

**METABOLIC AND MOLECULAR FACTORS ASSOCIATED WITH
HEAT-INDUCED LEAF SENESCENCE IN *AGROSTIS* (ssp.)**

By

DAVID JESPERSEN

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ABSTRACT OF THE DISSERTATION

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By DAVID JESPERSEN

Dissertation Director:
Dr. Bingru Huang

Heat stress is a major abiotic stress affecting many plants world wide. Bentgrasses (*Agrostis* spp.) are a genus which contains several cool-season perennial grass species which are used on high value turf areas such as golf course green. During summer months when temperatures are elevated bentgrasses frequently experience heat-induced leaf senescence characterized by alterations to metabolism and degradation of cellular constituents including membranes, pigments and proteins. Better understanding of physiological and molecular processes which affect heat-induced leaf senescence will aid in the developments of more heat tolerant bentgrasses. Research goals were to look at physiological, biochemical, molecular genetic difference during heat stress events play roles in delaying heat-induced senescence. This was accomplished in several studies which included a comparison in physiological responses of multiple cultivars exposed to heat stress; proteomic analysis of membrane proteins affected by heat stress as membranes are one of the first sites of heat damages; analysis of the effects of several compounds include cytokinin, an ethylene inhibitor, and nitrogen and how they delay heat-induced senescence and their effects on the plants proteome and metabolome; quantitative trait loci analysis to identify genetic regions associated with important heat

tolerance traits; development of candidate gene markers associated with important metabolic functions related to heat-induced senescence and their association with physiological traits related to heat tolerance; investigation of chlorophyll biosynthesis and degradation pathways to help elucidate the cause of chlorophyll loss from leaves during heat-induced senescence. The integration of this information will not only expand our knowledge of heat-induced senescence in cool season turfgrasses but may be used for marker-assisted selection to develop improved cultivars with delayed stress-induced senescence and maintain plant health and growth during heat stress events.

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LITERATURE REVIEW

Introduction

Predictive models forecast that future summers will increasingly see record breaking high temperatures, with today's highest temperatures potentially become the future's norm, as global warming continues to cause temperatures to rise (Battisti and Naylor, 2009). It is predicted that elevated temperatures will result in decreased plant prosperity world wide (Lobell and Field, 2007; Battisti and Naylor, 2009) which further necessitates the need for a better understanding of mechanisms of plant adaptation to heat stress to improve plant growth and productivity in increasing temperature environments. Even relatively short periods of high temperatures during key developmental phases of a plant's life cycle can have significant negative impacts on plant growth and production, as reported in various crop species. The adverse impact of elevated temperatures on annual crop yields in certain regions in the past few decades have already been observed, including rice (*Oryza sativa*) in China (Peng et al. 2004), maize (*Zea mays*) in Africa (Lobell et al. 2011), as well as other major grain crops (Lobell and Field, 2007). High temperatures also can be detrimental to the growth of perennial plants, such as turfgrasses, particularly for C₃, cool-season species (DiPola and Beard, 1992; Fry and Huang, 2004). The level of heat damage depends on a number of factors including plant species, developmental stage, duration, as well as the intensity of heat during the stressful period (Wahid et al. 2007). The differences in levels of heat tolerance also exist within an individual species. It is important to understand the underlying effects and responses to supra-optimal temperature of different turfgrass species.

Turfgrasses and the *Agrostis* Genus

Turfgrasses are a collection of species from the Poaceae family which are adapted to form continuous ground cover and are able to withstand regular mowing which are used for home lawns, parks, golf courses and athletic fields, as well as ground cover to prevent erosion. Commonly used turfgrass species include around 40 species which includes both C3 (cool-season) and C4 (warm-season) plants (Turgeon, 1999). Turfgrasses cover approximately 40 million acres in the United States making it one of the largest irrigated crops by area (Emmons and Rossi, 2014). In 2005 the turfgrass industry had an estimated economic output of over 60 billion dollars (Haydu et al., 2006). In addition to economic value turfgrasses also provide numerous other benefits including environmental benefits of reducing erosion and filtering runoff water, functional value as managed green spaces where people meet and participate in athletics, as well as adding aesthetic value to homes and neighborhoods (NTRI, 2003).

Agrostis is a genus of cool-season grasses with approximately 200 species, with several members of this genus being valuable turfgrass species, including creeping bentgrass (*Agrostis stolonifera*) and colonial bentgrass (*Agrostis stolonifera*) (MacBryde, 2005). These two species are particularly valuable for high value turf areas such as golf course greens and fairways due to their fine leaf texture and ability to withstand low mowing heights and form dense uniform canopies. These turfgrasses originated from pasture grasses collected from central Europe in the areas of Austria, southern Germany, and Hungary (Duich, 1985). Both creeping bentgrass and colonial bentgrass are allotetraploids containing 28 chromosomes ($2n=4x=28$) which share one genome in common, with the genome designations of $A_2A_2A_3A_3$ and $A_1A_1A_2A_2$ respectively

(Warnke, 2003). Currently the complete genome sequence of either species is not available although a number of genetic markers have been developed for both species including RFLP, AFLP, RAPD, SSR, SCAR and EST-specific markers (Chakraborty et al., 2005; Kubick et al., 2011; Golembiewski et al., 1997; Rajasekar et al., 2007; Rotter et al., 2009; Scheef et al., 2001; Vergara and Bughrara, 2003). More resources have been devoted to improving creeping bentgrass because of its more common use on turf areas such as golf course greens. Bentgrasses have low to moderate tolerance of abiotic stresses such as heat and drought and additionally are susceptible to a number of diseases such as dollar spot caused by *Sclerotinia homoeocarpa* or brown patch caused by *Rhizoctonia solani* (Fry and Huang, 2004; Warnke, 2003). Creeping bentgrass has been noted to decline during the heat in summer months, and has reduce growth and performance in elevated temperatures (Carrow, 1996; Xu and Huang, 2000). Recently new cultivars have been developed which demonstrate enhanced disease or stress tolerance, however there is still a need to develop improved cultivars which. Creeping and colonial bentgrasss are able to form interspecific hybrids which may be useful for developing improved cultivars, although the hybridization frequencies are fairly low (Belanger et al., 2003). Understanding the physiology and underlying molecular biology responsible for heat-tolerance and heat-induced leaf senescence will allow of the development of elite cultivars with enhanced abiotic stress tolerance.

Physiological and Metabolic Responses to Heat Stress

Various processes changes occur at physiological, cellular, and molecular levels when plants are exposed to elevated temperatures and most frequently these changes are

greatest in cool-season or temperate plant species. These include changes in membrane stability, carbon metabolism, protein metabolism, antioxidant metabolism, and hormone metabolism (Jespersen and Huang, 2014). These processes can be interrelated and interactively affect plant response and tolerance to heat stress. One of the typical heat stress symptoms is leaf chlorosis or senescence, which is a major contributor to decline in turf quality of cool-season grass species during summer months. This section reviews research advances in mechanisms of heat tolerance associated with leaf senescence, with focus on membrane stability, carbon metabolism, protein metabolism, antioxidant metabolism, and hormone metabolism. It is through this understanding that we can begin to develop more heat tolerant plants and practices to help negate the effects of ever increasing temperatures and maintain plant growth and productivity.

Carbon Metabolism

Photosynthesis is a two stage process of capturing light energy and fixing carbon, known as the light-reactions and dark-reactions of photosynthesis respectively, where plants capture and store energy in molecular bonds for later use. Photosynthesis is also one of the first processes to be damaged by increased temperatures due to many of its components being highly thermolabile, as found in various plant species (Al-Khatib and Paulsen, 1999; Camejo et al. 2005; Ciu et al. 2006). The inhibition of photosynthesis can cause detrimental shifts in carbon balance resulting in plants without the energy reserves to put towards heat tolerance mechanisms or recovery during post stress periods. While not all aspects of photosynthesis are equally damaged by heat stress, key components of both the light and dark reactions are damaged in ways which effect both the capturing of

light energy as well as the fixing of carbon. Both of these steps are needed for plants to complete the photosynthetic pathway and generate energy for metabolic functions of the cell.

Chlorophylls being the pigment molecules responsible for the capturing of light energy are essential for the process of photosynthesis. The decline in chlorophyll content or leaf chlorosis is one of the earliest symptoms of heat-induced leaf senescence. Under prolonged heat stress chlorophyll levels decline. Along with the decline in the levels of chlorophyll the activities of chlorophyll-degrading enzymes, such as chlorophyllase and chlorophyll peroxidases increase, while chlorophyll biosynthesis enzymes including 5-aminolevulinic, dehydratase, porphobilinogen deaminase Mg-chelatase, S-adenosyl Met methyl transferase and Mg-protoporphyrin monoester-cyclase exhibit reduced activities (Tewari and Tripathy, 1998; Todorov et al. 2003; Yamauchi et al. 2004). Both heat-accelerated chlorophyll degradation and heat-inhibition of chlorophyll synthesis may occur simultaneously, causing leaf chlorosis. However, the relative contribution of these two processes to heat-induced leaf chlorosis may vary with the level of stress and phases of leaf development (young vs. old leaves). Ultimately the loss of chlorophyll is due to an unfavorable shift in the balance between chlorophyll synthesis and degradation, with both aspect playing important roles. However recent literature has proposed that the inhibition of protein synthesis may be the more imperative factor leading to photoinhibition and subsequent loss of chlorophyll during heat induced senescence (Murata et al. 2007; Takahashi et al. 2008). Another early sign of heat damage to photosynthetic machinery is the Photosystem II (PS II) reaction center housed in the thylakoid membranes of the chloroplasts. The PS II complex is the first protein-pigment

complex involved the light-dependent reactions of photosynthesis and is responsible for splitting water into hydrogen and oxygen, as well as generating electrons for transport to other photosynthetic machinery and generating hydrogen ions to create the proton gradient needed for ATP synthesis (Taiz and Zeiger, 2010). Many studies have found that the PS II complex is highly thermolabile so even moderate increases in temperature may damage it and lead to a reduction in photosynthesis (Havaux 1993; Haldiman and Feller 2005). Chlorophyll fluorescence uses the light reemitted from chlorophyll molecules to estimate quantum yield and is widely used to estimate the photosynthetic health of light-dependent reactions (Yamada et al. 1996; Maxwell and Johnson, 2000). Quantum yield is a measure of how efficiently the photosynthetic complexes are capturing light energy to use for electron transport. Under severe stress PS II damage may accumulate to such a level that thylakoids are unable to restore light-dependent functions to pre-stress levels (Sharkova, 2001). The loss of PS II activity has been attributed to a number of functional changes which alter the structure or behavior of the protein complex. Damages to chloroplast membranes and increases in membrane fluidity caused by temperature stress may lead to the integral membrane proteins becoming dislodged from their location in the thylakoid. The idea that loss of PS II function is due to a dissociation with the thylakoid membrane is supported by studies which have shown that increased thylakoid stability is associated with better maintenance of photosynthetic capability (Thomas et al. 1986; Sharkey 2005; Ristic et al. 2007). Another theory as to the loss of PS II activity is that certain components within the protein complex become denatured. This denaturation can be the result of direct damage due to high temperatures or the creation of oxygen radicals which then damage cellular components by oxidizing them. The oxygen-evolving

complex responsible for the splitting of water molecules into hydrogen and oxygen is a major site of damage within the PS II complex (Bukhov et al. 1999; Toth et al. 2005). Yamane et al. (1998) have shown that the manganese-stabilizing protein needed to split water molecules dissociates from the oxygen evolving complex under high temperatures. Furthermore it has been demonstrated that the recovery of PS II during post-stress periods relies the synthesis of new protein components of the complex which may be further hindered by the altered protein metabolism associated with heat stress (Toth et al. 2005; Murata et al. 2007). Another aspect of the light-dependent reactions altered under high temperatures is increased activity of Photosystem I (PS I) another protein complex responsible for absorbing light energy in the chloroplasts (Havaux et al. 1996). This commonly observed phenomenon has been attributed to alteration of the thylakoid membrane and conformational changes of the b6f cytochrome protein, which is a membrane bound protein essential for electron transport (Thomas et al. 1986). This conformation change has been associated with the exposure of additional electron acceptor sights which would help facilitate the increased electron flow. This increased activity has been associated with cyclic electron flow, a pathway in the thylakoids which does not involve PS II but instead cycles electrons through PS I, as well as plastoquinone and cytochrome b6f. This increase in cyclic electron flow is believed to act a protective mechanism which acts as a sink for excess energy as well as a means to generate additional ATP (Munekage et al. 2004; Sharkey 2005).

The dark reaction, also known as Calvin cycle of photosynthesis, includes three major processes: carbon fixation, carbon reduction, and ribulose bisphosphate regeneration, which works coordinately to produce carbohydrates and intermediate metabolites

supporting plant growth. Under heat stress conditions it has been found that there is a reduction in the level of metabolites associated with the Calvin cycle which demonstrates a reduced level of carbon-fixation activities (Law and Crafts-Brandner, 1999). The rate of ribulose biphosphate regeneration and carbon reduction processes in the Calvin cycle are inhibited by heat stress (Wise et al. 2004). High temperatures also reduced many essential proteins of the Calvin cycle including both large and small subunits of Ribulose-1,6-bisphosphate carboxylase oxygenase (Rubisco), as well as Rubisco binding proteins and rubisco activase (Demirevska-Kepova et al. 2005). Rubisco which is essential for the photosynthetic process is estimated to be the most abundant protein on earth and is the enzyme responsible for fixing carbon dioxide to longer chain carbon molecules to create sugars which act as energy stores for later use.

It has been shown that under elevated temperatures that heat-inhibition of carbon assimilation is largely due to a reduction in the activation state of Rubisco (Law and Crafts-Brandner, 1999; Salvucci and Crafts-Brandner, 2004). This activation state refers to the conformational shape of the protein which needs to be open and free of sugar residues or other inhibitors to be able to bind and enzymatically react with carbon dioxide. When Rubisco is in an inactive state it will be unable to assimilate the carbon needed to complete photosynthesis. Rubisco activase is the protein which is responsible for returning Rubisco from the inactive to the enzymatically functional active state. Rubisco activase is believed to be more thermolabile than Rubisco itself (Crafts-Brandner et al. 2000). As temperatures rise Rubisco activase activity decreases which in turn leads to a higher percentage of Rubisco molecules being in the inactive state opposed to being active, which in turns leads to decreases in carbon fixation and reduces photosynthesis.

This limited carbon assimilation is in part due to increased photorespiration. Rubisco has the ability to bind both with the carbon dioxide needed to complete the photosynthetic process as well as oxygen; it is this binding of oxygen which leads to the photorespiratory pathway. Photorespiration is a much more serious concern in C₃ plants because they lack the carbon dioxide concentrating mechanisms of C₄ plants which act to reduce the possibility of Rubisco binding with oxygen. This is due to the fact that at increasing temperatures the solubility of carbon dioxide and oxygen shift in a way which favors oxygen binding as well Rubisco having a decreased specificity for preferential carbon dioxide binding or during certain abiotic stresses which create stomatal limitations (Jordan and Ogren, 1984). Once oxygen has bound to Rubisco the cell must expend energy resources to remove the oxygen molecule. Although photorespiration has widely been viewed as a wasteful process leading to reduced photosynthesis, there is the belief that it is valuable as a means a stress protection because it can potentially act as an energy sink to prevent photoinhibition from excess light energy absorbed, as well as the production of useful metabolites (Wingler et al. 2000).

In contrast to photosynthesis responses to increasing temperature, increases in respiration rates have been found in a number of plant species (Sato et al. 2000; Liu and Huang, 2001; Rizhsky et al. 2002; Rizhsky et al. 2004). A study investigating effects of heat stress on transcription in tobacco (*Nicotiana tabacum*) found that most photosynthetic gene were down-regulated (except a PSI and cytochrome gene which may be related to cyclic electron flow), whereas respiration-related genes were largely up regulated (Rizhsky et al. 2002). The increases in respiration and decreases in photosynthesis can cause carbohydrate depletion, particularly during prolonged periods

of heat stress. Decreases in starch accumulation as well as activities of starch related enzymes is a typical symptom of heat stress (Wilhelm et al. 1999; Chaitanya et al. 2001; Majoul et al. 2003). Additionally there is a decreased export of carbon from leaf tissues to roots under high temperature conditions (Dinar et al. 1983; Lafta and Lorenzen, 1995). This decrease in carbon mobilization coupled with the fact that respiration also increases in root tissues, which relies entirely on carbon generated from photosynthesis can be detrimental for plant growth under heat stress (Gunn and Farrar, 1999; Lyons et al. 2007). Maintaining balanced photosynthesis and respiration and carbohydrate accumulation is critically important for plant survival of long-term heat stress.

Membrane Thermostability

Membranes are an essentially structural component for all cell life and also are of key importance for a host of cellular functions. Membranes contain many proteins integrated onto their surface or into their structure. These proteins are responsible for a wide array of metabolic processes, from signal recognition and transduction, transporter proteins, to proteins in the thylakoid membranes of chloroplasts responsible for photosynthetic processes and those in the mitochondria responsible for the break-down of sugars to generate energy in the form of ATP. It is because of their importance in function as well as their sensitivity to fluctuations in temperatures that make them an important component of tolerance to increased temperatures.

Membrane stability is a commonly-used indicator of cellular integrity and viability in plant tolerance to various stresses (Shanahan et al. 1990; Marcum 1998; Liu and Huang, 2000; Sung et al. 2003; Camejo et al. 2005). Membrane stability can be

estimated by measuring the leakage of electrolyte from a cell or tissue. It has been shown to have strong positive correlations to plant heat tolerance (Blum et al. 2001).

As temperatures increase membranes have increased fluidity as the thermal energy disrupts bonds between adjacent fatty acid molecules. This fluidity lowers the stability and functions of membrane integral processes as heat increases (Savchenko et al. 2002). One of the main factors believed to affect membrane stability is lipid composition of the membrane. Lipids contain unsaturated fatty acid molecules which have at least one double bond between carbon molecules which introduce a slight bend in the fatty acid chain. The more saturated the fatty acid with hydrogen molecules the less double bonds will be present resulting in a straighter fatty acid chain. This presents greater opportunity to get near and interact to form bonds with the adjacent fatty acid molecules, resulting in a more rigid lipid layer. Increased levels of saturation have been shown to increase as temperature rises and increased saturation is associated with membrane stability at high temperatures (Thomas et al. 1986; Vigh et al. 1989; Larkindale and Huang, 2004). Due to the relative ease of isolating chloroplasts numerous studies have looked at the effects of lipid saturation on thylakoid membranes, demonstrating that improved membrane stability can have beneficial effects on the maintenance of photosynthetic capability by maintaining thylakoid integrity as well as the function of thylakoid bound proteins such as the pigment-protein complexes needed to harvest light (Raison et al. 1982). Membranes are also sites of temperature stress recognition, leading to signaling cascades which alter gene expression (Vigh et al. 1998). It is clear that whether through maintaining cell integrity, signaling of stressors or through important metabolic functions like molecule transport between cells or the generation of energy in the chloroplast through

photosynthesis, membrane integrity is imperative for maintaining cellular functions, particular since their integrity is vulnerable to changes in structure caused by changes in temperature but also is a major site of damage by reactive oxygen species as discussed in the next section.

Reactive Oxygen Species and Antioxidant Metabolism

Reactive oxygen species (ROS) are a group of oxygen containing molecules where the oxygen is in a reduced state causing the molecule to be highly reactive. These reactive radicals can then interact with cellular components leading to oxidative damage. These ROS can take many forms and commonly includes such molecules as super oxide (O_2^-), singlet oxygen (1O_2), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($OH^\cdot$). ROS production is frequently associated with photosynthesis in the chloroplasts, respiration in the mitochondria, as well as metabolic activities in peroxisomes. ROS are produced in the peroxisomes from the catabolism of fatty acids and other organic compounds (Gill and Tuteja, 2010). In the chloroplasts ROS production is induced when there is over excitation of electron transport associated with the light dependent reactions or CO_2 fixation is limited (Suzuki and Mittler, 2006). This leads to electrons not moving through the normal chain of photosynthesis but instead energizing oxygen molecules, which leads them to form into radicals and damage other molecules. Additionally the photorespiration pathway can lead to creation of ROS in peroxisomes through the action of glycolate oxidase (Mittler et al. 2004). In mitochondria heat stress leads to an uncoupling of respiration with ATP generation, which can lead to a depletion of ATP, and eventually to possible cell death, and similarly to ROS production in chloroplast, it causes an over-

reduction of electron transport leading to the formation of more these damaging radicals (Tiwari et al. 2002).

When these highly chemically reactive ROS are produced and the cell cannot detoxify them quickly, ROS accumulation causes oxidation of cellular components. Lipids, proteins and DNA molecules can all be damaged by ROS leading to their destruction and impairments of cell functions (Gill and Tuteja, 2010). The production of ROS in response to abiotic stresses, including heat stress, is well documented. Lipid peroxidation is a commonly used method to estimate overall oxidative damage due to the relative ease in the ability to measure the resulting compounds produced when ROS damage membrane lipids. Heat stress increases oxidative damage associated with ROS in various plant species (Sairam et al. 2000; Huang et al. 2001; Chaitanya et al. 2002; Larkindale and Knight, 2002). These damages lead to impairment of metabolic functions including photosynthesis, respiration and protein metabolism. The accumulation of these damages eventually leads to apoptosis, programmed cell death.

To prevent oxidative damages from ROS cells have developed antioxidant pathways to scavenging radicals before they can damage the cell. These pathways include non-enzymatic mechanisms, where various compounds including flavanoids, carotenoids, alkaloids and other phenolic compounds are able to scavenge ROS and react with them to limit the amount of free radicals present to cause damages. Other metabolites like ascorbic acid and glutathione also have the ability to accept the free electrons from ROS and are also key substrates for enzymatic antioxidant pathways such as ascorbate peroxidase cycle and glutathione reductase cycle (Alscher et al. 1989; Gechev et al. 2006). In the roots of maize plants exposed to heat stress an increase in glutathione was

found, which was associated with increased antioxidant metabolism (Nieto-sotelo and Ho, 1986). Antioxidant enzymes consist of a wide range of gene families common among plant species with an array of functions and expression profiles (Mittler et al. 2004).

Super oxide dismutase (SOD) is one of the first enzymes required for antioxidant pathways as it is responsible for converting superoxide into hydrogen peroxide, which is then further broken down by other antioxidants. Due to its unique ability to neutralize superoxide it is often expressed in the cellular compartments mentioned like chloroplasts where superoxides are generated, and during heat stress events expression and subsequent activity of SOD are increased to tolerate heat induced ROS damages (Alscher et al. 2002). Catalase (CAT) is a family of enzymes which are able to convert hydrogen peroxide directly to water and increases in activity have also been associated with more active antioxidant systems which confer heat tolerance (Sairam et al. 2000; Chaitanya et al. 2002). Hydrogen peroxide is also broken down through two other major pathways, the ascorbate-glutathione cycle and the glutathione-peroxidase cycle (Apel and Hirt, 2004). During the ascorbate- glutathione cycle hydrogen peroxide is converted to water and ascorbate is converted to monodehydroascorbate and then dehydroascorbate by their respective reductase enzymes resulting in glutathione disulfide which is then reduced to glutathione by the enzyme glutathione reductase. In the glutathione peroxidase cycle hydrogen peroxide and glutathione are converted to water and glutathione disulfide by glutathione peroxidase, which is then again converted back to glutathione by glutathione reductase.

When comparing tolerant and sensitive lines of wheat (*Triticum aestivum*), tolerant lines were found to have greater increases in SOD, CAT, ascorbate peroxidase

(APX), glutathione reductase (GR) and peroxidase (POX) activity, which in turn was associated with lower accumulations of membrane damages and maintaining higher levels of chlorophyll (Sairam et al. 2000, Almeselmani et al. 2006). The alteration of antioxidant activities is a well-documented response during exposure to high temperatures and has been noted in various plant species (Rainwater et al. 1996; Chaitanya et al. 2002; Gulen and Eris, 2004; Almeselmani et al. 2006). Potatoes (*Solanum tuberosum*) transgenically modified to increase expression of SOD and APX in response to stress had increased heat tolerance, demonstrating the importance of antioxidants for heat stress tolerance (Tang et al. 2006). It is however possible for these pathways to become overwhelmed and stress damages accumulate beyond their control. Studies involving arabidopsis (*Arabidopsis thaliana*) and creeping bentgrass (*Agrostis stolonifera*) showed that while antioxidant activities may increase during moderate stress, as temperature and the level of stress continue to increase there is an eventual decrease in the levels of these enzymes and their associated activities (Panchuk et al 2002; Xu and Huang, 2004). Decreases of antioxidant activity are then accompanied by increases in ROS damages (Jiang and Huang, 2001). Additionally decreases in SOD and CAT activities have been associated with onset of leaf senescence (Dhindsa et al. 1981).

While ROS can be injurious to plants, they are also important signal molecules important for regulation of cell growth and development, and of abiotic and biotic stress responses ROS have been found to influence various types of signal cascades including histidine and mitogen-activated protein kinases (Apel and Hirt, 2004). Additionally ROS are believed to interact with a number of transcription factors to regulate gene expression in response to abiotic stress. Large gene families of transcription factors are regulated in

response to heat stress and in turn effect the transcriptional response to heat stress including the regulation of genes involved in ROS pathways. WRKY and ZAT proteins are two such transcription factors, some of which are activated under heat stress and can lead to the production of antioxidants such as APX (Miller et al. 2008). Heat shock factors (HSF) are thought to be able to sense ROS and also lead to changes in signal pathways. An arabidopsis mutant was shown to have increased APX activity associated with increased HSF expression (Panchuk et al. 2002). Plants pretreated with hydrogen peroxide prior to heat stress had higher antioxidant activity (Uchida et al. 2002) which demonstrate that transient increases in ROS may activate antioxidant systems, which in part may explain how pre-treating plants with a minor heat stress event may confer higher tolerance levels during more extreme events. In some situations, however, ROS can signal the production of more ROS, most likely through NADPH oxidases (Gechev et al. 2006). As ROS accumulate this will eventual signal the cell to undergo programmed cell death. Antioxidant enzymes are just one group of proteins whose expression is altered in response to heat stress.

Protein Metabolism

Decreased levels of protein is a well-documented response to heat stress (Thomas, 1978; Ferguson et al. 1990; Ferguson et al. 1994; Gulen and Eris, 2004; Xu and Huang, 2004), which is one of the major characteristics of heat-induced leaf senescence (Thomas and Stoddart, 1980). Heat stress inhibits protein synthesis but accelerates protein degradation, Duncan and Hershey (1989) found that decrease protein synthesis is partly due to decreased ribosome activity and increased protein phosphorylation. Similarly

Ferguson et al. (1994) found that as heat stress temperatures increased, decreased ribosome activity, mRNA levels, as well as decreases in the uptake of radio-labeled methionine into new proteins demonstrated that proteins had decreased synthesis. One of the most common responses to high temperatures is the increase in free amino acid content, which may be in part to increased protein degradation (Guy et al. 2008). Although not all amino acids are accumulated equally under heat stress, which may demonstrate perturbations in some metabolic pathways or preferential accumulation of certain amino acids for protective functions (Mayer et al. 1990).

Proteases are proteins which are responsible for the catabolism of other proteins and polypeptides. During heat stress the level of proteases in the cell often increases and are likely responsible, at least in part, for protein degradation. Two major classes of proteases found to be up-regulated during heat stress are cysteine proteases and serine proteases (Palma et al. 2002; Schaller 2004). Additionally protease inhibitors have been found to reduce the amount of protein degradation during heat stress and inhibit cell death, confirming the regulatory role of proteases (Tiwari et al. 2002). Ubiquitin mediated protein degradation is also another major pathway for protein destruction. Ubiquitins are small highly conserved proteins which act to target proteins for degradation. Ubiquitins are ligated to proteins to be degraded, and the subsequent addition of additional ubiquitin units results in a polyubiquitin chain. The addition of the polyubiquitin chain onto the protein targets it to a proteasome complex which degrades the protein to smaller polypeptides (Belknap and Garbarino, 1996). In wheat roots exposed to heat, protein degradation was associated with decreases in the pool of free ubiquitin but an increase in ubiquitin conjugations demonstrating that ubiquitin mediated

degradation is an important element of protein degradation during heat stress (Ferguson et al. 1990).

Proteomic profiling by two-dimensional electrophoresis and mass spectrometry is a powerful tool for quantification of protein abundance and identification of specific proteins that change in response to a variety of events, such as heat stress. In response to heat stress, proteins associated with protein biosynthesis such as initiation factors and ribosomal proteins were found to be down regulated, while proteins such as cysteine protease associated with protein degradation were up-regulated (Majoul et al. 2004; Zou et al. 2011). Changes related to carbon metabolism were also noted, with many glycolysis proteins down-regulated but a few such as glyceraldehyde-3-phosphate dehydrogenase were upregulated by heat stress, and many photosynthetic proteins were down-regulated as well, with the exception of Rubisco activase, which as previously mentioned may play a role in maintaining the activity of the Calvin cycle (Majoul et al. 2004; Zou et al. 2011). In two turfgrass species increased heat tolerance was associated with maintenance of higher levels of photosynthetic proteins, such as rubisco and rubisco activase, and proteins related to carbon metabolism, such as glyceraldehyde-3-phosphate dehydrogenase, and malate dehydrogenase (Xu and Huang, 2010). Proteins in PSII components in photosynthesis declined while PSI components increased in response to heat stress (Ferreria et al. 2006). Alteration of proteins imparting critical cellular functions is an important mechanism of plant adaptation to heat stress.

When plants are exposed to elevated temperatures proteins are broken down to either be reformed as new proteins or transported to other areas of the plant so the nutrients can be reused elsewhere. Heat stress can directly damage proteins by altering

the three dimensional shape of proteins so they are no longer in active conformations. These damaged proteins then often aggregate together. Proteins may be damaged by ROS produced during heat stress. The oxidative damage inflicted by ROS will also render proteins unable to carry out their metabolic functions. Proteins with altered conformations due to damage caused directly by high temperatures or through oxidation caused by ROS are selectively targeted to be degraded. . Damaged proteins not only tie up and make unusable nutrients in the cell but are also potentially damaging by inhibiting metabolic functions and generating additional ROS.

Another important change in protein metabolism in response to heat stress is the accumulation of heat shock proteins (HSPs). HSPs can be found in almost all organisms, and although they were originally discovered as heat induced proteins, they can be induced by many other stresses and some are functional during non-stress, steady state conditions (Feder and Hofmann, 1999). HSPs are often induced by high temperature and are strongly associated with heat tolerance (Vierling 1991). HSPs can range in size from small proteins less than 15kd to larger ones over 100kd in size. Acting as chaperone proteins HSPs assist in the folding, or refolding of proteins into their active conformations (Hendrick and Hartl, 1995). Additionally some members of this protein family also act as signal molecules effecting signal transduction and eventual expression of genes (Wang et al. 2004).

Small HSPs are those that are under 40 kd in size and while it is a diverse group of proteins they all contain a highly conserved α -crystallin domain (Sun et al. 2002). Small HSPs are important for stabilizing proteins and assist in chaperone functions by binding to denatured proteins to prevent further denaturation and targeting them to be

refolded by other chaperones (Wang et al. 2004). It is believed that small HSPs bind with the hydrophilic domains of proteins which become exposed as a protein denatures but before protein aggregates form (Basha et al. 2011). These proteins are expressed during heat stress events, as well as in response to many other stressors. Expression patterns may change based on the level and duration of stress but it is believed that several heat shock factors (HSF) may control the expression of small HSPs (Schoffl et al. 1998). Analysis of HSF structure confirms that they can interact with DNA to influence transcriptional regulation (Nover et al. 1996). Three classes with over 20 different HSFs have been identified in plants demonstrating the diversity of this family of proteins responsible for influencing the genetic regulation of HSP and other heat responses (Kotak et al. 2007). While many details of the pathway still need to be determined it is believed that HSFA1 is a major factor responsible for leading to further responses to high temperatures, while HSFB1 which belongs to a different class of HSF can be induced by HSFA1 and is an important co-regulator which interacts with other transcriptional regulatory units (Baniwal et al. 2004).

The HSP 100 protein family is closely related to caseinolytic protease family (CLP) of proteins and is believed to make up sub-units of CLP proteases (Agarwal et al. 2001). Additionally HSP 100 proteins have been found to play a role in breaking apart aggregated proteins so then other chaperones can refold or degrade them (Glover and Lindquist, 1998). In Maize roots mutants lacking HSP 101 were shown to have deficient thermotolerance (Nieto-Sotelo et al. 2002). Similar results were found in Arabidopsis mutants lacking HSP 101 expression having reduced heat tolerance, additional mutants

over expressing this gene had increased tolerance compared to controls (Queitsch et al. 2000).

Other major families of HSPs include HSP 60, 70 and 90. HSP 60, sometimes referred to as chaperonin, has been shown to prevent the inactivation of proteins under high temperatures (Martin et al. 1992). HSP 70 has been shown to interact with HSP 100 to refold proteins which have aggregated (Glover and Lindquist, 1998) but in addition to its chaperone functions HSP 70 are not only important for stress tolerance but have roles in regulating cell development (Su and Li, 2008). In maize exposed to heat stress HSP 70 was shown to be involved in the regulation of anti-oxidant defenses (Hu et al. 2010). HSP 90, has the chaperone functions of refolding proteins similarly to other HSPs however many of its targets are involved in signal transduction, including steroid receptors and protein kinases, which shows that HSP 90 influence the regulation of cell responses to stress (Krishna and Gloor, 2001). Regardless the size of HSP, the induction of HSP generally is positively associated with heat tolerance in plants.

Hormone Metabolism

Plant hormones play key roles regulating plant growth and development. Hormones are also important for inducing responses to stress and the processes of acquired thermotolerance (Larkindale and Huang, 2004; Kotak et al. 2007). Several hormones that are reported to have major effects on heat stress responses in plants are discussed here.

Cytokinins regulate cell division and cell differentiation and play roles in plant tolerance to heat stress (Hare et al. 1997). Cytokinin content decrease under heat stress

(Cheikh and Jones, 1994). Exogenous applications of cytokinins has been found to elevate endogenous cytokinin levels, suppressing leaf senescence and heat-inhibition of photosynthesis, leading to improvement in heat tolerance (Tetley and Thimann, 1974; Caers et al. 1985; Veerasamy et al. 2007; Zhang and Ervin, 2008). Applications of cytokinins has been shown to delay heat induced senescence in creeping bentgrass leading to improvements in photosynthetic processes, including the maintenance of chlorophyll content and photochemical efficiency, as well as improved antioxidant function and an associated reduction in oxidative damage (Liu et al. 2002; Veerasamy et al 2007; Xu and Huang, 2009a) Increases in cytokinin production through genetic transformation has also been reported to be effective to improve plant tolerance to heat stress. Transformation of tobacco with an isopentenyl-transferase gene, the enzyme of the rate limiting step of cytokinin biosynthesis, on a heat shock promoter resulted in increases of stress tolerance related genes, and decreases in heat- induced senescence (Smart et al. 1991; Harding and Smigocki 1994). Another study which used an isopentenyl-transferase gene expressed under control of a senescence activated promoter found increased heat tolerance in creeping bentgrass, manifested as maintenance of higher chlorophyll content, as well as improved growth both above and below ground in the roots (Xing et al. 2009; Xu et al. 2009b). Investigations of protein changes associated with these transgenic bentgrasses have found that plants with delayed senescence due to increased cytokinins have alter accumulations of photosynthetic proteins, HSPs and antioxidant proteins which reflect important physiological pathways of thermotolerance (Xu et al. 2009 b). Increases of cytokinins in a plant have been associated with a delay in

the expression of senescence-associated genes and an up regulation of genes associated with photosynthesis (Gan and Amasino, 1996).

Ethylene accumulation has been associated with heat-induced leaf senescence and an important signal regulating the timing of senescence and activation of stress-related genes, as well as the associated break-down of cellular components including proteins, membranes and pigments (Davies et al. 1989; Lim et al. 2007;). Periods of high temperatures have been shown to induce endogenous accumulations of ethylene (Morgan and Drew, 1997; Balota et al. 2004; Hays et al. 2007). In oat leaves applying an ethylene precursor ACC stimulated senescence while inhibiting ethylene synthesis reduced senescence (Gepstein and Thimann, 1981). In arabidopsis similar effects were seen where exogenous applications of ethylene lead to an induction of senescence-associated genes while an ethylene insensitive mutant had the opposite effect of delayed expression of senescence associated genes and the maintenance of photosynthetic genes (Grbic and Bleecker, 1995). In other plant species similar results were found where blocking ethylene production or reception ameliorated the effects of heat stress (Hays et al. 2007; Xu and Huang, 2009a). In creeping bentgrass the application of aminoethoxyvinylglycine, a compound which inhibits the synthesis of ethylene, led to improved heat tolerance through the maintenance of higher chlorophyll content as well as improved antioxidant functions (Xu and Huang 2009a)

Absciscic Acid (ABA) is believed to be responsible for general stress responses and has been found to increase in response to many stresses, including heat stress (Daie and Campbell, 1981; Cheikh and Jones, 1994). ABA signaling pathways leads to the induction of transcription factors which then in turn lead to the expression of genes which

confer tolerance to stress condition including heat (Kim et al. 2004). ABA was shown to act synergistically with heat to increase the expression of stress-associated genes, demonstrated by increased expression of genes controlled by a stress responsive promoter when exposed to both heat and exogenous ABA (Xiong et al. 1999). Exogenous applications of ABA has been shown to increase heat tolerance, which has been associated with an increase in antioxidant metabolism resulting in higher activities of important antioxidant enzymes including APX, CAT, and SOD (Larkindale and Knight, 2002; Agarwal et al. 2005). Larkindale and Huang (2004) showed that an ABA insensitive mutant had a greater sensitivity to heat stress as demonstrated by increased accumulations of oxidative damage, which further demonstrates ABA's role in acquired thermotolerance particular relating to antioxidant pathways. Additionally ABA has been further implicated in acquired stress tolerance by increased accumulations of HSP proteins in response to exogenous applications of ABA (Heikkila et al. 1984; Campbell et al. 2001).

Salicylic acid (SA) increases during heat stress and may also play a role in the signaling of general stress responses, ultimately leading to increases in heat tolerance (Dat et al. 1998; Horvath et al. 2007). While exogenous applications of SA result in increases in hydrogen peroxide, possibly as part of the ROS signaling pathway, it was also shown to increase thermotolerance associated with increases in antioxidant enzyme activity (Dat et al. 2000). Increases in thermotolerance, as indicated as improved survival, increases in antioxidant function and maintenance of photosynthetic health, associated with exogenous applications of SA has been demonstrated in a number of species, although frequently application of higher rates leads to declines in thermotolerance

showing the delicate balance needed for appropriate stress signaling (Senaratna et al. 2000; He et al. 2005; Shi et al. 2006). Furthermore transgenic arabidopsis with inhibited SA production was shown to have increased oxidative damage and severely decreased survival during exposure to heat stress, while conversely mutants with increased SA accumulation were shown to have enhanced thermotolerance (Clarke et al. 2004; Larkindale and Huang, 2004). SA has been implicated in the genetic regulation of stress tolerance through experiments that demonstrate it can induce small nuclear proteins which then bind to known stress associated genes, as well SA leading to increased accumulation of HSPs (Goldsbrough et al. 1993; Clarke et al. 2004). Although stress-signaling pathways are far from being fully elucidated it is clear that plant hormones play a vital role in the signaling and coordination of responses to heat stress.

Use of Quantitative Genetics to Improve Plant Productivity

One way to develop improved cultivars is through the use of marker assisted selection. Marker assisted selection (MAS) is the use of specific genetic markers to guide selection of plant materials to use in breeding programs. MAS has the potential to improve the efficiency, effectiveness, and cost of plant breeding efforts ultimately developing improved cultivars more quickly (Collard et al., 2005). A number of potential methods exist to discover genetic markers to use for MAS. These include the discovery of quantitative trait loci (QTL) or using a candidate gene approach. QTL analysis involves developing a population and a corresponding linkage map which represents the organism genome. After screening the population for a trait of interest, specific regions of the linkage map can be associated with the trait of interest. Markers in the region of the

linkage map which are validated to be linked to the trait of interest can then be used in MAS (Haley and Andersson, 1997). QTL analysis has been used to identify genetic regions associated with heat tolerance in major agronomic crops, such as rice (*Oryza sativa*) (Ye et al., 2012; Zhang et al. 2009) and wheat (*Triticum aestivum*) (Mason et al., 2011; Paliwal et al., 2012; Vijayalakshmi et al., 2010).). However to date most QTL analysis in turfgrasses have dealt with biotic stresses caused by the disease such dollar spot in creeping bentgrass (Chakraborty et al., 2014; Honig et al., 2014), gray leaf spot in perennial ryegrass (*Lolium perenne*) (Curley et al., 2005) or Italian ryegrass (*Lolium multiflorum*) (Takahashi et al., 2014), and crown rust on perennial ryegrass (Sim et al., 2007). Recently QTL have also been identified for drought tolerance in creeping and colonial bentgrasses (Merewitz et al., 2012; Merewitz et al., 2014) The candidate gene approach is one where markers are developed for specific genes which believed to play an important role in the trait of interest (Pfielger et al., 2001). Candidate gene markers have been developed for important traits such as accumulation of pigments or polyphenols (Thorup et al., 2000; Verdu et al., 2014), or disease resistance (Faris et al., 1999; Dracatos et al., 2009). Additionally the candidate gene approach has been used to identify markers which are associated with drought for abiotic stress tolerance (Pelleschi et al., 1999; Tondelli et al., 2005; Yu et al., 2013, Thudi et al., 2014). However relatively little work has been done turfgrasses using candidate gene markers for heat tolerance.

Increasingly high-throughput sequencing technologies are being used to screen the entire genome for SNP polymorphisms, which allows researchers to screen thousands of markers to use in association mapping approaches. However a bentgrass genome has not been completely sequenced and relatively less resources are devoted to turfgrass species

compared to more agronomically important species. QTL mapping and the candidate gene approach are two potential ways to utilize MAS to develop improved bentgrass cultivars despite its complex genome structure and relative fewer resources available to researchers.

Objectives

The main goals of the presented research were to better understand the physiology, molecular biology, and genetics involved in regulating heat-induced leaf senescence and ultimately responsible for tolerance to elevated temperatures in bentgrasses. This was accomplished through several inter-related projects presented in this dissertation which include:

1) Physiological Traits and Genetic Variations Associated with Drought and Heat Tolerance in Creeping Bentgrass. Objectives were to compare several commercial creeping bentgrass cultivars' tolerance to abiotic stress and determine which physiological factors are most correlated with overall stress tolerance

2) Membrane Proteins Associated with Heat-Induced Leaf Senescence in a Cool-Season Grass Species. The objective was to analyze membrane proteins altered by heat stress in a tolerant and sensitive individual of a colonial x creeping bentgrass hybrid backcross population to understand protein changes which may be associated with delaying heat-induced senescence in membrane fractions.

3) Proteins Associated with Heat-Induced Leaf Senescence in Creeping Bentgrass as Affected by Foliar Application of Nitrogen, Cytokinins, and an Ethylene Inhibitor. Objectives include confirming the roles of exogenously applied compounds roles in

delaying leaf senescence and determining proteomic changes responsible for increased levels of heat tolerance.

4) Metabolite Responses to Exogenous Application of Nitrogen, Cytokinin, and Ethylene Inhibitors in Relation to Heat-Induced Senescence in Creeping Bentgrass. The objective was to explore metabolomic changes associated with exogenously applied compounds to determine how metabolism was being altered in association with delayed heat-induced senescence.

5) Quantitative Trait Loci Associated with Physiological Traits for Heat Tolerance in Creeping Bentgrass. The objective of this project was to screen a creeping bentgrass mapping population for several important physiological traits related to heat tolerance to perform QTL analysis and identify specific regions of the genome related to heat tolerance.

6) Candidate Genes and Molecular Markers Associated with Heat Tolerance in Colonial Bentgrass. Molecular markers were developed for candidate genes to confirm these genes roles in heat tolerance mechanisms as well as their potential for use in marker assisted selection.

7) Chlorophyll Loss Associated with Heat-Induced Senescence in Bentgrass. Metabolic factors associated with chlorophyll synthesis and degradation were explored to determine key steps which regulate chlorophyll loss during heat-induced leaf senescence using real-time PCR and enzymatic assays.

These projects may ultimately lead to a better understanding of the physiological, molecular, and genetic aspects involved in heat-induced leaf senescence so that we can

develop improved cultivars which have increased heat tolerances and maintain better performance with fewer inputs during summer months.

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**CHAPTER 1: Physiological Traits and Genetic Variations Associated with Drought
and Heat Tolerance in Creeping Bentgrass**

Jespersen, D., W.A. Meyer, B. Huang. 2013 Inter. Turf. Soc. Res. J. 12:459-464

INTRODUCTION

Drought and heat stress are two major environmental stresses causing turfgrass quality decline in many areas with limited water availability, particularly during summer months. Drought and heat stress causes various cellular and physiological damages, such as changes in membrane stability, loss of chlorophyll, inhibition of photosynthesis, and leaf dehydration, as well as induction of oxidative stress (Bohnert et al., 1995; Wahid et al., 2007). Tolerances to drought and heat stress have been related to maintaining leaf cellular hydration, membrane stability and photosynthesis, as well as less oxidative damage in various non-turfgrass species (Teulat et al. 1997; Blum et al., 2001; Dash and Mohanty, 2001; Dias et al., 2010; Hu et al., 2010) and turfgrass species (Huang et al., 1998; Huang and Gao 1999; Jiang and Huang, 2001; Abraham et al., 2004; Cui et al., 2006; Liu et al., 2008; Merewitz et al., 2011).

Drought and heat stress are known to have effects on common physiological processes as mentioned above, but injury symptoms induced by either stress alone or mechanisms tolerance to either stress may be distinctly different. For example, a typical drought stress symptom in turfgrass is leaf wilting and desiccation while leaf senescence is a primary symptom of heat stress, although both can result in eventual loss of turf quality (Beard 1973; Aronson et al., 1987; DiPaola and Beard, 1992; Marcum, 1998; Fry and Huang, 2004). Therefore, a distinctly different set of physiological traits may be associated with drought tolerance from those for heat tolerance although some common traits may also be possible. In addition, cultivars within a turfgrass species vary in drought or heat tolerance (Carrow, 1996; Qian et al., 1997; Marcum, 1998; McCann and Huang, 2008; Soliman et al, 2012), which provides genetic materials for improving

turfgrass stress tolerance (Fry and Huang, 2004). However, most of previous studies in turfgrass examined plant responses to heat stress or drought alone in individual experiments (Beard 1989; Qian, 1997; Marcum, 1998; Liu and Huang, 2000; Xu and Huang 2001; McCann and Huang, 2008), and furthermore a few studies performed the comparative analysis of differential physiological traits for drought and heat stress for turfgrasses exhibiting a wide range of genetic variations. By comparing genetic variations in physiological responses to drought and heat stress and examining the interrelationships among different physiological traits for stress tolerance, differential physiological traits associated with plant tolerance to drought or heat stress and the same traits related to both stresses may be determined. Understanding major or primary physiological traits linked to genetic variations in drought and heat stress tolerance will allow specific evaluation criteria to be created to aid in the development of improved varieties.

The objectives of this study were to compare cultivars variations in drought and heat tolerance of creeping bentgrass, a widely-used cool-season turfgrass sensitive to drought and heat stress, and to determine major physiological traits associated with either drought or heat stress and with both stresses through performing correlation and regression analysis.

MATERIALS AND METHODS

Plant Materials and Growth Conditions.

Sods of eight cultivars of creeping bentgrass ('007', 'Declaration', 'Kingpin', 'L93', 'Penncross', 'Pro-as7', 'Shark', and 'Tyee') were collected from 1-year-old field plots from the turfgrass research farm at Rutgers University, New Brunswick, NJ and transplanted into plastic containers (10 cm in diameter and 40 cm deep) filled with a sand and loamy soil mixture (1:2, v/v). Plants were watered daily and fertilized twice a week with 50 ml of half strength Hoagland's nutrient solution (Hoagland and Arnon, 1950). Plants were allowed to establish for 30 d in a greenhouse before being transferred to growth chambers and allowed to acclimate for one week before treatment conditions were induced.

Treatments and Experimental Design.

The experiment consisted of three treatments: 1) non-stress control: plants were well watered at the optimal growth temperature (20/15 °C, day/night); 2) drought stress: plants were maintained at 20/15°C but water was withheld for 21 d; 3) heat stress: plants were exposed to 35/30 °C (day/night) temperatures and were maintained well watered. Soil volumetric water content was maintained at 30% in the non-stress control and heat stress treatment, and dropped down to 4% by 21 d of drought stress. Soil water content was monitored with the time domain reflectometry in the upper 20-cm soil of the pot using a 20 cm long probe (Trase, Soil Moisture Equipment, Santa Barbara, CA). Each treatment had four replicates of each genotype that were arranged in a completely randomized block design in four growth chambers. Growth chambers had a 14 hour photoperiod with 500 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetic photon flux density and were

maintained at 60% relative humidity. Heat stress treatments ran from February 1 through March 8 2010, and drought stress treatments ran from April 12 through May 3 2010.

Physiological Evaluations of Stress Tolerance.

Turf quality (TQ) was visually rated as an indication of overall turf performance or plant health on a scale of 1-9 based on turf color, density, and uniformity, with 1 being dead turf and 9 being the best in all three quality components (Turgeon, 1999).

Two parameters related to leaf senescence and photosynthesis were evaluated, leaf photochemical efficiency (Fv/Fm) and chlorophyll content (CHL). Photochemical efficiency was estimated by measuring the ratio of variable fluorescence to maximum fluorescence (Fv/Fm) for leaves dark adapted for 30 min by using a fluorescence induction monitor (ADC BioScientific Ltd., Herts, UK). Chlorophyll content was measured using a method described by Hiscox and Israelstam (1979) with modification. Leaves chlorophyll was extracted in dimethylsulfoxide and the absorbance of the extraction solution was measured using a spectrophotometer (Spectronic Instruments, Inc., Rochester, NY) at wavelengths of 663 nm and 645 nm. Chlorophyll content of the samples was calculated using the equations described by Arnon (1949).

Membrane stability was measured with electrolyte leakage (EL). Electrolyte leakage was determined by measuring the initial electrical conductance (Ci) of solution with fresh leaves incubated in water and the maximum conductance (Cmax) of the solution with leaves killed in an autoclave at 140 °C for 20 min. Electrolyte leakage was calculated as a percentage, of Ci to Cmax (Blum and Ebercon, 1981).

Lipid peroxidation is an effect of oxidative damage in membranes (Zhang and Kirkham, 1994) and was estimated by measuring malondialdehyde (MDA) content, a

byproduct of lipid peroxidation, as described by Dhindsa et al. (1981). Leaf tissues were frozen in liquid nitrogen at the time of sampling and remained frozen at -20 °C. Tissue was ground and then homogenized with a phosphate extraction buffer. Samples were centrifuged and the supernatant was mixed with 2 mL of 20 % trichloroacetic acid containing 0.5% thiobarbituric acid. Samples were heated in a 95 °C water bath for 30 min and quickly cooled on ice, and once again centrifuged and the resulting supernatant's absorbance was measured at 532 and 600 nm. The concentration of MDA was calculated using an extinction coefficient of $155 \text{ mM}^{-1} \text{ cm}^{-1}$ (Health and Packer, 1968).

Leaf hydration status was measured using relative water content (RWC). Relative water content was measured by weighing fresh leaf tissue (FW), then soaking the samples in de-ionized water for 24 hours. Samples were then pat dried and weighed again to obtain the turgid weight (TW), and then put in an 80°C oven to dry for 72 hours and weighed again to obtain the dry weight (DW). Relative water content was obtained using the formula $\text{RWC} = (\text{FW}-\text{DW}) / (\text{TW}-\text{DW}) \times 100$ (Barrs, 1962)

Statistical Analysis.

The physiological responses of cultivars over the duration of drought or heat stress were compared for the above mentioned measurements using the analysis of variance test and significant differences between cultivars were tested with the Fisher's protected LSD at a probability of 0.05. Correlation analysis was performed to determine the relationship between turf quality (overall drought or heat tolerance index) and each physiological parameter and among physiological traits to determine interrelated traits for stress tolerance. A backward elimination regression analysis was used to determine major physiological traits contributing to genetic variations in turf quality under drought

or heat stress. The above analyses were performed using the Statistical Analysis System (SAS v 9.2, SAS Institute, Cary, NC).

RESULTS AND DISCUSSION

Correlation Analysis Between Physiological Parameters and Turf Quality

Under Drought or Heat Stress.

Turf quality is a commonly-used indicator of overall turf performance and stress tolerance in turfgrass (Turgeon, 1999; Fry and Huang, 2004). The correlation analysis between TQ and physiological traits for eight cultivars of creeping bentgrass demonstrated that under drought stress, turf quality was most highly correlated with RWC, EL, and CHL (had highest correlation coefficients), which was followed by MDA and Fv/Fm (Table 1). Leaf RWC, CHL, and Fv/Fm were positively correlated with TQ while EL and MDA were negatively correlated with TQ. Backward elimination regression analysis was used to determine what physiological traits were the most responsible for variations in TQ among the eight cultivars under drought stress. The results showed that differences in EL and RWC among cultivars were two major factors associated with variations in TQ under drought stress ($TQ = 4.23 - 4.38 EL + 3.42 RWC$).

Maintenance of leaf hydration status as indicated by high RWC is essential for cellular metabolic functions and leaf turgidity (Beard, 1989; Carrow 1996, Qian et al., 1997). Conversely, drought-induced dehydration can lead to damages of cellular membranes, and therefore, the maintenance of membrane stability during drought stress is critical for cell integrity and organelle compartmentation and functions (Scwhab and Heber, 1984; Bohnert et al., 1995, Zhai et al., 2012). Leaf RWC and EL have been considered important parameters for drought tolerance in various non-turfgrass plant species (Schonfeld et al., 1988; Matin et al., 1989; Premachandra et al., 1990; Blum, 1999; Tripathy et al., 2000; Loutfy et al., 2012). Our results suggested EL and RWC

could be useful physiological traits for screening germplasm of creeping bentgrass for improved drought tolerance.

A typical symptom of heat injury in turfgrass is leaf senescence, which is characterized by loss of chlorophyll and photosynthetic activities (Fry and Huang, 2004). Under heat stress, TQ had the strongest correlations with Fv/Fm, CHL, and EL followed by MDA, while RWC had the lowest correlation with TQ (Table 2). Backward elimination regression analysis demonstrated that leaf Fv/Fm ratio and chlorophyll content were the major contributors to the cultivar variations in TQ under heat stress ($TQ = -3.73 + 11.17 \text{ Fv/Fm} + 0.13 \text{ CHL}$). The photosynthetic machinery or chloroplast containing chlorophyll is one of the most sensitive sites to heat stress damages which involve thylakoid membrane disruption and loss of chlorophyll (Wise et al., 2004; Wang et al., 2010; Gomez Selvaraj et al., 2011). Loss of chlorophyll can lead to the decline in light absorption and photochemical efficiency for photosynthesis (Wahid et al., 2007). Maintaining higher chlorophyll levels or stay-green trait and efficient photosynthesis, therefore, is critically important for heat tolerance, particularly for creeping bentgrasses which are typically mowed at a low height with small amounts of leaf area available for photosynthesis.

Our results suggested that different physiological parameters may be used for the selection of drought tolerance vs. for heat tolerance in creeping bentgrass. Although drought and heat stress both cause declines in turf quality, different physiological traits were involved in genetic variations in drought tolerance vs. heat tolerance. This study found that creeping bentgrass adaptations to drought stress were mainly related to dehydration tolerance by maintaining turgid leaves and cell membrane integrity while

heat tolerance was predominantly due to physiological traits associated with leaf senescence and photosynthesis.

While RWC and EL were mainly correlated with drought tolerance and CHL and Fv/Fm were mainly correlated with heat tolerance in creeping bentgrass, membrane lipid peroxidation was correlated with TQ under both drought and heat stress in creeping bentgrass, although the correlation was not as strong as for the other four physiological parameters. Both drought and heat stress can cause the production of reactive oxygen species, which in turn damage membranes resulting in increases in MDA content. The level of lipid peroxidation has been associated with plant tolerance to various abiotic stresses in non-turfgrass species (Zhang and Kirkham, 1994; Sairam et al., 2000). Lowering membrane lipid peroxidation is considered an efficient defense system protecting membranes from drought and heat stress (Jiang and Huang, 2001; DaCosta and Huang, 2007; Soliman et al., 2011; Chen et al, 2012). Our results suggested that the measurement of MDA content could also be used as a selection parameter for improving plant tolerance to both drought and heat stress.

Cultivar Variation in Drought and Heat Tolerance.

The data for three major or primary traits associated with drought tolerance (TQ, RWC, EL) and three traits associated with heat tolerance (TQ, CHL, Fv/Fm) as discussed above are presented in Fig 1 and 2, respectively, to depict the cultivar variations in drought or heat tolerance. Eight cultivars exhibited significant variations in drought tolerance as indicated by TQ, RWC, and EL (Fig. 1) and significant variations in heat tolerance as illustrated by TQ, CHL, and Fv/Fm (Fig. 2).

Based on the combined results of all three traits (TQ, RWC, and EL), the eight cultivars were divided into three groups by the level of drought tolerance: ‘007’, ‘Pro-as7’ and ‘Declaration’ as most drought tolerant exhibiting the highest values of TQ and RWC and lowest EL; ‘Penncross’, ‘Tyee’, ‘L-93’ and ‘Kingpin’ were of intermediate tolerance and ‘Shark’ was the most sensitive with the lowest TQ and RWC and highest EL (Fig. 1). ‘L-93’ was previously reported to have better drought avoidance and ‘Declaration’ had better drought tolerance compared to ‘Penncross’ (McCann and Huang, 2008). No previous reports were found on drought tolerance in the relative tolerance of the other cultivars.

The three traits (TQ, CHL, and Fv/Fm) associated with heat tolerance showed that among the eight cultivars, most heat tolerant cultivars were ‘Declaration’, ‘L-93’, ‘Tyee’ and ‘007’ with highest TQ, CHL, and Fv/Fm; ‘Shark’ and ‘Pro-as7’ were of intermediate tolerance, while ‘Penncross’ and ‘Kingpin’ were most sensitive to heat stress with lowest values for all three parameters. Previous studies also reported ‘L-93’, ‘Declaration’ and ‘Shark’ was more tolerant to heat than ‘Penncross’ and ‘Kingpin’ (Liu and Huang, 2000; Xu and Huang, 2000; Xu et al., 2007). Information was not previously available on the relative heat tolerance for the other cultivars examined in this study.

In summary, our results demonstrated a range of genetic variations in drought or heat tolerance in creeping bentgrass, with some newer cultivars demonstrating improved drought and heat tolerance. The new cultivars, ‘007’ and ‘Declaration’, had superior tolerance to both drought and heat stress relative to the older cultivars, such as ‘Penncross’. Drought tolerance in creeping bentgrass was mainly associated with improved RWC and lower EL while heat tolerance was due to the improvement in CHL

and F_v/F_m . Incorporating physiological traits such those identified in this study in breeding programs may further improve stress tolerance in creeping bentgrass and other turfgrass species.

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Table 1. Correlation matrix for physiological parameters of eight creeping bentgrass cultivars ('Declaration', 'Kingpin', 'L93', 'Penncross', 'PRO-as7', 'Shark', 'Tyee', and '007') at 0, 7, 14, and 21 d of drought stress.

	TQ	Fv/Fm	EL	RWC	CHL	MDA
TQ	1.000					
Fv/Fm	0.749***	1.000				
EL	-0.897***	-0.772***	1.000			
RWC	0.905***	0.816***	-0.933***	1.000		
CHL	0.850***	0.655***	-0.800***	0.821***	1.000	
MDA	-0.848***	0.729***	0.867***	-0.901***	-0.802***	1.000

*** Correlation coefficients significant at $P < 0.001$

Abbreviations: TQ, turf quality; Fv/Fm, photochemical efficiency; EL, electrolyte leakage; RWC, relative water content; CHL, chlorophyll content; MDA, malondialdehyde content.

Table 2. Correlation matrix for physiological parameters of eight creeping bentgrass cultivars ('Declaration', 'Kingpin', 'L93', 'Penncross', 'PRO-as7', 'Shark', 'Tyee', and '007') at 0, 7, 14, 21, 28 and 35 d of heat stress.

	TQ	Fv/Fm	EL	RWC	CHL	MDA
TQ	1.000					
Fv/Fm	0.853***	1.000				
EL	-0.819***	-0.851***	1.000			
RWC	0.543***	0.570***	-0.622***	1.000		
CHL	0.850	0.824***	-0.888***	0.534***	1.000	
MDA	-0.763***	-0.644***	0.734***	-0.412***	-0.778***	1.000

*** Correlation coefficients significant at $P < 0.001$

Abbreviations: TQ, turf quality; Fv/Fm, photochemical efficiency; EL, electrolyte leakage; RWC, relative water content; CHL, chlorophyll content; MDA, malondialdehyde content.

Fig 1. Cultivar variations in turf quality (TQ), relative water content (RWC), and electrolyte leakage (EL) at 21 d of drought stress. Cultivars within a circle were not significantly different in one or more parameters based on LSD test at $p = 0.05$. Cultivars in different circles exhibited significant differences in one or more parameters based on LSD test at $p = 0.05$.

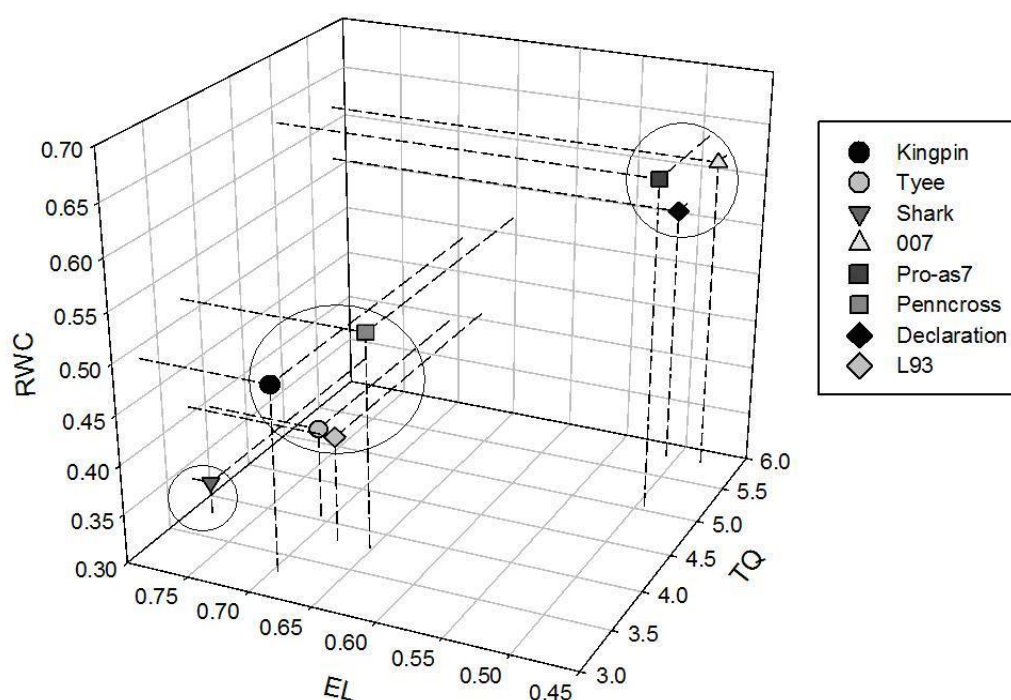
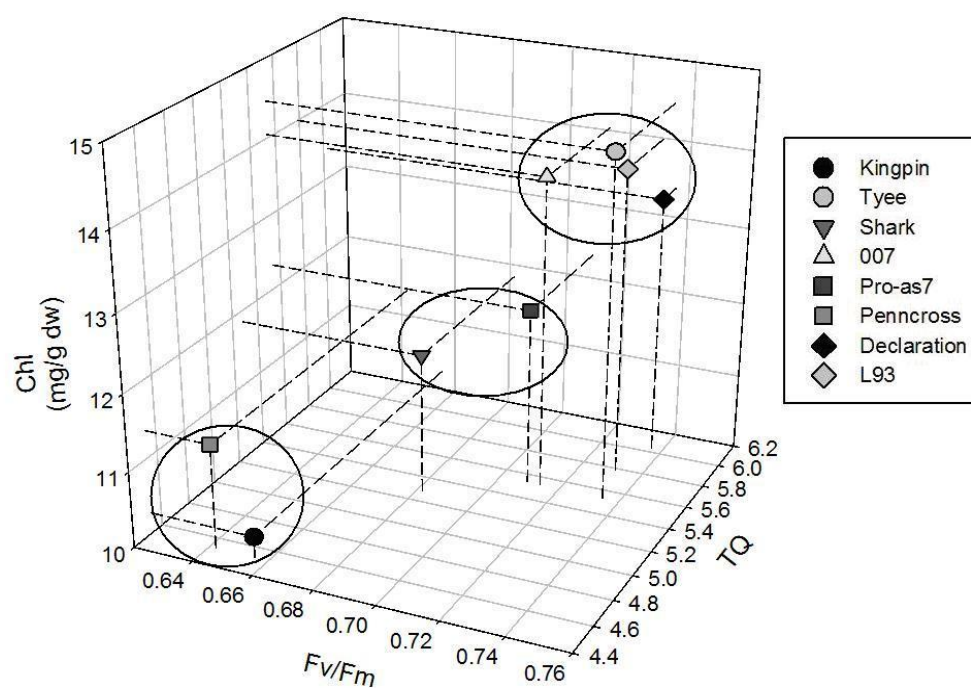


Fig 2. Cultivar variations in turf quality (TQ), leaf chlorophyll content (CHL), and photochemical efficiency (Fv/Fm) at 35 d of heat stress. Cultivars within a circle were not significantly different in one or more parameters based on LSD test at $p = 0.05$. Cultivars in different circles exhibited significant differences in one or more parameters based on LSD test at $p = 0.05$.



**CHAPTER 2: Membrane Proteins Associated with Heat-Induced Leaf Senescence
in a Cool-Season Grass Species**

Jespersen, D., C. Xu, B. Huang. 2015 Crop Sci. 55:837-850

INTRODUCTION

Heat stress is a major abiotic stress affecting many aspects of plant growth and productivity, particularly in cool-season plant species (Wahid et al., 2007). Prolonged durations of high-temperature stress typically induce leaf senescence, characterized by degradation or biosynthetic inhibition of cellular macromolecules such as chlorophyll and proteins (Ferguson et al., 1994; Jochum et al., 2007; Lim et al., 2007). Downstream effects of heat stress involve photosynthetic inhibition caused by degradation of photosynthetic machinery (Salvucci and Crafts-Brandner, 2004; Sato et al., 2000) and increased oxidative damage due to reactive oxygen species (ROS) production (Miller et al., 2008; Sairam et al., 2000). Delaying the onset or suppressing the extent of leaf senescence is important for improving whole-plant tolerance to heat stress. However, specific mechanisms regulating heat-induced leaf senescence are not well understood and need to be described further.

Many plant processes are altered during stress-induced leaf senescence and changes to membrane-based functions such as signal transduction, transport, and photosynthesis may constitute the initial response of a plant cell to deleterious environmental stresses (Marcum, 1998; Saidi et al., 2009; Vigh et al., 2007). Cellular membranes contain integral proteins which serve numerous functions including metabolite or solute transport, signaling, light harvesting, electron transfer, ATP generation, as well as stress response and defense (Taiz and Zeiger, 2010). Heat-induced leaf senescence may be associated with denaturation of these integral membrane proteins which hinders efficiency of various cellular functions associated with specific proteins (Blum et al., 2001; Xu et al., 2006; Yamane et al., 1998). Faster rates of soluble-protein

degradation linked to leaf senescence during heat stress have been reported in various plants species, including *Triticum aestivum* and *Nicotiana tabacum* (Al-Khatib and Paulsen, 1984; Ueda et al., 2000; Zavaleta-Mancera et al., 1999). However, most of the previous research focused on highly-abundant soluble proteins involved in photosynthetic biochemical reactions (i.e., carboxylase enzymes), respiratory reactions (i.e., dehydrogenase enzymes), and amino acid synthesis (Ferreira et al., 2006; Lee et al., 2007; Xi et al., 2006; Xu and Huang, 2010; Xu et al., 2010). Genomic studies estimate that 20-30% of genes encoding for proteins are membrane proteins, with cellular concentrations being much less abundant compared to soluble proteins (Wallin and Von Heijne, 1998; Ward, 2001). Changes to specific membrane proteins associated with leaf senescence or chlorophyll maintenance in leaves of cool-season plant species during prolonged heat stress are far less known. Knowledge of which membrane proteins are involved in delaying leaf senescence will aid in the elucidation of important mechanisms of the stay-green trait and provide information needed to help develop plants with lessened stress-induced senescence.

Bentgrasses (*Agrostis* spp.) are widely-utilized cool-season perennial turfgrass and forage species which consistently succumb to heat-induced damages typically expressed as leaf senescence, further leading to a decline in overall turf quality and functionality during prolonged periods of heat stress (Fry and Huang, 2004). Onset of heat-induced leaf senescence is dependent upon degree of heat stress, which occurred after 20 d at 30 °C and 8 d at 35 °C for *A. stolonifera* (cv. Penncross) (Huang and Gao, 2000; Huang et al., 1998). Improving the stay-green trait or developing germplasm with

lesser leaf senescence is critically important for maintaining high-quality turfgrass during summer months.

The objective of this study was to examine changes to membrane-bound proteins governing heat-induced leaf senescence in response to prolonged heat stress for two bentgrass genotypes contrasting in overall heat tolerance and to identify membrane-bound proteins and gene transcripts associated with the lesser extent of leaf senescence during heat stress. Preliminary studies showed that two genotypes ('COLXCB169' and 'COLXCB190') generated from a creeping bentgrass (*A. stolonifera*) x colonial bentgrass (*A. capilaris*) hybrid backcross population exhibited differential visual responses to heat stress with leaves of 'COLXCB169' staying greener for a longer duration of heat stress (Jespersen and Huang, unpublished data). To accomplish the objectives of this study three commonly-used physiological parameters for leaf senescence (turf quality, leaf chlorophyll content, and leaf membrane stability) were analyzed to assess heat-induced leaf senescence. Membrane proteins associated with heat-induced leaf senescence were separated and identified using two-dimensional electrophoresis (2-DE) and mass spectrophotometry. Additionally, transcript levels of genes encoding identified membrane proteins were examined using quantitative real-time PCR (q-PCR) to further confirm the role of these genes in leaf-senescence.

MATERIALS AND METHODS

Plant materials and Growth Conditions

Clonal plants of two genotypes ('ColxCB169' and 'ColxCB190') generated from a colonial bentgrass (*Agrostis capillaris*) x creeping bentgrass (*Agrostis stolonifera*) hybrid backcross population at Rutgers University were examined. Plants were selected based on preliminary research which showed distinct differences in leaf senescence rates between the two genotypes during prolonged heat stress (Jespersen and Huang, unpublished data). Plants were established in pots (15 cm in diameter and 20 cm deep) filled with a mixture of 30% sand and 70% peat moss in a greenhouse for 6 weeks. Plants were irrigated three times per week and fertilized once per week with half-strength Hoagland's nutrient solution (Hoagland and Arnon, 1950). Plants were transferred to controlled-climate growth chambers (Conviron, Winnipeg, Canada) set to 20/15 °C (day/night), 14 h photoperiod, and photosynthetically active radiation (PAR) at 610 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for 7 d for acclimation prior to imposition of heat stress treatment.

Treatments and Experimental Design

Plants were subjected to either heat stress (38/33 °C day/night) or non-stress control (20/15 °C day/night) conditions in growth chambers for 28 d. Other environmental conditions in growth chambers were unchanged at 60% relative humidity, 14 h photoperiod, and PAR of 610 $\mu\text{mol m}^{-2} \text{s}^{-1}$. Plants were irrigated twice per day and fertilized weekly with half-strength Hoagland's nutrient solution during the 28 d treatment period.

Plants were arranged in a split-plot design with temperature treatment as the main plot and genotype as sub-plots. Each temperature treatment was replicated in four growth

chambers. Each genotype was replicated in four pots with multiple plants in each pot which were exposed to either the control temperature or heat stress repeated in four growth chambers.

Physiological Measurements

Susceptibility to heat stress or degree of leaf senescence due to heat stress was evaluated by three common physiological indicators. Turf quality (TQ), leaf membrane stability via electrolyte leakage, and chlorophyll content measurements which were conducted every 7 d during the 28 d heat stress treatment period. Turf quality is a visual rating of turfgrass color, density, and uniformity used for evaluating overall turfgrass performance on a 1 to 9 scale with 9 being healthy grass and 1 being brown dead grass (Beard, 1973).

Leaf membrane stability was estimated by measuring cellular electrolyte leakage every 7 d during the 28 d heat stress treatment period. Approximately 0.1 g of leaf tissue was collected and placed in a test tube containing 35 ml deionized water. The tubes were agitated on a conical flask shaker for 16 h and an initial conductance (C_i) reading was taken using a conductivity meter (YSI Incorporated, Yellow Springs, OH). Leaf tissue was then killed by autoclaving at 121 °C for 20 min. After cooling, test tubes were agitated for an additional 16 h and then a final conductance (C_{max}) recorded. Electrolyte leakage was calculated using the formula $(\%) = (C_i/C_{max}) \times 100$ (Blum and Ebercon, 1981).

Leaf chlorophyll content was measured every 7 d during the 28 d heat stress treatment period using the method described by Hiscox and Israelstam (1979).

Approximately 0.1 g leaf tissue was collected and total chlorophyll extracted in 10 ml

dimethylsulfoxide in complete darkness for 72 h. The absorbance of extraction solution was measured at 663 nm and 645 nm (Spectronic Instruments, Inc., Rochester, NY). Leaf tissue was then dried in an oven for 72 h and chlorophyll content calculated according to the equations described by Arnon (1949).

Protein Extraction and Separation

The second fully-expanded leaves were collected from plants in four replicates per treatment and per genotype at 0, 14 and 28 d of heat stress treatment, immediately frozen in liquid nitrogen, and stored at -80 °C for protein analysis. Protein extraction was based off the differential protein solubilization protocol described by Molloy et al. (1998) with modifications. About 0.5 g of tissue was ground in liquid nitrogen and further homogenized with 2 ml of 40 mM Tris-base (pH 7.6) and 0.15 M NaCl extraction buffer on ice. The homogenate was centrifuged at 10,000 g for 15 min at 4 °C and the supernatant containing soluble proteins was removed. The remaining pellet was washed three times with 40 mM Tris-base (pH 7.6) and 0.15M NaCl extraction buffer. Two ml of extraction buffer containing 40 mM Tris-base, 7 M urea, 2 M thiourea, 2% 3-[(3-Cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) and 1% 2-mercaptoethanol was added to the pellet and sonicated for 30 minutes to homogenize the pellet. The resulting homogenate were centrifuged at 10,000 g for 15 min at 4 °C. The supernatant containing membrane and hydrophobic proteins was then precipitated at -20 °C in 8 ml acetone with 0.07% 2- mercaptoethanol for 12 h and then centrifuged for 15 min at 8500 g at 4 °C. The resulting pellet was then re-suspended in 8 ml ice-cold acetone containing 0.07% 2- mercaptoethanol and stored at -20 °C for 2 h. Samples were then washed with 0.07% 2- mercaptoethanol acetone solution and vacuum dried for 20

minutes. Dried pellets were then re-suspended in a resolubilization solution containing 8 M urea, 2 M thiourea, 2% CHAPS, 1% dithiothreitol (DTT) and 1% 3/10 biolytes.

Aliquots were then used to determine protein concentration according to Bradford (1976) using a commercial dye reagent (Bio-Rad Laboratories, Hercules, CA) with a bovine serum albumin standard.

Protein separation was performed according to Xu et al. (2008) with modifications. Immobilized pH gradient strips (pH 3-10, linear gradient, 13 cm) were used in an IPGPhor apparatus (GE Healthcare, Piscataway, NJ). Strips were rehydrated in 250 μ L of rehydration buffer containing 8 M urea, 2 M thiourea, 2% w/v CHAPS, 1% v/v IPG buffer, 1% DTT, and 0.002% bromophenol blue containing 250 μ g of extracted proteins. Voltage settings for first-dimension isoelectric focusing were 50 V for 14 h, 500V for 1 h, 1000 V for 1 h, 5000 V for 1 h and 8000 V to a total of 80kVh.

Following isoelectric focusing, strips were denatured in a buffer containing 6 M urea, 30% glycerol, 2% sodium dodecylsulfate, 0.002% bromophenol blue, 50 mM Tris-Base (pH 8.7), and 1% DTT. Strips were then incubated in a similar buffer in which DTT was replaced with 2.5% iodoacetamide. The second dimension gel electrophoresis was performed using a Hoefer SE 600 Ruby electrophoresis unit (GE Healthcare, Piscataway, NJ) and a 12.5% SDS-polyacrylamide gel. Gels were stained with a colloidal Coomassie blue stain (Neuhoff et al., 1988), scanned with a Personal Densitometer (GE Healthcare, Waukesha, WI, USA) and analyzed using SameSpots software (Nonlinepear, Newcastle upon Tyne, UK). Spot volumes were normalized as a percentage of total spot volume to correct for potential variation due to staining. Normalized spot volumes for heat stress gels were compared against controls gels to calculate changes in abundance due to

treatment effects, and an ANOVA was used to determine spots which had significantly altered accumulations. Protein spots with corresponding p-values of 0.05 or less were selected for further analysis and protein identification using mass spectrometry.

Selected spots were excised from the gel and a Trypsin digestion and Ziptip desalting were used prior to mass spectrometry analysis. Peptides were analyzed with MALDI-TOF-MS using a 4800 MALDI TOF/TOF analyzer (Applied Biosystem, Framingham, MA, USA) as described by Xu et al. (2008). MS and MS/MS spectra were compared to the NCBI green plant database using a local MASCOT search engine (V1.9, Matrix Science, Boston, MA, USA) on a GPS server (V. 3.5, Applied Biosystems, Framingham, MA, USA). Proteins with a confidence interval value >95% for at least two peptides were considered to be identified. The obtained sequence was also manually assigned to perform another search in the Swiss-Prot and TrEMBL databases (Universal Protein Resource, UniProt Consortium, 2011) using the FASTA format.

Quantitative Real-time PCR (qPCR) Analysis of Gene Transcript Levels

RNA was extracted from second fully-expanded leaves from plants in four replicates per treatment and per genotype at 14 d of heat stress using Trizol reagent (Life Technologies, Carlsbad, CA, USA), treated with a Turbo DNase kit (Life Technologies, Carlsbad, CA, USA), and then RNA clean up was performed using a RNeasy kit (Qiaagen, Venlo, Netherlands). The resulting RNA was converted to cDNA using a high capacity cDNA synthesis kit (Life Technologies, Carlsbad, CA, USA). Following cDNA synthesis, qPCR primers were designed using PrimerExpress software (Life Technologies, Carlsbad, CA, USA) with sequences based off of available EST sequences on the NCBI database

(Table 1). Actin was used as the endogenous control and relative gene expression was determined using the delta-delta-CT method (Schmittgen and Livak, 2008) using SYBR green dye with a StepOnePlus real-time PCR machine (Life Technologies, Carlsbad, CA, USA).

Statistical Analysis

Treatment effects for physiological parameters, relative gene expression levels, and normalized protein spot abundance and relative accumulation were determined using ANOVA according to the general linear model procedure of SAS (version 9.2; SAS Institute, Cary, NC). Differences between means were separated by Fisher's protected least significance difference test at the 0.05 probability level.

RESULTS AND DISCUSSION

Genotypic Variations in Heat-Induced Leaf Senescence

One of the major characteristics of leaf senescence is loss of chlorophyll, as discussed in the introduction. Chlorophyll content decreased rapidly during heat stress from 7 d onward in ‘COLXCB190’, whereas it did not change significantly during heat stress in ‘COLXCB169’ until 28 d of heat stress (Fig. 1A). Furthermore, chlorophyll content of two hybrid genotypes did not differ significantly prior to heat stress but by 14d of stress ‘COLXCB190’ had significantly lower chlorophyll content than ‘COLXCB169’ and at 28 d of heat stress, chlorophyll content of ‘COLXCB169’ was 156% greater than that of ‘COLXCB190’ (Fig. 1A). These data demonstrated that ‘COLXCB169’ exhibited both delayed and lesser degree of degradation of chlorophyll under heat stress or greater stay-green ability compared to ‘COLXCB190’. Maintaining chlorophyll levels in leaves is critically important for cool-season grass survival during heat stress, but chlorophyll degradation is among the earliest symptoms of heat-stress in cool-season grass species (Veerasamy et al., 2007; Xu and Huang, 2007). The decreases in chlorophyll content may be due to inhibition of chlorophyll synthesis and increases in catabolism during leaf senescence (Tewari and Tripathy, 1998; Wahid et al., 2007). In spinach leaves (*Spinacia oleracea*) the degradation of chlorophyll has been associated with damages in thylakoid membranes of chloroplasts which are extremely sensitive to heat stress (Bukov et al., 1999). Whether the greater stay-green ability of ‘COLXCB169’ was due to maintenance of chlorophyll synthesis or slower chlorophyll degradation deserves further investigation.

Leaf EL, as an indicator of cell membrane stability, increased in both genotypes throughout the stress treatment, indicating heat induced cellular membrane damages.

However, EL was significantly lower in ‘COLXCB169’ than that in ‘COLXCB190’ during the entire heat stress period (28 d) (Fig.1B). These results indicated that ‘COLXCB169’ experienced a lesser degree of leaf senescence by maintaining greater membrane stability under heat stress, compared to ‘COLXCB190’. Loss of cellular membrane stability is another common attribute of heat-induced leaf senescence, and has been observed in a wide range of plant species including *Solanum lycopersicum*, *Poa pratensis*, and *Triticum aestivum* (Camejo et al., 2005; Liu and Huang, 2000; Marcum, 1998; Shanahan et al., 1990; Wahid et al., 2007).

Turf quality rating is a commonly-used parameter for evaluating overall turf performance (Turgeon, 1999). TQ declined during 28 d of heat stress for both genotypes, but decreased at a much slower rate in ‘COLXCB169’ than ‘COLXCB190’ (Fig. 1C). Similar to chlorophyll content, ‘COLXCB169’ was only significantly lower than control at 28d of heat stress. The initial TQ prior to heat stress was 8.5 and 7.5 for ‘COLXCB169’ and ‘COLXCB190’ respectively. TQ at 28 d of heat stress declined to 6.5 in ‘COLXCB169’, whereas the TQ of ‘COLXCB190’ decreased drastically to a rating of 2.2, with a majority of leaves becoming brown and senescent (Fig. 1C). The data for TQ agreed with the results for leaf chlorophyll content and membrane stability, as discussed above. Leaf chlorophyll content and membrane stability are correlated to TQ or whole-plant heat tolerance, which are considered major traits for selecting heat-tolerant germplasm of turfgrass species (Jespersen et al., 2013).

All three parameters demonstrated the differential levels of heat-induced leaf senescence in the two genotypes, with ‘COLXCB169’ exhibiting a lesser degree of leaf senescence or staying green for a longer duration of heat stress than ‘COLXCB190’,

suggesting genotypic variation in the level of heat-induced leaf senescence in bentgrass. Genetic variability in the level of heat-induced leaf senescence, as manifested by differences in turf quality, chlorophyll content, and membrane stability, have been previously reported in other genotypes and species of turfgrasses (Lyons et al., 2006; Xu and Huang, 2007).

Differential Accumulation of Membrane Proteins in Response to Heat Stress

The 2-DE technique allows for the separation and quantification of a vast array of proteins expressed in an organism, and the identification of specific proteins responsive to an external stimuli, such as abiotic stresses (Kottapalli et al., 2009; Yan et al., 2006). Proteomic analysis with 2-DE detected 56 heat-responsive soluble proteins in creeping bentgrass (Xu and Huang, 2010). In this study, a total of 19 differentially-accumulated membrane proteins were found between heat-stressed and control plants for both genotypes, out of which 18 were successfully identified to a single protein identity using mass spectrometry (Table 2). An example of a representative gel with labeled spots which correspond to differentially-accumulated proteins is depicted in Figure 2.

Differentially-expressed membrane proteins in the two genotypes in response to heat stress were grouped into functional categories according to Bevan et al. (1998). Out of the identified proteins, the majority (69%) were grouped in the energy category, 16% in stress defense, 5% in metabolism, and 5% belonging to the signaling category, with the remaining protein spot being placed in unknown functions (Fig. 3). Among 19 membrane proteins, 17 exhibited decline in their abundance (down-regulated) and 2 proteins showed increases in their abundance (up-regulated) during heat stress in both genotypes (Fig. 4, Fig. 5a,b). These down-regulated proteins were mainly involved in the

functions of energy metabolism, stress defense, and signaling and the up-regulated proteins were involved in energy metabolism and stress defense. A number of spots were found to have the same protein identity in this study which is a common phenomenon in 2-DE proteomics, which may be due to a number of factors, including multiple gene copies in the genome producing several isoforms or post-translational modification of proteins (Giavalisco et al., 2005; Sarnighausen et al., 2004; Xu et al., 2009). These changes in protein accumulation are in alignment with physiological and metabolic changes which occur during stress-induced senescence, including decreases in photosynthesis, an imbalance in energy relations, increased oxidative damage, and altered signaling (Baniwal et al., 2004; Hays et al., 2007; Larkindale and Huang 2004). Previous studies which investigated the changes in membrane proteins in response to cold or salt stress also found changes in proteins related to signaling, energy, and stress defense (Kawamura and Uemura, 2003; Lee et al., 2004), although membrane protein changes under heat stress were not previously reported.

Membrane Proteins Involved in Energy Metabolism

Several membrane proteins involved in energy metabolism exhibited differential levels between ‘COLXCB169’ and ‘COLXCB190’ under heat stress. Those included ATP synthases (ATPase), chloroplast oxygen-evolving enhancer protein, and cytochrome b6f complex. These three membrane proteins play critical roles in ATP production in photosynthesis and respiration processes (Hopkins, 1999).

ATPase are key membrane-bound enzyme complexes for ATP generation, responsible for converting ADP to ATP by using trans-membrane proton gradients in the electron transport process in both photosynthesis and respiration (Boyer, 1997). The

ATPase complex consists of alpha-subunits and beta-subunits forming the catalytic core of the enzyme complex with the beta subunits involved in catalytic activities and the alpha subunits being regulatory (Walker et al., 1982). The abundance of ATPase CF1 alpha-subunits decreased in both genotypes, but the decline in its abundance occurred later (not until 28 d of heat stress) and to a lesser extent in ‘COLXCB169’ than ‘COLXCB190’ for several proteins spots (Fig. 5). The decline in the abundance of all alpha-subunits of ATPase may indicate the impairment of regulatory functions of this enzyme for ATP production under heat stress. The maintenance of greater abundance of alpha-subunits of ATPase in ‘COLXCB169’ could contribute to more active regulatory activity for ATP production under heat stress. Unlike the alpha-subunits of ATPase, the abundance of the beta-subunits of ATPase increased in ‘COLXCB169’ while it did not change in ‘COLXCB190’ in response to heat stress (Fig. 5). As the beta-subunit is the key element for catalytic functions of ATPase, the greater accumulation of beta-subunits in ‘COLXCB169’ could facilitate the maintenance of catalytic activities of ATPase for ATP production under heat stress. Both ‘COLXCB169’ and ‘COLXCB190’ experienced a decline in the transcript level for ATP synthase, as quantified with q-PCR. ‘COLXCB190’ experienced the decline to a significantly greater degree with a 3.4-fold decrease compared to a 2.1-fold decrease in ‘COLXCB169’ (Fig. 6). Several other studies in *Populus euphratica*, *Triticum aestivum*, or *Oryza sativa* found that ATP synthase was impaired by heat stress (Ferreira et al., 2006; Lee et al., 2007; Majoul et al., 2004). The interruption of ATPase function for ATP production is a major culprit of heat stress damages in plants as many processes, including stress defense and repair mechanisms depend on energy availability for plant survival of long-term heat stress

(Crafts-Brandner and Salvucci, 2000; Suss and Yordanov 1985). Impaired ATP generation is also believed to lead to the production of reactive oxygen species (ROS) caused by electron transport mechanisms having excess energy (Tiwari et al., 2002). Both proteomic and transcript results suggested that ATP synthase was maintained at a greater transcript level in 'COLXCB169', potentially resulting in the generation of more ATP to use for stress defense and repair mechanisms, leading to lesser leaf senescence.

The abundance of chloroplast oxygen evolving enhancer protein decreased during heat stress in both genotypes. The decline in the abundance of chloroplast oxygen evolving enhancer protein was delayed and less pronounced in 'COLXCB169' (2.2-fold reduction) than 'COLXCB190' (2.6-fold reduction) by 28 d of heat stress (Fig. 5). Both genotypes exhibited declines in the transcript level for the oxygen evolving enhancer protein, with a 2.8-fold and 2.3-fold reduction in 'COLXCB169' and 'COLXCB190', respectively (Fig. 6). However, the difference in the transcript levels was not statistically significant between the two genotypes. Many studies which look at both gene transcription and protein accumulation find results where transcription does not directly relate to protein levels due to a number of complex factors (Heazlewood and Millar, 2003; Hedge et al., 2003). The chloroplast oxygen-evolving enhancer protein is an important protein in the thylakoid membranes of photosystem II complex, which plays a role in oxidizing water generating electrons needed for the electron transport chain of photosynthesis and hydrogen ions needed for ATP synthesis (Hankamer and Barber, 1997). Heat-induced reductions in protein abundance for chloroplast oxygen evolving enhancer have been reported in other plant species (Bukhov et al., 1999; Takeuchi and Thornber, 1994; Toth et al., 2005). This is believed to be due to a dissociation from the

photosystem complex in the thylakoid membrane under high temperatures (Yamane et al., 1998). The decreases in both protein abundance and gene transcript level of the chloroplast oxygen-evolving enhancer under heat stress suggested that heat stress caused damages in light reactions of photosynthesis at both protein and gene levels, but to a lesser degree at the protein level in ‘COLXCB169’.

The decline in the abundance of Cytochrome b6f complex was detected at 14 d of heat stress in ‘COLXCB190’, but was not apparent in ‘COLXCB169’ until 28 d of heat stress (Fig. 5). This could represent an earlier or more severe decline in photosynthetic machinery associated with heat-induced senescence. The q-PCR analysis found that the transcript level of cytochrome b6 decreased by 1.7 fold after 14 d of heat stress in ‘COLXCB190’ but remained unchanged in ‘COLXCB169’(Fig. 6). Cytochrome b6f complex is a protein complex in thylakoid membranes involved in electron transport during the light-dependent reactions of photosynthesis and related protein complexes are also found in the mitochondria for generating energy (Cape et al., 2006). The abundance of this protein decreased significantly with heat stress in both genotypes, suggesting that heat stress could interrupt the function of cytochrome b6f complex in the electron transport process for ATP production in photosynthesis and respiration. Loss of cytochromes is believed to represent one of the early stages of leaf senescence (Schottler et al., 2006). In addition, as previously mentioned, disruptions to electron transport can lead to an excess of energy and ultimately the formation of ROS. The consistent trend of proteomic and transcript changes suggested that the decline of cytochrome b6f was delayed in the stay-green ‘COLXCB169’, which indicated less damage to thylakoid membranes and a better maintenance of photosynthetic processes and energy generation.

Pyruvate dehydrogenase kinase regulates metabolic processes by inactivating pyruvate dehydrogenase, a key protein involved in the citric acid cycle and energy production in the mitochondria (Lee et al., 2007; Popov et al., 1993). Pyruvate dehydrogenase kinase was found to decrease both sooner and to a greater extent in 'COLXCB190', implicating greater perturbations to energy production. By 28d of heat stress 'COLXCB190' had a 5-fold decrease in pyruvate dehydrogenase kinase compared to a 1.67 fold decrease in 'COLXCB169' (Fig. 5). Gene expression levels as determined by q-PCR showed slight decreases in the expression levels in 'COLXCB169' but a significantly greater decrease in 'COLXCB190' (Fig. 6). The transcript results have a similar trend to the protein results with a greater decline being seen in 'COLXCB190', indicating that both transcription and protein synthesis and degradation play a role in the level of accumulation of this protein. Pyruvate dehydrogenase has been found to both increase or decrease in response to abiotic stress, depending on plant species and kind of stress (Lee et al., 2007; Sweetlove et al., 2002). Pyruvate dehydrogenase kinase has been found to increase in response to a number of abiotic stresses in *Oryza sativa* (Rabbani et al., 2003), indicating altered metabolism involved in glycolysis and energy production during stress events. The greater decline in both transcript levels and protein accumulations of pyruvate dehydrogenase kinase in 'COLXCB190' indicated increased perturbations to energy generation via respiration resulting from heat-induced leaf senescence.

Membrane Proteins Involved in Stress Defense

Efficient antioxidant metabolism, involving antioxidant enzymes such as catalase and peroxidase, is important to scavenge ROS to reduce membrane lipid peroxidation and

other cellular damages under environmental stresses, including heat stress (Mittler, 2002). Membrane-bound catalases can be found in peroxisomes, which are responsible for the conversion of hydrogen peroxide to water in antioxidant metabolism (Sheptovitsky and Bruvig, 1996). Peroxidases (POD) are heme-containing glycoproteins in multiple isoenzyme forms in plants and involved in various physiological processes, including ROS scavenging, which detoxify hydrogen peroxide or lipid peroxides, suppressing lipid peroxidation of membranes (Hiraga et al., 2001). They can be found in plasma membranes and glyoxysomes membranes in plants (Bunkelmann and Trelease, 1996; Onsa et al., 2003). Both catalase and peroxidase exhibited significant decreases in their abundance in ‘COLXCB190’, while there was no significant decrease found in ‘COLXCB169’ (Fig. 5). Heat stress also causes reduction in the catalytic activity of these antioxidant enzymes, leading to oxidative damages, particularly in heat-sensitive species and cultivars (Dhinsda et al., 1981; Xu and Huang, 2004). The maintenance or increase in antioxidant proteins and their enzymatic activity have been widely recognized as important stress defense mechanisms in various plant species (Almeselmani et al., 2006; Chaitanya et al., 2002; Sairam et al., 2000). The sustained abundance of CAT and POD proteins seen in ‘COLXCB169’ could contribute to less heat-induced membrane lipid peroxidation. Using q-PCR it was found that both genotypes maintained similar levels of catalase expression after 14 d of heat stress (Fig. 6). This may reveal that changes in the level of catalase are not due to changes in transcription but perhaps the degradation of the protein. However, the expression levels of the antioxidant peroxidase remained similar to the control after 14 d of heat stress in ‘COLXCB169’ but experienced a significant decrease (2.11 fold) in ‘COLXCB190’(Fig. 6). This is in line with the proteomics results,

possibly indicating that ‘COLXCB169’ actively maintained certain antioxidant enzymes during heat stress which reduces the damages caused by ROS, and that antioxidant metabolisms may at least be partially under transcriptional regulation.

Heat shock proteins (HSPs) are a family of diverse proteins that are expressed during high temperature and other abiotic stress and often have chaperone functions influencing protein folding (Vierling, 1991). Certain members of the small heat shock protein family have been found to be localized in endomembranes (Helm et al., 1995). The abundance of the membrane-bound small HSP (HSP20) in our study increased with heat stress in ‘COLXCB190’, but did not change in ‘COLXCB169’ (Fig. 5). Some HSPs, particularly small HSPs (15-40 kD), are sensitive to heat stress and some are heat inducible (Rizhsky et al., 2002; Tian et al., 2009). Small HSPs may act as chaperones binding to denatured proteins to aid in their refolding after stress damages. The induction of HSP20 in ‘COLXCB190’ may reflect greater sensitivity of plants to heat stress, with chaperones being activated to repaired damaged proteins while the unchanged HSP20 expression in ‘COLXCB169’ indicated that such a protection mechanism was not induced by heat stress in leaves with a lesser degree of senescence. The transcript level of HSP20 did not differ significantly between the two genotypes (Fig. 6), indicating HSP20 may not be involved in the differential leaf responses to heat stress in the two genotypes at the transcript level.

Membrane Proteins Involved in Hormone Signaling

The induction of stress tolerance involves many different signal molecules, including signals from various plant hormones. Auxin is one of the major hormones regulating plant growth and development, as well as stress responses through signaling

pathways (Peleg and Blumwald, 2011). Auxin binding proteins (ABP) specifically interact with auxins (Ohmiya, 2002), which may act in defense signaling pathways regulating plant responses to heat stress (Carter and Thomburg, 1999; Qin et al., 2008). The abundance of the membrane-bound ABP decreased markedly in response to heat stress for both genotypes at 14 d heat stress; by 28 d of heat stress ABP returned to control levels in ‘COLXCB169’ but remained lower in ‘COLXCB190’ (Fig. 5). The transcript level of membrane-bound ABP gene exhibited decreases in ‘COLXCB169’ and increase in ‘COLXCB190’ (Fig. 6). The decrease in ABP abundance could lead to the decrease in the binding activity of the ABP with increasing temperatures, as reported by Löbner and Klämbt (1985). The results suggested that ‘COLXCB169’ could be better able in maintaining ABP binding activity in regulating heat responses, compared to ‘COLXCB190’, despite the lower level of gene expression.

Membrane Proteins Involved in Metabolism

Glycolate oxidase, a protein involved in photorespiration, exhibited differential changes at the protein level between the two genotypes in response to heat stress. At 28 d of heat stress, protein abundance of glycolate oxidase decreased in ‘COLXCB190’ whereas it did not change in ‘COLXCB169’ (Fig. 5), and no significant changes were detected at the transcript level for either genotype under stress (Fig. 6). During photorespiration glycolate is transported from the chloroplast to the peroxisome where glycolate oxidase converts it to glyoxylate and hydrogen peroxide (Apel and Hirt, 2004). This hydrogen peroxide is typically converted to water via catalase; however, if the balance between hydrogen peroxide production and conversion is disturbed this can possibly lead to an accumulation of ROS (Mittler, 2002). Glycolate oxidase is essential

for the flow of metabolites through the photorespiratory pathway and to prevent the build-up of glycolate which may in turn have phytotoxic effects and inhibit photosynthesis (Eisenhut et al., 2008; Gonzalez-Moro et al., 1997). The reduction in protein abundance of glycolate oxidase may lead to phytotoxic accumulations of glycolate, while the maintenance of this protein in ‘COLXCB169’ may prevent oxidative damages.

Conclusions

Heat stress induced changes in 19 membrane proteins mainly involved in energy production, metabolism, and stress defense in two *Agrostis* genotypes differing in the level and rate of leaf senescence. Membrane proteins responsible for maintained ability to produce energy and metabolism, the maintenance of efficient photorespiratory pathways, and antioxidant metabolism could serve important roles in regulating leaf senescence and whole-plant responses to heat stress in cool-season grass species. Future work includes confirming the importance of key membrane proteins and their associated metabolic processes through at the examination of enzymatic activity in leaves exhibiting a differential degree of leaf senescence under heat stress. Such information will provide further insights into metabolic factors regulating leaf responses to heat stress and potentially whole-plant tolerance to heat stress in cool-season grass species.

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Table 1. Primers for real-time PCR analysis of gene expression

Gene of interest	accession number	forward sequence	reverse sequence
ABP20 (auxin binding protein)	DV856487	CGGTGAAGAAGCTC AAGTCC	ATTAACCCGACCACG ACCTT
Actin	DY543529	CCTTTTCCAGCCATC TTTCA	GAGGTCCTTCCTGATA TCCA
ATP synthase CF1 alpha subunit	JU112622.1	ACACTAATGGCAAC CCTTCG	CACTTGAACCTACGCG ACCAA
catalase	DV868119	AAGGAGAACAACCTT CAAGCAG	GAATCGCTCTTGCCTT GCC
cytochrome b6f	DV864368.1	GCGGACCTGCACCT CTGT	ACAAAGACGACCTTG CCATCA
glycolate oxidase	DV854183	CCGCACACTGAGCT GGAA	GATCGGCATAGTGGT GATGC
HSP20	DV866831	CCTTTGGGATTTGGT TTGAA	AACGAATTGCCTGGA CTGAC
OEE (oxygen evolving enhancer)	DV867322	TCGAACTAGAGCAG AGTGAATTAAGC	TCCTCTCCGCCGATCA AC
peroxidase	FE527944	CCCAACCTACAGGA CATCGT	GGAAGCAGTCGTGGA AGAAG

Table 2. Membrane proteins differentially accumulated under heat stress

Functional category	Spot #	Protein:	Accession #	MW/PI
Energy	1	ATP synthase CF1 alpha subunit	118430299	55kD/6.1
	2	ATP synthase CF1 alpha subunit	309322117	55kD/5.9
	3	ATP synthase CF1 alpha subunit	118430299	55kD/6.1
	4	ATP synthase CF1 alpha subunit	48478769	55kD/5.8
	5	ATP synthase CF1 alpha subunit	48478769	55kD/5.8
	6	ATP synthase CF1 alpha subunit	118430299	55kD/6.1
	7	ATP synthase CF1 alpha subunit	194033146	55kD/6.3
	8	AtpA	51556908	55kD/6.0
	9	ATP synthase CF1 beta subunit	194033156	54kD/5.2
	10	chloroplast oxygen-evolving enhancer protein 1	147945622	34kD/6.1
	11	chloroplast oxygen-evolving enhancer protein 1	147945622	34kD/6.1
	12	Cytochrome b6-f complex iron-sulfur subunit, chloroplastic	68566191	24kD / 8.5
	13	pyruvate dehydrogenase kinase	115453191	33kD/6.2

Metabolism	14	glycolate oxidase	222636449	40kD/8.5
Stress Defense	15	catalase	90264977	57kD/6.3
	16	putative peroxidase	51038244	57kD/4.8
	17	HSP20 protein	86439765	17kD/4.7
Signaling	18	Possible: auxin-binding protein ABP20 precursor	195616892	20kD/6.0
unclear	19	unknown	-	-

Spot numbers correspond to spot numbers in Figure 2. Changes in accumulation are presented as relative fold changes in Figure 5.

Fig 1. Changes in chlorophyll content (A), and electrolyte leakage (B), and turf quality (C) of ‘COLXCB169’ and ‘COLXCB190’ during a 28-d treatment period. Vertical bars indicate values of the least significance difference test at $p = 0.05$ for comparison between the two genotypes at a given day of heat stress. Bars on the right represent values of the least significance difference test at $p = 0.05$ for comparison over time for a given genotype during heat stress.

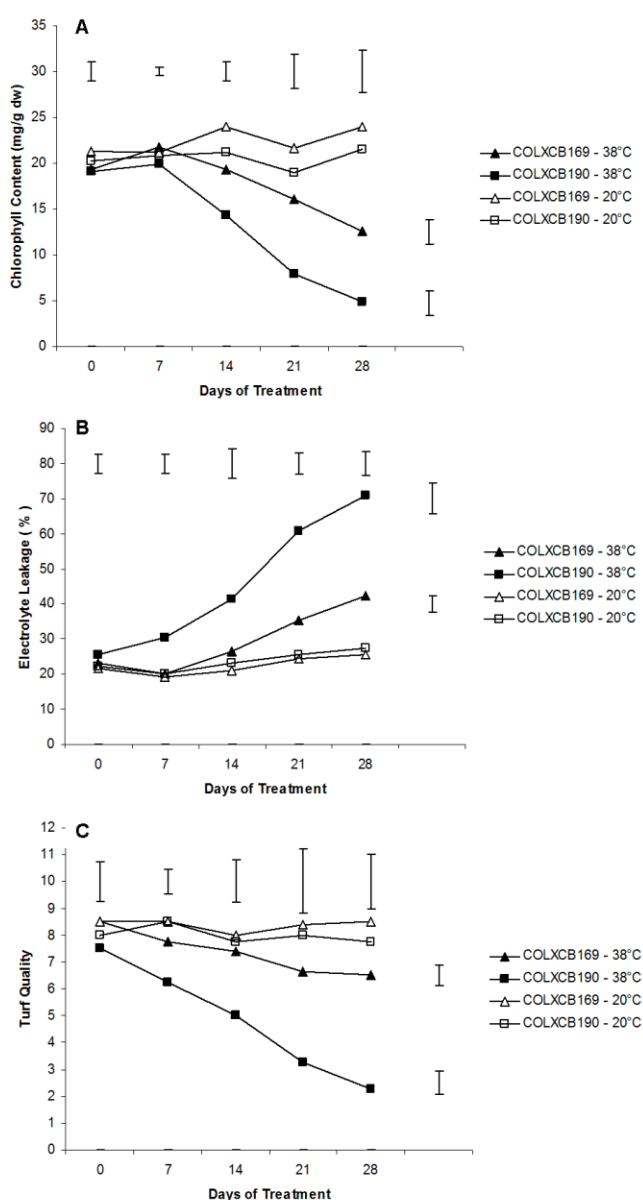


Fig. 2. An example of representative 2-D electrophoresis gels from the membrane protein fraction stained with coomassie blue. Labeled spot numbers correspond with the spot numbers in Table 2 of proteins exhibiting significant changes in abundance in response to heat stress.

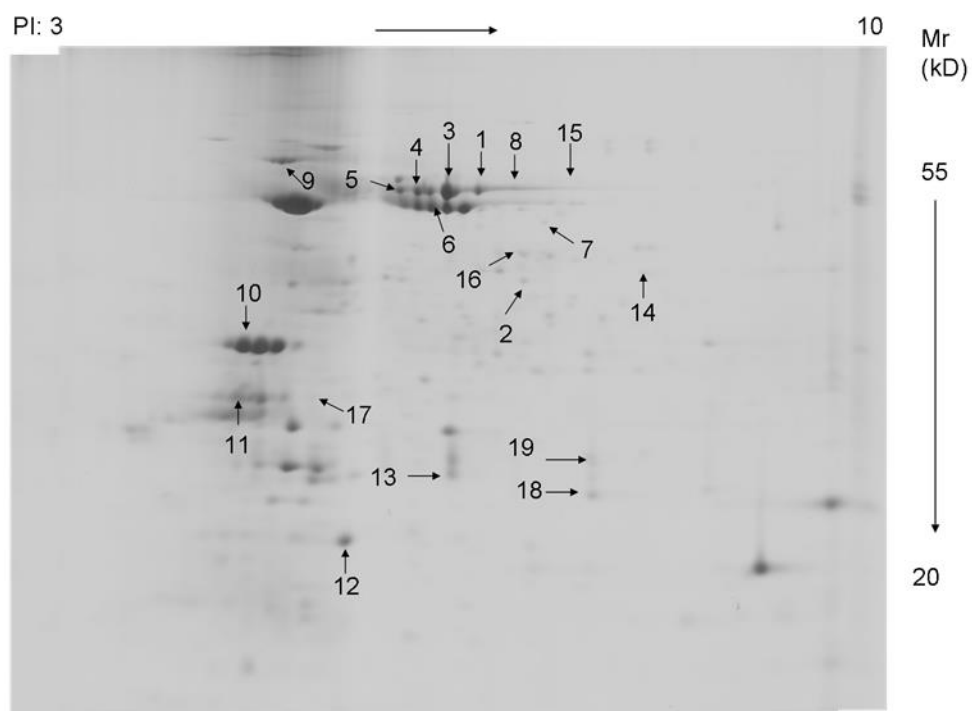


Fig. 3. Functional classification and the percent of proteins with differential responses to heat stress in two genotypes ('COLXCB169' and 'COLXCB190') in each functional category.

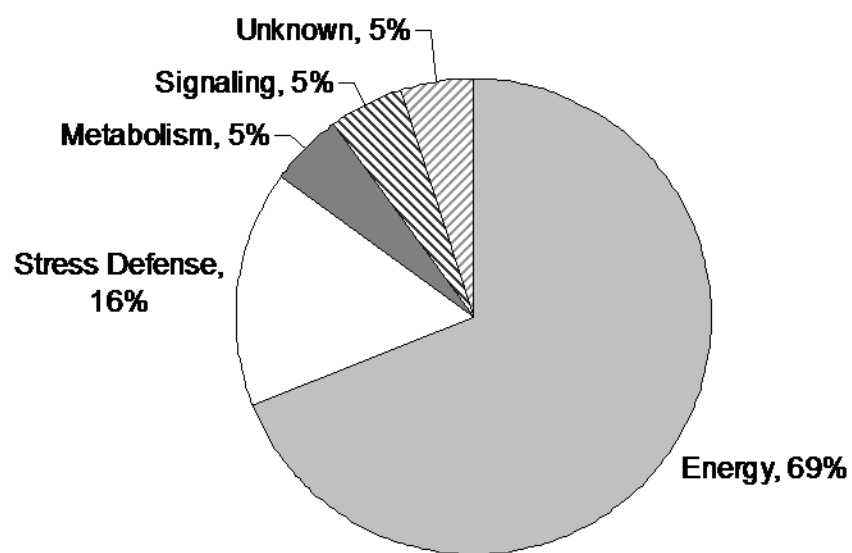


Fig. 4. Examples of membrane protein spots showing differential abundance between the two genotypes at 0, 14, and 28 d of heat stress. Spot numbers correspond to Table 2.

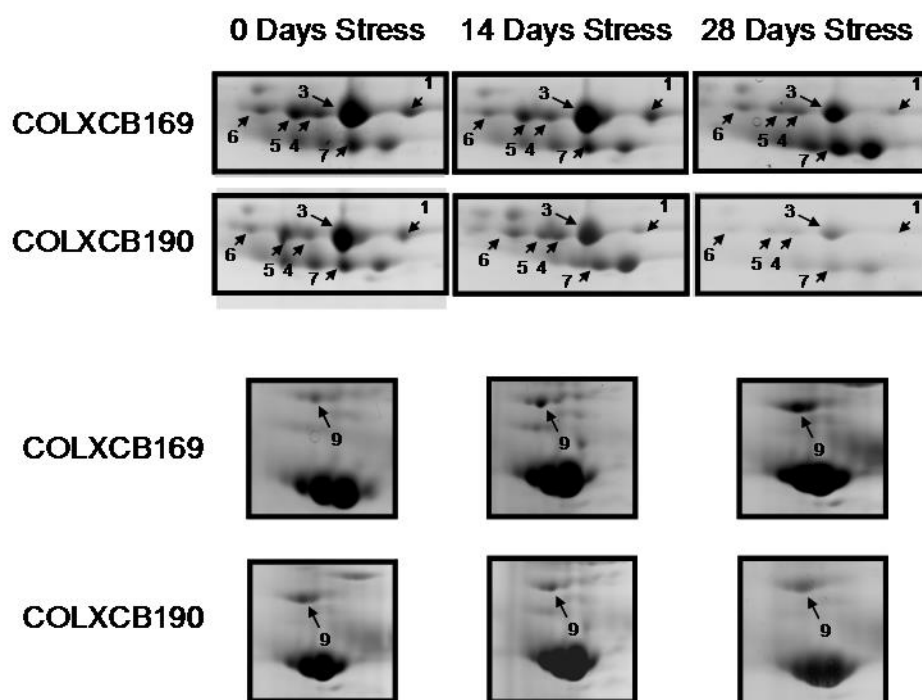


Fig. 5. Significant changes in membrane protein abundance in response to heat stress at 14 d of heat stress (A) and 28 d of heat stress (B) for two genotypes ('COLXCB169' and 'COLXCB190') relative to the non-stress control. Positive data indicate increases in protein abundance or up-regulation under heat stress and negative data indicate decreases in protein abundance or down-regulation under heat stress. Only protein spots which had significantly altered accumulations compared to control gels are represented in the graph ($p = 0.05$); bars represent standard error and asterisks indicate that there is a significant difference in relative change between the two genotypes according to Fisher's LSD (at $\alpha=0.05$). Numbers correspond to spot numbers in Table 2. ATP α : ATP synthase alpha subunit, ATP β : ATP synthase beta subunit, OEE: oxygen evolving enhancer protein, Cyt b6f: Cytochrome b6f, PDK: pyruvate dehydrogenase kinase, GlyOx: glycolate oxidase, CAT: catalase, HSP20: heat shock protein 20, Perox: peroxidase, ABP20: auxin binding protein 20.

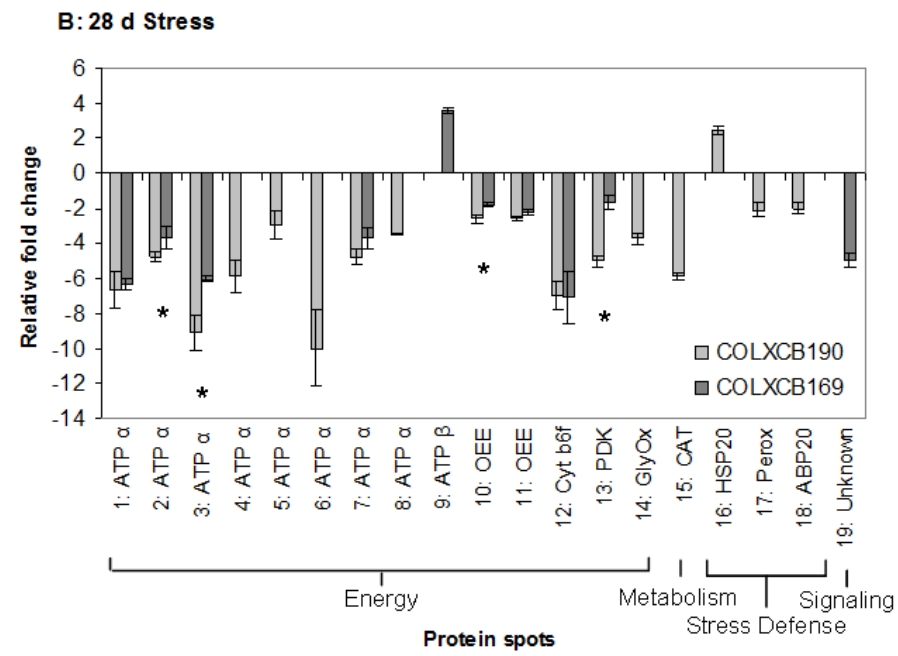
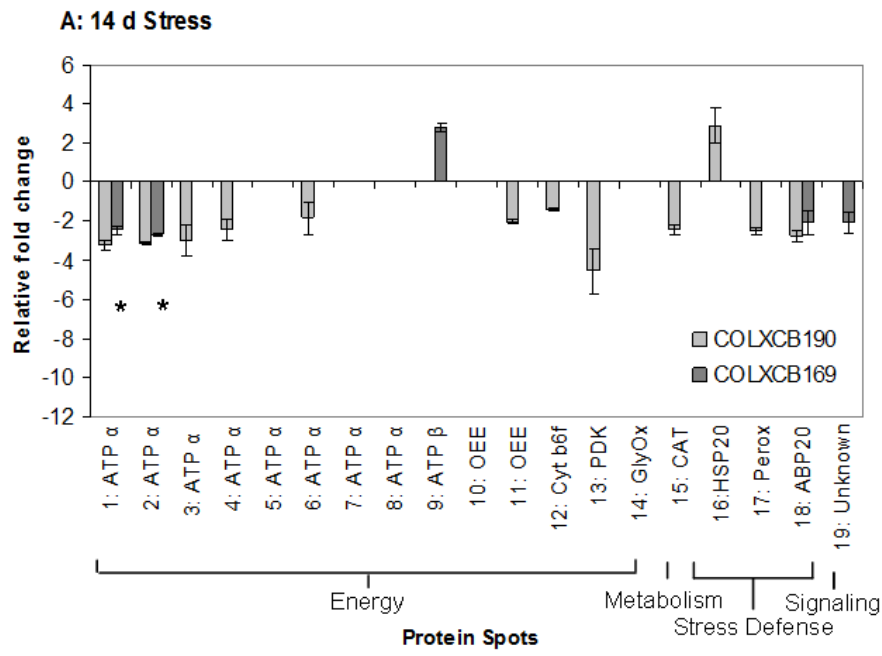
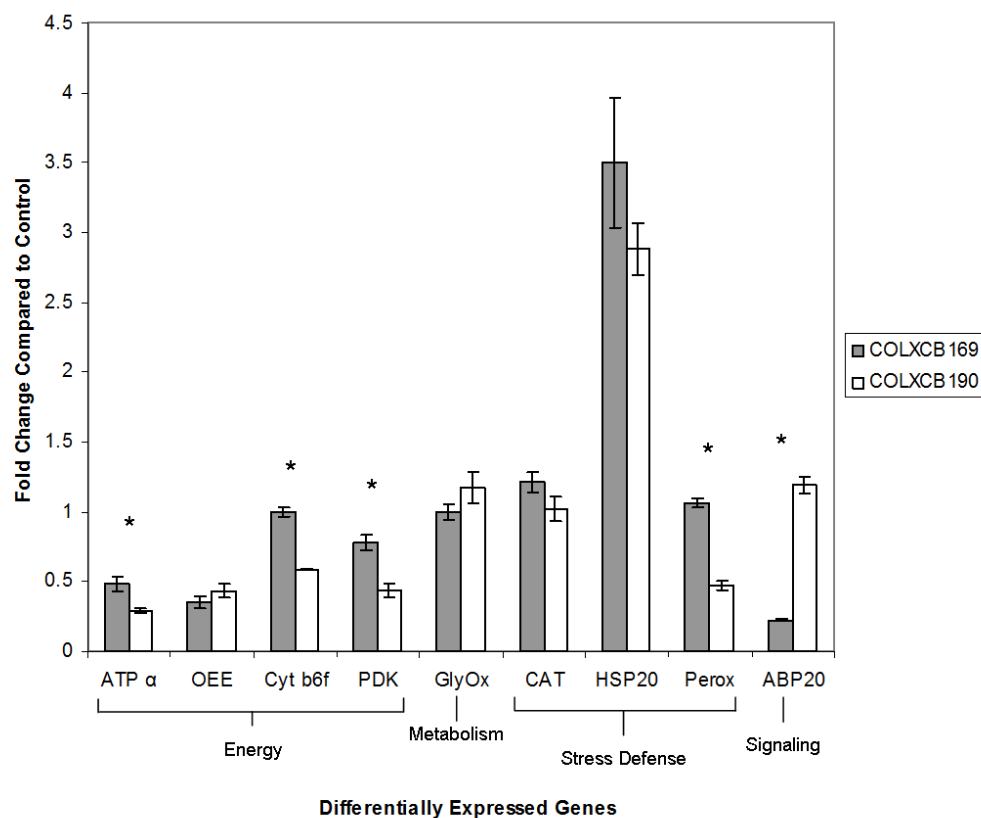


Fig. 6. Changes in relative transcript level of genes encoding differentially-expressed proteins in two genotypes ('COLXCB169' and 'COLXCB190') at 14 d of heat stress. Bars represent standard error of four replicates for each treatment and asterisks indicate that there is a significant difference in relative expression between the two genotypes according to Fisher's LSD (at $\alpha=0.05$).



**CHAPTER 3: Proteins Associated with Heat-Induced Leaf Senescence in Creeping
Bentgrass as Affected by Foliar Application of Nitrogen, Cytokinins, and an
Ethylene Inhibitor**

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INTRODUCTION

One of the typical symptoms of heat stress in cool-season grass species is premature leaf senescence. Heat-induced leaf senescence is characterized by loss of chlorophyll and proteins, and weakened metabolic activities, as well as oxidative damages (Wahid et al., 2007). Factors controlling chlorophyll or protein synthesis and degradation, such as nitrogen and plant hormones, such as cytokinins and ethylene may regulate heat-induced leaf senescence (Wolfe et al., 1988; Lim et al., 2007). However, major proteins and metabolic processes associated with heat-induced leaf senescence that are regulated by nitrogen, cytokinins, and ethylene are not well understood.

Nitrogen is one of the major plant nutrients indispensable for plant growth and function as an essential component of many cellular constituents including chlorophyll, nucleic acids, and proteins (Taiz, and Zeiger, 2010). Exogenous application of N compounds suppressed leaf senescence induced by heat stress in various plant species, creeping bentgrass (Fu and Huang, 2003; Wang et al., 2012) tall fescue (*Festuca arundinacea*) (Zhao et al., 2008), and maize (*Zea mays*) (Heckathorn et al., 1996). Exogenous applications of nitrogen resulted in increases in antioxidant activity (Wang et al., 2012), heat shock proteins (Heckathorn et al., 1996), or photosynthetic activities (Zhao et al., 2008) under heat stress conditions. However, specific proteins involved in N-suppression of heat-induced leaf senescence are yet to be determined.

Ethylene is a hormone known for its effects on inducing leaf senescence (Bleecker and Kende, 2000; Morgan and Drew, 1997). Increased levels of ethylene are associated with senescence and ethylene has been shown to increase during abiotic stress including drought, heat, salinity and oxidative stress (Balota et al., 2004; Gepstein and Thimann,

1981; Hayset et al., 2007). Ethylene biosynthesis is inhibited by aminoethoxyvinylglycine (AVG) (Even-Chen et al., 1982). The use of AVG to reduce ethylene levels and delay senescence has been demonstrated in a number of species, including mung bean (*Vigna radiata*) under ozone stress (Wenzel et al., 1995), winter wheat (*Triticum aestivum*) under drought stress (Beltrano et al., 1999), creeping bentgrass (*Agrostis stolonifera*) under heat stress (Xu and Huang, 2009) and oats (*Avena sativa*) (Gepstein and Thimann, 1981) and rice (*Oryza sativa*) during natural senescence (Kao and Yang, 1983). How the ethylene inhibitor suppresses leaf senescence induced by heat stress is still unclear.

Cytokinins are a class of plant hormones involved in cell division and differentiation, as well as chloroplast development, which plays important roles in delaying leaf senescence (Hare et al., 1997; Werner and Schmulling, 2009). Unlike ethylene, cytokinins have an inverse relationship to senescence where higher levels of cytokinins are associated with lower levels of senescence (Caers, et al., 1985; Mayak and Halevy, 1970; Nooden et al., 1990). Cytokinins have been shown to reduce heat damages either through exogenous applications (Xu and Huang, 2009; Liu and Huang, 2002; Zhang and Ervin, 2008) or through transgenic methods resulting in increased accumulations of cytokinins (Smart et al., 1991; Xu et al., 2009). However, specific proteins and associated metabolic processes, which are regulated by cytokinins, contributing to cytokinin-suppression of leaf senescence are not well documented.

Creeping bentgrass is a heat-sensitive, cool-season turfgrass species, and heat-induced leaf senescence is a major culprit leading to summer decline in turf quality (Fry and Huang, 2004). Minimizing heat-induced leaf senescence is critically important for maintaining high quality turf of creeping bentgrass during summer months. The objective

of this study was to determine major proteins and metabolic processes associated with the suppression of heat-induced leaf senescence in creeping bentgrass caused by foliar application of nitrogen, cytokinin, and an ethylene inhibitor. Leaf senescence was evaluated using three commonly-used indicators, including visual rating of turf quality, chlorophyll content and cell membrane stability. Proteins and associated metabolic processes controlling heat-induced leaf senescence that may be regulated by nitrogen, cytokinin, and the ethylene inhibitor were identified using two-dimensional electrophoresis (2-DE) and mass spectrometry analysis.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Sods of creeping bentgrass (cv. Pennncross) were collected from field plots at the turfgrass research farm at Rutgers University, North Brunswick, NJ and transplanted into plastic containers (15 cm in diameter and 20 cm deep) filled with fine sand. Plants were irrigated three times per week, received Hoagland's nutrient solution weekly (Hoagland and Arnon, 1950), and were trimmed weekly during establishment in a greenhouse. After 30-d establishment, plants were transferred to controlled environment growth chambers (Conviron, Winnipeg, Canada) set at 20/15 °C (day/night), 60% relative humidity, a 14-h photoperiod, and 610 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetically active radiation at the canopy level. Plants were maintained under those conditions for 7 d to allow for acclimation to the controlled-environment conditions before exposed to treatments.

Treatments and Experimental Design

Plants were treated with an ethylene inhibitor, aminoethoxyvinyl glycine (AVG), at 25 μM , cytokinin (zeatin riboside, ZR) at 25 μM , nitrogen (N) (carbonyldiamide, urea) at 18 mM, and water (untreated control) daily for 3 d prior to heat stress treatments, and then were applied at a 7-d interval for the remainder of the 28-d of heat treatment. The concentrations were selected based on preliminary tests showing positive effects on suppressing leaf senescence under heat stress. Additionally, all treatments contained 0.05% Tween 20. All treatments were applied as foliar spray at a volume which saturated the canopy (approximately 375 ml m^{-2} per pot).

Following 3-d treatment with water, AVG, N, or ZR, plants were exposed to two temperature treatments for 28 d: 20/15 °C (day/night) as the temperature control, or

35/30 °C (heat stress). Other growth chamber conditions were the same as described above. During the treatment period plants were watered daily, and fertilized twice per week with ¼ strength Hoagland's nutrient solution to maintain adequate hydration and nutrient status.

The experimental design was a split-plot design, with temperature treatments as the main plots, and exogenous treatments as the sub-plots. Each temperature treatment was repeated in four growth chambers. Each exogenous treatment had four replicates (four pots with multiple plants in each pot) within each temperature treatment. Plants were relocated among four growth chambers every 5 d to avoid potential confounding effects of environmental variations among different growth chambers.

Physiological Evaluation of Leaf Senescence

Effects of exogenous application of N, AVG, and ZR on leaf senescence were evaluated using three commonly-used physiological indicators, turf quality (TQ), leaf chlorophyll content, and membrane stability. Each parameter was taken at 7-d intervals during 28 d of heat stress.

Turf quality (TQ) is widely used as an indicator of overall turf health and vigor (Beard, 1973). It was visually rated on four replicates for each treatment using a 1-9 scale based on turf color, density, and uniformity, with 9 being the best in all three quality components and 1 being completely brown turf.

Chlorophyll content of leaves was measured using a dimethyl-sulfoxide extraction method described by Hiscox and Israelstam (1979). About 0.1 g of fresh leaf tissues collected from multiple plants in each replicate for each treatment was placed in a test tube containing 10 ml of dimethyl-sulfoxide and incubated in the dark for 72 h. The

absorbance of resulting solution was measured at 663 nm and 645 nm using a spectrophotometer (Spectronic Instruments, Inc., Rochester, NY). The leaf tissues were then dried in an oven at 80 °C for 72 h to obtain dry weight. Chlorophyll content was calculated using the equations described by Arnon (1949).

Membrane stability of leaves was estimated by measuring electrolyte leakage using the methods described by Blum and Ebercon (1981). About 0.1 g of leaf tissues collected from multiple plants in each replicate for each treatment was placed in a test tube with 35 ml de-ionized water, and incubated on a shaker for 16 h. Following this an initial reading of the conductance of the incubation solution was taken (C_{initial}) using a conductivity meter (YSI Incorporated, Yellow Springs, OH). Leaf tissue contained in the tubes was then killed by autoclaving at 140 °C for 20 min. Test tubes were placed back on the shaker for an additional 16 h and then a final conductance reading was taken (C_{max}). Electrolyte leakage, as an estimate of membrane stability, was calculated as a percentage of C_{initial} to C_{max} .

Protein Extraction and Separation Using 2D-PAGE

The second and third fully expanded leaves collected from each replicate for each treatment at 28 d of heat stress were immediately frozen in liquid nitrogen and stored at -80 °C for proteomic analysis. Protein extraction was performed using the TCA/Acetone method described by Xu et al., (2008) with modifications. Tissue weighing 0.5 grams was homogenized in 8ml of a solution containing 10% TCA and 0.07% 2-mercaptoethanol in acetone. Proteins were allowed to precipitate overnight at -20 °C. Following precipitation, centrifugation pelleted the proteins, and the resulting protein pellet was washed using cold acetone containing 0.07% 2-mercaptoethanol. The pellet

was then air dried and re-suspended in a solution containing 8M urea, 2 M thiourea, 2% 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 1% dithiothreitol (DTT), and 1% ampholytes. The resulting protein extraction was quantified with the methods describe by Bradford (1976) using a commercial dye reagent (Bio-Rad Laboratories, Hercules, CA) using a bovine serum albumin protein standard.

First dimension isoelectric focusing was performed using an IPGPhor apparatus (GE Healthcare, Piscataway, NJ). Immobilized pH gradient (IPG) strips (3-10pH, linear gradient, 13cm), were rehydrated with 250 μ L of a 8 M urea, 2 M thiourea, 2% w/v CHAPS, 1% v/v IPG buffer, 1% DTT, and 0.002% bromophenol blue rehydration buffer which contained 300 μ g of sample protein. IPG strips were subjected to an active rehydration step of 50 V for 14 h, followed by 500V for 1 h, 1000 V for 1 h, 5000 V for 1 h and finally 8000 V till a total of 80kVh was reached. Following isoelectric focusing strips were placed in a buffer containing 6 M urea, 30% glycerol, 2% sodium dodecylsulfate, 0.002% bromophenol blue, 50 mM Tris-Base (pH 8.7) and 1% DTT for 20 minutes, followed by a similar buffer in which DTT was replaced with 2.5% iodoacetamide for 15 minutes to ensure that all proteins were fully reduced and denatured. A Hoefer SE 600 Ruby electrophoresis unit (GE Healthcare, Piscataway, NJ) was used for the second dimension gel electrophoresis using 12.5% SDS-polyacrylamide gels. Gels were subsequently stained with a colloidal coomassie blue stain (Neuhoff et al., 1988) and scanned using a Personal Densitometer (GE Healthcare, Piscataway, NJ). The resulting images were analyzed using the SameSpots software (Nonlinear Durham, NC.). Protein spots were normalized to the total volume of spots on the gel and gels were compared against the water control treatment to look at the effects of exogenous

treatments under heat stress. Each treatment had 4 replicate gels included in the analysis. Following the selection of spots with significantly altered accumulations, spots were manual excised from the gel and subjected to a trypsin digestion followed by peptide identification via MALDI-TOF-MS as described in Xu and Huang (2008). MS and MS/MS spectra were compared to the NCBI green plant database using a local MASCOT search engine (V1.9, Matrix Science, Boston, MA, USA) and proteins with a confidence interval value >95% for at least two peptides were considered to be successfully identified.

Statistical Analysis

Treatment effects and interactions were analyzed using a general linear model with the ANOVA test and significant differences between treatments were tested by Fisher's protected LSD at $\alpha=0.05$ to separate the means. Statistical analysis was performed using SAS v9.2 (SAS Institute Inc, Cary, NC).

RESULT

Suppression of Heat-Induced Leaf Senescence Through Foliar Application of N, ZR, and AVG

Turf quality, chlorophyll content, and EL were unchanged during the experimental period (28 d) under the control temperature (20 °C) (Fig. 1A, B, C). No significant differences were detected between foliar applications of N, AVG, or ZR and the untreated control under control temperature for TQ, chlorophyll content, and EL.

Turf quality declined in all foliar treatments during heat stress, to a greater degree in untreated control plants (Fig. 1D). By 28 d of heat stress, TQ ratings declined to 7.3, 7.1 and 6.8 for N, AVG, and ZR treatments respectively, while the untreated control dropped down to 5.1, which is below the minimum acceptable level of TQ (6.0). Foliar applications effectively enhanced TQ, beginning at 7 d of heat stress for N treatment, at 14 d for AVG treatment, and at 21 d for ZR treatment, compared to the untreated control.

Chlorophyll content also declined in all treatments during heat stress, to a greater degree in the untreated control plants (Fig. 1E). By 28 d, chlorophyll content declined by 43% in AVG, ZR, and N treatments, while that of untreated control plants decreased by 60%. Foliar application of AVG, ZR and N maintained significantly higher chlorophyll content compared to the untreated control plants at 21 and 28 d of heat stress.

Electrolyte leakage (EL) increased in all treatments throughout the 28 d of heat stress, and by 28 d EL increased to 33, 36, and 34% in AVG, ZR, and N treatments, respectively, while it increased to 52.4% in the untreated control plants. At 21 and 28 d of stress treatment, AVG, ZR and N treatments had significantly lower EL compared to the untreated control plants (Fig. 1F).

The physiological results suggested that while leaf senescence was minimal under non-stress conditions, the application of N, ZR, and AVG provided no benefits; however, when plants under heat stress exhibited leaf senescence, foliar application of N, ZR, and AVG was effective in suppressing heat-induced leaf senescence.

Proteomic Responses to N, ZR, and AVG Application Under Heat Stress

Proteins were analyzed only in leaves of plants exposed to heat stress that demonstrated significant physiological effects of N, ZR, and AVG applications, but not for leaves exposed to the control temperature due to the lack of significant physiological effects of those treatments. The positive physiological effects of N, ZR, and AVG application under heat stress could be associated with alterations of the abundance level of proteins involved in different metabolic processes, as discussed below.

The 2-DE analysis identified a total of 49 protein spots differentially expressed in plants treated with AVG, ZR, or N compared to the untreated control under heat stress (Table 1). A representative 2D gel with labeled protein spots altered by exogenous treatments under heat stress is shown in Figure 2. Out of those 49 spots, 25 spots changed in the abundance levels in response to two or three treatments and some proteins were specifically responsive to only N, ZR or AVG application, with 9 spots being uniquely regulated by AVG, 3 spots by ZR, and 12 spots by N application (Fig. 3). Proteins exhibiting increases (up-regulated) or decreases (down-regulated) in abundance in responses to N, AVG, or ZR under heat stress are mainly classified into the following functional categories: energy, metabolism, stress defense, regulatory, and unknown (Fig. 4, 5).

In response to AVG application, abundance levels of 18 proteins increased (up-regulated) and 11 decreased (down-regulated). Of the 18 up-regulated proteins, the proportion of proteins involved in photosynthesis, respiration, metabolism, stress defense, and regulatory functions were 27.8, 22.2, 27.8, 16.7, and 5.6%, respectively (Fig. 5A). Of the 11 down-regulated proteins, a large proportion was associated with respiration functions (45.5%), and 9.1% were associated with regulatory functions or metabolism, and the remaining (36.4%) were unidentified or had unknown functions (Fig. 5B).

The application of ZR caused up-regulation of 12 proteins and down-regulation of 10 proteins (Fig. 5C, D). Of the 12 up-regulated protein spots 33.3% were involved with metabolism, 33.3% were associated with photosynthetic functions, 16.7% had regulatory functions, 8.3% were associated with respiration and the final 8.3% were proteins related to stress defense functions (Fig. 5C). Out of the 10 protein spots down-regulated by ZR, the majority were associated with respiration (60%), 10% were associated with photosynthetic functions, 10% had regulatory functions, and the remaining 20% had unknown functions (Fig. 5D).

In response to N application, 18 proteins were up-regulated and 16 proteins were down-regulated (Fig. 5E, F). Of the 18 up-regulated proteins, 33.3% were classified into metabolic functions, 27.8% were involved in photosynthesis, 22.2% had stress defense functions, 11.1% were associated with respiration, and 5.6% were proteins with regulatory functions (Fig. 5E). Among the 16 down-regulated proteins, 50% were associated with respiration, 12.5% had photosynthetic functions, 6.3% had regulatory functions, 6.3% were involved in metabolism, and the remaining 25% had unknown functions (Fig. 5F).

Significant increases and decreases for a number of important proteins were found in response to treatment with AVG, ZR, or N under heat stress (Fig. 3, 6). Energy proteins related to both photosynthesis and respiration had changes in abundance in response to exogenous treatment. The abundance of chlorophyll a/b binding protein increased by 1.2 and 2.3 fold due to AVG and N treatments, respectively, compared to the untreated control plants under heat stress. AVG, ZR, and N resulted in a 1.4, 1.3, and 1.4 fold increase for Rubisco large subunit and a 4.4, 1.4 and 3.3 fold increase of Rubisco small subunit respectively. Both ZR and N caused a 1.7 fold increase in phosphoribulokinase. In this study, the abundance level of cytochrome b6f was reduced by -2.2 and -1.9 fold, respectively, with ZR and N application, compared to that of untreated control plants. Treatment with ZR and N resulted in a -1.8 and -1.4 fold decrease in ferredoxin respectively. AVG caused a 1.5 fold increase in Rubisco activase. AVG and ZR caused a 1.6 and 1.2 fold increase in carbonic anhydrase while N treatment caused a -1.6 fold decrease.

A number of energy related proteins most strongly associated with respiration also had significant changes during heat stress. These include enolase which had a -1.4 fold decrease in accumulation in response to both AVG and ZR, and a -1.6 fold decrease in response to N. AVG, ZR, and N treatment cause a -1.5, -1.3, and -1.4 fold decrease in glyceraldehyde-3-phosphate dehydrogenase respectively. During heat stress succinate dehydrogenase had a -1.6 fold decrease in response to AVG, a -1.5 fold decrease with ZR, and a -2.4 fold decrease in response to N. Fructose biphosphate aldolase had a 1.3 fold increase in response to AVG and conversely a -1.7 fold decrease in response to N. Another protein spot identified as chloroplastic aldolase had a 2.3 fold increase in

response to N. Triosephosphate-isomerase, involved in glycolysis, was down-regulated by 1.5 fold by N application, which was not altered in response to other treatments. Phosphoglycerate kinase was found to have a 1.4, 1.1, and 1.3 fold increase in response to AVG, ZR, and N respectively. Treatment with ZR resulted in a 2.2-fold decrease in phosphoglycerate mutase compared to the untreated control under heat stress. AVG caused a 1.2, and 1.6 fold increase in ATP synthase alpha and beta subunits respectively. Additionally N treatment cause a -1.8 fold decrease in alcohol dehydrogenase under heat stress when compared to the untreated control.

Proteins related to metabolic functions with significant changes during heat stress include alanine aminotransferase which had a 1.5, 1.2, and 1.8 fold increase in response to AVG, ZR, and N respectively. Aminomethyltransferase which had a 2 and 2.2 fold increase in response to AVG and N respectively. Glycine decarboxylase P subunit which has a 2.2 fold increase in response to AVG and 1.8 fold increase in response to ZR. Ferredoxin-dependent glutamate synthase which experienced a 1.9 and 4.7 fold increase in response to AVG and N respectively. An Ftsh-Pftf precursor was found to have a 1.7 fold and 2 fold increase in response to AVG and ZR treatment, and a UDP-sulfoquinovose synthase protein was found to have a 1.3 and 1.4 fold increase in response to ZR and N treatments respectively. Peptidylprolyl isomerase had a -3 fold and -3.2 fold decrease in response to AVG and N. N treatment resulted in a 1.5 fold increase in alpha-glucan phosphorylase and glyoxylase was up-regulated 2.3 fold by N.

Defense related proteins with altered accumulations in response to exogenous treatments under heat stress include catalase which had a 1.5 and 1.3 fold increase in response to AVG and N. Glutathione-s-transferase, ascorbate peroxidase, and

chaperonin-60 which had a 1.9 fold, 1.3 fold and 1.6 fold increase with N treatment respectively. HSP70 had a 1.3-fold increase with ZR treatment and peroxiredoxin was found to have a 1.5 fold increase with AVG treatment when compared to the untreated control plants under heat stress

Regulatory proteins altered by exogenous treatments include mitochondrial elongation factor Tu which was down-regulated by all three exogenous treatments by -1.9 fold, -1.7 fold, and -2.2 fold decreases with AVG, ZR, and N treatment respectively. GTP-binding nuclear protein had a 1.6 and 1.2 fold increases in response to AVG, and ZR respectively. Histone protein H2B had a 1.6 fold increase in response to N and an RNA-binding protein had a 1.2 fold increase in response to ZR under heat stress.

Several protein spots which were unable to be clearly identified also had significant changes in accumulation under heat stress in response to exogenous treatments. These changes range from a -5.3 fold decrease to a 1.2 fold increase, with the majority of the unidentified spots experiencing down-regulation with exogenous treatment.

DISCUSSION

Our results demonstrated that there was a down-regulation in proteins related to energy production in glycolysis and electron transport chain of respiration (Fig. 7), and an up-regulation of proteins involved in both light-dependent and light-independent reactions of photosynthesis (Fig. 7), metabolism, and stress defense by exogenous application of N, ZR or AVG. Changes in proteins regulating those metabolic pathways could be associated with the suppression of leaf senescence induced by heat stress, as manifested by physiological changes. It is important to note that although protein levels are expressed relative to the untreated control, changes in levels of protein abundance may be due to changes in biosynthesis and degradation rates in both treated and untreated plants and to fully understand the effects these compounds have on protein regulation will require further work. The metabolic functions of proteins differentially or commonly affected by the application of N, ZR, and AVG are discussed in details below.

Metabolic Functions of Proteins Commonly-Regulated by Two or Three of the Exogenous Applications (N, AVG, and ZR) Under Heat Stress

Photosynthesis and Respiration

One of the predominant factors associated with heat stress is the inhibition of photosynthesis and stimulation of respiration, which can result in the depletion of carbohydrates for energy use and the generation of reactive oxygen species caused by imbalanced energy pathways (Tiwari et al., 2002; Suzuki and Mittler, 2006). The positive carbon balance between photosynthesis and respiration has previously been shown to be

an important mechanism for delaying heat-induced senescence and improving whole-plant heat tolerance (Lyons et al., 2007; Xu and Huang, 2000). Proteins related to either photosynthesis or respiration had differential accumulations in response to the application of N, AVG, and ZR under heat stress, which could at least partially explain the physiological effects of those compounds on the level of heat-induced leaf senescence related to carbon metabolism. Figure 7 highlights important pathways related to energy metabolism with proteins that were regulated by N, AVG, or ZR.

Chlorophyll a/b binding protein is part of the light-harvesting complex bound to thylakoids in chloroplasts (Paulsen, 1995). This photosynthetic protein is responsible for binding to chlorophyll pigment molecules in light harvesting complexes and transferring excitation energy during photosynthesis (Bassi et al., 1990). An increase in abundance of chlorophyll a/b binding protein was found in both AVG and N treatments compared to the untreated control plants under heat stress. Cytochrome b6f is also a thylakoid bound protein that is an essential component of the electron transport chain and serves as the final electron acceptor in the cyclical electron flow pathway of photosynthetic light-reactions (Cramer et al., 2006; Munekage et al., 2004). The cyclical electron flow driven solely by PSI has been found to be stimulated by heat stress (Schrader et al., 2004; Sharkey, 2005). Both ZR and N had reduced accumulation of cytochrome b6f compared to untreated control plants, suggesting that the capacity of cyclical electron flow could be limited and more electrons could be diverted to electron flow by the way of both PSII and PSI under heat stress. The increase in chlorophyll a/b binding protein for light harvesting due to AVG and N application and the decrease in cytochrome b6f for electron transport

by ZR and N suggested that the application of those compounds may enhance light harvesting capacity and electron transport efficiency during heat stress.

Ribulose-1,5-bisphosphate carboxylase/oxygenase (Rubisco) is the most abundant soluble protein in leaf tissues controlling carbon fixation, which often exhibits decline in its abundance level during heat stress (Demirevska-Kepova et al., 2005; Xu and Huang, 2010). In this study, foliar application of AVG, ZR, and N resulted in the up-regulation of Rubisco large subunits and Rubisco small subunits under heat stress.

Phosphoribulokinase (PRK) is also a critical enzyme in the Calvin cycle responsible for the regeneration of ribulose-1,5-bisphosphate, and its accumulation is associated with greater rate of photosynthesis under stress conditions (Laing et al., 2001; Takahashi and Murata, 2005). The application of ZR and N resulted in an increase in PRK abundance (Fig. 4, 6). Carbonic anhydrase is most commonly associated with aiding in the fixation of CO₂ in the Calvin cycle, but has also been implicated in electron transport and regulating pH (Tiwari et al., 2005). Carbonic anhydrase was up-regulated by AVG and ZR. The increase in Rubisco, PRK, and carbonic anhydrase by exogenous treatment with AVG, ZR and N may enhance carboxylation, carbon fixation, and RUBP regeneration during light-independent reactions of photosynthesis.

Respiration rate typically increases under heat stress, which may exceed photosynthetic rate, leading to a depletion of carbohydrates and an energy imbalance. Previous studies demonstrated that down-regulation of respiration was positively related to improved heat tolerance in bentgrass species (Lyons et al., 2007; Rachmilevitch and Huang, 2006). In contrast to the up-regulation of proteins for carbon fixation, proteins involved in respiration were mainly down-regulated by AVG, ZR, and N treatment under

heat stress, including enolase, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), succinate dehydrogenase, and ferredoxin. Both enolase and GAPDH are important enzymes involved in glycolysis of respiration (Plaxton, 2006). This down-regulation of glycolytic enzymes may lead to the suppression of respiration and slow the consumption of sugars which serve as energy reserves during heat stress. Succinate dehydrogenase is a membrane bound enzyme involved in linking the citric acid cycle to electron transport in the mitochondria (Hederstedt and Rutberg, 1981). Ferredoxins are also electron transport proteins involved in many important redox reactions (Takubo et al., 2003). The down-regulation of succinate dehydrogenase and ferredoxins suggested that electron transport of respiration could also be suppressed in leaves treated with AVG, ZR and N under heat stress. The down-regulation of respiratory proteins involved in either glycolysis or electron transport may result in lowered respiratory consumption of carbohydrates that support active leaf growth under heat stress.

Metabolism

Changes in amino acid metabolism is a common response to heat stress due to changes in protein metabolism and the need to maintain protein synthesis, as well as the accumulation of amino acids for protective functions (Guy et al., 2008; Mayer et al., 1990). Proteins for amino acid metabolism were mainly up-regulated by AVG, ZR and N treatment under heat stress, including alanine aminotransferase, aminomethyltransferase, glycine decarboxylase, and ferredoxin-dependent glutamate synthase. Alanine aminotransferase is involved in the generation and break down of alanine and plays roles in nitrogen and amino acid metabolism (Kikuchi et al., 1999). Alanine aminotransferase was up-regulated by all three compounds. The increase in alanine aminotransferase

caused by exogenous treatment with AVG, ZR and N demonstrated an improved capacity to synthesize alanine and may lead to changes in amino acid and nitrogen metabolism, resulting in delayed heat-induced senescence.

Aminomethyltransferase was up-regulated in AVG and N treatments, and glycine decarboxylase was up-regulated by AVG and ZR. Aminomethyltransferase and glycine decarboxylase are both part of the glycine-cleavage system which plays an important role in breaking down the glycine generated during photorespiration (Bourguignon et al., 1988). Ferredoxin-dependent glutamate synthase was up-regulated in both the AVG and N treatments, which is involved in nitrogen metabolism, particularly during photorespiration which generates large amounts of ammonia that need to be re-assimilated for the plant to survive (Kendall et al., 1986). The up-regulation of those proteins indicated that the application of all three compounds could facilitate the maintenance of more active nitrogen and amino acid metabolism under heat stress.

UDP-sulfoquinovose synthase is an enzyme involved in the synthesis of the anionic glycolipid sulfoquinovosyldiacylglycerol (Sanda et al., 2001). This glycolipid is an essential component of thylakoid membranes for properly functioning photosynthetic apparatus (Frentzen, 2004). The up-regulation of UDP-sulfoquinovose synthase in both ZR and N treatments may help maintain integrity of photosynthetic membranes during heat stress.

Stress Defense

Catalases are a large family of antioxidant proteins which neutralize H_2O_2 to water, and have been found to decrease during leaf senescence leading to higher levels of ROS (Dhindsa et al., 1981). The increased levels of ROS under stresses can result in

oxidative damages to cellular constituents, and ultimately lead to cell death (Mittler, 2002). Both the AVG and N treatment had increases in catalase accumulation. The increase in catalase levels likely results in the delay of senescence through the neutralization of ROS to protect cellular components.

Regulatory Functions

Several proteins related to the regulation of important processes, such as transcription or translation, were altered by exogenous treatments under heat stress. Both AVG and ZR led to the up-regulation of RAN-GTP nuclear binding protein. This nuclear protein is involved in regulating the cell cycle and has been implicated in mitotic spindle formation which is important for cellular division (Carazo-Salas et al., 2001; Vernoud et al., 2003). The up-regulation of RAN-GTP nuclear binding protein indicated that leaves treated with AVG and ZR could remain more active growth or experienced lesser extent of senescence during heat stress compared to the untreated control plants.

Metabolic Functions of Proteins Uniquely-Regulated by N, ZR, or AVG Alone

Nitrogen-Responsive Proteins

Several proteins involved in respiration or carbohydrate metabolism were uniquely regulated by N, but were not altered by other treatments for creeping bentgrass exposed to heat stress, including triosephosphate-isomerase, alcohol dehydrogenase (ADH), and α -glucan phosphorylase. Triosephosphate-isomerase involved in glycolysis was down-regulated by N application, but was not altered in response to other treatments. The ability of N to down-regulate triosephosphate-isomerase is another example of reducing glycolysis to lower respiration rates during heat stress. ADH is a key enzyme in

anaerobic respiration, which produces toxic ethanol and is inefficient for energy production. ADH content has been found to increase during stresses including UV-stress, hypoxia, and pathogen related signals (Matton et al., 1990; Umeda and Uchimiya, 1994). There was a -1.8 fold down-regulation of ADH in the N treatment (Fig. 4, 7), indicating the suppression of potential stress-induced anaerobic respiration. α -glucan phosphorylase is involved in the breakdown of starch in plants, and it has been shown to possibly play a role in certain abiotic tolerances such as salt or dehydration stress by helping regulate starch and sugar metabolism (Buchner et al., 1996; Zeeman et al., 2004). The up-regulation of this protein with N treatment may be related to the activation of starch breakdown to support plant growth during heat stress. These results suggested that N may suppress heat-induced leaf senescence by regulating carbohydrate utilization and availability.

Another major unique aspect of N effects is the up-regulation of stress protective proteins, including glyoxalase, glutathione-s-transferase (GST) and ascorbate peroxidase (APX). Glyoxalase is a protein involved in the detoxification of methylglyoxal, a reactive compound produced by several metabolic processes including glycolysis (Hossain et al., 2001). Glyoxylase was up-regulated by N, which may lead to reduced damages to cellular components caused by the accumulation of the by-product methylglyoxal under heat stress. Both GST and APX increased in response to N treatment. GST plays important roles in glutathione metabolism, but one of its more important roles is in antioxidant pathways (Marrs, 1996). Plants with higher GST expression tend to be more heat tolerant (Roxas et al., 2000). Similarly APX plays an important role in antioxidant metabolism and has been found to increase in response to stress (Almeselmani et al.,

2006; Koussevitzky et al., 2008). The application of N could have strengthened the antioxidant defense systems, suppressing heat-induced leaf senescence.

The N treatment also lead to an increase in chaperonin-60, which is a chaperone protein that help prevent unwanted protein aggregation and ensure correct protein folding (Boston et al., 1996). This protein is most widely associated with the assembly of Rubisco sub-units in the chloroplasts, but has also been show to interact with other proteins (Ellis and van der Vies, 1988; Salvucci, 2008). Additionally studies have shown that chaperonin-60 has increased expression during heat shock events (Demirevska-Kepova et al., 2005; Salvucci, 2008). Increases in chaperonin-60 may help plants maintain active conformations of proteins during heat stress.

Foliar application of N under heat stress also resulted in an up-regulation of histone protein H2B. Histones are involved in the organization of DNA within the nucleus and can play roles the cell cycle or in the epigenetic regulation of stress responses (Boyko and Kovalchuk, 2008). The up-regulation of this protein may indicate a more active cell cycle in creeping bentgrass exposed to heat stress due to N treatment.

ZR-Responsive Proteins

Proteins uniquely regulated by ZR included phosphoglycerate mutase, heat shock proteins (HSP), and RNA-binding proteins.

Phosphoglycerate mutase is an enzyme in the glycolysis pathway which is responsible for the conversion of 3-phosphoglyerate to 2-phosphoglycerate (Huang et al., 1993). Treatment with ZR resulted in a decrease in phosphoglycerate mutase compared to the untreated control under heat stress. The decrease in the abundance of this protein is

another example of glycolytic enzymes being down-regulated during heat stress to potentially improve carbon balance under heat stress.

Heat shock proteins, such as HSP70, are involved in the prevention of protein aggregates and the refolding of damaged proteins (Glover and Lindquist, 1998). The protective role of HSP70 has been found in many plant species with increases in HSP70 abundance being positively associated with levels of thermotolerance (Hu et al., 2010; Vierling, 1991). HSP70 increased in response to treatment with ZR compared to the untreated control during heat stress. The enhanced HSP70 abundance by application of ZR may strengthen the protective roles of this protein to suppress heat damages in leaves.

RNA-binding proteins play roles in the transport, stability and translation of mRNA (Sahi et al., 2007). ZR treatments resulted in an increase in RNA-binding proteins under heat stress compared to the untreated control. The up-regulation of this protein by ZR may help maintain protein synthesis during heat stress.

AVG-Responsive Proteins

Foliar application of AVG uniquely altered the abundance of two proteins involved in photosynthesis and energy generation, including Rubisco activase and ATP synthase, and a stress-protective protein, peroxiredoxin.

Rubisco activase maintains Rubisco in the active state so it can bind CO₂ for carboxylation in photosynthesis (Salvucci and Crafts-Brandner, 2004). Rubisco activase has been found to be highly liable to heat stress, and the decline in Rubisco activase activity and abundance results in heat-inhibition of photosynthesis (Salvucci and Crafts-Brandner, 2004). Rubisco activase abundance was up-regulated by AVG treatment under

heat stress which may result in improved photosynthesis by better maintaining the active state of Rubisco during heat stress.

ATP synthase is a transmembrane protein which uses H^+ gradients to generate ATP. ATP synthase has been found to be damaged by heat stress in a number of species (Lee et al., 2007; Majoul et al., 2004). Additionally impaired ATP synthase may result in less ATP available for energy dependent stress defense mechanisms (Süss and Yordanov, 1986). Treatment with AVG resulted in the up-regulation of both ATP synthase alpha and beta subunits. The up-regulation of ATP synthase may represent improved energy metabolism under heat stress.

Peroxioredoxin belongs to the family of peroxidases which help detoxify peroxide radicals (Dietz, 2003). The abundance of peroxiredoxin was increased by to AVG treatment under heat stress. The up-regulation of this protein with AVG treatment is another example of increased antioxidant enzymes, which can protect cells from ROS toxicity during heat stress.

Conclusions

Foliar applications of AVG, ZR, or N effectively suppressed heat-induced leaf senescence in creeping bentgrass, as indicated by improved TQ, chlorophyll content, membrane stability. The 2-DE analysis revealed that 49 protein spots differentially responded to a single compound application or were commonly regulated by two or three different compounds. The most common metabolic pathways in which proteins were either up-regulated or down-regulated by single compounds alone or shared between two or three compounds included energy metabolism (photosynthesis and respiration), stress defense (antioxidant metabolism and heat shock protection), as well as amino acid and

protein metabolism. Those N, AVG, or ZR-responsive metabolic pathways could play key roles in the regulation of heat-induced leaf senescence and whole-plant heat tolerance in creeping bentgrass and other cool-season grass species. Future work will explore specific metabolites and genes involved in N, AVG, or ZR- regulation of key metabolic pathways controlling heat-induced leaf senescence.

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Table 1. Spot numbers correspond to labeled spots in figure 2. Accession numbers correspond to GI numbers in the NCBI database. Mw/Pi represent the theoretical molecular weight in kilodaltons and isoelectric point respectively. Score is the protein score and PM is the number of unique peptides matched. Numbers represent significant changes in protein accumulation in each treatment compared to the control treatment at 28 d of heat stress according to Fischer's protect LSD ($p < 0.05$). Positive values indicate the fold increase, and negative numbers the fold decrease of a protein spot compared to the control. Significance level of LSD test were shown as p values, * < 0.05 , ** < 0.01 , *** < 0.001

Spot #	Protein	Accession #	Mr/pI	Score	PM	Fold Change Compared to Control			LSD test
						AVG	ZR	N	
Function Category- Energy									
1	Chloroplast chlorophyll a/b-binding protein precursor	149392272	28kD/5.7	213	3	n.s.	n.s.	2.3	**
2	Chloroplast PS type III chlorophyll a/b-binding protein	125656346	12kD/9.2	264	3	1.2	n.s.	n.s.	**
3	RuBisCO large subunit	32966580	53kD/6.2	600	6	1.4	1.3	1.4	**
4	RuBisCO small subunit	6409343	19kD/8.6	103	2	4.4	1.4	3.3	*
5	Phosphoribulokinase	125578	44kD/6.0	394	4	n.s.	1.7	1.7	*
6	Cytochrome b6-f complex iron-sulfur subunit	68566191	24kD/8.5	138	2	n.s.	-2.2	-1.9	*
7	Enolase	90110845	48kD/5.4	762	7	-1.4	-1.4	-1.6	*
8	Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	120680	37kD/6.7	473	6	-1.5	-1.3	-1.4	**

9	GAPDH A	115458768	43kD/7.6	167	3	-1.4	n.s.	n.s.	*
10	GAPDH B	120663	48kD/7.6	512	7	-1.9	-1.7	-2.2	*
11	Succinate dehydrogenase	297797713	70kD/5.9	193	2	-1.6	-1.5	-2.4	*
12	Phosphoglycerate kinase	21396683	31kD/4.9	347	4	1.4	1.1	1.3	*
13	Phosphoglycerate kinase	3328122	50kD/7.7	329	3	n.s.	n.s.	3.2	**
14	Fructose-bisphosphate aldolase	8272480	42kD/9.0	192	3	1.3	n.s.	-1.7	**
15	Ferredoxin	6561891	41kD/7.6	465	6	n.s.	-1.8	-1.4	*
16	Triosephosphate-isomerase	11124572	27kD/5.4	164	2	n.s.	n.s.	-1.5	**
17	Phosphoglycerate mutase	551288	61kD/5.3	244	3	n.s.	-2.2	n.s.	*
18	RuBisCO activase	225434859	52kD/5.5	228	3	1.5	n.s.	n.s.	*
19	ATPA	51556908	55kD/6.0	853	9	1.2	n.s.	n.s.	*
20	ATPase, beta subunit	194033156	54kD/5.2	818	9	1.6	n.s.	n.s.	*
21	Carbonic anhydrase	729003	35kD/8.9	213	2	1.6	1.2	-1.6	***
22	Chloroplastic aldolase	218115	42kD/7.6	225	3	n.s.	n.s.	2.3	**
23	Class III Alcohol dehydrogenase	1675394	41kD/6.8	171	2	n.s.	n.s.	-1.8	**
Function Category- Metabolism									
24	Alanine aminotransferase	29569153	54kD/8.0	609	8	1.5	1.2	1.8	**
25	Aminomethyltransferase	15460656	44kD/8.5	286	3	2	n.s.	2.2	**
26	Glycine decarboxylase P subunit	710308	111kD/6.5	500	8	2.2	1.8	n.s.	*
27	Ferredoxin-dependent glutamate synthase	50508366	175kD/6.4	273	3	1.9	n.s.	4.7	*
28	FtsH- Pftf precursor	52075838	72kD/6.4	338	4	1.7	2	n.s.	*
29	UDP-sulfoquinovose synthase	115463645	53kD/8.5	136	2	n.s.	1.3	1.4	*
30	Peptidylprolyl isomerase	21593963	28kD/9.4	231	4	-3	n.s.	-3.2	*
31	Alpha-glucan phosphorylase	14916632	94kD/7.3	349	5	n.s.	n.s.	1.5	*
32	Glyoxalase	46485858	30kD/5.0	94	2	n.s.	n.s.	2.3	***

Functional Category – Defense									
33	Catalase-1	2493543	57kD/6.5	571	6	1.5	n.s.	1.3	**
34	Catalase	90264977	57kD/6.3	304	4	1.4	n.s.	n.s.	*
35	Glutathione-s-transferase	11385471	22kD/5.0	101	2	n.s.	n.s.	1.9	**
36	Peroxisome ascorbate peroxidase	15080682	32kD/7.8	116	2	n.s.	n.s.	1.3	**
37	Chaperonin- 60	2493650	53kD/4.9	861	8	n.s.	n.s.	1.6	*
38	Heat shock protein 70	1143427	79kD/5.4	239	3	n.s.	1.3	n.s.	*
39	Peroxiredoxin	115435844	21kD/7.8	94	2	1.5	n.s.	n.s.	*
Functional Category - Regulatory									
40	Mitochondrial elongation factor Tu	1149571	52kD/5.5	299	3	-1.9	-1.7	-2.2	**
41	GTP-binding nuclear protein	1172835	25kD/6.4	451	6	1.6	1.2	n.s.	*
42	Histone H2B	302829292	17kD/938	124	2	n.s.	n.s.	1.6	**
43	RNA-binding protein	3850621	42kD/7.7	309	4	n.s.	1.2	n.s.	*
Functional Category - Unknown									
44	Unclear	N/A	N/A			n.s.	n.s.	-2.4	*
45	Unclear	N/A	N/A			-4.2	-4.8	5.3	**
46	Unclear	N/A	N/A			n.s.	-2.7	2.9	**
47	Unclear	N/A	N/A			-1.5	n.s.	1.5	*
48	Unclear	N/A	N/A			-1.5	n.s.	n.s.	*
49	Unclear	N/A	N/A			1.2	n.s.	n.s.	*

Fig 1. Changes in turf quality, chlorophyll content or electrolyte leakage during 28 d of 20/15 °C control conditions (A, B, and C respectively) or during 28 d of 35/30 °C heat stress (C, D, and F respectively) when treated with AVG, ZR, N, or a water control. Vertical bars represent least significance difference values between exogenous treatments at $p=0.05$.

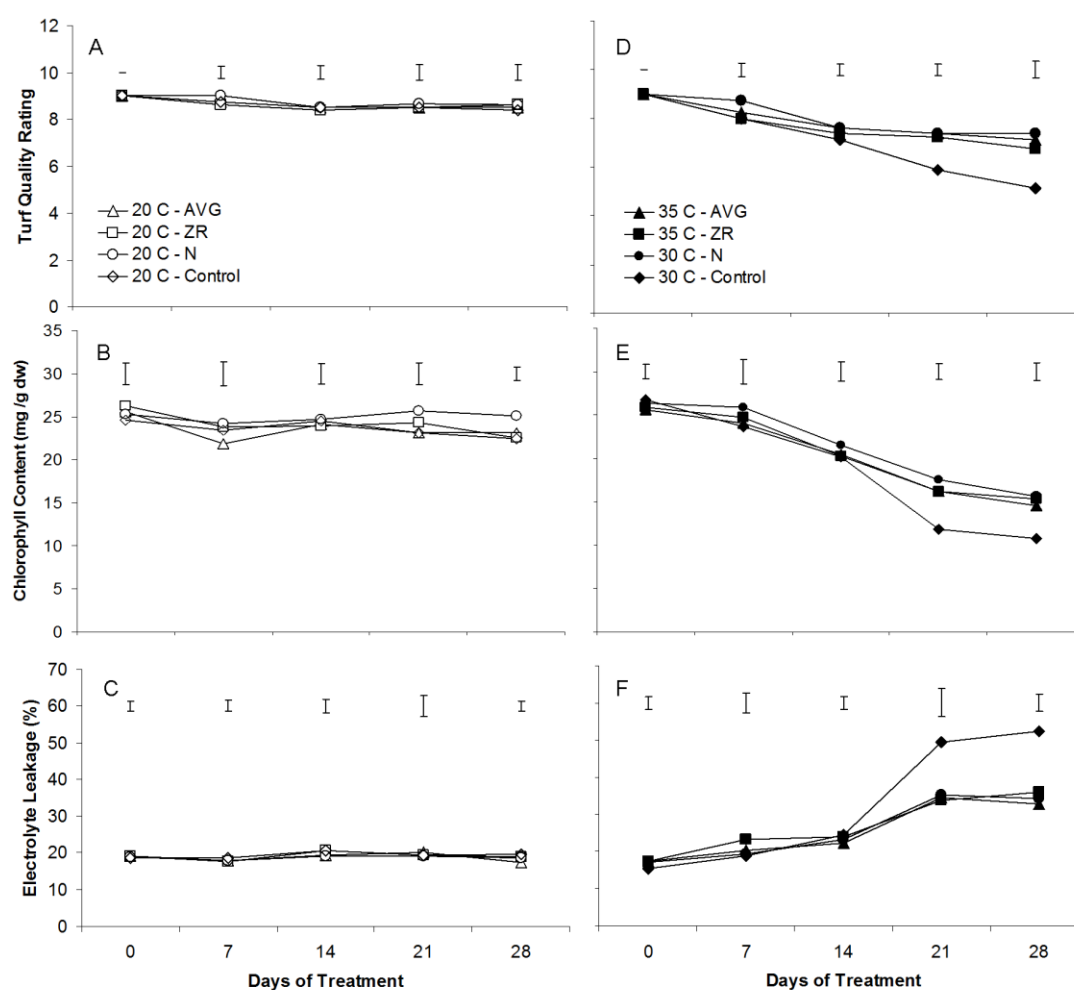


Fig 2. Representative 2-DE gel stained with coomassie blue. Labeled spot numbers correspond to the spot numbers in table 1 for proteins which had significantly different accumulations compared to the control at 28 d heat stress, and a close up of spot 3, and 7 showing representative spots for all four treatments under heat stress.

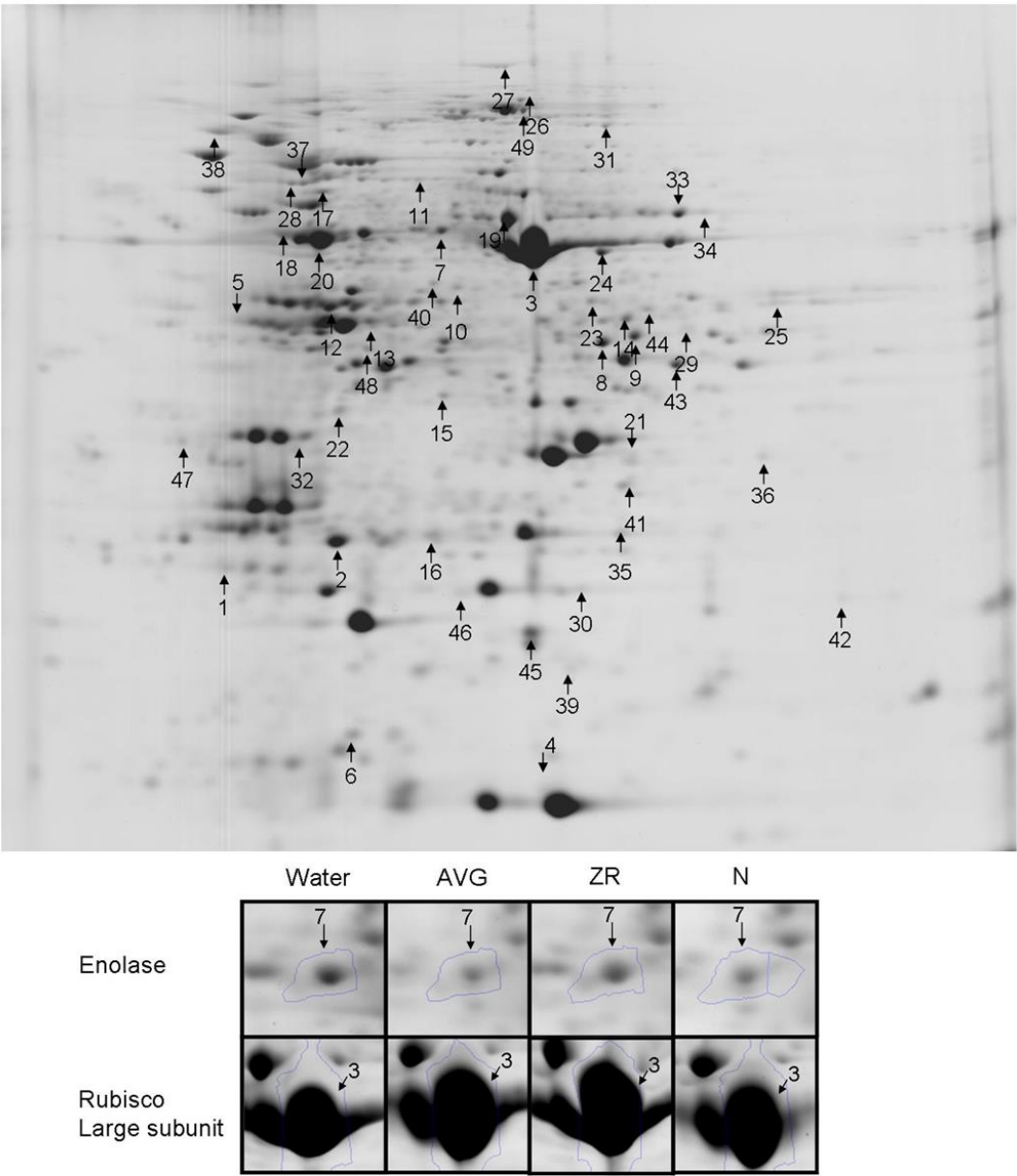


Fig 3. Up-regulation and down-regulation of protein spots in response to AVG, ZR, or N at 28 d of heat stress represented as fold change compared to the control treatment. Green represents a positive fold increase or up-regulation and red represents a negative fold decrease or down regulation. Black squares represent proteins spots not significantly altered by exogenous treatments compared to the control under heat stress. Spot numbers correspond to the spot numbers in table 1 and figure 2 which are the protein spots with significantly altered accumulations according Fischer's LSD ($p=0.05$).

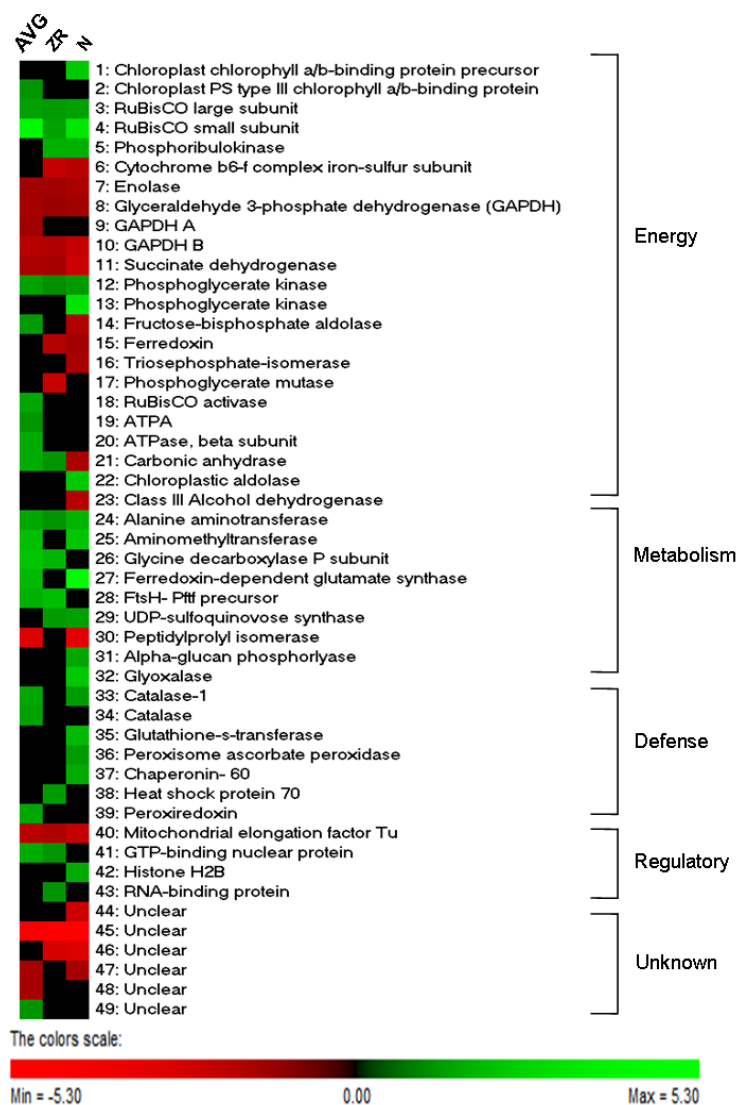


Fig 4. Venn diagram showing the number of protein spots with significantly altered accumulations by AVG, ZR, or N treatments compared to the control at 28 d of heat stress.

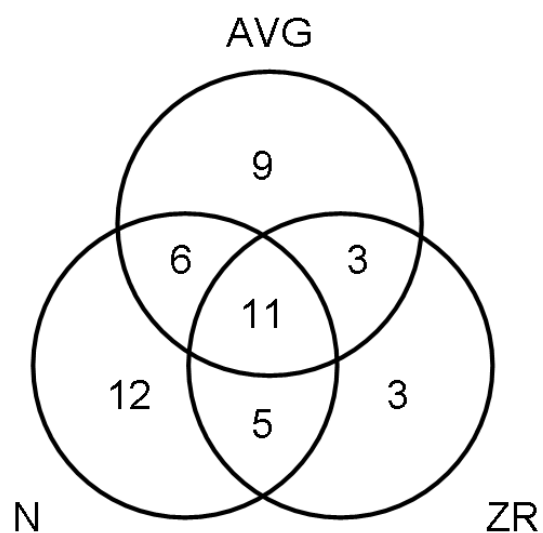


Fig 5. Percentage of protein spots significantly up or down-regulated and their functional categories in response to AVG (A/B), ZR (C/D), or N (E/F) compared to control plants during 28 d heat stress.

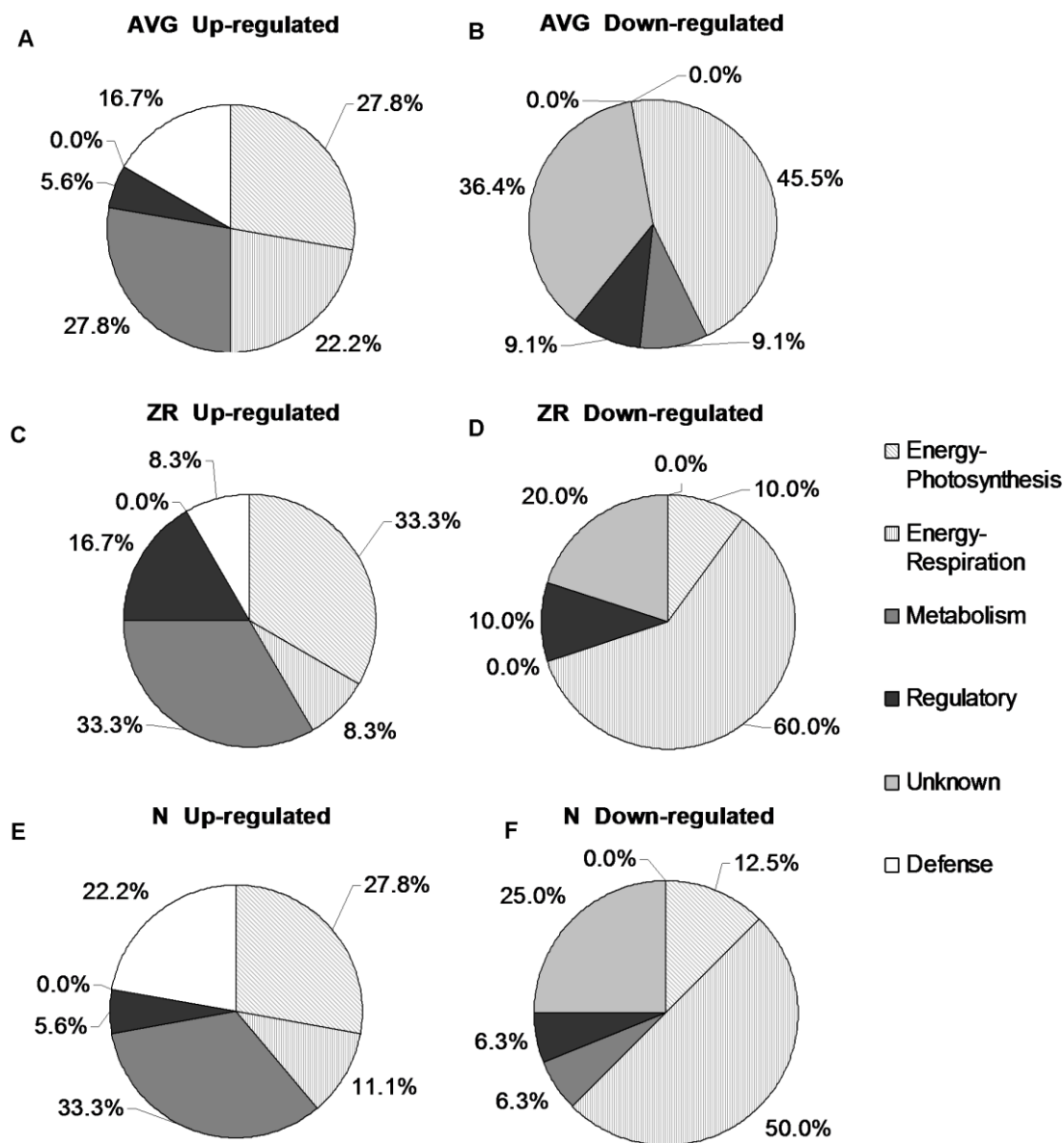


Fig 6. Up-regulation and down-regulation of protein spots in response to AVG, ZR, or N at 28 d of heat stress represented as fold change compared to the control treatment. Charts are organized by the functional category of protein spots and includes energy (A), metabolism (B), defense (C), regulatory (D) and unknown (E) functions. Spot numbers correspond to the spot numbers in table 1 and figure 2 which are the protein spots with significantly altered accumulation ($p=0.05$).

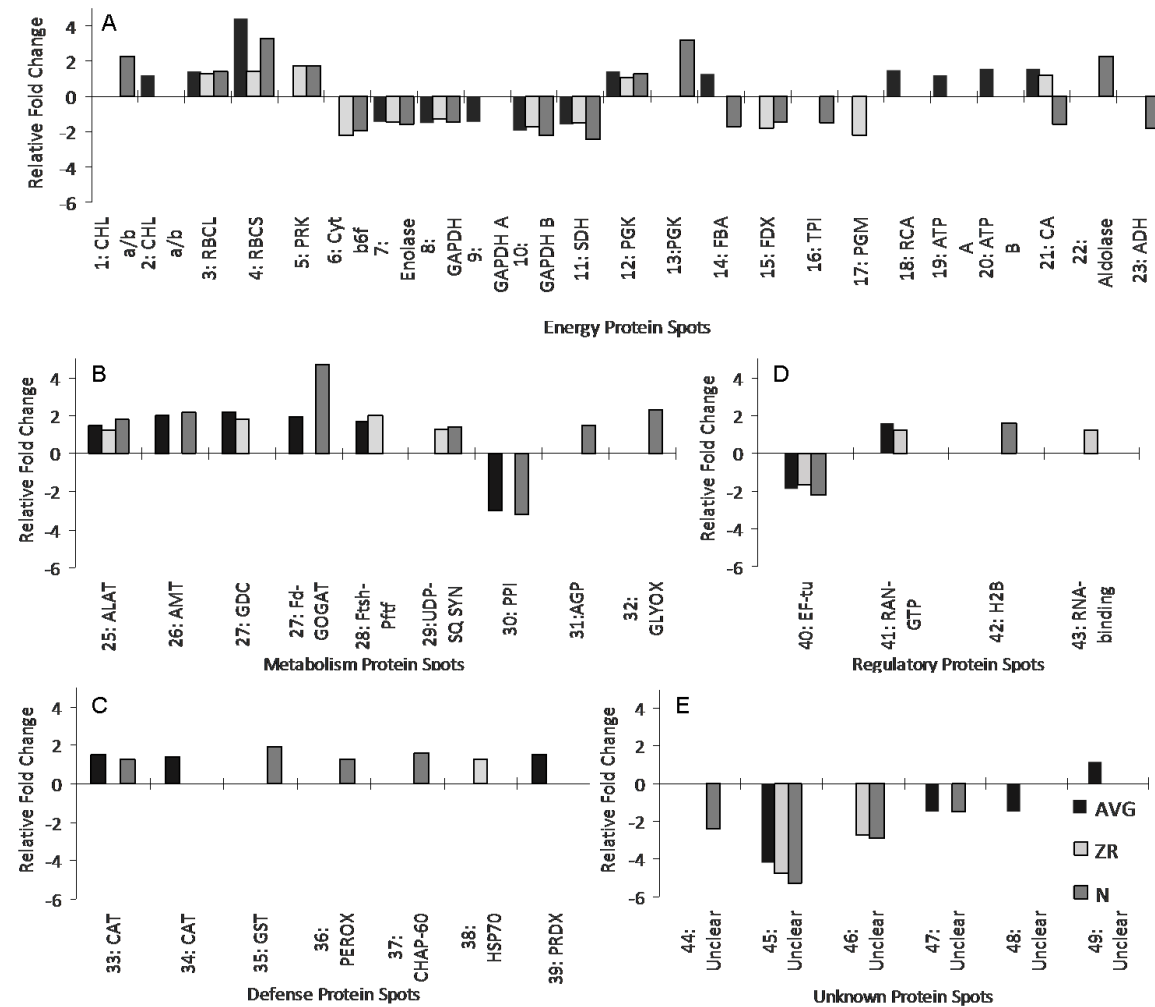
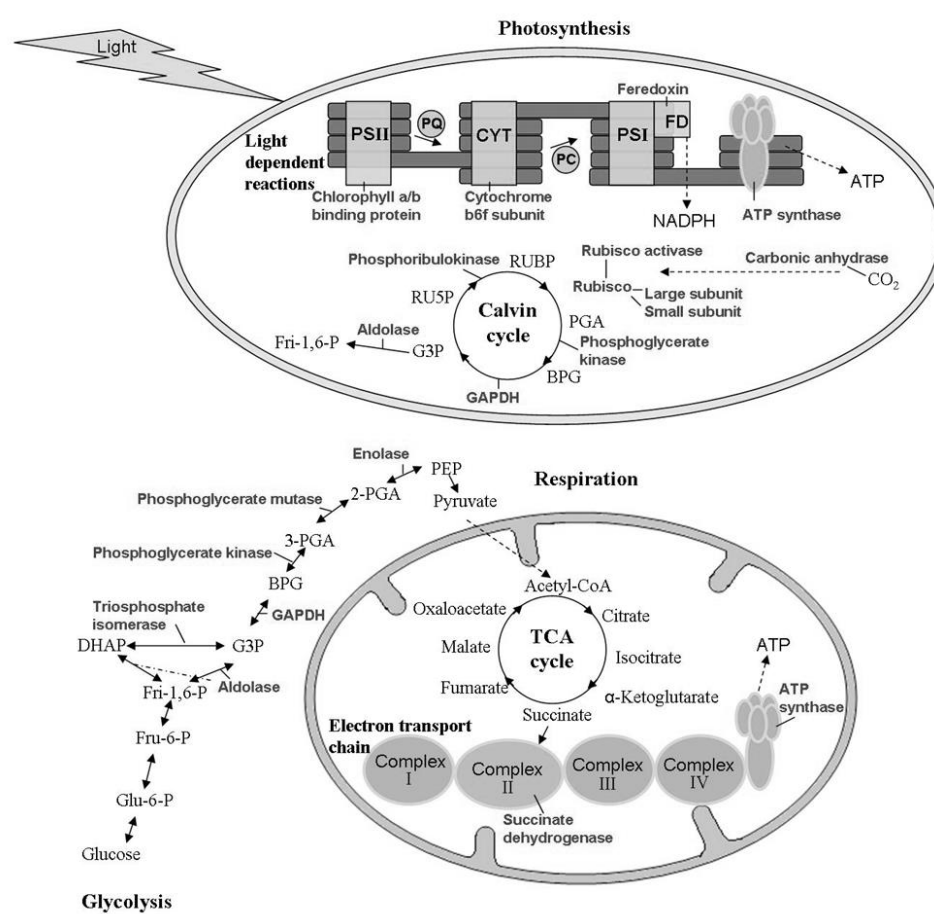


Fig 7. Diagram of important metabolic pathways relating to energy metabolism highlighting the key steps of photosynthesis, glycolysis and respiration with proteins being altered by AVG, R or N . Proteins with the abundance significantly altered by exogenous application of AVG, ZR, or N compared to the control during heat stress are labeled.



**CHAPTER 4: Metabolite Responses to Exogenous Application of Nitrogen,
Cytokinin, and Ethylene Inhibitors in Relation to Heat-Induced Senescence in
Creeping Bentgrass**

Jespersen, D., J. Yu, and B. Huang. 2015. PlosONE 10: e0123744.

INTRODUCTION

Heat is a major abiotic stress which leads to premature leaf senescence in many plant species, including cool-season grass species. Stress-induced senescence is associated with metabolic changes and shifts in carbon relations, the production of damaging reactive oxygen species, as well as the degradation of cellular constituents including proteins, lipids, and pigment molecules such as chlorophyll (Wahid et al., 2007). Many approaches have been used to alleviate heat stress-induced senescence and improve whole-plant stress tolerance, including exogenous application of plant growth regulators or hormones, such as ethylene inhibitors and cytokinins and nutrients, such as nitrogen (Xu and Huang B, 2009; Fu and Huang, 2007; Jespersen and Huang, 2015). However, metabolic mechanisms associated with heat-induced leaf senescence that are regulated by those external compounds are not well understood.

Ethylene is a gaseous plant hormone affecting plant stress tolerance and regulating senescence (Bleecker and Kende, 2000; Morgan and Drew, 1997). An increased level of ethylene is one of the main signals for inducing senescence (Grbić and Bleecker, 1995). Ethylene levels have also been shown to increase in response to a number of abiotic stresses, including heat, drought, and salinity (Balota et al., 2004; Hays et al., 2007). Decreased levels of ethylene production have been associated with improved stress tolerance in a cool-season grass species (Xu and Huang, 2007). Aminoethoxy vinyl glycine (AVG) is an ethylene inhibitor which reduces ethylene levels by inhibiting ethylene biosynthesis (Even-Chen et al., 1982). The use of AVG to reduce endogenous ethylene levels delay senescence has been demonstrated in a number of species including oat (*Avena sativa*), wheat (*Triticum aestivum*), and creeping bentgrass

(Xu and Huang B, 2009; Gepstein and Thimann, 1981; Beltrano et al., 1999). However, specific metabolites regulated by an ethylene inhibitor that may suppress heat-induced leaf senescence are yet to be determined.

Cytokinins are another class of plant hormones which play important roles in cell differentiation and division (Werner and Schmulling, 2009). Cytokinins also play a role in stress response and delaying leaf senescence (Hare et al., 1997). Both exogenous applications of cytokinins, as well as genetic transformation to increase endogenous cytokinins levels have been shown to delay senescence and increase abiotic stress tolerance in creeping bentgrass (Xu and Huang B, 2009; Merewitz et al., 2010; Xu et al., 2009; Zhao et al., 2008). It is unclear how exogenous application of cytokinins may control leaf senescence induced by heat stress at the metabolic level.

Nitrogen is a major growth limiting nutrient for plants which is an important constituent of biomolecules such as nucleic acids and proteins (Taiz and Zeiger, 2010). Nitrogen is well known for its effects on reducing leaf senescence during heat stress conditions (Tawfik et al., 1996). Nitrogen has been shown to improve heat tolerance by increasing antioxidant and photosynthetic activities in perennial grass species (Zhao et al., 2008; Wang et al., 2012), and resulting in elevated accumulations of heat shock proteins in *Zea mays* (Heckathorn et al., 1996). However, metabolic responses to additional nitrogen related to leaf senescence under heat stress are not well documented.

We have previously reported alteration of proteomic profiles by the exogenous application of ethylene inhibitors, cytokinins, or nitrogen in relation to the suppression of heat-induced leaf senescence in a cool-season grass species, creeping bentgrass (*Agrostis stolonifera*) (Jespersen and Huang, 2015). The previous study reported that the alleviation

of heat-induced senescence by N, AVG, or ZR was associated with enhanced protein abundance in photosynthesis and amino acid metabolism and stress defense systems (heat shock protection and antioxidants), as well as suppression of those imparting respiration metabolism. However, specific metabolites responsive to N, AVG, or ZR involved in those important metabolic pathways, such as photosynthesis and respiration, identified through the proteomic analysis are unknown. Metabolomic profiling is a powerful approach for identifying metabolites and metabolic pathways regulating plant growth and responses to external stimuli or stresses (Guy et al., 2008). Creeping bentgrass is a widely used forage and turf grass species in temperate regions, but sensitive to high temperatures, and understanding mechanisms of improving heat tolerance is important for enhancing the productivity and quality of cool-season grass species in environments with increasing temperatures. The objective of this study was to identify metabolites and associated metabolic pathways affected by the exogenous application of an ethylene inhibitor, cytokinin, nitrogen compounds that may contribute to their effects on the suppression of heat-induced leaf senescence and plant tolerance to heat stress in a cool-season perennial grass species, creeping bentgrass (*Agrostis stolonifera*).

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Sod plugs of creeping bentgrass (cv. 'Penncross') were collected from mature field plots at the Rutgers University Hort Farm II research facility, North Brunswick, NJ. Plants were transplants in to plastic pots filled with fine sand (15 cm in diameter and 20 cm deep) and allowed to establish in a greenhouse. During the 30-d establishment, plants were watered three times each week, received Hoagland's nutrient solution weekly (Hoagland and Arnon, 1950), and were trimmed to maintain a 5-cm canopy height. Plants were then transferred to controlled environment growth chambers (Conviron, Winnipeg, Canada) set at 20/15 °C (day/night temperature), a 14-h photoperiod with 610 $\mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetically active radiation (PAR) and allowed to acclimate for one week before the beginning of treatments.

Treatments and Experimental Design

Plants were treated with an ethylene inhibitor, aminoethoxyvinyl glycine (AVG), at 25 μM , cytokinin (trans-zeatin riboside, ZR) at 25 μM , nitrogen (N) (carbonyldiamide, urea) at 18 mM, and water (untreated control) daily for 3 d prior to heat stress treatments, and then were applied at a 7-d interval for the remainder of the 28-d of heat treatment. AVG and N were prepared in water, and ZR was dissolved in trace amount of 1N NaOH, before being diluted to appropriate concentrations with water. The ZR and AVG concentrations were selected based on preliminary tests showing positive effects on suppressing leaf senescence under heat stress (Xu and Huang B, 2009; Jespersen and Huang, 2015). The N rate was selected based on the common recommendation of N rate for foliar application in creeping bentgrass used as golf turf. Chemicals were obtained

from Sigma-Aldrich (St. Louis, MO). Additionally, all treatments contained 0.05% Tween 20. All treatments were applied as foliar spray at a volume which saturated the canopy (approximately 375 ml m⁻²).

Following 3-d treatment with water, AVG, N, or ZR, plants were exposed to two temperature treatments for 28 d: 20/15 °C (day/night) as the temperature control, or 35/30 °C (heat stress). Other growth chamber conditions were the same as described above. During the treatment period plants were watered daily, and fertilized twice per week with ¼ strength Hoagland's nutrient solution to maintain adequate hydration and nutrient status.

The experimental design was a split-plot design, with temperature treatments as the main plots, and exogenous treatments as the sub-plots. Each temperature treatment was repeated in four growth chambers. Each exogenous treatment had four replicates (four pots with multiple plants in each pot) within each temperature treatment. Plants from each temperature treatment were relocated among four growth chambers every 5 d to avoid potential confounding effects of environmental variations among different growth chambers.

Physiological Measurements

Leaf senescence and whole-plant heat tolerance was evaluated using three commonly-used indicators, including visual ratings of turf quality, chlorophyll content and cell membrane stability. Measurements were taken every 7-d starting at the onset of temperature treatments to assess treatment effects. Turf grass quality (TQ) was visually rated as an indicator of overall plant vigor based on color, density and uniformity of

grass canopy on a 1-9 scale, with 9 being the best in all three quality components and 1 being dead plants (Beard, 1973).

Chlorophyll content was measured using a modification of the methods described by Hiscox and Israelstam (1979). 0.1 gram of fresh leaf tissue was incubated in 10 ml of dimethyl-sulfoxide in the dark for 72 hours to extract chlorophyll from the leaf tissue. The resulting solution was measure on a spectrophotometer (Spectronic Instruments, Inc., Rochester, NY) at 663nm and 645nm. Leaf tissue was then dried for 72 h in a 80 °C oven to obtain dry weights. Chlorophyll content was calculated on a fresh weight basis using the equations described by Arnon (1949).

Membrane stability was estimated as electrolyte leakage using the methods described by Blum and Ebercon (1981). About 0.1g of fresh leaf tissue was placed in a tube with 35 ml de-ionized water and placed on an orbital shaker for 16h. An initial conductance reading (C_{initial}) of the incubated solution was taking using a conductivity meter (YSI Incorporated, Yellow Springs, OH). Tubes were then autoclaved at 120 °C for 20 min to kill all contained leaf tissue. Tubes were places back on the shaker for an additional 16 h and a final conductance reading was measured (C_{max}). Electrolyte leakage was calculated as a percentage of C_{initial} to C_{max} . to determine the percent of relative damage.

Metabolite Analysis

Extraction and Derivatization of Metabolites

Metabolites associated with heat-induced leaf senescence that may be regulated by nitrogen, cytokinin, and the ethylene inhibitor were identified using gas chromatography and mass spectrometry analysis. The procedure was conducted

following the method used by Du et al. (2011). Leaf tissue samples from 28 d of heat treatment were harvested and immediately frozen in liquid nitrogen and stored at -80 °C for later metabolite analysis. The extraction was modified from previously described protocols (Roessner et al., 2001; Rizhsky et al., 2004). For each sample, frozen leaves were lyophilized using a FreeZone 4.5 system (Labconco, Kansas City, MO) then ground to a fine powder, 25 mg of leaf tissue powder was transferred into a 10 ml microcentrifuge tube, and extracted in 1.4 ml of 80% (v/v) aqueous methanol at ambient temperature under 200 rpm for 2 h. Ribitol (10 μ l of a 2 mg ml⁻¹ solution) was added to each sample as an internal standard prior to incubation. Then samples were extracted in a water bath for 15 min at 70 °C. Tubes were centrifuged for 30 min at 12,000 rpm, the supernatant was transferred into new tubes, and 1.4 ml of water and 0.75 ml of chloroform were added. The mixture was thoroughly vortexed and centrifuged for 5 min at 5,000 rpm. 2 ml of the polar phase (methanol/water) was decanted into 1.5 ml HPLC vials and dried in a Centrivap benchtop centrifugal concentrator (Labconco, Kansas City, MO). The dried polar phase was methoximated with 80 μ l of methoxyamine hydrochloride (20 mg ml⁻¹) at 30 °C for 90 min and was trimethylsilylated with 80 μ l N-Methyl-N-trimethylsilyltri-fluoroacetamide (with 1% trimethylchlorosilane) for 60 min at 70 °C.

Gas Chromatography Mass Spectrometry (GC-MS) Analysis

GC-MS analysis followed the procedure described in Qiu et al. (2007). The derivatized extracts were analyzed with a PerkinElmer gas chromatograph coupled with a TurboMass-Autosystem XL mass spectrometer (PerkinElmer Inc., USA). Extract aliquots of 1 μ l were injected into a DB-5MS capillary column (30 m $\times$ 0.25 mm $\times$ 0.25 μ m, Agilent J & W Scientific, Folsom, CA). The inlet temperature was set at 260 °C. After a

6.5 min solvent delay, initial GC oven temperature was set at 60 °C; 1 min after injection, the GC oven temperature was raised 5 °C min⁻¹, and finally held at 280 °C for 15 min. The injection temperature was set to 280 °C and the ion source temperature was adjusted to 200 °C. The helium carrier gas had a constant flow rate of at 1ml min⁻¹. The measurements were made with electron impact ionization (70 eV) in the full scan mode (m/z 30-550). Turbomass 4.1.1 software (PerkinElmer Inc., USA) coupled with commercially available compound libraries: NIST 2005, Wiley 7.0 was used to identify the detected metabolites. For GC/MS results, compounds were identified based on retention times and comparison with reference spectra in mass spectral libraries. Peaks areas of metabolites were integrated with the Genesis algorithm, and relative quantities were calculated using the ribitol internal standard.

Statistical Analysis

Treatment effects were analyzed using an ANOVA test and treatments found to have significant difference were tested by Fisher's protected LSD at $\alpha=0.05$ to separate the means. Principle component analysis (PCA) using a correlation matrix to standardize the variables was performed to identify components which contributed the greatest variance between treatment groups. Additionally partial least squares regression (PLS) was used to model the relationship between metabolites and physiological measurements for overall heat tolerance. Variable importance project (VIP) scores were calculated for the PLS model to determine which metabolites according to Wold's criterion had higher contributions to delaying leaf senescence as estimated by chlorophyll content (Sauer, 2007). This approach accounts for both predictors and response variables to determine

metabolites contribution to the model. Statistical analysis was performed using SAS v9.2 (SAS Institute Inc, Cary, NC).

RESULTS

Effects of Exogenous Treatments on Suppressing Heat-Induced Leaf Senescence

All growth and physiological measurements, TQ, chlorophyll content, and EL, remained relatively unchanged during the experimental period (28 d) under non-stress temperature (20/15 °C) (Fig. 1A, B, C). At this temperature, no significant differences in TQ, chlorophyll content or EL were detected between treatments of N, ZR, or AVG and the untreated water control (Fig. 1A, B, C).

Turf quality declined in all treatments during heat stress but the decline happened sooner and to a greater degree in the untreated plants (Fig. 1D). By 28 d of heat stress, TQ rating had dropped to 5.3 for untreated plants, but remained significantly higher in the N, ZR, and AVG treatments than the untreated control, with TQ being 7.1, 6.8, and 7.0, respectively.

Leaf chlorophyll content also declined in all treatments during heat stress (Fig. 1E). By 28 d of heat stress, chlorophyll content declined by 59% in the untreated plants, whereas that for N, ZR, and AVG treatments declined by 41, 41, and 43% respectively. Plants treated with N, ZR, or AVG exhibited significantly greater chlorophyll content than the untreated plants at 21 and 28 d of heat stress.

Electrolyte leakage (EL) increased in all treatments during heat stress, indicating a loss of membrane stability (Fig. 1F). By 28 d of heat stress, EL increased to 33.0, 36.0, and 34.0% in AVG, ZR, and N treatments, respectively, while it increased to 52.4% in the untreated plants. Plants treated with N, ZR, or AVG had significantly lower EL at 21 and 28 d of heat stress compared to the untreated control.

Metabolite Responses to Exogenous N, ZR, or AVG Application Under Heat Stress

Results of metabolic profiling are presented here for plants exposed to heat stress conditions, but not for leaves exposed to the non-stress temperature due to the lack of significant physiological effects of N, ZR, and AVG applications compared to control for non-stress treatments. The differential responses of metabolites to N, ZR, and AVG application during heat stress may highlight the metabolic processes or pathways associated with the positive physiological effects of N, ZR, and AVG application in suppressing leaf senescence and improving whole-plant heat tolerance, as discussed below.

A total of 41 polar metabolites were quantified in leaves of creeping bentgrass exposed to heat stress and treated with N, ZR or AVG, out of which 11 were organic acids, 20 were sugars or sugar alcohols, and 10 were amino acids (Table 1). Out of the 41 metabolites, 38 had significantly altered levels in N, ZR, or AVG treated plants compared to untreated plants (Fig 2, Table 2). The application of N resulted in an increase in the content of 24 metabolites, a decreased content for 8 metabolites, and no significant effects on 9 metabolites relative to the untreated control at 28 d of heat stress (Fig 2). The application of ZR caused increases in the content of 15 metabolites, decreased content for 12 metabolites, and no significant effects for 14 metabolites (Fig 2). The application of AVG led to increases in the content of 15 metabolites and decreases in the content of 13 metabolites and did not have significant effects on 13 metabolites (Fig 2).

The application of N, AVG, or ZR resulted in significant increases in the content of a number of organic acids at 28 d of heat stress. All three exogenous treatments resulted in increased levels of aconitic acid, citric acid, and malic acid (Fig. 3). Both AVG and ZR treatments caused an increase in oxalic acid, while AVG or N treatment

resulted in an increase in pyruvic acid. Additionally, treatment with ZR or N lead to significant increases in galacturonic acid and glucaric acid.

For sugars, application of N increased the level of cellobiose, glucose, and L-threitol compared to the untreated plants (Fig. 4, 5, 6). Treatment with AVG resulted in the increase of lactose and glycerol content. Treatment with N or AVG also caused an increase in galactinol and sucrose. The application of ZR or N caused an increase in myo-inositol content. The application of ZR or AVG reduced the level of glucose, and lyxose. The content of glucose increased with N-treatment during heat stress compared to control plants. All three exogenous treatments caused an increase in mannobiose, melibiose, and sorbitol. Six sugars (arabinofuranose, fructose, galactose, maltose, sorbopyranose, and xylulose) exhibited decline in their content with N, AVG or ZR treatment.

The application of N, ZR and AVG had differential effects on individual amino acids. Treatment with AVG resulted in decreases of threonine and gamma-aminobutyric acid (GABA), while N treatment caused an increase in the content of GABA (Fig. 7). Both ZR and N treatment resulted in an increase in serine and isoleucine content. All three exogenous treatments resulted in increases in the content of aspartic acid, glutamic acid, and glycine.

Classification of Major Metabolites Responsive to N, ZR, or AVG and Associated Contributions to Suppression of Heat Injury

Principle component analysis (PCA) separated metabolites responsive to N, ZR, or AVG under heat stress into different principle components, and determined the contribution of each component to the overall variations in metabolite accumulation due to the effects of N, ZR, or AVG (Fig. 8). PCA was also used to identify metabolites

which had the greatest contribution to the differences between treatment groups.

Metabolites with the highest eigenvalues for a principle component (top 15%) were considered to have the greatest impact for a component. The first and second principle component accounted for 44.2% and 31.4% of variations in metabolite responses to N, ZR, or AVG treatments, respectively. Metabolite responses to untreated control were distinctly separated from those responsive to N, ZR, or AVG application within the first component of PCA while those responsive to N were distinctly separated into a group from those responsive to AVG or ZR treatment within the second component of PCA analysis.

Partial least squares regression (PLS) was performed to confirm specific metabolites which may play a role in overall heat tolerance by modeling the relationship between metabolite levels and chlorophyll content as an indicator of heat-induced leaf senescence, subsequent variable importance projections were plotted (Fig 9).. A number of metabolites, which are of potential importance for differences between treatments groups resulting in suppressed leaf senescence, were identified by both PCA and PLS analyses including sugars (arabinofuranose, fructose, galactose, melibiose, and sorbopyranose), sugar alcohol (sorbitol), and organic acids (citric acid). Other metabolites of potential importance were identified by one of the two analyses, including glycine (in PCA analysis) and sucrose (in PLS analysis).

DISCUSSION

Physiological results demonstrated that exogenous applications of AVG, ZR, or N resulted in the suppression of heat-induced leaf senescence or improvement in heat tolerance of creeping bentgrass, as manifested by increased TQ, chlorophyll content and membrane stability. The positive physiological effects of those compounds that could be associated with metabolic changes found through metabolic profiling are discussed below.

Accumulation of Sugars and Sugar Alcohols as Affected by AVG, N, and ZR in Relation to Heat-Induced Leaf Senescence

The most abundant disaccharide found in this study was sucrose. Sucrose is a major soluble sugar in plants for the storage and transport of carbon fixed through photosynthesis (Wold, 1994). Higher levels of sucrose have been associated with enhanced heat tolerance in grass species (Du et al., 2011; Xu et al., 2013). PLS analysis also associated sucrose levels with delayed leaf senescence as indicated by chlorophyll content levels in this study, indicating that it may be an important metabolite for stress tolerance. In addition to its primary role in metabolism, sucrose can also act as a signaling molecule effecting the regulation of other metabolic pathways (Koch, 2004). Other disaccharide species, including lactose and melibiose accumulated during stress events can act as protective osmolytes which help maintain the integrity of membranes and proteins, as well as maintain cell hydration levels (Dracup et al., 1986; Leshem and Kuiper, 1996). The effects of AVG, ZR, and N on heat stress in creeping bentgrass were previously found to result in the up-regulation photosynthetic proteins such as ribulose-1,5-bisphosphate carboxylase oxygenase and chlorophyll a/b binding protein potentially resulting in an increase in carbohydrate synthesis (Jespersen and Huang, 2015). Increased

levels of cellobiose, lactose, mannobiose, melibiose, and sucrose found with the AVG and N treatment reflected active photosynthetic supply of carbohydrates and increased carbohydrate reserves as disaccharides, which could play roles in protecting leaves of creeping bentgrass from prolonged periods of heat stress.

Monosaccharides, such as glucose and fructose produced through photosynthesis are readily utilized in respiratory metabolism for energy production, which is critically important for plant survival of stresses (Nilsen and Orcutt, 1996). With the exception of increases in the content of glucose with N treatment, the content of all other monosaccharides decreased with exogenous application of N, ZR, or AVG under heat stress. During heat stress respiration rate and respiratory demand for monosaccharides typically increase, which may lead to reduced accumulations of those sugars (Wahid et al., 2007; Lyons et al., 2007). Decreases in content of monosaccharides, including glucose and fructose, during heat stress have previously been reported in grass species (Du et al., 2011; Xu et al., 2013; Yu et al., 2012). The maintenance of respiration levels may represent more actively growing tissue, as well as generation of ATP for important stress defense mechanisms such as antioxidative functions, although increased amount of sugars may be consumed or simple sugar content may decline (Couee et al., 2006; Saradadevi and Raghavendra, 1992). Both PCA and PLS analysis associated fructose levels with differences in heat tolerance between treatment groups, supporting that it may be an important factor affecting heat tolerance. The decrease in the content of monosaccharides (arabinose, fructose, galactose, glucose, lyxose, sorbopyranose, and xylulose) in N, AVG or ZR-treated plants compared to the untreated control during heat

stress may reflect an increase in carbohydrate consumption or utilization for the maintenance of respiratory metabolism under heat stress.

Sugar alcohols or polyols, such as galactinol, glycerol, myo-inositol, and sorbitol are reduced sugars containing several hydroxyl groups, which act as compatible solutes regulating osmotic adjustment and protecting cells from dehydration damages (Shen et al., 1999; Taji et al., 2002; Wang and Stutte, 1992; Williamson et al., 2002). Sugar alcohols also have the ability to act as antioxidants which neutralize reactive oxygen species (Nishizawa et al., 2008; Smirnoff and Cumbes, 1989). Additionally myo-inositol has a role in signal transduction, as it binds to other molecules to form secondary messengers (Gillaspy, 2011). The greater accumulation of sugar alcohols in plants treated with AVG, ZR, or N compared to the untreated control under heat stress suggested that those sugar alcohols could be involved in the suppression of heat-induced leaf senescence by AVG, ZR, or N.

Accumulation of Organic Acids as Affected by AVG, N, and ZR in Relation to Heat-Induced Leaf Senescence

Exogenous treatment with AVG, ZR or N resulted in higher accumulations of organic acids (aconitic acid, citric acid, galacturonic acid, glucaric acid, malic acid, oxalic acid, phosphoric acid, and pyruvic acid) compared to the untreated plants exposed to heat stress. Many of these organic acids with higher accumulation are intermediates of the tricarboxylic acid (TCA) cycle of respiration, including citric acid, aconitic acid, malic acid, and pyruvic acid. The increase in those TCA intermediates may represent more active mitochondrial respiration for the generation of ATP, but many of these intermediates can also feed into other metabolic pathways, which are important for

regulating various cellular functions, such as nitrogen assimilation and redox balance (Sweetlove et al., 2010). The increased accumulation of organic acids in AVG, ZR, or N-treated plants corresponded with the decreased content of monosaccharides as substrates in respiration, which together suggested that the application of N, AVG, and ZR could help leaves of creeping bentgrass maintain more active energy metabolism and also reflected the positive effects of those treatments on the alleviation of leaf senescence or damages induced by heat stress.

Accumulation of Amino Acids as Affected by AVG, N, and ZR in Relation to Heat-Induced Leaf Senescence

The application of N, ZR, and AVG had differential effects on different amino acids content. The application of N resulted in increases in the content of GABA, glycine, aspartic acids, glutamic acid, and serine. Amino acids are major nitrogen containing cellular constituents (Taiz and Zeiger, 2010). The increase in amino acid content with exogenous applications of N may be due to higher levels of nitrogen available for the plant to assimilate into amino acids. Nitrogen status has previously been shown to affect free amino acid content (Darrall and Wareing, 1981; Barneix et al., 1984). GABA is a non-protogenic amino acid which has also been found to increase in response to stresses (Kinnersley and Turano, 2000) potentially serving as a signaling molecular, and also participates in other metabolic processes, including carbon and nitrogen balance (Bouché and Fromm, 2004; Fait et al., 2008). The increased accumulation of GABA with N treatment under heat stress suggested that this metabolite could play protective roles of leaves from heat-induced leaf senescence, although the underlying mechanisms deserve further investigation.

Higher accumulations of aspartic acid, glutamic acid, and glycine were observed with AVG, ZR, and N treatment under heat stress. Aspartic acid is a major precursor to many other amino acids (Azevedo et al., 2006) including isoleucine and glycine and its accumulation may represent an important shift in amino acid metabolism for enhanced heat tolerance. Similar to aspartic acid, glutamic acid is another amino acid which is a precursor to many other amino acids (Beale et al., 1975). Additionally glutamic acid plays important roles in nitrogen metabolism as well as chlorophyll biosynthesis (Forde and Lea, 2007). The increase in glutamic acid may improve heat tolerance by improving chlorophyll production, as well as improving the integration of nitrogen into other cellular molecules. Glycine is a product of photorespiration and is the precursor for the synthesis of glutathione, purines, and porphyrins (Meister, 2012; Noctor et al., 1997). PCA analysis found glycine to be one of the metabolites responsible for a greater percent of variance between treatment groups. Increased accumulations of glycine may represent a more active photorespiration cycle averting heat stress damages.

Both ZR and N treatments also enhanced the accumulation of isoleucine and serine in plants exposed to heat stress. Serine is another important amino acid involved the recycling of metabolites during the photorespiratory cycle which has been associated with improved stress tolerance (Moreno et al., 2005). Isoleucine can feed into the TCA cycle, as well as help generate redox potential, to help maintain respiration rates (Kirma et al., 2012), and has also shown to play roles in jasmonate signaling (Staswick and Tiraki, 2004). The increase in isoleucine may potential increase heat tolerance by helping maintain signaling pathways or TCA cycle activity. Proteomic profiling of plants treated with AVG ZR or N during heat stress found increases in proteins, such as glycine

decarboxylase or aminomethyltransferase associated with photorespiratory glycine cleavage, or ferredoxin dependent glutamate synthase support shifts in metabolism associated with these amino acids (Fu and Huang, 2007). Metabolite results indicated that the enhanced accumulation of those amino acids associated with nitrogen balance, photorespiration or which are important biosynthetic precursors may represent important shift in metabolism resulting in delayed heat-induced senescence.

Conclusions

Exogenous treatment of creeping bentgrass with AVG, ZR or N resulted in improved heat tolerance compared to an untreated control, demonstrated by improved membrane stability, chlorophyll content and overall quality. In general metabolic changes included increased amino acid, organic acid, disaccharide and sugar alcohol content, and lower monosaccharide content with exogenous treatment with AVG, ZR or N compared to the untreated control under heat stress, which are involved in osmoregulation, antioxidant metabolism, carbon and nitrogen metabolism, as well as stress signaling molecules. Future research will identify underlying mechanism how changes in individual metabolites affected by AVG, N, or ZR may be involved in the suppression of heat-induced leaf senescence in cool-season grass species. Such information will provide further insights into metabolic and molecular factors controlling heat-induced leaf senescence and also is useful to identifying metabolites that may be incorporated into chemical products alleviating heat-induced leaf senescence through exogenous applications in cool-season turfgrass management.

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Table 1. The 41 metabolites identified by GC-MS at 28 d heat stress and there respective retention times (RT).

	Metabolite	RT
1	Acetic acid	9.33
2	Aconitic acid	26.36
3	Citric acid	27.84
4	Galacturonic acid	46.26
5	Glucaric acid	27.78
6	Gluconic acid	31.66
7	Glyceric Acid	16.28
8	Malic acid	20.37
9	Oxalic acid	11.06
10	Phosphoric acid	12.07
11	Pyruvic acid	8.62
12	Arabinofuranose	26.61
13	Cellobiose	43.45
14	Fructose	28.91
15	Galactinol	46.99
16	Galactose	29.27
17	Glucose	29.41
18	Glycerol	14.79
19	Gulose	34.87
20	Lactose	34.36
21	L-Threitol	26.15
22	Lyxose	24.79
23	Maltose	40.17
24	Mannobiose	42.54
25	Melibiose	48.91
26	Myo-inositol	33.09
27	Sorbitol	30.05
28	Sorbopyranose	27.71
29	Sucrose	42.22
30	Trehalose	43.69
31	Xylulose	22.44
32	Alanine	10.05
33	Aspartic acid	21.13
34	GABA	21.32
35	Glutamic acid	23.50

36	Glycine	10.57
37	Isoleucine	15.28
38	Proline	15.35
39	Serine	17.08
40	Threonine	17.75
41	Valine	13.14

Table 2: Metabolite levels during heat stress for AVG, ZR, N-treated and control plants.

Relative quantities of metabolites and statistical groupings for AVG, ZR, N and Control treatments at 28 days heat stress. Relative values are calculated from an internal ribitol standard. Letters represent LSD groupings for a given metabolite with treatments sharing a letter not being significantly different at $p=0.05$.

	AVG		ZR		N		Control		LSD value
Metabolite:	Relative value	LSD group	Relative value	LSD group	Relative value	LSD group	Relative value	LSD group	
Acetic acid	0.215	A	0.232	A	0.129	B	0.234	A	0.025
Aconitic acid	3.699	A	2.658	B	2.129	C	1.781	D	0.275
Citric acid	1.316	A	1.219	B	1.134	C	0.889	D	0.063
Galacturonic acid	0.165	C	0.218	B	0.242	A	0.171	C	0.015
Glucaric acid	0.368	C	0.436	B	0.544	A	0.368	C	0.037
Gluconic acid	0.176	A	0.163	A	0.056	A	0.164	A	0.356
Glyceric Acid	0.670	A	0.869	A	2.162	A	0.629	A	2.385
Malic acid	2.674	A	2.754	A	3.063	A	1.820	B	0.472
Oxalic acid	2.703	A	2.338	B	2.024	C	2.122	C	0.187
Phosphoric acid	0.021	AB	0.025	AB	0.026	A	0.015	B	0.011
Pyruvic acid	0.015	A	0.006	BC	0.008	B	0.004	C	0.004
Arabinofuranose	1.008	C	1.157	B	1.217	B	1.484	A	0.074
Cellobiose	0.189	B	0.199	B	0.248	A	0.181	B	0.020
Fructose	11.667	C	11.228	C	13.231	B	16.624	A	0.668
Galactinol	2.506	AB	2.304	BC	2.634	A	2.038	C	0.289
Galactose	1.678	D	2.604	C	3.721	B	4.484	A	0.330
Glucose	10.009	C	10.438	C	13.104	A	11.634	B	0.778
Glycerol	2.538	A	2.411	AB	2.385	AB	2.021	B	0.486
Gulose	9.778	A	8.773	A	10.504	A	8.274	A	2.238
Lactose	2.097	A	1.501	B	1.486	B	1.424	B	0.399
L-Threitol	0.227	B	0.243	B	0.350	A	0.227	B	0.030
Lyxose	0.182	C	0.229	B	0.233	AB	0.242	A	0.012
Maltose	0.743	C	0.676	D	0.937	B	1.018	A	0.033
Mannobiose	0.150	B	0.141	C	0.176	A	0.124	D	0.004
Melibiose	3.808	A	3.580	AB	3.306	B	2.540	C	0.285
Myo-inositol	2.232	BC	2.772	A	2.523	AB	2.128	C	0.314
Sorbitol	0.120	A	0.095	B	0.095	B	0.083	C	0.004
Sorbopyranose	0.646	C	0.876	B	0.860	B	1.505	A	0.106
Sucrose	66.112	A	56.702	B	65.171	A	56.317	B	3.079
Trehalose	0.196	A	0.131	C	0.163	B	0.203	A	0.011
Xylulose	0.257	B	0.276	B	0.272	B	0.325	A	0.031
Alanine	0.339	B	0.363	B	0.440	A	0.464	A	0.026
Aspartic acid	0.069	C	0.072	B	0.082	A	0.046	D	0.003
GABA	0.755	C	0.998	B	1.433	A	1.008	B	0.046

Glutamic acid	0.371	B	0.398	B	0.441	A	0.242	C	0.036
Glycine	0.034	B	0.038	A	0.023	C	0.018	D	0.002
Isoleucine	0.015	B	0.022	A	0.023	A	0.014	B	0.002
Proline	0.428	B	0.316	C	0.649	A	0.661	A	0.029
Serine	0.359	C	0.549	B	0.644	A	0.359	C	0.034
Threonine	0.254	C	0.308	BC	0.442	A	0.361	B	0.057
Valine	0.144	D	0.152	C	0.195	A	0.165	B	0.004

Fig. 1. Physiological effects of AVG, CK, and N in creeping bentgrass. Changes to turf quality (A), chlorophyll content (B), and electrolyte leakage (C) during 28 d of 20/15 C or 35/30 C when treated with AVG, ZR, N or water as a control. Vertical bars represent least significance difference values between exogenous treatments at $p=0.05$.

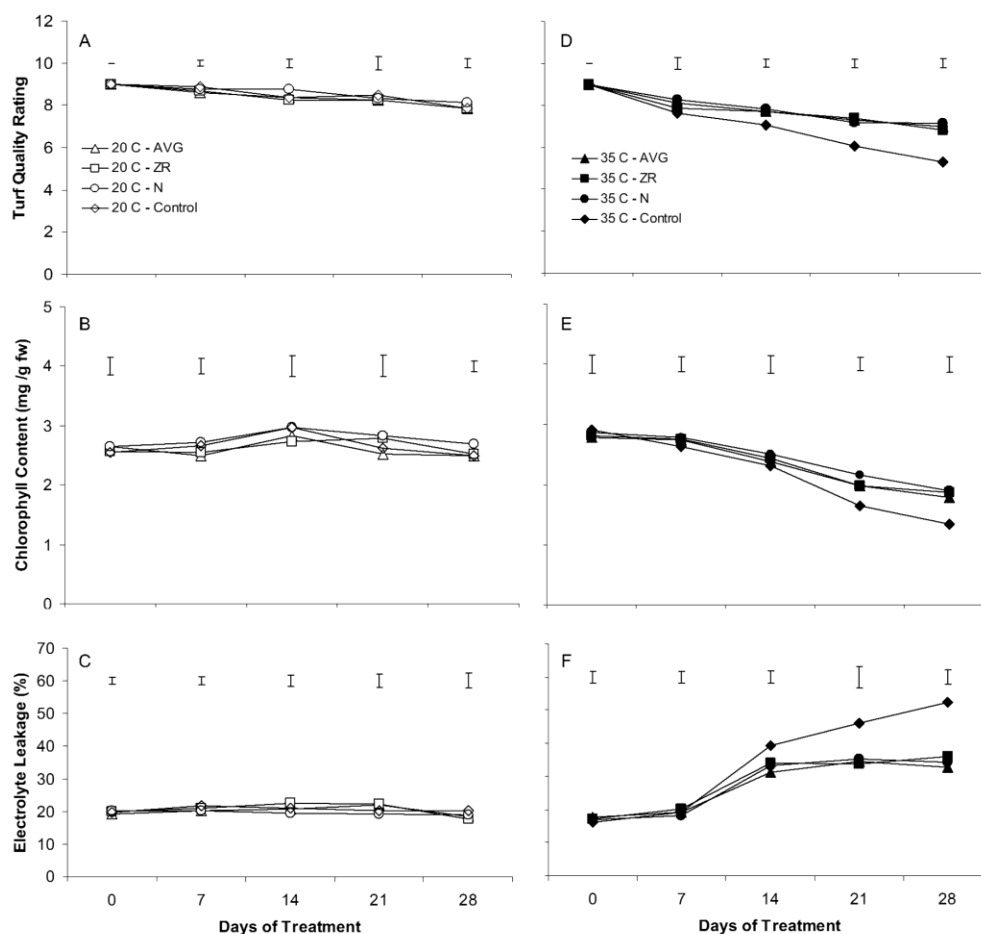


Fig. 2. Heat map of changes in metabolite levels in AVG, ZR, and N-treated plants at 28-d heat stress compared to the control. Heat map showing the fold change of each metabolite for AVG, ZR, and N-treated plants when compared against the control at 28-d Heat stress. Green indicates an increase in metabolite fold number or up-regulation, and red indicates a down-regulation of a specific metabolite compared to the control.

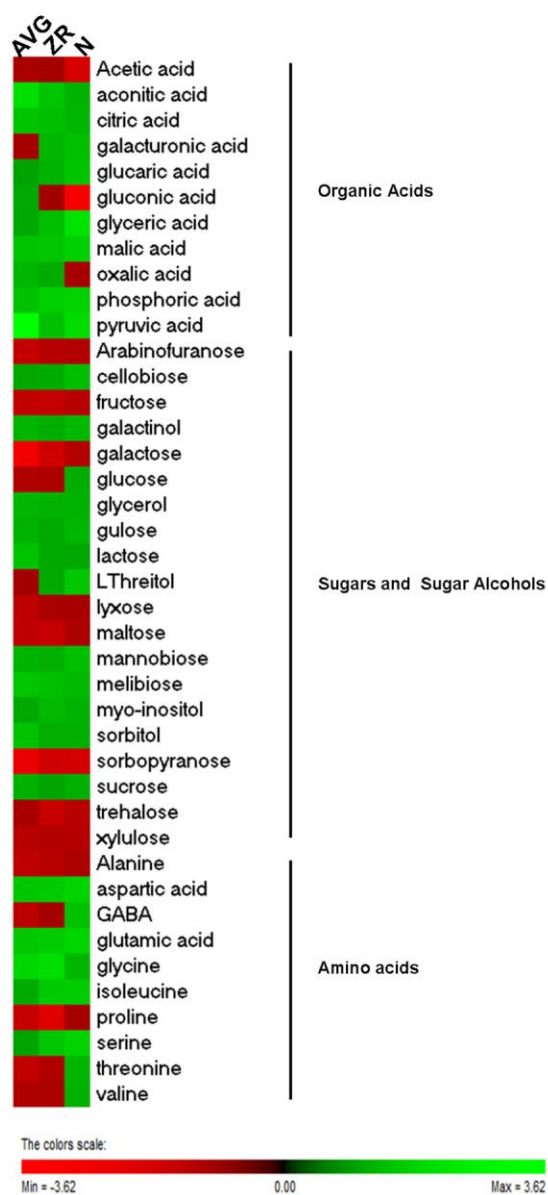


Fig. 3. Organic acid levels during heat stress for AVG, ZR, and N-treated plants, and the water-treated control. Relative quantities of organic acids at 28-d heat stress for AVG, ZR, and N-treated plants, and the water-treated control. Error bars indicate standard deviations and letters are statistical groups according to Fisher's protected LSD ($p = 0.05$), with groups not containing the same letter being significantly different. Only metabolites which had at least one group significantly different from the others are presented.

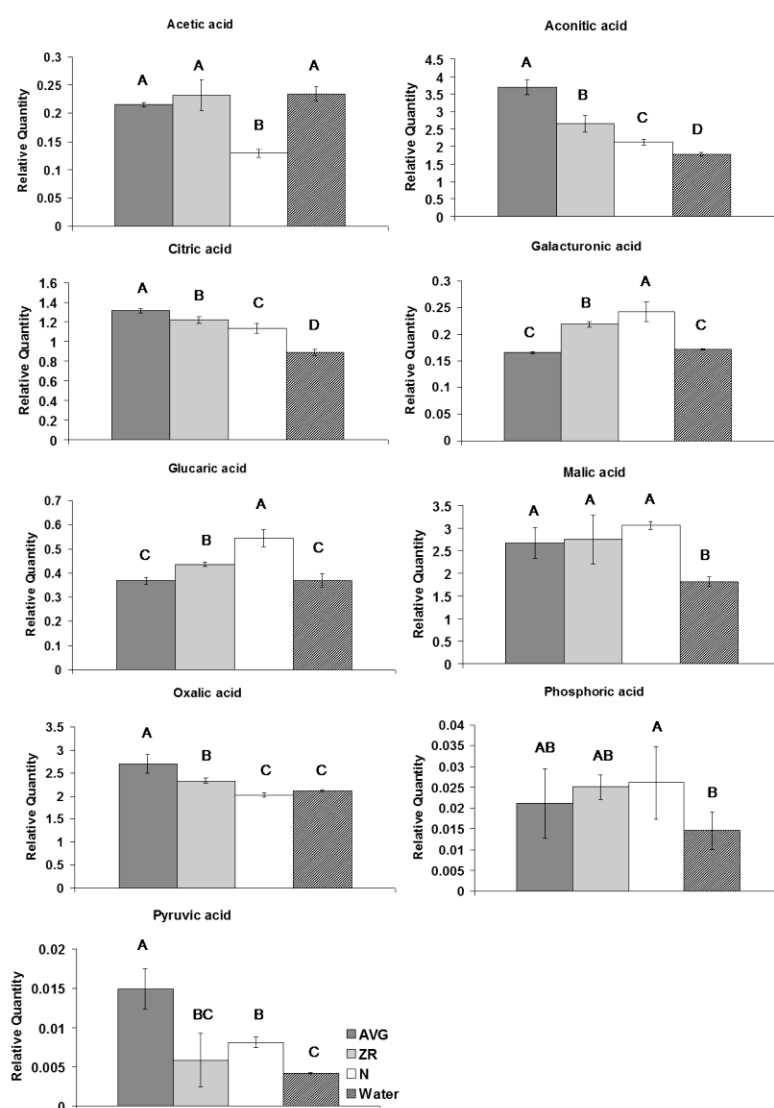


Fig. 4. Disaccharide levels during heat stress for AVG, ZR, and N-treated plants, and the water-treated control. Relative quantities of disaccharides at 28-d heat stress for AVG, ZR, and N-treated plants, and the water-treated control. Error bars indicate standard deviations and letters are statistical groups according to Fisher's protected LSD ($p = 0.05$), with groups not containing the same letter being significantly different. Only metabolites which had at least one group significantly different from the others are presented.

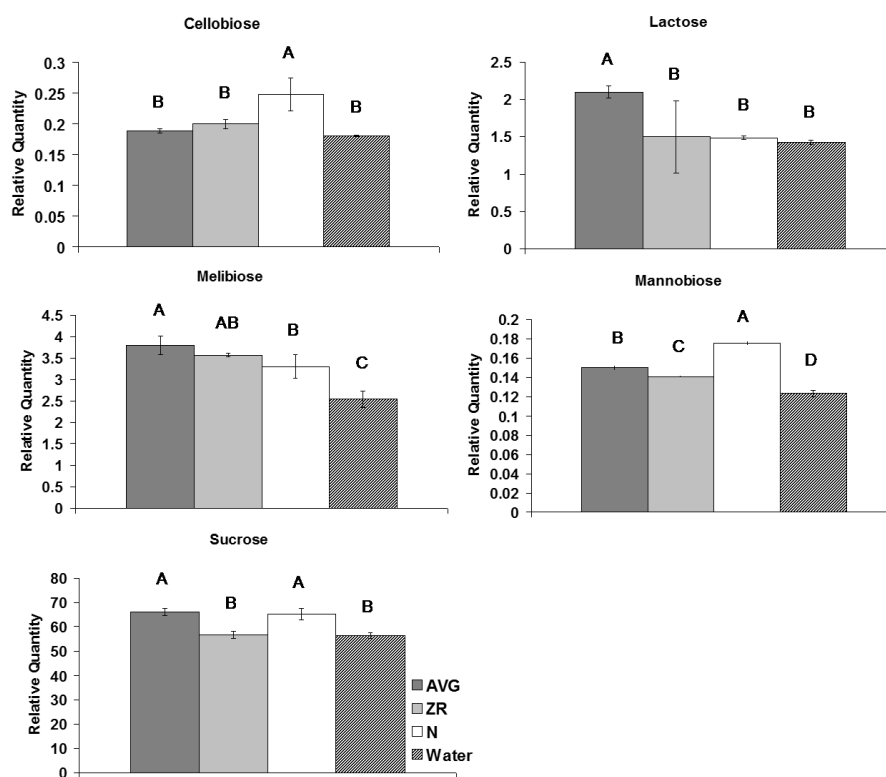


Fig. 5. Monosaccharide levels during heat stress for AVG, ZR, and N-treated plants, and the water-treated control. Relative quantities of monosaccharides at 28-d heat stress for AVG, ZR, and N-treated plants, and the water-treated control. Error bars indicate standard deviations and letters are statistical groups according to Fisher's protected LSD ($p = 0.05$), with groups not containing the same letter being significantly different. Only metabolites which had at least one group significantly different from the others are presented.

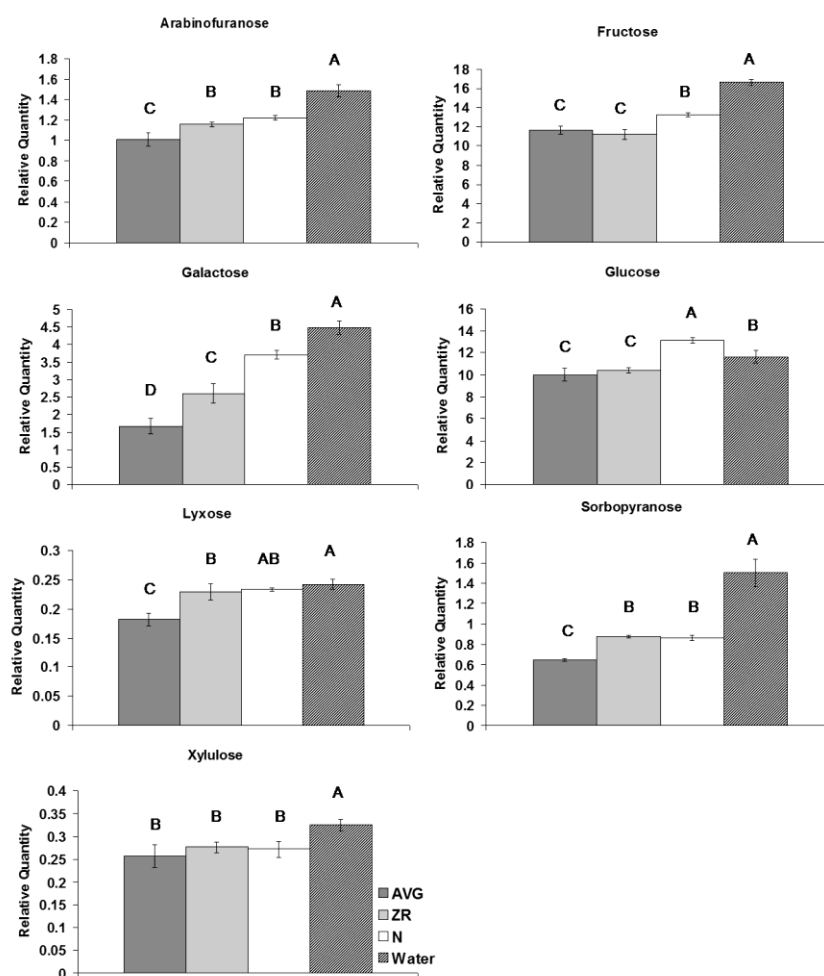


Fig. 6. Sugar alcohol levels during heat stress for AVG, ZR, and N-treated plants, and the water-treated control. Relative quantities of sugar alcohols at 28-d heat stress for AVG, ZR, and N-treated plants, and the water-treated control. Error bars indicate standard deviations and letters are statistical groups according to Fisher's protected LSD ($p = 0.05$), with groups not containing the same letter being significantly different. Only metabolites which had at least one group significantly different from the others are presented.

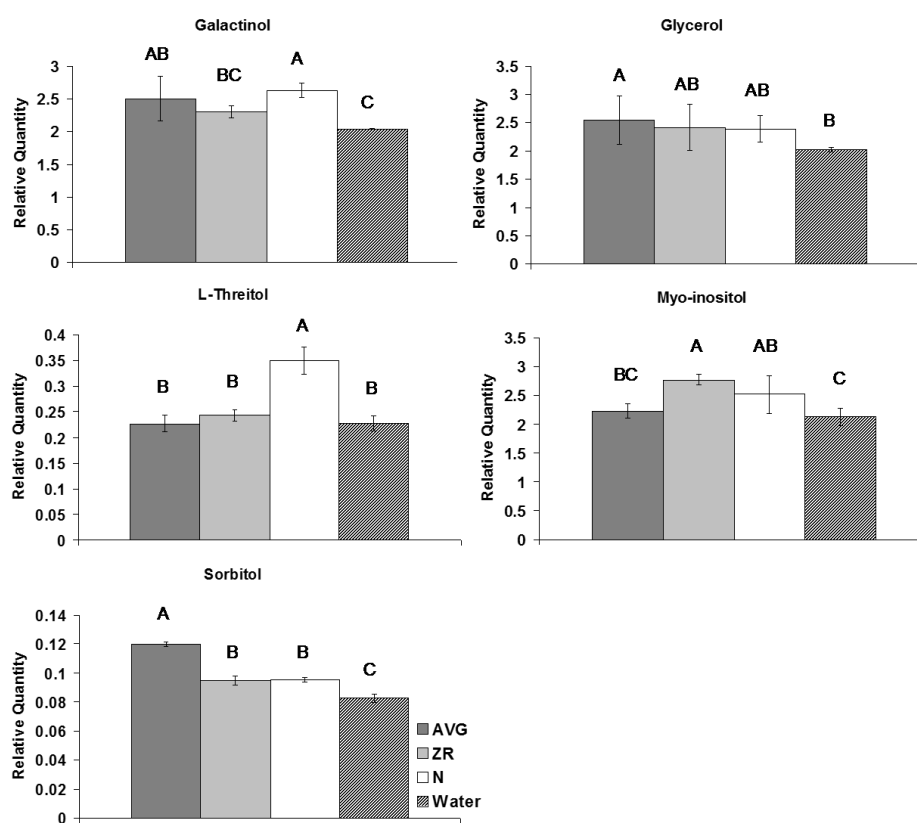


Fig. 7. Amino acid levels during heat stress for AVG, ZR, and N-treated plants, and the water-treated control. Relative quantities of amino acids at 28-d heat stress for AVG, ZR, and N-treated plants, and the water-treated control. Error bars indicate standard deviations and letters are statistical groups according to Fisher's protected LSD ($p = 0.05$), with groups not containing the same letter being significantly different. Only metabolites which had at least one group significantly different from the others are presented.

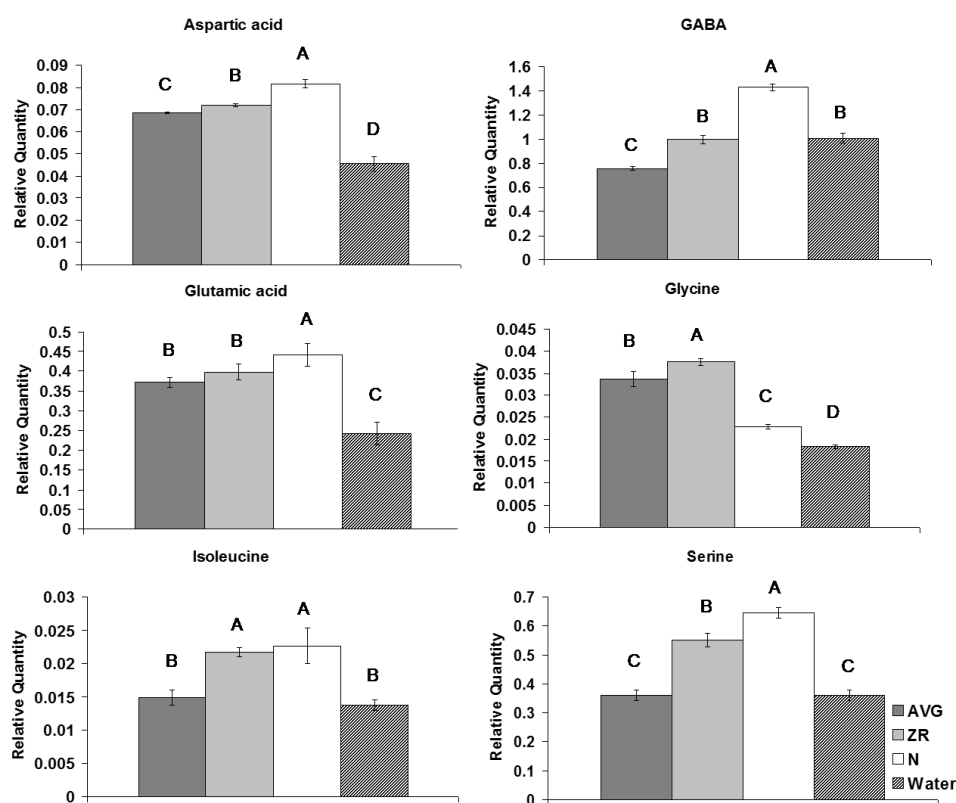


Fig. 8. Principle component plot of AVG, ZR, N or water treatment groups during heat stress. Principle component analysis based on metabolite levels during 28 d heat stress for plants treated with AVG, ZR, N, or the water control. Component one is represented on the X-axis and accounted for 44.2% of variance, and component 2 is represented on the Y axis and accounted for 32.4% of variance.

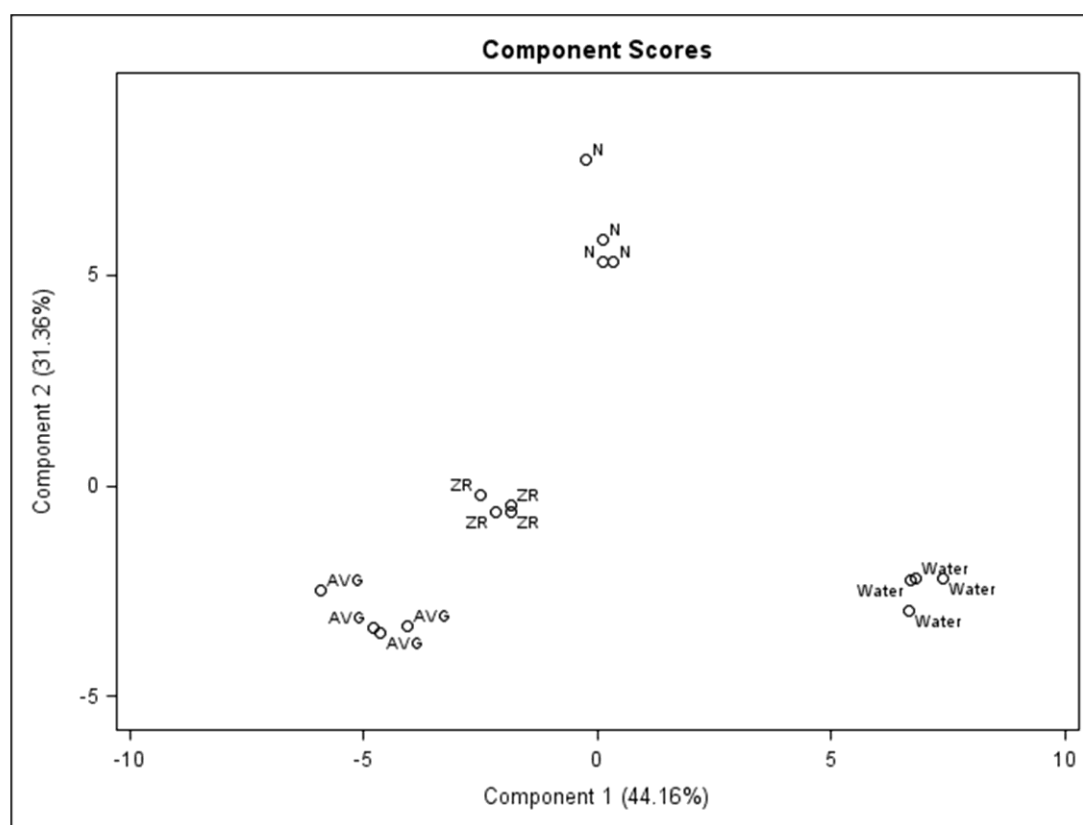
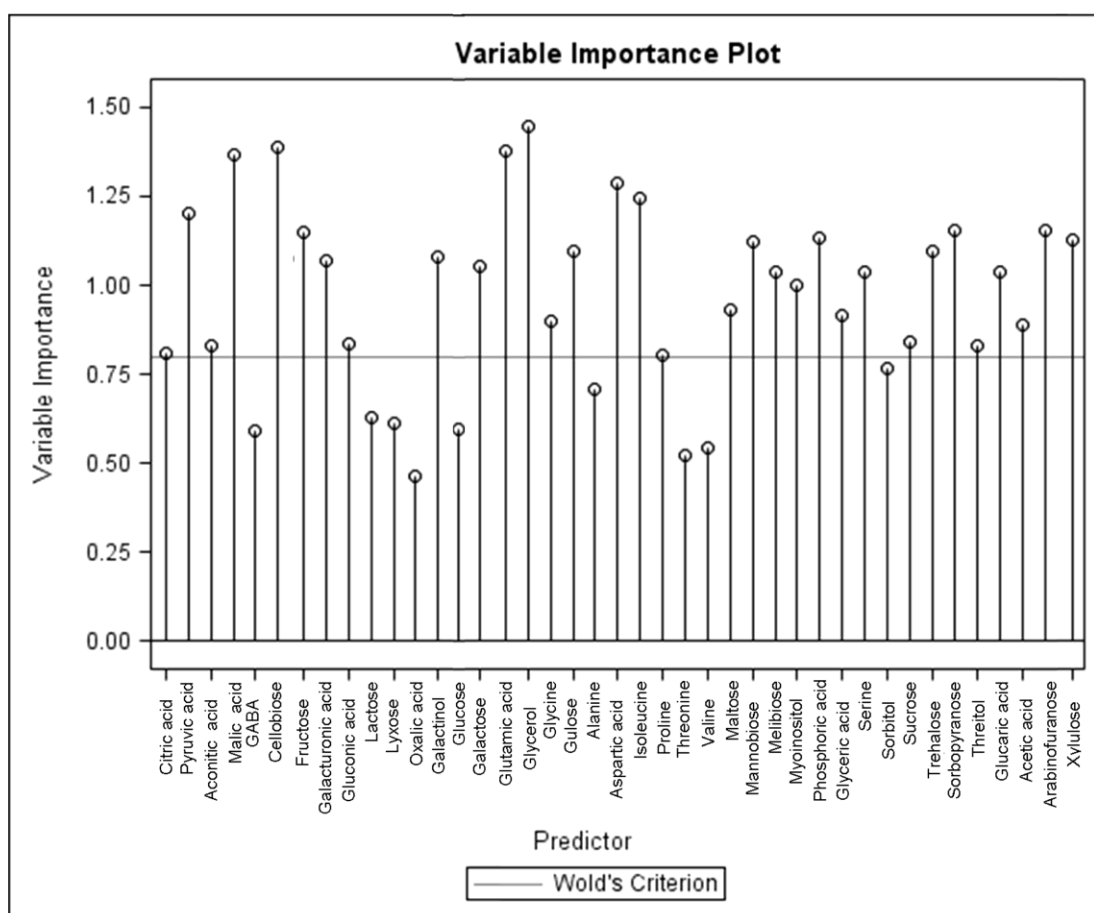


Fig. 9. Variable importance plot of metabolites during heat stress. Variable importance projection plot generated from the partial least squares regression highlighting which metabolites may potentially play a larger role in heat tolerance as predicted by the model. Wold's criterion is denoted by a horizontal line.



**CHAPTER 5: Quantitative Trait Loci Associated with Physiological Traits for Heat
Tolerance in Creeping Bentgrass**

Jespersen, D., E.B. Merewitz, Y. Xu, J. Honig, S.A. Bonos, W.A. Meyer. 2015. Crop Sci.
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INTRODUCTION

Creeping bentgrass (*Agrostis stolonifera*) is a cool-season turfgrass widely-used on golf courses, but it is sensitive to heat stress, often exhibiting turf quality decline during summer months when temperatures are elevated (Carrow, 1996; Fry and Huang, 2004). At the canopy level, high temperatures lead to a reduction in plant growth and thinning and yellowing or senescence of the turf canopy (Carrow, 1996). Physiological damages caused by heat stress include inhibition of transpirational cooling due to stomatal closure and the degradation of important cellular constituents, such as chlorophyll and cellular membranes, leading to leaf senescence (Wahid et al., 2007). The creation of improved varieties is the most feasible and sustainable way to increase plant tolerance to heat stress.

Traditional breeding efforts have resulted in moderate improvements to abiotic stress tolerance in bentgrasses, however, progress has been slow (Bonos and Huang, 2006). This is largely due to the complexity of abiotic stress tolerance, including heat stress, which is a polygenic trait controlled by many genes. Additionally the polyploid nature of creeping bentgrass, which is an outcrossing allotetraploid ($2x=4n=28$), further increases the difficulty of breeding efforts (Chakraborty, 2005). DNA markers have been created for creeping bentgrass, including RFLP, AFLP, RAPD and SSR markers (Chakraborty et al., 2005; Kubick et al., 2011; Golembiewski et al., 1997; Vergara and Bughrara, 2003), but a fully annotated genome for creeping bentgrass is not currently available. The use of marker assisted selection has the potential to greatly improve breeding efficiency and create improved lines for polygenic traits such as heat tolerance while maintaining other valuable characteristics associated with turf quality such as

density, color, or establishment (Fei, 2008). One way to select markers for marker assisted selection is through the identification of quantitative trait loci (QTL).

Using linkage maps created from molecular markers and screening mapping populations for a trait of interest, specific regions of the genome can be associated with quantitative traits, such as stress tolerance (Staub et al., 1996). QTL are regions of the genome statistically associated with these traits of interest (Jones et al., 1997). QTL analysis has been used to find genetic regions associated with heat tolerance in major agronomic crops, such as rice (*Oryza sativa*) (Ye et al., 2012; Zhang et al. 2009) and wheat (*Triticum aestivum*) (Mason et al., 2011; Paliwal et al., 2012; Vijayalakshmi et al., 2010). However there are relatively less genetic resources available to turfgrass species compared to major agronomic species and relatively fewer QTL have been identified. To date most QTL regions identified in cool-season turfgrasses have been related to disease resistance, including dollar spot in creeping bentgrass (Chakraborty et al., 2014; Honig et al., 2014), gray leaf spot in perennial ryegrass (*Lolium perenne*) (Curley et al., 2005) and Italian ryegrass (*Lolium multiflorum*) (Takahashi et al., 2014), and crown rust on perennial ryegrass (Sim et al., 2007). Recently Merewitz et al. (2012) identified several QTL regions associated with drought tolerance in a hybrid creeping x colonial (*Agrostis capillaris*) bentgrass population. To our knowledge, QTL markers linked to physiological traits for heat tolerance in cool-season turfgrass species have not been well documented.

The objective of this study was to identify QTL regions and genetic markers associated with heat tolerance in creeping bentgrass. A previously developed mapping population and linkage map, which was used for the identification of QTL associated with dollar spot and drought tolerance (Honig et al., 2014; Merewitz et al., 2014), was

used in this study. To assess phenotypic variation in heat tolerance, a number of commonly used physiological measurements associated with heat stress were taken, including visual turf quality, chlorophyll content, membrane stability, and canopy temperature depression under different environmental conditions (two-year field trials, greenhouse, and control-environment growth chambers). Identified QTL regions could be potentially used for marker assisted selection and ultimately aid in the creation of more heat tolerant bentgrasses through marker assisted selection and help better understand the underlying genetics of heat tolerance in bentgrass.

MATERIALS AND METHODS

Mapping Population and Linkage Map

A creeping bentgrass mapping population previously described by Honig et al. (2014), which showed a range of phenotypic responses to abiotic stress, was used. The mapping population was developed by crossing a dollar spot resistant clone of the cultivar L93 (L93-10) with a dollar spot sensitive clone (7418-3) collected from Piping Rock Golf Course in Long Island, NY. The population used for linkage map creation consisted of 180 pseudo F_2 individuals and linkage maps were created using 200 simple sequence repeat markers (SSR), 153 amplified fragment length polymorphisms (AFLP), 33 conserved intron-scanning primers (CISP), and 59 intron length polymorphism (ILP) markers. Linkage maps were created for each parent (L93-10 and 7418-3) and each consisted of 14 linkage groups corresponding to 7 pairs of homoeologous chromosomes. Linkage groups are numbered by syntenic relationships to rice and the previously developed creeping bentgrass map by Chakraborty et al. (2005). Additional details on the creation of the mapping population and linkage maps as well as marker designations can be found in Honig et al. (2014).

A group of 100 individuals selected from the pseudo F_2 population were used to screen for heat tolerance and identify QTL in this study. The relatively smaller number of individuals used for this population may result in an overestimation of identified QTL effects but unfortunately greater numbers could not be used due to space limitations in field and growth chamber environments. This same subset of individuals has previously been used for the identification of QTL regions associated with drought tolerance (Merewitz et al., 2014). Phenotypic screening for heat stress responses was performed

using this subset of individuals with four trials across three different environments, including field trials in summer 2008 and 2009, an environmental controlled growth chamber trial in 2009, and a greenhouse trial in summer 2010.

Field Trials

Field trials were conducted in mowed spaced-plant plots planted with 100 individuals of the mapping population at the Rutgers University turfgrass research farm, Hort Farm II, in North Brunswick, NJ. Each plant was cloned to obtain 4 replicates of each of the 100 progeny and the parents. The soil type was a fine-loamy mixed mesic type Hapludult soil. Measurements were taken monthly during summer months from June through August of both years, and included a visual turf quality rating, chlorophyll content, electrolyte leakage, canopy temperature depression, and multispectral radiometer readings for normalized difference vegetative index and leaf area index as described below. A weather station approximately 130 m away from the field tracked the temperature during the trials. The 2008 field trial had an average temperature of 23.3 °C with an average maximum temperature of 31.3 °C across July and August. Temperatures for the 2009 field trial had an average temperature of 23.6 °C with an average maximum temperature of 28.3 °C averaged across July and August. Applications of a 16-4-8 (N-P-K) fertilizer were made in July and November of both 2008 and 2009 at a rate of 0.29 g N m². The turf was mowed weekly to maintain a canopy height of approximately 5.0 cm and was maintained at well watered conditions throughout the summer trials. Pesticide applications were made as part of standard preventative or curative regimes using the labeled rates and frequencies for bentgrasses. Treatments included weed control using Dithiopyr (S,S-dimethyl 2-(difluoromethyl)-4-(2-methylpropyl)-6-(trifluoromethyl)-3,5-

pyridinedicarbothioate; Dimension, Dow AgroSciences), dicamba (3,6-dichloro-2-methoxybenzoic acid; Banvel; BASF), and clopyralid (3,6-dichloropicolinic acid; Lontrel; Dow AgroSciences), to control fungal pathogens flutolanil (N-[3-(1-methylethoxy)-phenyl]-2-(trifluoromethyl)-benzamide; ProStar 70 WDG, AgrEvo TM), mefenoxam (R,S-2-[(2,6-dimethylphenyl)-methoxyacetylaminol]-propionic acid methyl ester; Subdue GR, Syngenta), and chlorothalonil (tetrachloroisophthalonitrile; Daconil Ultrex) were used, and to control insects bifenthrin (2-Methyl-3-phenylphenyl)methyl (1S,3S)-3-[(Z)-2-chloro-3,3,3-trifluoroprop-1-enyl]-2,2-dimethylcyclopropane-1-carboxylate; Wisdom; Amvec) was used.

Growth Chamber Trial

Plants of 100 clones from the mapping population were initially established in 10 cm plastic pots filled with sterilized sand in a greenhouse with 3 vegetative replicates of each clone. Plants were then moved into controlled-environmental growth chambers (Conviron, Winnipeg, Canada) one week prior to the imposition of heat stress to allow for plants to acclimate. Growth chambers were set to a 14-h photoperiod with $650 \mu\text{mol m}^{-2} \text{s}^{-1}$ photosynthetically active radiation (PAR), 60% relative humidity and set to 20°C during the acclimation period. Throughout the establishment period and the trial period, plants were well watered, fertilized weekly with half-strength Hoagland's nutrient solution (Hoagland and Arnon, 1950) and cut to a height of 5 cm. Temperatures were increased to 35/30 °C day/night temperatures for 6 weeks to screen for heat tolerance in three growth chambers (three replicates for heat stress for each clone). Plants were rotated among the environmentally control growth chambers to avoid any potential confounding effects. Measurements were taken at 0, 3 and 6 weeks of heat stress and included a visual turf

quality rating, chlorophyll content, electrolyte leakage, and canopy temperature depression.

Greenhouse Trial

Three vegetative replicates of 100 clones from the mapping population were established in 10 cm plastic pots filled with sterilized sand in a greenhouse. Plants were well watered, fertilized weekly with half-strength Hoagland's nutrient solution, and trimmed to a height of 5 cm weekly. Light was provided by natural sunlight supplemented with automated sodium lamps to maintain 800 to 1000 $\mu\text{mol m}^{-2} \text{s}^{-1}$ PAR. Measurements were taken in July of 2010 after 5 weeks of elevated temperatures and included a visual turf quality rating, chlorophyll content, electrolyte leakage, and canopy temperature depression. The monthly average air temperature in July was approximately 30 ° C in the greenhouse. Plants were randomly moved throughout the benches in the greenhouse every 5 d to avoid confounding effects of different locations within the greenhouse.

Phenotypic Analysis of Heat Tolerance

Phenotypic variation in heat tolerance among the 100 clones of the mapping population were evaluated for a number of parameters, including a visual rating of turf quality, leaf chlorophyll content, leaf membrane stability, and canopy temperature depression. Turf grass quality (TQ) was visually rated as a subjective indicator of overall plant health based on color, density and uniformity of grass canopy on a 1-9 scale reflecting the levels of leaf senescence in the turf canopy, with 9 being the best in all three quality components representing a actively growing dense green canopy and 1 being completely dead plants (Beard, 1973). Chlorophyll content was measured using the procedures described by Hiscox and Israelstam (1979). Chlorophyll was extracted by

placing approximately 0.1 grams of fresh leaf tissue into 10 ml of dimethylsulfoxide, and allowed to incubate in darkness for 72 hours. The resulting extract was measured at 663 nm and 645 nm on a spectrophotometer (Spectronic Instruments, Inc., Rochester, NY). Chlorophyll content was calculated using the equations described by Arnon (1949) on a dry weight basis.

Membrane stability was estimated as electrolyte leakage using the methods described by Blum and Ebercon (1981). About 0.1g of fresh leaf tissue was placed in a 50 ml conical tube with 35 ml de-ionized water and incubated on a shaker for 16 h. A conductivity meter (YSI Incorporated, Yellow Springs, OH) was used to take an initial conductance reading ($C_{initial}$) of the incubated solution. Tubes were then autoclaved at 120 °C for 20 min to kill the leaf tissue. Tubes were incubated on the shaker for an additional 16 h and a final conductance reading was measured (C_{max}). Electrolyte leakage was calculated as a percent relative damage by finding the percentage of $C_{initial}$ to C_{max} .

Canopy temperature depression was measured by using a handheld infrared thermometer (Telatemp, Fullerton, CA) to compare the canopy temperature to the ambient air temperature. At least three measurements were taken per replicate and averaged to ensure consistent readings.

For both the 2008 and 2009 field trials, canopy reflectance values for visible and near-infrared spectrums were measured using a multispectral radiometer (MSR; model MSR-16, Cropscan Inc., Rochester, MN). A built in sensor integrates ambient light information with reflectance data to ensure consistent readings. Additionally, readings were only taken on cloudless days and always around the same time of day

(approximately 2 pm). The ratio of near infrared to red reflectance values was used to estimate ground cover or leaf area index (LAI) (Asrar et al., 1984; Trenholm et al., 1999). Additionally, normalized difference vegetative index (NDVI) is a commonly used method to detect green vegetation and was used to assess green leaf biomass and was calculated as $(\text{reflectance at } 935 - \text{reflectance at } 661) / (\text{reflectance at } 935 + \text{reflectance at } 661)$. NDVI using MSR has previously been used to measure turf health in response to abiotic stress in a number of studies (Fenstermaker-Shaulis et al. 1997; Jiang and Carrow, 2005; Xu and Huang, 2010).

Experimental Design and Statistics

All trials were a randomized complete block design, with 4 replicates of the 100 individuals in the 2008 and 2009 field trials and 3 replicates in both the growth chamber and greenhouse trials. Differences between individuals of the mapping population were analyzed using Proc GLM in SAS v9.2 using an ANOVA test and treatments found to have significant difference were tested by Fisher's protected LSD at $\alpha=0.05$ to separate the means, and Proc Corr was used for correlation analysis (SAS Institute Inc, Cary, NC). QTL analysis was performed using MapQTL 5.0 software (Kyazma software; van Ooijen, 2004). Interval mapping and Kruskal-Wallis analysis was used to identify potential QTL regions and markers associated with heat tolerance related traits. QTL for heat tolerance traits were identified for each individual trial separately. Permutation analysis was performed with 1000 iterations to determine the genome-wide threshold for LOD significance at $p < 0.05$ for each trait. Additionally automatic cofactor selection was used to identify cofactors ($p < 0.02$) and multiple QTL mapping (MQM) procedures was performed. Loci which surpassed the genome wide threshold for LOD significance were

classified as QTLs. Loci which did not meet the required LOD for significance but had a LOD score of > 3.0 and was associated with a marker significant at $p < 0.05$ by Kruskal-Wallis analysis was deemed a putative QTL. QTL regions were defined by ± 1 LOD to either side and were characterized as major ($R^2 > 10$) or minor ($R^2 < 10$) (Collard et al., 2005).

RESULTS

Phenotypic Variation in Heat Tolerance

A summary of mean, standard deviation, and ranges for phenotypic traits, including turf quality (TQ), chlorophyll content (CHL), electrolyte leakage (EL), canopy temperature depression (CTD), green leaf biomass (NDVI) and leaf area index (LAI) can be found in Table 1. There existed significant differences according to the ANOVA test for traits within the population showing segregation for phenotypic responses to heat stress, as well as differences between trials which experienced varying levels of heat stress and significant trait x trial interactions. These differences between trials could be due to numerous factors including humidity, light conditions, variability in temperature, wind, soils and other factors which are particularly variable under field conditions when compared to controlled environment conditions. These differences in environmental conditions could all affect the onset and duration of heat stress ultimately leading to differences in responses to heat stress during different trials.

Turf quality of 100 creeping bentgrass clones exhibited a wide range of variation during summer months of the field trials or under heat stress in controlled environment trials, ranging from 2.3 which represents severely damaged plants with much dead or senesced tissue, to 9.0 which represent very healthy plants with a dense green actively growing canopy, with an average of 7.06 for the 2008 field trial, from 2.5 to 8.75 with an average of 6.15 for the 2009 field trial, from 4.0 to 7.5 with an average of 5.63 for the growth chamber trial, and from 4.25 to 7.3 with an average of 5.94 for the greenhouse trial (Fig. 1).

A large amount of the variation in heat responses for CHL content were found across 100 creeping bentgrass clones (Fig. 2). Leaf CHL content ranged from 8.42 to 16.04 mg g⁻¹ with an average of 11.19 mg g⁻¹ for the 2008 field trial, 6.32 to 17.04 mg g⁻¹ with an average of 11.37 mg g⁻¹ for the 2009 field trial, 6.73 to 14.59 mg g⁻¹ with an average of 11.32 mg g⁻¹ for the growth chamber trial, and 9.05 to 16.25 mg g⁻¹ with an average of 12.11 mg g⁻¹ for the greenhouse trial (Fig. 2). For most environments, the population approached a normal distribution.

Leaf membrane stability as measured by electrolyte leakage was also variable among 100 creeping bentgrass clones (Fig. 3). The population average for leaf EL was lower in both years of the field trial, 19.78% and 21.48% for the 2008 and 2009 field trials respectively, when compared to the growth chamber trial which had an average of 30.84% and the greenhouse trial which had an average of 27.04%. Leaf EL ranged from 14.76 to 31.93 % for the 2008 field trial, 15.44 to 61.14 % for the 2009 field trial, 16.94 to 56.38% for the growth chamber trial, and 16.37 to 50.07% for the greenhouse trial (Fig. 3).

The CTD, which measured the difference between air and canopy temperature, was also variable among the clones in the mapping population (Fig. 4). However, the variation was greater in the field trials compared to the greenhouse and growth chamber trials (Fig. 4). The CTD values ranged from -3.18 to 6.90 for the 2008 field trial, -3.95 to 7.75 for the 2009 field trial, -0.65 to 3.28 for the growth chamber trial, and -3.22 to -0.55 for the greenhouse trial.

Canopy characteristics measured by canopy reflectance in the field trials showed NDVI index ranged from 0.39 to 0.87 and 0.63 to 0.80 for the 2008 and 2009 field trials

respectively, while LAI ranged from 2.41 to 14.19 in 2008 and 4.49 to 9.35 in 2009 (Fig. 5). Both measurements resulted in more variability in 2008 compared to 2009. The MSR used in the field trial requires uniform, high light conditions and specific distance of the scanner from the canopy to accurately measure canopy reflectance, which cannot accurately measure canopy reflectance in the growth chamber or greenhouse, and therefore these measurements were not taken in the growth chamber and greenhouse trials.

Identification of QTL Regions Associated with Heat Tolerance

A total of 19 QTL were found for the L93-10 parental linkage map, of which 8 were putative QTL (Table 2). All QTL were classified as major QTL since their phenotypic variance (R^2) was greater than 10 (Collard et al., 2005). The percent of explained variance for QTL regions ranged from 13.1% for NDVI in the 2008 field trial to 73.2% for LAI for the 2008 field trial, both associated with Os06g_05090CN(481) on linkage group 7.2. These QTL represent a total of 17 different regions across 9 linkage groups (Fig. 6). Linkage group 4.1 of the L93-10 parental linkage map had the highest density of QTL regions with a total of 7 QTL regions associated with 3 different traits, CHL, CTD, and TQ. Additionally overlapping QTL regions were found for CHL at 33-37 cM, CHL and TQ from 82-86 cM on linkage group 4.1, and for NDVI and LAI from 10-12 cM on linkage group 4.2.

A total of 13 QTL regions were found on the 7418-3 parental linkage map, of which five were putative QTLs (Table 3). This represents a total of 11 different regions across eight linkage groups (Fig. 6). All QTL were classified as major QTL since their phenotypic variance (R^2) was greater than 10. The percent of explained variance ranged

from 11.7% for a QTL region associated with the marker BRSC1_008P(420) for EL on linkage group 1.1 in the 2008 field trial to 66.5% for a QTL associated with EL in the 2008 field trial, associated with the marker GT1080(206) on linkage group 4.1. Linkage group 3.1 from the 7418-3 parental linkage map had the highest density of QTLs with 3 traits (CTD, NDVI, and TQ) representing 3 separate regions on the linkage map. On linkage group 3.1 from 48-49 cM, there was an overlapping QTL region for the traits TQ and CTD. Additionally from 74-75cM on Linkage group 5.1 there was an overlapping QTL region for CHL and LAI.

In addition to QTL regions identified by MQM interval mapping, a number of markers significantly associated with the measured heat tolerance traits were identified with non-parametric mapping using Kruskal-Wallis analysis, which does not take into account the linkage between markers. A total of 52 markers were significantly associated with traits at the $p = 0.001$ level across all four trials on the L93-10 parental linkage map, and 15 markers were significantly associated with traits at the $p = 0.001$ on the 7418-3 parental linkage map (Table 4).

DISCUSSION

A wide range of genetic variation in heat tolerance was observed among the 100 clones of the creeping bentgrass mapping population as measured through physiological traits (CHL, EL, CTD), phenotypic traits for turf color and density (NDVI and LAI), as well as an estimate of overall turf performance (TQ). As with most mapping populations, there was a normal distribution with some individuals having high levels of heat tolerance, and some with extremely low levels of heat tolerance, but with the majority falling somewhere in between. Summer bentgrass turf quality decline is highly associated with leaf senescence, which is characterized by leaf chlorosis and deterioration of cell membranes of leaves (Carrow, 1996; Marcum, 1998). Leaf chlorophyll content and membrane stability as estimated by CHL and EL are highly correlated to turf quality for plants exposed to heat stress and positively associated with heat tolerance (Marcum, 1998; Jespersen et al., 2013). Another aspect of heat stress that is associated with turf quality decline is the induction of stomatal closure, which leads to the increases in leaf or canopy temperature due to the loss of transpirational cooling effects (Walter et al., 2012). Plants that can maintain lower canopy temperature than air temperature or with lower or more negative CTD values are generally more heat tolerant (Bonos and Murphy, 1999; Reynolds et al., 1994). Canopy NDVI, and LAI reflect canopy greenness and density, and NDVI and LAI values are highly correlated to turf quality (Jiang and Carrow, 2005; Xu and Huang, 2010). Many of the measured physiological traits are correlated as the changes occur concurrently and may influence each other (Marcum, 1998; Jiang and Carrow, 2005; Jespersen et al., 2013). For example, a loss of chlorophyll may also affect NDVI and TQ ratings due to a loss of color from the turf canopy which will be reflected

in these measurements. The above mentioned traits may be important mechanisms for heat tolerance and play important roles in overall plant performance during heat stress resulting in differences in overall TQ ratings. The overlap between QTL for TQ and other traits, as discussed below, support that overall quality is influenced by these factors. The wide range of variation in all physiological and phenotypic traits (TQ, CHL, EL, CTD, and NDVI and LAI) for plant tolerance to heat stress, demonstrated that the mapping population segregated for heat tolerance and heat-tolerance traits associated with leaf senescence (CHL, EL), canopy cooling (CTD), and canopy color and density (TQ, NDVI, and LAI) could be genetically controlled although they could also be influenced by environmental factors, as demonstrated by not only significant difference between genotypes but also significant differences between trials. The power of a QTL to predict a trait is determined by how closely a marker is associated with that trait. The genotypic information coefficient (GIC) ranges from 0 to 1 with a score closer to one indicating that there is greater marker information and the marker is closer to the QTL region (van Ooijen, 2004). In this study, 18 of the 32 QTL regions found on both linkage maps had high GIC values close to one (> 0.9). These results indicated these markers could be closely associated with QTL regions for traits such as CHL, EL, NDVI and LAI and may be useful for marker assisted selection in improving heat tolerance.

Comparison of QTL in related species with greater genetic resources available may help identify syntenous regions which are important for heat tolerance. Linkage groups 4 on the L-93 map and 3 on the 7418-3 map had the greatest number of QTL and the highest density of QTL associated with heat tolerance traits. These linkage groups correspond with rice chromosomes 1 and 3, respectively (Honig et al., 2014). QTL

associated with agronomic traits related to heat tolerance in rice such as spikelet fertility and seed set have been found on rice chromosomes 1 and 3 (Ye et al., 2012; Zhang et al. 2009). QTL on rice chromosomes 1 and 3 which were associated with heat tolerance have also been found to be associated with other abiotic stresses, such as drought and salinity (Jagadish et al., 2010). Additionally, QTL for other traits which may be important for heat tolerance, such as membrane stability and chlorophyll content have been found on rice chromosomes 1 and 3, confirming that these syntenous regions are important for abiotic stress tolerance (Tripathy et al., 2000; Wang et al., 2008). Analysis across different studies and species may be useful for identifying QTL and the underlying genes responsible for heat tolerance.

Several markers related to important heat tolerance traits as identified by QTL analysis are associated with genes which may play roles in delaying leaf senescence. Os09g34190FN which was associated with CTD and TQ was identified as acyl-coenzyme A thioesterase. Acyl-coenzyme A thioesterase is involved in lipid metabolism (Hunt and Alexson, 2002). Membrane composition and fluidity are important factors affecting heat tolerance, as membrane structure is essential for energy pathways such as photosynthesis, as well as signaling functions (Sangwan et al., 2002). Os07g04240AV which was associated with TQ and CHL was identified as succinate dehydrogenase. Succinate dehydrogenase is a membrane bound enzyme which ties the citric acid cycle to electron transport chain (Hederstedt and Rutberg, 1981). Changes in respiration metabolism can lead to energy imbalances and the production of damaging reactive oxygen species during heat stress (Wahid et al., 2007). The ability for plants to alter lipid profiles and mitochondrial activity may be two important pathways for increased

tolerance to elevated temperatures. However, further research is needed to confirm if these genes or other candidate genes are responsible for QTL effects.

A number of QTL regions identified in this study were associated with multiple traits, including CHL and TQ on group 4.1, and NDVI and LAI on 4.2 for the L93-10 linkage map. Additionally adjacent overlapping QTL for CHL were found in two environments on linkage group 4.1. On the 7418-3 linkage maps QTL for multiple traits were found for TQ and CTD on linkage group 3.1, and CHL and LAI on group 5.1. Additionally the QTL region on linkage group 5.1 of 7418-3 linkage map for CHL and LAI appeared in both the 2008 and 2009 field trials. No QTL regions were found across all trials indicating that differences in environmental factors are a large contributor to the identified QTL. Environmental factors which may contribute to differences in response to heat stress include relative humidity, light intensity, as well as differences in the duration and severity of heat stress. Multiple traits for different physiological functions associated with the same QTL suggested selecting for the combined traits could be effective in improving heat tolerance. The results of QTLs found in multiple environments or linked to multiple traits indicated that those QTL regions could contain multiple candidate genes positively associated with multiple physiological traits for heat tolerance, and could be the most reliable QTLs for marker assisted selection of heat tolerant germplasm.

QTL regions associated with drought stress in the same mapping population used in this study have been identified in our previous study (Merewitz et al. 2014). A number of common QTL regions which were found to be associated with traits for drought tolerance in the previous study were also linked to heat tolerance traits in this study. On the L93-10 parental linkage map on linkage group 4.1 marker GA1104 which was

associated with TQ for heat stress conditions in the 2009 field trial in this study was also associated with TQ for drought stress (Merewitz et al., 2014). In addition, on the 4.1 linkage group three markers associated with TQ (abCG_CabG_170.59, GA1104), CTD (abCC_CTab_174.84), and TQ and CHL (Os07g_04240AV), respectively, for heat tolerance in this study were all found to be associated with TQ and CTD under drought stress conditions (Merewitz et al., 2014). On the 7418-3 linkage map, a marker associated with TQ and NDVI, GA1486A, was also associated with NDVI during drought stress. Turf quality from both heat and drought studies shared the QTL loci GA1104 on linkage group 4.1. In addition, correlation analysis of turf quality for the same mapping population exposed to drought stress (Merewitz et al., 2014) and heat stress in this study demonstrates a significant correlation between heat and drought performance (R^2 value of 0.716) (Fig. 7), indicating many clones which had greater heat tolerance also possessed greater drought tolerance and the mechanisms used by these individual may be shared between heat and drought responses as part of a general abiotic stress response.

Those QTL and associated markers that were found in this study with heat stress and the drought study by Merewitz et al. (2014) are of particular interest, which could be used to select creeping bentgrass for improving both drought and heat tolerance and also suggest the heat and drought tolerance could share common mechanisms or traits. Heat and drought stress may cause similar damages in turfgrasses, including reduced photosynthesis and the accumulation of reactive oxygen species; additionally heat and drought often occur simultaneously during summer months (Jiang and Huang, 2001). QTLs which are associated with multiple physiological traits linked to both drought and heat tolerance are particularly useful for selecting turfgrasses tolerant to the combined

heat and drought stress, which commonly occurs during the summer months in many areas. Additionally fine mapping of identified QTL regions could help uncover underlying genes and mechanisms responsible for improved heat tolerance in creeping bentgrass, which deserves further investigation.

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Table 1. Summary of physiological data. Mean, F value, and range for turf quality (TQ), chlorophyll (CHL) content, electrolyte leakage (EL), canopy temperature depression (CTD), green leaf biomass (NDVI), and leaf area index (LAI) measured at the end of heat stress during each trial when segregation among 100 clones was the greatest, as well as the ANOVA significance levels comparing the different trials and the trait by trial interaction.

	Field 2008			Field 2009			Growth Chamber			Green House			Trial	Trait x Trial
Trait	Mean	F-Val.	Range	Mean	F-Val.	Range	Mean	F-Val.	Range	Mean	F-Val.	Range	ANOVA	ANOVA
TQ	7.06 ****	3.41	2.33 : 9.00	6.15 ****	3.97	2.50 : 8.75	5.63 ****	11.6	4.00 : 7.50	5.94 ***	3.2	4.25 : 7.33	****	****
CHL	11.19 ****	2.88	8.42 : 16.04	11.37 ***	1.70	6.32 : 17.04	11.32 ****	4.9	6.73 : 14.59	12.11 **	1.61	9.05 : 16.25	*	****
EL	19.78 **	1.7	14.76 : 31.93	21.48 ***	2.1	15.44 : 61.14	30.84 ****	3.2	16.94 : 56.38	27.04 ***	2.3	16.37 : 50.07	****	**
CTD	-0.63 ****	2.41	-3.18 : 6.90	0.63 ***	1.57	-3.95 : 7.75	0.59 ***	1.56	-0.65 : 3.28	-1.70 ***	1.79	-3.22 : -0.55	****	****
NDVI	0.72 ****	9.45	0.39 : 0.87	0.73 ****	1.93	0.63 : 0.80	-	-	-	-	-	-	**	****
LAI	7.26 ****	8.47	2.41 : 14.19	6.64 ****	1.99	4.49 : 9.35	-	-	-	-	-	-	****	****

F-Val. = F statistic value. TQ =turf quality, CHL=chlorophyll content as mg/g dry weight,

EL=electrolyte leakage as % damage, CTD=canopy temperature depression,

NDVI=green leaf biomass, and LAI=leaf area index.

ANOVA significance ** = $P > 0.01$, *** = $P > 0.001$, **** = $P > 0.0001$

Table 2. Heat tolerance QTL on the L93-10 parental linkage map.

Data presented is from the MQM analysis as well as the marker most closely associated with the QTL. If the same QTL is identified multiple times within a trial only the most significant QTL was presented. Group represents the linkage group number the QTL is found on, Pos. is the position on the group in centimorgans. LOD is the logarithm of odds score which is compared against the results from the permutation test to determine significance. Expl. Var. is the percent variance of the trait explained by the QTL. GIC is the genotypic information coefficient where scores closer to one represent a marker more closely associated with the QTL. K* is the K-stat from Kruskal-Wallis analysis, and KW sig. is the level of significance as determined by Kruskal-Wallis analysis

Group	Pos.	Marker	Trait	Trial	LOD	LOD threshold	Expl. Var.	GIC	K*	KW sig.
1.1	52.04	GT1387(131)	CHL	Field 08	5.56	3.3	22.4	1	22.72	*****
2.1 ^P	36.569	abCC_CTab_294.4	CHL	Field 09	3.34	3.6	41.6	0.346	10.686	****
2.2	0.37	COL343F(195)	EL	Chamber	3.09	3.0	13.9	1	9.342	****
4.1	42.827	GA1104(154)	TQ	Field 09	4	3.5	17.7	0.982	13.017	*****
4.1 ^P	33.641	GA263(133)	CHL	Field 09	3.45	3.6	40.8	0.502	12.628	*****
4.1 ^P	37.404	COL349bV(169)	CHL	Chamber	3.18	3.5	13.8	0.984	11.319	*****
4.1	63.987	abCG_CabG_170.59	TQ	Field 09	4.83	3.5	20.6	0.976	18.494	*****
4.1	75.826	abCC_CTab_174.84	CTD	Field 09	5.86	4.2	42.1	0.672	14.08	*****
4.1	83.272	Os07g_04240AV(716)	TQ	Field 09	5.51	3.5	19.3	0.992	22.096	*****
4.1 ^P	83.272	Os07g_04240AV(716)	CHL	Field 09	3.45	3.6	19.9	0.746	13.666	*****
4.2	11.038	GT253(155)	NDVI	Field 09	3.79	3.3	16.3	1	14.769	*****
4.2	11.038	GT253(155)	LAI	Field 09	3.73	3.2	16.1	1	14.108	*****
5.1	73.374	Os09g_34190FN(347)	CTD	Chamber	3.29	3.2	13.4	0.984	13.248	*****
5.1	134.073	Os09g_34190FN(345)	TQ	GH	4.26	3.4	17.3	0.986	15.097	*****
6.2 ^P	15	GA1835N(149)	LAI	Field 08	5.12	7.1	62.9	0.278	10.744	****
7.1	52.574	GA1346(102)	LAI	Field 09	3.67	3.2	17.9	0.866	15.16	*****

7.2 ^p	31.109	Os06g_05090CN(481)	NDVI	Field 08	3.09	3.3	13.1	0.998	12.797	*****
7.2 ^p	39.515	Os06g_05090CN(481)	LAI	Field 08	5.36	7.1	73.2	0.061	12.621	*****
7.2 ^p	82.349	abCC_CabT_353.93	CTD	Field 08	4.06	5.1	21.4	0.813	13.677	*****

KW sig. P = *:0.1 **:0.05 ***:0.01 ****:0.005 *****:0.001 *****:0.0005

*****:0.0001 ^p = putative QTL

Table 3. Heat tolerance QTL on the 7418-3 parental linkage map. Data presented is from the MQM analysis as well as the marker most closely associated with the QTL. If the same QTL is identified multiple times within a trial only the most significant QTL was presented. Group is the linkage group number the QTL is found on, Pos. is the position on the group in centimorgans. LOD is the logarithm of odds score which is compared against the results from the permutation test to determine significance. Expl. Var. is the percent variance of the trait explained by the QTL. GIC is the genotypic information coefficient. K* is the K-stat from Kruskal-Wallis analysis, and KW sig. is the level of significance as determined by Kruskal-Wallis analysis

Group	Pos.	Marker	Trait	Trial	LOD	LOD threshold	Expl. Var.	GIC	K*	KW sig.
1.1 ^P	82.616	BRSC1_008P(420)	EL	Field 08	3.06	8.5	11.746	0.994	6.815	***
2.1	38.331	Os01g_16520AN(234)	CTD	Chamber	4.18	3.5	18.4	0.993	14.875	*****
3.1 ^P	49.224	GA1486A(152)	TQ	Field 08	6.47	7.3	67	0.133	7.015	***
3.1 ^P	49.224	GA1486A(152)	NDVI	Field 08	6.32	7.1	66.8	0.133	8.592	****
3.1 ^P	59.015	GA1643(237)	CTD	Field 08	3	3.4	12.5	0.953	14.028	*****
3.1 ^P	73.511	GA1643(235)	CTD	Field 08	3	3.4	12.9	0.986	11.893	*****
3.2	39.208	GA1675N(192)	EL	GH	4.8	4.4	61.3	0.992	3.781	*
4.1	-5.96	GT1080(206)	EL	Field 08	17.14	8.5	66.5	0.788	3.4	*
4.2	8.949	GT253(146)	CHL	Chamber	3.35	3.3	30.3	0.494	5.583	**
5.1	69.152	Os09g_34190FN(336)	TQ	GH	3.69	3.6	16.6	0.98	17.066	*****
5.1	74.7	ACG_CAG_137.64	CHL	Field 09	4.09	3.7	17.9	0.945	10.383	****
5.1	75.7	ACG_CAG_137.64	LAI	Field 08	3.45	3.4	26.6	0.443	8.618	****
6.1	127.504	ACG_CAC_91.3	TQ	Chamber	3.59	3.3	16.2	0.832	10.43	****

KW sig. P = *:0.1 **:0.05 ***:0.01 ****:0.005 *****:0.001 *****:0.0005

*****:0.0001 ^P = putative QTL

Table 4. Additional significant markers identified by Kruskal-Wallis analysis. Markers significantly associated with heat tolerance traits significant at $P \leq 0.001$ from both the L93-10 and 7418-3 parental linkage maps.

Map	Group	Position	Marker	Trait	Trial	K*	Signif.
L93-10	1.1	34.354	GA147(204)	CHL	Field 2008	12.082	*****
L93-10	1.1	23.419	abCC_CTC_96.55	TQ	Field 2008	11.295	*****
L93-10	1.1	23.419	abCC_CTC_96.55	CHL	Field 2008	14.571	*****
L93-10	1.1	41.03	PRSC1_001(227)	CHL	Field 2008	16.509	*****
L93-10	1.1	42.489	PRSC1_001F(227)	CHL	Field 2008	16.346	*****
L93-10	1.1	44.986	GT283(70)	CHL	Field 2008	12.3	*****
L93-10	1.1	46.135	GA165(160)	CHL	Field 2008	15.588	*****
L93-10	1.1	48.429	GT283(92)	CHL	Field 2008	12.606	*****
L93-10	1.1	50.402	Os01g_40860EF(242)	CHL	Field 2008	15.102	*****
L93-10	1.1	52.04	GT1387(131)	CHL	Field 2008	22.72	*****
L93-10	1.1	53.939	P394(215)	CHL	Field 2008	14.253	*****
L93-10	1.1	56.542	GA1643(248)	CHL	Field 2008	16.598	*****
L93-10	1.1	57.902	GA1486B(171)	CHL	Field 2008	21.162	*****
L93-10	1.2	24.472	GA1528(223)	CHL	Chamber	11.118	*****
L93-10	2.1	32.569	abCC_CTab_294.4	TQ	Field 2009	11.522	*****
L93-10	2.2	42.624	KSU104N(181)	CTD	Field 2008	11.268	*****
L93-10	4.1	20.857	SRSC9_020V(370)	TQ	Field 2009	14.898	*****
L93-10	4.1	34.056	GA263(133)	CTD	Field 2009	12.124	*****
L93-10	4.1	34.056	GA263(133)	TQ	Field 2009	14.193	*****
L93-10	4.1	34.056	GA263(133)	CHL	Field 2009	12.628	*****

L93-10	4.1	35.113	GA501(176)	TQ	Field 2009	12.059	*****
L93-10	4.1	36.013	GT1350(183)	TQ	Field 2009	13.382	*****
L93-10	4.1	37.404	COL349bV(169)	CHL	Chamber	11.319	*****
L93-10	4.1	37.404	COL349bV(169)	TQ	Field 2009	13.733	*****
L93-10	4.1	37.723	COL349bV(147)	TQ	Field 2009	14.769	*****
L93-10	4.1	38.926	ORSC8_003P(524)	CTD	Field 2009	13.003	*****
L93-10	4.1	38.926	ORSC8_003P(524)	TQ	Field 2009	13.666	*****
L93-10	4.1	38.926	ORSC8_003P(524)	CHL	Field 2009	12.357	*****
L93-10	4.1	40.385	GT1419(168)	TQ	Field 2009	11.694	*****
L93-10	4.1	40.385	GT1419(168)	CHL	Field 2009	13.196	*****
L93-10	4.1	40.66	GT1419(191)	TQ	Field 2009	14.863	*****
L93-10	4.1	40.66	GT1419(191)	CHL	Field 2009	13.676	*****
L93-10	4.1	42.827	GA1104(154)	TQ	Field 2009	13.017	*****
L93-10	4.1	50.464	Os03g_02350V(411)	CTD	Field 2009	11.274	*****
L93-10	4.1	50.464	Os03g_02350V(411)	TQ	Field 2009	11.896	*****
L93-10	4.1	50.464	Os03g_02350V(411)	CHL	Field 2009	11.689	*****
L93-10	4.1	63.987	abCG_CabG_170.59	CTD	Field 2009	18.435	*****
L93-10	4.1	63.987	abCG_CabG_170.59	TQ	Field 2009	18.819	*****
L93-10	4.1	68.378	abGG_CTC_132.82	CTD	Field 2009	12.639	*****
L93-10	4.1	68.378	abGG_CTC_132.82	TQ	Field 2009	13.843	*****
L93-10	4.1	77.773	abCC_CTab_174.84	CTD	Field 2009	14.08	*****
L93-10	4.1	77.773	abCC_CTab_174.84	TQ	Field 2009	15.045	*****
L93-10	4.1	82.272	Os07g_04240AV(716)	CHL	Field 2009	13.666	*****
L93-10	4.1	82.272	Os07g_04240AV(716)	CTD	Field 2009	21.627	*****
L93-10	4.1	82.272	Os07g_04240AV(716)	TQ	Field 2009	22.096	*****
L93-10	4.2	0	abCG_CabC_276.14	NDVI	Field 2008	12.1	*****
L93-10	4.2	0	abCG_CabC_276.14	LAI	Field 2008	12.424	*****

L93-10	4.2	6.203	GT253(167)	NDVI	Field 2009	13.986	*****
L93-10	4.2	6.203	GT253(167)	LAI	Field 2009	13.762	*****
L93-10	4.2	11.038	GT253(155)	NDVI	Field 2009	14.769	*****
L93-10	4.2	11.038	GT253(155)	LAI	Field 2009	14.108	*****
L93-10	4.2	52.015	GT1393(231)	NDVI	Field 2009	11.796	*****
L93-10	4.2	52.015	GT1393(231)	LAI	Field 2009	11.086	*****
L93-10	5.1	73.374	Os09g_34190FN(347)	CTD	Chamber	13.248	*****
L93-10	5.1	134.073	Os09g_34190FN(345)	TQ	Greenhouse	15.097	*****
L93-10	7.1	52.574	GA1346(102)	NDVI	Field 2009	12.046	*****
L93-10	7.1	52.574	GA1346(102)	LAI	Field 2009	15.16	*****
L93-10	7.1	55.415	SRSC2_028P(287)	NDVI	Field 2009	11.158	*****
L93-10	7.1	55.415	SRSC2_028P(287)	LAI	Field 2009	14.373	*****
L93-10	7.1	65.841	abCG_CabC_228.11	LAI	Field 2009	12.285	*****
L93-10	7.2	10.946	GA1668V(233)	CHL	Field 2008	11.284	*****
L93-10	7.2	17.247	GT1369B(146)	CHL	Field 2008	12.348	*****
L93-10	7.2	31.109	Os06g_05090CN(481)	CHL	Field 2008	13.482	*****
L93-10	7.2	31.109	Os06g_05090CN(481)	NDVI	Field 2008	12.797	*****
L93-10	7.2	31.109	Os06g_05090CN(481)	LAI	Field 2008	12.621	*****
L93-10	7.2	38.515	GA1548(160)	LAI	Field 2008	10.99	*****
L93-10	7.2	38.515	GA1548(160)	CHL	Field 2008	18.863	*****
L93-10	7.2	40.439	GA1057(158)	CHL	Field 2008	11.677	*****
L93-10	7.2	41.095	GA1548(157)	EL	Field 2009	11.237	*****
L93-10	7.2	41.095	GA1548(157)	CHL	Field 2008	13.652	*****
L93-10	7.2	42.47	KSU073N(700)	CHL	Field 2008	12.742	*****
L93-10	7.2	45.484	GA1545(288)	CHL	Field 2008	12.095	*****
L93-10	7.2	47.823	GT558(138)	EL	Field 2009	11.901	*****
L93-10	7.2	47.823	GT558(138)	CHL	Field 2008	12.892	*****

L93-10	7.2	49.416	Os03g_02410V(677)	CTD	Field 2008	12.107	*****
L93-10	7.2	55.27	CAG361(220)	CHL	Field 2008	14.406	*****
L93-10	7.2	55.912	CAG336(218)	CHL	Field 2008	14.167	*****
L93-10	7.2	63.014	P771(197)	CHL	Field 2008	15.348	*****
L93-10	7.2	65.255	GT586(169)	CTD	Field 2008	11.355	*****
L93-10	7.2	65.255	GT586(169)	EL	Field 2009	11.095	*****
L93-10	7.2	81.349	abCC_CabT_353.93	CTD	Field 2008	13.677	*****
Map	Group	Position	Marker	Trait	Trial	K*	Signif.
7418-3	2.1	38.331	Os01g_16520AN(234)	CTD	Chamber	14.875	*****
7418-3	2.1	73.203	CA12(122)	TQ	Chamber	10.899	*****
7418-3	3.1	52.979	GA1643(264)	CTD	Field 2008	11.191	*****
7418-3	3.1	59.015	GA1643(237)	CTD	Field 2008	14.028	*****
7418-3	3.1	67.437	GA1643(262)	TQ	Field 2009	11.472	*****
7418-3	3.1	73.511	GA1643(235)	CTD	Field 2008	11.893	*****
7418-3	4.1	26.918	GT423(120)	CTD	Field 2009	11.305	*****
7418-3	5.1	18.226	Os09g_34190FN(345)	TQ	Greenhouse	15.097	*****
7418-3	5.1	69.152	Os09g_34190FN(336)	TQ	Greenhouse	17.066	*****
7418-3	5.2	66.364	ACT_CAC_186.09	EL	Field 2009	15.519	*****
7418-3	6.1	65.982	GA996(161)	EL	Greenhouse	10.852	*****
7418-3	7.1	45.232	GA337(217)	TQ	Chamber	13.764	*****
7418-3	7.2	0	GT1074(205)	CHL	Field 2008	11.566	*****
7418-3	7.2	26.471	GA1668V(233)	CHL	Field 2008	11.284	*****
7418-3	7.2	42.895	Os06g_01650BN(285)	TQ	Greenhouse	11.247	*****

TQ turf quality, CHL chlorophyll content, EL electrolyte leakage, CTD canopy

temperature depression, NDVI green leaf biomass, LAI leaf area index. KW sig. P =

*****:0.001 *****:0.0005 *****:0.0001

Fig 1. Distribution of turf quality scores for the (A) 2008 field, (B) 2009 field, (C) growth chamber, and (D) green house trial demonstrating the segregation of heat tolerance in the population. Replicates were averaged for the average rating of a line within the mapping population. Data presented is from the end of heat stress when damage was most severe for the trial.

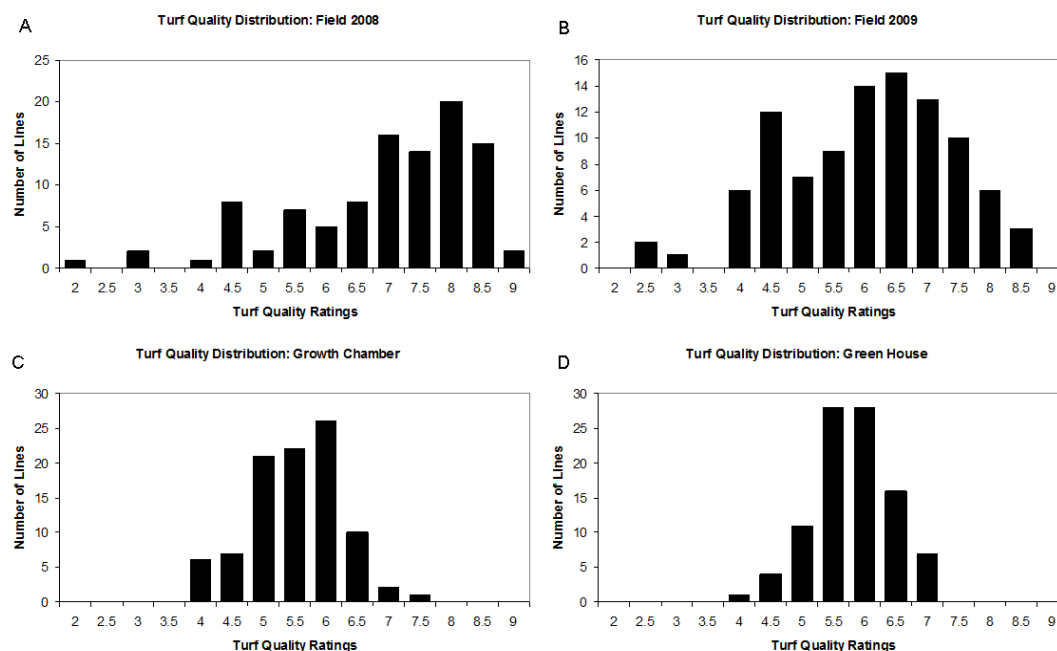


Fig 2. Distribution of chlorophyll content for the (A) 2008 field, (B) 2009 field, (C) growth chamber, and (D) green house trial demonstrating the segregation of heat tolerance in the population. Replicates were averaged for the average content of a line within the mapping population. Data presented is from the end of heat stress when damage was most severe for the trial.

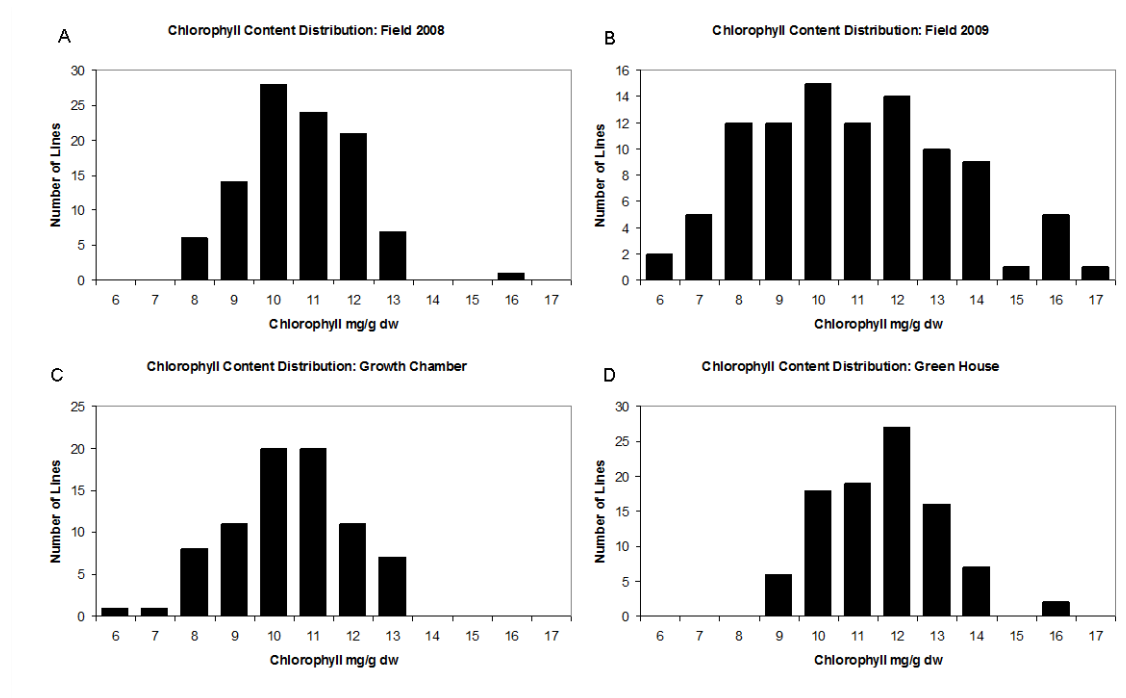


Fig 3. Distribution of membrane stability represented by percent relative damage as measured by electrolyte leakage for the (A) 2008 field, (B) 2009 field, (C) growth chamber, and (D) green house trial demonstrating the segregation of heat tolerance in the population. Replicates were averaged for the average content of a line within the mapping population. Data presented is from the end of heat stress when damage was most severe for the trial.

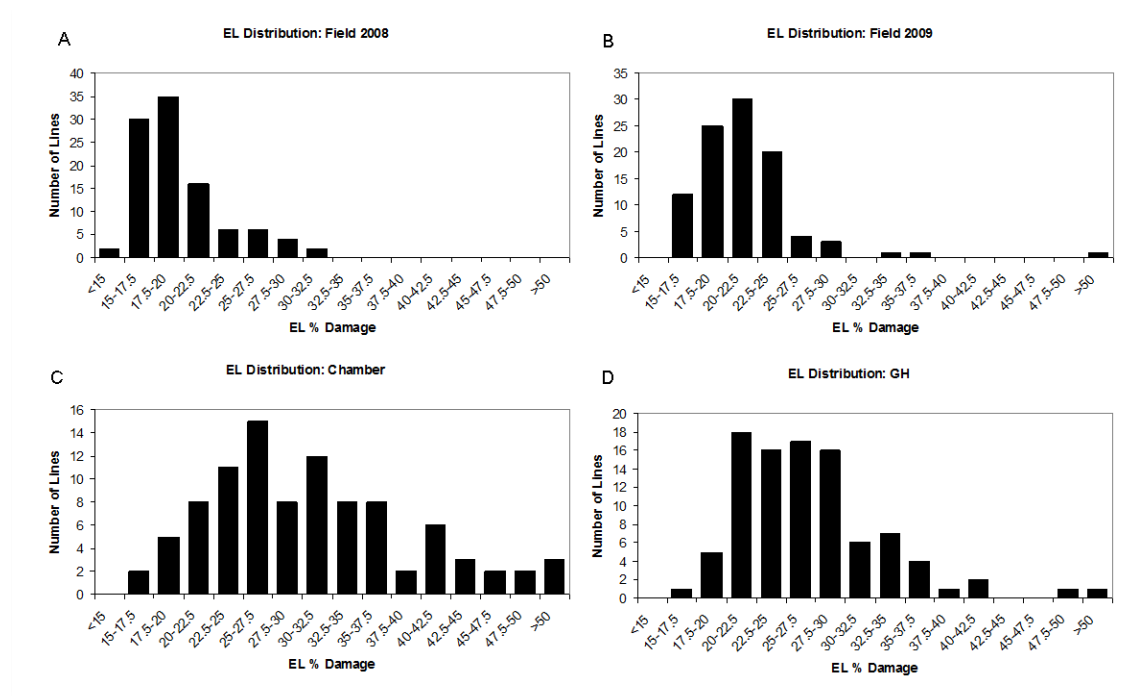


Fig 4. Distribution of canopy temperature depression for the (A) 2008 field, (B) 2009 field, (C) growth chamber, and (D) green house trial demonstrating the segregation of heat tolerance in the population. Replicates were averaged for the average content of a line within the mapping population. Data presented is from the end of heat stress when damage was most severe for the trial.

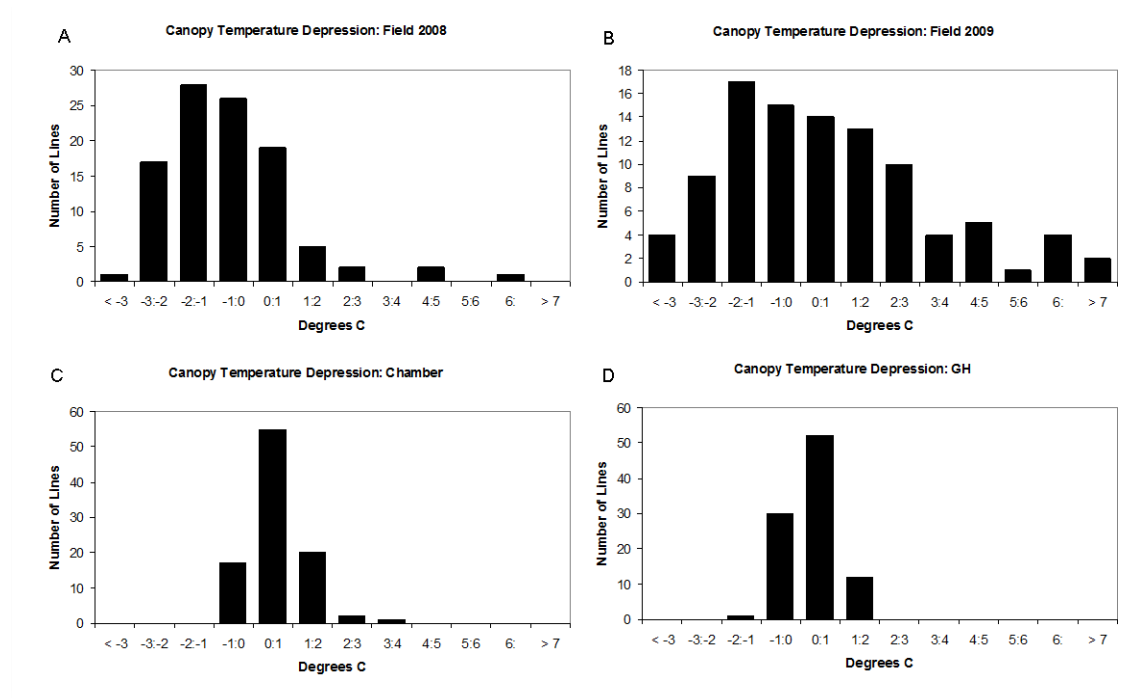


Fig 5. Distribution of MSR data for NDVI (A) for the 2008 field and (B) 2009 field trial, and LAI for the (C) 2008 field and (D) 2009 field trials demonstrating the segregation of heat tolerance in the population. Replicates were averaged for the average content of a line within the mapping population. Data presented is from the end of heat stress when damage was most severe for the trial.

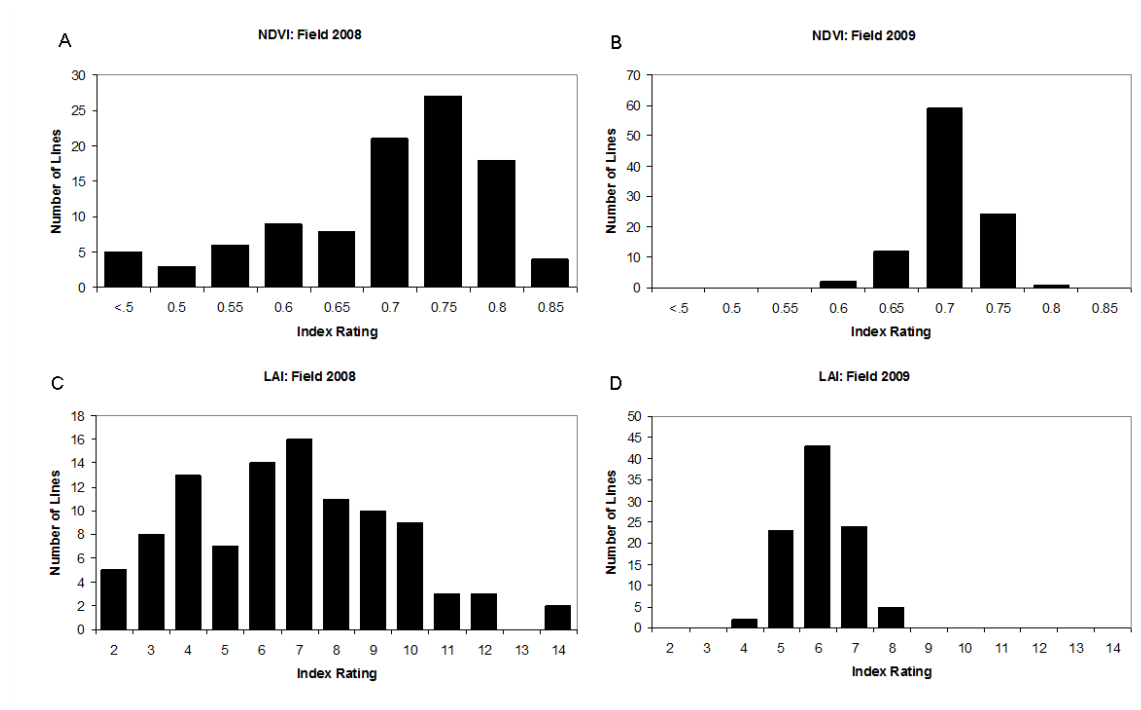
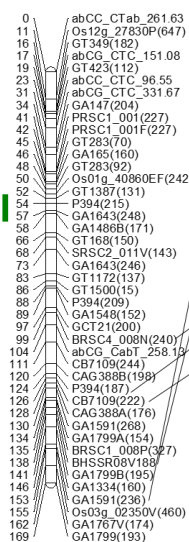
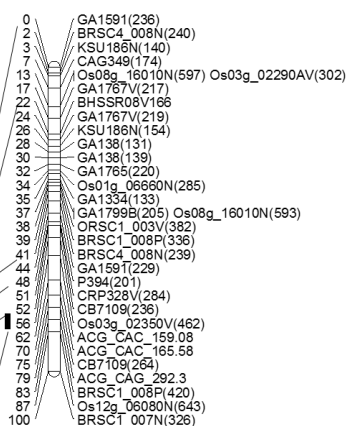


Fig 6. Linkage groups (A) 1 through 3 (B) 4 and 5 (C) 6 and 7, and quantitative trait loci (QTL) detected for creeping bentgrass on the L93-10 and 7418-3 parental linkage maps. The centimorgan (cM) distance scale is located to the left of each individual linkage group and corresponding marker name is located to the right. Numbers in parenthesis indicate allele size in creeping bentgrass. Connecting lines indicate marker associations between homologous (solid) and homoeologous (dashed) linkage groups. *linkage group numbers for syntenous wheat chromosomes based on rice-wheat relationships presented by Sorrells et al. (2003). TQ, turf quality; CHL, chlorophyll content; EL, electrolyte leakage; CTD, canopy temperature depression; ; NDVI, normalized difference vegetative index (green leaf biomass); LAI, leaf area index.

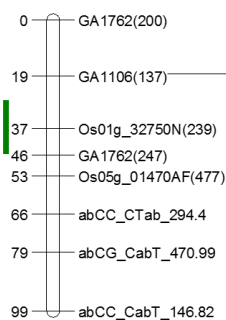
1.1 (L93-10) *3



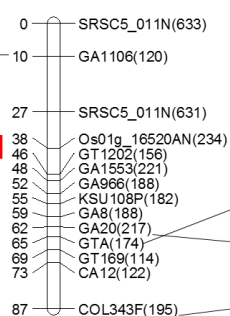
1.1(7418-3) *3



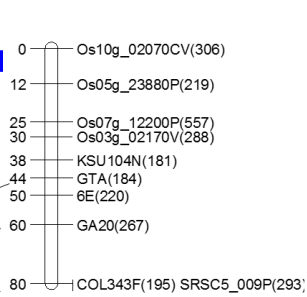
2.1 (L93-10) *1



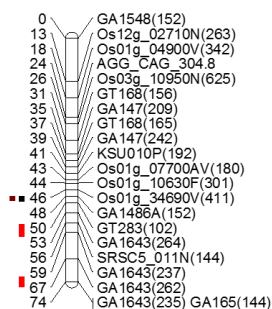
2.1 (7418-3) *1



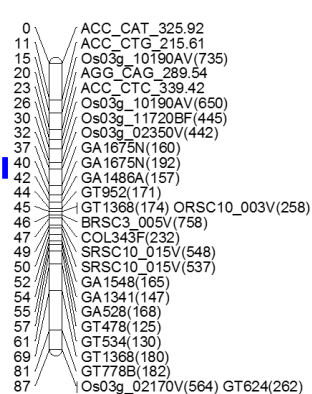
2.2 (L93-10) *1



3.1 (7418-3) *3



3.2 (7418-3) *4



TQ =

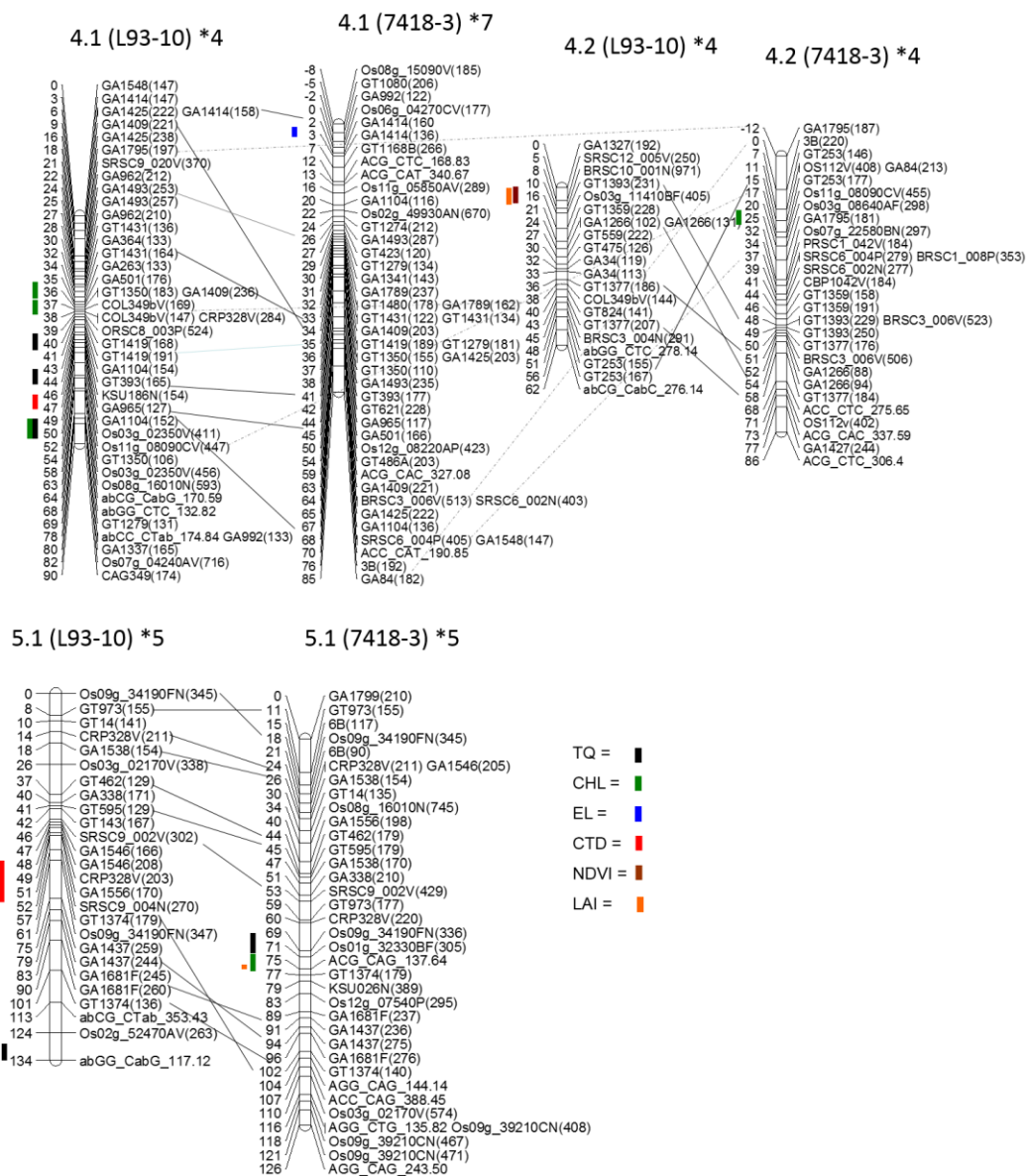
CHL =

EL =

CTD =

NDVI =

LAI =



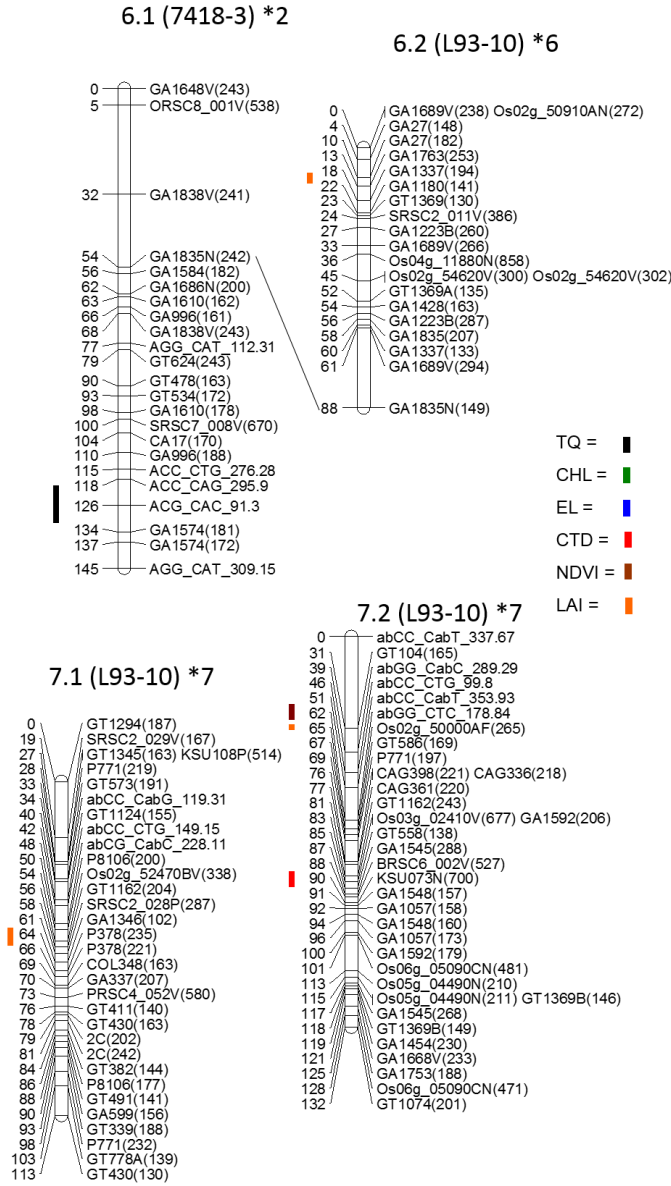
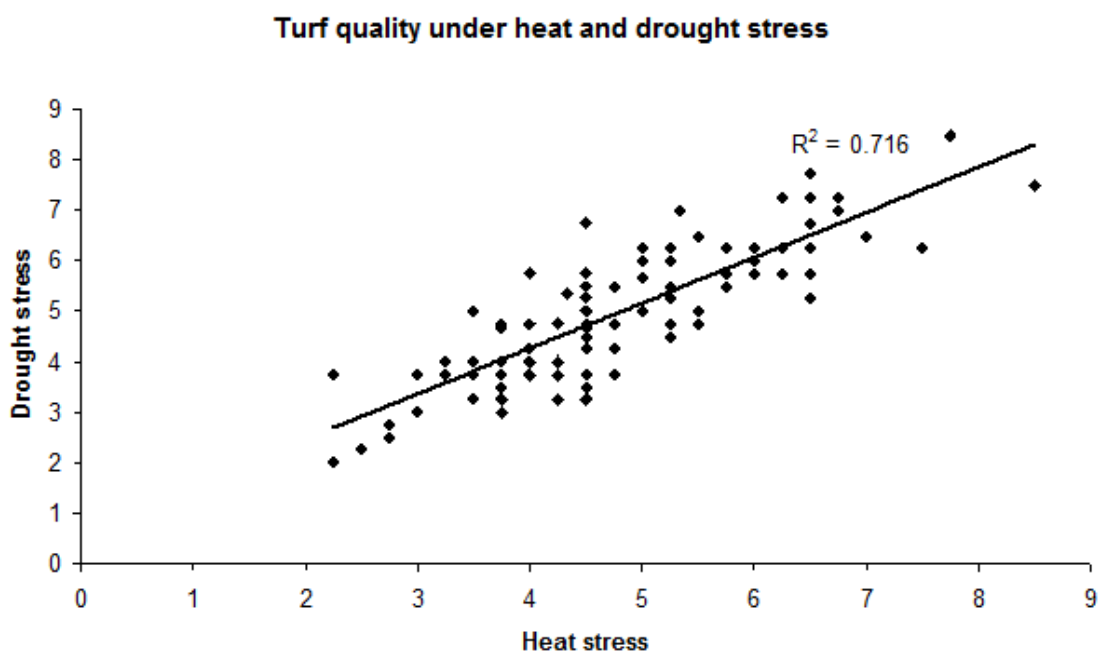


Fig 7. Correlation of heat tolerance and drought tolerance among mapping population lines as represented by turf quality scores from the summer 2009 field trial for heat stress and fall 2009 field trial for drought stress (see Merewitz et al.,2014).



**CHAPTER 6: Candidate Genes and Molecular Markers Associated with Heat
Tolerance in Colonial Bentgrass**

INTRODUCTION

Heat stress is one of the most detrimental abiotic stresses restricting the growth of cool-season plant species during the summer months. Heat stress causes changes in various physiological and metabolic processes, such as the production of reactive oxygen species leading to oxidative damage of DNA, lipids, and proteins, altered energy metabolism resulting in a loss of carbon stores, and the degradation of proteins (Wahid et al., 2007). Plants develop a number of mechanisms for combating heat stress, including increased antioxidant capacity to scavenge reactive oxygen species, synthesis of chaperone proteins to maintain proper protein conformation, and maintenance of active energy metabolism (Miller et al., 2008; Vierling, 1991; Wahid et al., 2007).

Numerous genes involved in heat tolerance mechanisms have been proposed through genomic, transcriptomic, and proteomic analysis in various plant species (Thudi et al., 2014; Qin et al., 2008; Rizhsky et al., 2002; Xu and Huang, 2010; Lee et al., 2007). The candidate gene approach has also been used to identify key genes which may control drought tolerance in various plants species, such as annual crops and perennial grasses (Pelleschi et al., 1999; Tondelli et al., 2005; Yu et al., 2013, Thudi et al., 2014). Most markers developed for perennial grasses have been ALFP, RFLP, SCAR or SSR markers used in QTL analysis to identify loci associated with disease resistance or drought tolerance (Chakraborty et al., 2014; Honig et al., 2014; Merewitz et al., 2012). However other marker types such as CAPs markers of allele-specific markers have successfully been developed for candidate genes in other species to be used for marker assisted selection to develop improved varieties (Guo and Qiu, 2013; Quint et al., 2002).

Bentgrasses, including creeping bentgrass (*Agrostis stolonifera*) and colonial bentgrass (*Agrostis capillaris*), are cool-season perennial grasses widely used as turfgrass due to their superior density and tolerance to low mowing height, but exhibit poor to moderate heat tolerance (Turgeon, 1999). Colonial bentgrass was reported to exhibit better recuperative potential from drought damages compared to creeping bentgrass upon re-watering (DaCosta and Huang, 2007) and superior resistance to the fungal pathogen *Sclerotinia homoeocarpa* (Belanger et al., 2004). In bentgrass species proteomic and enzymatic studies have identified a number of genes or gene products which may play important roles in heat tolerance and may be useful as candidate genes which control heat tolerance (Xu and Huang, 2004; He et al., 2005; Xu and Huang, 2010; Jespersen et al., 2015). The genes identified included stress defense proteins such as antioxidant enzymes and chaperone proteins.

Interspecific hybridization between colonial bentgrass and creeping bentgrass followed by backcrossing to creeping bentgrass could transfer beneficial traits to creeping bentgrass (Belanger et al., 2003, 2004). A colonial bentgrass x creeping bentgrass hybrid and some progeny from a backcross to creeping bentgrass population were found to develop improved heat tolerance (Jespersen et al., 2015) and drought tolerance (Merewitz et al., 2012), as well as disease resistance (Rotter et al., 2009). Several QTLs associated with growth and physiological traits linked to drought tolerance (Merewitz et al., 2012) were detected through analysis of the backcross hybrid population. The availability of the backcross population provides the opportunity to determine if differences in specific genes inherited from colonial bentgrass ultimately result in phenotypic differences for improved heat stress tolerance.

The goal of this study was to develop DNA markers for candidate genes associated with improved heat stress tolerance in bentgrass species. The backcross mapping population was screened for heat tolerance in addition to segregation for gene specific markers. Development of candidate gene markers associated with heat stress tolerance in colonial bentgrass could potentially be used for marker-assisted selection in intraspecific as well as interspecific breeding programs for cool-season perennial grasses.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

An interspecific colonial and creeping bentgrass hybrid backcross population was used to examine variations in heat tolerance in this study. The hybrid backcross population was created by crossing a colonial x creeping bentgrass hybrid (TH15) with a creeping bentgrass plant (9188) (Rotter et al., 2009). The population consisted of 93 individuals in addition to the hybrid parent (TH15), creeping bentgrass parent (9188), and the creeping bentgrass grandparent that was used to generate the hybrid (5061). This backcross population previously exhibited phenotypic segregation for drought tolerance (Merewitz et al., 2012), and two population individuals (ColxCB169 and ColxCB190) which displayed differential levels of heat tolerance were used in additionally analysis.

Plants were established in pots (15 cm in diameter and 20 cm deep) filled with a mixture of 50% soil mix (fine-loamy, mixed mesic Typic Hapludult type soil) and 50% peat moss in a greenhouse for 6 wk. Three replicate pots of each population individual were transferred to environmentally control growth chambers (Conviron, Winnipeg, Canada) set to 20/15°C (day/night), a 14 h photoperiod, with photosynthetically active radiation (PAR) of 600 $\mu\text{mol m}^{-2} \text{s}^{-1}$, 60% relative humidity and allowed to acclimate for 7 d. Heat stress was induced by raising the chamber temperatures to 38/33°C (day/night) and maintained for 6 weeks to measure the effects of heat stress on the population. The treatment had four replicates in four growth chambers. Throughout establishment and during the imposition of heat stress plants were maintained well watered and received half-strength Hoagland's nutrient solution weekly (Hoagland and Arnon, 1950). The trial was repeated with 4 replicates of two population individuals that represented a top

(ColxCB169) and bottom (ColxCB190) performing genotype to confirm differences in heat tolerance and for use in gene expression assays.

Physiological Screening

Plants were exposed to heat stress for 6 wks and were measured every 2 wks for common assessments of heat tolerance or accumulation of stress damages which included turf quality, membrane stability, chlorophyll content, photochemical efficiency, and normalized difference vegetative index (NDVI). Turf quality (TQ) is a visual rating of overall plant performance based on color, density and uniformity, with 9 being completely healthy plants and 1 being completely dead plants (Beard 1973).

Membrane stability was estimated via electrolyte leakage (EL) according to the methods of Blum and Ebercon (1981). Approximately 0.1g of fresh leaf tissue was placed in a tube with 35 ml de-ionized water and shaken at room temperature for 16 h. An initial conductance reading (C_{initial}) of the incubated solution was taken using a conductivity meter (YSI Incorporated, Yellow Springs, OH). Tubes were then autoclaved at 120 °C for 20 min to kill all contained leaf tissue and placed back on the shaker for an additional 16 h after which a final conductance reading was measured (C_{max}). Electrolyte leakage was calculated as a percentage of C_{initial} to C_{max} to determine the percent of relative damage.

Chlorophyll content was measured using a modification of the methods described by Hiscox and Israelstam (1979). Approximately 0.1g of fresh leaf tissue was placed in 10 ml of dimethyl sulfoxide and allowed to incubate in the dark for 5 d to extract chlorophyll from the leaf tissue. The extracted dimethyl sulfoxide solution was measured on a spectrophotometer (Spectronic Instruments, Inc., Rochester, NY) at 663 and 645 nm

Leaf tissue was then dried for 72 h in an 80 °C oven to obtain dry weights and chlorophyll content was calculated on a dry weight basis using the equations described by Arnon (1949)

Photochemical efficiency was estimated by chlorophyll fluorescence using a fluorescence induction monitor (Fim 1500, Dynamax, Houston, TX). Leaves were dark adapted for 30 minutes and photochemical efficiency was estimated by the ratio of variable fluorescence (Fv) to maximal fluorescence (Fm). Three subsamples per replicate were taken for each round of measurements. Normalized difference vegetative index (NDVI) estimates the green leaf biomass of the canopy based on the reflectance of red and near infrared spectral bands and can be used to estimate plant health and density (Fenstermaker-Shaulis et al., 1997). NDVI was measured using a TCM 500 NDVI meter (Spectrum Technologies, Aurora, IL).

Marker Development and Screening

Potential candidate genes involved with abiotic stress tolerance in bentgrasses were identified from previous research involving proteomic analysis and gene expression assays (Xu and Huang, 2004; Tian et al., 2009; Xu and Huang, 2010). Allele specific markers were developed by aligning bentgrass EST sequences from the NCBI database for a gene of interest in the program Mega5 (Tamura et al., 2011). Exploitable polymorphisms were manually identified in the 3' untranslated region and primers were designed to amplify only specific identified alleles. The 3' UTR was selected due to its higher variability and polymorphisms were often identified as short indels differing between colonial and creeping bentgrasses. Primers were designed to be ~20 basepairs, have a Tm of 60 °C, and amplify a product of 200-600 bp. Additionally, cleavage

amplified polymorphisms (CAPS) markers were also used to identify differences in candidate genes within the population. ESTs for genes of interest from the NCBI database were uploaded into the program SNP2CAPS (Thiel et al., 2004) which was used to identify polymorphic restriction sites. PCR products containing the polymorphic restriction site were amplified as described above and incubated with the restriction enzyme as per the manufacturer's instructions. The resulting products of both allele specific markers and CAPs markers were run on a 3.5% MetaPhor (Cambrex BioScience Rockland, Inc., Rockland, ME) agarose gel and imaged using a CCD imaging system (Gel Doc XR, BioRad, Hercules CA). Table 1 contains additional information on primer sequences and marker design.

Real-Time PCR

Leaves of the same developmental age were harvested at 0 and 14 d of heat stress and flash frozen in liquid nitrogen for later use in gene expression assays. RNA was extracted using Trizol reagent (Life Technologies, Carlsbad, CA), treated with a Turbo DNase kit (Life Technologies) and further purification was performed with the RNeasy kit (Qiaqen, Venlo, Netherlands). A high-capacity cDNA synthesis kit (Life Technologies) was used to generate cDNA and qPCR primers were designed using PrimerExpress software (Life Technologies). qPCR was performed on a StepOnePlus real-time PCR machine using SYBR green master mix (Life Technologies). In addition to biological replicates, 3 technical replicates were used per sample, along with no template negative controls. Actin was used as the endogenous control gene and relative gene expression was determined using the delta-delta-CT method (Schmittgen and Livak, 2008). The qPCR primer sequences are given in Table 2.

Statistically Analysis

Physiological data and gene expression data was assessed using ANOVA to determine significant differences between population individuals and means were separated using Fisher's protected LSD using SAS v9.2 (SAS institute 1992) proc GLM. Marker association to physiological parameters was performed using the non-parametric Kruskal-Wallis test using proc npar1way in SAS v9.2. Location of markers on genetic linkage maps was determined using JoinMap 3.0 (van Ooijen and Voorrips, 2001) using the parameters described in Rotter et al. (2009).

RESULTS

Genetic Variation in Growth and Physiological Traits Associated with Heat Stress

Heat stress resulted in decline of all measurements, including TQ, EL, CHL, NDVI, and Fv/Fm, in parents and backcross population (Table 3). The average TQ score declined from 8.9 under control conditions to 5.3 by the end of heat stress. EL increased from 22.2% under control condition to 41.8 % by the end of heat stress. CHL decreased by 51.6% and Fv/Fm decreased by 11.5 % due to heat stress. NDVI declined from 0.33 under control conditions to 0.24 by the end of the trial.

The phenotypic variation within the population is demonstrated by the distribution curves in Figure 1. Average TQ ratings for individuals of the population at the end of heat stress ranged from 1.3 to 6.7. EL ranged from 25.6% to 96.8%. CHL ranged from 1.03 mg/g dry wt to 16.99 mg/g dw. Fv/Fm ratios ranged from 0.46 to 0.76, and NDVI ranged from 0.12 to 0.39 by the end of heat stress. These data demonstrate the population segregated for heat tolerance. Differences in the level of heat tolerance were found between the parents of the population, with the hybrid parent being more heat tolerant than either the creeping bentgrass parent or grandparent, as demonstrated by significant differences in TQ, NDVI, Fv/Fm and CHL during the trial. By the end of the trial the hybrid parent TH15 had a TQ score of 6.66, NDVI index rating of 0.27, Fv/Fm ratio of 0.773, EL of 29.7%, and CHL content level of 14.9 mg/g dw, while the creeping bentgrass parent 9188 had a TQ score of 5.33, NDVI index rating of 0.21, Fv/Fm ratio of 0.686, ET at 36.2%, and CHL content level of 10.9 mg/g dw. The creeping bentgrass grandparent 5061 had a TQ score of 5.33, NDVI index rating of 0.16, Fv/Fm ratio of 0.727, EL at 32.4%, and CHL content level of 13.3 mg/g dw.

Two individuals that exhibited either heat tolerance or sensitivity were selected from the backcross population for additional tests. In this additional trial that only tested the heat sensitive ColxCB190 and the more tolerant ColxCB169, similar trends were found to the trial which tested the entire population, with ColxCB169 significantly outperforming ColxCB190 in terms of heat tolerance. By two weeks of heat stress, ColxCB169 had maintained a TQ rating of 8 while ColxCB190 TQ declined to 4.5 (Fig 2a). CHL and EL did not significantly change by 2 weeks of heat stress for ColXCB169 but CHL decreased by 53% (Fig. 2b) and EL increased 138% (Fig. 2c) in ColXCB190. These results confirm that ColXCB169 had significantly greater heat tolerance than ColXCB190.

Differential Expression of Selected Candidate Genes in Response to Heat Stress

Comparing the Two Hybrid Individuals Contrasting in Heat Tolerance

qPCR was used for gene expression assays to determine the differential expression of selected candidate genes in response to heat stress comparing the two population individuals with contrasting heat tolerance (heat-tolerant ColXCB169 and heat-sensitive ColXCB190) (Fig. 3). The transcript levels of antioxidant genes (*GST* and *CAT*) and heat-shock proteins (*HSP26*, *HSP70*, *HSP101*), cysteine protease (*CP*) for protein degradation, and expansin (*EXP*) controlling cell expansion exhibited differential responses to heat stress between the heat-tolerant and the heat-sensitive line when exposed to heat stress. The expression levels of *GST*, and *HSP26* significantly increased in response to heat stress, but the extent of increase differed between the heat-tolerant and the heat-sensitive line. The transcript levels exhibited a 3.41-fold and 1.69-fold increase for *GST*, and 1.69-fold increase and 4.39-fold increase for *HSP26* in the tolerant and the

sensitive line, respectively. *HSP70* had differential expression between the heat tolerant and sensitive line when exposed to heat stress. *HSP70* expression levels increased by 2.72 fold in the sensitive line, but decreased by 1.72 fold in the tolerant line in response to heat stress when compared to the control. Conversely *HSP101* expression levels significantly increased in the heat tolerant genotype compared to control conditions by 1.75 fold but significantly decreased by 1.32 fold in the heat sensitive line resulting in greater transcript levels for *HSP101* in the heat tolerant genotype. The expression levels of *CAT* and *EXP* in the heat tolerant genotype did not change significantly between heat stress and control conditions but *CAT* and *EXP* expression declined significantly by 1.53 fold and 5.68 fold when the heat sensitive genotype was exposed to heat stress resulting in significantly less expression for both genes when compared to the heat tolerant genotype. The transcript level of *CP* of the heat-sensitive genotype under heat stress had a 2.31-fold increase over control conditions, while it did not change significantly in the tolerant genotype in response to heat stress resulting in the sensitive genotype have a significant increase in *CP* expression compared to the tolerant genotype. The transcript levels of *GAPDH* involved in energy metabolism decreased under heat stress in both genotypes when compared to control conditions, but did not significantly differ between the two genotypes.

DNA-Markers Linked to Selected Candidate Genes for Heat-Tolerance Traits

Candidate gene markers were mapped to linkage groups 2A1, 3A1, 4A1, 4A2, and 7A1 of the colonial bentgrass linkage map using the linkage group naming of the previously developed map (Rotter et al., 2009) (Fig. 4). All except the cysteine protease marker mapped to the expected colonial bentgrass linkage group based on the

relationship of cool-season grasses to rice (Sorrels et al., 2003). The markers for *GST* and *GAPDH* were not successfully mapped to a linkage group, which could be due to the statistical thresholds set for adding markers to the maps or the need for greater genetic information surrounding the candidate gene markers. Kruskal Wallis analysis found candidate gene markers associated with TQ, EL, CHL, Fv/Fm and NDVI (Table 4). The marker for *CP* was significantly associated with different distributions for TQ and NDVI. *Expansin* and *CAT* markers were both significantly linked to EL and NDVI. Lines with the marker for *GST* had significant differences in response to the photosynthetic parameters of CHL and Fv/Fm compared to lines which did not contain the markers according to Kruskal Wallis analysis. Both *GAPDH* and *HSP70* markers were significantly associated with EL. Lines with the marker for *HSP26* had significantly different distributions for CHL, EL and NDVI within the population when analyzed with the Kruskal Wallis test demonstrating a significant association between the *HSP26* marker and these traits. The marker for *HSP101* was not significantly related to any of the tested traits in this population.

DISCUSSION

The mapping population used in this study exhibited a wide range of heat tolerance as demonstrated by the segregation for TQ, membrane stability estimated by EL, photosynthetic traits (CHL and Fv/Fm ratio), and green leaf biomass measured as NDVI. Maintenance of photosynthesis and chlorophyll content, and membrane stability has been linked to improved heat tolerance in turfgrasses (Carrow, 1996; Marcum, 1998). EL, CHL and Fv/FM are highly correlated with overall heat tolerance in creeping bentgrass (Jespersen et al., 2013). The wide range of genetic variation in heat-tolerant traits across the creeping and colonial bentgrass hybrid backcross mapping population is of great value for the identification of candidate genes and DNA markers lined to heat tolerance.

Candidate gene markers largely exploited the genetic differences between colonial and creeping bentgrasses and physiological traits related to heat tolerance were frequently associated with colonial bentgrass alleles. The use of these or similar markers may help breeders with the introgression of colonial bentgrass genes associated with improved stress tolerance into creeping bentgrasses with desirable turf characteristics. The potential utility of an interspecific breeding program for the introgression of colonial bentgrass genes to increase stress tolerance is further supported by the hybrid parent TH15 having higher stress tolerance levels than the creeping bentgrass progenitors 9188 and 5061. The genetic variation in the aforementioned growth and physiological traits could be associated with the differential expression levels of related candidate genes and molecular markers involved in several major metabolic processes, including cell expansion, energy metabolism and protein metabolism, as well as antioxidant defense, as discussed below.

Antioxidant metabolism is an important defense mechanism for scavenging reactive oxygen species induced by stresses, including heat stress (Miller et al., 2008). The elevated expression of antioxidant genes and the accumulation of antioxidant proteins, including peroxidase, CAT, and GST, have been positively associated with heat tolerance in various plant species, including creeping bentgrass (Rizhsky et al., 2002; Xu and Huang, 2008; Xu and Huang, 2010). A previous proteomic study found that the heat-tolerant ColxCB169 had significantly greater abundance of CAT than the heat-sensitive ColXCB190 under heat stress (Jespersen et al., 2015). In this study, the heat-tolerant ColxCB169 had significantly higher transcript levels of both *CAT* and *GST* than the heat-sensitive ColxCB190 under heat stress, demonstrating the positive roles of *CAT* and *GST* in heat tolerance of bentgrass at the transcript level. The population individuals which possessed the candidate gene marker for the antioxidant genes, *CAT* and *GST*, had significantly different distribution patterns for EL, NDVI, CHL and Fv/Fm when compared to individuals who did not contain the candidate gene markers. The candidate gene markers for *CAT* and *GST* were associated with increased heat tolerance, which may be of use in selecting for improved antioxidant capacity to defend against stress damages in cool-season grasses.

Heat shock proteins are a large family of proteins which can be induced by heat and other abiotic stresses and have been strongly linked to heat tolerance (Vierling, 1991). HSPs of different molecular weights may play various roles in stress protection although most HSPs serve as chaperones (Hendrick and Hartl, 1995). Small HSPs, such as HSP26, are involved in stabilizing other proteins, HSP101 is involved in unfolding improperly folded proteins to aid in the return to proper conformations, and HSP70 helps to prevent

protein aggregation and is involved in signal transduction (Wang et al., 2004). Proteomic studies for bentgrasses showed an accumulation of HSPs in response to heat stress including HSP70 and HSP90 (Xu et al., 2010; Xu and Huang, 2010). In this study, the transcript levels of *HSP101* and *HSP26* were significantly greater in the heat-tolerant ColxCB169 compared to those in the heat-sensitive ColxCB190. Kruskal-Wallis analysis showed *HSP26* was associated with several physiological traits, including EL, CHL, and NDVI. However, the expression levels for HSP70 were lower in ColxCB169. *HSP101* was significantly more up-regulated in the heat-tolerant genotype than in the sensitive genotype, but the candidate gene marker did not have significant associations with any of the physiological traits measured. However when the candidate gene markers were added to the existing linkage map the marker for *HSP101* co-localized to a QTL region associated with drought tolerance on linkage group 2A1 (Merewitz et al., 2012) further supporting a potential role in abiotic stress tolerance. Among the HSPs examined in this study, *HSP26* could play major stress protective roles and the marker linked to *HSP26* could be valuable in creating bentgrasses with improved heat stress tolerance based on changes to gene expression and association with important physiological traits for heat tolerance.

Unlike HSPs, most soluble proteins degrade or are denatured under heat stress, which disrupts various cellular functions involving protein enzymes and contributes to stress-induced leaf senescence (Thomas and Stoddart, 1980; Vierling, 1991). Cysteine proteases are a class of enzymes that breakdown proteins and are involved in tissue senescence (Chen et al., 2002). A decline in soluble protein content and increases in protease activities have been observed in creeping bentgrass demonstrating symptoms of

leaf senescence under heat stress (Veerasamy et al., 2007) In this study, the heat-tolerant individual had a significantly lower level of *CP* expression compared to the sensitive individual under heat stress, which was corresponded to the greater TQ, CHL, and Fv/Fm ratio, as well as lower EL, and these results indicated that *CP* could serve as a negative regulator for heat tolerance. In addition, the marker for cysteine protease was linked to TQ and NDVI. The close link between *CP* gene expression and the marker association with phenotypic traits suggested that cysteine protease could be used as a candidate gene for genetic modification and useful marker for breeding selection of bentgrass germplasm with improved heat tolerance.

Respiration metabolism play essential roles in plant adaptation to heat stress by providing energy to activate various metabolic processes (Wahid et al., 2007). *GAPDH* is a major enzyme in respiration catalyzing the breakdown processes of sugars during glycolysis (Plaxton, 1996). *GAPDH* expression has been found to be increased during heat shock conditions in *Arabidopsis* (Yang et al., 1993). The protein abundance of *GAPDH* has been found to be positively associated with heat tolerance in creeping bentgrass plants (Jespersen and Huang, 2015). In this study *GAPDH* expression was significantly decreased in both the tolerant and sensitive genotypes by heat stress, however there was no significant difference in the level of down-regulation between the two genotypes under heat stress. The marker for *GAPDH* was significantly associated with membrane stability (measured as EL). Both gene expression and marker association of *GAPDH* with physiological traits in the backcross population suggested that maintaining higher levels of *GAPDH* could sustain the supply of ATP needed for stress defense and it could serve as a molecular marker to select heat-tolerance germplasm of

cool-season grasses. Expansins are a family of proteins involved in loosening cell walls and regulating cellular expansion (Cosgrove, 2000). Expansins have been found to be inducible by hormone signals in addition to being induced by abiotic stress (Zhou et al., 2010). Increased expression of *expansins* has been positively related to heat stress tolerance in different plant species, including creeping bentgrass (Xu et al., 2007) and wheat (Qin et al., 2008). In this study, the heat-sensitive genotype had significantly greater expression levels of *EXP* in response to heat stress compared to the heat-tolerant genotype, and the *EXP* gene marker was associated with membrane stability (EL) and green leaf biomass (measured as NDVI). These results indicated that *EXP* could be a useful candidate gene and marker for improving heat tolerance either through genetic transformation or molecular-assisted breeding.

In summary, the hybrid backcross population showed a range of genetic variations for heat tolerance as determined by overall visual quality, EL and CHL. Additionally when looking at candidate gene expression in a heat tolerant and heat sensitive member of the population most candidate genes had differential expression between the two individuals under heat stress supporting that they may play roles in heat tolerance. Several candidate gene markers developed in this study (*CAT*, *CP*, *EXP*, *GAPDH*, *GST*, *HSP26* and *HSP70*) were found to be linked to important physiological traits for heat tolerance such as TQ, EL, NDVI, CHL and Fv/Fm. The successful implementation of candidate gene markers associated with heat tolerance will allow for improved selection and aid in the creation of improved turfgrass cultivars with increased abiotic stress tolerances.

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Table1. Sequences used for candidate gene markers

Gene		Sequence	Accession	Marker Type
<i>CAT</i>	F	GTGGAAGCATACTGACCCTA	DV856768	Allele Specific
	R	GGTTCATTAATCAGGGTGCATA		
<i>CP</i>	F	GTCCGCCATGCACACCTCA	DV858986	Allele Specific
	R	GCGTACGGAAGCAAATGTATT		
<i>EXP</i>	F	CTCTCGCAGGAGAATCTTCA	DV853008	Allele Specific
	R	AACTGCAAGACGACGATCACACAA		
<i>GAPDH</i>	F	CCAATGGAAGTGAACGCACG	DV856708	Allele Specific
	R	GTTCGATACTTCGAATCATTATT		
<i>GST</i>	F	GGGCTCAACTTCTTGGCAATA	DV857137	Allele Specific
	R	CACCGAAACACACATGACACA		
<i>HSP26</i>	F	CTGGTGGACCCGATGTCG	DV857713	Allele Specific
	R	CACGCCGTTCTTGAGCTCA		
<i>HSP70</i>	F	ACAAGATGAAGGAGCTCGAG	DV853545	CAPS - AgsI ^a
	R	CAGGTGATATGATTGGCACC		
<i>HSP101</i>	F	TTCTTTCTCTGTTCGCTCTTTT	DV858769	Allele Specific
	R	CTGTTTGATTTACATGGCAATTT		

^aAgsI restriction enzyme was obtained from Sibenzyme (Academtown, Russia) and restriction reactions were performed as per the manufacturer's instructions

Table 2. Primers used for real-time PCR

Gene		Sequence
<i>Catalase:</i>	F	AAGGAGAACAACCTTCAAGCAG
	R	GAATCGCTCTTGCCTTGCC
<i>Cysteine protease:</i>	F	GGTTGATGAGGAACAGATTGC
	R	CAGATGTATGGGCACGACAC
<i>Expansin:</i>	F	CCCCTCTCGCAGGAGAATC
	R	TCTTGCTCAAGCTCCAAATCAA
<i>Glutathione-S-transferase:</i>	F	ACACGGTCGCCAGGTTCTT
	R	ATGACGCCGTGCAGAGGAT
<i>Glyceraldehyde-phosphate-dehydrogenase</i>	F	TCGTGGTTCAGGTCTCCAAGA
	R	GTCACGGAACGCCTGGTT
<i>Hsp 101:</i>	F	CAGCGTCATCGAGAAGGGA
	R	GACCAGGCTCTTGATCCTG
<i>HSP 26:</i>	F	GCCGTGGGACATCATGGA
	R	CTTCACTTCGTCCCGTGACA
<i>HSP 70:</i>	F	GATGGCGTTCTCTGGGTTGA
	R	GCATGGGCGGCTACATGT
<i>Actin:</i>	F	CCTTTTCCAGCCATCTTTCA
	R	GAGGTCCTTCCTGATATCCA

Table 3. Summary of physiological measurements and ANOVA statistics from non-stress conditions and the final measurement day of heat stress for the 93 individual in the backcross population.

	Control				6 weeks of heat stress			
Parameter	Mean	Range	F-statistic	P-Value	Mean	Range	F-statistic	P-Value
Turf Quality	8.9	8 – 9	2.21	0.0001	5.3	1.3 – 6.7	3.39	<0.0001
NDVI	0.33	0.22 - 0.47	2.04	0.0001	0.24	0.12 – 0.39	2.03	<0.0001
Electrolyte Leakage	22.2%	16.2% - 26.2%	0.96	N.S.	41.8%	25.6% - 96.8%	3.07	<0.0001
Chlorophyll Content mg/ g dw	23.7	15.12 – 29.2	2.00	0.0001	11.46	1.03 – 16.99	2.06	<0.0001
Chlorophyll Fluorescence	0.80	0.77- 0.83	0.98	N.S.	0.71	0.46 – 0.76	1.97	<0.0001

Table 4. Summary of Kruskal-Wallis statistics linking candidate gene markers to physiological traits

Candidate Gene	Physiological Trait	K* statistic	Significance: P-value ^a
<i>CAT</i>	EL	4.2	0.041
	NDVI	4.4	0.036
<i>CP</i>	NDVI	8.9	0.003
	TQ	6.2	0.012
<i>EXP</i>	EL	3.9	0.042
	NDVI	3.3	0.050
<i>GAPDH</i>	EL	3.6	0.045
<i>GST</i>	CHL	4.5	0.035
	Fv/Fm	3.3	0.050
<i>HSP26</i>	CHL	7.1	0.008
	EL	4.1	0.043
	NDVI	5.3	0.021
<i>HSP101</i>	-	-	N.S.
<i>HSP70</i>	EL	3.7	0.048

^a Table presents the most significant data if multiple sampling days were found to be significantly associated with a given trait

Fig 1. Distribution curves from the final day of heat stress when differences in the population were greatest showing variations in 93 individuals of the mapping population for heat stress for (A) TQ, (B) EL, (C) CHL, (D) NDVI, and (E) Fv/Fm. Triangles represent parental lines with solid black being TH15, gray being 9188 and white being 5061

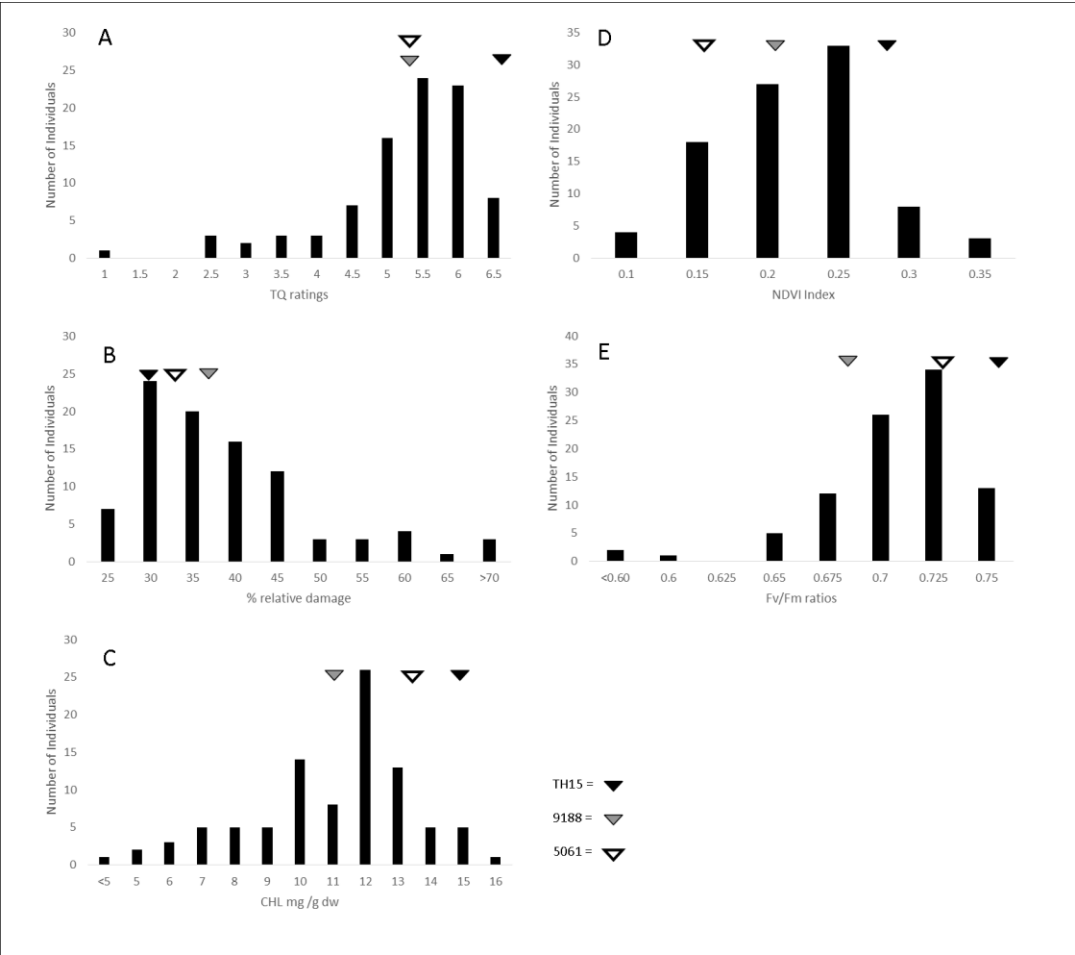


Fig 2. Physiological differences between the heat tolerance ColXCB169 (black diamonds) and heat sensitive ColXCB190 (gray boxes) for 2 weeks of heat stress for (A) TQ, (B) CHL, and (C) EL. Bars represent standard deviations

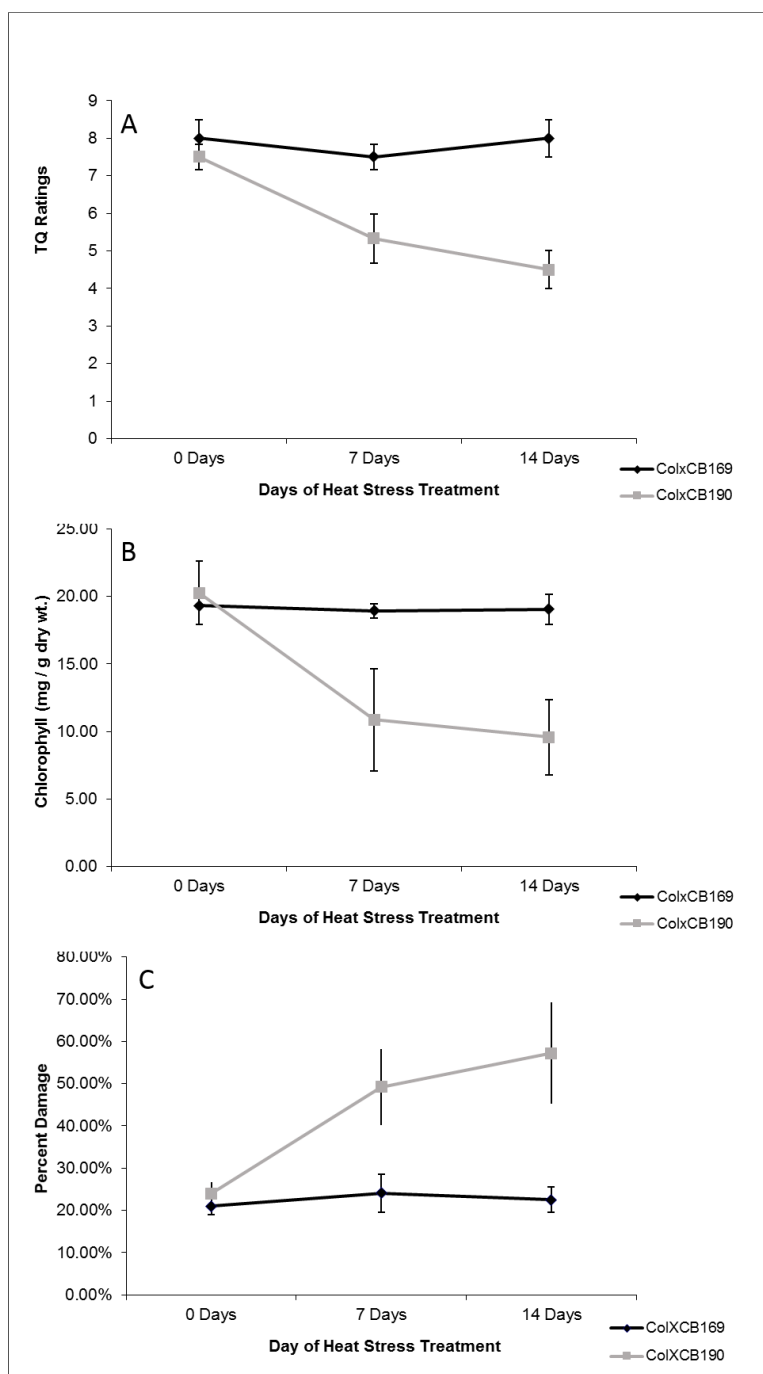


Fig 3. Differences in gene expression using qPCR for the heat tolerance ColXCB169 (black bars) and heat sensitive ColXCB190 (gray bars). Fold changes are changes in gene expression levels compared to control conditions (20/15 °C day/night). Actin was used as the endogenous control for the delta delta CT method. Bars represent standard deviations

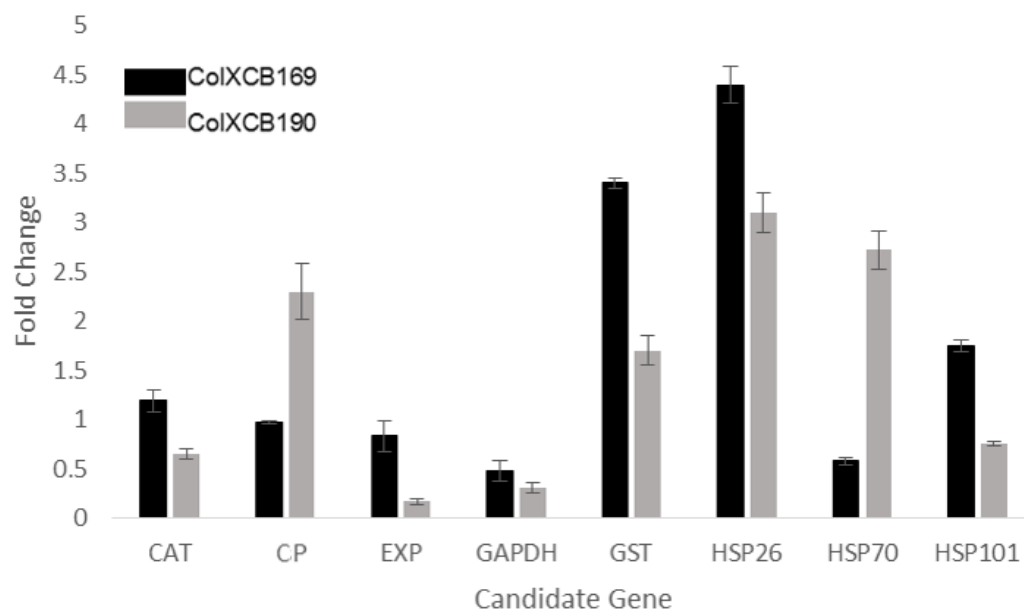
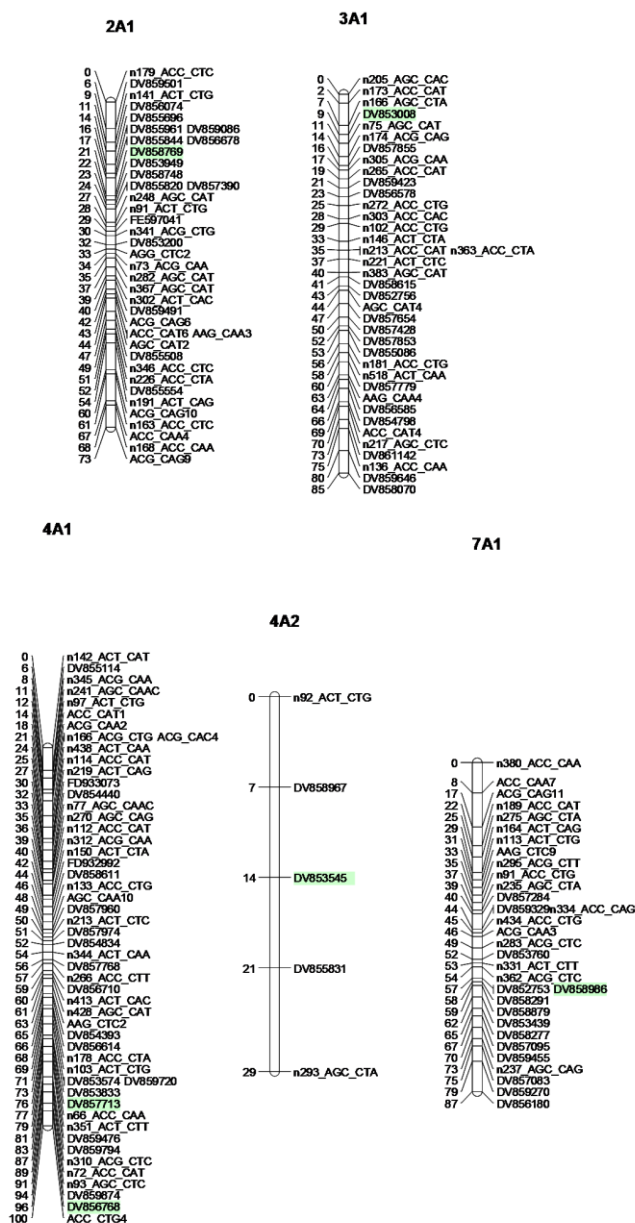


Fig 4. Linkage maps for colonial bentgrass showing the linkage groups to which the candidate gene markers successfully were added. Added markers are highlighted and named by their accession numbers



**CHAPTER 7: Chlorophyll Loss Associated with Heat-Induced Senescence in
Bentgrass**

INTRODUCTION

Heat stress is a major abiotic factor which affects cool-seasons plants and frequently limits growth during summer months. Heat stress damage in plants is characterized by leaf senescence associated with the loss of macromolecules, including chlorophyll pigments (Wahid, 2007). Heat-induced chlorophyll loss has been noted in a number of species, including sorghum (*Sorghum bicolor*), wheat (*Triticum aestivum*), Kentucky bluegrass (*Poa pratensis*), and creeping bentgrass (*Agrostis stolonifera*) (Djanaguiraman et al., 2014; Ristic et al., 2006; He and Huang, 2007; Liu and Huang, 2000). The metabolic factors underlying heat-induced loss of chlorophyll molecules in associated with heat-accelerated leaf senescence are generally not fully understood.

Chlorophyll is the main pigment involved in the absorption of light energy for use in photosynthesis in the thylakoids (Taiz and Zeiger, 2010). Its structure consists of a porphyrin ring with a magnesium ion bound in the middle and a phytol chain (Tanaka and Tanaka, 2007). Several important enzymes involved in chlorophyll synthesis include porphobilinogen deaminase (PBGD) which is responsible for combining four porphobilinogen subunits into a ring structure (Jones and Jordan, 1994); Mg-chelatase (MG-CHT) which is responsible for inserting the magnesium ion into the tetrapyrrole ring (Papenbrock et al., 2000); and protochlorophyllide reductase (POR) which is generates chlorophyllide in the presence of light (Forreiter et al., 1991). Additionally a number of pathways for the initial steps of chlorophyll degradation have been proposed including chlorophyllase (CHLASE) which cleaves the phytol chain for chlorophyll molecules (Holden, 1961); pheophytinase which cleaves the phytol chain from pheophytin, a chlorophyll molecule with the magnesium ion removed (Schelbert et al.,

2009); and chlorophyll-degrading peroxidases (CHL-PRX) which oxidizes chlorophyll in the presence of H₂O₂ and phenolic compounds (Yamauchi et al., 2004). Recently several advances have allowed for a greater understanding into chlorophyll metabolism and how it differs from similarly structured heme proteins (Eckhardt et al., 2004). However, changes in chlorophyll metabolism and in particular how they relate to chlorophyll loss during stress-induced senescence are not well documented. In a number of species abiotic factors have been shown to alter chlorophyll synthesis. PBGD and MG-CHT in wheat (*Triticum aestivum*) and cucumber (*Cucumis sativus*) had decreased activity due to heat or chilling stress (Tewari and Tripathy, 1998). Aminolevulinic acid deaminase activity was found to be decreased in sunflower (*Helianthus annuus*) due to salt stress (Santos, 2004). Protochlorophyllide-reductase (POR) has also been found to be a regulatory step that is down-regulated in senescent leaves (Forreiter, 1991). It has also been found that chlorophyll loss may be due to changes in chlorophyll degradation with several pathways being proposed as being responsible for losses in chlorophyll including CHLASE, PPH, or CHL-PEROX (Kaewsuksaeng et al., 2011; Hortensteiner and Krautler, 2011)

Bentgrasses (*Agrostis*) are a genus of cool-season perennial grasses which contain several species with fine textured leaves that are well adapted for use as turfgrasses on high value turf areas (Beard, 1973). Bentgrasses have low to moderate levels of heat tolerance which results damage to turf areas during summer months (Turgeon, 1999). As with other cool-season plants, one of the major symptoms of heat stress in bentgrasses is a loss of chlorophyll associated with pre-mature leaf senescence (Xu and Huang, 2000). Two important bentgrass species are colonial bentgrass (*A. capillaris*) and creeping bentgrass (*A. stolonifera*). A colonial (*A. capillaris*) x creeping (*A. stolonifera*) bentgrass

hybrid backcross population has previously been shown to have a range of phenotypic responses under abiotic stress conditions such as drought and heat (Merewitz et al., 2012; Jespersen et al., 2015). Some backcross individuals exhibited superior stay-green phenotypes or lesser extent of heat-induced leaf senescence than others (Jespersen et al., 2015). The question of whether heat-induced leaf senescence associated with loss of chlorophyll is related to heat-inhibition of chlorophyll synthesis and/or heat-accelerated chlorophyll degradation in cool-season grass species is unknown. Understanding the key factors regulating chlorophyll metabolism responsible for the loss of chlorophyll during heat-induced senescence could allow for the development of more stress-tolerant genotypes with stay-green traits either through marker assisted selection or transgenic approaches. It is hypothesized that the loss of chlorophyll in bentgrass is due to alteration in gene expression or metabolic activities of chlorophyll synthesis and chlorophyll degradation enzymes, contributing to the overall decline in turf quality during summer months. The objectives of this study were to examine genetic variations in the level of heat-induced leaf senescence in individuals of a colonial x creeping bentgrass population contrasting in heat tolerance, and determine whether loss of leaf chlorophyll during heat-induced leaf senescence was due to suppression of gene expression and enzymatic activities involved in chlorophyll synthesis or stimulation of genes expression or enzymatic activities for chlorophyll degradation. Looking at both chlorophyll synthesis and degradation pathways will help elucidate the key metabolic processes responsible for the loss in chlorophyll associated with heat induced senescence, which could facilitate the development of improved cultivars with stay-green characteristics.

MATERIALS AND METHODS

Clonally propagated plants of two hybrid backcross individuals ('ColxCB169' and 'ColxCB190') from a colonial bentgrass (*Agrostis capillaris*) x creeping bentgrass (*Agrostis stolonifera*) hybrid backcross population generated at Rutgers University and previously shown to contrast in heat tolerance were examined in this study (Rotter et al. 2009; Jespersen et al., 2015). Plants were established in plastic pots (15 cm in diameter and 20 cm deep) filled with a mixture of 50% soil (fine-loamy, mixed mesic Typic Hapludult type soil) and 50% peat moss in a greenhouse for 6 weeks before being transferred to environmentally controlled growth chambers (Conviron, Winnipeg, Canada) controlled at 20/15 °C (day/night), 14-h photoperiod, and photosynthetically active radiation (PAR) at 600 $\mu\text{mol m}^{-2} \text{s}^{-1}$ for a 7 d acclimation period prior to imposition of heat stress. Plants were maintained well-watered and fertilized weekly with half-strength Hoagland's nutrient solution (Hoagland and Arnon 1950) during plant establishment and heat-stress periods.

Plants of both genotypes were exposed to either heat stress conditions of 38/33°C (day/night) or optimal temperature of 20/15°C (day/night) for 28 d. Each temperature was repeated in four growth chambers and each genotype had four pots of plants which were randomly placed in four different growth chambers. The experiment design was a randomized split-plot design with temperature treatments as main plots and genotypes as subplots, with each treatment and line having four replicates.

Physiological Analysis of Heat-Induced Leaf Senescence

The extent of leaf senescence was evaluated using commonly-used parameters, including chlorophyll content, chlorophyll fluorescence, and membrane stability. In

addition, the content of a chlorophyll precursor, 5-aminolevulinic acid (5-ALA), was measured to assess metabolic changes of chlorophyll as affected by heat stress. Whole-plant responses to heat stress was evaluated by visually rating turf quality (TQ) on a 1-9 scale based on leaf color, density and uniformity to assess plant health and levels of leaf senescence with 1 representing dead plants and 9 representing completely healthy plants (Beard 1973). All measurements were taken every 7 d during the 28-d heat stress period.

Chlorophyll content (CHL) was measured the methods described by Hiscox and Israelstam (1979) with modification. Approximately 0.1g of fresh leaf tissue was placed in 10 ml of dimethyl sulfoxide and incubated in the dark for 5 d to extract chlorophyll from the tissue. The extracted solution was measured spectrophotometrically at 663 and 645 nm (Genesys 2, Spectronic Instruments, Inc., Rochester, NY). Leaf tissue was then filtered from the remaining solution and dried for 72 h in an 80°C oven to obtain dry weights. Chlorophyll content was calculated on a dry weight basis using the equations described by Arnon (1949).

The content of 5-ALA was estimated using a modification of the methods described by Beale and Castelfranco (1974). A 0.1g of frozen leaf tissue was ground in sodium acetate buffer (1M pH 4.6) and centrifuged at 12,000 g for 10 min. A 0.2 ml of the resulting supernatant was mixed with 0.2 ml water and 50 µl of acetylacetone and boiled for 10 minutes. A 0.5 ml of a modified Ehrlich's reagent consisting of 2% p-dimethylaminobenzaldehyde, 6% perchloric acid, 88% acetic acid was added to the solution boiled extract. After a brief incubation period the solution was measured at 555 nm to quantify 5-ALA.

Chlorophyll fluorescence (Fv/Fm) was measured to estimate photochemical efficiency and the health of the PSII reaction center. Leaves were dark adapted for 30 minutes prior to measurement and the ratio of variable fluorescence (Fv) to maximal fluorescence (Fm) was measured using a fluorescence induction monitor (Fim 1500, Dynamax, Houston, TX). Three subsamples per replicate were taken for each measurement.

Membrane stability (EL) was estimated using electrolyte leakage as described by Blum and Ebercon (1981). Approximately 0.1 g fresh tissue was placed in 35 ml of deionized water and incubated on a shaker for 16 h. An initial conductance reading (C_{initial}) of the solution was taken with a conductivity meter (YSI Incorporated, Yellow Springs, OH) and the samples were autoclaved at 120 °C for 20 minutes to kill all containing leaf tissue and placed back on the shaker for an additional 16 h. A final conductance reading (C_{max}) was measured and percent relative damage was calculated as $C_{\text{initial}} / C_{\text{max}} \times 100$.

Real-Time PCR Analysis of Gene Expression of Chlorophyll-Synthesizing or Chlorophyll-Degrading Enzymes

Leaves (the second and third fully-expanded leaves of a plant) were collected at 28 d of either heat stress or non-stress temperature treatment for gene expression analysis, at which time heat-induced leaf senescence was significantly different for the two backcross individuals. Leaf tissues were flash frozen in liquid nitrogen, and stored in a – 80 °C freezer for further analysis. RNA was extracted with an RNeasy kit (Qiaagen, Venlo, Netherlands) and treated with a Turbo DNase kit (Life Technologies) to removed DNA

contamination. A high-capacity cDNA synthesis kit (Life Technologies) was used to generate cDNA and qPCR primers were designed using Primer3Plus software (Rozen and Skaletsky, 2000). qPCR was performed on a StepOnePlus real-time PCR machine using SYBR green master mix (Life Technologies). In addition to biological replicates, 3 technical replicates were used per sample, along with no template negative controls. Actin was used as the endogenous control gene and relative gene expression was determined using the delta-delta-CT method (Schmittgen and Livak 2008). The qPCR primer sequences are given in Table 1.

Bioassays of Activities of Chlorophyll-Synthesizing and Chlorophyll-Degrading Enzymes

Purified chlorophyll pigments for use in enzyme assays were obtained by using a dioxane precipitation resulting in an acetone solution with a chlorophyll concentration of 500 $\mu\text{g ml}^{-1}$ (Iriyama et al., 1974). Pheophytin was produced by adding 60 μl of 0.1N HCL per 1 ml of the chlorophyll acetone solution and letting it incubated for 5 min. Enzyme extraction was performed by grinding 0.3g of frozen leaf tissue into an enzyme extraction buffer consisting of 0.1M phosphate buffer (pH 7), 1% Triton x-100, 1mM PMSF, and 3% PVPP. Extractions were centrifuged at 4°C for 20 minutes at 9,000 g and the resulting supernatant was used for enzyme assays. Protein concentration was quantified using a protein assay dye reagent (Bio-Rad, Hercules, California) and enzyme activity was expressed on a per unit protein basis.

Porphobilinogen deaminase (PBGD) activity was measured using the methods described in Jones and Jordan (1994). Enzyme extraction was incubated at 37°C in a 0.1M Tris buffer (pH7.5) containing 2.5 mM EDTA, 15 mM mmMgCl₂, 0.1% BSA, and

2 mM porphobilogen. To this reaction 0.25 ml of 5N HCL and 0.1 ml 0.1% benzoquinone were added and the absorbance was measured at 405nm using a spectrophotometer.

Chlorophyll-degrading peroxidase (CHL-PRX) activity was estimated by measuring the decrease in chlorophyll in the presence of H₂O₂ and a phenolic compound (Aiamla-or et al., 2010). The enzyme extraction (50µl) was added to 1ml 100mM phosphate buffer (pH 7) with 0.1 ml 1% H₂O₂, 0.1 ml 25 mM p-coumaric acid, 0.1 ml 1% Triton X-100 and 0.1 ml chlorophyll solution and the decrease in absorbance at 668 nm was measured over the course of 10 minutes; one unit of activity was defined as a decrease of 0.1A per min.

Chlorophyllase (CHLASE) activity was estimated using a modification of the procedures presented in Fang et al. (1998). The reaction consisted of 0.1ml enzyme extraction, 0.3 ml acetone, 0.7 ml 50 mM Tris buffer (pH 7), 20 µl ascorbate (0.1M) and 0.2 ml chlorophyll solution. The reaction was incubated for 60 minutes at 35°C in the dark. The reaction was then added to a tube containing 1ml 0.5M Tris (pH 9), 1 ml acetone, and 2 ml hexane. The tube was shaken vigorously to extract the remaining chlorophyll to the hexane phase and chlorophyllide to the aqueous phase. The tube was briefly centrifuged to separate the two phases and the production of chlorophyllide was measured using a spectrophotometer at 665 nm.

Pheophytinase (PPH) was estimated using a modification of the procedures presented in Kaewsuksaeng et al. (2011). The reaction consisted of 0.2 ml enzyme extraction, 0.6 ml 20mM HEPES buffer (pH 7.75), 0.1 ml 1% Triton X-100, and 0.1 ml pheophytin solution. The reaction was incubated at 30°C in the dark for 1 hour and the

reaction was added to a tube containing 1ml 0.5M Tris (pH 9), 1 ml acetone, and 2 ml hexane. The tube was shaken vigorously to extract the remaining pheophytin to the hexane phase and. The tube was briefly centrifuged to separate the two phases and the production of pheophorbide was measured using a spectrophotometer at 665 nm.

Confirmation of PPH Expression Related to Heat-Induced Leaf Senescence Using an *Arabidopsis thaliana* Mutant

Wild type (Col-0) and T-DNA insertion lines (at5g13800-1, SALK_000095) were obtained from the Arabidopsis Biological Resource Center (ABRC) (Alonso et al., 2003). The T-DNA insertion line was previously demonstrated to be a mutant line which lacked PPH expression and confirmed used RT-PCR (Zhang et al., 2015). Plants were growth in 10 cm² pots filled with peat soil in environmentally controlled growth chambers set to 22 °C with a 12-h photoperiod with PAR of 350 $\mu\text{mol m}^{-2} \text{s}^{-1}$ and plants were maintained well watered and fertilized weekly with half-strength Hoagland's nutrient solution (Hoagland and Arnon, 1950). To assess heat tolerance in the wild type and mutant line, 22-d old Arabidopsis plants were maintained in non-stress control conditions or exposed to heat stress conditions of 45/40 °C (day/night) for 3 d and then returned to non-stress control conditions to recover for 3 d. Physiological assessment included El, Fv/Fm and CHL performed as previously described. Each treatment included six individual plants as six replicates. In conjunction to physiological measurements leaf samples were collected and frozen to be used for qPCR and PPH activity assays as previously described (Table 2). Additionally 5-d old seedlings grown on MS media were exposed to 43°C heat stress conditions for 3 h and then transferred back to non-stress conditions for 3 d. The number

of seedlings which survived was counted to calculate survival ratio. Three replicate plates were assessed using this method.

Statistically Analysis

Physiological data, gene expression data, and enzyme assays were assessed using analysis of variance to determine significant differences between temperature treatments and hybrid backcross genotypes. Means were separated using Fisher's protected LSD using a statistical analysis program (SAS v9.2; SAS institute 1992).

RESULTS

Physiological Characterization of Heat-Induced Leaf Senescence

Heat stress caused chlorophyll levels to decrease significantly in both lines (Fig 1). At 28 d of non-stress control temperature, plants had similar total CHL levels for the heat-tolerant ColxCB169 and heat-sensitive ColxCB190. Under heat stress conditions both lines experienced a loss in chlorophyll; however, ColxCB169 had a much less severe decline (by 11.6%) compared to ColxCB190 (by 38.7%). Chlorophyll a (CHL A) and chlorophyll b (CHL B) had similar trends of change in response to heat stress, with heat-induced decline in both CHL A and CHL B being more pronounced in ColxCB190 than in ColxCB169. At 28 d of stress treatment, ColxCB190 had a 12% and 10% decline in the levels of CHL A and CHL B respectively, compared to a 41% decline for CHL A and a 27% decline for CHL B in ColxCB169. This loss of chlorophyll also ultimately caused a significant difference in CHL A:CHL B ratio between ColxCB169 and ColxCB190 at 28 d of heat stress, with the tolerant line maintaining a ratio of approximately 4.8 while the sensitive line's ratio declined from 4.6 to 3.7. The content of 5-ALA did not differ significantly between ColxCB169 and ColxCB190 in non-stress control conditions, and heat stress did not alter 5-ALA content in ColxCB169, while 5-ALA content of ColxCB190 had a 91.7% increase at 28 d of heat stress (Fig. 2).

For Fv/Fm, no significant differences were detected between the two genotypes under non-stress control conditions. By 28 d heat stress, both genotypes had significant declines compared to non-stress control conditions (Fig. 3). The decline in Fv/Fm for ColxCB169 was to a lesser degree than that for ColxCB190 when exposed to heat stress, declining to 0.778 and 0.710 respectively (Fig 3A).

There were also significant differences in EL when comparing ColxCB169 and ColxCB190 during heat stress. Heat stress treatment did not result in any changes for ColxCB169 when compared to plants in the non-stress treatment. ColxCB190 experienced a significant increase in EL by 28 d of heat stress with heat stress plants having 41.9% relative membrane damage compared to only 24.7 % for non-stress plants (Fig. 3B).

Under non-stress control conditions there were no differences in TQ between the two genotypes. Under heat stress conditions, TQ of both genotypes declined significantly, but ColxCB190 declined quicker and to a significantly greater extent dropping to 4.6, compared to 7.0 for ColxCB169 by 28 d of heat stress (Fig. 3C).

Gene Expression Changes of Chlorophyll-Synthesizing and Chlorophyll-Degrading

Enzymes in Response to Heat Stress

Expression levels for two genes involved in chlorophyll synthesis, *PBGD* and *Mg-CHT*, did not significantly change in response to 28 d of heat stress compared to non-stress control conditions, and were not significantly different between the two genotypes (Fig 4A). *POR* exhibited significant increases in the expression level at 28 d of heat stress, with a 1.5- and 1.7-fold increase for ColxCB169 and ColxCB190, respectively (Fig 4A). However, the changes in expression levels of this gene were not significantly different between the two genotypes at 28 of heat stress.

A number of differences were found between ColxCB169 and ColxCB190 when comparing the expression levels of genes involved in chlorophyll degradation (Fig 4B). *CHLASE* expression did not change in ColxCB169 when exposed to heat stress, but increased by 7.6 fold in ColxCB190, resulting in significantly higher expression of

CHLASE transcript levels, compared to ColxCB169. *PPH* expression had a similar pattern to *CHLASE* in that ColxCB169 did not have a significant change in expression in response to heat stress, but ColxCB190 had a 3.4-fold increase in expression levels, resulting in the sensitive genotype having increased *PPH* gene expression compared to the tolerant genotype. Conversely, peroxidase expression increased by 2.7 fold in ColxCB169 under heat stress compared to the non-stress control but did not change for ColxCB190. ColxCB169 had significantly greater fold changes in peroxidase expression levels compared to ColxCB190 when exposed to heat stress.

Enzymatic Activity of Chlorophyll-Synthesizing and Chlorophyll-Degrading Enzymes in Response to Heat Stress

PBGD activity was not significantly altered by heat stress in either genotype (Fig 5A). However, ColxCB190 had higher activity for PBGD in both the heat and non-stress control conditions compared to ColxCB169 in either temperature treatment. *CHLASE* activity was not significantly altered by heat stress for either of the two genotypes, although ColxCB190 line had approximately 20% higher activity when compared to ColxCB169 in either temperature treatment (Fig 5B). *PPH* activity did not significantly increase in ColxCB169 exposed to heat stress (Fig 5C). However there was a significant increase in ColxCB190 at 28 d heat stress with *PPH* activity increasing by 15% compared to non-stress conditions, which was 11% greater than ColxCB169 *PPH* activity under heat stress conditions. For *CHL-PRX* activity, heat stress did not cause changes in the activity in ColxCB169 (Fig 5D). In ColxCB190, the activity of *CHL-PRX* decreased by 61% under heat stress compared to non-stress plants. Comparing the two genotypes under

heat stress, ColxCB169 had significantly higher CHL-PRX activity compared to ColxCB190.

Confirmation of PPH Expression Related to Heat-Induced Leaf Senescence Using an *Arabidopsis thaliana* Mutant

The roles of PPH in regulating heat-induced senescence and associated chlorophyll loss could be confirmed using *pph* mutant. This study used an available *pph* mutant of the model plant *Arabidopsis thaliana* due to lack of such resource in perennial grass species and the wide application of the model species. Physiological responses of the *pph* mutant and WT *Arabidopsis* to heat stress and associated changes in PPH gene expression and enzymatic activity were performed and compared. .

Heat stress caused significant declines in EL, Fv/Fm and CHL in both the WT plants and *pph* mutant line; however, the decline was to a significantly greater extent in the WT plants (Fig. 6). Heat stress increased EL to 52.7% in the WT compared to 43.9% in the *pph* mutant line. Fv/Fm declined from 0.83 in non-stress conditions to 0.63 for the WT, but only 0.76 for the *pph* mutant line during heat stress. In a similar trend CHL declined by 43.9% in the WT under heat stress condition while in the *pph* mutant CHL decline by 31.6% having maintained significantly higher CHL. This difference between the WT and mutant lines was also found to persist after 3 d recovery with the mutant line maintaining significantly improved EL, Fv/Fm and CHL.

PPH gene expression and enzyme activity was measured during heat stress and was found to correlate with chlorophyll loss in the WT *Arabidopsis* plants (Fig. 7). *pph* expression was significantly increased by 11.3 fold during 3 d of heat stress, and then declined to 5.0 fold above non-stress levels during the 3-d recovery still being

significantly higher than the non-stress control baseline. In vitro enzyme activity for PPH matched expression levels with PPH activity, exhibiting an increase of 44% during 3 d of heat stress compared to non-stress conditions. PPH activity then declined during the 3-d recovery. After heat stress of 5-d old seedlings for 3 h, only 16.7% of the WT plants survived compared to a significantly greater proportion of 37.2% survival rate for the *pph* mutant plants (Fig. 8).

DISCUSSION

Heat stress accelerated leaf senescence in both genotypes of the colonial x creeping bentgrass population, as demonstrated by the decline in leaf chlorophyll content and leaf photochemical efficiency, as well as increases in membrane stability, which could contribute to the decline in overall turf quality. Significant genetic variations in the level of heat-induced leaf senescence and TQ were observed between two genotypes in this study. These results were in agreement with previous studies for the same two genotypes (ColxCB169 and ColxCB190), which found that superior heat tolerance of ColxCB169 (Jespersen et al., unpublished data; Jespersen et al., 2015). Proteomic analysis of the two genotypes found that the improved heat tolerance of ColxCB169 was associated with increased expression of stress defense genes, such as antioxidant or heat shock proteins and accumulation of proteins involved in energy metabolism, photosynthesis and stress defense (Jespersen et al., unpublished data; Jespersen et al., 2015). Further analysis of metabolic and transcript factors underlying heat-accelerated decline in chlorophyll content in this study found that increased gene expression and metabolic activities of enzymes involved in chlorophyll degradation could be the main factor contributing to loss of chlorophyll and associated leaf senescence in the cool-season bentgrass species.

PBGD, involved in combining four porphobilinogen subunits to generate a porphyrin ring, MG-CHT, which inserts a magnesium ion into the porphyrin ring, and POR controlling one of the final steps in chlorophyll synthesis to generate chlorophyllide, are major enzymes regulating chlorophyll synthesis (Bollivar, 2006). Gene expression levels of PBGD, MG-CHT, and POR did not change significantly in response to heat

stress, nor varied between the two hybrid backcross genotypes. MG-CHT and POR enzymatic activities were not measured in this study. The enzymatic activity of PBGD exhibited the same pattern as the expression level of this gene in terms of heat effects and genetic variations. Those results suggested that PBGD, MG-CHT, and POR may not be the controlling metabolic factors of chlorophyll synthesis that lead to heat-induced decline in CHL content and genetic variations in the loss of chlorophyll in bentgrass species. Tewari and Tripathy (1998) found that heat stress resulted in decreases in PBGD and MG-CHT activity in both cucumber or wheat seedlings exposed to 42°C heat stress for 24 – 48 hours, resulting in reduced CHL accumulation. Heat-inhibition of chlorophyll synthesis may be dependent on the severity of stress and variations in heat-sensitivity of different plant species. Additionally we measured the content of 5-ALA which is the major precursor feeding into chlorophyll synthesis (Eckhardt et al., 2004). The hybrid backcross genotype that exhibited greater decline in chlorophyll content (ColxCB190) had higher content of 5-ALA under heat stress than the line with lower level of leaf senescence. These data further supported the notion that chlorophyll loss was not caused by a lack of carbon feeding into chlorophyll synthesis pathways in bentgrass plants exposed to heat stress.

The results of changes in chlorophyll degradation pathways in response to heat stress and associated with the genetic variations are particularly worth noting in this study. CHLASE was one of the earliest enzymes discovered, which breaks down chlorophyll molecules by removing the phytol chain to generate chlorophyllide (Holden, 1961). A previous study in *Arabidopsis* has shown that in vitro CHLASE activity was increased by heat stress (Todorov et al., 2003). Under heat stress conditions CHLASE gene expression

were significantly increased in the heat-sensitive ColxCB190 but not in the heat-tolerant ColxCB169. In addition, ColxCB190 also had higher CHLASE activity when compared to ColxCB169 under non-stress and heat stress conditions, although no changes to CHLASE activity caused by heat stress conditions in either genotype. The function of CHLASE as the enzyme responsible for chlorophyll degradation was proposed to be species or potentially tissue dependent, which may reflect species variations in leaf senescence (Azoulay-Shemer et al., 2011; Guyer et al., 2014). The greater CHLASE expression under heat stress and enzymatic activity in ColxCB190 than ColxCB169 under both non-stress and heat stress conditions suggested that CHLASE could be involved in both natural leaf senescence and heat-induced leaf senescence in bentgrass species, since leaf senescence is not only accelerated by heat stress but also occurs naturally in perennial grass species.

The chlorophyll-degrading gene PPH cleaves pheophytin which is a chlorophyll molecular with the magnesium ion removed (Schelbert et al., 2009). In the current study the heat-sensitive ColxCB190 had both increased expression and enzyme activity for PPH compared to the tolerant ColxCB169 in response to heat stress. The genetic differences and consistent pattern of heat responses at both gene expression and enzymatic levels of PPH indicated that PPH could be is one of the key enzymes regulating chlorophyll loss and heat-induced leaf senescence in bentgrass species in this study. Schelbert et al. (2009) found that chlorophyll degraded at a normal rate in CHLASE mutants of *Arabidopsis* and has proposed PPH is the main enzymatic pathway for chlorophyll degradation. PPH gene expression was also found to correlate with chlorophyll loss in some plant species, such as broccoli (*Brassica oleracea*), pear (*Pyrus*

bretschneider) and cabbage (*Brassica rapa*) (Büchert et al., 2011; Cheng and Guang, 2014; Zhang et al., 2011). Additionally PPH activity was found to increase coordinately with chlorophyll loss, further supporting its role in chlorophyll degradation, as found in broccoli and perennial ryegrass (*Lolium perenne*) (Aiamla-or et al., 2012; Zhang et al., 2015). In this study, a *pph* mutant of Arabidopsis exhibited stay-green phenotypes or suppressed leaf senescence as demonstrated by the maintenance of higher CHL content and Fv/Fm, as well as lower EL compared to the WT Arabidopsis plants. Additionally mutant plants had a higher survival rate compared to WT plants, further supporting that *pph* mutant plants had improved heat tolerance. In the WT Arabidopsis, PPH gene expression levels and enzyme activity both increased in response to heat stress, which corresponded with a decline in chlorophyll levels, similar to what was found in the bentgrass plants exposed to heat stress. Together these results support that PPH could be a key regulator of chlorophyll loss during heat-induced senescence.

Chlorophyll peroxidases oxidize chlorophyll in the presence of H₂O₂ and phenolic compounds to generate C13-hydroxychlorophyll a (Yamauchi, 2004). Funamoto et al., (2003) had previously found that CHL-PRXs were involved in post-harvest chlorophyll degradation. However, the role of CHL-PRX role in chlorophyll degradation, which selectively use chlorophyll as their substrates has been questioned (Matile et al., 1999). In this study, qPCR showed that ColxCB169 had increased peroxidase gene expression while ColxCB190 had no significant changes. However CHL-PRX activity assays showed that ColxCB169 had no changes in enzyme activity, while ColxCB190 had a significant decrease in enzyme activity. Both qPCR and enzyme activity precludes that

CHL-PRX were responsible for increased chlorophyll degradation in the more senescent ColxCB190 during heat stress.

In summary, expression levels of several key genes involved in chlorophyll synthesis were not inhibited by heat stress, and along with enzymatic and spectrophotometric assays support that inhibition of chlorophyll synthesis associated with the enzymes PBGD, MG-CHT, or POR was not the major contributor to declines in chlorophyll level induced by heat stress and could not account for the genetic variations in heat-induced leaf senescence. It should be noted that other enzymes in the chlorophyll synthesis were not examined and that many genes are post-transcriptionally regulated so there may have been disturbances to chlorophyll synthesis not detected in this study. Several different enzymatic pathways have been proposed to be responsible for the initial steps of chlorophyll catabolism including CHLASE, PPH, and CHL-PRX. The current study support that PPH may have increased activity during heat stress leading to higher rates of chlorophyll degradation and may ultimately be one of the key steps responsible for the loss of chlorophyll during stress-induced senescence. Arabidopsis mutants lacking *pph* expression further confirmed that PPH may play important roles in heat-induced senescence. Future research will include further analysis of other enzymes in chlorophyll metabolism caused by heat stress, as well as using transgenic approaches or marker assisted selection of utilizing stay-green genes such as *pph* to aid in creating improved cultivars with stay-green traits that maintain chlorophyll levels during stress events.

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Table 1. Primers used for qPCR to measure gene expression levels in bentgrass plants

Gene:	Accession:	Forward primer:	Reverse Primer:
Porphobilinogen deaminase	DV861883	TAGCGCTGCGGATTAG AACT	GAAGGATAACGAAC CGCTGA
Mg-Chelatase	GR278806	CATCAGGGCGGATAG AGAGA	TCTGCCACAATCAGC TTCAG
Protochlorophyllide-reductase	DV854057	GCGTCTACTGGAGCTG GAAC	GTCACTTCATGCAGG TCACG
Chlorophyllase	DV859235	GGTCGCATTCCTGAGG TCTA	ATCATATTCAACCGG GTCCA
Pheophytinase	JU113198	GAATGTCATTGCCGTC TGAA	CAATGAAATGCTGGA CCTGA
Peroxidase	FE527944	CCCAACCTACAGGACA TCGT	GGAAGCAGTCGTGGA AGAAG
Actin	DY543529	CCTTTTCCAGCCATCT TTCA	GAGGTCCTTCCTGAT ATCCA

Table 2. Primers used to assess gene expression using qPCR and RT-PCR in *Arabidopsis* plants.

Gene	Forward Primer	Reverse Primer	Method used for
Pheophytinase (<i>AtPPH</i>)	CCCTACTGAAGATCCT ACTACC	TCCAGAGATCCAGAGA GAATAC	qPCR
Actin2	CGCTGACCGTATGAG CAAAGA	GCAAGAATGGAACCAC CGATC	qPCR
Pheophytinase (<i>AtPPH</i>)	CCCAAAGCTAGCACG TCTCT	GCAGACTTCCCTCCAA ACACC	RT-PCR
Actin2	CGCTGACCGTATGAG CAAAGA	GCAAGAATGGAACCAC CGATC	RT-PCR

Fig 1. Total chlorophyll, chlorophyll A and chlorophyll B content for ColxCB169 and ColxCB190 at 28 d of either heat stress or non-stress control conditions. Bars represent standard error. Letters represent LSD groupings with treatments containing the same letter are not significantly different. LSD comparisons are only within a chlorophyll measurement group using capital letters for total chlorophyll, lower case letters of chlorophyll A, and italicized letters for chlorophyll B.

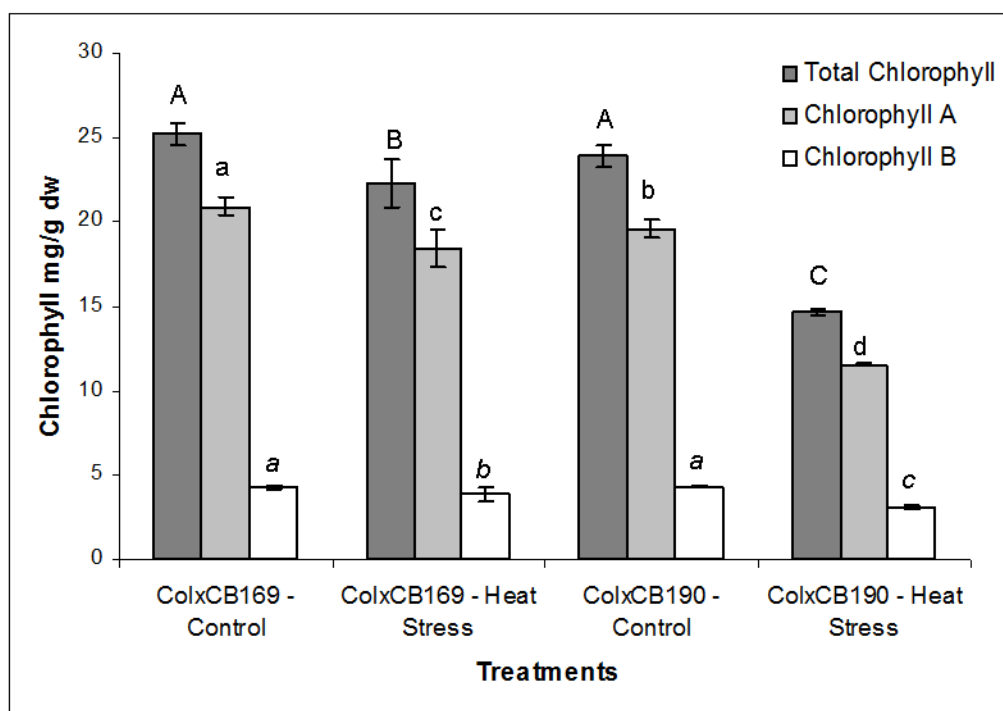


Fig 2. 5-ALA content for ColxCB169 and ColxCB190 for both heat stress and non-stress control conditions at 28 d of treatment. Bars represent standard error; letters represent LSD groups with treatments containing the same letter being not significantly different.

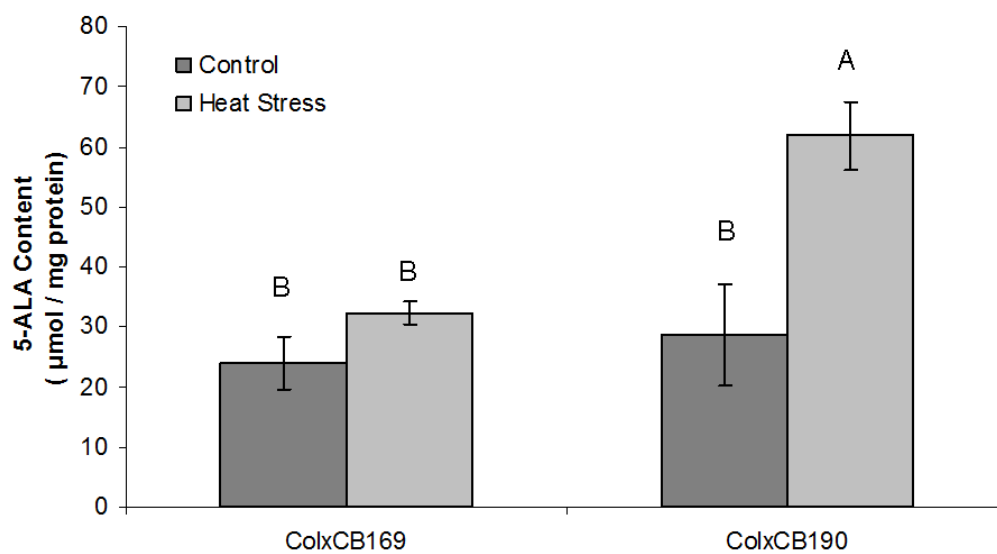


Fig 3. Fv/Fm (A), EL (B), and TQ (C) for the tolerant ColxCB169 and sensitive ColxCB190 for both heat stress and non-stress control conditions at 28 d of treatment.

Bars represent LSD values for $p=0.05$.

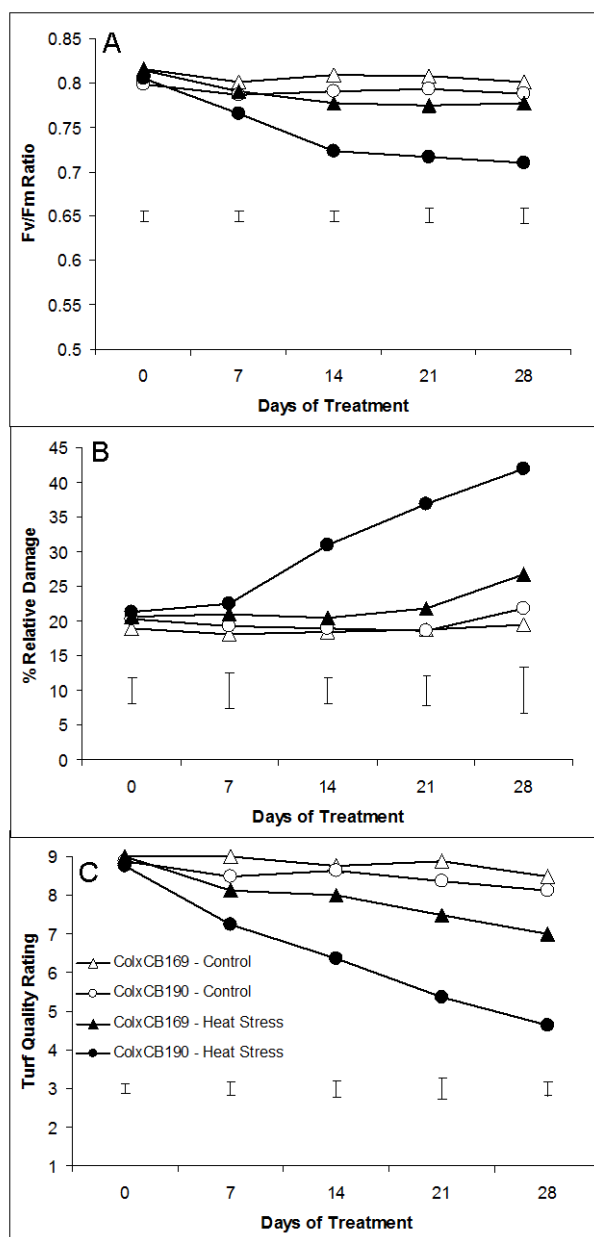


Fig 4. Gene expression levels as measured by qPCR for chlorophyll synthesis (A) and chlorophyll degradation (B) genes. Fold changes are calculated based on the change in expression for the tolerant or sensitive line at 28d heat stress compared to 28 d non-stress control plants of the same genotype. Bars represent standard error, and asterisks indicate that there are significant differences in the fold change between the tolerant and sensitive line.

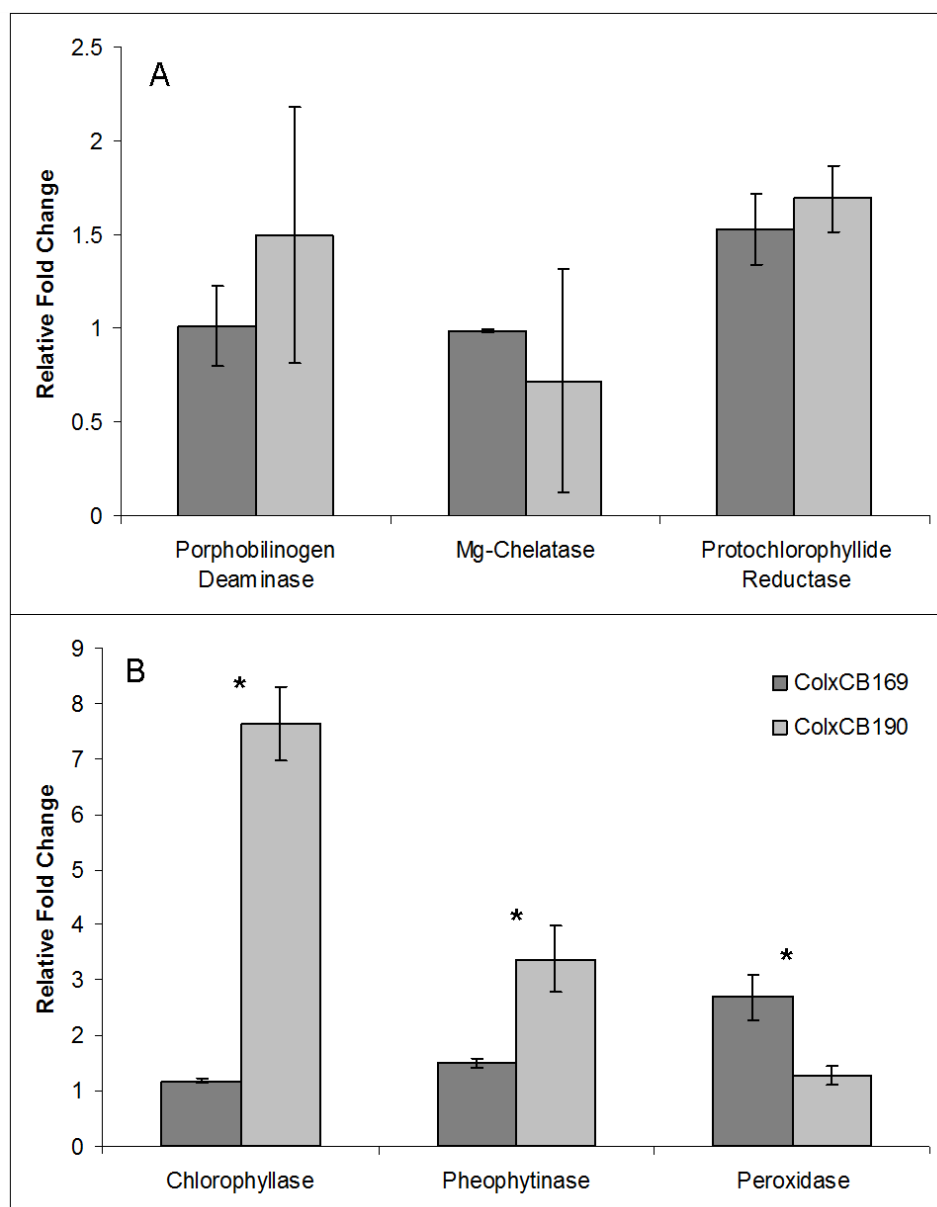


Fig 5. Enzyme activities of ColxCB169 and ColxCB190 for both heat stress and non-stress control conditions at 28 d of treatment for PBGD (A), CHLASE (B), PPH (C), and CHL-PEROX (D). Bars represent standard error; letters represent LSD groups with treatments containing the same letter being not significantly different.

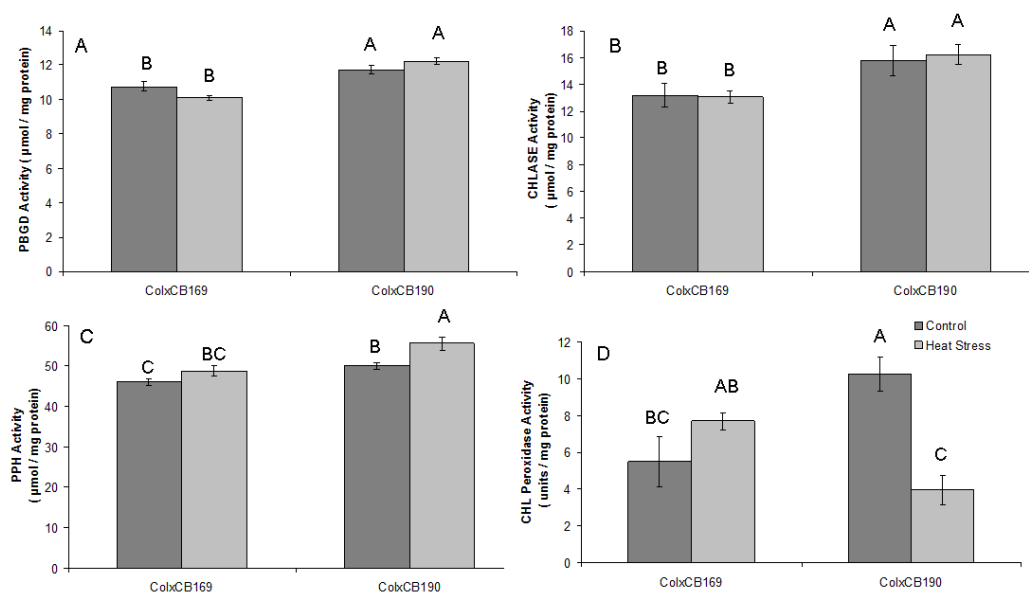


Fig 6. EL (A), Fv/Fm (B), and CHL (C) for wild type and mutant *Arabidopsis* plants under non-stress control conditions, 3 d heat stress, and 3 d recovery, as well as images (C) showing the differences between plants. Bars represent standard error; and asterisks indicate that there are significant between the wild type and mutant line for a given sampling day.

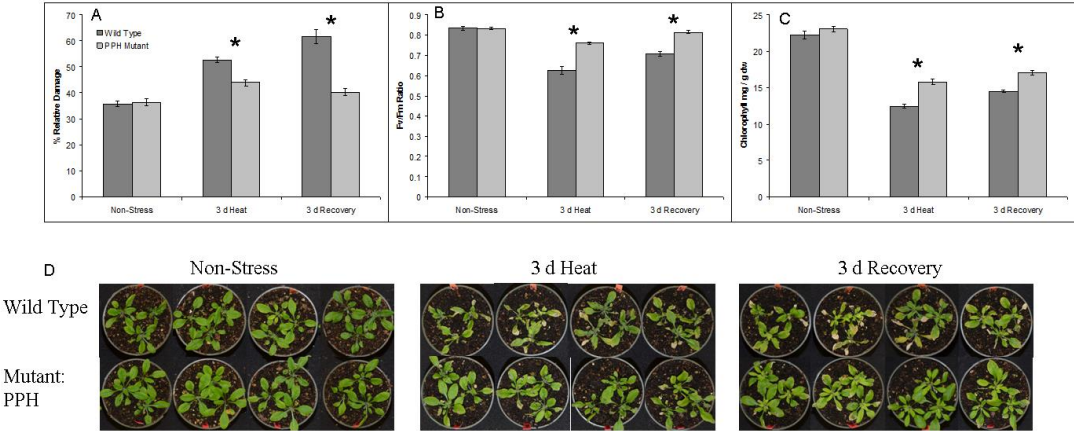


Fig 7. PPH gene expression as measured by qPCR (A), and enzyme activity (B) for *Arabidopsis* plants under non-stress control conditions, 3 d heat stress, and 3 d recovery. Gene expression and activity were not determined for the mutant line. Bars represent standard error; letters represent LSD groups with treatments containing the same letter being not significantly different.

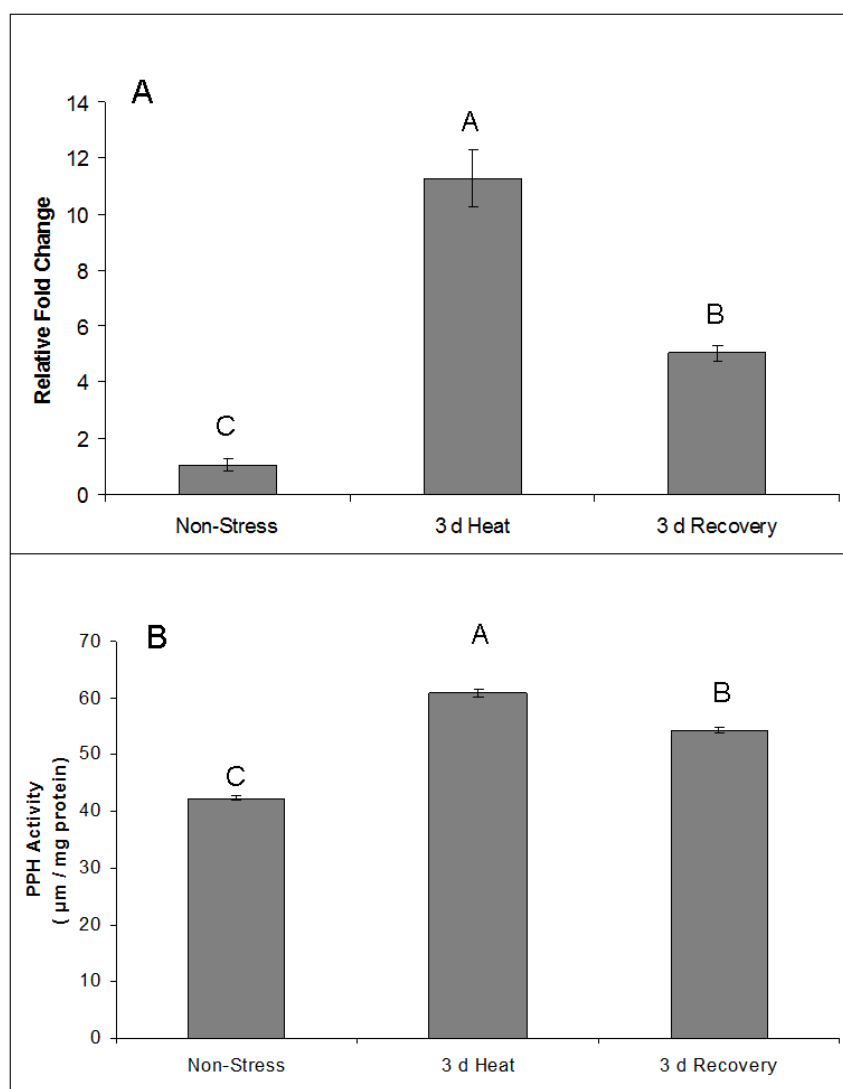
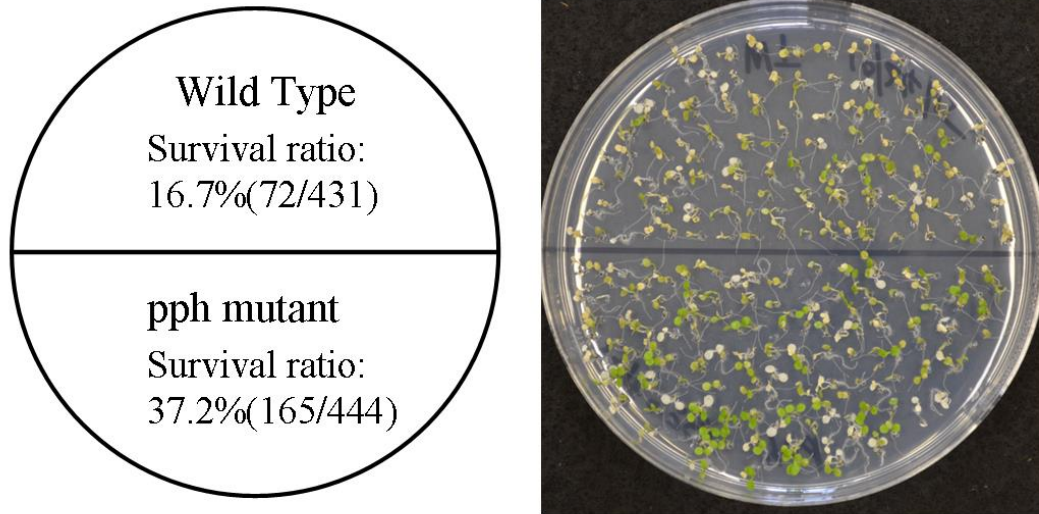


Fig 8. Representative plate showing the survival of 5 d old seedlings of both the wild type and *pph* mutant line when exposed to 3 hr heat shock.



CONCLUSIONS

The goal of this dissertation research was to explore physiological, metabolic and molecular factors associated with heat-induced leaf senescence in *Agrostis* species, combining plant physiology with the underlying molecular biology to better understand the damages caused by heat stress and mechanisms which are important for avoiding these damages. One of the key approaches was to compare genotypes which differed in abiotic stress tolerance to analyze the differences which result in premature leaf senescence during heat stress conditions. The initial study found that a range of abiotic stress tolerance exists in creeping bentgrass cultivars and that the genetic differences associated with these cultivars may be related to a number of physiological parameters such as membrane stability or photosynthetic damages such as reductions in chlorophyll content of photochemical efficiency.

Proteomic analysis of membrane proteins found that proteins such as ATP-synthase, chloroplast oxygen evolving enhancer protein, and pyruvate dehydrogenase kinase, as well as antioxidant proteins such as catalase and peroxidase were down-regulated to a greater extent in heat sensitive plants indicating differences in ATP metabolism, light harvesting, and photosynthetic photochemical reactions as well as proteins for efficient processing of photorespiratory products and reactive oxygen species may serve important roles in regulating leaf senescence in bentgrass under heat stress. Exogenous applications of a cytokinin, ethylene inhibitor, or nitrogen were found to delay heat-induced senescence in creeping bentgrass and proteomic and metabolomics approaches were used to understand how these compounds increased heat tolerance. Proteomic analysis uncovered that maintaining higher accumulations of photosynthetic

proteins such as rubisco and chlorophyll a/b binding protein, metabolic proteins such as alanine aminotransferase, and stress defense proteins such as HSP70 or catalase, in addition to lower accumulations of respiratory proteins such as succinate dehydrogenase may be important mechanisms through which these compounds delay heat-induced senescence. These exogenously applied compounds also resulted in increased accumulations of certain organic acids such as citric acid, the disaccharide sucrose, and decreased accumulations of monosaccharaides such as glucose and fructose indicating potential changes in carbon or nitrogen metabolism or osmoregulation during heat stress.

Using both QTL and candidate gene approaches a number of potential markers have been identified which may be of use in breeding programs for marker assisted selection. Genetic regions and linked markers were identified in a number of different environments to be associated with important traits related to heat-induced leaf senescence which included chlorophyll content, membrane stability, and leaf canopy density and health. Additionally candidate gene markers associated with important stress defense mechanisms including antioxidant proteins such as catalase or glutathione-S-transferase, or protein metabolism involving chaperone proteins such and heat shock protein 26, or protein degradation proteins such as cysteine protease were developed in a colonial bentgrass hybrid backcross population. These candidate genes were demonstrated to be differentially regulated in heat tolerant and sensitive individuals and were associated with important heat tolerance traits such and maintained membrane stability, and chlorophyll content.

Lastly chlorophyll loss during heat-induced senescence was examined to determine how perturbations to chlorophyll synthesis and degradation resulted in the

decline in chlorophyll levels seen during heat stress events. Real-time PCR and enzymatic assays indicated that increased chlorophyll degradation is the major contributor to decreased chlorophyll levels and that pheophytinase may be one of the key enzymes catabolizing chlorophyll during leaf senescence. The potential role of pheophytinase's involvement in heat mediated chlorophyll degradation was confirmed using *Arabidopsis* mutants.

This research indicates that genetic differences between individuals may result in different expression levels of key genes and differential accumulations of proteins and metabolites which ultimately affect the level of leaf senescence induced by heat stress. Future research may include confirmation of and further exploration of important pathways responsible for delaying heat-induced leaf senescence, such as those involved in photosynthesis and energy metabolism or stress defense mechanisms. Additionally, increased understanding of regulation of key metabolic processes will also help in our understanding of differences in abiotic stress tolerance and induction of leaf senescence seen between different genotypes. Lastly, confirmation and utilization of molecular markers for marker assisted selection will allow for the development of elite cultivars with improved abiotic stress tolerances which will maintain higher quality, require fewer inputs, and potentially have stay green traits during stress events.