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BIOCHEMICAL TRAITS AND PATHWAYS ASSOCIATED WITH HEAT

TOLERANCE IN FINE FESCUE

By

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ABSTRACT OF THE DISSERTATION

Biochemical Traits and Pathways Associated with Heat Tolerance in Fine Fescue (*Festuca* ssp.)

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Heat stress is a major factor that causes the summer decline in turfgrass especially for the cool-season species. Understanding the heat tolerance mechanism would provide basis for effectively managing heat damage during summer time and breeding heat tolerant cultivars. In this thesis, the variance in heat tolerance among fine fescue species and cultivars were evaluated; key factors, in the physiological, biochemical and proteomic aspects, associated with the heat tolerance were identified and the heat tolerance mechanism in fine fescue was elucidated.

The dissertation is mainly composed of four parts. In the first part, a total of 26 fine fescue cultivars were evaluated for their heat tolerance and drought tolerance by physiological parameters. The result indicated that heat stress is much more detrimental to fine fescue species compared to drought stress. Additionally, several cultivars with good heat tolerance or drought tolerance were selected, the heat tolerant cultivars include 'Blue Ray', 'Spartan II', 'MN-HD1', 'Shoreline', 'Navigator II', 'Azure', 'Beacon',

'Aurora Gold', 'Reliant IV', 'Marco Polo', 'Garnet', 'Wendy Jean', 'Razor', and 'Cindy Lou', while the drought tolerant cultivars include 'Spartan II', 'MN-HD1', 'Reliant IV', 'Garnet', 'Azure', and 'Aurora Gold'. These cultivars could be used in hot and/or dry environments and as breeding germplasms for developing heat and/or drought-tolerance.

To further elucidate the heat tolerance mechanism in fine fescue, in the second part, the differential membrane composition (fatty acids, sterols and membrane proteins) change under heat stress was compared between heat tolerant fine fescue cultivar 'Reliant IV' and heat sensitive fine fescue cultivar 'Predator'. This experiment found that the better heat tolerance in 'Reliant IV' is associated with greater increase of ethyl sterols (sitosterol, stigmasterol, avenasterol and fucosterol), unsaturated long chain fatty acids (18:1 and 18:2), less severe down-regulation of membrane proteins involved in photosynthesis, protein modification and signaling and greater up-regulation of heat responsive proteins, including Rubisco activase and disease resistance protein 1.

In the third part, the differential response of free amino acids and soluble proteins to heat stress were compared between 'Reliant IV' and 'Predator'. This experiment found that the heat tolerant 'Reliant IV' exhibited greater accumulation of seven essential amino acids (histidine, glutamine, glutamate, proline, threonine, aspartate and tryptophan) and several soluble proteins, including glyceraldehyde 3-phosphate dehydrogenase, triosephosphate isomerase, dihydrolipoyl dehydrogenase, malate dehydrogenase, Rubisco large subunit binding protein subunit alpha, protein disulfide-isomerase, catalase, calcium-transporting ATPase, lectin-domain containing receptor kinase, stromal 70 kDa heat shock-related protein, 20 kDa chaperonin, actin, tubulin beta-2 chain, aspartate aminotransferase, formate dehydrogenase and UDP-sulfoquinovose synthase. These

differentially accumulated free amino acids and soluble proteins could be associated with the genetic variation in heat tolerance of fine fescue.

In the last part, the differential change of phenolic composition under heat stress was compared between 'Reliant IV' and 'Predator'. A total of 12 phenolic acids were identified in the leaves of fine fescue cultivars. The result indicated that homovanillic acid and caffeic acid were more up-regulated in 'Reliant IV' under short-term heat stress, while 3, 4-dyhydroxybenzoic acid showed greater accumulation in 'Reliant IV' under long-term heat stress. These greater accumulated phenolic acids could account for the better heat tolerance in 'Reliant IV' and be potentially used as heat stress reliever in cool-season grass species.

In summary, these studies identified a series of components (metabolites or proteins) that associated with heat tolerance in fine fescue. These identified components could potentially be incorporated into bio-stimulant product to relieving heat damage or serve as basis to develop molecular marker in assisting heat-tolerant germplasms selection.

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CHAPTER 1. Introduction

1.1. Characteristics and types of fine fescue species

The grass is a large family of nearly 10,000 plant species and plays an important role in environment, agriculture and industry. Turfgrasses are a subset of the grasses that form a continuous ground cover in parks, home lawns and golf courses. These species are mainly members of three subfamilies as *Pooideae*, *Chloridoideae*, and *Panicoideae*. Turfgrasses plays important role in environment and human activity by providing playing ground for exercise and relaxation, preventing runoff and erosion of topsoil, and providing cooling effect for environment and absorbing greenhouse gas carbon dioxide.

Within the *Pooideae* subfamily, *Festuca* genus contains approximately 450 species, including fine fescue. Fine fescue is composed of two complexes as red fescue complex (*Festuca. rubra*) and sheep fescue (*Festuca. ovina*) complex. The red fescue complex includes strong creeping red fescue (*Festuca. rubra* ssp. *Rubra*), slender creeping red fescue (*Festuca. rubra* ssp. *Rubra*), slender creeping red fescue (*Festuca. rubra* ssp. *Rubra*), slender creeping red fescue (*Festuca. rubra* ssp. *litoralis*) and Chewings fescue (*Festuca. rubra* ssp. *comutata*), while sheep fescue complex includes sheep fescue (*Festuca. ovina* ssp. *hirtula*) and hard fescue (*Festuca. trachyphylla*). In addition, hard fescue, sheep fescue and Chewings fescue are bunch type grasses, while strong creeping red fescue and slender creeping red fescue are rhizomatous type grasses (Ruemmele, Wipff, et al., 2003).

Fine fescue is characterized by its fine and narrow leaf. Fine fescue was first used as golf turf back to sixteenth century and since then several important fine fescue cultivars were breed and used throughout the world (Ruemmele, Wipff, et al., 2003). Fine fescue has been used on the putting greens, fairways and roughs on golf course and other natural

areas (Christians, 2000, Dernoeden, 1998). It forms attractive appearance with well adaptation to low-input environment, shade and acid soil conditions (Roberts, 1990, Ruemmele, Brilman, et al., 1995, Ruemmele, Wipff, et al., 2003)

Fine fescues are cool-season species widely adapted to temperate climate regions (Beard, 1973). Genetic variability in abiotic stress tolerance exists among fine fescue species and subspecies. For example, hard fescue performs best under low maintenance conditions (reducing fertilization and irrigation), followed by Chewings fescue, strong creeping red fescue and tall fescue (Tate, Smith, et al., 2012). Slender creeping red fescue showed better salinity tolerance compared to other fine fescue species (Friell, Watkins, et al., 2012). While limited information is available about the genetic variations in heat tolerance of fine fescue species, although heat stress is a primary abiotic stress limiting the growth of cool-season turfgrass species.

1.2. Heat stress affecting the growth of cool-season turfgrass species

Heat stress is generally defined as the rise in temperature exceed the temperature threshold and last for a period of time that sufficient to cause damage on plants. Usually, an increase of 10 to 15 °C above the optimal temperature would cause heat damage on plants and be considered as heat stress. However, heat stress is a complex process that is greatly influenced by heat intensity (degree of temperature) and duration. At extreme high temperature, catastrophic collapse of cellular organization would be induced within minutes leading to severe cell damage and cell death (Schöffl, Prandl, et al., 1999). While, long time exposure to moderate heat stress would also lead to heat damages, including the inactivation of enzymes involved in metabolic pathway, inhibition of

protein synthesis, induction of protein denaturation and aggregation, increase of membrane fluidity and lose of membrane integrity (Wahid, Gelani, et al., 2007). These heat damages eventually lead to the imbalance of energy, inhibition of growth, production of toxic compounds and even plant death (Howarth, 2005). The heat damage on plants would also depend on plant development stage. For example, heat stress would lead to reduced seed set and weight during seed filling stage, while lead to induced sterility during anthesis stage (Wahid, Gelani, et al., 2007). In addition, the great genetic variation of heat tolerance exists among different plant species and cultivars. Based on their temperature requirement, plants can be divided into cool-season plants and warm-season plants. Most cool-season plants have moderate to high cold tolerance, but are susceptible to extended heat stress.

In the last century, conventional breeding greatly contributed to the turfgrass improvement with improved attractiveness, less input requirement and better biotic and abiotic stress tolerance. Recently, efforts have been devoted to improve plant heat tolerance by genetic transformation and molecular marker assisted selection (Duncan and Carrow, 1999, Grover, Agarwal, et al., 2000, Iba, 2002). Therefore, a more complete and thorough understanding of heat tolerance mechanism is in need, which will provide the basis for heat tolerance improvement by molecular breeding or biotechnology. In next section, some major heat tolerance mechanisms will be discussed.

1.3. Heat tolerance mechanism

Plants' survival under heat stress relies on the quick perception of stress condition and induction of heat tolerance mechanism. The increased cell membrane fluidity is an initial stress signaling and triggers the influx of Ca^{2+} under heat stress condition. The sharp increase of cytosolic Ca^{2+} results in the activation of mitogen activated protein kinase (MAPK) and calcium dependent protein kinase (CDPK) (Sangwan and Dhindsa, 2002). The accumulated ROS under heat stress is also important signaling molecules that activating MAPK cascade. Activation of these cascades leads to adaptive responses in plants, these responses involved in energy production, assimilate partitioning, cell membrane stability, hormone change, antioxidants and stress protein expression (Wahid, Gelani, et al., 2007). While my thesis work mainly focuses on the heat tolerance mechanism in the aspects of lipid metabolism, protein metabolism, amino acid metabolism and secondary metabolism.

1.3.1. Lipid metabolism

Cellular membrane plays important role in plant, including selective permeability of material, signaling perception and transduction and cellular function regulation. While the cellular membrane has been long identified as a primary site of heat stress damage (Blum and Ebercon, 1981). Heat stress accelerates the kinetic energy and the movement of membrane components, thus causing increased membrane fluidity and permeability (Wahid, Gelani, et al., 2007). In addition, heat stress cause direct damage on cell membrane components leading to lipid peroxidation, membrane protein denaturation and dysfunction (Huang, 2006). The heat damage on membrane function, as indicated by increased loss of electrolytes, has been observed in various plant species and widely used as a major indicator for heat tolerance (Wahid, Gelani, et al., 2007). To cope with this situation, plants tend to change membrane composition to retard the increased membrane

fluidity and permeability caused by heat stress, these changes including increased desaturation level of fatty acid and increased sterol content (Huang, 2006). The detail about membrane composition change and its relationship with heat tolerance will be discussed below.

Lipid is a major component of cell membrane and is mainly composed of phospholipids, glycolipids and sterols. Phospholipids and glycolipids have very similar structures, both consisting of a fatty acid tail, a three-carbon backbone, and a polar head group. Maintenance of proper fatty acid composition in phospholipids and glycolipids is of great importance for plants to adapt to stress conditions (Berglund, Larsson, et al., 2004, Gigon, Matos, et al., 2004). In response to heat stress, the increased saturation level of fatty acid was observed in various plant species, leading to increased cell membrane melting temperature and therefore retarding the increase of membrane fluidity and permeability caused by heat stress (Huang, 2006). Previous research also found that this increased fatty acid saturation level is mainly caused by the decreased abundance of unsaturated fatty acids (Larkindale et al., 2004). To further illustrate the function of membrane fatty acids in plant heat tolerance, transgenic and mutant plants were used. The transgenic tobacco containing low level of unsaturated fatty acid shows improved tolerance to heat stress (Murakami, Tsuyama, et al., 2000), suggesting a close relationship between fatty acid saturation level and heat tolerance. Similar result has also been found in the soybean (*Glycine max*) that a mutant with higher saturation level of fatty acid showed significantly improved heat tolerance compared to wild type plants (Alfonso, Yruela, et al., 2001). The mutation and transgenic analysis eluciate the importance of proper fatty acid composition for heat tolerance.

Sterol is another important lipid class, which only accounts a small amount of lipid composition on cell membrane, but plays important role in regulating membrane fluidity and permeability by serving as membrane reinforcers (Dufourc, 2008). Sterols are mainly found in three forms as free sterol, steryl ester and steryl glucoside with free sterols as the most abundant existing form. A typical plant membranes sterol profile has been previously illustrated by pea (*Pisum sativum*) seedling, which consists of 50% sitosterol, 25% stigmasterol, <10% each campesterol, isofucosterol and cholesterol (Nomura, Kitasaka, et al., 1999). The protecting function of sterols has been widely explored in salt tolerance. For example, a significantly higher sterol/phospholipid ratio has been reported in halophytes, suggesting a potential function of sterol in protecting plants from salt stress (Blits and Gallagher, 1990, Wu, Seliskar, et al., 1998). In common with salinity tolerance of halophytes, tomato (Lycopersicon esculentum) calii lines with high saline tolerance also confer higher sterol/phospholipid ratio compared with salinity sensitive tomato calii lines. (Kerkeb, Donaire, et al., 2001). In addition, increased sterol/phospholipid ratio has been observed under salt stress and interpreted as salt adaption mechanism (Kuiper, 1985, Mansour, Salama, et al., 2002). The protecting role of sterols in plants under salt stress may be related to its function in inducing membrane rigidity (Singer and Wan, 1975). The function of sterols in regulating membrane stability under heat stress has been investigated on model membrane, suggesting an ordering effect on membrane under heat stress (Dufourc, 2008). While limited information has been provided on the sterols change in response to heat stress and their potential function in the aspect of membrane regulating and heat tolerance in plants.

Recent works revealed direct interaction between these two membrane lipid family (fatty acid and sterol) (Phillips, Ursell, et al., 2009). For example, the content of sterol and fatty acid are interrelated as the promoter of several genes for the enzymes involved in the biosynthesis of fatty acid contain sterol-responsive elements (Thewke, Kramer, et al., 2000). In addition, it has been long known that the hydrophobic thickness of the lipid bilayers is defined by fatty acid length, but recent research found the presence of sterols also greatly influence the membrane thickness (Cornelius, 2001, Lee, 2004). However, few studies have investigated and compared the interaction of these two major membrane constituents in relation to heat stress tolerance.

We hypothesized that the differential quantity or proportion of these two major membrane lipid family (fatty acid and sterol) could account for the genetic variations in membrane stability under heat stress. Therefore, in my thesis work, the differential changes in fatty acid and sterol composition under heat stress were compared in two hard fescue cultivars contrasting in heat tolerance, trying to identify major membrane constituents that are associated with the genetic variations in membrane stability and heat tolerance.

1.3.2. Protein metabolism

The plant acclimation process to heat stress involves profound change at transcription, proteomic and metabolic level. Previous research indicates that the gene expression change at transcription level do not always correspond to that at proteomic level (Bogeat-Triboulot, Brosché, et al., 2007). Since proteins are the direct effectors of plant activity, such as enzymes in metabolism pathway, regulators and components of

transcription and translation machinery, and components for plasma membrane, cell cytoskeleton and intracellular compartments (Kosová, Vítámvás, et al., 2011), therefore investigation of proteomic change under heat stress is highly important. Analysis of plant proteomes under contrasting treatments is widely used to identify heat-responsive proteins and elucidate pathways that are crucial for heat tolerance. Previous research on proteomic analysis has illustrated the major protein changes in the aspect of chaperone function, redox homeostasis and energy metabolism (Kosová, Vítámvás, et al., 2011).

Heat stress is usually associated with increased risk of protein denaturation, aggregation and misfolding. To cope with this situation, plants tend to accumulate proteins with chaperone function under heat stress. Among accumulated these proteins, the heat shock protein (HSP) is most well known (Kosová, Vítámvás, et al., 2011). Generally, the HSP serves as molecular chaperones preventing protein aggregation and assisting protein refolding under heat stress (Huang and Xu, 2008, Tripp, Mishra, et al., 2009). The HSP can be classified into five groups, according to their molecular weight, such as HSP100, HSP90, HSP70, HSP60 and small heat shock proteins (sHSP) (Trent, 1996). The HSP100 family is known to solubilize the aggregated proteins and release it to be refolded by the HSP70 family (Hsp104, 1998, Wang, Vinocur, et al., 2004). The HSP90 family plays an important role in protein folding, degradation and trafficking (Wang, Vinocur, et al., 2004). The HSP60 family mainly assists with folding of newly synthesized or trans-located proteins (Huang and Xu, 2008). Among all HSP families, the sHSP family is the most prevalent HSP in plants and binds to partially unfolded proteins to prevent irreversible aggregation (Sun, Van Montagu, et al., 2002). A positive relationship has been observed between sHSP accumulation and thermotolerance (Downs, Heckathorn, et al., 1998). In addition to HSP, up regulation of other proteins with chaperone function has also been reported under heat stress, including chaperonin 60 β subunit, chaperonin 10 and chloroplast chaperonin (Ferreira, Hjernø, et al., 2006, Zhang, Li, et al., 2010). Overall these chaperone proteins play overlapping and complementary roles together to achieve or maintain proper protein structure and function.

Oxidative damage is another common damage under heat stress (Apel and Hirt, 2004). The reactive oxygen species (ROS) are continuously produced as byproducts of oxidizing metabolic activity or electron flow in chloroplasts, mitochondria and microbodies (Mittler, Vanderauwera, et al., 2004). Under normal conditions, the generated ROS is scavenged by antioxidant defense systems (Alscher, Donahue, et al., 1997). Under stress conditions, the balance between ROS generation and scavenging is disturbed leading to accumulation of ROS. The excessive accumulation of ROS is cytotoxic and can cause oxidative damage to almost all cell components, including protein, DNA and lipids (Apel and Hirt, 2004). Plants develop a series of antioxidant systems to scavenge excessive ROS both enzymatically and non-enzymatically. The proteomic study revealed the accumulation of proteins involved in ROS scavenging process in response to heat stress, these proteins including glutathione S-transferase, dehydroascorbate reductase, cytosolic thioredoxin h-type, cytosolic Cu/Zn-SOD and Mn-peroxidase (Huang and Xu, 2008, Lee, Ahsan, et al., 2007).

The heat acclimation processes is associated with biosynthesis of heat protective compounds and therefore requires increased energy input. Maintenance of balance between carbohydrate anabolism and catabolism is important for plants survival under the stress conditions and mainly based on the balance of photosynthesis and respiration. Previous proteomic studies revealed large amount of proteins responding to heat stress are involved in these three energy related processes (photosynthesis, dark respiration and photorespiration) (Han et al., 2009; Lee et al., 2007; Li et al., 2013; Zhang et al., 2013). In addition, better maintenance of proteins involved in energy metabolism was found to be associated with better heat tolerance in plants (Xu and Huang, 2010).

As discussed above, most of the previous research focuses on the change of highly abundant soluble proteins under abiotic stress. However genomic analysis reveals that around 20% to 30% genes encoding membrane proteins are less abundant compared to soluble proteins but play important roles in plant activity including water and solutes transportation, signaling, light harvesting, electron transfer and energy production (Taiz and Zeiger, 2010, Ward, 2001). Heat stress causes dissociation or denaturation of membrane proteins and therefore hinders their normal function. Previous research has reported the decreased abundance of several membrane proteins related to photosynthesis under heat stress, including 33-kDa manganese (Mn)-stabilizing protein (Yamane, et al. 1998), oxygen evolving complex (OEC) (De Ronde, et al. 2004) and D1, D2 protein of reaction center (De Las Rivas and Barber 1997). A more recent study observed a lesser or later decrease of membrane proteins in response to heat stress in a heat tolerant line of bentgrass (Agrostis spp.) compared to a heat sensitive line, including those categorized to energy metabolism (ATP-synthase, Cytochrome b6f, chloroplast oxygen-evolving enhancer protein, and pyruvate dehydrogenase kinase) and antioxidant processes (catalase and peroxidase) (Jespersen et al., 2015). Limited information is provided on membrane proteins and the identification of more membrane proteins involved in heat tolerance is needed.

In my thesis work, a thorough analysis of both soluble proteins and membrane proteins was conducted to compare the proteomic changes under heat stress between two hard fescue cultivars contrasting in heat tolerance. The identification of soluble and membrane proteins involved in better heat tolerance would aid in a better understanding of heat tolerance mechanisms and provide information to help to develop heat tolerant plants.

1.3.3. Amino acid metabolism

Altered metabolism is another key adaptive mechanism for heat tolerance. Recently, comprehensive metabolism investigation has been done on heat stress and identified accumulation of free amino acids as the major metabolism response to heat stress (Caldana, Degenkolbe, et al., 2011, Du, Wang, et al., 2011, Kaplan, Kopka, et al., 2004).

Free amino acids is a primary metabolite family and plays an important role in stress tolerance (D'Mello, 2015). Their central role depend not only on being constituents of protein but also on their role as regulatory and signaling molecules and precursors for numerous secondary metabolites (D'Mello, 2015). The 20 proteinogenic amino acids can be divided into five different families according to their structure and synthesis pathway, including pyruvate family (alanine, valine and leucine), glutamate family (glutamate, glutamine, histidine, proline and arginine), aspartate family (aspartate, asparagine, lysine, threonine, isoleucine and methionine), serine family (serine, glycine and cysteine) and aromatic amino acid family (tyrosine, phenylalanine and tryptophan).

Different amino acid families serve different roles under abiotic stress. For example, glutamate family pathway is strongly activated under stress conditions to accumulate

stress related amino acids as proline and beta-aminobutyric acid (Forde and Lea, 2007). Great accumulation of amino acids in the pyruvate family is observed in anoxic condition resulting from pyruvate accumulation (Good and Muench, 1993). The altered aspartate family pathway, especially the stimulated lysine catabolism under stress conditions, is essential to generate energy (D'Mello, 2015). Aromatic amino acids serve as precursors for important secondary metabolites, including phytoalexins, alkaloids, lignins, flavonoids, isoflavonoids and hydroxycinnamic acid (Dixon, 2001).

A strong response of free amino acids was observed under heat stress. Under heat shock, several amino acids involved in the pyruvate family (alanine, valine and leucine) and aspartate family (asparagine, isoleucine and threonine) showed increase in Arabidopsis (Kaplan, Kopka, et al., 2004). Increased synthesis rate of alanine, valine, leucine, isoleucine, proline and γ -aminobutyrate under heat shock was observed in cowpea (*Vigna unguiculata*) cells (Mayer, Cherry, et al., 1990). While under prolonged heat stress, free amino acids in the pyruvate family (alanine and valine), aspartate family (asparagine, lysine, isoleucine, methionine and threonine) and serine family (glycine) were up regulated, with greater up regulation in heat tolerant C4 plants compared to those of heat sensitive C3 plants (Du, Wang, et al., 2011).

Among all these free amino acids, proline gained the most focus and showed dramatic accumulation under heat stress (Krasensky and Jonak, 2012). Proline can protect plants from stress damage by acting as an osmolyte, a ROS scavenger, and a molecular chaperone (Szabados and Savoure, 2010). While its function in heat stress is still under debate as different proline response has been observed under heat stress. For example, heat induced increase of proline content was detected in Kentucky bluegrass

(*Poa pratensis*) (Du, Wang, et al., 2011), while no heat response was detected in Arabidopsis and tobacco (*Nicotiana tabacum*) (Dobra, Motyka, et al., 2010, Rizhsky, Liang, et al., 2004).

However, major amino acids conferring heat tolerance in cool-season grass species are not well documented. The identification of major amino acids associated with the genetic variations in heat tolerance in two cultivars of hard fescues will complement the previous findings with other species and enhance further understanding of the mechanisms of heat tolerance in cool-season turfgrass species.

1.3.4. Secondary metabolism

Plant secondary metabolites are composed of an enormous variety of chemicals accounting to more than 100,000 and is characterized with diverse chemical types, including aliphatic, aromatic, hydroaromatic, and heterocyclic (Edreva, Velikova, et al., 2008, Hadacek, 2002). Most of the secondary metabolites are synthesized from intermediates of the primary metabolism pathway using phenylpropanoid, shikimate, mevalonate or methyl erythritol phosphate pathways (Wahid and Ghazanfar, 2006). Unlike primary metabolites, such as amino acids, nucleotides, simple carbohydrates and membrane lipids, the secondary metabolites do not have direct function in growth, development and reproduction. However many secondary metabolites have been found to have important regulating and protecting roles in abiotic and biotic stress tolerance.

Carotenoid is a secondary metabolites family containing more than 600 compounds and is known to protect cellular structures under abiotic stress conditions (Havaux, 1998, Wahid and Ghazanfar, 2006). Some carotenoid compounds (zeaxanthin, violaxanthin, antheraxanthin) interact with cell membrane, particularly thylakoid membrane to decrease membrane fluidity and protect membrane from lipid peroxidation under heat stress (Havaux, 1998, Horton, 2002). Another important secondary metabolites family in abiotic stress tolerance is isoprene. Isoprene has been proved to protect photochemical system II from ROS damage and a positive relationship has been found between the isoprene production and maintenance of photosynthesis under heat stress (Sharkey, 2005, Velikova and Loreto, 2005). Phenolic compounds are a large metabolite family that consist of more than 8,000 compounds with diverse structure ranging from single aromatic ringed compounds to large and complex polyphenols (Marinova, Ribarova, et al., 2005). Phenolic compounds derive from phenylopropanoid metabolism (Crozier, Clifford, et al., 2008), which is a particularly interesting metabolism pathway that large amount of fixed carbon (around 20%) flows through this pathway under normal conditions (Dí'az, Bernal, et al., 2001) and is greatly induced under stress conditions (Keleş and Öncel, 2002, Leyva, Jarillo, et al., 1995, Oh, Trick, et al., 2009). The phenolic compounds have been found to protect plants from stress conditions. For example, the accumulation of flavonoid is observed after infection by fungal endophyte and pathogen and plays a role in defense again biotic stress (Koskimäki, Hokkanen, et al., 2009). Anthocyanins are found to accumulate under abiotic stress, including drought, low temperature, UV-B exposure and nutrient deficiency and protect plants by direct shielding and scavenging free radicals (Lee and Gould, 2002, Wahid and Ghazanfar, 2006). Ligning have important roles in mechanical support for plants, water transport in xylem and defense response to pest and microorganisms and the altered lignin biosynthesis has also been reported under various biotic and abiotic stress conditions (Moura, Bonine, et al., 2010).

Phenolic acids are an important group of phenolic compounds and are particularly interesting regarding their potential function in abiotic stress. They are the direct products of phenylalanine ammonia lyase (PAL), which is a control enzyme at the branch point from primary metabolism to phenylopropanoid metabolism, catalyzing the transformation, from phenyalanine to cinnamic acid, which serves as intermediary for other phenolic acid synthesis (Camm and Towers, 1973). The induction of PAL activity was reported under heat stress and considered as an important cell acclimation mechanism to heat stress (Oh, Trick, et al., 2009, Rivero, Ruiz, et al., 2001). In addition, water-soluble phenolic acids process sufficient chemical structure for antioxidant activity and has been proved to scavenge hydrogen peroxide (H_2O_2) and 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical in vitro (Sroka and Cisowski, 2003). One phenolic acid, salicylic acid, has been identified as signaling molecule inducing stress acclimation and protecting plants from stress damage (Horváth, Szalai, et al., 2007). A transient increase of salicylic acid at the initial stage of heat stress was observed in various plant species and enhanced antioxidant system in plants to improve heat tolerance and survival (Horváth, Szalai, et al., 2007). All these previous researches points out that the phenolic acids have great potential in protecting plants from heat stress. However, very limited studies have investigated the response and potential roles of these phenolic acids in heat stress tolerance.

Our study examined differential changes in the quantity and composition of phenolic acids in response to short-term heat shock and long-term heat stress in two hard fescue

cultivars contrasting in heat tolerance and identify major constituents of phenolic acids associated with the genetic variations in heat tolerance.

1.4. Thesis research goal and objectives

The overall goal of my research is to investigate physiological and biochemical mechanisms associated with heat tolerance in fine fescue species. This goal was addressed by conducting four projects with the following specific objectives:

1) To evaluate the heat tolerance and drought tolerance among different fine fescue species and cultivars, and to identify major physiological traits that are related to heat tolerance and drought tolerance in fine fescue

2) To compared differential membrane composition (fatty acid, sterol and membrane proteins) changes under heat stress between heat-tolerant cultivar 'Reliant IV' and heat-sensitive cultivar 'Predator', and to identify major membrane composition that associated with variation in heat tolerance in fine fescue

3) To investigate differential proteomic and free amino acid changes under heat stress between heat-tolerant cultivar 'Reliant IV' and heat-sensitive cultivar 'Predator', and to identify soluble proteins and free amino acids that are associated with different heat tolerance in fine fescue

4) To investigate differential phenolic acid composition change under heat stress between heat-tolerant cultivar 'Reliant IV' and heat-sensitive cultivar 'Predator', and to identify phenolic acid components that are associated with different heat tolerance in fine fescue

CHAPTER 2. Differential Physiological Responses and Genetic Variations in Fine Fescue Species for Heat and Drought Stress

2.1. Background

The optimal growing temperature for cool-season grass species ranges from 18 to 23 °C, whereas air temperatures typically exceed 30 to 35 °C for daytime and 23 to 28 °C for nighttime during summer months in the transition zone (Kunkel, Stevens, et al., 2013). Drought stress is another major limiting factor for turfgrass growth, particularly during the summer months. The decline in turf quality (TQ) of fine fescues, which is commonly observed during the summer, is typically associated with heat and/or drought and is referred as summer decline (Turgeon, 1996). Evaluating the stress-induced TQ decline caused by heat or drought and comparing responses across cultivars would offer a better understanding of the summer decline in fine fescues.

Healthy turfgrass stands are characterized by uniform and dense canopy, dark-green leaf color, and active growth (Beard, 1972). Extensive reports have shown that stress-related leaf senescence is associated with disruption or degradation of cellular membranes with downstream effects on photosynthetic carbohydrate synthesis (Huang, DaCosta, et al., 2014, Wahid, Gelani, et al., 2007). Prolonged heat stress typically induces lipid peroxidation and membrane instability with subsequent effects on chlorophyll integrity and net photosynthetic rates in cool-season grass species, including creeping bentgrass (*Agrostis stolonifera*), Kentucky bluegrass and perennial ryegrass (*Lolium perenne*) (Jiang and Huang, 2001, Liu and Huang, 2000). Alternatively, drought stress caused by decreased rainfall or limited irrigation is another major problem leading

to steady TQ decline of cool-season turfgrass stands during the summer months. While drought stress similarly imposes negative effects on cellular membrane stability, photochemical efficiency and chlorophyll integrity, it also induces significant decreases in leaf water potential in Kentucky bluegrass (Abraham, Huang, et al., 2004, Jiang and Huang, 2000). Similar effects of drought stress have been detected in other cool-season grasses including tall fescue (*Festuca arundinacea*), creeping bentgrass and perennial ryegrass (Carrow and Duncan, 2003, Karcher, Richardson, et al., 2008, McCann and Huang, 2008, Wang and Bughrara, 2008). Given that drought and heat stress typically occur together under field conditions, it is important to determine which stress is more detrimental so the proper management can be taken to prevent or control summer decline in fine fescues.

The fine fescue family is comprised of several species and subspecies, including strong creeping red fescue, slender creeping red fescue, Chewings fescue, hard fescue and sheep fescue. Fine fescue species are cool-season grasses widely utilized in home lawns and golf courses throughout cool-temperate climates. They form attractive turf stands that are characterized by narrow and fine leaf textures (Christians, 2000, Christians and Engelke, 1994). They are well adapted to poor soil fertility, moderate shade, and acidic soil conditions; however, little is known regarding their tolerance to heat and drought stress (Turgeon, 2011). The objectives of this study were to 1) examine whether heat or drought stress (dry down by withholding irrigation) is more detrimental to fine fescues, 2) determine genotypic variations of heat and drought tolerance within fine fescues, and 3) identify physiological parameters that can be used as indicators for heat and drought tolerance in fine fescues.

2.2. Materials and Methods

2.2.1. Plant material and growth conditions

A total of 26 cultivars of fine fescue were evaluated in the study: seven hard fescues (Blue Ray, Beacon, Spartan II, Predator, MN-HD1, Reliant IV, Aurora Gold), eight Chewings fescues (Zodiac, Intrigue II, Radar, Fairmount, Rushmore, 7 Seas, Columbia II, Longfellow), seven strong creeping red fescues (Navigator II, Boreal, Lustrous, Garnet, Wendy Jean, Razor, Cindy Lou), two sheep fescues (Azure, Marco Polo) and two slender creeping red fescues (Shoreline, ASR-050). Seeds for each cultivar were sterilized in 1% (v/v) sodium hypochlorite solution for 1 min, rinsed with sterile water, and sown at 19.5 g seed per m^2 . The seeds for heat stress and its corresponding control were sown in sterile sand (autoclaved at 121 °C, 124.1 kPa, 60 min) in plastic pots (15 cm diameter x 14 cm depth) on April 2, 2014. The seeds for drought stress and its corresponding control were sown in sterile fritted clay medium (Profile Products, Deerfield, IL) in plastic pots (10 cm diameter \times 40 cm depth) on April 2, 2014. Plants were maintained in the greenhouse for 48 d and treated with Heritage (Active ingredient: azoxystrobin, 0.032 mg per m²; Syngenta Crop Protection LLC, Greensboro, NC) and Segway (Active ingredient: cyazofamid, 0.055 mg per m^2 ; FMC corp., Philadelphia, PA) every 21d to prevent pathogen infection. Greenhouse environmental conditions were 23/20 °C (day/night), 700 $mmol \cdot m^{-2} \cdot s^{-1}$ photosynthetically active radiation from sunlight and supplemental lighting, 60% relative humidity, and 14 h photoperiod. Plants were irrigated daily to maintain well-irrigated conditions, trimmed twice per week to maintain a 7 cm canopy height, and applied with half strength Hoagland's nutrient solution every 4 d during
establishment. Following the establishment period, plants were transferred to controlled environment growth chambers (Environmental Growth Chambers, Chagrin Falls, OH) at 21/18 °C (day/night), 650 mmol·m⁻²·s⁻¹ photosynthetically active radiation, 60% relative humidity, and 14 h photoperiod for 7 d to allow plants to acclimate to growth chamber conditions prior to stress imposition.

2.2.2. Treatment and experimental design

Following establishment and acclimation to growth chamber conditions, 416 containers (26 cultivars x 4 treatments x 4 replicates) were subjected to heat, drought, or two non-stress control treatments on May 26, 2014. Two distinct sets of non-stress control plants respective to heat or drought stress treatments were used. For drought treatment, irrigation was withheld for 28 d and volumetric soil water content (SWC) began to decrease to below the control level at 4 d of water withholding, and decreased to 7.0% by 28 d of drought treatment, while SWC of non-stress control containers was maintained at the pot capacity (~29%) by daily irrigation. During drought treatment, all environmental conditions were the same as those previously described during the chamber acclimation period. For heat treatment, plants were subjected to heat stress for 28 d by increasing the growth chamber day/night temperatures to 38/33 °C, while non-stress controls containers were maintained at 21/18 °C (day/night). All other environmental conditions were the same as those previously described during the chamber acclimation period.

The experiment was arranged in a split-plot treatment arrangement, with stress treatment (heat, drought, or non-stress control) as the main plot and plant cultivar (within

each species) as the sub-plot. Each main plot (drought, heat, or non-stress control) was replicated in four different growth chambers with one growth chamber as one replicated main plot. Each cultivar (sub-plot) was replicated in four containers, which were placed across four different growth chambers of heat, drought, or non-stress treatment (main plots), with one container per chamber. All cultivars (sub-plots) were arranged randomly within each growth chamber. Plants were relocated or re-randomized within each of the four growth chambers every 3 d to minimize possible edge effects of the environmental conditions within a chamber.

2.2.3. Soil water content and physiological analysis

The SWC was monitored using a time reflectometer (Trase System1; Soilmoisture Equipment Corp., Santa Barbara, CA). Three waveguide probes, each measuring 30 cm in length, were inserted into the root zone and SWC was measured for drought and non-stress treatments every day (Topp, Davis, et al., 1980).

The relative water content (RWC) was measured to determine leaf hydration status at 4, 14, 21, and 28 d of drought treatment. Approximately 0.2 g leaf tissue was collected and fresh weight (FW) was measured immediately after harvesting. Leaves were then submerged in deionized water for 12 h at 4 °C, blotted dry, and again weighed for turgid weight (TW). Leaves were then dried in an oven at 80 °C for 3 d and weighed to determine dry weight (DW). Leaf RWC was calculated using the formula [(FW – DW)/(TW –DW)] * 100 (Barrs and Weatherley, 1962).

Leaf membrane stability was estimated by measuring electrolyte leakage (EL) at 4, 14, 21, and 28 d of drought treatment and at 7, 14, 21, and 28 d of heat treatment.

Approximately 0.2 g leaf tissue was collected, rinsed with deionized water, and placed in a test tube containing 30 mL deionized water. Tubes were agitated on a shaker for 12 h and the initial conductance (C_i) of the incubation solution was measured using a conductivity meter (YSI, Yellow Springs, OH). Leaf tissue was then killed by autoclaving at 121 °C for 20 min, agitated for 12 h, and the maximal conductance (C_{max}) of incubation solution was measured. Leaf EL was calculated using the formula (C_i/C_{max}) * 100 (Blum and Ebercon, 1981).

The chlorophyll content (Chl) was determined according to the methods described by Hiscox and Israestem (Hiscox and Israelstam, 1979) with modifications. The measurement was taken at 4, 14, 21, and 28 d of drought treatment and at 7, 14, 21, and 28 d of heat treatment. Leaf tissue (0.1 g) was collected and incubated in 10 mL dimethyl sulfoxide in darkness for 72 hours to extract chlorophyll from tissue. The resulting solution was analyzed on a spectrophotometer (Spectronic Instruments, Inc., Rochester, NY) at 663 and 645 nm. The remaining tissue was filtered and dried in an oven at 80 °C for 72 h to obtain dry weights. Chlorophyll content was then calculated on a dry weight basis according to the equations described by Arnon (Arnon, 1949). The ratio of chlorophyll content was calculated using the formula [(chlorophyll content at stress condition) / (chlorophyll content at control condition)].

The F_v/F_m was measured as a ratio of the variable fluorescence (F_v) value to the maximum fluorescence (F_m) value using a chlorophyll fluorescence meter (Fim 1500; Dynamax, Houston, TX). Leaf clips were first used to dark-adapt the leaves for 30 mins and then the F_v/F_m was determined with the fluorescence meter. The measurement was

taken at 4, 14, 21, and 28 d of drought treatment and at 7, 14, 21, and 28 d of heat treatment. Two subsample measurements were taken per plant per sampling day.

Visual evaluation of TQ was performed to evaluate overall turfgrass performance on a scale of 1 to 9, with 1 being brown and desiccated turf, 6 being the minimal acceptable level, and 9 being green and healthy turf. Ratings were based on canopy uniformity, visual attractiveness, leaf color, and canopy density.

2.2.4. Statistical analysis

General linear model analysis was performed within each fine fescue species using Statistics Analysis System (SAS) (version 9.3; SAS Institute, Cary, NC) to determine differences between cultivars within a species in response to treatment. The cultivar differences were separated by the least significance difference (LSD) test at the 0.05 probability level. Correlation analysis, analysis of variance (ANOVA) and Ward's cluster analysis were performed across all fine fescue cultivars using the JMP statistical discovery software (SAS Institute). Ward's analysis provides an overall ranking of heat or drought tolerance on all evaluated fine fescue cultivars.

2.3. Results

2.3.1. Overall turf performance and physiological responses to heat and drought stress

A significant TQ decline was detected beginning from 7 d of heat stress in Chewings fescues, slender creeping red fescues, and strong creeping red fescues, while not until 14 d for hard fescues and sheep fescues (Fig. 2.1). By the end of the heat treatment (28 d), TQ of the hard fescues were 2.8 to 4.0, sheep fescues, slender creeping red fescues, or

strong fescues were 2.7 to 3.5, 2.3 to 3.3, or 2.7 to 3.5 respectively, while TQ of Chewings fescues were 1.3 to 2.0. Under drought stress, a significant decline in TQ was detected beginning at 7 d of drought treatment in all fine fescue species (Fig. 2.2). By the end of 28 d drought treatment, TQ of Chewings fescues were 3.7 to 4.2, hard and sheep fescues were 5.5 to 8.2 and 6.5 to 7.8, respectively, and slender creeping and strong creeping red fescues were 4.7 to 5.3 and 4.2 to 7.0, respectively.

A significant increase of EL in response to heat stress was detected as early as 7 d of heat treatment in all fine fescue species and cultivars (Fig. 2.3). Chewings fescues showed a rapid EL increase during heat stress and reached 70% to 87% at the end of heat treatment (28 d), while hard fescues, sheep fescues, slender creeping red fescues, and strong creeping red fescues reached 55% to 75%, 63% to 69%, 65%, and 60% to 69%, respectively, at the end of heat treatment. A significant EL increase can be detected beginning from 21 d of drought treatment for Chewings fescues and slender creeping red fescues and detected at 28 d for hard fescues, sheep fescues, and strong creeping red fescues reached 37% to 47% and 24% to 57%, respectively, at the end of drought stress, while EL of hard fescues, sheep fescues reached 19% to 32%, 21% to 23%, and 29% to 33%, respectively, at the end of drought stress.

A significant decline in F_v/F_m was detected beginning at 14 d of heat treatment for hard fescues and sheep fescues, while the decline was detected as early as 7 d for Chewings fescues, slender creeping red fescues, and strong creeping red fescues (Fig. 2.5). Leaf F_v/F_m of Chewings fescues declined to 0.605 to 0.708, hard fescues and sheep fescues declined to F_v/F_m at 0.643 to 0.685 and 0.639 to 0.671, respectively, and strong creeping red fescues and slender creeping red fescues declined to 0.646 to 0.720 and 0.635 to 0.703, respectively, at 28 d of heat treatment. For drought stress, a significant F_v/F_m decrease was detected beginning from 14 d of drought treatment for Chewings fescues and strong creeping red fescues, while only a transient decline was detected at 14 d for slender creeping red fescues, hard fescues and sheep fescues (Fig. 2.6). At 28 d of drought treatment, the F_v/F_m of Chewings fescues declined to 0.588 to 0.729, strong creeping red fescues and slender creeping red fescues declined to 0.560 to 0.798 and 0.746 to 0.768, respectively, while hard fescues and sheep fescues declined to 0.733 to 0.823 or 0.775 to 0.796, respectively.

The Chl showed a transient increase at the early stage (7 d and 14 d) of heat stress and then a slight decrease to lower level compared to respective control at 28 d of heat stress (Fig. 2.7). Under drought stress, no consistent change of Chl was detected (Fig. 2.8). However, a transient increase of chlorophyll content was detected compared to respective controls at 14 d drought stress and ultimately was maintained at a similar level to that of controls despite the prolonged stress.

In response to drought stress, RWC of leaf tissue began declining at 4 d of drought treatment in fine fescue cultivars (Fig. 2.9). Most cultivars of Chewings fescue had RWC dropped below 50% (37% to 50%) beginning at 21 days of drought stress. Most hard fescues, sheep fescues, slender creeping red fescues, and strong creeping red fescues maintained RWC above 50% (53% to 77%, 67% to 79%, 62% to 66%, or 47% to 71% respectively) at 21 d of drought stress. At the end of drought treatment, the RWC of Chewings fescues were 27% to 35%, slender creeping red fescues and strong creeping

red fescues were 45% to 49% or 37% to 66%, respectively, while the RWC of hard fescues and sheep fescues were 49% to 77% or 51% to 63%, respectively.

2.3.2. Genotype variation under heat and drought stress

There were significant effects due to heat treatment (TRT) for TQ, EL, F_v/F_m , and due to drought treatment (TRT) for TQ, EL, RWC, F_v/F_m , Chl, indicating these parameters respond to heat and/or drought stress (Tables 1, 2). The interaction effect of TRTxDuration of treatment (D) and TRTxGenotype (G) were significant for all parameters under both heat stress and drought stress, indicating stress response was affected by stress duration and genotypic variation.

Correlation analysis was performed using TQ and physiological data at 21 d heat stress and 28 d drought stress (Tables 3, 4), since great stress responses were observed under these dates. Correlation analysis based on results of 21 d heat stress showed that EL, F_v/F_m , and Chl were significantly correlated to TQ with respective correlation coefficients of -0.86, 0.87, and 0.77. This result showed that leaf Chl, EL, and F_v/F_m are good indicators for turf performance under heat stress in fine fescues. Correlation analysis based on result of 28 d drought stress showed that EL, RWC, and F_v/F_m were significantly correlated to TQ with respective correlation analysis based on result of 28 d drought stress showed that EL, RWC, and F_v/F_m were significantly correlated to TQ with respective correlation coefficients as -0.74, 0.94, and 0.80, while no significant correlation was detected between Chl and TQ. This result showed that EL, RWC, and F_v/F_m are good indicators for turf performance under drought stress in fine fescues.

The genetic variation of heat tolerance in fine fescues was determined by Ward's cluster analysis using TQ, EL and F_v/F_m . All 26 fine fescue cultivars were classified into

four groups (Fig. 2.10). Several cultivars with good heat tolerance were selected, including Blue Ray, Spartan II, MN-HD1, Shoreline, Navigator II, Azure, Beacon, Aurora Gold, Reliant IV, Marco Polo, Garnet, Wendy Jean, Razor, and Cindy Lou. The genetic variation of drought tolerance in fine fescues was determined by Ward's cluster analysis using TQ, RWC, F_v/F_m and EL (Fig. 2.10). Several cultivars with good drought tolerance were selected, including Spartan II, MN-HD1, Reliant IV, Garnet, Azure, and Aurora Gold.

2.4. Discussions

Under heat stress, decreased Chl content, loss of membrane stability, and declined F_v/F_m has been reported in various cool-season grass species (Abraham, Huang, et al., 2004, Larkindale and Huang, 2004, Wang, Cui, et al., 2009). In this study, EL showed the most dramatic change under heat stress, suggesting cell membranes are major sites for heat damage. The increase of EL indicates loss of membrane integrity and partial dysfunction of membrane selective permeability (Bajji, Kinet, et al., 2002). Sustaining the function of cell membranes is critical in maintaining cellular activities under stress conditions and therefore greatly influence the stress tolerance of plants (Wahid, Gelani, et al., 2007). Heat-induced leaf senescence is characterized by limited photosynthetic capacity caused by declined Chl content and F_v/F_m (Abraham, Huang, et al., 2004, Cui, Li, et al., 2006, Watkins, Huang, et al., 2007). In this study, significantly change of Fv/Fm and EL was observed in response to heat stress and strong correlation between TQ and these physiological parameters were detected, suggesting these traits may serve as indicators for evaluation of heat tolerance in fine fescues.

Under drought stress, decreased Chl, decreased Fv/Fm, decreased RWC and increased EL have been reported in various cool-season grass species (Bian and Jiang, 2009, Fu and Huang, 2001, Huang and Gao, 1999). In this study, leaf RWC showed the most dramatic change and greatest variation in response to drought stress, suggesting the importance of maintaining leaf water content during prolonged drought stress. The improved maintenance of RWC under drought stress could be contributed by improved water-uptake ability at low soil water conditions (Volaire, Thomas, et al., 1998), and improved dehydration resistance of tissues and organs (Volaire and Lelievre, 2001). It is well documented that drought-tolerant cultivars exhibit higher leaf RWC compared to drought-sensitive cultivars under prolonged drought conditions, as demonstrated in various cool-season grass species, including Kentucky bluegrass (Abraham, Huang, et al., 2004), tall fescue (Cross, Bonos, et al., 2013), and perennial ryegrass (Jiang and Fry, 1998). In addition, cell membrane stability is a critical factor to maintain efficient cellular activities and EL is a common indicator for assessing drought tolerance (Blum and Ebercon, 1981, Marcum, 1998). In this study, significant change of EL, RWC and F_v/F_m was observed in response to drought stress and strong correlation between TQ and these physiological parameters were detected under drought stress, suggesting these traits could serve as indicators for evaluation of drought tolerance in fine fescues.

In summary, the TQ and physiological parameters results demonstrated that fine fescues were more sensitive to heat stress than drought stress, and there were greater genotypic variations in heat tolerance than drought tolerance within fine fescue species. Cross et al. (Cross, Bonos, et al., 2013) examined whether genotypic variations in tall fescue summer turf performance is related primarily to heat tolerance or drought tolerance for 24 tall fescue selections and concluded that top-performing tall fescue cultivars during summer stress was mainly due to superior heat tolerance. Our results suggested that there was a greater potential for improving heat-tolerance than for drought tolerance in the fine fescues due to greater sensitivity to heat stress and greater genotypic variation of heat tolerance. Several cultivars with high heat tolerance were selected, as Blue Ray, Spartan II, MN-HD1, Shoreline, Navigator II, Azure, Beacon, Aurora Gold, Reliant IV, Marco Polo, Garnet, Wendy Jean, Razor, and Cindy Lou. And several cultivars with high drought tolerance were selected, as Spartan II, MN-HD1, Reliant IV, Garnet, Azure, and Aurora Gold. The better heat tolerance in these cultivars would be contributed by better maintenance of photochemical efficiency and membrane stability under heat stress. In addition to photochemical efficiency and membrane stability, the better drought tolerance would also be associated with better maintenance relative water content under drought conditions. These traits would be used as indicators for heat and/or drought tolerance in fine fescues.



Fig. 2.1. Turf quality of (A) chewings fescue, (B) hard fescue, (C) sheep fescue and slender creeping red fescue, and (D) strong creeping red fescue as affected by heat stress compared to control. Turf quality was performed visually to evaluate overall turfgrass performance on a scale of 1 to 9, with 1 being brown and desiccated turf and 9 being green and healthy turf. Control line shows the averaged value of all cultivars. Vertical bars of the figure indicate least significant difference (LSD) values (P \leq 0.05) for comparison at a given day of treatment.



Fig. 2.2. Turf quality of (A) Chewings fescue, (B) hard fescue, (C) sheep fescue and slender creeping red fescue, and (D) strong creeping red fescue as affected by drought stress compared to control. Turf quality was performed visually to evaluate overall turfgrass performance on a scale of 1 to 9, with 1 being brown and desiccated turf and 9 being green and healthy turf. Control line shows the averaged value of all cultivars. Vertical bars of the figure indicate least significant difference (LSD) values (P \leq 0.05) for comparison at a given day of treatment.



Fig. 2.3. Electrolyte leakage of (A) Chewings fescue, (B) hard fescue, (C) sheep fescue and slender creeping red fescue, and (D) strong creeping red fescue as affected by heat stress compared to control. Control line shows the averaged value of all cultivars. Vertical bars of the figure indicate least significant difference (LSD) values ($P \le 0.05$) for comparison at a given day of treatment.



Fig. 2.4. Electrolyte leakage of (A) Chewings fescue, (B) hard fescue, (C) sheep fescue and slender creeping red fescue, and (D) strong creeping red fescue as affected by drought stress compared to control. Control line shows the averaged value of all cultivars. Vertical bars of the figure indicate least significant difference (LSD) values ($P \le 0.05$) for comparison at a given day of treatment.



Fig. 2.5. Photochemical efficiency of (A) Chewings fescue, (B) hard fescue, (C) sheep fescue and slender creeping red fescue, and (D) strong creeping red fescue as affected by heat stress compared to control. Control line shows the averaged value of all cultivars. Vertical bars of the figure indicate least significant difference (LSD) values ($P \le 0.05$) for comparison at a given day of treatment.



Fig. 2.6. Photochemical efficiency of (A) Chewings fescue, (B) hard fescue, (C) sheep fescue and slender creeping red fescue, and (D) strong creeping red fescue as affected by drought stress compared to control. Control line shows the averaged value of all cultivars. Vertical bars of the figure indicate least significant difference (LSD) values ($P \le 0.05$) for comparison at a given day of treatment.



Fig. 2.7. Relative change of chlorophyll content under heat stress compared to control of (A) Chewings fescue, (B) hard fescue, (C) sheep fescue and slender creeping red fescue, and (D) strong creeping red fescue. Vertical bars of the figure indicate least significant difference (LSD) values ($P \le 0.05$) for comparison at a given day of treatment.



Fig. 2.8. Relative change of chlorophyll content under drought stress compared to control of (A) Chewings fescue, (B) hard fescue, (C) sheep fescue and slender creeping red fescue, and (D) strong creeping red fescue. Vertical bars of the figure indicate least significant difference (LSD) values ($P \le 0.05$) for comparison at a given day of treatment.



Fig. 2.9. Relative water content (%) of (A) Chewings fescue, (B) hard fescue, (C) sheep fescue and slender creeping red fescue, and (D) strong creeping red fescue as affected by drought stress compared to control. Control line shows the averaged value of all cultivars. Vertical bars of the figure indicate least significant difference (LSD) values ($P \le 0.05$) for comparison at a given day of treatment.



Fig. 2.10. Ward's cluster analysis of 26 fine fescue cultivars based on photochemical efficiency (Fv/Fm), electrolyte leakage (EL) and turf quality (TQ) at the 21 day of heat treatment.



Fig. 2.11. Ward's cluster analysis of 26 fine fescue cultivars base on photochemical efficiency (Fv/Fm), electrolyte leakage (EL), relative water content (RWC) and turf quality (TQ) at the 28 day of drought treatment.

Table 2.1. Summary of the ANOVA for treatment (TRT), duration of treatment (D) or genotype (G) effects and their interactions on turf quality (TQ), electrolyte leakage (EL), chlorophyll content (Chl) and photochemical efficiency (Fv/Fm) measured at 7, 14, 21, and 28 d of heat treatment.

Source of variance	TQ	EL	Chl	Fv/Fm	df
TRT	**	**	ns	**	1
D	**	**	**	**	3
G	**	**	**	**	25
TRT x D	**	**	**	**	3
TRT x G	**	**	**	**	25
D x G	**	**	**	**	75
TRT x D x G	**	**	**	**	75

** Significant at *P*≤0.01

* Significant at 0.01<*P*≤0.05

Table 2.2. Summary of the ANOVA for treatment (TRT), duration of treatment (D) or genotype (G) effects and their interactions on turf quality (TQ), electrolyte leakage (EL), chlorophyll content (Chl), photochemical efficiency (Fv/Fm) and relative water content (RWC) measured at 7, 14, 21, and 28 d of drought treatment.

Source of variance	TQ	EL	Chl	Fv/Fm	RWC	df
TRT	**	**	*	**	**	1
D	**	**	**	**	**	3
G	**	**	**	**	**	25
TRT x D	**	**	**	**	**	3
TRT x G	**	**	**	**	**	25
D x G	**	**	**	**	**	75
TRT x D x G	**	**	**	**	**	75

** Significant at *P*≤0.01

* Significant at 0.01<*P*≤0.05

	Fv/Fm	EL	TQ	Relative change of Chl
Fv/Fm	1			
EL	-0.87**	1		
TQ	0.87**	-0.86**	1	
Relative change of Chl	0.82**	-0.81**	0.77**	1

Table 2.3. Correlation between turf quality (TQ), electrolyte leakage (EL), photochemical efficiency (Fv/Fm) and relative change of chlorophyll content (Chl) at 21 d of heat stress.

** Significant at P≤0.01

* Significant at 0.01<*P*≤0.05

Table 2.4. Correlation between turf quality (TQ), electrolyte leakage (EL), relative water content (RWC), photochemical efficiency (Fv/Fm) and Relative change of chlorophyll content (Chl) at 28 d of drought stress.

	TQ	EL	RWC	Fv/Fm	Relative change of Chl
TQ	1				
EL	-0.74**	1			
RWC	0.94**	-0.79**	1		
Fv/Fm	0.8**	-0.57**	0.79**	1	
Relative change of Chl	ns	ns	ns	ns	1

** Significant at P≤0.01

* Significant at 0.01<*P*≤0.05

CHAPTER 3. Differential Profiles of Membrane Proteins, Fatty Acids, and Sterols Associated with Genetic Variations in Heat Tolerance for a Perennial Grass Species, Hard Fescue (*Festuca trachyphylla*)

3.1. Background

Heat stress is one of the most detrimental stresses limiting growth of temperate plant species (Pradhan et al., 2012; Sermons et al., 2012; Wahid, 2007). Cellular membranes are sensitive to changes in temperature, which has been identified as a major site for heat damage (Quinn, 1988). Heat stress causes loss of membrane stability and integrity, causing cell dysfunction; therefore, sustaining integrity and function of cellular membrane is critically important for plant tolerance to heat stress (Blum and Ebercon, 1981, Marcum, 1998). Membrane stability and integrity is largely controlled by the chemical composition of membranes, which mainly include three classes of constituents, phospholipids or fatty acids, sterols, and proteins (Taiz and Zeiger, 2002). The differential changes of membrane constituents in response to heat stress play critical roles in plant stress tolerance.

Phospholipids forms the bilayers of the membrane, which mainly consists of fatty acids in saturated or unsaturated forms, and proper fatty acid composition is critical for maintaining membrane stability during plant adaptation to stress conditions (Gigon, Matos, et al., 2004, Levitt, 1980, Levitt, 1980). Heat stress causes reduction of unsaturated fatty acid content and increase in saturated fatty acid content, leading to increased saturation level of fatty acids, which has been positively associated with heat tolerance (Larkindale and Huang, 2004, Raison, Roberts, et al., 1982). A soybean mutant

with higher saturation level of fatty acid [increased content of palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1) and linoleic acid (18:2) and reduced content of palmitoleic acid (16:1) and linolenic acid (18:3)] showed unusual tolerance to heat stress (Alfonso, Yruela, et al., 2001). The fatty acid number notation takes the form C:D, where C is the number of carbon atoms and D is the number of double bonds in the fatty acid. In addition, enhanced heat tolerance was observed in Arabidopsis *fabB* mutant deficient in unsaturated 16:0 fatty acid (Kunst and Somerville, 1989) and in Arabidopsis *fabC* mutant deficient in 16:1 and 18:1 unsaturation (Hugly, Kunst, et al., 1989). Furthermore, lower content of 16:3 and 18:3 caused by silencing omega-3 fatty acid desaturase rendered more heat tolerance in transgenic tobacco (Murakami, Tsuyama, et al., 2000). The mutation and transgenic analysis eluciate the importance of proper fatty acid composition for heat tolerance.

Membrane proteins are embedded in the lipid bilayers, which play important roles in water and solute transport, signaling, light harvesting, electron transfer and energy production (Taiz and Zeiger, 2010). Denaturation or dissociation of membrane proteins related to photosystem II, including 33-kDa manganese (Mn)-stabilizing protein (Yamane, Kashino, et al., 1998), oxygen evolving complex (OEC) (De Ronde, Cress, et al., 2004) and D1, D2 proteins of the reaction center (De Las Rivas and Barber, 1997) has been reported under heat stress. A lesser or later decrease of membrane proteins in response to heat stress was observed in a heat tolerant line of bentgrass (*Agrostis* spp.) compared to a heat sensitive line, including those categorized to energy metabolism (ATP-synthase, Cytochrome b6f, chloroplast oxygen-evolving enhancer protein, and pyruvate dehydrogenase kinase) and antioxidant processes (catalase and peroxidase)

(Jespersen, Xu, et al., 2015). Although proteomic analysis of soluble protein responses to heat stress has been well documented (Wahid, 2007; Zhou and Abaraha, 2007), limited information is available on changes in membrane proteins for plant adaptation to heat stress, particularly on specific membrane proteins that could be up-regulated conferring membrane thermostability and plant tolerance to heat stress.

Sterols in cellular membranes serve as regulators for membrane fluidity and permeability (Hartmann, 1998, Schaller, 2003). A typical plants membrane sterol profile, as found in pea (Pisum sativum) seedlings, consist of 50% sitosterol, 25% stigmasterol, and less than 10% each of campesterol, fucosterol and cholesterol (Nomura, Kitasaka, et al., 1999). Different sterol components exhibited different biological functions, including restricting the mobility of membrane phospholipids (Schuler, Duportail, et al., 1990, Schuler, Milon, et al., 1991), modulating activities of membrane located enzymes (e.g. ATPase) (Grandmougin-Ferjani, Schuler-Muller, et al., 1997) and serving as precursors to brassinosteroids (Schaller, 2003). Different responses of individual sterol components were observed under different abiotic stress. Under salinity stress, all sterol components (sitosterol, stigmasterol, campesterol and cholesterol) showed increased content (Kerkeb, Donaire, et al., 2001), while under cold stress sitosterol and fucosterol showed increased content and cholesterol, campesterol and stigmasterol showed decreased content (Palta, Whitaker, et al., 1993). Increased total sterol content has been reported under salinity stress and cold stress with a positive relationship between increased level of total sterol content and stress tolerance (Blits and Gallagher, 1990, Kerkeb, Donaire, et al., 2001, Palta, Whitaker, et al., 1993). The function of sterols has been investigated on a model membrane system and showed an ordering effect on membrane at temperatures above its

phase transition temperature and a disordering effect on membrane at temperatures below its phase transition temperature (Dufourc, 2008). However, limited information is available on how sterols change in response to heat stress or are involved in maintaining membrane stability and heat tolerance.

Recent works revealed direct interaction among major membrane components (fatty acid, sterol and membrane proteins) (Phillips, Ursell, et al., 2009). The hydrophobic thickness of the lipid bilayers, defined by the fatty acid length, tends to match the hydrophobic thickness of the membrane proteins. In the extreme mismatch the membrane proteins would be excluded from the lipid bilayers (Lee, 2004). The optimal fatty acid chain length for membrane proteins would also be affected by existence of sterol. For example, the optimal chain length for Na⁺, K⁺-ATPase is C22 in the absence of cholesterol but C18 in the presence of cholesterol (Cornelius, 2001). In addition, the lipid environment also affects the function of membrane proteins. For example, the open probably of bacterial mechanosensitive channel is dependent on the fatty acid length that lower pipette pressure is required to open the channel in short chain fatty acids (pipette pressure required: C16 < C18 < C20) (Perozo, Kloda, et al., 2002). A greatly increased activity of Na⁺, K⁺-ATPase was observed in the presence of cholesterol (Cornelius, 2001). The content of sterol and fatty acid are interrelated as the promoter of several genes for the enzymes involved in the biosynthesis of fatty acid containing sterol-responsive elements (Thewke, Kramer, et al., 2000). However, few studies have investigated and compared the interaction of these three major membrane constituents in relation to heat stress tolerance.

We hypothesized that the differential quantity or proportion of differential classes of membrane constituents (sterol, fatty acid and proteins) could account for the genetic variations in heat tolerance. Therefore, the objectives of this study were to examine differential changes in membrane constituents (sterol, fatty acid and membrane protein) in response to heat stress for a cool-season perennial grass species, hard fescue (*Festuca longifolia*) and identify major membrane constituents associated with the genetic variations in heat tolerance for two varieties contrasting in heat tolerance.

3.2. Materials and Methods

3.2.1. Plant materials and growth conditions

Thirty tillers of each variety ('Reliant IV' and 'Predator') were planted in plastic containers (15 cm in depth and 14 cm in diameter) filled with sterile sand autoclaved at 121°C for 60 min. Plants were established for 56 d from 4 May 2015 to 29 June 2015 in a greenhouse with an average day/night temperature of 23/20 °C and at least 710 µmol m⁻²·s⁻¹ photosynthetically active radiation (PAR) from sunlight and supplemental lighting. Plants were irrigated daily, trimmed twice per week to maintain 7-cm canopy height, and fertilized every 4 d with half-strength Hoagland's nutrient solution (Hoagland and Arnon, 1950). Following the establishment period, plants were transferred to controlled-environment growth chambers (Environmental Growth Chambers, Chagrin Falls, OH) controlled at 22/18 °C (day/night), 650 µmol m⁻²·s⁻¹ photosynthetically active radiation, 60% relative humidity, and 12-h photoperiod for 7 d to allow for plant acclimation to growth chamber conditions prior to stress imposition.

3.2.2. Treatment and experimental design

Four containers of plants for each variety were exposed to heat stress at 38/33 °C (day/night) or maintained under non-stress control conditions at 22/18 °C (day/night). The experimental design was a split-plot design with temperature as the main plots and varieties as the subplots. Each temperature treatment was repeated in four growth chambers. All varieties were arranged randomly within each growth chamber and were relocated among the four growth chambers used for same temperature treatment every 3 d to avoid possible confounding effects of unique growth chamber environmental variations from occurring.

3.2.3. Physiological measurements

Turf quality (TQ), electrolyte leakage (EL) and photochemical efficiency (Fv/Fm) were measured at 0 d (6 July 2015), 5 d (11 July 2015), 10 d (16 July 2015) and 15 d (21 July 2015) of heat stress. The TQ rating was performed to evaluate overall turfgrass performance on a scale of 1 to 9, with 1 being brown and desiccated turf, 6 being the minimal acceptable level, and 9 being green and healthy turf. Ratings were based on parameters such as uniformity, visual attractiveness, leaf color, and canopy density (Beard, 1973).

Leaf membrane stability was estimated by measuring EL. Approximately 0.2 g leaf tissue was collected, rinsed with deionized water, and placed in a test tube containing 30 mL deionized water. Tubes were agitated on a shaker for 12 h and initial conductance (C_i) of the incubation solution measured using a conductivity meter (YSI, Yellow Springs, OH). Leaf tissue was then killed by autoclaving at 121 °C for 20 min, agitated

for 12 h, and maximal conductance (C_{max}) of the incubation solution was measured. Plant EL was calculated using the formula (C_i/C_{max}) x100 (Blum and Ebercon, 1981).

Plant Fv/Fm was measured as a ratio of the variable fluorescence (Fv) value to the maximum fluorescence (Fm) value using a chlorophyll fluorescence meter (Fim 1500; Dynamax, Houston, TX). Leaf clips were first used to dark-adapt the leaves for 30 mins and then the Fv/Fm was determined with the fluorescence meter. Two subsamples were collected per plant per sampling day.

3.2.4. Fatty acid extraction and analysis

Fatty acid composition was determined by the method of Browse (Browse, McCourt, et al., 1986) with some modifications. Fresh leaf samples (0.2 g) were incubated in 1N H₂SO₄ containing heptadecanoic acid as an internal standard at 80 °C for 90 min. After cooling to room temperature, 1.5 ml 0.9% NaCl and 400 µl hexane was added to the mixture. The upper hexane layer contained the fatty acids. The upper hexane layer contained the fatty acids methyl esters (FAME), that were separated by gas chromatography (GC, Agilent 6890, Wilmington, DE) equipped with Econo-CapTMECTM-WAX capillary column (length 30 m, internal diameter 0.25 mm, phase polyethyleneglycol, film 0.25 μ m, Alltech, Deerfield, IL) and quantified by using a Flame Ionization Detector (FID, Agilent 6890, Wilmington, DE). The GC was programmed to begin at 60 °C for 1 min and increase to 180 °C at the rate of 5 °C per minutes for 20 min. The inlet temperature and detector temperature were 230 °C and 250 °C, respectively. The levels of the major fatty acids were calculated by using heptadecanoic acid as an internal standard. Pure standards (product number: CRM47885, Sigma-Aldrich Corporation) were used for peak identification.

3.2.5. Sterol extraction and analysis

Freeze-dried leaf samples were ground to powder, and incubated in 25 ml 12.5 g potassium hydroxide dissolved in 60% alcohol for 1 h at 70 °C with cholestane as an internal standard. After cooling to room temperature, sterols were extracted with hexane for three times. The sterol extract was then evaporated and subject to silylation by commercial kit (Sil-Prep Kit, Fisher Scientific). Sterols were separated by gas chromatography (GC, Agilent 6890, Wilmington, DE) equipped with Econo-CapTMECTM-WAX capillary column (length 30 m, internal diameter 0.25 mm, phase polyethyleneglycol, film 0.25 µm, Alltech, Deerfield, IL) and quantified by using a Flame Ionization Detector (FID, Agilent 6890, Wilmington, DE). The GC was programmed at 260 °C for 1 min and then increases to 300 °C at the rate of 3 °C per minute. The levels of the sterols were calculated by using cholestane as internal standard. Pure standards were used for peak identification. All standards were purchased from Sigma-Aldrich Corporation.

3.2.6. Protein extraction and analysis

Protein extraction was based on the method by Molloy (Molloy, Herbert, et al., 1998) with modifications. Approximately 0.5 g leaf tissue was homogenized with 2 mL of 40 mM Tris-base (pH 7.6) and 0.15 M NaCl extraction buffer. The homogenate was centrifuged at 10,000 g for 15 min at 4 °C. The pellet was washed three times with 40 mM Tris-base (pH 7.6) and 0.15 M NaCl extraction buffer. 2 ml of extraction butter (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, sonicated for 30 min and then centrifuged for 15 min at 10,000g, 4 °C. The membrane

protein were precipitated at -20 °C in 8 mL of acetone with 0.07% 2-mercaptoethanol for 12 h and then centrifuged for 15 min at 8500 g at 4 °C. 8 mL of ice- cold acetone containing 0.07% 2-mercaptoethanol was add to the resulting pellet and stored at -20 for 2h. The pellet was washed three times with 0.07% 2-mercaptoethanol-acetone solution and then resuspended in 1ml 8 M of urea, 2 M of thiourea, 2% CHAPS, 1% dithiothreitol (DTT), and 1% 3/10 biolytes. The protein concentration was determined based on method by Bradford (Bradford, 1976) using a commercial dye reagent (Bio-Rad Laboratories, Hercules, CA).

Proteins were separated according to Xu (Xu, Xu, et al., 2008) with modifications. Immobilized pH gradient strips (pH 3–10; linear gradient, 13 cm) were first rehydrated in 250 ml rehydration buffer containing 250 ° µg extracted proteins (8 M of urea, 2 M of thiourea, 2% w/v CHAPS, 1% v/v IPG buffer, 1% DTT, and 0.002% bromophenol blue). The strips were put in an IPGPhor apparatus (GE Healthcare, Piscataway, NJ) and run 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. After the isoelectric focusing, the strips were equilibrated twice for 15 min in a buffer containing 6 M urea, 30% glycerol, 2% sodium dodecyl- sulfate, 0.002% bromophenol blue, 50 mM Tris-Base (pH 8.7), and 1% DTT and then incubated in the same buffer replacing DTT with 2.5% iodoacetamide. Gel electrophoresis of the second dimension was performed in a 12.5% SDS-polyacrylamide gel using a Hoefer SE 600 Ruby electrophoresis unit (GE Healthcare, Piscataway, NJ). The voltage conditions were 5mA for 30 min and 20 mA for 6h per gel. The gel was then stained by Coomassie blue stain (Neuhoff, Arold, et al., 1988) and analyzed using SameSpots software (Nonlinear, Newcastle on Tyne, UK). Normalized spot volumes of heat stress gels were compared

with that of the control gel and to calculate the abundance change under heat stress. Proteins spots with p-value lower than 0.05 were selected and identified using liquid chromatography and tandem mass spectrum (LC-MS/MS) by N-cell company (Hong Kong, China).

3.2.7. Statistical analysis

Treatment effects and the interaction between temperature treatment and varieties were determined by analysis of variance using the general linear model procedure of SAS (version 9.3; SAS Institute, Cary, NC). Differences between treatment means were separated by Fisher's protected least significance difference (LSD) test at the 0.05 probability level.

3.3. Results

3.3.1. Differential physiological responses to two varieties of hard fescue to heat stress

Overall turf performance of hard fescues was evaluated by TQ, while the cell membrane stability and integrity was evaluated by EL and photosystem II efficiency was measured by Fv/Fm. A significant decline in TQ was detected as early as 5 d of heat treatment in both hard fescue varieties (Fig. 3.1A). Significant differences in TQ between 'Reliant IV' and 'Predator' were detected at 10 and 15 d of heat treatment, with 'Reliant IV' exhibiting higher TQ than 'Predator'. At the end of heat stress, TQ of 'Reliant IV' and 'Predator' decreased to 7.0 and 5.3, respectively.

As shown in figure 3.1B, a significant increase of EL was detected as early as 5 d of heat treatment in 'Predator' but not until 10 d of heat treatment in 'Reliant IV'.

Significant differences in EL between 'Reliant IV' and 'Predator' were detected at 5, 10 and 15 d of heat treatment, with 'Reliant IV' exhibiting lower EL than 'Predator'. At the end of heat stress, EL of 'Reliant IV' and 'Predator' increased to 25% and 37% respectively. As shown in figure 3.1C, a significant decrease of Fv/Fm was detected beginning from 5 d of heat treatment in both hard fescue varieties. Significant differences in Fv/Fm between 'Reliant IV' and 'Predator' were detected at 5, 10 and 15 d of heat treatment, with 'Reliant IV' and 'Predator' were detected at 5, 10 and 15 d of heat treatment, with 'Reliant IV' exhibiting higher Fv/Fm than 'Predator'.

3.3.2. Sterol composition associated with heat tolerance

A total of 5 sterols (avenasterol, fucosterol, campesterol, stigmasterol and sitosterol) and 1 sterol precursor (cycloartenol) were detected in leaves of the two hard fescue varieties. Among them, sitosterol (56%) accounted for the majority of sterol in hard fescue, followed by cycloartenol (18%), campesterol (18%), fucosterol (9%) and avenasterol (5%), while stigmasterol only accounted for 1% of total sterol content (Fig. 3.2A). The content of all sterols (sitosterol, campesterol, stigmasterol, avenasterol and fucosterol) increased, while the content of sterol precursor (cycloartenol) decreased in both varieties under heat stress (Fig. 3.2B).

All ethyl-sterols (sitosterol, stigmasterol, avenasterol and fucosterol) showed greater increases under stress from their respective non-stress controls in 'Reliant IV' than 'Predator', while campesterol, which is a methyl-sterol, did not show significant difference between the two varieties either by relative change to the control or by actual content (Fig. 3.2B). Under optimal temperature, 'Reliant IV' and 'Predator' showed similar content of stigmasterol, fucosterol and avenasterol (Fig. 3.3). While under heat
stress the content of stigmasterol, fucosterol and avenasterol increased to a greater level in 'Reliant IV' than in 'Predator'. For sitosterol, similar content was detected under heat stress between 'Reliant IV' and 'Predator', while lower content of sitosterol in 'Reliant IV' under optimal temperature indicates greater capacity of sitosterol up-regulation in 'Reliant IV'.

3.3.3. Fatty acid composition associated with heat tolerance in hard fescue

A total of 6 fatty acids, including two 16-carbon fatty acids (16:1 and 16:0) and four 18-carbon fatty acids (18:3, 18:2, 18:1, 18:0) were detected in this study. Among them, 18:3 fatty acid accounted for the majority (64%), followed by 16:0 fatty acid (18%) and 18:2 fatty acid (14%), while 16:1, 18:1 and 18:0 fatty acid only accounted for 2%, 1% and 1%, respectively (Fig. 3.4A).

Under heat stress, the content of 16:0, 18:0, 18:1 and 18:2 fatty acids increased with greater up regulation of 18:1 and 18:2 in 'Reliant IV', while the content of 18:3 fatty acid decreased with greater decrease in 'Reliant IV' (Fig. 3.4B). Under optimal temperature, 'Reliant IV' and 'Predator' showed similar content of 18:1 and 18:2 fatty acids (Fig. 3.5). While under heat stress the content of 18:1 and 18:2 increased to a greater level in 'Reliant IV' than in 'Predator'. In contrast, 18:3 fatty acid showed a significant decrease under heat stress. Although no significant differences in the content of 18:3 was detected under heat stress between the two varieties, higher content of 18:3 under optimal temperature in 'Reliant IV' indicates greater down-regulation of this fatty acid under heat stress in 'Reliant IV'.

3.3.4. Membrane proteins associated with heat tolerance

A representative gel image for proteins extracted from membranes in leaves of hard fescue was shown in figure 3.6. A total of 13 differentially expressed membrane proteins under heat stress compared to control were identified in either hard fescue varieties (Fig. 3.7).

Seven membrane proteins exhibited declined protein abundance under heat stress (Fig. 3.7A). These decreased membrane proteins were categorized into the functional groups of photosynthesis (ATP synthase subunit alpha, ATP synthase subunit beta, ATP synthase subunit gamma, cytochrome b6-f complex iron-sulfur subunit, ferredoxin-NADP reductase), signaling (lectin-domain containing receptor kinase A4.2), and protein modification (S-acyltransferase) (Fig. 3.7A). The decrease of all three ATPase subunits (alpha, beta, and gamma) was detected in 'Predator', while a less severe decrease of ATPase subunit alpha and no significant decrease of ATPase subunit beta and gamma were detected in 'Reliant IV' under heat stress. The decrease of S-acyltransferase was only detected in 'Predator', while no significant change was detected in 'Reliant IV' than 'Predator'. The ferredoxin-NADP reductase and lectin receptor-like kinase did not differ in the down-regulated level between 'Reliant IV' and 'Predator'.

Six proteins exhibited up-regulation under heat stress in both hard fescue varieties (Fig. 3.7B). They were categorized into functional groups of photosynthesis [Rubisco activase, oxygen-evolving enhancer protein 1 (OEE1)], stress defense (stromal 70 kDa heat shock-related protein, disease resistance protein 1), protein folding [chaperonin 60 (CPN 60)] and protein degradation (ATP-dependent zinc metalloprotease protein).

Among them, two membrane proteins (Rubisco activase and disease resistance protein 1) increased to a greater degree in 'Reliant IV' compared to 'Predator'. One the contrary, the abundance of CPN 60 increased to a greater degree in 'Predator' compared to 'Reliant IV'. OEE1, stromal 70 kDa heat shock-related protein and ATP-dependent zinc metalloprotease 2 showed similar up-regulated level between 'Reliant IV' and 'Predator'.

3.4. Discussions

Loss of membrane stability and integrity, as indicated by increased EL, and impaired photosystem II function, as indicated by decreased Fv/Fm, are important attributors to heat-induced leaf senescence (Wahid, Gelani, et al., 2007). A strong positive correlation between whole-plant performances with membrane stability and integrity and between whole-plant performances with photosystem II efficiency under heat stress has previously been reported (Jespersen, Xu, et al., 2015, Marcum, 1998). The physiological analysis of EL, Fv/Fm, and TQ in this study demonstrated that 'Reliant IV' showed greater heat tolerance compared to 'Predator'.

Sterols are considered as membrane reinforcers, and increasing sterol content leads to increased membrane rigidity (Dufourc, 2008b). In this study, membrane sterol content increased during heat stress in both varieties. Similar increases of sterol content has been observed under salinity stress (Blits and Gallagher, 1990) and a positive correlation between increased sterol content and salinity tolerance has been suggested previously (Kerkeb, Donaire, et al., 2001). Increased sterol content might be an important heat acclimation mechanism based on its function in inducing membrane rigidity, which could retard heat-induced membrane permeability. Furthermore, all ethyl-sterols (sitosterol, stigmasterol, avenasterol and fucosterol) increased to a greater extent in response to heat stress from their respective non-stress controls in 'Reliant IV' than 'Predator', while the methyl-sterol campesterol did not show significant difference between the two varieties. The previous research conducted on model membrane systems suggested additional ethyl group on sterols might lead to a stronger ordering effect on membranes under heat stress (Dufourc, 2008, Dufourc, 2008). Our results indicated that ethyl-sterol instead of methylsterol could contribute to the difference in the membrane stability between 'Reliant IV' and 'Predator' exposed to heat stress. The regulating role of individual sterol components on cell membrane thermostability in grass species deserves further investigation.

The saturation level of fatty acids influences membrane fluidity, and non-linearity of fatty acid chains caused by double bonds could inhibit tight packing of lipid molecules, thereby reducing rigidity of membranes (Geuther, 1977, Vigh, Maresca, et al., 1998). In this study, the increased content of saturated fatty acids (16:0 and 18:0) or with lower numbers of double bonds (less saturated 18:1 and 18:2) and the decreased content of highly unsaturated fatty acids (18:3) suggested an increased saturation level of fatty acids under heat stress. The increased saturation level of fatty acids could retard the increased membrane fluidity caused by heat stress. Similar results were previously reported in bentgrass, in which 18:1 and 18:2 fatty acid increased under heat stress with higher increased levels of 18:2 fatty acid in heat tolerant varieties than that of heat sensitive varieties (Larkindale and Huang, 2004). Higher 18:1 and 18:2 fatty acid content in transgenic tobacco and an Arabidopsis mutant caused by deficiency in the 18:2 unsaturation process was also associated with enhanced heat tolerance and improved

photosynthetic function under heat stress (Alfonso, Yruela, et al., 2001, Hugly, Kunst, et al., 1989, Murakami, Tsuyama, et al., 2000). Our results suggested that the accumulation of the less unsaturated fatty acids or more saturated long chain (18:1 and 18:2) fatty acids may contribute to the maintenance of better membrane stability in the leaves of 'Reliant IV' compared to 'Predator'.

Membrane proteins are involved in various processes, including signaling, electron transfer, nutrient transport, and metabolic activities, as well as stress protection, but some proteins can be damages due to accelerated protein degradation or dissociation from the membrane under heat stress while some proteins exhibit up-regulation in response to stress (Kosová, Vítámvás, et al., 2011, Yamane, Kashino, et al., 1998). In this study, membrane proteins exhibited decline in the abundance under heat stress were mainly involved in photosynthesis (ATP synthase subunit alpha, ATP synthase subunit beta, ATP synthase subunit gamma, cytochrome b6-f complex iron-sulfur subunit, ferredoxin-NADP reductase,), signaling (lectin-domain containing receptor kinase A4.2), and protein modification (S-acyltransferase).

ATPase is a key membrane-bound enzyme that converts ADP to ATP in both photosynthesis and respiration (Boyer, 1997). Three subunits are major constituents for ATPase catalytic core with subunit alpha and subunit beta forming hexamer and gamma subunit inside it (Leslie and Walker, 2000). The interruption of ATPase activity caused by heat stress contributes to the decline in photosynthetic efficiency (Crafts-Brandner and Salvucci, 2000). The impaired ATP production can also lead to accumulation of ROS and shortage of energy, which is required for stress defense and long-term survival under stress conditions (Wahid, Gelani, et al., 2007). The cytochrome b6f iron-sulfur subunit is

an important component of cytochrome b6f, which is an electron carrier that connects photosystem I and photosystem II (Whitelegge, Zhang, et al., 2002). The disruption of cytochrome b6f could jeopardize the electron transport process for ATP production and lead to excess of energy and ROS accumulation (Schöttler, Flügel, et al., 2007). Damage to cytochrome complexes and related electron flow has been suggested to be a major effect of heat stress (Sharkey, 2005). The lesser degree of down-regulation of cytochrome b6f iron-sulfur subunit and all three subunits of ATPase in 'Reliant IV' relative to 'Predator' could help maintain electron transport and energy production during photosynthesis under heat stress. Ferredoxin-NADP reductase is the enzyme that catalyzed the last step of light driven electron transfer to form NADPH (Arakaki, Ceccarelli, et al., 1997). Our results suggested that ferredoxin-NADP reductase were sensitive to heat stress, but the changes in the abundance of this membrane protein was not related to the genetic variation in heat tolerance for hard fescue.

S-acyltransferase catalyzes the reversible lipid modification of protein Cys residues (Hemsley and Grierson, 2008), which is an important secondary modification that can regulate protein stability (Valdez and Pelham, 2005), trafficking (Rocks, Peyker, et al., 2005) and function (Gubitosi-Klug, Mancuso, et al., 2005). The critical role of S-acyltransferase in salt tolerance in regulating membrane protein localization and activity has been suggested in Arabidopsis (Zhou, Li, et al., 2013). Lectin receptor-like kinases are transmembrane proteins with amino-terminal extracellular domains and carboxyl-terminal intracellular kinase domains (Shiu and Bleecker, 2001). They are involved in various signaling pathways including disease resistance, hormone signaling, wounding stress responses and salinity stress response (Barre, Hervé, et al., 2002, Joshi, Dang, et

al., 2010). S-acyltransferase and lectin receptor-like kinase could play positive roles in maintaining membrane protein stability in the heat-tolerant varies of hard fescue exposed to heat stress.

In this study, six membrane proteins exhibited up-regulation in response to heat stress in both varieties of hard fescue. They were categorized into functional groups of photosynthesis (Rubisco activase, disease resistance protein 1, OEE1), stress defense (stromal 70 kDa heat shock-related protein, disease resistance protein 1, CPN 60) and protein degradation (ATP-dependent zinc metalloprotease protein). Rubisco activase is well known as a regulator of Rubisco by releasing inhibitor sugar phosphates from the active site of Rubisco (Portis, 1995). Its important roles under heat stress has been suggested in protecting protein synthesis machinery (thylakoid-bound ribosome) (Rokka, Zhang, et al., 2001) and regulating Rubisco activity (Salvucci, 2008, Salvucci and Crafts-Brandner, 2004). Heat-induced increases in the abundance of Rubisco activase have been reported previously (Law and Crafts-Brandner, 2001, Salvucci and Crafts-Brandner, 2004). The greater induction level of Rubisco activase could help activate or protect Rubisco to sustain photosynthesis under heat stress. The disease resistance proteins can recognize avirulence proteins from pathogen and induce defense responses (Martin, Bogdanove, et al., 2003). The crosstalk between biotic and abiotic stress has been suggested with transcription factors, kinases, hormones and ROS serving as convergence points (Fujita, Fujita, et al., 2006, Xiong and Yang, 2003), however no information has been provided for the potential role of disease resistance protein 1 in abiotic stress tolerance. Our result indicated the greater up regulation of Rubisco activase and disease

resistance protein 1 in 'Reliant IV' compared to 'Predator' could contribute its greater heat tolerance.

OEE1 together with OEE2 and OEE3 constitute chloroplast oxygen-evolving enhancer protein, which is bound to photosystem II on the luminal side of the thylakoid membrane (Sugihara, Hanagata, et al., 2000). Previous research reported the role of OEE1 in oxygen evolution and PSII stability (Mizobuchi and Yamamoto, 1989). The induction of OEE1 may be one heat tolerance mechanism by maintaining PSII capacity. HSP70 is a set of prominent cellular machines that prevent aggregation, assist refolding and facilitate proteolytic degradation of unstable proteins (Kotak, Larkindale, et al., 2007). The up-regulation of HSP70 under heat stress was previously reported in rice (Oryza sativa L.) leaves (Lee, Ahsan, et al., 2007) and a positive correlation between Hsp70 and heat tolerance has been suggested (Sung and Guy, 2003). The ATP-dependent zinc metalloprotease is known as a membrane-bound protease with ATP-dependent zinc metalloprotease 2 as the most abundant FtsH protein (Sinvany-Villalobo, Davydov, et al., 2004). The previous study suggested the involvement of ATP-dependent zinc metalloprotease in the degradation of unassembled proteins and cleavage of the reaction center-binding protein D1 of photosystem II under light stress (Lindahl, Spetea, et al., 2000, Ostersetzer and Adam, 1997). Our results indicated the up-regulation of OEE1, stromal 70 kDa heat shock-related protein and ATP-dependent zinc metalloprotease 2 could facility heat stress defense, but was not associated with the genetic variation in heat tolerance between the two hard fescue varieties.

The major role of chaperonin is involved in the folding of newly synthesized proteins and prevent misfolding of protein structures under both normal and stress conditions (Hartl, 1996, Wang, Vinocur, et al., 2004). However the function of CPN 60 in plants especially under abiotic stress is still unclear. The more significant induction of CPN 60 in 'Predator' may be caused by greater sensitivity of plants to heat stress, with chaperones being activated to bind and repair damaged proteins.

Pervious works revealed a direct link between lipid fatty acid environment and membrane protein stability (Phillips, Ursell, et al., 2009). The hydrophobic thickness of the lipid bilayers, defined by fatty acid length, tends to match the hydrophobic thickness of the membrane proteins, while in the extreme mismatch the membrane proteins would be excluded from the lipid bilayers (Lee, 2004). The decreased membrane thickness, as indicated by increased total content of C16 fatty acids and similar level of total content of C18 fatty acids under heat stress compared to control (S. 1) was observed in response to heat stress and would contribute to denaturation or dissociation of membrane proteins under heat stress. The optimal fatty acid chain length for membrane proteins would also be affected by existence of sterol, as the optimal chain length for Na⁺, K⁺-ATPase is C22 in the absence of cholesterol but C18 in the presence of cholesterol (Cornelius, 2001). In this study, increased sterol content under heat stress could play positive role in maintaining membrane proteins and the greater increase in 'Reliant IV' compared to 'Predator' could account for less severe decrease or better maintenance of membrane proteins in 'Reliant IV'.

In summary, this study demonstrated that heat tolerance in hard fescue, as evaluated by overall turf performance and membrane stability, as well as leaf photochemical efficiency was associated with greater increase of ethyl sterols (sitosterol, stigmasterol, avenasterol and fucosterol), unsaturated long chain fatty acids (18:1 and 18:2), as well as less severe

down-regulation of membrane proteins involved in photosynthesis, protein modification and signaling and greater up-regulation of heat responsive proteins, including Rubisco activase and disease resistance protein 1 (Fig. 3.8). Modification of those membrane constituents could lead to improvement in heat tolerance for hard fescue and other coolseason grass species. Those membrane constituents could also be used as biochemical markers to select for heat-tolerant germplasm due to their contribution to heat tolerance.



Fig. 3.1. (A) Turf quality and (B) electrolyte leakage and (C) photochemical efficiency of 'Reliant IV' and 'Predator' affected by heat stress. Turf quality was rated on the scale of 1 to 9, with 1 being worst and 9 being the best. Vertical bars indicate Fisher's protected LSD values (P \leq 0.05) for comparison between two temperature treatments and varieties at a given day of treatment.



Fig. 3.2. (A) Sterol composition of 'Reliant IV' at optimal temperature. (B) Assignment of the 6 detected sterols to sterol metabolic pathways.



Fig. 3.3. Content of (A) cycloartenol (B) campesterol (C) sitosterol (D) fucosterol (E) stigmasterol and (F) avenasterol of 'Reliant IV' and 'Predator' at 15 d of heat stress. Columns marked with different letters indicate significant difference between two temperature treatments and varieties according to Fisher's protected LSD ($P \le 0.05$).



Fig. 3.4. (A) Fatty acid composition of 'Reliant IV' at optimal temperature. (B) Assignment of the 6 detected fatty acids to fatty acid metabolic pathways.



Fig. 3.5. Content of (A) 16:0 (B) 16:1 (C) 18:0 (D) 18:1 (E) 18:2 and (F) 18:3 fatty acid of 'Reliant IV' and 'Predator' at 15 d of heat stress. Columns marked with different letters indicate significant difference between two temperature treatments and varieties according to Fisher's protected LSD ($P \le 0.05$).



Fig. 3.6. A representative 2D gel image of membrane proteins.



Fig. 3.7. Relative changes of the abundance of membrane proteins in response to heat stress compared to the non-stress control for 'Reliant IV' and 'Predator'. (A) '-' values indicate a negative fold-change for protein spots down-regulated by heat stress and (B) '+' values indicate positive fold-changes for protein spots up-regulated by heat stress. '*' Indicates there is a significant difference in relative change between 'Reliant IV' and 'Predator' (P<0.05).



Fig. 3.8. Total content of (A) C 16 fatty acids and (B) C 18 fatty acids of 'Reliant IV' and 'Predator' at 15 d of heat stress. Columns marked with different letters indicate significant difference between two temperature treatments and varieties according to Fisher's protected LSD ($P \le 0.05$).

CHAPTER 4. Differential Responses of Amino Acids and Soluble Proteins to Heat Stress Associated with Genetic Variations in Heat Tolerance for Hard Fescue (*Festuca Trachyphylla*)

4.1. Background

Heat stress is detrimental to plant growth and productivity in most plants, especially for cool-season species. Plant adaptation to heat stress involves profound changes in metabolic, physiological, and molecular processes (Wahid et al., 2007). Amino acid and protein metabolism are among major metabolic processes going through adjustment during plant adaptation to heat stress (Du et al., 2011; Kaplan et al., 2004; Yamakawa and Hakata, 2010). Understanding differential changes in both amino acids and proteins between different varieties of plants contrasting in heat tolerance will enable the identification of key metabolic processes controlling genetic variations in heat tolerance.

Free amino acids are constituents of proteins and play regulatory roles in abiotic stress responses as signaling molecules, precursor for numerous secondary metabolites, protein chaperone and osmotic protectants (D'Mello, 2015). Different amino acids exhibited different roles and respond differently to abiotic stress. For example, proline protects plants from stress damage by serving as compatible osmolyte, regulator for redox homeostasis and molecular chaperone (Verbruggen and Hermans 2008, Szabados and Savoure 2010). The aromatic amino acids (tyrosine, phenylalanine and tryptophan) serve as precursor for numerous metabolites that are involved in stress defense, including auxin, melatonin, phenolic compounds and alkaloids (Dixon, 2001). Glycine is known to be substrate for respiration and also serve as a precursor for glycine betaine, which is a

well know stress protector (Holmström et al., 2000; Oliver et al., 1990; Sakamoto and Murata, 2002). Glutamate may act as a signaling molecular in root architecture (Walch-Liu et al., 2006), N and C metabolism (Lam et al., 2006), and interaction with the ABA signaling system (Kang et al., 2004). In addition, a previous metabolic study reported the accumulation of some amino acids in response to heat stress, including alanine, valine, leucine, asparagine, lysine, methionine, isoleucine and threonine (Du, Wang, et al., 2011; Kaplan, Kopka, et al., 2004). However, major amino acids conferring heat tolerance in cool-season grass species are not well documented.

Proteins play a central role in heat tolerance of plants, such as serving as enzymes in metabolism pathway, regulators and components of transcription and translation machinery, and components for plasma membrane, cell cytoskeleton and intracellular compartments (Kosová et al., 2011). Most previous analysis of proteome has been applied on plant response to short term heat shock (hours) (Han et al., 2009; Lee et al., 2007; Li et al., 2013; Zhang et al., 2013). The proteomic response to heat shock has been illustrated as up regulation of proteins involved in various processes, such as energy metabolism (UDP-glucose pyrophosphorylase UGPase, pyruvate dehydrogenase and transketolase), chaperone function (HSP110, HSP90, HSP70, HSP60 and small HSP) and redox homeostasis (dehydroascorbate reductase, thioredoxin h-type and chloroplast precursors of SOD) (Baniwal et al., 2004; Kosová, Vítámvás, et al., 2011; Lee, Ahsan, et al., 2007; Li, Wei, et al., 2013). While many studies have found increased or decreased content or abundance of different proteins in response to heat stress, however, few studies have examined both amino acids and proteins in relation to genetic variations for heat

tolerance, although amino acids are constituents of proteins and their content is closely related to protein metabolism.

Fine fescue species (*Festuca* ssp.) are widely used turfgrass in low maintenance areas due to its superior abiotic stress tolerance, including heat stress, among cool-season turfgrass species. Physiological analysis of 26 varieties of five fine fescue species demonstrated a wide range of genetic variability in heat tolerance among fine fescue species and varieties; hard fescue was among the most tolerant of the five fine fescue species and varieties also varied in heat tolerance within the species (Wang et al., 2017a). Analysis of membrane constituents has identified some membrane proteins, fatty acids, and sterol associated with the genetic variations in heat tolerance between two varieties ('Reliant IV' and 'Predator' of hard fescue contrasting in heat tolerance (Wang et al., 2017b). The objective of this study was to identify major amino acids and soluble proteins associated with genetic variations in heat tolerance in two varieties of hard fescues. Such information will complement previous findings with physiological traits, membrane proteins, and lipid metabolism and enhance further understanding mechanisms of heat tolerance in cool-season turfgrass species.

4.2. Materials and Methods

4.2.1. Plant material and growth conditions

A total of 30 tillers of two hard fescue varieties ('Reliant IV' and 'Predator') were planted in plastic containers (15 cm in depth and 14 cm in diameter) filled with sterile sand autoclaved at 121°C for 60 min on March 1, 2015. Plants were established for 56 d in a greenhouse with an average day/night temperature of 23/20 °C and 710 μ mol^{·m^{-2·}s⁻¹} photosynthetically active radiation (PAR) from sunlight and supplemental lighting from March 1, 2015 to April 25, 2015. Plants were irrigated daily, trimmed twice per week to maintain 7-cm canopy height, and fertilized every 4 d with half-strength Hoagland's nutrient solution (Hoagland and Arnon, 1950). Following the establishment period, plants were transferred to controlled-environment growth chambers (Environmental Growth Chambers, Chagrin Falls, OH) controlled at 22/18 °C (day/night), 650 μ mol^{-m⁻²·s⁻¹} photosynthetically active radiation, 60% relative humidity, and 12-h photoperiod for 7 d to allow for plant acclimation to growth chamber conditions prior to stress imposition.

4.2.2. Treatments and experimental design

Plants for both varieties were exposed to heat stress at 38/33 °C (day/night) or maintained under non-stress control conditions at 22/18 °C (day/night) for 28 days from May 4, 2015 to June 1, 2015. Each temperature treatment was repeated in four growth chambers and each variety had four replicates (pots), which were randomly placed, within each growth chamber. The experimental design was a split-plot design with temperature as the main plots and varieties as the subplots. All varieties were arranged randomly within each growth chamber and were relocated among the four growth chambers used for same temperature treatment every 3 d to avoid possible confounding effects of unique growth chamber environmental variations from occurring.

4.2.3. Physiological measurements

Turf quality (TQ) and electrolyte leakage (EL) were measured at 7, 14, 21 and 28 d of heat stress. The TQ rating was performed to evaluate overall turfgrass performance on

a scale of 1 to 9, with 1 being brown and desiccated turf, 6 being the minimal acceptable level, and 9 being green and healthy turf. Ratings were based on parameters such as uniformity, visual attractiveness, leaf color, and canopy density.

Leaf membrane stability was estimated by measuring EL. Approximately 0.2 g leaf tissue was collected, rinsed with deionized water, and placed in a test tube containing 30 mL deionized water. Tubes were agitated on a shaker for 12 h and initial conductance (C_i) of the incubation solution measured using a conductivity meter (YSI, Yellow Springs, OH). Leaf tissue was then killed by autoclaving at 121 °C for 20 min, agitated for 12 h, and maximal conductance (C_{max}) of the incubation solution was measured. Plant EL was calculated using the formula (C_i/C_{max}) x100 (Blum and Ebercon, 1981).

4.2.4. Protein extraction and separation

Protein extraction was based on the method by Molloy (Molloy et al., 1998) with modifications. Approximately 0.5 g leaf tissue was homogenized with 2 mL of 40 mM Tris-base (pH 7.6) and 0.15 M NaCl extraction buffer. The homogenate was centrifuged at 10,000 g for 15 min at 4 °C. The supernatant contained the soluble proteins, while the pellet contained the membrane protein. The soluble protein were precipitated at -20 in 8 mL of acetone with 0.07% 2-mercaptoethanol for 12 h and then centrifuged for 15 min at 8500 g at 4 °C. 8 mL of ice-cold acetone containing 0.07% 2-mercaptoethanol was added to the resulting pellet and stored at -20 °C for 2 h. The pellet was washed three times with 0.07% 2-mercaptoethanol-acetone solution. The pellet was then re-suspended in 1 ml 8 M of urea, 2 M of thiourea, 2% CHAPS, 1% dithiothreitol (DTT), and 1% 3/10 biolytes.

The protein concentration was determined based on method by (Bradford, 1976) using a commercial dye reagent (Bio-Rad Laboratories, Hercules, CA).

Proteins were separated according to (Xu et al., 2008) with modifications. Immobilized pH gradient strips (pH 3–10; linear gradient, 13 cm) were rehydrated in 250 ml rehydration buffer containing 250 mg extracted proteins (8 M of urea, 2 M of thiourea, 2% w/v CHAPS, 1% v/v IPG buffer, 1% DTT, and 0.002% bromophenol blue). The strips were put in an IPGPhor apparatus (GE Healthcare, Piscataway, NJ) and run 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 80 kVh. After the isoelectric focusing, the strips were equilibrated twice for 15 min 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 and then incubated in the same buffer replacing DTT with 2.5% iodoacetamide. Gel electrophoresis of the second dimension was performed in a 12.5% SDS-polyacrylamide gel and by Hoefer SE 600 Ruby electrophoresis unit (GE Healthcare, Piscataway, NJ). The voltage conditions were 5 mA for 30 min and 20 mA for 6 h per gel. The gel was then stained by Coomassie blue stain and analyzed using SameSpots software (Nonlinepear, Newcastle on Tyne, UK). Normalized spot volumes of heat stress gels were compared with that of the control gels to calculate the abundance change under heat stress. Proteins spots with p-value lower than 0.05 were selected and identified using liquid chromatography and tandem mass spectrum by N-cell company (Hong Kong, China).

4.2.5. Free amino acids extraction and analysis

Free amino acids were extracted by grinding frozen leave material with liquid nitrogen and 4 ml of 70% ethanol. After storage overnight in 4°C, the samples were centrifuged (10500 rpm, 10 minutes). The supernatants were collected, filtered through a membrane (0.45 um PTFE membrane), concentrated under vacuum, and stored in -20°C (Rozan et al., 2000).

The free amino acids content of the extract was analyzed by an HPLC gradient system with precolumn phenylisothiocyanate (PITC) derivatization (Khan et al., 1994). Buffer A consisting of 0.1 M ammonium acetate and buffer B consisting of 0.1 ammonium acetate, acetonitrile, and methanol (44:46:10 v/v) were used (Rozan, Kuo, et al., 2000). For sample derivatization, 100 μ l of the extract was removed and dried under vacuum (37°C). 20 μ l of the first reagent [methanol, water, triethylamine (2:2:1 v/v)] was added and then dried under vacuum. Then the sample was reacted with 30 µl of the PITC reagent [methanol, PITC, water, triethylamine (7:1:1:1 v/v)] at room temperature for 20 minutes before drying under vacuum. The derivatized samples were then redissolved in 1 ml of buffer A. Sample was injected into Agilent 1100 series HPLC/MSD trap equipped with an autodegasser, quaternary pump, autosampler, column thermostat, and a diode array detector. The gradients started with 10 % B at 0 min, up to 17.5% at 3 min, 21% at 5.5min, 35% at 8 min, and 100% at 28 min, followed by 10 min column equilibration with the starting mobile phase proportion. The column used was Zorbax Eclipse XDB-C8, 150×4.6mmm, 5µm, set at 28 °C. The absorbance at 254 nm was used for the calculations. Individual standards were purchased from Sigma and prepared as above.

4.2.6. Statistical analysis

Treatment effects, variety variations, and the interaction between temperature treatment and varieties were determined by analysis of variance using the general linear model procedure of SAS (version 9.3; SAS Institute, Cary, NC). Differences between treatment means and varieties were separated by Fisher's protected least significance difference (LSD) test at the 0.05 probability level.

4.3. Results

4.3.1. Differential physiological response to heat stress between heat tolerant and heat sensitive varieties

Overall turf performance was evaluated by turf quality (TQ), while cell membrane stability and integrity was evaluated by electrolyte leakage (EL). A significant decrease of TQ was detected at 7 d of heat treatment for 'Predator', while not until 14 d for 'Reliant IV' (Fig. 4.1A). Significant differences in TQ between 'Reliant IV' and 'Predator' were detected at 7, 14, 21, 28 d of heat treatment, with 'Reliant IV' exhibiting higher TQ than 'Predator'. At the end of heat treatment, TQ of 'Reliant IV' and 'Predator' dropped to 5.6 and 3.8, respectively.

A significant increase of EL was detected at 14 d of heat treatment for 'Predator', while not until 21 d for 'Reliant IV' (Fig. 4.1B). Significant differences in EL between 'Reliant IV' and 'Predator' were observed from 14 d of heat treatment, with 'Reliant IV' exhibiting lower EL than 'Predator'. At the end of heat treatment, EL of 'Reliant IV' and 'Predator' increased to 37% and 43%, respectively.

4.3.2. Differential response of free amino acids to heat stress between heat tolerant and heat sensitive varieties

Variation in free amino acid content existed in hard fescue varieties under control condition, therefore, the content of free amino acids under heat stress was normalized by that under control condition; the data were expressed as percentage of control in all figures. Total free amino acid content increased to 164% and 143% of the control under heat stress in 'Reliant IV' and 'Predator', respectively, while changes of individual amino acid content in response to heat stress varied between the two varieties (Fig. 4.2). Most amino acids showed increased content under heat stress compared to those of the non-stress control, including phenylalanine, tyrosine, tryptophan, serine, glycine, alanine, leucine, valine, lysine, aspartate, threonine, isoleucine, glutamine, histidine, arginine, glutamate and proline (Fig. 4.3). Among them, seven amino acids showed greater increase in 'Reliant IV' compared to 'Predator', including glutamine, glutamate, proline, histidine, tryptophan, threonine and aspartate. Decreased content of asparagine was detected under heat stress, with greater decrease in 'Predator' compared to 'Reliant IV'.

4.3.3. Differential response of soluble proteins to heat stress in heat tolerant and heat sensitive varieties

Variation in soluble protein content existed in hard fescue varieties under control condition, therefore, the content of soluble protein under heat stress was normalized by that under control condition; the data were expressed as percentage of control. Total soluble protein content decreased to 76% and 66% of the non-stress control under heat stress for 'Reliant IV' and 'Predator', respectively (Fig. 4.4).

A total of 30 soluble proteins showing differential expression level in plants exposed to heat stress compared to the control plants in either hard fescue varieties were identified (Table 1). Out of the identified protein spots, 34% were categorized into photosynthesis, 13% in signaling, 10% in metabolism, 10% in stress defense, 7% in cell organization, 7% in redox homeostasis, 7% in protein folding, 3% in DNA processing, 3% in protein degradation, 3% in protein synthesis and 3% in unknown category (Fig. 4.5). Several soluble proteins were more up-regulated by heat stress in 'Reliant IV' than in 'Predator', including those involved in photosynthesis (Glyceraldehyde 3-phosphate dehydrogenase, triosephosphate isomerase, dihydrolipoyl dehydrogenase, malate dehydrogenase, Rubisco large subunit binding protein subunit alpha), protein folding (protein disulfideisomerase), redox hemostasis (catalase), signaling (calcium-transporting ATPase, lectindomain containing receptor kinase), stress defense (stromal 70 kDa heat shock-related protein and 20 kDa chaperonin), cell organization (actin, tubulin beta-2 chain), and metabolism (aspartate aminotransferase, formate dehydrogenase, UDP-sulfoquinovose synthase). Some soluble proteins were down regulated under heat stress, to a lesser extent in 'Reliant IV' than in 'Predator', including those involved in carboxylation in photosynthesis (Rubisco subunits), protein synthesis (50S ribosomal protein L12-2) and signaling (Serine/threonine-protein kinase) (Table 1).

4.4. Discussions

Physiological analysis of 26 fine fescue varieties for their responses to heat stress found that 'Reliant IV' exhibited greater heat tolerance compared to 'Predator' (Wang et al., 2017a), and our results in this study confirmed the previous results. Our previous studies also identified some membrane proteins, such as those involved in electron transport of photosynthesis (ATP synthase subunits and cytochrome b6-f complex iron-sulfur subunit), signaling (lectin-domain containing receptor kinase A4.2), protein modification (S-acyltransferase), and stress defense (disease resistance protein 1) could contribute to the differential level of heat tolerance between the two varieties of hard fescues (Wang et al., 2017). As membrane proteins and soluble proteins play distinct roles in plant growth and stress adaptation, it is also important to understand major soluble proteins and associated amino acids in relation to heat tolerance.

In this study, we have found less decreased total soluble protein content and greater increased total amino acid content under heat stress in 'Reliant IV' compared to 'Predator', indicating the differential accumulation of soluble proteins and free amino acids could be associated with the genetic variations in heat tolerance in hard fescue. The decreased total protein content (Chaitanya et al., 2001; Gulen and Eris, 2004) and increased free amino acids content (Kaplan, Kopka, et al., 2004; Mayer et al., 1990) has been previously observed in non-turfgrass species exposed to heat stress. The inhibition of protein synthesis and accelerated protein proteolysis into free amino acids under heat stress may contribute to the accumulation of free amino acids and the decline in protein content under heat stress (Levitt, 1980). Proteolysis degrades damaged proteins into amino acids under stress conditions (Chinnusamy et al., 2007; Dubey, 1999; Roy-

Macauley et al., 1992). Amino acid induction can also be due to enhanced amino acid synthesis activities (Yamakawa and Hakata, 2010). Nevertheless, increased total content of free amino acid under heat stress may help plants to survive heat stress by providing organic nitrogen sources to support various nitrogen-demanding metabolic activities. The metabolic functions of these amino acids and soluble proteins related to heat tolerance are discussed below.

4.4.1. Free amino acids associated with heat tolerance in hard fescue

Individual amino acids exhibited differential responses to heat stress in two hard fescue varieties, but most have showed increased content under heat stress in this study. The free amino acids can be categorized into five different families according to their biosynthesis pathway, including aromatic amino acids family (phenylalanine, tyrosine and tryptophan), serine family (serine, glycine and cysteine), pyruvate family (alanine, leucine and valine), aspartate family (asparagine, aspartate, lysine, threonine, isoleucine and methionine) and glutamate family (glutamate, histidine, glutamine, proline and arginine) (Fig. 4.6).

An overall up-regulation of amino acids in glutamate family has been observed, with greater extent of increase in 'Reliant IV' for glutamate, histidine, glutamine and proline. Glutamate and glutamine play important roles in amino acid synthesis as they are directly involved in ammonium assimilation and transfer the amino group to all other amino acids (Lea and Ireland, 1999). Proline plays important role in stress tolerance, as compatible osmolyte, regulator for redox homeostasis and molecular chaperone (Szabados and Savoure, 2010; Verbruggen and Hermans, 2008). Arginine serves as precursor of polyamines, which acts as ROS-scavenging and as membrane protectors in stress

tolerance (Alcázar et al., 2006; Lea et al., 2007). Histidine is required for plant growth and development and serves as metal chelators (Stepansky and Leustek, 2006). Our result indicated up-regulation of amino acids in glutamate family (glutamate, glutamine, proline and histidine) could play positive roles in regulating heat tolerance in hard fescue.

The content of aspartate and threonine was greater in 'Reliant IV' than 'Predator' under heat stress. Aspartate is precursor for three metabolic pathways, which leads to synthesis of asparagine, NAD+ and aspartate derived amino acid (lysine, methionine, threonine and isoleucine) respectively (D'Mello, 2015). Asparagine is a major nitrogen transport compounds in xylem and phloem (Lea, Sodek, et al., 2007). The synthesis of asparagine from aspartate is catalyzed by asparagine synthetase through an ATP-dependent transfer of amide group from glutamate to aspartate (D'Mello, 2015). The content of several aspartate derived amino acids, asparagine, lysine, threonine and isoleucine, also increased under heat stress. Our result indicated that the accumulation of aspartate and threonine was positively associated with heat tolerance in hard fescue.

Three amino acids categorized into aromatic amino acid family, including tyrosine, phenylalanine and tryptophan, exhibited differential responses to heat stress for the two hard fescue varieties, with greater accumulation of tryptophan and less accumulation of tyrosine and phenylalanine in 'Reliant IV' compared to 'Predator'. All these three amino acids are derived from shikimate pathway and serve as precursor for numerous metabolites. Tryptophan is precursor for most indole compounds, including two plant hormone auxin and melatonin (Tzin and Galili, 2010), which promote plant tolerance to various abiotic stress (Arnao and Hernández-Ruiz, 2013; Lei et al., 2004; Peyrot and Ducrocq, 2008; Zhang et al., 2013). Phenylalanine is a precursor for phenylpropanoid

biosynthesis pathway, which generate numerous phenolic compounds (Vogt, 2010). Phenylalanine ammonia lyase (PAL), the enzyme at the gateway from phenylalanine to phenolic compounds, is highly induced under abiotic stress conditions and is considered as a major stress acclimation process (Christie et al., 1994; Dixon and Paiva, 1995; Oh et al., 2009). Tyrosine is a precursor of isoquinoline alkaloids, tyramine and tocochromanols (vitamin E) (Radwanski and Last, 1995). The greater accumulation of tryptophan and less accumulation of tyrosine and phenylalanine in 'Reliant IV' could facilitate its superior heat tolerance to 'Predator' by involving in hormone and secondary metabolism.

In contrast to the results discussed above, the content of several amino acids including glycine, alanine, valine and leucine, exhibited greater increases in 'Predator' than in 'Reliant IV' under heat stress. Glycine is an important substrate for respiration and also serves as a precursor for glycine betaine, which is known to accumulate in responses to various abiotic stresses (Holmström, Somersalo, et al., 2000; Oliver, Neuburger, et al., 1990; Sakamoto and Murata, 2002). Increased content of alanine, valine and leucine in response to heat stress has been previously reported in Arabidopsis (Kaplan, Kopka, et al., 2004) and has been suggested as a consequence of stress induced pyruvate accumulation (Good and Muench, 1993; Good and Zaplachinski, 1994; Rocha et al., 2010). Our finding that 'Predator' showed greater increase of glycine, alanine, leucine and valine under heat stress compared to 'Reliant IV' may reflect its greater level of physiological stress or sensitivity in response to heat stress.

4.4.2. Soluble proteins associated with heat tolerance in hard fescue

While total content of proteins in general decreased in both verities of hard fescue under heat stress, the content of some individual proteins either maintained unchanged, increased, or decreased and also exhibited differential response between the two varieties under heat stress. Therefore, identification of specific soluble proteins with important biological functions is of great significance for better understanding of metabolic pathways or processes underlying genetic variations in heat tolerance.

A large proportion of differential-expressed soluble proteins involved in energy metabolism, including Ribulose-1, 5-bisphosphate carboxylase/oxygenase (Rubisco) large and small chain, Rubisco large subunit binding protein subunit alpha, dihydrolipoyl dehydrogenase, malate dehydrogenase, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and triosephosphate isomerase. Rubisco large subunit and small subunit are the major components for Rubisco (Spreitzer and Salvucci, 2002), while Rubisco binding protein serves as a chaperon in Rubisco assembly and in stabilization of Rubisco under heat stress (Parsell and Lindquist 1993, Demirevska-Kepova, Holzer et al. 2005). The better maintenance of Rubisco under heat stress in 'Reliant IV' could assist in maintaining photosynthesis under heat stress. Respiration provided the energy for biosynthesis, cellular maintenance and active transport in plants and also for stress defense under stress conditions (Taiz and Zeiger, 2010). More importantly, the respiration can also provide carbon skeleton for various metabolites, including amino acids (Fig. 4.6). In this study, greater up regulation of dihydrolipoyl dehydrogenase, malate dehydrogenase, GAPDH and triosephosphate isomerase were detected under heat stress in 'Reliant IV' compared to 'Predator'. The triosephosphate isomerase and

GAPDH are the key enzymes in glycolysis, which catalyze the converting from dihydroxyacetone phosphate to glyceraldehyde 3-phosphate and from glyceraldehyde 3phosphate into 1,3-bisphosphoglycerate, respectively. Dihydrolipoyl dehydrogenase and malate dehydrogenase are involved in TCA cycle. The dihydrolipoyl dehydrogenase is a primary component of pyruvate dehydrogenase complex, which involved in transferring carbon from glycolysis to TCA cycle (Reid et al., 1977). Malate dehydrogenase catalyzes the reversible reaction from malate to oxaloacetic acid (Hodges, 2002). A greater increase of enzymes involved in glycolysis and TCA cycle in 'Reliant IV' could be associated with its better heat tolerance by providing carbon skeleton for amino acids synthesis for stress defense.

Another characteristic feature of heat stress is oxidative damage leading to reactive oxygen species (ROS) production that can cause denaturation and dysfunction of almost all cellular components (Apel and Hirt, 2004). The up regulation of two enzymes (superoxide dismutase and catalase) involved in ROS scavenging were detected under heat stress, with greater up regulation of catalase in 'Reliant IV' compared to 'Predator'. Superoxide dismutase catalyzes the conversion from O_2 . to H_2O_2 and the generated H_2O_2 was subsequently split into water and oxygen by catalase (Ahmad et al., 2010). The superoxide dismutase and catalase showed increased enzyme activity in response to heat stress (Chaitanya et al., 2002; Gill and Tuteja, 2010) with a positive relationship between up-regulation level of catalase activity and heat tolerance (Xu et al., 2015). The greater up regulation of catalase in 'Reliant IV' would protect plants from oxidative damage, contributing to better tolerance to heat stress.

It has been long known that heat leads to increased expression of proteins with chaperone functions, such as heat shock proteins (HSP). The 70 kDa HSP is a prominent family that has essential function in preventing aggregation, assisting refolding and facilitating proteolytic degradation of non-native proteins under stress conditions (Wang et al., 2004). The 20 kDa chaperone, as a small heat shock protein, involves in stabilizing and preventing non-native protein aggregation through hydrophobic interaction (Lee and Vierling, 2000; Veinger et al., 1998). In addition, compared to 'Predator', 'Reliant IV' had greater accumulation of other proteins involved in protein folding, such as protein disulfide isomerase (Hatahet and Ruddock, 2009; Pemberton, 2006; Wilkinson and Gilbert, 2004) and protein synthesis, such as the chloroplast 50S ribosomal protein L12-2 (Wittmann, 1982) which involved in the synthesis of subunits of photosystem I, photosystem II, ATPase and cytochrome b6f and Rubisco large subunit (Ridley et al., 1967). The greater up-regulation of HSP70, HSP20, protein disulfide isomerase, and chloroplast 50S ribosomal protein L12-2 in 'Reliant IV' suggested the importance of those proteins in protecting hard fescue plants from heat damages. For the protein assisting in pre-ribosomal RNA processing and RNA stabilization (Zchut et al., 2003), glycine rich RNA-binding protein exhibited increased abundance level in response to heat stress in both hard fescue varieties, although no variety difference were detected in this study. The up regulation of these proteins has been reported under various stress conditions, including water, cold, light and wounding (Sachetto-Martins et al., 2000). Our results indicated that the up regulation of glycine rich RNA-binding protein could play potential roles in heat responses, but would not account for variation of heat tolerance in hard fescue.

An increased accumulation of proteins for enzymes involved in metabolism (UDPsulfoquinovose synthase, formate dehydrogenase and aspartate aminotransferase) indicated metabolic adjustment in response to heat stress in hard fescue. The UDPsulfoquinovose synthase is a soluble enzyme located in chloroplast stroma and plays a regulatory role in the synthesis of sulfolipid (Shimojima, 2011), which stabilizes protein complexes, including photosystem II (Minoda et al., 2003) and CF0-CF1 ATPase (Taran et al., 2000). The formate dehydrogenase is a mitochondrial, NAD-dependent enzyme, which catalyzes the oxidation of formate (des Francs-Small et al., 1993). The formate dehydrogenase is strongly induced under abiotic stress (drought, chilling and dark) at both transcription and translation level, and the resulting formate respiration would complement classic respiration under stress conditions (Hourton-Cabassa et al., 1998). The aspartate aminotransferase mediates aspartate synthesis by catalyzing the reversible transamination between glutamate and oxaloacetate to generate aspartate and 2oxoglutarate (de la Torre et al., 2014). The increased protein abundance of aspartate aminotransferase is consistent with the increase in the content of aspartate and aspartatederived amino acids observed in 'Reliant IV', as discussed above. Altogether, the greater accumulation of UDP-sulfoquinovose synthase, formate dehydrogenase, aspartate aminotransferase under heat stress in 'Reliant IV' compared to 'Predator' indicated metabolic adjustment for enhanced synthesis of amino acids and proteins playing roles in stabilizing protein complex and respiratory energy metabolism is important for hard fescue tolerance to heat tolerance.

Cytoskeleton is a complex network that helps to maintain cell shape, cell signaling and intracellular transportation (Taiz and Zeiger, 2010). The greater up regulation of two
major components of cytoskeleton (beta tubulin and actin) was detected under heat stress in 'Reliant IV'. Similar increase was also reported under heat stress in *P. euphratica* suggested as a result of cytoskeleton reorganization (Ferreira et al., 2006). Therefore, it is intriguing to speculate cytoskeleton rebuilding under heat stress in hard fescue plants.

Stress signaling and transduction are key stress defense responses. Four proteins involved in signaling, including receptor kinase (lectin-domain containing receptor kinase A4.2. serine/threonine protein kinase and leucine-rich repeat receptor-like serine/threonine protein kinase) and calcium transporting ATPase, exhibited upregulation in response to heat stress in this study. The receptor-like kinase connects cell wall, plasma membrane and cytoskeleton, and plays central role in signaling transduction by accepting external signal and converting it into appropriate outputs such as changes in gene and protein expression and in metabolism (Fujita et al., 2006). Several membrane of serine/threonine protein kinase family is induced by stress conditions (Diédhiou et al., 2008; Joshi et al., 2010). Calcium transporting ATPase is ATP-dependent calcium pump that transport calcium against its concentration (White and Broadley, 2003). Transient increase of calcium ions has been observed in response to heat stress and suggested to be involved in signaling transduction (Gong et al., 1998). The greater up regulation of Lectin-domain containing receptor kinase A4.2 and calcium transporting ATPase under heat stress in 'Reliant IV' might assist in signaling transduction. However, how the expression level of these two proteins and signaling transduction are related to heat tolerance is not well known.

The up regulation of DNA helicase was detected upon heat stress with higher upregulation level in 'Predator'. DNA helicase catalyzes the unwinding of duplex DNA, which is critical step for replication, repair, recombination, transcription and translation (Matson et al., 1994). The induction of DNA helicase has been reported under cold and salinity stress and suggested to be involved in stress signaling (Vashisht et al., 2005). The greater extent of up-regulation of DNA helicase in heat-sensitive 'Predator' indicated higher levels of DNA damages due to heat stress that may require more abundant DNA helicase for repairing damaged DNA, although the functions of DNA helicase in heat tolerance deserves further investigation.

In summary, greater accumulation of total free amino acid content and less severe decrease of total soluble protein content was observed under heat stress in 'Reliant IV' compared to 'Predator'. Furthermore, 'Reliant IV' showed greater increase of six essential amino acids (histidine, glutamine, proline, threonine, aspartate, tryptophan) and several soluble proteins, including glyceraldehyde 3-phosphate dehydrogenase, triosephosphate isomerase, dihydrolipoyl dehydrogenase, malate dehydrogenase, Rubisco large subunit binding protein subunit alpha, protein disulfide-isomerase, catalase, calcium-transporting ATPase, lectin-domain containing receptor kinase, stromal 70 kDa heat shock-related protein, 20 kDa chaperonin, actin, tubulin beta-2 chain, aspartate aminotransferase, formate dehydrogenase and UDP-sulfoquinovose synthase. The differential accumulation of those amino acids and soluble proteins under heat stress between 'Reliant IV' and 'Predator' could be associated with the variation of heat tolerance in hard fescue. The more up-regulated amino acids in 'Reliant IV' exposed to heat stress could potentially be incorporated into biostimulant products used in managing stressed turfgrass. The direct involvement in heat tolerance of the differentially accumulated amino acids and soluble proteins between the two varieties deserves further investigation.



Fig. 4.1. (A) Turf quality and (B) electrolyte leakage of 'Reliant IV' and 'Predator' as affected by heat stress compared to control under 7, 14, 21 and 28 day (d). Turf quality was rated visually to evaluate overall turfgrass performance on the scale of 1 to 9, with 1 being worst and 9 being the best. Vertical bars of the figure indicate least significant difference values ($P \le 0.05$) for comparison at a given day of treatment.



Fig. 4.2. Total amino acid content percentage of control (%) in 'Reliant IV' and 'Predator' at 21 day of heat stress. Columns marked with different letters indicate significant difference between 'Reliant IV' and 'Predator' according to least significant difference ($P \le 0.05$). Dotted line indicates 100% at control.



Fig. 4.3. Free amino acids content percentage of control (%) in 'Reliant IV' and 'Predator' at 21 day of heat stress. Columns marked with different letters indicate significant difference between 'Reliant IV' and 'Predator' according to least significant difference ($P \le 0.05$).



Fig. 4.4. Total soluble protein content percentage of control (%) in 'Reliant IV' and 'Predator' at 21 day of heat stress. Columns marked with different letters indicate significant difference between 'Reliant IV' and 'Predator' according to least significant difference ($P \le 0.05$). Dotted line indicates 100% at control.



Fig. 4.5. Function classification of differential expressed soluble proteins at 21 day of heat stress compared to control in either 'Reliant IV' or 'Predator'.



Fig. 4.6. Assignment of the 18 detected free amino acids and the 5 soluble proteins to amino acids metabolic pathways.

Table 4.1. Fold changes of protein abundance under 21 day of heat stress compared to control in 'Predator' and 'Reliant IV'. Heat stress significantly decreased (-) or increased (+) protein abundance compared to control according to least significant difference ($P \le 0.05$).

Protein Description	Uniprot	Protein	Heat Effect compared	(fold change to control)
-	Accession No.	Score	Predator	Reliant IV
ganization				
Actin	B1P763	366.19	ns ^z	2.5
Tubulin beta-2 chain	M8AFS1	107.3	3.5	5.1
processing				
DNA helicase	M0UEL0	176.19	6	5.2
olism				
Aspartate aminotransferase	W5G5A6	916.86	ns	2.6
Formate dehydrogenase, mitochondrial	N1R356	376.76	2.2	2.4
UDP-sulfoquinovose synthase, chloroplastic	R7W2K9	114.29	ns	2.9
y metabolism				
Dihydrolipoyl dehydrogenase	I1HF32	71.21	ns	3
Dihydrolipoyl dehydrogenase	I1HLI3	736.6	1.3	2.4
Glyceraldehyde-3-phosphate dehydrogenase	C98527	1818.27	2.2	2.4
Glyceraldehyde-3-phosphate dehydrogenase	F2CUW2	141.67	ns	3.7
Malate dehydrogenase	M0Z0D3	243.45	ns	2.5
Malate dehydrogenase	A0A096UKG	33.27	2.3	2.2
	6			
Ribulose bisphosphate carboxylase large chain	B0YIE1	492.78	-2	-1.8
Ribulose bisphosphate carboxylase small chain	Q9SBU5	142.71	-2.2	-1.5
RuBisCO large subunit-binding protein subunit alpha, chloroplastic	P08823	5848.41	ns	3.2
Triosephosphate isomerase	A0A077RSI3	136.62	1.6	2.3
n degradation				
E3 ubiquitin-protein ligase UPL4	M7YJY4	198.75	2.3	1.8
n folding				
Peptidyl-prolyl cis-trans isomerase	I1HN82	46.54	1.5	1.5
Protein disulfide-isomerase	A0A024FR39	1103.44	ns	2.8
n synthesis				
50S ribosomal protein L12-2, chloroplastic	M8BN49	125.53	-2.9	-1.5
homeostasis				
Catalase	I1HWC4	589.18	ns	2.2

^z ns indicating not significant at 0.05 probability level

CHAPTER 5. Heat-Induced Changes in Phenolic Acid Composition in Two Hard Fescue Cultivars and Their Relationship to Heat Tolerance

5.1 Background

Plant secondary metabolites play important role in plant stress adaptation and defense (Kaplan, Kopka, et al., 2004). Phenolic acid is an important group of secondary metabolites and derives from phenylopropanoid metabolism (Crozier, Clifford, et al., 2008). The phenylalanine ammonia lyase (PAL) is a control enzyme at the branch point from primary (shikimate pathway) to secondary (phenylopropanoid) metabolism, catalyzing the transformation, by deamination, from phenyalanine to cinnamic acid, which serves as intermediary for phenolic acid synthesis (Camm and Towers, 1973). Induction of PAL activity was reported under heat stress and considered as one of a major cell acclimation to stress conditions (Oh, Trick, et al., 2009, Rivero, Ruiz, et al., 2001). The resulting accumulation of total phenolic acid content was also reported under heat stress (Rivero, Ruiz, et al., 2001). However, the association of phenolic acid accumulation and composition with heat tolerance has not been well documented.

Phenolic acids can be divided into two classes according to their structure as derivatives of benzoic acid (e.g., gallic acid, benzoic acid and salicylic acid) and derivatives of cinnamic acids (e.g., coumaric acid, caffeic acid, ferulic acid and sinapic acid). Previous research indicates water-soluble phenolic acids can protect plants from oxidative damage, which is a major consequence in heat stress, by scavenging hydrogen peroxide (H_2O_2) and 1,1-Diphenyl-2-picrylhydrazyl (DPPH) radical (Sroka and Cisowski, 2003). Different phenolic acids exhibit different antioxidant activity, which is positively

correlated with the number of hydroxyl groups bonded to aromatic rings. For example, the phenolic acids with three hydroxyl groups (gallic acid) showed highest antioxidant activity, followed by two hydroxyl groups (caffeic acid, dihydroxybenzoic acid), while the compounds with one hydroxyl group (salicylic acid and 3-hydroxybenzoic acid) showed lowest antioxidant activity (Sroka and Cisowski, 2003). Another important role phenolic acids process in plants is signaling transduction. Salicylic acid is a well studied phenolic acid and identified as signaling molecule inducing heat acclimation and protecting plants from heat damage (Horváth, Szalai, et al., 2007). A transient increase of salicylic acid at the beginning (several hours) of heat stress was observed in various plant species and enhanced antioxidant system in plants to improve heat tolerance and survival (Horváth, Szalai, et al., 2007). The other phenolic acids shared similar structure and biosynthesis pathway with salicylic acid; therefore we suspect that they may have similar function as salicylic acid. In addition, the function of these phenolic acid as signaling molecules has been widely explored in plant-microbe symbioss, as inducer of various genes involved in symbiosis establishment (Mandal, Chakraborty, et al., 2010). However, very limited studies have investigated the response and potential roles of these phenolic acids in heat stress tolerance.

We hypothesized that the differential quantity or proportion of different phenolic acid could be associated with the genotypic variations in heat tolerance. Therefore, the objectives of this study were to examine differential changes in the quantity and composition of phenolic acids in response to short-term heat shock and long-term heat stress for a cool-season perennial grass species, hard fescue and identify major constituents of phenolic acids associated with the genetic variations in heat tolerance for two cultivars contrasting in heat tolerance.

5.2 Materials and Methods

5.2.1 Plant Materials and Growth Conditions

Tillers of two hard fescue cultivars ('Reliant IV' and 'Predator') were transplanted into plastic containers (15 cm in depth and 14 cm in diameter) filled with sterile sand autoclaved at 121 °C for 60 min to minimize potential soil-born disease infection. Plants were established in a greenhouse with an average day/night temperature of 23/20 °C and 710 μ mol.m⁻².s⁻¹ photosynthetically active radiation (*PAR*) from sunlight and supplemental lighting from March 1, 2017 to May 25, 2017. Plants were irrigated daily, trimmed twice per week to maintain 7 cm canopy height, and fertilized every 4 d with half-strength Hoagland's nutrient solution (Hoagland and Arnon, 1950). Following the establishment period, plants were transferred to controlled-environment growth chambers (Environmental Growth Chambers, Chagrin Falls, OH) controlled at 22/18 °C (day/night), 650 μ mol.m⁻².s⁻¹ *PAR*, 60% relative humidity, and 12-h photoperiod for 7 d to allow for plant acclimation to growth chamber conditions prior to stress imposition.

5.2.2 Treatments and Experimental Design

Plants for both cultivars were exposed to heat stress at 38/33 °C (day/night) or maintained under non-stress control conditions at 22/18 °C (day/night) for 28 d. Each temperature treatment was repeated in four growth chambers and a total of eight growth chambers were used simultaneously. Each cultivar had four replicates (pots), which were

randomly placed within each growth chamber. The experimental design was a split-plot design with temperature as the main plots and cultivars as the subplots. Plants were arranged randomly within each growth chamber and were relocated among the four growth chambers used for same temperature treatment every 3 d to avoid possible confounding effects of unique growth chamber environmental variations from occurring.

5.2.3 Physiological Measurements

Turf quality (TQ), electrolyte leakage (EL) and photochemical efficiency (Fv/Fm) were measured at 7, 14, 21 and 28 d of heat stress. The TQ rating was performed to evaluate overall turfgrass performance on a scale of 1 to 9, with 1 being brown and desiccated turf, 6 being the minimal acceptable level, and 9 being green and healthy turf. The ratings were based on parameters such as uniformity, visual attractiveness, leaf color, and canopy density.

Leaf membrane stability was estimated by measuring EL. Approximately 0.2 g leaf tissue was collected, rinsed with deionized water, and placed in a test tube containing 30 mL deionized water. Tubes were agitated on a shaker for 12 h and initial conductance (Ci) of the incubation solution measured using a conductivity meter (Yellow Springs Instrument, Yellow Springs, OH). Leaf tissue was then killed by autoclaving at 121 °C for 20 min, agitated for 12 h, and maximal conductance (Cmax) of the incubation solution was measured. Plant EL was calculated using the formula (Ci/Cmax) x100 (Blum and Ebercon, 1981).

The Fv/Fm was measured as a ratio of the variable fluorescence value to the maximum fluorescence value using a chlorophyll fluorescence meter (Fim 1500;

Dynamax, Houston, TX). Leaf clips were first used to dark-adapt the leaves for 30 mins and then the Fv/Fm was determined with the fluorescence meter. Two subsample measurements were taken per plant per sampling day.

5.2.4 Phenolic Acid Extraction and Analysis

Free amino acids were extracted by grinding frozen leaf material with liquid nitrogen and 4 mL of 70% ethanol. After storage overnight in 4 °C, the samples were centrifuged (10500 g, 10 min). The supernatants were collected, filtered through a membrane (0.45 μ m PTFE membrane), concentrated under vacuum, and stored in -20 °C (Rozan, Kuo, et al., 2000). The 800 μ L supernatants were dried under nitrogen and re-suspend in 300 μ L 45% methanol and 0.1% Formic acid.

The instrument was Agilent 1290 Infinity II ultra performance liquid chromatography (UPLC) coupled with 6470 triple-quad mass spectrometry (MS) and electrospray ionization interface (Agilent Technologies, Santa Clara, CA). The software was MassHunter Workstation, with Data Acquisition of version B.08.00, Qualitative Analysis B.07.00, Quantitative Analysis B.07.01, and Optimizer B.08.00 (Agilent Technologies). The analytical column was Waters ACQUITY UPLC BEH C18 1.7 μ m, 2.1 × 50 mm, protected with a Waters 2.1 × 5 mm guard column of the same stationary phase (Waters Corporation, Milford, MA). Mobile phase A was water with 0.2% acetic acid, and mobile phase B was acetonitrile with 0.1% acetic acid, with a flow rate of 0.4 mL/min. The gradient was 3% B from 0 to 1min, 3 to 10% B from 1 to 3 min, 10 to 30% B from 3 to 10 min, 30 to 70% from 10 to 10.5 min, and held at 70% until 13 min. Liquid chromatography eluent before 1 min and after 9.5 min was split into waste. The column

thermostat was set at 25 °C and equilibrated for 2 min with 3% B between injections. The injection volume was 0.4 μ L. For MS, high purity nitrogen was used as nebulizing and drying gas, sheath gas, and used for collision-induced dissociation. The nebulizer pressure was set at 30 psi, the drying gas was set at 350 °C with a flow rate of 12 L/min, and the sheath gas was 200 °C of 12 L/min. The scanning polarity was negative, with a capillary voltage of 2500 V and nozzle voltage of 2000 V. Multiple reaction monitoring was used as the scanning mode, with one primary transition as qualifier and a secondary transition as qualifier for each of compounds, as shown in Table 1. The transitions were automatically optimized by the optimizer software or manually optimized. Each transition had a dwell time of 15 ms, with a total cycle time of 546 ms. The accelerator voltage was set at 4.

5.2.5 Statistical Analysis

Treatment effects and the interaction between temperature treatment and varieties were determined by analysis of variance using the general linear model procedure of SAS (version 9.3; SAS Institute, Cary, NC). Differences between treatment means were separated by Fisher's protected least significance difference (LSD) test at the 0.05 probability level.

5.3.1 Differential physiological responses to heat stress between two hard fescue cultivars

Overall turf performance was evaluated by TQ, while cell membrane stability and photosystem II efficiency were evaluated by EL and Fv/Fm, respectively. The TQ showed a significant decline from 14 d of heat stress in both hard fescue cultivars (Fig. 1A). 'Reliant IV' exhibited significantly higher TQ compared to 'Predator' at 14, 21, 28 d of heat stress. At the end of heat stress, the TQ of 'Reliant IV' and 'Predator' declined to 7.0 and 5.5, respectively.

A significant increase of EL was detected at 7 d of heat stress in both hard fescue cultivars (Fig. 1B). The significant difference in EL between two hard fescue cultivars was detected at 14, 21 and 28 d of heat stress, with 'Reliant IV' exhibiting lower EL compared to 'Predator'. At the end of heat stress, the EL of 'Reliant IV' and 'Predator' increased to 27.0 and 41.8, respectively.

A significant decrease of Fv/Fm was detected from 7 d of heat stress in both hard fescue cultivars (Fig. 1C). The significant different of Fv/Fm between two hard fescue cultivars was detected at 14, 21 and 28 d of heat stress, with 'Reliant IV' showing significant higher Fv/Fm compared to 'Predator'. At the end of heat stress, the Fv/Fm of 'Reliant IV' and 'Predator' decreased to 0.69 and 0.61, respectively.

5.3.2 Phenolic acid composition associated with heat tolerance

The significant increase of total phenolic acids compared to the initial level was detected at 7 h and 21 d of heat stress in 'Predator', while the increase was only detected

at 21 d of heat stress in 'Reliant IV' (Fig. 2). The total phenolic acids did not differ significantly between the two cultivars at any sample collection dates (0 h, 7 h and 21 d of heat stress).

A total of 12 phenolic acids were identified and quantified in leaves of two hard fescue cultivars (Fig. 3). Among 12 phenolic acids, ferulic acid (31.2%), caffeic acid (21.7%), syringic acid (18.3%) and coumaric acid (9.7%) were the most abundant in hard fescue, followed by vanillic acid (4.3%), 3,4-dyhydroxybenzoic acid (4.3%), salicylic acid (4.1%), homovanillic acid (3.1%) and 4-hydroxybenzoic acid (1.8%), while gallic acid (0.9%), cinnamic acid (0.4%) and benzoic acid (0.2%) accountedfor less than 1% of total content of 12 phenolic acids.

In response to short-term (7 h) of heat shock, the content of salicylic acid, homovanillic acid, caffeic acid and ferulic acid increased significantly while the content of most phenolic acids (4-hydroxybenzoic acid, cinnamic acid, benzoic acid, 3, 4-dyhydroxybenzoic acid, gallic acid, coumaric acid, vanillic acid, syringic acid) remained the same level as the initial control (Fig. 4). The increase of homovanillic acid and caffeic acid were only detected in 'Reliant IV'. While the increases of ferulic acid and salicylic acid were detected in both cultivars, to a higher abundance in 'Reliant IV' for ferulic acid and to higher abundance in 'Predator' for salicylic acid.

Under long-term heat stress (21 d), the content of most phenolic acids (4hydroxybenzoic acid, 3, 4-dyhydroxybenzoic acid, coumaric acid, gallic acid, cinnamic acid, benzoic acid and vanillic acid) showed significant increase compared to the initial level, and was higher in 'Predator' compared to 'Reliant IV' for 4-hydroxybenzoic acid, coumaric acid, gallic acid, cinnamic acid, benzoic acid and vanillic acid and was higher in 'Reliant IV' compared to 'Predator' for 3, 4-dyhydorxybenzoic acid (Fig. 4). Salicylic acid and syringic acid and syringic acid remained the same content as the initial level, while the content of homovanillic acid, caffeic acid and ferulic acid showed significant decrease, to similar levels in both cultivars.

5.4 Discussions

Loss of membrane stability and integrity as indicated by increased EL and impaired photosystem II as indicated by decreased Fv/Fm are commonly-used parameters to evaluate the level of heat stress tolerance in plants (Wahid, Gelani, et al., 2007). In this study, hard fescue cultivar 'Reliant IV' exhibited higher TQ, higher Fv/Fm and lower EL under heat stress compared to 'Predator. Previous studies identified major proteins and amino acids associated with the genotypic variations in heat tolerance between 'Reliant IV' and 'Predator' (Wang, Burgess, et al., 2017, Wang, Juliani, et al., 2017). This study first found that phenolic acids could also play positive roles in plant responses to both short-term heat shock and long-term heat in cool-season turfgrass species.

Salicylic acid is a well-studied phenolic acid for its roles in the induction of defense systems against biotic and abiotic stress, which is considered a stress signaling molecule and involved in heat acclimation or acquired thermotolerance (Horváth, Szalai, et al., 2007). The transient increase of salicylic acid content at the initiation of heat stress exposure was reported in various plant species, which can induce or activate downstream stress-protective systems (Horváth, Szalai, et al., 2007). Salicylic acid was shown to enhance heat tolerance by activating various antioxidant enzymes, such as ascorbate peroxidase (APX) (Larkindale and Huang, 2004), superoxide dismutase (SOD), and

catalase (CAT) (He, Liu, et al., 2005). In this study, a transient elevation in salicylic acid content was detected at 7 h of exposure to high temperature in both hard fescue cultivars, indicating salicylic acid could act as a common heat-stress signaling molecule in both cultivars of hard fescue.

Compared to salicylic acid, little is known of the functions of the other three phenolic acids (homovanillic acid, caffeic acid and ferulic acid) that also exhibited transient increases in response to short-term heat shock in hard fescue. Some studies pointed out that ferulic acid and homovanillic acid can act as signaling molecules that induces *Agrobacterium* virulence system (Melchers, Regensburg-Tuïnk, et al., 1989). In this study, the increase of ferulic acid was detected in both hard fescue cultivars, while the increase of homovanillic acid, caffeic acid was detected only in 'Reliant IV'. The transient nature of homovanillic acid, caffeic acid and ferulic acid, same as shown in salicylic acid, suggested that those three phenolic acids could also act as signaling molecules in hard fescue and homovanillic acid and caffeic acid may be unique signaling molecules in 'Reliant IV' accounting for its better heat tolerance compared to 'Predator'.

Under long-term heat stress, seven phenolic acids exhibited increases in their content, including 4-hydroxybenzoic acid, 3, 4-dyhydroxybenzoic acid, coumaric acid, gallic acid, cinnamic acid, benzoic acid and vanillic acid, and the content of 4-hydroxybenzoic acid, coumaric acid, gallic acid, cinnamic acid, benzoic acid and vanillic acid accumulated to a higher level in 'Predator' compared to 'Reliant IV'. The greater increases in the above six phenolic acids in 'Predator' suggested that those molecules may signify the sensitivity of plants to heat stress. Similar results were found in drought stress responses with studies reporting a strong negative relationship between total

phenolic acid accumulation and drought tolerance in various plant species, suggesting that the accumulation of some phenolic acids may be the result of drought damage and reflect the sensitivity to drought stress (Hura, Grzesiak, et al., 2007, Hura, Hura, et al., 2008, Sánchez-Rodríguez, Rubio-Wilhelmi, et al., 2010).

Some phenolic acids may act as antioxidants, protecting plants form stress damages. The antioxidant efficiency of phenolic acids depends on the number and position of hydroxyl groups linked to aromatic ring (Sroka and Cisowski, 2003). The 3, 4-dyhydroxybenzoic acid and gallic acid are strong antioxidant molecules; the 4hydroxybenzoic acid, coumaric acid, and vanillic acid have low antioxidant activity; while cinnamic acid and benzoic acid has no antioxidant activity based on its structure that no hydroxyl group linked to aromatic ring (Sroka and Cisowski, 2003). In this study, the content of 3, 4-dyhydroxybenzoic acid increased under long-term heat stress, and to a higher level in 'Reliant IV' than in 'Predator'. The 3, 4-dyhydroxybenzoic acid is a strong antioxidant molecule (Sroka and Cisowski, 2003). The greater accumulation of 3, 4-dyhydroxybenzoic would be associated with better heat tolerance in 'Reliant IV'. However, the direct relationship of the accumulation of 4-hydroxybenzoic acid, 3, 4dyhydroxybenzoic acid, coumaric acid, gallic acid, cinnamic acid, benzoic acid and vanillic acid to antioxidant activities in hard fescue and other cool-season grass species deserves further investigation.

In summary, this study demonstrated that the differential heat tolerance in hard fescue cultivars was associated with greater increase of homovanillic acid and caffeic acid at the early stage of heat stress and with greater accumulation of 3, 4dyhydroxybenzoic acid under long-term heat stress. These more up-regulated phenolic acids in 'Reliant IV' exposed to heat stress could be potentially incorporated into the biostimulant products used in relieving heat stress in turfgrass. The metabolic and molecular mechanism of phenolic acids regulating heat tolerance deserves further investigation.



Fig. 5.1. (A) Turf quality and (B) electrolyte leakage and (C) photochemical efficiency of 'Reliant IV' and 'Predator' hard fescue affected by heat stress. Turf quality was rated on the scale of 1 to 9, with 1 being worst and 9 being the best. Vertical bars indicate Fisher's protected LSD values (P \leq 0.05) for comparison between two temperature treatments and varieties at a given day of treatment.



Fig. 5.2. Total content of phenolic acids of 'Reliant IV' and 'Predator' hard fescue at 0 h, 7 h and 21 d of heat stress. Columns marked with different letters indicate significant difference between two temperature treatments and varieties according to Fisher's protected LSD ($P \le 0.05$).



Fig. 5.3. Phenolic acid composition of 'Reliant IV' hard fescue at optimal temperature.



Fig. 5.4. Content of phenolic acids of 'Reliant IV' and 'Predator' hard fescue at 0 h, 7 h and 21 d of heat stress. Columns are marked with different letters indicating significant difference between 'Reliant IV' and 'Predator' according to least significant difference ($P \le 0.05$). The specific type of phenolic acid is labeled on the top left corner of each sub-figure.



Fig. 5.5. Changes of 12 phenolic acids in the metabolic pathways at 0 h, 7 h and 21 d of heat stress for 'Reliant' and 'Predator' hard fescue.

Table. 5.1 the list of phenolic acids used in this study, together with their retention time (RT), precursor, fragmentor voltage, quantifier, collision energy, limit of detection (LOD), limit of quantification (LOQ) and calibration curve.

c	RT		Fragmentor	a,	Collision	LOD	ГОО	Calibrat	tion curve	
Compound	(min)	Frecursor	voltage	Quantifier	Energy	(lm/gn)	(lm/gn)	Equation	R²	Range (ng/ml)
protocatechuic acid	1.610	153.0	86	109.1	12	5.57	5.57	Y=11.167X-198.605	0.9944	$5.57 \sim 713.50$
gallic acid	2.200	169.0	80	125.0	12	15.03	30.06	Y=2.584X+1.074	0.9987	$15.03\sim962.00$
4-hydroxybenzoic acid	2.664	137.0	74	93.1	16	1.36	2.72	Y=119.563X-140.193	6666.0	$2.72 \sim 1393$
vanillic acid	3.424	167.0	80	152.0	12	1.64	3.27	Y=21.915X-91.697	8666.0	$1.64 \sim 838.00$
3-hydroxybenzoic acid	3.540	137.0	74	93.1	8	4.00	4.00	Y=43.497X+167.28	9666.0	$4.00 \sim 1026.00$
caffeic acid	3.552	179.0	86	135.1	16	7.74	7.74	Y=15.413X-244.995	0.9936	$7.74 \sim 495.5$
syringic acid	3.823	197.0	125	182.0	6	4.41	4.41	Y=11.886X+17.237	0.9993	$4.41 \sim 564.00$
homovanillic acid	3.921	181.1	54	137.1	4	4.64	9.28	Y=13.89X-312.84	866.0	$4.64 \sim 1188.00$
coumaric acid	4.638	163.0	80	119.1	12	0.97	1.94	Y=110.04X-665.807	0.9994	$1.94 \sim 991.00$
ferulic acid	5.270	193.1	88	134.0	16	3.43	3.43	Y=16.144X-74.951	0.9993	$3.43 \sim 880.00$
salicylic acid	5.412	137.0	80	93.0	17	2.20	4.40	Y=142.715X-1530.571	0.9994	$4.40 \sim 1128.00$
benzoic acid	5.783	121.0	70	77.1	6	1.34	2.67	Y=21.97X+1156.248	0.9978	$2.67 \sim 684.00$
sinapic acid	5.871	223.1	115	164.0	13	15.21	30.43	Y=0.9594X-6.553	0.9846	$30.43 \sim 487.00$
2-hydroxycinnamic acid	6.510	163.0	20	0.911	6	1.87	1.87	Y=110.142X-205.007	1666.0	$1.87 \sim 479.00$
cinnamic acid	8.577	147.0	60	103.0	6	0.52	1.03	Y=42.256X-16.142	1666.0	$1.03 \sim 530.00$

CHAPTER 6. Conclusions and Future Work

My thesis research focused on the investigation of heat tolerance by evaluating performance of twenty-six fine fescue cultivars under heat stress, by comparing the proteomic profile, cell membrane composition and phenolic acids composition of hard fescue cultivars contrasting in heat tolerance. The evaluation of heat tolerance among different fine fescue cultivars could serve as guidance for fine fescue breeding. The identified heat responsive elements could serve as a basis for development of molecular markers, which would greatly facilitate selection of heat tolerant fine fescue cultivars.

Our result indicated that hard fescue cultivars showed the most heat tolerance than the other species and subspecies with better maintenance of membrane stability, photosynthesis ability and overall turf quality. While Chewing fescue cultivars showed the least heat tolerance with the lowest overall turf quality and greatest physiological damages during evaluation period. Among the evaluated hard fescue cultivars, 'Reliant IV' was the most heat tolerant hard fescue cultivar, while 'Predator' was the most heat sensitive hard fescue cultivar.

To better understand the mechanism of heat tolerance and to identify key factor in heat tolerance, the proteomic profiles of heat tolerant 'Reliant IV' and heat sensitive 'Predator' were compared after heat stress treatment. Generally, the identified heat responsive proteins are mainly involved in photosynthesis, glycolysis, stress defense, redox homeostasis, signaling, protein folding and degradation. In this work, the heat tolerance among different cultivars was correlated with the induction of proteins involved in redox homeostasis, stress defense and protein folding and degradation. The higher levels of catalase, chaperone 20, HSP 70, formate dehydrogenase, malate dehydrogenase and UDP-sulfoquinovose in heat tolerant cultivar 'Reliant IV' could contribute to its superior thermo tolerance compared to the heat sensitive cultivar 'Predator'.

The variances in heat tolerance among different cultivars were also correlated with their membrane compositions. Our research found heat tolerance was positively correlated with the decrease of 18:03 fatty acid and accumulation of ethyl-sterol at heat stress. The maintenance or less severe down-regulation of membrane proteins involved in photosynthesis and signaling and greater up-regulation of several heat responsive proteins involved in stress defense, protein folding and degradation during the heat treatment of heat tolerant cultivar 'Reliant IV' could contribute to superior heat tolerance.

Another metabolic process that has been related to the heat tolerance is phenolic acids composition. Our result demonstrated that the differential heat tolerance in hard fescue cultivars is associated with greater increase of homovanillic acid and caffeic acid at the early stage of heat stress and with greater accumulation of 3, 4-dyhydroxybenzoic acid under long-term heat stress. These more up-regulated phenolic acids in 'Reliant IV' exposed to heat stress could be potentially incorporated into the biostimulant products used in relieving heat stress in turfgrass.

Future work should focus on confirmation of identified heat responsive protein at both transcription and translation level and develop molecular marker for heat tolerance based on the identified heat responsive elements. It would also be interesting to investigate the direct involvement of the identified metabolites and their potential use as biostimulant to relieve heat damage.

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