerant cultivars can be resistant in both humid and arid conditions.

Conventional breeding contributed substantially to the genetic improvement of turfgrasses in the last century (Humphreys, 1999). In most cases, germplasm screening for heat tolerance relies on field and whole-plant techniques, which are less efficient and sensitive due to environmental interactions (Marcum, 1998). Continuous efforts have been devoted to developing rapid and accurate procedures that allow simultaneous screening of large numbers of genotypes in order to breed heat-tolerant C_3 turfgrasses for use in hot and humid areas (Wehner and Watschke, 1981). The recent progress in genetic manipulation of plants open up opportunities for incorporating cellular and molecular techniques into turfgrass improvement (Duncan and Carrow, 1999). Particularly, the

technology exists to make pinpoint genetic changes to turfgrasses using marker-assisted selection or transgenic technology (Sticklen and Kenna, 1998). Therefore, a more complete and through understanding of heat injury and mechanisms for heat tolerance in turfgrasses at the whole-plant, cellular and molecular levels is needed, which will facilitate the improvement of heat tolerance in cool-season turfgrass species utilizing molecular breeding or biotechnology.

MECHANISMS FOR HEAT TOLERANCE

Plant adaptation to heat stress involves mechanisms at various levels, including changes in molecular, cellular, biochemical, physiological, and growth responses (Sung et al., 2003; Wahid et al., 2007). This section will focus on the discussion of some major heat tolerance mechanisms, including photosynthesis, carbon and nitrogen metabolism, membrane thermostability, antioxidant response, metabolite production, protein alteration and gene expression, as well as hormone regulation.

Photosynthesis

Photosynthesis is among the most sensitive processes to elevated temperatures. Alterations in various photosynthetic attributes under heat stress are often used as indicators of thermotolerance of the plant as any constraint in photosynthesis can limit plant growth under high temperature (Wahid et al., 2007).

The sensitivity of C_3 photosynthesis to heat is primarily due to the damage to components of photosystem (PS) II located in the thylakoid membranes of the chloroplast (Al-Khatib and Paulsen, 1999; Thebud and Santarius, 1982). PS II may lose its capacity of oxygen evolution under heat stress, leading to restricted electron transport and limited generation of reducing powers for metabolic functions (Albertini et al., 2005; Toth et al., 2005). Thylakoids harbor chlorophyll, a pigment that absorbs light energy to drive photosynthesis. Heat-induced damage of thylakoids and photosynthetic function is characterized by loss of chlorophyll, known as leaf senescence (John et al., 1995; Rajcan et al., 1999; Weis and Berry, 1988). Chlorophyll fluorescence yield reflects the homestate of thylakoid membrane, which is widely used as a sensitive indicator for heat-induced inhibition of photosynthesis (Bolhar-Nordenkampf et al., 1989; Schreiber and Berry,

1977). Reduction in net photosynthetic rate as well as chlorophyll content and fluorescence responding to supraoptimal temperatures has been shown in numerous C_3 turfgrasses, including members of bluegrass (*Poa* spp.), ryegrass (*Lolium* spp.), fescue (*Festuca* spp.) and bentgrass (*Agrostis* spp.) (Huang et al., 1998; Jiang and Huang, 2001; Wehner and Watschke, 1981; Xu and Huang, 2001). The ability of a plant to retain chlorophyll under stress, so called "stay-green", is a favorable trait the breeders pursue (Thomas and Howarth, 2000). Introduction of this character from the pasture grass meadow fescue (*Festuca pratensis*) to annual ryegrass (*Lolium multiflorum*) has been performed, resulting in indefinite greenness on senescing leaves of the plants (Thomas et al., 1999).

Inhibition of C_3 photosynthesis is closely related to the inactivation of ribulose-1, 5-bisphosphate carboxylase/oxygenase (Rubisco) (Kobza and Edwards, 1987). Although the catalytic activity of Rubisco increases with temperature, the low affinity of the enzyme for CO_2 and its dual nature as an oxygenase at high temperature limit the possible rise in C_3 net photosynthetic rate (Salvucci and Crafts-Brandner, 2004). The reduced affinity of Rubisco for CO_2 in C_3 grasses as temperature increases has been attributed to reduced amount of large and small subunits of Rubisco and Rubisco binding proteins, as well as inhibition of Rubisco activase that is responsible for Rubisco regeneration (Kepova et al., 2005; Salvucci and Crafts-Brandner, 2004).

The ability to sustain leaf gas exchange under heat stress also has a direct relationship with heat stress (Wahid et al., 2007). Decreases in C_3 photosynthetic capacity may also be partially dependent on temperature-induced irreversible reductions in the quantum yield for CO_2 uptake (Monson et al., 1982). Wherley and Sinclair (2009)

recently reported that the commonly observed decline in growth of C_3 grasses such as creeping bentgrass (*Agrostis stolonifera*) and perennial ryegrass (*Lolium perenne*) at elevated temperature may include a sensitivity to elevated vapor pressure deficit confounded with elevated temperature, resulting in restricted transpiration rate and limited leaf CO_2 uptake. The dissipation of excess absorbed light energy or nonphotochemical quenching of chlorophyll fluorescence under stress is critical to the prevention of photooxidative damage of the photosynthetic apparatus (Monson et al., 1982; Niyogi et al., 1998).

Carbon and Nitrogen Metabolism

High temperature affects carbohydrate metabolism, including its accumulation, allocation and utilization. The major source of carbohydrate is photosynthesis, which is inhibited by heat stress as previously discussed. The reduction in source activities can be reflected by heat-induced down-regulation of Rubisco (Weis and Berry, 1988). Leaf concentrations of 3-phosphoglycerate, fructose-6-phosphate, and glucose-6-phosphate all declined whereas RuBP content increased in C_3 grass species subjected to heat stress, indicating an inhibition of metabolite flow through Rubisco (Kobza and Edwards, 1987; Law and Crafts-Brandner, 1999). Meanwhile, as a major sink for carbohydrates, respiration increases with temperatures in most plant species (Lambers, 1985), including C_3 grasses such as oat (*Avena sativa*) (Tetley and Thimann, 1974) and sandberg bluegrass (*Poa secunda*) (Monaco et al., 2005). Increased carbon consumption in accordance with reduced carbon production may cause low to moderate carbon depletion under heat stress (Dinar et al., 1983). Dramatic increase in the daily carbon consumption to production

ratio was observed in creeping bentgrass subjected to heat stress, whose respiration rate exceeded its photosynthesis rate when temperature was elevated to 34 or 38 °C (Huang and Gao, 2000). The greater consumption of carbon in the sink under heat stress requires higher carbon allocation efficiency from source to sink (Tahir and Nakata, 2005). Carbohydrate allocation is fundamentally important for plant root growth and function, because roots are completely dependent on carbon assimilates supplied by leaves (Lambers et al., 1999). The disturbance of carbohydrate metabolism in roots has been suggested as a primary factor responsible for growth inhibition and dysfunctions of roots grown at high soil temperature (Du and Tachibana, 1994). A heat-tolerant cultivar of creeping bentgrass was found to maintain higher carbon allocation rate from shoots to roots during summer months compared to a heat-sensitive cultivar (Xu and Huang, 2006).

Nitrogen is the most commonly used mineral nutrient, which plays a pivotal role in many critical functions in the plant. It serves as an alternative source of assimilates and its economy in plants closely relates to leaf senescence trigged by various abiotic stresses, including heat. Heat stress reduced the remobilization efficiency of nitrogen, as reported by Tahir and Nakata (2005) that there exists a positive correlation between the rate of chlorophyll loss from the flag leaf of winter wheat and nitrogen remobilization efficiency under heat stress. Retention of green leaf area under heat stress is a favorable trait for turfgrass as that increases the aesthetic value of the plant. At the whole plant level, stay-green can be viewed as a consequence of the balance between nitrogen demand and supply (Borrell et al., 2001). Decreased total nitrogen concentration and accumulation of nitrate that indicates an imbalance between net absorption and assimilation rates under heat stress was reported in tall fescue (Cui et al., 2006). Xu and Huang (2006) reported

that a heat-tolerant cultivar of creeping bentgrass maintained higher nitrogen uptake rate and nitrate reductase activity in roots. Fu and Huang (2003) found foliar application of NH_4NO_3 alleviated heat injury and delayled leaf senescence in creeping bentgrass. The delayed leaf senescence resulting from the increase in leaf nitrogen content may allow carbon and nitrogen to be allocated to roots and maintain a greater capacity to extract nitrogen from the soil (Borrell et al., 2001).

Membrane Thermostability

Cellular membranes are composed of two layers of phospholipids consisting of unsaturated and saturated fatty acids. High temperature accelerates the kinetic energy and movement of molecules composing membrane and loosens chemical bonds within the molecules, thus causing reduction in saturation level of fatty acids (Savehenko et al., 2002). Such alterations increase the fluidity of the lipid bilayer and the permeability of membranes, as evidenced by increased electrolyte leakage and lipid peroxidation level (Blum and Ebercon, 1981; Suss and Yordanov, 1986).

Cell membranes are the site for many biological activities of the plant. Cellular membrane disruption under heat stress can impact photosynthetic and respiratory activity, and decrease the ability of the plasmalemma to retain solutes (Lin et al., 1985). Kim and Portis (2005) found transgenic arabidopsis plants having less polyunsaturation of thylakoid lipids exhibited better membrane integrity under heat stress. Thebud and Santarius (1982) reported photosynthesis was inactivated at temperatures far below those causing dramatic changes in the integrity of the tonoplast and plasmalemma.

Assessing cellular membrane thermalstability (CMT) has become a wildly used technique in breeding programs for evaluating genotypic differences in heat tolerance of various plant species, including C_3 grasses such as wheat (*Triticum aestivum*) (Blum and Ebercon, 1981; Fokar et al., 1998; Ibrahim and Quick, 2001; Saadalla et al., 1990), whose grain yield and quality are positively correlated to its CMT under high temperature. Using CMT to predict whole-plant heat tolerance among turfgrass cultivars was first reported by Marcum (1998). They detected a negative correlation between CMT and leaf firing rate ($r = –0.80$) as well as a positive correlation between CMT and shoot dry weight ($r = 0.75$) of Kentucky bluegrass subjected to heat stress.

Antioxidant Response

The production and consumption of energy is often unbalanced under heat stress due to more severe delay in the dark reactions of photosynthesis than the absorption of light and the transport of electrons (Weis and Berry, 1988). The electron flow driven by PS I was stimulated in contrast to sharp inhibition of that mediated by PS II (Dash and Mohanty, 2002). Reactive oxygen species (ROS) are produced in response to heat-induced oxidative stress, including radical (O^{2-}), hydrogen peroxide (H_2O_2), hydroxyl free radical (OH), and singlet oxygen ($O^1{}_2$). The rates and cellular sites of their production during temperature stress could play a central role in stress perception and protection (Dash and Mohanty, 2002; Suzuki and Mittler, 2006). However, they are highly cytotoxic and can react with unsaturated fatty acids to cause peroxidation of essential membrane lipids in plasmalemma and intercellular organelles.

Plant cells develop scavenging mechanisms to protect cells from the attack of ROS through a complex antioxidant system, an important contributor to the survival of a plant during heat stress. Plants possess non-enzymic and enzymic antioxidants. Antioxidant enzymes include superoxide dismutase (SOD), catalase (CAT), peroxidase (POX or POD), ascorbate peroxidase (APX), and glutathione reductase (GR). Heat-induced oxidative stress has been reported in creeping bentgrass (Liu and Huang, 2000), tall fescue (Cui et al., 2005) and Kentucky bluegrass (Jiang and Huang, 2001). Liu and Huang (2000) found the activities of SOD and CAT decreased and POD activity increased in both leaves and roots of creeping bentgrass during heat stress, leading to increased lipid peroxidation level and leaf senescence. Jiang and Huang (2001) reported heat stress reduced the activities of APX, CAT and GR in tall fescue and Kentucky bluegrass. Cultivar variations in antioxidant enzyme activities were associated with the differences in heat tolerance of turfgrasses and indicated a positive correlation between chlorophyll content and the antioxidant enzymes, and a negative correlation between membrane injury index and the antioxidant enzymes (Almeselmani et al., 2006; Cui et al., 2005; Huang et al., 2001). Metabolites like glutathione, ascorbic acid, α-tocopherol and carotenoids can function as non-enzymic antioxidants (Almeselmani et al., 2006). Increased levels of foliar ascorbate and glutathione were reported in wheat seedlings in response to heat stress (Dash and Mohanty, 2002).

Metabolite Production

Metabolites are the intermediates and products of metabolism, which may be involved in plant adaptation to heat stress through various functions. Except for those

mentioned in the previous section that function as antioxidants, metabolites may act as signaling and regulatory agents, and compatible solutes.

A well-known group of metabolites having hormone-like functions as primary messengers in signal transduction are sugars, in addition to their essential roles as substrates in carbon and energy metabolism (Koch, 1996; Rolland et al., 2002). Hexoses and sucrose have been recognized as important signal molecules in source-sink regulation, and their signaling pathways may integrate with those responding to plant hormones, phosphate, light, and stress-related stimuli (Roitsch, 1999). After studying the effects of the metabolic regulator D-glucose and different stress-related stimuli on photosynthesis, sink metabolism, and defense response, Ehness et al. (1997) suggested that carbohydrate signals and stress-related stimuli independently activate different intracellular signaling pathways that ultimately are integrated to coordinately regulate source and sink metabolism and activate defense responses.

Organic acids are low molecular weight compounds found in all organisms and characterized by the possession of one or more carboxyl groups. Some of the organic acids are involved in energy production as intermediates in the tricarboxylic acid cycle (ex. citrate, aconitic acid), and others are primarily present in cells for cation charge balancing or for maintaining osmotic potential (ex. malate, malonate, oxalate) (Jones, 1998). Some of them were found to play an important role in plant responses to abiotic stresses such as nutrient deficiency (ex. citrate, malate) (Jones and Darrah, 1995) and anaerobic stress (ex. lactic acid) (Xia and Roberts, 1994).

Another group of essential metabolites is the amino acids. In addition to their primary roles as intermediates in the synthesis and catabolism of proteins, many of them

may function as defensive agents against abiotic stresses. Good and Zaplachinski (1994) detected linear increases in amino acid levels with the induction of drought stress in rapeseed (*Brassica napus*) leaves. Fougere et al. (1991) reported that salt stress induced great increases in proline and asparagine levels in alfalfa (*Medicago sativa*). Sharma and Dietz (2006) found that cysteine is a central metabolite in antioxidant defence and metal sequestration under heavy metal stress.

Particularly, low-molecular-weight compatible solutes from different major metabolite groups could accumulate under heat stress, including amino acids (ex. proline), polyamines (ex. putrescine), carbohydrates (ex. sucrose), and sugar alcohols (ex. mannitol), as heat stresses may eventually cause cellular dehydration in plants (Rajam et al., 1998; Rizhsky et al., 2004). Wahid (2007) detected immediate reduction in leaf water potential of sugarcane (*Saccharum officinarum*) responding to heat stress; however, the effect was offset by early synthesis of free proline, glycinebetaine and soluble sugars, indicating crucial roles of these metabolites in improving net assimilation and heat tolerance. Rizhsky et al. (2004) found that sucrose replaced proline as the major osmoprotectant in arabidopsis (*Arabidopsis thaliana*) during combined drought and heat stress. Kaplan et al. (2004) highlighted the action of multiple compatible solutes that exert additive or synergistic effects during the induction of thermotolerance.

Protein Alteration and Gene Expression

Heat stress decreases protein accumulation and alters protein composition (Monjardino et al., 2005; Thomas et al., 2002). Maintenance of higher protein content and less severe protein degradation during prolonged periods of heat stress were related to

thermotolerance (He and Huang, 2007). Moderate heat response involves proteins functioning in lipid biogenesis, cytoskeleton structure, sulfate assimilation, amino acid biosynthesis and nuclear transport (Ferreira et al., 2006), as well as proteins that correspond to antioxidant enzymes (Kepova et al., 2005).

Studies with a number of higher plants indicate that, while synthesis of most normal proteins and mRNAs is inhibited, the transcription and translation of a small set of proteins, called heat shock proteins (HSPs), may be induced or enhanced when plants are exposed to elevated temperatures (Vierling, 1991; Viswanathan and KhannaChopra, 1996). HSPs are generally classified into two groups: the high-molecular-mass HSPs (60-110 kD) and low-molecular-mass HSPs (15-30 kD), which are also called small HSPs (sHSPs). Most, but not all, heat shock proteins are molecular chaperones, which bind and stabilize proteins at intermediate stages of folding, assembly, degradation, and translocation across membranes. Chemical agents such as amino acid analogues, arsenite, cadmium, and ethanol could induce HSP synthesis in the absence of heat stress, and subsequently induce heat tolerance (Belanger et al., 1986). He and Huang (2007) reported better heat tolerance in the Kentucky bluegrass was associated with induction of HSPs in the cytoplasm and membranes during the early phase of heat stress. Park et al. (1996) detected heat-induced synthesis of HSPs in creeping bentgrass and found heat tolerance of the grass is linked to the presence of a few additional HSP25 polypeptides. Induction of certain HSPs during heat acclimation was also related to enhanced thermotolerance in creeping bentgrass (He et al., 2005).

In addition to the induction of heat stress-related protein, induced expression of dehydration stress-related proteins were also found under high temperature, due to the

fact that heat stress may cause cellular dehydration in plants as previously mentioned. For instance, a dynamic change in dehydrin homolog proteins was reported in both warm-season turf species such as bermudagrass (*Cynodon* spp.), and cool-season turf species such as perennial ryegrass and bentgrass cultivars (Zhou and Abaraha, 2007).

The expression of many genes with regulatory functions is altered in response to heat stress, including transcription factors, key enzymes such as kinases and phosphatases, and other proteins involved in protein protection and degradation. Many responses are common to different types of abiotic stresses so stress-signaling pathways are often targeted to a common set of genes. They may be not only involved in regulating downstream stress responses, but also in stress perception and signaling (Kant et al., 2008). Hays et al. (2004) identified a few quantitative trait loci (QTLs) regulating heat tolerance in wheat, which were associated with optimal photosynthesis/stomatal conductance, suppression of signaling molecules that promote leaf senescence, and prevention of seed abortion. Ottaviano et al. (1991) found six QTLs accounting for 53% of the genetic variability for inherent cellular membrane thermostability in maize (*Zea mays*). Zhang et al. (2005) detected higher expression levels of the genes involved in cell maintenance, chloroplast association, photosynthesis, protein synthesis, stress signaling, and transcriptional regulation in the heat-tolerant genotype of fescue under heat-stress conditions, while the genes related to metabolism and stress had higher expression in the heat-sensitive genotype. Xu et al. (2008) also detected up-regulated genes in bentgrass species involved in stress defense pathways or tolerance mechanisms, such as cell wall elasticity, secondary metabolism, regulatory functions, and protein synthesis.

Hormone Regulation

Extensive research has demonstrated that plant growth and development, as well as adaptation to environmental stress, are regulated by some endogenous or natural signaling molecules or chemicals that are produced at specific tissues or organs of a plant, and are present in extremely low concentrations in the plant (Nemhauser et al., 2006). These chemicals are collectively called the plant hormones or phytohormones, and are also considered plant growth regulators. It is generally accepted that there are five major classes of plant hormones, including cytokinins (CKs), abscisic acid (ABA), gibberellins or gibberellic acids (GAs), auxin or indoleacetic acid (IAA), and ethylene. Several other plant growth regulators that exist naturally, but are not grouped into the five major hormone classes, include jasmonic acid (JA), salicylic acid (SA), brassinosteroids (BRs), polyamines, and nitric oxide.

Exploring mechanisms underlying hormonal regulation of plant stress tolerance has been a major subject in plant stress research. Historically, the effects of a hormone have been defined largely by exogenous application of the hormone. More recently, the isolation of hormone biosynthetic and response mutants has provided new powerful tools for identifying the mechanisms of various hormones in plant growth and development (Gray, 2004). A single hormone can regulate diverse cellular and developmental processes, whereas a single process or mechanism is often influenced by multiple hormones. Each hormone has their unique functions, but in most cases, it may interact with other hormones in regulating the same physiological events or processes, indicating a complex interaction between plant hormones and physiological responses (Gray, 2004). Hormonal interaction can also be reflected by the fact that the production of one hormone

may enhance or inhibit the production of another hormone. Therefore, the output of hormone action often depends on the interactive, synergistic, or antagonistic effects of different hormones rather than the independent activities of each one (Weiss and Ori, 2007).

This section will introduce and discuss the mechanisms of three major hormones that may be involved in the regulation of plant tolerance to heat stress, including CKs, ethylene, and GAs. For an orderly discussion, the following section is organized to discuss each class of hormones and the mechanisms involved in mediating plant tolerance to heat stress.

Cytokinins (CKs)

CKs are known for their regulation of various processes in plant growth and development such as cell division, cell growth and differentiation (Taiz and Zeiger, 2006). The endogenous content of CKs decreases in natural senescing tissues or senescence induced by environmental stresses. The decline in CKs content could be due to a decrease in biosynthesis or an increase in catabolism (Nooden and Leopold, 1988). Additionally, stresses could also inhibit CK transport from roots to shoots and the reduced supply may lead to shoot senescence, as CKs are primarily produced in roots. A study imposed in wheat reported the content of CKs decreased in shoots and accumulated in roots simultaneously as a result of temperature increase (Farkhutdinov et al., 1997), which indirectly supported this perspective.

Changes in endogenous content of CKs may regulate plant tolerance to environmental stress. Two main approaches have proved effective for altering CKs

synthesis or production. One approach is the exogenous application of CK solutions to the plant itself or soil where it grows. Using this approach, Adedipe et al. (1971) found that applying benzyladenine (BA), a synthetic substance that is similar to CK in structure and function, to the leaves of bean (*Phaseolus vulgaris*) helped maintain high chlorophyll content thus delaying leaf senescence. Similarly, Liu et al. (2002) reported that applications of 1 and 10 mM zeatin riboside (ZR), a major form of CKs, to the root zone of creeping bentgrass increased endogenous CK content and mitigated heat stress injury in both shoots and roots, and the effects were concentration-dependent. However, the efficacy of exogenous applications is limited, because it is difficult to control the absorption and transport of CKs in the plants (Clark et al., 2004; Khodakovskaya et al., 2006). Therefore, the second method, incorporation of transgenes affecting endogenous hormone production in plants through the use of biotechnology has attracted more attention recently. An *ipt* gene that codes for isopentenyltransferase, a key enzyme in CK biosynthesis pathway, has been incorporated into various plant species to modify CK production, such as annual wormwood (*Artemisia annua*) (Geng et al., 2001), sugarbeet (*Beta vulgaris*) (Ivic et al., 2001) and arabidopsis (Zhang et al., 2000). Attempts have been made to use the *ipt* gene under the control of various stress-activated promoters including those induced by heat, drought and light. Among the most widely-used inducible promoters is *SAG12*, a promoter of senescence-associated gene encoding a cysteine protease isolated and cloned from arabidopsis (Swartzberg et al., 2006). The *SAG12-ipt* construct has three important features: temporal regulation, spatial regulation, and quantitative regulation that can auto-regulate CK synthesis (Gan and Amasino, 1995; Gan and Amasino, 1996). The retention of chlorophyll in *ipt*-transgenic plants has been

reported under various stress conditions, such as dark incubation (Khodakovskaya et al., 2006), waterlogging (Huynh et al., 2005) and freezing stress (Hu et al., 2005). Another direct effect of the *ipt* insertion is a marked accumulation of *ipt* transcripts and CKs. Heat induction of *ipt*-transgenic arabidopsis caused the *ipt* mRNA to accumulate and increased the level of zeatin by 52-fold, ZR by 23-fold and ZR 5'-monophosphate by 2-fold (Medford et al., 1989). Geng et al. (2001) detected a 2 to 3-fold increase of isopentyl-type CKs in *ipt*-transgenic annual wormwood.

Cytokinins promote antioxidant activities or suppress oxidative damage in various plant species. For example, Liu and Huang (2002) reported that injection of CK to the root zone of creeping bentgrass significantly suppressed the decline in SOD and CAT activities, slowed leaf senescence and alleviated heat-induced lipid peroxidation of cell membranes. Incubation of wheat leaves in 10^{-4} M 6-benzylaminopurine (a synthetic CK) increased the activities of CAT and APX and reduced the levels of reactive oxygen species, resulting in delay of dark senescence (Zavaleta-Mancera et al., 2007). Exogenous seaweed extracts and humic acid containing cytokinins had also enhanced endogenous antioxidant levels in Kentucky bluegrass under low soil moisture conditions and might contribute to turfgrass drought tolerance (Zhang and Schmidt, 1999). The heat-shock-inducible *ipt* gene expression in transgenic tobacco plants led to enhanced cytokinin biosynthesis and lower lipid peroxidation compared with controls under non-stressed and copper-stressed conditions (Thomas et al., 2005).

Exogenous application of ZR on protein metabolism associated with heat tolerance has been investigated. Veerasamy et al. (2007) found that treatments with ZR helped maintain higher leaf chlorophyll content and soluble protein content in creeping

bentgrass under heat stress, as well as enhanced expression of 32 and 57-kDa HSPs under heat stress conditions, indicating CKs may have helped alleviate heat injury by induction or up-regulation of HSPs. In addition, ZR-treated plants had less severe degradation of Rubisco and lower protease activity than untreated plants exposed to heat stress.

Ethylene

Ethylene production in plant tissues increases in response to various environmental stresses including heat stress (Borisova et al., 2001; Shirazi et al., 1993). The production of ethylene influences many aspects of plant growth and development, including leaf senescence and abscission, flowering and fruit ripening (Grbic and Bleecker, 1995 165). Enhanced ethylene production has been associated with reduced growth and accelerated processes characteristic of leaf senescence, such as the decline in chlorophyll and increases in activity of hydrolytic enzymes, in response to environmental stresses (Grbic and Bleecker, 1995). It is also supported by the observation that a stress-susceptible winter wheat cultivar produced significantly greater ethylene than a stress-resistant cultivar under high temperature (38ºC) (Balota et al., 2004).

Delayed leaf senescence has been achieved by transgenic approaches that perturb endogenous ethylene-response signal transduction pathways (Suzuki et al., 2005), as observed in ethylene-deficient tomato (*Lycopersicon esculentum*) (John et al., 1995). Similar consequences have been seen with the use of ethylene synthesis or action inhibitors such as aminoethoxyvinylglycine (AVG) and 1-methylcyclopropane (1-MCP) (Hays et al., 2007; Jiao et al., 2006). However, complete blocking of ethylene production may also be detrimental to heat tolerance. Larkindale and Knight (2002) reported that

ethylene was necessary in protection against heat-induced oxidative damage in arabidopsis because ethylene-insensitive mutant *etr-1* showed increased susceptibility to heat.

Ethylene regulation on heat tolerance may not directly relate to HSPs production, as suggested by Larkindale et al. (2005) when they investigated the performance of ethylene signaling mutants (*ein2* and *etr1*) of arabidopsis under heat stress. All mutants showed certain defects in acquired thermotolerance of root growth and seedling survival, but they still accumulated wild-type levels of HSP101 and sHSPs. However, there were other studies indicating their regulatory effects on HSPs. Proteomic analysis showed ethylene precursor aminocyclopropane carboxylic acid (ACC) induced stress-related proteins including HSPs in barrel medic (*Medicago truncatula*) roots during nodulation (Prayitno et al., 2006).

Gibberllic acids (GAs)

GA promotes growth and elongation of cells so it stimulates rapid stem and root growth in plants. Krauss and Marschner (1984) reported that shoots of potato (*Solanum tuberosum*) plants growing under high temperatures showed increased GA levels, which might be involved in the interaction between high soil temperature and the carbohydrate metabolism of the affected tubers. Grindstaff et al. (1996) found the endoplasmic reticulum membranes of GA-treated barley (*Hordeum vulgare*) aleurone layers became less heat-tolerant. Chen et al. (1986) studies the effect of GA on the thermotolerance of etiolated mung bean (*Vigna radiata*) seedlings. They found GA enhanced the heat-shock

effect at non-lethal temperature, which then made the seedlings tolerate to the potentially lethal temperature and improved seedling recovery from heat injury.

Plants treated with GA-biosynthesis inhibitors are shorter and usually more tolerant to a range of environmental stresses (Sarkar et al., 2004; Vettakkorumakankav et al., 1999). There are four different groups of GA-biosynthesis inhibitors. One GA inhibitor, trinexapac-ethyl (TE), belongs to the group of GA-inhibitors having structural similarities with 2-oxoglutaric acid, which is the co-substrate of dioxygenases that catalyze late steps of GA formation (Rademacher, 2000). More specifically, TE blocks the conversion of GA_{20} to GA_1, the final step in GA biosynthesis pathway (Adams et al., 1992; King et al., 1997). One main application of TE is growth control in turfgrasses, such as clipping reduction, seedhead suppression of annual bluegrass (*Poa annua*) and improvement of overall turf quality in various turfgrass species (Borger, 2008; Fagerness and Yelverton, 2001; Lickfeldt et al., 2001).

TE has been used for improving turfgrass tolerance to abiotic stresses such as shade (Ervin et al., 2004; Goss et al., 2002), freezing (Fagerness et al., 2002), and combined drought and heat stress (McCann and Huang, 2007). The modulation of GA levels results in a sequence of events that subsequently lead to stress tolerance, including inhibition of cell expansion in sheaths and basal regions of leaves, altered root to shoot ratio, and increased levels of photosynthetic pigments and antioxidant enzymes (Fletcher and Gilley, 2000; Kaufmann, 1986). The effects of TE on promoting chlorophyll content and shoot density were reported in perennial ryegrass (Ervin and Koski, 1998), Kentucky bluegass (Ervin and Koski, 2001; Stier and Rogers, 2001), and creeping bentgrass (Fagerness and Yelverton, 2001). It was also reported that TE affected photoassimilate

partitioning to adjacent tillers and total non-structural carbohydrate accumulation (Han et al., 1998).

PLANT MATERIALS

Creeping Bentgrass

Creeping bentgrass (*Agrostis stolonifera*) is a cool-season grass species native to Northern Europe and parts of the Mediterranean region. It is best known for its fine texture and tolerance to low mowing heights, which makes it well suited for use on high quality golf course tees, greens, and fairways (Warnke, 2002).

The landmark cultivar of the species is Penncoss, which was released by Dr. H. B. Musser from the Pennsylvania State University in 1955. Since then, breeding efforts have focused on increasing turf quality and widening the adaptability of creeping bentgrass (Warnke, 2002). The use of creeping bentgrass in warmer climatic zones is increasing due to the higher quality of putting surface it forms than bermudagrass. Turf researchers have tried to develop better cultivars that can tolerate high ambient and soil temperatures in order to expanding the geographical range of its use. Major improvement of the species was achieved in the 1990s for the release of new heat-tolerant cultivars such as Cato, Crenshaw, SR1020 and L93 (Engelke, 1998).

Proper management of creeping bentgrass tolerance to heat stress requires a basic understanding of the plant response to high temperature. Genetic variations in heat tolerance among creeping bentgrass cultivars in shoot physiological responses to high temperature may relate to different levels of reduction in net photosynthesis and elevation in respiration rates (Huang et al., 1998). Summer root decline in creeping bentgrass was associated with decreased new root production and increased root mortality, so that the differential root production and mortality among cultivars may contribute to the

variations in their aboveground performance during summer (Huang and Liu, 2003). However, a much more through understanding of the genetic mechanisms behind selection criteria for creeping bentgrass tolerance to heat stress is required for use of such genetic-based approaches for marker-assisted selection in future genetic improvement of the species.

Genetic transformation of favorable genes into creeping bentgrass can provide useful genetic variation for cultivar improvement. Transgenic creeping bentgrass has been generated by microprojectile bombardment (Xiao and Ha, 1997; Zhong et al., 1993), or agrobacterium (*Agrobacterium tumefaciens*)-mediated transformation (Aswath et al., 2005; Luo et al., 2004). Foreign genes used for transformation experiments to introduce biotic or abiotic stress tolerance into creeping bentgrass include *PR5K* for dollar spot resistance (Guo et al., 2003), *VuNCED1* for NaCl and drought tolerance (Aswath et al., 2005), *bar* and *cp4* for herbicide resistance (Asano et al., 1998; Chai et al., 2008; Fei and Nelson, 2007).

Thermal Rough Bentgrass

One approach to understanding the mechanisms of plant stress tolerance has been to focus on plants adapted to extreme environments. A C_3 perennial grass species, rough bentgrass (*Agrostis scabra*), was identified in Yellowstone National Park, which occurs both in thermal and nonthermal habitats (Stout and Al-Niemi, 2002). The thermal form of *A. scabra* grows actively in the chronically hot soils. It differs from the nonthermal form in its shorter stature, more rapid growth and annual habit; seeds of thermal *A. scabra*

populations germinate from December to January, whereas nonthermal *A. scabra* populations do not initiate new growth from their perennial roots until late June (Tercek et al., 2003).

Thermal *A. scabra* may have adopted both heat avoidance and tolerance strategies (Stout and Al-Niemi, 2002). There have been a few recent publications from our lab describing some physiological traits associated with superior thermotolerance of the species at the whole-plant level. Lyons et al. (2007) found the canopy photosynthesis and respiration rates in thermal *A. scabra* could be maintained responding to short-term soil temperature elevation. Root thermotolerance of *A. scabra* to high soil temperature may be associated with high proportion of alternative respiration (Rachmilevitch et al., 2007), low maintenance and ion uptake costs (Rachmilevitch et al., 2006b), as well as efficient expenditure and adjustment of carbon and nitrogen allocation patterns between growth and respiration (Rachmilevitch et al., 2006a). However, limited information is available on the biochemical and molecular mechanisms of this species to tolerate high temperature, which is desirable for improving heat tolerance of the heat-sensitive *Agrostis* species such as creeping bentgrass if understood.

OVERALL GOAL AND OBJECTIVES

The overall goal of my dissertation research was to explore mechanisms associated with heat tolerance in cool-season grass species, particularly creeping bentgrass.

This goal was achieved by conducting seven projects with the following specific objectives:

1) To evaluate leaf senescence induced by heat stress for the two *Agrostis* species contrasting in heat tolerance, and to determine whether heat-induced leaf senescence in both *Agrostis* species were associated with changes in the three major senescence-related hormones (ethylene, abscisic acid and cytokinins).

2) To examine temperatures and duration required for HSP expression in the two *Agrostis* species contrasting in heat tolerance, and to evaluate whether genetic variation in heat tolerance for *Agrostis* species was related to differential HSP expression patterns.

3) To determine similarities and differences in metabolic responses between thermal *A. scabra* and heat-sensitive creeping bentgrass, and to identify key metabolites essential for plant tolerance to high temperature.

4) To determine whether foliar application of an ethylene synthesis inhibitor (AVG) and a synthetic cytokinin (ZR) would enhance creeping bentgrass tolerance to heat stress associated with the suppression of leaf senescence.

5) To investigate whether foliar application of TE and two biostimulants containing seaweed extracts would alleviate decline in creeping bentgrass growth during summer months, and to examine the effects of TE and the biostimulants on leaf senescence and root growth.

6) To investigate the effects of expression of *SAG12-ipt* on shoot and root growth and leaf senescence in creeping bentgrass subjected to heat stress.

7) To identify protein changes associated with increases in endogenous CK production through *ipt* transformation in creeping bentgrass, and determine proteomic mechanisms underlying CK-regulation of creeping bentgrass responses to heat stress.

Experimental designs and research findings on each of the projects are described in the following seven chapters, respectively.

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CHAPTER 1. Heat-Induced Leaf Senescence and Hormonal Changes for Thermal Bentgrass and Turf-type Bentgrass Species Differing in Heat Tolerance

INTRODUCTION

High temperature is a major factor limiting growth of cool-season plant species. One of the typical symptoms of heat injury for many plant species is leaf senescence (Thomas and Stoddart, 1980). Leaf senescence is characterized by loss of chlorophyll and photosynthetic activities in leaves (John et al., 1995). Heat-induced leaf senescence and associated changes in physiological activities have been reported in various plant species (Thomas and Stoddart, 1980; Xu et al., 1995; Yeh and Hsu, 2004). Xu et al. (1995) reported that increases in temperature during maturation of wheat (*Triticum aestivum*) enhanced leaf senescence, accentuated the loss of chloroplast integrity, and accelerated the decline of PSII-mediated electron transport. Cool-season turfgrass species, such as creeping bentgrass (*Agrostis stolonifera*), are sensitive to heat stress, and quickly lose color and suffer from a series of physiological injuries when exposed to high temperatures above 30 ºC. Leaf senescence was observed after 20 d at 30 ºC and only 8 d at 35 ºC for *A. stolonifera* cv. Penncross (Huang and Gao, 2000; Huang et al., 1998).

Many physiological and biochemical factors, such as plant hormones, can be related to leaf senescence. In many non-turf plant species, it has been found that both genetically- and environmentally-regulated senescence are associated with hormonal changes, with either up-regulating or down-regulating effects (Nooden and Leopoid, 1988). Ethylene, abscisic acid (ABA) and cytokinins are three major phytohormones that mediate signaling events involved in plant senescence (Chang et al., 2003). It has been

reported that exogenous ethylene can accelerate processes characteristic of leaf senescence, such as the decline of chlorophyll and increases in activity of hydrolytic enzymes (Grbic and Bleecker, 1995). In addition, delayed leaf senescence was observed in transgenic ethylene-deficient tomato (*Lycopersicon esculentum*) plants (John et al., 1995). ABA is involved in many plant responses to biotic and abiotic stresses. Rapid ABA accumulation has been observed when plants are subjected to drought, salinity, and extreme temperatures (Xiong et al., 2002). Some studies also found that ABA accumulation may induce leaf senescence. Spraying ABA reduced leaf chlorophyll content in rice (*Oryza sativa*) (Yang et al., 2002). Becker and Apel (1993) reported that senescence-associated mRNAs were induced by ABA in detached leaves of barley (*Hordeum vulgare*). In contrast to the actions of ethylene and ABA, cytokinins retard senescence in vegetative and floral tissues (Nooden and Leopoid, 1988). In transgenic P_{SAG12}-IPT tobacco (*Nicotiana tabacum*) plants, enhanced cytokinin synthesis targeted to senescing leaves significantly delayed leaf senescence; chlorophyll content and photosynthetic photon absorption were maintained, and nitrogen translocation to non-senescing leaves was reduced (Jordi et al., 2000). Applying benzyladenine (BA), a synthetic substance that is similar to cytokinin in structure and function, to the leaves of bean (*Phaseolus vulgaris*) plants helped maintain high chlorophyll content, thus delaying leaf senescence under normal environmental conditions (Adedipe, 1971).

The mechanisms of heat-induced leaf senescence are largely unknown. Identification of physiological or metabolic factors associated with senescence has practical value for turfgrass management and is important for revealing basic mechanisms of plant heat tolerance. Recently, a heat-tolerant C_3 perennial grass species,

Agrostis scabra (thermal rough bentgrass), has been identified growing in geothermally heated areas in Yellowstone National Park (Stout and Al-Niemi, 2002). It survives or even thrives in the chronically hot soils with temperatures up to 45 ºC (Tercek et al., 2003). When exposed to 35 ºC in controlled environment chambers, these plants maintain green leaves longer than creeping bentgrass (*A. stolonifera*), a widely-cultivated cool-season grass whose optimal growth temperature is between 10 to 18 ºC. Our objectives were to evaluate leaf senescence induced by heat stress for these two *Agrostis* species contrasting in heat tolerance, and to determine whether heat-induced leaf senescence in both *Agrostis* species was associated with changes in the three major senescence-related hormones (ethylene, ABA and cytokinins). Turf quality, photochemical efficiency, and the content of two pigments (chlorophyll and carotenoid) were measured to evaluate the degree of heat tolerance and leaf senescence. Quantitative changes in ethylene, ABA, and two major forms of cytokinins, trans-zeatin/zeatin riboside (Z/ZR) and isopentenyl adenosine (IPA) during heat stress were determined to examine their relationship with heat-induced leaf senescence.

MATERIALS AND METHODS

Plant Materials

Creeping bentgrass (cv. Penncross) plugs were collected from field plots at Hort Farm II, Rutgers University, NJ. Plants of *A. scabra*, originally collected from geothermally heated areas in Yellowstone National Park, Wyoming, were propagated in a greenhouse at Rutgers University. Both species were planted in well-drained plastic pots (15 cm in diameter and 20 cm deep) filled with sterilized sand and fertilized weekly with full-strength Hoagland's solution. Plants were cut weekly to maintain a canopy height of approximately 5 cm. After one month of establishment in the greenhouse, plants were transferred into controlled-environment growth chambers (Conviron, Winnipeg, Canada) with a temperature of 20 ºC/15 ºC (day/night), 14-h photoperiod, 50% relative humidity, and 400 μmol m^{-2} s^{-1} photosynthetic photon flux density at the canopy height. Plants were acclimated to growth chamber conditions for one week prior to exposure to different temperature treatments.

Treatments and Experimental Design

Plants of both species were exposed to 35 ºC/30 ºC (day/night) (high temperature) or 20 ºC/15 ºC (day/night) (control) for 35 d. Plants were watered twice daily to prevent water deficit in the high temperature treatment. Each temperature treatment was repeated in four growth chambers as four replicates. Species were arranged randomly inside each chamber. The experiment consisted of two factors (temperature and species), which were arranged as a completely randomized block design.

Measurements

Turf quality was evaluated based on color, density, and uniformity of the grass canopy using a 0~9 scale, with 9 representing fully green, dense turf canopy and 0 representing completely dead plants. Leaf photochemical efficiency of photosystem II (PSII) was measured with a fluorescence induction monitor (FIM 1500, Analytical Development Company LTD.) following a 30 min dark adaptation period. Photochemical efficiency was expressed as the ratio of Fv/Fm, which in fully healthy plants is approximately 0.8. A lower value of Fv/Fm indicates that a proportion of PSII reaction centers are damaged (Fracheboud, 2003).

Leaf chlorophyll and carotenoid were extracted from leaves (0.2g fresh weight) using dimethyl sulfoxide. The absorbance of leaf extracts was determined using a spectrophotometer (Spectronic Genesys2, Spectronic Instruments, Rochester, N.Y.). Chlorophyll content was calculated based on the absorbance at 663 nm and 645 nm, and carotenoid content was calculated based on the absorbance at 663 nm, 645 nm, and 470 nm using the formulas described by Arnon (1949).

Ethylene production of leaves was determined using a gas chromatograph (GC-8A, Shimadzn). An airtight system was designed to collect ethylene gas evolved from leaves. Five to seven attached leaves were grouped and sealed inside a 10-mL syringe (Becton, Dickinson and Company) with a rubber stopper around leaf base. Vacuum grease and Teflon tape were used to prevent leaks. Four groups of leaves from each pot were randomly selected and sealed in syringes as four sub-samples. A 0.5 mL of gas sample from each syringe was taken through the rubber stopper every 2 h and injected into GC to determine ethylene concentration. The average of hourly production of

ethylene was calculated based on changes in ethylene concentration over time and a standard curve.

ABA and two forms of cytokinin (trans-zeatin/zeatin riboside and isopentenyl adenosine) were quantified by an indirect competitive enzyme-linked immunosorbent assay. Extraction and quantification of hormones followed the method described by Setter et al. (2001) with some modifications (Wang and Huang, 2003). Briefly, samples were extracted in 80% [v/v] methanol and purified with reverse phase C_{18} columns. Hydrophilic contaminants were washed out with 200 μL of 20% solvent [20% methanol, 80% aqueous TEA (10 mM triethylamine, PH3.5)]. The cytokinin-containing fraction was eluted using 200 μL of 30% solvent (30% methanol, 70% aqueous TEA), and the ABA-containing fraction was eluted using 150 μL of 55% methanol.

Statistical Analysis

Analysis of variance and correlation was based on the general linear model procedure of SAS (SAS Institute Inc., Cary, NC). Effects of species, temperature and their interactions were tested separately for each sampling date for all the parameters. Results of analysis of temperature effects on each species were shown in separate figures to exhibit how each species responded to different temperatures. Differences between treatment means were separated by Fisher' protected least significant difference (LSD) test at the 0.05 probability level.

RESULTS

Changes in physiological parameters associated with leaf senescence and heat tolerance

Turf quality (TQ) of both species gradually declined over time at 35 ºC (Fig. 1). Turf quality for *A. stolonifera* declined to below the control level, beginning at 14 d of treatment, while a significant decline in *A. scabra* was not observed until 35 d. By 35 d of heat stress, turf quality decreased to 81% of the control for *A. scabra* and 56% of the control for *A. stolonifera*.

Under high temperature, plants of both species exhibited lower photochemical efficiency (Fv/Fm) than the control, beginning at 14 d of treatment (Fig. 2). The decline in Fv/Fm became more dramatic with stress duration and more pronounced for *A. stolonifera* than *A. scabra* at 35 ºC.

Leaf chlorophyll content for *A. stolonifera* decreased below the control level at 7 d of high temperature treatment, while that for *A. scabra* was maintained at the control level until 21 d of treatment (Fig. 3). *A. stolonifera* exhibited greater extent of decline in chlorophyll content than *A. scabra* with the progression of heat stress. Species variation and the change in carotenoid content with stress duration followed a similar pattern to that of chlorophyll content (Fig. 4). In *A. scabra* plants exposed to high temperature, carotenoid content was maintained at the control level for 28 d before a significant decrease occurred. For *A. stolonifera* plants, the decrease started at 7 d and became significant after 14 d. The decline in carotenoid content for *A. stolonifera* was more pronounced than for *A. scabra* during the entire experimental period of the high temperature treatment.

Changes in ethylene, ABA and cytokinin content

Ethylene production rate increased with treatment duration in both species under high temperature, and was 2-3 times of the initial level for both species at 35 d of treatment (Fig. 5). The timing of ethylene increase varied with species. In *A. scabra*, the rate of ethylene production at 35 ºC did not increase significantly above the control level until 35 d, whereas a significant increase occurred at 14 d of heat treatment for *A. stolonifera*.

At 35 ºC, ABA content increased to the highest level at 14 d for *A. stolonifera* and at 21 d for *A. scabra*, and declined thereafter. However, ABA content was significantly higher than the control level after only 7 d of treatment for *A. stolonifera* and after 21 d for *A. scabra* (Fig. 6). In addition, the peak values of ABA varied with species, which was 77.4 ρmol g^{-1} fresh weight (FW) for *A. stolonifera* (5.9 times of the control) and 64.6 ρmol g^{-1} FW for *A. scabra*, which was equal to 4.0 times of the control value.

The content of both forms of cytokinins declined with the progression of heat stress for both species (Fig. 7 and 8). For IPA content, the decrease in *A. scabra* became significantly after 14 d whereas in *A. stolonifera* it became significant after only 7 d (Fig. 7). At 35 d of heat stress, the content of IPA for *A. scabra* was reduced to 30% of the initial level, whereas that in *A. stolonifera* was reduced to 22%. The decrease in Z/ZR content became significant after 14 d of heat stress for *A. scabra* and 7 d for *A. stolonifera*. At the end of treatment period, Z/ZR cytokinin declined by 65% for *A. stolonifera* and by 50% for *A. scabra* (Fig. 8).

Correlation between hormones and senescence parameters

In general, ethylene and ABA contents were negatively correlated to TQ, leaf Fv/Fm, Chl and carotenoid (Crd) contents, while both forms of cytokinin contents had positive correlations with these parameters for both species (Table 1). For *A. scabra*, the correlation coefficients between all three hormones and senescence parameters were highly significant, which ranged from -0.51 (Fv/Fm) to -0.83 (Crd) for ethylene, -0.51 (Fv/Fm) to -0.75 (Chl) for ABA, 0.60 (TQ) to 0.79 (Fv/Fm) for IPA and 0.63 (TQ) to 0.89 (Fv/Fm) for Z/ZR (Table 1A). For *A. stolonifera*, there were significant correlations of ethylene and cytokinins with all senescence parameters, with higher correlation coefficients for ethylene (ranging from -0.90 to -0.96) than cytokinins (ranging from 0.72 to 0.94) (Table 1B). There were no significant correlations of ABA in *A. stolonifera*.

DISCUSSION

The decline of turfgrass quality under heat stress was observed three weeks earlier in *A. stolonifera* compared to *A. scabra*. Changes in chlorophyll and carotenoid contents exhibited consistent differences in the timing and severity of leaf senescence induced by heat treatment between the two species. Both chlorophyll and carotenoid contents were maintained at the control level for approximately 14 d in *A. scabra*, without any significant decrease until 21 and 28 d, respectively. The decline in TQ, chlorophyll and carotenoid content was less severe for *A. scabra* than *A. stolonifera*. These results demonstrated that heat tolerant *A. scabra* exhibited delayed and less severe leaf senescence under heat stress. Previous studies on root response to high temperatures for these two species also found that *A. scabra* exhibited higher tolerance to high soil temperature than *A. stolonifera*, with smaller decreases in root growth rate, cell membrane stability, maximum root length, and nitrate uptake (Lyons et al., 2007; Rachmilevitch et al., 2006).

Enhanced ethylene production has been associated with reduced growth and accelerated senescence in response to environmental stresses (Balota et al., 2004). In our studies, ethylene production rate of both bentgrass species increased significantly under heat stress when there was a 20% decline in chlorophyll content (Fig. 3). It has been reported in winter wheat that the stress-resistant cv. Dropia produced significantly less ethylene than the stress-susceptible cv. Delia under high temperature (38 ºC) (Balota et al., 2004). We did not observe significant differences in ethylene accumulation between the two bentgrass species tested. Ethylene production increased to a similar extent for both species after 35 d of heat treatment. However, the initiation of the increase was 14 d

later in *A. scabra* than in *A. stolonifera* under stress conditions. This delay of ethylene accumulation in *A. scabra* was consistent with the delay of leaf senescence as manifested by turf quality and chlorophyll and carotenoid contents.

ABA accumulation in response to heat shock and other stresses has been reported in several plant species, including maize (*Zea mays*), durum wheat (*Triticum durum*) and pea (*Pisum sativum*) (Musatenko et al., 2003; Shakirova et al., 1995; Veselov et al., 1998). In our study, ABA accumulation occurred at 35 ºC for both species, exhibiting a peak at 14 and 21 d for *A. stolonifera* and *A. scabra*, respectively. Similar results have recently been reported for grape (*Vitis vinifera*), where ABA content drastically increased within one hour after heat treatments (38 ºC) (Wang et al., 2005). However, the increase in *A. scabra* was delayed for 14 d and the maximum accumulation at the peak was smaller compared to *A. stolonifera*. These results suggested that *A. scabra* could maintain its ABA content for a longer period of time and hold a less pronounced increase when exposed to prolonged heat treatment, which could possibly be associated with less stress injury and better heat tolerance.

Both Z/ZR and IPA production consistently decreased under heat stress, as demonstrated in both bentgrass species in our study and many non-turf plant species in various other studies. One potential explanation for cytokinin reduction in shoots is due to inhibition of cytokinins transport from roots to shoots under elevated temperatures, as cytokinins are primarily produced in roots. This perspective is supported by a study imposed in wheat which reported that the content of cytokinins decreased in shoots and accumulated in roots simultaneously as a result of temperature increase (Farkhutdinov et al., 1997). Additionally, the decline of endogenous cytokinins in senescing tissues may be

due to a decrease in biosynthesis or an increase in metabolism (Nooden and Leopoid, 1988). It would be necessary to examine specific changes in cytokinin synthesis, degradation, and immobilization to reveal the actions of cytokinin in heat tolerance of plants, including turfgrass. In terms of species variation, the decreases of both forms of cytokinins were delayed for 7 d and less severe after 35 d of heat stress in *A. scabra* than in *A. stolonifera*, suggesting that maintenance of a higher level of endogenous cytokinin for a longer period of time may contribute to better heat tolerance.

Data from correlation analysis demonstrated that endogenous ethylene and ABA production were positively correlated and cytokinin production was negatively correlated with heat-induced leaf senescence; however, the correlation with ABA was not significant for *A. stolonifera*. Thus, approaches that can increase endogenous cytokinin levels or suppress ethylene production may lead to improved heat tolerance and delayed foliar senescence. Exogenous spray of cytokinin, or its derivatives, may be one possible method. Liu et al. (2002) reported that applications of 1 and 10 mM ZR to the root zone of creeping bentgrass increased cytokinin content in leaves and roots and mitigated heat stress injury in both shoots and roots. Endogenous cytokinin levels may also be increased by transgenic approaches. Teplova et al. (2000) transformed tobacco plants with the *ipt* gene that codes for isopentenyltransferase, which was subsequently expressed after a heat-shock treatment. Elevated temperature resulted in a decrease in the transpiration of wild-type plants, whereas the transpiration rate was maintained at high levels in transgenic plants. Conversely, since ethylene production was negatively correlated with heat-induced senescence, delayed leaf senescence may also be achieved by transgenic approaches that can perturb endogenous ethylene-response signal transduction pathways

(Suzuki et al., 2005). However, blocking ethylene production may be detrimental to heat tolerance. Larkindale and Knight (2002) reported that ethylene was necessary in protection against heat-induced oxidative damage in arabidopsis (*Arabidopsis thaliana*) because ethylene-insensitive mutant *etr-1* showed increased susceptibility to heat. These studies indicate the complex interaction of hormones and stress tolerance.

In summary, heat-tolerant *A. scabra* exhibited delayed and less severe leaf senescence during heat stress, compared to heat-sensitive *A. stolonifera*. The increases in ethylene and ABA, and decreases in cytokinins, could contribute to heat-induced leaf senescence and differences in heat tolerance between the two bentgrass species. Ethylene and cytokinins were more closely correlated to physiological parameters associated with leaf senescence and heat tolerance than ABA, especially for heat-sensitive *A. stolonifera*. This suggested that approaches that can suppress endogenous ethylene, or increase cytokinin levels might be used to delay foliar senescence, and ultimately improve heat tolerance.

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Table 1 Correlations of ethylene, abscisic acid (ABA), isopentenyl adenosin (IPA) and trans-zeatin/zeatin riboside (Z/ZR) with senescence parameters (turf quality, Fv/Fm, chlorophyll and caroteonid contents) under heat stress (35 ^{o}C). The significance of correlation coefficients was tested at $P \leq 0.05$ (*) and $P \leq 0.01$ (**).

A. scabra

	Ethylene	ABA	IPA	Z/ZR
Turf quality	-0.81**	-0.53*	0.60**	0.63**
Fv/Fm	-0.51*	-0.51*	0.79**	0.89**
Chlorophyll	-0.77**	-0.75**	0.77**	0.77**
Carotenoid	-0.83**	-0.56*	0.67**	0.68**

A. stolonifera

	Ethylene	ABA	IPA	Z/ZR
Turf quality	-0.90**	-0.25	0.72**	0.79**
Fv/Fm	-0.92**	-0.29	0.73**	0.74**
Chlorophyll	-0.96**	-0.42	0.88**	0.94**
Carotenoid	-0.90**	-0.45	0.87**	0.93**

Fig. 1 Changes in turf quality with temperatures (20 ^{o}C, solid lines and filled symbols and 35 ^{o}C, dotted lines and open symbols) for creeping bentgrass (*Agrostis stolonifera*) (**A**) and *Agrostis scabra* (**B**). Turf quality was evaluated using '0 - 9' scales (9 representing fully green, dense turf canopy, and 0 representing completely dead plants) Vertical bars on the bottom indicate LSDs ($P \leq 0.05$) for treatment comparison at a given day of treatment.

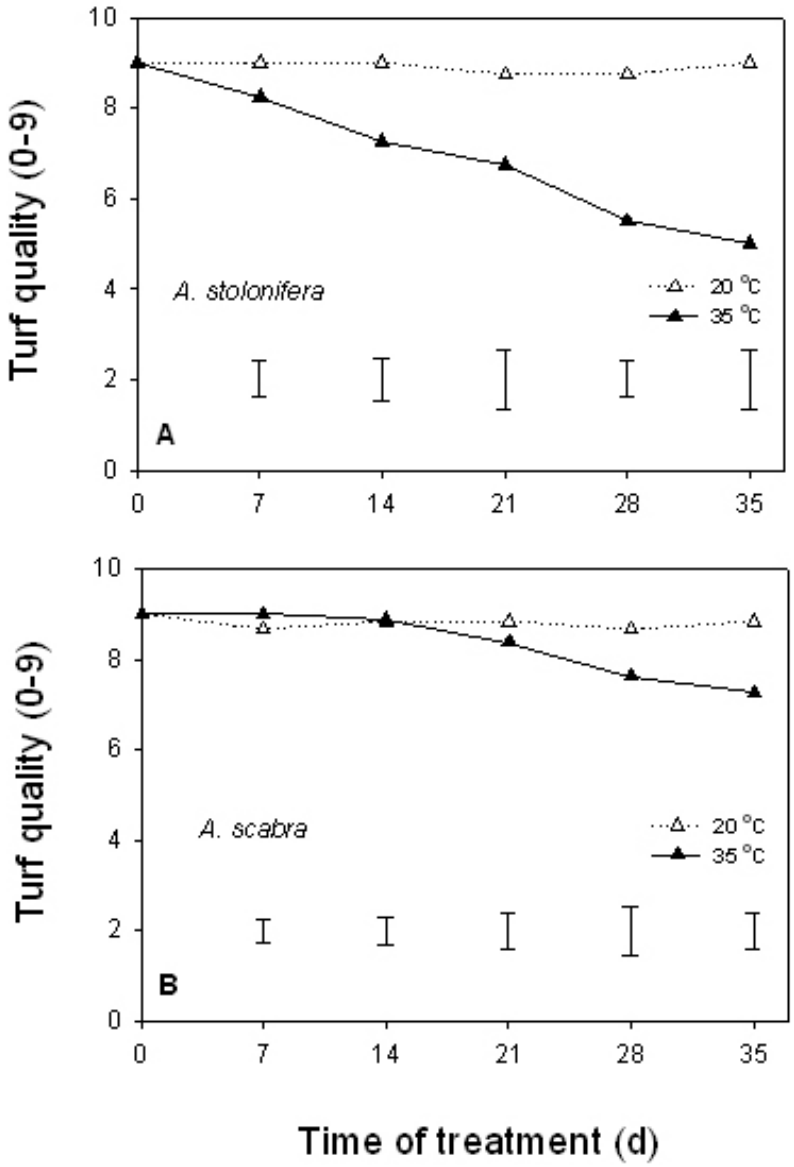


Fig. 2 Changes in leaf photochemical efficiency (measured as Fv/Fm ratio) with temperatures (20 ^{o}C, solid lines and filled symbols and 35 ^{o}C, dotted lines and open symbols) for creeping bentgrass (*Agrostis stolonifera*) (**A**) and *Agrostis scabra* (**B**). Vertical bars on the bottom indicate LSDs ($P \leq 0.05$) for treatment comparison at a given day of treatment. Fv/Fm= (Fm-Fo)/Fm. Fo is the ground state value of fluorescence, and Fm is the maximum value of fluorescence.

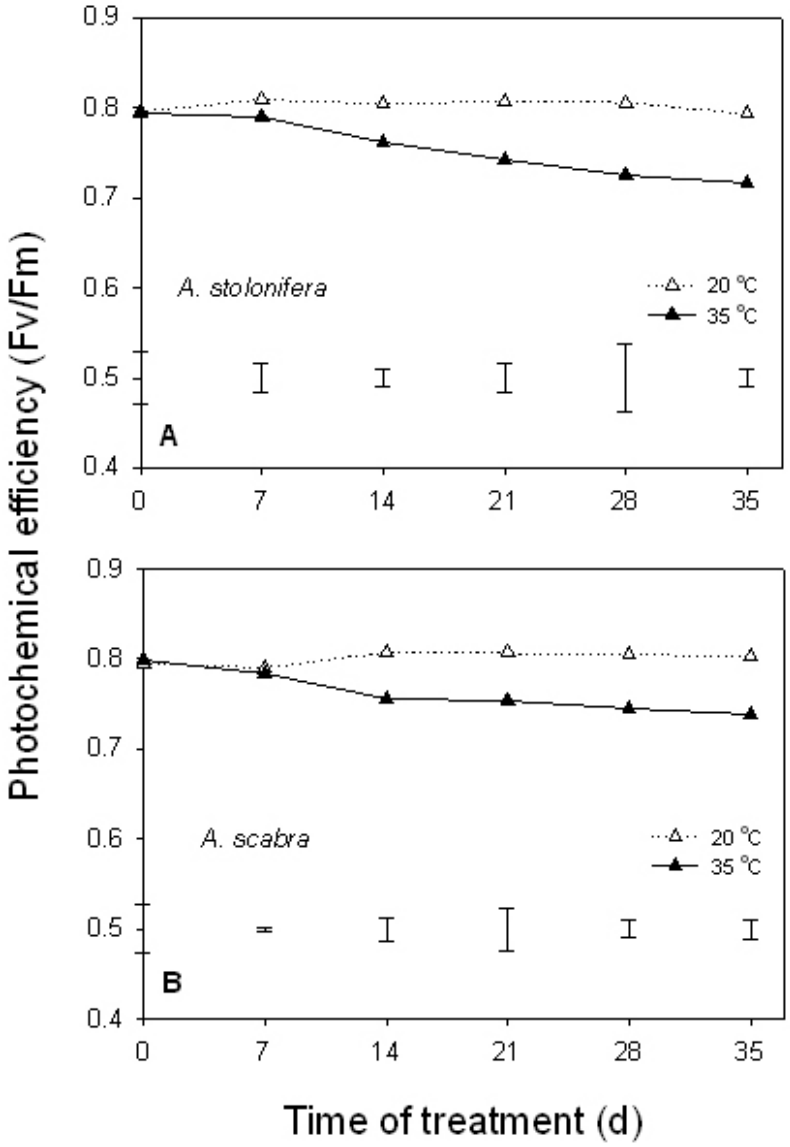


Fig. 3 Changes in leaf chlorophyll content (mg g^{-1} DW) with temperatures (20 ^{o}C, solid lines and filled symbols and 35 ^{o}C, dotted lines and open symbols) for creeping bentgrass (*Agrostis stolonifera*) (**A**) and *Agrostis scabra* (**B**). Vertical bars on the bottom indicate LSDs ($P \leq 0.05$) for treatment comparison at a given day of treatment.

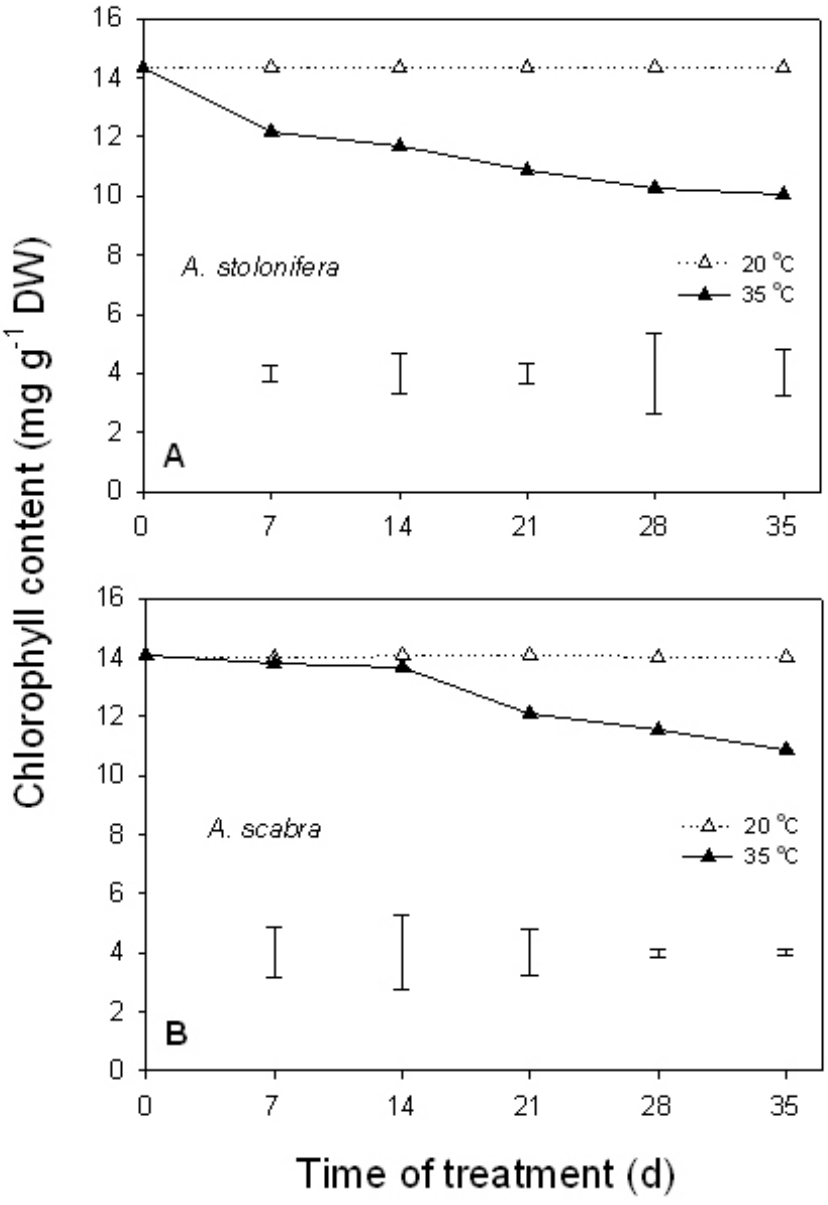


Fig. 4 Changes in carotenoid content (mg g^{-1} DW) with temperatures (20 ^{o}C, solid lines and filled symbols and 35 ^{o}C, dotted lines and open symbols) for creeping bentgrass (*Agrostis stolonifera*) (**A**) and *Agrostis scabra* (**B**). Vertical bars on the bottom indicate LSDs ($P \leq 0.05$) for treatment comparison at a given day of treatment.

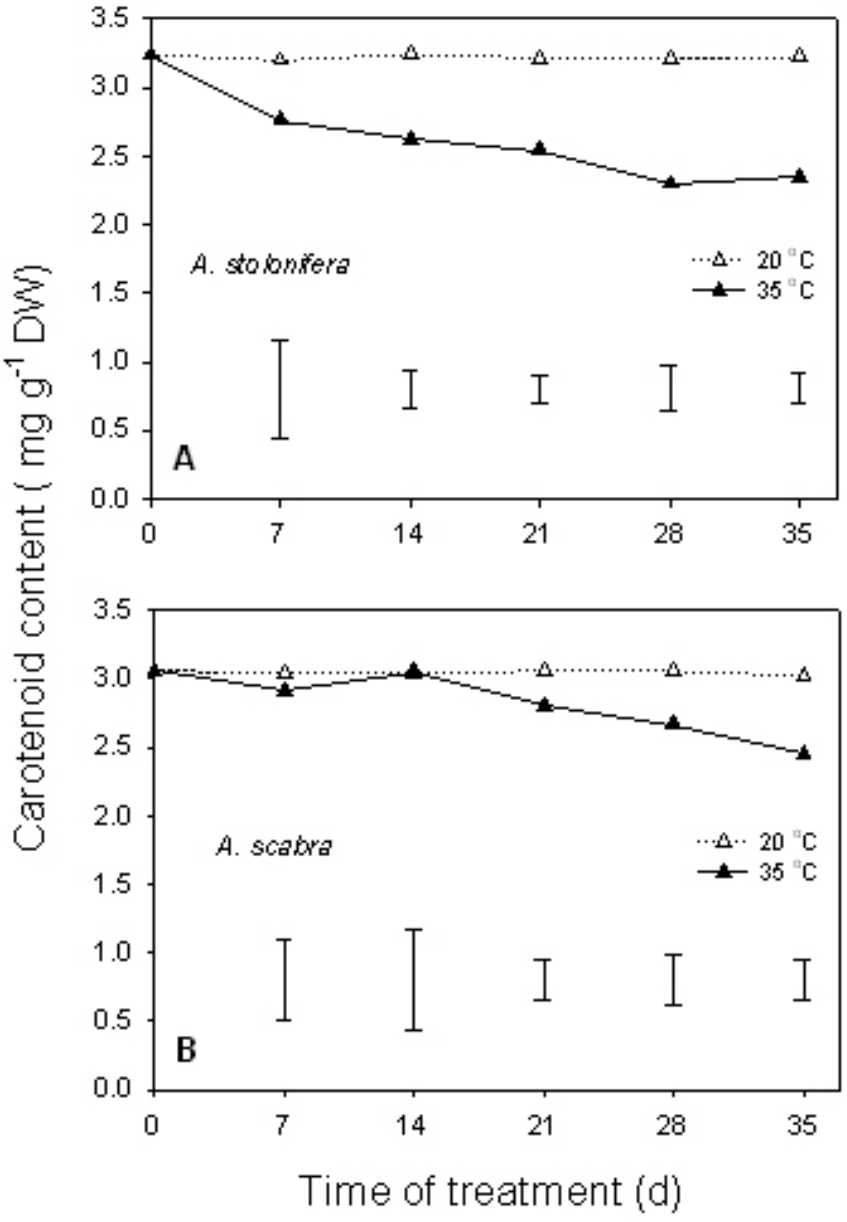


Fig. 5 Changes in leaf ethylene production rate (nL g^{-1} FW h^{-1}) with temperatures (20 ^{o}C, solid lines and filled symbols and 35 ^{o}C, dotted lines and open symbols) for creeping bentgrass (*Agrostis stolonifera*) (**A**) and *Agrostis scabra* (**B**). Vertical bars on the bottom indicate LSDs ($P \leq 0.05$) for treatment comparison at a given day of treatment.

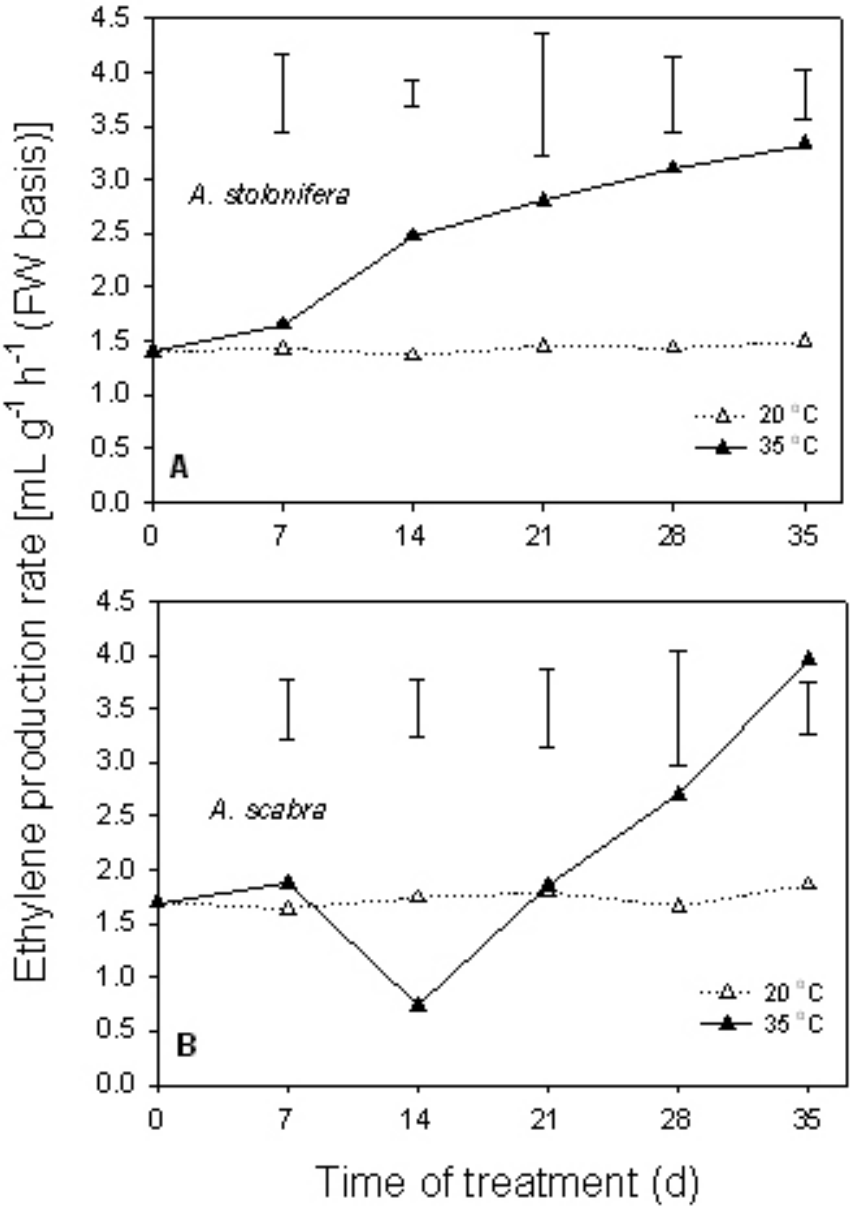


Fig. 6 Changes in leaf abscisic acid content (ρmol g^{-1} FW) with temperatures (20 ^{o}C, solid lines and filled symbols and 35 ^{o}C, dotted lines and open symbols) for creeping bentgrass (*Agrostis stolonifera*) (**A**) and *Agrostis scabra* (**B**). Vertical bars on the bottom indicate LSDs ($P \leq 0.05$) for treatment comparison at a given day of treatment.

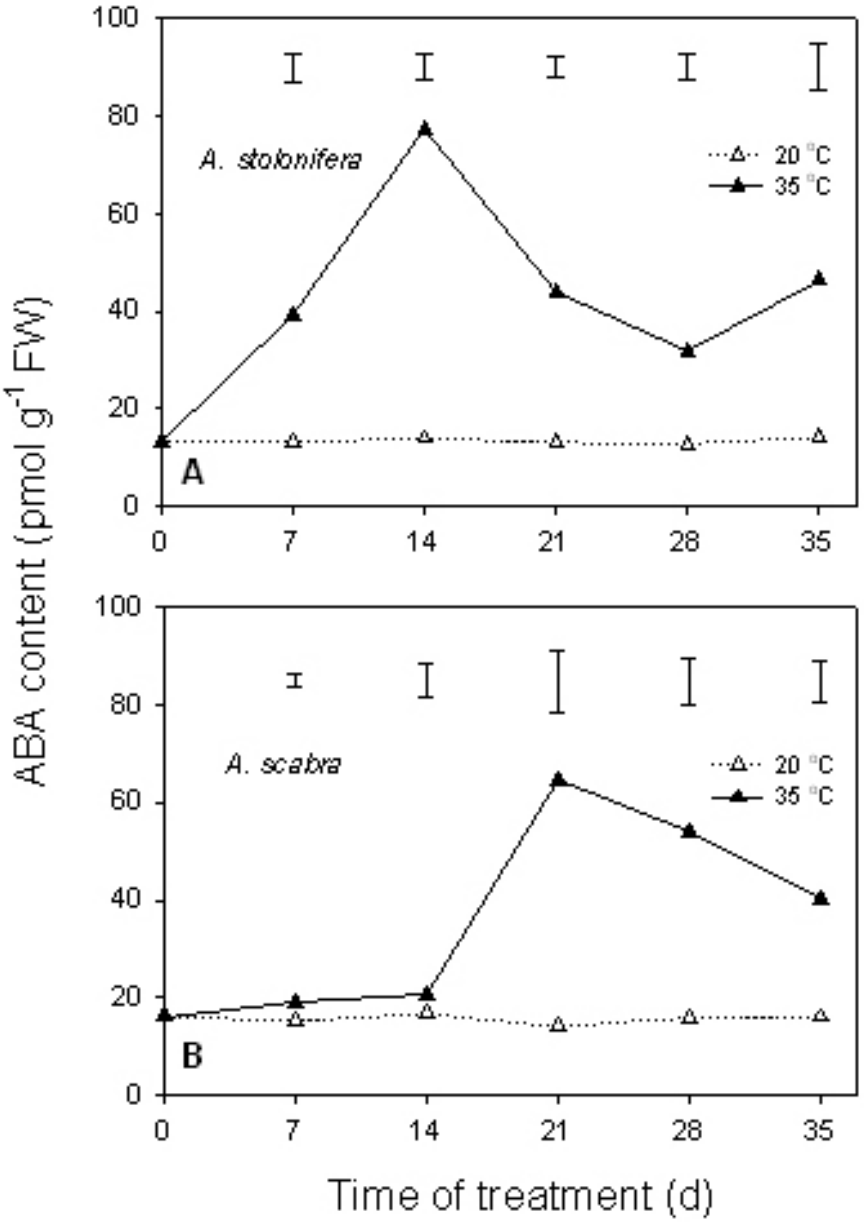


Fig. 7 Changes in isopentenyl adenosine (IPA) content (ρmol g^{-1}FW) with temperatures (20 ^{o}C, solid lines and filled symbols and 35 ^{o}C, dotted lines and open symbols) for creeping bentgrass (*Agrostis stolonifera*) (**A**) and *Agrostis scabra* (**B**). Vertical bars on the bottom indicate LSDs ($P \leq 0.05$) for treatment comparison at a given day of treatment.

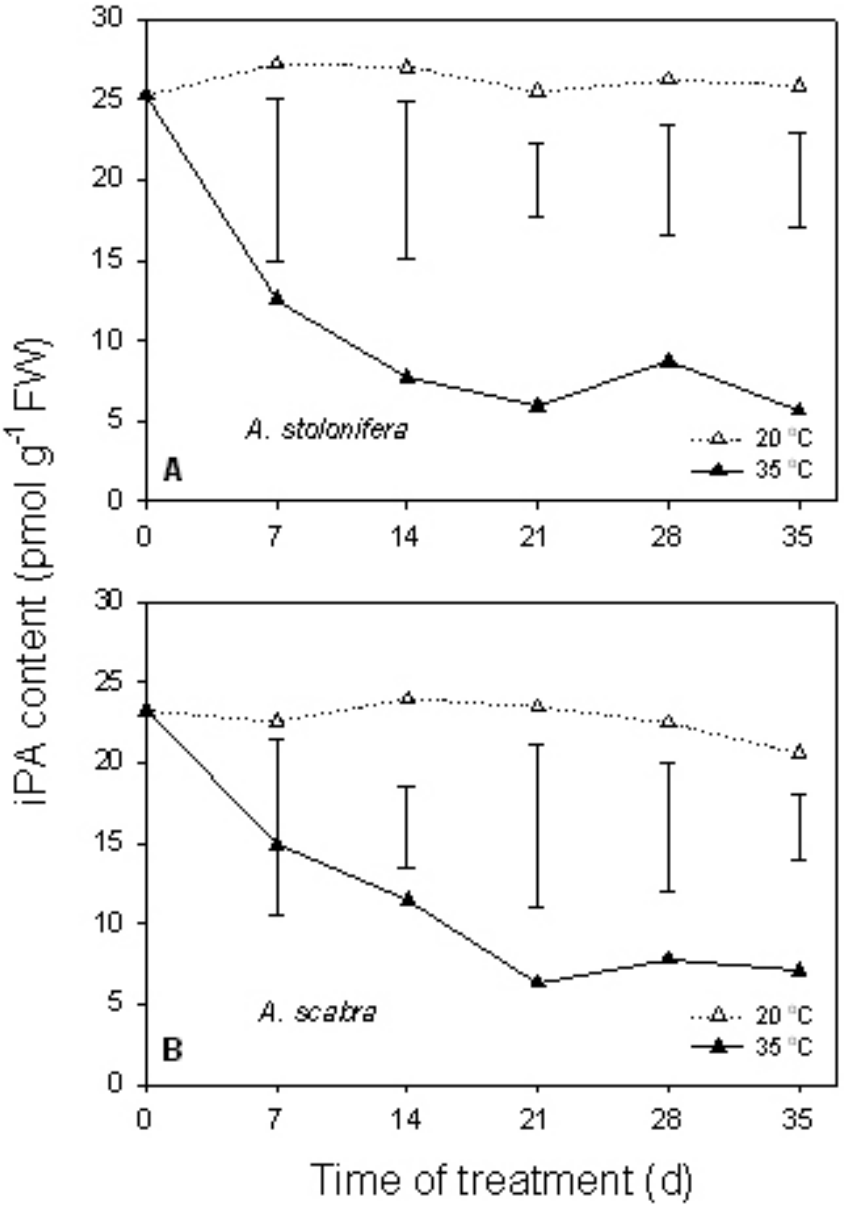
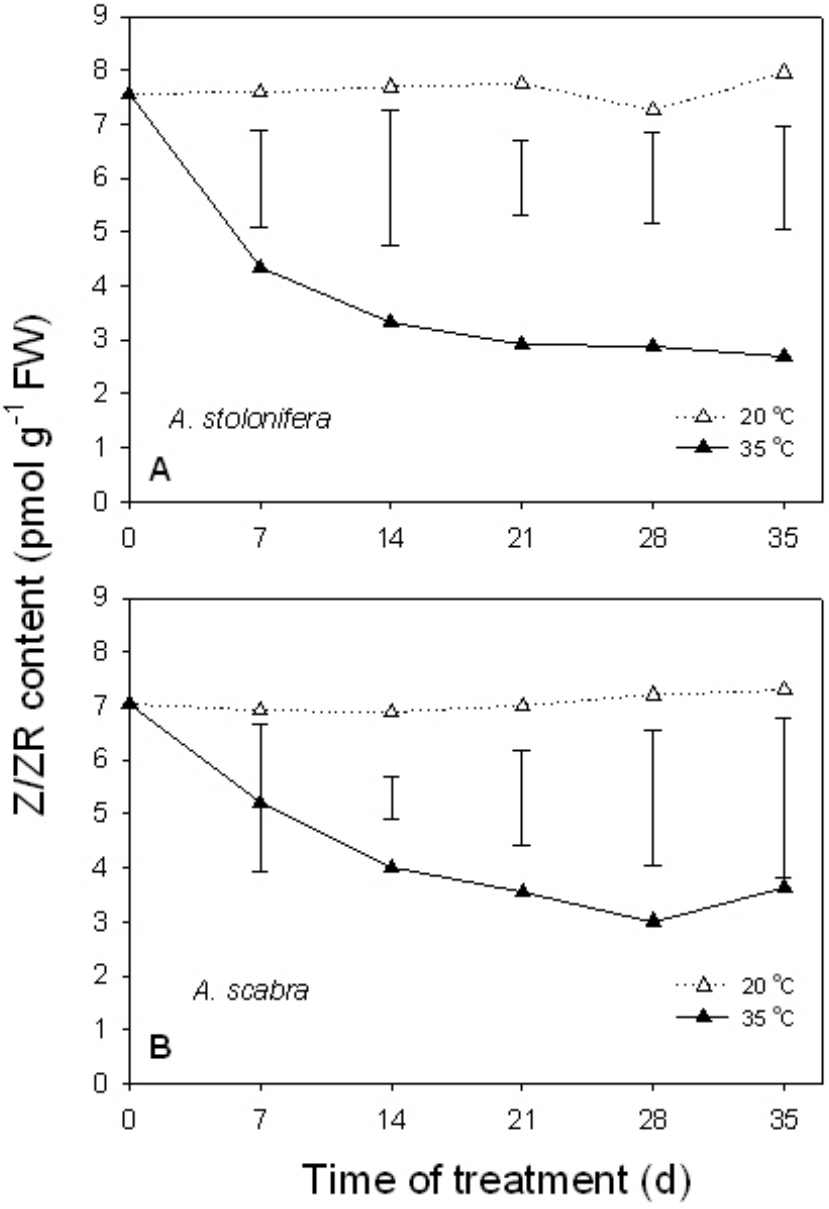


Fig. 8 Changes in trans-zeatin/zeatin riboside (Z/ZR) content (ρmol g^{-1} FW) with temperatures (20 ^{o}C, solid lines and filled symbols and 35 ^{o}C, dotted lines and open symbols) for creeping bentgrass (*Agrostis stolonifera*) (**A**) and *Agrostis scabra* (**B**). Vertical bars on the bottom indicate LSDs ($P \leq 0.05$) for treatment comparison at a given day of treatment.



CHAPTER 2. Differential Protein Expression for Geothermal *Agrostis scabra* and Turf-type *Agrostis stolonifera* Differing in Heat Tolerance

INTRODUCTION

Temperatures above the optimal growth range for plants can affect many metabolic processes, including protein metabolism. Studies with a number of higher plants indicate that, while synthesis of most normal proteins and mRNAs is inhibited, the transcription and translation of a small set of proteins, called heat shock proteins (HSPs), may be induced or enhanced when plants are exposed to elevated temperatures (Ahn et al., 2004; Pareek et al., 1998; Park et al., 1996). HSPs are generally classified into two groups: the high-molecular-mass HSPs (60-110 kD) and low-molecular-mass HSPs (15-30 kD), which is also called small HSPs (Park et al., 1996; Vierling, 1991).

The expression of HSPs has been correlated with acquisition of thermotolerance (Katterman, 1990; Vierling, 1991). Previous studies provide evidence on the relationship between HSPs and heat tolerance (Park et al., 1997; Sung and Guy, 2003; Vierling, 1991). For example, Sung and Guy (2003) reported that reduced HSC70-1 expression in arabidopsis (*Arabidopsis thaliana*) increased plant sensitivity to heat shock (44 ºC for 10 min), whereas overexpression of HSC70-1 improved plant tolerance to heat shock. Ristic et al. (1998) confirmed the association between specific HSPs and the heat tolerance phenotype in maize (*Zea mays*). They crossed a heat-tolerant line that synthesized a 45 kD HSP with a heat-sensitive line that did not synthesize it, and observed the synthesis of the 45 kD HSP in F2 plants that displayed an increased ability to recover from heat stress (45 ^{o}C). Heat-tolerant plants are capable of repairing damage caused by heat and

resuming normal metabolic functions faster than non-tolerant plants (Park et al., 1997). Krishnan et al. (1989) conducted research on wheat (*Triticum aestivum*) varieties exhibiting distinct levels of acquired thermaltolerance and observed significant quantitative differences between a heat-tolerant cv. Mustang and a heat-sensitive cv. Sturdy in the expression of 16, 17, 22, 26, 33, and 42 kD HSPs and the synthesis of the small subunit of ribulose 1, 5-bisphosphate carboxylase/oxygenase (Rubisco).

A C_3 perennial grass species, thermal rough bentgrass (*Agrostis scabra*), has recently been identified in Yellowstone National Park (Stout and Al-Niemi, 2002). It grows actively in the chronically hot soils that are permeated by hot steam with temperatures up to 45 ^{o}C (Tercek et al., 2003). In contrast, temperatures above 24 ^{o}C have been considered detrimental for common C_3 grasses used as turf or forage in cool climates, including creeping bentgrass (*Agrostis stolonifera*) (Beard, 1973). By comparing *A. scabra* with the heat-sensitive *A. stolonifera*, Rachmilevitch et al. (2006) found that acclimation of respiratory carbon metabolism played an important role in root survival of *A. scabra* at chronically high soil temperatures. Maintaining higher root viability, cell membrane stability, and nutrient uptake activity also contributed to better heat tolerance of *A. scabra* than *A. stolonifera* (Lyons et al., 2007; Rachmilevitch et al., 2006). Xu and Huang (2007) reported that delayed and less severe leaf senescence in *A. scabra* than in *A. stolonifera* under heat stress were related to the delayed and lower magnitude of increases in ethylene and ABA production, as well as the delayed and less severe decline in the accumulation of cytokinins. The superior heat tolerance of *A. scabra* may also be related to changes in protein degradation and synthesis, especially HSPs, in response to increasing temperature. Stout and Al-Niemi (2002) reported that enhanced

HSP expression in roots, particularly small molecular HSPs, was associated with the adaptation of *Dichanthelium lanuginosum* to geothermal soils with temperatures above 40 ^{o}C in Yellowstone National Park. However, such information is not yet documented in *A. scabra*.

The objectives of this study were to examine temperatures and duration required for HSP expression in the thermal *A. scabra*, compared with that in heat-sensitive *A. stolonifera*, and to evaluate whether genetic variation in heat tolerance for *Agrostis* species was related to differential HSP expression patterns. Protein changes were also compared for two cultivars of *A. stolonifera* differing in heat tolerance: moderate heat-tolerance cv. L-93 and heat-sensitive cv. Penncross.

MATERIALS AND METHODS

Plant Materials

Plugs of two creeping bentgrass (*A. stolonifera)* cultivars, 'Penncross' and 'L-93', were collected from turfgrass field plots at Hort Farm II, Rutgers University, New Brunswick, NJ. Clonal plants of thermal *A. scabra* were collected from geothermally heated areas in Yellowstone National Park, Wyoming. They were planted in sterilized sand and maintained in a research greenhouse. After four weeks of establishment, plants were transferred into controlled-environment growth chambers (Conviron, Winnipeg, Canada), with a day/night temperature of 20 ºC, a 14-h photoperiod, 60% humidity, and a photosynthetic active radiation of 450 µmol m^{-2} s^{-1} at canopy height. Plants were allowed to acclimate to growth chamber conditions for one week before treatments were imposed.

Treatments and Experimental Design

Plants were exposed to 20, 30, 35, 40, or 45 ºC for 14 d at each temperature in five growth chambers. Each temperature treatment had four pots as replicates. Plants of two species and cultivars were arranged randomly inside each chamber. Every three days, plants at each temperature were moved to a different chamber to eliminate effects of other environmental conditions. During the treatment period, plants were watered twice daily and fertilized weekly with 100 mL half-strength Hoagland's solution.

Measurements of Physiological Parameters

Leaf photochemical efficiency was estimated by measuring chlorophyll fluorescence in the form of Fv/Fm ratio, with a fluorescence induction monitor (FIM 1500, Analytical Development Company LTD., Hoddedson, United Kingdom). Samples were collected at 0, 3, 7, 10 and 14 d for each temperature treatment. Intact leaves were covered in specially designed leaf clips, which served to dark-adapt the leaf for 30 min before Fv/Fm was measured.

Leaf chlorophyll and carotenoid were extracted from 0.2 g fresh leaves after 0, 3, 7 and 14 d for each temperature treatment. Samples were soaked in dimethyl sulfoxide for 48 h in darkness. The absorbance of leaf extracts was determined with a spectrophotometer (Spectronic Genesys2, Spectronic Instruments, Rochester, N.Y.). Chlorophyll content was calculated based on the absorbance at 663 nm and 645 nm. Carotenoid content was calculated based on the absorbance at 663 nm, 645 nm, and 470 nm (Arnon, 1949).

Protein Extraction and Quantification

Extraction and quantification of soluble protein from leaves followed the methods described by Shimoni et al. (1997) with modifications. 0.5 g frozen leaf tissues was ground with a mortar and pestle in liquid nitrogen to fine powder and extracted in 3 ml of extraction buffer [0.1 mM Tris-HCl (pH 7.6), 0.15 M NaCl] on ice. The suspension was centrifuged twice at 4 ºC for 15 min at 12000 rpm. The resulting supernatant was regarded as salt-soluble proteins and collected for analysis. Protein content was determined using the method of Bradford (1976). A 100 μL of protein extraction (diluted

8 times) was mixed with 3 mL of color reagent (0.01% coomassie brilliant blue G, 4.75% ethanol, and 8.5% phosphoric acid). The absorbance was measured on the spectrophotometer at 595 nm between 5 and 30 min after reaction. A standard curve was made from bovine serum albumin.

SDS-PAGE and Western Blot

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with leaf protein extracts as described by He et al. (2005) using 6% stacking gel and 12% running gel. Protein extracts were diluted with extraction buffer to an equal concentration. 18 μg of protein from each sample was loaded in each well. Protein profiles were separated on a mini-gel electrophoresis apparatus (MP3 Electrophoresis System, Bio-Rad, CA). Protein bands were visualized by staining with coomassie brilliant blue R-250.

For western blotting, separated proteins on the unstained gel were transferred to a nitrocellulose membrane (Trans-Blot Transfer Medium, Bio-Rad, CA) following the method of (Towbin et al., 1979). Immunoblotting was performed as described by Lennon et al. (1997). HSP60 and HSP70 mouse monoclonal antibodies (Stressgen Biotech. Corp., Victoria, Canada) were used at 1:4000 and 1:1000 dilutions, respectively. A goat anti-mouse IgG conjugated with alkaline phosphatase (AP) (Sigma, St. Louis, MO) was used as a secondary antibody at a 1:5000 dilution. Bound antibodies were detected using AP conjugate substrate kit (Bio-Rad, Richmond, CA).

Statistical Analysis

The experiment was considered to be a completely randomized split-plot design, with temperature as the main plots, and species and cultivars as the sub-plots. Analysis of variance was based on the general linear model procedure of SAS (SAS Institute Inc., Cary, NC). Main effect of species/cultivar and temperature was tested at each sampling day for all the parameters. Results of analysis of temperature effects on each cultivar were shown in separate figures to exhibit how each species/cultivar responded to heat stress. Differences between treatment means were separated by least significant difference (LSD) test at 0.05 probability level.

RESULTS

Photochemical efficiency

Photochemical efficiency, expressed as Fv/Fm ratio, decreased with elevated temperature from 20 to 45 °C for both *Agrostis* species (Fig. 1). The decline was observed at 10 d of 35 °C and at 3 d of 40 and 45 °C for both species. At 35 °C, it decreased to 89% of the initial level in *A. scabra* and 'L-93', and 86% in 'Penncross' after 10 d of treatment. At 40 and 45 °C, thermal *A. scabra* exhibited less severe decline in Fv/Fm than both cultivars ('L-93' and 'Penncross') of *A. stolonifera* during the entire 14-d treatment period, except at 7 d of 45 °C, Fv/Fm declined to zero for both species. Photochemical efficiency for thermal *A. scabra* was maintained at 78% and 72% of the initial level at 14 d of 40 °C and 3 d of 45 °C, respectively (Fig. 1A). For *A. stolonifera*, photochemical efficiency of both cultivars decreased to 54% at 14 d of 40 °C. It decreased dramatically to 38% and 8% of the initial level at 3 d of 45 °C in 'L-93' and 'Penncross', respectively (Fig. 1C and B).

Pigment content

Significant decreases in chlorophyll content were detected at 35, 40 and 45 °C for both species while no significant change was observed at 30 °C, compared to that at 20 °C (Fig. 2). The decline began at 7, 3, and 3 d of 35, 40, and 45 °C, respectively, for thermal *A. scabra*, and at 3 d for all three temperatures in both cultivars of *A. stolonifera*. It was more severe in both *A. stolonifera* cultivars than thermal *A. scabra*. By 14 d of 35 °C, chlorophyll content decreased to 72% of the control level (20 °C) in *A. scabra* and

62% in both cultivars of *A. stolonifera*. By 14 d of 40 °C, chlorophyll content decreased to 39, 36, and 21% of their control levels in *A. scabra*, 'L-93' and 'Penncross', respectively. By 14 d of 45 °C, chlorophyll content was only 12% of the control in both species.

Decrease in carotenoid content with increasing temperature and duration followed the same pattern as chlorophyll content (Fig. 3). At 14 d of treatment, it decreased to 80, 69, and 65 % of the control level at 35 °C, and to 47, 44, and 29% at 40 °C in *A. scabra*, 'L-93' and 'Penncross', respectively. Over 96% decline in carotenoid content was detected at 45 °C by 14 d of treatment. At 3 and 7 d at 45 °C, *A. scabra* maintained higher carotenoid content than both 'Penncross' and 'L-93'.

Soluble protein content

Soluble protein content declined as temperature was elevated to 40~45°C for *A. scabra* and 30-45 °C for both cultivars of *A. stolonifera* (Fig. 4). Protein content decline in *A. scabra* was detected at 7 d of 40 °C and 3 d of 45 °C. For both cultivars of *A. stolonifera*, significant decreases were observed at 14 d of 30 °C, 7 d of 35 °C, and 3 d of 40 °C and 45 °C, for both cultivars. Although protein contents declined dramatically at 45 °C for both species, more proteins were maintained in *A. scabra* (41% and 16% of the initial amount after 3 and 7 d) than in both cultivars of *A. stolonifera*, 'L-93' (25% and 10% of the initial amount) and 'Penncross' (21% and 10% of the initial amount).

Protein profiles

Species variations in protein profiles were observed at 3 d of treatment (Fig. 5). The most pronounced change was the severe degradation of a 55 kD protein, the large subunit of Rubisco, at 45°C in both cultivars of *A. stolonifera*, 'Penncross' (Fig. 5B, Lane 5) and 'L-93' (Fig. 5C, Lane 5). There was no obvious degradation of this protein in *A. scabra* at the same time and temperature (Fig. 5A, Lane 5). Two high molecular weight proteins (~180 and 200 kD) partially degraded at 45 °C for both species, but the extent was less severe in *A. scabra* than in 'Penncross' and 'L-93'. Up-regulation of an 83 kD protein was detected in *A. scabra* at 40 and 45 °C (Fig. 5A, Lane 4 and 5) and in both cultivars of *A. stolonifera* at 40 °C (Fig. 5B, C; Lane 4). In addition, expression of a 68 kD protein was enhanced in both cultivars of *A. stolonifera* at 35 and 40°C (Fig. 5B, C; Lane 3 and 4), but not in *A. scabra*.

At 7 d of 45 °C, protein content decreased to less than 15% for both species and protein bands were un-detectable (not shown in Fig. 6). Degradation of the 200 kD protein was observed at 40 °C for both species (Fig. 6A, B and C; Lane 4). Expression of the 83 kD protein was enhanced at 35 and 40 °C in 'L-93' (Fig. 5C, Lane 3 and 4), and 40 °C in *A. scabra* and 'Penncross' (Fig. 5A, B; Lane 4). Up-regulation of a 23 kD protein was detected in 'L-93' at 7 d of 35 °C and 40 °C (Fig. 6D, Lane 3 and 4). There was no severe degradation of the 55 kD protein at 7 d of 20-40 °C in both species.

At 14 d, the 83 kD protein was up-regulated at 35 and 40 °C in *A. scabra* and 'L-93', and at 40 °C in 'Penncross' (Fig. 7). Enhanced expression of the 23 kD protein can

be detected at 35 and 40 °C in ‘L-93’ (Fig. 7C, Lane 3 and 4) and *A. scabra* (Fig. 7A, Lane 3 and 4), but only at 40 °C in ‘Penncross’ (Fig. 7B, Lane 4). A 66 kD protein was strongly induced at 40 ^{o}C in *A. scabra* (Fig. 7A, Lane 4), and weakly induced in ‘L-93’ or ‘Penncross’ (Fig. 7B and C, Lane 4). There was another 36 kD protein being up-regulated in both cultivars of *A. stolonifera* at 40 °C (Fig. 7B and C, Lane 4), but not in *A. scabra*. In addition, a 32 kD protein was down-regulated at 40 °C in *A. scabra* and ‘L-93’ (Fig. 7A, C; Lane 4), and at 35 and 40 °C in ‘Penncross’ (Fig. 7B, Lane 3 and 4).

Western blot

Increases in expression of both HSP60 and HSP70 were detected after 3 d of elevated temperature treatments (Fig. 8). HSP70 was constitutively expressed under control temperature (20 °C) and enhanced at 40 °C and 45 °C for both species. The expression was strongest in *A. scabra*, intermediate in ‘L-93’ and least in ‘Penncross’ at 40 °C and 45 °C. Basal HSP60 expression was low at 20 °C in both species, but it was up-regulated as temperature was elevated to 35-45 °C. The enhanced expression was strongest in *A. scabra*, intermediate in ‘L-93’ and least in ‘Penncross’ at 40 °C and 45 °C.

DISCUSSION

One of the major effects of supraoptimal temperature on higher plants is dysfunction of photosynthesis, which is associated with pigment degradation and thermosensitivity of photochemical reactions occurring in the thylakoid membrane system (Weis and Berry, 1988). Our results demonstrated that the heat-induced damage of the photosynthetic system in both *Agrostis* species occurred when growth temperature was elevated to 35 ºC, as manifested by significant declines in three photosynthetic parameters, leaf photochemical efficiency, chlorophyll and carotenoid contents (Fig. 1-3). However, the declines in all three parameters under high temperatures (35-45 °C) were the least severe in *A. scabra*, intermediate in 'L-93' and the greatest in 'Penncross', suggesting that *A. scabra* was more heat tolerant than both cultivars of *A. stolonifera*. The results were consistent with the species and cultivars variations in heat tolerance previously reported by Rachmilevitch et al. (2006a) and Lyons et al. (2007) based on measurements of carbon metabolism and root growth.

Different heat tolerance between the two species and cultivars could be associated with changes in protein abundance and expression. The decline in soluble protein content did not become significant in thermal *A. scabra* until the temperature was elevated to 40 ºC, but it was detected at 30 ºC in both cultivars of *A. stolonifera* (Fig. 4). SDS-PAGE revealed that most proteins were significantly degraded at 3 d of 45 °C in both cultivars of *A. stolonifera*, while thermal *A. scabra* still maintained strong expression in most protein bands at this temperature and stress duration (Fig. 5). Specifically, a 32 kDa protein started to degrade at 35 ^{o}C in 'Penncross', but did not until 40 ^{o}C in *A. scabra* and 'L-93' (Fig. 7). In particular, the large subunit of Rubisco, with a molecular weight of 55

kDa, exhibited significant down-regulation in both cultivars of *A. stolonifera*. The down-regulation of Rubisco indicated that CO_2 assimilation may be limited at high temperatures (Weis and Berry, 1988). Reduction in single-leaf photosynthetic rate and Rubisco activity was reported in both 'L-93' and 'Penncross' when exposed to 35 ^{o}C (Xu and Huang, 2001). For *A. scabra*, there was no significant degradation of the Rubisco protein at the same temperature and stress duration. The results suggested that *A. scabra* was able to tolerate higher temperatures by maintaining higher protein thermostability, including Rubisco enzymes associated with photosynthesis.

The induction or up-regulation of HSPs has been found in various plant species exposed to heat stress, as previously discussed. Proteins of different sizes can be induced in different plant species due to the genetic variation and differences in temperature level or duration. Our study found the induction or up-regulation of both high-molecular-mass and low-molecular-mass proteins under heat stress, and the level of up-regulation of those different proteins varied with species/cultivars, temperature, and stress duration. The earlier induction of HSPs in both cytoplasm (64, 78, and 85 kDa) and membranes (39, 45, and 66 kDa) has been associated with better heat tolerance in two heat-tolerant cultivars of Kentucky bluegrass ('Eagleton' and 'Midnight'), compared to that in a heat-sensitive cultivar ('Brilliant') (He and Huang, 2007). In this study, up-regulation of an 83 kDa protein was consistently observed in both species during the entire treatment period at 40 ^{o}C and at 14 d of 35 ^{o}C. The induction of this HSP83 has been reported by Park et al. (1996) in both heat-tolerant and non-tolerant variants of 'Penncross' after 1.5 h at 40 °C . Enhanced expression of a HSP83 from arabidopsis by elevated temperature was also reported by Conner et al. (1990). However, we noticed that this protein band was visible

in *A. scabra*, but disappeared in both cultivars of *A. stolonifera* at 3 d of 45 °C. Biochemical studies of HSP83 showed that it forms complexes with several proteins, including members of the steroid hormone receptor superfamily, casein kinase II, eukaryotic initiation factor-2 a-kinase, actin, and tubulin (Lindquist and Craig, 1988). One of the major functions of HSP83 is its involvement in steroid hormone receptors (Conner et al., 1990). Therefore, the distinct maintenance of the HSP83 in *A. scabra* at extremely high temperature (45 ^{o}C) may contribute to its better heat tolerance by affecting hormonal regulation pathways. HSP66 was induced at 14 d of 40 ºC in both species (Fig. 7). The expression was very strong in *A. scabra* but mild in both cultivars of *A. stolonifera*. Expression of HSP60 and HSP70 examined by western blotting also reflected species/cultivar variation in heat tolerance. Heat-tolerant *A. scabra* and 'L-93' exhibited stronger expression of both HSP60 and HSP70 under high temperatures (40 and 45 ^{o}C) than the heat-sensitive cultivar 'Penncross'. The two most-studied families, HSP60 and HSP70, are proposed to act as molecular chaperones, assisting in a large variety of protein folding processes in almost all cellular compartments (Bukau and Horwich, 1998; Gething, 1997). The much stronger induction of these proteins may play a key role in the superior heat tolerance of *A. scabra* over *A. stolonifera*.

Small HSPs are found to be particularly important in plant adaptation to heat stress (Vierling, 1991). Stout and Al-Niemi (2002) reported that the expression of small HSPs (14-21 kDa) was enhanced in root extracts from *D. lanuginosum* experiencing soil temperatures above 40 °C, and suggested that the up-regulation of these proteins were closely related to the degree of heat adaptation to geothermal soils. HSP23 was up-regulated at lower temperatures in heat-tolerant *A. scabra* and moderately heat-tolerant

‘L-93’ than in heat-sensitive cultivar ‘Penncross’. It was induced at both 35 and 40 ºC in ‘L-93’ and *A. scabra* beginning at 7 and 14 d, respectively, but only induced at 40 ºC in ‘Penncross’ after 14 d. The up-regulation of HSP23 has also been reported in tomato (*Lycopersicon esculentum*) plants exposed to heat stress (Liu and Shono, 1999). The 23 kDa proteins are located in Photosystem II (PSII), which are involved in the light-driven oxidation of water in photosynthesis (Seidler, 1996). The up-regulation of 23 kDa proteins in PSII at lower temperature and shorter stress duration for heat-tolerant *A. scabra* and ‘L-93’ could contribute to their maintenance of higher photochemical efficiency for a longer period of heat stress and at higher temperatures.

In summary, the induction of HSPs, particularly small HSPs (23 kDa), at a lower level of heat stress and earlier stress coupled with higher protein thermostability, particularly high molecular weight proteins (83 kDa and large units of Rubisco), could contribute to superior heat tolerance of *A. scabra* adapted to chronically hot geothermal soils. Further study is underway to identify specific proteins involved in heat adaptation in the heat-tolerant *A. scabra* and the moderately tolerant creeping bentgrass cultivar ‘L-93 using 2-dimensional electrophoresis techniques. Those highly up-regulated HSPs in heat-tolerant plants may be used as protein molecular markers in the selection for heat-tolerant grass plants.

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Fig. 1 Changes in photochemical efficiency, measured as Fv/Fm, in *Agrostis scabra* (A), *A. stolonifera* 'Penncross' (B) and *A. stolonifera* 'L-93' (C) with increasing temperatures. Vertical bars on the bottom indicate LSDs (P = 0.05) for treatment comparison at a given day of treatment.

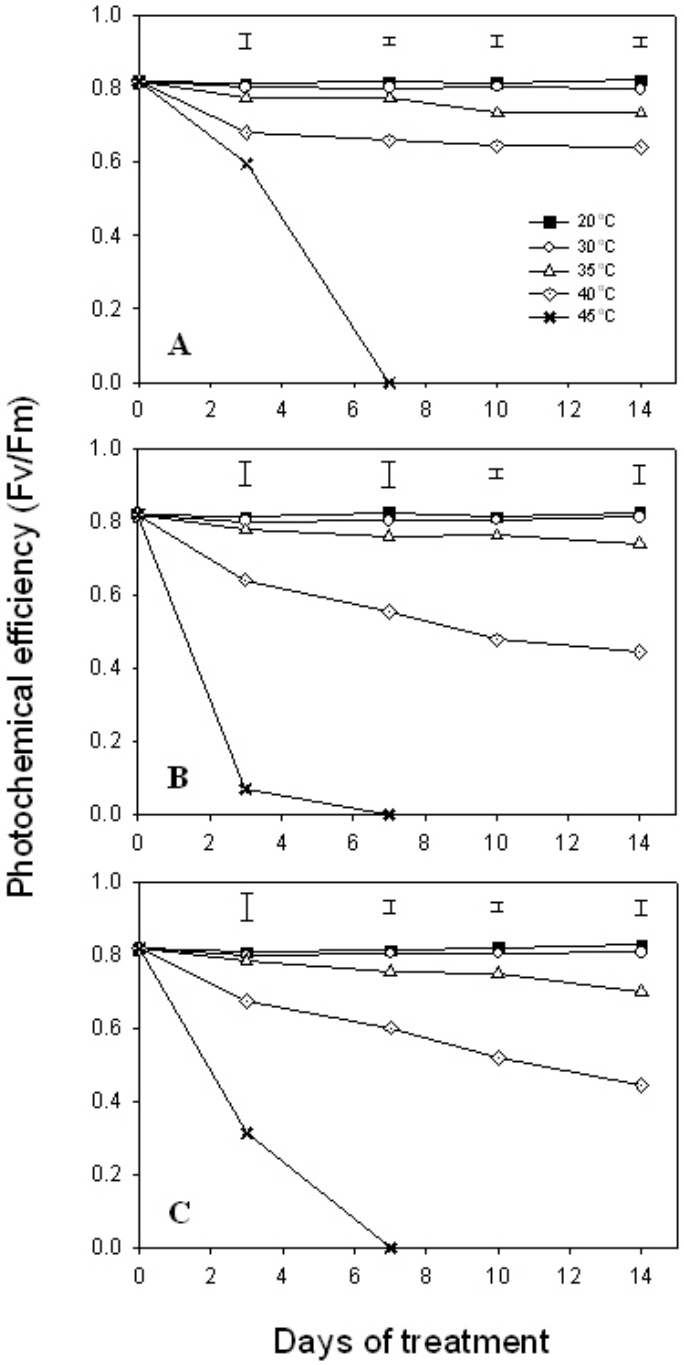


Fig. 2 Changes in chlorophyll content (mg g^{-1}dw) of *Agrostis scabra* (A), *A. stolonifera* 'Penncross' (B) and *A. stolonifera* 'L-93' (C) with increasing temperatures. Vertical bars on the bottom indicate LSDs (P = 0.05) for treatment comparison at a given day of treatment.

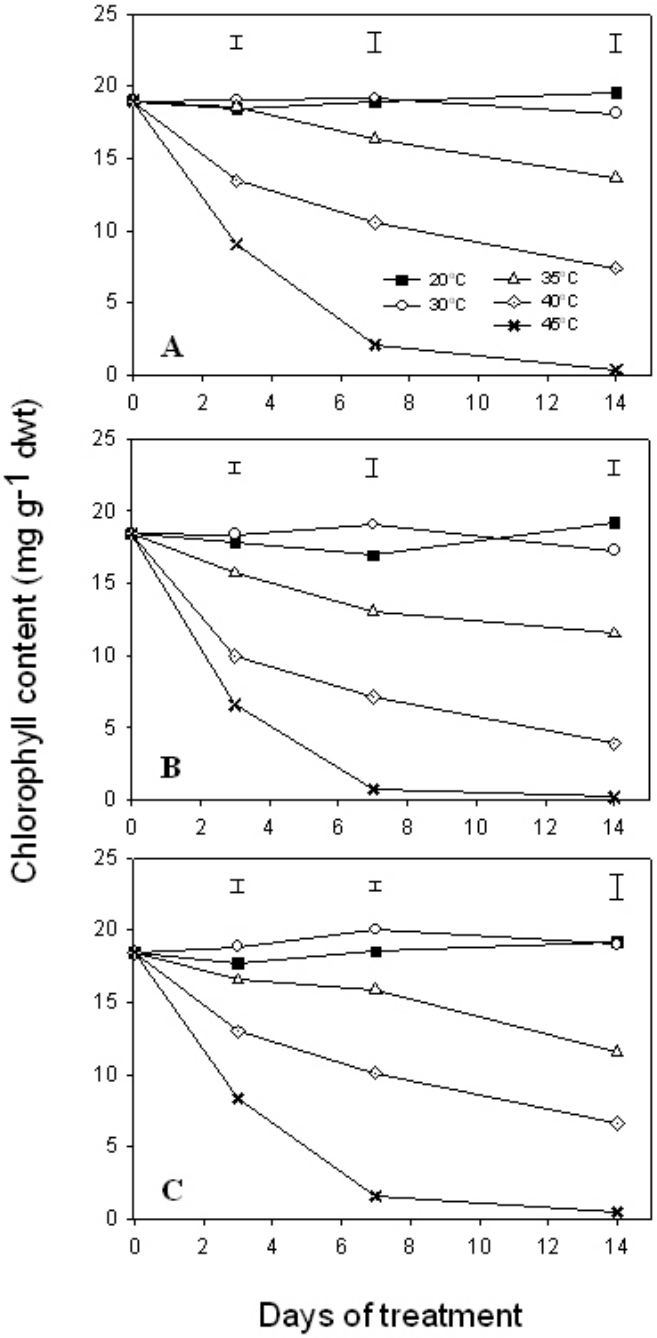


Fig. 3 Changes in carotenoid content (mg g^{-1}dw) of *Agrostis scabra* (A), *A. stolonifera* 'Penncross' (B) and *A. stolonifera* 'L-93' (C) with increasing temperatures. Vertical bars on the bottom indicate LSDs (P = 0.05) for treatment comparison at a given day of treatment.

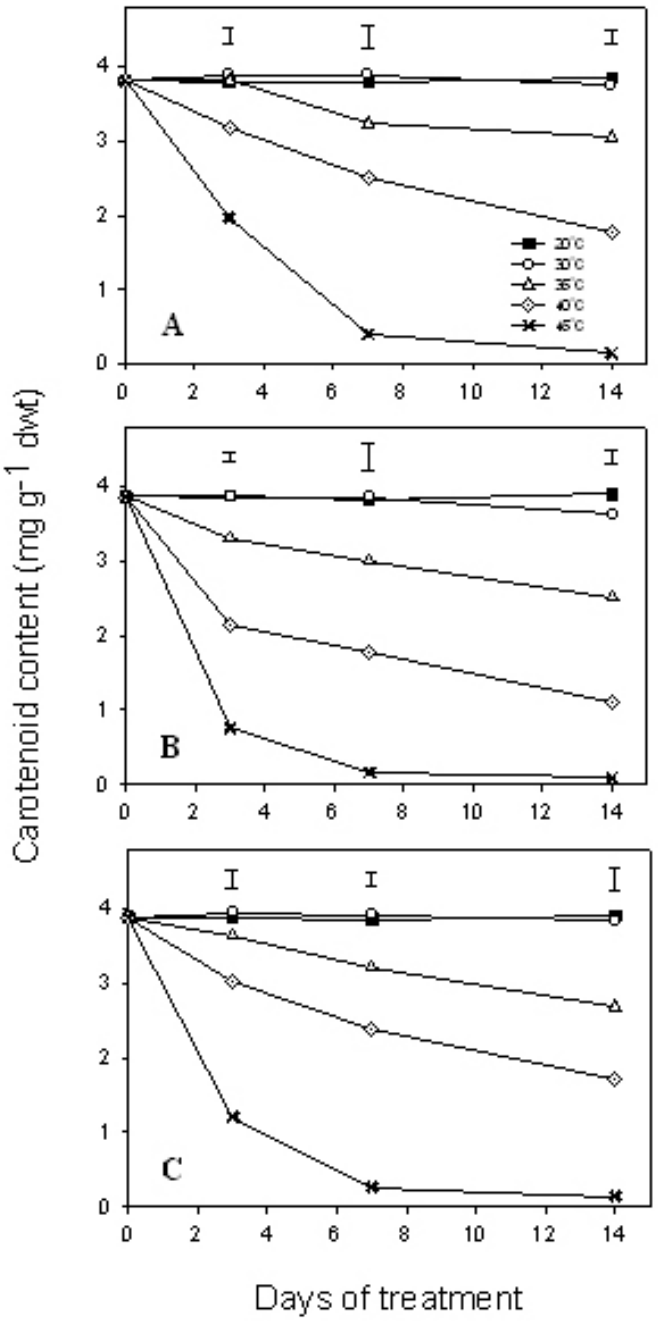


Fig. 4 Changes in total soluble protein content (mg g^{-1}dw) of *Agrostis scabra* (A), *A. stolonifera* 'Penncross' (B) and *A. stolonifera* 'L-93' (C) with increasing temperatures. Vertical bars on the bottom indicate LSDs (P = 0.05) for treatment comparison at a given day of treatment.

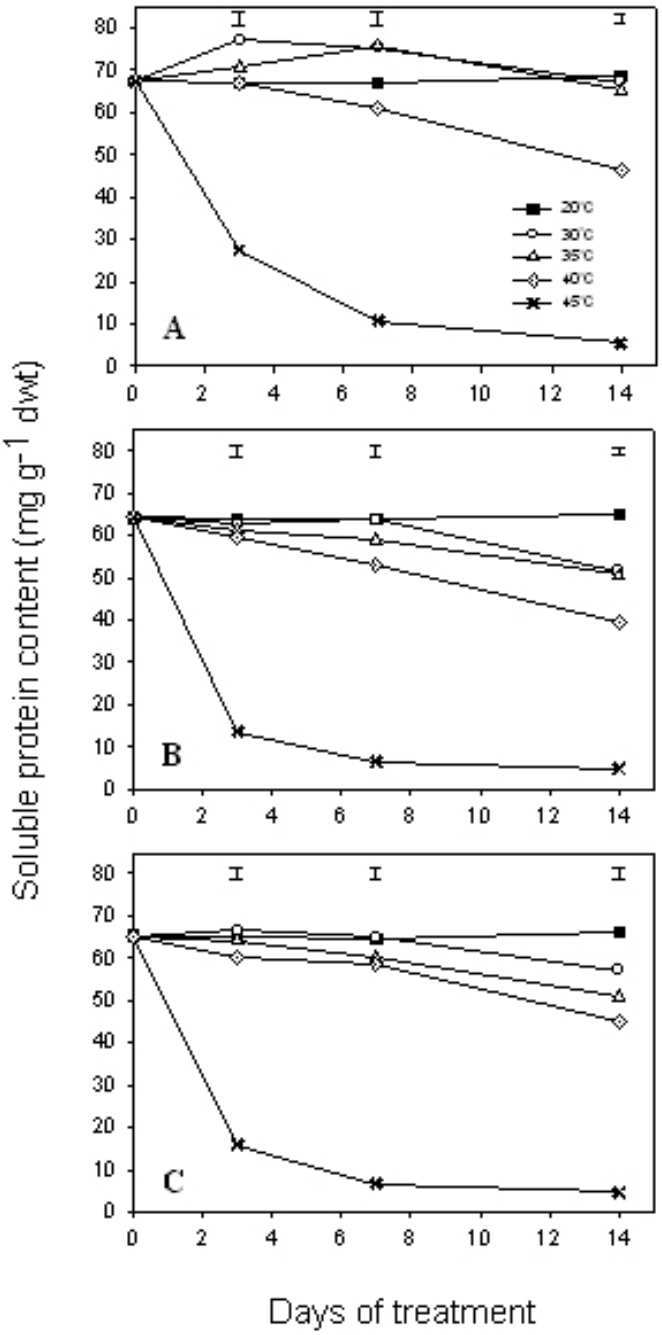


Fig. 5 Soluble protein profile of *Agrostis scabra* (A), *A. stolonifera* 'Penncross' (B) and *A. stolonifera* 'L-93' (C) after 3 d of different temperature treatments. Equal amounts of protein (18 g) were loaded in each lane. Arrows indicate newly induced proteins. M=standard protein marker with standard molecular weight.

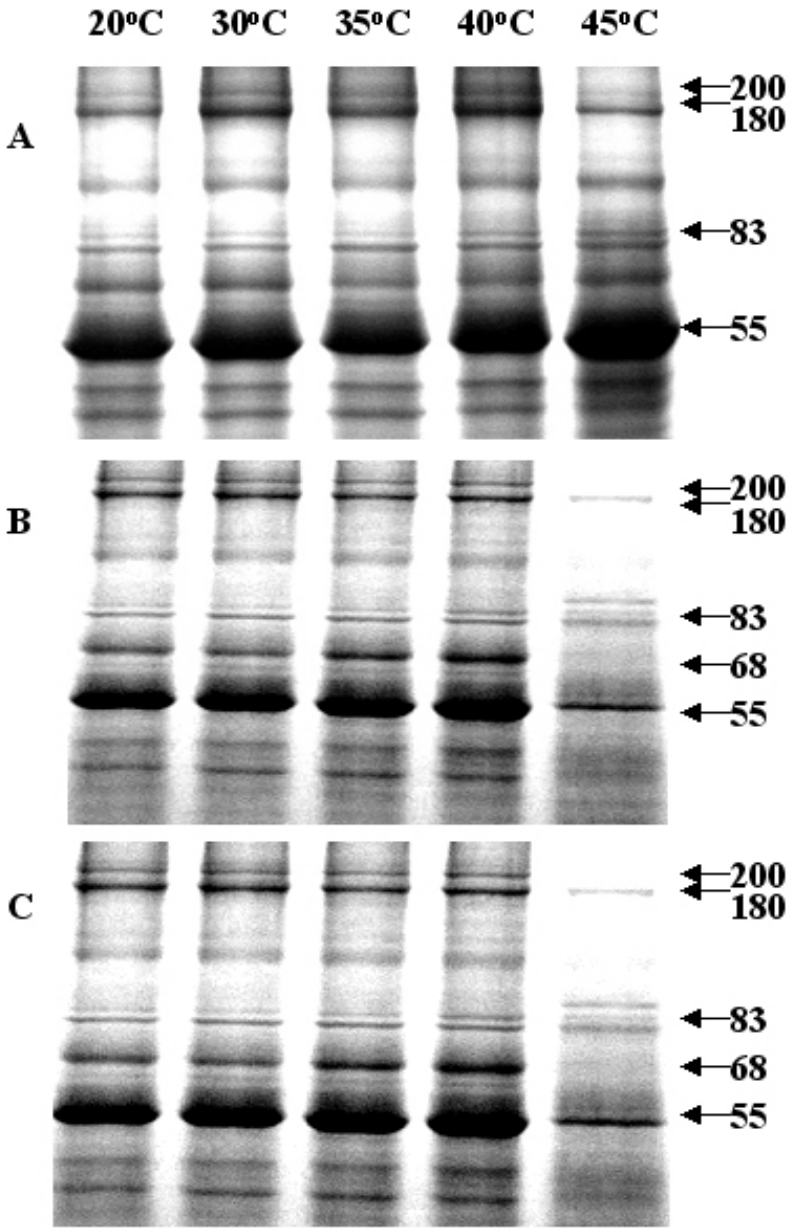


Fig. 6 Soluble protein profile of *Agrostis scabra* (A), *A. stolonifera* 'Penncross' (B) and *A. stolonifera* 'L-93' (C and D) after 7 d of different temperature treatments. Equal amounts of protein (18 g) were loaded in each lane. Arrows indicate newly induced proteins. M=standard protein marker with standard molecular weight.

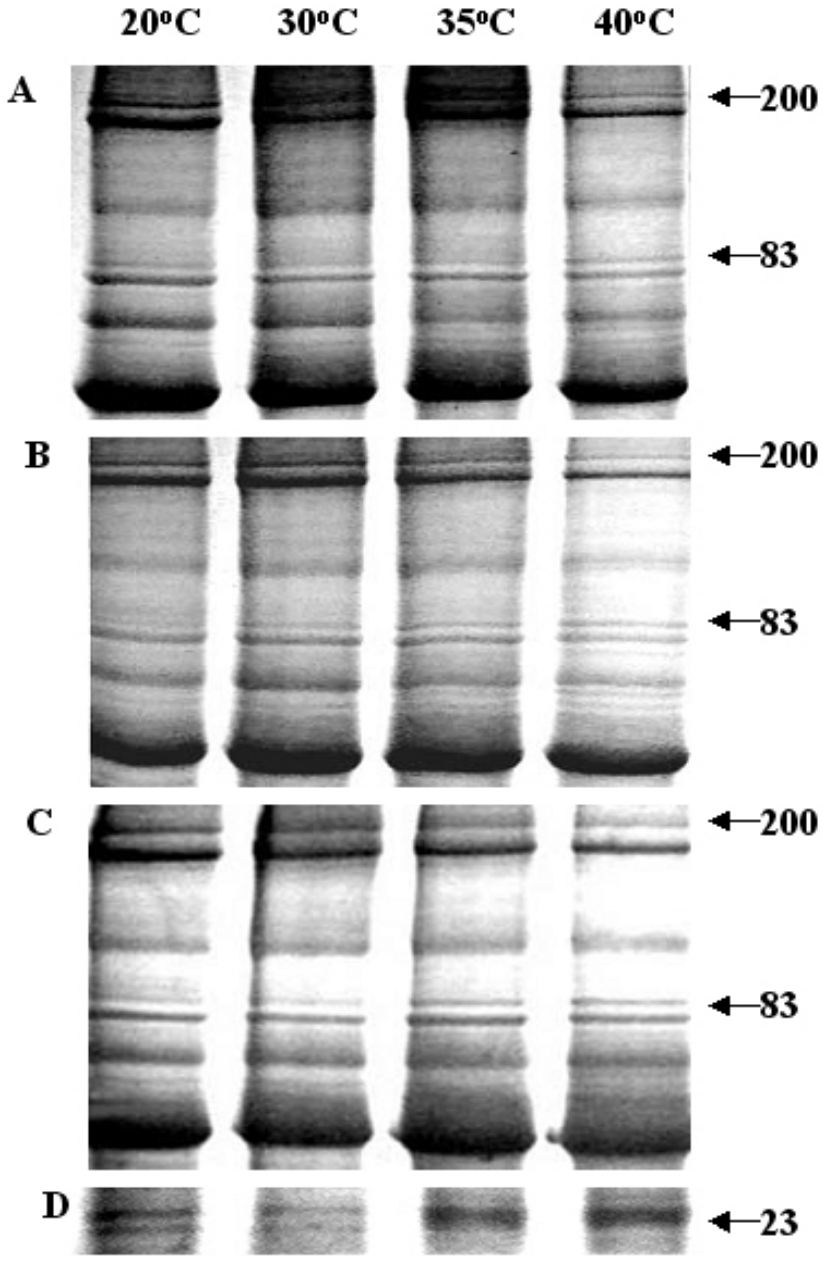


Fig. 7 Soluble protein profile of *Agrostis scabra* (A), *A. stolonifera* 'Penncross' (B) and *A. stolonifera* 'L-93' (C) after 14 d of different temperature treatments. Equal amounts of protein (18 g) were loaded in each lane. Arrows indicate newly induced proteins. M=standard protein marker with standard molecular weight.

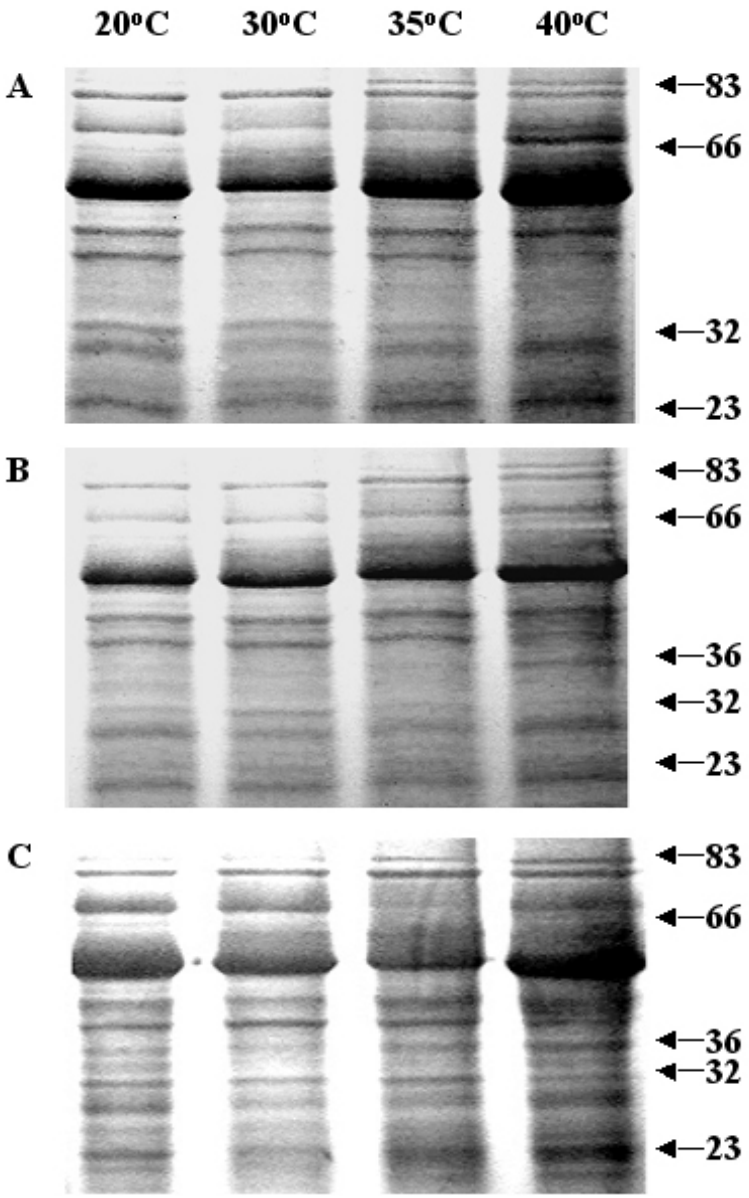
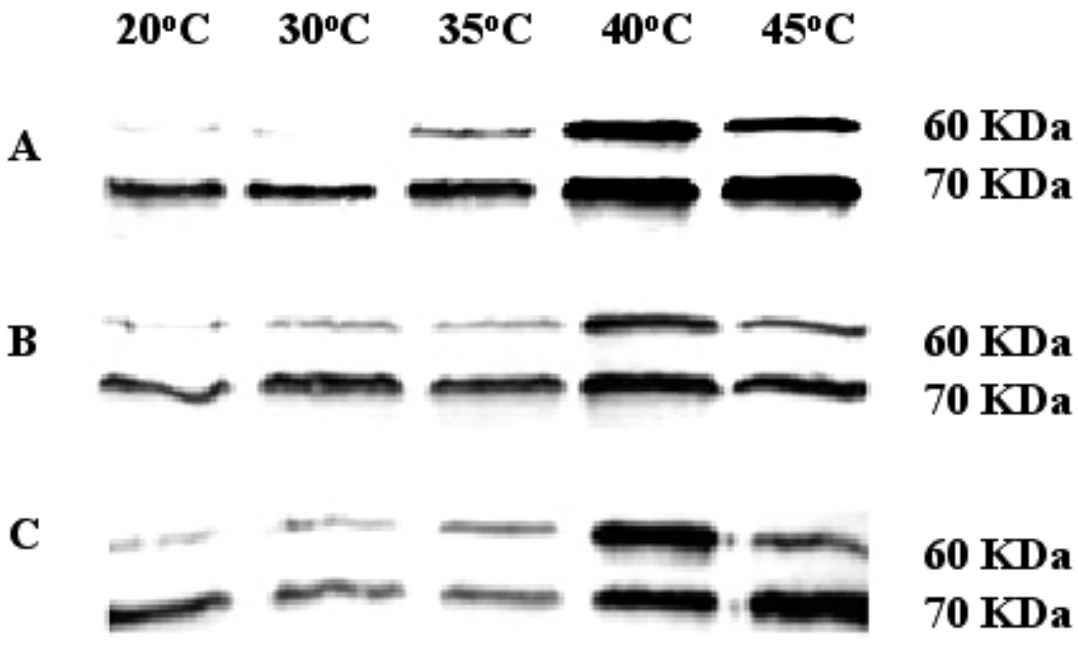


Fig. 8 Immunoblots of HSP60 and HSP70 of *Agrostis scabra* (A), *A. stolonifera* ‘Penncross’ (B) and *A. stolonifera* ‘L-93’ (C) after 3 d of different temperature treatments with HSC70 and HSP60 antibodies. Equal amounts of protein (18 g) were loaded in each lane. M=standard protein marker with standard molecular weight.



CHAPTER 3. Metabolic Profiling for Geothermal *Agrostis scabra* and Turf-Type *Agrostis stolonifera* Contrasting in Heat Tolerance

INTRODUCTION

High temperature is a detrimental environmental factor limiting growth and productivity of cool-season plants, which can disturb a great number of metabolic pathways such as photosynthetic and respiratory activities, energy production and antioxidant metabolism (Sairam et al., 2000; Zobayed et al., 2005). The accumulation of some metabolites and the reduction of others under heat stress may lead to imbalances that are difficult to correct, especially when the levels of regulatory metabolites are forced outside of normal ranges so that regulatory homeostasis cannot function properly to restore normal levels (Taiz and Zeiger, 2006).

One of these imbalances is found in the production and consumption of energy, due to more severe delay in the dark reactions of photosynthesis than the absorption of light and the transport of electrons. The dissipation of excess absorbed light energy under stress is critical to the prevention of photooxidative damage of the photosynthetic apparatus (Niyogi et al., 1998). Metabolites like glutathione, ascorbic acid, α-tocopherol and carotenoids can function as antioxidants and protect plants from the cytotoxic effects of reactive oxygen species (ROS) induced by heat stress that disrupt the cellular homeostasis (Almeselmani et al., 2006). Increased level of foliar ascorbate and glutathione was reported in wheat (*Triticum aestivum*) seedlings in response to heat stress (Dash and Mohanty, 2002).

There is also accumulation of low molecular weight compatible solutes under heat stress, including amino acids (ex. proline), polyamines (ex. putrescine), carbohydrates (ex. sucrose), and sugar alcohols (ex. mannitol), as most abiotic stresses may eventually cause cellular dehydration in plants (Rajam et al., 1998; Rizhsky et al., 2004). Kaplan et al. (2004) highlighted the roles of signaling molecules and the action of a compatible solute network in arabidopsis (*Arabidopsis thaliana*) adaptation to thermal stress and found a combination of compatible solutes that exerts additive or synergistic effects during the induction of thermotolerance. The ability of plants to control the rates of metabolic processes in response to changes in the internal or external environment was considered necessary for conserving the stability of the intracellular environment and essential for maintaining an efficient functional state (Plaxton and McManus, 2006).

A C_3 perennial grass species, thermal rough bentgrass (*Agrostis scabra*), has recently been identified in Yellowstone National Park, Wyoming (Stout and Al-Niemi, 2002). It survives in the chronically hot soils that are permeated by hot steam with temperatures up to 45 ^{o}C (Tercek et al., 2003). On the contrary, common C_3 grasses used as turf or forage in cool climates, including creeping bentgrass (*A. stolonifera*), often suffers from temperatures above 24 ^{o}C (Beard, 1973). Superior thermal tolerance of *A. scabra* has been related to efficient expenditure and adjustment of carbon and nitrogen allocation patterns between growth and respiration (Lyons et al., 2007; Rachmilevitch et al., 2006a). The advent of high throughput profiling technology including genomics, proteomics, and metabolomics has revolutionized the study of plant metabolism and responses to environmental stimuli (Duran et al., 2003; Plaxton and McManus, 2006). By constructing a suppression subtractive cDNA library to compare *A. scabra* with *A.*

stolonifera, Tian et al. (2009) identified hundreds of heat-responsive genes, including a great number of genes involved in stress/defense and protein metabolism. Consistently, up-regulation of heat shock proteins, sucrose synthase, and antioxidant enzymes such as glutathione S-transferase and superoxide dismutase in *A. scabra* was confirmed at the protein level (Xu and Huang, 2008a; Xu and Huang, 2008b). However, a comprehensive profiling analysis that allows simultaneously analysis of metabolites in a complex extract in this thermal grass species is still lacking, which is desirable in order to complement current understanding of heat-tolerant mechanisms at the gene and protein levels.

Hence, we performed a global metabolite-profiling analysis using gas chromatography-mass spectrometry (GC-MS) with the objectives to determine similarities and differences in metabolic responses between thermal *A. scabra* and heat-sensitive *A. stolonefera*, and to identify key metabolites essential for plant tolerance to high temperature. Unveiling and understanding the unique metabolic mechanisms that the thermal grass has evolved to adapt to extreme temperature environments are of great value for improving heat tolerance of other grass species that are sensitive to above-optimum temperature.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

A. stolonifera (cv. Penncross) plugs were collected from field plots at Hort Farm II, Rutgers University, North Brunswick, NJ. Clonal plants of thermal *A. scabra* (ecot. Ntas) were collected from geothermally heated areas in Yellowstone National Park, Wyoming. Both species were planted in well-drained plastic pots (15 cm in diameter and 20 cm deep) filled with sterilized sand and fertilized weekly with 100 mL half-strength Hogland's solution. Plants were cut weekly to maintain a canopy height of approximately 5 cm. After one month of establishment in the greenhouse, plants were transferred into controlled-environment growth chambers (Conviron, Winnipeg, Canada) with a temperature of 20 ºC/18 ºC (day/night), 14-h photoperiod, 65% relative humidity, and 400 μmol m^{-2} s^{-1} photosynthetic photon flux density at the canopy height. Plants were acclimated to growth chamber conditions for one week prior to exposure to different temperature treatments.

Treatments and Experimental Design

Plants of both species were exposed to 40 ºC/38 ºC (day/night) (high temperature) or 20 ºC/18 ºC (day/night) (control) for 2 d (short-term treatment) or 10 d (long-term treatment). Plants were watered twice daily to prevent water deficit in the high temperature treatment. Each temperature treatment was repeated in four growth chambers as four replicates. Species were arranged randomly inside each chamber. The experiment consisted of two factors (temperature and species), which were arranged as a completely randomized block design.

Measurements of Physiological Parameters

Leaf photochemical efficiency of photosystem II (PSII) was measured at the end of each temperature treatment with a fluorescence induction monitor (FIM 1500, Analytical Development Company LTD., Hoddedson, United Kingdom) following a 30 min dark adaptation period. Photochemical efficiency was expressed as the ratio of Fv/Fm, which in fully healthy plants is approximately 0.8. A lower value of Fv/Fm indicates that a proportion of PSII reaction centers are damaged (Fracheboud and Leipner, 2003).

Leaf chlorophyll was extracted from 0.2 g fresh leaves at the end of the temperature treatment. Samples were soaked in dimethyl sulfoxide for 48 h in darkness. The absorbance of leaf extracts was determined with a spectrophotometer (Spectronic Genesys2, Spectronic Instruments, Rochester, NY). Chlorophyll content was calculated based on the absorbance at 663 nm and 645 nm using the formulas described by Arnon (1949).

Extraction, Derivatization and Analysis of Metabolites Using GC-MS

Leaves and roots of each individual plant were harvested separately at the end of the temperature treatment and immediately frozen in liquid nitrogen and stored at –80 ºC until further analysis. Extraction of polar metabolites was performed following the methods of Roessner et al. (2000) and Rizhsky et al. (2004) with modifications. Frozen samples were ground into fine powder with mortars and pestles in liquid nitrogen. Approximately 100 mg powder of each sample was transferred into a 10 mL centrifuge tube and mixed with 1.4 mL 80% (v/v) aqueous methanol. The homogenate was

centrifuged at 200 rpm for 2 h at room temperature, then incubated in a water bath set at 70 °C for 15 min. 100 μL of ribitol solution (2 mg mL^{-1} water) was added as an internal standard prior to the incubation. Afterwards, the extracts were centrifuged at 12000 rpm for 30 min and the supernatants were decanted into new culture tubes. 1.4 mL of water and 0.75 mL of chloroform were added into the tubes. The mixture was vortexed thoroughly and centrifuged at 5000 rpm for 5 min. 300 μL of the polar phase (methanol/water) was decanted into 1.5 mL HPLC vials and dried in a Centrivap benchtop centrifugal vacuum concentrator (Labconco Corporation, Kansas City, MO) overnight. The dried residue was redissolved and derivatized for 90 min at 30 ºC in 80 μL of 20 mg mL^{-1} methoxyamine hydrochloride and then trimethylsilylated for 30 min at 37 ºC with 40μL N-methyl-N-(trimetylsilyl) trifluoroacetamide (MSTFA) (with 1% trimethylchlorosilane).

The chemically modified extracts were subsequently subject to GC-MS analysis following the procedure of Qiu et al. (2007) with modifications. The derivatized extracts were analyzed with a PerkinElmer gas chromatograph coupled with a TurboMass-Autosystem XL mass spectrometer (PerkinElmer Inc., USA). 1 μL aliquot of the extract was injected into a DB-5MS capillary column (30 m × 0.25 mm × 0.25 μm, Agilent J&W Scientific, Folsom, CA). The inlet temperature was set at 260 ºC. After a 5-min solvent delay, initial GC oven temperature was set at 80 ºC; 2 min after injection, the GC oven temperature was raised to 280 ºC with 5 ºC min^{-1}, and finally held at 280 ºC for 13 min. The injection temperature was set to 280 ºC and the ion source temperature was adjusted to 200 ºC. Helium was used as the carrier gas with a constant flow rate set at 1 mL min^{-1}. The measurements were made with electron impact ionization (70 eV) in the full scan

mode (m/z 30-550). Within each GC-MS chromatogram, the peak area of each compound was normalized through being divided by the peak area of the internal standard (ribitol) to get a response ratio. The response ratios were further converted to relative metabolite contents through being divided by dry weights of the samples.

The metabolites were identified by Turbomass 4.1.1 software (PerkinElmer Inc., USA) coupled with commercially available mass spectrum libraries: NIST 2005 (Fisons, Manchest, UK) and Wiley 7.0 (Palisade Cooperation, Yonkers, NY).

Statistical Analysis

The experiment was considered to be a completely randomized split-plot design, with temperature as the main plots and plant lines as the sub-plots. Analysis of variance was based on the general linear model procedure of SAS (SAS Institute Inc., Cary, NC). For GC/MS results, compounds were identified based on retention time and comparison with reference spectra in mass spectral libraries. Peak areas of compounds were integrated with the Genesis algorithm. Treatment means and differences between *A. scabra* and *A. stolonifera* for each physiological parameter and peak area of each metabolite were separated by the least significant difference (LSD) test at 0.05 probability level.

RESULTS

Responses of photochemical efficiency and chlorophyll content to heat stress in A. scabra and A. stolonifera

Plants of both species exhibited significant decline in photochemical efficiency after short-term (2 d) and long-term (10 d) heat stress. After 2 d at 40 ^{o}C, photochemical efficiency declined to similar levels in *A. scabra* (5%) and *A. stolonifera* (6%), compared to their respective values under optimum temperature (20 ^{o}C). However, after 10 d of at 40 ^{o}C, the decline of photochemical efficiency was less greater in *A. scabra* (7%) than in *A. stolonifera* (11%).

There was no significantly change in chlorophyll content in both species after short-term heat stress. After long-term heat stress, chlorophyll content declined in both species compared to their respective values under optimum temperature, and the decline was much greater in *A. stolonifera* (41%) than in *A. scabra* (29%).

Metabolic responses to heat stress in A. scabra and A. stolonifera

Representative GC-MS traces for leaf and root samples are shown in Fig. 2A and 2B, respectively. A total of 50 compounds in the polar extracts of both leaves and roots were identified. The identity as well as retention time of each identified compound was listed in Table 1. Among these 50 metabolites, there were 16 organic acids, 12 sugars and sugar alcohols, 12 amino acids, and 10 other compounds.

Leaf metabolic responses to heat stress in A. scabra and A. stolonifera

The production of 10 identified organic acids was significantly affected by heat stress in the leaves of at least one *Agrostis* species as shown in Fig. 3. The levels of seven metabolites were increased in both species under either short-term or long-term heat stress. Among them, the increase in five metabolites (lactic acid, threonic acid, glyceric acid, aminomalonic acid and α-linolenic acid) was greater in *A. stolonifera*, whereas the increase in the other two metabolites (itaconic acid and gluconic acid) was greater in *A. scabra*. Aconitic acid level was increased only in *A. scabra* (1.6-fold) under long-term heat stress and citric acid level was increased only in *A. stolonifera* under both short-term (1.6-fold) and long-term (1.9-fold) heat stress. Shikimic acid level was decreased in both species, but the decline was less severe in *A. scabra* (58 and 50%) after both short- and long-term heat stress compared to *A. stolonifera* (62 and 77%).

We identified significant changes in the levels of four sugars and two sugar alcohols in leaf response to heat stress (Fig. 4). The production of three sugars (fructose, glucose and floridoside) was significantly reduced in *A. stolonifera* under heat stress, but not in *A. scabra*. Conversely, turanose content increased in both species, to a greater extent in *A. stolonifera* than in *A. scabra* after both 2 and 10 d of heat stress. For the two sugar alcohols, glycol content significantly increased to a similar extent in both species after 2 d of heat stress; mannitol content increased after 2 d of heat stress only in *A. stolonifera*, but in both species after 10 d of heat stress with a greater extent in *A. scabra*.

Ten identified amino acids responded to heat stress in the leaves of both species, all of which exhibited significant increases in their levels (Fig. 5). Nine of them (Val, Ser, Ala, Thr, Gly, 5-Oxoproline, Ile, GABA and Asn) increased after both short- and long-term heat stress, except for Asp that increased only after short-term heat stress. Among

them are two branched-chain amino acids (Val and Ile). Val content increased by 3.3- and 6.2-fold in *A. scabra*, and 2.8- and 3.8-fold in *A. stolonifera* under short- and long-term heat stress, respectively; Ile content also increased greater in *A. scabra* (9.0-fold) than *A. stolonifera* (5.0-fold) after 10 d of heat stress.

Changes in the levels of six other leaf metabolites were also detected in response to heat stress (Fig. 6). Five metabolites were increased in both species under heat stress, except for putrescine, which was increased only in *A. scabra* but not in *A. stolonifera*. Among the five metabolites, deanol content increased by 9.7-fold in *A.scabra* whereas 4.6-fold in *A. stolonifera* after 10 d of heat stress; urea content increased by 4.4- and 3.6-fold in *A. scabra* and *A. stolonifera*, respectively; the contents of phosphoric acid and myo-inositol increased by 3.2- and 4.1-fold in *A. scabra*, but only 1.7-and 2.1-fold in *A. stolonifera*.

Root metabolic responses to heat stress in A. scabra and A. stolonifera

There were eight identified organic acids exhibiting significant changes in the roots of at least one species under heat stress (Fig. 7). Four of them (itaconic acid, aconitic acid, glyceric acid, and citric acid) were decreased in both species under heat stress. Another three organic acids (caffeic acid, malic acid, and gluconic acid) were decreased only in *A. stolonifera* but not in *A. scabra* under heat stress. Acetic acid level was increased in *A. scabra* after 2 and 10 d of heat stress, but only after 2 d of heat stress in *A. stolonifera*.

Six sugars were detected with significant changes in their production in root response to heat stress (Fig. 8). Three of them (fructose, glucose, and galactose) were decreased whereas two of them (sucrose and turanose) accumulated in both species under

heat stress, with greater increases in *A. stolonifera*. Melibiose content was increased only in *A. stolonifera* after 10 d of heat stress.

We identified changes in ten amino acid levels in the roots of *Agrostis* species responding to heat stress (Fig. 9). Seven of them (Ala, Pro, Thr, 5-Oxoproline, Asp, GABA, and Asn) were increased in both species under heat stress. However, significant increases of the other three amino acids (Val, Gly and Ile) were only detected in *A. scabra* but not in *A. stolonifera* under heat stress.

There were six other root metabolites responding to heat stress (Fig. 10). Three metabolites (deanol, 2-pyrrolidinone, and myo-inositol) were increased where as two metabolites (phosphoric acid monomethyl ester and ethanolamine) were decreased in both species under heat stress. Urea content was increased only in *A. stolonifera* but not in *A. scabra* under heat stress.

DISCUSSION

High temperature stress often causes dysfunction of photosynthesis, as indicated by pigment degradation and sensitivity of photochemical reactions in thylakoid membranes (Weis and Berry, 1988). We detected significant declines in chlorophyll content and photochemical efficiency in both *Agrostis* species after being exposed to 40 ^{o}C for 10 d, but thermal *A. scabra* had less dramatic reduction in both parameters under heat stress compared to heat-sensitive *A. stolonifera*. Previous studies also detected higher root viability, cell membrane stability and nutrient uptake activity in *A. scabra* than *A. stolonifera* (Lyons et al., 2007; Rachmilevitch et al., 2006b). The consistent variations in the physiological parameters between the two species validated the feasibility to integrate information on metabolic profiling obtained in this study with previous findings at the gene and protein levels to determine key players in thermotolerance of *Agrostis* species.

Differential responses of organic acids to heat stress in the two Agrostis species

Organic acids are low molecular weight compounds found in all organisms and characterized by the possession of one or more carboxyl groups. Some of the them are involved in energy production as intermediates in the tricarboxylic acid (TCA) cycle, and others are primarily present in cells for cation charge balancing or for maintaining osmotic potential (Jones, 1998).

Citric acid and aconitic acid are two major organic acids in the TCA cycle, which is a hub of metabolism with central importance in both energy production and biosynthesis. It is crucial for the cell to regulate concentrations of TCA cycle metabolites

in the mitochondria to retain homeostasis of cellular metabolism. In the current study, there was significant accumulation of aconitic acid in the leaves of *A. scabra* when citric acid levels were not affected under prolonged heat stress. Conversely, in the leaves of *A. stolonifera*, citric acid levels were increased under short- and long-term heat stress when no significant changes in aconitic acid levels were detected. The fact that aconitic acid can be synthesized by thermal dehydration of citric acid suggests the net flux from citric acid to aconitic acid may favor the adaptation of the thermal species to hot environments.

Shikimic acid is involved in the biosynthesis of various aromatic plant primary metabolites including the aromatic amino acids, which then participate in the production of numerous secondary plant products such as anthocyanins, lignin, growth regulators, and phenolics. The lower reduction in shikimic acid in *A. scabra* under heat stress indicated less damage of heat to this fundamental metabolic pathway, which holds the supply of many important metabolites derived from it for plant's survival under heat stress.

Threonic acid has been reported as a natural constituent in the leaves of only a few plant species and been related to amino acid catabolism and glyceric acid production (Helsper and Loewus, 1982). Accumulation of threonic acid might be related to ascorbic acid breakdown (Pedreschi et al., 2009). The accumulation of glyceric acid under stress may be associated with stomatal opening as reported by Pallas and Wright (1973) that its content increased with decreases in stomatal resistance in the epidermis of *Vicia faba*. We observed less accumulation of both threonic acid and glyceric acid in *A. scabra* under heat stress, which may indicate less severe amino acid breakdown and higher stomatal resistance for water conservation under heat stress.

Itaconic acid and gluconic acid levels were increased more in the leaves of *A. scabra* and decreased less in the roots of *A. scabra* under heat stress compared to *A. stolonifera*. Itaconate has been found to reduce the incidence of germination, onset of germination, and growth of the embryo axis as well as the specific activity of isocitrate lyase in seedlings of *Zea mays, Vigna glabra, Glycine hispida, Vigna sinensis, Trigonella foenumgraecum, Lens culinaris*, and *Medicago sativa* (Khan and McFadden, 1979). Considerable increase of gluconic acid may indicate the activation of the pentose phosphate pathway (Pedreschi et al., 2009).

Lactic acid is a typical fermentation product whose concentration does not increase until the rate of lactate production exceeds the rate of lactate removal. The earlier and greater accumulation of lactic acid in *A. stolonifera* under heat stress indicates higher heat sensitivity of such governing factors as monocarboxylate transporters and lactate dehydrogenase (LDH), resulting in shifts from the normal cellular metabolism of the respiratory pathways to the energetically far less efficient fermentation pathways (Pedreschi et al., 2009).

In the roots, production of malic acid was increased in *A. scabra* but decreased in *A. stolonifera* transiently. A decrease of malic acid indicated a reduced metabolic activity at the level of the Krebs cycle (Pedreschi et al., 2009), whereas the intensified synthesis of malate has been related to ROS defense, constituting a reserve of reduced carbon equivalents (Rzepka et al., 2009). Caffeic acid level was decreased only in the roots of *A. stolonifera* but not in *A. scabra* under heat stress. Caffeic acid has been found to enhance the activities of peroxidases and polyphenol oxidases, which relates to lignification and phenolic metabolism during rhizogenesis of mung bean (*Phaseolus aureus*) (Batish et al.,

2008). Increased caffeic acid concentration also contributed to the accumulation of important defense compounds in root periderm of sweet potato (*Ipomoea batatas*) under drought stress (Harrison and Peterson, 2006).

Response of sugars and sugar alcohols to heat stress in the two Agrostis species

Soluble sugars, especially sucrose, glucose, and fructose, play an obviously central role in plant structure and metabolism at the cellular and whole-plant levels (Couee et al., 2006). Reduced fructose and glucose production was detected in the leaves and roots of both *Agrostis* species under heat stress, with less decrease in *A. scabra*. Floridoside content significantly decreased in the leaves of *A. stolonifera* but not in *A. scabra* under heat stress. Sucrose is a well-known signaling molecule and compatible solute during abiotic stresses. A quick rise in sucrose content was observed in arabidopsis in response to heat stress (Kaplan et al., 2004). Less accumulation of sucrose and other soluble sugars including turanose and melibiose may indicate less heat-induced injuries in *A. scabra* under heat stress, such as starch degradation or cell wall breakdown due to enzymatic processes or chemical reactions of hydroxyl radicals (Rizhsky et al., 2004)).

Sugar alcohols can mimic the structure of water and maintain an artificial sphere of hydration around the macromolecules because of their water-like hydroxyl groups, so that they may protect macromolecules from rapid osmotic changes and oxidative stress (Tarczynski et al., 1993). They may also function as scavengers of ROS and prevent peroxidation of lipids and the subsequent cell damage (Smirnoff and Cumbes, 1989). We detected transient increase of glycol content in the leaves of both *Agrostis* species under heat stress, which did not last when heat stress was prolonged. Short-term heat stress

triggered early accumulation of mannitol in the leaves of *A. stolonifera*; however, when heat stress is prolonged, the accumulation was greater in *A. scabra*. Mannitol plays an important role in storage of carbon and energy, regulation of co-enzymes, osmoregulation and free-radical scavenging (Stoop et al., 1996). Prabhavathi et al. (2002) found that transgenic eggplants (*Solanum melongena*) containing bacterial mannitol-1-phosphate dehydrogenase (*mtlD*) gene showed increased tolerance to abiotic stresses.

Response of amino acids to heat stress in the two Agrostis species

In addition to their primary roles as intermediates in the synthesis and catabolism of proteins, many of the amino acids may function as defensive agents against abiotic stresses. A linear increase in amino acid levels with the induction of drought stress was detected in rapeseed (*Brassica napus*) leaves by Good and Zaplachinski (1994).

We detected higher accumulation of two branched-chain amino acids, Ile and Val, in *A. scabra*. They are known to serve as precursors for such metabolites as cyanogenic glycosides, which are derived from secondary metabolism and act in defense mechanisms (Kaplan et al., 2004). The greater accumulation of Ile and Val in both leaves and roots of *A. scabra* under heat stress may support increased production of secondary metabolites as part of a defense response. Increased synthesis rates of branched-chain amino acids were also reported in cowpea (*Vigna unguiculata*) subjected to heat shock (Mayer et al., 1990).

5-Oxoproline levels accumulated more in both leaves and roots of *A. scabra* under heat stress. Conversion of 5-oxoproline to glutamate is required in the regeneration of glutathione, an essential component of the cellular antioxidative defense system (Meister, 1983). The higher 5-Oxoproline levels in *A. scabra* may indicate a more

responsive and efficient glutathione-mediated detoxification pathway. This accumulation corresponds with the enhancement of different transcripts involved in glutathione biosynthesis under heat stress (Rizhsky et al., 2004). More significant up-regulation of three glutathione-S-transferase proteins was previously detected in the roots of this thermal species compared to *A. stolonifera* under heat stress (Xu and Huang, 2008a).

It is worth noting the significant accumulation of GABA in leaves and roots of both *Agrostis* species under heat stress. Rapid accumulation of GABA in the cytosol of plant cells was found in various environmental stresses including heat stress (Kinnersley and Turano, 2000; Rizhsky et al., 2004). GABA is transported into mitochondria and metabolized through a pathway known as GABA shunt, leading to the production of Ala (Taiz and Zeiger, 2006). GABA shunt has been associated with various physiological responses, including the regulation of cytosolic pH, carbon and nitrogen metabolism, protection against pathogens and oxidative stress, osmoregulation and signaling (Bouche and Fromm, 2004; Fait et al., 2008). The collective accumulation of GABA and Ala we detected in both *Agrostis* species under heat stress may indicate an active role for this pathway in relieving heat injuries on metabolism. Coordinate increases in GABA and Ala synthesis rates were previously reported in cowpea (Mayer et al., 1990) and arabidopsis (Kaplan et al., 2004) subjected to heat treatment.

Response of other metabolites to heat stress in the two Agrostis species

The accumulation of a polyamine, putrescine, was only detected in thermal *A. scabra* but not in *A. stolonifera* under prolonged heat stress. Coordinate accumulation of putrescine and other metabolites with protective roles were detected in the arabidopsis

metabolome (Kaplan et al., 2004). The variations in putrescine were found to parallel the constitutive variation of antioxidant enzymes and oxidant resistance in wheat (Ye et al., 1997).

Myo-inositol, a polyol, can function as an osmolyte and its accumulation was reported in many plants subjected to various environment stresses that can cause cellular dehydration (Ishitani et al., 1996; Klages et al., 1999). Higher levels of myo-inositol, was detected in both leaves and roots of *A. scabra* under heat stress, which may lead to better osmoprotection in this species.

In summary, the superior heat tolerance of *A. scabra* over *A. stolonifera* was detected at the metabolite level, as manifested by differential accumulation of certain organic acids, sugars and sugar alcohols, and amino acids under heat stress. Specifically, the production of a few organic acids involved in the TCA cycle (ex. citric acid, aconitic acid), soluble sugars that play a central role in plant structure and metabolism (ex. fructose, glucose), and amino acids involved in the antioxidative defense system (ex. 5-Oxoproline) were either up-regulated to a greater extent or down-regulated to a less severe extent in *A. scabra* responding to heat stress.

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Table 1 Identities of the 50 metabolites detected by GC-MS and their respective retention time (RT) in the leaves and roots of *A. scabra* and *A. stolonifera* growing under optimal temperature (20 ^{o}C) or heat stress (40 ^{o}C) for 2 d and 10d.

No.	Compound name	RT (min)	No.	Compound name	RT (min)
	Organic acids		27	Glycerol	10.985
1	Pyruvic acid	5.524	28	Mannitol	26.099
2	Lactic acid	5.718			
3	Acetic acid	6.082		**Amino acids**	
4	2-hydroxy Propenoic acid	6.336	29	Val	6.396
5	Succinic acid	12.057	30	Ala	6.671
6	Glyceric acid	12.433	31	Gly	7.148
7	Itaconic acid	12.765	32	Ile	11.476
8	Aminomalonic acid	15.960	33	Pro	11.520
9	Malic acid	16.459	34	Ser	13.207
10	Threonic acid	18.175	35	Thr	13.846
11	Aconitic acid	22.570	36	5-Oxoproline	17.147
12	Shikimic acid	23.836	37	Asp	17.218
13	Citric acid	23.935	38	GABA	17.389
14	Gluconic acid	27.735	39	Asn	20.682
15	Caffeic acid	30.130	40	Tyr	26.335
16	α-linolenic acid	31.579			
				Others	
	Sugar and sugar alcohols		41	Deanol	4.367
17	Fructose	25.022	42	2-pyrrolidinone	7.685
18	Galactose	25.367	43	Phosphoric acid monomethyl ester	8.481
19	Glucose	25.537	44	Urea	10.258
20	Mannose	25.793	45	Ethanolamine	10.733
21	Maltose	27.170	46	Phosphoric acid	10.890
22	Sucrose	38.391	47	2-Furanone, 3,4-dihydroxytetrahydro	13.491
23	Melibiose	43.273	48	6-hydroxy-2-aminohexanoic acid	19.594
24	Floridoside	46.197	49	Putrescine	22.082
25	Turanose	53.259	50	Myo-inositol	29.143
26	Glycol	4.314			

Fig. 1 Changes in photochemical efficiency (A) and chlorophyll content (B) in response to short-term (2 d) and long-term (10 d) heat stress in *A. scabra* and *A. stolonifera*. "*" was given when a significant temperature effect exists in a given species on a given date.

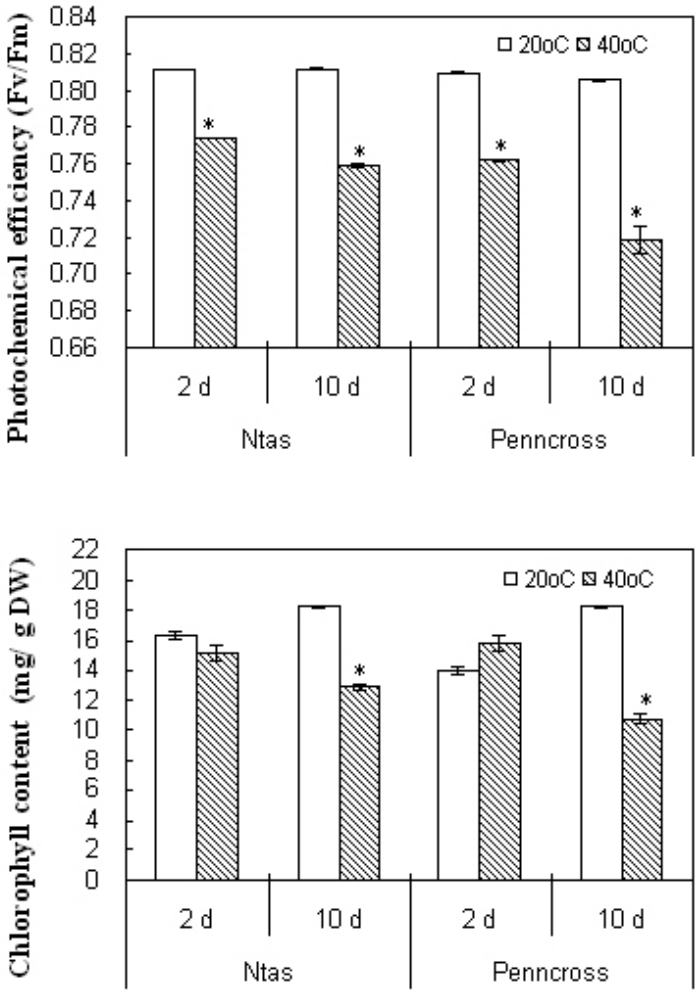


Fig. 2 GC-MS profiles of polar extracts obtained from leaves (A.) and roots (B.) of the *Agrostis* species.

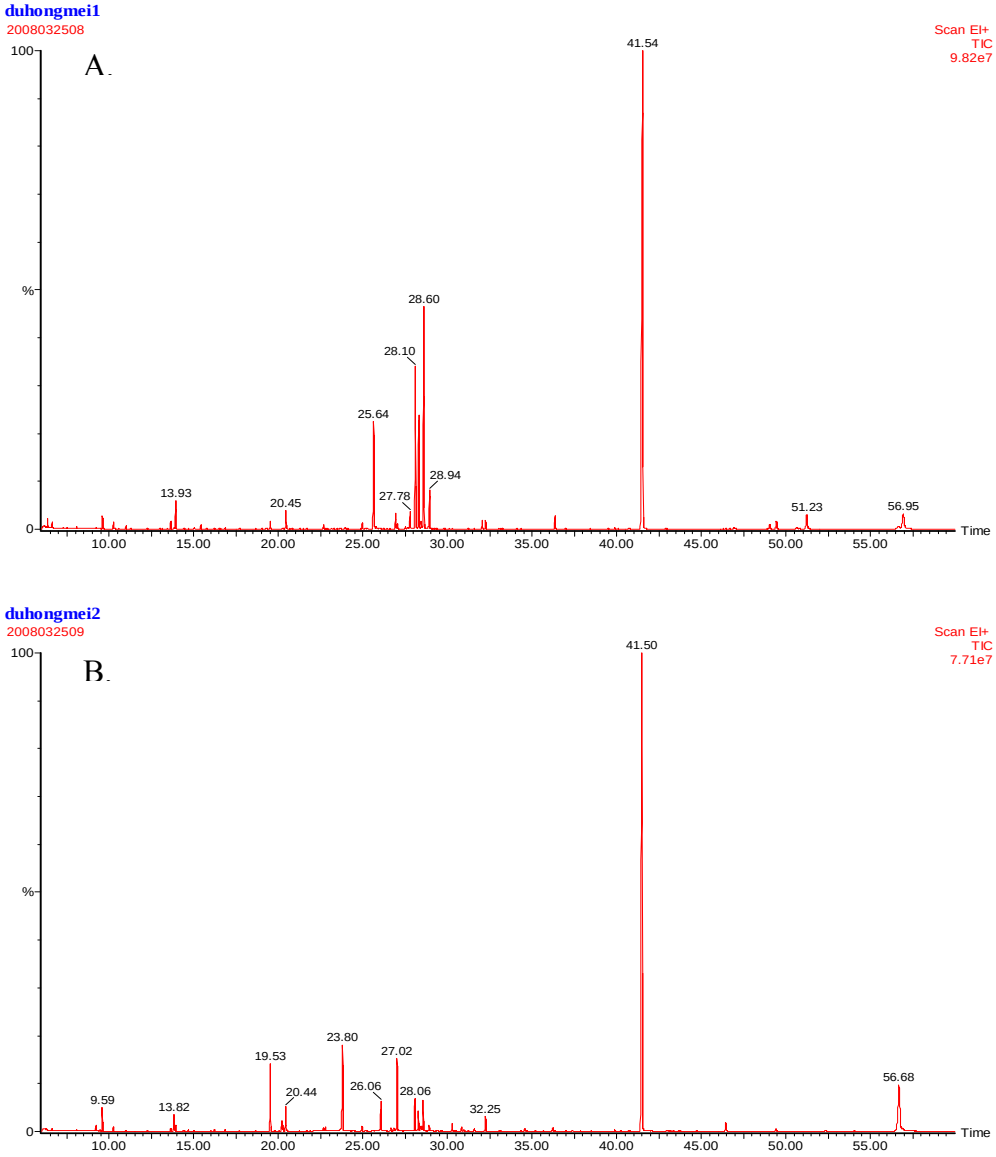


Fig. 3 Metabolic responses of organic acids to short-term (2 d) and long-term (10 d) heat stress in the leaves of *A. scabra* and *A. stolonifera*.

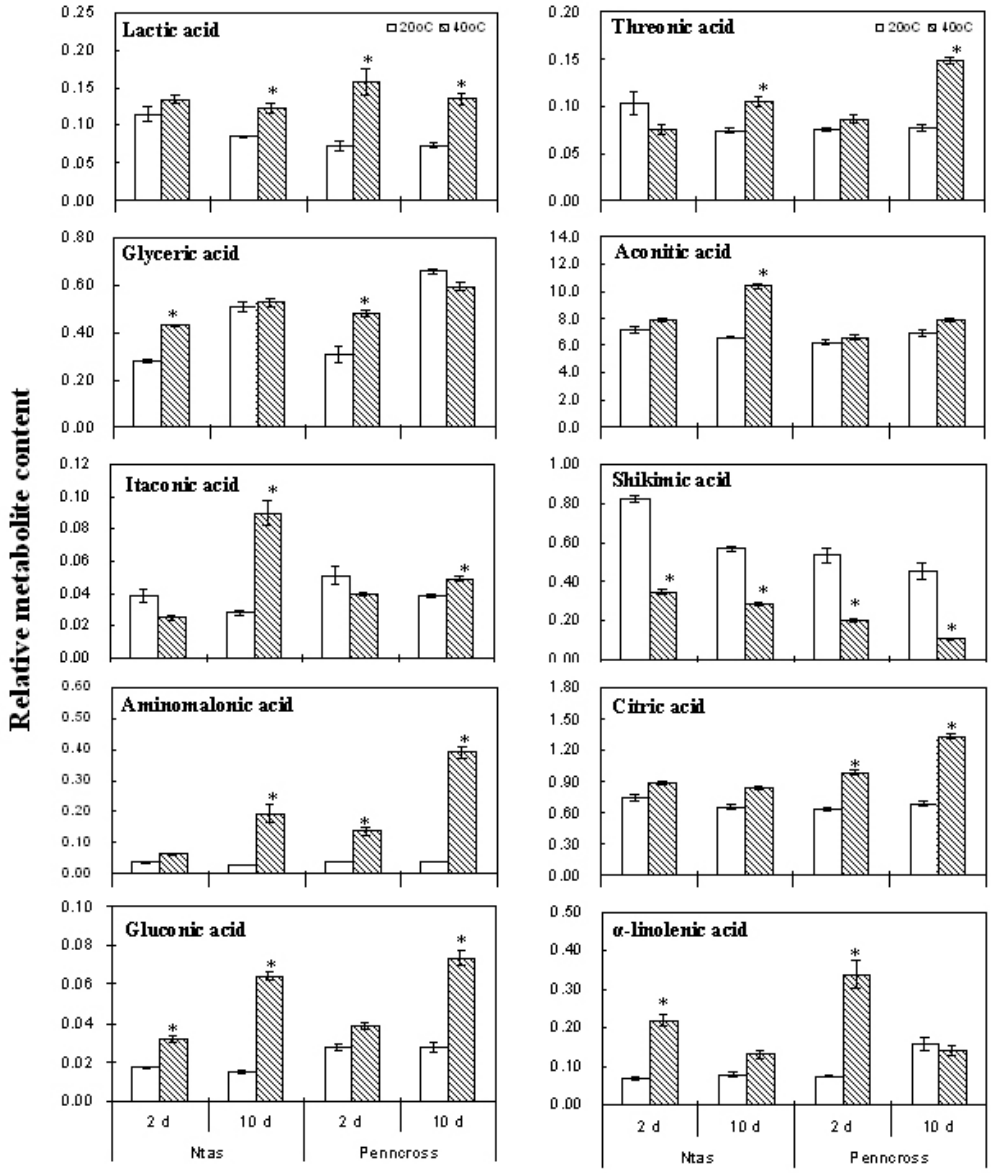


Fig. 4 Metabolic responses of sugars and sugar alcohols to short-term (2 d) and long-term (10 d) heat stress in the leaves of *A. scabra* and *A. stolonifera*.

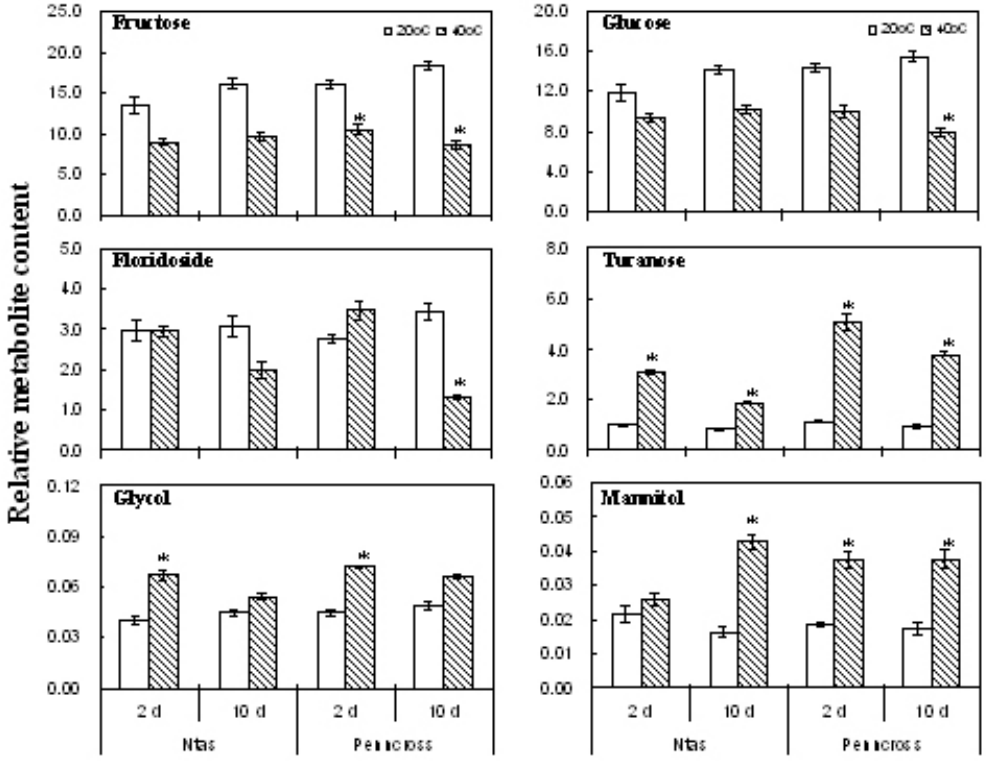


Fig. 5 Metabolic responses of amino acids to short-term (2 d) and long-term (10 d) heat stress in the leaves of *A. scabra* and *A. stolonifera*.

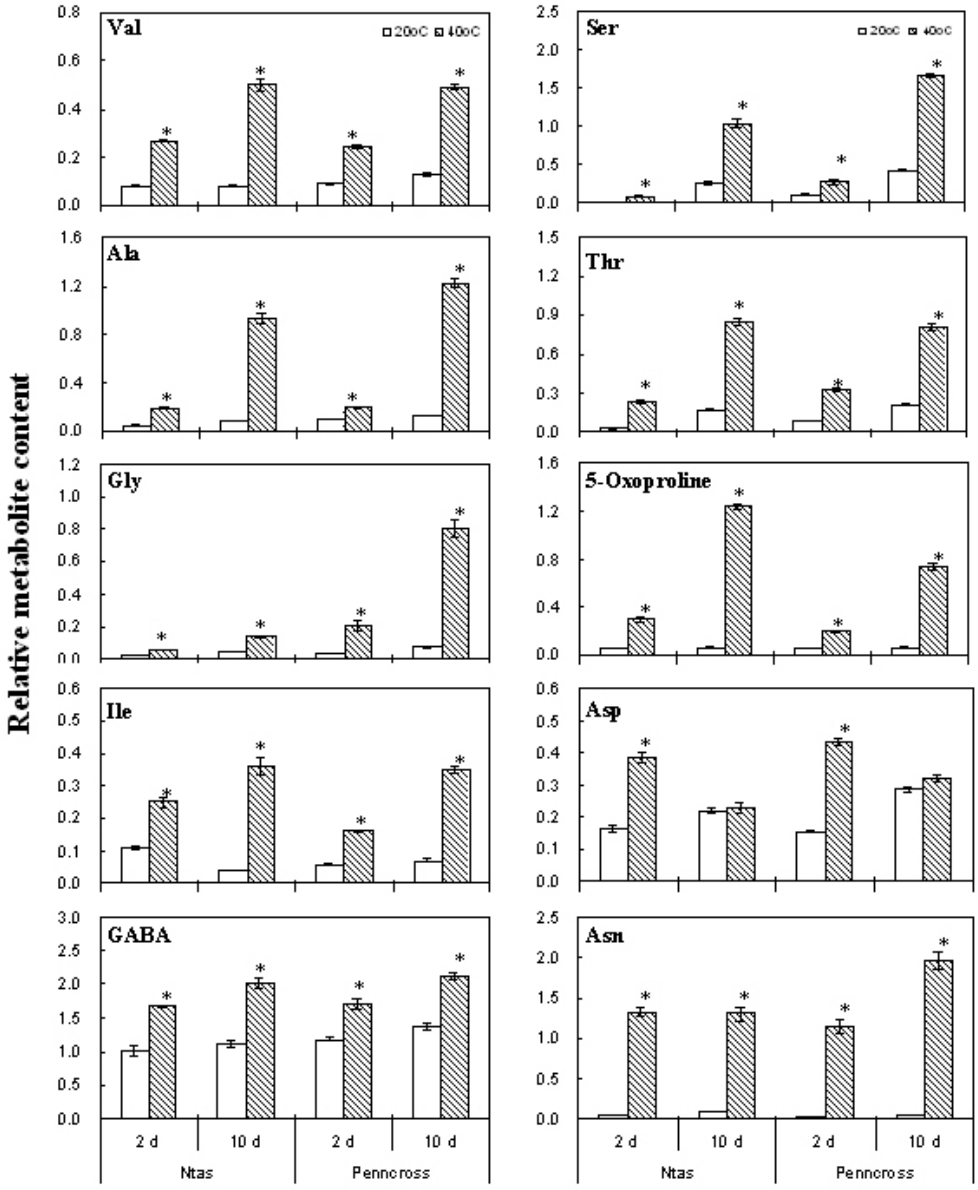


Fig. 6 Metabolic responses of other compounds to short-term (2 d) and long-term (10 d) heat stress in the leaves of *A. scabra* and *A. stolonifera*.

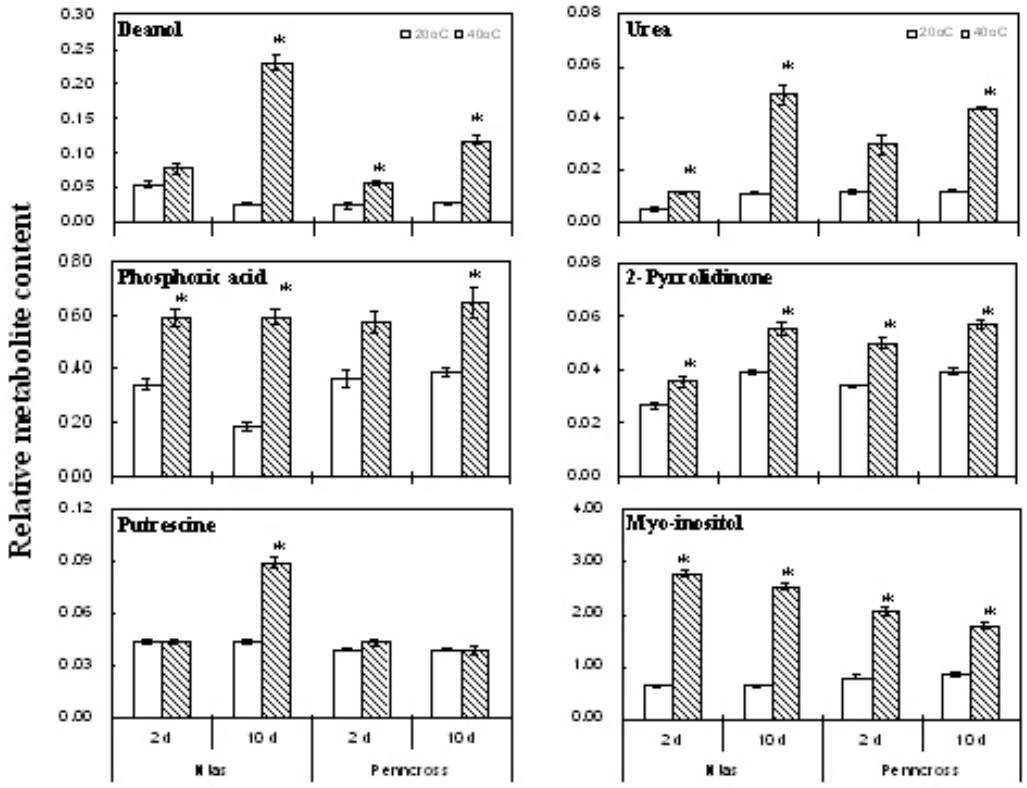


Fig. 7 Metabolic responses of organic acids to short-term (2 d) and long-term (10 d) heat stress in the roots of *A. scabra* and *A. stolonifera*.

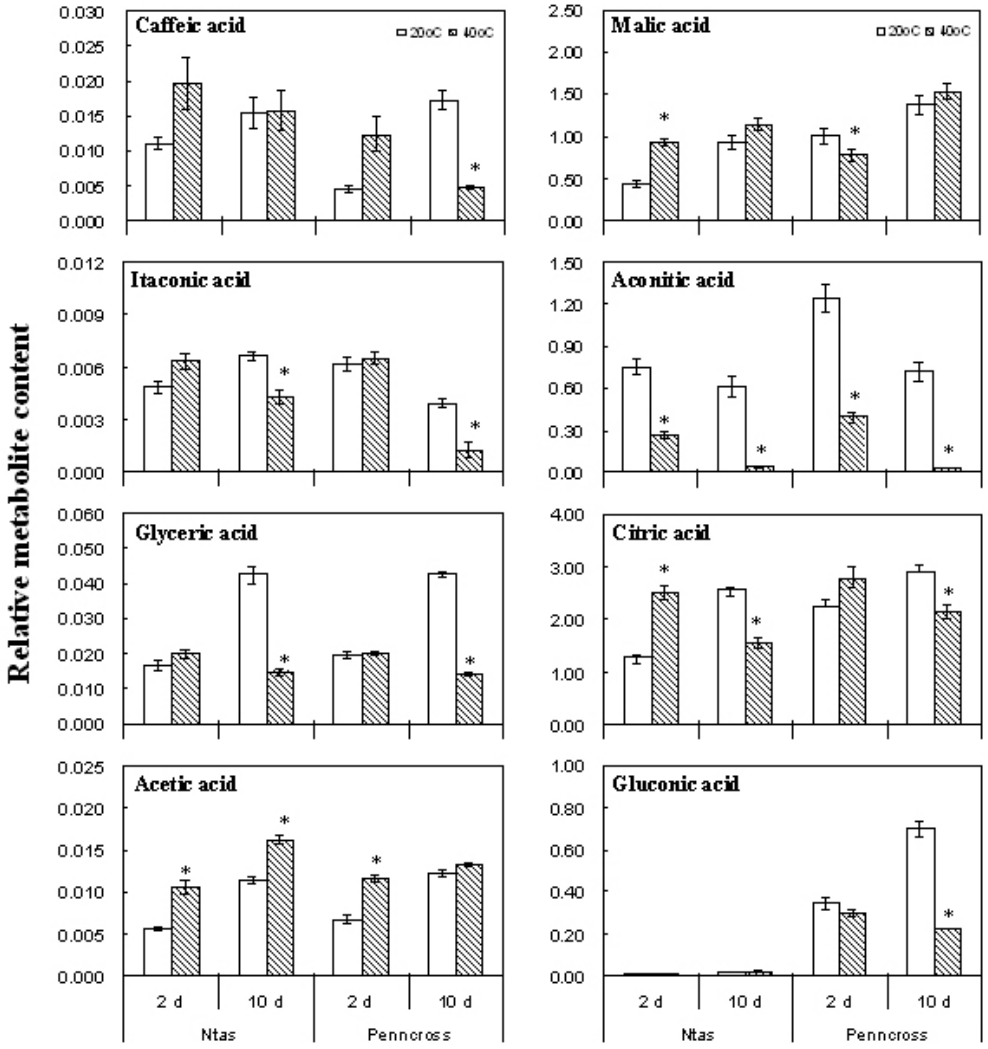


Fig. 8 Metabolic responses of sugars and sugar alcohols to short-term (2 d) and long-term (10 d) heat stress in the roots of *A. scabra* and *A. stolonifera*.

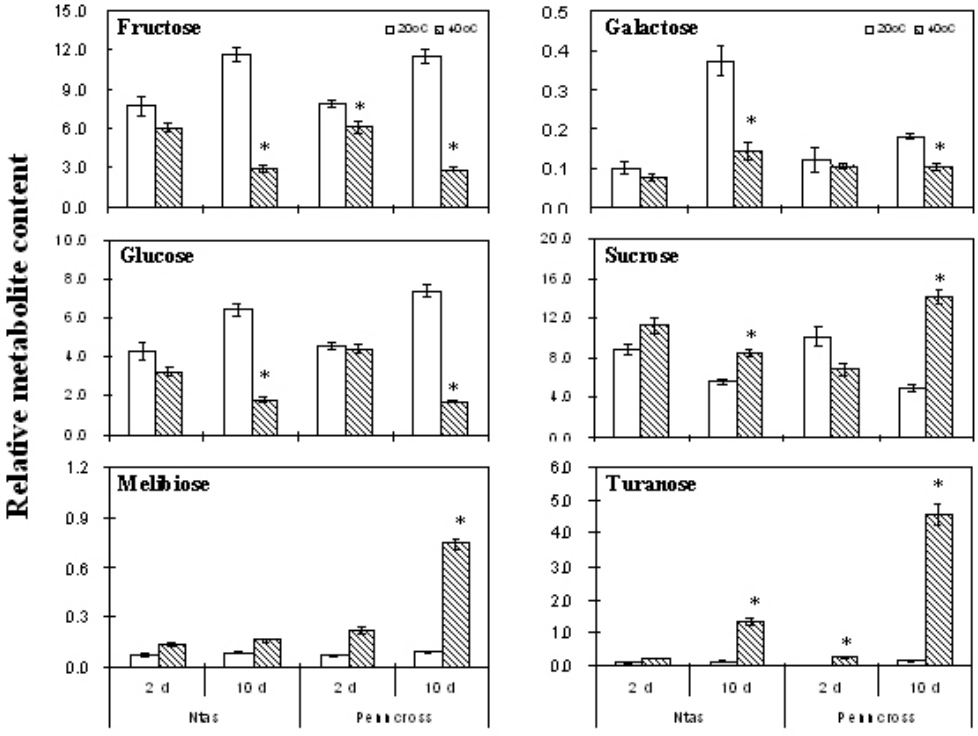


Fig. 9 Metabolic responses of amino acids to short-term (2 d) and long-term (10 d) heat stress in the roots of *A. scabra* and *A. stolonifera*.

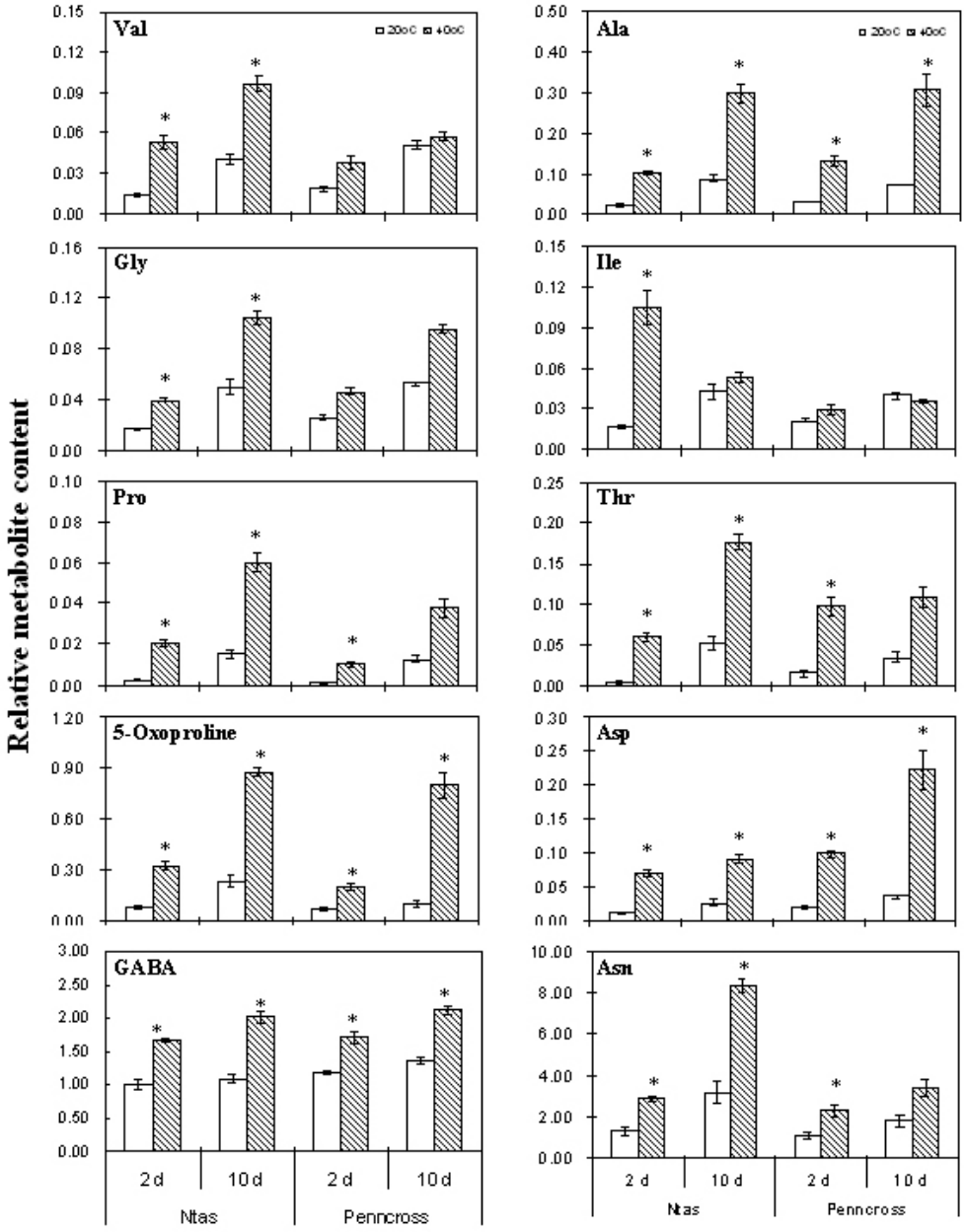
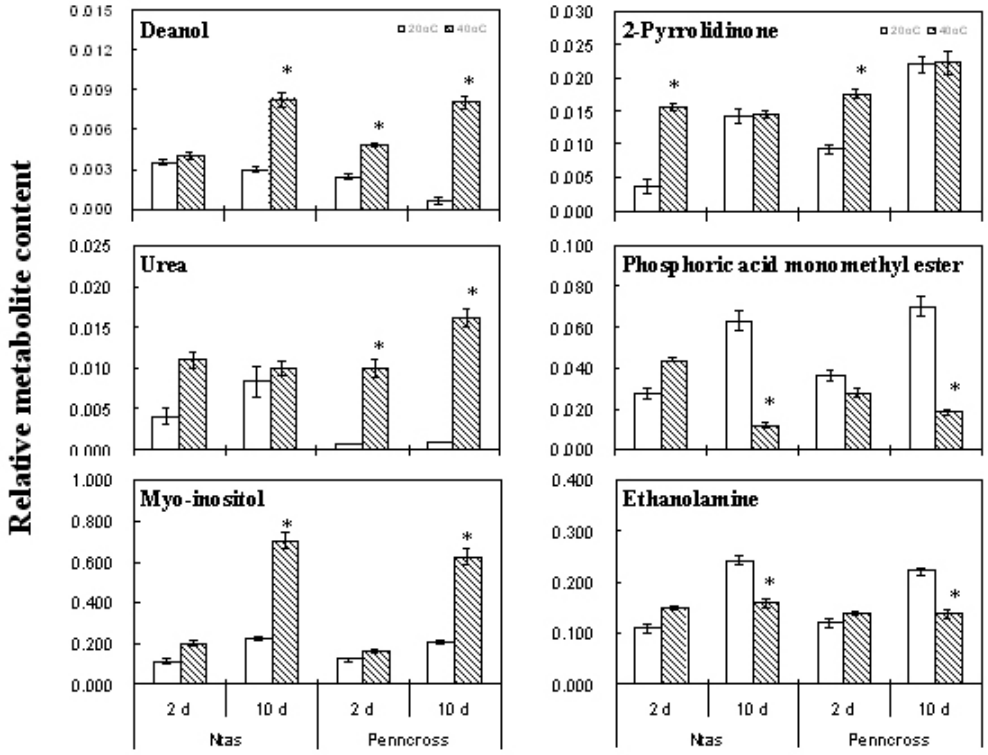


Fig. 10 Metabolic responses of other compounds to short-term (2 d) and long-term (10 d) heat stress in the roots of *A. scabra* and *A. stolonifera*.



CHAPTER 4. Effects of Foliar Applied Ethylene Inhibitor and Synthetic Cytokinin on Creeping Bentgrass to Enhance Heat Tolerance

INTRODUCTION

Leaf senescence is one of the typical symptoms of heat stress injury in various plant species. It has been associated with declines in chlorophyll content and an imbalance between antioxidant defenses and the generation of activated oxygen species that could induce oxidative damage (Munne-Bosch and Penuelas, 2003; Prochazkova and Wilhelmova, 2007). Previous studies suggest that hormone metabolism may play an important role in regulating heat-induced leaf senescence (Lim et al., 2006; Quirino et al., 2000). Cytokinins and ethylene are two main groups of senescence-related hormones (Gapper et al., 2005). Ethylene production in plant tissues increases in response to various environmental stresses, including heat stress (Borisova et al., 2001; Shirazi et al., 1993). The production of ethylene influences many aspects of plant growth and development, including the stimulation of leaf senescence, the ripening of fruit, the opening of flowers and the abscission of leaves (Grbic and Bleecker, 1995). In contrast, the production of cytokinins in leaves decreases under various stress conditions, such as heat stress (Farkhutdinov et al., 1997; Wang et al., 2004). Cytokinins are considered as senescence inhibitors, which are mainly involved in cell division, cell growth and differentiation (Werner et al., 2001). Xu and Huang (2007) recently reported that ethylene production rate is positively correlated whereas cytokinin content is negatively correlated to the degree of leaf senescence, when creeping bentgrass was exposed to heat

stress (35^{o}C). Delayed leaf senescence might be related to low production of ethylene and maintenance of cytokinins synthesis under supraoptimal temperature conditions.

Regulation of heat-induced leaf senescence by altering endogenous production of hormones has been studied in various plant species, such as common bean (*Phaseolus vulgaris*) (Udomprasert et al., 1995), arabidopsis (*Arabidopsis thaliana*) (Larkindale and Knight, 2002), maize (*Zea mays*) (Cheikh and Jones, 1994) and wheat (*Triticum aestivum*) (Balota et al., 2004). For instance, Burkhanova et al. (2001) reported that incubation of rosette leaf disks of arabidopsis in N^6-benzyladenine retarded senescence induced by a brief heat treatment (37 ^{o}C for 3 h). Moreover, transgenic tobacco (*Nicotiana tabacum*) plants carrying the *ipt* gene that codes for a key enzyme isopentenyltransferase in cytokinin biosyntheisis pathway accumulated cytokinins and maintained significantly higher transpiration rates than wild-type plants after heat shock (Teplova et al., 2000). Root zone injection of a synthetic cytokinin, zeatin riboside (ZR), has been reported to be effective in mitigating heat stress injury to shoots and roots of creeping bentgrass by increasing endogenous cytokinin contents (Liu et al., 2002).

The immediate precursor of ethylene is 1-aminocyclopropane-1-carboxylic acid (ACC), hence ethylene synthesis can be suppressed by preventing the conversion of S-adenosylmethionine to ACC or the conversion of ACC to ethylene (Bradford et al., 1982; Wenzel et al., 1995). Available chemicals that function in the first conversion include aminoethoxyvinylglycine (AVG) and aminooxyacetic acid, whereas cobalt chloride ($CoCl_2$) acts in the second conversion (Bradford et al., 1982; Romera and Alcantara, 1994). Jiao et al. (2006) studied the impacts of AVG and $CoCl_2$ on the leaves of broad bean (*Vicia faba*) under heat stress (45 ^{o}C for 12 h). They found that watering the plants

with 20 μM AVG or $CoCl_2$ solutions increased the contents of polyamines, enhanced the activities of several antioxidant enzymes, and mitigated the reduction of membrane stability. Ethylene action inhibitors are represented by CO_2, Ag^{2+} and 1-methylcyclopropane (1-MCP) (Philosophhadas et al., 1994). A recent study found that the application of 1-MCP to a heat susceptible wheat cultivar reduced heat-induced kernel abortion and prevented the reduction in kernel weight, which validated the link between ethylene in regulating susceptibility to heat stress and senescence in certain wheat genotypes (Hays et al., 2007).

Creeping bentgrass is a widely used cool-season turfgrass species on golf courses. However, heat-induced turf quality decline associated with leaf senescence is a major problem in turfgrass culture. Delaying or suppressing leaf senescence would improve plant tolerance to heat stress, and increase the atheistic aspects and functions of turfgrasses. Although extensive research has been conducted in foliar application of plant hormones, such as ethylene inhibitors and cytokinins for controlling leaf senescence in annual crops, the use of these chemicals in turfgrass management is limited. In addition, how application of these chemicals may affect heat-induced leaf senescence in cool-season turfgrass species is not clear. The objective of this study was to determine whether foliar application of an ethylene synthesis inhibitor (AVG) and a synthetic cytokinin (ZR) would enhance creeping bentgrass tolerance to heat stress associated with the suppression of leaf senescence. Heat tolerance and leaf senescence were examined by measuring turf quality, leaf chlorophyll content, antioxidant enzyme activities and the level of lipid peroxidation. Effects of foliar application of AVG and ZR on endogenous production of cytokinins and ethylene were also evaluated.

MATERIALS AND METHODS

Plant Materials

Creeping bentgrass cv. Penncross sod plugs were collected from 4-year old turfgrass field plots at Hort Farm II, Rutgers University, NJ. They were planted in plastic pots (15 cm in diameter and 20 cm deep) filled with sterilized sand. Plants were watered daily and fertilized weekly with full-strength Hoagland's solution. Plants were cut weekly to maintain a canopy height of approximately 5 cm. After one month of establishment in the greenhouse, plants were transferred into controlled-environment growth chambers (Conviron, Winnipeg, Canada) with a temperature of 20 ºC, 14-h photoperiod, 50% relative humidity, and 500 μmol m^{-2} s^{-1} photosynthetic photon flux density at the canopy height. Plants were acclimated to growth chamber conditions for one week prior to exposure to different treatments.

Treatments and Experimental Design

Plants were treated with AVG, ZR, or water (as the control) the day before exposure to two different temperatures and then weekly during the temperature treatment. Eight plants were sprayed with 50 mL of 25 μM AVG and eight plants were sprayed with 50 mL of 25 μM ZR, whereas another eight plantswere sprayed with 50 mL of distilled water. Concentrations were determined based on a previous study (Liu et al., 2002) and a preliminary test, in which four concentrations (10, 25, 50, and 100 μM) of each treatment were applied to creeping bentgrass and the rate with optimum turf performance (50 μM AVG and 25 μM ZR) was selected for this study. Four pots of each AVG, ZR, or water-treated plants were exposed to 35 ºC (heat stress) or 20 ºC (control) in growth chambers

for 35 d. Plants were arranged randomly inside each chamber and rotated every three days. Plants were watered twice a day to prevent water deficit during the heat stress period, and fertilized weekly using the Hoagland's nutrient solution. The whole experiment was repeated for three times in different growth chambers to minimize other environmental variations.

The experiment consisted of two factors (temperature and chemical treatments), which were arranged as a split-plot design with temperature treatment as the main plot and the chemical treatment as the sub-plot, with four replicates for each combination of temperature and chemical treatments.

Evaluation of Leaf Senescence and Antioxidant Activities

Turf quality was visually rated based on color, density, and uniformity of the grass canopy using a 0 to 9 scale, with 9 representing the best quality with uniform green, dense turf canopy and 0 representing the lowest quality with completely dead plants.

Leaf chlorophyll was extracted from about 0.2 g fresh leaves using dimethyl sulfoxide. The absorbance of leaf extracts was determined using a spectrophotometer (Spectronic Genesys2, Spectronic Instruments, Rochester, N.Y.). Chlorophyll content was calculated based on the absorbance at 663 nm and 645 nm using the formulas described by Arnon (1949).

For the analysis of antioxidant enzyme activities and the level of lipid peroxidation, leaf samples (0.5 g fresh leaves) were collected from each pot at each sampling date, frozen in liquid nitrogen immediately, and stored at –80^{o}C until use. For extraction, the frozen sample was ground on ice with 4 mL of 50 mM cold phosphate

buffer (pH 7.0). The homogenate was centrifuged at 12000 g for 30 min. The supernatant was collected for the assays of antioxidant enzyme activity and content of malondialdehyde (MDA, the final product of lipid peroxidation).

The activity of superoxide dismutase (SOD) was determined according to the methods of Giannopolitis and Ries (1977) by measuring its ability to inhibit the photoreduction of nitro blue tetrazolium (NBT). Enzyme extract (100 μL) was added to a 3 mL reaction solution containing 50 μM NBT, 1.3 μM riboflavin, 13 mM methionine, 75 nM EDTA, 50 mM phosphate buffer (pH 7.8). The solution was placed under a bank of fluorescent light at 80 $\mu mol^{-1}\ m^{-2}\ s^{-1}$ to react for 20 min. The reaction was then stopped by incubating the solution in dark for 5 min. The absorbance of the reacted solution at 560 nm was determined with a spectrophotometer (Spectronic Instruments, Rochester, NY). One unit of SOD activity was defined as the amount of enzyme that inhibited 50% of NBT photoreduction.

Catalase (CAT) activity was measured as a decline in absorbance at 240 nm for 1 min using the method of Chance and Maehly (1955) with modification. A 100 μL enzyme extract was added to a 3 mL reaction solution containing 15 mM H_2O_2 and 50 mM phosphate buffer (pH 7.0).

The content of MDA was measured using the method of Dhindsa et al. (1981). A 1 mL MDA extract was added to 2 mL reaction solution containing 20% w/v trichloroacetic acid and 0.5% w/v thiobarbituric acid. The solution was incubated in water bath at 95^{o}C for 30 min then cooled quickly in ice-water bath. The solution was centrifuged at 12000 g for 15 min and the supernatant was collected. The absorbance of the supernatant was measured at 532 and 600 nm. The concentration of MDA was

calculated by subtraction of OD600 from OD532 and an extinction coefficient of 155 mm^{-1} cm^{-1} (Heath and Packer, 1968).

Analysis of Endogenous Production of Ethylene and Cytokinins

Ethylene production of leaves was determined using a gas chromatograph following the method described in Larkindale and Huang (2004). An airtight system was designed to collect ethylene gas evolved from leaves. Five to seven leaves of similar ages attached to the plant were sealed inside a 10-mL syringe (Becton, Dickinson and Company) with a rubber stopper around the leaf base. Vacuum grease and Teflon tape were applied to the leaf base to prevent air leakage. A 0.5 mL of gas sample from each syringe was taken through the rubber stopper every 2 h and injected into a gas chromatograph (GC-8A, Shimadzn) to determine ethylene concentration. The average of hourly production of ethylene was calculated based on changes in ethylene concentration over time and a standard curve. Four groups of leaves from each pot were analyzed as four sub-samples.

Two forms of cytokinin (ZR and isopentenyl adenosine (iPA)) were quantified using an indirect competitive enzyme-linked immunosorbent assay. Extraction and quantification of hormones followed the method described by Setter et al. (2001) with some modifications (Wang and Huang, 2003). Leaf samples were extracted in an extraction buffer containing 80% [v/v] methanol, 1% acetic acid, and 10 mg/L butilated hydroxytoluene in a ratio of 1:10. Then the extracts were passed through an elute C18 column (Extract-Clean, 100 mg/1.5 mL, Alltech, IL). The elution (200 μL) was vacuum-dried and re-dissolved in 150 mL aqueous triethylamine (TEA) (10 mM, pH 3.5). The

samples then were put on a reverse phase C_{18} column (Discovery DSC-18 SPE-96 plate, 25 mg, Supelco, PA). Hydrophilic contaminants were washed out with 200 μL of 20% solvent [20% methanol, 80% aqueous TEA]. The cytokinin-containing fraction was eluted using 200 μL of 30% solvent (30% methanol, 70% aqueous TEA). Monoclonal antibodies against ZR and iPA were developed by Eberle et al. (1986). A goat anti-mouse IgG conjugated with alkaline phosphatase (Sigma, St. Louis, MO) was used as a secondary antibody. Bound antibody was detected using *p*-Nitrophenyl Phosphate (Dicyclohexylammonium salt).

Statistical Analysis

Analysis of variance was based on the general linear model procedure of SAS 9.1 (SAS Institute Inc., Cary, NC). Effects of chemical treatments, temperature, and their interactions were tested using analysis of variance (ANOVA). Treatments were compared separately for each sampling date when the interactions of treatment and treatment duration were significant for either temperature or chemical treatment. Treatment differences were separated by Fisher's protected least significant difference (LSD) test at the 0.05 probability level. LSD bars are present in the figures when significant chemical effects were detected.

RESULTS

Changes in turf quality and chlorophyll content

Turf quality (Fig. 1) and leaf chlorophyll content (Fig. 2) of AVG-treated, ZR-treated and the water-treated plants did not differ over time at the control temperature (20ºC), so data of chemical treatments were pooled for plants grown at 20 ^{o}C. However, both parameters declined during heat stress (35 ºC) with the greatest reduction in the water-treated control. By 35 d of heat stress, turf quality of AVG-, ZR-, and water-treated plants decreased by 21, 29, and 33%, respectively, compared to their respective control plants at 20 ^{o}C (Fig. 1); leaf chlorophyll content of the AVG-, ZR-, and water-treated plants at 35 ºC declined by 14, 16, and 26%, respectively (Fig. 2). AVG-treated and ZR-treated plants had significantly higher turf quality than water-treated plants at 21-35 d and 28 d of heat stress, respectively (Fig. 1). Leaf chlorophyll content was significantly higher in both AVG- and ZR-treated plants than water-treated plants from 14 to 35 d of heat stress (Fig. 2).

Changes in activity of antioxidant enzymes and MDA content

Application of AVG or ZR had no effects on CAT or SOD activity at 20 ^{o}C (Fig. 3 and 4) and data of chemical treatments were pooled. Both CAT and SOD activities decreased during heat stress in all plants, regardless of chemical treatments (Fig. 3 and 4). The decrease in water-treated plants became significantly greater than in AVG- or ZR-treated plants, beginning at 7 and 21 d for CAT activity, and at 7 and 14 d for SOD activity, respectively.

Data of chemical treatments were also pooled for MDA content because no

significant chemical effects were detected among AVG-treated, ZR-treated, and water-treated plants at 20 ^{o}C (Fig. 5). MDA content in all plants increased during heat stress, reaching 200, 209 and 249% of the initial level after 35 d in AVG-, ZR- and water-treated plants, respectively (Fig. 5). The content of MDA was significantly higher in water-treated plants than in the AVG-treated plants at 28-35 d, and than ZR-treated plants at 28 d of heat stress.

Changes in ethylene and cytokinin production

Plants exposed to 20 ^{o}C maintained constant ethylene production rate during the study period, regardless of chemical treatments thus data of chemical treatments were pooled at this temperature (Fig. 6). Ethylene production rate gradually increased in AVG-, ZR-, and water-treated plants under heat stress (Fig. 6). However, the magnitude of that increase was significantly smaller after 7 d of heat stress in ZR-treated plants, and after 14 and 21 d of heat stress in AVG-treated plants, compared to water-treated plants exposed to the same temperature. By 21 d of heat treatment, ethylene production rate was 25 and 11% lower in AVG- and ZR-treated plants than that in the water-treated plants, respectively.

Endogenous ZR content (Fig. 7) and iPA content (Fig. 8) of the plants exposed to 20 ^{o}C did not change significantly during 21 d of the treatment period for water-treated, AVG-, or ZR-treated plants. Data of chemical treatments were also pooled. Under heat stress, endogenous ZR and iPA contents in water-treated plants decreased by 62 and 75% of their respective initial levels after 21 d. A 4.4 fold accumulation of leaf ZR content was detected after 7 d of heat stress in ZR-treated plants, followed by a gentle fallback to

a level comparable to that in the plants maintained under normal temperature. Accordingly, leaf iPA content in ZR-treated plants consistently accumulated with prolonged heat treatment and reached 2.6 times of the level in the plants at normal temperature. Plants treated with AVG also maintained significantly higher ZR content than water-treated plants at 14 and 21 d of heat stress (Fig. 7). However, there were no such difference in leaf iPA content between AVG- and water-treated plants under heat stress (Fig. 8).

DISCUSSION

Foliar spray of the ethylene inhibitor (AVG) effectively altered the rate of ethylene production in creeping bentgrass under heat stress. Ethylene is typically present in low quantity in healthy plant tissues, but its production increases during leaf senescence and can be triggered by diverse stresses (Mackerness, 2000). In this study, ethylene evolution consistently increased to more than two-fold of the controls (water-treated plants) after 21 d of heat treatment compared to the plants growing under normal temperature. The magnitude of this increase was comparable to our recent study, in which we also detected a 2-3 fold increase at 35 d of 35 ^{o}C for creeping bentgrass (Xu and Huang, 2007). AVG treatment suppressed 25% of ethylene accumulation after 21 d of heat stress in creeping bentgrass compared to the water-treated control plants in the same conditions. Saltveit (2005) reported that a tissue concentration as low as 5 μm AVG was active in reducing ethylene biosynthesis. Nevertheless, the fact that AVG treatment did not completely block stress ethylene accumulation indicated that stress ethylene may not be formed by normal pathway and there exist other factors that control its production (Wang et al., 1990). Another point worthy of mention is that AVG had no significant effects on ethylene evolution for plants growing under control temperature. This was consistent with the finding of Nandwal et al. (2000) when they treated chickpea (*Cicer arietinum*) plants with 10 μM AVG.

Enhanced ethylene evolution has been associated with reduced growth and accelerated senescence in response to environmental stresses (Balota et al., 2004). Thus, the prevention of stress ethylene production should alleviate these adverse effects. We found AVG treatment did delay leaf senescence of creeping bentgrass induced by heat

stress, as reflected by mitigated declines in turf quality and leaf chlorophyll content compared to water-treated controls. Turf quality has been positively correlated to the protection of antioxidant defending system under heat stress (Xu and Huang, 2003; Zhang and Ervin, 2008). Heat stress often induces oxidative stress in plants, as manifested by significant disruption to the function of various antioxidant enzymes and elevated lipid peroxidation levels (Gong et al., 1998; Kurganova et al., 1997; Xu and Huang, 2003). We observed that AVG-treated creeping bentgrass had significantly less severe declines in the activities of CAT and SOD than the water-treated plants did during prolonged periods of high temperature. CAT and SOD are two major scavenging enzymes. They suppress the production of active oxygen species by quenching super oxygen free radicals and H_2O_2, respectively (Liu and Huang, 2002). Our results suggest that foliar application of AVG may suppress heat-induced leaf senescence through partially recovering the activities of these enzymes and reducing free radicals and H_2O_2 content. Lipid peroxidation is another parameter sensitive to oxidative damage and responsible for impairment of membrane function. MDA is the product of peroxidation of unsaturated fatty acids. The level of MDA in AVG-treated plants was brought down compared to water-treated plants under heat stress, though it was still higher than the plants growing at normal temperature. This suggested that heat-induced lipid peroxidation in creeping bentgrass may be mediated through ethylene evolution and the damage to the cell membrane is reversible. Lieberman (1979) proposed that lipid peroxidation might be associated with ethylene formation. This was in agreement with our findings and supported by an experiment conducted by Munne-Bosch et al. (2004). They found 100 nL L^{-1} airborne ethylene altered antioxidant defense systems in holm oak

(*Quercus ilex*) under heat stress as indicated by enhanced MDA and lower α-tocopherol and ascorbate levels; thus it caused higher visual leaf area damage and greater limitations of photosynthesis compared to the controls. Additionally, Saltveit (2005) found that the ability of AVG to reduce ethylene production in excised tomato (*Lycopersicon esculentum*) pericarp discs was highly correlated (R2=0.98) to its ability to reduce protein synthesis. Beltrano et al. (1999) also found the treatment with 50 μm AVG augmented the protein content above the control under drought stress in wheat. Actually, like many other inhibitors, AVG may affect other metabolic pathways because it is not a specific inhibitor of ethylene biosynthesis and other pyridoxal phosphate-dependent enzymatic reactions may also be affected (Wenzel et al., 1995).

Endogenous cytokinin production in the leaves of water-treated creeping bentgrass decreased dramatically when heat treatment was prolonged, in the form of both iPA and ZR. The levels of cytokinins were consistent with our findings in a couple of previous studies (Liu et al., 2002; Wang et al., 2003; Wang et al., 2004). The reduction in leaf cytokinin content may result from the inhibition of cytokinins transport from roots to shoots under elevated temperatures, as cytokinins are synthesized primarily in root tips and transported via the xylem to the shoots to exert major regulatory influences on growth and development (Farkhutdinov et al., 1997; Haberer and Kieber, 2002). Foliar application of 25 μm ZR solution elevated cytokinin levels in creeping bentgrass leaves under heat stress. Zhang and Ervin (2008) has recently reported similar findings that application of 10 μm t-ZR increased leaf ZR concentration by 28% in creeping bentgrass at 14 d of heat stress, but they did not compare the variation in the accumulation patterns between iPA and ZR that was observed in this study. Quantity of endogenous ZR

responded rapidly to the foliar spray of ZR, which increased to more than four times the initial level after 7 d of treatment, but declined to a lower level afterwards; however, it was still higher than in water-treated plants. On the contrary, endogenous iPA content continually accumulated over the whole treatment period. The iPA was found to be the predominant type of cytokinin in creeping bentgrass in previous studies (Wang et al., 2003; Xu and Huang, 2007), which may also serve as the reservoir for efficient utilization of cytokinins as indicated in the current study.

Increased leaf cytokinin content in creeping bentgrass due to foliar application of ZR appeared to play important roles in suppressing leaf senescence and improving heat stress, as we observed less declines in turf quality and leaf chlorophyll content in ZR-treated plants compared to water-treated controls. Exogenous application of cytokinin also improved wheat tolerance to combined heat and drought stresses, as measured by the increases in grain yield and cell membrane and chlorophyll stability (Gupta et al., 2000). Cheikh and Jones (1994) also believed that the maintenance of high levels of cytokinins in maize kernels during heat stress provided yield stability of maize and favored thermo tolerance. Moreover, there have been a few studies reporting the antioxidant responses in the presence of cytokinins under different abiotic stresses. Zavaleta-Mancera et al. (2007) reported that wheat leaves incubated in 10^{-4} M 6-benzylaminopurine (a synthetic cytokinin) increased the activities of antioxidant enzymes (CAT and ascorbate peroxidase) and reduced the levels of reactive oxygen species, resulting in delay of dark senescence. Exogenous seaweed extracts and humic acid containing cytokinins have also enhanced endogenous antioxidant levels in Kentucky bluegrass (*Poa pratensis*) under low soil moisture conditions and might contribute to turfgrass drought tolerance (Zhang

and Schmidt, 1999). Synkova et al. (2006) reported that transgenic *Pssu-ipt* tobacco plants with a significant increase of cytokinin contents throughout the ontogeny exhibited elevated activities of antioxidant enzymes particularly including glutathione reductase, SOD and APX. We observed similar effects on activating the antioxidant system after ZR treatment in creeping bentgrass exposed to supraoptimal temperature.

It is interesting that application of ZR also affected stress ethylene accumulation whereas application of AVG reversed the decline in ZR but not iPA in creeping bentgrass under heat stress. This may indicate a direct correlation between the actions of cytokinin and ethylene on senescence. Kudryakova et al. (2001) reported 10^{-6}M cytokinin (benzyladenine) suppressed ethylene action on the dark-induced senescence in detached arabidopsis leaves. Taverner et al. (1999) found that ethylene production promotes inactivation of cytokinin and its degradation, which may facilitate the Petunia corolla senescence. Hence, a combined application of exogenous cytokinin and ethylene inhibitor may function simultaneously on promoting heat tolerance of creeping bentgrass. In practice, biostimulants with cytokinins as active ingredients such as seaweed extract products are already widely used on golf courses and other fields for turfgrass management. AVG-based plant growth regulator has been used to manage ripening and storage of fruits. Recently, a turf company is interested in funding our research to develop certain ethylene inhibitors as stress reducers.

In summary, our results demonstrated that foliar spray of the ethylene inhibitor AVG or cytokinins was effective at suppressing leaf senescence of creeping bentgrass by increasing endogenous production of cytokinins or inhibiting ethylene production during heat stress. Both treatments had similar effects. The inhibition of ethylene production and

stimulation of cytokinin accumulation may lead to the increases in leaf chlorophyll content and antioxidant activities and lower lipid peroxidation, contributing to the improvement in turf quality under heat stress. Our results suggest that AVG or ZR could be used to alleviate heat injury in cool-season turfgrasses. However, it should be noted that the results from controlled-environmental growth chambers need to be confirmed under field conditions.

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Fig. 1 Effects of AVG, ZR and water treatments on turf quality under normal temperature (20ºC) and heat stress (35ºC). Vertical bars indicate LSDs ($P \leq 0.05$) for treatment comparison at a given day of treatment.

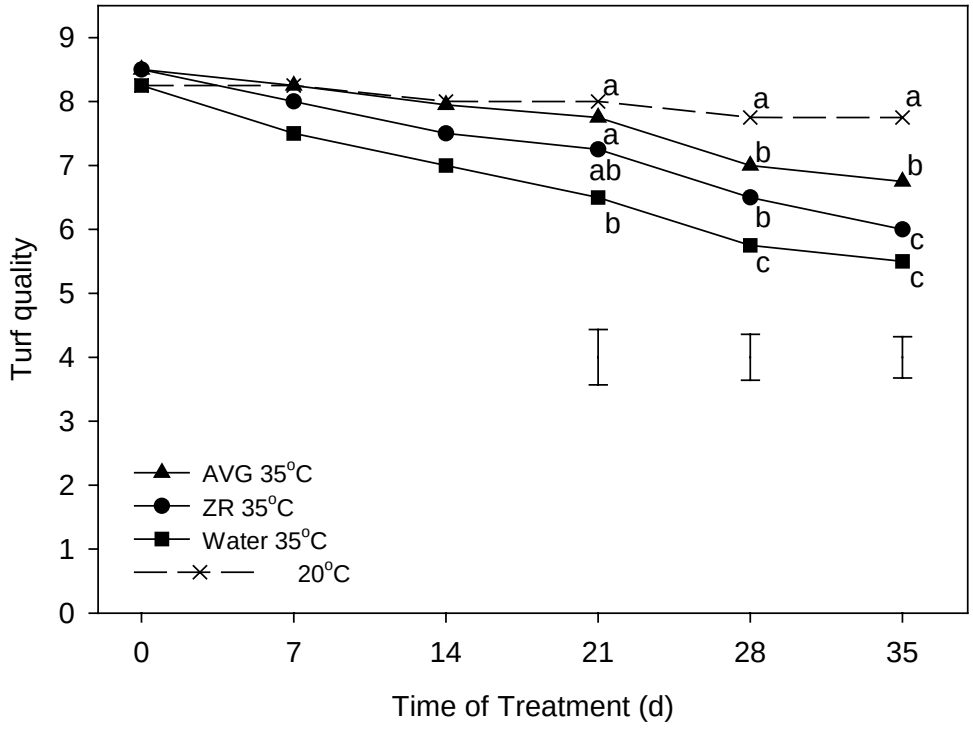


Fig. 2 Effects of AVG, ZR and water treatments on chlorophyll content (mg g^{-1} FW) under normal temperature (20ºC) and heat stress (35ºC). Vertical bars indicate LSDs ($P \leq 0.05$) for treatment comparison at a given day of treatment.

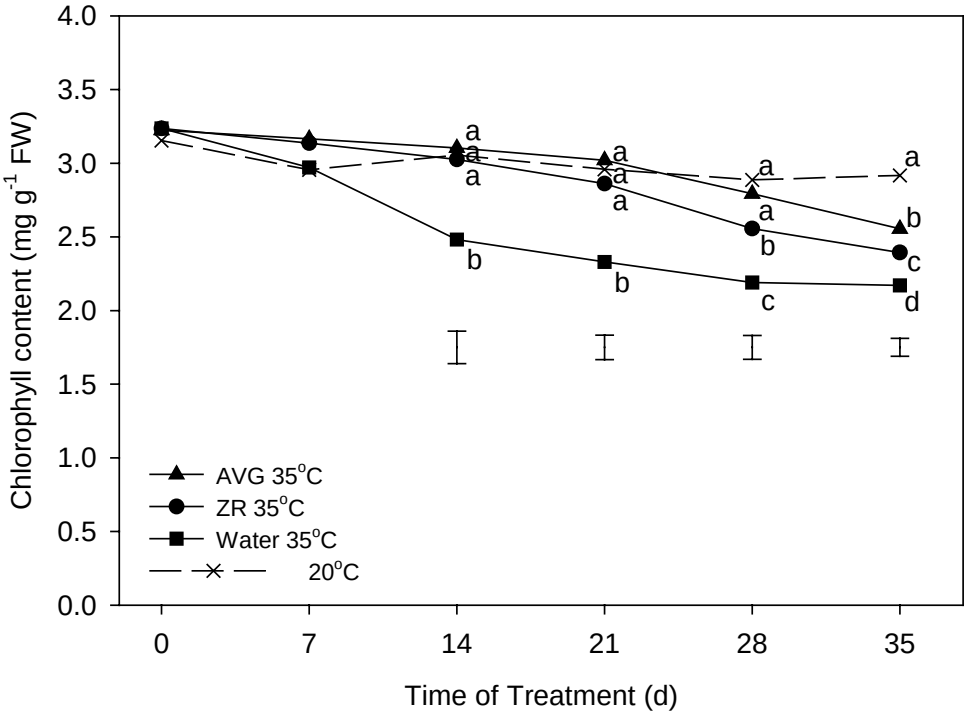


Fig. 3 Effects of AVG, ZR and water treatments on CAT activity (Unit g^{-1} FW) under normal temperature (20ºC) and heat stress (35ºC). Vertical bars indicate LSDs ($P \leq 0.05$) for treatment comparison at a given day of treatment.

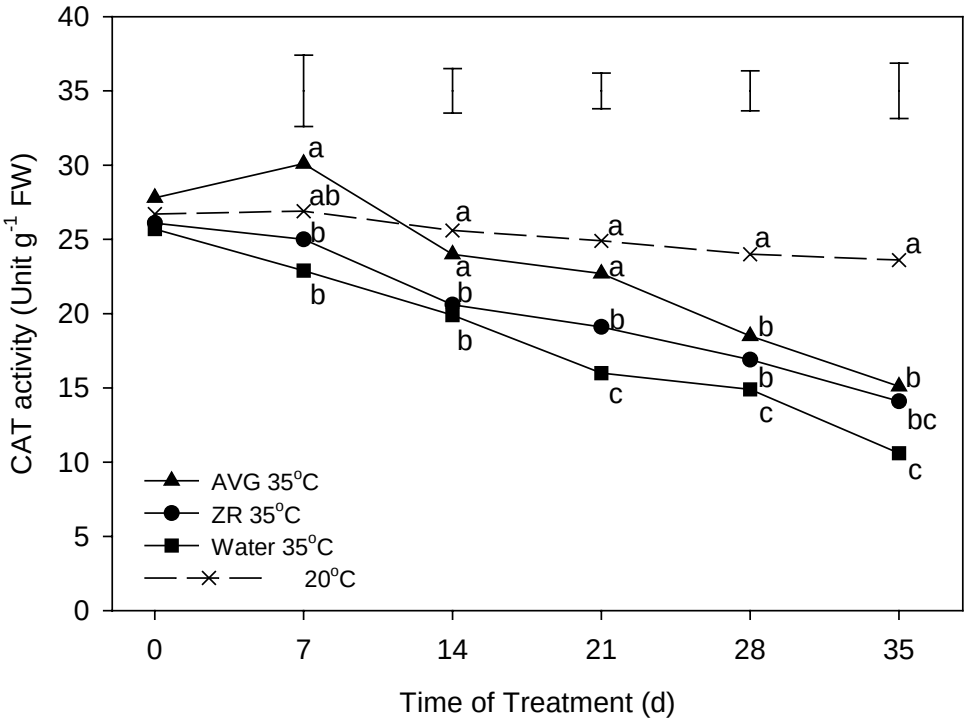


Fig. 4 Effects of AVG, ZR and water treatments on SOD activity (Unit g^{-1} FW) under normal temperature (20ºC) and heat stress (35ºC). Vertical bars indicate LSDs ($P \leq 0.05$) for treatment comparison at a given day of treatment.

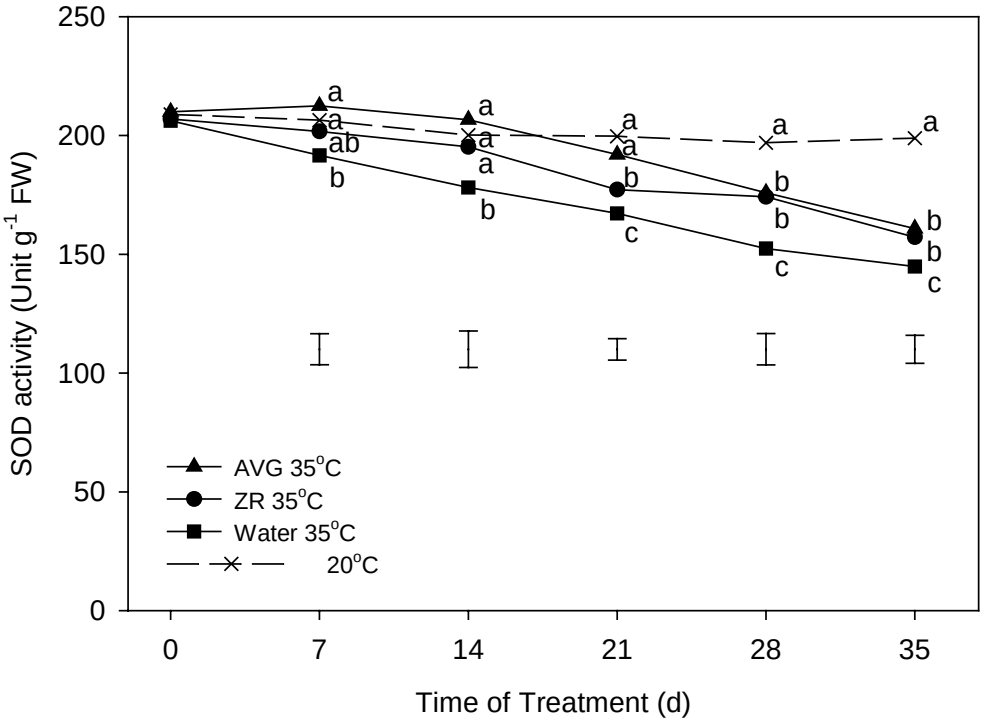


Fig. 5 Effects of AVG, ZR and water treatments on MDA content (nmol g^{-1} FW) under normal temperature (20ºC) and heat stress (35ºC). Vertical bars indicate LSDs ($P \leq 0.05$) for treatment comparison at a given day of treatment.

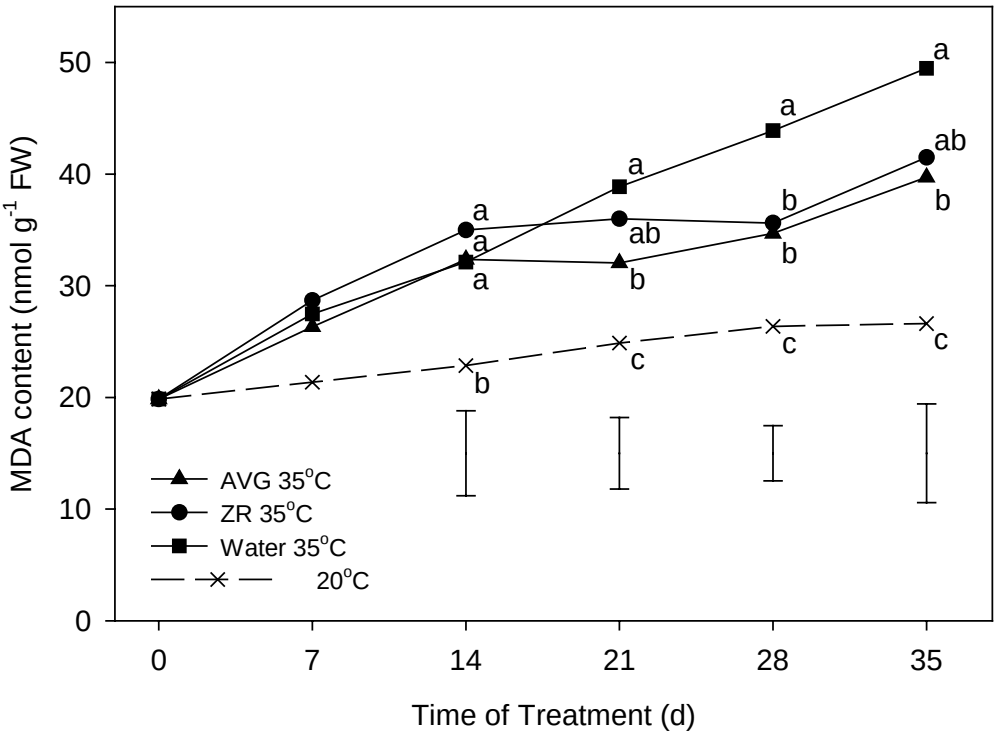


Fig. 6 Effects of AVG, ZR and water treatments on ethylene production rate (nl h^{-1} g^{-1} FW) under normal temperature (20ºC) and heat stress (35ºC). Vertical bars indicate LSDs ($P \leq 0.05$) for treatment comparison at a given day of treatment.

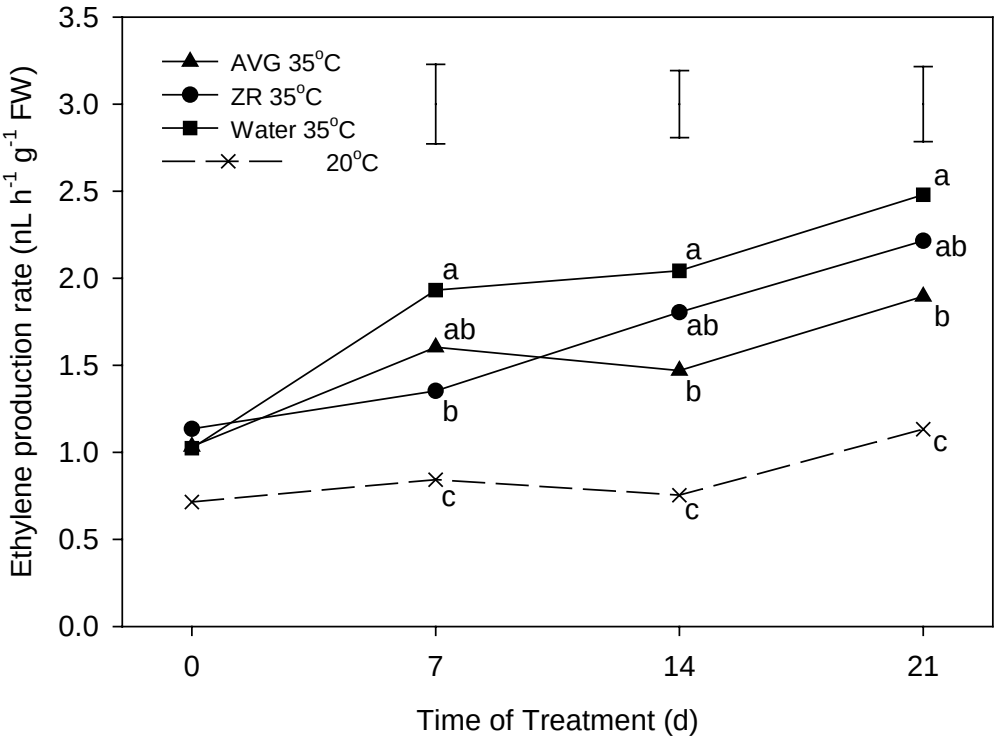


Fig. 7 Effects of AVG, ZR and water treatments on ZR content (pmol g^{-1} DW) under normal temperature (20ºC) and heat stress (35ºC). Vertical bars indicate LSDs ($P \leq 0.05$) for treatment comparison at a given day of treatment.

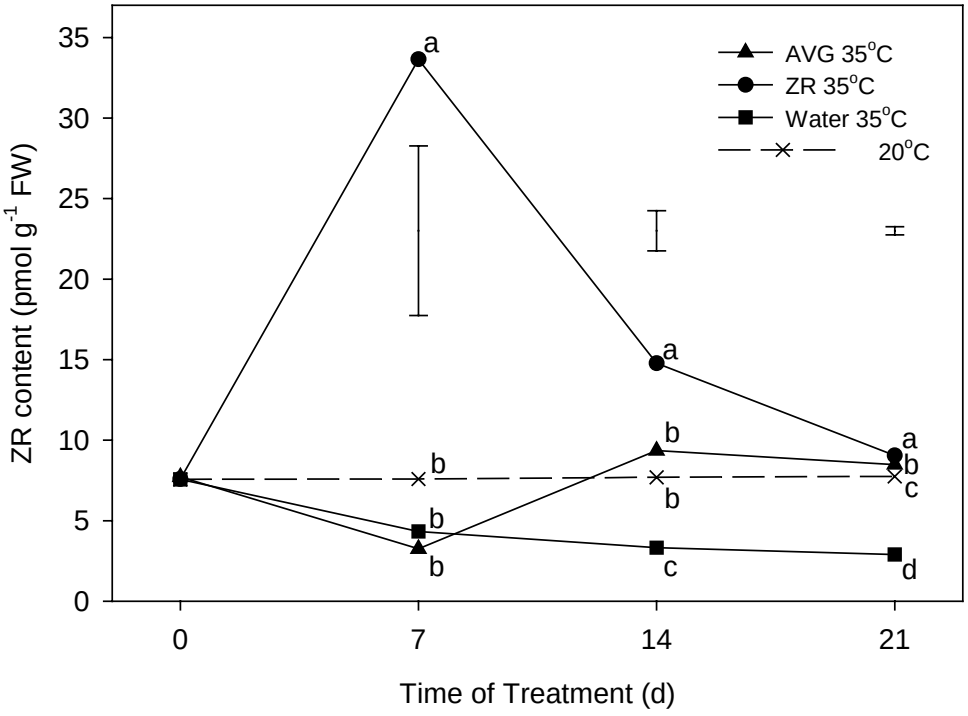
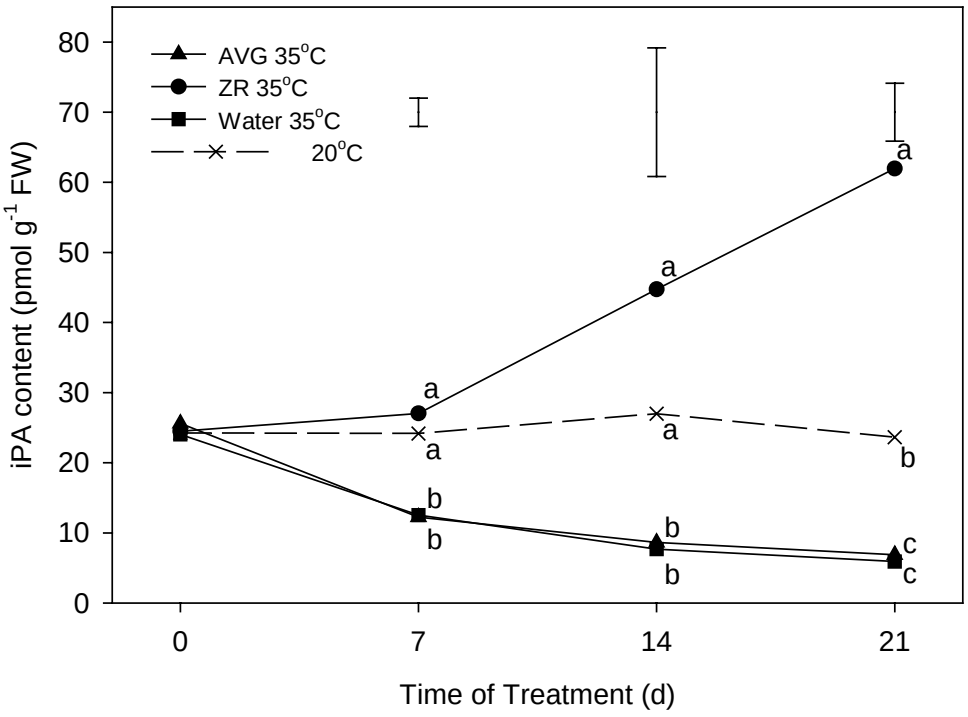


Fig. 8 Effects of AVG, ZR and water treatments on iPA content (pmol g^{-1} DW) under normal temperature (20ºC) and heat stress (35ºC). Vertical bars indicate LSDs ($P \leq 0.05$) for treatment comparison at a given day of treatment.



CHAPTER 5. Responses of Creeping Bentgrass to Trinexapac-Ethyl and Biostimulants under Summer Stress

INTRODUCTION

Creeping bentgrass (*Agrostis stolonifera*) is a widely used cool-season grass species for golf course putting greens. It grows vigorously during spring and fall when growth temperatures are 18-24 ^{o}C for shoots and 10-18 ^{o}C for roots (Beard, 1973). Turf quality declines on creeping bentgrass greens during summer months when temperature exceeds the optimum, which is characterized by thinning turf canopy, leaf senescence and root dieback (Carrow, 1996). Heat stress is found to be the primary factor leading to summer bentgrass decline (Huang, 2001). Many physiological factors have been associated with heat stress injury in cool-season grass species, including inhibition of photosynthesis, reduction in water and nutrient uptake, and hormone synthesis (Huang and Xu, 2000; Fry and Huang, 2004; Liu and Huang, 2005). Root growth has been found to be more sensitive to heat stress than shoots and root dieback precedes decline in turf quality for creeping bentgrass (Beard and Daniel, 1966; Xu and Huang, 2000). Root dieback inhibits the production of cytokinins, a class of plant hormones that are primarily produced in roots, which in turn affect shoot growth and senescence (Adeldipe et al., 1971; Henson and Wareing, 1976; Udomprasert et al., 1995). Incorporation of management practices such as use of natural products or plant growth regulators that may promote shoot and root growth would favor creeping bentgrass survival in the summer.

A plant growth regulator (PGR), trinexapac-ethyl (TE, Syngenta Crop Protection, Greensboro, NC), has been widely used in turfgrass management for clipping reduction,

seedhead suppression of annual bluegrass (*Poa annua*) and improvement of overall turf quality in various turfgrass species (Borger, 2008; Fagerness et al., 2002; Lickfeldt et al., 2001). It belongs to gibberellic acid (GA) inhibitors and blocks the conversion of GA_{20} to GA_1, the final step in GA biosynthesis pathway (Adams et al., 1992; King et al., 1997), leading to the inhibition of cell expansion in sheaths and basal regions of leaves (Kaufmann, 1986). Recently, TE has been found to be effective in improving turf performance under such adverse conditions as shade (Ervin et al., 2004; Goss et al., 2002), freezing (Fagerness et al., 2002), heat tolerance (Wang et al., 2006), and combined drought and heat stress (McCann and Huang, 2007). Despite the wide use of TE in bentgrass green management, the physiological effects of TE application associated with summer bentgrass decline are not well documented. TE has been reported to cause increases in chlorophyll content and shoot density in various turfgrass species (Ervin and Koski, 1998; Ervin and Koski, 2001; Fagerness and Yelverton, 2001; Stier and Rogers, 2001). As discussed earlier, the typical symptoms of summer bentgrass decline are leaf senescence and a reduction in overall canopy leaf density. Therefore, it is hypothesized that foliar application of TE may alleviate summer bentgrass decline by suppressing leaf senescence and promoting a denser turf canopy during summer months.

In addition to PGRs, some biostimulant products were developed to improve turfgrass quality, especially in turf that is under environmental or cultural stress (Karnok, 2000). Among a variety of active ingredients in biostimulants, one ingredient common to many biostimulant products is seaweed extract, which is rich in organic and mineral compounds and often exhibit activity of plant hormones such as cytokinins and auxin (Sanderson et al., 1987; Tay et al., 1985; Wells et al., 2003). Some biostimulant products

may increase soil microbial density and activity by incorporating microbial inoculums, which in turn enhances turfgrass quality through increased organic matter decomposition and improved nutrient availability (Mueller and Kussow, 2005). Exogenous application of seaweed extracts has been observed to improve growth, yield and stress tolerance of many crops such as wheat (*Triticum aestivum*) (Bokil et al., 1974), tomato (*Lycopersicon esculentum*) (Crouch and van Standen, 1992), and soybean (*Glycine max*) (Rathore et al., 2008). Zhang et al. (2003) found seaweed extract could be a beneficial supplement for reducing standard fertilizer and fungicide inputs while maintaining adequate creeping bentgrass health. However, knowledge of effects of seaweed-based biostimulants on turfgrass growth under heat stress conditions is still limited and the mechanisms for the effects remain largely unknown. With the increasing use of biostimulants on creeping bentgrass putting greens, the information on whether and how the biostimulants affect creeping bentgrass summer performance would help turf managers develop more efficient summer stress management practices.

The objectives of this study were to investigate whether foliar application of TE and two biostimulants containing seaweed extracts would alleviate decline in creeping bentgrass growth during summer months and to examine the effects of TE and the biostimulants on leaf senescence and root growth. Shoot growth and leaf senescence of creeping bentgrass were examined by measuring turf quality, turf density, leaf chlorophyll content, and canopy net photosynthetic rate. Root growth was examined by measuring total root surface area and root biomass.

MATERIALS AND METHODS

Plant Materials and Growing Conditions

The experiment was performed on a 'Penncross' creeping bentgrass green built on a root-zone mixture consisting of medium-sized sand meeting USGA size guidelines (Green Section Staff, 1993) and sphagnum peat (9:1 in volume) at Hort Farm II, North Brunswick, NJ. An on-site weather station was located approximately 130 m from the experimental site to record daily meteorological parameters. The green was mowed six days per week at 4 mm and clippings were removed. It was irrigated daily to replace 100% evapotranspiration water loss. A 16-4-8 (N-P_2O_5-K_2O) fertilizer was applied in April, June, and September at a rate of 122 kg ha^{-1} of nitrogen in 2007 and 2008 to maintain adequate soil nutrient status. Fungicides (Spectro 90WDG, Daconil Ultrex, Pentathlon and Banner Maxx) were applied on a curative basis mainly to control dollar spot and brown patch.

Treatments and Experimental Design

The two biostimulants used were CPR (Emerald Isle Solutions, Ann Arbor, MI) and TurfVigor (Novozymes Biologicals Inc., Salem, VA). CPR is a blend of natural sea plant extract, micronutrients, and a surfactant agent. It contains 4% N, 1% K_2O, 0.53% Mg, 1% S, 2% Fe, 0.25% Mn and 0.2% Zn. TurfVigor is a formulation containing 0.014% patented microbial strains (*Bacillus* spp. and *Paenbacillus* spp.) along with kelp extract and macro- and micro-nutrients. This product contains 9% N, 3%P_2O_5, 6% K_2O, 0.6% Fe, 0.05% Mn and 0.05% Zn. All three products were applied following their respective manufacturer recommended rates: 1) TE (120 g a.i. L^{-1} emulsifiable

concentrate): 0.05 kg a.i. ha^{-1}; 2) TurfVigor: 47.75 L ha^{-1}; 3) CPR: 19.10 L ha^{-1}. The water volume applied for control and carry volume for TurfVigor, CPR and TE was 2 gallon per 1000 ft^2. Water (control) treatment was also included in the experiment. Each control plot was treated with the same volume of water as the volume of TE or biostimulant solutions sprayed on treated plots. Treatments were applied using a CO_2-pressurized backpack sprayer on June 23, July 6, July 25, Aug. 8, Aug. 24 and Sep. 7 in 2007, and on June 11, June 27, July 11, July 28, Aug. 13 and Aug. 27 in 2008.

The experiment was a completely randomized design with four replicates or plots (0.76 × 1.22 m each plot) for each treatment.

Measurements

All measurements were taken 2 weeks after each spray was applied. Overall turf performance was evaluated by rating turf quality (TQ) based on color, density, and uniformity of the grass canopy using a 0-9 scale (9 representing fully green, dense turf canopy and 0 representing completely dead plants) (Beard, 1973). A rating of 6 was considered to be the minimum acceptable TQ for a putting green.

Leaf chlorophyll was extracted from approximately 0.1 g of fresh leaves incubated in 10 mL dimethyl sulfoxide in the dark for 72 h. The absorbance of the leaf extracts was determined using a spectrophotometer (Spectronic Genesys2, Spectronic Instruments, Rochester, N.Y.). Chlorophyll content (CHL) was calculated based on the absorbance at 663 nm and 645 nm using the formulas described by Arnon (1949).

Canopy reflectance characteristics of turf plots were measured with a hand-held multispectral radiometer (MSR) (Cropscan, Rochester, MN) on clear and sunny days between 1100 and 1400 h. The MSR scanned a fixed surface area of each plot (a circular

area of approximately 0.7 m^2), providing an additional measurement to visual estimates of turf quality by spectral assessment of canopy characteristics. The ratio of near infrared (IR) (935 nm) to red (R) (661 nm) is correlated to turf visual quality, shoot density, and stress injury level in warm-season and cool-season turfgrass species (Jiang and Carrow, 2005 and 2007; Trenholm, et al., 1999) and used to estimate leaf area index or shoot density in crops (Asrar et al., 1984; Hatfield et al., 1983) and turfgrasses (Trenholm, et al., 1999).

Canopy Pn was measured using a gas exchange analyzer with a canopy chamber constantly providing 400 μL L^{-1} CO_2 (LI-COR 6400, LI-COR Biosciences, Lincoln, NE). The canopy chamber consisted of an acrylic cylinder (10 cm diameter and 8 cm height), which was pressed into the ground approximately 3 cm to provide an adequate seal for canopy gas exchange measurements (DaCosta and Huang, 2006). Photosynthesis measurements were performed on clear and sunny days between 1100 and 1400 h at times of maximal solar radiation.

Root samples were collected from three soil cores (15 cm deep, 76 cm^3 soil core) randomly located within each plot. Roots were washed free of soil and scanned on a flatbed color scanner. Total root surface area (including root length and diameter) per square meter of turf canopy (m^2 m^{-2} turf canopy) was quantified using WinRhizo software (Regent Instruments, Quebec). Roots were then oven-dried at 80 ^oC for 7 d and measured for dry weight (DW). Root biomass was expressed as root DW per square meter of turf canopy (g DW m^{-2} turf canopy).

Statistical Analysis

Analysis of variance was based on the general linear model procedure of SAS 9.1 (SAS Institute Inc., Cary, NC). Effects of chemical treatments were tested using analysis of variance (ANOVA). Treatments were compared separately for each sampling date in each year. Treatment differences were separated by Fisher's protected least significant difference (LSD) test at the 0.05 probability level. LSD bars were present in the figures when significant chemical effects were detected.

RESULTS

Yearly interactions were significant for all the parameters so that the 2007 and 2008 data for each parameter were presented separately.

Turf quality

In 2007, TQ in all plots gradually declined from July 3 to Sep. 4 and showed partial recovery on Sep.17 (Fig. 1A). Plots sprayed with CPR and TurfVigor consistently maintained 22-100% higher TQ than the control plots on all sampling dates. TQ of TE-treated plots were not different from that of the control plots between July 3 and Aug. 1. But on Aug.14, Sep. 4 and Sep. 19, TE treatment improved TQ by 37, 62 and 29%, respectively, compared to the control plots.

In 2008, TQ declined from June 24 to Aug. 9 and recovered to some extent after Aug. 26 in all plots (Fig. 1B). CPR and TurfVigor treatments consistently increased TQ by 6-28% on all sampling dates, compared to the control plots. Plots treated with TE maintained 8-18% higher TQ than the control plots on all sampling dates except June 24.

Turf density

Turf density was estimated as the ratio of R_{935}/R_{661}. The ratio of R_{935}/R_{661} for all plots declined from July 3 to Sep. 4 and then increased to above the July level on Sep.17 in 2007 (Fig. 2A). The R_{935}/R_{661} ratio of CPR-treated plots was 11, 14 and 16% higher than that in the control plots on July 17, Aug. 1 and 14, respectively. Plots sprayed with TE also maintained 9 and 14% higher R_{935}/R_{661} ratio on Aug. 1 and 14, compared to the

control plots. The ratio of R_{935}/R_{661} in TurfVigor-treated plots were 16-27% higher from July 17 to Aug. 14, and also recovered more quickly and maintained 27% higher on Sep. 19 compared to the control plots.

The R_{935}/R_{661} ratio in 2008 followed a similar pattern of changes as that in 2007, with a graduate decline from June 24 to Aug. 26 for all plots and then recovered to above the June level on Sep.10 (Fig. 2B). The ratio was 12-18% higher in plots sprayed with TurfVigor than the ratio of the control plots. TE-treated plots also maintained 12 and 14% higher R_{935}/R_{661} on Aug. 26 and Sep. 10 than the control plots. There was no significant difference in R_{935}/R_{661} between CPR-treated plots and the control plots in 2008.

Chlorophyll content

In 2007, CHL in all plots declined from July 3 to Sep. 4 and increased to the same or above the July level on Sep.17 (Fig. 3A). TurfVigor-treated plots consistently maintained 22-76% higher CHL than the control plots. CPR treatment resulted in 53 and 61% higher CHL content on Aug. 14 and Sep. 19, respectively, compared to the control plots. Plots sprayed with TE maintained 53% higher CHL than the control plots on Aug. 14.

In 2008, CHL of control plots exhibited significant decline from June 24 to Aug. 9, whereas CHL in all other treatments declined till July 25 and then recovered to above the June level on Sep. 10 (Fig. 3B). CPR-treated plots maintained 79, 22 and 22% higher CHL than the control plots on Aug. 9, Aug. 26 and Sep.10, respectively. Plots sprayed

with TurfVigor had 67 and 29% higher CHL than the control plots on Aug. 9 and Aug. 26. TE treatment increased CHL by 47% on Aug. 9 compared to the control plots.

Canopy net photosynthetic rate

In 2007, canopy Pn gradually declined from July 3 to Aug. 14 for all plots, followed by a fully recovery in CPR and TurfVigor-treated plots and a partial recovery in TE-treated plots and control plots on Sep. 4 (Fig. 4A). TurfVigor-treated plots maintained 12-64% higher Pn than the control plots on all sampling dates. TE-treated plots exhibited 18% higher Pn only on Sep. 4, compared to the control plots. CPR treatment had no significant effects on canopy Pn on any sampling date.

In 2008, canopy Pn declined from June 24 to Aug. 9 and then recovered to above the June level for all plots (Fig. 4B). The greatest Pn reduction was observed on Aug. 9 in the control plots (35% of the initial level in June), and the smallest reduction was in TurfVigor-treated plots (18% of the initial). Statistically significant treatment effects on Pn was only detected in TE-treated plots on June 24 and in TurfVigor-treated plots on July 25 compared to the control plots. There was no significant difference in Pn between CPR-treated and the control plots.

Root growth

TurfVigor treatment resulted in significantly larger root surface area than control treatment on Aug. 14 in 2007 and Sep. 10 in 2008 (Fig. 5A, B). CPR and TE treatments did not affect root surface area. Root biomass gradually decreased for all plots during the whole experimental period in both years. It declined by 54% on Sep. 19 in 2007 and 67% on Sep. 10 in 2008, respectively, in the control plots. In 2007, plots sprayed with

TurfVigor and TE maintained greater root biomass than the control plots on July 17 and Aug. 14 (Fig. 6A). In 2008, greater root biomass was observed in CPR-treated plots on Sep. 10 and in TurfVigor-treated plots on Aug. 26 and Sep. 10, compared to the control plots (Fig. 6B).

DISCUSSION

Overall turf performance of creeping bentgrass evaluated by TQ declined in both summers in 2007 and 2008. The decline was more severe in 2007 than in 2008, which could be due to relatively higher maximum daily air temperature, especially from mid-July to early September in 2007 (Fig. 7). TQ decline was associated with leaf senescence, as demonstrated by decline in leaf chlorophyll content and photosynthetic rate, and decline in turf density, estimated by canopy reflectance ratio (R_{935}/R_{661}). In addition, root surface area and root biomass declined during summer months in both years, indicating less root production and more root death occurring with increasing temperature. These observations were consistent with previous findings that summer bentgrass decline was associated with leaf senescence, decline in photosynthetic activities, and increases in root mortality (Liu and Huang, 2000; Xu and Huang, 2006).

The two biostimulants significantly improved visual quality of creeping bentgrass putting green during the summer. Higher TQ in TurfVigor or CPR-treated plots was observed on all sampling dates in both years compared to the control plots. Leaf senescence during summer was alleviated, as manifested by suppression of chlorophyll loss and increased canopy density in plots treated with either product. The maintenance of higher chlorophyll and more photosynthetically active leaves enabled the maintenance of higher canopy photosynthesis in creeping bentgrass treated with TurfVigor or CPR during summer months in both years. In addition, there were some positive effects of both biostimulants on root growth of creeping bentgrass. TurfVigor-treated plots exhibited larger root surface area on Aug. 14 in 2007 and Sep. 10 in 2008 and higher root

biomass on July 17 and Aug. 14 in 2007, and Aug. 26 and Sep. 10 in 2008. CPR-treated plots exhibited greater root biomass on Sep. 10 in 2008.

The growth promoting effect of seaweed-extract based biostimulants is thought to be due to various organic compounds present in the seaweed extract, and more specifically, due to the presence of relatively high levels of cytokinins (Steveni et al., 1992). Cytokinins are known for their functions of suppressing leaf senescence and promoting tillering (Gan and Amasino, 1995; Xu et al., 2009). Zhang and Ervin (2008) recently compared the effects of seaweed-based cytokinins to a cytokinin standard (10 μM ZR) on creeping bentgrass under heat stress (35/25 ^{o}C, day/night) and found that endogenous cytokinin contents increased to comparable levels for the two treatments. Therefore, application of seaweed-based biostimulants could affect the hormone status within plants. Additionally, the adverse effects of high soil temperatures on shoot growth could be attributed to decreased nutrient uptake by roots (Fry and Huang, 2004), whereas cytokinins are related to nitrogen mobilization and partitioning (Goicoechea et al., 1996). Rathore et al. (2008) studied the effects of foliar applications of a seaweed extract on nutrient uptake, growth and yield of soybean without the application of chemical fertilizers. They did observe enhanced yield as well as improved nutrient uptake (N, P, K and S) with seaweed extract applications. Some studies suggested that the effects of seaweed extracts were independent of the addition of macro- and microelements (Mueller and Kussow, 2005; Wrightman and Thimann, 1980). Beckett et al. (1994) investigated the effect of the seaweed concentrate 'Kelpak' on the yield of tepary bean (*Phaseolus acutifolius*) grown under conditions of varying nutrient supply and found Kelpak significantly increased the yield of plants growing at all concentrations of nutrient supply,

suggesting that seaweed extract did not act simply as a fertilizer. Alternatively, some other studies suggested the micronutrients in a seaweed-based biostimulant formulation may act mainly as enzyme catalysts (Silva et al., 2008). Rayorath et al. (2008) found that *A. nodosum* extract induced amylase activity in barley (*Hordeum vulgare*). Zhang et al. (2003) reported that seaweed extract applications increased superoxide dismutase activity and improved physiological activity of creeping bentgrass, irrespective of fertilization regimes. The microbial strains in TurfVigor may have some additional beneficial effects, but need further testing. Mueller and Kussow (2005) found the root-zone microbial community did respond to summer decline of bentgrass roots and concomitant decreases in quantities of root exudates, but the five biostimulants they tested did not effectively alter the putting green microbial community in terms of enzyme activity or substrate utilization.

TE treatment significantly improved TQ of creeping bentgrass from mid-August to mid-September in 2007 and from early July to mid-September in 2008 in this study. The improvement in TQ was associated with increases in green color and turf density. TE has been shown to increase total chlorophyll content per unit leaf tissue and canopy density as measured through tiller counts or visual ratings (Ervin and Koski, 1998; Ervin and Koski, 2001; Fagerness and Yelverton, 2001; Stier and Rogers, 2001). We observed higher CHL on certain sampling dates (Aug. 1 in 2007 and Aug. 9 in 2008), as well as denser turf canopy as reflected by higher R_{935}/R_{661} ratio on Aug. 1 and 14 in 2007, and Aug. 26 and Sep. 10 in 2008, in TE-treated plots. The effects of TE on promoting maintenance of canopy leaf area and chlorophyll content are most likely due to a combination of decreased leaf senescence and increased tillering capability (Breuninger

and Watschke, 1989; Heckman et al., 2001). Ervin and Zhang (2007) found that sequential TE treatment significantly increased leaf cytokinin (trans-zeatin riboside, t-ZR) content of creeping bentgrass, Kentucky bluegrass (*Poa pratensis*), and hybrid bermudagrass (*Cynodon dactylon* × *C. transavaalensis*) sods grown in flats under a greenhouse mist system. Han et al. (1998) reported that TE affected photoassimilate partitioning to adjacent tillers and total non-structural carbohydrate accumulation. We observed positive effects of TE on photosynthetic activities, as manifested by higher canopy net photosynthetic rates in TE-treated plots on some sampling dates, suggesting TE may increase photosynthetic capacity that could favor creeping bentgrass survival under summer stress.

TE effects on root growth were not consistent in 2007 and 2008. Greater root biomass was observed on two of the six sampling dates in 2007 but not observed in 2008. Beasley and Branham (2007) reported that TE-treated Kentucky bluegrass showed no significant difference in total root length or surface area compared to control plants under two temperature regimes (23/18 ^{o}C and 30/25 ^{o}C, day/night). Temperature and TE interactive effects on root growth were inconclusive in previous studies with other turfgrass species. Han et al. (1998) reported TE increased root growth and root carbohydrate levels in 'Penncross' creeping bentgrass. However, in another study conducted by , TE did not affect root biomass of the same turf cultivars during most of the stress period and recovery. Additionally, Goss et al. (2002) found the increase in the number of tillers by TE significantly lowered the root to shoot ratio, because additional tillers had the same total root mass per unit area.

In summary, TE treatment significantly elevated TQ of creeping bentgrass under summer stress by alleviating leaf senescence but had limited effects on promoting root growth. The two seaweed-based biostimulants significantly improved visual quality of creeping bentgrass putting green in summer by promoting both shoot and root growth. Application of TE and selected biostimulants following their respective label rates in a 2-week interval may be effective to improve summer performance of creeping bentgrass.

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Fig. 1 Effects of TE, CPR, TurfVigor and control treatments on turf quality of creeping bentgrass during the experimental period in 2007 (A) and 2008 (B). Vertical bars indicate LSDs ($P \leq 0.05$) for treatment comparison at a given day of treatment. LSDs ($P \leq 0.05$) for comparison among the samplings dates in 2007 are 0.71, 0.53, 0.44 and 0.73 in TE, CPR, TurfVigor and control treatments, respectively; LSDs ($P \leq 0.05$) for comparison among the samplings dates in 2008 are 0.44, 0.44, 0.28 and 0.29 in TE, CPR, TurfVigor and control treatments, respectively. The LSD bars were not presented on sampling dates when treatment effects was not significant.

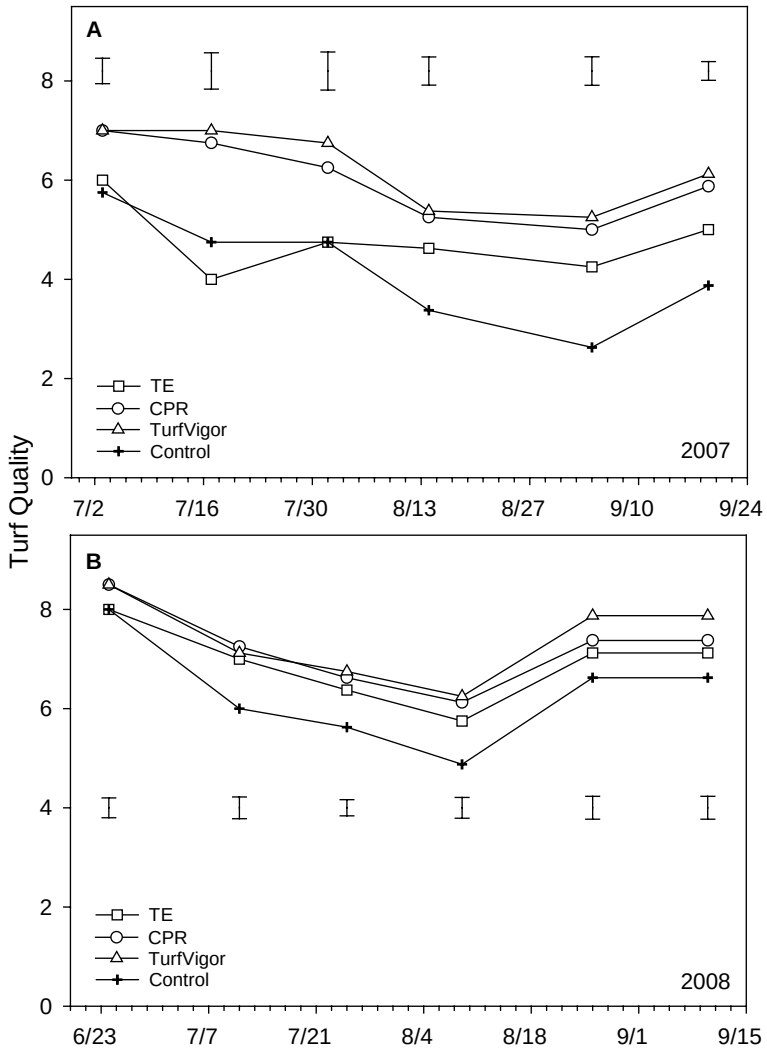


Fig. 2 Effects of TE, CPR, TurfVigor and control treatments on turf density estimated as R_{935}/R_{661} of creeping bentgrass during the experimental period in 2007 (A) and 2008 (B). Vertical bars indicate LSDs ($P \leq 0.05$) for treatment comparison at a given day of treatment. LSDs ($P \leq 0.05$) for comparison among the samplings dates in 2007 are 0.59, 0.76, 0.82 and 0.37 in TE, CPR, TurfVigor and control treatments, respectively; LSDs ($P \leq 0.05$) for comparison among the samplings dates in 2008 are 0.64, 0.68, 0.75 and 0.34 in TE, CPR, TurfVigor and control treatments, respectively. The LSD bars were not presented on sampling dates when treatment effects was not significant.

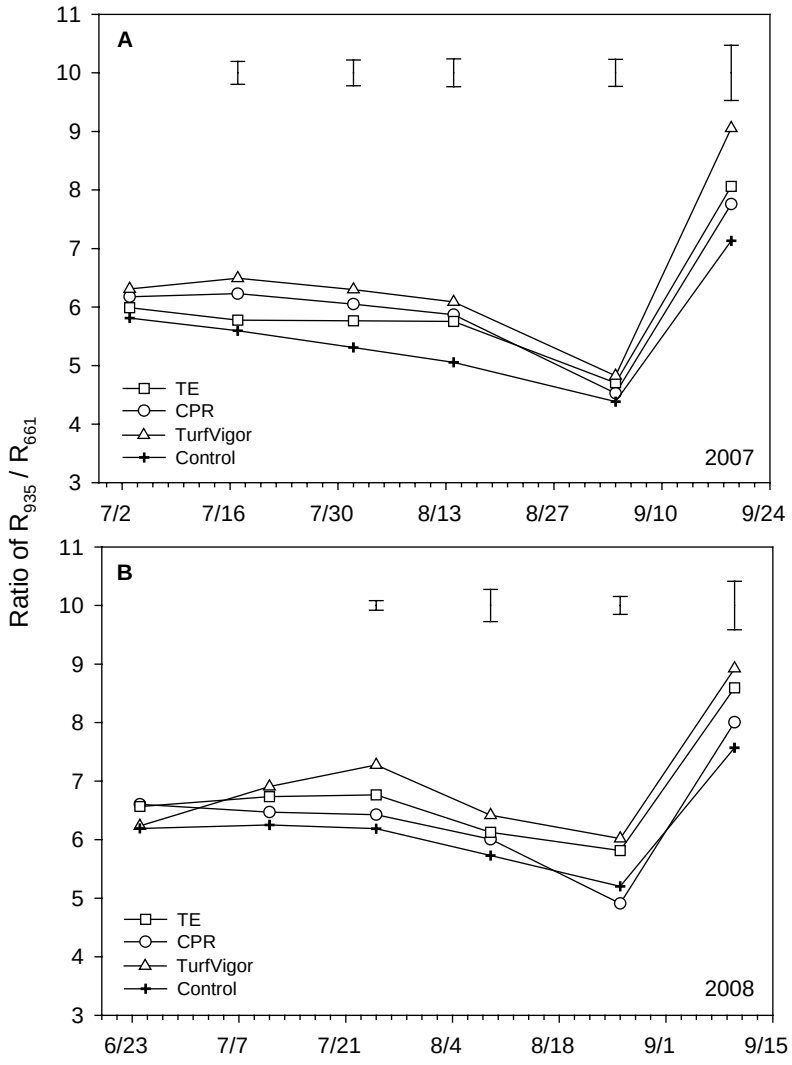


Fig. 3 Effects of TE, CPR, TurfVigor and control treatments on chlorophyll content (mg g^{-1} FW) of creeping bentgrass during the experimental period in 2007 (A) and 2008 (B). Vertical bars indicate LSDs ($P \leq 0.05$) for treatment comparison at a given day of treatment. LSDs ($P \leq 0.05$) for comparison among the samplings dates in 2007 are 0.29, 0.41, 0.30 and 0.30 mg g^{-1} FW in TE, CPR, TurfVigor and control treatments, respectively; LSDs ($P \leq 0.05$) for comparison among the samplings dates in 2008 are 0.47, 0.36, 0.33 and 0.25 mg g^{-1} FW in TE, CPR, TurfVigor and control treatments, respectively. The LSD bars were not presented on sampling dates when treatment effects was not significant.

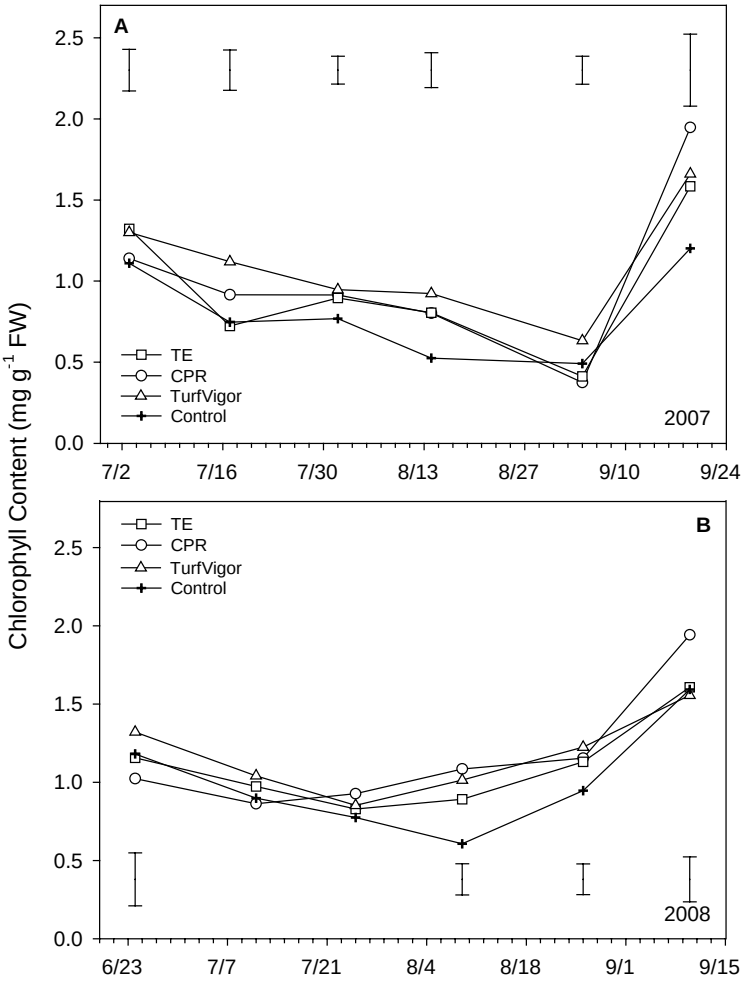


Fig. 4 Effects of TE, CPR, TurfVigor and control treatments on canopy Pn (CO_2 μmol m^{-2} s^{-1}) of creeping bentgrass during the experimental period in 2007 (A) and 2008 (B). Vertical bars indicate LSDs ($P \leq 0.05$) for treatment comparison at a given day of treatment. LSDs ($P \leq 0.05$) for comparison among the samplings dates in 2007 are 0.48, 0.51, 0.86 and 0.37 CO_2 μmol m^{-2} s^{-1} in TE, CPR, TurfVigor and control treatments, respectively; LSDs ($P \leq 0.05$) for comparison among the samplings dates in 2008 are 1.31, 1.85, 1.36 and 1.47 CO_2 μmol m^{-2} s^{-1} in TE, CPR, TurfVigor and control treatments, respectively. The LSD bars were not presented on sampling dates when treatment effects was not significant.

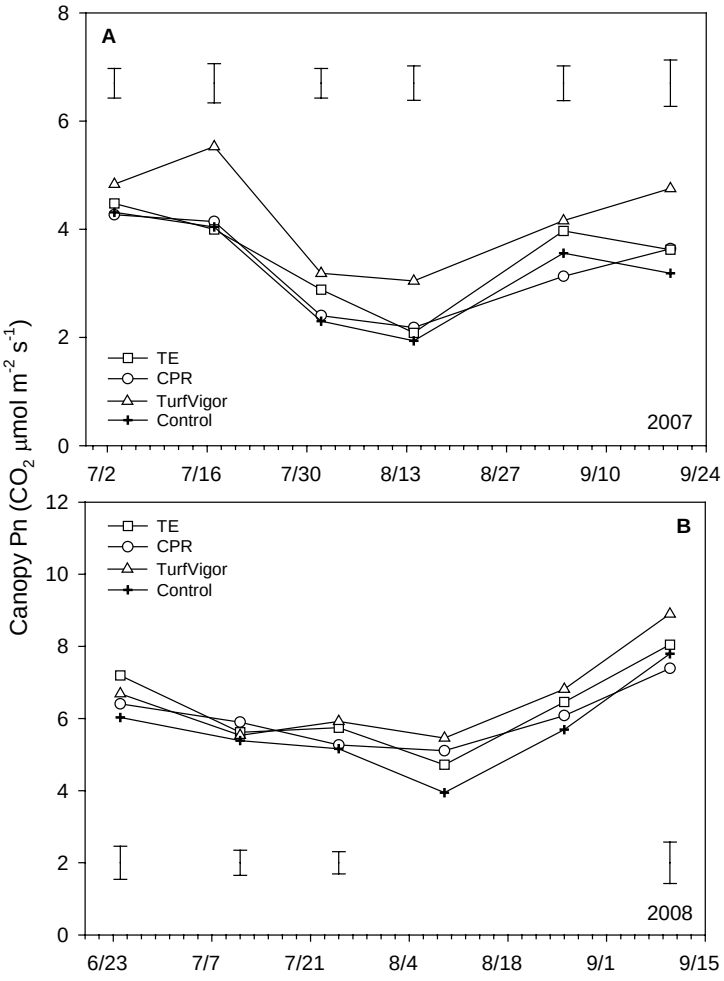


Fig. 5 Effects of TE, CPR, TurfVigor and control treatments on total root surface area (cm^2) of creeping bentgrass during the experimental period in 2007 (A) and 2008 (B).Vertical bars indicate LSDs ($P \leq 0.05$) for treatment comparison at a given day of treatment. LSDs ($P \leq 0.05$) for comparison among the samplings dates in 2007 are 65.19, 65.54, 97.71 and 72.89 cm^2 in TE, CPR, TurfVigor and control treatments, respectively; LSDs ($P \leq 0.05$) for comparison among the samplings dates in 2 008 are 115.07, 88.94, 96.23 and 111.91 cm^2 in TE, CPR, TurfVigor and control treatments, respectively. The LSD bars were not presented on sampling dates when treatment effects was not significant.

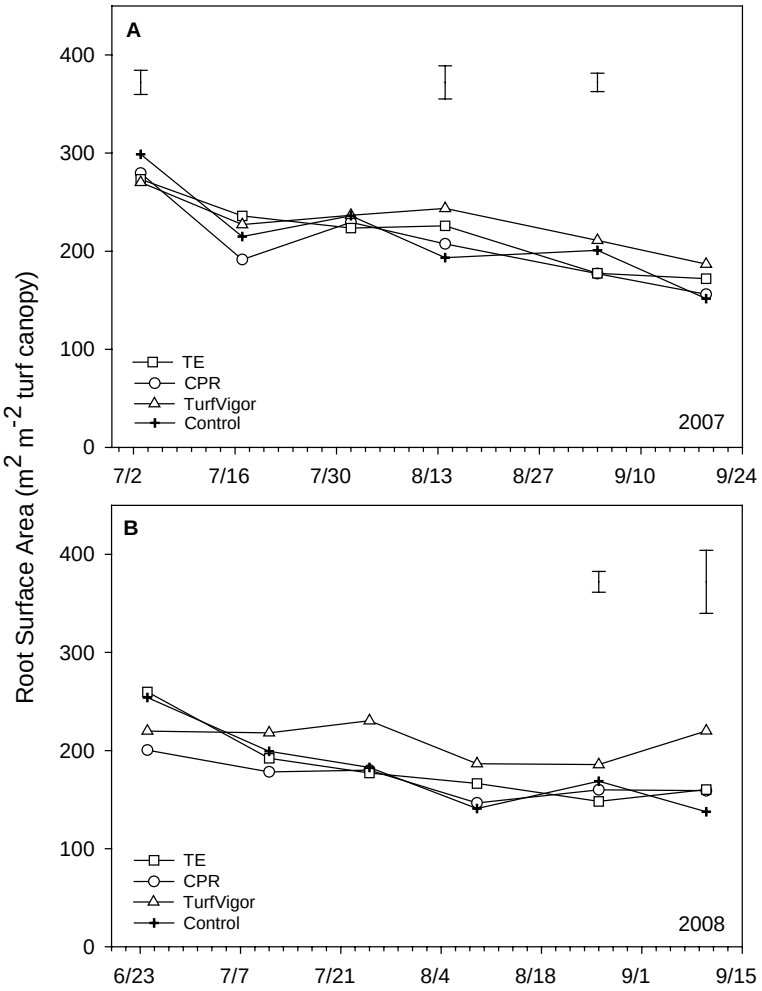


Fig. 6 Effects of TE, CPR, TurfVigor and control treatments on root biomass (g) of creeping bentgrass during the experimental period in 2007 (A) and 2008 (B). Vertical bars indicate LSDs ($P \leq 0.05$) for treatment comparison at a given day of treatment. LSDs ($P \leq 0.05$) for comparison among the samplings dates in 2007 are 0.046, 0.039, 0.061 and 0.054 g in TE, CPR, TurfVigor and control treatments, respectively; LSDs ($P \leq 0.05$) for comparison among the samplings dates in 2008 are 0.069, 0.054, 0.044 and 0.050 g in TE, CPR, TurfVigor and control treatments, respectively. The LSD bars were not presented on sampling dates when treatment effects was not significant.

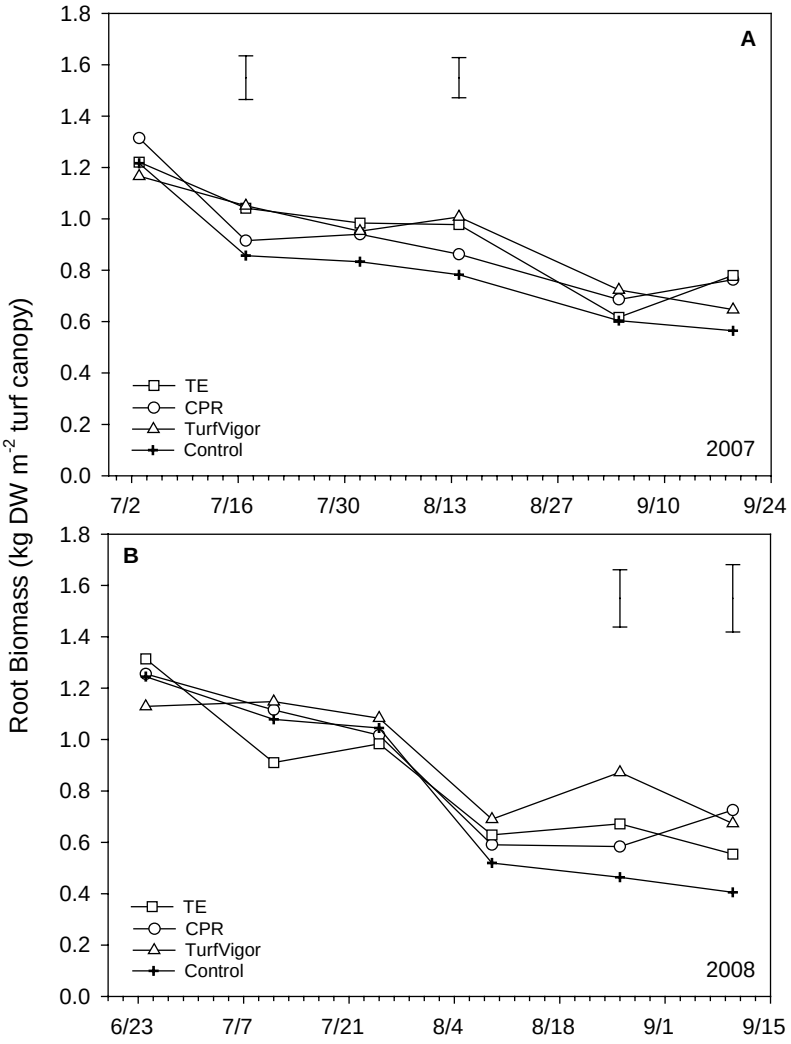
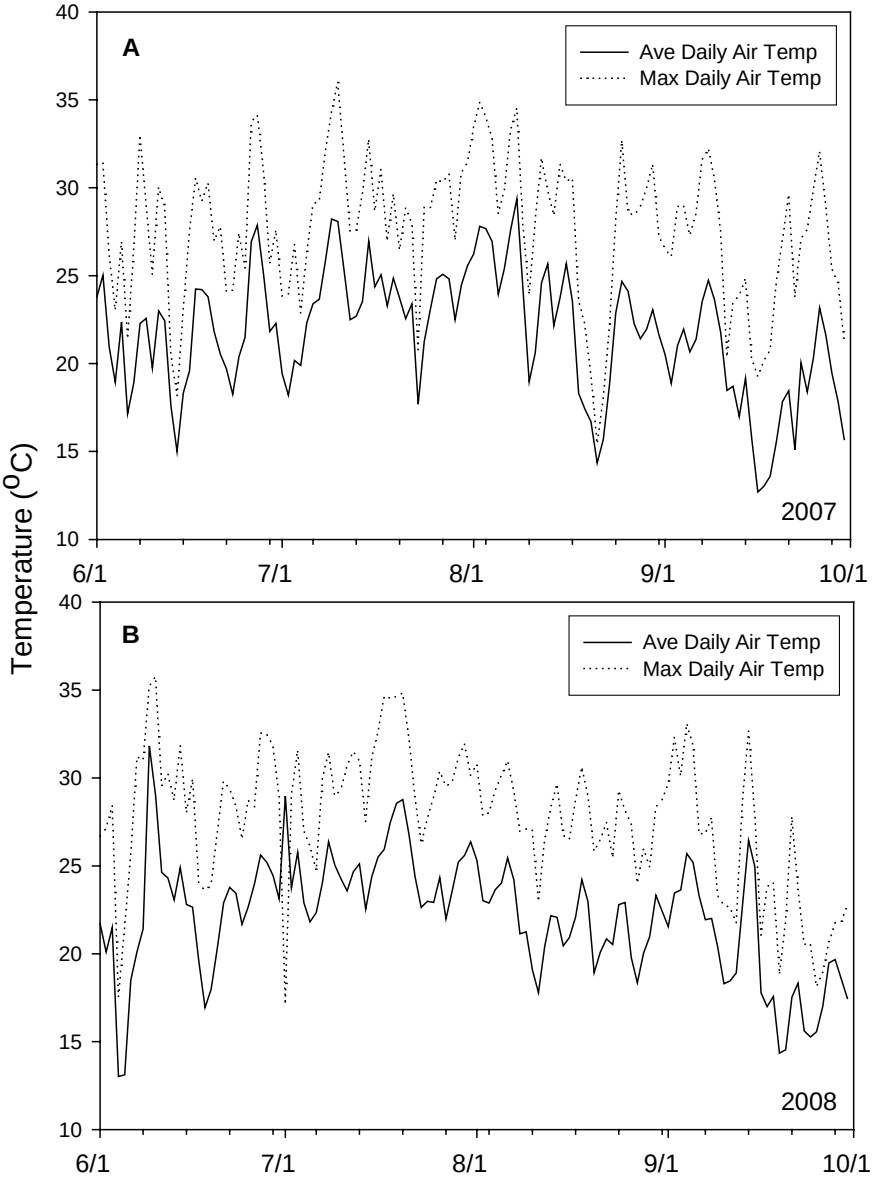


Fig. 7 Daily average and maximum air temperatures during the experimental period in 2007 (A) and 2008 (B). Data was recorded from an onsite weather station located at Horticulture Farm II, North Brunswick, NJ approximately 130 m from the experimental field plots.



CHAPTER 6. Effects of *SAG12-ipt* Expression on Cytokinin Production, Growth and Senescence of Creeping Bentgrass (*Agrostis stolonifera* L.) under Heat Stress

INTRODUCTION

Cytokinins are known for their regulation of various processes in plant growth and development, including leaf senescence (Sakakibara, 2006). A negative correlation between the concentration of cytokinins and the magnitude of leaf senescence has been reported in various plant species including *Agrosits* grass species (Xu and Huang, 2007). There are two main approaches that have been used to study the effects of cytokinins on plant senescence: exogenous application of cytokinins to leaves or soil, and transgenic manipulation of cytokinin biosynthesis (McCabe et al., 2001). Delay or suppression of leaf senescence through exogenous application of cytokinins has been reported in many plant species such as tobacco (*Nicotiana tabacum*) (Singh et al., 1992), rose (*Rosa hybrida*) (Mayak and Halevy, 1970) and potato (*Solanum tuberosum*) (McGrady et al., 1986). Foliar or root-zone application of cytokinins to creeping bentgrass has also been proved effective on delaying leaf senescence induced by various environmental stresses. Zhang and Ervin (2004) reported that foliar spray of seaweed extracts containing cytokinins alleviated drought-induced leaf senescence of creeping bentgrass. Liu et al., (2002) found that injection of cytokinin into the root-zone of creeping bentgrass suppressed leaf senescence and enhanced heat tolerance. However, the efficacy of exogenous applications is limited, because it is difficult to control the absorption and transport of cytokinin in the plants (Clark et al., 2004; Khodakovskaya et al., 2006).

Recent developments in molecular biology provide new tools to assess the roles of cytokinins in plant stress responses. An enzyme called adenine isopentenyl transferase (IPT) catalyzes the rate-limiting step in cytokinin biosynthesis (Gan and Amasino, 1995). A gene isolated from *Agrobacterium tumefaciens* that encodes the IPT enzyme has been available for transgenic research for more than a decade. The *ipt* gene has been introduced into different plant species, mostly dicotyledonous crop plants (Geng et al., 2001; Ivic et al., 2001; Khodakovskaya et al., 2005; Zhang et al., 2000). Different morphological and physiological changes have been observed in individual *ipt* transgenic plants due to the overproduction of cytokinins. Luo et al. (2005) reported that expression of the *ipt* gene ligated to the AGPase S1 promoter, which is active exclusively in sink tissues in tomato (*Lycopersicon esculentum*), resulted in the retention of chlorophyll and cytokinin in excised leaves or discs, as well as the greater formation of unbranched roots on cuttings compared to the wild type. In transgenic *ipt* tobacco plants under the control of a promoter for the small subunit of Rubisco (*Pssu-ipt*), overproduction of endogenous cytokinins caused stress responses such as over-accumulation of phenolic compounds, synthesis of pathogenesis related proteins and increase in peroxidase activities (Schnablova et al., 2006). Ivic et al. (2001) found that some *Pat- ipt* (*ipt* gene fused to a patatin gene promoter) transgenic sugar beet (*Beta vulgaris*) lines exhibited elevated glucose levels in the leaves and reduced sucrose concentration in the taproots.

Attempts have been made to use the *ipt* gene under the control of various stress-activated promoters including those inducible by heat, drought and light. Among the most widely used inducible promoters is *SAG12*, the promoter from an arabidopsis (*Arabidopsis thaliana*) senescence-associated gene encoding a cysteine protease

(Swartzberg et al., 2006). The mRNA of *SAG12* can be detected only in senescent tissues, which may result from natural senescence or stress-induced senescence (Gan and Amasino 1995). The *SAG12-ipt* construct has three important features: temporal regulation, spatial regulation, and quantitative regulation (Gan and Amasino, 1995; Gan and Amasino, 1996) and has the function of auto-regulation of cytokinin synthesis (Clark et al., 2004). Briefly, the expression of *SAG-ipt* is activated only at the onset of senescence, resulting in increases in cytokinin content, which prevents further tissue senescence. The suppression of senescence will in turn result in the attenuation of the SAG12 promoter, preventing further accumulation of cytokinins to a level that may interrupt normal plant development and cause morphological deformation.

In the past few years, the potential to improve plant growth and stress tolerance by incorporating the *ipt* gene into forage and turf grasses has received some attention. Studies have been conducted on a limited number of grass species such as tall fescue (*Festuca arundinacea*) for cold tolerance (Hu et al., 2005) and ryegrass (*Lolium multiflorum*) for delayed natural leaf senescence (Li et al., 2004). However, the effects of expression of *ipt* on heat tolerance of cool-season grasses have not been investigated. Decline in tiller and root growth and leaf senescence are typical symptoms of heat stress in cool-season grass species. Heat-induced leaf senescence is characterized by loss of chlorophyll, leading to the reduction in leaf photosynthetic capacity and whole-plant growth (John et al., 1995). In the case of a horticultural plant such as turfgrass, chlorosis can decrease its aesthetic appearance, resulting in a decrease in its salability and value in a landscape (Clark et al., 2004). This study was conducted to investigate the effects of expression of *SAG12-ipt* on shoot and root growth and leaf senescence in a heat-sensitive

turfgrass species, creeping bentgrass (*Agrostis stolonifera*), exposed to heat stress. Such information will provide better understanding of the roles of cytokinins in regulating cool-season grass tolerance to high temperature stress.

MATERIALS AND METHODS

Tissue Culture, *Agrobacterium*-Mediated Transformation, and Plant Regeneration

A single stolon from a single plant of creeping bentgrass (cv. Penncross) was collected and cut into pieces (50 mm in length) for the production of calli. Calli were produced in darkness at 24 ˚C on a tissue culture media containing 4.3 g MS salts, 500 mg casein hydrolysate, 100 mg myo-inositol, 6.6 mg dicamba, 2.5 mg benzyladenine, 30 g sucrose and 2 g Gell-Gro in 1 L of water at pH 5.7. The media was changed every 3 weeks.

The plasmid pSG516 containing *SAG12-ipt* and the *nos* termination sequence was obtained from R.M Amasino (University of Wisconsin). pSG516 was cut with *SpeI* to release *SAG12-ipt-nos*. This fragment was cloned into the *XbaI* site of the binary vector pCAMBIA1301 (CAMBIA, Canberra, Australia; GenBank accession no.: 234297) and transformed into *A. tumefaciens* strain LBA 4404 via electroporation. Hygromycin resistance from the *hptII* gene in pCAMBIA1301 was used as the selective marker. Calli were subjected to co-cultivation with *A. tumefaciens* containing pCAMBIA1301-SAG12-ipt. After callus selection on hygromycin-containing media and plant regeneration, the presence of an intact gene construct was confirmed by PCR with the 720 bp *ipt* gene cDNA fragment. The *A. tumefactions*-infected calli were co-cultured on tissue culture media with the addition of the antibiotic cefotaxime (250mg L^{-1}) for one week and then transferred to a selection media with the addition of hygromycin (175mg L^{-1}). Surviving calli were then moved to regeneration media. Seedlings that survived antibiotic screening were considered transgenic and selected for further analysis.

All calli were derived from a single stolon from a single plant. The *SAG12-ipt* transgenic plants and a transgenic control line (containing the pCAMBIA1301 vector without the *SAG12-ipt* insert) are therefore genetically identical. Eight independent transgenic control lines were generated and the transformation has been confirmed with southern blotting. A transgenic line exhibiting desirable turf quality traits and that was similar in growth rate and growth habit to the wild type was chosen for comparison to the *SAG12-ipt* plants in this study. Eight *SAG12-ipt* transgenic lines of creeping bentgrass and a transgenic control line were propagated in a greenhouse for this study. The greenhouse had natural light averaging 600 μmol m^{-2} s^{-1} photosynthetic photon flux density at the canopy height for 14 h photoperiod and an average air temperature of 21 ^{o}C/14 ^{o}C (day/night). The transgenic plants and the untransformed plants were started from the same number of tillers and established under the same conditions. Five tillers of the same size for each *SAG12-ipt* line as well as the control line were transplanted into plastic tubes (5 cm in diameter and 40 cm in length) filled with sterilized sand. Holes at the bottom of the tubes provided good drainage. Plants were watered daily and fertilized twice a week with full-strength Hoagland's solution (Arnon, 1949). Plants were established in the greenhouse for 42 d and then transferred to controlled-environment reach-in growth chambers (Conviron, Winnipeg, Canada) with a temperature of 20 ºC/15 ºC (day/night), 14-h photoperiod, 50% relative humidity, and 500 μmol m^{-2} s^{-1} photosynthetic photon flux density at the canopy height.

Treatments and Experimental Design

After 42 d from transplant establishment, plants were allowed to acclimate to growth chamber conditions for 7 d before temperature treatments were imposed. Four tubes of plants for each line (four replicates) were sampled for initial measurements as the control. Six tubes of plants for each line (six replicates) were exposed to 35 ºC (high temperature) for 14 d in three growth chambers. Plants were watered twice daily until free drainage occurred from the bottom of the tube to prevent water deficit during the heat treatment period. Three tubes of plants for each line in each chamber were relocated every three days to different chambers so that plants were exposed to four different chambers to eliminate potential effects of growth chamber environments. The experiment consisted of two factors (temperature and plant lines), which were arranged as a split-plot design with temperature as the main factor and plant line as the sub-factor.

Measurements

This study focused on the examination of physiological changes of transgenic plants during exposure to heat stress and the comparison between the transgenic plants and the untransformed plants under heat stress conditions. Therefore, plants before and after heat stress treatment were compared. All of the following measurements were made after 49-d of establishment or 0 d of heat stress and at 14 d of heat stress.

Growth characterization

Total tiller number per plant was counted individually for six plants in each transgenic line. Leaf chlorophyll was extracted using dimethyl sulfoxide (DMSO). 0.2 g of fresh leaves (a combination of leaves of different ages representing the overall greenness of the plant) was incubated in 10 mL DMSO for 48 h in darkness. Original leaf

extracts were diluted eight times, and then the absorbance of the diluted extracts was determined using a spectrophotometer (Spectronic Genesys2, Spectronic Instruments, Rochester, N.Y.). Chlorophyll content was calculated based on the absorbance at 663 nm and 645 nm using the formulas described by Arnon (1949). A separate set of leaf samples were taken from the same plant at the same time of sampling for chlorophyll extraction, and fresh weight was measured immediately. The samples were then dried in an oven at 80 ^{o}C to measure dry weight. The fresh weight to dry weight ratio was calculated and used to convert fresh weight of samples used for chlorophyll extraction to dry weight. Leaf chlorophyll content was expressed as mg g^{-1} dry weight to eliminate the variation in water content among samples.

Roots were washed free from sand and root number was counted individually for each plant. Roots were then scanned on a flatbed color scanner. Total root length for each plant was measured using WinRhizo software (Regent Instruments, Quebec, Canada).

Cytokinin analysis

Two major forms of cytokinin, trans-zeatin/zeatin riboside (Z/ZR) and isopentenyladenine/adenosine (iP/iPA), were quantified by an indirect competitive enzyme-linked immunosorbent assay (ELISA). Extraction and quantification of hormones followed the method described by Setter et al. (2001) with some modifications (Wang et al., 2003). Briefly, samples were extracted in 80% [v/v] methanol and purified with reverse phase C_{18} columns. Hydrophilic contaminants were washed out with 200 μL of 20% solvent [20% methanol, 80% aqueous TEA (10mM triethylamine, pH3.5)]. The cytokinin-containing fraction was eluted using 200 μL of 30% solvent [30% methanol,

70% aqueous triethylamine (TEA)]. An indirect competitive ELISA was used for quantification of Z/ZR and iP/iPA as previously decribed by Setter et al. (2001). Monoclonal antibodies against Z/ZR and iP/iPA were developed by Eberle et al. (1986).

Northern blot of ipt expression

Total RNA was extracted from shoot samples using TRIZOL Reagent (Invitrogen, USA). A 10 µg of RNA was size fractionated in 1.2% (w/v) agarose gel for 3 hours at 65 volts and transferred to a nylon membrane using a capillary blot method. The membranes were UV-cross linked. Different gene fragments were separately labeled using a random primed labeling kit (Ambion, USA). The labeled probes were purified using NICK^{TM} columns (GE Healthcare, Sweden). Hybridization was carried out at 42 °C overnight in a NorthernMax prehybridization/hybridization buffer (Ambion, USA). The membranes were washed with 2×SSC, 0.2% (w/v) SDS at 42°C for 10 min, and then with 0.1×SSC, 0.1% SDS at 42 °C for 10 min. Membranes were exposed to X-ray film (Fuji photo film, Japan) at –80 °C for signal detection. The strength of each band was quantified by GeneTool software (Syngene, Frederick, MD).

The northern blots were replicated three times for each sample. The best representative image from the three replicates was selected and included in the paper.

Statistical Analysis

Analysis of variance was based on the general linear model procedure of SAS (SAS Institute Inc., Cary, NC). Effects of transgenic lines, temperature and their interactions were tested separately for each sampling date for all the parameters.

Differences between treatment means were separated by Fisher’ protected least significant difference (LSD) test at the 0.05 probability level.

RESULTS

Tiller formation and leaf greenness of *SAG12-ipt* transgenic creeping bentgrass in response to heat stress.

Five *SAG12-ipt* transgenic lines (S32, S37, S40, S41, and S43) had significantly more tillers than the control line at both 0 and 14 d after heat treatment (Table 1). S25 had more tillers than the control at 0 d, but not at 14 d. S16 had more tillers than the control at 14 d, but not at 0 d. S7 did not significantly differ from the control in tiller production. The number of tillers did not change in the control line, but increased in six *SAG12-ipt* lines (S16, S32, S37, S40, S41, and S43) after 14 d of heat stress.

Six *SAG12-ipt* lines (S16, S25, S32, S37, S41, and S43) had significantly higher leaf chlorophyll content at 0 and 14 d of heat stress (Table 1). S7 maintained higher chlorophyll content than the control after 14 d of heat stress, but not at 0 d. S40 had higher chlorophyll content than the control only at 0 d. The control line and S16, S25, S32, S40, S41, and S43 exhibited a decline in chlorophyll content during 14 d of heat stress, whereas S7 and S37 maintained chlorophyll content at the initial level after 14 d of heat stress. However, the decline in chlorophyll content of the control line was greater than most of the *SAG12-ipt* lines, with a reduction of 32, 18, 21, 18, 40, 17, and 19% in the control line, S16, S25, S32, S40, S41, and S43, respectively.

Root number and growth of SAG12-ipt transgenic creeping bentgrass in response to heat stress

Four *SAG12-ipt* lines (S32, S40, S41, and S43) had significantly more roots than the control line at both 0 and 14 d of heat treatment (Table 2). Three *SAG12-ipt* lines (S7,

S16, and S37) did not differ from the control at 0 d, but had a greater number of roots at 14 d of heat stress. S25 had more roots at 0 d, but not at 14 d of heat stress compared to the control. There was no significant change in root number in the control line and S16, S25, S40, but significant increases ranging from 65% to 83% of the initial root number were detected in S7, S32, S37, S41, and S43 after 14 d of heat stress.

The *SAG12-ipt* lines S25, S41, S43, had significantly longer roots than the control line at both 0 and 14 d of heat stress (Table 2). The *SAG12-ipt* lines S32, S37, S40 did not differ from the control at 0 d, but had longer roots at 14 d of heat stress. The control and two *SAG12-ipt* lines (S7, S16) had significantly shorter roots after 14 d of heat stress compared to their respective initial length. No significant changes in root length were observed in line S25, S32, S37, S40, S41 and S43 following 14 d of heat stress, compared to their respective initial values.

Expression pattern of the ipt gene in SAG12-ipt transgenic creeping bentgrass in response to heat stress

Expression of the *ipt* gene was enhanced by heat stress in six transgenic lines (S16, S25, S32, S37, S41, S43) compared to the initial level of each line at 20 ^{o}C (Fig. 1). All six lines had stronger expression of the *ipt* gene than the control line under heat stress, and three of them (S32, S41 and S43) also had higher initial expression of *ipt* than the control line at 20 ^{o}C. There was no up-regulation of *ipt* gene expression in S7 by heat stress; however, the expression was stronger than the control at both 20 and 35 ^{o}C.

Expression of the *ipt* gene was low in S40 at 20 and 35 ^{o}C, and not different from the control line.

Cytokinin production in SAG12-ipt transgenic creeping bentgrass in response to heat stress

Five *SAG12-ipt* lines, S25, S32, S37, S41, S43, which had increased tiller and root growth, as well as strong *ipt* expression, were selected for analysis of cytokinin content. Differences between the control and *ipt* lines and changes during heat stress for shoot iPA, root iPA, shoot ZR, and root ZR content are illustrated in Fig.2-5.

Under normal growth temperature (20 ^{o}C) or at 0 d of heat stress, no difference in shoot iPA content was detected between the control and *SAG12-ipt* lines, except for S41 with a lower iPA content (Fig. 2). At 14 d of heat stress, all *SAG12-ipt* lines except S43 had significantly higher iPA content than the control line. Shoot iPA content decreased significantly in the control line after 14 d of heat stress, whereas in all the *ipt* lines, iPA content did not change during heat stress.

Root iPA content was not different between the control and *SAG12-ipt* lines at 0 d of heat stress. Five of the *SAG12-ipt* lines had 2.5-3.5 times more iPA in their roots after 14 d of heat stress compared to their initial levels at 0 d (Fig. 3). In contrast, root iPA content in the control line decreased by 20% at 14 d of heat stress and was significantly lower than in all transgenic lines.

ZR content in the shoots of three *SAG12-ipt* lines (S25, S37, S41) was not significantly different from the control line at 0 or 14 d of heat stress (Fig. 4). S32, which had the same ZR content as the control line at 0 d, maintained more ZR in shoots at 14 d of heat stress. Root ZR content was not different between the control and all *SAG12-ipt* lines at 14 d of heat stress (Fig. 5). Two *SAG12-ipt* lines (S25, S32) had lower root ZR content than the control line at 0 d.

Table 3 shows total iPA and ZR content of whole plants. Two *SAG12-ipt* lines (S41, S43) had lower iPA and the other three (S25, S32, S37) showed no difference from the control at 0 d. At 14 d of heat stress, total iPA declined in the control line, but increased in all *SAG12-ipt* lines and was significantly higher than in the control line. Three *SAG12-ipt* lines (S25, S41, and S43) had lower initial ZR and two others (S32, S37) were comparable to the control at 0 d. At 14 d of heat stress, all *SAG12-ipt* lines except S25 had significantly more ZR than the control. Total ZR content decreased significantly at 14 d of heat stress in the control line, while it remained unchanged in *SAG12-ipt* lines S25, S32, S37 and S41, and increased in S43.

DISCUSSION

Leaf senescence characterized by loss of chlorophyll can be induced by many environmental factors, including high temperatures and wounding, and by internal factors such as aging and reproductive development (Gan and Amasino 1997). In the present study, leaf chlorophyll content declined significantly after 14 d of heat stress at 35 ^{o}C in the control plants. However, most *SAG12-ipt* lines exhibited less severe decline and maintained significantly higher chlorophyll content than the control line after 14 d of heat stress. Some *SAG12-ipt* lines also had higher initial chlorophyll content than the control line prior to heat exposure (0 d of heat stress) or during the phase of plant establishment (after 49 d of tiller transplanting). *SAG12* is senescence specific, which can be induced during natural and stress-induced senescence (Gan and Amasino 1995). The retention of chlorophyll has been reported in *ipt*-transgenic plants exposed to various stress treatments, such as dark incubation (Khodakovskaya et al., 2006), waterlogging (Huynh et al. 2005) and freezing stress (Hu et al., 2005). The suppression of leaf senescence in *SAG12-ipt* grass plants during heat stress could be related to the activation of *SAG12-ipt* expression by heat-induced leaf senescence. The retention of chlorophyll during plant establishment in transgenic plants could be due to the activation of *SAG12-ipt* by wounding-induced senescence associated with tiller transplanting or by natural senescence signals associated with tissue aging in mature plants. It is worth mentioning that some of the transgenic lines contained rather lower amounts of iPA or ZR compared to the control line prior to heat treatment. This could be explained as there exists other forms of cytokinins than the two that haven been measured such as dihydrozeatin, which significantly contributed to the total amount of cytokinins. There is also a possibility that

zeatin could be transformed to the corresponding storage O-glucoside(s) in order to maintain cytokinin homeostasis, which could escape from the ELISA analysis.

A marked accumulation of *ipt* transcripts in the leaves of the *SAG12-ipt* lines during heat treatment was detected in the study. Coordinately, total iPA content increased in all five *SAG12-ipt* lines examined, whereas in the control line iPA decreased after 14 d of heat stress; total ZR content was maintained at the original level in the *SAG12-ipt* lines whereas that in the control line decreased after 14 d of heat stress. This association between the increase of cytokinin and the up-regulation of *ipt* gene transcription indicated that expression of the *ipt* gene in creeping bentgrass plants could produce a functional isopentenyl transferase enzyme when *SAG12-ipt* was activated by heat stress. Huynh et al. (2005) had come to a similar conclusion in a study with *SAG12-ipt* arabidopsis plants exposed to flooding stress. In addition, cytokinins are primarily produced in roots in the form of iPA, which is the direct product of IPT enzyme activity. All five transgenic lines accumulated 2.5-3.5 times more iP/iPA in the roots after 14 d of heat stress compared to their initial levels, while the control line lost 20% of its initial iPA. A comparable increase in iP/iPA (1.75-4 times) in *ipt*-transgenic tall fescue plants driven by the maize ubiquitin promoter after plant exposure to low temperature was previously reported by Hu et al. (2005). Another study conducted on transgenic annual wormwood (*Artemisia annua*) also reported 2- to 3-fold increase in iP/iPA (Geng et al., 2001). However, we did not observed any significant increase of Z/ZR in the transgenic *SAG12-ipt* creeping bentgrass, in contrast to studies on tobacco (Jordi et al., 2000; Thomas et al., 1995) or arabidopsis (Huynh et al., 2005). Instead, we observed a steady maintenance of Z/ZR at their original levels in most *SAG12-ipt* transgenic lines in contrast to the decline in the

control line. This may indicate limitations in the capacity of creeping bentgrass to hydroxylate iP/iPA to Z/ZR compared to other systems. It may be critical to sustain the balance between iP/iPA and Z/ZR-type cytokinins to ensure normal growth and development of the transgenic creeping bentgrass, which is actively controlled by *SAG12* promoter in response to stress signals.

The expression of *SAG12-ipt* induced by natural and heat-induced leaf senescence in perennial grass plants may have resulted in the promoting effects on shoot growth of creeping bentgrass. Six of the *ipt* lines developed more tillers than the control line during the 49-d period of plant establishment under normal growth temperatures, suggesting that transformation with the *SAG12-ipt* promoted tiller formation in creeping bentgrass even under non-stress temperature conditions. This could be related to the activation of *SAG12-ipt* expression by wounding-induced senescence associated with tiller transplanting or by natural senescence signals associated with tissue aging in mature plants, as leaves have fast turnover rate in perennial grasses. Following 14 d of heat stress, the production of tillers continued in six of the *SAG12-ipt* lines, whereas it ceased in the control line, and the number of tillers were several times more in the transgenic than in the control plants (Table 1). This may be due to the enhanced expression of the *ipt* gene in response to heat-induced senescence signals. The activation of *SAG12-ipt* expression at the onset of natural or heat-induced senescence may stimulate cytokinin synthesis as discussed above, thereby promoting tiller formation. It has been previously reported that the elevated cytokinin production in *ipt*-transgenic plants often resulted in the release of apical dominance, in the form of excessive lateral branching or flower buds

in many dicot plant species (Clark et al., 2004; Khodakovskaya et al., 2006), and in the form of increased tillering ability in tall fescue grass (Hu et al., 2005).

Many other studies reported decreased root branching after stress treatment in *ipt*-transgenic dicot plants (Clark et al., 2004; Luo et al., 2005), and severe reductions in root growth in plants transformed with *ipt* driven by constitutive promoters (Hewelt et al., 1994; Van Loven et al., 1993). In contrast, most of the transgenic creeping bentgrass with the senescence-inducible promoter produced more roots, and had greater total root length than the control line under both normal growth temperature and heat stress. The promotive effects of *SAG12-ipt* expression on root formation of creeping bentgrass may result from increased tiller production. When we compared the number of roots and total root length on a per tiller basis, no differences between the *SAG12-ipt* lines and the control line were found. For example, root number/tiller is 1.95 for *SAG-ipt* S43 compared to 1.47 for the control line; root length/tiller is 59.0 mm for *SAG-ipt* S43 versus 58.1 mm for the control line at 0 d of heat stress. However, under heat stress, total root length declined significantly for the control line, while most *SAG12-ipt* lines (6 out of 8 lines) did not exhibit root length decline. Our results suggest that the increased root production may be the result of an indirect effect of *SAG12-ipt* transformation on tillering in creeping bentgrass while the maintenance of root length under heat stress are at least in part due to enhanced root survival and growth due to the expression of *SAG12-ipt* and the resulting elevated level of cytokinins. These results are in agreement with other studies indicating that cytokinins were positively associated with the maintenance of root viability under heat stress (Liu et al. 2002; Liu and Huang 2004).

In summary, most *SAG12-ipt* transgenic creeping bentgrass established faster under normal growth temperature and had increased tiller and root growth and suppressed leaf senescence under heat stress, compared to the control line plants. The promotive effects of *SAG12-ipt* expression on creeping bentgrass growth, and the suppression of heat-induced leaf senescence, were associated with increased *ipt* gene transcription and elevated cytokinin production. Our results indicated that increased endogenous production of cytokinin through genetic transformation using the *ipt* gene may play an important role in regulating cool-season grass growth and tolerance to heat stress. Further research will be conducted to identify the downstream genes that are responsive to *ipt* expression and elevated cytokinins, in improved shoot and root growth of cool-season grass species under heat stress.

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Table 1 Tiller number (per plant) and chlorophyll content (mg g^{-1} DW) for the control line (C) and eight *SAG12-ipt* transgenic lines at 0 d (20 ^{o}C) and 14 d of heat stress (35 ^{o}C).

Line number	Tiller number (per plant)		Chlorophyll content (mg g^{-1} DW)	
	0 d	14 d	0 d	14 d
C	23.2 c A *	30.7 d A	10.5 c A	7.1 b B
S7	22.7 c A	33.3 cd A	10.9 bc A	9.9 a A
S16	27.5 bc B	52.5 ab A	12.5 a A	10.2 a B
S25	36.8 a A	43.8 bcd A	12.9 a A	10.2 a B
S32	32.1 ab B	48.6 abc A	12.2 ab A	10.0 a B
S37	31.8 ab B	48.2 abc A	12.9 a A	11.4 a A
S40	39.1 a B	64.8 a A	13.0 a A	7.8 b B
S41	36.2 a B	55.7 ab A	12.8 a A	10.6 a B
S43	31.9 ab B	56.5 ab A	13.2 a A	10.7 a B

* Means (n = 4 for data at 0 d; n = 6 for data at 14 d) within the row for each parameter followed by the same upper case letters are not significantly different based on LSD test at P=0.05. Means (n = 4 for data at 0 d; n = 6 for data at 14 d) within the column for each parameter followed by the same lower case letter are not significantly different based on LSD test at P=0.05.

Table 2 Root number (per plant) and length (mm per plant) for the control line (C) and eight *SAG12-ipt* transgenic lines at 0 d (20 ^{o}C) and 14 d of heat stress (35 ^{o}C).

Line number	Root number (per plant)		Root length (cm per plant)	
	0 d	14 d	0 d	14 d
C	34.0 c A *	42.6 c A	134.8 bc A	74.6 c B
S7	44.0 bc B	79.0 ab A	147.2 abc A	85.7 c B
S16	51.3 bc A	79.0 ab A	180.3 abc A	101.2 bc B
S25	60.0 ab A	63.6 bc A	199.3 a A	202.7 a A
S32	61.7 ab B	101.8 a A	133.6 bc A	147.7 ab A
S37	48.0 bc B	85.2 ab A	136.3 bc A	151.7 ab A
S40	81.0 a A	101.2 a A	124.1 c A	144.2 ab A
S41	59.0 b B	107.7 a A	184.9 ab A	151.8 ab A
S43	62.3 ab B	103.8 a A	188.3 ab A	171.2 a A

* Means (n = 4 for data at 0 d; n = 6 for data at 14 d) within the row for each parameter followed by the same upper case letters are not significantly different based on LSD test at P=0.05. Means (n = 4 for data at 0 d; n = 6 for data at 14 d) within the column for each parameter followed by the same lower case letter are not significantly different based on LSD test at P=0.05.

Table 3 Total iPA and ZR contents (pmol g^{-1} DW) for the control line (C) and five *SAG12-ipt* transgenic lines at 0 d (20 ^{o}C) and 14 d of heat stress (35 ^{o}C).

Line number	Total iPA (pmol g^{-1} DW)		Total ZR (pmol g^{-1} DW)	
	0 d	14 d	0 d	14 d
C	78.5 a A *	53.7 d B	27.4 a A	17.2 c B
S25	66.1 ab B	100.9 c A	20.2 bc A	17.8 bc A
S32	57.8 ab B	140.8 ab A	23.6 ab A	24.7 a A
S37	70.4 ab B	168.1 a A	22.1 ab A	23.5 a A
S41	53.0 b B	136.2 b A	20.1 bc A	23.8 a A
S43	52.8 b B	105.0 c A	15.9 c B	21.5 ab A

* Means (n = 4 for data at 0 d; n = 6 for data at 14 d) within the row for each parameter followed by the same upper case letters are not significantly different based on LSD test at P=0.05. Means (n = 4 for data at 0 d; n = 6 for data at 14 d) within the column for each parameter followed by the same lower case letter are not significantly different based on LSD test at P=0.05.

Fig. 1 Northern confirmation of *ipt* gene expression for the control line (C) and eight *SAG12-ipt* transgenic lines at 0 d (20 ^{o}C) and 14 d of heat stress (35 ^{o}C). RNA gel stained with ethidium bromide is shown on the bottom as the loading control.

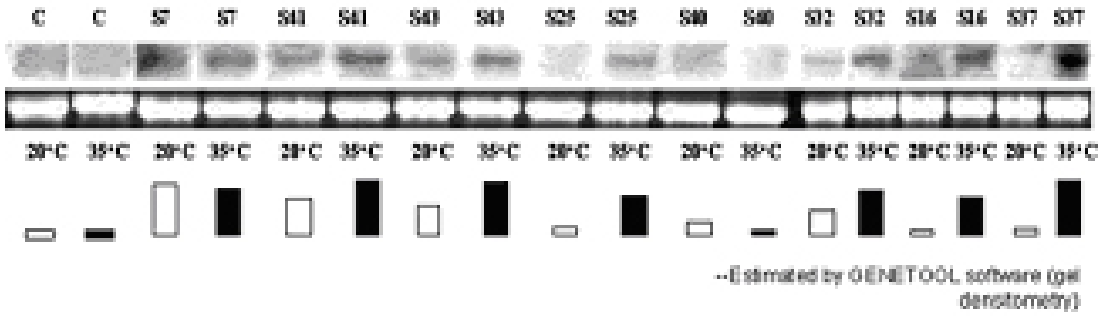


Fig. 2 iPA content (pmol g^{-1} DW) in the shoots of the control line (C) and five *SAG12-ipt* transgenic lines at 0 d (20 ^{o}C) and 14 d of heat stress (35 ^{o}C). Columns (means of four replicates for data at 0 d; means of six replicates for data at 14 d) followed by the same letters are not significantly different based on LSD test at P=0.05.

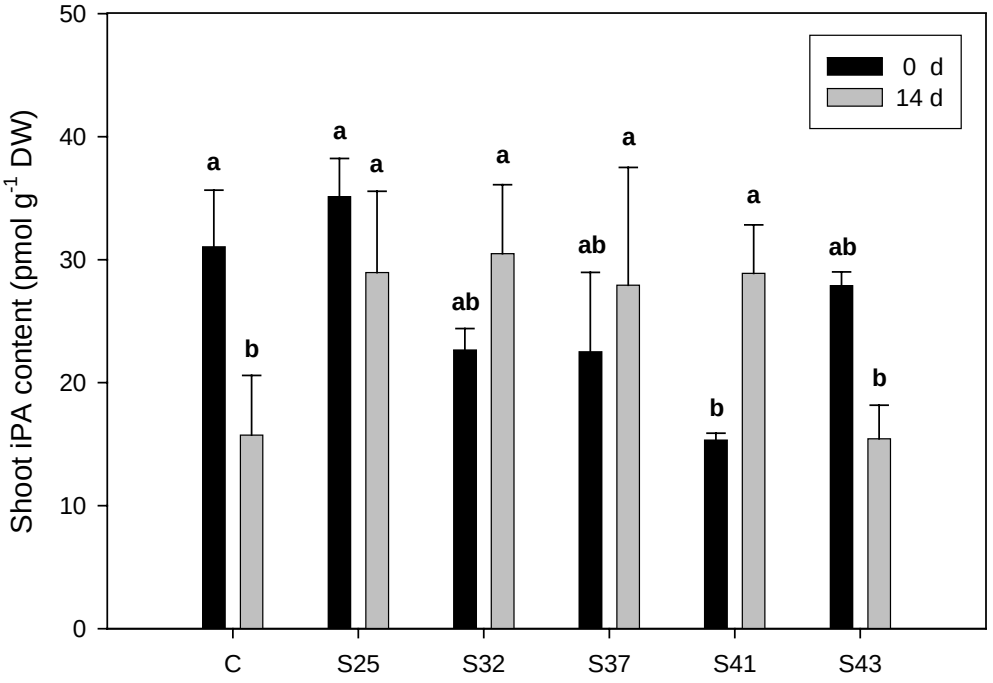


Fig. 3 iPA content (pmol g^{-1} DW) in the roots of the control line (C) and five *SAG12-ipt* transgenic lines at 0 d (20 ^{o}C) and 14 d of heat stress (35 ^{o}C). Columns (means of four replicates for data at 0 d; means of six replicates for data at 14 d) followed by the same letters are not significantly different based on LSD test at P=0.05.

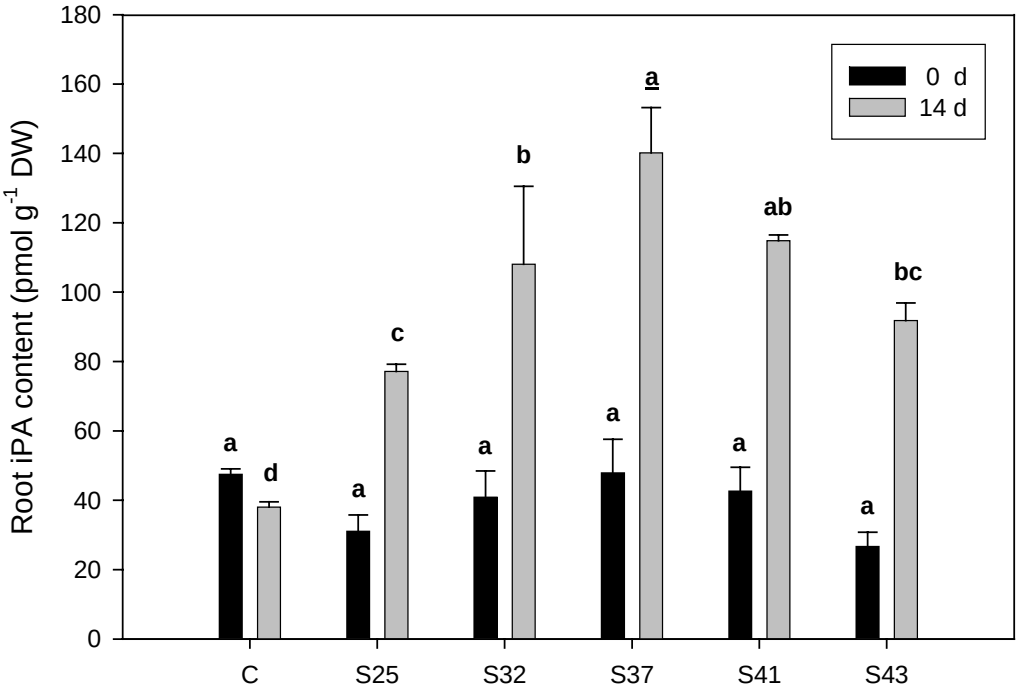


Fig. 4 ZR content (pmol g^{-1} DW) in the shoots of the control (C) and five *SAG12-ipt* transgenic lines at 0 d (20 ^{o}C) and 14 d of heat stress (35 ^{o}C). Columns (means of four replicates for data at 0 d; means of six replicates for data at 14 d) followed by the same letters are not significantly different based on LSD test at P=0.05.

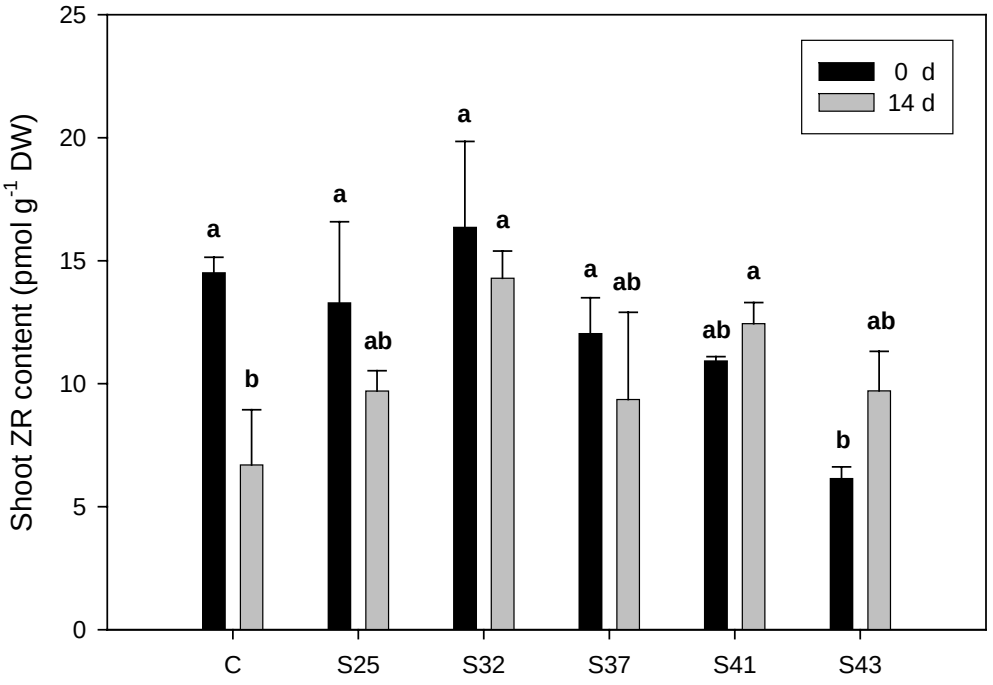
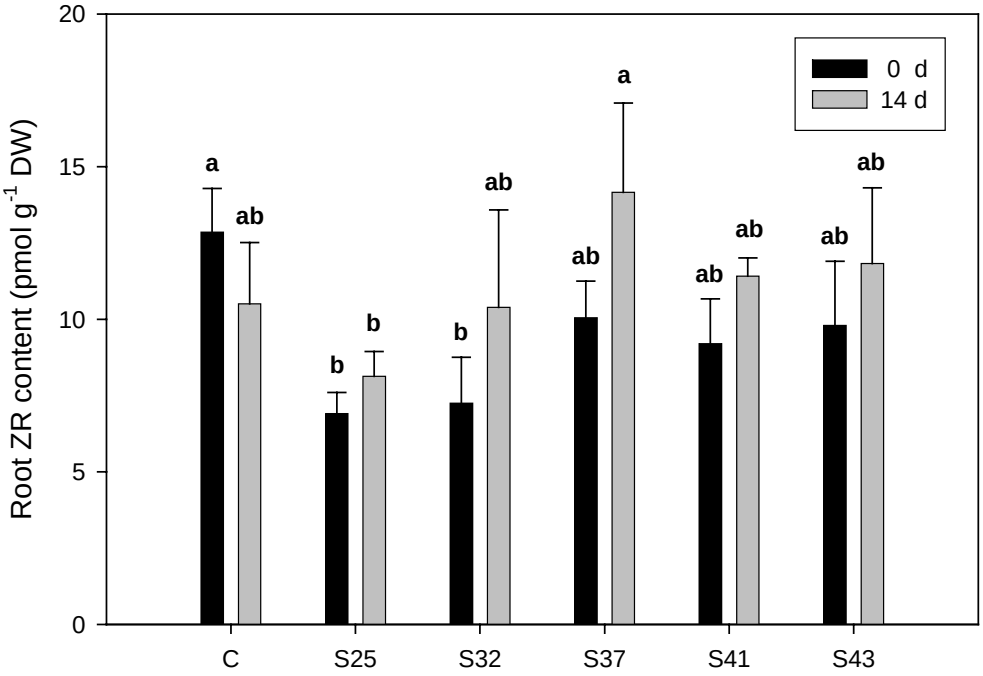


Fig. 5 ZR content (pmol g^{-1} DW) in the roots of the control line (C) and five *SAG12-ipt* transgenic lines at 0 d (20 ^{o}C) and 14 d of heat stress (35 ^{o}C). Columns (means of four replicates for data at 0 d; means of six replicates for data at 14 d) followed by the same letters are not significantly different based on LSD test at P=0.05.



CHAPTER 7. Proteomic Changes Associated with Expression of a Gene (*ipt*) Controlling Cytokinin Synthesis for Improving Heat Tolerance in Creeping Bentgrass

INTRODUCTION

Heat stress is a major factor influencing the growth of cool-season plant species during summer. Plant adaptation to heat stress involves changes in various processes, including hormone metabolism (Wahid et al., 2007). Heat stress inhibits synthesis and causes degradation of cytokinins (CK), a hormone known to regulate various growth and development processes, including cell division, leaf senescence, and root growth (Heintz et al., 2006; Hoth et al., 2003; Nooden and Leopold, 1988). CKs may play important roles in regulating plant responses to heat stress.

Exogenous application of CKs has been found effective to suppress leaf senescence (Singh et al., 1992) and mitigate root mortality and root electrolyte leakage, leading to improved heat tolerance (Liu et al., 2002; Xu and Huang, 2009; Xu et al., 2009). Over-expression of a gene isolated from agrobacterium (*Agrobacterium tumefaciens*) that encodes the enzyme adenine isopentenyl transferase (*ipt*), which catalyzes the rate-limiting step in CK biosynthesis, has also demonstrated positive effects of elevated levels of CK in delaying leaf senescence and improving stress tolerances in various plant species (Clark et al., 2004; Gan and Amasino, 1995; Veselov et al., 1995), including heat tolerance in perennial grass species (Xu et al., 2009). The expression of *ipt* gene in creeping bentgrass (*Agrostis stolonifera*), a heat-sensitive C_3 perennial grass species, improved heat tolerance, as manifested by increases in tiller formation and root

production and delay in leaf senescence under heat stress (Xu et al., 2009). In spite of physiological studies demonstrating the positive effects of CK on stress tolerance, the biochemical and molecular mechanisms of CK regulation of plant stress tolerance, particularly heat tolerance is largely unknown.

Recent advances in proteomics have made it possible to perform large-scale, quantitative measurements of protein composition in plants, providing a powerful approach to discovering the genes and pathways that are crucial for stress responsiveness and tolerance (Chen and Harmon, 2006; Yoshimura et al., 2008). Proteomic analysis using two-dimensional gel electrophoresis (2D-GE) and mass spectrometry has identified many stress-responsive proteins in various plant species in response to a wide range of abiotic stresses, including heat stress (Ferreira et al. 2006; Lee et al. 2007; Swidzinski et al., 2004; Valcu et al., 2008; Xu and Huang, 2008). Previous studies have also detected some proteins responsive to changes in CK production through exogenous application of CK or over-expression of *ipt* gene for increasing CK synthesis. Wingler et al. (1998) reported that *ipt* transgenic tobacco plants that produced more CK delayed the decline in Rubisco content in senescent tissues. Zavaleta-Mancera et al. (2007) reported that incubation of wheat leaves (*Triticum aestivum*) with 6-benzylaminopurine reduced the degradation of Rubisco large and small subunits during dark-induced senescence. However, a large-scale analysis of proteomic changes associated with changing endogenous production of CK, particularly in relation to heat stress tolerance is lacking. Knowledge of proteins conferring stress tolerance that may be regulated by CKs may provide further insights into molecular mechanisms of CK-regulated stress tolerance.

The objectives of this study were to identify protein changes associated with increases in endogenous CK production through *ipt* transformation in creeping bentgrass and determine proteomic mechanisms underlying CK-regulation of C_3 perennial grass responses to heat stress. Specifically, the study was conducted to compare differentially-expressed proteins in leaves and roots between *ipt*-transgenic creeping bentgrass lines and a non-*ipt* transgenic control line (a null transformant containing the vector without *ipt*) exposed to heat stress. Transgenic lines were generated using two inducible promoters from arabidopsis (*Arabidopsis thaliana*) (*SAG12* and *HSP18*) to avoid over-production of CK with constitutive promoters. The *SAG12-ipt* construct has an autoregulatory feature in that the transcription of *SAG12-ipt* is activated in response to leaf senescence, leading to CK production, which in turn suppresses senescence; the *SAG12* promoter then attenuates *ipt* transcription and subsequent enzyme production, which prevent over-production of CK (Gan and Amasino, 1995; Gan and Amasino, 1996). Rivero et al. (2007) used the promoter from a senescence-associated receptor protein kinase gene (*SARK*) as a promoter for *ipt* and found expression of *SARK-ipt* delayed drought-induced leaf senescence in tobacco. The small heat shock protein gene promoter in the *HSP18-ipt* line is heat-inducible with an optimum induction temperature at 35-37 ^{o}C (Takahashi et al., 1992; Yoshida et al., 1995). HSP promoters have been used to control gene transcription for increasing CK synthesis in tobacco (Schmulling et al., 1989; Smart et al., 1991; Smigocki, 1991; Van Loven et al., 1993). In this study, 2D-GE followed by matrix-assisted laser desorption/ ionization-time of flight-mass spectrometry (MALDI-TOF MS) was used to identify proteins that are differentially expressed between the *ipt*-lines and the null transformant (NT) after plants were exposed to 10 d of heat

stress at 35 ^{o}C. Putative roles of the identified proteins in senescence and heat tolerance of the plants are discussed.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Transgenic creeping bentgrass cv. Penncross expressing *SAG12-ipt* or *HSP18-ipt* was generated from stolons of a single plant in order to produce transgenic plants with identical genetic background. The *ipt* gene is from the Ti plasmid of agrobacterium. The construct pCAMBIA1300-SAG12-ipt was created from pSG516 (Gan and Amasino, 1995) and pCAMBIA 1300, a binary plasmid containing the gene for hygromycin resistance. The construct pCAMBIA1301-HSP18-ipt-GUS was created from pUC-HSP18, pSG516 and pCAMBIA 1301, a binary plasmid containing the genes for hygromycin and GUS. Both constructs were introduced into agrobacterium LBA4404 by electroporation. The pCAMBIA1300 and pCAMBIA1301 without ipt were used to generate NT control lines. See Xing et al. (2009) and Xu et al. (2009) for details. Two *ipt*-transgenic lines, *SAG12-ipt* (S41), and *HSP18-ipt* (H31), were selected after Northern and CK analyses confirmed *ipt* expression and elevated CK production under stress, compared to a NT line containing the pCAMBIA1301 vector without *ipt*. These lines were chosen because they were representative of typical heat stress responses among nine transgenic lines evaluated from each category Xing et al. (2009).

Plants of S41, H31 and NT were vegetatively propagated into plastic pots (15 cm in diameter and 20 cm deep) filled with sterilized sand and established in a greenhouse. The greenhouse had natural light averaging 600 μmol m^{-2} s^{-1} photosynthetic photon flux density at the canopy height for 12-h photoperiod and an average air temperature of 21 ^{o}C/14 ^{o}C (day/night). Plants were watered daily and fertilized twice a week with half-

strength Hoagland's solution (Arnon, 1949). After 42 d of establishment in the greenhouse, clonal plants were transferred to controlled-environment growth chambers (Conviron, Winnipeg, Canada) with a temperature of 20 ºC/15 ºC (day/night), 12-h photoperiod, 60% relative humidity, and 500 µmol m^{-2} s^{-1} photosynthetic photon flux density at the canopy height. Plants were allowed to acclimate to growth chamber conditions for 7 d before temperature treatments were imposed.

Treatments and Experimental Design

Plants of S41, H31, and NT were exposed to 35 ºC/30 ºC (day/night) (heat stress) or 20 ºC/15 ºC (day/night) (control) in growth chambers for 10 d. Each temperature treatment was repeated in three growth chambers. Plants were arranged randomly inside each chamber and relocated within and among chambers every three days to minimize environment differences among and within the chambers. Plants were watered twice daily until free drainage occurred from the bottom of the container to prevent water deficit during the heat treatment period and fertilized weekly using half-strength Hoagland's nutrient solution (Hoagland and Arnon, 1950).

Physiological Evaluation

Leaf chlorophyll content was measured to evaluate leaf senescence in transgenic and NT plants. Leaf chlorophyll was extracted from about 0.2 g fresh leaves using dimethyl sulfoxide. The absorbance of leaf extracts was determined using a spectrophotometer (Spectronic Genesys2, Spectronic Instruments, Rochester, NY). Chlorophyll content was calculated based on the absorbance at 663 nm and 645 nm using the formulas described by Arnon (1949).

Leaf IPA, the form of CK whose production was directly controlled by the *ipt* gene, was quantified by an indirect competitive enzyme-linked immunosorbent assay (ELISA). Extraction and quantification of hormones followed the method described by Setter et al. (2001) with some modifications (Wang et al., 2003). Briefly, samples were extracted in 80% (v/v) methanol and isolated with reverse phase C_{18} columns. Hydrophilic contaminants were washed out with 200 μL of 20% solvent [20% methanol, 80% aqueous triethylamine (TEA, 10 mM, pH 3.5)]. The CK-containing fraction was eluted using 200 μL of 30% solvent (30% methanol, 70% aqueous TEA). A mouse monoclonal antibody against IPA (Agdia, Inc., Elkhart, IN) and a goat anti-mouse IgG conjugated with alkaline phosphatase (AP) (Sigma, St. Louis, MO) were used as the primary and secondary antibodies, respectively.

At the end of the treatment, root growth response to heat stress was evaluated. The root system was washed free of sand and organic matters. Root fresh weight was measured after blotting dry.

Northern Blot of *ipt* Expression

Total RNA was extracted from leaves using TRIZOL Reagent (Invitrogen, USA). A 10 μg of RNA was size fractionated in 1.2% (w/v) agarose gel for 3 h at 65 volts and transferred to a nylon membrane using a capillary blot method. The membranes were UV-cross linked. Different gene fragments were separately labeled using a random primed labeling kit (Ambion, USA). The labeled probes were purified using NICK^{TM} columns (GE Healthcare, Sweden). Hybridization was carried out at 42 °C overnight in a northernMax prehybridization/hybridization buffer (Ambion, USA). The membranes

were washed with 2×SSC, 0.2% (w/v) SDS at 42 °C for 10 min, and then with 0.1×SSC, 0.1% SDS at 42 °C for 10 min. Membranes were exposed to X-ray film (Fuji photo film, Japan) at –80 °C for signal detection. The northern blots were repeated three times for each sample. The best representative image from the three replicates was presented.

Protein Extraction and Quantification

Shoot and root samples from each pot were harvested at the end of the treatment (10 d) and immediately frozen in liquid nitrogen. Each sample was carefully selected to ensure it represents the nature and average of different parts along the leaf and root elongation zone. The samples were then ground into fine powder and stored at –80 ^{o}C before analysis. Proteins were extracted using the trichloroacetic acid/acetone method described by Xu et al. (2008). About 0.5 g leaf or 1 g root samples were homogenized on ice in 10 mL of precipitation solution (10% trichloroacetic acid and 0.07% 2-mercaptoethanol in acetone) for 10 min and incubated at –20 ^{o}C for 2 h. The protein pellet was collected and washed with cold acetone containing 0.07% 2-mercaptoethanol until the supernatant was colorless. The pellet was then vacuum-dried and suspended in resolubilization solution (8 M urea, 2 M thiourea, 2% CHAPS, 1% dithiothreitol and 1% pharmalyte). The suspension was centrifuged at 21000 g_n for 20 min and the supernatant was collected for protein quantification.

Protein content was determined using the method of Bradford (1976). A 10 μL of protein extract was mixed with 0.5 mL of a commercial dye reagent (diluted 5 times) (Bio-Rad Laboratories, Hercules, CA). The absorbance was measured

spectrophotometrically at 595 nm between 5 and 30 min after reaction. A standard curve was made from bovine serum albumin.

2D-GE and Image Analysis

An IPGPhor apparatus (GE Healthcare, Piscataway, NJ) was used for isoelectric focusing (IEF). Portions of the extracts containing 300 μg of protein were subjected to IEF in immobilized pH gradient strips (pH 3.0-10.0, linear gradient, 13 cm), formed by rehydrating the strips for 12 h at room temperature in 250 μL of rehydration buffer (8 M urea, 2 M thiourea, 2% CHAPS, 1% dithiothreitol, 1% pharmalyte and 0.002% bromophenol blue). The voltages for IEF were 500 V for 1 h, 1000 V for 1 h, 5000 V for 1 h and 8000 V for 80 kVh. Following IEF, the strips were equilibrated for 15 min twice at room temperature in equilibration buffer I (50 mM Tris-HCl pH 8.8, 6 M urea, 30% glycerol, 2% SDS and 1% dithiothreitol), then transferred to equilibration buffer II (50 mM Tris-HCl pH 8.8, 6 M urea, 30% glycerol, 2% SDS and 2.5% iodoacetamide). The second dimension electrophoresis was performed on a 12.5% SDS-polyacrylamide gel using a Hoefer SE 600 Ruby electrophoresis unit (GE Healthcare, Piscataway, NJ). The running conditions were 5 mA /strip for 30 min followed by 20 mA /strip for 5 h. The gels were stained with coomassie brilliant blue (CBB) G-250 and scanned using a Personal Densitometer (GE Healthcare, Piscataway, NJ).

Gel images were analyzed using Progenesis software (Nonlinear Dynamics, Durham, NC). Manual correction and editing of spot features created by automatic default spot analysis settings were included. The spot volumes were normalized as a percentage of the total volume of all spots on the gel in order to correct the variability due

to staining. Data were subjected to analysis of variance to test the treatment effects on each transgenic line. Means were separated by Fisher's protected least significance difference (LSD) test ($P<0.05$).

Protein Identification

Selected protein spots were manually excised from gels and subjected to digestion with trypsin. The peptides were analyzed by MALDI-TOF-MS as described by (Xu and Huang, 2008). Data were searched against the NCBI database using a local MASCOT search engine (V1.9) on a GPS (V. 3.5, ABI) server. Proteins containing at least two peptides with confidence interval values larger than 95% were considered as being identified. The obtained sequence was also manually assigned to perform another search in the Swiss-Prot and TrEMBL databases using FASTA.

Statistical Analysis

The experiment was considered to be a completely randomized split-plot design, with temperature as the main plots and plant lines as the sub-plots. Analysis of variance was based on the general linear model procedure of SAS (SAS Institute Inc., Cary, NC). Treatment means and differences between transgenic and NT control plants for each physiological parameter were separated by LSD test at 0.05 probability level.

RESULTS

Growth and physiological responses to heat stress

Both *ipt*-transgenic lines produced had significantly more tillers than the NT plants after 10 d of heat treatment at 35 ^{o}C (Fig. 1). No significant differences in leaf chlorophyll content (CHL) were observed among the three lines grown at 20 ^{o}C. After 10 d at 35 ^{o}C, CHL in S41 and H31 was maintained at the respective control levels (20 ^{o}C); however, CHL in NT declined by 30%, and was 29 and 32% lower than that in S41 and H31, respectively (Fig. 2A).

Leaf IPA content was comparable among the three lines when they were grown at 20 ^{o}C (2B). Heat treatment (35 ^{o}C for 10 d) led to 53% decrease in leaf IPA content in NT. On the contrary, there was no significant decline in leaf IPA content in H31, and the content was even increased by 31% in S41 after the same treatment. Both transgenic lines maintained significantly higher leaf CK (IPA) content than the NT plants under heat tress.

Total root weight did not differ among the three lines when grown at 20 ^{o}C, but significantly decreased in all three lines when temperature was increased to 35 ^{o}C for 10 d (Fig. 2C). Heat-induced reduction in root growth was the most severe in NT (73%), followed by S41 (54%) and H31 (40%). Both transgenic lines had significantly greater root weight than the NT plants under heat tress.

Differential ipt gene expression in response to heat stress between NT and transgenic lines

Low levels of *ipt* gene expression were detected in S41 and H31 when they were grown at optimum temperature (20 ^{o}C), and the expression levels were enhanced by heat stress (35 ^{o}C for 10 d) in both transgenic lines (Fig. 3). No *ipt* gene expression was detected in the NT plants grown at optimum temperature or under heat stress.

Differential proteomic responses to heat stress in leaves between NT and transgenic lines

A total of approximately 300 protein spots were detected on each gel for each leaf sample. They were reproducibly observed in three independent replications. No significant differences were observed in the expression levels of all proteins among the three lines at 20 ^{o}C. However, 15 protein spots exhibited differential response patterns to heat stress at 35 ^{o}C between transgenic lines and the NT control line. These 15 spots were excised from the gels for protein identification in order to examine differential protein changes caused by the *ipt* transformation in response to heat stress.

Fourteen of the differentially-expressed proteins were identified by MALDI-TOF-MS and their respective positions were marked in the protein map shown in Fig. 4. The identities of these proteins and the responding patterns of their expression levels to heat stress in each line were summarized in Table 1. The normalized volume (abundance) of each spot was used to analyze the differential level of protein expression, presented as the % volume of each protein spot in heat-stressed plants compared to unstressed plants. The expression levels of four protein spots (#22, 40, 110 and 192) at 20 and 35 ^{o}C in each line were shown in Fig. 5 as examples. Spot #22 was identified as a heat shock protein (Hsp) 90, whose expression level was increased only in S41, but not changed in H31 or NT

under heat stress (Fig. 5A). Spot #40 was identified as an Hsp70; its expression level was increased in both S41 and H31, but not changed in NT under heat stress (Fig. 5A). Spot #110 (Fig. 5B) and 192 (Fig. 5C) were identified as glycolate oxidase and a putative oxygen-evolving complex protein, respectively; the expression levels of both proteins were decreased in NT, not changed in S41, and increased in H31 under heat stress.

The functions of the 14 identified proteins were categorized using the criteria described in Bevan et al. (1998) and summarized in Fig. 8A. Among 15 proteins, 60% of the proteins belonged to the energy category, mainly for glycolysis (enolase, GAPDH) and photosynthesis (Rubisco large subunit, Rubisco small subunit, rubisco large subunit-binding protein subunit α, chloroplast chlorophyll a-b binding protein precursor, oxygen-evolving enhancer protein 2, putative oxygen-evolving complex, and photosystem II polypeptide); 20% of the proteins belonged to the category of protein destination and storage, including two Hsp90s and one Hsp70; 13% of the proteins belonged to the category of disease/defense, these were two antioxidant enzymes (catalase-1 and glycolate oxidase). One protein (7%) belonged to the category of unclear classification.

Differential proteomic responses to heat stress in roots between NT and transgenic lines

Over 600 protein spots were reproducibly detected and clearly separated by 2D-GE in roots of creeping bentgrass. A total of 43 protein spots were found differentially expressed among the three lines in response to 10 d of heat treatment and their positions were marked in the protein map shown in Fig. 6. Among the 43 proteins, 38 proteins were successfully identified by MALDI-TOF-MS. Their identities as well as the

responding patterns of their expression levels to heat stress in each line were summarized in Table 2. The expression levels of six spots (#118, 264, 267, 316, 322 and 324) at 20 and 35 ^{o}C in each line were shown in Fig. 7 as examples. Spot #316 and 322 were identified as two glutathione S-transferases, whose expression levels were only increased in S41, but not changed in NT or H31 under heat stress (Fig. 7A). Spot #324 was identified as a small GTP-binding protein (RAN); its expression level was only increased in NT, but not changed in the transgenic lines under heat stress (Fig. 7A). Spot #118 was identified as an Hsp90, whose expression level was increased only in S41, but not changed in NT or H31 (Fig. 7B). Spot #264 and 267 were identified as two GAPDHs; their expression levels were increased only in H31, but not changed in NT or S41 under heat stress (Fig. 7C).

The functions of the 43 proteins were summarized in Fig. 8B. Sixteen percent of the proteins fell in the category of metabolism, such as ferredoxin-dependent glutamate synthase (Fd-GOGAT) and methionine synthase; 26% of the proteins belonged to the category of energy and most of them play roles in glycolysis (GAPDH, triosephosphate isomerase, fructose-bisphosphate aldolase and glucose-6-phosphate isomerase), respiration (NADH dehydrogenase) and tricarboxylic acid (TCA) pathway (NAD-dependent isocitrate dehydrogenase and aconitate hydratase); 16% of the proteins were related to protein destination and storage, including two Hsps and one calreticulin; 9% of the proteins belonged to the category of disease/defense, including three antioxidants (ascorbate peroxidase and two glutathione S-transferases). Proteins in the other categories included transcription (5%), protein synthesis (5%), intracellular traffic (2%), cell structure (2%), signal transduction (2%), secondary metabolism (5%), and unclear

classification (2%).

Predicted subcellular localization of the differentially-expressed proteins in leaves and roots of the three lines under heat stress

The subcellular location of each identified protein was predicted based on computer analysis using the Swiss-Prot protein knowledge base (http://ca.expasy.org/sprot/). Among the 15 differentially-expressed proteins in leaves between NT and the *ipt*-transgenic lines, the largest portion was localized in the chloroplast (53%), followed by the cytoplasm (20%), peroxisome (13%), plastid (7%) and unknown (7%) (Fig. 9A). Among the 43 differentially-expressed proteins in roots between NT and the *ipt*-transgenic lines, the largest portion was localized in the cytoplasm (33%), followed by plastid (16%), nucleus (14%), unknown (14%), endoplasm (9%), mitochondrion (9%) and peroxisomse (5%) (Fig. 9B).

DISCUSSION

The two *ipt*-transgenic lines were able to maintain shoot growth and exhibited no leaf senescence following 10 d of heat stress at 35 ^{o}C (Fig. 1). Both S41 and H31 had maintained higher leaf chlorophyll content and root mass than NT after 10 d of heat stress. Our results suggested that the transformation of creeping bentgrass with the *ipt* gene using *SAG12* or *HSP18* promoter improved heat tolerance associated with suppression of leaf senescence and promotion of root growth. These results could be related to the expression of *ipt* transcripts and increased production of CKs (Xing et al., 2009; Xu et al., 2009). Consistently, enhanced expression of *ipt* transcripts in both transgenic lines under heat stress was confirmed by northern blots in the current study. Leaf IPA, the form of CK whose production directly controlled by the *ipt* gene, was maintained or even increased in the two transgenic lines under heat stress, in contrary to the decrease in the NT plants, indicating the active functioning of the *ipt* gene ligated to either promoter in stimulating CK production under heat stress. No difference in the levels of leaf IPA was detected among the three lines when they were grown at optimal temperature, though low expression of *ipt* transcripts was detected in both *ipt*-transgenic lines. It suggests that both inducible promoters functioned effectively to prevent overexpression of *ipt* transcripts when there was no senescence or heat shock signal and CK contents were adequate.

Chloroplast proteins represent a major fraction (53%) of the differentially expressed proteins in leaves detected in the present study and most of them had relatively higher expression levels after heat stress in at least one *ipt*-transgenic line. Caers et al. (1985) studied the effects of heat stress on photosynthetic activity and chloroplast

ultrastructure in correlation with the endogenous CK concentration in maize (*Zea mays*) seedlings, and found the inhibition of photosynthetic activity and chlorophyll accumulation by heat stress could be reversed when levels of CKs were elevated by adding benzyladenine.

Root tissue was more severely affected by heat stress. Xu and Huang (2000) reported that high soil temperature was more detrimental than high air temperature and roots may mediate shoot responses to high temperature stress in creeping bentgrass. We observed 28 more differentially-expressed proteins between NT and the *ipt*-transgenic lines in the roots of the three lines than in the leaves under heat stress. The differentially-expressed proteins detected in roots included a high percentage (33%) of proteins located to cytoplasm, suggesting the importance of cytosolic proteins in root tolerance to heat stress. Differentially-expressed proteins in leaves or roots in responses to heat stress between the transgenic lines and the NT control are discussed below according to functional categories.

Leaf proteomic changes associated with ipt-transformation and affected by heat stress

Energy category

Many identified proteins in this category are related to photosynthesis. Photosynthesis is among the most sensitive processes to elevated temperature. Changes in various transcripts and proteins involved in photosynthetic reactions has been reported in response to heat stress (Allakhverdiev et al., 2008; Kim and Portis, 2005; Pushpalatha et al., 2008).

The oxygen-evolving complex (OEC) along with the associated cofactors in PSII and carbon fixation by Rubisco are two primary targets of heat damage in plants (Allakhverdiev et al., 2008). We identified two OEC-related protein spots (#178 and 192). Spot #178 was OEE 2 that contained a conserved domain of the extrinsic protein PsbP. Its expression level was reduced 30% only in NT, but not in S41 or H31 under heat stress. Spot #192 was a putative OEC precursor that contained a conserved domain of the extrinsic protein PsbQ. Its expression level was decreased 70% in NT, not changed in S41, and increased in H31 (1.9-fold) under heat stress. Protection of OEC by the extrinsic proteins of PSII was shown to be essential for development of cellular thermotolerance (Kimura et al., 2002), thus maintenance or up-regulation of the extrinsic proteins in PSII may attribute to the superiority of the two transgenic lines over the NT control line growing under heat stress.

Rubisco large and small subunits often degrade with leaf senescence or maturation (Wilson et al., 2002). Wingler et al. (1998) reported that the senescence-related decline in Rubisco was delayed in transgenic tobacco plants that produced more CK. Zavaleta-Mancera et al. (2007) also found incubation of wheat leaves (*Triticum aestivum*) with 6-benzylaminopurine reduced the degradation of Rubisco large and small subunits during dark-induced senescence. We observed decreased expression levels of both Rubisco large and small subunits in NT and S41 under heat stress. There was no reduction in the expression level of Rubisco small subunit in H31, indicating improved thermal stability of Rubisco protein in this line. The difference could be due to the direct response of the *HSP18* promoter in H31 to heat stress on chloroplast protein levels by effectively controlling the *ipt* gene to regulate CK production under heat stress, compared

to NT without the *ipt* gene or S41 with the *SAG12* promoter that responded to senescence signals caused by heat stress.

The expression level of a chloroplast chlorophyll a-b binding protein (LHCP) belonging to the light-harvesting complex was increased the most in NT (2.2-fold), followed by H31 (1.2-fold), and not changed in S41 under heat stress. This protein functions as a light receptor that captures and delivers excitation energy to PS II and I. Under heat stress, carbon fixation is inhibited, which does not require much light energy and may result in oxidative damage to the photosynthetic apparatus (Dinar et al., 1983). Therefore the lower or no induction of LHCP in H31 and S41 compared to NT indicated reduced light absorption capacity to avoid excessive excitation energy under heat stress that could damage the photosynthetic apparatus. An alternative explanation can be in the *ipt*-transgenic plants there is still a balance between NADPH production in the light reactions and its use in the Calvin cycle thus there is no need for more LHCP, while the increase in LHCP by NT may be an attempt to dissipate excess light energy. The regulatory role of CKs in LHCP was reported by Flores and Tobin (1988), who detected a decline in the mRNA level of LHCP during dark-induced senescence whereas CK pretreatment slightly increased the mRNA abundance. There may exist a discrepancy between the abundance of LHCP mRNA and protein as reported by Barak et al. (2000) in the chlorophyll-deficient tobacco mutants, which accumulated normal levels of LHCP transcript but failed to accumulate the protein.

Several proteins functioning in the glycolytic pathway were differentially expressed between NT and the *ipt*-transgenic lines, including enolase and glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Enolase and GAPDH are enzymes catalyzing the

ninth and sixth steps of glycolysis, respectively. A proteomic study conducted by Ferreira et al. (2006) in *Populus euphratica* leaves found abundance of enolase and GAPDH was transiently increased after 30 h of heat stress, followed by a reduction after 54 h of heat stress, suggesting an early acceleration of the glycolytic pathway upon exposure to high temperature. In our study, the expression level of a leaf cytosolic enolase was increased in S41 (2.0-fold), maintained in H31, but decreased in NT (0.7-fold) after 10 d of heat stress; the expression level of a cytosolic GAPDH was increased to similar extents in NT (1.5-fold), S41 (1.4-fold) and H31 (1.5-fold), respectively. The higher enolase expression in the leaves of both transgenic lines carrying the *ipt* gene than in the NT control line after 10 d of heat stress may have resulted from CK activation of the glycolytic pathway. The response of enolase to CK was also found in the leaves of common ice plants (*Mesembryanthemum crystallinum*) by Forsthoefel et al. (1995), who detected increased enolase transcripts upon treatment of unstressed plants with 6-benzylaminopurine.

Protein destination and storage category

We identified a plastid Hsp90, a cytoplasmic Hsp90 and a chloroplast Hsp70 in this category. Proteins in Hsp90 family act as chaperones with ATPase activity and interact with proteins involved in transcription regulation and signal transduction pathways (Majoul et al., 2003). Hsp70 has known function in preventing protein aggregation and assisting refolding of non-native proteins under both normal and stress conditions. The expression of Hsps has been correlated with the acquisition of thermotolerance in many plant species (Vierling, 1991), including creeping bentgrass (He et al., 2005; Park et al., 1996). In the current study, we observed an increase in the

abundance of a plastid Hsp90 in S41 (2.8-fold) and a decrease in the abundance of a cytoplasmic Hsp90 in NT (70%) under heat stress. A chloroplast Hsp70 was up-regulated 1.5-fold and 2.0-fold of in S41 and H31, respectively, but not in NT. These results suggest a regulatory role of CK in Hsp metabolism in the leaves of the *ipt*-transgenic lines under heat stress, which may be related to the inhibition of Hsp degradation or activation of Hsp production. Consistently, Veerasamy et al. (2007) previously reported foliar spray of zeatin riboside increased the content of several Hsps and alleviated heat-induced leaf senescence in creeping bentgrass.

Disease/defense category

We identified two proteins belonging to this category, which are glycolate oxidase (GOX) and catalase (CAT-1). GOX is a key enzyme involved in the photorespiratory pathway, which helps dissipate excessive energy and protect photosynthetic membranes. Catalase, whose primary function is to catalyze the decomposition of H_2O_2, is essential for the protection of GOX against photoinactivation (Schafer and Feierabend, 2000). Both GOX and CAT represent the major constituents of the peroxisomal matrix in photosynthetic tissues. Ludt and Kindl (1990) found both GOX and CAT mRNAs decreased during leaf senescence in lentil (*Lens culinaris*). Transgenic *Pssu-ipt* tobacco with elevated content of endogenous CKs showed increased GOX activity compared to non-transformed control plants (Synkova et al., 2004). We observed the expression level of GOX significantly decreased by 70% in NT, whereas it increased in H31 (1.7-fold) but did not change in S41 under heat stress. These results indicate improved heat tolerance regulated by CK in the transgenic lines could involve maintenance or enhancement of

expression of such glycolate pathway enzymes as GOX. The expression level of CAT-1 was increased to a greater extent in NT (2.3-fold) than in S41 (1.4-fold) and H31 (1.6-fold) under heat stress. Stress-induced CAT-1 expression was related to the triggering of H_2O_2 production, as proved by the observation that CAT-1 expression was induced by applied H_2O_2 (Xing et al., 2007). Thus the lower CAT-1 expression in the *ipt*-transgenic lines could reflect less severe oxidative damage caused by heat stress than that in the NT control line. This down-regulatory role of CKs in CAT was also reported by Toyama et al. (1995) in a large-scale differential hybridization study, which detected a decreased level of CAT cDNA in etiolated cotyledons of cucumber (*Cucumis sativus*) treated with N^6-benzyladenine.

Root proteomic changes associated with ipt-transformation and affected by heat stress

Metabolism category

Ferredoxin-dependent glutamate synthase (Fd-GOGAT) is an enzyme functioning in the glutamine synthetase (GS)/GOGAT ammonium assimilation pathway, which regulates nitrogen assimilation (Tempest et al., 1970). We observed a significant decline in the expression level of Fd-GOGAT protein in roots of NT and H31, but not in S41 under heat stress. The maintenance of Fd-GOGAT in roots of S41 could sustain active assimilation of nitrogen, suggesting that the *SAG12* promoter was effective in responding to the signals triggered by impaired nutrient uptake under heat stress, which correlates with our recent findings that *SAG12-ipt* expression suppressed leaf senescence induced by N deficiency in creeping bentgrass (Zhang et al., 2010).

Serine hydroxymethyltransferase (SHMT) and phosphoserine aminotransferase (PSAT) are two enzymes functioning in one-carbon compound metabolic pathway. Both were detected in soybean (*Glycine max*) root nodules and involved in the purine biosynthetic pathway required for transport of the assimilated nitrogen from the nodules (Mitchell et al., 1986; Reynolds and Blevins, 1986). Xu and Huang (2008) recently reported the expression level of SHMT decreased whereas that of PSAT increased under heat stress in two *Agrostis* grass species. In the current study, the decline of SHMT was greatest in NT (80%), intermediate in H31 (60%), and least in S41 (30%) after 10 d of heat stress. Increased PSAT expression was only observed in NT (1.5-fold), but not in S41 or H31. The less severe decline of SHMT and more stable production of PSAT in both transgenic lines may improve nitrogen transport and utilization to enable better growth under heat stress compared to the NT control line.

The expression level of a nucleotide-sugar dehydratase was decreased 80% only in NT under heat stress, but not changed in either *ipt*-transgenic line. Production of this enzyme may assist in maintaining adequate sugar metabolism for growth of the transgenic lines under heat stress. This may relate to decreased sugar sensitivity resulting from activation of CK signaling as reported by Franco-Zorrilla et al. (2005).

Energy category

Increased expression levels of two phosphorylated cytoplasmic GAPDHs were detected only in roots of H31 (1.2- and 1.7-fold, respectively) under heat stress. GAPDH has been regarded as a target of CK action in regulating glycolytic activity by modifying the phosphorylation state of GAPDH (Heintz et al., 2006). Glucose-6-phosphate

isomerase (GPI) and triose phosphate isomerase (TPI) are enzymes catalyzing the second and fifth steps of glycolysis, respectively. We observed an increased level of a cytoplasmic GPI expression only in the roots of H31 (1.7-fold) under heat stress. The expression level of a cytoplasmic TPI was increased in all three lines after heat treatment, but the increase was greater in both transgenic lines (2.1-fold) than in NT (1.5-fold). Dorion et al. (2005) has reported cytoplasmic TPI activity and protein levels appeared to be regulated during development and are important in the supply of carbon to respiratory and biosynthetic pathways during active growth of potato. Hence, CK regulation of GPI and TPI could play a role in the balance of metabolic fluxes in plant primary metabolism that is required for sustained plant growth under stress conditions.

NADH dehydrogenase is an enzyme located in the mitochondrion that catalyzes the transfer of electrons from NADH to coenzyme Q in the respiratory pathway. We identified two NADH dehydrogenase proteins whose expression levels were both decreased only in H31 under heat stress. Rachmilevitch et al. (2006) reported total root respiration rate and specific respiratory costs for maintenance and ion uptake increased with increasing soil temperatures in two *Agrostis* grass species and the increases were less pronounced in the tolerant species than in the sensitive species. The lower NADH in H31 than in NT may reflect the inhibitory effects of CKs on respiratory carbon metabolism, which could lower respiratory carbon consumption in H31, an important factor controlling root survival under high temperature. The repression of the respiratory rise by CKs such as kinetin and BAP was also reported by Tetley and Thimann (1974). Moreover, Miller (1980) suggested that CK-inhibited point may be located between NADH dehydrogenase and cytochrome b of the electron transport system.

NAD-dependent isocitrate dehydrogenase (IDH) is an enzymes functioning in the TCA cycle and catalyzes the oxidative decarboxylation of isocitrate while converting NAD^+ to NADH. It has been proposed that mitochondrial IDH can serve as the enzymatic origin of 2-oxoglutarate, the carbon skeleton required for plant ammonium assimilation through the GS/GOGAT pathway (Hodges et al., 2003). Lancien et al. (1999) found that IDH mRNA levels were increased by the addition of nitrate or NH_4^+ to N-starved tobacco roots. We detected decreased levels of IDH expression in roots of NT and H31 under heat stress while that level in S41 was maintained. The relatively higher level of IDH expression in roots of S41 could reflect better assimilation of nitrogen for sustained growth under heat stress compared to the NT control line, which is consistent with the maintenance of Fd-GOGAT expression in the same line under heat stress as discussed above.

The expression level of a ferredoxin-NADP reductase precursor (FNR) was reduced 60% in the roots of NT, but maintained in both transgenic lines under heat stress. FNR in the non-photosynthetic tissues probably support ferredoxin-dependent biosynthetic processes such as nitrogen assimilation (Green et al., 1991; Morigasaki et al., 1990). In that case, maintained FNR expression under heat stress could also reflect a superior nitrogen assimilation capacity of the transgenic lines over the NT control line that facilitates their growth under heat stress, in accordance with our findings on Fd-GOGAT and IDH expression.

Protein destination and storage category

The expression levels of a protein disulphide-isomerase (PDI), two Hsp90s and a putative t-complex protein 1 theta chain were increased in roots of S41 under heat stress. PDI catalyzes the formation, cleavage and isomerisation of disulphide bonds, and is involved in regulating the folding and deposition of storage proteins (Johnson and Bhave, 2004). Up-regulation of PDI as well as Hsp90s expression in S41 may be related to protein folding and deposition of damaged proteins. Function of the putative t-complex protein 1 theta chain in stress tolerance was not well documented.

Cyclophilins catalyze the isomerization of peptide bonds from *trans* form to *cis* form at proline residues and facilitate protein folding. Pan et al. (2008) reported that cyclophilin A is required for activation, export and translocation of some important nuclear proteins. In this study, the expression level of a cyclophilin A-2 protein was increased 3.7-fold in both transgenic lines and 2.7-fold in NT under heat stress. Greater accumulation of this protein under heat stress could contribute to improved heat tolerance of the transgenic lines by reinforcing proper protein export and translocation.

Calreticulin is a multifunctional protein that binds Ca^{2+} ions. It binds to misfolded proteins and prevents them from being exported from the endoplasmic reticulum to the golgi apparatus. Increased level of calreticulin expression was observed only in roots of H31 (1.4-fold) under heat stress, which may also support the survival of this transgenic line under heat stress by facilitating proper protein export and translocation.

Disease/defense category

Heat stress induces the production of reactive oxygen species (ROS). The induction of ROS-related enzymes are involved in the protection of root tissues from

oxidative damage under stress conditions (Yoshimura et al., 2008). Among them are glutathione S-transferase (GST) and ascorbate peroxidase (APX). We detected increased expression of two GSTs only in S41 (1.4-fold), and decreased expression of an APX only in NT (80%). The accumulation of GST and maintenance of APX may be partially responsible for the capability of transgenic lines to sustain root growth under heat stress through activating the ROS-scavenging system. Similarly, Roxas et al. (2000) reported that overexpression of GST in tobacco seedlings increased glutathione-dependent peroxide scavenging and alterations in glutathione and ascorbate metabolism that led to reduced oxidative damage and enhanced growth under heat stress.

The expression level of a molybdenum cofactor (Moco)-containing protein was increased in S41 (1.6-fold) under heat stress. Moco-containing enzymes play roles in basic metabolic reactions in the nitrogen, sulfur, and carbon cycles, catalyzing oxygen atom transfer in a two-electron transfer redox reaction mediated by Moco (Kisker et al., 1997). Four major groups of them have been found in plants, which are nitrate reductase, catalyzing the key step in inorganic nitrogen assimilation, aldehyde oxidase, catalyzing the last step in the biosynthesis of abscisic acid, xanthine dehydrogenase, involved in purine catabolism and stress reactions, and sulphite oxidase, involved in detoxifying excess sulphite (Mendel and Hansch, 2002). Although the function of the Moco protein we identified in S41 has not been specified, up-regulation of this Moco protein by CK may facilitate plant survival by assisting in the oxidation-reduction processes involved in heat stress responses.

Transcription category

The expression level of a putative heterogeneous nuclear ribonucleoprotein (hnRNP) A2 was decreased only in roots of NT (50%) but maintained in both *ipt*-transgenic lines under heat stress. hnRNPs are complexes formed by the nuclear precursors of mRNAs and specific proteins, which are major constituents of the nucleus and are important elements in the posttranscriptional pathway of the expression of genetic information (Swanson and Dreyfuss, 1988). The maintenance of this hnRNP in transgenic plants could enable proper gene expression for plants growing under stress conditions.

Decreased expression level of a nuclear RNA binding protein A-like protein was observed only in H31 (30%) under heat stress. RNA-binding proteins participate in synthesizing, processing, editing, modifying and exporting RNA molecules from the nucleus (Fedoroff, 2002). Some of the RNA-binding proteins have been reported to be hormone receptors and mediate a subset of hormone actions (Razem et al., 2006). How nuclear RNA binding proteins are involved in stress tolerance is not well understood.

Summary

Transformation with *ipt* induced protein changes involved in multiple functional groups. The diversity of the differentially expressed proteins in response to heat stress suggests a regulatory role of CK in various metabolic pathways for heat tolerance. Among the differentially expressed proteins, a remarkably high percentage of them function in energy, protein destination and storage, and disease/defense categories in both leaves and roots. The expression levels of many of these proteins were maintained or increased in at least one *ipt*-transgenic line under heat stress while they were decreased in

the NT control line. CK regulation of heat tolerance of creeping bentgrass could involve complex mechanisms operating at the transcriptional, posttranscriptional and posttranslational levels. Further research may be conducted to confirm the expression of differentially-expressed proteins using Western blot analysis and identify genes encoding those proteins altered by *ipt* expression in order to reveal specific metabolic pathways and molecular mechanisms of CK regulation of heat tolerance in perennial grass species.

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Table 1 Differentially expressed shoot proteins identified by mass spectrometry among NT, S41 and H31 under heat stress (35 ^{o}C) compared with those at normal temperature (20 ^{o}C). Spot #: protein spot number corresponding to Fig. 4; Protein ID [source]: protein identification based on the highest score in an alignment with this protein; Subcellular localization: predicted subcellular location of the proteins based on computer analysis; Mr/PI: hypothetical molecular weight / isoelectrical point; PS: protein score; AccN: accession number.

* Differential expression level of each protein was shown as the % volume of the spot in heat-treated plants compared unstressed plants. Values <1 represent significant down-regulation of that protein under heat stress, whereas values >1 represent significant up-regulation of that protein under heat stress. Label "-" indicates no significant difference existed in the abundance of the protein between the two temperatures.

Spot #	NT	S41	H31	Protein ID [source]	Subcellular localization	Mr/pI	PS	AccN
02. Energy								
67	0.7	2.0	-	Enolase (2-phosphoglycerate dehydratase) [Oryza sativa]	cytoplasm	47973/5.41	925	gi|90110845
73	2.8	-	-	Rubisco large subunit-binding protein subunit alpha [Triticum aestivum]	chloroplast, plastid	57521/4.83	863	gi|134102
81	0.7	0.6	0.6	Rubisco large subunit [Triticum aestivum]	chloroplast	52791/6.22	600	gi|32966580
132	1.5	1.4	1.5	Glyceraldehyde-3-phasphate dehydrogenase (GAPDH) [Hordeum vulgare]	cytoplasm	36514/6.67	632	gi|120680
172	2.2	-	1.2	Chloroplast chlorophyll a-b binding protein precursor [Oryza sativa]	chloroplast	27565/5.69	213	gi|149392272
178	0.7	-	-	Oxygen-evolving enhancer protein 2 [Triticum aestivum]	chloroplast, plastid	27270/8.84	606	gi|131394
192	0.3	-	1.9	Putative oxygen-evolving complex [Triticum aestivum]	chloroplast	21139/9.72	424	gi|134290407
198	0.8	0.6	-	Rubisco small subunit [Avena clauda]	chloroplast	18857/8.29	606	gi|6409335
204	0.3	0.5	0.6	Photosystem II polypeptide [Triticum aestivum]	chloroplast	34575/5.82	64	gi|37783281
06. Protein destination and storage								
22	-	2.8	-	Heat shock protein 90 [Secale cereale]	plastid	88117/4.90	249	gi|556673
23	0.3	-	-	Heat shock protein 90 [Oryza sativa]	cytoplasm	80214/4.98	208	gi|39104468
40	-	1.5	2.0	Heat shock protein 70 [Cucumis sativus]	chloroplast	78635/5.10	248	gi|1143427
11. Disease/defense								
59	2.3	1.4	1.6	Catalase-1 [Triticum aestivum]	peroxisome	56808/6.52	633	gi|2493543
110	0.3	-	1.7	Glycolate oxidase [Oryza sativa]	peroxisome	40850/9.38	429	gi|115455773
12. Unclear classification								
191	3.0	11.5	8.0	No confident ID	/	/	/	/

Table 2 Differentially expressed root proteins identified by mass spectrometry among NT, S41 and H31 under heat stress (35 ^{o}C) compared with those at normal temperature (20 ^{o}C). Spot #: protein spot number corresponding to Fig. 6; Protein ID [source]: protein identification based on the highest score in an alignment with this protein; Subcellular localization: predicted subcellular location of the proteins based on computer analysis; Mr/PI: hypothetical molecular weight / isoelectrical point; PS: protein score; AccN: accession number.

* Differential expression level of each protein was shown as the % volume of the spot in heat-treated plants compared unstressed plants. Values <1 represent significant down-regulation of that protein under heat stress, whereas values >1 represent significant up-regulation of that protein under heat stress. Label “-” indicates no significant difference existed in the abundance of the protein between the two temperatures.

Spot #	NT	S41	H31	Protein ID [source]	Subcellular localization	Mr/pI	PS	AccN
01. Metabolism								
15	0.7	-	0.5	Ferredoxin-dependent glutamate synthase; Fd-GOGAT [*Hordeum vulgare*]	plastid,chloroplast	48618/5.27	173	Q08258
74	0.8	0.6	0.9	Methionine synthase [*sorghum bicolor*]	cytoplasm	83788/5.93	211	Q8W0Q7
79	0.5	0.6	0.6	Methionine synthase [*sorghum bicolor*]	cytoplasm	83788/5.93	351	Q8W0Q7
82	0.7	0.5	0.5	Methionine synthase [*Catharanthus roseus*]	cytoplasm	84857/6.10	190	S57636
199	0.2	0.7	0.4	Serine hydroxymethyltransferase [Oryza sativa]	plastid, mitochondrion	61354/8.82	597	Q7Y1F0
241	1.5	-	-	Phosphoserine aminotransferase [Oryza sativa]	plastid, mitochondrion	44931/8.53	370	Q8LMR0
262	0.2	-	-	Nucleotide-sugar dehydratase [Arabidopsis thaliana]	plastid	38621/8.58	504	F84688
02. Energy								
53	-	0.6	-	Aconitate hydratase (Aconitase) [*Cucurbita maxima*]	cytoplasm	98005/5.74	251	P49608
57	-	0.6	0.6	Putative aconitate hydratase (Aconitase) [*Oryza sativa*]	cytoplasm	98083/5.67	342	Q6YZX6
70	1.5	2.1	2.1	Triosephosphate isomerase [Hordeum vulgare]	cytoplasm	26720/5.39	270	gi|2507469
165	-	-	1.7	Glucose-6-phosphate isomerase (GPI)	cytoplasm	62237/6.96	195	P49105
192	-	-	0.4	NADH dehydrogenase [Arabidopsis thaliana]	mitochondrion	53504/8.46	526	Q9FNN5
196	-	-	0.2	NADH dehydrogenase [Arabidopsis thaliana]	mitochondrion	53504/8.46	551	Q9FNN5
244	-	-	0.9	Fructose-bisphosphate aldolase [*Oryza sativa*]	cytoplasm	38719/6.55	545	Q40676
252	0.6	-	0.7	NAD-dependent isocitrate dehydrogenase [Oryza sativa]	mitochondrion	40629/8.14	386	Q6ZI55
264	-	-	1.2	GAPDH (phosphorylation) [*Hordeum vulgare*]	cytoplasm	33235/6.20	850	P08477
267	-	-	1.7	GAPDH (phosphorylation) [*Hordeum vulgare*]	cytoplasm	33235/6.20	880	P08477
291	0.4	-	-	Ferredoxin-NADP reductase precursor [Zea mays]	plastid,chloroplast	36375/8.37	210	S53305
04. Transcription								
213	-	-	0.7	Nuclear RNA binding protein A-like [*Oryza sativa*]	nucleus	11066/9.88	119	Q5JM99
230	0.5	-	-	Putative heterogeneous nuclearribonucleoprotein A2 [*Oryza sativa*]	nucleus	39419/5.55	221	Q6YVH4
05. Protein synthesis								
107	-	0.5	-	Poly(A)-binding protein [*Triticum aestivum*]	nucleus	70823/6.60	186	P93616
347	-	-	3.1	Elongation factor 2 [*Beta vulgaris*]	cytoplasm	93738/5.93	385	O23755
06. Protein destination and storage								
54	-	2.5	-	Endoplasmin homolog (HSP90) [Hordeum vulgare]	endoplasm	92859/4.86	1030	P36183
109	1.6	-	-	Stress-inducible protein [Glycine max]	nucleus	63585/5.81	369	Q43468
118	-	1.5	-	Endoplasmin homolog (HSP90) [Hordeum vulgare]	endoplasm	92859/4.86	854	P36183
153	-	1.4	-	Putative t-complex protein 1 theta chain [Oryza sativa]	cytoplasm	60265/6.16	537	Q653F6
163	-	-	1.4	Calreticulin; calcium-binding protein [Hordeum vulgare]	endoplasm	47359/4.48	543	Q40041
256	-	1.1	-	Putative disulphide-isomerase [Oryza sativa]	endoplasm	56854/5.01	256	Q53LQ0
336	2.7	3.7	3.7	Cyclophilin A-2 [Triticum aestivum]	nucleus	18379/8.52	108	Q93XQ6
08. Intracellular traffic								
324	1.4	-	-	Ran (small GTP-binding protein) [*Oryza sativa*]	nucleus	25038/6.66	601	Q9XJ45
09. Cell structure								
186	-	-	0.6	Beta-5 tubulin [*Triticum aestivum*]	cytoplasm	50309/4.73	840	Q9ZRA8
10. Signal transduction								
350	-	0.8	-	Nucleoside diphosphate kinase [Lolium perenne]	mitochondrion	16501/6.30	576	Q9LKM0
11. Disease/defense								
218	-	1.6	-	Molybdenum cofactor containing proteins [Oryza sativa]	peroxisome	43780/8.14	301	Q8LP96
305	0.3	-	-	Ascorbate peroxidase [*Hordeum vulgare*]	peroxisome	31708/7.76	349	Q94IC3
316	-	1.4	-	Glutathione S-transferase [Triticum aestivum]	plastid, mitochondrion	23338/5.79	238	Q9SP56
322	-	1.4	-	Glutathione S-transferase [Triticum aestivum]	plastid, mitochondrion	23338/5.79	176	Q9SP56
20. Secondary metabolism								
98	-	-	0.6	Phenylalanie ammonia-lyase [*Hordeum vulgare*]	cytoplasm	54073/5.73	285	T05968
272	0.7	-	-	dDTP-glucose 4-6-dehydratases-like protein [*Arabidopsis thaliana*]	undefined	38389/7.09	297	T45701
12. Unclear classification								
77	0.3	-	0.3	Possible: OSJNBa0019J05.7 [*Oryza sativa*]	/	106067/8.72	/	Q7XW87
78	0.1	0.2	-	Possible: hydroxyproline-rich glycoprotein-like protein [*Oryza sativa*]	/	106021/9.26	/	Q6YS91
224	-	1.8	1.5	Possible: At1g30580 [Arabidopsis thaliana]	/	44471/6.35	342	Q9SA73
285	-	0.7	-	No confident ID	/	/	/	/
342	-	-	1.7	Os03g0737000 [Oryza sativa]	/	22307/9.18	293	B9FBP6

Fig. 1 Morphological difference in shoot production of NT, S41 and H31 after 10 d of heat treatment at 35 ºC.

Fig. 2 Chlorophyll content (mg g^{-1} DW) (**A.**), leaf IPA content (pmol g^{-1} FW) (**B.**) and root weight (g FW) (**C.**) of NT, S41 and H31 after 10 d of treatment at normal temperature (20 ºC) or heat stress (35 ºC). Uppercase letters are for comparison between the two temperatures in a given transgenic line. Lowercase letters are for comparison among the three transgenic lines at a given temperature. The same letters indicate no significant difference existed between temperatures or among lines at P=0.05.

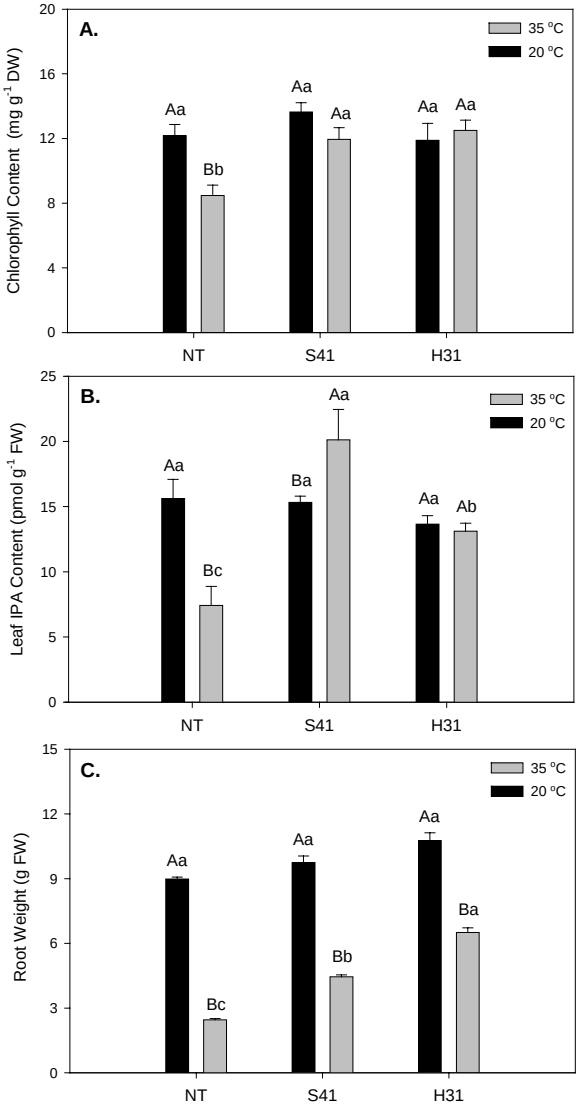


Fig. 3 Northern confirmation of the *ipt* gene expression levels in the leaves of NT, S41 and H31 after 10 d of treatment at normal temperature (20 ºC) or heat stress (35 ºC). The bottom panel shows the ribosomal RNA bands loaded as a control.

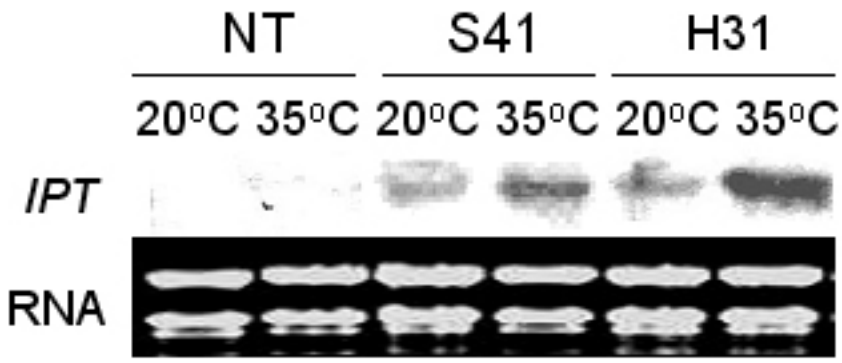


Fig. 4 A representative coomassie-stained 2D polyacrylamide gel of separated shoot proteins from NT grown at normal temperature (20 ºC) treatment. Identified proteins with significant difference among NT, S41 and H31 are indicated. Molecular weight (Mr) is marked.

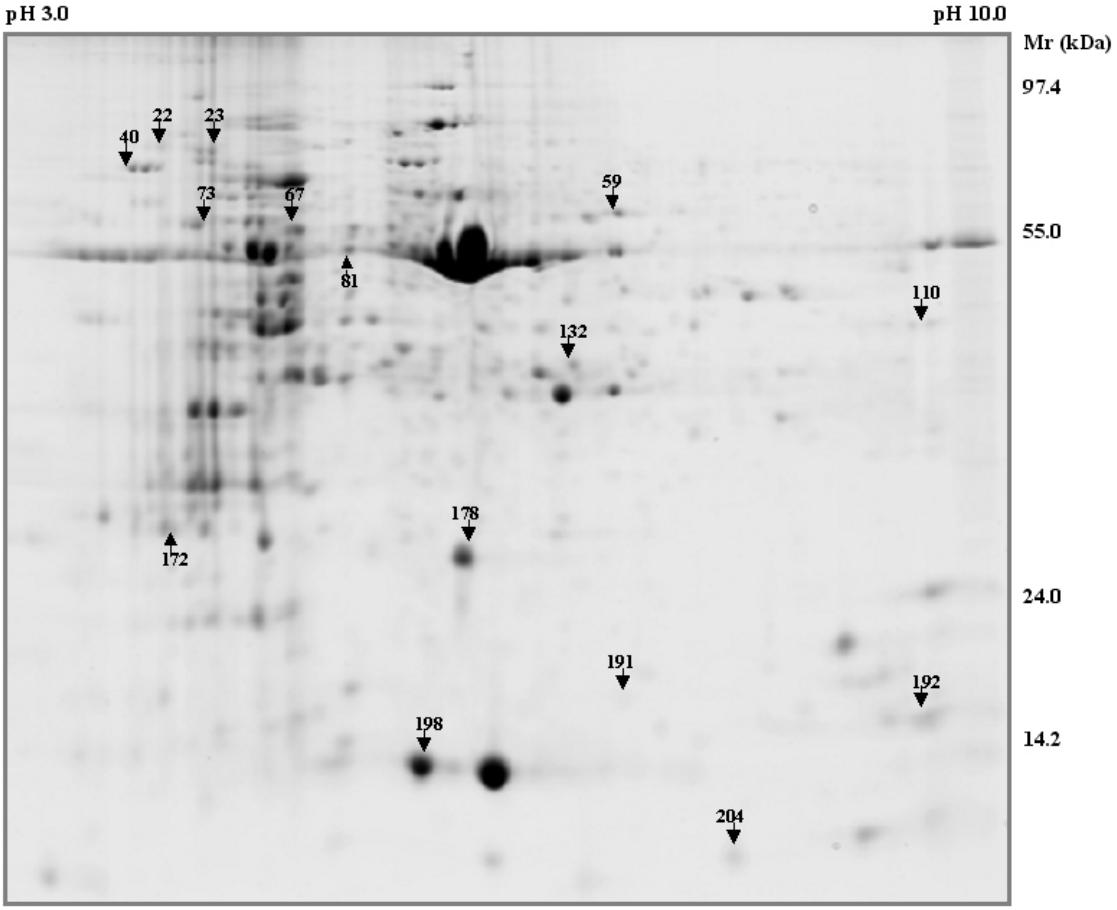


Fig. 5 Selected differentially expressed protein spots (**A**. #22, 40; **B.** #110; **C**. #192) in shoots of NT, S41 and H31 after 10 d of treatment at normal temperature (20 ºC) or heat stress (35 ºC).

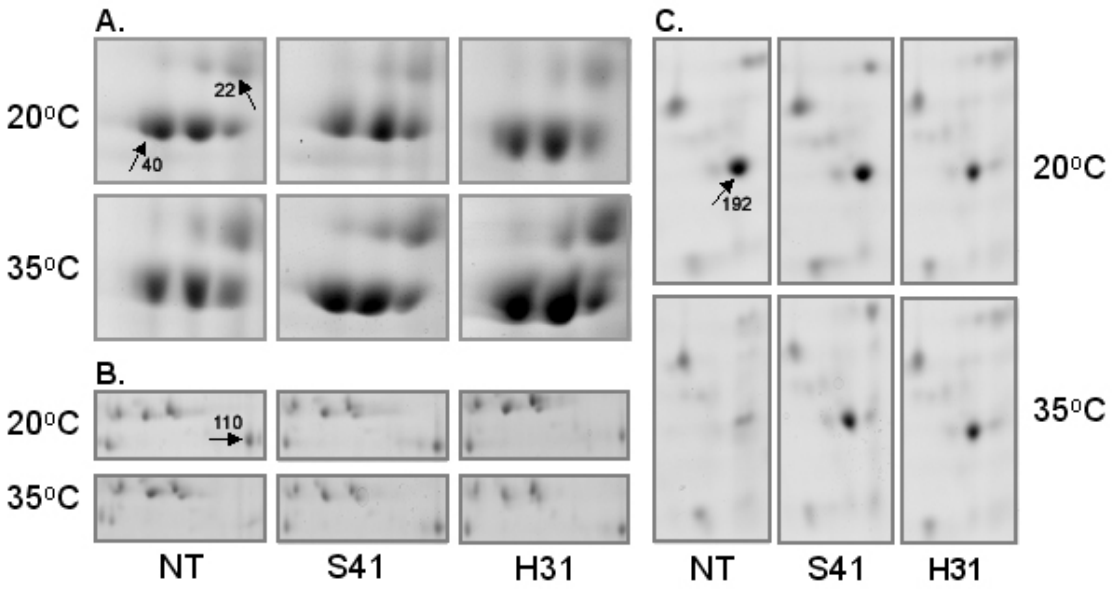


Fig. 6 A representative coomassie-stained 2D polyacrylamide gel of separated root proteins from NT grown at normal temperature (20 ºC) treatment. Identified proteins with significant difference among NT, S41 and H31 are indicated. Molecular weight (Mr) is marked.

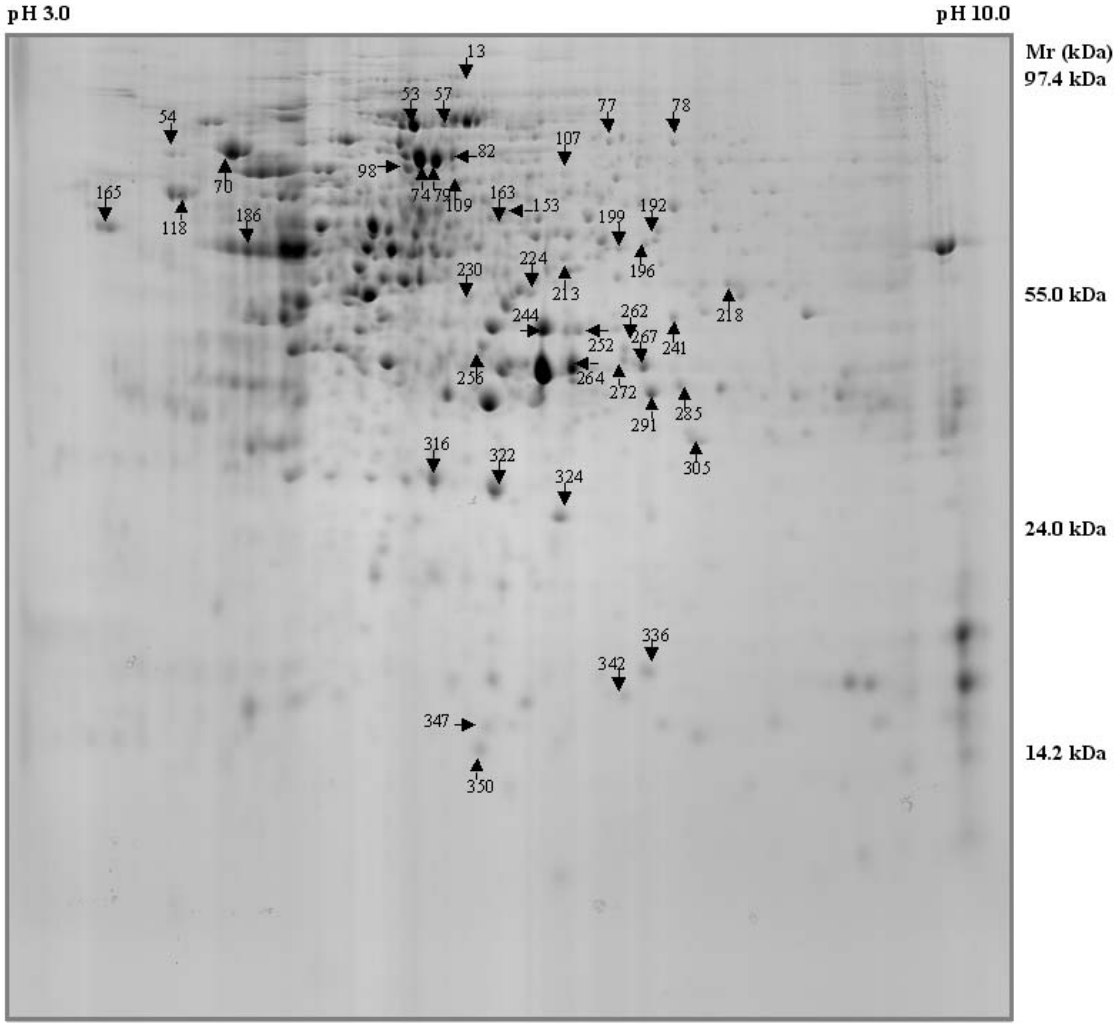


Fig. 7 Selected differentially expressed protein spots (**A.** #316, 322, 324; **B.** #118; **C.** #264, 267) in roots of NT, S41 and H31 after 10 d of treatment at normal temperature (20 ºC) or heat stress (35 ºC).

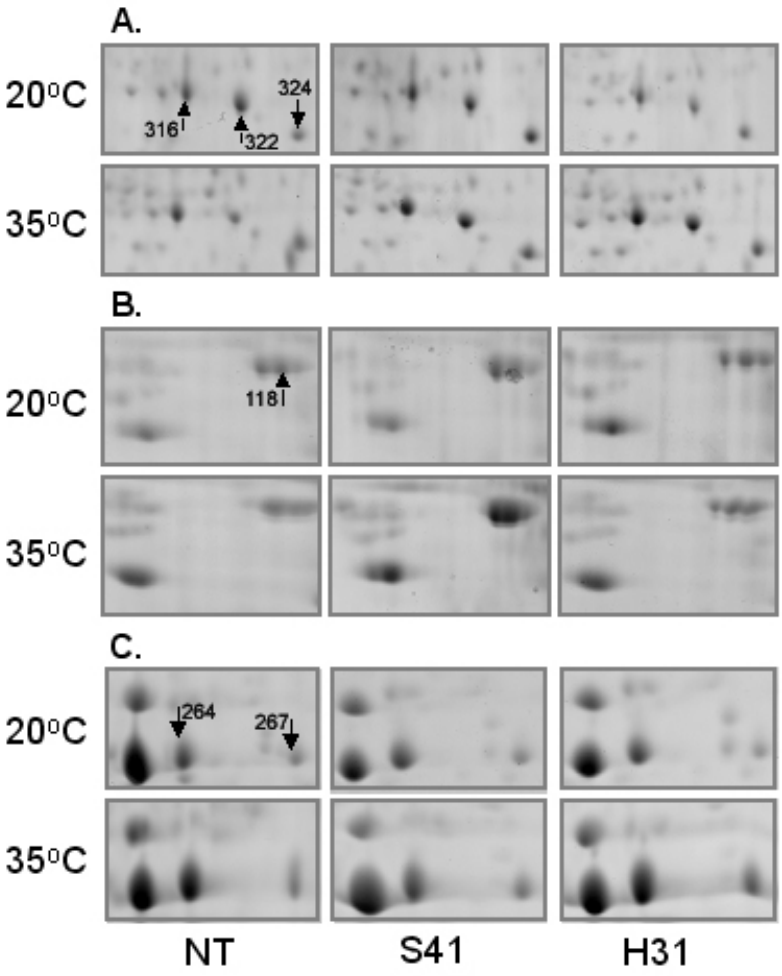


Fig. 8 Pie charts showing functional classes of differentially expressed proteins in shoots (**A.**) and roots (**B.**) of NT, S41 and H31 in response to heat stress. The functional classification was based on the nomenclature described by Bevan et al. (1998).

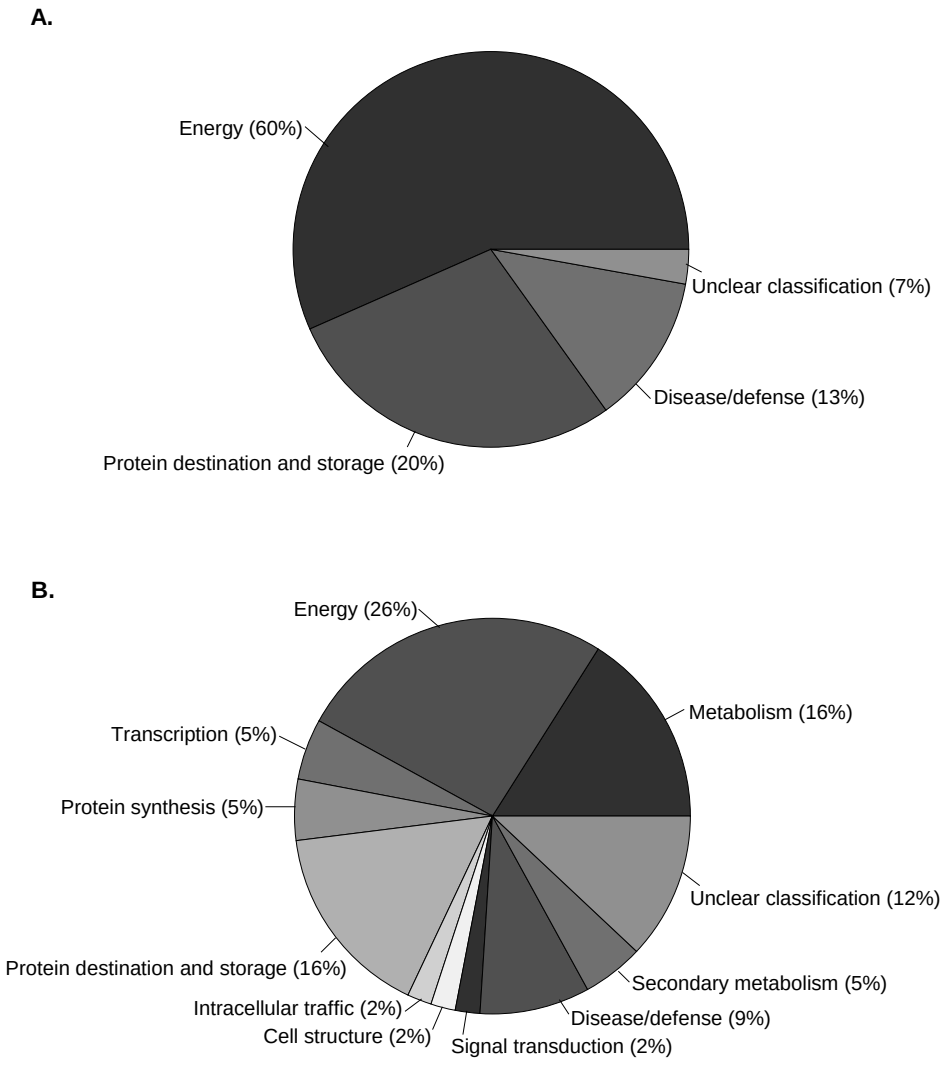
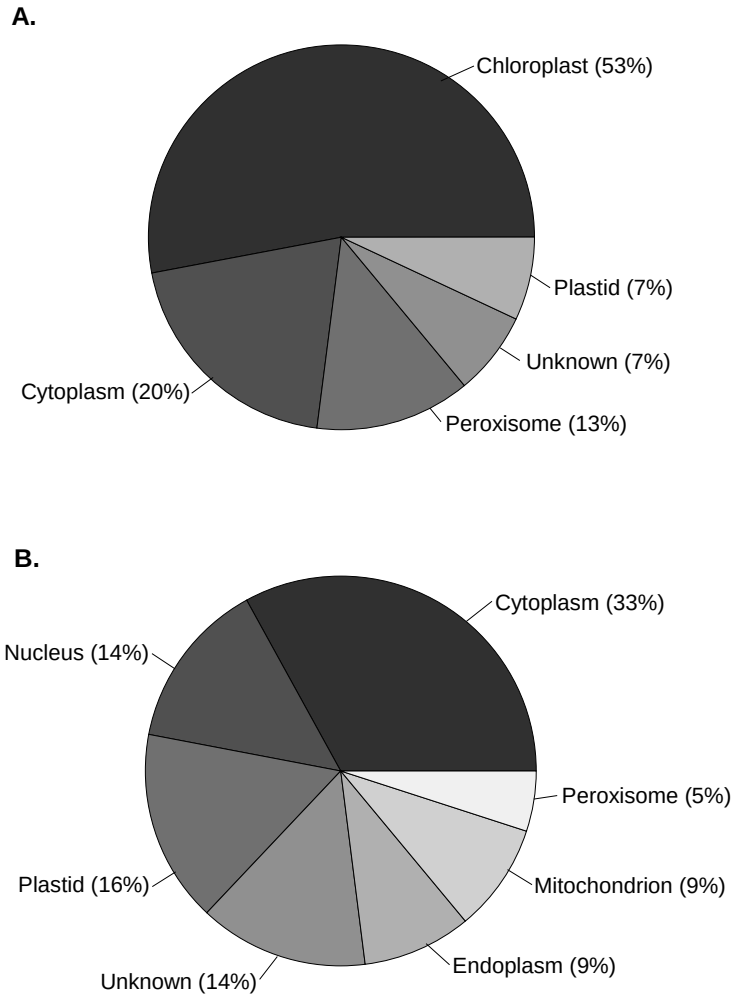


Fig. 9 Pie charts showing predicted organelle distributions of differentially expressed proteins in shoots (**A.**) and roots (**B.**) of NT, S41 and H31 in response to heat stress.



CONCLUSIONS

My dissertation research focused on the investigation of heat tolerance mechanisms by comparing two bentgrass species contrasting in heat tolerance, or by comparing non-transgenic creeping bentgrass with transgenic creeping bentgrass having improved heat tolerance, or by exogenous application of plant growth regulators and biostimulants. Seven projects including both growth chamber and field studies were performed. Our results lead to the following general conclusions.

Heat-tolerance of *Agrostis* grass species was negatively correlated to the accumulation of senescence-promoting hormones such as ethylene, and positively correlated to the production of senescence-inhibiting hormones such as cytokinins. Thus, approaches that can suppress ethylene production, or increase endogenous cytokinin levels might be used to delay foliar senescence and improve heat tolerance.

Heat tolerance of *Agrostis* grass species and cultivars were also attributed to the early induction of HSPs, particularly small molecular weight protein (23 kDa) at a lower level of heat stress, and the maintenance of protein thermostability, particularly high molecular weight proteins (83 kDa and large units of Rubisco). At the metabolite level, heat tolerance of *Agrostis* grass species were related to the maintenance or up-regulation of certain organic acids involved in the TCA cycle (ex. citric acid, aconitic acid), soluble sugars that play a central role in plant structure and metabolism (ex. fructose, glucose), and amino acids involved in the antioxidative defense system (ex. 5-Oxoproline).

Transformation with the *ipt* gene controlling cytokinn biosynthesis maintained or even increased cytokinin production in creeping bentgrass subjected to heat stress. The expression of *SAG12-ipt* stimulated tiller formation and root production, and delayed leaf

senescence in creeping bentgrass subjected to heat stress. Transformation with *ipt* also resulted in protein changes in both leaves and roots of creeping bentgrass, which were involved in multiple functions, particularly in energy metabolism, protein destination and storage, and stress defense. The diversity of proteins altered in the *ipt*-transgenic plants suggests a regulatory role of cytokinin in various metabolic pathways associated with heat tolerance in *Agrostis* grass species.

Applying exogenous hormones or hormone inhibitors can modify endogenous hormone levels under heat stress. Foliar spray of an ethylene inhibitor (AVG) or synthetic cytokinin (ZR) in controlled-environments was effective at suppressing leaf senescence of creeping bentgrass induced by heat stress. Under field conditions, application of the gibberellic acid inhibitor (TE) and two seaweed-based biostimulants (CPR and TurfVigor) that contain cytokinins following their respective label rates in a 2-week interval were effective in improving summer performance of creeping bentgrass.

Further research that may be conducted includes confirmation of the expression patterns of the heat-responsive genes, proteins and metabolites identified in this dissertation, in order to reveal specific metabolic pathways and molecular mechanisms of hormone regulation on stress tolerance. It would also be interesting to study the interactive, synergistic, or antagonistic effects of multiple hormones on a specific physiological, biochemical, or molecular process relating to stress tolerance.

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PUBLICATIONS

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